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Metabolism of Vitamins D and A

Hector F. DeLuca

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_3 of high specific activity was also obtained by a biosynthetic route (Imrie *et al.*, 1967).

Using physiologic quantities of radioactive vitamin D_3 it was possible to detect metabolites of vitamin D that had been

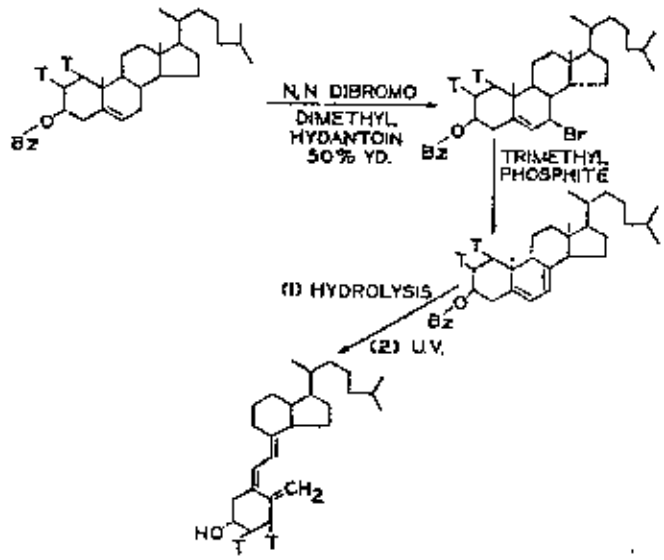

Figure 1. Synthesis of 1,2-^{3}H vitamin D_3 of high specific activity 26,000 d.m.p./I.U.

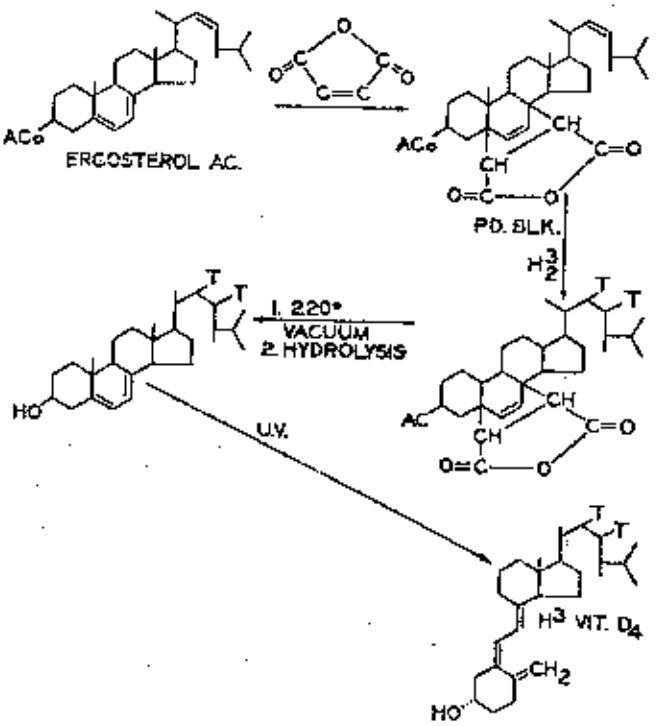

Figure 2. Synthesis of 22,23-^{3}H vitamin D_4 150,000 d.p.m./I.U.

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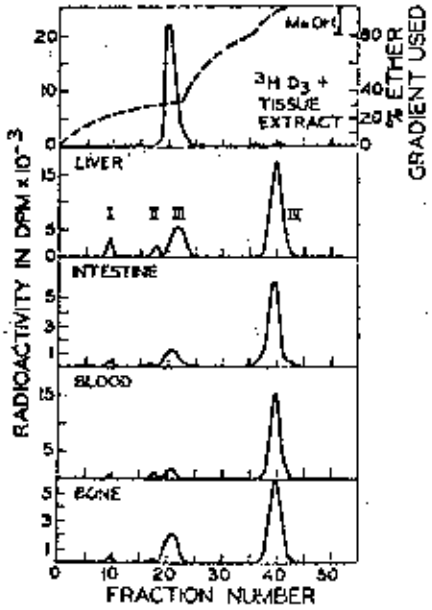

Figure 3. Silicic acid column chromatography of methanol-chloroform extracts of tissues from rats given 0.25 μg. of 1,2-^{3}H vitamin D$_3$ intravenously 12 hours previously

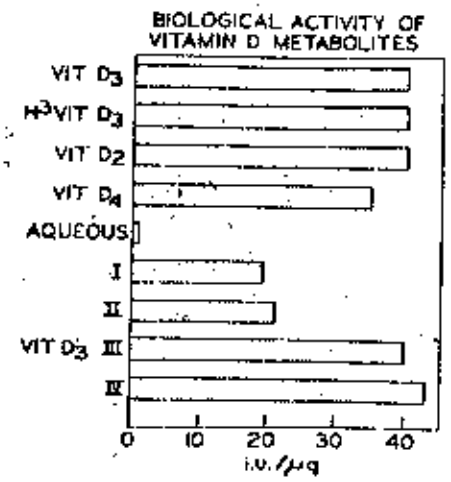

Figure 4. Biological activity of vitamin D$_3$ and its metabolite fractions

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

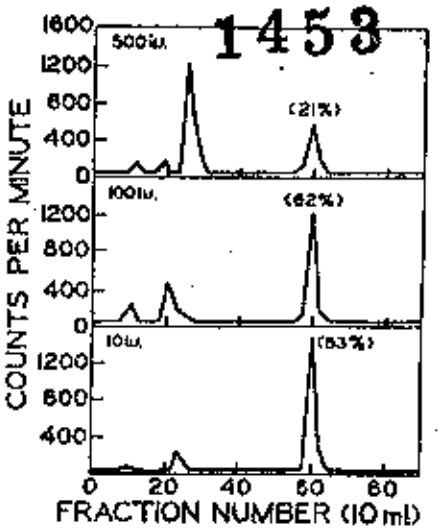

Figure 5. Effect of dose on distribution of ^{3}H among metabolite fractions after 1,2-^{3}H vitamin D$_3$

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 radioactivity 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$_3$

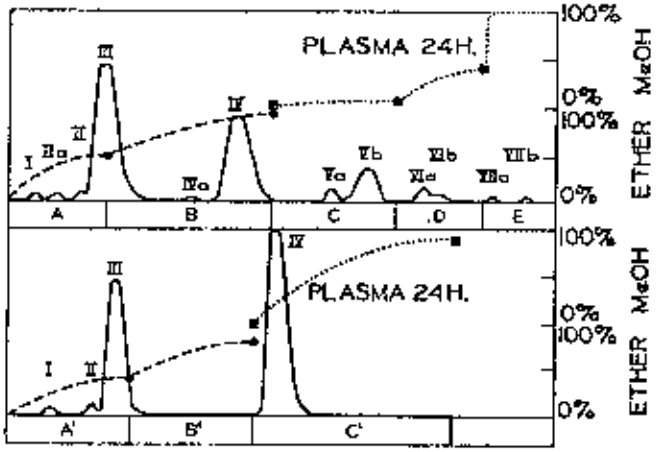

Figure 6. Chromatography of plasma extract on silicic acid using a refined gradient system

---- 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

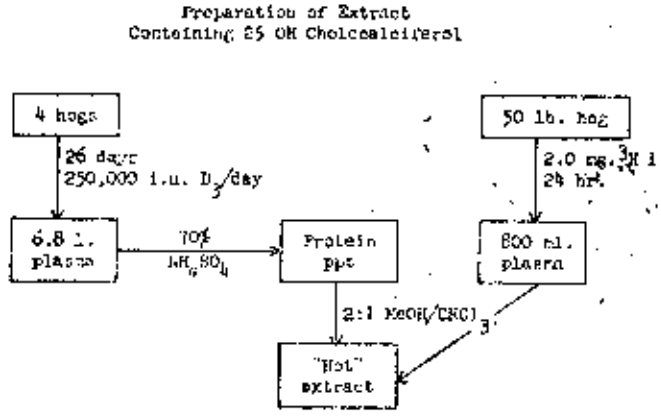

Figure 7. Preparation of extract for isolation of 25-hydroxycholecalciferol

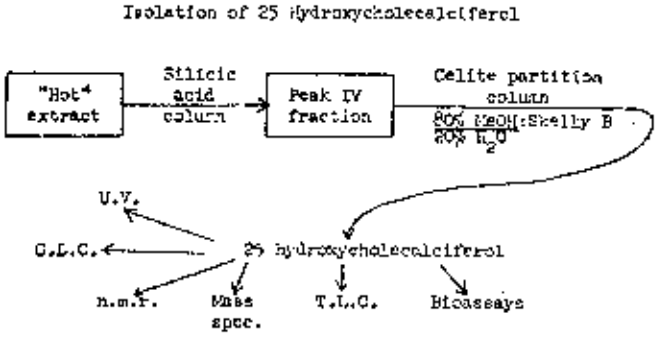

Figure 8. Flow diagram of isolation of 25-hydroxycholecalciferol from porcine plasma

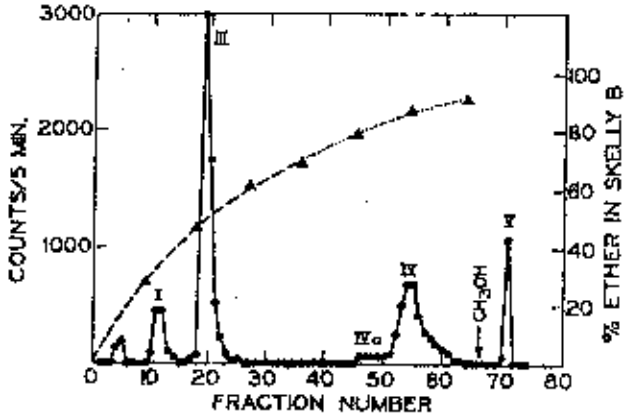

Figure 9. Silicic acid chromatography in isolation of 25-hydroxycholecalciferol from porcine plasma

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 ^{3}H 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

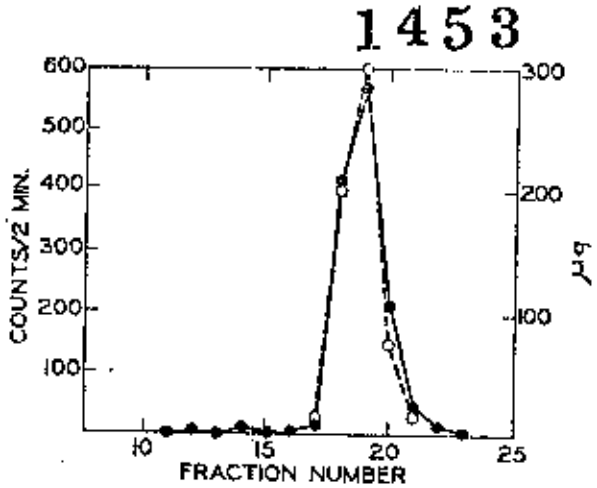

Figure 10. Partition chromatography of 25-hydroxycholecalciferol on Celite using 80% methanol, 20% water equilibrated with a petroleum fraction b.p. 65–67°C. ○ μg. 25 OH D_3 based on spectrum. ● Radioactivity

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.

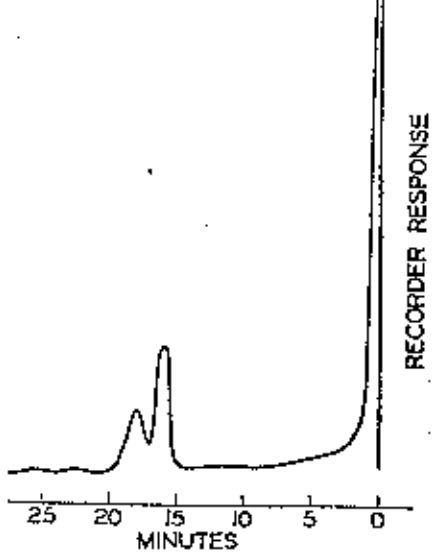

Figure 11. Gas-liquid chromatography of 25 - hydroxycholecalciferol demonstrating purity of isolated material from porcine plasma

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_2 (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.

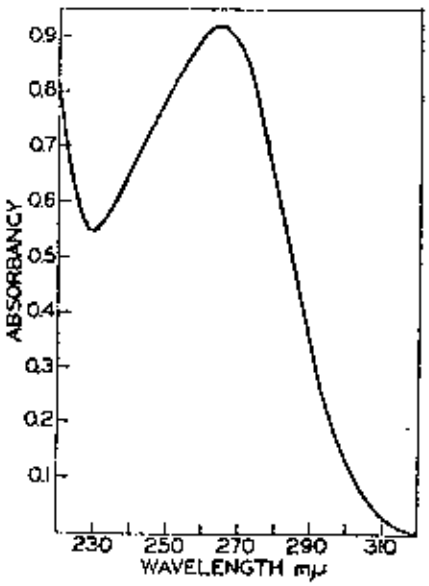

Figure 12. Ultraviolet spectrum of isolated 25-hydroxycholecalciferol in diethyl ether

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

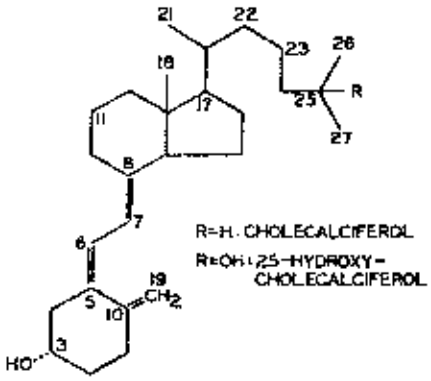

Figure 13. Structure of 25-hydroxycholecalciferol, believed to be metabolically active form of vitamin D_3

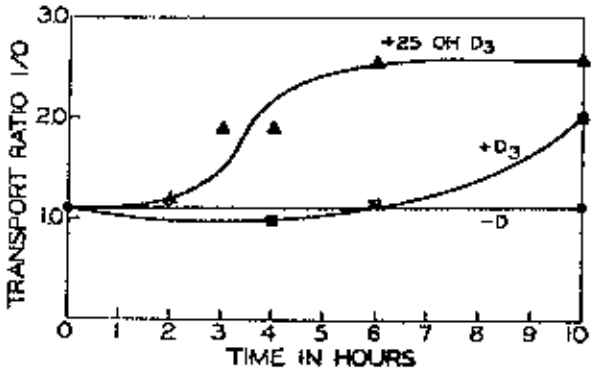

Figure 14. Response of calcium transport system of rats to 0.25 μg. intravenously of 25-hydroxycholecalciferol or vitamin D_3

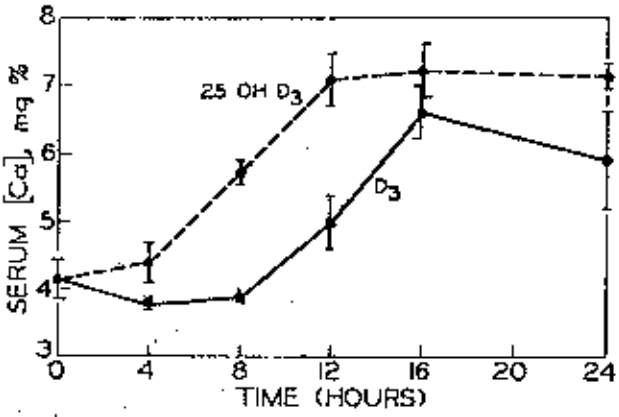

Figure 15. Serum calcium response of vitamin D-deficient rats in a low-calcium diet to 25-hydroxycholecalciferol or vitamin D_3

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

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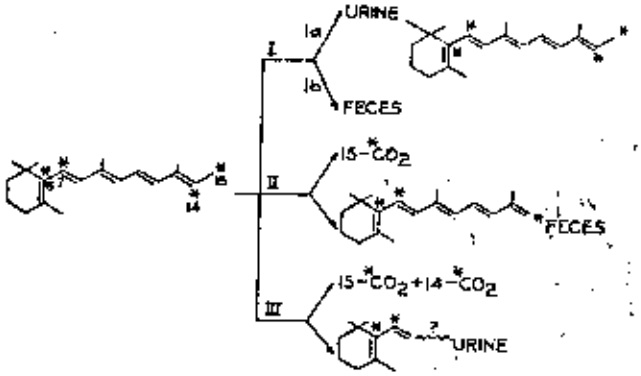

Figure 16. Proposed pathways of retinol or retinoic acid metabolism

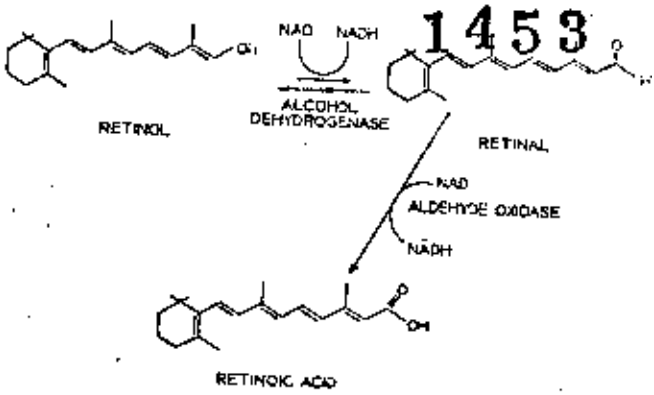

Figure 18. Conversion of retinol to retinoic acid

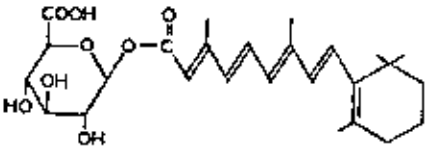

Figure 17. Retinoyl β-glucuronide

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.

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.

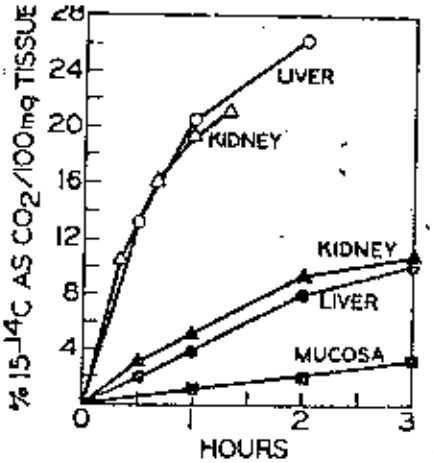

Figure 19. Rate of C-15 decarboxylation of retinoic acid by tissue slices

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

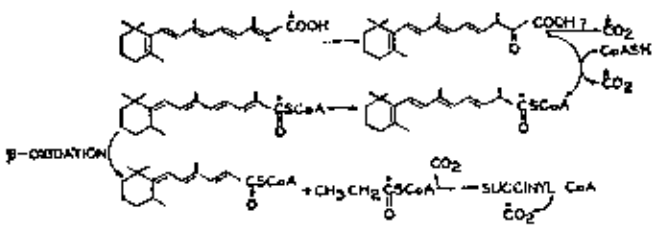

Figure 20. Proposed pathway of metabolism of 14-^{14}C retinoic acid in tissue slices

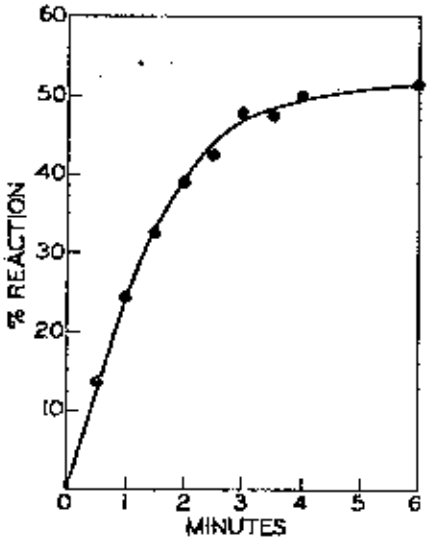

Figure 21. Rate of C-15 decarboxylation of retinoic acid by microsomes from rat liver

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 $^{14}CO_2$ by Kidney Slices[a]

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

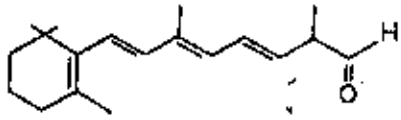

Figure 22. Proposed product of oxidative decarboxylation of retinoic acid

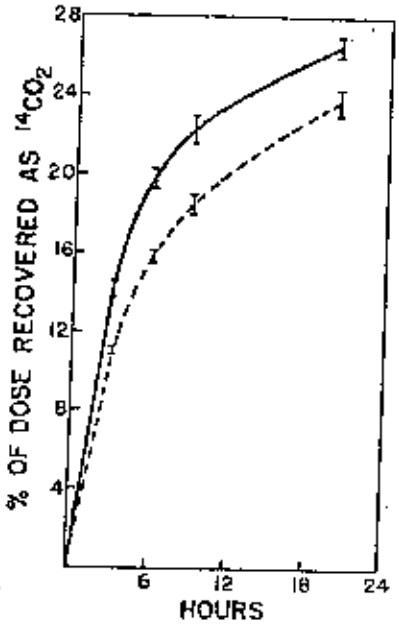

Figure 23. Effect of DPPD on in vivo decarboxylation of 15-^{14}C retinoic acid

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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PARATHYROID GLANDS, ULTRASTRUCT.
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PARATHYROID HORMONE: ITS SUBCELLULAR ACTIONS AND ITS RELATIONSHIP TO VITAMIN D

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For many years, work in our laboratory has been concerned with the mechanisms of vitamin D action. At first the metabolism of citrate under the influence of vitamin D appeared to provide an important new lead. During the course of our experiments we found that administration of the vitamin to rats had a marked protective effect on the stucture of isolated kidney mitochondria (DeLuca *et al.*, 1960). The action of vitamin D, therefore, in decreasing the citrate oxidation by mitochondria (DeLuca *et al.*, 1957; DeLuca, Gran, and Steenbock, 1957) appeared to be caused by a reduced penetration of the triply ionized substrate into the isolated mitochondria. More importantly, however, it showed a basic relationship between vitamin D and the structure and function of a subcellular membrane system. As converging evidence the vitamin had long been known to increase the absorption of calcium from the intestinal tract, a relationship that has been reaffirmed by experiments using various *in vitro* techniques (Dowdle, Schachter, and Schenker, 1960; Harrison and Harrison, 1960a). From this and other physiological evidence it seemed quite clear that vitamin D must play a basic role in the transport of Ca and P_i across cellular and subcellular membranes. These two lines of evidence suggested that the mitochondrial membrane might well provide a model for a study of Ca and P_i transport as influenced by vitamin D and PTH.

Because much of the following discussion concerns ion transport by mitochondria, which is dependent upon the energy of oxidative phosphorylation, it may be helpful to review briefly the essentials of this process. The electron

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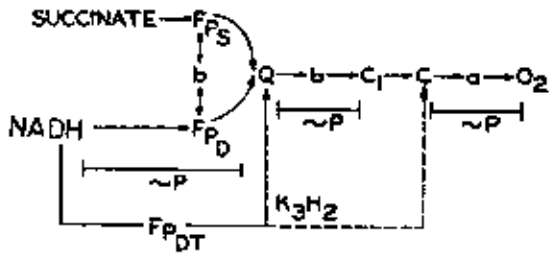

FIG. 1. *Simplified electron transport scheme for mammalian mitochondria. Cytochrome a is the combination of cytochrome a and a_3; F_{P_S} is succinate flavoprotein; F_{P_D} is NAD flavoprotein; $F_{P_{DT}}$ is diaphorase; K_3H_2 is reduced vitamin K_3.*

transport chain for mammalian mitochondria is depicted in Fig. 1. The reduced pyridine nucleotides are oxidized through the electron transport chain by the pathways shown, while succinate is oxidized directly through a flavine enzyme which taps into the chain at the coenzyme Q stage. High-energy intermediates are generated at the NADH-flavine, cytochrome b-cytochrome C_1 and the cytochrome a – a_3 – O_2 sites. CN^- and azide inhibit the cytochrome a–a_3; antimycin A inhibits between cytochrome b and C_1; rotenone and Amytal block the oxidation of NADH; and 10^{-7} M dicumarol blocks the DT-diaphorase pathway. The following reactions indicate the simplest hypothetical pathway for coupling the electron transport chain (Fig. 1) to the synthesis of ATP.

$$AH_2 + B + Y \rightleftharpoons A \sim Y + BH_2 \quad (1)$$

$$A \sim Y + P_i \rightleftharpoons Y \sim P + A \quad (2)$$

$$Y \sim P + ADP \rightleftharpoons ATP + Y \quad (3)$$

A and B are sequential members of the electron transport chain. Reduced A reacts with B in the presence of some factor we will call Y, forming a nonphosphorylated intermediate called A ~ Y (Reaction 1). This then reacts with inorganic phosphate (P_i) to form Y ~ P, a phosphorylated high-energy intermediate (Reaction 2), which in turn reacts with ADP to form ATP (Reaction 3). Uncouplers such as DNP and dicumarol, and the antibiotics Dianemycin and Nigericin, inhibit Reaction 1. The uncouplers undoubtedly act in a much different manner from the antibiotics, perhaps causing a discharge of A ~ Y.

Considering the over-all process, the flow of electrons through the electron transport chain as shown here is [illegible] linked to the synthesis of ATP. [illegible] ADP is available to accept the high energy phosphate generated by the [illegible] transport chain, respiration is shut down. Addition of ADP causes [illegible] in respiration accompanied by the synthesis of ATP. [illegible] of oxidative phosphorylation will also stimulate respira-[illegible] they destroy the requirement for coupling respira-

tion to phosphorylation. Of course, when the uncoupler stimulates respiration, there is no corresponding synthesis of ATP. A very useful inhibitor in this area of metabolism, oligomycin, was first described by Lardy, Johnson, and McMurray (1958). It blocks either Reaction 3 or, more likely, Reaction 2. This has no effect on the coupling process and results in very low respiration rates comparable to the levels obtained even in the absence of ADP. Uncouplers together with oligomycin will nevertheless stimulate respiration but, again, no ATP is synthesized. If, in the presence of oligomycin, a substance stimulates respiration, two interpretations of the data are possible. Either the substance is an uncoupler, or it harnesses the energy of oxidative phosphorylation via an intermediate prior to the appearance of ATP. As will be demonstrated later, PTH is to fit the latter possibility.

At first it was essential to define conditions for the transport of Ca and P_i into the mitochondria. These early experiments (Vasington and Murphy, 1961; DeLuca and Engstrom, 1961) were followed by a host of investigations making this a very popular subject for mitochondrial experts to pursue (Brierley, Murer, and Green, 1963; Rossi and Lehninger, 1963). As the dust settled, the following facts emerged (Engstrom and DeLuca, 1964; Vasington and Murphy, 1962; Brierley *et al.*, 1962). Calcium is taken up by mitochondria mainly as a phosphate salt; it is deposited as an amorphous precipitate (not hydroxyapatite) in granules in mitochondria (Peachey, 1962). This translocation is powered by the energy of oxidative phosphorylation as illustrated in Fig. 2.

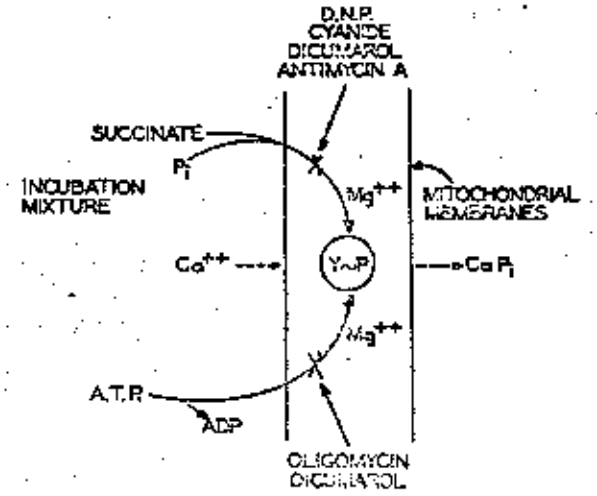

FIG. 2. *Schematic diagram for calcium phosphate translocation into mitochondria. (From Fig. 6, Engstrom and DeLuca, Biochemistry, 3:332. Reproduced by courtesy of the publisher.)*

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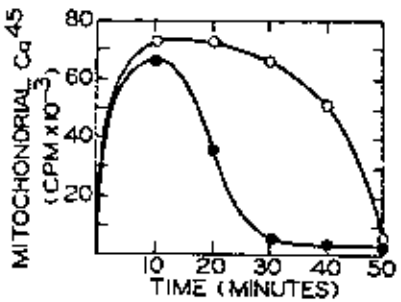

FIG. 3. *Vitamin D-stimulated release of Ca by mitochondria from PTX vitamin D-deficient rats* (○—○). *The animals were fed a high Ca, low P diet for 15 days. Vitamin D_3, 50 μg in 0.1 ml of propylene glycol was added to the vessel* (●—●). *The medium contained 0.4 μmoles ATP and 0.9 μmoles of Ca^{45}-Ca^{45} (70,000–75,000 cpm). (From Fig. 3, DeLuca, Engstrom, and Rasmussen, Proc. nat. Acad. Sci.* [Wash.], *48:1607. Reproduced by courtesy of the publisher.)*

When an oxidizable substrate (succinate) is provided, Mg^{++} and P_i are required to generate a high-energy intermediate, $Y \sim P$. This intermediate then supplies energy for the translocation of Ca and P_i. The hypothetical intermediate, and its energy, may also be generated by reversing the oxidative phosphorylative chain by the addition of ATP. In this case, P_i is not required because it is supplied by the hydrolysis of ATP; Mg^{++}, however, is required. Either ATP or an oxidizable substrate can supply the energy needed for the translocation. Inhibition by well-known inhibitors would therefore depend upon which energy supply is used. For example, oligomycin, which inhibits a terminal reaction in the phosphorylative sequence, blocks calcium phosphate uptake by mitochondria when ATP is the energy source but it has no effect when succinate is used to power the reaction. In a reverse manner, uncouplers of oxidative phosphorylation such as DNP or respiratory inhibitors such as CN^- or antimycin A do not block Ca uptake when ATP is supplied. Ba^{++}, Sr^{++}, and Mn^{++} are also transported into the mitochondria by similar mechanisms. Recent evidence suggests that Ca^{++}, Mg^{++}, and Mn^{++} can be transported by these mechanisms in the absence of P_i transport, suggesting that these translocations primarily involve a cation rather than phosphate activation. Regardless of the energy source, the rate and absolute amount of Ca uptake are not stimulated by either vitamin D or PTH (DeLuca and Engstrom, 1961; Engstrom and DeLuca, 1963) added *in vitro* or given *in vivo*, at least under the conditions described to date.

In Fig. 2 the Ca uptake mechanism is postulated: if mitochondria take up Ca in the presence of substrate and ATP without P_i, then upon exhaustion of the ATP with added hexokinase-glucose no further uptake is possible because the energy supply is cut off from both substrate and ATP. ATP can be added [illegible] to bind the added Ca, thereby eliminating the need

for hexokinase and glucose. Under these conditions, the reverse process, i.e., the Ca passage out of the mitochondria, can be examined. It is on this process that vitamin D exerts its marked effect (Engstrom and DeLuca, 1962; 1964a) as shown in Fig. 3.

The vitamin, whether given *in vivo* or added *in vitro*, greatly stimulates calcium phosphate release. Recent evidence from our laboratory suggests that a vitamin D metabolite may well be the active form in this and other Ca transport processes. PTH added *in vitro* acts synergistically with vitamin D (DeLuca, Engstrom, and Rasmussen, 1962) (Fig. 4). The hormone also stimulates the release of Ca but only in mitochondria isolated from rats receiving vitamin D. In the absence of vitamin D, the hormone has no effect whatsoever even when added at the highest concentration tested. This observation agrees with that of Harrison, Harrison, and Park (1958) who demonstrated that the response of serum Ca to PTH was greatly reduced in vitamin D deficiency. These studies provided a very important insight into at least a portion of the vitamin D-PTH interrelation.

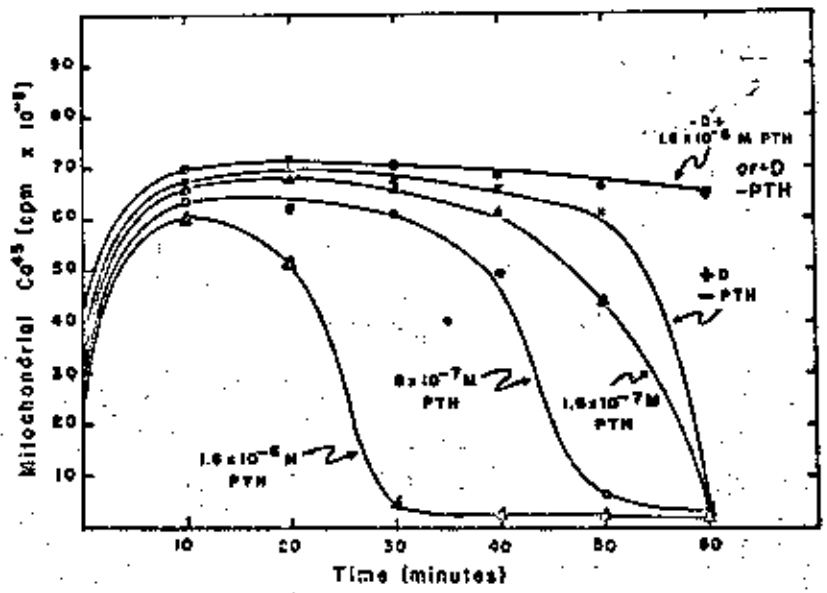

FIG. 4. *PTH stimulation of Ca released from mitochondria of rats fed vitamin D. The reaction mixture included 0.2 μmoles ATP and 0.6 μmoles Ca containing 70,000 cpm of Ca^{45}. (Redrawn from Fig. 4, DeLuca, Engstrom, and Rasmussen, Proc. nat. Acad. Sci.* [Wash.], *48:1607. Reproduced by courtesy of the publisher.)*

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To study Ca release, it is essential to examine it in mitochondria having intact membranes (Engstrom and DeLuca, 1964a). For this reason, Ca release can only be studied in mitochondria supplied with adequate substrate and an iso- or hypertonic sucrose solution. Inhibition of oxidative phosphorylation with CN^-, or DNP, or the elimination of substrate will cause an immediate release of Ca, independent of both vitamin D and PTH (Engstrom and DeLuca, 1964a). Other hormones, e.g., lysine vasopressin, lipid mobilizing hormone, adrenocorticotropin, glucagon, insulin, and growth hormone, as well as PTH modified by acetylation, have been examined for a comparable effect, but the results indicate a high degree of specificity for PTH in this system (DeLuca, Engstrom, and Rasmussen, 1962).

The striking effects of PTH on Ca release suggested that the hormone may have other, more profound influence on mitochondrial physiology and function. There was also the long-standing suggestion that PTH uncouples oxidative phosphorylation. This question was therefore re-examined using Rasmussen's highly purified PTH (Rasmussen and Craig, 1962a). A possible effect of vitamin D had already been examined and had been excluded (DeLuca *et al.*, 1957). PTH added *in vitro* depressed oxidative phosphorylation (Table 1), but unexpectedly in some instances it stimulated respiration even in the presence of excess phosphate acceptor.

This increased respiration in the presence of excess phosphate acceptor (ADP) suggested that possibly the hormone might be stimulating some system to bring about an increase in non-phosphorylative oxidation. To examine this possibility, oligomycin was added to block respiration coupled to ATP synthesis. Under these conditions (Fang *et al.*, 1963), the hormone greatly stimulated respiration, as revealed in Fig. 5. This respiration was not accompanied by the synthesis of ATP, and represented some non-phosphorylative oxidation. Inhibitor studies were quick to reveal that this respiration nevertheless traversed the expected sequence in the electron transport chain and at least a part of the phosphorylative machinery. CN^-, antimycin A, and rotenone (only with NAD-linked substrates) which inhibit at various points along the electron transport sequence, and DNP, dicumarol, and gramicidin S which inhibit or uncouple early in the phosphorylative chain all block the PTH-stimulated respiration. Oligomycin did not block this respiration, at least when ATP was present. Dicumarol at 10^{-7} M, which blocks the DT-diaphorase system, also failed to inhibit the PTH-stimulated respiration, thereby ruling out this pathway.

TABLE 1

Effect of PTH on Oxidative Phosphorylation by Liver Mitochondria

Substrate	PTH	O_2 μmoles	ΔP μmoles	Δ P/O
Glutamate	−	6.8	19.7	2.9
	+	10.0	18.0	1.8
Succinate	−	12.0	22.8	1.9
	+	11.4	13.7	1.2

[illegible] 40 μmoles succinate buffer pH [illegible] μmoles Mg Cl, 6.0 [illegible] 100 μg PTH. Average time, [illegible] from the [illegible].

TABLE 2

Requirements for PTH Stimulation of Mitochondrial Respiration

Alteration to Medium	PTH-dependent μl O_2/40 min
None	78
No substrate	0
No ATP	0
No $MgCl_2$	0
No P_i	0
No vitamin D	80
No sucrose (KCl)	75
Plus Ca^{++} (0.2 mmole)	78

Tabulated in part from data of Fang *et al.*, 1963.

Of greatest importance was the discovery that PTH-stimulated respiration required not only substrate but P_i and Mg^{++} (Table 2). This immediately suggested that the hormone might be stimulating magnesium phosphate uptake by mitochondria, consuming the energy represented by the increase in non-phosphorylative respiration. The ATP requirement shown here is found only when glutamate and α-ketoglutarate are used as substrates, and is related to the supply of phosphate acceptor to the substrate level-phosphorylation system which is PTH-insensitive (Sallis, Martin, and DeLuca, unpublished). Note also the absence of a vitamin D requirement in contrast to the Ca release system.

Experiments by Sallis subsequently confirmed the belief that the hormone stimulates the uptake of P_i (Sallis, DeLuca, and Rasmussen, 1963a, b). The reaction is quite rapid, going to completion within 20 minutes, and resulting in as much as 1 μmole of P_i/mg of mitochondrial protein. Virtually any tricarboxylic acid cycle substrate, e.g., pyruvate-malate, citrate, α-ketoglutarate, succinate, or other substrates oxidized by mitochondria will support this transport. Mg^{++} is also transported along with P_i and quantitative measurements indicate a Mg^{++}/P_i ratio of 1.5:1.0, suggesting that it is deposited as $Mg_3(PO_4)_2$. Table 3 summarizes the requirements of this system.

The hormonal-dependent transport requires an oxidizable substrate, Mg^{++}

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TABLE 3

Requirements for PTH-stimulated P_i Uptake by Mitochondria

Addition to Medium	PTH-dependent μmoles P_i Accumulated
None	2.4
ATP	1.9
Oligomycin	0.6
Oligomycin + ATP	1.9
No $MgCl_2$	0.0
No glutamate	0.0
KCl replacing sucrose	0.0

Derived from data of Sallis, DeLuca, and Rasmussen (1963b).

and P_i. KCl did not satisfactorily substitute for sucrose in this system, presumably because membrane damage prevented the accumulation of the magnesium phosphate salt. It will also be observed that with glutamate as substrate, oligomycin greatly inhibits the hormone-dependent transport of phosphate. A more complete inhibition may be obtained if α-ketoglutarate is used but the influence of the hormone can subsequently be restored by the addition of ATP, ADP, or AMP.

The requirement of these nucleotides is due to the lack of sensitivity of the substrate level-phosphorylation to PTH. In the absence of suitable phosphate acceptor or substrate for ATPase, i.e., ATP, no phosphate acceptor is available for that phosphorylation. Moreover, the substrate level-phosphorylation is not consumed by PTH action. Respiration or oxidation of α-ketoglutarate and consequently glutamate is limited by a lack of acceptor for this phosphorylation system. This can be corrected by the addition of ATP, ADP, or AMP.

Additional experiments show that this translocation, like the Ca system, is closely related to oxidative phosphorylation. The effects of inhibitors in this system are summarized in Table 4. The response is blocked by CN^-, antimycin A, and rotenone when NAD substrates are used. Of the inhibitors of the phosphorylative chain, DNP, dicumarol, Nigericin, and Dianemycin inhibit while oligomycin, triethyltin (not shown), and aurovertin (not shown) do not. If the transport reaction is carried out in the absence of O_2, as one [illegible] PTH stimulated transport can be observed. The action of [illegible] the transport of phosphate is highly specific for this pep[illegible] with the previous systems the addition of many other hormones at [illegible] concentrations has no influence upon phosphate transport [illegible] Rasmussen, 1963). As little as 10 μg will give a measur[illegible] phosphate assay such as [illegible] is used. This system [illegible] to assay hormone fractions during its purification.

The PTH-dependent translocation reactions are not completely specific for P_i (Rasmussen *et al.*, 1964). Figure 6 indicates that both $HAsO_4^=$ and $SO_4^=$ can substitute for P_i. The hormone stimulates the transport of these ions although not as effectively as it does P_i. These three ions are mutually competitive in that the presence of one inhibits the transport of another. As shown in Fig. 7, the transport of $HAsO_4^=$ and $SO_4^=$ also is accompanied by increased respiration just as is the transport of $HPO_4^=$. Qualitatively, then, these anions appear to be transported in much the same manner as P_i.

These experiments raise the question of whether the hormone influences sulfate and arsenate metabolism *in vivo*. Evidence for sulfate is already available (Bernstein and Handler, 1958; Bronner, 1960; Bradford *et al.*, 1959) but additional experiments testing this possibility would nevertheless be extremely interesting.

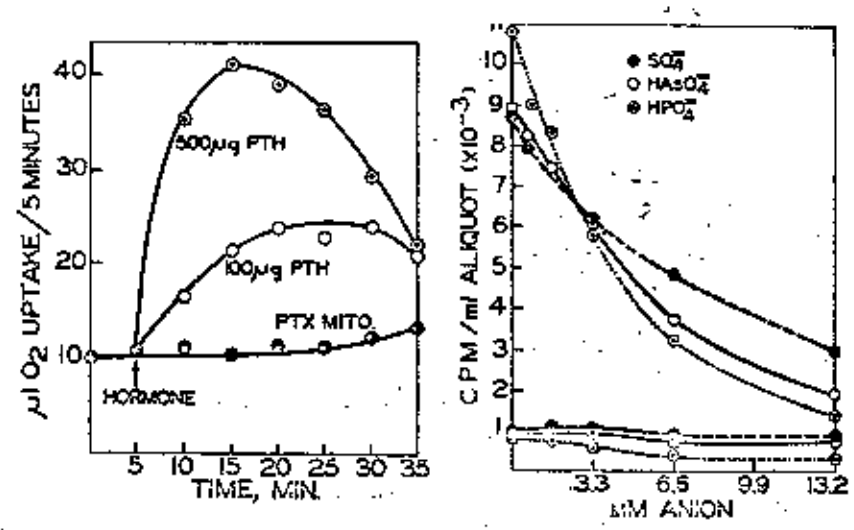

FIG. 5. *PTH stimulation of glutamate oxidation by oligomycin-inhibited mitochondria from PTX rats. Mitochondria (MITO) from 200 mg of liver were incubated at 30° C with [illegible] μmoles phosphate buffer, pH 7.3, 2 μmoles ATP, 30 μmoles glutamate, 20 μmoles $MgCl_2$, [illegible] μmoles sucrose and 100 μg oligomycin in a total vol. of 3 ml. (Redrawn from Fig. 10, Rasmussen and DeLuca, Ergebn. Physiol., 53:151. Reproduced by courtesy of the publisher.)*

FIG. 6. *Effect of anion concentration upon P^{32}-P_i uptake at pH 7.2. The lower 3 curves represent uptake of P^{32} in the absence of PTH and the upper 3 curves in its presence, 150 μg/flask. The mitochondria were incubated for 20 min at a constant amount of [illegible] concentrations of specific anion. When either arsenate or sulfate was [illegible] P_i was present. (From Fig. 3, Rasmussen et al., Endocrinology, [illegible]. Reproduced by [illegible] of the publisher.)*

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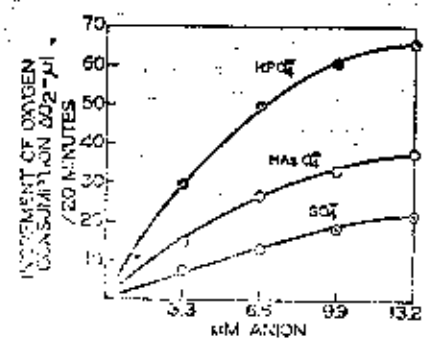
It must be emphasized that our experiments do not distinguish between Mg^{++} and P_i or other anion translocation. Although the anion has been the measured ion, Mg^{++} has always been transported with the anion. In no instance have we been able to substitute another cation for Mg^{++}. It is already quite clear that other anions can substitute for P_i.

TABLE 4

Effect of Metabolic Inhibitors on PTH-dependent P_i Uptake

Addition to Medium	Inhibitor Concentration (moles)	PTH-dependent μmoles P_i Accumulated
Dianemycin	1 μg/3.0 ml	0.1
Nigericin	1 μg/3.0 ml	0.1
Rotenone	10^{-6}	0
Fluoride	10^{-3}	2.1
Phloridzin	10^{-4}	1.6
PCMB	10^{-5}	2.4
Warfarin	7×10^{-5}	1.7
Gramicidin	7×10^{-5}	0
DNP	2×10^{-6}	0
Dicumarol	7×10^{-6}	0
Antimycin	10^{-7}	0
CN^-	10^{-3}	0
Azide	10^{-3}	0.5
N_2	.....	0
Control	.....	2.4

Derived in part from data of Sallis, DeLuca, and Rasmussen (1963b).



[illegible] PTH, [illegible] 20-min period as a function [illegible] H_2P. [illegible] et al., *Endocrinology*, [illegible]. Reproduced [illegible]

Unlike the calcium phosphate system, it has not been possible to induce PTH-dependent magnesium phosphate transport using ATP as the energy source. However, as revealed in Table 5, we have been able to demonstrate a mitochondrial ATPase which is hormone-dependent (Sallis and DeLuca, 1964). This ATPase has a pH optimum in the neighborhood of 6.7 and an optimum Mg^{++} concentration of 1 μmole/ml. Perhaps the most peculiar observation

TABLE 5

PTH-dependent ATPase

Addition	μmoles Pi Liberated/15 min
None	0.80
PTH	3.40

Mitochondria equivalent to 0.28 mg N were incubated in a medium containing [illegible] μmole Tris, [illegible] μmole cacodylate, 3.1 μmole acetate buffer pH 6.7, 3 μmole ATP, 1 μmole $MgCl_2$, 50 μmole sucrose and 75 μg of PTH. Incubation was at 30° C for 15 min. (From Sallis and DeLuca, 1964).

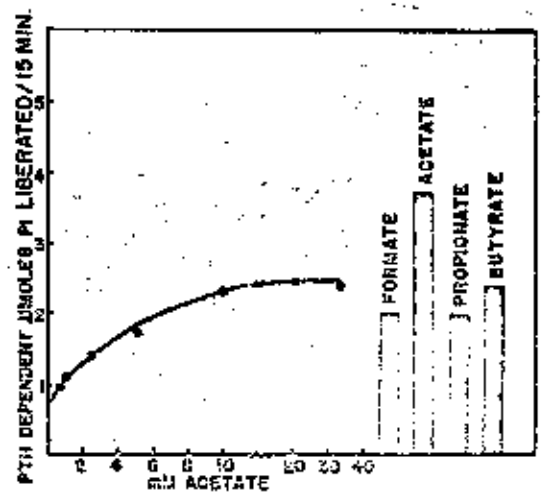


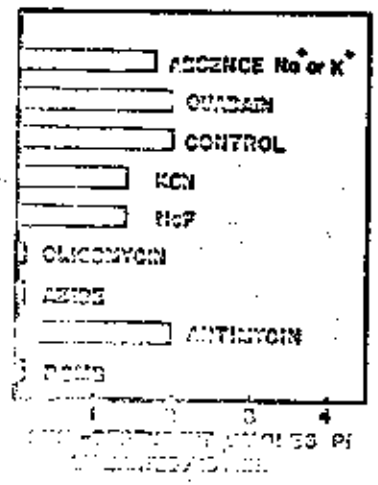
FIG. 8. *Acetate dependence of PTH-stimulated ATPase. The curve indicates effect of acetate concentration on PTH-stimulated ATPase. The vertical bars compare the effect of [illegible] anions with that of acetate at 33 mmole. Reactions carried out as described in Table 5. (Taken in part from data of Sallis and DeLuca, 1964.)*

was that this ATPase required the presence of acetate or some short-chain fatty acid for maximal stimulation (Fig. 8). At present, the acetate requirement remains an unsolved problem and is currently under study. The large amounts required suggest that it is carrying out some non-catalytic role.

Valuable information has been gained from inhibitor studies of the ATPase activity. The PTH-dependent ATPase is insensitive to ouabain (Fig. 9) and proceeds very well in the absence of Na^+ or K^+ which rules out an ATPase generally associated with ion transport. That it is blocked by oligomycin is strong evidence that this ATPase is related to the reversal of the oxidative phosphorylation chain. Interestingly enough, this ATPase is not blocked by Nigericin and Dianemycin which are believed to interact with the phosphorylative chain very near the electron transport chain.

The amount of PTH required for a measurable response in the ATPase system has been shown to be in the neighborhood of 2 μg/ml (Sallis and DeLuca, 1964). This is a lower concentration than that observed necessary for PTH-dependent phosphate transport and for PTH-dependent increase in substrate oxidation, where responses have been observed with 3–7 μg/ml.

The ATPase response is highly specific for PTH in that a number of other hormones at higher or equal concentrations were not effective in this system. Moreover, the oxidation of PTH with peracetic acid eliminates the hormone's effectiveness in this system as it does in the whole animal. The ATPase data



[illegible] PTH-stimulated ATP [illegible] liver mitochondria. Inhibitor concentra- [illegible] 1 μg/ml azide, 10^{-5} M; [illegible] (Derived from [illegible])

TABLE 6

Influence of PTH on the ATP-P_i^{32} Exchange Reaction of Mitochondria

Addition	Per Cent Label as ATP
None	23
PTH 25 μg/ml	7

Medium contained [illegible] mmole P_i^{32}, 1 mmole $MgCl_2$, 36 mmole Tris-cacodylate acetate buffer, pH 6.7, 50 mmole sucrose, and 1.1 mg mitochondrial protein. (From Sallis and DeLuca, 1964).

added to the transport experiments offer very strong evidence that PTH interacts in some fashion with the oxidative phosphorylation chain of mitochondria. By using inhibitor data, it is now possible to map its site of interaction. This type of analysis suggests that the hormone interacts between the DNP-, Nigericin-, and Dianemycin-sensitive sites and the aurovertin-, oligomycin-, and triethyltin-sensitive sites. The first three agents act prior to the entrance of P_i into the phosphorylative chain; the last three presumably act upon the last two steps in ATP synthesis, i.e., the formation of $Y \sim P$ or the conversion of the phosphorylated intermediate to ATP. The hormone could be acting either at the non-phosphorylated high-energy intermediate or at the phosphorylated intermediate stage. On the basis of the postulated site of oligomycin and aurovertin inhibition, the former appears more likely. Presumably one of these intermediates supplies energy for the various PTH-stimulated translocation reactions.

Further evidence that PTH interacts with the phosphorylative chain is shown in Table 6. If inorganic phosphate is labeled with P^{32} and the exchange reaction between P_i and ATP in mitochondria is measured, then some estimate of this portion of the phosphorylative chain can be obtained. Using this approach it was demonstrated that the presence of PTH markedly depressed this exchange reaction. Even when complete analyses of the reaction mixture for P_i, AMP, ADP, and ATP were carried out and the results expressed on a specific activity basis, PTH markedly depressed the exchange reaction.

The magnesium phosphate transport system responds to PTH equally well with mitochondria from rats severely deficient in vitamin D or fed adequate amounts of the vitamin (Sallis, DeLuca, and Rasmussen, 1963b). This contrasts sharply with the Ca-release system which is absolutely dependent upon vitamin D. Figure 10 depicts schematically the two basic transport systems involving PTH: (1) the Ca-release system which is absolutely vitamin D dependent; and (2) the phosphate-uptake system which is completely [illegible]

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dependent of vitamin D. These results provide a new outlook on the PTH-vitamin D interrelation (Rasmussen, DeLuca, Engstrom, and Sallis, 1963).

When rats are fed an adequate Ca and P diet severely deficient in vitamin D, they develop low serum Ca concentrations; if they are maintained on these diets for 6 weeks or longer, a large number will develop tetany and die. Parathyroidectomy of such animals at the third week, as shown in Fig. 11, did not lower the plasma Ca concentration further but resulted in a rapid rise in plasma P, to approximately 20 mg/100 ml followed by tetany and death. Administering 150 U of purified PTH to these animals did not change plasma Ca levels but prevented the rise in serum P_i. In animals receiving vitamin D and a low Ca diet, PTX produced a drop in plasma Ca and a somewhat delayed rise in plasma P, to 20 mg/100 ml followed by tetany and death. PTH injection prevented both these plasma changes. The lack of plasma Ca response to PTH in vitamin D deficiency had been reported by Harrison, Harrison, and Park (1958). Conflicting reports were published by Nichols, Schartum, and Vaes (1963), and Toverud (1964). The reason for the divergent reports on the vitamin D-PTH interrelation is, largely, the lack of proof of extreme vitamin D deficiency. This can best be illustrated in Table 7 which shows that a total of 0.3 U of vitamin D per rat is sufficient to produce a plasma Ca response to PTH. This amount of vitamin D would not even register on the line test method of vitamin D assay, which has been considered the most sensitive method of detection known. The necessity of extreme vitamin D deficiency for these studies cannot be overemphasized. This can be induced only if colonies of rats are maintained on extremely low vitamin D and fed purified diets such as those described by Steenbock and Herting (1955), and DeLuca *et al.* (1961).

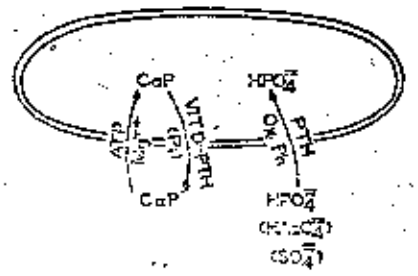

FIG. 10. *The effect of vitamin D and PTH on Ca and P_i movements in mitochondria. Both [illegible] the release of [illegible] phosphate, and the presence of the vitamin is required for PTH [illegible] D stimulates $HPO_4^{=}$ uptake. This is coupled to a stimulation of res- [illegible] PTH stimulates $SO_4^{=}$ and $HAcO_4^{-}$ uptake. (From Fig. 1, Rasmussen et al., [illegible] by courtesy of the publisher.)*

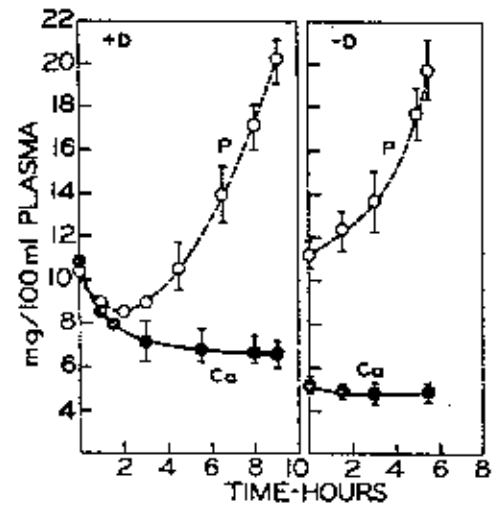

FIG. 11. *The sequential changes in plasma Ca and P, after PTX of vitamin D-fed rats maintained on a low Ca diet (left) and of D-deficient rats maintained on a normal Ca diet (right). The fall in plasma P, observed 2 hr after PTX (left) was significant ($p < 0.01$) when compared with the initial value. (From Fig. 2, Rasmussen et al., J. clin. Invest., 42:1942. Reproduced by courtesy of the publisher.)*

TABLE 7

Minimal Vitamin D Requirements for Parathyroid Responsiveness

Vitamin D	Plasma Calcium		Plasma Phosphate	
	Control	PTX	Control	PTX
−D	4.9	4.8	10.9	12.9*
+0.05 U×6	7.4†	4.6‡	9.3	10.8*
+0.50 U×6	10.5†	8.7‡	9.5	11.2*
+100 U	10.6†	8.6‡	9.9	11.6*

Ca and P values expressed as mg/100 ml. PTX—parathyroidectomy, control—sham operated (from Rasmussen *et al.*, 1963).

* A significant ($p < 0.01$) rise in plasma P after PTX in all groups. Each group contained 8 animals.

† Significant ($p < 0.01$) rise in plasma Ca as compared with −D animals.

‡ Significant ($p < 0.01$) fall in plasma Ca as a result of PTX.

The 150 U of vitamin D was administered by mouth 18 hr before blood was withdrawn.

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SUMMARY

The mitochondrial systems provide, for the first time, a subcellular membrane system which responds specifically to PTH. In that capacity they may well supply key information about the action of the hormone at the molecular level, especially in view of our knowledge of mitochondrial structure and function. Moreover, the present experiments reveal the startling possibility that PTH, and perhaps other hormones as well, may control intracellular ion concentrations and intracellular ion pumps. This may in turn result in diverse metabolic changes already reported for many hormones.

That the present *in vitro* experiments are physiologically significant is more than substantiated by the high degree of specificity displayed for the hormone molecule in the four systems studied to date. The remarkable parallel between the mitochondrial observations and the hormonal effects on ion transport in the renal tubule provide additional support. For example, the hormone increases Ca reabsorption while increasing P_i excretion in the renal tubules. In a parallel fashion it increases Ca efflux from the mitochondria but increases P_i influx. Perhaps the most powerful argument is the recent advance in the elucidation of the vitamin D-PTH interrelation as a result of the *in vitro* experiments.

Despite these arguments, some unanswered problems necessitate cautious interpretation of the mitochondrial results in the physiological sense. Rather high doses of PTH are used for the *in vitro* experiment; changes in mitochondrial accumulation of ions cannot in themselves account for the hormone's physiological effects; the liver has never been shown to be a target organ for PTH. These problems, although troublesome, are not serious but they certainly demand continued investigation and consideration.

It is important at this stage to regard the mitochondrial systems as a tool of great value in determining the molecular mechanism of PTH action. In addition, we would like to speculate that the systems of mitochondria which are responsive to the hormone are present in other subcellular membranes and, upon their identification, an understanding of the hormone's physiological actions at the molecular level may well be within our grasp.

Arch. Dis. Child. 36: 373-380 (1961)

HYPERVITAMINOSIS D WITH OSTEOSCLEROSIS

BY

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Administration of vitamin D in high dosage produces extraosseous calcification, renal failure and usually hypercalcaemia, with a characteristic set of symptoms (Jeans, 1950). Its effects on bone have been alluded to in a number of publications (Jeans, 1950; Ross, 1952; Debré, 1948; Anning, Dawson, Dolby and Ingram, 1948; Ross and Williams, 1939; Tumulty and Howard, 1942; Danowski, Winkler and Peters, 1945; Howard and Meyer, 1948; Freeman, Rhoads and Yeager, 1946; Thatcher, 1931). In adults the characteristic roentgenologic bone finding is rarefaction. In children rarefaction is also reported, but with the additional finding of increased density of the zone of provisional calcification, and periosteal thickening (Ross, 1952).

The following case report demonstrates that excess ingestion of vitamin D may be accompanied by a severe degree of osteosclerosis, which is reversible, in addition to its better known effects.

Case History

A 5½-year-old white boy was admitted to the Orthopaedic Hospital Clinic complaining of pain in the right leg and flat feet. At the age of 3 he was considered elsewhere to have rickets on the basis of a 'peculiar feeling to the bones of the skull' and radiographs of the wrists. He was given two capsules of vitamin D daily, each containing 50,000 units, in addition to two tablespoons or more of cod liver oil and multivitamin drops. A few months after this he developed nausea and nonfender recurrent lumps the size of half a lemon, over both tibias. His physician prescribed vitamin C and the lumps disappeared. In addition to the vitamin therapy at home, he was given a teaspoonful of cod liver oil daily at nursery school. After nine months of this therapy he become irritable, restless and nauseated, and the vitamins were stopped by the family. At the age of 4½, a diagnosis of rheumatic fever and anaemia was made and treatment was started with ferrous sulphate and sodium salicylate. His appetite was good, but he continued to be irritable and restless, sleeping only five hours at night. During the ensuing year his refusal to participate in school activities was ascribed by his teacher to stiffness in the extremities.

His birth weight was 8 lb. 8 oz. He was the sort of a young woman who was married and separated before the child was born. Labour and delivery were normal. He was breast fed for eight months. He sat at 5 months, stood at 10 months, and walked at 11 months. His first tooth erupted at 4 months.

He was brought up chiefly by the mother's parents, while the mother worked. At an early age he had a tonsillectomy, adenoidectomy and circumcision. He also had measles.

Physical examination revealed an alert, hyperactive, well-developed and nourished white boy (Fig. 1). There was increased A-P diameter of the head with a suggestion of frontal and parietal bossing. The eyes, ears, nose and throat were normal. Dentition was normal, and the teeth appeared healthy. The thyroid gland was not

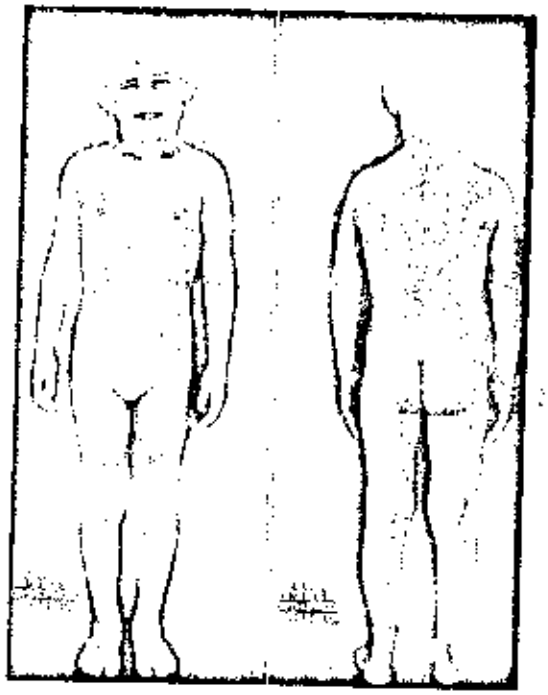

FIG. 1. The patient at 5½ years of age

FIG. 2.—Radiograph at initial examination. Note alternating bands of decreased and increased density at ends of radius and ulna.

FIG. 3.—Skull showing generalized increase in density.

palpable. The thorax showed beading of costochondral junctions. The heart, lungs and abdomen showed no abnormalities. The genitalia were normal for a boy of 5. The testes were in the scrotum.

The epiphyseal regions of the long bones were stocky and broad. There was no tenderness or pain. There was eversion of the os calcis and loss of the longitudinal arch bilaterally. There was some spasm of the left rectus femoris muscle and 20-degree flexion contractures of the knees. The skin was clear. There were no palpable calcific deposits anywhere. There was a healed ecchymosis on the lateral aspect of the right thigh.

A complete blood count showed 3·70 million erythrocytes, 12·8 g. haemoglobin, 10,400 leucocytes, with 40% polymorphs, 52% lymphocytes, 4% eosinophils, and 4% transitional forms. Urine analysis showed pale yellow urine of specific gravity 1,005, acid reaction, no sugar, acetone, albumin or formed elements. Urine calcium was 0·19 g. in a 24-hour volume of 1,750 ml. Serum calcium was 17 mg. per 100 ml., phosphorus 5·2 mg. per 100 ml., alkaline phosphatase 19 King-Armstrong units, acid phosphatase 5·3 King-Armstrong units, and protein-bound iodine 6·8 µg. %. Radiographs showed generalized increase in density of the bones. Alternating bands of decreased and increased density were present in the metaphyses, best seen at the wrist (Fig. 2). The cortices of the metatarsals, metacarpals, and phalanges were thickened. Metaphyseal areas were a little wider than usual and remodelling had not proceeded normally. There was some encroachment on the medullary canals at the ends of the long bones and in the shaft, caused possibly by thickened cortex. The base of the skull and cranial vault were increased in density (Fig. 3). The petroclinoid ligaments were heavily calcified. Periarticular calcifications were present about the feet, hips, hands, ankles and shoulders. Kidney shadows were dense but not markedly so. The lumbar spine showed dense and square appearing vertebral bodies (Fig. 4). Intervertebral discs were not calcified. Soft tissue calcification or periosteal new bone could be made out along the sternum.

A chest film made two years previously showed no abnormalities in the lung fields, and normal bone density. Films of the wrists and skull made one and a half years previously showed normal bone density (Figs. 5-6). Zones of provisional calcification were maintained. The epiphyseal lines were not widened. Bony maturation corresponded satisfactorily with the given chronological age of 4 years. The skull showed no abnormalities. There was no roentgen evidence of rickets.

The family was instructed to stop administering vitamin D and presumably did so. They did not return for follow-up observation and care except sporadically, as he was under private care for general paediatric supervision.

Radiographs were obtained at seven, eight and 10 months after initial examination. An antero-posterior view of the pelvis after seven months showed a thin zone of decreased density about the pelvic bones, probably representing new growth (Fig. 7). Femoral

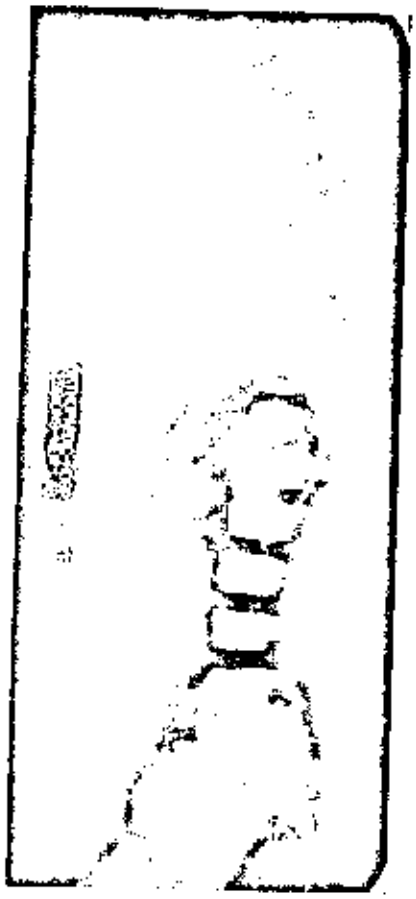

FIG. 4.—Note the dense square-appearing vertebral bodies.

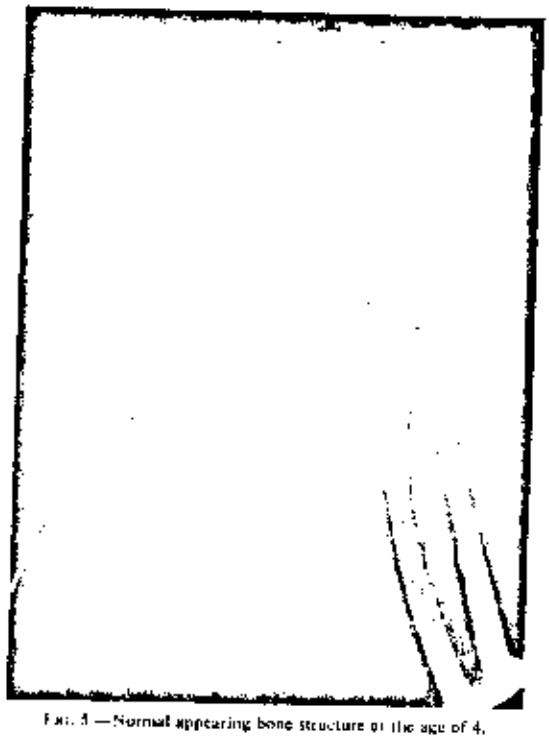

FIG. 5.—Normal appearing bone structure at the age of 4.

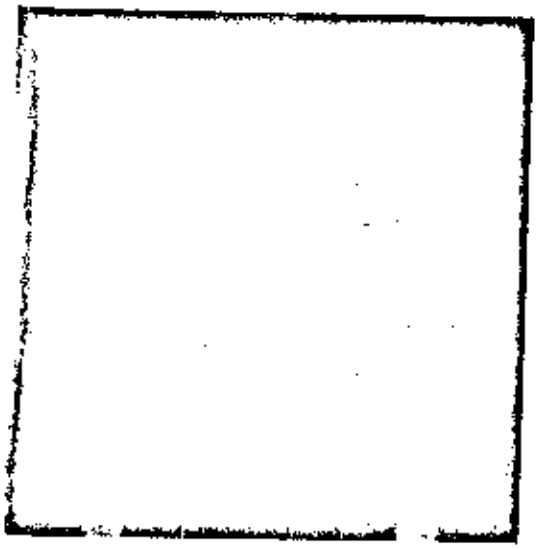

FIG. 7.—Pelvis seven months after discontinuing vitamin D.

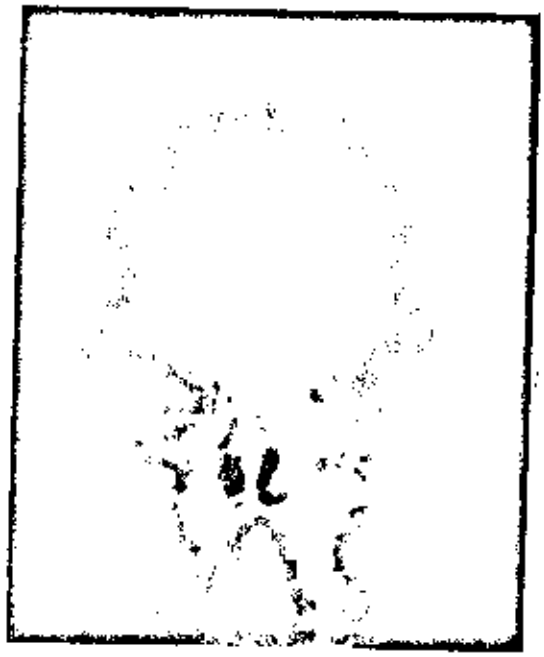

FIG. 6.—Normal appearing skull at the age of 4.

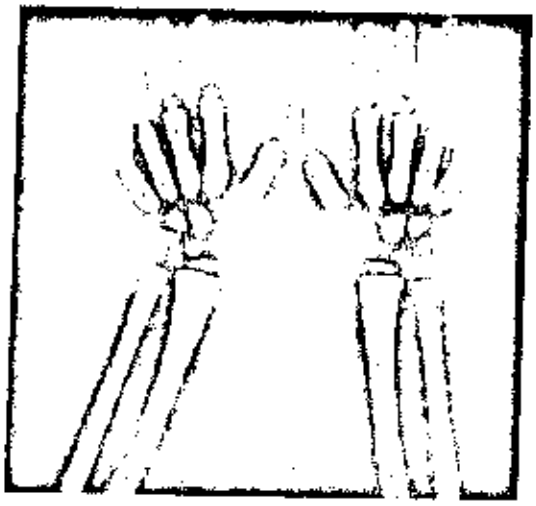

FIG. 8. Note new bone growth at ends of radius and ulna.

arteries were seen to be delicately outlined by calcium. A month later, films of the wrists showed widened zones of relatively decreased density about the ends of the long bones, presumably representing bone laid down

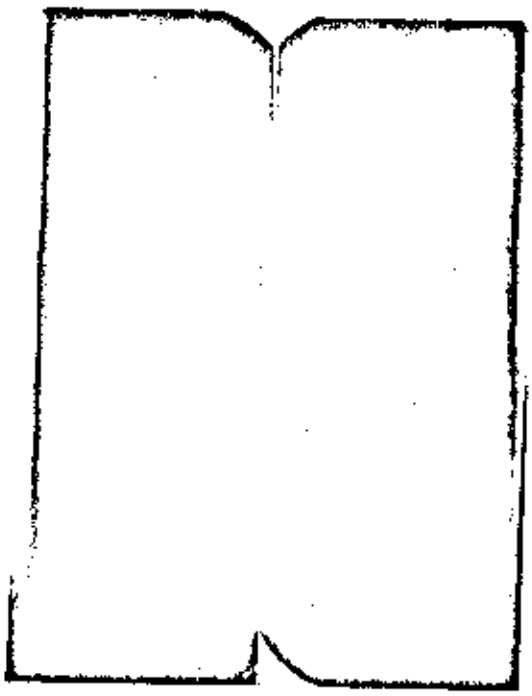

FIG. 9a. Lower extremities at 5 years.

since the patient was taken off massive doses of vitamin (Fig. 8). Periarticular calcification persisted. Bony maturation was about 4 years, definitely retarded for the chronologic age of 6 years and 2 months. Two months later zones of lessened density were seen about the metaphyses adjacent to the knees and ankles (Fig. 9a).

Fifteen months after our first examination, he was taken to his private paediatrician, who kindly provided us with data concerning his course during the next several months. He was kept in hospital at this time because of fever, swelling of face and abdomen, and haematuria, after an upper respiratory infection. He had slight fever, tachycardia, a blood pressure of 180/120 mm. Hg, and moderate oedema of face and abdomen. Treatment was instituted with strict bed rest, penicillin and moderate salt restriction. Parenteral reserpine and "apresoline" (hydrallazine) were given for one week, with a drop in blood pressure to 130/85 mm. Hg. An intercurrent viral gastro-enteritis was followed by loss of facial oedema and ascites. Blood pressure remained between 130/90 and 110/80 mm. Hg without further hypotensive drugs. His weight had dropped to 40 lb from 48·5 lb. on admission. His urine which had many red cells on admission, cleared to 20-25 per h.p.f. and he was discharged home.

Radiographs during this period revealed bone density to be less than previously. Calcifications were present in the falx cerebri and tentorium. Periarticular calcifications were still evident about the hips. The transverse diameter of the heart was at the upper limit of normal. Renal shadows were moderately dense. The tip of the 12th rib was apparently fractured. Bony maturation was about 4 years, compared with the chronological age of 6 years and 10 months.

Laboratory studies during this period and for the next several months are tabulated (Table I). The blood pressure remained near 120/90 mm. Hg on hypotensive therapy. The urine continued to show albumin, red blood cells and casts. The blood sedimentation rate and non-protein nitrogen remained elevated.

The family did not follow the rigid routine of bed rest prescribed, and sent him to school without medical permission.

When he was seen by the author one year after being in hospital under Dr. Wollmann, he was an alert, 7-year-old boy with a uraemic odour and marked pallor. Blood pressure was 190/130 mm. Hg. The fundus showed moderate crossing changes but no haemorrhages or papilloedema. Lungs were clear. The heart was enlarged to percussion and had loud tones of poor quality. There was a late diastolic sound suggestive of a presystolic murmur. The abdomen showed no abnormalities. There was no ascites or oedema. Skeletal survey by radiograph at this time showed continued loss of bone density (Fig. 9b). Bone of more normal density had been laid down since vitamin dosage had been discontinued. The overall decrease in density had been spectacular. Soft tissue calcifications were still demonstrated in the tissues about the hips. Chest film showed the heart to be moderately enlarged. The renal shadows had decreased in size and were increased in

TABLE 1

LABORATORY FINDINGS DURING 1957

Date (1957)	Urinalysis	Haemoglobin (g./100 ml.)	Non-protein Nitrogen (mg./100 ml.)	Sedimentation Rate (mm./hr.) (Wintrobe)
February 28	4 + albumin; 10-13 R.B.C.; 3-4 W.B.C.; granular casts	12	76	40
March 28	4 + albumin; 12-15 R.B.C.; 10-14 W.B.C.; hyaline casts	13·4	76	44
May 16	3 + albumin; 8-10 R.B.C.; 10-12 W.B.C.; granular casts	13·4	72	[illegible]
September 12	4 + albumin; 6-8 R.B.C.; 10-12 W.B.C.; granular casts	10·7	103	92

density, although no definite calcifications were identified.

An electrocardiogram was within normal limits. Blood studies and urine analysis are tabulated (Table 2). A grave prognosis was given and a low protein diet omitting milk was advised. In spite of this, however, the family insisted he attend school if at all possible. The next few weeks saw development of widespread ecchymosis, oozing of blood from the nose, and increased depth of breathing with gallop rhythm. The family called in a physician living near them, who supervised his home care. He died soon after being readmitted to hospital.

Autopsy. Autopsy revealed small amounts of fluid in the peritoneal and pericardial cavities. The heart weighed 268 g. The left ventricle measured 14 mm. in thickness. There were some atherosclerotic changes in the first part of the aorta. The coronary ostia were patent. Both lungs were dark red and firm posteriorly, appearing juicy, bloody, and mucopurulent on cut section. The gastrointestinal tract showed haemorrhagic spots in the stomach and first portion of the duodenum. There was an intestinal intussusception about 6·5 cm. in length, approximately 90 cm. above the ileocaecal valve. The liver was abnormally heavy at 850 g. Cut surface showed fatty change. The spleen, pancreas and adrenals were normal.

Both kidneys were embedded in a small amount of fat. The right kidney weighed 47 g. (normal 74 g.) and the left kidney weighed 53 g. (normal 75 g.). On cut surface the capsule stripped with difficulty. After removal of the capsule the surface of both kidneys appeared granular. The pelvis appeared normal.

Lymphoid tissue was grossly normal. The thyroid showed cystic change. The parathyroid glands appeared normal. The bone marrow was deep red-brown in

TABLE 2

TERMINAL LABORATORY STUDIES

Laboratory Studies	Findings
Urinalysis	Sp. gr. 1,012, pH 6·0, albumin 4 +; sugar negative; 15-25 W.B.C., 4-6 R.B.C., occasional epithelial cell, occasional broad coarse casts, occasional coarse granular cast, occasional broad W.B.C. cast
Complete blood count	Hb 5·9 g./100 ml., W.B.C. 10,000; polymorphs 65, lymphocytes 22, basophils 1, monocytes 9, eosinophils 3, haematocrit 18 %
Serum creatinine	6·0 mg./100 ml.
Blood urea nitrogen	108 mg./100 ml.
Serum K	4·7 mEq./litre
Serum alkaline phosphatase	8·0 Bodansky units
Serum inorganic phosphorus	9·2 mg./100 ml. (5·3 mEq./litre)
Serum calcium	8·4 mg./100 ml. (4·1 mEq./litre)

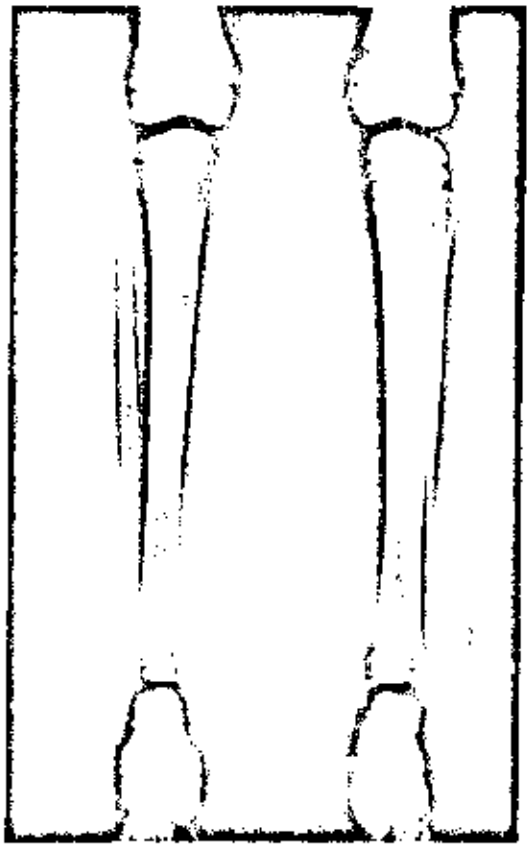

FIG. 9b.—Lower extremities at 7½ years, showing extent of sclerotic bony process.

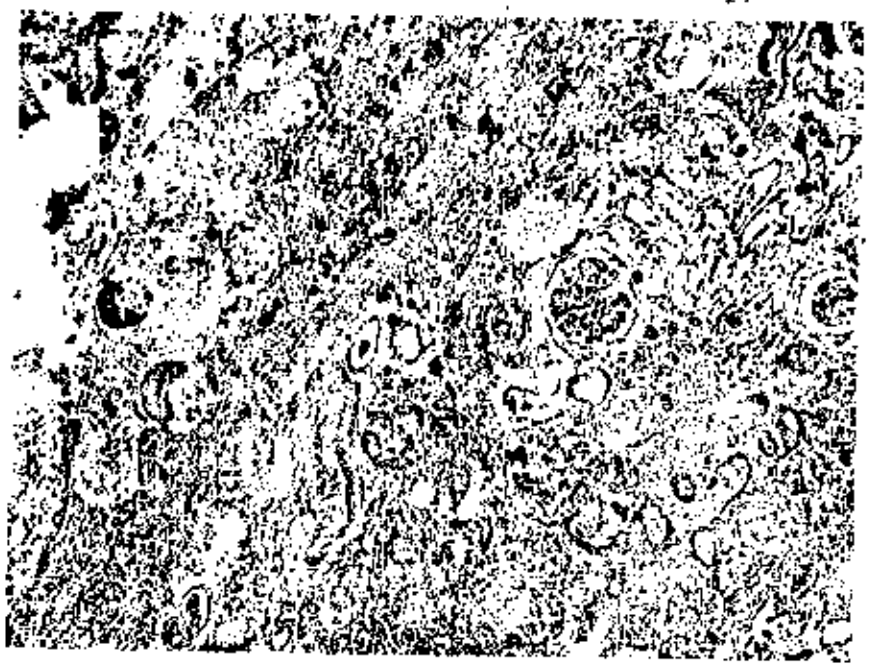

FIG. 10.—Autopsy section of kidney. (× 75.)

FIG. 11.—Autopsy section of bone marrow. (× 34.)

colour. The brain was grossly normal, but was increased in weight to 1,365 g. The pituitary gland was normal.

On microscopic section, the heart showed hypertrophy and coronary atherosclerosis. The lungs showed metastatic calcification of the alveolar wall and peribronchial tissue. There was uraemic pneumonitis. The gastro-intestinal tract showed haemorrhage in the small intestine and multiple metastatic calcification of gastric glands. There was minimal fatty change in the liver. Metastatic calcification was present in the adrenal glands, but not in the spleen or pancreas. The pathologist's description of the kidney sections follows:

'Practically all glomeruli are fibrotic and there is very extensive atrophy of tubules. What glomeruli remain show at times adhesions and at times crescent formation. There is a marked interstitial fibrosis and inflammatory reaction with striking deposition of calcium throughout the renal parenchyma. Calcium is found in both cortex and medulla. At times it lies within tubular cells, at other times within glomeruli and most frequently appears to lie interstitially. There is extensive intimal proliferation of both small arteries and arterioles.' (See Fig. 10.)

The bone marrow (Fig. 11) showed osteoporosis. Parathyroid tissue was hyperplastic.

Discussion

Increased density of bone is seldom seen and always presents an interesting problem in diagnosis. The possibility of marble-bone disease was quickly dismissed when the serum calcium level was reported. It appeared highly likely that the excessive ingestion of vitamin D was the cause of his presenting complaints and striking findings. It is not possible to exclude hypervitaminosis A. Vitamins A and D are combined in most dose forms, and information regarding exact dose form is not available in the present instance.

That the excess deposit of mineral is actually a result of excessive vitamin ingestion seems likely in view of the following considerations. First, the history and findings apart from the skeleton are consistent with the known syndrome of vitamin D intoxication. Second, the dose and duration of ingestion of vitamin D were greatly excessive. Third, reported cases of vitamin D intoxication with bone demineralization were mostly older people and patients with rheumatoid arthritis. It is plausible to believe that skeletal maturity does not favour deposition of mineral salts. Furthermore, metabolic forces against skeletal mineralization exist in rheumatoid arthritis, an inflammatory disease with catabolic, or at least anti-anabolic effect on bone and other supporting structures.

It seems reasonable that a child's growing skeleton would react to a high concentration of circulating calcium by incorporating it in the skeleton at areas of epiphyseal and membranous growth. The distribution of excess mineral deposit in long bones is consistent with this type of orderly deposition. Known actions of vitamin D relate to improved absorption of calcium and phosphorus, rather than to specific effects at the level of bone mineralization. Thus it should be possible to correlate skeletal calcification with the level of serum calcium. A study of the ionized calcium level would have been valuable.

A striking parallel to the present case is seen in the cases reported as 'idiopathic hypercalcaemia' (Lowe, Henderson, Park and McCreal, 1954; Creery and Neill, 1954; Creery, 1953; Payne, 1952; Lightwood, 1952; Fellers and Schwartz, 1958; Franconi, Girardet, Schlesinger, Butler and Black, 1952). The main clinical and laboratory features are similar, except for the onset and duration, and the presence of the 'elfin facies' and associated sequelae.

An interesting feature of the present case is the prompt reversal of bone density on discontinuance of vitamin D therapy. This began before evidence of renal failure appeared. Later progressive bone demineralization should properly be ascribed to renal failure with accompanying acidosis. A finding supporting this view is the presence at autopsy of osteoporosis in the bone sections, representing loss of more than the expected amount of bone substance. Closer observation of calcium metabolism might have changed the outcome. It is known that renal damage secondary to hypervitaminosis D is reversible. The presence of a constant steady stream of calcium from bone to blood stream was undoubtedly responsible for progressive unrelenting renal damage. It was not possible to test the effect of cortisone or other drugs on the serum calcium level.

The chief value of the present case report appears to be the demonstration of a mechanism for quantitative increase in bone mineral in humans. Many interesting studies could be planned on similar cases. It is hoped that the report of this case will stimulate others to make further careful observations in the rarely available similar situation.

Summary

A case report of a young boy who ingested large quantities of vitamin D over a period of one year demonstrates that reversible increase in bone density may occur, with severe calcinosis and fatal renal failure.

The extent and distribution of the osteosclerosis differs from that reported in vitamin D overdosage in children and resembles somewhat the picture seen in the severe cases of 'idiopathic hypercalcaemia'.

I am indebted to R. S. Cleland, M.D., of the Children's Hospital of Los Angeles for permission to reproduce microscopic slides and to paraphrase and quote from the autopsy report. Credit is also due to Richard Schreiber, M.D., of the Orthopaedic Hospital, for aid in describing x-ray findings. George R. Wollmann, M.D., of the Children's Hospital staff was kind enough to provide data from his office files.

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ADDRESSES AND ORIGINAL ARTICLES

THE HORMONES AND THEIR CHEMICAL RELATIONS *

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LECTURE II

Synthetic Compounds resembling Œstrin

The constitution of the œstrus-producing hormone and the pharmacologically active derivatives have already been discussed. As pointed out, the actual synthesis of the œstrin molecule is fraught with the very greatest difficulty, and it was decided to investigate the activity of synthetic organic compounds whose structure resembled in one direction or another that of œstrin (Cook and Dodds, 1933; Cook, Dodds, and Hewett, 1933; Cook, Dodds, Hewett, and Lawson, 1934; Cook, Dodds, and Greenwood, 1934).

Since œstrin contains a partly hydrogenated phenanthrene system containing oxygen groups, it seemed natural first to investigate simpler compounds of this type. Phenanthrene itself was found to be completely inactive, but the compound 1-keto-1 : 2 : 3 : 4-tetrahydro-phenanthrene was found to possess definite œstrogenic activity; this compound is represented by the formula shown in Fig. 7. When this is injected in doses of 100 mg. into castrated rats, it was found that within some 40 hours full œstrus had developed, including uterine changes. The œstrus is of very prolonged duration, lasting in some cases for 20 days. It would appear therefore that it is possible to reproduce all the known phenomena of œstrus in the rat and mouse with this compound. Moreover, when it is injected into immature female mice, premature puberty is induced. In the immature female rat and mouse the vagina is represented by a solid cord of cells which become canalised either at puberty or following the injection of œstrin. This keto-tetrahydro-phenanthrene compound is capable of causing canalisation of the vagina, and of inducing œstrus in these animals.

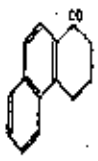


FIG. 7
1-Keto-1 : 2 : 3 : 4-tetrahydrophenanthrene.

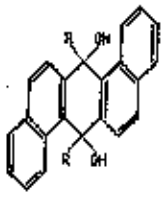


FIG. 8
Dibenzanthracene diols.

Another series of compounds have been very carefully studied and these include derivatives of 1 : 2 : 5 : 6-dibenzanthracene in which the ring-system is partly reduced and hydroxyl groups introduced.

It will be observed that the molecules of these substances are symmetrical, and that each half of the molecule is of the same type as 1-keto-1 : 2 : 3 : 4-tetrahydrophenanthrene. A series of diols having the general constitution 9 : 10-dihydroxy-9 : 10-dialkyl-9 : 10-dihydro-1 : 2 : 5 : 6-dibenzanthracene have been investigated; the graphic formula is seen in Fig. 8.

Now if the di-methyl compound be made, this, when injected, is without activity in doses of 100 mg. The di-ethyl compound, however, shows evidence of activity in doses as small as 1 mg. It is thus over 100 times as potent as the keto-tetrahydro-phenanthrene compound.

Ascending the alkyl series, we next encounter the di-n-propyl, and the activity of this compound is so great that 0·025 mg. will produce all the signs of œstrus in œstrated rats. It is thus 40 times as active as the di-ethyl, and 4000 times as active as the phenanthrene compound. This substance is considerably more active than the tri-hydroxy-œstrin, which is one of the natural œstrus-producing agents.

Ascending the series still further we find the next homologue in di-n-butyl; this shows less activity than the di-n-propyl in that 0·1 mg. represents the effective unit. That is to say, it has about quarter the activity of the di-n-propyl compound.

Increasing the length of the side-chain shows that the di-n-amyl homologue is inactive, whilst the six carbon atom side-chain in the next homologue, the di-n-hexyl, is also inactive. Table I. shows the relative activity of members of this series.

TABLE I.—*The Activity of Members of the Series*

9 : 10-dihydroxy-9 : 10-dialkyl-9 : 10-dihydro-1 : 2 : 5 : 6-dibenzanthracene.

Substance.	No. of animals.	Dose in mg.	Per cent. positive.	Remarks.
Di-methyl ..	Rats, 5	100	nil	—
Di-ethyl ..	,, 5	100	100	Œstrus lasted 13 days.
	,, 5	10	100	,, ,, 13 ,,
	,, 5	1·0	100	,, ,, 6 ,,
Di-n-propyl	,, 5	5	100	—
	,, 5	1·0	100	—
	,, 5	0·1	60	—
	,, 10	0·05	80	—
	,, 10	0·025	40	—
Di-n-butyl	,, 10	100	100	Œstrus lasted 28 days, animals then killed.
	,, 9	1·0	50	Œstrus lasted 18 hours.
	,, 11	0·1	50	,, ,, 36 ,,
	Mice 11	1·0	100	Animals in œstrus when killed on 10th day.
	Rats, 10	10	90	Suspension in water per os.
	,, 10	1·0	10	
Di-n-amyl ..	,, 5	100	nil	—
Di-n-hexyl ..	,, 3	100	nil	—
1-keto-1 : 2 : 3 : 4-tetrahydro-phenanthrene	Rats, 36	100	100	Œstrus lasted 20 days, suspension in water administered per os.
	,, 5	100	nil	

This is a very instructive series, since it shows how the chemist can manufacture what might be termed a skeleton key with which to open the œstrus lock. The real key is ketohydroxy-œstrin, which is produced by the body. The skeleton key is the dibenzanthracene compound which we have indicated. By building up artificial teeth, so to speak, on the key, it is possible to open the lock with difficulty with the di-ethyl; but by putting on another tooth in the di-propyl, the lock slides back with ease, in fact, with just as much ease as with one of the real keys provided by nature. Another tooth in the di-butyl compound makes the key a little stiff, whilst yet another in the di-amyl makes it jam. It is also possible to show that these compounds, when administered, give an increase in the size of the uterus similar to that which occurs in natural œstrus.

In order to prove that the lock has been genuinely opened and not forced, it can be shown by a further series of experiments the effect of two of these compounds on the plumage of the capon (Cook, Dodds, and Greenwood, 1934). It is a well-known fact that the brown leghorn

* The Goulstonian lectures for 1934, delivered before the Royal College of Physicians of London on March 8th, 9th, and 15th. Lecture I. appeared in our last issue and Lecture III. will be published next week.

capon retains its male plumage, and if large doses of oestrin are injected subcutaneously into such a bird, the plumage changes from male to female. We were able to show that with two of these compounds—namely, 1-keto-1 : 2 : 3 : 4-tetrahydro-phenanthrene and 9 : 10-dihydroxy-9 : 10-di-n-butyl-9 : 10-dihydro-1 : 2 : 5 : 6-dibenzanthracene—even this lock was capable of responding to our synthetic key. Fig. 9 shows the effect on the plumage of injecting these compounds. The birds were brown leghorn capons whose plumage showed male characteristics. On injecting the compounds mentioned it can be seen that in the growing feathers there is a rapid change from the male to the female type. This is particularly well shown in feathers from the breast region. The markings in the illustration indicate the male and female areas in the feathers, but the striking character of these changes cannot of course be fully appreciated without coloured reproduction.

We can state, therefore, that, in so far as is known up to the present, these compounds possess all the known activity of the natural oestrus-producing hormone.

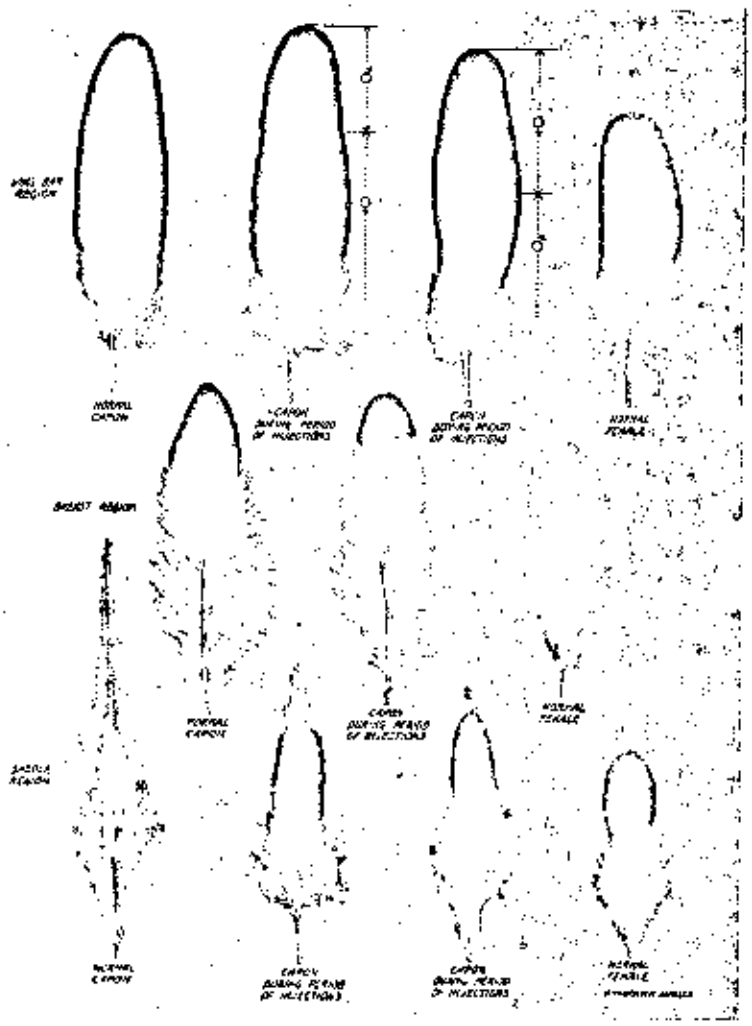


FIG. 9.—Effect of 1-Keto-1 : 2 : 3 : 4-tetrahydrophenanthrene on plumage of capons.

Specificity of Hormones

The significance of these observations is considerable, since they throw grave doubt on the question of specificity of hormones. The original conception postulated that hormone action was one of the most specific. Whilst it is known that para-hydroxy-phenols and their derivatives are capable of causing a slight rise of blood pressure, it would have been deemed incredible that so complicated a series of events as occur in the oestrus cycle could be initiated and maintained by any other than the naturally occurring hormone. Here, however, we see compounds of somewhat differing structures which are capable of reproducing the whole phenomenon, not only in mammals, but also in birds. It appears justifiable to assume that there are certain parts of the oestrin molecule which are necessary to reproduce the physiological activity, and that these compounds of ours also possess this.

The significance of this to the organic chemist is great, since it may mean that it is not necessary to synthesise the whole of the elaborate molecules that occur in nature, but that skeleton keys may possibly be found for many of the other hormones. At the present time it is impossible to state the exact groupings necessary in the substances for this activity. In all the active compounds the phenanthrene nucleus is to be seen. We now know, however, that even this is not essential, since we have found evidence of oestrogenic activity with the derivative of anthracene—namely, 1-keto-1 : 2 : 3 : 4 : 5 : 6 : 7 : 8-octa-hydro-anthracene—which is represented in Fig. 10. Research has been extended on these lines, and it is possible to state from unpublished data (Cook, Dodds, and Lawson) that definitely active anthracene derivatives may be obtained. The important factors seem to be a certain degree of unsaturation in a condensed carbon ring-system together with polar groups. These are probably necessary for a high order of activity, although definite activity of a low order was shown by some compounds which contain no oxygen. A great many compounds were tested with negative results.

These results provide a possible explanation for the very remarkable experiments carried out by Aschheim and Hohlweg (1933), who show that the extraction of a wide variety of bituminous substances is capable of yielding products with very definite oestrogenic activity.

Certain coals, mineral oils, &c. were used as starting material in the preparation of the oestrogenic substance. Aschheim and Hohlweg advanced the very attractive theory that this oestrogenic substance in coal was in effect that variety of the oestrus hormone that is known to be present in plants, and which was later called tokokinin. It has been shown that tokokinin has the same constitution

as keto-hydroxy-œstrin, and according to Aschheim and Hohlweg's ingenious theory, the œstrogenic activity of extracts from coal could be attributed to the tokokinin of primeval forests which had been preserved in the form of coal. In view, however, of our results it would appear that such an assumption is not necessary, since it is well known that extracts of coal will contain compounds of the same type of structure as those which have been proved to be capable of giving rise to œstrus. We ourselves have shown that certain of the ulmins of coal possess definite œstrogenic activity.

In order to complete a review of compounds capable of causing œstrus apart from the naturally occurring hormone, it is necessary to refer to the statement made by Coke (1930) that clupanodonic acid possesses marked œstrus-producing activity. Now this compound is a straight chain, unsaturated fatty acid obtained from certain fish oils. A very careful investigation has been made into this problem, but it has been found impossible to confirm the œstrus-producing activity of this compound. We were able, however, to demonstrate low-grade activity in certain highly unsaturated fatty acids of the general formula of clupanodonic acid.

This raises the very interesting possibility that perhaps the body possesses the power of converting the straight chain into a carbon ring compound. From the chemical point of view it has been proved that cyclisation of such a highly unsaturated compound does occur (Edisbury, Morton, and Lovern, 1933). As, however, the activity is so small, the other possibility—namely, that the fish oils contained a trace of œstrin which was not eliminated in the purification process—must be borne in mind. Up to the present, therefore, it is safe to say that all the synthetic œstrus-producing substances appear to belong to the condensed carbon ring system, and that the most powerful possess the phenanthrene nucleus.

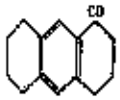


FIG. 10
1-Keto-1 : 2 : 3:4:5:6:7:8-octa-hydro-anthracene.

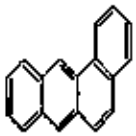
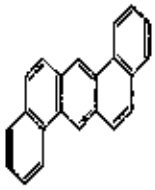
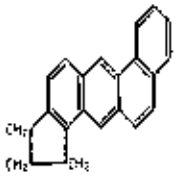


FIG. 11.—1 : 2-Benzanthracene.



1 : 2 : 5 : 6-Dibenz-anthracene

1 : 2-Benz-pyrene.



5 : 6-cyclo-Pentene-1 : 2-benzanthracene.

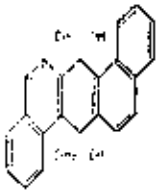


FIG. 12
9 : 10-Dihydroxy-10 : 10-di-n-propyl-9 : 10-dihydro-1 : 2 : 5 : 6-dibenzanthracene.

Carcinogenesis and Œstrogenesis

The hydrogenated phenanthrene ring type of compound, as already pointed out, has only recently been recognised as a normal constituent of the body. A list of the typical compounds of the body belonging to this group has been given earlier together with the structural formulæ. Mainly through the work of E. L. Kennaway and his co-workers at the Cancer Hospital, it has been proved that a carcinogenic agent present in tar also belongs to this particular type of chemical compound (Kennaway, 1924, 1925, 1930; Cook, Hieger, Kennaway, and Maynord, 1932; Cook, 1932). It will here be necessary to consider the question of carcinogenesis in some detail.

The observations of industrial cancer led to the assumption that tar was capable of producing malignant disease when applied to the skin. This was first proved by Yamagiwa and Ichikawa (1915, 1917, 1918) who, in 1915, were able to demonstrate experimentally that on application of tar to the skin of rabbits an epitheliomatous condition in a large proportion of cases resulted. Tsutsui (1918) repeated these experiments with mice in 1918, and this was confirmed by Passey in 1921, who used soot (Passey, 1922). It was found necessary to continue the experimental painting for very long periods, and nine months to a year was frequently necessary in order to produce a malignant tumour.

It was at first assumed that the tar acted by producing a chronic irritation which later became the subject of malignant change. Doubt, however, was cast on this view by the observation that not all tars were capable of causing malignant changes. In a very important investigation Kennaway (1924, 1925) showed that the carcinogenic agent could be produced by heating acetylene or isoprene in the presence of hydrogen. This proved conclusively that it was possible to obtain a carcinogenic agent which contained only carbon and hydrogen. Maynord in 1927 studied the fluorescent properties of these synthetic mixtures, and observed the similarity of the spectrum to that of the carcinogenic tar. This work, which was continued by Hieger, suggested that the mixtures contained hydrocarbons of the polycyclic aromatic type. An investigation was accordingly commenced on the synthesis of a series of these compounds and a study of them from the point of view of their ability to produce carcinoma when painted on to the skin of mice. In a number of papers the various (Cook, 1932; Cook, Hewett and Hieger, 1932; Cook, Hieger, Kennaway, and Maynord, 1932) showed that a series of compounds related to 1 : 2-benzanthracene possessed carcinogenic properties. The only compounds of this series that we need consider at the present time are 1 : 2 : 5 : 6-dibenzanthracene, 1 : 2-benzpyrene, and 5 : 6-cyclopentene-1 : 2-benzanthracene (Fig. 11). It can be seen at a glance that these carcinogenic compounds bear some resemblance to the group of substances that we discussed earlier.

The gradual elucidation of the structure of cholesterol, vitamin D, and the sex hormones has increased the theoretical importance of the work of Kennaway and his colleagues, since prior to these investigations it might have been argued that the production of tar cancer was of purely academic interest, as the carcinogenic agents apparently bore no relationship to substances taking part in the composition of the body. Their extensive investigations, however, have shown that the carcinogenic hydrocarbons belong to this form of condensed carbon ring compounds, and, as has been pointed out by Cook, the processes which would be entailed by the formation of these compounds from the naturally occurring sterols and bile acids would involve reduction, dehydration, and dehydrogenation, and might readily occur in the animal body; it seems possible that these carcinogenics might result from these processes operating upon degradation products of sterol metabolism (Cook, 1933).

The most potent synthetic œstrus-producing agent obtained by us is 9 : 10-dihydroxy-9 : 10-di-n-propyl-9 : 10-dihydro-1 : 2 : 5 : 6-dibenzanthracene which, it can be seen, is a derivative of 1 : 2 : 5 : 6-dibenzanthracene, the carcinogenic hydrocarbon (Fig. 12).

In view of the resemblance of the molecular structure of these two compounds, it was decided to investigate the carcinogenic hydrocarbons to see whether they possessed any œstrogenic activity. Accordingly a series of tests were made in which 1 : 2 : 5 : 6-dibenzanthracene, 1 : 2-benzpyrene, and 5 : 6-*cyclo*penteno-1 : 2-benzanthracene were used. With 1 : 2 : 5 : 6-dibenzanthracene completely negative results were obtained; but with the 1 : 2-benzpyrene and the 5 : 6-*cyclo*penteno-1 : 2-benzanthracene it was found possible to produce a full œstrus response by the injection of 100 mg. of the compound. The usual strict criteria for true œstrus was applied rigorously in these two instances, and the net results of these investigations establish the fact that these two compounds possess dual activity—namely, the power of carcinogenesis and œstrogenesis (Cook and Dodds, 1933). It must, however, be pointed out that the œstrogenic action, though definite, is only feeble, and that the main pharmacological action of these compounds lies in their carcinogenic powers. For example, the dibenzanthracene molecule is carcinogenic in the aromatic state, but œstrogenic when suitably hydrogenated and supplied with hydroxyl groups.

I believe that this is the first instance of the description of two entirely separate actions of a single molecule, and this again throws considerable doubt on the whole conception of hormone activity; not only have we already proved the lack of specificity of hormones, but have proved now that a single substance of known constitution may have at least two entirely separate actions initiating such differing processes as œstrus and the production of carcinoma. There is not at the moment sufficient evidence to warrant a great deal of speculation, but two possible thoughts arise to the mind: either the phenomena of œstrus and carcinoma are closely related or, alternatively, the carcinogenic hydrocarbons bear the same relationship to the production of cancer that the synthetic œstrus-producing agents bear to the œstrus phenomenon—namely, that they are both skeleton keys. It must be admitted that the excessive cellular proliferation occurring in the early stages of œstrus bears a superficial resemblance to certain phases of carcinoma.

Œstrogenic Activity of Vitamin D

As has already been mentioned, the condensed carbon ring group of compounds also includes vitamin D or calciferol. The vast amount of chemical work that has been pursued on this subject has shown that it is possible to take the inert sterol, ergosterol, and by irradiation with ultra-violet light under suitable conditions, to confer upon it the activity associated with vitamin D. By the application of chemical methods it has been possible to isolate in crystalline form the vitamin from the products of irradiation, but the exact constitution of this substance is not yet determined. The formula of ergosterol is shown in Fig. 13. From what is known of its constitution at the present time it can be seen that it is at any rate closely related to the group of compounds that we have discussed.

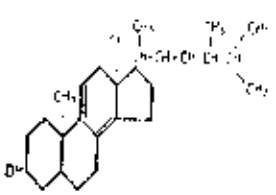


FIG. 13.—Ergosterol.

Bearing in mind the very remarkable observations on the dual activity of benzpyrene and 5 : 6-*cyclo*penteno-1 : 2-benzanthracene, it was felt that a careful trial of sterols related to ergosterol and calciferol for œstrus-producing activity would be interesting. Again, the same elaborate precautions were taken to be sure that any effects produced represented the whole of the œstrus phenomenon. The ovariectomised animals were injected with a solution of the sterol in oil, and 100 mg. were administered in each case. It was found that the greatest activity was possessed by neo-ergosterol; 40 per cent. of the animals injected with this compound developed full œstrus. In the case of calciferol full œstrus was developed in 20 per cent. of the animals, while with ergosterol 20 per cent. of the animals also showed definite evidence of œstrus-producing activity.

In all cases a number of animals were killed at the height of œstrus, and the characteristic enlargement and distention of the uterus was found. In the case of those injected with calciferol, the animals died at a later date with the characteristic symptoms of hypervitaminosis, but it must be emphasised that the œstrus changes occurred before there was any sign of intoxication. It must of course be borne in mind that the amount of calciferol required to produce œstrus is quite out of proportion to that required to protect an animal against rickets.

An interesting point was brought out by running a series of control animals which were injected with parathormone. These animals developed a hypercalcæmia similar to that obtained in hypervitaminosis, but no œstrus resulted. One can therefore definitely state that elevation of blood calcium does not produce œstrus, and that the œstrogenic activity of vitamin D appears to be quite apart from its power to raise the blood calcium content.

We have therefore a very striking confirmation of the observation that a single molecule may possess at least two entirely separate physiological effects, in this case antirachitic and œstrogenic activity. Until this observation was made this dual activity had only been observed with the synthetic carcinogenic hydrocarbons. In the case of vitamin D, however, we were able to reproduce the same phenomena with a naturally occurring substance. Again, it is too early to speculate upon the theoretical process underlying these surprising observations. It seems impossible to imagine that all these groups of compounds are converted in the body of the ovariectomised animal into œstrin, and there is certainly no evidence for such an assumption at the present time. In any case, these observations taken all together indicate the extreme importance of the

TABLE II.—*Relative Potencies of Sterols*

Substance.	No. of rats	Dose in mg.	Per cent. positive.	Remarks.	Method of Preparation.
5 : 6-*cyclo*penteno-1 : 2-benzanthracene ..	10	100	30	—	Cook (1931).
1 : 2-benzpyrene	10	100	30	—	Cook, Hewett, Hieger (1933). Inhoffen (1932).
Neo-ergosterol ..	10	100	40	—	
Calciferol	10	100	20	3 died.	—
Ergosterol	10	100	20	All died.	—
1 : 9-dimethylphenanthrene ..	5	100	60	—	Haworth and Mavin (1932).
Cinnamodonic acid (after treatment with bromine)	5	50	60	1 died.	—
Coal tar	5	100	40	—	—

sterol group in the metabolism, not only of animals, but also of human beings. Whether the phenomena associated with rickets, malignant disease, and sex activity are all related, or whether all the locks are capable of being opened by skeleton keys, remains yet to be proved. It can be seen that in this particular sterol group there is no oestrogenic activity with cholesterol, but the potency increases with the increasing degree of unsaturation rising to a maximum in the case of neo-ergosterol. Table II. indicates the relative potencies of vitamin D, &c.

The Thyrotropic Hormone

It will be remembered that in Lecture I. the various hormones secreted by the anterior lobe of the pituitary were discussed, and amongst them appeared the thyrotropic hormone. It would seem that this hormone bears a very similar relationship to the thyroid to that which prolan bears to the ovary. We have already seen that prolan acts upon the ovary and stimulates it to produce oestrin and allied compounds. The thyrotropic hormone stimulates the thyroid to produce its own hormone, thyroxine. It will perhaps be advisable to review very briefly the experimental evidence of the existence of the thyrotropic hormone.

As in the case of prolan in relation to the ovary, the evidence produced by a study of cases of Simmonds's disease indicates the connexion between the anterior lobe of the pituitary and the thyroid gland. It has also been

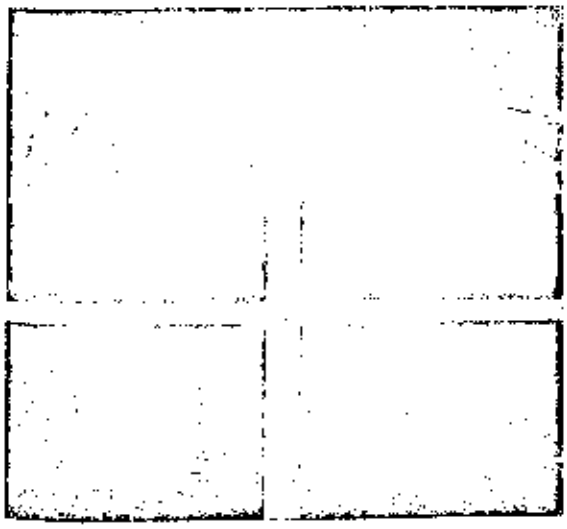


FIG. 14.—Exophthalmos in the guinea-pig. Reproduced from the *Bulletin* of the Johns Hopkins Hospital.

shown that removal of the pituitary gland causes either reduction of development or atrophy of the thyroid (Adler, 1914; Allen, 1916; Smith, 1916; Foster and Smith, 1926; Smith, 1927; Richter and Wislocki, 1930). Several of these observers noted the decrease in basal metabolic rate, and Foster and Smith (1926) and also Richter and Wislocki (1930) noted that the changes can be prevented by injection of an extract of the anterior lobe. Moreover, the addition of an extract of the anterior lobe of the pituitary to the fluid in which axolotls are kept initiates a metamorphosis. The metamorphosis is preceded by hyperplasia of the thyroid gland.

In 1929 Max Aron published an account of the action of an extract of the anterior lobe of the hypophysis upon the thyroid of the guinea-pig. In a later series of papers in 1930 he reported a very careful study of this hormone and showed that there was great hypertrophy of the parenchyma of the gland associated with liquefaction and final disappearance of the colloid; if the injections be discontinued the thyroid gland returns to its normal state (Aron, 1929 and 1930[1,2]).

Krogh, Lindberg, and Okkels (1932) made a very careful histological investigation of the thyroids of guinea-pigs following prolonged treatment with anterior lobe extract; they maintained that whilst the glands gave the appearance of having returned to normal after cessation of injections, it was found that the Golgi apparatus still showed the hyperplasia in increase which is characteristic of Graves's disease.

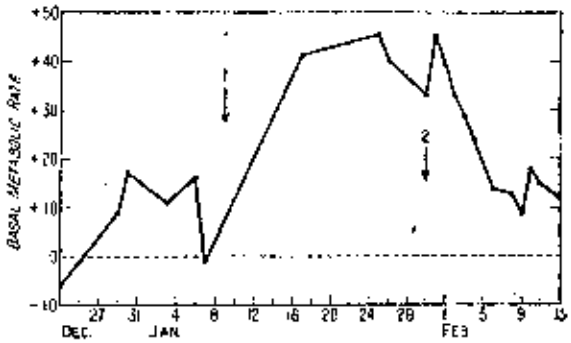


FIG. 15.—Chart showing effect of thyrotropic hormone on the basal metabolic rate of a woman (case 27). Administration of the hormone, in doses of 600 mouse units daily, was begun at the point marked 1 (Jan. 9th) and was stopped at 2 (Jan. 30th).

Some of the first observers to study the effects of the hormone upon the basal metabolic rate of animals were Verzár and Wahl (1931). They showed that it is possible to increase the basal metabolic rate to a very considerable level and that this falls on withdrawing the extract. They further made the interesting observation that this rise in basal metabolic rate could be controlled by the administration of potassium iodide. It was also found impossible to produce hyperactivity of the thyroid in an animal receiving large doses of potassium iodide. This observation was also made independently in my laboratory.

It is very interesting to compare this phenomenon with the well-known effect of iodine in reducing the high basal metabolic rate in persons with thyrotoxicosis. In general it may be stated that the increase in basal metabolic rate is directly dependent upon the amount injected.

A still further effect of this hormone is the production of exophthalmos in animals. This has been successfully demonstrated by Hogben (1922–23) and by Spaul (1923–24); it has also been observed in ducks and guinea-pigs by Schoekaert (1931[1,2] and 1932) and by Loeb and Friedman (1931–32). The following illustration is reproduced from a paper by Friedgood (1934) and shows very clearly the marked exophthalmos occurring in a guinea-pig (Fig. 14.)

It would appear that here we have an experimental method of producing Graves's disease, and as in the case of Graves's disease the condition can be controlled by the administration of iodine.

With regard to the chemical properties of the extract, very little at present is known. It is apparently of a protein character and so readily destroyed by heat and in alkaline solution. It seems, therefore, to belong to the protein group and to correspond in properties roughly with hormones such as prolan and insulin. The accompanying chart shows a typical experiment made in my laboratory upon a volunteer with this material (Fig. 15).

Guinea-pigs were used for the animal experiments and their oxygen consumption was measured by a modified Krogh apparatus.

I have made a very careful study of the urine of patients suffering from Graves's disease and thyrotoxicosis in the hope of finding a hormone in the urine in the same manner that one finds prolan in the case of pregnancy, but so far no conclusive evidence has been obtained. By a modification of the method of Dickens (1930) for the separation of prolan, it was possible to obtain definite though small yields of a thyrotropic substance. It has, of course, been conclusively shown by many observers that this hormone is without effect upon the basal metabolic rate of animals from whom the thyroid has been removed. We can see, therefore, that the relationship of the pituitary gland to the thyroid resembles in many respects the relationship that it holds to the ovary.

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ROYAL FREE HOSPITAL. At the annual meeting recently, Lord Falmouth, the president, stated that during the past year an average of 1000 patients had been treated daily, including 100 children in the Dentaire Dental Clinic. The nurses' home had been completed at a cost of about £150,000; two new wards were opened for eye, nose, and throat cases, with 30 beds and a receiver; the pay beds have been increased to 23. The Albert Levy wing at the Queen Mary Hospital, comprising an obstetric and gynæcological out-patients department and an electrotherapeutic and massage department, has been brought into full operation. Polyclinic lectures have been revised to bring them into accord with modern ideas. Sir Hilton Young, Minister of Health, congratulated the governors upon the mortality in the maternity wards being only 2·8 per 1000, "the lowest in London, and probably in the whole country."

INTERNATIONAL COÖPERATION IN PUBLIC HEALTH

ITS ACHIEVEMENTS AND PROSPECTS *

By Sir GEORGE S. BUCHANAN, C.B., M.D., F.R.C.P. Lond.

The International Sanitary Convention, 1926

AFTER the war, as I have already shown, the impulse to establishment of greater confidence between countries was growing, while it had to be realised that the latest convention, though as recent as 1912, now related to a pre-war political geography that was extinct, and to States whose governments as well as whose frontiers had been radically changed. Meanwhile, also, a good deal more had been learned from the war years about international coöperation over infectious diseases, and it was clear that if any general new convention was made it could no longer be limited to cholera, plague, and yellow fever. Several countries had, for example, adopted a policy of their own to control the entry of typhus and small-pox infection at their sea or land frontiers, and action in regard to these two diseases at least required some mutual understanding in the interests of traffic and of rational hygiene. We were in great difficulties also in consequence of the way in which rat plague had spread from port to port by means of shipping, often causing human plague and, in any case, great expense in its eradication. The danger was being met by efficient methods of effectively destroying rats, particularly by cyanide fumigation, but shipping was finding itself in the impossible position of having to submit to deratisation and all the delay and cost that it involved, at one port after another, undertaken merely because the vessel had originally come from an infected country, and without regard to the previous deratisations or fumigations to which she might have been subjected in course of the same voyage. It may well be supposed that when the fumigation of a ship on which there was no evidence of any plague infection was found to cost several hundreds of pounds without producing a single dead rat, the owners became eloquent.

The difficulties of amending the 1912 Convention and adjusting it to new conditions, political and medical, were very great. Nothing could have seemed more contradictory, for example, than the post-war policies of the health services of the United States, Japan, and Great Britain on many of the questions at issue. Indeed, at one time it seemed likely that no new world-wide agreement would be possible, and that the whole matter would have to be left to particular agreements negotiated between countries or to regional agreements. This view was strongly contested by countries like our own with a large world trade, and fortunately with good effect. The convention which at present regulates all these matters—the International Sanitary Convention of Paris, 1926—drawn up by a plenipotentiary conference working on the drafts prepared by the Office International d'Hygiène publique—is world-wide in its application, and was able to be so by its being based on the adoption of new principles of international coöperation. The rat difficulty, for example,

* The Milroy Lectures delivered before the Royal College of Physicians of London on Feb. 27th and March 1st, 1934, with a few subsequent additions of detail.

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Nutrition Abst. & Rev 27, 1957

DEFICIENCY DISEASES: VITAMIN D

1117

DONATH, W. F., DE JONGE, H., TEN THIJE, J. H. and DE WAART, A. An experimental study of the influence of vitamin D upon the metabolism of calcium. *Koninkl. Nederl. Akad. Wetenschappen. Proc., Ser. C*, 1956, 59, 111–122; 123–135.

Vol. 27, No. 3

Rabbits received to appetite a diet of barley husk meal 28·5, extracted soya bean meal 10, oatmeal 28·5, wheat flakes 28·5, yeast 2, cod liver oil 0·75 and minerals 0·75 per cent., with water and some green feed. After a control period groups of them received weekly intravenous injections of a solution of a product of irradiated ergosterol, or pure vitamin D_2, or pure vitamin D_3, or pure vitamin D_2 with vitamin A, or one of the organic solvents used for the vitamins.

The first injection of a vitamin, but not of solvent alone, was always followed by loss of appetite, but the effect was less after later injections. The results of biochemical tests are shown in tables. Statistical investigation showed that all 4 vitamin D preparations could be considered together. They had no significant effect on temperature. Slight falls of Hb and small but significant falls of red cell count suggested incipient anaemia. There were significant increases of serum cholesterol and alkaline phosphatase, highly significant increases of serum, Ca, N.P.N. and urea and probably significant increases of inorganic P. Total P rose for from 2 to 6 weeks and then fell, sometimes to below the control levels. Loss of weight was considerable, especially in the rabbits given vitamin A.

Percentages of Ca in dry matter of aorta, kidney, femur and skull are shown in a table. In rabbits given vitamin D the mean Ca contents of aorta and kidney were 6·9 and 2·7 per cent. compared with less than 0·04 per cent. in controls. Ca values in femur and skull were low in the experimental animals; Ca had apparently been withdrawn from the skeleton and deposited in soft tissues. Calcification of soft tissue was demonstrated histologically by independent observers. There was very significantly more severe calcification in the rabbits given pure vitamin D_2 or D_3 than in those given the ergosterol product or vitamin D_2 with vitamin A. Deposition of calcium was not related to duration of life. The largest amounts were in aorta, stomach, kidney, lung and heart and no Ca was found in liver, brain, adrenal, pancreas and some other tissues.

Rabbits which died showed the syndrome of anaemia. The dangers of overdosage with vitamin D in man are discussed.—D. Duncan.

Am. J. Dis. Child. 53: 754-59, (1937)

COMPARISON OF THE ANTIRACHITIC EFFECTS ON HUMAN BEINGS OF VITAMIN D FROM DIFFERENT SOURCES

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TORONTO, CANADA

Shortly after the discovery of the production of vitamin D by the irradiation of ergosterol, the observation was made that the antirachitic effect on chickens of the irradiated ergosterol differed markedly from that of cod liver oil, rat unit for rat unit of vitamin D.[1] This raised the question of the relative efficacy in human beings of the various

TABLE 1.—*Potency of Various Vitamin D Products Commonly Used*

Product	Daily Amount Usually Prescribed	Approximate No. of U. S. P. Units of Vitamin D
Cod liver oil	3 tsp.	1,000
Viosterol	10 drops	2,000
10 D codliver oil*	1 tsp.	1,000
Halibut liver oil	1 tsp.	2,000
Halibut liver oil and viosterol	10 drops	2,000
Percomorph liver oil	10 drops	2,000
Cod liver oil and percomorph liver oil	1 tsp.	3,100
Irradiated fresh milk	12-40 oz.	57-190
Irradiated evaporated milk	5-20 oz.	60-196

* A mixture of cod liver oil and viosterol.

vitamin D-containing substances and the dosage of each necessary for the prevention and cure of infantile rickets. For some years it has been customary to administer daily for the prevention of rickets 3 teaspoonfuls of cod liver oil, which amount contains approximately 1,000 U. S. P. units of vitamin D, or 10 drops of standard viosterol (10,000 U. S. P. [international] units of vitamin D per gram), which dose contains approximately 2,000 units. In table 1 are given most of the commonly used vitamin D products, with the number of U. S. P. units contained in the daily amount usually administered for the prevention of infantile rickets. This paper is a presentation of observations on the antirachitic

Read at the Forty-Eighth Annual Meeting of the American Pediatric Society, Bolton Landing, N. Y., June 11, 1936.

From the Department of Paediatrics, University of Toronto Faculty of Medicine, and the Hospital for Sick Children, under the direction of Dr. Alan Brown.

1. Hess, A. F., and Supplee, G. C.: Proc. Soc. Exper. Biol. & Med. **27**: 609, 1930. Massengale, O. N., and Nussmeier, M.: J. Biol. Chem. **87**:423, 1930. Mussehl, F. E., and Ackerson, C. W.: Poultry Sc. **9**:334, 1930.

TABLE 2.—*Development of Rickets in Infants, Aged 13 Months and Younger, Who Received Different Amounts of Vitamin D in Various Forms Throughout Five Winter Months*

Year of Observation	Source of Vitamin D	No. of U. S. P. Units of Vitamin D Given Daily	No. of Infants	Age, Months: Less Than 1	1	2	3	4	5	6	7	8	Percentage of Infants: Maximum Degree of Rickets Subsequent to Initial Examination: None	Extremely Slight	Mild	Moderate and Marked	Actively Advancing Rickets with No Evidence of Healing at End of Period of Observation
1932-34	1 teaspoonful cod liver oil	800	42	0	1	8	5	4	5	5	10	3	81	12	7	0	0
1932-34	2 teaspoonfuls cod liver oil	700	42	0	2	6	6	10	4	7	8	1	73	10	7	2	2
1933-34	3 teaspoonfuls cod liver oil	1,050	50	0	7	5	5	5	8	6	10	2	74	14	8	4	4
1933-34	1½ drops viosterol	270	50	0	9	10	11	4	3	6	4	2	52	16	2	0	0
1933-34	2½ drops viosterol	540	44	1	10	9	10	3	5	2	5	0	63	23	7	0	2
1933-34	5 drops viosterol	1,080	46	0	12	13	2	6	3	1	1	2	78	20	2	0	2
1933-34	10 drops viosterol	2,100	49	1	5	13	7	8	4	4	3	1	76	11	7	0	4
1933-34	20 ounces (592 cc.) irradiated fresh milk	95	71	0	8	16	11	8	9	8	7	2	69	22	6	0	8
1933-34	30-40 ounces (892-1,184 cc.) irradiated fresh milk	70	70	0	0	5	4	13	15	12	11	0	53	15	3	0	8
1933-34	No vitamin D	0	53	1	11	30	7	10	9	4	3	1	55	22	15	8	16
1934-35	10-40 ounces (296-1,184 cc.) irradiated fresh milk	47-160	108	0	5	14	17	29	21	13	0	0	76	13	11	0	0
1934-35	6-20 ounces (177.6-592 cc.) irradiated evaporated milk	60-196	105	0	19	30	24	17	19	4	0	0	84	12	17	0	0
1934-35	No vitamin D	0	104	1	19	23	30	16	13	5	0	0	47	19	27	26	16
1935-36	Irradiated yeast	500	60	0	0	0	0	13	27	23	5	0	57	4	8	0	0
1935-36	0.43 teaspoonful cod liver oil	150	74	0	12	21	13	17	6	4	0	0	60	19	12	0	3
1935-36	0.78 drops percomorph liver oils	150	60	0	7	11	4	12	11	8	1	1	75	15	10	0	5
1935-36	Irradiated cholesterol	150	77	0	15	12	21	17	10	3	0	0	82	8	10	0	1
1935-36	No vitamin D	0	75	1	12	19	15	10	7	4	0	0	51	15	20	13	31

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value of various amounts of cod liver oil, viosterol, irradiated fresh milk, irradiated evaporated milk, irradiated cholesterol, irradiated yeast and a mixture of fish liver oils of high potency.

PROCEDURE

Observations were conducted during the winter months of the past three years, commencing in October or November and continuing until March or April—in every case a total of five months. Twelve hundred and twenty-eight infants were observed. These babies represented a cross-section of the infant population of Toronto. They were practically all of British and northern European stock. With the exception of some of those seen during the first winter,[2] all the infants were artificially fed with dilutions of cow's milk and added carbohydrate. The antirachitic substances used and the number of U. S. P. units of vitamin D administered daily, together with the ages of the infants at the time of the initial observation, are given in table 2. The presence or absence of

TABLE 3.—*Development of Rickets in Infants Who Received No Vitamin D Throughout Five Winter Months*

		Percentage of Infants				
		Maximum Degree of Rickets Subsequent to Initial Examination				Actively Advancing Rickets with No Evidence of Healing at End of Period of Observation
Year of Observation	Total No. of Infants	No Rickets	Extremely Slight Rickets	Mild Rickets	Moderate and Marked Rickets	
1923-24	65	55	22	15	8	18
1924-25	104	47	10	27	16	16
1925-26	75	51	16	20	13	21
Total, 1923-26	244	50	15	22	13	21

rickets was determined solely on the basis of roentgenograms of the wrists which were taken at the beginning at the middle and at the end of the periods of observation.

Dr. Martha Eliot, assistant chief of the Children's Bureau, United States Department of Labor, Washington, D. C., interpreted all the roentgenograms. The plates were read not only to ascertain the degree of the rachitic process but also to learn whether the process was actively advancing or if there were evidences of healing. Table 2 shows the maximum degrees of rickets which developed during the periods of observation in infants of all the groups studied, together with the percentage of infants in each of the groups who at the end of the periods of observation showed evidence of an actively advancing rachitic process.

In table 3 is given the maximum degree of rickets observed at any time subsequent to the initial examination in infants who received no vitamin D preparation. In this table is also given the percentage of infants showing evidence of an actively advancing rachitic process at the final examination.

2. Drake, T. G. H.; Tisdall, F. F., and Brown, A.: Canad. M. A. J. 31: 368, 1934 (table XII).

COMMENT

It is to be noted that of the 244 infants receiving no vitamin D, 13 per cent had rickets of a moderate or marked degree during the periods of observation. Also at the end of the five month period, 21 per cent of the 244 infants showed an actively advancing rachitic process without evidence of healing.

During the first year of the observations, in view of the facts that the customary daily dose of cod liver oil is 3 teaspoonfuls (1,000 U. S. P. units) and that the usual dose of viosterol is 10 drops (2,000 U. S. P. units) daily, it was decided to observe the effect of the daily administration of one, two and three teaspoonfuls of cod liver oil and one and one-quarter, two and one-half, five and ten drops of viosterol. It was thought that the lower amounts of each of the products would probably be too small to be effective in the prevention of rickets and thus would afford a means of making a fairly accurate comparison of their antirachitic effects. Surprisingly, however, it became evident that, as shown in table 2, one and one-quarter drops of viosterol (270 U. S. P. units) was just as effective as 10 drops of viosterol (2,160 U. S. P. units) in the prevention of rickets. Also no evidence was found that 3 teaspoonfuls of cod liver oil (1,000 U. S. P. units) was any more effective than 1 teaspoonful (330 U. S. P. units); in fact, the results obtained with 1 teaspoonful were better than those obtained with either 2 or 3 teaspoonfuls, which can be explained only by taking account of the difficulty in administration of the larger amounts. Every possible care was taken to insure the regular administration of the amounts prescribed, and in any instance in which evidence of maladministration was found the case was promptly excluded from this series. It also is to be noted that the daily administration of 500 U. S. P. units of vitamin D in the form of irradiated yeast was slightly more effective in the prevention of rickets than were the varying amounts of viosterol used. This is no doubt accounted for by the fact that, for ease of administration, the irradiated yeast was mixed with cereal and given only to those children who were old enough to be receiving cereal; hence the average age of the infants in this group was slightly greater than that of the babies in the other groups.

During the past winter the amount of cod liver oil administered daily was reduced to slightly less than ½ teaspoonful (150 U. S. P. units). It was seen that the antirachitic effect of this very small amount of cod liver oil was as great as that secured with amounts varying from 1 to 3 teaspoonfuls (table 2). For purposes of comparison, a daily dose of 150 U. S. P. units of vitamin D in the form of a mixture of fish liver oils of high potency (percomorph liver oil) was administered to the infants in another group, and the same number of U. S. P.

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units in the form of irradiated cholesterol was given daily to the babies in still another group.[3] In no instance did moderate or marked rickets develop. The numbers of infants with mild rickets and with extremely slight rickets were approximately the same in the different groups. It is evident, therefore, that rat unit for rat unit equally good results were obtained with cod liver oil, percomorph liver oil and irradiated cholesterol.

Some observers have expressed the belief that irradiated milk is a more effective antirachitic substance for human use than are other forms of vitamin D, rat unit for rat unit. In this series of cases irradiated fresh milk has been administered to babies in one group and irradiated evaporated milk to infants in another group in the amounts in which they required milk. The irradiated milk was the sole source of vitamin D given to these infants, and it furnished from 47 to 196 U. S. P. units daily (table 2). The infants received increasing amounts of vitamin D as, with advancing age, they needed greater amounts of milk. However, the babies in another group received exactly 20 ounces (592 cc.) of irradiated fresh milk daily (95 U. S. P. units) throughout the five month period of observation. It is of interest to note that the antirachitic effect of the daily dose of 95 U. S. P. units of vitamin D in the form of irradiated fresh milk was approximately equal to that obtained when 150 U. S. P. units of vitamin D in the form of other substances was administered. This means that probably the dosages have not yet been reached at which it is possible to make the most accurate comparison of the antirachitic values of the different substances tested. However, I believe there is sufficient evidence to indicate that there is no essential difference in the antirachitic potency of vitamin D in the form of cod liver oil, of mixed fish liver oils of high potency, or irradiated cholesterol and of irradiated milk. Although in this study viosterol was not administered in doses as low in content of vitamin D as were the doses of the other substances used, the results indicate that the antirachitic effectiveness of viosterol in human beings is not appreciably less, rat unit for rat unit, than that of the other forms of vitamin D.

During the past two years my associates and I have been able to observe the curative effect of comparatively small amounts of irradiated cholesterol and of irradiated yeast on patients with rickets. During the winter of 1935 it was observed that in 8 patients the daily administration of 750 U. S. P. units of vitamin D in the form of irradiated cholesterol produced definite healing of rickets of moderate or marked

3. Mead Johnson & Co. furnished the mixture of fish liver oils of high potency (percomorph liver oil), and E. I. Du Pont de Nemours & Co. furnished the irradiated cholesterol.

degree in from two to four weeks. During the winter of 1936 similar results were obtained in 3 consecutive patients who received only 300 U. S. P. units of vitamin D daily in the form of irradiated cholesterol and also in 4 consecutive patients who received 500 U. S. P. units daily in the form of irradiated yeast.

CONCLUSIONS

No evidence was obtained of any difference in the antirachitic effectiveness in human beings, rat unit for rat unit, of vitamin D administered in the form of cod liver oil, of a mixture of fish liver oils of high potency (percomorph liver oil), of irradiated cholesterol, of irradiated fresh milk or of irradiated evaporated milk.

No evidence was observed of any difference in the antirachitic effectiveness in human beings between the daily administration of 150 U. S. P. units of vitamin D in the form of any of the other substances mentioned and that of 270 U. S. P. units of vitamin D in the form of viosterol. As smaller amounts of viosterol were not administered, it is entirely possible that this substance is as effective in human beings, rat unit for rat unit, as are the others.

The administration of vitamin D in amounts as low as 95 U. S. P. units daily prevented the development of rickets of a moderate or marked degree in every case. No evidence was obtained that this extremely small amount was less effective in preventing rickets, as measured by roentgenograms, than were the larger amounts usually administered.

Rapid healing of rickets of moderate or marked degree resulted from the daily administration of as little as 300 and 500 U. S. P. units of vitamin D. The 300 U. S. P. units of vitamin D was administered in the form of irradiated cholesterol, and the 500 U. S. P. units, in the form of irradiated yeast.

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Vitamin D Stimulated Calcium Binding Protein from Rat Intestinal Mucosa. Purification and Some Properties*

D. Drescher and H. F. DeLuca†

ABSTRACT: Rat intestinal calcium binding protein made in response to vitamin D has been purified to homogeneity. Two main purification procedures have been employed, each of which is suitable for preparative purposes. The calcium binding activity from the supernatant fraction of intestinal mucosa has been purified several 100-fold by means of column chromatography with Sephadex G-100 followed by CM-Sephadex. A second procedure involving gel filtration on Bio-Gel P series resins also yields a homogeneous calcium binding protein. Using the latter procedure another protein having a similar charge as the calcium binding protein has been isolated, and is thought to be a precursor of the calcium binding protein. Homogeneity of the calcium binding protein obtained by these methods has been established by column chromatography, urea disc gel electrophoresis, and ultracentrifugation. A molecular weight of between 8000 and 9000 has been estimated by sedimentation equilibrium ultracentrifugation measurements while a molecular weight of 13,000 has been estimated by means of gel filtration and disc gel electrophoresis.

The appearance of a calcium binding protein was first observed in the supernatant fraction from intestines of rachitic chickens given vitamin D (Taylor and Wasserman, 1965; Wasserman and Taylor, 1966). The calcium binding activity was assayed by means of a chelating resin which competes for radioactive calcium with the soluble binding protein. Calcium binding proteins of a similar nature have since been found in the intestinal mucosa of the rat (Kallfelz *et al.*, 1967; Schachter *et al.*, 1967), dog (Taylor *et al.*, 1968), and monkey and calf (Wasserman, 1970). These binding proteins have been implicated by some investigators as the carriers responsible for the transport of calcium in the small intestine in response to vitamin D (Wasserman and Corradino, 1967; Wasserman *et al.*, 1969).

In initial studies (Wasserman and Taylor, 1966; Kallfelz *et al.*, 1967), both chick and rat calcium binding proteins were partially purified by heat fractionation followed by Sephadex G-100 chromatography. Recently, higher degrees of purity have been obtained by preparative disc electrophoresis of partially purified calcium binding fractions (Wasserman *et al.*, 1968; G. Eilon, unpublished communication, 1970). Some physical properties of the purified chick calcium binding protein have been reported; 1 mole of protein binds 1 mole of calcium ion, and the molecular weight by sedimentation equilibrium is about 25,000 (Wasserman *et al.*, 1968). The molecular weight of the vitamin D dependent rat calcium binding protein has been estimated by calibrated gel filtration to be about 13,000 (Schachter, 1970). There is also a calcium binding protein in rat intestinal mucosa which appears at the void volume of a Sephadex G-100 column and which is present in vitamin D deficient rats (Kallfelz *et al.*, 1968). Thus, two calcium binding proteins, of which only the smaller appears in response to vitamin D, have been observed in our and other laboratories (Kallfelz *et al.*, 1968; Schachter, 1970; J. E. Harrison, unpublished communication 1970). Ooizumi *et al.* (1970) have fractionated two calcium binding proteins of estimated molecular weights of 24,000 and 145,000, both of which they report, appear in response to vitamin D. Since their fractionation techniques were quite different from those of other investigators, further work must be done to explain their apparently unique results.

The present study reports the purification, by means of ion exchange and gel filtration chromatography, of the calcium binding protein which appears in rat intestinal mucosa in response to vitamin D.

A preliminary description of some of the properties of the purified material is presented. Also described in this paper is a protein, devoid of calcium binding activity, which is of special interest because of its similarities to the calcium binding protein in size and charge characteristics.

Materials and Methods

Animals. Male 21-day-old albino rats were obtained from the Holtzman Co., Madison, Wis. They were maintained in hanging wire cages and given food and water *ad libitum*. The rats were fed a purified, vitamin D free diet 24 (1.2% calcium-0.12% phosphorus) described by Guroff *et al.* (1963) which induces rickets in the animals. After 5–6 weeks on this diet the animals became severly rachitic and were suitable for use in the preparation of calcium binding protein.

Chemicals. Crystalline vitamin D_3 (cholecalciferol) was obtained from N. V. Philips-Duphar, Weesp, The Netherlands. Calcium-45 used in the binding assays was from International Chemical and Nuclear Corp., City of Industry, Calif. Coomassie brilliant blue stain for disc electrophoretic gels was a product of Colab Laboratories, Inc., Chicago Heights, Ill. Acrylamide, bisacrylamide, and other reagents used for preparing the polyacrylamide gels were Eastman grade, obtained from Distillation Products Industries, Rochester, N. Y. All other chemicals were of analytical reagent grade.

Column Chromatography. The gel filtration resins used were Sephadex G-100 (Pharmacia Fine Chemicals, Uppsala, Sweden) and the Bio-Gels P-60 and P-20, 50–150 mesh (Bio-Rad Laboratories, Richmond, Calif.). The ion-exchange resin used was CM-Sephadex C-25. All resins were allowed to

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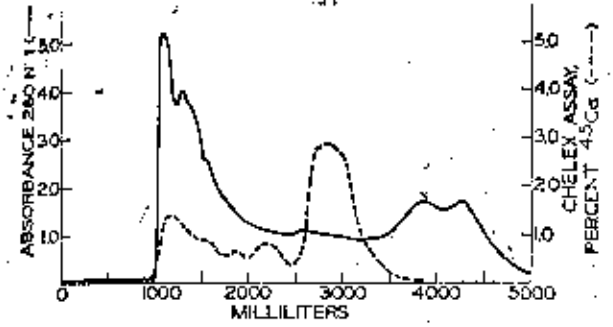

FIGURE 1: Sephadex G-100 chromatogram of the supernatant fraction of intestinal homogenate from rats given vitamin D. Forty rachitic rats were given 250 μg (10,000 IU) vitamin D_3 each 72 hr before sacrifice. The supernatant fraction was prepared and applied to the column as described under Materials and Methods. Column bed dimensions: 7.5 × 70 cm; flow rate: 2 ml/(cm² hr). The column was equilibrated and eluted with 0.08 M NaCl, 0.008% β-mercaptoethanol, and 0.08 M Tris-HCl (pH 7.0). Absorbance at 280 nm (- - -) or per cent ^{45}Ca by the Chelex assay for calcium binding protein (—) (see text) is plotted *vs.* milliliters effluent. Vitamin D dependent calcium binding activity is found at about 2.7 void volumes.

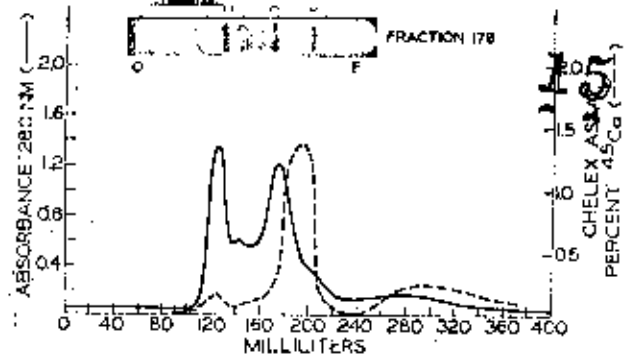

FIGURE 3: Bio-Gel P-60 column chromatogram of proteins associated with calcium binding activity on CM-Sephadex (see Figure 2). Both the peak and shoulder region from the 0.1 M phosphate (pH 7.5) area of a CM-Sephadex chromatogram were combined and concentrated to 5 ml. This was applied to the P-60 column (2 × 97 cm) and eluted with 0.04 M NaCl, 0.008% β-mercaptoethanol, and 0.04 M Tris-HCl (pH 7.0) at a flow rate of 2.3 ml/(cm² hr). The inset is a photo of an electrophoretic gel of the fraction at 178 ml. The origin and front of the gel are marked O and F, respectively.

swell, the fine particles were removed by floatation in deionized water, and were equilibrated with their initial eluent buffer.

Specific dimensions and flow rates of individual columns used in the fractionations are found in the legends to Figures 1–5.

Preparation of Calcium Binding Protein. The vitamin D deficient rats were each given 250 μg of vitamin D as described in Figure 1. Seventy-two hours later they were killed by decapitation and 12 cm of small intestine was excised, slit lengthwise, and immediately rinsed in ice-cold isotonic saline. Washed intestines were quickly frozen to −70° in a beaker submerged in Dry Ice–acetone. A mixture of frozen intestines and an equal amount of Dry Ice was placed in a large mortar, and the tissue was ground to the consistency of fine gravel. The material was homogenized 1.5 min at high speed in a Waring Blendor in a solution of 0.08 M NaCl, 0.008% β-mercaptoethanol, and 0.08 M Tris-HCl (pH 7.0). Approximately 5 ml of solution/intestine was used. The homogenate was centrifuged for 30 min at 40,000*g* in a refrigerated centrifuge and the supernatant retained. The sediment was resuspended and the extraction procedure repeated. The second supernatant was combined with the first, and concentrated in a 400-ml Amicon ultrafiltration cell at 0° using Diaflo UM2 membrane (Amicon Corp., Lexington, Mass.). Final sample volume was 100 ml, which was applied to the Sephadex G-100 column of Figure 1. In all the chromatographic separations the recovery of protein was 90–100%.

Assay for Calcium Binding Protein. The Chelex method for assay of calcium binding protein (Wasserman *et al.*, 1968) was used. The assay depends upon the competition between the chelating resin Chelex-100 (Bio-Rad Laboratories, Richmond, Calif.) and the soluble calcium binding protein for added ^{45}Ca (Wasserman *et al.*, 1968). In practice, 1 ml of sample was pipetted into a 3-ml centrifuge tube. A 0.2-ml volume of rapidly stirred, suspended resin (1:1, wet resin buffer) was pipetted into the tube and immediately swirled several times on a rotary mixer. The buffer was the same as

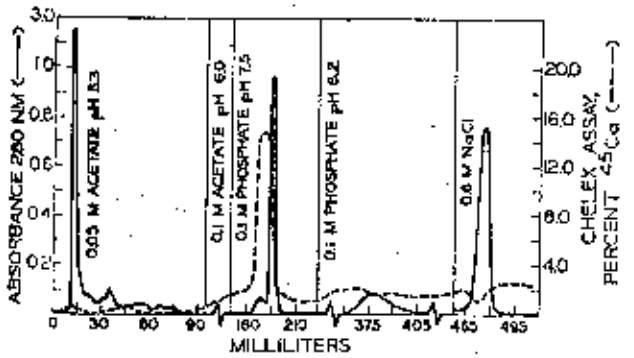

FIGURE 2: Chromatography of crude calcium binding protein from a Sephadex G-100 column on CM-Sephadex. Ordinate and abscissa are marked in the same manner as in Figure 1. Material from the Sephadex G-100 region showing calcium binding activity (Figure 1) was applied to a 1 × 36.5 cm column equilibrated with 0.05 M acetate (pH 5.3). Sample volume was 1 ml and the flow rate was 9 ml/(cm² hr). Stepwise elution was carried out with various buffers as indicated by vertical lines in the figure. All buffers were 0.008% in β-mercaptoethanol. Flow rate was doubled after the activity had been eluted.

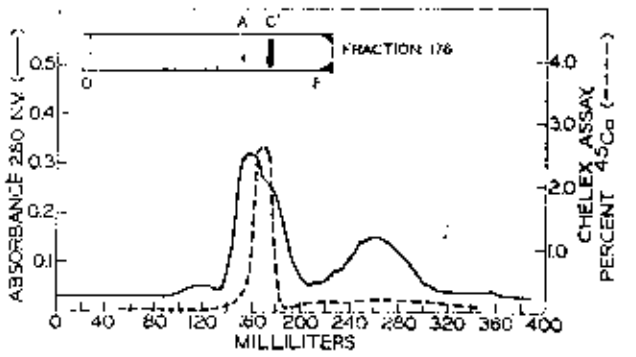

FIGURE 4: Bio-Gel P-60 elution diagram of the region of calcium binding activity from previous P-60 column (Figure 3). The procedure was similar to that described in Figure 3. The sample applied to the disk gel (see inset) was the fraction at 176 ml; bands A and C' are marked as before. Letters O and F mark the gel origin and front, respectively.

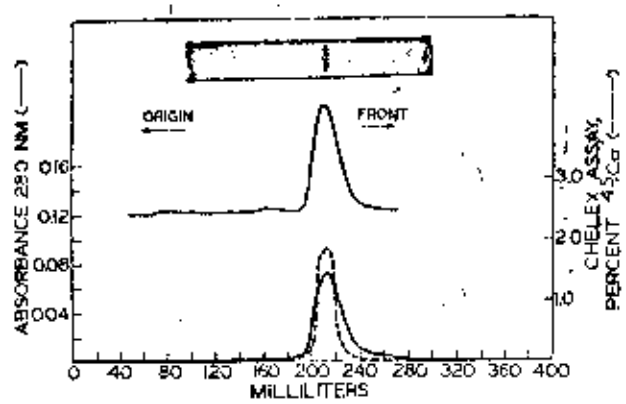

FIGURE 5: Bio-Gel P-20 column chromatography of calcium binding protein from the Bio-Gel P-60 column (Figure 4). A sample of 2 ml volume was applied to the P-20 column (2.2 × 100 cm) and eluted with 0.04 M NaCl, 0.008% β-mercaptoethanol, 0.04 M Tris HCl (pH 7.0) at a flow rate of 1.6 ml/(cm² hr). A photo of the disk gel and densitometric tracing (not in the same scale) of the calcium binding activity peak (band C′, Figure 4) are included. Orientation of the gel is indicated.

that used for the Sephadex G-100 column (Figure 1). Next, 100 μg of ^{45}Ca solution of approximately 10 μCi/ml was added, and the contents of the tube mixed thoroughly for 60 sec. The resin was sedimented by centrifugation and samples of the supernatant fluid were transferred onto circles of Whatman No. 5203-A filter paper (23 mm diameter). The circles of filter paper were dried in an oven at 80°, placed in counting vials containing a toluene-counting solution, and the ^{45}Ca was determined by means of a Packard Tri-Carb Model 3003 liquid scintillation counter. The counting solution consisted of 0.5% 2,5-diphenyloxazole and 0.025% 1,4-bis[2(4-methyl-5-phenyloxazolyl)]benzene dissolved in toluene. No quench corrections were found necessary.

Aliquots of supernatant fluid from tubes containing Chelex plus buffer in place of sample gave a low level of radioactivity which represents the ^{45}Ca not bound by the resin (Wasserman *et al.*, 1968). This blank radioactivity equalled the amount found in the case of fractions lacking calcium binding activity. The total ^{45}Ca present was determined by adding 100 μg of ^{45}Ca solution of 1.1 ml of buffer, without Chelex, and counting the resulting solution. Then the calcium binding activity, in units of % supernatant ^{45}Ca, could be calculated from the expression [(S) − (B)]100%/(T) where S, B, and T are the supernatant, blank, and total ^{45}Ca, respectively. Specific activity calculations are discussed in Table I. Protein concentration was determined on the basis of absorbancy at 280 nm with a Beckman DB-G spectrophotometer.

Disc Gel Electrophoresis. The electrophoretic system used to monitor purification was a 7% acrylamide, 9 M urea gel described by Neville (1967). The components other than the acrylamide and bis acrylamide were weighed out into a Pyrex test tube marked at 40 ml. Ten milliliters of a 0.004% aqueous riboflavin solution was added and the mixture was heated to 80° in a water bath with swirling to dissolve the urea. The acrylamide and bisacrylamide were weighed out in a separate test tube and just enough water added to effect solution. The acrylamide solution was quickly poured into the urea catalyst solution, the volume brought to 40 ml, and dissolved air was removed by means of an aspirator. The gel solution was poured to a height of 90 mm into mounted 8 × 130 mm Pyrex tubes and overlaid with 25 μg of water. The acrylamide was polymerized by means of a fluorescent light. The spacer gel was prepared in a 10-ml volume in a similar manner, and 2.5 ml of 0.004% riboflavin was added. After removing water from the top of the separation gel and rinsing with unpolymerized spacer gel solution, 0.1 ml of the spacer gel was added per tube. The spacer gel was partially polymerized under fluorescent light and no water overlay was used.

Samples for disc electrophoresis were prepared as described by Neville (1967). The apparatus was similar to that of Davis (1964), and electrophoresis was run 6–7 hr under the conditions described by Neville (1967) until the marker dye reached the end of the gel. The gels were removed and exposed for 30 min to 0.05% coomassie blue in 12.5% trichloroacetic acid to stain the proteins as described by Chrambach *et al.* (1967). Removal of the excess coomassie blue from the gels was

TABLE I: Increase in Specific Activity of Calcium Binding Protein with Fractionation.[a]

Fractionation	Sp Act. (%/(mg ml))	Rel Sp Act.
Mucosal supernatant fraction from rats given vitamin D (250 μg)	0.8	1.0
Sephadex G-100 column (Figure 1)	8.8	11.4
First Bio-Gel P-60 column (Figure 3)	11.1	14.4
Second Bio-Gel P-60 column (Figure 4)	31.8	41.2
Bio-Gel P-20 column (Figure 5)	44.1	57.4
Calcium binding fraction from vitamin D deficient rats after Sephadex G-100 fractionation	2.3	
CM-Sephadex columns, initial fractions from calcium binding fraction (Figure 2)	101.3, 795.9	

[a] Fractions were assayed by the Chelex method described in the text. For each chromatogram, the activity in the peak tube was used for calculation of specific activity. Protein concentration was estimated by the expression $A = Ecl$, where A is the absorbance at 280 nm, c is concentration in milligrams per milliliter, and l is the light path length (1 cm). The specific extinction coefficient, E, is set equal to 1.5 mg/(ml cm); that is, the estimate assumes that a protein concentration of 1.0 mg/ml has an absorbance of 1.5 at 280 nm. Specific activity was calculated by dividing the per cent of total ^{45}Ca above background in the supernatant of the Chelex-100 assay tube by the concentration in milligrams per milliliter of protein in the same tube. The mucosal supernatant in the table represents material from rats dosed as described in Figure 1 and prepared as described in the Materials and Methods section. The purification steps involving columns previously illustrated are so designated. Only the calcium binding protein which appears in response to vitamin D is reported in the tabulated results. The supernatant fraction from vitamin D deficient rats reveals a lowered specific activity in the calcium binding protein region of the Sephadex G-100 profile and is included in the table for comparison. The initial fractions of the calcium binding peak from CM-Sephadex yielded low amounts of protein of high specific activity; two representative activity values are listed.

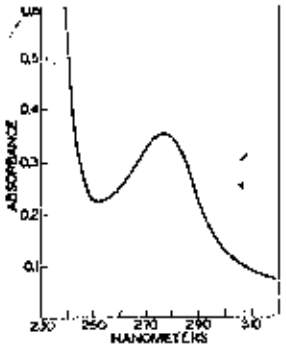

FIGURE 6: Ultraviolet spectrum of purified calcium binding protein. The sample was taken from the active peak eluted from the Bio-Gel P-20 column (Figure 5).

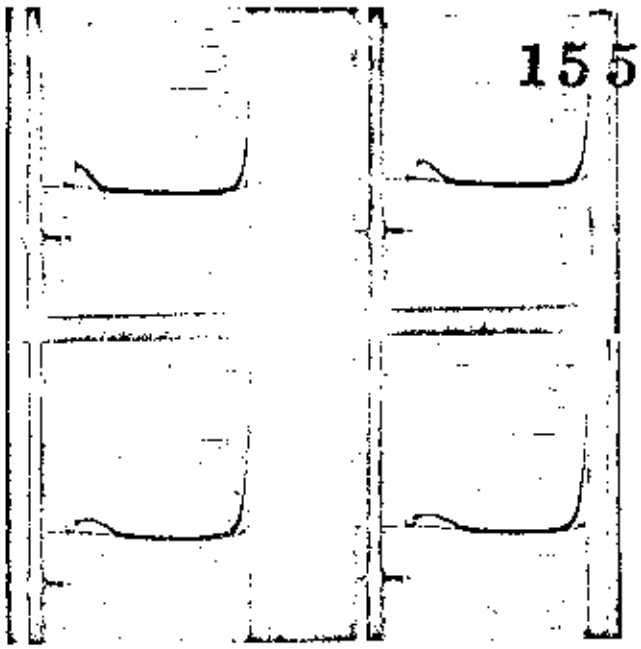

FIGURE 7: Sedimentation of purified calcium binding protein in the analytical ultracentrifuge. The sample used was concentrated material from the Bio-Gel P-20 peak (Figure 5). Exposures of plates (left to right) were taken at 0, 12, 36, and 48 min at 59,780 rpm at 20° in 0.04 M NaCl, 0.008% β-mercaptoethanol, and 0.04 M Tris-HCl (pH 7.0) bar angle 60°.

accomplished with 10% trichloroacetic acid at 45° for 6–12 hr. The gels could be kept in excellent condition for 1 year or more by equilibrating and storing them with an aqueous solution of 7% acetic acid–35% ethylene glycol at −10°. After storage, proteins on the gels can be restained as described above.

Photographs. Gels were positioned in small corked test tubes completely filled with 12.5% trichloroacetic acid, and illuminated from behind with fluorescent light. A Nikomat camera with a Micro-Nikkor Auto 1:3.5 lens, f = 55 m (Nikon, Japan) at a distance of 24 cm and Kodak Plus-X Pan film were used.

Ultraviolet Spectra. Ultraviolet spectra were recorded with a Beckman DB-G recording spectrophotometer.

Analytical Ultracentrifuge Runs. A Spinco Model E analytical ultracentrifuge was used for all measurements. The conditions of the sedimentation velocity experiment are listed in Figure 6 and those of the equilibrium experiment (Yphantis, 1964) in Figure 7. In the determination of molecular weight, fringes were measured with a Gaertner microcomparator.

Results

Preparation of Crude Calcium Binding Protein. The first step in purification of calcium binding protein described by others (Kallfelz *et al.*, 1967; Wasserman *et al.*, 1968) using heat denaturation was avoided because of the possibility of altering the native state of the calcium binding protein. The next step in the purification of calcium binding protein from supernatant was similar to that described by Kallfelz *et al.* (1967) using Sephadex G-100. The 40,000*g* intestinal supernatant was immediately concentrated and directly applied to the large preparative Sephadex G-100 column (Figure 1). A peak of calcium binding activity as a result of vitamin D supplementation was eluted at approximately 2.7 void volumes, as shown by previous investigators for heat-treated supernatant fraction (Kallfelz *et al.*, 1967; Avioli *et al.*, 1969).

Purification of Calcium Binding Protein with CM-Sephadex. A portion of the calcium binding protein peak from the G-100 Sephadex fraction shown in Figure 1 was applied to a CM-Sephadex column. The elution profile (Figure 2) reveals only one calcium binding peak. A large amount of protein lacking calcium binding activity was eluted at one void volume, which on disc electrophoresis reveals several proteins.

Of the two protein peaks eluted with 0.1 M phosphate (pH 7.5) only the smaller one possesses calcium binding activity. Material from the initial fraction of the smaller peak showed a single protein band (C′) on disc gel electrophoresis (see Figure 3). The last fractions of the smaller peak contained significant amounts of another protein (band A on electrophoresis, see Figure 3). The larger peak contained mostly another protein (band H on electrophoresis, Figure 3). Thus the order of elution of the three proteins on CM-Sephadex was C′, A, and H.

Further development of the CM-Sephadex column (Figure 2) with 0.1 M phosphate (pH 8.2) eluted another peak, probably hemoglobin because of its reddish color. The last peak from the CM-Sephadex column was eluted with 0.6 M NaCl. At this point all of the applied protein had been accounted for.

Separation of Proteins Associated with Calcium Binding Activity by Means of Gel Filtration. Figure 3 shows the chromatogram resulting from the application of the calcium binding fractions from the CM-Sephadex column to a Bio-Gel P-60 column. Calcium binding activity was located in the shoulder region at about 200-ml elution volume (Figure 3). All three of the major proteins detected by disc electrophoresis of the calcium binding region from the CM-Sephadex chromatogram (bands C′, A, and H) again appeared in the P-60 chromatogram (inset Figure 3). The calcium binding activity region of Figure 3 was rechromatographed on another Bio-Gel P-60 column and its elution profile is shown in Figure 4. Only two major staining proteins remained, *i.e.*, electrophoretic bands A and C′. The protein which eluted at 260 ml did not stain with coomassie Blue. Calcium binding activity appeared to be associated with the second, lower molecular weight protein, *i.e.*, band C′.

Isolation of a Single Protein with Calcium Binding Activity. Figure 5 shows a further purification of the calcium binding protein on Bio-Gel P-20. Activity was now associated with one chromatographic peak. The peak was shown to be essentially one component on disc gel electrophoresis (band C′). The

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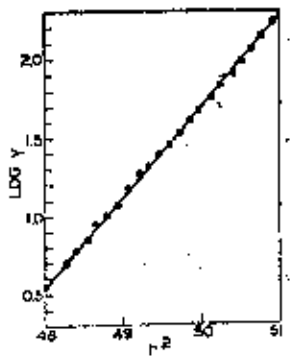

FIGURE 8: High-speed sedimentation equilibrium of purified calcium binding protein. Log y is plotted *vs.* r^2 after the method of Yphantis (1964), where y is the fringe displacement. The sedimentation equilibrium was performed at 12° in a double-sector cell. Equilibrium was achieved after 20 hr at 50,740 rpm. The concentration of protein was 0.5 mg/ml in 0.04 M NaCl, 0.008% β-mercaptoethanol, and 0.05 M Tris-HCl (pH 7.0). Molecular weight was calculated by means of the expression $2RT(2.303)(\text{slope})/(1 - \bar{v}\rho)(\omega^2)$ with the assigned values of $\bar{v} = 0.73$ and $\rho = 1.0$. By this method, the molecular weight of calcium binding protein is found to be 8200.

densitometric tracing (Figure 5) of disc gels in which relatively high concentrations of protein were electrophoresed indicated very small amounts of two higher molecular weight proteins. At lower sample concentrations, these components were not visible.

Ultraviolet Spectrum. Figure 6 shows an ultraviolet absorption spectrum of the purified calcium binding protein of Figure 5. There is a well-defined peak centering at 275 nm, with a slight shoulder at 290 nm.

Sedimentation velocity photographs of the calcium binding protein are shown in Figure 7. Even with the maximum rotor speeds available (59,780 rpm) the separation of the protein boundary from the meniscus failed to occur. However, it appeared that no higher molecular weight proteins were present in the sample.

Molecular Weight by Sedimentation Equilibrium. A sedimentation equilibrium measurement on the binding protein was also performed. A plot of log y *vs.* r^2 (Figure 8) yielded essentially a straight line, indicating the presence of a single component. The molecular weight value obtained from the data was 8200, assuming a partial specific volume of 0.73.

Specific Activity Increase with Purification. Attainment of increased purity as revealed by disc electrophoresis was accompanied by an increase in specific calcium binding activity. Table I summarizes the increase in specific activity of the calcium binding protein accompanying purification. The data concern only the lower molecular weight calcium binding protein which appears following vitamin D administration. Included in the table is the specific activity of the calcium binding region from the G-100 profile for intestinal supernatant taken from vitamin D deficient rats. The specific activity in this case is one-fourth to one-fifth the value for corresponding rats given vitamin D. The purifications illustrated in Figures 1–5 actually represent two routes. A very high degree of purification is achieved by the CM-Sephadex procedure and at least a portion of the isolated protein is homogeneous on disc electrophoresis. However, the yield of protein of this purity is very low. The gel filtration steps with Bio-Gel columns succeed in purifying a larger quantity of material, although the specific activity of the final product is lower, due in part to the limited stability of the calcium binding protein with time.

Discussion

Preparation of Crude Calcium Binding Protein without Ammonium Sulfate or Heat. The Sephadex G-100 chromatographic procedure used in this study as the initial fractionation step appeared superior to the heat or ammonium sulfate fractionation methods used by other investigators because the latter procedures result in an accelerated loss of binding activity during subsequent purification. Such activity loss is a serious problem for the isolation of the protein from rats since the yield in this case is low (perhaps 20–50 μg/rat in these experiments). Also, we have observed that heat and ammonium sulfate treatments result in the artifactual appearance of a new peak at about 1.6 void volumes on Sephadex G-100 chromatograms, apparently the result of disaggregation of large proteins into subunits. Such observations suggest that these fractionation methods may be capable of altering the native state of proteins with calcium binding activity. Accordingly, one possible explanation for the results of Ooizumi *et al.* (1970) may be that they observed a 24,000 molecular weight disaggregation product of the larger (>100,000 molecular weight) calcium binding protein, after heating to 60° for 5 min. Their use of a Diaflo UM-20E membrane for protein concentration indicates that they are studying mainly proteins above 15,000 molecular weight, according to the retention characteristics of the UM-20E membrane (Amicon Publication No. 403, 1970). As mentioned earlier, the work in this paper deals solely with the smaller (13,000 or lower molecular weight) calcium binding protein.

Purification of the Calcium Binding Protein with CM-Sephadex. The CM-Sephadex procedure is very effective in separating calcium binding protein from other contaminating proteins. As can be surmised from Figure 2, the CM-Sephadex method alone can be used for preparing quite pure calcium binding protein if used subsequent to the Sephadex G-100 procedure.

At least five void volumes of each of the acetate buffers must be used for successful operation of the CM-Sephadex column. The use of a lower pH for the first 0.1 M phosphate elution, such as pH 7.0 instead of pH 7.5, results in a more pronounced resolution of the proteins in the calcium binding region. However, it has not been possible to separate the smaller peak completely from the larger, and the calcium binding protein is usually contaminated to some extent with a closely related protein (band A on disc electrophoresis).

Purification by Gel Filtration. The gel filtration procedures using the Bio-Gel columns provide the advantage that proteins similar in size to the calcium binding protein can readily be isolated. This is particularly important since a protein (A on disc gel electrophoresis) believed to be a precursor of the calcium binding protein (Drescher and DeLuca, 1971) can be isolated. That this protein is similar in size and charge to the calcium binding protein is shown by its behavior on CM-Sephadex, Bio-Gel columns, and disc electrophoresis.

The purified calcium binding protein (Figure 5) had only minor impurities which increased on standing at 4°. Minor impurities can readily be detected on the gels since the urea disc electrophoretic system and the staining procedure employed are both extremely sensitive analytical methods (Neville, 1967; Chrambach *et al.*, 1967). The urea used in the gels was passed through a mixed bed ion-exchange resin and recrystallized before use. Purification of the urea in this fashion had no effect on the disc gel electrophoretic results.

Molecular Weight of Calcium Binding Protein. Data from the ultracentrifuge velocity measurement (Figure 7) and equi-

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librium measurement (Figure 8) indicate that the molecular weight of the calcium binding protein is surprisingly small. Making the assumptions listed in Figure 8, a value of 8200 is obtained. On the other hand, the estimate of molecular weight by position of activity on calibrated Sephadex G-100 columns and other gel filtration columns, both in ours and other laboratories, has consistently given values around 13,000. A similar value is obtained by means of calibrated urea disc gels in our laboratory. The question arises as to the reason for the discrepancy between molecular weight values obtained by gel filtration *vs.* ultracentrifugation. The striking linearity of the data of Figure 8 argues against a lower molecular weight contaminant in the ultracentrifuge sample. We therefore investigated the possibility of asymmetry of the molecule.

The calcium binding protein is retained by Amicon Diaflo UM-2 membranes, which are rated to retain proteins of at least 2000 molecular weight (Amicon Corp., 1970). However, the protein is not retained by Diaflo PM-10 membranes which are rated to retain molecules the size of cytochrome *c* (12,400 molecular weight, from horse heart) and above. Yet the rat calcium binding protein migrates behind cytochrome *c* on gel filtration and disc gel systems. When cytochrome *c* standard (horse heart, Sigma, St. Louis, Mo.) was run in exactly the same manner as calcium binding protein for determination of molecular weight, a value very close to its true molecular weight was obtained, further supporting the validity of the low molecular weight value for calcium binding protein from ultracentrifuge data.

The results of molecular weight determination for calcium binding protein can be explained in two ways. First, the molecule may have an ellipsoidal shape, as do the postulated bacterial transport carrier proteins (Pardee, 1968). Secondly, the partial specific volume may be higher than the assumed value of 0.73, as might result from bound lipid. At present, the first hypothesis is favored because of analogy to bacterial membrane carriers, but the question has yet to be resolved.

Specific Activity of Purified Calcium Binding Protein. The increase in specific activity of the calcium binding protein with purification by the gel filtration columns is similar to that described for purification of the chick calcium binding protein (Wasserman *et al.*, 1968). The larger apparent increase in specific activity at the CM-Sephadex step may be due to the following causes. First, the sampling of low concentrations of protein can give overestimates of specific activity. Secondly, the shorter time period involved in two *vs.* five column steps allows less opportunity for loss of activity of the calcium binding protein.

It should also be noted that specific activities are compared to the base value of supernatant fraction and not to whole homogenate. This was done because of the great variability experienced in measuring calcium binding activity in whole homogenate using the Chelex assay. There is also present a relatively large amount of a high molecular weight calcium binding protein not related to vitamin D in rat intestinal homogenate, as discussed earlier. Hence the first actual purification step, namely the 40,000*g* centrifugation, is not included in the calculation of relative specific activity increases. Compared to supernatant values, at least a 57-fold purification of calcium binding activity has been achieved by gel filtration, and several 100-fold purification by ion exchange.

Acknowledgment

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On the Site of Discrimination of Chicks against Vitamin D_2[1]

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Chicks are capable of converting both vitamins D_2 and D_3 at approximately equal rates to the biologically active polar metabolite (peak IV) believed to be the metabolically active form of the vitamin. However, the peak IV metabolite from vitamin D_2 has little vitamin D activity in chicks while that from D_3 is more biologically active than D_3 itself. The peak IV both from D_2 and from D_3 is fully antirachitic in rats suggesting that the chick discriminates against vitamin D_2 at the level of the peak IV metabolite.

In a recent report from this laboratory, it was shown that chicks respond poorly to vitamin D_2 versus D_3 because D_2 is rapidly deleted from blood and other tissues and excreted into the intestine via the bile (1). It was suggested that vitamin D_2 or a metabolite thereof is rapidly rejected as a foreign material reducing its effective lifetime in the animal. In other experiments carried out in this laboratory, the existence of a metabolically active form of the vitamin has been established (2, 3). This metabolite is the major vitamin D–active substance in the body when truly physiologic doses of the vitamin are given (2, 4). Furthermore, it is formed in large amounts before physiologic expression of the vitamin's action is obvious (4). It not only mimics the actions of vitamin D in elevating serum calcium concentration and stimulating intestinal calcium transport, but its effects are more rapid than those of the parent vitamin (3). It is also the major vitamin D–active substance in the nucleus where the vitamin is believed to function to initiate events ultimately leading to increased calcium transport (4, 5). In this report it will be shown that this metabolite is formed from both D_2 and D_3 in chicks but the amount from D_2 in the chick remains at a low level whereas it is usually at high levels in the case of D_3. In addition it will be shown that this metabolite fraction is fully biologically active in rats whether it is derived from D_2 or D_3 from both chicks and rats. However the metabolite from D_2 is only slightly active in chicks whether it is prepared from rat or chick tissues. On the other hand, the metabolite from D_3 is more biologically active in chick intestinal absorption than is the parent vitamin. These results strongly suggest that the discrimination by chicks against vitamin D_2 is at the level of the suggested metabolically active form of the vitamin. A preliminary report of this work was presented before the Federated Societies for Experimental Biology and Medicine (6).

MATERIALS AND METHODS

Preparation of chicks. One day-old white Leghorn cockerels were obtained from the Sunnyside Hatcheries, Oregon, Wisconsin, and housed in an incubator maintained at 37°. They were fed a purified rachitogenic diet as previously described (1). The chicks become severely rachitic on this diet and were used at 4 weeks of age.

Preparation of rats. Young male weanling rats

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were obtained from Sprague-Dawley, Madison, Wisconsin, and were maintained in hanging wire cages. They were fed *ad libitum* a rachitogenic diet (7). After 3 weeks the rats became rachitic and were suitable for the line test assay as described in the U.S. Pharmacopeia (8) as well as for the preparation of vitamin D metabolites.

Biological assays. The amount of each metabolite to be tested was determined on the basis of radioactivity assuming each metabolite to possess the same specific activity as the parent vitamin D_3; i.e., 122 dpm/IU for ^3H D_3 and 98 dpm/IU for ^{14}C D_3.

Rat rickets cure test. This was performed exactly as described for the line test method in the U.S. Pharmacopeia (8). The doses were prepared in ether, dissolved in cotton seed oil (Wesson) and the ether removed with a stream of N_2. Each rat received no more than 0.1 ml of oil.

Chick calcium-absorption assay. The chick bioassay used was similar to that described by Migicovsky (9) with certain modifications. Twenty-eight-day-old rachitic Leghorn cockerels weighing 180-210 g were dosed orally with 0.1 ml of the material to be assayed dissolved in Wesson oil at a concentration of 100 IU/ml. Controls were given the same volume of Wesson oil. Dosed chicks were fasted for 24 hr after which each chick was orally administered 2.3 μCi of ^{45}Ca (Nuclear Science and Engineering Co., Pittsburgh, Penna.) in a volume of 0.2 ml. This ^{45}Ca solution was also 1% in nonradioactive $CaCl_2$. Chicks were sacrificed by ether suffocation exactly 2 hr after administration of label. The tibia of each chick was then removed and dissected until clean. Bones were placed in 25×148-mm Pyrex test tubes and weighed.

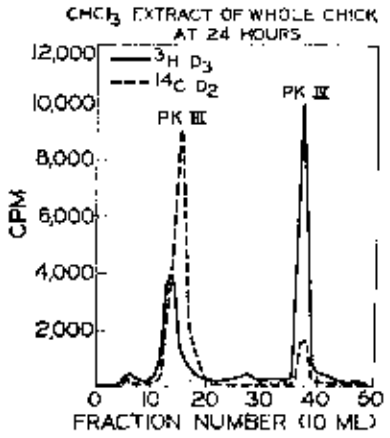

FIG. 1. Silicic acid column profile of the chloroform extract of the entire chick that received either 0.25 μg [1, 2-^{3}H] vitamin D_3 (---) or 0.25 μg U-^{14}C vitamin D_2 (- - - - - -) 24 hr before. Chromatography was carried out as described by Lund and DeLuca (2).

TABLE I

DISTRIBUTION OF ADMINISTERED RADIOACTIVITY FROM 0.25-μg DOSES OF ^3H D_3 OR ^{14}C D_2 AMONG VITAMIN D AND ITS METABOLITES

Time (hr)	Fraction	Vitamin D_3 (% dose)	Vitamin D_2 (% dose)
1	III	80.2 ± 6.8[a]	70.2 ± [illegible]
	IV	8.4 ± 2.1	8.3 ± [illegible]
24	III	10.4 ± 3.2	10.4 ± [illegible]
	IV	42.5 ± 3.8	13.2 ± [illegible]

[a] Standard deviations. There were at least 4 chicks per group. The recovered radioactivity is from the entire chick whose feathers and gastrointestinal tract had been removed. The chromatography was carried out as described previously and shown in Fig. 1.

Radioactive vitamins D_3 and D_2. [1, 2-^{3}H] vitamin D_3 (specific activity 20,000 dpm/IU) was prepared as previously described (10) as was [U-^{14}C] vitamin D_2 (30,000 dpm/IU) (1). For experiments dealing with the metabolic conversion of the vitamins to metabolites, 0.25 μg dissolved in 0.05 ml 95% ethanol was administered intrajugularly to each rachitic chick. For experiments dealing with the isolation of metabolites for a study of their biological activity in both rats and chicks, the radioactive vitamins were diluted with the appropriate nonradioactive vitamin to give a specific activity of 122 dpm/IU for D_3 and 98 dpm/IU for D_2. These were dissolved in ether to which was added Wesson oil. The ether was removed with a stream of N_2 to give a solution containing 2 mg ^3H D_3 or ^{14}C D_2 in 1 ml Wesson oil. One-tenth milliliter of this solution was given orally to a rat or a chick.

Extraction and chromatography of vitamin D metabolites. In the experiments concerned with the proportion of either ^3H vitamin D_3 or ^{14}C vitamin D_2 and their metabolites in the chick after a 0.25 μg dose, chicks were killed by ether suffocation at the indicated times after dosage. The gastrointestinal tract and feathers were removed and the remaining carcass was ground in a meat grinder and then extracted with methanol-chloroform using a Waring Blendor (11). The entire chloroform extract was then chromatographed on silicic acid columns as described previously (2). Then 5 ml of concentrated nitric acid was added to each tube and bones were digested over heating coils. After cooling, each sample was brought to a volume of 25 ml with a

tent solution consisting of 30 vol water, 10 vol ethanol, and 1 vol of the detergent Triton X-100. One-milliliter aliquots were then taken and placed in scintillation counting vials with 15 ml of dioxane counting solution. Vials were counted at 18% gain with a window of 50–1000.

Dioxane counting solution. The ^{45}Ca counting solution was made by dissolving 7.0 g PPO, 300 mg dimethyl POPOP, and 150 g naphthalene in 1500 ml of dioxane. Two hundred fifty milliliters of water containing 18 mg disodium EDTA was then added.

Radioactivity counting. All radioisotopes were counted in a Packard Tri-Carb liquid scintillation counter, Model 3003 (Packard Instruments Corp., LaGrange, Ill.), employing an external standard for counting efficiency determinations. Dioxane counting solution was used for counting ^{45}Ca, while ^{14}C and ^{3}H were counted using toluene counting solution (12).

RESULTS

It has previously been shown in rats that 24 hr after a 0.25 μg dose of 1,2 ^{3}H vitamin D_3 most of the remaining radioactivity is found in the peak IV metabolite fraction. Figure 1 demonstrates that this is the case in chicks as well. This profile also serves as a reference point for the experiments to be reported in this communication.

At 1 hr after injection of 0.25 μg of either ^{3}H D_3 or ^{14}C D_3, 70–80% of the dose is recoverable as unchanged vitamin D (peak III, Fig. 1) while only a small amount appears as peak IV metabolites (Table I). After 24 hr, however, there is little unchanged vitamin D_3 or vitamin D_2. Rather large amounts of peak IV remain in the case of D_3 while small amounts of peak IV are found in the case of D_2. Thus it is clear that peak IV is the major form of the original ^{3}H D_3 administered. These results agree with previous results which showed that the ^{14}C from ^{14}C D_3 disappeared more rapidly from chicks than does ^{3}H from ^{3}H D_3. The major difference lies not in the disappearance of peak III or unchanged vitamin D but rather in the rate of disappearance of peak IV. These data suggest that the chick does not discriminate between D_2 and D_3 at the level of conversion to peak IV but rather at the rate of peak IV catabolism and excretion.

To test this hypothesis directly, peak IV fractions were prepared from both D_2 and D_3 from both rats and chicks. As expected, the peak IV fractions from all these sources were all fully antirachitic by the rat line test assay (Table II) with values of 40 IU/μg.

A biological assay was devised using rachitic chicks as the test animal. That the assay is valid is shown by the response curve of chicks to physiologic dose of vitamin D_3 (Fig. 2). The response recorded was essentially linear between 1–10 IU of vitamin D_3. It is also important to note that the

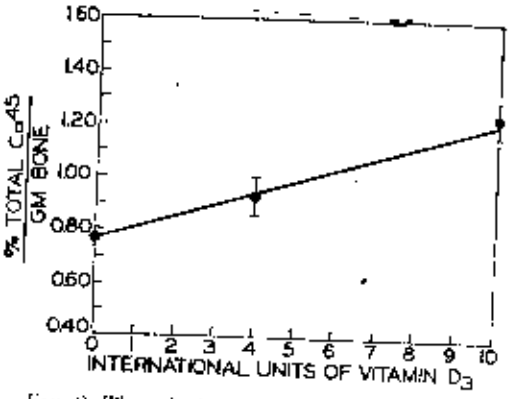

FIG. 2. The relationship of vitamin D_3 dosage and bone uptake of orally administered ^{45}Ca. Vertical bars denote standard error of the mean. The exact method is described in the text.

TABLE II

ANTIRACHITIC EFFECTIVENESS OF PEAK IV METABOLITES IN RATS[a]

Group Vegetable oil control	IU/μg
D_3	40
D_3 Peak IV from rat	40
D_3 Peak IV from chick	42
D_2	40
D_2 Peak IV from rat	39
D_2 Peak IV from chick	41

[a] The scores represent group averages where each group consisted of at least 5 and usually 8 rats. The assays were carried out according to the U. S. Pharmacopeia (8) in which each rat was given the equivalent of 4 IU of antirachitic activity. The data are expressed as international units (IU) of antirachitic activity per microgram of compound tested. Standard cholecalciferol gave 40 IU/μg.

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TABLE III

ASSAY OF VITAMIN D ACTIVITY OF PEAK IV METABOLITES IN CHICKS

Group	% Total $CaCO_3$ bone			Number of animals	IU/μg
	Mean	±	Standard deviation[a]		
Vegetable oil control	0.77	±	0.22	32	- -
D_3 (0.1 μg)	0.93	±	0.27	14	40
D_3 (0.25 μg)	1.21	±	0.25	15	40
D_3 Peak IV (0.25 μg) from rat	1.36	±	0.26	8	50
D_3 Peak IV (0.25 μg) from chick	1.11	±	0.43	9	57
D_2 (10 0.25 μg)	1.02	±	0.18	16	21
D_2 Peak IV (0.25 μg) from rat	0.96	±	0.25	11	16
D_2 Peak IV (0.25 μg) from chick	0.83	±	0.13	5	6

[a] Standard deviations were calculated by means of the expression

$$\left[\frac{\Sigma d^2}{N} - \left(\frac{\Sigma d}{N}\right)^2\right]^{1/2}$$

The figures in parentheses represent the amount of compound given to each animal in the assay group. The data are calculated in terms of international units of antirachitic activity per microgram of compound. Standard cholecalciferol gives 40 IU of antirachitic activity per microgram.

mean response remained quite constant from experiment to experiment.

When the peak IV fractions were assayed for vitamin D activity by the above-indicated assay the data shown in Table III resulted. Of great interest is the fact that the peak IV fractions from vitamin D_3 derived from both rats and chicks were more active than vitamin D_3 itself. However, the peak IV fractions from both chicks and rats obtained from vitamin D_2 were only slightly active in the chick assay. As would be expected, vitamin D_2 itself was also less active. It is interesting to note that peak IV from D_2 isolated from chicks was less active than that isolated from rats. This suggests that the peak IV from chicks may contain some different metabolites than those from rats which may differ in their potency in chicks. However, further confirmation of this will be necessary before such a conclusion can be made.

DISCUSSION

The results of the present study confirm the conclusion reached earlier that vitamin D_2 or a metabolite thereof is rapidly excreted thereby reducing its effective half-time. In the course of this study, the biologically active fraction of peak IV has been positively identified as 25-hydroxycholecalciferol in the case of vitamin D_3 (13, 14). The metabolite which derives from D_2 has not yet been identified, however. Furthermore, strong evidence has been advanced that the 25-hydroxy derivative is the metabolically active form of the vitamin (15). Assuming the 25-hydroxy derivatives as the metabolically active form of the vitamins, it is now possible to suggest a possible mechanism for the discrimination against D_2 by chicks.

It is apparent that the chick can convert both D_2 and D_3 to the biologically active peak IV metabolites. Both are fully active in the rat which does not discriminate between D_2 and D_3. However, the same peak IV metabolite preparation from D_2 is only slightly effective in the chick, suggesting that the peak IV metabolite from D_2 is rapidly degraded and/or excreted. Assuming the 25-hydroxy derivatives as the metabolically active form of the vitamins, it is apparent that the derivative from D_2 would have only a short lifetime in the chick thus accounting for the poor biological activity of D_2 in the chick. Work is now in progress to isolate and identify the biliary excretion products of both D_2 and D_3 in the chick. Preliminary results support the above hypothesis and it appears likely that the curious discrimination against vitamin D_2 by chicks will soon be elucidated at the molecular level.

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In Vitro Studies of Vitamin D-Induced Aortic Calcification[1] (34148)

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The arterial calcification induced by the administration of large doses of vitamin D has been known and studied for many years. In the course of these studies, a great deal of morphological, histochemical, and biochemical data have accumulated which have been largely descriptive in nature (1-4). More recently, as new data have accumulated on the metabolism of Vitamin D and its mode of action, the manner in which this vitamin exerts its effects on osseous and calcium metabolism has been greatly clarified (5, 6). But with regard to the arterial calcification induced by hypervitaminosis D, two central problems remain. The first is whether the vitamin or one of its metabolites exerts a direct effect on the artery or whether the calcification is entirely a consequence of secondary changes in the composition of the extracellular fluids. The second is what factors are responsible for the peculiar and predictable distribution of arterial disease which hypervitaminosis D has in common with many other arteriopathies including human atherosclerosis (2). One approach to the study of this problem might be to assess the effects of sera of various compositions on arterial segments *in vitro*, isolated from general body metabolism. We here report the results of such a study which disclosed that the serum levels of calcium and vitamin D are both important determinants of the accumulation of calcium by such arteries and that the susceptibility of different arterial segments to calcification *in vitro* paralleled their susceptibility *in vivo* to some extent.

Materials and Methods. Two hundred g male Sprague-Dawley rats were used in all experiments except those involving rachitic animals. Sera were obtained by aortic puncture from animals lightly anesthetized with ether. Hypervitaminosis D was produced by injecting 200,000 units of Vitamin D_3 intraperitoneally three times weekly for 3 weeks. These rats, which were hypercalcemic and had extensive aortic calcification were the source of hypervitaminotic serum. Rachitic rat serum was obtained from 75 g male rats maintained for 3 weeks on Steenbock's rachitogenic diet no. 2 (7). Control sera for these experiments were from pair-fed animals of comparable weight on a stock diet. Two types of experimental systems were used. The first was an organ culture method in which 2-mm thick rings of thoracic aortas of rats were dissected free of surrounding connective tissue and placed on strips of cellulose acetate surgical burn dressing overlying metal rafts in Falcon organ culture dishes containing 2.2 m of a nutrient medium consisting of 40% rat serum and 60% NCTC 109 tissue culture medium supplemented with 1000 units of penicillin and 1000 units of streptomycin/ml. The serum was from normal rats or rats with hypervitaminosis D. The cultures were refed three times weekly and were maintained at 37° in an atmosphere of 95% O_2 and 5% CO_2. After 1 week in culture, some rings were fixed in formalin, embedded in paraffin, and sections were stained with hematoxylin and eosin or the von Kossa reagent. Others were dried to constant weight at 104°, weighed, and ashed in sulfuric acid. Calcium was measured with an atomic absorption spectrophotometer and phosphate by the method of Fiske and Subba Row (8).

Since it soon became apparent that simple incubation of arteries in serum yielded results essentially similar to those obtained with organ culture, the method of Martin *et al.* (9) was used for most of the experiments. In this technique, segments of aortic arch,

[1] Supported in part by grants from the U. S. Public Health Service (HE 06713) and the Otho S. A. Sprague foundation

thoracic aorta, or abdominal aorta were dissected free of surrounding connective tissue, weighed and placed in 0.2 ml of serum/mg of wet weight of tissue. The serum contained 1000 units of penicillin and 1000 units of streptomycin/ml. The tubes in which the experiment was done were gassed with 95% O_2–5% CO_2, capped, and incubated at 37°. After the test period was complete the arteries were removed and chemical and histological studies were done in a manner identical to that used for the organ cultures.

Results. Organ cultures. With this technique the aortas appeared to be histologically viable, although small areas of focal necrosis were found even in control specimens, particularly at the sites of intercostal artery origin. Calcification was uncommon in control specimens cultured in medium supplemented with normal rat serum. In specimens cultured in medium supplemented with the serum of rats with hypervitaminosis D, calcification was uniformly much more extensive than in controls and occurred mostly in elastic tissue fibers and later in adjacent smooth muscle cells which were necrotic. Calcification was not observed in viable areas of the explant. There was no significant proliferative cellular response to these calcified areas which were largely in the media, often near the adventitial surface, and particularly advanced at sites of branching of intercostal arteries. This localization was perhaps related to the injury produced by the trauma of dissecting the surrounding periadventitial connective tissue during preparation. In keeping with the histological observations, chemical measurement showed that there was much more calcium in cultures maintained in medium containing serum from rats with hypervitaminosis D than in control cultures (Fig. 1).

Serum incubations. In these preparations histological study indicated that by the second day of incubation, necrosis had begun and after 72 hr was virtually complete. When a timed study of calcium accumulation was done, the greatest increment of calcium accumulation occurred between 48 and 72 hr after incubation was begun if normal rat serum was used. In keeping with the observations of Martin *et al.* (9), calcification was virtually restricted to elastic tissue lamellae when the vessels were studied histologically. Arteries incubated in serum from rachitic rats accumulated less calcium and phosphate than those incubated in control sera. Table I shows the results of this experiment. Except for experiments using rachitic serum, the results depicted in each Tables I–IV were reproduced in three repeated experiments. The PO_4 values varied similarly to those of calcium. When serum from normal rats and those with hypervitaminosis D were compared, it was found that if incubation was performed in serum from hypervitaminotic rats there was greater accumulation of both calcium and phosphate than with control serum (Table II).

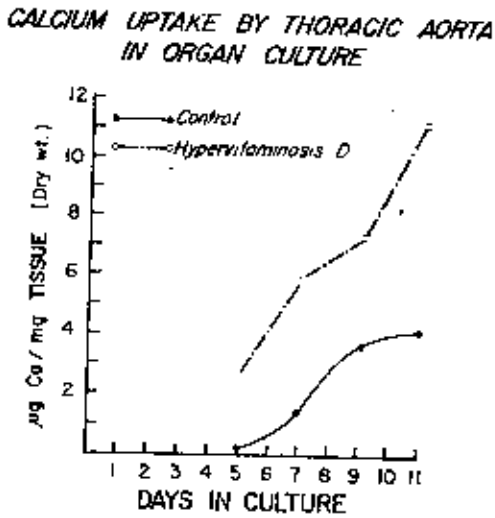

FIG. 1. Arteries maintained in organ culture in medium supplemented with serum from rats with hypervitaminosis D accumulate more calcium than those maintained in medium supplemented with normal rat serum.

TABLE I.[a]

Serum	No. of samples	Mean[b]	SD[c]
Rachitic	4	0.0019	0.00037
Normal	4	0.0065	0.0022

[a] Thoracic aortas of normal rats were incubated in serum for 72 hours. They accumulated more calcium from normal than from rachitic serum.

[b] (mg of Ca/mg of dry wt).

[c] Standard deviation.

TABLE II.[a]

Serum	No. of samples	Mean[b]	SD[c]
Normal	6	0.0017	0.0011
Hypervitaminosis D	6	0.0073	0.0018

[a] If thoracic aortas of normal rats were incubated for 72 hr in serum from normal rats or rats with hypervitaminosis D, they accumulated more calcium from hypervitaminotic serum.

[b] (mg of Ca/mg of dry wt).

[c] Standard deviation.

When large amounts of Vitamin D_3 (50 or 100 units/ml of serum dissolved in 0.01 ml of EtOH) were added to the serum and these sera compared with serum containing an equal weight of cholesterol dissolved in a similar amount of alcohol, no difference in the amount of calcium accumulated by either thoracic aorta or aortic arch was demonstrable. In agreement with the data of Martin *et al.* (9), addition of sufficient $CaCl_2$ to raise the level of serum calcium by 5 mg/100 ml also did not increase the amount of calcium accumulation. However, when both vitamin D and calcium were added to the serum in these amounts and comparisons made with serum containing equivalent amounts of cholesterol and NaCl, arteries incubated in the serum with added Vitamin D and calcium accumulated more calcium and phosphate than those incubated in control sera (Table III). Here, measurements were made both 48 and 72 hr after incubation was begun and the differences were present at both time intervals.

In experiments in which different segments of the aorta were compared, it was found that when segments of aortic arch, thoracic aorta or abdominal aorta were incubated in either normal serum or serum from rats with hypervitaminosis D and calcium measured in the arteries after 72 hr of incubation, aortic arch accumulated the most calcium, thoracic aorta slightly but not significantly less, abdominal aorta the least (Table IV). Calcium was measured in one set of arteries before incubation and no differences in calcium content of the three segments were found. Since histological studies indicated that calcification was virtually restricted to elastic tissue in these preparations, the amount of elastic tissue was measured in these portions of the aorta. This was done by the method of Hass (10) which uses warm 88% formic acid to digest the tissue elements which are not elastic. The isolated elastic tissue was then blotted dry, weighed, and the value was expressed as a percentage of the wet weight of the tissue. Table IV shows that the amount of elastic tissue in these three aortic segments paralleled the calcium accumulation in this incubation system.

Discussion. Superficially, at least, these data appear to provide clear-cut evidence that the composition of the serum is of primary importance in the pathogenesis of the arterial calcification induced by hypervitaminosis D and that both the hypercalcemia

TABLE III.[a]

	48 hr			72 hr		
	No. of samples	Mean[b]	SD[c]	No. of samples	Mean[b]	SD[c]
Serum + NaCl + cholesterol	6	0.0014	0.002	6	0.0018	0.0007
+ Ca + cholesterol	6	0.0015	0.0016	6	0.0023	0.0005
+ Vit. D + NaCl	6	0.0007	0.0005	6	0.0023	0.001
+ Ca + Vit. D	6	0.0048	0.0005	6	0.0042	0.0007

[a] As described in the text, addition of Vitamin D or calcium alone to serum did not influence the accumulation of calcium by thoracic aortas when incubated in such serum. If both calcium and Vitamin D were added, the aortas bound more calcium after either 48 or 72 hr of incubation.

[b] (mg of Ca/mg of dry wt).

[c] Standard deviation.

TABLE IV.[a]

Segment	Calcium accumulation			Elastica (%)		
	No. of samples	Mean[b]	SD[c]	No. of samples	Mean[b]	SD[c]
Arch	8	0.007	0.0001	5	70.0	6.4
Thoracic	8	0.006	0.0001	5	62.4	7.4
Abdominal	8	0.002	0.0005	5	23.8	6.0

[a] The amount of calcium accumulation by different arterial segments parallels the amount of elastin they contain.

[b] (mg of Ca/mg of dry wt).

[c] Standard deviation.

induced by Vitamin D and a direct local action of the vitamin itself are synergistic in contributing to the development of the lesion. There are, however, several qualifying points which detract from the strength of the data presented here. The first is the nature of the experimental system used. In it, whether simple serum incubation or an organ culture system was used, calcification was virtually restricted to areas of necrosis and even here was found restricted to elastic tissue fibers. In addition, even in the organ cultures, the distribution of calcification was primarily in the deep media in contrast to what is seen *in vivo*, where calcification usually begins in the inner elastic lamnelae in major arteries (2). Even *in vivo*, however, calcification of viable smooth muscle cells is an uncommon event in the arteries which calcify under the influence of Vitamin D and elastic fibers are the first elective site of calcification (3). The differences in the mural pattern of calcification *in vivo* and *in vitro* are perhaps related to hemodynamic factors. Since the rat aorta contains no vasa vasora (11), its nutrition is derived from the luminal surface in an intact animal. In our systems, there was no flow or pressure and the nutrition of the tissue was by diffusion from all its surfaces. The adventitial trauma produced during dissection may also have contributed to the localization of the disease.

The observation that serum from hypervitaminotic animals or normal sera enriched with both vitamin D and calcium induced increased accumulation by the arteries as early as 48 hr after incubation was begun suggests that this was a true metabolic effect rather than something more nonspecific because necrosis was not yet advanced at this time.

The dosages of vitamin D added to the serum in these experiments was massive, and it might be argued that they are too far beyond the physiological range to be meaningful. However, this study was not designed to investigate the normal action of vitamin D but its toxic effects. It is more pertinent to point out that it has now been established that Vitamin D is normally converted in the body to 25-hydrocholecalciferol, a compound which has now been isolated and shown to be much more metabolically active than vitamin D itself *in vivo* (5) as well as in studies of organ cultures of bones (6). If it were used in a system such as this one, it might be possible to demonstrate an effect on arteries in lower dosage, and perhaps even without the addition of excess calcium to the serum.

In vitro, the arteries exhibited much the same sequence of calcification as they do *in vivo*, where calcification begins in the aortic arch and then spreads centrifugally (2). As expected, since the calcification was localized in the elastic tissue, the amount of elastic tissue in the artery paralleled the amount of calcium accumulation. The segmental distribution of many arterial diseases has often been considered to be related to local hemodynamic differences (11). These data, as do some others in the literature in studies of other arterial diseases (11) suggest that part of the hemodynamic effects on the distribution of arterial diseases are not primary, but are related to the morphological modifications

which pressure, flow, or turbulence induce in arteries both during embryonic development and in the adult. For example, elastic tissue develops most extensively in areas of pulsatile flow, and there is experimental data indicating that pulsatile flow through tubes inserted under rat skins can induce alignment of proliferated connective tissue elements into an organized structure having some of the morphological organization of an artery (12).

Summary. Arterial segments incubated in a culture system containing serum from rats with hypervitaminosis D accumulate more calcium than segments incubated in a system containing normal rat serum. If such arterial segments are incubated in serum, they accumulate more calcium from normal serum than uremic serum and more from serum from rats with hypervitaminosis than from normal serum. Addition of either vitamin D or calcium alone to normal serum does not result in excess calcium accumulation by arteries incubated in such serum, but addition of both does. Different aortic segments accumulate different amounts of calcium, apparently in relation to the proportion of elastic tissue in the segments.

We wish to thank Dr. G. M. Hass for his encouragement and support.

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DAIRY PRODUCTS

Chemical Method for the Determination of Vitamin D in Evaporated Milk With Elimination of Cholesterol by Digitonin Precipitation

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The colorimetric method of Jones *et al.* for the determination of vitamin D in evaporated milk, based on the USP XVI method, was modified to obtain a simpler colorimetric measurement and more accurate results. The main modification is the elimination of nearly all cholesterol, according to the procedure of Den Heeder for the determination of the sterol content of fats, by precipitation with digitonin in the saponified mass. The Florex column, used in the procedure of Jones *et al.* for the elimination of decomposition products of vitamin A, was omitted since it gave high blank values; an alumina column was substituted. The resulting method, when applied to samples of evaporated milk to which a known quantity of vitamin D had been added, showed a recovery of 78% with a standard deviation of 6%, using a calibration curve based on crystalline vitamin D. Data from these measurements, as well as data from vitamin D determination on several brands of fortified and unfortified evaporated milk, are presented.

In the method by Jones *et al.* (1) for the determination of vitamin D in evaporated milk, the proteins are dissolved and the vitamin D content of the extracted fat is determined by a slightly modified USP procedure for multivitamin preparations. The USP method (2) proceeds as follows: isolation of the unsaponifiable matter, according to Wilkie *et al.* (3); partition chromatography on a Celite-polyethylene glycol 600-isooctane column, eliminating vitamin A, according to Theivagt and Campbell (4); adsorption chromatography on a Florex XXS column to eliminate decomposition products of vitamin A, according to Schmall *et al.* (5); colorimetric determination, using acetic anhydride as color inhibitor for the reaction of antimony trichloride with vitamin D, together with internal standards and measurement at a second wavelength to correct for interferences in the color development and a strong background color, according to Wilkie *et al.* (3). This USP method is rather complicated and several authors, in examining vitamin preparations, found varying results and proposed alterations (2, 6, 7).

In preliminary determinations with the modified USP procedure (1) in evaporated milk, we found a continuously rising absorbance for the antimony trichloride color reaction; this resulted in too high and rather varying results for the vitamin D content. Since Morton (8) has noted that cholesterol, ergosterol, and other common congeners of vitamin D also develop a color with antimony trichloride, although more slowly, the increasing color in the vitamin D determinations in evaporated milk was ascribed to a large amount of cholesterol in relation to vitamin D. This supposition was confirmed by determinations on solutions of pure vitamin D and cholesterol; *see* Table 1.

Another disturbing factor was that the benzene extract of the Florex used gave a red-brown color with antimony trichloride.

We tried the procedure of Barua and Rao (9) in an attempt to eliminate the Florex column and

* J. Ass. Offic. Anal. Chem.

Table 1. Color reaction with antimony trichloride of pure cholesterol-vitamin D solutions

Time after Adding Reagent, sec	Extinctions; Cholesterol:Vit. D, mg:IU/ml				
	40:128	20:128	4:128	0.4:128	0.04:128
15	0.46	0.32	0.180	0.173	0.170
20	0.59	0.37	0.187	0.173	0.171
30	0.79	0.47	0.201	0.175	0.171

to obtain an assay sample solution with less cholesterol. In this method only one column of deactivated alumina containing 8% water is used. After development a specific band containing vitamin D is taken from the column; vitamin A is converted to anhydrovitamin A before chromatography. The results were not entirely satisfactory; the elimination of vitamin A and/or its decomposition products, as well as elimination of cholesterol, was insufficient.

We then investigated whether the cholesterol could be precipitated with digitonin in the soap solution of the second saponification, according to the procedure of Den Herder (10–12), without co-precipitation of vitamin D. It appeared that vitamin D is precipitated only at higher concentrations and does not co-precipitate with cholesterol digitonide in the low concentrations in evaporated milk. A larger (1.5 times) amount of digitonin, as prescribed by Den Herder (11, 12), proved to be useful for a more complete precipitation of cholesterol.

Determinations with this digitonin precipitation, followed by chromatography on Celite-polyethylene glycol and alkaline alumina (2), gave satisfactory assay sample preparations. In the colorimetric measurements nearly no alteration in absorbance was found after the color with vitamin D had reached its maximum intensity.

The blank values in this procedure were considerably lower than with the original method. Therefore, it seemed advantageous for increased accuracy to omit the internal standard in the colorimetric measurement (7) and to double the final concentration (2) by dissolving the assay sample preparation in 2.5 instead of 5 ml.

METHOD

Apparatus

(a) *Distillation apparatus.*—Fitted with 250 and 500 ml evaporation flasks for vacuum distillation.

(b) *Columns.*—*Celite column:* 2.5 × 25 cm glass tube, with flared top section, constricted to 0.8 cm diameter at the lower end and fitted with glass stopcock. *Alumina column:* 1.3 × 17 cm glass tube, with 250 ml bulb at upper end, constricted to 0.8 cm diameter at lower end and fitted with glass stopcock. A constriction should be made 6 cm above stopcock.

(c) *Ultraviolet lamp.*—Should have weak radiation in 300 mμ region.

(d) *Spectrophotometer.*—Coleman Universal.

Reagents

(a) *Potassium hydroxide solution.*—50%. Dissolve 780 g KOH in distilled water and dilute to 1 L.

(b) *Digitonin solution.*—1.5%. Dissolve 3.75 g digitonin in 250 ml 80% alcohol.

(c) *Celite 545.*—Johns-Manville, N.Y.

(d) *Alumina.*—Basic (Woelm), for chromatography.

(e) *Standard solutions.*—*Vitamin D solution:* Dissolve 25 mg crystalline vitamin D_2 (calciferol, British Drug House) or vitamin D_3 (cholecalciferol) in 100 ml isooctane (standard I). Pipet 5 ml of this solution into 50 ml volumetric flask and dilute to mark with isooctane (standard II). One ml contains about 1000 IU vitamin D (1 μg vitamin D equals 40 IU or USP units). *Vitamin A solution:* Saponify 50 mg synthetic vitamin A (Dohyfral-A-palmitate, Philips-Duffat) in 30 ml 96% alcohol with 2 ml KOH solution by boiling 30 min under reflux in electric heating mantle. Dilute with 30 ml distilled water and cool to room temperature. Transfer solution to separatory funnel and extract three times with 50 ml ether each time. Combine ether extracts and wash with distilled water (50 ml portions) until neutral to phenolphthalein. Dry ether extract with anhydrous Na_2SO_4. Evaporate to dryness under vacuum in current of nitrogen. Dissolve residue in 25 ml isooctane. These standard solutions should be stored in refrigerator.

(f) *Color reagent.*—*Solution A:* Dissolve contents of 100 g bottle of dry, crystalline antimony trichloride in 1,2-dichloroethane and dilute to 443 ml. *Solution B:* Add 10 ml acetyl chloride to 40 ml 1,2-dichloroethane and mix. *Color reagent:* Mix 45 ml solution A and 5 ml solution B. This mixture should be used within seven days.

(g) *Color inhibitor.*—Mix equal volumes of acetic anhydride and 1,2-dichloroethane.

Preparation of Columns

Swirl 100 ml isooctane and 25 g Celite 545 in screw-capped, wide-mouth bottle until slurry is formed. Add dropwise, with shaking, 10 ml polyethylene glycol-600. Close bottle and shake vigorously. Insert plug of cotton wool at point of constriction of 2.5 × 25 cm tube. Open stopcock and transfer half of slurry to tube. Use glass rod to remove any

air pockets which may have formed. Then apply gentle suction, at the same time firmly packing column with 20 mm disk plunger, until nearly dry. Remove vacuum and add remainder of slurry. Apply suction, using disk plunger as before. Remove vacuum when column is nearly dry. Finally press with disk plunger to form solid surface. Add isooctane until saturation; maintain 0.5 cm isooctane above surface.

Weigh 10 g alumina into conical flask provided with ground-glass stopper and add 0.8 ml distilled water (8%). Close flask, shake until powder is free-flowing again, and cool. Fill column with isooctane to bulb and insert plug of cotton wool at constriction of tube. Pour alumina in slow stream into tube and allow to settle. Open stopcock and let isooctane drain; maintain 0.5 cm isooctane above surface of column.

Columns should be prepared shortly before use.

Procedure

The entire analysis should be done in dimmed light for best results.

First saponification.—Weigh 400 g sample of evaporated milk into 800 ml beaker. Add successively small quantity of hydroquinone, 100 ml 96% ethanol, and 50 ml 50% KOH solution. Heat under nitrogen in electric mantle at temperature not to exceed 60°C. Stir continuously until liquid turns dark brown. Cool in running water and transfer liquid quantitatively to separatory funnel. Extract successively with 250 and 200 ml ether. Evaporate solvent under vacuum in water bath at 30–40°C in current of nitrogen.

Second saponification and digitonin precipitation of sterols.—Add small quantity of hydroquinone and 16 ml 50% KOH solution to the remaining 40–50 ml fat and alcohol. Fill flask with nitrogen and saponify sample a second time by boiling gently 20 min under reflux in electric mantle. Swirl flask occasionally to remove some ether until liquid is homogeneous. Immediately after saponification dilute with 60 ml 96% ethanol. Transfer quantitatively to 750 ml conical flask with 120 ml distilled water and 300 ml 96% ethanol. Heat this diluted solution to 50°C, add 30 ml 1.5% digitonin solution, and mix. Slowly cool liquid and store in refrigerator overnight.

Isolation of remaining unsaponifiables.—Filter precipitate of cholesterol digitonide by suction on Büchner funnel and dilute filtrate with 750 ml water. Rinse flask, wash precipitate, and transfer to 3 L separatory funnel with part of this water. Extract diluted solution three times with 500, 200, and 100 ml ether, shaking 45 sec each time. Collect extracts in 1 L separatory funnel and then wash with water as follows: Add 200 ml water to ether extracts (add water so that it settles through ether, rather than pouring down side). Do not swirl or shake. Draw off aqueous layer. Add 10 ml water, shake, add additional 100 ml water without shaking as before, and draw off aqueous layer. Wash extract with 50 ml water, shake well, and add, as above, additional 100 ml water. Without further shaking, draw off aqueous phase. Repeat, washing extract as above. Continue to wash extract but with 100 ml portions of water, with shaking, until neutral to phenolphthalein. Dry washed extract with ca 25 g anhydrous Na_2SO_4, preferably overnight in refrigerator. Decant dried solution into distillation flask and wash Na_2SO_4 with some ether. Evaporate to dryness under vacuum under nitrogen in water bath at 30–40°C. Completely dry residue with stream of nitrogen; add, if necessary, some acetone to facilitate removal of traces of water.

Chromatography on Celite.—Dissolve residue in 4 ml isooctane, add 1 ml standard vitamin A solution, and mix. Open stopcock of Celite column. Just as isooctane enters column, quantitatively transfer sample solution onto column. Rinse flask with three 5 ml portions of isooctane, adding each succeeding portion as preceding one enters column. Collect effluent in 250 ml evaporation flask. Continue adding isooctane until front of fluorescent band (UV light) of vitamin A is 0.5 cm from bottom of column (usually 100–125 ml isooctane is required). Maintain flow rate between 2 and 5 ml/min, using stopcock. Heat collected effluent in water bath below 50°C and evaporate solvent in vacuum under nitrogen until volume is ca 5 ml.

Chromatography on alumina.—After cooling, quantitatively transfer entire 5 ml onto alumina column and rinse flask with three 5 ml portions of isooctane in same way as indicated with Celite column. Fill bulb of tube with 200–250 ml isooctane, depending on position of vitamin D on column. Adjust flow rate at 2–3 ml/min, using stopcock.

Development of column gives three colored zones, from top to bottom: (a) yellow-orange zone 1 cm long, at 2 cm from top; (b) faint pink zone 0.5 cm long, at 1 cm below zone (a); (c) pale yellow band, 0.2–0.5 cm below pink zone and 3 cm from bottom of column. (With some types of isooctane, separation of zones a and b is sometimes incomplete.)

Under UV light, the yellow-green fluorescent zone, overlapping the pink zone to a large extent, contains all the vitamin D. The extract of this zone, treated with antimony trichloride, shows a disappearing blue color, followed by the yellow-rose color of vitamin D, which reaches its maximum in 30 sec. The extract of the yellow-orange zone and a part of the adjoining colorless zone, treated with antimony

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trichloride, shows an increasing red color, indicating the presence of cholesterol.

Isolation and colorimetric determination of vitamin D.—Remove most of solvent by suction and take fluorescent zone from column with small glass spatula by scooping up successive portions beginning at top of column. Transfer this section quantitatively to small beaker and elute with three 5 ml portions of ether. Collect eluates in 25 ml conical flask and evaporate to dryness on water bath under nitrogen at 30–40°C. Dissolve residue in 2.5 ml 1,2-dichloroethane and immediately pipet 1 ml solution into each of two matched colorimeter tubes (19 mm od). Then pipet 1 ml 1,2-dichloroethane into tube 1 and 1 ml color inhibitor into tube 2. Quickly add 5 ml color reagent to tube 1 and mix. Exactly 30 sec after addition of color reagent, determine absorbance at 500 mμ against 1,2-dichloroethane; exactly 90 sec after addition of color reagent determine absorbance at 550 mμ. Determine absorbance of solution of tube 2 similarly. Designate absorbances found without color inhibitor (tube 1) as A^1_{500} and A^1_{550} and those with color inhibitor (tube 2) as A^2_{500} and A^2_{550}. Calculate corrected value of absorbance at 500 mμ (A_{500}) by formula:

$$A_{500} = (A^1_{500} - A^2_{500}) - 0.67(A^1_{550} - A^2_{550}).$$

Evaluate corresponding amount of vitamin D (IU/ml) from calibration curve. Calculate vitamin D content of sample as:

IU/fluid oz = IU/ml × 0.196
IU/100 g = IU/ml × 0.625
μg/100 g = IU/ml × 0.0156

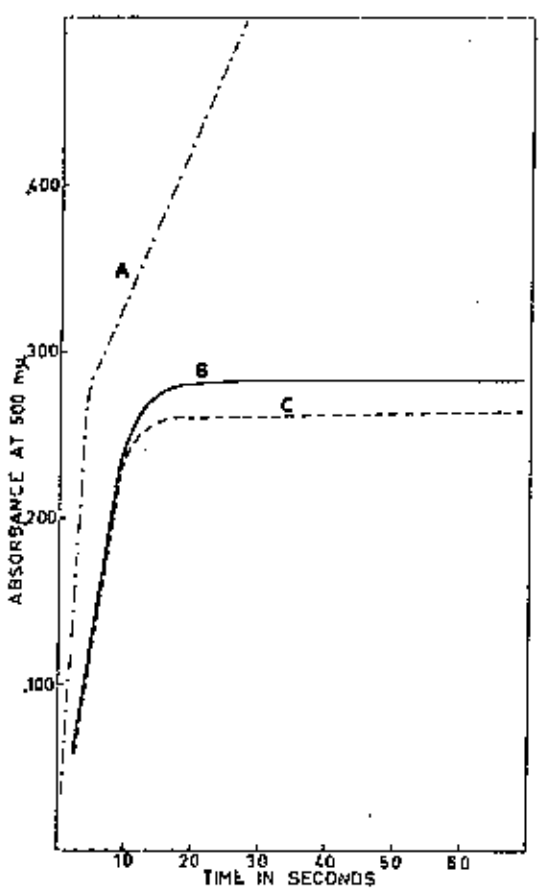

FIG. 1—Color development of pure and assay solutions of vitamin D with antimony trichloride: A, assay solution from Jones et al. method; B, pure vitamin D solution; C, assay solution from modified method.

Calibration Curve

Pipet 1 ml standard II into small beaker; evaporate to dryness under nitrogen. Dissolve residue in 5 ml 1,2-dichloroethane. Check potency of this solution by spectrophotometry at 270 mμ against 1,2-dichloroethane, using two matched 1 cm cells:

A_{270} × 4500 = IU vitamin D_2/ml standard
A_{270} × 4400 = IU vitamin D_3/ml standard

Pipet 0.0, 0.1, 0.2, 0.3, and 0.4 ml standard II in duplicate into ten previously matched colorimeter tubes (19 mm od). Determine corrected absorbance for quantity of vitamin D as follows: Evaporate solvent of two corresponding tubes under nitrogen; dissolve residue of one tube in 2 ml 1,2-dichloroethane and residue of other tube in 1 ml 1,2-dichloroethane and 1 ml color inhibitor. Quickly add 5 ml color reagent to one tube and measure absorbances as described above. Determine absorbances of other tube similarly. Calculate corrected absorbance at 500 mμ as indicated above. Plot values of A_{500} against IU vitamin D in 0.0, 0.1, 0.2, 0.3, and 0.4 ml standard II. Plot should be linear with concentration; deviations should be less than 7%.

Results and Discussion

Development

The color development with antimony trichloride (A^1_{500}) of a solution of pure vitamin D and of assay sample preparations of fortified evaporated milk is shown in Fig. 1. Assays by the original and the modified method are compared. The assay sample prepared according to the modified procedure gives nearly the absorbance curve of a pure vitamin D solution. The somewhat lower values must be ascribed to losses in the cleanup procedure. The absorbances for the assay sample prepared according to the original method are higher and show a strong continuous rise.

The pure vitamin D solution shows a maximum absorbance in 30 sec, while the absorbance of the assay sample is nearly constant 25 sec after the

Table 2. Recovery of added vitamin D to unfortified evaporated milk by proposed method

Sample			Units Vit. D/fl. oz				
No.	Date	Factory	Unfort. Prod., Found	Added	Fort. Prod., Found	Rec.,	%
1[a]	6/21/68		0	84	74	74	88
2	7/ 4/68	A	1	38	33	32	84
3	7/10/68	B	5	40	37	32	80
4	7/20/68	B/C	6	81	67	61	75
5	7/23/68	C	7	53	45	38	72
6	7/25/68	C	7	92	78	71	77
7	8/22/68	D	3	25	24	21	84
8	8/29/68	F	7	28	28	21	75
9	12/24/68	G	1	56	40	39	70
10	1/13/69	G	2	56	44	42	75
Av. recovery							78
Standard deviation[b]							6

[a] Butterfat sample.

[b] $s = \sqrt{\frac{\Sigma(x - \bar{x})^2}{n - 1}}$

addition of antimony trichloride reagent. Therefore, the 30 sec absorbance reading was taken as a measure for the vitamin D content. The absorbance/time curve of assay sample preparation is not always so perfectly horizontal as in the graph. In many cases a continuous slow rise is observed, and the absorbance is (somewhat) higher than that of a corresponding pure vitamin D solution. We ascribed this to impurities caused by a less than perfect separation on the alumina column. These varying quantities of impurities necessitate a correction for non-vitamin D color. The colorimetric measurement at 500 and 550 mμ, without and with acetic anhydride as color inhibitor (3), was therefore retained.

Recovery

Recoveries were tested with unfortified evaporated milk from seven factories. The results of single determinations on the unfortified product and the product to which a known quantity of pure vitamin D was added are chronologically listed in Table 2. According to these figures the recovery is 78% with a standard deviation of 6%. The recovery is less than those found by Jones *et al.* (103%), but seems acceptable in view of the many steps in the procedure. The standard deviation of the recovery is, however, distinctly lower than that by the Jones *et al.* method, viz., 6% against 12%.

The recovery experiments indicate that extremes of 7 units of vitamin D/fluid ounce can be expected between duplicates of a sample of normal fortified evaporated milk containing 25 IU/fluid ounce. The difference will probably be smaller because the standard deviation of the recovery is for differences between vitamin D content of fortified and unfortified products. Values of duplicate determinations given in Table 3 support this supposition.

Natural Vitamin D Content of Evaporated Milk

Table 4 gives a chronological listing of the results of vitamin D determinations with the modified method on nonfortified evaporated milk samples of several Netherlands factories, including samples from the recovery experiments. The dates of determination are generally a few weeks after production. The values in the table are generally rather high compared to those found for cows' milk in animal tests (13). The range of these values in the literature is, however, the same as found in our chemical tests. Karg (13)

Table 3. Reproducibility of proposed method, duplicate determinations

Sample			
No.	Factory	Units Vit. D/fl. oz	
130/131	A	31	31
141/142	B	25	29
149/152	C	27	29
151/154	D	31	34
155/156	E	25	22
157/158	F	25	26
Standard deviation[a]		1.8	

[a] $s = \sqrt{\frac{\Sigma(x_1 - x_2)^2}{2n}}$

Table 4. Vitamin D content of several brands of unfortified evaporated milk, according to proposed method

Sample			Fat Cont., %	Vit. D, Units/fl. oz
No.	Date	Factory		
94	6/27/68	A	7.8	3
97	7/ 8/68	A	7.8	4
100	7/16/68	B	9.4	5
101	7/22/68	C	9.0	7
105	8/22/68	D	7.8	3
108	8/29/68	F	8.0	7
117	9/13/68	G	7.8	2
129	11/19/68	H	7.8	4
132	11/26/68	H	7.8	3
140	12/24/68	G	7.8	1
143	1/13/69	G	7.8	2
146	2/12/69	I	9.0	4.5
Average				3.8

has summarized the data found in the literature as follows:

Cows' Milk Obtained From:	*IU Vitamin D/kg*
Cows indoors in winter	2.8–20
Cows outdoors in summer	20–48
Cows outdoors in summer, Alpine pastures	48–108

These figures of Karg correspond largely with those given in 1965 by the American Dairy Science Association (14), viz., winter milk average 13.0 IU/quart (range 3–23), summer milk average 31.2 IU/quart (range 12–43).

Since 1 L of regular milk with 3.8% fat and 8.7% fat-free solids (density 1.03) gives 16 fluid ounces (at 29.57 ml or 31.35 g) of evaporated milk with 7.9% fat and 18.0% fat-free solids (density 1.06), the range of vitamin D values for cows' milk, given by Karg, corresponds with 0.2–7 IU/fluid ounce for evaporated milk. An average of 13.0 IU/quart (=13.7 IU/L) for winter milk corresponds with 1 IU/fluid ounce of evaporated milk in winter; an average of 31.2 IU/quart (=33.0 IU/L) for summer milk corresponds with 2 IU/fluid ounce of evaporated milk in summer.

Vitamin D Content of Fortified Evaporated Milk

Table 5 gives results of vitamin D determinations according to the proposed method on a number of fortified evaporated milk samples of several Netherlands factories, along with the declared content. Taking into account that the recovery of the proposed method is about 80%, it appears that, with two exceptions, the samples contained at least the declared quantity of vitamin D. In several cases the experimentally found vitamin D contents were distinctly higher than those recorded on the label. This is probably caused by the fact that an excess of vitamin D is added to the milk before evaporation to compensate for supposed losses in the manufacture, and the fact that the natural vitamin D content is neglected.

Vitamin D Content of Other Products

Determinations in fortified milk powders gave values which agreed well with the known added quantities. With these products the first saponification can be substituted by an extraction of the fat from the wetted product and from the sodium sulfate redried product with ether or petroleum ether. Incidental determinations in sweetened condensed milk and milk powder with carbohydrates (baby foods) indicated that the proposed method can also be used for these products. Products without milk fat have not been analyzed until now. Because the substance that gives the yellow-green fluorescent band in UV light and

Table 5. Vitamin D content of several brands of fortified evaporated milk

Sample			Units Vit. D/fl. oz	
No.	Date	Factory	Label	Found
104	7/29/68	A	≥25[c]	26
133	12/ 3/68	A	fortified	25
134	12/ 4/68	A	33 (added)[b]	25
139	12/18/68	A	≥25[c]	28
3.V	8/28/68	B	≥25[c]	30
122	10/15/68	D	≥33[b]	36
124	10/ 2/68	C	–	32
128	10/11/68	D	25[c]	25
130	11/25/68	E	≥25[c]	31
131	11/28/68	E	≥25[c]	31
135	12/ 6/68	F	≥33[b]	29
137	12/12/68	F	≥33[b]	22
136	12/12/68	G	27[d]	28
139	12/18/68	G	25 (added)[e]	30

[a] Or 400 USP units/reconstituted quart (half milk-half water).

[b] Or when 1 part is diluted with 1½ parts water, each quart so reconstituted will provide a minimum of 500 IU.

[c] Or 400 USP units/pint.

[d] Calculated from 850 IU/kg.

[e] Calculated from 840 IU/L.

indicates the position of vitamin D on the alumina column will be lacking, these products must be further investigated.

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Vitamin D and Urinary Amino Acid Excretion in the Rat.* (26859)

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An elevated excretion of amino acids in the urine of rachitic children was first observed by Hottinger(1). Freudenberg and Goetz(2) later demonstrated that this could be reversed by a single massive dose of Vit. D. However, Jonxis *et al.*(3), who also reported

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an increase in free and bound urinary amino acids in rachitic children, found that this increased excretion did not return to normal for 3 to 4 weeks after treatment with Vit. D. It was therefore obvious that this effect of the vitamin lags far behind its effect on the calcium and phosphorus levels of plasma and initiation of healing of rickets. Jonxis and Huisman(4) more recently concluded that the increased excretion was not the result of a metabolic disturbance but rather of a defective tubular reabsorption, since plasma amino acid levels were unchanged in rickets. Nevertheless these investigators considered the aminoaciduria to be a primary effect of Vit. D deficiency. Many studies on amino acid excretion have since appeared and have been carefully reviewed elsewhere (Lathem *et al.*(5), Jonxis(6), Evered(7), Harrison and Harrison(8), and Harris(9)).

In this laboratory, the search for a metabolic action of Vit. D has led to a study of amino acid metabolism in rats fed various diets with and without Vit. D. This paper reports results which show clearly that aminoaciduria does not occur in rats deficient in Vit. D.

Methods. Thirty-day-old male albino rats of the Holtzman strain weighing 65-85 g were used in all experiments. They were housed in overhanging wire cages during the first 3-day period and given food and distilled water *ad libitum*. They were then placed in wire metabolism cages and were transferred to separate feeding cages for 3 one-hour periods daily during which time they had access to unlimited quantities of food unless otherwise indicated. Urine collections were made during the 7th-9th, 10th-12th, and 13th-15th days of experiment. Urine was collected under a layer of toluene and feces were retained on wire screens attached to the bottom of the cage. At the end of an experiment, the rats were killed by a blow on the head followed by severing of the carotid artery. When desired, blood was collected in 15 ml centrifuge tubes. Finally, the rachitic state of the rats was estimated by femur ash determinations and by silver nitrate staining of sectioned radii(10).

Free alpha amino nitrogen was measured by the method of Van Slyke *et al.*(11), serum alpha amino nitrogen according to the method of Hamilton and Van Slyke(12), urinary phosphorus by the method of Fiske and Subbarow(13) following digestion with concentrated nitric and perchloric acids, and creatinine according to the method of Owen *et al.*(14). Individual urinary amino acids were identified and determined quantitatively by ion exchange chromatography according to the method of Moore *et al.*(15) with the Beckman/Spinco Amino Acid Analyzer(16).

All rats were fed essentially the basal diet of Bellin and Steenbock(17) which contains D-glucose monohydrate (Cerelose) 67%, steamed egg white 18%, cottonseed oil (Wesson) 10%, roughage (Celluflour) 3%, calcium and phosphorus free salts 2%, and crystalline vitamins. In addition the rats received orally a fat soluble vitamin mixture dissolved in cottonseed oil (Wesson) which supplied 70 μg of β carotene, 875 μg of α tocopherol, and 105 μg of menadione to each rat per week. In each experiment, one-half the rats received orally 75 i.u. of crystalline Vit. D_2 (calciferol) in cottonseed oil per rat every 3 days. The desired levels of calcium and phosphorus in the diet were achieved by addition of $CaCO_3$ and/or an equimolar mixture of KH_2PO_4 and K_2HPO_4 at the expense of the Cerelose. Diet 11 was non-rachitogenic (0.45% Ca and 0.3% P), diets 23 (0.45% Ca and 0.02% P) and 34B (0.02% Ca and 0.02% P) were strongly rachitogenic while diet 11D (0.02% Ca and 0.3% P) produced the low calcium Vit. D deficiency syndrome in agreement with the results of Steenbock and Herting(10). Diet 24 (1.5% Ca and 0.12% P) was another strongly rachitogenic diet in which vitamin-free casein (General Biochemicals Inc.) plus 0.2% L-cystine served as the protein source. Severe rickets was produced by this diet in agreement with the results of Guroff(18).

Results and discussion. Although there have been numerous reports of aminoaciduria in rachitic infants and dogs, studies with rats have been lacking. In the many experiments conducted, we were unable to demonstrate a significant elevation of free alpha amino nitrogen in urine of rachitic rats above

TABLE I. Urinary Free Alpha Amino Nitrogen from Rats Fed Various Diets with and without Vitamin D.*

		mg α-amino nitrogen/100 g rat/3 days				mg α-amino nitrogen/mg creatinine/3 days			
		Ad libitum		Pair fed		*Ad libitum*		Pair fed	
		−D	+D†	−D	+D†	−D	+D†	−D	+D†
Diet 24	(1.5% Ca + .12% P)‡	3.3	3.7	3.1	3.3	.6	.4	.6	.4
" 23	(.45% Ca + .02% P)	6.3	5.3	—	—	.6	.5	—	—
" 34B	(.02% Ca + .02% P)	4.1	4.4	4.7	4.2	.6	.5	.6	.5
" 11D	(.02% Ca + .3% P)	2.1	3.3	1.9	2.1	.3	.4	.3	.3
" 11	(.45% Ca + .3% P)	3.3	4.0	3.1	4.4	.5	.6	.5	.6

* Values are avg from at least 6 animals fed the various diets for 12 days.
† Seventy-five i.u. of vit. D_2 (calciferol) in Wesson oil was given each rat every 3 days.
‡ Protein source was casein plus 0.2% L-cystine.

that in the urine of their Vit. D supplemented controls, in contrast to a previous report(19). Typical results of those obtained are shown in Table I. It is clear that only slight elevations were found in urinary alpha amino nitrogen when the potent rachitogenic diets 23 and 34B were fed. In a further effort to simulate dietary conditions associated with infantile rickets more closely, the protein source was changed to casein plus cystine and calcium level was elevated to insure rachitogenesis. Again, no aminoaciduria was evident (diet 24). These results contrast sharply with those obtained with human rickets in which a 3- to 4-fold elevation of alpha amino nitrogen occurs(20).

The problem of changes in food consumption and animal size is a major consideration in studies such as these despite the expression of amino nitrogen values on an equivalent rat weight basis. To overcome such criticism, rats on the various diets tested were pair-fed with their Vit. D supplemented controls and amino nitrogen excretion was expressed on either a rat weight basis or a urinary creatinine basis. In these experiments (Table I) it was even more evident that aminoaciduria does not occur in rat rickets (diets 23, 34B or 24) or when either a non-rachitogenic (No. 11) or a low Ca (No. 11D) diet is fed. Our results, therefore, do not support the belief of Jonxis *et al.*(4) that aminoaciduria is a primary effect of Vit. D deficiency.

The difference between human and rat rickets with regard to aminoaciduria aroused some interest. The existence of a defective tubular reabsorption of phosphate in rachitic infants and dogs has been known for some time(21). In agreement with others(22), results shown in Table II demonstrate that excessive urinary excretion of phosphate is not found in rachitic rats. It may be a coincidence that rats and humans differ in both amino acid and phosphate reabsorption in rickets; however, a possible relationship between phosphate and amino acid reabsorption in the kidney tubules may provide insight into this problem.

It appeared possible that aminoaciduria in rat rickets might be masked by excessive excretion of a single amino acid which might be unaffected by Vit. D. The amino acids from rachitic and Vit. D supplemented rats were therefore determined qualitatively and quantitatively by ion exchange chromatography. Jonxis and Huisman(23) have reported large losses of serine, threonine, glycine, alanine, histidine, lysine, and bound glutamic acid in urine of rachitic children. Our results shown in Table III clearly reveal that in rat rickets no marked increases occur that might be classified as an aminoaciduria. It should be noted that the relatively large increase in cystine excretion occurred with the diet (No. 24) supplemented with 0.2%

TABLE II. Urinary Phosphorus of Rats Fed Rachitogenic Diets with and without Vitamin D.*

		mg/rat/6 days	
		−D	+D†
Diet 23	(.45% Ca + .02% P)	.8	1.0
" 34B	(.02% Ca + .02% P)	1.0	1.0

* Values are avg from 8 rats fed their respective diets for 12 days.
† Seventy-five i.u. of vit. D_2/rat every 3 days.

TABLE III. Urinary Amino Acids from Rats Fed Rachitogenic Diets with and without Vitamin D.*

	μmoles/100 g rat/3 days			
	Diet 23		Diet 24‡	
	−D	+D†	−D	+D†
Aspartic acid	3.4	3.1	1.4	2.1
Threonine	3.5	3.3	5.0	4.5
Serine, glutamine & asparagine	9.2	5.9	7.9	6.8
Glutamic acid	11.0	12.0	6.0	7.0
Proline, citrulline	4.1	2.9	4.8	4.4
Glycine	14.7	17.5	10.9	11.9
Alanine	6.8	6.7	6.7	6.6
Methionine	2.3	1.0	3.0	4.0
Valine	1.4	.8	1.1	.9
Cystine	—	2.2	47.7	23.2
Isoleucine	1.8	1.3	2.2	1.3
Leucine	1.6	1.6	1.5	1.3
Tyrosine	1.2	.9	1.1	1.0
Phenylalanine	1.2	.9	.8	1.1
Lysine, ornithine	1.7	3.2	4.6	6.0
Histidine	5.7	5.6	1.4	2.2
Arginine	3.0	3.2	2.7	3.2
Methionine sulfoxides	16.0	12.7	3.0	3.0
Total	86.6	85.4	111.6	87.5

* Values represent one analysis of a pooled sample from 10 rats fed their respective diets for 30-42 days.

† Seventy five i.u. of vit. D_2/rat every 3 days.

‡ Casein plus 0.2% cystine served as protein source.

L cystine. Since no significant effect of Vit. D deficiency on excretion of this amino acid occurred with all the other diets tested, which contain adequate amounts of cystine in the protein form, this was not looked upon as an aminoaciduria associated with rickets. Similar results, not shown, were also obtained with the other diets (Nos. 34D, 11 and 11D) tested.

Summary. Free alpha amino nitrogen as well as individual amino acids have been determined in urine of rats fed various diets with and without Vit. D. In contrast to infantile rickets, no aminoaciduria occurs in Vit. D deficiency whether strongly rachitogenic or non-rachitogenic diets are fed. Furthermore, a rachitogenic casein diet also produced no aminoaciduria.

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Spectrophotometric Determination of Vitamins D in Presence of Vitamin A

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The difficulties and limitations associated with the available physicochemical methods for the determination of vitamin D in oil samples, including those containing vitamin A, led to the present investigation. The proposed method utilizes a two-step chromatographic process for the separation of vitamin D from the other nonsaponifiable oil components. In the first step an activated earth, Superfiltrol, is used to remove the vitamin A, related carotenoids, pigments, and some sterols. In the second step activated alumina is used to remove certain polyenes, as well as other residues from the oil and Superfiltrol itself. The vitamin D is ultimately determined by utilizing its absorption maximum at 265 mμ as a measure of the amount present. The method has been successfully applied, within the limitations stated, to samples of irradiated ergosterol in corn oil, crystalline vitamin D_2 and vitamin A acetate in corn oil, irradiated ergosterol and vitamin A palmitate in corn oil, some fish liver oils, and some miscellaneous samples.

FOR several years work in these laboratories has been directed toward the determination of vitamins D present in various oils using chromatographic techniques to separate the vitamin D from other nonsaponifiable oil constituents. Prior to this work two methods were proposed: the first by Ewing, Kingsley, Brown, and Emmett (*5*), and the second by Ewing, Powell, Brown, and Emmett (*6*).

The latter method, developed to determine the amount of vitamin D_2 present in samples of irradiated ergosterol in corn oil, employed a single chromatographic step using Superfiltrol as the adsorbent. The ultimate determination of vitamin D_2 was carried out by measuring absorbancy at 265 mμ. Neither method was completely satisfactory with samples containing less than 50,000 U.S.P. units of vitamin D_2 per gram.

In order to determine the vitamin D_2 present in oil samples containing less than the above amount of vitamin D_2, a modification of the method utilizing the absorbancy at 265 mμ was investigated. The method involves a two-step chromatographic process. In the first step a Superfiltrol column is used to remove vitamin A, carotenoids, pigments, some sterols, and irradiation products of ergosterol other than vitamin D_2. The use of this and similar adsorbents for like separations has been well established (*1, 5, 6, 9-11*), and recently has been employed by Green (*7, 8*) for the determination of vitamin D_2 in crude irradiation mixtures, and vitamin D, in some fish liver oils.

The second chromatographic step uses an activated alumina to remove certain unsaturated compounds of the squalene type, certain vitamin A decomposition products, residual impurities from the Superfiltrol adsorbent, and other materials present in the nonsaponifiable portion of vegetable oils. This adsorbent has been used by previous investigators for similar separations (*2, 3, 8*), but for the most part these studies have not dealt with the quantitative aspects of the separation.

Vitamin D which is eluted from the adsorbent column is finally determined by measuring the absorbance at its 265 mμ maximum rather than by the use of antimony chloride reagent. The shape of the absorption curve of vitamin D in the region of 265 mμ is a valuable criterion of the purity of the compound. Marked distortion of the absorption curve is indicative of the probable presence of an impurity with the vitamin D and may introduce an error.

The method has been successfully applied, within the stated limits, to samples of crystalline vitamin D_2 and vitamin A acetate in corn oil, irradiated ergosterol and vitamin A palmitate in corn oil, some fish liver oils, and some miscellaneous samples.

REAGENTS AND APPARATUS

Hexane. Skellysolve B (obtained from the Skelly Oil Co., 3711 California Ave., Chicago, Ill., and having a boiling range of 65° to 68° C.) was passed through a column of activated silica gel 5 cm. in diameter by 75 cm. long. Only the first 500 to 600 ml., having an absorbancy value of less than 0.035 at 230 mμ as measured against air, was used. The silica gel (obtained from the Davison Chemical Corp., Baltimore) was used as received. It could be re-used by washing out the adsorbed organic material with distilled water, drying, and heating 24 hours in a muffle furnace at 250° to 255° C.

Absolute Alcohol. C.P. absolute ethyl alcohol was treated with 20 grams of C.P. potassium hydroxide and 10 grams of silver nitrate per liter, either for 48 hours with frequent shaking, or for 8 hours under a reflux. It was distilled from an efficient column, and the first 50 ml. and the last 150 ml. were discarded. The distillate was dried using amalgamated aluminum foil which was left in contact with it for 48 hours with frequent shaking, or for 8 hours under a reflux. It was then distilled from an efficient column as in the first step. It was required to have an absorbancy value of less than 0.075 at 230 mμ and less than 0.025 at 265 mμ.

Alcoholic Potassium Hydroxide. This was made up as used by adding 28 grams of C.P. potassium hydroxide to 1 liter of absolute alcohol as prepared above, making a 0.5*N* solution.

Anhydrous Ether. C.P. absolute diethyl ether was distilled from a mixture of C.P. anhydrous sodium sulfite and sodium hydroxide in an efficient column just before use. The distillate was required to have an absorbancy value of less than 0.20 at 230 mμ and less than 0.025 at 265 mμ.

Superfiltrol was obtained from the Filtrol Corp., 315 W 9th, Los Angeles, Calif. Not all lots were satisfactory.

Alumina. Two grades of activated alumina obtained from the Chemical Division, Aluminum Co. of America, Pittsburgh, Pa., were used: F-20, minus 160 mesh, and F-20, minus 80 mesh. Other similar aluminas have also been used satisfactorily.

Crystalline Vitamin D_2 was obtained from the Winthrop Chemical Co., Inc., New York, N. Y.

Crystalline Vitamin A Acetate (potency 2,000,000 U.S.P. units per gram) was obtained from Merck & Co., Inc., Rahway, N. J.

Spectrophotometer. The absorption spectra were all obtained using a Model DU Beckman spectrophotometer equipped with a hydrogen source and employing 1-cm. quartz cells. The sensitivity was adjusted so that the slit varied from about 1 mm. at 230 mμ to 0.4 mm. at 200 mμ.

Adsorption Tubes consisted of a convenient length (20 to 25 cm.) of borosilicate glass tubing 8 mm. in internal diameter, to which a short piece of 6-mm. glass tubing had been joined. This type of tube was used for both chromatographic steps.

PROCEDURE

The sample for assay should contain at least 4000 U.S.P. units of vitamin D and not more than 70,000 to 75,000 U.S.P. units of vitamin A. The ratio of vitamin A to vitamin D should not exceed 10 to 1.

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1. A weighed 1-gram sample of the oil is placed in a 125-ml. Erlenmeyer flask and 10 ml. of alcoholic potassium hydroxide is added. The sample is saponified on a water bath at 70° C. for 30 minutes to 1 hour with occasional swirling (the former time is usually sufficient for complete saponification). A short-stemmed funnel placed in the mouth of the flask serves as a condenser.

2. When saponification is complete, the funnel and sides of the flask are washed down with 20 ml. of water. The nonsaponifiable portion of the oil is extracted with one 40-ml., followed by three 30-ml. portions of ether. The combined ether extracts are washed with five 50-ml. portions of water (the first three without shaking). The interface should be clean, and the aqueous layer should not be alkaline to phenolphthalein at this point. The washed ether extract is dried by filtering it through a sintered-glass funnel containing 10 grams of anhydrous c.p. sodium sulfate. The separatory funnel is rinsed with 5 ml. of ether, and the funnel containing the drying agent is rinsed with 25 ml. of ether in small portions.

3. The ether extract is then evaporated to dryness on a water bath at about 45° C. under a reduced pressure of 30 to 40 mm. of mercury.

4. The dry residue is taken up in 3 ml. of a solvent composed of 50 parts of hexane and 10 parts of ether by volume (v./v.). This is preparatory to chromatographing on the appropriate Superfiltrol column.

5. Superfiltrol Column Step. PROCEDURE A. This procedure is recommended for use with samples that contain vitamin D in corn oil, or for other types of oil samples where the ratio of vitamin A to D does not exceed 5 to 1. The total amount of vitamin A introduced onto this column is important and should not exceed 40,000 to 45,000 U.S.P. units. This is the saturation level of the adsorbent, and if it is exceeded the desired separation will not be completely effected. This can be qualitatively ascertained by noting how far down the column the blue coloration, due to the presence of vitamin A, has progressed upon completion of the elution; complete traversal indicates that the saturation level has been exceeded.

The Superfiltrol column is packed to a height of 4 cm. using the method of Ewing *et al.* (*5*). The column is prewashed with 15 ml. of 50 hexane–10 ether (v./v.) under a sufficient pressure differential so that the rate of flow is close to 1 ml. in 2.5 minutes; this rate of flow is maintained throughout. The eluate obtained up to this point is discarded, and just before the top of the column becomes dry the dissolved residue from step 4 is added to the column. The flask which contained the dissolved residue is rinsed with 3 ml. of the above solvent, and this is added to the column in a similar manner. Finally 10 ml. of the above solvent is added to the column to elute the vitamin D completely. The entire eluate thus obtained is evaporated to dryness under the conditions noted in step 3.

PROCEDURE B. This procedure is designed for use in cases where the vitamin A to D ratio does not exceed 10 to 1 but, in general, is in excess of 5 to 1. The amount of vitamin A introduced onto this column should not exceed 70,000 to 75,000 U.S.P. units. The means for determining whether or not the saturation level has been exceeded is discussed in Procedure A.

The Superfiltrol column is packed to a height of 6 cm. as described in Procedure A. The column is prewashed with 15 ml. of 50 hexane–10 ether (v./v.) under a sufficient pressure differential so that the rate of flow is close to 1 ml. in 2.5 minutes; this rate of flow is maintained throughout. Just before the top of the column becomes dry, the dissolved residue from step 4 is added to the column. The flask which contained the dissolved residue is rinsed with 2 ml. of the above solvent, and this rinse is then added to the column in a similar manner. When this last addition has just entered completely on the column, the entire eluate thus far obtained is discarded. The vitamin D is finally eluted with 15 ml. of 50 hexane–10 ether (v./v.), and the eluate obtained is evaporated to dryness under the conditions noted in step 3.

6. The alumina chromatographic column step is the same for samples treated by procedure A or B.

The alumina column is prepared by first adding to it 0.5 gram of activated alumina of minus 100-mesh size. This is packed by merely tapping the sides of the column, which is under slightly reduced pressure. Then there is added 3.5 grams of activated alumina of minus 80-mesh size. A column so prepared yields readily reproducible results, and has the advantage of ease in controlling the rate of flow, which is very important on this column. The column is standardized by prewashing with 10 ml of a solvent which contains 0.6% ethyl alcohol in ether (v./v.) at a flow rate of 1 ml in 1.5 minutes, this rate of flow being maintained throughout. Just before the top of the column becomes dry, 5 ml. of hexane is added to the column to remove the prewash solvent. Then the dry residue from Procedure A or B, dissolved in 3 ml. of hexane, is added to the column in a similar manner. This is followed by the addition of 3 ml. of hexane used to rinse the flask.

The column is developed with 12 ml. of a solvent composed of 70 ether–30 hexane (v./v.) followed by 2 ml. of 0.6% alcohol in ether (v./v.). The entire eluate obtained to this point is discarded. The vitamin D is then eluted with 18 ml. of 0.6% alcohol in ether (v./v.). The eluate thus obtained is evaporated to dryness under the conditions noted in step 3.

7. The dry residue obtained from step 6 is taken up in a known volume of hexane such that the absorption maximum at 265 mμ will appear on the density scale somewhere between 0.250 and 0.450, which embraces the range of maximum sensitivity of the instrument. It is advisable to obtain the absorption curve over the range of 230 to 290 mμ, as marked distortion of this curve from that of pure vitamin D_2 or D_3 is indicative of a determination probably possessing a large positive error, particularly where small amounts of vitamin D are concerned.

8. Various approaches are possible for converting spectrophotometric readings to units of vitamin D. In the studies reported here this was done by multiplying the $E^{1\%}_{1\,cm.}$ 265 mμ by 100,000 to give U.S.P. equivalent units of vitamin D per gram. This was on the basis of data from a number of samples of irradiated ergosterol in corn oil which had previously been assayed spectrophotometrically by the method of Ewing *et al.* (*6*) and also bioassayed using the method and reference standard of U.S.P. XIII.

The present U.S.P. XIV specifies crystalline vitamin D_2 as reference standard and by definition establishes this as containing 40,000,000 U.S.P. units per gram. On the basis of the latest reported $E^{1\%}_{1\,cm.}$ for crystalline vitamin D_2 (*12*) the factor for converting spectrophotometric readings to U.S.P. equivalent units, assuming no loss of vitamin D in carrying out the various steps of the above described assay procedure, would be as follows:

$$\frac{40{,}000{,}000\ (\text{units of vitamin D per gram of vitamin } D_2)}{485\ (E^{1\%}_{1\,cm.}\ 265\ m\mu\ \text{vitamin } D_2)} = 82{,}474$$

Theoretically, this would hold only if vitamin D_2 was assayed. For assaying samples known to contain vitamin D_3, the $E^{1\%}_{1\,cm.}$ of which is 460, the correct calculated factor for converting spectrophotometric readings to units would be 87,000.

NOTES ON PROCEDURE

The following general information was acquired in the development of the method presented (*11*).

The Superfiltrol used was selected from various available lots; not all lots examined were satisfactory. In general, the whiter grades were more effective than the darker grades for the desired separation. Attempts to standardize the material by heating, or by washing followed by drying and heating, yielded an adsorbent unsuitable for use; the material was used as received. Suitability of a lot for use can most easily be determined by treating a known mixture of vitamins A and D as described, and noting whether or not the desired separation is obtained. Moisture has pronounced adverse effect on the adsorbent, and must be completely excluded.

The relative positions of the chromatographic zones for vitamin D and the other nonsaponifiable oil components were not found to vary appreciably under different eluent compositions. As the column length was increased, the threshold volume for vitamin D increased, as did the total volume required for complete elution. When pure, crystalline vitamin D_2 and the procedure outlined were used, the recovery was 98% from the 4-cm. Superfiltrol column and 96% from the 6-cm. column. The vitamin A and related carotenoids are irreversibly adsorbed on this column.

Because the activity of different samples of alumina varies (often over a considerable range), it was necessary to find some convenient method of standardizing the adsorbent with respect to the threshold volume of vitamin D, the banding of the vitamin D for a given solvent system, and rate of flow. As the commercially available alumina adsorbents usually adsorb vitamin D too strongly, the problem is one of controlled deactivation. The method used was to prewash the adsorbent with a definite volume (10 ml.) of a solvent composed of varying amounts of alcohol in ether, introduce pure, crystalline vitamin D_2 in hexane onto the column, and determine its threshold volume using 70 ether–30 hexane (v./v.) as the eluant. It is desirable to standardize the alumina so that the threshold volume is 12 to 15 ml., using 70 ether–30 hexane (v./v.), and so that not more than 20 to 25 ml. of the ether-alcohol solvent (preferably having the same composition as the prewashing solvent) is required to elute the vitamin D completely. Using the procedure outlined, the recovery of pure, crystalline vitamin D_2 from this column was found to be 96 to 98%. The interfering nonsaponifiable oil components present are much less strongly adsorbed than the vitamin D, and are eluted prior to it on the column.

The rate of flow on this column is very important, as this controls the degree of equilibrium attained. In the procedure outlined this factor was controlled by utilizing aluminas of two different mesh sizes, the finer mesh being at the bottom. As an alternative, a single alumina could be chosen with a particle size such that the indicated rate of flow could be maintained using a small pressure differential (10 to 20 mm. of mercury).

RESULTS

In Table I are listed the results obtained for various dilutions of a commercially prepared irradiated ergosterol in corn oil. Procedure A for the Superfiltrol column step was used for these samples. The original oil had been carefully bioassayed previously and found to contain 500,000 U.S.P. XIII units of vitamin D_2 per gram. This potency was also checked several times using the spectrophotometric method of Ewing *et al.* (*5*), which gave an average value of 474,000 units. Using the former value, dilutions of the original oil were made with corn oil to give the calculated potencies indicated as U.S.P. units. This resulted in a series of samples ranging in potency from 44,000 to 2980 vitamin D units per gram. One or more replicate samples of each group having similar potencies were assayed.

Table I. Potency of Samples of Irradiated Ergosterol in Corn Oil

Sample No.	U.S.P. Units of Vitamin D_2/G.	Observed U.S.P. Equivalent Units of Vitamin D_2/G.	Deviation, %	Mean Deviation, %
1	44,000	36,800	−16.2	
2	44,000	39,600	−10.0	
3	44,000	38,400	−12.6	−12.9
4	25,620	21,350	−16.7	
5	25,620	21,600	−15.6	
6	25,620	28,200	+10.0	
7	23,300	21,300	− 8.6	− 7.6
8	12,420	11,050	−11.1	
9	12,420	10,500	−15.1	
10	12,420	10,850	−12.6	
11	12,150	10,450	−14.6	
12	12,150	10,300	−15.1	
13	12,150	10,500	−13.6	
14	12,150	11,310	− 7.1	
15	10,630	10,600	− 0.3	
16	10,630	9,950	− 6.4	−10.7
17	9,200	8,350	− 9.1	
18	8,700	8,100	− 7.0	
19	8,040	8,350	+ 6.5	
20	8,040	7,100	−11.7	
21	8,040	7,100	−11.7	
22	8,040	7,070	−12.0	
23	8,040	7,200	−10.4	
24	8,040	7,920	− 1.4	− 7.1
25	6,150	5,700	−10.8	
26	6,150	5,810	− 5.6	
27	5,980	6,150	+ 3.3	
28	5,980	5,940	− 0.6	
29	5,980	6,360	+ 6.3	
30	5,980	5,300	−11.4	
31	5,980	5,000	−16.3	− 5.0
32	5,075	4,800	− 5.4	
33	5,075	4,920	− 3.0	
34	5,075	5,200	+ 2.5	
35	5,070	4,370	−13.8	
36	5,010	5,500	+ 9.2	
37	4,450	4,300	− 3.3	
38	4,070	4,170	+ 1.3	− 1.4
39	2,980	3,680	+23.5	
40	2,980	3,780	+21.8	
41	2,980	3,370	+13.8	
42	2,980	2,790	− 6.3	
43	2,980	2,970	− 0.3	+11.4

Table II. Potency of Samples Containing Both Vitamins A and D[a]

Sample No.	U.S.P. Units of Vitamin A/G.	U.S.P. Units of Vitamin D/G.	Observed U.S.P. Equivalent Units of Vitamin D/G.	Deviation, %	Mean Deviation, %
Irradiated Ergosterol and Vitamin A Palmitate in Corn Oil					
1	27,600	6,525	5,900	+ 6.9	
2	41,400	8,675	8,280	+ 6.9	
3 (0.05 g.)	810,000	162,000	162,000	0.0	
Crystalline Vitamin D_2 and Vitamin A Acetate in Corn Oil					
4	25,600	20,400	18,500	− 9.3	
5	25,600	20,400	17,000	−13.3	
6	28,800	19,800	17,130	−10.4	
7	28,800	19,300	17,540	− 9.2	−10.5
8	27,600	11,200	9,650	−13.8	
9	27,600	11,200	10,100	− 9.8	
10	27,600	11,200	10,100	− 9.8	
11	25,600	12,000	10,650	−11.3	
12	26,600	13,700	13,200	− 3.6	− 9.7
13	46,600	12,200	11,000	− 9.9	
14	47,800	11,150	10,700	− 4.0	− 6.9
15 (0.1 g.)	135,600	30,880	29,800	− 3.6	
16 (0.1 g.)	136,600	30,880	29,600	− 4.2	
17 (0.1 g.)	135,600	30,880	28,900	− 6.5	− 4.7
18	22,100	4,490	5,260	+17.5	
19	22,100	4,490	4,700	+ 4.7	
20	22,100	4,490	4,600	+ 2.5	+ 8.2
21	28,800	6,400	6,100	− 5.0	
22	28,800	6,100	5,500	− 9.8	
23	28,800	6,100	6,300	+ 3.3	
24	28,800	6,100	6,500	+ 7.4	− 1.0
25	59,400	10,850	11,800	+ 8.7	
26	59,400	10,850	12,300	+13.3	
27	59,400	10,850	9,820	− 9.5	
28	59,400	10,850	10,850	0.0	+ 3.1
29[b]	30,000	20,000	20,700	− 3.5	
30[c]	33,800	250,000	201,600	−19.3	

[a] Procedure A for Superfiltrol column step used for these samples.
[b] Tuna liver oil.
[c] Irradiated ergosterol in fish liver oil.

In Tables II and III are listed the results obtained for the following sample types: (1) crystalline vitamin D_2 and vitamin A acetate in corn oil, (2) irradiated ergosterol and vitamin A palmitate in corn oil, (3) some fish oils, and (4) some miscellaneous samples. For type 1, samples of the indicated potency were made up in corn oil using pure crystalline vitamin D_2 and crystalline vitamin A acetate (potency given as 2,000,000 U.S.P. units per gram). For type 2, an oil containing 810,000 U.S.P. units of vitamin A palmitate and 162,000 U.S.P. units of vitamin D_2 per gram (present as a bioassayed irradiated ergosterol) was diluted as required with corn oil. Where it was desired to alter the ratio of vitamin A to D in this oil, either irradiated ergosterol or vitamin A palmitate of a known potency was added as required.

In the columns headed U.S.P. units of vitamin A or D per gram of oil are given the assay values for the original oil, or those calculated from the known weight dilutions of these original oils. Unless otherwise indicated after the sample number, all the determinations listed were made on 1-gram samples.

DISCUSSION

The tables list determinations made on samples of irradiated ergosterol in corn oil, crystalline vitamin D_2 and vitamin A in corn oil, irradiated ergosterol and vitamin A palmitate in corn oil, two fish liver oils, and some miscellaneous samples. Of the 110 determinations listed, only five differ from the bioassay value for

the bioassay value calculated from the dilution factor) by more than 20%.

In Figure 1 are given some typical absorbancy curves for the indicated samples in Table III. The maximum absorbancy is at 265 mμ, identical with that for pure vitamin D_2 and D_3. The distortion of the absorption spectra, although very slight in the critical region of 260 to 270 mμ, is more marked in the region of 230 to 240 mμ, especially for samples containing smaller amounts of vitamin D.

Three principal factors must be considered in stating the range of application for the proposed method. For samples containing less than about 4000 U.S.P. units of vitamin D per gram of oil the error begins to rise rapidly, and this value may be taken as a lower limit for the satisfactory determination of vitamin D in oil samples. Furthermore, the ratio of vitamin A to D is important, and for the procedure outlined the error begins to rise rapidly when this ratio exceeds 10 to 1. Some limited work using an 8-cm. Superfiltrol column indicates that oils with a somewhat higher ratio of A to D may be satisfactorily determined (in the neighborhood of a 15 to 1 ratio). Lastly, the amount of vitamin A introduced onto the Superfiltrol column is important, and should not exceed the saturation levels given in the procedure. Within the above stated limitations, very satisfactory determinations may be made, and for the results given the mean deviation varied from −12.9% to +9.3%.

The interferences which determine the range of application of the method stem from two sources. The first is due to a small amount of residue in the oil itself which cannot be completely removed. Its contribution to the total light absorption in the final step becomes more pronounced as the amount of vitamin D decreases and consequently the error increases. Where the oil content per gram is low in relation to the vitamin D present, smaller amounts of vitamin D can be satisfactorily determined (around 3000 U.S.P. units). The second source of interference is the decomposition product(s) of vitamin A formed on the Superfiltrol column. When present in large amounts, a portion of it tends to be eluted with the vitamin D from the alumina column. Its absorption curve is similar to that obtained by Embree and Shantz (4) for subvitamin A and kitol. Green also observed similar absorption curves in his work with vitamin A on floridin earths (7). A larger Superfiltrol column, however, will allow a greater amount of vitamin A to be introduced onto the column, and will yield satisfactory results at higher vitamin A to D ratios.

Attempts to apply the method to halibut liver oils met with failure due to the presence of an interfering substance whose chromatographic behavior on both columns, under widely differing conditions, was identical with that of vitamin D.

Table III. Potency of Samples Containing Both Vitamins A and D[a]

Sample No.	U.S.P. Units of Vitamin A/G.	U.S.P. Units of Vitamin D/G.	Observed U.S.P. Equivalent Units of Vitamin D/G.	Deviation, %	Mean Deviation, %
		Irradiated Ergosterol and Vitamin A Palmitate in Corn Oil			
1	16,700	9,340	8,770	− 6.1	− 9.6
2	16,700	9,340	8,610	− 8.8	
3	16,700	9,340	8,110	−13.1	
4	16,700	9,340	8,160	−12.4	
5	44,700	8,950	8,160	− 8.7	
6	44,700	8,950	7,880	−11.9	
7	44,700	8,950	7,890	−11.7	
8	44,700	8,950	8,080	− 9.6	−10.4
9	44,300	6,070	6,080	0.0	− 6.2
10	44,300	6,070	5,610	− 7.6	
11	44,300	6,070	5,270	−13.1	
12	44,300	6,070	5,490	− 9.5	
13	44,300	6,070	6,010	− 1.0	
14	69,300	6,610	8,350	+26.2	+ 3.2
15	69,300	6,610	7,340	+11.0	
16	69,300	6,610	5,700	−13.7	
17	69,300	6,610	5,800	−10.8	
		Crystalline Vitamin D_2 and Vitamin A Acetate in Corn Oil			
18	48,700	5,080	5,790	+14.0	+ 9.3
19	48,700	5,080	5,030	− 1.0	
20	48,700	5,080	5,670	+11.8	
21	48,700	5,080	5,710	+12.3	
22	42,900	6,200	6,030	− 2.7	− 8.0
23	42,900	6,200	5,420	−12.6	
24	42,900	6,200	5,470	−11.5	
		Cod Liver Oil Fortified with Irradiated Ergosterol and Vitamin A Palmitate			
25	42,400	8,340	7,500	−10.1	− 7.1
26	42,400	8,340	8,020	− 3.8	
27	42,400	8,340	7,740	− 7.3	
		Tuna Liver Oil			
28	30,000 (estd.)	20,000	20,580	+ 2.9	+ 1.8
29	30,000	20,000	20,100	+ 0.7	
		Tuna Liver Oil Fortified with Irradiated Ergosterol (75% D_3, 25% D_2)			
30	12,200	10,630	10,600	− 0.1	− 3.4
31	12,200	10,630	9,930	− 6.7	
		Abdec Drops[b]			
32 (2.0 g.)	8,800	1,760	2,000	+13.8	+13.0
33 (2.0 g.)	8,800	1,760	2,160	+22.8	
34 (2.0 g.)	8,800	1,760	1,620	− 7.9	
35 (2.0 g.)	8,800	1,760	2,170	+23.3	
		Abdol Capsules[c]			
36 (3.0 g.)	5,650	1,100	1,200	+ 9.1	+11.4
37 (3.0 g.)	5,650	1,100	1,250	+13.7	

[a] Procedure B for Superfiltrol column step used for these samples.
[b] Parke, Davis & Co. water-miscible multivitamin preparation.
[c] Parke, Davis & Co. multivitamin capsule.

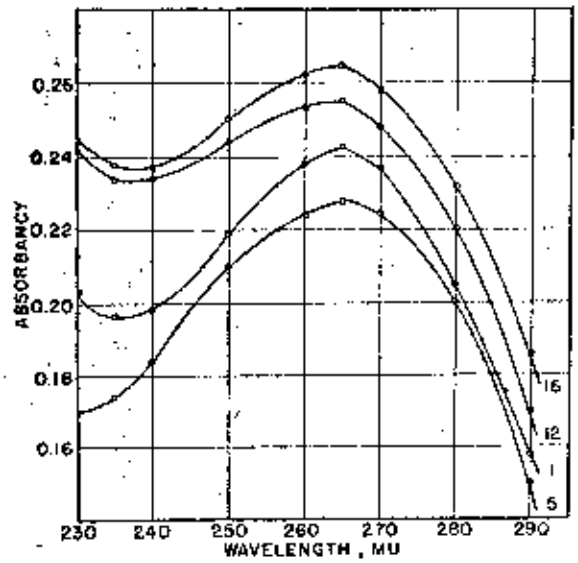

Figure 1. Typical Absorbancy Curves for Indicated Samples in Table III

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7/4

26 December 1973

1732

Dr Humphrey Sassoon
TRACOR-JITCO
1300 E Gude Drive
Rockville, Md 20851

Dear Dr Sassoon

UK REGULATIONS ON VITAMIN D

1. These regulations are currently under review and my advisers in London were reluctant to send you obsolescent information: hence the delays we have experienced.

2. First of all, food. It would appear that the only articles fortified by law with Vitamin D are margarine and National Dried Milk. This milk is fortified under a regulation brought in as a wartime measure; it is now hardly used at all in Britain, accounting for less than one quarter of a per cent of the national intake of milk products.

3. However, many other items are fortified and the regulations lay down that in such cases the packaging or label must show the minimum quantity of the additive in the product. This applies to Vitamin D fortified baby foods and also condensed milk and other products. However flour and bread are not fortified with Vitamin D.

4. The National Food Survey indicates that 35 to 40 per cent of our Vitamin D intake comes from margarine; fish is the second major source with 18 to 20 per cent. Milk products and milk account for 8 to 10 per cent and other foods such as baby foods make up the remainder.

5. The sale of medicinal products containing Vitamin D is currently controlled only by the Medicines (Exemption from Licence) (Food and Cosmetics) Order 1971 which subjects several defined categories of products (and particularly those providing more than 250 units of anti-rachitic activity per day) to the provisions of a licensing system operated under the Medicines Act 1968. Thus the safety of all products on the market - and also those proposed for marketing - will be considered individually by the Committee on Safety of Medicines having regard to their recommended dosage and their proposed indications.

6. In addition, the recommendations of the Medicines Commission on products intended for general over-the-counter sales (other than in pharmacies) have now been published. If these are accepted, only those preparations with a recommended maximum daily dose not exceeding 400 units are recommended as suitable for general sale, and these will be required to carry an indication that only half the dose should be given to children receiving dried milk feeds.

7. Copies of the relevant Statutory Instruments on Food and Drugs and on Medicines are enclosed, also a report by the Medicines Commission.

8. I hope you managed to spend Christmas in England and that you had a good one.

Yours sincerely

Patrick Fallon

P J Fallon

Encl.

ENCLOSURES

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ETIOLOGY OF THE SEVERE FORM OF IDIOPATHIC HYPERCALCEMIA OF INFANCY*

A Defect in Vitamin D Metabolism

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WITHIN the past decade, a new syndrome has been described by Fanconi et al.[1] in infants showing retardation of growth. Mental retardation, renal dysfunction and osteosclerosis, in addition to dwarfism, characterize this clinical entity, and hypercalcemia, hypercalciuria and azotemia are the major biochemical abnormalities.

At about the same time, Lightwood[2,3] reported a large series of infants showing idiopathic hypercalcemia in whom failure to thrive, anorexia, vomiting and constipation were often associated with thirst and polyuria. Hypercalcemia and azotemia were also found in these infants, but osteosclerosis and mental retardation were not observed. In contrast to this mild, reversible form, only a few reports of the more severe disease have appeared.

Since few detailed metabolic observations have been made in this disease, the following cases and studies are reported — they show a consistent alteration in calcium and phosphorus metabolism and a relation of the abnormality to vitamin D metabolism.

CASE REPORTS

The growth curves and laboratory data for the 3 cases are given in Figure 1 and Table 1.

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CASE 1.* A.S. (43-54-27), a female infant born on July 1, 1954, weighed 2.6 kg. and measured 52 cm. in length. She was given a regular whole-milk formula and a vitamin preparation,† 1 teaspoonful daily, until the age of 10 months. She took solid food from 3 to 6 months, but after 7 months she became a severe feeding problem, with intermittent vomiting, and intake actually averaged only 500 ml. of milk a day. Growth was retarded and at the age of 5 months she weighed 5.76 kg. Her 1st tooth erupted at 8 months. At the age of 13 months she was admitted to the hospital for evaluation of a left-sided facial paralysis of 1 week's duration. The family history was noncontributory. At this time she weighed 6.7 kg., and the head circumference was 43 cm. Initial laboratory studies showed a hemoglobin of 10.4 gm. per 100 ml.; urinalysis revealed a specific gravity of 1.012 and a trace to + test for albumin. The blood nonprotein nitrogen was elevated at 74 mg. per 100 ml. Because she failed to sit up or support her weight and made no effort at vocalization, she was considered to be mentally retarded. X-ray study of the skull showed microcrania, with increased density of the bone.

She was readmitted to the hospital on January 9, 1956, for diagnostic studies. On physical examination the blood pressure was 110/60. She was small, appeared malnourished, weighed 6.9 kg., measured 74 cm. and had a head circumference of 43.5 cm. She was irritable and whined constantly. She followed objects with her eyes, but did not reach for them. A Grade 1 systolic murmur was heard in the 2d right costal interspace. The liver edge was palpated 1.5 cm. below the right costal margin. The remainder of the physical examination was not remarkable.

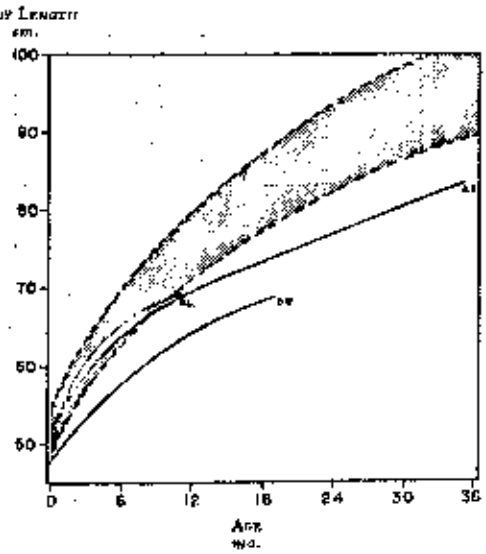

FIGURE 1. *Growth in Severe Hypercalcemia.*
The shaded area represents average 3d to 97th percentiles.

Examination of the blood showed a hemoglobin of 10.0 gm. per 100 ml.; the maximum serum calcium was 3.5 millimols per liter, the alkaline phosphatase was normal, and the liver flocculation studies were negative. Urinary alpha-amino nitrogen was 19.3 mg. per 24 hours. X-ray study showed markedly increased density, with areas of irregular decreased calcification at the ends of the long bones. Nephrocalcinosis was evident in the abdominal film. The patient was discharged on a low intake of vitamin D, with sodium phytate, 0.5 gm. twice daily. In the interval before the 3d admission, in July, 1957, she began to eat better and was able to take some strained food; however, she had frequency of urination.

*The radiologic features of this case have been reported previously.[4]
†Available as Vi-Daylin from Abbott Laboratories, North Chicago, Illinois.

At the time of the third admission for balance and renal-function studies at the age of 36 months, she weighed 8.8 kg. and measured 84.5 cm. in length, and the head circumference was 45 cm. The blood pressure was 116/80. The remainder of the examination confirmed the marked degree of mental retardation. Laboratory studies showed a hemoglobin of 11.0 gm. per 100 ml., and urinalysis was unremarkable. Two 5-day balance collections for calcium and nitrogen were obtained, after which discrete inulin and para-aminohippurate (PAH) renal-clearance studies were done (Table 2).

CASE 2.‡ D.W. (44-41-71), a male infant, was born on August 21, 1955, after an uncomplicated pregnancy. He weighed 3.3 kg. and measured 48 cm. in length. He was given a whole-milk formula and vitamins,§ 0.6 ml. daily. At 3 months of age he weighed 4.8 kg. He smiled at 4 months. At 5 months, his physician noted that he was small for his age. He weighed 5.6 kg. at 6 months. After this his food intake decreased: he vomited occasionally and failed to grow. During a hospital admission at the age of 8 months an elevated blood urea nitrogen and an abnormal urinalysis were found. Bone age was determined to be at the 3-month level. He was then referred to the Children's Medical Center for evaluation. In the 3-week interval he received 15 mg. of thyroid daily.

On admission at the age of 9 months he was well proportioned, but small. He weighed 6.0 kg., his length was 63.5 cm., and his head was 40.5 cm. in circumference. The blood pressure was 140/86 with a 5-cm. cuff. He had a peculiar "elfin" facies, and a right-lower-facial-nerve paralysis was apparent at times. A Grade 2 systolic murmur was heard along the lower left sternal border. The remainder of the physical examination was negative although the patient did not sit or make efforts at locomotion. Urinalysis was abnormal. Anemia (hemoglobin of 8.7 gm. per 100 ml.), mild azotemia and hypercalcemia were found. Urine culture yielded several organisms on three occasions. X-ray studies showed a definite increase in density in the metaphyseal area of the long bones, at the base of the skull and in the pelvic and vertebral bones. An intravenous pyelogram showed poor excretion of the dye. The kidneys did not appear small, and no nephrocalcinosis was noted. Biopsies of rib, liver and kidney have been reported in detail elsewhere.[5] In particular, the kidney had an atrophic cortex and tubules. He was given sulfisoxazole for the urinary-tract infection and discharged on his regular intake.

At the age of 13 months he weighed 6.4 kg. and measured 65 cm., and the blood pressure was still elevated (150/60). He was taking approximately 900 ml. of whole milk daily and some solid foods. He was then started on sodium phytate, 1 gm. daily, and the vitamin D was omitted from his vitamin intake. Two months later he was started on a milk containing no added vitamin D as well, which was continued to the age of 19½ months.

When he was admitted to the hospital for special metabolic studies at this time, he weighed 6.7 kg. and measured 69 cm., and his head circumference was 41.5 cm. The blood pressure was 140/110. There had been no significant change in his condition. He continued to grow slowly. Both azotemia and hypercalcemia persisted. Two 5-day balance studies were obtained and discrete renal function was evaluated by the technic of inulin and PAH clearance with phosphate loading. Further x-ray studies showed marked progression of the osteosclerosis.

CASE 3. R.L. (46-26-79), a male infant, was born on January 26, 1957; he weighed 3.2 kg., and measured 48.3 cm. He took 800 to 1000 ml. of a whole-milk formula daily. In addition, he received 0.8 ml. of a standard vitamin preparation containing 1000 units of vitamin D per 0.6 ml. Development was normal during the 1st 3 months, when he weighed 5.4 kg. and measured 59.7 cm. At 4½ months he had 2 teeth.

During the next 2 months he failed to gain weight normally and did not seem to advance developmentally in learning to sit. He ate poorly. Examination of the blood showed

‡Reported previously in a clinicopathological conference.[5]
§Available as Tri-Vi-Sol from Mead Johnson and Company, Evansville, Indiana.

a normal hemoglobin and nonprotein nitrogen. A plain film of the abdomen disclosed increased density of bone, and an intravenous pyelogram indicated poor excretion of dye. He was admitted to the hospital on August 20 for evaluation. The family history was noncontributory. On admission he weighed 5.7 kg. and measured 67 cm. in length; his head circumference was 43 cm., and the blood pressure was 88/60. He appeared chronically ill, but physical examination was otherwise unremarkable. Examination of the blood showed a hemoglobin of 9.7 gm. per 100 ml. A random specimen of urine had a specific gravity of 1.009, and an overnight specimen for concentration had a specific gravity of 1.012. The maximum serum calcium was 3.3 millimols per liter. Creatinine clearance was depressed. X-ray study of the skull and long bones showed increased density, but not to a marked degree. He was given milk low in vitamin D, and other exogenous vitamin D preparations were discontinued. He continued to drink approximately 1 liter of milk per day at home and grew slowly. At the age of 11 months he weighed 11.8 kg. and measured 69.5 cm., and his head circumference was 47 cm.

TABLE 1. *Laboratory Observations in Idiopathic Hypercalcemia.*

Date	Age	Urine: Calcium	Urine: Magnesium	Urine: Phosphorus	Routine tests	Blood: Hemoglobin	Blood: Nonprotein nitrogen
	mo.	*millimols/24 hr.*	*millimols/24 hr.*	*millimols/24 hr.*		*gm./100 ml.*	*mg./100 ml.*
Case 1 (born 7/9/54):							
10/55	15				Specific gravity, 1.012; pH, 7.0; + test for albumin.	10.4	
12/55	17				pH, 6.5; + test for albumin; *Escherichia coli.*	9.5	75.4
1/56	18	0.73		1.7	pH, 5.0; + test for albumin; alpha-amino nitrogen, 13.3 mg./24 hr.	10.0	60
4/56	21						63.3
7/56	24	(+ + + Sulkowitch test)					67
10/56	27	(+ + + Sulkowitch test)			pH, 5.5; negative test for albumin.		83
11/56	28						100
2/57	31						56
4/57	33	2.25 (+ + Sulkowitch test)	1.60	14.3	Specific gravity, 1.005; pH, 6.5; + test for albumin.		69
7/57	36	1.28	0.70	8.4	Specific gravity, 1.003; pH, 6.0; trace test for albumin; *Esch. coli.*	11.0	74
Case 2 (born 8/21/55):							
6/56	9	0.98 (+ + + Sulkowitch test)	0.42	6.2	Specific gravity, 1.014; pH, 5.0; negative test for albumin; casts in sediment.	9.3	61.4
9/56	13	1.96 (+ + Sulkowitch test)	0.90	7.8	pH, 6.0; trace test for albumin; *Proteus vulgaris.*	8.0	82
11/56	15				pH, 5.5; negative test for albumin; granular casts in sediment.		73
1/57	17	1.99 (+ + + Sulkowitch test)	0.25	10.7	pH, 5.0; trace test for albumin; clumps of white cells in sediment.		71.6
4/57	20	0.50	0.70	6.3	pH, 5.5; trace test for albumin; *Esch. coli.*	10.6	74
Case 3 (born 1/26/57):							
7/57	6					10.5	28
8/57	6½	0.94 (+ + + Sulkowitch test)	0.74		Specific gravity, 1.012; pH, 6.6; negative test for albumin.	9.7	29
10/57	8½	1.30					29
11/57	9½						48
12/57	11						66

METHODS

Balance studies were carried out on a metabolic ward. Intake was determined from the analysis of duplicate diets and of refusals. Vomitus was analyzed separately. Stool periods were marked with a standard 350-mg. carmine dye orally. Daily specimens of urine were collected under oil in an iced container and pooled for analysis in five-day periods. Blo-

samples taken at the start of each period and at other times were allowed to clot for one hour, when serum was removed and frozen. Serum lipoprotein determinations were analyzed immediately.

Infant foods, formula, homogenized stool and urine were weighed in aliquots and digested with concentrated nitric acid and heat for forty minutes. The samples were brought to volume and filtered if necessary. For phosphorus and nitrogen analysis, original aliquots were digested separately with concentrated sulfuric acid. Sodium and potassium were determined by internal standard flame photometry;[8] chloride by a modified Volhard titration,[9] phosphorus by the Fiske and Subbarrow[9] technic and calcium and magnesium by initial oxalate precipitation followed by magnesium ammonium phosphate precipitation. The precipitates were dissolved in perchloric acid with heat, and the cation titrated with ethylenedinitrilo-tetracetic acid with Cal-Red indicator[10] for calcium and erichrome-black-T[11] for magnesium. Serum magnesium was determined by a modified titan yellow photometric technic,[12] cholesterol by the Schoenheimer–Sperry method,[13] serum total protein by a micro-Kjeldahl digestion technic,[14] serum vitamin A and carotene by a modified Blacklan and May technic,[15] and citric acid by a modification of

TABLE 1 (*Concluded*).

Date	Serum: Calcium	Magnesium	Phosphorus	Total protein	Cholesterol	Alkaline phosphatase	Other
	millimols/liter	*millimols/liter*	*millimols/liter*	*gm./100 ml.*	*mg./100 ml.*	*Bodansky units*	
Case 1 (born 7/9/54):							
10/55							
12/55							
1/56	2.8-3.7		2.2	7.2	187	4.9-6.8	pH, carbon dioxide content, chloride, sodium, potassium & alpha-amino nitrogen normal
4/56	2.9		2.1				
7/56	2.6		2.1	6.8			
10/56	2.9-3.6		2.2	7.2		9.3	
11/56	2.9		2.5				
2/57	2.7		1.9				
4/57	3.2		2.3			6.2	
7/57	2.7-3.0	1.19	1.6	6.4	210		pH, carbon dioxide content, chloride, sodium & potassium normal
Case 2 (born 8/21/55):							
8/56	2.8-3.2		1.9	6.5	219	4.5-9.0	Carbon dioxide, chloride, sodium & potassium normal
9/56	3.0-3.3		2.2	6.9			
10/56	3.4	1.00	2.6	6.1			
1/57	2.5		2.4		213	13.4	
8/57	2.4-3.2	1.09	1.6	6.3	208	8.0	pH, carbon dioxide, chloride, sodium & potassium normal
Case 3 (born 1/26/57):							
4/57							
6/57	2.9-3.3		1.6	7.1	330		Carbon dioxide, 21 millimols/liter; pH, chloride, sodium & potassium normal.
8/57	3.6		1.7	6.2	260		
11/57	3.3		2.2	6.8	330		
12/57	3.3		2.2		290		

the method of Natelson.[16] Urinary organic acid was determined by an electrometric titration using the Van Slyke and Palmer method,[17] titratable acidity by titration to serum pH with a pH meter with externally shielded electrodes, and ammonia by Conway's[18] microdiffusion technic.

Vitamin D was determined by the standard AOAC bioassay method[19] as follows: normal weanling Sprague-Dawley rats, twenty-one days of age, were placed on a rachitogenic diet containing 1.2 per cent calcium for twenty days. At the end of this time, severe rickets was manifest. The material for analysis was mixed with skim-milk powder, which had been ether extracted. This was fed in two doses on the first and third days. The diet was offered for a ten-day period, during which body weight did not decrease. The rats were then killed, and the tibias were dissected from the soft tissues. A longitudinal section of the proximal epiphysis was rinsed in water, immersed in 2 per cent silver nitrate for one minute and rinsed again in water. The specimen was exposed to daylight or an ultraviolet-light source for one to three minutes. The degree of calcification as evidenced by black staining without discoloration in the uncalcified areas was immediately graded 1 to 6; Grade 4 represented a solid line across the metaphysis. Seven rats, all of which had readable grading results, were considered adequate for each determination.

Clearance procedures were determined by both the endogenous creatinine and the inulin and PAH method. Creatinine was determined according to the procedure of Hare and Hare.[20] Discrete clearance measurements were performed after a standard priming dose of inulin and PAH, followed by a sustaining infusion of both in isotonic saline solution.[21,22] Urine was obtained from an inlying catheter and collected under oil with a sterile-water bladder wash. At least two bloods per each three clearance periods were obtained for extrapolation of serum values. A buffered phosphate solution for phosphate loading contained sodium phosphate, 11.36 gm. (80 millimols), and sodium biphosphate, 2.76 gm. (20 millimols) in 1000 ml. of solution. This solution was approximately isotonic (280 milliosmols per liter) and contained 3 mg. of phosphorus per milliliter. The pH was checked after autoclaving and before administration and was 7.3 to 7.4. A priming dose and sustaining solution were given.

Results

The 3 infants were normal at birth, but by the age of four or five months evidence of a generalized growth disturbance appeared (Fig. 1). Each child had been first referred to the hospital for failure to thrive. The diagnosis of hypercalcemia was not recognized immediately in Case 1. However, the subsequent radiologic findings were diagnostic. Both Case 2 and Case 3 were recognized initially from the x-ray studies, which indicated the abnormal bone density.

Table 2. *Renal-Function Data.*

Case No.	Age	Surface Area	Inulin Clearance	Creatinine Clearance	Para-amino Hippuric Acid Clearance	Filtration Fraction	Maximum Secretion of Para-amino Hippuric Acid
	mo.	m^2	ml./min./m^2	ml./min./m^2	ml./min./m^2		mg./min./m^2
1	36	0.43	14.4	—	76.6	0.19	9.5
2	9	0.30	—	19.5	—	—	—
	19	0.34	16.7	—	66.2	0.25	—
	28	0.34	—	15.9	—	—	—
3	7	0.32	—	16.2	—	—	—
Normal range		1.00	76.0 ± 15.0		360.0 ± 70.0	0.21 ± 0.05	42.0 ± 10.0

Laboratory data are presented in detail in Table 1. The serum calcium concentrations were elevated (2.75 to 3.65 millimols per liter whereas the normal range is 2.40 to 2.75 millimols per liter), although occasionally a normal value was found. Magnesium concentration in the serum was slightly elevated on a few determinations, which may have been related to the depressed renal function. Serum phosphorus was variable, but tended to be normal. During a period of progressive azotemia in Case 1 the serum phosphorus increased, but later returned to normal levels with her general improvement.

Cholesterol values were only occasionally elevated. Serum total protein determinations were normal. Serum total lipoprotein was normal in Case 1, 260 mg. per 100 ml., and Case 3, 571 mg. per 100 ml. (normal, up to 800 mg. per 100 ml.). Serum vitamin A was consistently elevated in all 3 patients (94 to 187 microgm. per 100 ml.) and serum carotenoids were elevated in Case 3 only.

Serum citrate was increased in 2 patients, being 3.5 mg. per 100 ml. in Case 2 at eighteen months and 5.0 mg. per 100 ml. in Case 3 at eight months (normal, 1.6 to 2.8 mg. per 100 ml.). Alkaline-phosphatase levels in the serum remained in the normal range throughout.

The renal-function studies indicated significant impairment in all 3 infants (Table 2). Glomerular filtration was reduced to 20 to 25 per cent of normal. Renal plasma flow, determined in 2 of the patients by PAH clearance, was decreased to an equal degree, with a resultant normal filtration fraction. Tubular secretory function (PAH tubular maxima) in 1 patient (Case 1) was depressed to the same degree. During this study with a nonreabsorbable anion (PAH) load, potassium secretion was noted (the excreted to filtered ratios varied from 1.72 to 2.00).

An acid urine (pH of 5.08 to 6.62) was excreted by all the infants, and significant titratable acid and ammonia were found. Although maximal acidification was not evaluated after acid loading, an increase in titratable acidity was observed during phosphate loading. Urinary excretion of organic acid was normal, but urinary citrate was only 40 to 50 mg. per twenty-four hours (normal, 100 to 120 mg. per twenty-four hours) in each child. Excessive aminoaciduria was not found in 2 of the patients (Case 2, 9.2 mg., and Case 1, 13.3 mg. per day).

Phosphate excretion in Cases 1 and 2, determined during the clearance studies with inulin, is presented in Table 3. In both patients phosphate reabsorption varied from 66 to 90 millimols per 100 ml. of filtrate at normal serum phosphate levels. An acute phosphate load, which raised the serum phosphate from 1.5 to 2.3 millimols per liter, caused a twofold increase in phosphate reabsorption.

Table 3. *Renal Reabsorption of Phosphorus.**

Subject of Test	Inulin Clearance	Serum Phosphorus	Phosphorus Reabsorption
	ml./min./m²	*millimols/liter*	*millimols/100 ml. of filtrate*
Adults[22]	21-82	1.1	92
	11-20	1.1	56
	5-10	1.0	40
Patients with hypercalcemia:			
Case 1 (36 mo. of age)	15	1.5	90
Case 2 (19 mo. of age)	14	1.5	66
	18†	2.3	122
Normal infant (16 mo. of age)[16]	58	1.8	137
	73†	3.6	165
20 hr. after 600,000 units of vitamin D intramuscularly			
	64	1.9	143
	61†	3.9	208

*Reference data recalculated for purposes of comparison.
†During intravenous buffered phosphate loading.

Calcium excretion in the urine was elevated in all 3 infants and varied from 0.5 to 1.5 millimols per day. The calcium and nitrogen balance data are presented in Figure 2. In relation to the minimal nitrogen balance, which fluctuated insignificantly, the calcium balance was positive (13 and 18 per cent in Case 1 and 33 and 36 per cent of the intake in Case 2).

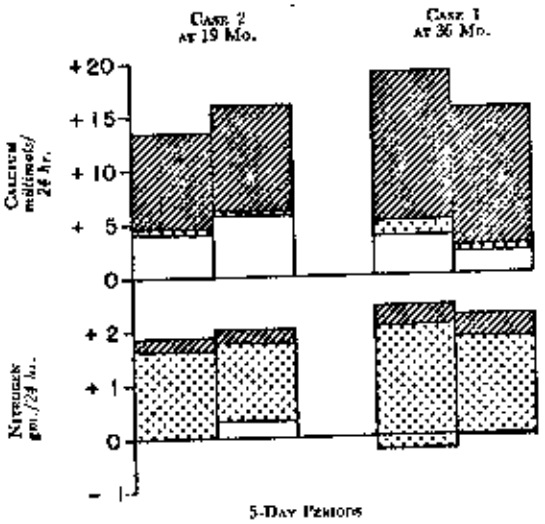

Figure 2. *Balance Data in Severe Hypercalcemia. Intake is plotted up from the baseline; stool (crosshatched areas) and urinary (stippled areas) excretion are plotted down from intake. A clear area represents positive balance; an area below the baseline represents negative balance.*

The results of the bioassay for serum vitamin D activity are presented in Table 4. The level of serum activity in 4 normal infants receiving standard multivitamin supplements varied from 100 to 200 units per 100 ml. of serum. In contrast, the levels in the 3 hypercalcemic infants were markedly elevated to twenty to thirty times the normal values. The serum of the hypercalcemic infants contained 1700 to 6000 units of vitamin D activity per 100 ml. These values are comparable to the results obtained in serum from 1 child with resistant rickets who received 62,500 units of vitamin D orally a day.

Discussion

Although an excessive vitamin D ingestion or an unusual sensitivity to this vitamin has been postulated as the cause of the mild form of idiopathic hypercalcemia of infancy,[2] no direct proof of this relation has been obtained. The high incidence in Great Britain, where over 200 cases were recognized in a two-year period, has diminished since excessive exogenous vitamin D intake has been curtailed there.[3] Previously, infants in Britain were estimated to receive as much as 4000 units daily, mainly as calciferol added to infant foods. An intake exceeding 1800 units daily has been shown by Jeans and Stearns[4] to retard optimal growth in young infants. The British Paedi-

atric Association recommended a decrease in the vitamin D content of infant foods as a result of these findings.

Infants with the mild form of hypercalcemia do not have severe constitutional signs, including mental

TABLE 4. *Serum Activity of Substance Similar to Vitamin D* in Normal Children and Adults (Rachitic-Rat Bioassay).*

Subject of Test	No. of Rats	Serum Aliquot	Activity	
		ml.	*healing units*	*U.S.P. units/100 ml. of serum*
	21	—	4.6	—
Normal children:				
R. (1 yr. of age)	5	2.0	4.5	185
N. (1 yr. of age)	5	2.0	4.5	195
McD. (2 yr. of age)	3	2.0	5.0	
	3	3.0	5.3	184
B. (2 yr. of age)	3	2.0	3.9	
	3	3.0	5.0	172
Children & adults[22]		1.5-4.0		110

*Reference *U.S.P.* standard in oil, 0.1 microgm. of vitamin D_2 = 4 units.

and growth retardation and osteosclerosis; moreover, the disease is usually self-limited and benign. Therapy of this form of the disease consists of removal of vitamin D from the diet, a reduction in calcium intake or

TABLE 5. *Serum Activity of Substance Similar to Vitamin D in 3 Hypercalcemic Infants and a Patient with Resistant Rickets (Rachitic-Rat Bioassay).*

Subject of Test	Age	No. of Rats	Serum Aliquot	Activity	
	mo		*ml.*	*healing units*	*U.S.P. units/100 ml. of serum*
Hypercalcemic infants:					
Case 1	33	7	0.05	1.8	3100
	36	7	0.10	1.4	1700
	42	7	0.10	3.1	2700
Case 2	14	8	0.08	3.4	6000
	19	7	0.05	2.2	3600
Case 3	7	7	0.10	2.3	2000
Patient with resistant rickets:					
G.T.	40	6	0.10	4.9	4300

the administration of cortisone, or all three. These cause the serum calcium to return to normal more promptly, with improvement in the clinical condition. Such treatment generally seems to be necessary for only a few weeks.

In contrast to the more frequently reported fulminant or mild form of hypercalcemia is the limited occurrence of the severe or Fanconi type, of which more than 15 cases have been reported. This appears to be an unrelated entity, although there are features common to both. The cases reported here are strikingly similar to the cases of Fanconi and Schlesinger (Table 5).

The early history and growth data indicate that the 3 infants described above were normal at birth although no chemical and radiologic studies were available then. The exact time of onset of the disease is unknown, and the possibility of an onset *in utero* related to some maternal influence cannot be excluded. The earliest signs were nonspecific and related mainly to growth retardation. Only careful radiologic and chemical studies clarified the diagnosis.

Renal impairment, evident readily as azotemia, is one of the principal findings (Table 6) in the severe form of hypercalcemia. There was significant impairment in glomerular filtration, renal plasma flow and tubular secretory function. The symmetrical reduc-

TABLE 6. *Clinical Features[1] of Severe Hypercalcemia.*

Features	Case 1	Case 2	Case 3
Dwarfism & mental retardation (with convergent strabismus)	±	±	±
Persistent hypercalcemia	+	+	+
Normal or elevated phosphatemia	+	+	+
Renal disturbances, albuminuria, azotemia & low clearances	+	+	+
Hypercalciuria (& hyperphosphaturia)	+	+	+
Osteosclerosis	+	+	+
Alkali reserve slightly lowered	±	±	±
Cholesterol slightly elevated	−	+	+
Serum sodium & chloride normal	+	+	+
Asymptomatic heart disease	+	+	−

tion to about a fourth of normal with a normal filtration fraction (glomerular filtration rate to renal plasma flow) and a normal effective tubular ratio (renal plasma flow to functional tubular tissue) indicates a loss of total nephron population. The renal biopsy in Case 2 at nine and a half months of age was consistent with these observations. Hypercalcemia per se may cause diminished renal function, which is reversible.[27] Since the hypercalcemia undoubtedly was present before diagnosis, the exact relation to the renal impairment found is not known. Certainly considerable renal damage was present at the time of diagnosis at eighteen, nine and seven months of age in each child, as was evident from abnormal urinalyses, azotemia and diminished clearances. Renal infection was found in 2 of the infants (Cases 1 and 2) as a complicating event.

An interpretation of phosphate clearance in these infants is difficult because of the advanced renal impairment. The control values, as compared with those of normal infants on a surface-area basis or

normal serum levels, were either normal or depressed. However, when corrected for the diminished filtered load (low glomerular filtration with normal serum concentrations), the tubular reabsorption was increased as compared to data from adults with similarly depressed function[23] (Table 3). The addition of a phosphate load (in Case 2) resulted in a twofold increase in reabsorption. This increase is similar in degree to that found by Klein and Gow[24] after parenteral vitamin D administration. Although the actual value for maximal tubular reabsorption (T_{mp}) as compared to values obtained during normal renal function is decreased, the ratio of T_{mp} to load was increased in this infant.

Balance studies in 2 of the infants were carried out while they were given their usual dietary calcium intake. Since both infants were retarded and inactive, prolonged balance studies were feasible. Nitrogen retention was negligible in both infants. In contrast, calcium balance was markedly positive, especially considering the absence of growth. The degree of net absorption, which varied from 17 to 35 per cent of the intake, was unusual and undoubtedly contributed to the marked osteosclerosis present in these infants, even though hypercalciuria occurred. Hypercalciuria, which is an important feature of the syndrome, may be related to the hypercalcemia, although increased calcium excretion has been described with normal serum calcium concentrations. Dent has suggested that vitamin D may have a local action on the kidney tubule to promote calcium excretion.[26]

Although no consistent pattern in serum cholesterol was observed, an abnormality in lipoprotein metabolism has been suggested. Salt and Wolff[30] have described 2 infants with severe hypercalcemia in whom elevated lipoproteins were found. In 2 of the cases reported above, lipoproteins were separated in the serum by ultracentrifugation. In 1 infant (Case 1), at four years of age, a normal distribution pattern was obtained. However, in the other (Case 3), at fifteen months of age, a total value of 571 mg. per 100 ml. was found, with 261 mg. per 100 ml. in the beta-lipoprotein fraction (S_f^{3-9}) (normal, up to 270 mg. per 100 ml.). Forfar et al.[31] postulated an abnormality in cholesterol metabolism with the production of a substance active in vitamin D, although a defect in lipoprotein metabolism was not considered.

The vitamin A levels, which were elevated in all 3 infants, also suggest an abnormality in lipoprotein metabolism. The vitamin A intake was estimated to be 5000 units daily, a dosage that does not usually result in increased serum values. Krinsky, Cornwell and Oncley[31] recently demonstrated the transport of vitamin A and carotene in lipoprotein fractions of human serum. A defect in the rate of clearing of serum lipids by an altered lipoprotein metabolism has not been demonstrated, however, in this disease.

The elevated serum citrate levels found in 2 of the infants during the early phase of the disease may have been related to the action of vitamin D. Carlsson[32] has suggested that vitamin D results in dissolution rather than accretion of bone, possibly through citrate metabolism. Furthermore, citrate has been increased in serum and tissues (other than the liver) by vitamin D administration.[33] Urinary citrate was low rather than elevated; however, the severe renal impairment limits citrate excretion.[34] The apparent paradox between the action of vitamin D described above and the radiologic finding of increased bone density may be explained by the other factors, including solubility of calcium salts and parathyroid activity, which also influence bone formation. Without dynamic measurements of calcium metabolism, relative rates of accretion and dissolution cannot be considered in detail.

The metabolic data obtained in these infants is consistent with the presently known actions of vitamin D. That excessive serum vitamin D activity is of primary importance in this syndrome is evident from the bioassay measurements, which indicate a consistent and highly significant increased activity of vitamin D in the serum of each of the three infants. The exclusion of exogenous vitamin D had no effect on the clinical and chemical condition. Since vitamin D had been given in a usual dosage up to 1500 units daily until the time of diagnosis, the contribution of exogenous vitamin to the pathologic process cannot be excluded. However, exogenous vitamin D loads would not be expected to produce acute biochemical changes in the presence of such high serum activity. Furthermore, activity has been found in the serum of these infants as long as fourteen months after the removal of exogenous vitamin D from the diet. This is well beyond the period during which exogenous vitamin might persist normally.

The nature of this substance active in vitamin D is unknown. The possibility exists that this is not the naturally occurring vitamin D_3, but another sterol. At least 8 compounds other than vitamins D_2 and D_3 have antirachitic properties.[35] A cholesterol derivative, an estrogen-related substance or an abnormal adrenal hormone could represent the unknown factor with vitamin D activity. Recently, vitamin D has been shown to be in the alpha$_1$-globulin and alpha$_2$-globulin fraction of the serum proteins,[36] the lipoprotein fraction. Regardless of the exact nature of this substance, the elevated lipoprotein values found in this syndrome further suggest the possibility of a defect in the transport of this substance similar to vitamin D. Thus, the etiology of the severe form of idiopathic hypercalcemia of infancy appears to be related to a defect in the metabolism of such a substance.

Summary and Conclusions

Three patients with the characteristic clinical and

chemical findings of severe idiopathic hypercalcemia of infancy are described.

The principal features of this syndrome have been shown to simulate the physiologic actions of excess vitamin D on the growing child.

Serum vitamin D activity determined by the rachitic-rat bioassay technic was found to be twenty to thirty times that in normal infants. This suggests that a defect in the metabolism of vitamin D or related substances is the primary etiologic factor in this rare syndrome, leading one to include this disease in the rapidly growing list of inborn errors of metabolism, or molecular diseases.

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Diphosphonates Inhibit Hydroxyapatite Dissolution in vitro and Bone Resorption in Tissue Culture and in vivo

Abstract. *Two diphosphonates containing the P–C–P bond, $Cl_2C(PO_3HNa)_2$ and $H_2C(PO_3HNa)_2$ retard the rate of dissolution of apatite crystals in vitro. They inhibit bone resorption induced by parathyroid extract in mouse calvaria in tissue culture and in thyroparathyroidectomized rats in vivo.*

Inorganic pyrophosphate inhibits the precipitation (*1, 2*) and the dissolution (*3*) of hydroxyapatite crystals in vitro. Because pyrophosphate is present in plasma (*4*), teeth (*5*), and bone (*6*), we have suggested (*2*) that it might regulate both the formation and destruction of mineralized tissues in vivo. Although pyrophosphate and longer-chain condensed phosphates can inhibit the deposition of calcium phosphate in chick embryo femurs in tissue culture (*7*) and in the aorta (*8*) and skin (*9*) of the rat, it has not yet been possible to demonstrate an effect on the resorption of living bone. This failure to influence bone resorption may be due to hydrolysis of the P–O–P bond locally in the bone by pyrophosphatase before it can reach its site of action.

Substances were therefore sought that would be related in structure to pyrophosphate but resistant to chemical and enzymatic hydrolysis. Various compounds containing the P–C–P bond have been synthesized and found to have an effect similar to that of condensed phosphates on the precipitation of hydroxyapatite in vitro and on calcification in vivo (*10*). In addition, they were also active when administered orally (*10*). We now describe the effect of two such compounds containing a P–C–P bond, namely, sodium dichloromethylenediphosphonate [$Cl_2C(PO_3HNa)_2$] and sodium methylenediphosphonate [$H_2C(PO_3HNa)_2$], on the dissolution of apatite crystals in vitro and on bone resorption induced by parathyroid extract in tissue culture and

Table 1. Effect of $Cl_2C(PO_3HNa)_2$, $H_2C(PO_3HNa)_2$ and $CH_3(CH_2)_4PO_3HNa$ on resorption of mouse calvaria in tissue culture. Resorption was assessed by a point-counting method with the use of a grid. The resorption in all groups was compared with those subjected to PTE alone, and the significances of the differences were determined by Student's *t*-test. Percentages are given as means ± S.E. The asterisk denotes differences significant at less than 2 percent level.

Dosage (μg of P per ml)	Calvaria (No.)	Total bone resorbed (%)
	No PTE	
	16	7.8 ± 0.6*
	PTE	
	85	21.3 ± 0.7
	PTE + $Cl_2C(PO_3HNa)_2$	
0.01	6	18.3 ± 2.5
0.1	13	10.1 ± 1.0*
1	11	10.2 ± 0.8*
	PTE + $H_2C(PO_3HNa)_2$	
0.1	10	19.4 ± 1.4
1	12	16.2 ± 2.4*
	PTE + $CH_3(CH_2)_4PO_3HNa$	
10	9	17.6 ± 1.9
1	13	24.0 ± 2.0

in living rats. A monophosphonate, sodium pentane-1-phosphonate [$CH_3(CH_2)_4PO_3HNa$], which contains only a single C–P bond, as well as pyrophosphate and polyphosphate, was used as a control.

Hydroxyapatite crystals similar to those used previously (*2, 3*) were equilibrated for 15 hours at 37°C in a solution of 0.155*M* KCl buffered at *p*H 7.0 with 0.01*M* barbital. A concentrated solution of $H_2C(PO_3HNa)_2$, $Cl_2C(PO_3HNa)_2$, $CH_3(CH_2)_4PO_3HNa$ or pyrophosphate dissolved in the same buffer was added slowly with stirring over 2 hours until the final amount added represented 5 percent of the total phosphate of the apatite present (*3*). The crystals were filtered and then resuspended in a similar buffer containing no calcium, phosphate, phosphonates, or pyrophosphate. Control crystals, treated identically except that no phosphonates or pyrophosphate had been added, were resuspended in a similar manner. Addition of the diphosphonates and pyrophosphate to apatite crystals resulted in reduction of the rate at which calcium and phosphate entered the solution (Fig. 1); the monophosphonate had no effect.

Calvaria from 3-day-old mice were cultured individually for 5 days in 2 ml of modified BGJ medium (*11*) containing heat-inactivated horse serum (5 percent). The medium was changed each day. Parathyroid extract (PTE Parathormone, Lilly) added at a concentration of 0.2 unit/ml induced detectable resorption in such bones. This resorption, as assessed quantitatively by a point counting method, could be inhibited by $Cl_2C(PO_3HNa)_2$ or $H_2C(PO_3HNa)_2$ at concentrations as low as 0.1 and 1.0 μg of P per milliliter, respectively (Table 1); $CH_3(CH_2)_4PO_3HNa$, in contrast, was not inhibitory even at concentrations as high as 10 μg of P per milliliter (Table 1). Pyrophosphate and polyphosphates also have no effect at similar concentrations. Histologic and electron microscopic surveys failed to reveal any toxic action of the phosphonates on the cultured bones; however, this does not exclude subtle metabolic effects.

Male Wistar rats (100 to 130 g) were thyroparathyroidectomized surgically and maintained for about 4 days on a diet poor in calcium and phosphate. The drinking water was supplemented with 2 percent (weight to volume) calcium gluconate. For 36 hours before the experiment the rats were allowed to drink distilled water only. On the day of the experiment a blood sample was taken, and the animals were then injected subcutaneously with 50 units of PTE per 100 g of body weight. Six hours later a second blood sample was taken, and its calcium concentration was compared with the first. Parathyroid extract induced a significant rise in plasma calcium (Table 2). This rise could be completely prevented by $Cl_2C(PO_3HNa)_2$ and $H_2C(PO_3HNa)_2$ at doses of 10 mg of P per kilogram of body weight, given subcutaneously daily for 3 days up to but not including the day on which the parathyroid extract was given

Table 2. Influence of $H_2C(PO_3HNa)_2$, $Cl_2C(PO_3HNa)_2$, and $CH_3(CH_2)_4PO_3HNa$, given at 10 mg of P per kilogram body weight per day, on the rise in plasma calcium induced by 50 units of parathyroid extract (PTE) per 100 g of body weight in thyroparathyroidectomized rats. The significances of the differences in plasma calcium between the animals treated with PTE alone and the other groups were determined by Student's *t*-test. SC, subcutaneous.

Treatment			Plasma calcium (mg per 100 ml) (mean ± SE)			
Material	Route	Animals (No.)	Before PTE	Six hours after PTE treatment	Mean change ± SE	*P*
No PTE		14	6.59 ± .26	6.27 ± .33	−0.32 ± .29	<.01
PTE alone		47	6.27 ± .15	8.22 ± .29	+1.95 ± .24	
PTE + $H_2C(PO_3HNa)_2$	SC	14	5.92 ± .41	6.10 ± .33	+0.18 ± .25	<.01
PTE + $H_2C(PO_3HNa)_2$	Oral	14	5.13 ± .35	6.29 ± .46	+1.16 ± .28	<.05
PTE + $Cl_2C(PO_3HNa)_2$	SC	14	5.41 ± .40	5.70 ± .34	+0.29 ± .16	<.01
PTE + $Cl_2C(PO_3HNa)_2$	Oral	14	6.05 ± .34	7.11 ± .48	+1.06 ± .26	<.05
PTE + $CH_3(CH_2)_4PO_3HNa$	SC	14	5.30 ± .40	7.13 ± .68	+1.83 ± .38	

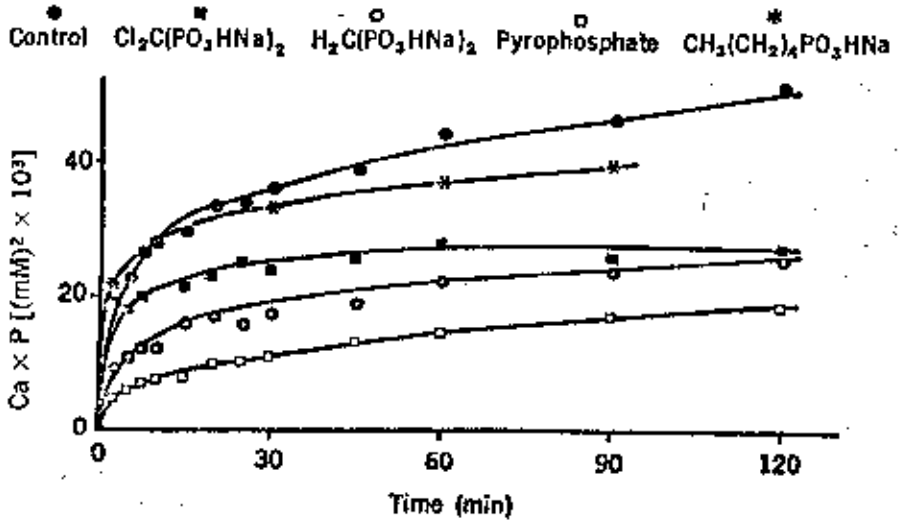

Fig. 1. Effect of pyrophosphate, two diphosphonates [$Cl_2C(PO_3HNa)_2$ and $H_2C(PO_3HNa)_2$] and a monophosphonate [$CH_3(CH_2)_4PO_3HNa$] on the dissolution of hydroxyapatite crystals in vitro.

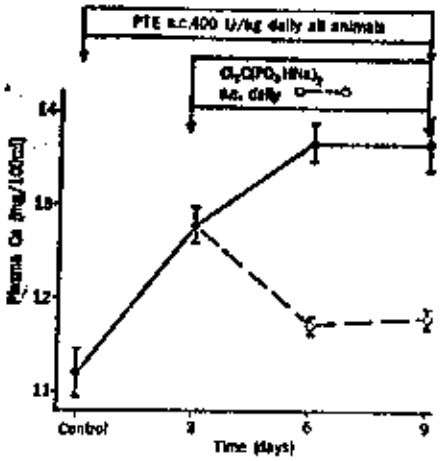

Fig. 2. Influence of $Cl_2C(PO_3HNa)_2$ (5 mg of P per kilogram of body weight, subcutaneously) on the hypercalcemia of rats treated for 9 days with parathyroid extract.

(Table 2). The compounds were also effective when given orally; $CH_3(CH_2)_4$-PO_3HNa had no significant effect on the increase in plasma calcium under similar conditions (Table 2). Pyrophosphate and polyphosphates given at 10 and 30 mg also have no effect. The $Cl_2C(PO_3HNa)_2$ can also reverse the hypercalcemia in rats given prior treatment with PTE for several days (*12*) (Fig. 2).

Our experiments indicate that diphosphonates and pyrophosphate retard the dissolution of hydroxyapatite crystals in vitro. The diphosphonates, but not pyrophosphate and polyphosphates, also inhibit the resorption of living bone in several experimental systems. For instance, diphosphonates can prevent the development of immobilization osteoporosis in rats (*13*). Furthermore, some diphosphonates reduce plasma calcium values in normal or thyroparathyroidectomized rats fed a low calcium diet (see for example Table 2). This indicates that the diphosphonates can reduce bone resorption both in the presence or the absence of parathyroid hormone.

The diphosphonates, which contain a P–C–P bond, differ from pyrophosphate and longer-chain condensed phosphates, which contain P–O–P bonds, in that the latter do not inhibit bone resorption in tissue culture and in vivo. It is possible that this difference is due to the hydrolysis of the condensed phosphates when they are given parenterally or added to tissue culture. They would therefore be unable to reach their potential site of action in bone. Pyrophosphatases from bone and other tissues are unable to split the phosphonates in vitro and it may be that phosphonates act on bone in tissue culture and in vivo because they are resistant to such hydrolysis. The simplest explanation of the mechanism of action of diphosphonates on bone resorption is that they retard crystal dissolution in a manner similar to that of pyrophosphate (*3*). Other mechanisms are, however, possible.

To our knowledge the diphosphonates are still the only substances, apart from thyrocalcitonin (*14*), that can significantly inhibit bone resorption in vivo, although fluoride (*15*), orthophosphate (*16*) and estrogens (*17*) have been studied for their potential therapeutic effect in this respect. Since diphosphonates appear to be relatively nontoxic they might prove valuable in the treatment of osteoporosis and other human diseases that involve increased resorption of bone.

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The Inhibitory Effect of Phosphonates on the Formation of Calcium Phosphate Crystals *in vitro* and on Aortic and Kidney Calcification *in vivo*

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Abstract. 1. Various phosphonates, which are compounds containing C-P bonds, have been studied to see whether they are able to inhibit, in a manner similar to that of pyrophosphate and the condensed phosphates, the crystallization of calcium phosphate *in vitro* and the pathological calcification of the aorta and the kidneys of rats given large amounts of vitamin D_3.

2. Six of the ten compounds studied markedly increased the minimum product, $[Ca] \times [P]$, required to induce the precipitation of calcium phosphate *in vitro* under physiological conditions of pH, ionic strength and temperature. Inhibition was observed at concentrations as low as 10^{-7}—10^{-8} M.

3. Most of the diphosphonates, particularly those possessing P-C-P bonds, showed some ability to inhibit the calcification of the aortas and kidneys of rats treated with large amounts of vitamin D_3. The most effective inhibitors were methylene diphosphonate (MDP), ethane-1 hydroxy-1-diphosphonate (EHDP) and dichloromethylene diphosphonate (Cl_2MDP).

4. The phosphonates that possess P-C-P bonds thus appear to have effects on the deposition of calcium phosphate *in vitro* and *in vivo* similar to those of inorganic pyrophosphate and the condensed phosphates, which possess P-O-P bonds. These phosphonates differ from the condensed phosphates in that they inhibit kidney calcification as well as aortic calcification and are active by mouth as well as parenterally. The wider spectrum of activity of the phosphonates *in vivo* may be due to the fact that they are more resistant to chemical and enzymic breakdown.

5. Phosphonates might be used therapeutically in man against diseases in which calcium salts deposit in soft tissues.

Key words: Calcification, phosphonates, pyrophosphate, calcium, phosphorus, nephrocalcinosis, arteriosclerosis.

Low concentrations of inorganic pyrophosphate inhibit both the precipitation [1] and growth [2] of hydroxyapatite crystals *in vitro*. Since pyrophosphate has been found to be present in plasma at concentrations similar to those required to produce inhibition *in vitro* [3], it has been suggested that pyrophosphate might be one of the physiological agents responsible for preventing deposition of calcium phosphate in soft tissues *in vivo* [3, 4]. At sites of mineralization it was supposed that the pyrophosphate would have to be removed enzymically before deposition of calcium phosphate could occur.

In support of this hypothesis pyrophosphate and long chain condensed phosphates administered parenterally have been shown to be able to inhibit certain types of experimentally-induced calcification in animals. Thus these compounds prevent the aortic calcification induced by vitamin D_3 in rats [5] and skin calcification induced by dihydrotachysterol in rats [6, 7]. At the same doses they are, however, unable to inhibit the kidney calcification produced by vitamin D_3 [6], and have no effect upon the mineralization of bone [8]. It was thought that the differing sensitivity of various tissues to inhibition of calcification by condensed phosphates might be related to the amount of pyrophosphatase they contained, tissues such as kidney and bone that are rich in pyrophosphatases and alkaline phosphatases, enzymes which are now recognized to be able to act as pyrophosphatases [9—12], would be capable of hydrolysing the pyrophosphate before it could act. Since the intestine contains a high amount of alkaline phosphatase [13], a similar explanation might account for the observation that the condensed phosphates only inhibit calcification when administered subcutaneously but not when given orally.

Since the condensed phosphates were ineffective by mouth and did not act against all forms of soft tissue calcification their potential clinical application against disorders of calcification seemed somewhat limited. What seemed to be required were compounds similar to pyrophosphate in their behaviour towards hydroxyapatite but resistant to hydrolysis by pyrophosphatase. Compounds that possess the P-C-P bond instead of the P-O-P bond exhibit these properties [14—16]. In this paper we report the effect of a number of phosphonates possessing C-P or P-C-P bonds on the precipitation and crystallization of hydroxyapatite *in vitro* and on the calcification of rat aorta and kidney *in vivo*. The results indicate that the diphosphonates have a wider spectrum of activity than the condensed phosphates. Thus, not only are they active against aortic calcification but also against renal calcification and when given by mouth. Preliminary results have been presented elsewhere [15, 16].

Table 1. *List of phosphonates studied*

Name of compound	Abbreviation	Formula (as acid)
1-pentanemonophosphonic acid	PMP	$CH_3(CH_2)_4—PO_3H_2$
ethane-1:2-diphosphonic acid	E-1:2-DP	$CH_2—PO_3H_2$ / $CH_2—PO_3H_2$
methylenediphosphonic acid	MDP	CH_2 (→ PO_3H_2, → PO_3H_2)
methylenehydroxydiphosphonic acid	MHDP	$CH(OH)$ (→ PO_3H_2, → PO_3H_2)
dichloromethylenediphosphonic acid	Cl_2MDP	Cl_2C (→ PO_3H_2, → PO_3H_2)
ethane-1-amino-1:1-diphosphonic acid	EADP	$CH_3—C(NH_2)$ (→ PO_3H_2, → PO_3H_2)
ethane-1-hydroxy-1:1-diphosphonic acid	EHDP	$CH_3—C(OH)$ (→ PO_3H_2, → PO_3H_2)
ethane-1-hydroxy-1:1:2-triphosphonic acid	E-1-HTP	$CH_2(PO_3H_2)—C(OH)(PO_3H_2)—PO_3H_2$
propane-1:1:3:3-tetraphosphonic acid	PTeP	$CH(PO_3H_2)—CH_2—CH(PO_3H_2)—PO_3H_2$
polyester chain condensate of EHDP with acetic anhydride	Condensate I	$CH_3—C(=O)—[O—C(PO_3H_2)(CH_3)—P(=O)(OH)]_{1.9}—O—C(PO_3H_2)—CH_3$

Materials and Methods

The phosphonates were synthesized by the Procter and Gamble Co., Cincinnati, Ohio, U.S.A., and were kindly supplied to us by Dr. M. D. Francis. Sodium salts of the compounds listed in Table 1 were used.

Effect of Phosphonates on the Formation of Apatite Crystals in vitro. The minimum product (formation product), $[Ca] \times [P_i]$, required to induce precipitation of calcium phosphate from solution was measured as described previously [1]. With this method 1.7 mM Ca is incubated with increasing amounts (0.5—8.0 mM) of phosphate in siliconized vessels on a shaker for 3 days at 37 °C. The solutions are buffered at pH 7.4 with 0.01 M barbital and the ionic strength is brought to 0.16 by adding the appropriate amount of KCl. After 3 days any crystals formed are removed by filtration through "Göttingen Membran" filters (0.45 μ pore size) and the amounts of calcium and phosphate

remaining in solution are compared with those present at the beginning of the incubation, thus making it possible to determine the minimum [Ca] × [P] product required toinduce precipitation.

Prevention of Calcification of Aortas and Kidneys in Rats. Calcification of aortas and kidneys was induced in female Wistar rats, weighing 150--200 g, by giving them 75,000 IU (1.875 mg) vitamin D_3/kg body wt. per day by mouth for five days. The experimental procedure was similar to that described previously [5]. The phosphonate compounds were adjusted to pH 7.4 and were given daily at doses of 1 or 10 mg P/kg body wt., subcutaneously (s.c.) or orally (p.os), from two days before starting vitamin D_3 up to eight days after the end of administration of vitamin D_3, when the animals were killed. The concentrations of the compounds were such that the daily dose was given subcutaneously or orally in 0.2 ml/100 g body wt. Each treatment group contained 10 animals. There were two control groups: one consisted of 60 animals that received vitamin D_3 but instead of phosphonates received daily subcutaneous injections of 0.2 ml/100 g body wt. of 0.9% (w/v) NaCl, the other consisted of 20 animals that received no treatment whatsoever. All treatments were allocated to individual animals at random and each cage contained five animals. All animals were fed a standard diet (No. 194 from Nafag, Gossau, Switzerland).

In order to see whether or not the phosphonates had any effect on the hypercalcaemia induced by vitamin D_3, venous blood was collected from the orbit of each animal 24 hours after the last dose of the vitamin, when the hypercalcaemia due to the vitamin should be at its peak [5]. After sacrifice the aortas and kidneys were removed and their weights were determined after drying to constant weight at 105 °C. The organs were then ashed in a muffle oven at 600 °C for eight hours. The residues were dissolved in 0.5 N-HCl and the calcium determined. The calcium of the aortas and plasma was determined by photometric titration with EDTA using calcichrome as an indicator [17] and the calcium of the kidneys by atomic absorption spectroscopy (Perkin Elmer Model 290 B).

Statistical Analyses. The differences between the vitamin D_3 group and each treatment group (Tables 3, 4 and 5) were examined statistically for evidence of treatment effects, i.e. a difference in mean calcium content compared with vitamin D_3 control. In the case of aorta and kidney calcium the values in each treatment group followed a skewed distribution so the logarithms of the calcium values were used for statistical analysis. It was assumed throughout that the variation between animals receiving the same treatment was not dependent on the treatment. The standard errors of treatment means were calculated from a pooled estimate of the variance, and the significance of differences in mean compared with vitamin D_3 control determined by two-sided t tests.

Since the primary interest lies in detecting compounds which inhibit calcification, i.e. in compounds that cause a reduction in mean calcium compared with vitamin D_3 control, a one-tailed test would be more appropriate. However, the possibility that a compound may cause an increase in mean calcium should also be investigated and for this reason the results of two-tailed tests have been given. In fact there were only two significant increases in mean calcium, with PMP and E-1:2-DP both on plasma. Neither of these, when studied with the other plasma calcium results for these compounds, is regarded as sufficient evidence for a real treatment effect.

Significant reductions in mean calcium were not automatically interpreted as indicative of treatment activity. The choice of the 5% significance level, although customary, is nevertheless arbitrary. Also as 120 t tests were performed some reductions are expected to achieve significance by chance alone. An additional criterion was therefore used in forming conclusions about treatment activities. It was expected that, if a compound was active in inhibiting calcification, 10 mg/kg doses should be more active than 1 mg/kg doses and that applications given s.c. should be more active than applications given p.os. We felt justified in interpreting significant reductions as evidence for treatment activity if these constraints were satisfied. This applies also to differences significant at the 10% level only provided they were reinforced by significant differences in other applications of the same compound. The fact that these constraints were satisfied in all but three cases (PTeP on aorta calcium, MHDP and EADP on kidney calcium) gives reassuring support to the validity of the whole experiment.

Results

Effect of Phosphonates on the Precipitation of Calcium Phosphate in vitro. Table 2 shows the effect of the phosphonates on the minimum product, [Ca] × [P], required to form crystals *in vitro*. EHDP appeared to be the most effective inhibitor of precipitation and exerted a strong effect at 10^{-7} M. EHDP, EADP, MHDP, MDP, Condensate I and Cl_2MDP were all good inhibitors at 10^{-6} M. The remaining compounds, E-1-HTP, E-1:2-DP, PMP and PTeP had only minimal effects. At concentrations greater than 10^{-4} M the calcium salts of some of the phosphonates precipitated from solution.

Effect of the Phosphonates on Vitamin D_3-induced Calcification of Aortas and Kidneys and on Plasma Calcium. Tables 3, 4 and 5 summarize the effect of the various phosphonates on the calcium content of aortas, kidneys and plasma of rats treated with vitamin D_3. Vitamin D_3 significantly increased the calcium content of aortas, kidneys and plasma of animals treated with vitamin D_3 alone compared with untreated control animals. The numbers of animals surviving the duration of the experiment are shown in

Table 2. *Influence of various phosphonates on the minimum product [Ca] × [P] in mM² (formation product) necessary to induce precipitation of calcium phosphate from solution. Incubation for three days at pH 7.4 and 37° C. Control values obtained from 71 incubation without phosphonates were 4.84 ± 0.12 (mean ± SE, [Ca] × [P] in mM²). The results shown here represent the increase in minimum [Ca] × [P] product observed in the presence of the phosphonate compared with the minimum [Ca] × [P] product required for crystal formation in a parallel experiment but in the absence of the phosphonate. Number of experiments in parentheses*

Compound	Mean increase in formation product over control value		
	Concentration of phosphonate		
	10^{-5} M	10^{-6} M	10^{-7} M
PMP	0.38 (6)	0.47 (8)	—
E-1-2-DP	0.50 (1)	0.20 (1)	—
MDP	—	2.21 (7)	0.62 (5)
MHDP	—	3.10 (1)	0.40 (1)
Cl_2MDP	6.30 (1)	1.10 (1)	—
EADP	—	4.90 (1)	0.30 (1)
EHDP	—	>7.30 (6)	1.88 (5)
E-1-HTP	0.70 (1)	−0.10 (1)	—
PTeP	0.20 (1)	−0.50 (1)	—
Condensate I	—	1.90 (1)	0.70 (1)

Table 3. The survival proportions of the untreated control group and the vitamin D_3 control, 17/20 and 53/60, are almost identical. Clearly there is no evidence that treatment with vitamin D_3 itself caused an increase in mortality. The survival proportions for each treatment group were compared with that for vitamin D_3 control using the two-sided t test. There was evidence (significant at the 1% and 5% level respectively) that MHDP and MDP, both at 10 mg P/kg s.c., caused an increase in mortality. A survival proportion of 6/10 differs from one of 53/60 at the 9% significance level. There is therefore a suggestion that EADP and E-1-HTP, at 10 mg P/kg s.c., may also cause an increase in mortality.

The only effect of the phosphonates on the body weight was that the animals who received 10 mg s.c. of MDP, EADP, EHDP, Condensate I, E-1-HTP and PTeP weighed less at the end of the experiment than the animals given Vitamin D alone. The s.c. administration of 1 mg P/kg and the oral administration had no effect.

Aorta Calcium. All the phosphonates except PMP had some effect in reducing aortic calcium when given at 10 mg P/kg s.c. (Table 3). All compounds except PMP, E-1-HTP and Condensate I were also effective at 1 mg P/kg s.c. Fewer compounds were effective by the oral route; MDP, Cl_2MDP, EHDP, E-1-HTP and Condensate I were effective at 10 mgP/kg p.os., whereas only MDP and perhaps PTeP were effective at 1 mg P/kg p.os.

Kidney Calcium. Seven of the ten phosphonates tested caused significant reductions in kidney calcium (Table 4). All of the significant differences listed in Table 4 can reasonably be interpreted as indicating real treatment effects, but there are two anomalies. The reductions in mean calcium when both MHDP and EADP were given at 10 mg P/kg s.c. were not significant whereas the reductions with 1 mg P/kg s.c. were in both cases highly significant. With MHDP at 10 mg P/kg given p.os there was also a highly significant reduction. As only 2 and 3 determinations respectively are available in the 10 mg P/kg s.c. treatment groups the evidence on balance favours the conclusion that these two treatments are both active.

In conclusion, there was evidence that the following compounds caused some inhibition of kidney calcification: MDP, MHDP, Cl_2MDP, EADP, EHDP, PTeP and Condensate I.

Table 4. *Influence of various phosphonates on the amount of calcium in the aortas of rats that received 75,000 IU vitamin D_3 per kg body weight daily for 5 days. Aorta calcium as mean $\log_{10}$ (mg Ca/g dry weight) ± SE of mean. Number of determinations in parentheses*

Compound	1 mg P/kg s.c.	Survival	1 mg P/kg p. os	Survival	10 mg P/kg s.c.	Survival	10 mg P/kg p. os	Survival
PMP	4.44 ± 0.19 (9)	10/10	4.57 ± 0.19 (9)	9/10	4.5[illegible] ± 0.19 (9)	10/10	4.66 ± 0.20 (8)	9/10
E-1-2-DP	4.13 ± 0.21[b] (7)	9/10	4.39 ± 0.21 (7)	8/10	3.75 ± 0.19[c] (9)	10/10	4.45 ± 0.20 (8)	9/10
MDP	4.16 ± 0.20[b] (8)	10/10	3.90 ± 0.18[b] (10)	10/10	3.11 ± 0.28[c] (4)	4/10	3.17 ± 0.19[c] (9)	10/10
MHDP	3.62 ± 0.21[c] (7)	9/10	4.58 ± 0.21 (7)	9/10	3.66 ± 0.56[a] (1)	2/10	4.47 ± 0.23 (6)	9/10
Cl_2MDP	3.38 ± 0.19[c] (9)	9/10	4.51 ± 0.18 (10)	10/10	3.39 ± 0.21[c] (7)	9/10	3.25 ± 0.21[c] (7)	10/10
EADP	3.79 ± 0.19[c] (9)	9/10	4.42 ± 0.19 (9)	10/10	3.56 ± 0.25[c] (5)	6/10	4.57 ± 0.19 (9)	10/10
EHDP	3.95 ± 0.18[c] (10)	10/10	4.33 ± 0.23 (6)	7/10	3.33 ± 0.19[c] (9)	9/10	3.38 ± 0.23[b] (6)	10/10
E-1-HTP	4.70 ± 0.20 (8)	9/10	4.58 ± 0.19 (9)	10/10	4.05 ± 0.23[b] (6)	6/10	4.25 ± 0.19[a] (9)	10/10
PTeP	4.22 ± 0.20[b] (8)	10/10	4.28 ± 0.19[a] (9)	10/10	3.88 ± 0.21[c] (7)	7/10	4.41 ± 0.18 (10)	10/10
Condensate I	4.42 ± 0.23 (6)	6/10	4.82 ± 0.19 (9)	10/10	3.52 ± 0.19[c] (9)	9/10	4.26 ± 0.19[a] (9)	10/10

Vitamin D_3 only: 4.64 ± 0.09 (51) | Untreated control: 3.31 ± 0.14 (16) } difference significant at 1% level. Survival { 53/60 | 17/20

[a] Denotes that difference in mean aortic calcium compared with vitamin D_3 Control is significant at 10% level.
[b] Denotes that difference in mean aortic calcium compared with vitamin D_3 Control is significant at 5% level.
[c] Denotes that difference in mean aortic calcium compared with vitamin D_3 Control is significant at 1% level.

Table 4. *The influence of various phosphonates on the amount of calcium in the kidneys of rats that received 75,000 IU vitamin D_2 per kg body weight daily for 5 days. Kidney calcium as mean $\log_{10}$ (mg Ca/g dry weight) ± SE of mean. Number of determinations in parentheses*

Compound	1 mg P/kg s.c.	1 mg P/kg p. os	10 mg P/kg s.c.	10 mg P/kg p. os
PMP	3.81 ± 0.19 (5)	4.03 ± 0.11 (9)	3.83 ± 0.16 (7)	3.94 ± 0.15 (8)
E-1:2-DP	3.71 ± 0.19 (5)	3.83 ± 0.17 (9)	3.93 ± 0.17 (6)	3.82 ± 0.16 (7)
MDP	3.16 ± 0.27[b] (6)	3.71 ± 0.16 (7)	3.38 ± 0.19[b] (5)	3.23 ± 0.19[b] (5)
MHDP	3.11 ± 0.16[b] (7)	3.96 ± 0.16 (7)	3.60 ± 0.31 (2)	2.30 ± 0.17[b] (6)
Cl_2MDP	3.18 ± 0.17[b] (6)	3.41 ± 0.16[a] (7)	2.97 ± 0.14[b] (9)	2.99 ± 0.13[b] (10)
EADP	3.23 ± 0.17[b] (6)	3.99 ± 0.15 (8)	3.86 ± 0.24 (3)	3.61 ± 0.13 (10)
EHDP	3.36 ± 0.15[b] (8)	3.77 ± 0.17 (6)	3.12 ± 0.17[b] (6)	3.02 ± 0.17[b] (6)
E-1-HTP	3.69 ± 0.16 (7)	3.96 ± 0.13 (10)	3.77 ± 0.17 (6)	3.55 ± 0.17 (6)
PTeP	3.70 ± 0.24 (3)	3.89 ± 0.16 (7)	3.14 ± 0.10[a] (6)	4.01 ± 0.19 (5)
Condensate I	3.54 ± 0.17 (6)	3.95 ± 0.15 (8)	3.24 ± 0.19[b] (5)	3.86 ± 0.17 (6)

Vitamin D_2 only: 3.83 ± 0.07 (37)
Untreated control: 2.73 ± 0.11 (15)
difference significant at 1% level.

[a] Denotes that difference in mean compared with vitamin D_2 Control is significant at 5% level.
[b] Denotes that difference in mean compared with vitamin D_2 Control is significant at 1% level.

Table 5. *Influence of various phosphonates on the plasma calcium of rats that received 75,000 IU vitamin D_2 per kg body weight daily for 5 days. The values were taken 24 hours after the last dose of vitamin D_2. Plasma calcium as mg per 100 ml ± SE of mean. Number of determinations in parentheses*

Compound	1 mg P/kg s.c.	1 mg P/kg p. os	10 mg P/kg s.c.	10 mg P/kg p. os.
PMP	15.90 ± 0.54[c] (9)	13.51 ± 0.57 (8)	14.29 ± 0.67 (8)	14.79 ± 0.54 (9)
E-1:2-DP	14.51 ± 0.61 (7)	14.53 ± 0.66 (6)	15.01 ± 0.57[a] (8)	14.66 ± 0.54 (9)
MDP	15.02 ± 0.54 (9)	14.41 ± 0.54 (9)	11.82 ± 0.57[c] (8)	14.04 ± 0.54 (9)
MHDP	14.36 ± 0.61 (10)	14.73 ± 0.61 (7)	14.00 ± 0.93 (3)	15.10 ± 0.61 (7)
Cl_2MDP	14.08 ± 0.66 (6)	15.08 ± 0.51 (10)	12.98 ± 0.66[b] (6)	12.94 ± 0.54[b] (9)
EADP	14.10 ± 0.66 (6)	14.63 ± 0.72 (5)	12.70 ± 0.61[b] (7)	14.09 ± 0.54 (9)
EHDP	14.80 ± 0.51 (10)	14.30 ± 0.66 (6)	13.07 ± 0.61[a] (7)	14.10 ± 0.54 (9)
E-1-HTP	14.76 ± 0.61 (7)	14.66 ± 0.51 (10)	14.62 ± 0.57 (8)	13.98 ± 0.66 (6)
PTeP	15.24 ± 0.61 (7)	14.83 ± 0.66 (6)	15.35 ± 0.66 (6)	15.50 ± 0.80 (4)
Condensate I	13.57 ± 0.80 (4)	14.79 ± 0.54 (9)	14.69 ± 0.57 (8)	14.78 ± 0.72 (5)

Vitamin D_2 only: 14.36 ± 0.25 (41)
Untreated Control: 10.84 ± 0.39 (17)
difference significant at 1% level.

[a] Denotes that difference in mean compared with vitamin D_2 Control is significant at 10% level.
[b] Denotes that difference in mean compared with vitamin D_2 Control is significant at 5% level.
[c] Denotes that difference in mean compared with vitamin D_2 Control is significant at 1% level.

Plasma Calcium. Only 3 of the phosphonates, MDP, Cl_2MDP and EADP, showed evidence of being able to reduce the hypercalcaemia induced by vitamin D_2 (Table 5). These 3 compounds were effective when they were given at 10 mg P/kg s.c. Cl_2MDP was also effective at this dose when given p.os.

Discussion

These experiments show that various phosphonates act in a similar manner to pyrophosphate and condensed phosphates on the inhibition of crystallization of hydroxyapatite *in vitro* and on the prevention of aortic calcification in rats given large doses of vitamin D_2. Some of these phosphonates have additional properties not shared with the condensed phosphates; these include the ability to prevent aortic calcification when given orally as well as subcutaneously and the ability to prevent kidney calcification. Furthermore, in opposition to condensed phosphates [8], large doses of EHDP (greater than 2.5 mg P/kg) have been found to inhibit mineralisation of rat and dog bones and to produce a rachitic and osteomalacic type appearance on X-ray and by histology. No such lesions were observed with similar and higher doses of Cl_2MDP[1, 2]. The inhibition of bone mineralisation ceased and calcification resumed within four weeks after stopping injections of EHDP[1]. The fact that, unlike the polyphosphates, the phosphonates are effective orally and are able to inhibit kidney calcification may be attributable to their marked resistance to spontaneous and enzymic hydrolysis. However, as would be expected, most of the active compounds were less effective by mouth than subcutaneously, suggesting that their absorption from the intestinal tract may not be complete. This is consistent with other studies in rats which have shown that, in the case of ^{14}C-labelled EHDP, only about 3% of an oral load is absorbed[3].

1 King, W. R., Michael, W. R.: Personal communication.
2 Fleisch, H., Russell, R. G. G., Muhlbauer, R. C.: Unpublished observations.
3 Francis, M. D., personal communication.

When the effect of any particular compound on aortic and kidney calcification at the various doses was considered, there was a fair correlation between the ability of any compound to inhibit crystal growth *in vitro* and its overall ability to inhibit calcification *in vivo*. This strengthens the view that the biological activity of these compounds could be a consequence of their observed action on crystal growth [14, 16]. In general, the compounds that have, so far, proved most active have contained the P-C-P bond. Since compounds that contain the P-C-P bond are related in structure to those that contain the P-O-P bond, the diphosphonates, pyrophosphate and polyphosphates may all act on crystal growth by similar mechanisms. In this study the three most effective inhibitors of calcification appeared to be MDP, Cl_2MDP and EHDP. The compounds PMP, which contains a single C-P bond, and E-1:2-DP, which contains a P-C-C-P bond, were less effective or inactive. It is not known whether any of the compounds are metabolized *in vivo* and to what extent this might alter their activity.

It is of interest that some of the diphosphonates (Cl_2MDP, MDP and EADP) can partially prevent the hypercalcaemia induced by vitamin D_3. This rise in plasma calcium after treatment with vitamin D_3 is probably the result of two processes, firstly an increase in intestinal absorption of calcium and secondly an increased resorption of bone. The ability of Cl_2MDP, MDP and EADP to reduce plasma calcium could be due to an interference with either of these processes. Alternatively it might be due to precipitation of circulating calcium as the insoluble calcium phosphonate. Since there is good evidence that these doses of phosphonates can inhibit bone resorption in living rats [15, 18, 19] it is probable that the effect on plasma calcium is at least partly attributable to inhibition of bone resorption.

It is clear, however, that any reduction in plasma calcium concentration cannot be the sole explanation of the ability of the phosphonates to reduce aortic and kidney calcification, since several compounds had no effect on plasma calcium, but were, nonetheless, good inhibitors of calcification. This is also true of the condensed phosphates which can inhibit calcification without changing plasma calcium [5]. One of the most effective inhibitors of calcification, namely EHDP, did not produce a hypocalcaemia but on the contrary a hypercalcaemia when tested shortly after its administration. The phosphonates do not seem to act either by an intestinal or kidney effect, since no diminished intestinal absorption or increased kidney excretion was seen in rats under balance studies [20]. It is possible, however, that the inhibitory effects of phosphonates on calcification may be due to other causes than crystal growth inhibition, for example, an inhibition of pyrophosphatases or inhibition of conversion of vitamin D_3 to its biologically more active 25-hydroxy metabolite. Our preliminary studies of the interaction of phosphonates with pyrophosphatases indicate that the phosphonates are relatively poor inhibitors of pyrophosphatases and act principally by the chelation of metal ions necessary for enzyme activity. The significance of possible inhibitory effects on pyrophosphatases *in vivo* cannot yet be assessed. The effects of these phosphonates on other metabolic processes in which pyrophosphate is involved are also not known.

The experiments carried out so far indicate that the diphosphonates might be used therapeutically in man against diseases that involve the abnormal deposition of calcium in soft tissues as previously suggested by Francis [14]. Preliminary experience in myositis ossificans in humans suggests that EHDP prevents the progress of the disease without disturbing the formation and mineralisation of normal bone [21].

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1845 BROOKS—WO 2-4171

U.S. DEPARTMENT OF
HEALTH, EDUCATION, AND WELFARE
Food and Drug Administration
Washington, D.C. 20204

BC DT

FOR RELEASE IN P.M. PAPERS
Friday, August 27, 1965

HEW-062

The Food and Drug Administration, Department of Health, Education, and Welfare, is publishing a proposal which, if adopted, would reduce the amount of vitamin D that may be added to food products. Purpose of the proposal is to prevent possible injury to infants.

Commissioner George P. Larrick said that last November Dr. Robert Cooke of Johns Hopkins University had expressed concern that the ingestion of excessive amounts of vitamin D was a possible cause of infantile hypercalcemia. This is a condition which in its severe form involves not only an increase of calcium in the blood but changes in the bony structure of the face, an effect upon the aortic valve of the heart, and mental abnormalities.

On the basis of Dr. Cooke's views, the Commissioner invited the Committee on Nutrition of the American Academy of Pediatrics and a joint Committee of the Council on Foods and Nutrition and the Council on Drugs of the American Medical Association to look into this problem.

Both Committees recommended that, while there has been no positive demonstration of a cause and effect relationship of vitamin D to this disease, there should be restrictions on the marketing of foods containing added vitamin D.

The Committees made clear that there is abundant scientific evidence to demonstrate that an excessive intake of vitamin D is of no value. Four hundred USP units per day will meet the full requirements of infants, children and nursing mothers. For years large therapeutic doses of vitamin D have been held to prescription sale.

"In the light of all we know about vitamin D," Mr. Larrick said, "I have .ched the conclusion that prudence calls for issuing a proposal based on the

(More)

: ecommendations of the expert committees since the restrictions suggested would not deprive the public of any needed nutrients."

The proposal would permit the continued addition of vitamin D to such foods s milk, milk products and infant formulas at a level of 400 USP units per quart. Over the counter vitamin D preparations would be limited to a dosage of 400 USP nits of vitamin D per day. The proposal would deny authority for the addition of vitamin D to standardized foods such as enriched flour, enriched corn meal, enriched rice, enriched macaroni products, enriched bread and margarine.

The proposal would amend further FDA's regulations regarding vitamin D supplements as follows:

1. Vitamin D would be permitted in food supplements supplying not more than 400 USP units per day.

2. Vitamin D preparations containing over 400 USP units per day would be sold only on prescription.

3. Any drug containing doses of more than 400 USP units per day of vitamin D with labeling bearing directions for self-medication of vitamin D deficiency (rickets) would be classed as misbranded under the Food, Drug, and Cosmetic Act.

4. Preparations supplying less than 400 USP units of vitamin D and represented for treatment of vitamin D deficiency by the lay public would be egarded as misbranded, since the layman is not qualified to diagnose or treat ich a deficiency.

The proposal will be published in the Federal Register of Saturday, August 3d, 1965. Sixty days is allowed for comment. Commissioner Larrick invites comment rom all concerned and particularly from any person who may have scientific evidence that an excess of vitamin D has been shown to be a factor in the development of ypercalcemia in infants and any person who may have scientific evidence that this vitamin is not a factor in the disease.

# # #

NEW NEWS RELEASE

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VITAMINS A and D PRODUCTS

FOR RELEASE: PLEASE OBSERVE EMBARGO
P.M.'s - Wednesday, December 13, 1972

The Food and Drug Administration today acted to limit the strength of Vitamin A and D products available for over-the-counter sale.

FDA's objective is to establish safe amounts of vitamins that can be effective as dietary supplements for use by consumers. Based on current scientific knowledge the Agency is proposing daily limits of 10,000 International Units (I.U.) for vitamin A and 400 I.U. for vitamin D.

The principal effect of the proposed regulations will be to have makers of multivitamins revise their formulas. Many multivitamin products are presently below the limits proposed for vitamins A and D. There are on the market, however, vitamin A products in dosages 10 times the recommended daily allowance (RDA) and vitamin D products 60 times the RDA.

High-dosage vitamin products prescribed by physicians for specific patients will be unaffected.

Reports of misuse of large doses of both vitamins A and D have prompted medical groups and FDA to seek limitations on them. Widespread promotion of both vitamins has resulted in excess use for conditions

-more-

such as acne, night blindness, and arthritis. Neither vitamin A nor D are proven effective for these conditions in well nourished people.

Excessive amounts of vitamin A taken over long periods can increase pressure within the human skull and may mimic a brain tumor. Large doses of this vitamin have also been shown to retard growth in children. Vitamin D has been known to retard mental and physical growth in children.

In commenting on the proposal, Charles C. Edwards, M.D., Commissioner of Food and Drugs, said: "Vitamins A and D are known to be toxic; and they are heavily promoted in high doses to the consumer; and we continue to accumulate evidence of adverse effects from excessive intake. The FDA has concluded, therefore, that consumer safety requires the action we are taking."

Interested persons may submit written comments within 60 days. Comments should be addressed to the Hearing Clerk, Department of Health, Education, and Welfare, Room 6-88, 5600 Fishers Lane, Rockville, Maryland 20852.

# # #

Food Protection Committee
Food and Nutrition Board, 1965

Chemicals Used in Food Processing

National Academy of Sciences,
National Research Council, Washington, D.C.

Food Protection Committee, 1972

Comprehensive GRAS Survey

National Academy of Sciences,
National Research Council

Italian Translation

Induction of Experimental Tumors with Vitamin D_2

Di Allessandro Franciosi (From the Institute of Anatomy and Pathological Histology of the Royal Modena University — Modena Section, Session of 9 December 1942)

Source: ~~Unknown (2)~~ Boll. Soc. Ital. Sper. 17: 701-702, 1942

Among the substances with possible blastomogenic properties on which research has been carried out in the past few years, vitamin D_2 has also been tested.

Using this substance on albino mice (mus musculus) B. Polettini (Tumori 12: 101, 1938) was able to obtain in 6 mice out of 15, after 13 months of skin painting, the formation of mammary adenomas exhibiting definite cytological signs of malignancy, whereas subcutaneous injections of vitamin D induced, after 4-11 months, a smaller percentage (in 4 out of 13 animals) of blastomas (tumors), but of greater histological variety.

Considering the fact that Polettini used in his tests animals in which spontaneous neoplastic processes (tumors) never occur, whereas such a phenomenon is not exceptional during the breeding of mice at our Institute, it seems possible that the eventual blastomogenic action of vitamin D_2 might be associated with the constitutional tendency on the part of these animals to develop tumors. The effect of this substance on rats and pigeons was also tested.

On 18 December 1939, the following tests were initiated:

1) Effects of skin painting of vitamin D_2 (Vigantol, Merck) on alternate days on mice and pigeons.

2) Effects of parenteral injection of vitamin D_2 on mice and rats.

3) Study of control mice allowed to age without any kind of treatment.

Results of our studies

1) In 15 mice painted with vitamin D_2, after 6-11 months, the formation of 3 neoplasms of different type (neoplastic leukemic lymphoadenoma, a mammary adenoma, and a subcutaneous spindle-cell sarcoma) were noted in 20% of the mice at points far removed from the site of treatment. In 6 pigeons painted with the same vitamin D_2 solution, no tumor formation was observed after more than 2 1/2 years.

2) In 15 mice receiving a subcutaneous dorsal injection of vitamin D_2, the formation of a tumor (Ehrlich-type mammary adenocarcinoma) was noted in 6.6% of the animals, after 11 months, at a point far removed from the injection site. No tumor formation was observed in 10 rats after 26 months of the same treatment.

3) In 30 control mice allowed to age, a single spontaneous tumor (Ehrlich-type mammary adenocarcinoma) was noted in 3.3% of the animals after 18 months.

The most that can be said about these results is that they agree with those obtained by Polettini. In fact, it cannot be denied that vitamin D_2 has promoted, in our tests, the formation of tumors in mice; in 20% of the animals when applied through the skin and in 6.6% when introduced parenterally, as compared to 3.3% in control animals. In addition, the formation of tumors occurred much more rapidly than in untreated mice. The effects (expressed in percent) obtained by Polettini are much greater than ours, namely 40% in animals painted with the vitamin and 32.5% in those receiving a subcutaneous injection. This difference in the percentage of tumor-carrying

animals noted in my tests and those of Polettini, although not reflecting on the merits of this noted researcher, suggests to our mind that different conditions of breeding (race), environment, nutrition, etc. may be the possible cause of such a discrepancy.

In order to explain the genesis of the tumors obtained, a selective action of vitamin D_2 cannot be invoked, both in view of the low percentage of tumor-bearing animals and because the tumors were not induced at the site of treatment and were not of the same histological type, whereas carcinogens with a definite action almost always induce tumors at the point of application and of the same histological type. I believe that in my tests vitamin D_2 has promoted, perhaps by affecting the humoral state of the host organism, the genesis (formation) of a larger number of tumors than the one observed in control animals; without such a promoting action, I cannot explain the pathogenetic mechanism of tumor formation.

After considering the action and the chemical structure of vitamin D_2, Polettini arrives at the following conclusion: "An interpretation is not easy, because too many factors can be claimed to intervene and cause modifications of a chemical or chemical-physical order in the general and local metabolism of the organism in relation to the physical or chemical nature of the substance used". We also want to stay away from such conclusions, while the fact of having obtained, also by skin painting, tumors of various histological types appears to be of interest.

Boll. Soc. Ital. Biol. Sper. 17: 701-702 (1942)

SULLA PRODUZIONE DI TUMORI SPERIMENTALI CON VITAMINA D_2.

Di Alessandro Franciosi.

(*Dall' Istituto di Anatomia e Istologia Patologica della R. Università di Modena*).

Sezione di Modena — Seduta del 9 dicembre 1942.

Fra le sostanze di cui in questi ultimi anni si sono cercate eventuali proprietà blastogene, è stata anche sperimentata la vitamina D_2.

Usando tale sostanza Polettini (1) ha ottenuto nel *mus musculus* albino, dopo 13 mesi di pennellature cutanee, l'insorgenza in 6 topi su 15 di adenomi mammari presentanti netti caratteri citologici di malignità, mentre per iniezioni sottocutanee ha riscontrato in 4-11 mesi una percentuale minore di blastomi (4 su 13), ma maggiore varietà istologica. Nei topi controllo trattati con colesterina non ha ottenuto alcun tumore.

Tenuto conto che il Polettini ha adoperato animali in cui mai insorgevano processi neoplastici spontanei, mentre nell'allevamento di topini del nostro Istituto, tale fenomeno non è eccezionale, ci apparve suggestivo unire l'eventuale azione blastogena della vitamina D_2 alla tendenza costituzionale per i tumori in questi animali. Fu sperimentata anche l'azione di questa sostanza sul ratto e sul colombo.

Il 18-12-'39 dava inizio agli esperimenti che così impostai:

1°) Effetti della pennellatura a giorni alterni con vitamina D_2 (Vigantolo Merk) nei topi e colombi.

2°) Effetti della inoculazione per via parenterale di vitamina D_2 in topi e ratti.

3°) Topi controllo tenuti ad invecchiare senza alcun trattamento.

Risultato delle ricerche. 1°) In 15 topi pennellati con vitamina D_2, insorsero in 6-11 mesi, in punti lontani dal luogo di trattamento, 3 neoplasie di tipo diverso (linfoadenosi leucemica neoplastiforme, adenoma mammario, sarcoma fusocellulare del sottocutaneo) con una percentuale del 20 %.

In 6 colombi sottoposti a pennellature della stessa soluzione di vitamina D_2 per più di due anni e mezzo non si notò insorgenza di tumori.

2°) In 15 topi in cui fu iniettata nel sottocute (dorso) la vitamina D_2 si ebbe l'insorgenza di un tumore (adenocarcinoma mammario tipo Ehrlich) pur esso lontano dal punto di inoculazione della sostanza, dopo 11 mesi di trattamento, nella percentuale del 6,6 %. In 10 ratti

anche dopo 26 mesi di esperimento non si ebbe la comparsa di alcun tumore.

3°) In 30 topi controllo che furono lasciati ad invecchiare insorse spontaneamente un solo tumore (adenocarcinoma mammario tipo Ehrlich) dopo 18 mesi di attesa nella percentuale del 3,3 %.

Di massima si può asserire che questi risultati concordano con quelli del Polettini. Infatti, non si può negare che la vitamina D_2 abbia favorito nei miei esperimenti l'insorgenza dei tumori nei topini; con una percentuale del 20 % usata per via percutanea e del 6,6 % introdotta per via parenterale, in contrapposto al 3,3 % degli animali controllo, inoltre i tumori insorsero molto più rapidamente che nei topi non trattati. In effetti la percentuale del Polettini è di gran lunga superiore 40 % nei pennellati e 32,5 % per iniezione sottocutanea. Questa differenza di percentuali dei miei esperimenti pur non infirmando quelle dell' illustre Ricercatore, ci porta a pensare a varie condizioni razza, ambiente, nutrizione, etc., possibili cause di questo divario.

Per spiegare la genesi dei tumori ottenuti non può essere invocata una azione elettiva della vitamina D_2, sia per la scarsa percentuale ottenuta sia perchè i tumori non sono insorti nel punto di trattamento e non sono stati dello stesso tipo istologico: mentre con le sostanze cancerigene ad azione definita si ottengono quasi sempre nel punto di applicazione e dello stesso tipo istologico. Penso che la vitamina D_2 nei miei esperimenti abbia favorito, incidendo forse sullo stato umorale dell'organismo ospite, la genesi dei tumori in maggior numero di quanto si è osservato in animali di controllo: senza con questo volerne spiegare il meccanismo patogenetico.

Polettini dopo considerazioni sull'azione e costituzione chimica della D_2 conclude: « L' interpretazione non è agevole perchè troppi fattori possono essere chiamati in causa ad intervenire a provocare modificazioni di ordine chimico o chimico-fisico nel ricambio generale e locale dell' organismo in rapporto alla natura fisica o chimica della sostanza adoprata ». Anche noi non vogliamo allontanarci da tale conclusione mentre ci pare interessante il fatto di avere ottenuto, anche per pennellature, tumori di tipo istologico vario.

(1) Polettini, B. *Tumori*, 1938, 12, 101.

Science 165: 1264, (1969)

Diphosphonates Inhibit Formation of Calcium Phosphate Crystals in vitro and Pathological Calcification in vivo

Abstract. *Two diphosphonates containing the P–C–P bond, $CH_3C(OH)(PO_3HNa)_2$ and $H_2C(PO_3HNa)_2$, inhibit the crystallization of calcium phosphate in vitro and prevent aortic calcification of rats given large amounts of vitamin D_3. The diphosphonates therefore have effects similar to those described for compounds containing the P–O–P bond but are active when administered orally.*

Low concentrations of pyrophosphate inhibit the precipitation of hydroxyapatite crystals in vitro (*1, 2*). Pyrophosphate is present in plasma at concentrations which are inhibitory in vitro (*3*), and therefore this compound might be one of the physiological agents responsible for preventing the deposi-

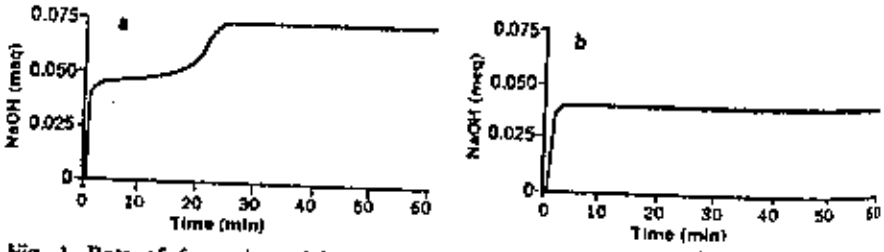

Fig. 1. Rate of formation of hydroxyapatite followed titrimetrically at pH 7.4 with a pH-stat instrument. (a) Control, no addition; (b) 2×10^{-4} M $CH_3C(OH)(PO_3HNa)_2$ present.

tion of calcium phosphate in soft tissues in vivo (*3*). In rats, pyrophosphate and longer-chain condensed phosphates (polyphosphates) indeed inhibit aortic calcification induced by excess vitamin D_3 (*4, 5*) and skin calcification induced by various other means (*6*). However pyro- and polyphosphates do not inhibit the nephrocalcinosis produced by the vitamin D_3 (*5*) and are not active when given orally. This is attributed to the hydrolysis of the P–O–P bond by pyrophosphatases in the kidney and intestine. Therefore, compounds related to pyrophosphate in structure but stable to chemical and enzymatic hydrolysis were sought and tested for their effect on precipitation of hydroxyapatite and on heterotopic calcification in vivo. Of the many compounds synthesized and tested, those that possess the P–C–P bond instead of the P–O–P bond seem to meet this requirement. Two such diphosphonates, namely sodium ethane-1-hydroxy-1,1-diphosphonate [$CH_3C(OH)(PO_3HNa)_2$] and sodium methylene diphosphonate [$H_2C(PO_3HNa)_2$], have been examined, and their effects were compared with those of a monophosphonate, sodium *n*-pentane-1-phosphonate [$CH_3(CH_2)_4PO_3HNa$], which possesses only a single C–P bond. We studied the effects of these compounds on the precipitation and crystallization of hydroxyapatite in vitro and on the calcification of rat aortas in vivo.

The rate of formation of hydroxyapatite was followed titrimetrically by the consumption of base by means of a radiometer *p*H-stat instrument. Solutions of calcium chloride and sodium phosphate were mixed to obtain a final concentration of 4 mmole/liter in each at zero time. The amount of NaOH required to keep the *p*H constant at 7.4 was then continuously recorded as a function of time. In this system precipitation and crystal growth occur in two distinct stages (Fig. 1a). The first, which corresponds to the initial rise in consumption of NaOH, represents the formation of amorphous calcium phosphate; the second, which corresponds to the second rise in consumption of NaOH, represents the crystal growth of hydroxyapatite. At one-tenth to one-fifteenth the initial concentrations of calcium or phosphate, $CH_3C(OH)(PO_3HNa)_2$ (Fig. 1b) and $H_2C(PO_3HNa)_2$ as well as inorganic pyrophosphate completely inhibit the second stage but do not influence the first. In contrast, the monophosphonate [$CH_3(CH_2)_4PO_3HNa$] and disodium ethylenediamine-

Table 1. Influence of $CH_3C(OH)(PO_3HNa)_2$, $H_2C(PO_3HNa)_2$ and $CH_3(CH_2)_4PO_3HNa$, given orally or subcutaneously, on aortic calcification induced by vitamin D_3 in rats. In all cases, the dosage was 10 mg of P per kilogram of body weight. Because the calcium values were not normally distributed, the significances (*P*) of the differences from the vitamin D_3 treated controls were determined by Student's *t*-test, with the use of the log calcium values. The numbers of animals are in parentheses. Control 1, no vitamin D_3; control 2, vitamin D_3 only. All other rats were treated with vitamin D_3 and phosphonates.

Substance	Calcium in aorta (μg/dry weight)		
	(μg/mg)	(log μg/g)	*P*
Control 1	5.4 ± 2.6 (26)	3.23 ± 0.10	<.001
Control 2	68.3 ± 7.6 (51)	4.64 ± 0.07	
	Subcutaneous administration		
$CH_3C(OH)(PO_3HNa)_2$	2.2 ± 0.4 (9)	3.33 ± 0.06	<.001
$H_2C(PO_3HNa)_2$	2.5 ± 1.1 (13)	3.18 ± 0.11	<.001
$CH_3(CH_2)_4PO_3HNa$	79.1 ± 25.3 (9)	4.59 ± 0.22	>.6
	Oral administration		
$CH_3C(OH)(PO_3HNa)_2$	2.7 ± 0.6 (6)	3.38 ± 0.09	<.001
$H_2C(PO_3HNa)_2$	3.8 ± 1.5 (17)	3.28 ± 0.11	<.001
$CH_3(CH_2)_4PO_3HNa$	64.0 ± 16.5 (8)	4.66 ± 0.15	>.9

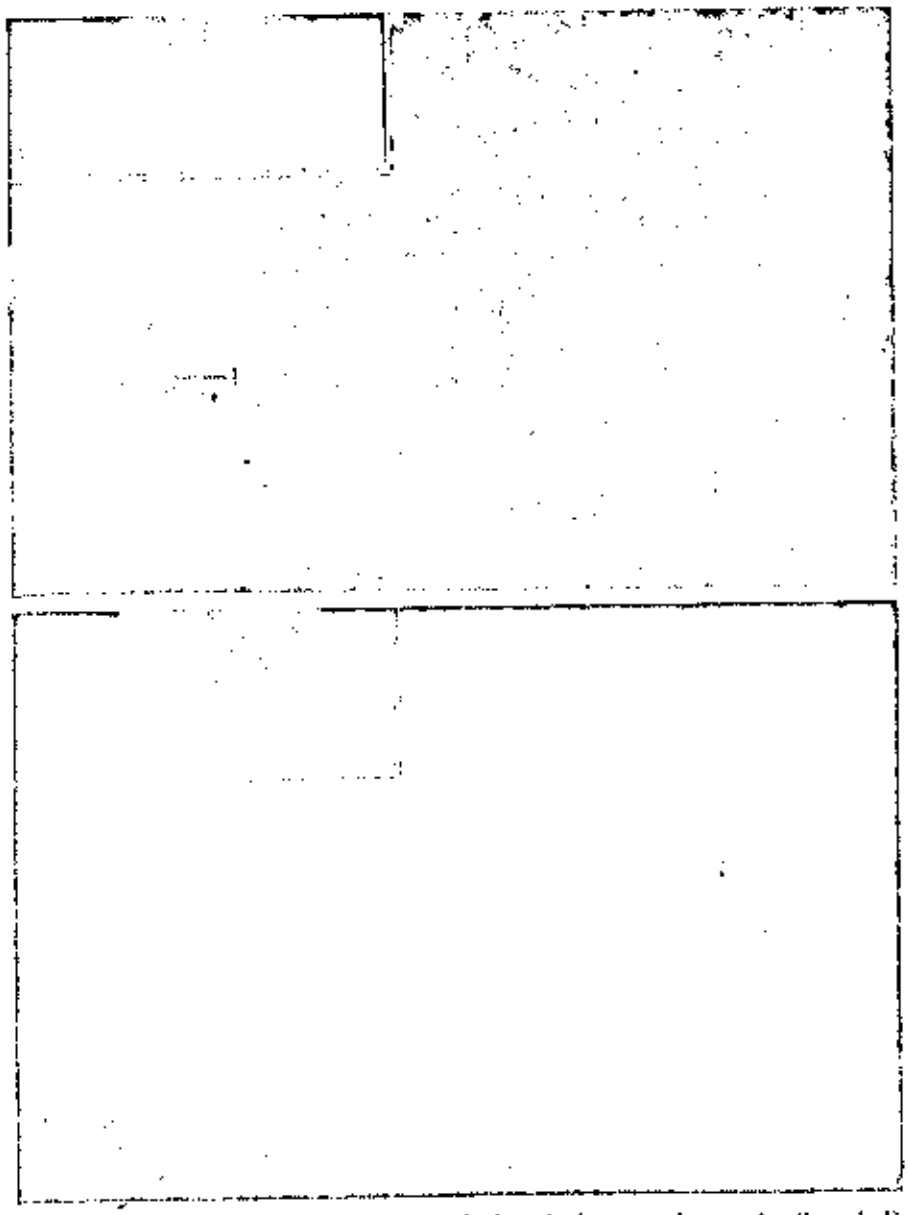

Fig. 2. Electron diffraction patterns (a and c) and electron micrographs (b and d) of freshly precipitated calcium orthophosphate. The precipitates were obtained at 22 hours from solutions of $CaCl_2$ and NaH_2PO_4 which, after mixing, were $4 \times 10^{-3}M$, $pH = 7.4$ and at 25°C; (a and b) with $2 \times 10^{-3}M$ $CH_3C(OH)(PO_3HNa)_2$; (c and d) without $CH_3C(OH)(PO_3HNa)_2$.

tetraacetate (EDTA) have no effect on either stage. Electron microscopy and electron diffraction showed that $CH_3C(OH)(PO_3HNa)_2$ (Fig. 2), $H_2C(PO_3HNa)_2$, and pyrophosphate inhibit the crystal growth of hydroxyapatite but that the monophosphonate and EDTA do not. The inhibitory effect of the diphosphonates on the crystal growth of hydroxyapatite is shown by the pattern obtained by electron diffraction and the gel-like appearance of the calcium phosphate in the electron microscope—both features typical of the amorphous form (*7, 8*).

Pyrophosphate increases the minimum product (Ca × P) required to induce precipitation of calcium phosphate from solution under defined conditions (*1*). The two diphosphonates have similar, striking effects at concentrations as low as $10^{-7}M$; the monophosphonate has only a small effect.

Aortic calcification was induced in rats by treating them by mouth daily with 75,000 I.U. of vitamin D_3 per kilogram of body weight for 5 days (*4, 5*). The phosphonates—$CH_3C(OH)(PO_3HNa)_2$, $H_2C(PO_3HNa)_2$, or $CH_3(CH_2)_4PO_3HNa$ (10 mg of P per kilogram of body weight)—were given subcutaneously or by mouth daily from 2 days before vitamin D_3 was administered and continued until 7 days after administration of the vitamin D; the animals were then killed. Calcification of the aortas was assessed by measuring calcium content of aortas ashed at 600°C. When given orally or subcutaneously, the two diphosphonates completely prevent calcification (Table 1); the monophosphonate does not prevent calcification by either route.

Our data show that diphosphonates have effects similar to those of pyrophosphate and condensed phosphates on the inhibition of crystal growth of hydroxyapatite in vitro (*1, 9, 10*) and on the prevention of aortic calcification in rats given large doses of vitamin D_3 (*5*). In addition, they possess properties not shared with the condensed phosphates. Thus, not only do they prevent aortic calcification when given by mouth rather than subcutaneously, but they also prevent kidney calcification (*10*) when given by either route.

Studies with other phosphonate compounds have shown that there is a good correlation between the ability of any single compound to prevent hydroxyapatite crystal growth in vitro and its ability to prevent calcification in vivo (*10*). This strengthens the view that the biological activity of these compounds and of pyrophosphate is a consequence of their observed action on crystal growth. The most likely mechanism of action of the diphosphonates both in vivo and in vitro is strong chemisorption on hydroxyapatite, as demonstrated with ^{14}C-ethanehydroxydiphosphonate on synthetic apatite (*8*). The compounds that have so far proved most active have all contained the P–C–P bond; $CH_3(CH_2)_4PO_3HNa$, which contains a single C–P bond, is ineffective. The fact that EDTA has no effect shows that the inhibition of crystal growth is not determined solely by the chelation properties of the P–C–P materials and of EDTA itself. Since the molecular configuration of the P–C–P bond is close to that of the P–O–P bond, the diphosphonates, pyrophosphate, and polyphosphates may all act on crystal growth by a similar chemisorption mechanism. The observation that the diphosphonates, unlike the polyphosphates, are effective when given orally and are able to inhibit kidney calcification may be attributed to their resistance to either chemical or enzymatic hydrolysis.

The diphosphonates might provide a convenient model for investigating the action of pyrophosphate in calcium homeostasis. Since the diphosphonates are of low toxicity, they might also be used against diseases in which calcium salts deposit in soft tissues. The administration of $CH_3C(OH)(PO_3HNa)_2$ to two patients in the acute phase of myositis ossificans (*11*) has been associated with an arrest in the progress of the disease.

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THE DIAGNOSIS AND MANAGEMENT OF THE VARIOUS TYPES OF RICKETS

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In the years following the suggestion that sunlight would prevent rickets[34] and the subsequent researches that culminated in the isolation of vitamin D[3, 31, 32, 45] there was a dramatic reduction in the incidence of rickets throughout the civilized world. Since that time the incidence of the disease has continued to fall, largely as a result of improvement in social and economic conditions, and of progress in the fields of public health and education. To-day, except in a few areas, vitamin D deficiency is no longer an important problem in pædiatrics.

Gratifying as has been this improvement, children with rickets still appear. In some a deficient intake of vitamin D is obvious, and the rickets heals rapidly on initiation of standard vitamin D therapy. There remains, however, a heterogeneous group of rachitic children in whom a history of vitamin deficiency cannot be elicited, and in whom active rickets persists despite the administration of conventional doses of vitamin D.

It is the purpose of this paper to outline an approach to the differential diagnosis of the various types of rickets met in medical and surgical practice, and to discuss certain present concepts concerning the management and prognosis of each type.

DEFINITION OF THE TERM "RICKETS"

Rickets may be defined as a generalized lesion of bone characterized by a failure of calcium salts to be deposited promptly in bone matrix

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TABLE 18. *Classification of Rickets*

	TYPE	ÆTIOLOGY	DESIGNATION PREFERRED BY AUTHORS	OTHER DESIGNATIONS FOR THE SYNDROME	NO. OF CASES H.S.C. 1946–55
Responsive to usual therapeutic doses of vitamin D.	I *Vitamin D deficiency*				
	i. Dietary deficiency of vitamin D		Vitamin D deficiency rickets	Rickets, classical rickets, infantile rickets, simple rickets, deficiency rickets.	70
	ii. Defective absorption of vitamin D and calcium due to steatorrhœa			Cœliac rickets.	
	II *Chronic renal insufficiency with secondary hyperparathyroidism.* (Chronic pyelonephritis, chronic glomerulonephritis, congenital renal abnormalities, late stage of types III, A, iii, iv, B, i)		Pannephritic osteodystrophy	Renal rickets, renal osteodystrophy, rickets due to glomerular insufficiency, glomerular rickets.	6
	III *Renal tubular insufficiency*				
	A. Renal tubular insufficiency resulting in phosphate depletion				
	i. Defective reabsorption of phosphate (± glucose)		Hypophosphatæmic vitamin D-refractory rickets of the simple type	Vitamin D-resistant rickets (with hypophosphatæmia), primary vitamin D-refractory rickets, tubular rickets, endogenous rickets, late rickets, rachitis tarda, phosphate diabetes, Milkman's syndrome (adult), idiopathic osteomalacia (adult).	14
	ii. Defective reabsorption of phosphate, glucose, many aminoacids (± protein)		Hypophosphatæmic vitamin D-refractory rickets with aminoaciduria	Fanconi syndrome, Debré-deToni-Fanconi syndrome, nephrotic glycosuric dwarfism with hypophosphatæmic rickets, amindiabetes.	2
	iii. Defective reabsorption of phosphate, glucose, many aminoacids, protein (± water), with defective conservation of fixed base		Hypophosphatæmic vitamin D-refractory rickets with aminoaciduria and acidosis		0
	iv. Generalized defect in cystine metabolism, with (? secondary) defective tubular reabsorption of phosphate, glucose, many aminoacids, protein, fixed base (± water)		Cystine storage disease with vitamin D-refractory rickets	Fanconi syndrome, Debré-deToni-Fanconi syndrome, Lignac's disease, Lignac-Fanconi syndrome, cystinosis, cystinuria with rickets.	4
	v. Defective reabsorption of phosphate, glycine and fixed base		Hypophosphatæmic vitamin D-refractory rickets with hyperglycinuria		0
	vi. Miscellaneous abnormalities with associated renal tubular reabsorption defects		Oculo-cerebro-renal syndrome with hypophosphatæmic vitamin D-refractory rickets Other rare conditions	Organic-aciduria, decreased renal ammonia production, hydrophthalmos and mental retardation.	0
	B. Defective handling of other ions by the renal tubules resulting in calcium depletion				
	i. Defective conservation of fixed base		Rickets due to renal tubular acidosis	Infantile renal acidosis, Lightwood's syndrome, hyperchloræmic renal acidosis (with nephrocalcinosis), Albright's syndrome (adult).	0
	ii. Defective calcium reabsorption			Rickets due to idiopathic hypercalcuria.	0
	IV *Miscellaneous*				
	i. Defective calcification of bone matrix associated with generalized depression of alkaline phosphatase activity		Hypophosphatasia	Osteodysmetamorphosis fœtalis, osteoblastic dysostosis, rickets with low alkaline phosphatase activity.	4

'Resistant' to usual doses of vitamin D

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(osteoid)[2] and in the pre-osseous cartilage at the zone of provisional calcification. The radiological and histological characteristics of this lesion are, of course, well known. The difficulties that frequently arise in interpreting the lesions in a given patient stem from the fact that the typical skeletal changes of rickets can be produced by several metabolically unrelated disturbances.

CLASSIFICATION OF RICKETS

In the past there was much disagreement concerning the fundamental disturbances giving rise to the various types of rickets, and much confusion regarding nomenclature. Although many of these problems cannot yet be resolved, the authors have undertaken to present a classification based upon current knowledge concerning the ætiology of the numerous known types of rickets (Table 18). The classification is not intended to encompass the various forms of osteomalacia that occur in adults.

Present evidence indicates that there are three main causes of rickets: (1) vitamin D deficiency, (2) chronic renal insufficiency, and (3) renal tubular insufficiency. For completeness, another category, (4) miscellaneous, has been added. To date, the latter includes only one condition—hypophosphatasia. The rachitic lesions attributable to the latter three categories do not respond to normal amounts of vitamin D.

By the use of standard diagnostic procedures, enumerated below, it is possible to fit any rachitic patient into the appropriate ætiological category and subdivision. As a result, a more rational definition of therapy and prognosis becomes possible.

NOMENCLATURE

In naming the various types of rickets, the authors have attempted to select for each a designation that is definitive. The large number of specific entities makes the task a difficult one and the terminology somewhat cumbersome. Proper names have been eliminated entirely, because their use caused much confusion in the past.

In particular, we concur with Professor Fanconi in eliminating "Fanconi syndrome,"[27] a designation that has been used indiscriminately to describe two entities now known to differ clinically, genetically and prognostically.

Although once a term of wider implication, "vitamin D-resistant rickets" has been used more recently to designate the specific syndrome defined under type III, A, i. We consider, as do others, that the alternative term "refractory" better expresses the present concepts of path-

ogenesis. We have chosen "hypophosphatæmic vitamin D-refractory rickets" as the explicit designation of the general group of rachitic lesions resulting from relatively intractable defects in the reabsorption of phosphate by the renal tubules (types III, A, i-vi).

GENERAL MEDICAL AND SURGICAL PROBLEMS IN WHICH THE DIAGNOSIS OF RICKETS SHOULD BE CONSIDERED

There is usually little difficulty in establishing the diagnosis of rickets, provided the condition is considered. However, the presenting manifestations of rickets may simulate a number of medical, surgical or neurological conditions.

The diagnosis of rickets should be considered in infants presenting with convulsions, tetany, general irritability, muscular weakness and delayed physical development. Because of the severe metabolic disturbances that accompany certain of the less common types (notably types III, A, iii, iv, vi), the diagnosis should be considered in infants and young children who fail to thrive.

In toddlers, rickets, especially types II, III and IV, should be considered in the differential diagnosis of such non-rachitic conditions as "physiological" bowing of the legs, tibial and femoral torsion, and osteochondritis tibiæ deformans (Blount's disease). In older children, dwarfism may be a major feature,[30] and the condition must be differentiated from achondroplasia.

PROCEDURE FOR CLASSIFYING AN ESTABLISHED CASE OF RICKETS

With rare exceptions, the diagnosis of rickets is readily established by (1) the typical radiographic appearance of the growing ends of the long bones, and (2) the demonstration of increased serum alkaline phosphatase activity. On very rare occasions histological studies of a costo-chondral biopsy may be required to prove the diagnosis of rickets.

On the other hand, the final categorization of an established case usually requires a number of diagnostic procedures, since no single symptom, physical sign, biochemical determination, radiographic appearance or histological characteristic can provide a reliable differentiation. The diversity of the possible manifestations of rickets places an equal obligation on physician and surgeon to understand the general principles of classification.

From both the therapeutic and prognostic standpoints, it is of prime importance to decide at the outset whether a given lesion is the result of vitamin D deficiency (type I) or whether it must be classified as one of the types of rickets that fail to respond to normal amounts of vitamin D (types II, III and IV). Usually the age of the

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TABLE 19. *Usual Biochemical Findings in the Various Types of Rickets*

TYPE	TYPE OF RICKETS	BLOOD CHEMISTRY					URINE FINDINGS			TISSUE CYSTINE
		Calcium	Phosphorus	Alk. phosphatase	Urea	Acidosis	Glucose	Protein	Abnormal aminoacids	
I	Vitamin D deficiency rickets	N or ↓	↓	↑	N	0	0	0	+	0
II	Osteodystrophy due to chronic renal insufficiency (Pannephritic osteodystrophy)	↓ or N	↓	↑	↑	+	0	+	0	0
III	Rickets due to renal tubular insufficiency:									
A.										
i.	Hypophosphataemic vitamin D-refractory rickets of the simple type	N	↓	↑	N	0	0 (rarely +)	0	0	0
ii.	Hypophosphatemic vitamin D-refractory rickets with aminoaciduria	N or ↓	↓	↑	N	0	+	0 or +	++	0
iii.	Hypophosphatemic vitamin D-refractory rickets with aminoaciduria and acidosis	N or ↓	↓	↑	N or ↑	+	+	+	++	0
iv.	Cystine storage disease with vitamin D-refractory rickets	N or ↓	↓ or ↑	↑	N or ↑	+	+	+	++	+
v.	Hypophosphatemic vitamin D-refractory rickets with hyperglycinuria	N	↓	↑	N	+	0	0	+ (Glycine only)	0
vi.	Oculo-cerebro-renal syndrome with hypophosphatemic vitamin D-refractory rickets	N	↓	↑	N	+	0 or +	+	++	0
B.										
i.	Rickets due to renal tubular acidosis	N	↓	↑	N	+	0	+	0	0
IV	Miscellaneous Hypophosphatasia	N or ↑	N	↓	N or ↑	0	0	0	+ (Phosphorylethanolamine only)	0

0 = absent. + = present. N = normal.

patient, history, physical examination and the biochemical and radiological findings serve to answer the question. Occasionally in an infant with a questionable feeding history it may be impossible to differentiate between the "classical" and the "refractory" types. In this situation it is necessary to evaluate the response to a one-month therapeutic trial of vitamin D, administered as a daily dose of 3000 I.U., preferably in a water-miscible form.

Having eliminated vitamin D deficiency as the cause of a given rachitic lesion, it is important to ascertain next whether the rickets is attributable primarily to dysfunction of the glomerulus or the tubule. In most cases a clear-cut distinction can readily be made by a determination of the blood urea and serum inorganic phosphorus concentrations. Elevations of both indicate the presence of extensive glomerular insufficiency; a normal urea level and hypophosphataemia indicate that the disturbance in renal function is localized to the tubules. However, secondary glomerular insufficiency may eventually develop in certain types of rickets in which tubular dysfunction is the primary lesion (types III, A, iii, iv, B, i); under these circumstances additional tests are required to complete the differentiation.

As The Hospital for Sick Children, Toronto, it is customary to carry out the following procedures on most rachitic patients at the time of the initial examination. Although additional procedures are occasionally required, those enumerated below usually sufficed to define the type of rickets with considerable precision. (1) History, including dietary and genetical aspects; (2) physical examination, including height and weight; (3) ophthalmoscopic examination of the cornea for presence of cystine crystals (see below); (4) routine urinalysis and urinary amino acid chromatography; (5) blood chemistry—total serum calcium, inorganic phosphorus and alkaline phosphatase; blood urea; serum electrolytes, base-combined bicarbonate and blood pH; (6) radiography—antero-posterior views of wrists and knees. Exposure settings are recorded for future reference. (7) Clinical photograph for future comparison.

The usual biochemical findings for the various types of rickets are indicated in Table 19.

INCIDENCE OF THE VARIOUS TYPES OF RICKETS

It is impossible to state with accuracy the incidence of the various types of rickets in the general population, but perhaps some idea of the relative incidence of the various syndromes may be had by noting the admissions to The Hospital for Sick Children during the 10-year period 1946-55 (Table 17).

Vitamin D deficiency rickets is still the most common type of

rickets in the predominantly urban population served by this hospital; of the lesions that persist despite the intake of normal amounts of vitamin D, the simple type of hypophosphataemic vitamin D-refractory rickets is the most prevalent. Perhaps contrary to expectation, pannephritic osteodystrophy is relatively uncommon in children.

PATHOGENESIS OF RICKETS: PHYSIOLOGICAL CONSIDERATIONS

The normal deposition of calcium in growing bone and pre-osseous cartilage is dependent on (1) the integrity of intrinsic mechanisms within the proliferating matrix that control mineral deposition, and (2) the maintenance of physiological concentrations of calcium and inorganic phosphate in the serum. A disturbance of either or both these factors may result in rickets.

The maintenance of normal serum concentrations of calcium and phosphorus depends upon a delicate balance between the absorption of each element from the intestine, their excretion by the kidneys and intestine, and their rates of movement into or out of bone. Many factors, including vitamin D and parathormone, are necessary for the maintenance of physiological concentrations of calcium and phosphorus in the serum.

Space does not permit a comprehensive review of the modern and changing concepts of the rôle of vitamin D in calcium and phosphorus homeostasis and of its action upon the intrinsic mechanisms governing calcification in organic matrix. It has long been accepted that vitamin D increases the intestinal absorption of calcium and phosphorus. Recently it has been demonstrated also that diminished tubular reabsorption of phosphate[24] and of amino acids[28] occurs in the vitamin D-deficient infant, and that correction of both these defects is brought about by administration of vitamin D. These findings emphasize the important inter-relation of vitamin D, renal tubule function, and the rachitic state. Like parathormone, vitamin D (at least in large amounts) mobilizes calcium from bone.[23] However, by using a technique similar to that described by Fraser *et al.*,[21] it has been shown that vitamin D is not essential for calcium accretion in the vitamin D-deficient human being.[20] Whether it plays any rôle in this process is still open to debate.[5]

As yet it is impossible to assess the relative importance of the various physiological actions of vitamin D. Each of the foregoing considerations must be carefully evaluated in attempting to define the pathogenesis of the various types of rickets. The lack of fundamental knowledge concerning many of the mechanisms offers a wide scope for clinical investigation in this field.

CLINICAL ASPECTS OF THE VARIOUS TYPES OF RICKETS

The various types of rickets are described briefly in the following sections, and the therapeutic measures are outlined. For further details, the reader is referred to the medical literature.

Type I. Vitamin D Deficiency Rickets

The clinical features and the therapy of vitamin D deficiency rickets are well known. Healing of the lesion is brought about readily by the daily administration of 2000 to 3000 I.U. of vitamin D. Special precautions should be taken to prevent hypocalcaemia during the early phases of therapy. For infants the recommended prophylactic dose of vitamin D is 400 to 800 I.U.

Recent studies in Great Britain[30, 33] and the United States[9] have produced evidence that vitamin D hypersensitivity plays a rôle in the ætiology of idiopathic hypercalcæmia of infants. The present authors suspect that the fortification of evaporated milk with vitamin D has had a desirable effect upon the incidence of rickets in North America, but they advise against the use of prophylactic doses in excess of 1000 I.U. and deprecate the unwarranted addition of vitamin D to other commercially prepared foods.

Type II. Osteodystrophy Due to Chronic Renal Insufficiency (Pannephritic Osteodystrophy)

PATHOGENESIS. Although it has long been recognized that chronic renal insufficiency can result in skeletal lesions, the mechanisms by which these lesions are produced remain uncertain. Until recently it was held that diminished glomerular filtration of phosphate resulted in hyperphosphataemia, which in turn resulted in secondary hyperparathyroidism of varying degree. Concomitant tubular damage has been thought to produce a failure of base economy which resulted in depletion of skeletal calcium and systemic acidosis.[42] However, the concepts of renal physiology are currently undergoing radical changes, and many of the ideas expressed above may require revision. At present it is probably safe to state that, in chronic renal insufficiency, the bones come under the influence of (1) a high concentration of parathormone, and (2) acidosis. In addition, recent evidence suggests the possible existence of (3) an unidentified factor that interferes with the intrinsic mechanisms of bone calcification.[47]

These various deleterious influences produce a skeletal lesion in which are combined, in varying degrees, the characteristic changes of both rickets and hyperparathyroidism.[18] We have named this lesion "osteodystrophy due to chronic renal insufficiency," and we suggest

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the term "pannephritic osteodystrophy" as a more convenient, but, in a strict sense, less accurate substitute. The term "renal rickets" is misleading on two counts, and should be abandoned. "Renal" fails to draw a distinction between pannephritic and tubular ætiology, and "rickets" only partially describes the osseous lesion.

Clinical Features. Theoretically, any abnormality that results in chronic renal insufficiency can cause pannephritic osteodystrophy. However, for some reason the latter condition is rarely seen in

Fig. 25. Osteodystrophy due to chronic renal insufficiency. Male: aged 14 years.

children, possibly because skeletal lesions develop slowly, and may not have become evident before terminal renal failure supervenes.

Symptoms and signs of chronic renal insufficiency (polyuria, hyposthenuria, albuminuria and nitrogen retention) antedate the onset of skeletal symptoms, usually by at least two years. Medical advice may be sought because of growth failure, bone pain, mild skeletal deformity (nearly always genu valgum) or hypocalcæmic convulsions.

The blood urea level is elevated, and the serum phosphorus and alkaline phosphatase concentrations are increased. The serum calcium concentration may be decreased or normal, acidosis is usually present, and aminoaciduria is absent (Table 18). The radiographic changes (Fig. 25) are those of (1) rickets (increased thickness of the epiphysial plate and fraying of the metaphysis in the region of the

zone of provisional calcification); (2) hyperparathyroidism (irregular disintegration of the sub-epiphysial metaphysis ("cotton wool appearance"), coarse irregular trabeculation, erosion or loss of definition of bone cortex in the long bones and metacarpals, and "tufting" of the terminal phalanges; and (3) generalized osteoporosis. The three lesions co-exist with varying degrees of prominence.

Treatment. Treatment consists in (1) appropriate medical and surgical management of the renal condition, and (2) measures directed toward improving the bone lesions.

Restriction of proteins to approximately 1 gm. per kilogram a day restricts phosphorus intake and thus tends to reduce the hyperparathyroidism. Phosphorus absorption is further decreased and calcium intake enhanced by giving calcium lactate (0.1 to 0.2 gm. per kilogram a day). Great caution should be exercised in controlling acidosis by the administration of alkalinizing potassium and sodium salts, since they may result in hyperkalæmia or tetany.

Promising results are currently being obtained by the cautious administration of increased amounts of vitamin D in pannephritic osteodystrophy.[14] During the early stages of therapy the patient should be observed carefully for evidence of increasing nitrogen retention, and the dose of vitamin D should be reduced considerably as soon as healing appears to be complete. In the present state of knowledge no conclusions can be reached concerning the effect of this new form of treatment upon the ultimate course of the underlying renal insufficiency.

Associated orthopædic problems should be treated conservatively; fatal uræmia may result from surgical intervention and prolonged immobilization.

Type III. Rickets Due to Tubular Insufficiency: General Remarks

It was recognized many years ago that certain cases of rickets failed to respond to usual therapeutic doses of vitamin D (2000 to 5000 I.U.). Some patients were shown to have chronic renal insufficiency (type II, pannephritic osteodystrophy, above). The remainder (type III) characteristically had no signs of glomerular failure, and many, except for their rachitic disabilities, were in excellent health. From a clinical standpoint the various syndromes included under type III presented a confusing picture which, until the adoption of modern biochemical techniques, defied logical explanation. However, during the past decade it has become increasingly evident that derangement in function of the renal tubule is present in all the syndromes included under types III, A and B.

Dysfunction of the renal tubule can result in rickets by two

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mechanisms. In rare instances, disturbances in renal tubular function can result in sufficient urinary losses of calcium to produce rickets (type III, B). In practice, however, it has been demonstrated that almost always the tubular dysfunction is manifested by an increased renal clearance of phosphate (type III, A).

The exact nature of the vitamin D refractoriness in the latter group of rachitic patients is not yet completely understood. It has been demonstrated that intestinal absorption of vitamin D takes place readily.[3, 4] The intrinsic mechanisms of mineral deposition apparently are unimpaired.[5, 21] Although, characteristically, the urinary excretion of calcium is less than normal,[7, 40, 44] the latter finding has a variety of possible explanations. It does not rule out the now widely held contention that excessive renal phosphate clearance is the most important[7, 23, 17, 37] and possibly the only primary defect in rickets of this type. Large doses of vitamin D have been shown to reduce the excessive phosphate clearance in these patients.[12, 37] The demonstration of anatomical and histological lesions in the proximal convoluted tubules of some cases offers presumptive evidence that excessive phosphate clearance is attributable to inadequate reabsorption of this ion by the tubule.[11]

Several subdivisions of type III, A, are necessary. In the most common entity, hypophosphatæmic vitamin D-refractory rickets of the simple type, decreased tubular reabsorption of phosphate is the only demonstrable renal disturbance. However, largely as a result of the pioneer studies of Dent,[13] it is now evident that any combination of the many known functions of the renal tubule may be deranged. In Table 18 we presented an ætiological classification of the more important entities caused by this "spectrum" of renal tubule defects. We did so with the realization that additional syndromes have been reported, and that others will undoubtedly be discovered in the future, and also with the realization that new concepts of tubule physiology may necessitate revision of the classification.

Type III, A, 1. Hypophosphatæmic Vitamin D-Refractory Rickets of the Simple Type[35]

PATHOGENESIS. As mentioned previously, increased clearance of phosphate is usually the only demonstrable renal defect in this syndrome. A familial incidence has been observed in many instances (Fig. 26), and recent studies suggest either sex-linked dominant[46] or autosomal dominant inheritance. Sporadic cases occur. Both sexes are involved.

CLINICAL FEATURES. In this condition the only apparent biochemical abnormality is hypophosphatæmia. All clinical signs are referable to the

skeleton; the patient is otherwise in excellent health and has a normal life expectancy.

Skeletal deformities usually become evident at the commencement of weight bearing. Genu varum with femoral and tibial torsion is the most common deformity, but others may occur. Moderate dwarfing is usually present when the patient is first seen. The costo-chondral junctions and the large joints are prominent, the carrying angle of the elbow is usually lost, and frontal prominence may be present. Un-

Fig. 26. Hypophosphatæmic vitamin D-refractory rickets of the simple type. Three brothers and a female first cousin.

fortunately, in the past, considerable time usually elapsed before the refractoriness of the condition was recognized by the practitioner (two years, on the average, in our series). During a period of this order, deformities and dwarfing gradually became much more marked.

The total serum calcium concentration is normal, and tetany does not occur. The serum inorganic phosphorus concentration is low, and alkaline phosphatase activity is increased. The blood urea level and the urinary amino acid excretion are normal. Renal glycosuria is present in rare instances (Table 18).

Radiographs of the bones show the typical changes of rickets. However, because the metabolic disturbance is relatively mild, but of long duration, it is often possible to make a provisional radiological differentiation from vitamin D deficiency rickets. The thickness of the epiphysial plate is moderately increased, fraying of the metaphysis is

visible (but often not striking), and coarseness of the trabecular pattern is prominent.

TREATMENT. In 1937 Albright, Butler and Bloomberg[1] reported the satisfactory response of a patient to very large doses of vitamin D. Until recently this has been the only form of therapy available. At first, therapeutic results seemed excellent, but it is now becoming increasingly evident both here and elsewhere that treatment using massive doses of vitamin D is not entirely satisfactory because, in most cases, the optimal therapeutic dose lies within the general range of the amount that produces vitamin D intoxication.

In order to minimize the risk of cumulative vitamin D overdosage, the authors have adopted a conservative procedure for establishing the optimal subtoxic dose of vitamin D. Patients receiving vitamin D are examined at monthly intervals until the maintenance dose is established, and thereafter every two or three months until the end of puberty. At each examination the parent is interviewed and the following determinations are made: height and weight; serum calcium, phosphorus, alkaline phosphatase and urea concentrations; urinalysis (including Sulkowitch test and specific gravity). Radiographs and clinical photographs are taken at intervals of about six to 18 months as required.

A normal balanced diet is prescribed. Synthetic vitamin D_2 (calciferol) is administered orally, starting with a daily intake of 40,000 to 50,000 I.U. (1.0 to 1.25 mg.). Initially, the daily dose is increased by 25,000 I.U. at intervals of not less than one month. Larger increments are added when the dose exceeds 150,000 I.U. a day.

In this manner the vitamin D dosage is increased until either (1) complete healing is attained or (2) mild intoxication is produced. At the earliest evidence of intoxication, vitamin D is discontinued for two weeks. If, after this interval, all signs of toxicity have disappeared, vitamin D therapy is resumed, giving a daily dosage 25,000 I.U. less than previously. From time to time minor adjustments in dosage are required during the growth period, since the objective of therapy is to maintain a maximal degree of healing without vitamin D intoxication.

Although it is conceivable that considerable reduction in dosage may be practicable after puberty, patients have been reported who suffered from active disease in childhood, became symptom-free in their twenties, but had severe osteomalacia in their later years.[15] Therefore, in patients past puberty, it would seem advisable to administer sufficient vitamin D to maintain a normal serum phosphatase activity and a serum phosphorus concentration above 2.5 mg. per 100 ml.

Although equal amounts of dihydrotachysterol (A.T.10) have been shown to be equally effective in the treatment of hypophosphatæmic

vitamin D-refractory rickets,[24, 39] the present authors doubt that this costly drug offers any advantages.

It can be surmised that the ideal therapeutic regimen would result in (1) establishment of physiological serum calcium, phosphorus and alkaline phosphatase concentrations; (2) normalization of the radiographs of the skeleton; and (3) establishment of a normal growth rate. The efficacy of any treatment must be assessed on the basis of these criteria of complete healing.

The criteria of early vitamin D intoxication are (1) anorexia, irritability, lassitude; (2) weight loss or cessation of normal weight gain; (3) polydipsia, polyuria (especially nocturia); (4) hypercalcæmia (above 11.5 mg. per 100 ml.); and (5) elevation in the blood urea level. These toxic manifestations are reversible if vitamin D is withheld promptly and copious amounts of fluids administered. However, if hypercalcæmia is allowed to continue, generalized vascular calcification and chronic renal insufficiency supervene.

It has been our experience that, with careful instruction, the parents can readily recognize the early signs of vitamin D intoxication. We no longer instruct the parents to use the Sulkowitch reagent. Gradual attainment of the optimal therapeutic dosage of vitamin D by the method described ensures rapid recovery from toxicity after vitamin D has been withheld. To date none of our patients has had recognizable sequelæ to the minor degrees of intoxication that have occasionally occurred.

In order to obtain warning of impending intoxication, Stearns and her associates have recently evolved a procedure that deserves further trial.[40] Since the urinary calcium output of children with untreated hypophosphatæmic vitamin D-refractory rickets has been shown to be less than that of normal children, they define the optimal therapeutic dose of vitamin D as that amount required to establish a calcium output that falls within the somewhat wide normal range established by Knapp.[27] To determine the proper dose, they merely require estimates of the patient's body weight and calcium intake, and a knowledge of the 24-hour urinary calcium output.

Saville *et al.* have combined oral phosphate supplements with dihydrotachysterol or calciferol in the treatment of this condition.[38] They state that these measures reduce the toxicity of vitamin D and maintain the serum inorganic phosphorus concentration above 4 mg. per 100 ml.

Results of Treatment. It is generally agreed that considerable benefit is to be expected from the use of large doses of vitamin D in the treatment of most cases of hypophosphatæmic vitamin D-refractory rickets of the simple type. However, in one of our patients vitamin D

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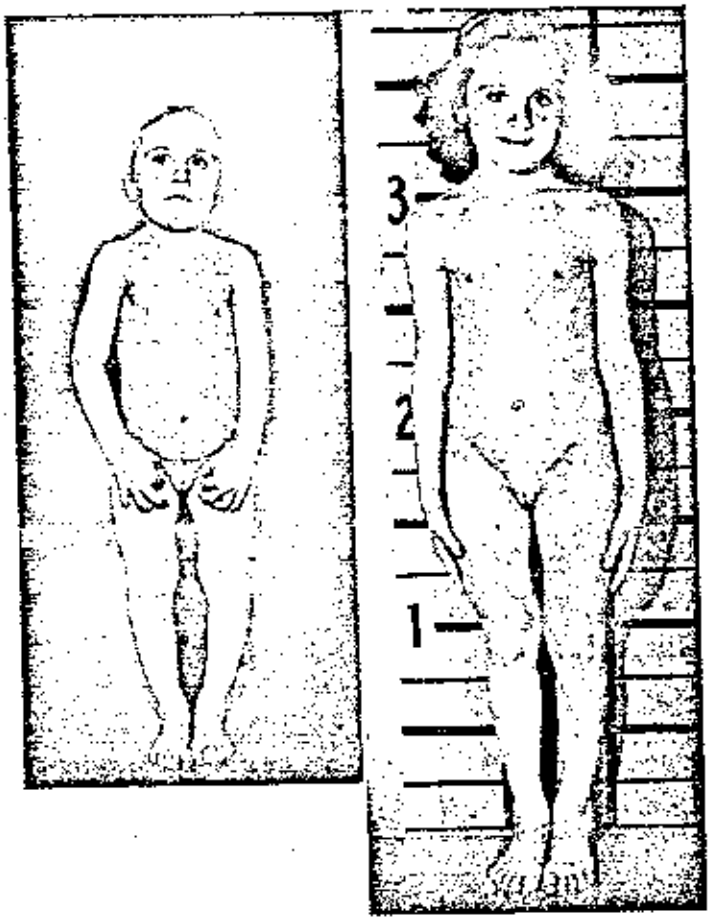
was of no value, because intoxication resulted from a dose that produced no clinical, biochemical or radiological evidence of healing. Our experience with 11 additional cases of this syndrome has shown that progression of the deformities was arrested by vitamin D therapy



A B

Fig. 27. Hypophosphatæmic vitamin D-refractory rickets of the simple type. A, Aged 4 years. No therapy. B, Aged 8 years. Note improvement while receiving optimal subtoxic doses of vitamin D.

in all but two patients; in most, steady orthopædic improvement occurred (Fig. 27).

On the other hand, although vitamin D therapy must exert some beneficial effect on growth, its efficacy in promoting good skeletal growth was disappointing. If one compares the growth of our patients with that of the normal population (Fig. 28), the following defects

become evident. None of the patients attained average height, although vitamin D intake had been gradually raised above, and maintained at, the maximal limit of tolerance (50,000 to 450,000 I.U.) in all but one case. The heights of one quarter of the patients reached the 3 percentile of normal, but the statures of the remainder fell below this range, some very much below. A corresponding lack of adequate response to optimal doses of vitamin D was reflected in the serum

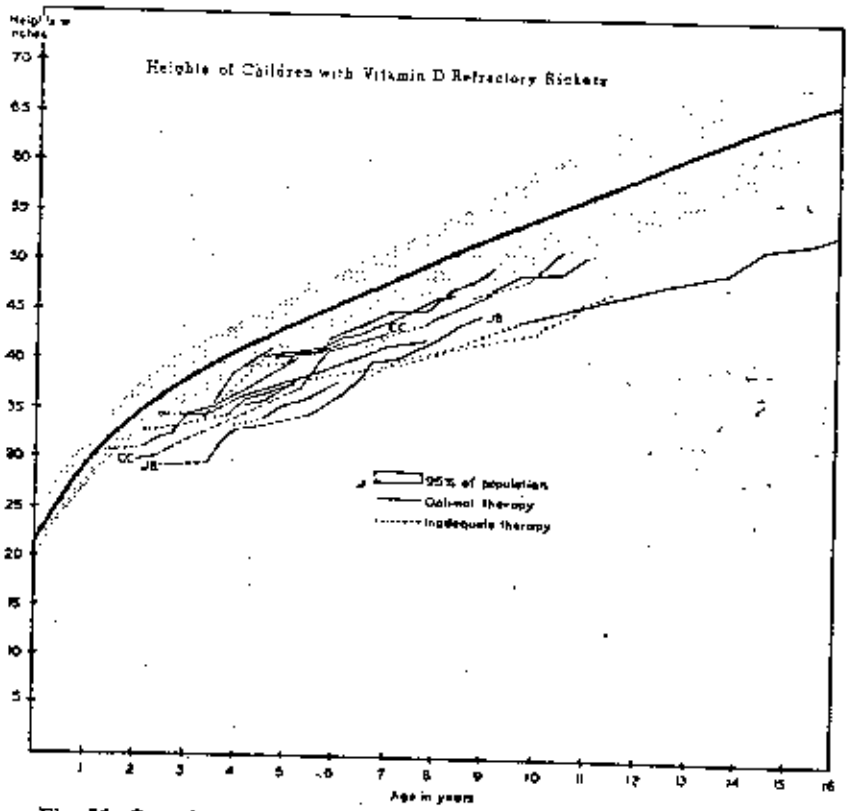


Fig. 28. Growth pattern of 14 children with vitamin D-refractory rickets.

phosphorus and alkaline phosphatase values of treated patients (Table 20) and in the persistence of coarse trabecular markings or minor epiphysial lesions in the radiographs of more than half the cases.

It has been stated, largely on the basis of radiographic evidence, that the establishment of a normal serum phosphorus concentration is not a prerequisite for healing in hypophosphatæmic vitamin D-refractory rickets.[22] On the contrary, we have observed, in studying the responses of the individual patients to therapy, that the radiographic picture provided an unreliable estimate of the patient's progress, but that the

TABLE 20. *Effect of Vitamin D Therapy on the Serum Chemistry of 15 Cases of Hypophosphatæmic Vitamin D-Refractory Rickets*

DIAGNOSIS	THERAPEUTIC STATUS	MEAN TOTAL CALCIUM (MG./100 ML.)	MEAN INORGANIC PHOSPHORUS (MG./100 ML.)	MEAN ALKALINE PHOSPHATASE (K.A UNITS)
Normal Range		9.5–11.0	4.3–6.0	8–16
Type IIIA,i (12 cases)	Before therapy	10.4	2.7	53
	During optimal vitamin D therapy	10.3	3.3	27
	During vitamin D intoxication (10 cases)	13.2	3.8	16
Type IIIA,ii (3 cases)	Before therapy	9.2	2.3	111
	During vitamin D therapy	10.6	4.8	19.9

biochemical data correlated well with the patient's growth. Experiments now in progress at this hospital suggest that the serum phosphorus concentration may prove to be the most important single factor in determining the status of the rachitic lesion. It is hoped that these experiments may eventually provide a basis for a safer and more effective management of this disease.

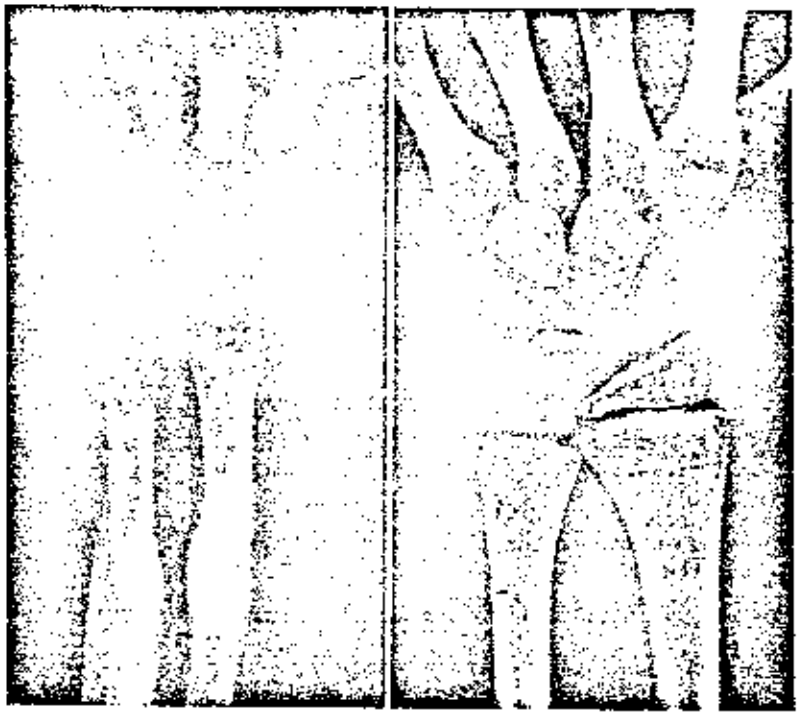
Type III, A, ii. Hypophosphatæmic Vitamin D-Refractory Rickets with Aminoaciduria

The clinical features of this rare syndrome are similar to those of Type III, A, i. However, in our experience the onset tends to be earlier, the rachitic lesion is very much more severe, and muscular weakness is prominent. Radiographs show evidence of florid rickets and often pathological fractures (Fig. 29, *A*). In our experience, deformities have not been severe, probably because the child is usually unable to bear weight.

Biochemical findings are indistinguishable from those of severe vitamin D deficiency rickets except that generalized aminoaciduria is more marked. Plasma amino acid concentrations are normal. Hypocalcæmia may be a feature. However, the condition is readily distinguished from vitamin D deficiency rickets by demonstrating a lack of response to normal therapeutic doses of vitamin D. The entity under discussion and the two that follow (types III, A, iii, iv) have frequently been referred to as the "Fanconi syndrome."

We consider that the differentiation of this disease from the simple type of hypophosphatæmic vitamin D-refractory rickets is of great therapeutic and prognostic importance. Our experience with three

cases indicates that, when large doses of vitamin D_2 (100,000 I.U. in each case) were administered, rapid and apparently complete radiographic healing of the epiphysial lesions took place without vitamin D intoxication (Fig. 29, *B*). The serum calcium, phosphorus and alkaline phosphatase determinations became normal in two cases treated for a four-year period (Table 19), pathological aminoaciduria disappeared completely, and deformities tended to regress favourably on adequate vitamin D therapy. It is our impression that there was a significant



A B

Fig. 29. Hypophosphatæmic vitamin D-refractory rickets with aminoaciduria. Wrist (*A*) at age 2 years. No therapy. (*B*) At age 8 years. Optimal dosage of vitamin D_2.

improvement in the growth rate during vitamin D therapy (Fig. 28, cases C.C. and J.B.).

From our limited experience we suggest that patients of this type probably have an excellent prognosis if the diagnosis is made at an early stage. In our third case, diagnosed at the age of eight years, some six years after the onset of symptoms, cirrhosis had already developed with a secondary malignant hepatoma. Similar hepatic changes have been reported previously in children and adults suffering from vitamin D-refractory rickets with aminoaciduria,[35, 41] but whether chronic amino acid losses are ætiological remains unconfirmed.

Type III, A, iii. Hypophosphatæmic Vitamin D-Refractory Rickets with Aminoaciduria and Acidosis[13]

In a certain group of patients tubular dysfunction may also affect acid-base regulation.

In infants the clinical features are similar to those of cystine storage disease (type III, A, iv), and there has been some argument whether these patients may, in fact, represent undiagnosed examples of the latter condition. We favour the opinion that the two entities are entirely distinct. Treatment wih alkalis and large doses of vitamin D probably offers a better prognosis than exists for cystine storage disease, but the metabolic derangements are complex, and prognosis should be guarded.

Cases with similar biochemical disturbances may present with manifestations of varying severity, and at any age from infancy to middle age. In some, diminished phosphatase activity has been demonstrated in the proximal tubules. Large doses of vitamin D decrease the abnormal renal clearance of phosphate, glucose and amino acids, and combined therapy using vitamin D and alkalis has yielded favourable results. Cirrhosis has been reported in a few long-standing cases.[41]

Type III, A, iv. Cystine Storage Disease with Rickets[8]

Pathogenesis. Although most of the early manifestations of cystine storage disease are directly attributable to renal tubular insufficiency, there exists a generalized defect in the metabolism of cystine whereby large amounts of this amino acid are deposited in various tissues of the body. From clinical observations it is probable, though not proved, that the derangement in cystine metabolism is the primary lesion and that the renal lesion is secondary. The disease appears to be determined by a heterozygous recessive gene,[9] but the biochemical explanation for the cystine deposition is unknown. In association with the renal dysfunction, a specific anatomical lesion of the proximal tubules has been noted.[11]

Clinical Aspects. The clinical manifestations of cystine storage disease apparently depend upon the severity of the renal lesions. In the acute form, failure to thrive, polyuria, renal glycosuria, dehydration, acidosis, hypokalæmia and emaciation are common features and usually appear within the first year of life. Rickets may be present or may not develop until later. The usual biochemical findings are shown in Table 18. The chronic form usually presents in the second or third year of life. The typical manifestations are dwarfism, rachitic deformities and often photophobia.

The presence of insoluble cystine crystals in various tissues is the pathognomonic feature that differentiates the disease from all other

forms of rickets. Birefringent crystals are visible in unstained bone marrow aspirate examined under Nicol prisms, and minute, shimmering, white particles can be seen in the cornea by slit-lamp examination.

A much more convenient, and in our experience a surprisingly reliable method of demonstrating the crystals in the cornea is by use of the +40 dioptre lens of the standard ophthalmoscope. By using this procedure the provisional diagnosis of cystine storage disease was correctly made in each of our six confirmed cases, and, to date, no falsely positive diagnoses have been made. This simple technique should be used in the routine physical examination of all patients with "resistant" rickets.

Treatment and Prognosis. Treatment consists in administering oral alkalis to correct the acidosis, potassium to correct or prevent hypokalæmia,* and large doses of vitamin D to improve the rachitic lesions. The risk of producing dangerous hypokalæmia during the performance of the glucose tolerance test should be borne in mind. Intensive therapy does much to improve the general condition of the patient, and usually produces healing of the rachitic lesions. However, the therapeutic measures at present available have no apparent effect on the disordered metabolism of cystine. Except in rare instances,[12] glomerular involvement eventually occurs, and death results from uræmic or hypokalæmic crises, usually before the age of 10 years.

Type III, A, v. Hypophosphatæmic Vitamin D-Refractory Rickets with Hyperglycinuria[15]

This recently described rare syndrome has its onset in adolescence or early adult life, and is characterized by severe osteomalacia, severe muscle weakness, hypophosphatæmia, hypercalcuria, acidosis and excessive urinary excretion of glycine. Response to large doses of vitamin D has been moderately satisfactory.

Type III, A, vi. Miscellaneous Abnormalities with Associated Renal Tubular Reabsorption Defects

Oculo-cerebro-renal Syndrome. In this rare condition are associated congenital glaucoma, mental deficiency, acidosis, aminoaciduria and usually hypophosphatæmic vitamin D-refractory rickets. For further details, the reader is referred to the original report.[29]

* The authors have given the following mixture[28] with satisfactory results:

Sodium citrate	50 gm.	Dose: 15–30 ml. mixed with orange juice 4 or 5 times daily as required.
Potassium citrate	50 gm.	
Citric acid	60 gm.	
Water to	1000 ml.	

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OTHER CONDITIONS IN WHICH HYPOPHOSPHATÆMIC RICKETS OR OSTEOMALACIA HAS OCCASIONALLY BEEN REPORTED. These include (*a*) hepato-lenticular degeneration,[43] (*b*) multiple myelomatosis,[16] (*c*) neuro-fibromatosis,[38] (*d*) chronic lead poisoning.[10]

Type III, B. Defective Handling of Other Ions by the Renal Tubules Resulting in Calcium Depletion

RICKETS DUE TO RENAL TUBULAR ACIDOSIS.[28] The various forms of renal tubular acidosis of infants and children are rare in North America, and the clinical features will not be described here. Protracted urinary losses of fixed base produce gradual depletion of calcium, but clinical rickets is rarely observed. Control of the acidosis is followed by healing of the rachitic lesions.

IDIOPATHIC HYPERCALCURIA. Adults are occasionally encountered who excrete large quantities of calcium in the urine despite normal serum calcium and acid-base determinations. Although in adults osteomalacia has occasionally resulted,[2] rickets due to this cause has not been reported in childhood.

Type IV. Miscellaneous

HYPOPHOSPHATASIA. Hypophosphatasia is a genetically determined metabolic disease characterized by three salient features: (1) abnormal mineralization of bone, (2) diminished alkaline phosphatase activity in serum and tissues, and (3) increased urinary excretion of phosphorylethanolamine. Although the bone lesions have certain similarities with those of the other types of rickets, thus justifying the inclusion of this disease in the general classification of rickets, the biochemical findings are dissimilar. The osseous lesions are considered to be due to a disturbance in the intrinsic mechanisms concerned with the elaboration of organic bone matrix or its calcification, or both.[19]

THE ORTHOPÆDIC MANAGEMENT OF RACHITIC DEFORMITIES

The common orthopædic deformities associated with the various long-standing types of rickets include genu varum, genu valgum, femoral torsion, anterior angulation of the femora, coxa vara, intrapelvic protrusion of the acetabulum, kyphosis and scoliosis. The upper extremities, though usually shortened, are less frequently deformed because they do not support body weight.

Because deformities are prominent in many types of rickets, the surgeon is frequently the first specialist to be consulted. He must, therefore, be fully cognizant of the differential diagnosis of the various rachitic disorders and must work in close association with the physician

responsible for the medical management. If the surgeon fails to recognize an underlying rachitic lesion, the deformities will invariably recur after surgical correction. On the other hand, once the rickets has been brought under satisfactory medical control, the deformities tend to regress considerably. This improvement can often be enhanced by appropriate splinting of the lower limbs during the sleeping hours. By using the splints during the night, continuous influence is exerted upon epiphysial growth for a period of at least 12 hours daily. This procedure usually corrects the less severe deformities in approximately six months, and we consider it preferable to the use of heavy awkward and less efficient day-braces.

If, in spite of adequate medical therapy and conservative orthopædic measures, severe rachitic deformities persist, operative correction is indicated. In such a case it is imperative to discontinue intensive vitamin D therapy at least three weeks pre-operatively in order to avoid the risk of severe hypercalcæmia, which otherwise would occur on immobilizing the patient. Surgical intervention is contra-indicated if chronic renal insufficiency is present.

Osteotomy of a curved or "dome" type is the most satisfactory operative procedure and should be performed through the cancellous metaphysial region of the long bone, not only because the deformity can be more accurately corrected at this site, but also because union occurs more rapidly. The patient is made ambulatory as early as possible, at which time vitamin D therapy is resumed.

SUMMARY

Although vitamin D deficiency is no longer a serious pædiatric problem, a heterogeneous group of rachitic infants and children still exists, many of whom fail to respond to conventional doses of vitamin D. The application of modern clinical methods has resulted in the delineation of several distinct types of rickets, and much is now known about the pathogenesis of each. Since current therapeutic measures frequently can offer the patient a great deal of benefit, it is incumbent upon both physician and surgeon to understand the classification of rickets.

In this paper the authors have presented a scheme to aid in establishing the nature of a given rachitic lesion. They have summarized the characteristic clinical features of the various types of rickets and some of the present concepts of medical and orthopædic management.

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THE RELATION BETWEEN INFANTILE HYPERCALCEMIA AND VITAMIN D—PUBLIC HEALTH IMPLICATIONS IN NORTH AMERICA

This report was written by Dr. Donald Fraser of the Department of Paediatrics, University of Toronto and The Hospital for Sick Children, Toronto, Ontario, Canada, in consultation with the Committee on Nutrition of the American Academy of Pediatrics. The position taken on the issue examined reflects the current opinion of the Committee and provides the scientific background for the publicly stated position of the Committee published in PEDIATRICS *(35:1022, 1965) and in correspondence with the Food and Drug Administration.*

FOR MORE than 40 years it has been known that vitamin D is essential for maintenance of normal calcium metabolism in man. Efforts over this period have been directed toward establishing an adequate intake of vitamin D throughout the population. National advisory committees in many countries have formulated recommended allowances for the various age groups, public health education programs have stressed the need for the vitamin and the value of sunshine, and the vitamin has been made available to the population at large through provision of inexpensive vitamin supplements and, more recently, the enrichment of milk and a number of commonly used foodstuffs. In consequence, vitamin D deficiency rickets, once a common pediatric problem, is now relatively rare in the United States and other parts of the world.

On the other hand, it has also long been known that vitamin D is potentially dangerous in very high dosage. It had been thought originally that the margin of safety between the requirement of vitamin D and its toxic dosage was very great. However, reports from Great Britain and Switzerland in 1952 drew attention to a group of infants with unexplained hypercalcemia.[1,2] Additional cases of infantile hypercalcemia began to appear throughout the British Isles, and patients with mild or severe manifestations have been reported in the United States, Canada, and elsewhere. On the basis of circumstantial evidence, vitamin D was implicated in the pathogenesis of the disease, and, as a result, there has been a growing question about the safety of vitamin D intakes which appreciably exceeded the 400 I.U. daily allowance currently recommended[3,4] for prevention of vitamin D deficiency during growth, pregnancy, and lactation.

In 1963, the Committee on Nutrition of the American Academy of Pediatrics published a report entitled "The Prophylactic Requirements and the Toxicity of Vitamin D."[5] The Committee summarized the evidence then available regarding the cause of infantile hypercalcemia and made certain recommendations designed to promote an intake of vitamin D by the population at large which would meet the recommended dietary allowances for prevention of rickets as well as minimize the likelihood of excessive intakes. Since the publication of that report, it has been shown that the severe form of infantile hypercalcemia frequently has its inception *in utero*. It has also been shown that there exists a seriously incapacitating syndrome of older children and adolescents which represents, at least sometimes, the late normocalcemic stage of previously unrecognized infantile hypercalcemia of the severe form.

The present report re-examines the rela-

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tion of vitamin D and infantile hypercalcemia in the light of these recent discoveries, and it attempts to assess the dangers of vitamin D to the population at large.

CLINICAL FEATURES OF INFANTILE HYPERCALCEMIA

The clinical aspects of infantile hypercalcemia (also referred to as idiopathic hypercalcemia of infancy) have been reviewed in detail elsewhere.[6-8] To summarize briefly, idiopathic hypercalcemia can be classified roughly into two forms—mild and severe. However, cases with intermediate manifestations are seen. Some authors consider that the two forms represent the ends of a spectrum of the same pathological process; others disagree. This important question is not yet settled.[8] It has been stated in Britain that at least 8% of the patients had the severely affected form,[9] but it is often impossible to categorize a given patient with certainty.

Mild Form

Hypercalcemia is the principal disturbance in patients with the mild form. Characteristically, there is failure to thrive, usually of sudden onset between 3 and 7 months of age. Mild azotemia occurs on occasion. These sequelae of hypercalcemia are normally completely reversible with therapy and probably even without treatment. However, a few patients have died during the acute hypercalcemic phase. Treatment consists of marked restriction of calcium intake, temporary elimination of both vitamin D and sunlight, and, occasionally, administration of cortisone. Long-term prognosis is excellent.

Severe Form

Infants with the severe form have hypercalcemia and failure to thrive of a degree similar to or greater than that observed in the mild cases. In addition, several other more serious features occur. A characteristic "elfin" facies, impairment of renal function, severe mental defect (often with strabismus), dense mineralization of the base of the skull and metaphyses of the long bones, and usually an aortic systolic murmur are present in varying degrees. Originally it was thought that the murmur was caused by thickening or calcification of the aortic valve cusps. However, it is now evident that the systolic murmurs heard in the upper parasternal region may frequently, perhaps always, denote supravalvular aortic stenosis or peripheral pulmonary artery stenoses.[10-12]

Recent publications have pointed out that, in contrast to the mild form, the severe form of infantile hypercalcemia frequently has its inception *in utero*,[8,13] and with further study it may turn out that this feature is a fundamental aspect. The low mean birthweight, the frequent detection of systolic murmurs in the neonatal period, the peculiar facies occasionally noted at birth, and the extent of the anatomical lesions at the time of diagnosis are consistent with a prenatal onset.

The severe form of infantile hypercalcemia has a bad prognosis. The mental defect is permanent and usually severe, cardiovascular and renal lesions are probably never reversible even with good therapy, and a number of patients have died.

The Late Normocalcemic Stage of Severe Infantile Hypercalcemia

Although formerly not distinguished from valvular aortic stenosis, a rare condition characterized by stenosis of the supravalvular segment of the aorta has recently been receiving increasing interest from cardiologists. It now appears that there are at least two syndromes with this vascular anomaly as a feature. In one syndrome, often familial, stenosis of the aorta is the only obvious abnormality.[13,14] In the other apparently more common syndrome, children and adolescents with supravalvular aortic stenosis also manifest severe mental defects, a peculiar facial appearance, and often abnormalities in tooth formation. This grouping of features in association with a history of unsatisfactory progress in infancy suggested to Black and Bonham-Carter,[15] Hooft, *et*

al.,[16] and Beuren, *et al.*[17] that the syndrome might represent the late manifestation of the severe form of infantile hypercalcemia. However, none of the patients were hypercalcemic at the age when diagnosed. Thus, this astute speculation remained unconfirmed until the report of Garcia, *et al.* in 1964.[18] These authors described a 9-month-old infant with supravalvular aortic stenosis, probable mental retardation, and typical elfin facies who had unquestionable hypercalcemia. Supravalvular aortic stenosis has subsequently been demonstrated in a few additional patients with severe infantile hypercalcemia;[8,11,12] in some of these patients hypoplasia of the pulmonary or renal arteries and other vascular lesions have also been found.

It is clear that the syndromes consisting of supravalvular aortic stenosis, severe mental defect, and peculiar facies may represent previously unrecognized severe infantile hypercalcemia in patients who have spontaneously become normocalcemic. However, such conditions as intra-uterine rubella can produce mental defect and pulmonary artery stenosis.[19] Thus, it is by no means certain that all instances of the juvenile syndrome can be attributed to the severe form of infantile hypercalcemia.

THE CAUSE OF INFANTILE HYPERCALCEMIA—THE POSSIBLE ROLE OF VITAMIN D

Of the several hypotheses advanced to explain the pathogenesis of infantile hypercalcemia, the one most seriously considered involves vitamin D. The prognoses of the mild and severe forms of infantile hypercalcemia differ greatly, and it is not yet proved that the two syndromes have a common etiology. Also, the severe form of the disease involves grave consequences, and the more common mild form can be expected to have no sequelae.[19] Therefore, an attempt has been made whenever possible in the present discussion to classify the data according to the diagnosis of the individual under consideration.

Epidemiological Evidence

VITAMIN D INTAKES OF INFANTS: By 1953 to 1954, approximately 200 cases of infantile hypercalcemia (mainly of the mild form) had been reported in Great Britain. Many suggestions were advanced to explain the increasing incidence of the condition. The most widely accepted of these implicated vitamin D as the underlying causative agent; in support of this possibility, British investigators pointed to the observation that, in the mid 1950's, the normal infant in Britain might readily acquire as much as 4,000 I.U. of vitamin D per day by ingesting nationally subsidized or commercial milk powders, infant cereals, and specific vitamin D supplements.[20]

Four thousand I.U. of vitamin D was 10 times the presently recommended prophylactic dosage of vitamin D. On the other hand, ingestion of this amount of vitamin D obviously was not the only factor, since the great majority of British infants receiving such intakes of vitamin D were unaffected and a considerable number of cases of infantile hypercalcemia were known to have occurred with intakes below 1,000 I.U. per day.[21] It was, therefore, proposed that hypersensitivity to the vitamin,[22] a defect in cholesterol metabolism,[23] or defective removal of vitamin D[24,25] were other possibilities to be considered in the etiology.

By the mid 1950's British health authorities had become alarmed at the high incidence of infantile hypercalcemia in the United Kingdom. As a result of the joint recommendations of the British Ministry of Health Subcommittee on Welfare Foods[26] and of the British Paediatric Association Committee on Hypercalcaemia,[20] and with the cooperation of industry, reductions were made in the vitamin D content of various infant foods and supplements. According to estimates at the time[27,28] and subsequently[9] the daily intake of British infants younger than 1 year of age had been approximately halved by 1957 to 1958. Editorials in the *British Medical Journal*[27] and *Lancet*[28] in 1960 stated that the incidence of idiopathic

hypercalcemia was believed by many British pediatricians to have decreased since the institution of vitamin D curtailment. Although it was generally inferred that a causal relation between vitamin D and infantile hypercalcemia had been established, epidemiological proof that there had been a reduction in incidence was not available at that time.

In 1964 the British Paediatric Association (BPA)[8] reported the results of three surveys of incidence of infantile hypercalcemia in Great Britain—one in 1953 to 1955 at the time when the greatest number of cases was being reported; one in 1959, approximately 1 to 2 years after the major reduction in vitamin D intake had taken place; and one in 1960 to 1961. The results of the BPA Committee on Hypercalcaemia survey are shown in Table I. The Committee's interpretation of the data was guarded:[9] "The marked decline in hypercalcaemia (1960 to 1961) does not correspond in time with the major reduction in vitamin D allowances which took place in 1957 to 1958, but followed it after an interval of two to three years. . . . Unfortunately, the results of these surveys do not provide a positive answer to the question of the part played by excessive intake of vitamin D in the pathogenesis of infantile hypercalcaemia. . . . It remains speculative whether the decrease in hypercalcaemia noted from 1960 is a consequence of reduced vitamin D intake."

On the other hand, not all authors have made such a non-committal assessment of these data. A leading article in the same issue of the *British Medical Journal*[29] stated: "[It] seems reasonable but perhaps not finally proved that excess of vitamin D is, in fact, the essential cause of hypercalcaemia." In a third article in that issue, Bransby, *et al.*[30] acknowledged the use of many data from the above survey but gave the impression that they had concluded that vitamin D intake and infantile hypercalcemia were causally related.

These epidemiological data apply in the main to the mild form of infantile hypercalcemia. For the purposes of the present consideration, it might be questioned whether a decline also occurred in the incidence of the severe form specifically. The BPA Committee was not able to decide this question. From what is now known of the usual prenatal onset of the severe form of infantile hypercalcemia, alteration in infant feeding practices might be expected to have little or no bearing upon the incidence of this form of the condition.

TABLE I

INCIDENCE OF INFANTILE HYPERCALCEMIA (ALL FORMS) IN GREAT BRITAIN[8]

Survey	Period	Months	Reported Cases	Cases per Month
1	1953 to 1955	30	216	7.2
	1957 to 1958*			
2	1959	12	82	6.8
3	1960 to 1961	17	50	3.0

* In 1957 to 1958, the overall intake of vitamin D intake was estimated to have been halved.

VITAMIN D INTAKES AND METABOLISM DURING PREGNANCY: Studies of vitamin D metabolism in normal human pregnancy have hitherto been almost completely neglected. Clinical experience in China has demonstrated a need for vitamin D during pregnancy in excess of the requirement of the normal adult. The total vitamin D allowance recommended for the later stages of pregnancy by advisory committees in the United States and Canada is 400 I.U. per day.[3,4] There is no question that this quantity is sufficient to protect the normal mother and fetus in all instances. However, the recommended allowance seems to have been arrived at empirically, for there is practically nothing known of the minimum requirements during the various stages of pregnancy.

The published case reports of infantile hypercalcemia have usually failed to record the specific vitamin D intakes of the mothers of affected infants. Referring to the mild form of the disease, Black[6] states without

further documentation, "thorough investigation of the maternal diet during pregnancy has never revealed an excessive intake of vitamin D."

Evidence that the severe form of infantile hypercalcemia may often commence *in utero* has recently focussed attention upon maternal vitamin D intake and metabolism during pregnancy. Although there must be many pregnant women who ingest much more than the recommended allowance of vitamin D (400 I.U./day), the evidence thus far does not prove that excessive maternal intake is a factor in causing the severe form of infantile hypercalcemia. The total vitamin D intakes of the mothers of two Canadian infants with the severe form of infantile hypercalcemia did not likely exceed 200 I.U. per day, since the dairy milk did not contain added vitamin D, the mothers did not regularly consume any of the vitamin D-enriched foods then on the market, and they did not take vitamin supplements during pregnancy. The infants each received a total of 800 I.U. vitamin D per day.[8] Kenny, *et al.*[31] calculated that the total daily intake of a mother who bore an asymptomatic hypercalcemic infant was 600 I.U. per day for the last 7 months of pregnancy. This mother had previously borne a severely affected infant[25] but the vitamin D intake during the earlier pregnancy was not stated. Black and Bonham-Carter[15] stated that the mothers of four normocalcemic juvenile patients with peculiar facies, mental defect, and supravalvular aortic stenosis had had normal vitamin D intakes during pregnancy. Thus, if vitamin D plays a role in the intra-uterine development of the severe form of infantile hypercalcemia, it must do so either by placental transfer of the vitamin from the mother to the excessively vitamin D-sensitive fetus or alternatively by producing a response in the excessively sensitive mother which is deleterious to the fetus. To date there have been no published studies of the vitamin D metabolism of the pregnant mothers of severely affected infants.

Biological Tests of Vitamin D Excess and Hypersensitivity in the Affected Infant

If epidemiological evidence for a direct causal relation between vitamin D and infantile hypercalcemia still remains inconclusive, so also does evidence obtained in individual cases by direct biochemical study of vitamin D concentrations of blood and metabolism of the vitamin. Two types of studies have been carried out in attempting to clarify this question: (1) measurement of vitamin D concentrations in serum and other tissues, yielding data which would indicate either excessive intake or abnormally slow disappearance of vitamin D, and (2) assessment of response of patients to vitamin D administration, yielding evidence regarding hypersensitivity.

Vitamin D Concentrations in Serum and Other Tissues: Most of the studies of serum vitamin D concentrations have been carried out on severely affected infants. However, Cuthbertson[32] and Thomas, *et al.*[38] carried out bioassays on the sera of six and two mildly affected infants, respectively, and found normal values in each. One mildly affected infant in Cuthbertson's series, who died during the hypercalcemic phase, had lower vitamin D concentration in the liver than that of a control subject of the same age.

In patients with the severe form of infantile hypercalcemia results of various studies are contradictory. Cuthbertson[32] could not detect an elevation in the plasma vitamin D concentration in any of five severely affected infants. One patient was studied while still hypercalcemic before restricting vitamin D. The intake was estimated to be 1,500 I.U. per day. A second patient had a normal vitamin D level and was still hypercalcemic when studied 10 days after instituting vitamin D restriction. A third severely affected infant had a normal vitamin D concentration and was still hypercalcemic 25 days after instituting restriction. Two others with normal vitamin D levels had been off vitamin D for 18 days and 200 days, but

they were normocalcemic when tested and these findings are, therefore, probably not significant.

Kenny, *et al.*[31] found a level of 3 units per ml (normal for their assay 0.7 to 3.1 units per ml) in the serum of the asymptomatic sibling of a patient with the severe form[23] while the former was receiving a low vitamin D intake. When the infant was given 400 I.U. vitamin D per day for 10 days, the vitamin D level was 4 units per ml. A subsequent value of 8 units per ml is in question, because technical difficulties were experienced with this assay.

On the other hand, several investigators have recorded findings at variance with the foregoing reports. Fellers and Schwartz[34] studied the concentrations of vitamin D (or vitamin D-like substance) in serum by rat bioassay in three patients with severe infantile hypercalcemia, each of whom appears to have been receiving approximately 1,200 to 1,500 I.U. vitamin D per day until the time of diagnosis. The concentrations of vitamin D were recorded as 31, 60, and 20 units per ml, respectively, at diagnosis; levels of 17 and 27 units per ml were recorded during a 14-month follow-up after discontinuing vitamin D in the first patient and a value of 36 was recorded after a 6-month follow-up in the second patient. Fellers and Schwartz quoted their normal range as 1.10 to 1.95 units per ml; they concluded that the patients' concentrations of vitamin D were abnormally high and persisted for an unduly long time. The severely affected hypercalcemic infant reported by Garcia, *et al.*[10] was considered to have a significantly elevated serum vitamin D concentration of 15.4 units per ml, as compared to a normal range of 0.6 to 4.0 units per ml. In one patient with the severe form who was receiving 4,000 I.U. per day, Smith, Blizzard, and Harrison[25] estimated a serum concentration of 23 units per ml, but this must be considered with some reservation since only four rats were used for the assay. After restriction of vitamin D intake for 6 months and 21 months, the values were 5 and 2 units per ml, respectively. They considered all these values to be elevated, but, for some unexplained reason, their normal range was 0.6 to 1.65 units per ml, compared to the range usually quoted (0.6 to 4.0 units per ml).

The reason for the discrepancies in the various results on patients with the severe form of hypercalcemia is not clear.

Response of Affected Infants to Vitamin D Administration: The response of patients to loading doses of vitamin D has not been extensively studied to date, and, when assessed, the effect on the calcium level in serum has been variable. To date, apparently, none of these investigations have been carried out in patients with the severe form of hypercalcemia.

Challenge with excessive doses of vitamin D in several studies on mildly affected patients produced no alteration in the clinical or biochemical status. Forfar, *et al.*[33] reported on a patient in the actively hypercalcemic phase who had no aggravation of symptoms, and the serum calcium appeared to decrease while receiving 50,000 to 100,000 I.U. per day for 1 week. Two 2-year-old patients of Hubble,[35] who were in complete remission, received 6,000 I.U. vitamin D per day for 90 days and 70,000 I.U. per day for 30 days, respectively, with no symptoms and no hypercalcemia. Similarly, the administration of 2,000 I.U. vitamin D per day to a patient with the mild form[36] for about 1 month produced inconsistent and probably insignificant alterations in serum calcium levels and did not result in exacerbation of symptoms.

By contrast, in the studies of Kenny, *et al.*[31] on the asymptomatic hypercalcemic sibling of a severely affected patient, the serum calcium level was normal on a vitamin D intake of 40 I.U. per day but rose to hypercalcemic levels after 10 days on 400 I.U. per day. Other investigations have yielded similar increases in the concentration of serum calcium levels, but the interpretation is not clear since the stressing dosages of vitamin D were unreasonably

excessive. Bonham-Carter, *et al.*[27] reported a rise of serum calcium levels from 10.6 to 11.9 mg/100 ml and deterioration in the clinical condition of a 9-month-old infant in remission during administration of 10,000 I.U. vitamin D per day for a 12-day period. The child was considered to have the mild form of the disease. A second, mildly affected patient of Forfar, *et al.*,[23] who was still actively hypercalcemic, showed an increase in the serum calcium level of approximately 2 mg/100 ml, an increase in the urine calcium output and a weight loss during a 1-week period while receiving 100,000 I.U. per day.

With the probable exception of the atypical patient of Kenny, *et al.*,[31] evidence of abnormal responsiveness of mildly affected patients to reasonable vitamin D challenges has been unconvincing.

Vascular Lesions Produced in Experimental Animals by Toxic Dosages of Vitamin D

Vitamin D, administered in high dosage to experimental animals, has long been known to produce lesions in various parts of the vascular system. In particular, the findings of Hass, *et al.*[38] and of Gillman and Gilbert[39] in mature rabbits and rats, and recently of Friedman and Roberts[40] in newborn rabbits, are of interest with respect to supravalvular aortic stenosis in the human.

In mature animals, doses of 25,000 to 125,000 I.U. per day for periods of up to 21 days produced various vascular lesions. Those in the aortas were of particular interest. In both species, the proximal aorta was found to be the most susceptible to damage; the lesion progressed distally from that point. Early signs consisted of subintimal edema and damage to the elastic lamina. More severe lesions were of greater depth and occasionally formed solid calcified or cartilage-like plaques in the deeper layers of the artery wall, but stenoses were not mentioned. Intimal proliferation or vegetative accumulations of polymorphonuclear leucocytes on the intimal surface sometimes occurred. Calcification, if it took place, frequently became resorbed after vitamin D had been withdrawn. Actual stenosis of the aorta has not been reported in studies on mature animals.

Working on the assumption that supravalvular aortic stenosis of humans occurs in early infancy, probably *in utero*, Friedman and Roberts[40] recently studied newborn offspring of rabbits fed very large doses (1.5 million units) of vitamin D throughout gestation. The finding of very elevated levels of vitamin D in the offspring demonstrated its transplacental passage. A significant number of the offspring had generalized vascular lesions; some animals showed aortic changes reminiscent of supravalvular aortic stenosis in humans.

Although serum calcium concentrations were not reported in the three studies cited, such large doses of vitamin D would almost certainly produce hypercalcemia. Therefore, it is not possible to be certain whether vitamin D *per se*, or the resultant hypercalcemia, produced the vascular lesions observed in the experimental animals or their offspring.

Assessment of the Evidence Impugning Vitamin D in the Pathogenesis of Infantile Hypercalcemia

There is no valid reason to give more weight to one of the foregoing studies of vitamin D metabolism than to another. Considering patients with either the mild or the severe form of infantile hypercalcemia, and taking all the data at face value, consistent results have not been obtained with any of the tests. Although there is a certain amount of circumstantial evidence that vitamin D may play an important role in causing infantile hypercalcemia, this hypothesis has not yet been proved for either form of the disease.

It has been suggested that the severe form of infantile hypercalcemia might be caused by an abnormality of maternal vitamin D intake or metabolism during pregnancy. Although animal studies have produced intriguing results, there is as yet no

direct clinical evidence for the hypothesis.

It is possible that application of more specific or more accurate methods for studying vitamin D metabolism, or more careful study of the importance of dietary calcium intake, may help to elucidate the pathogenesis of this disease in the future. On the other hand, it is also possible that vitamin D may eventually prove not to be directly involved in the pathogenesis of this bizarre symptom-complex, particularly in the severe form of infantile hypercalcemia.

THE INCIDENCE OF INFANTILE HYPERCALCEMIA

Overall Incidence

Although cases of infantile hypercalcemia have been reported from many parts of the world, the nationwide surveys conducted in Great Britain[6] provide the only quantitative estimates of the incidence of this condition. From the 1960 to 1961 survey, the computed incidence of infantile hypercalcemia for the British Isles was 35 cases per year. Since in 1960 there were 785,000 births in Great Britain, the incidence of infantile hypercalcemia of all forms was 0.045 cases per 1,000 live births, or approximately 1 case for every 20,000 live births.[6]

Clearly, these figures may not be applicable to North America. For one thing, about 8% of the cases in Great Britain were estimated to be severely affected, whereas almost all of the 50 odd cases reported in North America during the past 14 years have been classified as severely affected. However, the British figures serve to emphasize the overall rarity of the condition, since there is no reason to suggest that the incidence of infantile hypercalcemia is greater in any other country.

Incidence of the Severe Form

The obvious difference in the seriousness of the two forms of infantile hypercalcemia emphasizes the importance of attempting to derive some estimate of the incidence of the severe form of infantile hypercalcemia *per se*. The British reports point out the difficulty in clearly distinguishing between the two forms of the disease. However, if one accepts the suggestion made by the BPA Committee on Hypercalcaemia that approximately 8% of the British cases were severely affected, the incidence of the severe form of infantile hypercalcemia appears to have been approximately 0.0036 cases per 1,000 live births, or approximately 1 case for every 275,000 births.

The incidence of the severe form of infantile hypercalcemia can be arrived at in a different way by computing the incidence of supravalvular aortic stenosis in the pediatric population. The latter calculation was possible using data available from the Toronto Heart Registry and the Cardiology Service of The Hospital for Sick Children. By including all mentally defective patients with supravalvular aortic stenosis and peculiar facial appearance and all patients with the severe form of infantile hypercalcemia, a total of 11 presumptive cases were diagnosed in the course of studying 8,000 consecutive cases of congenital heart disease during a 16-year period. Accepting 6 cases per 1,000 births as an estimate of the incidence of all forms of congenital heart disease in the pediatric age group,[41] the approximate incidence of severe infantile hypercalcemia was calculated at 0.008 cases per 1,000 births.[8] This yields an incidence of 1 case per 120,000 births. Possibly this estimate is erroneously high since it is not yet proved that all mentally defective children with supravalvular aortic stenosis previously had severe infantile hypercalcemia.

It is obvious that the foregoing estimates are open to criticism. Nonetheless, they confirm the general impression that infantile hypercalcemia, especially in its more serious form, is an extremely rare condition. As a cause of heart disease in the population at large, infantile hypercalcemia plays a very small role. As a cause of mental defect, its contribution is much less than, for example, that of phenylketonuria, itself a rare condition in the population.

PROPHYLACTIC REQUIREMENTS OF VITAMIN D AT DIFFERENT AGES

The recommended dietary allowances for vitamin D, as set forth by the Committee on Nutrition of the American Academy of Pediatrics[5] are similar to those of the National Academy of Sciences and National Research Council Food and Nutrition Board in the United States[3] and the Canadian Council on Nutrition[4] and are based on present knowledge of the minimum requirements and the toxicity of vitamin D at different ages. These recommendations constitute suitable guidelines upon which to base public health nutrition policy. A total intake of 400 I.U. vitamin D per day is recommended for prematures, infants, children, and adolescents; an allowance of 400 I.U. per day is recommended for women during the later part of pregnancy and during lactation. It is clearly stated that the amounts recommended refer to the total ingested vitamin D from all sources. No supplemental vitamin D is considered necessary for adults, over and above that acquired from the ordinary diet and casual exposure to sunlight.

There is no question that these amounts of vitamin D are sufficient to meet the requirements of virtually all normal individuals, even in the absence of ultraviolet irradiation. Furthermore, it is considered from present evidence that intakes of this order carry no risk of toxicity for normal individuals at any age, whether during infancy, childhood, or adult life.[5] On the other hand, the lower limit of intake that can be depended upon to provide effective prophylaxis is very difficult to ascertain. In infancy, intakes of 100 I.U. per day have been shown to be sufficient to prevent deficiency.[12,13] In older children living under modern conditions, a daily intake of 400 I.U. apparently provides considerably more vitamin D than is necessary for prophylaxis. Vitamin D deficiency rickets is virtually non-existent beyond infancy in North America, despite the fact that the great majority of high school children and adolescents do not take specific vitamin supplements[14,15] (with the result that many children ingest a total of less than 100 I.U. per day without obvious consequence).

It is clear that the recommended allowance of 400 I.U. vitamin D per day amply provides for the total vitamin D requirements of normal infants, children, and pregnant women. From the standpoint of preventing rickets, there is no reason for the normal individual to ingest more than this total amount. From the standpoint of avoiding hypercalcemia in the population at large, there is no compelling necessity for physicians or public health authorities to strive for lower overall intakes.

PUBLIC HEALTH IMPLICATIONS

Early in the history of rickets prophylaxis, it became apparent in temperate climates that sunlight could not be relied on to provide protection against rickets during the winter months and, similarly, that the usual foods of infants (including breast milk, cow's milk, egg yolk, and butter) did not contain enough vitamin D to meet requirements in many instances. Therefore, the use of vitamin D supplements was promoted, and there was widespread acceptance of this public health measure in North America and Great Britain. It soon became evident, however, that rickets had not been entirely eliminated, and a decision was made to enrich milk with added vitamin D. This measure, along with other sociological factors, had outstanding success in reducing the incidence of infantile rickets in the United States[16] where practically all evaporated milk and an estimated 85% of all dairy milk now contains added vitamin D.[6] In Canada, where the incidence of rickets has also diminished greatly, only evaporated milk is systematically enriched. However, vitamin D deficiency still occurs with considerable frequency in some metropolitan areas; at least 100 proved infantile cases were admitted to Montreal and Toronto hospitals in a recent 12-month period.[17] There is no doubt that most cases of vitamin D deficiency could have been

prevented by a concerted program of fluid-milk enrichment.

It soon became apparent that vitamin D fortification of milk was an effective means of preventing rickets, and the success of this program gave an impetus for the enrichment of a variety of commonplace foods in the United States and, until recently, in Canada. As a result, the usual diet has recently contained variable and practically unavoidable amounts of vitamin D, and in many instances the amount of vitamin D may be considerable. In recent years, however, the suggestion that vitamin D might be a primary factor in causing infantile hypercalcemia has forced nutritionists and public health authorities to give careful reconsideration to national practices relating to this essential nutrient.

In 1963, the Committee on Nutrition of the American Academy of Pediatrics reviewed the information then available regarding the prophylactic requirements of vitamin D and the evidence for its toxicity.[5] It also made a survey of foods in the United States and Canada that then contained added vitamin D. On the basis of hypothetical food intakes, the Committee demonstrated the theoretical possibility that, by consuming only a reasonable selection of commercially enriched foods, some infants and children might consume several times the recommended daily allowance of 400 I.U. The validity of these hypothetical calculations has since been confirmed by two dietary surveys.[18,19]

At the time of the report, the Committee considered that the evidence linking vitamin D and infantile hypercalcemia was circumstantial. However, it proposed that measures should be taken which would bring about a reduction in the unnecessarily abundant dietary sources of vitamin D available to both children and adults and provide adequate intakes to prevent vitamin D deficiency in the population at large. The recommendations, which were reiterated and amplified in 1965[20] can be summarized as follows:

1. continuance of the enrichment of all types of milk with vitamin D at a level of 400 I.U. per (United States) quart,
2. discontinuance of vitamin D supplementation of all other foodstuffs,
3. adjustment of all vitamin D supplements to contain 400 I.U. per recommended daily dose,
4. assessment by the physician of the dietary intake of vitamin D before prescribing specific vitamin D supplements to the individual patient.

New information about the pathogenesis of infantile hypercalcemia and its incidence has become available since the Committee's report, and an appraisal of current national policies regarding vitamin D is again warranted. There is still a considerable lack of knowledge concerning the metabolism of vitamin D and the limits of its requirement and toxicity. On the one hand, the evidence that the severe form of infantile hypercalcemia may have its inception *in utero* now places a responsibility on physicians to be alert to possible prenatal factors. On the other hand, it is now obvious that both forms of infantile hypercalcemia are very rare. Furthermore, it is still not proved that vitamin D plays a central role in the pathogenesis of either the mild or the severe form of this disease.

The evidence concerning infantile hypercalcemia does not alone provide justification for extensive changes in national policies relating to vitamin D. The recommendations of the Committee on Nutrition, however, are appropriate in that they embody the principle of sensible moderation in consumption of vitamin D at all ages. Vitamin D is a potentially toxic substance and the possible long-term effects of intakes that exceed requirements by several orders of magnitude are still unknown. Implementation of the Committee's recommendations would have the effect of promoting intakes that closely approximate the recommended allowances. Careful consideration suggests that the Committee's proposals would not increase the incidence of vitamin D deficiency in the infants and children of North America, yet they would have the effect of minimizing the likelihood of high intakes by individuals in all popula-

tion groups, including infants and pregnant women.

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Preliminary Communication

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METABOLIC ABNORMALITIES IN HYPOPHOSPHATASIA

IN 1948 Rathbun[1] reported from this hospital a metabolic disorder which he designated "hypophosphatasia." His report concerned an infant in whom there was defective calcification of bone matrix, associated with levels of serum alkaline phosphatase considerably below the normal range. Since his original description three additional articles[2–4] and two new names ("rickets with deficiency of alkaline phosphatase activity," and "osteodysmetamorphosis fœtalis") have been added to the literature. These contributions have helped to establish the clinical features of the condition.

We are currently studying a female infant who developed a bulging anterior fontanelle at 3 months of age and who manifests the typical abnormalities in the skull, long bones, and serum.

The fundamental disturbance in hypophosphatasia remains a matter of conjecture. We record here two hitherto unreported observations which may prove helpful in elucidating the pathogenesis of the syndrome.

It has been shown previously that serum from a patient with this condition does not inhibit the alkaline-phosphatase activity of normal serum. We have confirmed this and have shown in addition that serum from our patient will produce in-vitro calcification in the cartilage of rachitic rats. We present this as further evidence that defective calcification in hypophosphatasia results from a primary disturbance in the metabolism of bone matrix.

The association in our patient of metabolic bone disease and mild renal insufficiency prompted us to study the urinary amino-acid pattern by paper partition chromatography as described by C. E. Dent. In addition to the usual amino-acids we have repeatedly found a ninhydrin-positive substance similar in every property so far tested to aminoethyl phosphoric ester (NH_2-CH_2-CH_2-O-PO(OH)2). The material is also present in the plasma of our patient, and this is unlikely to be a product of abnormal renal metabolism. We have learned from

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McCance and from Dent[5] that they too have found this material in the urine of an infant with hypophosphatasia. Their finding was made independently of ours and at about the same time.

The substance cannot be detected in the plasma of normal persons or in urine. Therefore we are now attempting to establish its identity. If it proves to be aminoethyl phosphoric ester, one might speculate on the possible relation of alkaline phosphatase and the above-mentioned phosphate ester in bone formation.

On the basis of studies of the activity of serum alkaline phosphatase in parents and siblings, other workers have suggested that hypophosphatasia is genetically determined. We have found low concentrations of the same abnormal ninhydrin-positive substance in the father's urine. This finding further supports the suggestion that hereditary factors operate in the aetiology of hypophosphatasia.

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Parathyroid hormone
parathyroid gland
vitamin D deficiency
aminoaciduria
phosphaturia
hypocalcemia
hypophosphatemia
rickets

Hyperparathyroidism as the Cause of Hyperaminoaciduria and Phosphaturia in Human Vitamin D Deficiency

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Extract

Thirty-nine infants with simple vitamin D deficiency were studied; three stages of deficiency were recognized. Stage I comprised hypocalcemia, usually as the sole important biochemical finding, while convulsions were a common clinical sign. Stage II revealed normocalcemia with hyperaminoaciduria, hypophosphatemia, and hyperphosphaturia; rickets was also in evidence. Stage III was comparable to stage II, but with recurrence of hypocalcemia and convulsions, the rickets was more severe. Patients progressed spontaneously from the early to the later stages of the deficiency syndrome; administration of parathyroid extract appeared to accelerate the rate of progression. Calcium infusion and vitamin D therapy each initially raised the serum calcium level in hypocalcemic patients; thereafter, aminoaciduria and hyperphosphaturia were suppressed.

These diverse observations were interpreted in accordance with current knowledge of vitamin D and parathyroid hormone interrelations. The acquired excretory abnormality involving amino acid and phosphorus is the result of impaired tubular absorption; this defect is considered to be dependent on the development of endogenous reactive hyperparathyroidism, and not dependent on cellular deficiency of vitamin D *per se*. The stimulus for the hyperparathyroidism is hypocalcemia induced by deficiency of vitamin D. Normocalcemia is restored if sufficient vitamin D is present in cellular membranes to amplify the stimulative action of parathyroid hormone on intestinal transport of calcium and its release from bone. Severe deficiency of vitamin D blocks this regulatory effect upon calcium, but does not block the inhibitory effect of parathyroid hormone on renal tubular transport of amino acids and phosphorus.

Speculation

An excess of parathyroid hormone rather than a simple deficiency of vitamin D at the renal tubular epithelial cell appears to cause the disturbance of transport affecting the absorption phosphorus, amino acids and other solutes. This impairment of function is the price paid in renal cellular economy for the conservation of calcium. The cellular mechanisms underlying this coexistent inhibitory effect of parathyroid hormone constitutes an important and fascinating subject for further investigation.

Introduction

Circumstances still exist in Canada which predispose to vitamin D deficiency in the human infant. Vitamin D supplementation of bottled dairy milk is uncommon in this country, and during the winter months infants acquire little vitamin D from exposure to sunlight. Since fluid milk constitutes the principal nutrient of infants in the poorer urban populations, it is not surprising, therefore, that many cases of vitamin D deficiency still occur in the spring months among infants born in the previous year.

Vitamin D deficiency can present with a wide variety of manifestations [23]. Among these, the abnormalities of urinary excretion involving phosphorus [1, 23, 28], amino acids [19, 25, 27] and bicarbonate [47, 48] have long been of interest to investigators. Hyperaminoaciduria has been recognized as a component of the human vitamin D deficiency syndrome for over 30 years [6, 8, 12, 19, 25, 27, 45]; this phenomenon, which occurs in some, but not in all infants with vitamin D deficiency [6, 19, 25, 45] attracted our particular attention. Thirty-nine untreated patients were studied and correlations made of biochemical, clinical and radiographic information. On the basis of the observations to be reported, we propose that the human vitamin D deficiency syndrome develops progressively in three 'stages': an early stage with hypocalcemia and minimal bone demineralization; an intermediate stage with normocalcemia, hypophosphatemia, hyperaminoaciduria and moderate rickets; and a later stage with hypophosphatemia, hyperaminoaciduria, reappearance of hypocalcemia and advanced rickets. We suggest these three stages reflect progressive vitamin D depletion, modified by resultant hyperparathyroidism, the latter being the factor which impairs renal tubular function.

Materials and Methods

Patients

Thirty-nine infants were investigated. The criteria for acceptance of a patient into the study were:

1. that all investigations were performed at admission on the same day
2. that no therapy (vitamin D or calcium) had been given prior to the investigation.
3. that rapid healing of rickets occurred subsequently on a therapeutic dosage of vitamin D_2 (5000 units/day) x 4 weeks, and without recurrence of rickets when the normal daily requirement (400 IU/day) was instituted therapeutically.

Determinations of total calcium, inorganic phosphorus and alkaline phosphatase were made on serum or plasma taken in the morning before feeding; the amino acid content of urine was measured, and roentgenograms of skull and long bones were obtained. The X-ray films were evaluated by one radiologist[1] without foreknowledge of the patient's biochemical and clinical status.

Analytical Methods

Calcium was determined by a titrimetric method [9], inorganic phosphorus by the molybdate method [17], and alkaline phosphatase activity by the method of KING and ARMSTRONG [30].

Urine samples were obtained by spontaneous voiding and the amino acid content assessed by ascending two-dimensional partition chromatography [13]. An aliquot equivalent to 250 μg total nitrogen was chromatographed on 10-inch square Whatman No. 4 filter paper. The chromatogram was first developed in an ammonia atmosphere with water-saturated phenol solvent (Mallinckrodt), which was then dried off overnight at 25°. The second solvent was 2,6-lutidine (95% Practical-Eastman Organic Chemicals) : water (2:1). After drying overnight, the chromatogram was dipped in a mixture of ninhydrin (0.25% w/v), isatin (0.01% w/v), 2,6-lutidine (1% v/v), in acetone[2], and heated at 80° for ten minutes. The chromatograms were viewed by transmitted light. The intensity of each spot was evaluated visually on an arbitrary scale (0–10 units) and the degree of aminoaciduria expressed as the arithmetic sum of the spot 'values'. The ratings were made by two observers reading independently, and the average of the two totals was used. The reliability of the visual scoring method was evaluated by comparing the summed 'spot units' with a quantitative measurement of total aminoaciduria. Urine samples from nine normal children and from four patients with vitamin D deficiency were analyzed on ground ion exchange resin columns using a Beckman Spinco Model-120 amino acid analyzer [42]. A linear relation between the two methods of estimating the aminoaciduria was observed (fig. 1) in the range encountered with the majority of samples in the study.

Endogenous amino acid clearance rates were measured quantitatively using elution chromatography on ion exchange resin columns as described previously [30].

Special procedures were done on additional patients (Part II). Prolonged intravenous infusion of calcium was performed using the technique described by FRASER *et al.* [10]. Inorganic phosphorus was infused intra-

[1] Dr. J.S. DUNBAR, Radiologist-in-Chief, Montreal Children's Hospital.

[2] SHAW, K.N.F.: personal communication.

venously in a buffered solution as described earlier [40]. Parathyroid extract[3] (PTE) was given subcutaneously in rotated sites. Blood samples in these special studies were always obtained in the fasting state in the morning to minimize the effect of diurnal and dietary variation; all serum calcium values were corrected to a standard specific gravity of 1.027 [14].

Part I
Clinical and Biochemical Correlations in the Human Vitamin D Deficiency Syndrome

The individual pretreatment values for serum calcium and phosphorus and aminoaciduria obtained from the thirty-nine patients with vitamin D deficiency are shown in figures 2A and 2B. Figure 2A indicates the relation of the initial calcium value to the urinary amino acid excretion; figure 2B shows the relation of initial phosphorus value to urinary amino acid excretion in the same patients. The shaded area in each figure represents the normal range for aminoaciduria and for serum calcium or serum phosphorus. For purposes of discussion of the results, each graph is divided into four areas by a horizontal line, which arbitrarily differentiates normal from hypocalcemia at 9.0 mg/100 ml and normal from hypophosphatemia at 4.0 mg/100 ml; and by a vertical line, which differentiates normal from hyperaminoaciduria at 15 units. The latter value was derived from a review of chromatograms obtained from the normal children (fig. 1).

Three groups of patients with the vitamin D deficiency syndrome are evident in figure 2; additional information on these patients is summarized in table I and as follows:

Group I

There were nine patients (solid circles, fig. 2), in whom the serum calcium was less than 9.0 mg/100 ml, and in whom the serum phosphorus level was normal. Of these, six had normal aminoaciduria, and in only three was minimal hyperaminoaciduria found. In six patients, the radiological signs (solid circles, fig. 3) were confined to a loss of definition of skull sutures and some demineralization of the calvarium; this was a characteristic roentgen sign of this stage of vitamin D deficiency; in the three other patients, mild rickety changes in long bones were also seen.

Six patients presented with convulsions and one with tetany; in none was vitamin D deficiency the initial diagnosis. The age on admission ranged from 2–12 months, with the peak at 4–6 months.

[3] Para-Thor-Mone, Lilly.

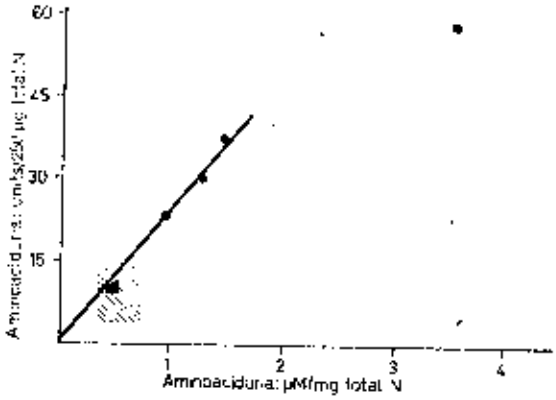

Fig. 1. Comparison of two methods of estimating aminoaciduria in nine normal infants (hatched area), and four vitamin D-deficient infants (solid circles). Quantitative values obtained by column chromatography are plotted on the abscissa; the results obtained by visual estimation of partition chromatograms (see text) are plotted on the ordinate.

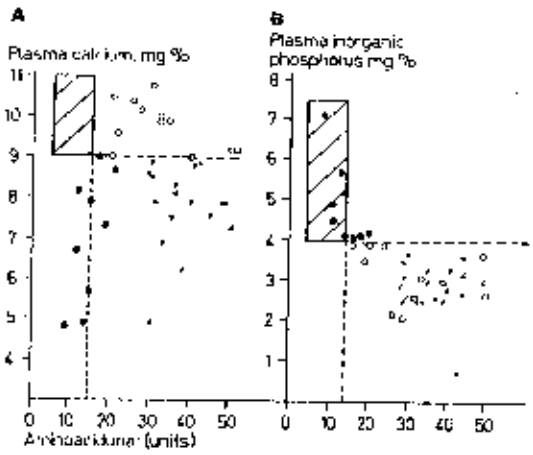

Fig. 2. A. Serum calcium values in thirty-nine vitamin D-deficient infants in relation to aminoaciduria (see text). Shaded area represents the normal range.
B. Serum phosphorus levels in the same patients plotted in relation to aminoaciduria. Solid circles, open circles and crosses, respectively designate early (stage I), moderate (stage II) and severe (stage III) deficiency. Shaded area represents the normal range.

Group II

There were 13 patients in this group (open circles, fig. 2). In all, the serum calcium level was normal (>9.0 mg/100 ml), the phosphorus level was below 4.0 mg/100 ml, and there was hyperaminoaciduria. All patients had typical radiological signs of moderate

Table I. Data obtained from 39 infants with the vitamin D deficiency syndrome

Stage of deficiency	Number of patients	Sex m:f	Serum Calcium mg/100 ml mean ± SD	Serum Phosphorus mg/100 ml mean ± SD	Aminoaciduria units mean ± SD	Alkaline phosphatase K.A. units % mean and range
I	9	6:3	7.0 ± 1.3	4.9 ± 1.2	14.8 ± 3.7	71.9 (35–130)
II	13	9:4	9.7 ± 0.6	3.2 ± 0.7	30.0 ± 10.9	99.9 (48–260)
III	17	12:6	7.8 ± 1.0	3.0 ± 0.4	37.5 ± 6.3	102.4 (44–220)

rickets in the long bones and costochondral junctions (open circles, fig. 3).

In only two patients was the diagnosis of rickets made purely on clinical signs and the history; in half of the patients, the admitting diagnosis was 'bronchopneumonia'. The age on admission ranged from 4–13 months, with a peak incidence between 6–8 months.

Group III

Seventeen patients had hypocalcemia, hypophosphatemia and hyperaminoaciduria (crosses, fig. 2). Roentgen signs of rickets were florid and unmistakable in most patients (crosses, fig. 3). In four patients, roentgen signs of hyperparathyroidism were found in long bones, phalanges or clavicles; these signs included subperiosteal erosion in phalanges and long bones and the 'rotting stump' sign in clavicles and tibia.

These infants exhibited the classical clinical signs of rickets and were diagnosed clinically. Three infants were admitted with convulsions. The age range was 4–40 months, with the peak between 6–12 months.

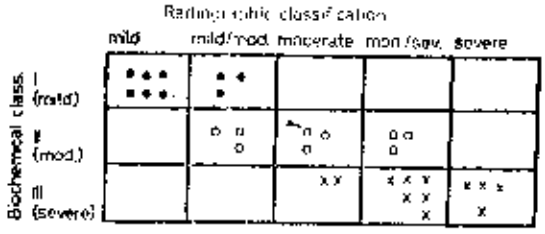

Fig. 3. Roentgen signs obtained in each of thirty patients correlated with the biochemical findings from the same patient; the latter are classified according to figure 2. Criteria for the roentgen classification are: mild = calvarium demineralized, mild-moderate = minimal costochondral and long bone findings; moderate = typical rickettic changes clearly apparent; moderate-severe = advanced rickety bone changes; severe = clear evidence of hyperparathyroidism in phalanges, clavicles, tibia etc. One untreated patient revealed progression of roentgen signs in a 13-day period (arrows).

Part II
Mechanism of Aminoaciduria and Phosphaturia in Human Vitamin D Deficiency

Hypothesis

The human vitamin D deficiency syndrome comprises three phases (Part I). This segregation can now be interpreted in terms of the new knowledge of vitamin D and parathyroid hormone interrelations [38]. Deficiency of vitamin D in the experimental animal initially impairs intestinal absorption of calcium and release of calcium from bone [2]. As a result, there is a decline in the extracellular concentration of calcium. Reactive hyperparathyroidism then occurs in response to the hypocalcemia [3]; the parathyroid response does not depend on the presence or absence of vitamin D itself. If sufficient vitamin D is still present in the tissues, parathyroid hormone can be effective in mobilizing calcium [2, 38]; thus calcium homeostasis is repaired. In response to the accelerated synthesis and release of parathyroid hormone, there is, however, the predicted renal tubular response, with rejection of solute [2]; this renal response is not dependent on the presence or absence of vitamin D. As the cellular depletion of vitamin D deficiency becomes further aggravated, maintenance of calcium homeostasis is again compromised [38], despite continuing reactive hyperparathyroidism; hypocalcemia thus may recur in severe vitamin D deficiency.

It is our contention that the hyperaminoaciduria and hypophosphatemia of *human* vitamin D deficiency are both directly dependent on endogenous hyperparathyroidism and not on cellular depletion of vitamin D itself. The following investigations were performed in vitamin D-deficient infants to test this hypothesis.

Results

Mechanism of the Hyperaminoaciduria

The endogenous renal clearance rates of amino acids were measured in the fasting stage in one patient

with stage II deficiency, and in another with stage III deficiency. The renal clearance rates of most amino acids were significantly elevated in the presence of normal plasma concentrations of the affected amino acids (table II). The hyperaminoaciduria of vitamin D deficiency is generalized and is the result of impaired tubular conservation. These data confirm the work of earlier investigators [6, 12, 27] concerning the mechanism and composition of the hyperaminoaciduria in human vitamin D deficiency.

Table II. Endogenous renal clearance rates of amino acids in vitamin D deficiency (ml/min 1.73 m²)

	Subject J. N.	Subject L. V.	
Stage of deficiency	II	III	
Sex	male	female	
Age (months)	8	9	
Endog. creatinine clearance ml/min/1.73 m²	102	97	normal range [39]
Aspartic acid	trace	6.0	trace–8.8
Threonine	3.9	4.3	0.5–2.5
Serine	7.6	9.3	1.2–3.4
Glutamine/asparagine	10.6	4.4	0.1–2.3
Proline	trace	trace	0–0.3
Glutamic acid	0.3	1.7	0.08–2.4
Glycine	12.0	27.3	1.2–8.6
Alanine	1.3	2.4	0.2–1.3
Valine	0.3	0.4	0.2–0.2
Methionine	3.7	1.5	1.0–3.4
Isoleucine	0.4	0.7	0.2–1.0
Leucine	0.6	0.9	0.2–0.9
Tyrosine	4.9	4.6	0.8–3.3
Phenylalanine	1.5	2.3	0.3–2.3
Ornithine	0.9	2.0	0.2–0.8
Lysine	3.6	10.9	0.3–2.4
Histidine	20.7	35.1	1.9–21.8
Arginine	0.2	1.2	0.1–1.2

Correlation of Aminoaciduria with Phosphaturia

The hypophosphatemia of human vitamin D deficiency is related to its high renal clearance [23, 28]. Inhibition of tubular absorption of phosphate, either by deficiency of vitamin D or by endogenous parathyroid hormone, has been proposed to account for this phenomenon [8, 21, 23, 43]. If the inhibition of tubular transport of phosphate and amino acids in patients with the vitamin D deficiency syndrome has a common antecedent [8], then a direct relation between the excretion rates of phosphorus and amino acids might be anticipated. The inverse relation between blood phosphorus and hyperaminoaciduria, which has been observed here (fig. 2B), and by others [8], would, in this case, also be anticipated.

A direct relation ($r = 0.72$) between hyperaminoaciduria and hyperphosphaturia was observed (fig. 4) in nineteen patients with various stages of the deficiency syndrome in whom the phosphorus and amino acid content of urine was measured in the same urine sample.

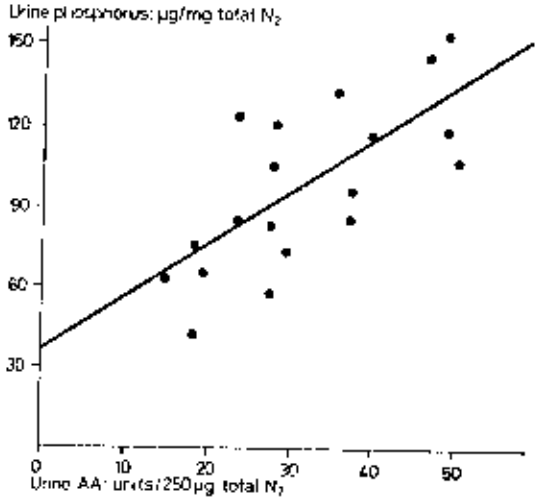

Fig. 4. Correlation of aminoaciduria and phosphaturia in nineteen patients with vitamin D deficiency. The correlation is significant ($r = 0.72$). Regression equation: $y = 36.8 + 1.92x$.

Spontaneous Evolution of the Vitamin D Deficiency Syndrome

Five infants with vitamin D deficiency were placed immediately at the time of diagnosis on a diet containing unenriched dairy milk; no vitamin D supplement was given. Four of these patients had hypocalcemia and were classified as stage I (early) deficiency. In each patient, the serum calcium rose slowly and spontaneously (fig. 5A); this was accompanied by an increase in the aminoaciduria and a decrease in the serum phosphorus level (fig. 5B). One of these patients also exhibited progression in roentgen signs over a thirteen-day period of observation (fig. 3). In effect, the four patients progressed spontaneously from stage I to stage II of the deficiency syndrome.

A fifth patient (fig. 5) was initially classified as stage II deficiency on the basis of biochemical and roentgen findings. In the subsequent six days the serum calcium level fell, and the aminoaciduria increased; his serum phosphorus level remained below normal. These findings suggest a progression of the deficiency syndrome to stage III.

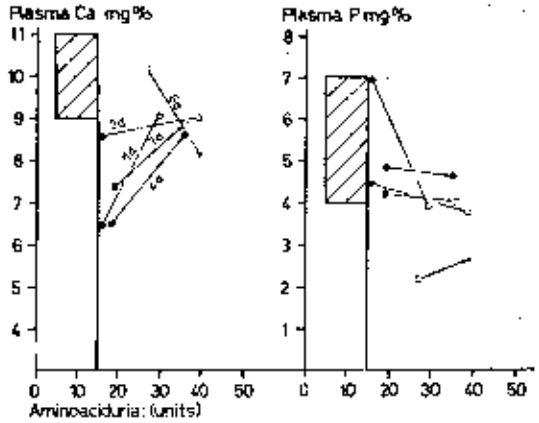

Fig. 5. Changes in biochemical data in untreated patients; numerals indicate days between initial and follow-up determinations. Note progression to next stage of deficiency during elapsed time; symbols used according to classification in figure 2 and text (Part I).

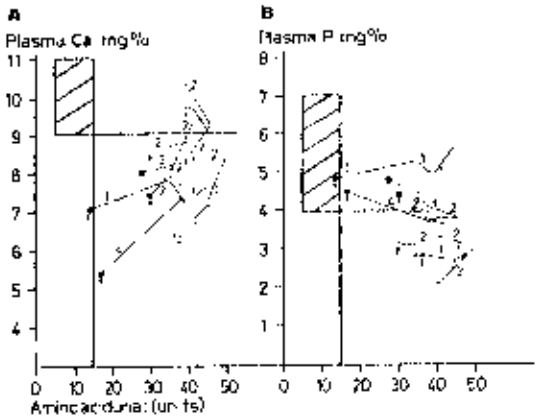

Fig. 6. Response to PTE, 50 units q 6 h. Note progression from one stage of deficiency to the next. Numerals indicate days elapsed between observations. See figure 2 for symbols.

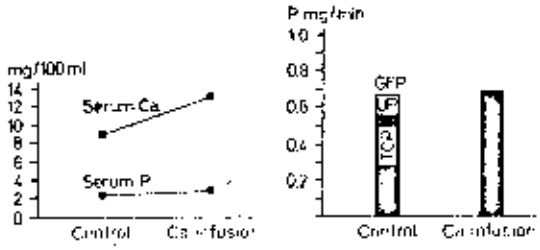

Fig. 7. Tubular absorption of phosphorus in patient J.A. (table IV), in the ninth hour after hypercalcemia had been induced by intravenous calcium infusion.

Response to Exogenous Parathyroid Hormone

Parathyroid extract was given intramuscularly (50 USP units, q 6 h × 4 days) to six untreated patients (fig. 6). Sufficient dosage of exogenous hormone might accelerate the transition from the existing to the next stage of the deficiency syndrome, if this transition is parathyroid dependent.

Three patients with stage I deficiency responded rapidly to PTE with increased serum calcium levels and augmented aminoaciduria; plasma phosphorus fell in two of the subjects (fig. 6). The response occurred more rapidly than in untreated patients (fig. 5), suggesting that comparable changes might not have occurred during the elapsed time, in the absence of the exogenous hormone. The sequential response in time of one patient with stage I deficiency, before, during and after PTE, and then with vitamin D_2 treatment, is presented in table III. Upon withdrawal of PTE, there is a reversion to pretreatment status.

Two patients with stage II deficiency, one of whom showed some spontaneous progression prior to injection, were also given PTE. This procedure raised the serum calcium, and aminoaciduria declined slightly during the period of calcium elevation. The existing low serum phosphorus levels did not change greatly.

In one patient with presumed stage III deficiency, the same dose of parathyroid extract used in the other patients failed to increase to serum calcium level to

Table III. Biochemical data from a patient[1] with stage I deficiency before, during and after intramuscular PTE (50 units 6q 6h × 13 doses) and then while on vitamin D_2 (5000 units/day).

Date	Regime	Serum Calcium mg %	Serum Phosphorus mg %	Aminoaciduria units/250 µg total N
12/4	pretreatment	5.4	3.8	17
15/4	8 doses PTE	6.6	2.4	
17/4	13 doses PTE	7.4	3.7	39
18/4	24 hours off PTE	6.1	3.7	
21/4	began vitamin D_2 5000 units/day	6.9	4.3	23
24/4	third day on vitamin D_2	8.9	4.9	
28/4	one week, vitamin D_2	9.3	5.7	12

[1] White female infant seven months of age. Considered to be at stage I of the deficiency syndrome on basis of biochemical, roentgen and clinical findings.

Table IV. Amino acid reabsorption in response to calcium infusion in vitamin D deficiency

Patient	Filtered load μmoles/min				Reabsorbed % of filt. load				Clearance rate ml/min/1.73 m²			
	J.A.		F.B.		J.A.		F.B.		J.A.		F.B.	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
Aspartic acid	1.6	2.1	0.4	0.6	100	100	100	100	0	0	Tr	Tr
Threonine	9.4	6.2	2.9	1.9	96.0	98.0	97.2	96.8	3.9	1.6	5.6	4.4
Serine	12.1	9.4	5.0	3.0	92.5	97.0	96.0	95.4	7.6	2.5	8.6	7.2
Glutamine and asparagine	12.7	21.4	9.6	6.5	89.5	97.5	96.1	98.6	10.6	2.0	8.5	2.1
Proline	12.5	6.8	6.1	–	100	100	100	–	0	0	Tr	
Glutamic acid	30.9	9.8	7.9	5.6	99.5	100	99.7	99.8	0.3	0	0.6	2.0
Glycine	14.6	15.5	6.7	5.2	88.5	98.0	87.3	90.7	12.0	1.8	26.9	14.0
Alanine	22.4	14.3	4.9	7.3	98.5	99.0	97.5	98.8	1.3	0.9	5.1	1.8
Valine	12.7	8.1	4.6	2.6	99.8	99.5	99.7	99.6	0.3	0.2	0.3	0.2
Methionine	1.1	0.9	0.4	0.7	96.0	99.0	100	100	3.7	0.8	Tr	Tr
Isoleucine	5.0	2.2	1.2	0.9	99.5	99.5	100	100	0.4	0.4	Tr	Tr
Leucine	6.5	4.2	2.3	1.5	99.5	99.5	99.6	100	0.6	0.7	Tr	Tr
Tyrosine	3.2	2.2	2.2	1.5	95.0	95.5	97.2	97.3	4.9	3.8	5.9	4.5
Phenylalanine	2.6	2.2	0.9	1.2	98.0	97.5	98.8	97.5	1.5	1.9	2.9	3.4
Serum calcium mg/100 ml	9.2	13.2	6.0	11.0								
Creatinine clearance ml/min	29.5	24.5	51.0	36.0								

Note: J.A.: patient with stage II vitamin D deficiency.
F.B.: patient with stage III vitamin D deficiency.
Pre and Post refer to condition prior to and during the 6th to 9th hour of infusion.

the normal range; on the other hand, the hypophosphatemia was exaggerated and the aminoaciduria increased further.

Response to Intravenous Calcium Infusion

Two vitamin D-deficient patients were infused with calcium. In one patient with stage I deficiency (J.A., table IV), the infusion produced unequivocal hypercalcemia; when this had been sustained for approximately six hours, the hyperaminoaciduria began to decline. Net tubular reabsorption of amino acids had increased greatly in the seventh, eighth and ninth hours of the infusion; complete phosphate reabsorption was achieved at the same time (fig. 7). In the second patient (F.B., table IV) with stage III deficiency, the infusion achieved only a normal serum calcium level, a finding in keeping with the more severe bone changes [10]. Renal clearance of amino acids was, however, reduced by this procedure, although net tubular absorption of amino acids was less improved because of an apparent fall in glomerular filtration rate during the procedure. Calcium infusion did not appreciably alter amino acid absorption and excretion in four control patients, each affected with one variety of the Fanconi syndrome.

Phosphorus Infusion

Many workers have proposed an association between phosphorus and amino acid transport [4, 8, 16]; some have suggested that the defect in phosphorus metabolism might, in itself, influence tubular absorption of amino acids [8]. One of our patients with type II deficiency was given a phosphorus infusion. The serum phosphorus level rose from 2.8 to 6.1 mg/100 ml, while the serum calcium level fell from 8.6 to 6.1 mg/100 ml. The filtered load of amino acids did not change significantly (preinfusion, 233.4 μmoles/min/1.73 m²; infusion, 226.12 μmoles/min/1.73 m²), whereas the reabsorbed fraction dropped from 94 % to 87.7 % during the infusion.

Response to Vitamin D Therapy

If hyperparathyroidism accounts for the aminoaciduria and phosphaturia of vitamin D deficiency, treatment with vitamin D should *first* enhance calcium homeostasis, thereby suppressing the hyperparathyroidism; *then* the hyperaminoaciduria and hypophosphatemia should return to normal. We followed the response to vitamin D therapy in patients with the deficiency syndrome (fig. 8). In two patients, serum cal-

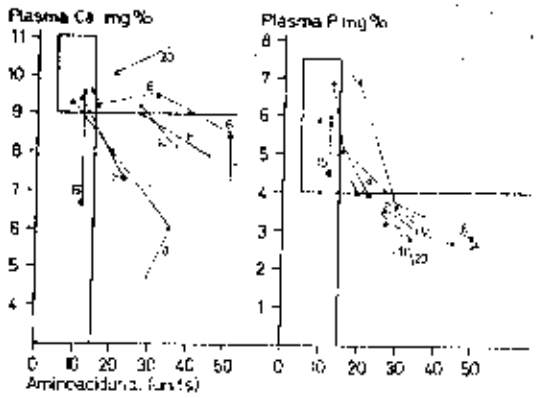

Fig. 8. Biochemical response to vitamin D_2 administration (5000 units daily) in deficient infants. Numerals indicate number of days elapsed between observations. See figure 2 for symbols.

cium levels rose immediately, clearly preceding any improvement in plasma phosphorus levels and in amino acid excretion. In no patient with prior hypocalcemia did the serum phosphorus level or amino acid excretion improve without a concomitant improvement in the serum calcium level.

Discussion

Vitamin D deficiency in the human infant can be divided into three stages on the basis of the clinical data obtained in the present study (part I). We propose that endogenous hyperparathyroidism, in response to the primary effect of vitamin D deficiency upon calcium metabolism, accounts for the biochemical and clinical findings peculiar to the three stages of vitamin D deficiency (Part II). Although the evidence for significant hyperparathyroidism in human vitamin D deficiency is circumstantial [5, 10, 21, 23, 26, 28, 34, 43, 45], it is quite convincing and can be summarized as follows:

1. The level of serum calcium during the three stages of deficiency (fig. 2, table I), varies in accordance with the predicted relation between PTH and vitamin D [2, 38] and their respective roles in the intestinal absorption of calcium and its release from bone.

2. Urinary excretion of phosphorus varies in direct proportion to the aminoaciduria in human vitamin D deficiency (fig. 4); plasma phosphorus levels and aminoaciduria (fig. 7 B) vary inversely [8]. These phenomena consistently reflect diminished tubular absorption [8, 23]. Parathyroid hyperactivity has been proposed on several occasions [10, 26, 31, 43, 45] to account for the hyperphosphaturia; this mechanism should also account for the aminoaciduria.

3. In human vitamin D deficiency, there is a natural tendency to progress in time from one stage of the biochemical syndrome to the next (fig. 5). The rate of progression apparently can be accelerated by the administration of PTH (fig. 6).

4. (a) Temporary elevation of the serum calcium concentration by intravenous infusion of calcium in the face of unchanged vitamin D nutrition temporarily suppresses the renal tubular dysfunction (fig. 7, table IV). (b) Vitamin D therapy first enhances the restoration of normal serum calcium levels if prior hypocalcemia exists; only thereafter does any apparent tubular dysfunction subside (fig. 8).

It has been suggested that many of the biochemical and physiological features of the vitamin D deficient syndrome depend on cellular depletion of the sterol itself [8, 23, 28]. However, the benefit obtained from temporary restoration of extracellular calcium levels by calcium infusion as demonstrated in this study and elsewhere [10], indicates that another mechanism for the tubular dysfunction may exist. LAVENDER and POLLMAN [31] have proposed that calcium ion may itself enhance tubular transport of phosphorus; they did not study the effect of this ion on amino acid absorption. However, the suppresive action of calcium upon synthesis and release of parathyroid hormone must also be considered [31, 36]. Experimental vitamin D deficiency in the mammal is associated with significant parathyroid hyperactivity [2, 3, 11, 20, 32, 35, 37]. Hyperplasia in explants of rat parathyroid glands and hypersecretion of calcium mobilizing hormone occur *in vitro* in response to diminished calcium concentration in the medium [36]. Hypertrophy and hyperfunction of parathyroid tissue also occur *in vivo* in the vitamin D-deficient rat [3]; the process is dependent on the hypocalcemia and not on deficiency of vitamin D *per se*. Tubular dysfunction in the vitamin D-deficient rat can be attributed to a direct effect of parathyroid hormone [2, 20]; the abnormality in the rat is not dependent on cellular depletion of vitamin D itself, and it is enhanced by exogenous PTH and suppressed by calcium infusion or parathyroidectomy [20].

One may thus believe by analogy that the tubular dysfunction of vitamin D deficiency in man is indeed parathyroid dependent; however, there are noteworthy differences between the species in their response to vitamin D deficiency. For instance, the rat, unlike the human and the dog, does not develop marked hypophosphatemia in advanced vitamin D deficiency which may explain the relative rarity of rickets in the rat exposed to simple vitamin D deficiency [22]. The tubular response also varies in different species; aminoaciduria appears relatively early (stage II) in the human deficiency syndrome, but only after severe hypocalcemia has been present for several weeks in the rat.

[33], and it apparently never occurs in the hypocalcemic, vitamin D-deficient dog[4].

The hypothesis could be strengthened if primary hyperparathyroidism in man were known to be accompanied by high renal clearance of amino acids and phosphorus. Documentation of hormone-dependent hyperaminoaciduria is rare in reports on primary hyperparathyroidism [24, 29]; others have stated it to be absent [28]. However, a review of the data on 127 patients with primary hyperparathyroidism from the series of DENT [15] revealed that some degree of renal hyperaminoaciduria is present in 44% of the total group; the incidence is higher (65%) in those first presenting with bone disease. The hyperaminoaciduria (and hyperphosphaturia) disappears after corrective surgical parathyroidectomy in most patients.

A number of interesting clinical features of the human vitamin D deficiency syndrome merit attention. In the present study, a sex ratio of 2:1 in favor of affected males was observed in vitamin D deficiency (table 1). A similar ratio has also been reported by others [7, 33]. The significance of this finding is not clear; there is, of course, at least one X-linked gene which is responsible for renal tubular conservation of phosphorus [49], but how this predisposes to the manifestation of vitamin D deficiency in males is unknown.

In severe vitamin D deficiency, PTE may be relatively ineffective in raising the serum calcium level; this was observed in the present study (fig. 6) and also by earlier investigators [28, 45]. The 'pseudohypoparathyroidism' proposed to explain this observation [45] can be reinterpreted as the requirement for vitamin D to mediate the action of PTH on intestinal absorption of calcium and on release of calcium from bone [23, 38]. Severe deficiency of the vitamin blocks the homeostatic effect of PTH; hence the fall in blood calcium levels and the poor response to PTE in raising serum calcium levels [2]. On the other hand, the tubular response to PTE does not require vitamin D [2, 20]; hyperaminoaciduria and the anticipated effects on phosphorus metabolism are thus observed following PTE injection in the deficient human infant (fig. 6).

The initial stage of the deficiency syndrome is important because of the clinical predilection for tetany or convulsions; its sole biochemical manifestation is hypocalcemia. Roentgen findings are minimal and effectively limited to the skull. Unless this stage of the syndrome, which is found in young infants in the early months of the year, is recognized, the infant will remain at risk until the second stage of deficiency evolves, or until the infant receives vitamin D. One of the most intriguing questions about the human deficiency syndrome concerns the existence at any time of stage I deficiency. Why does hypocalcemia appear and then persist for several days: why is there no immediate effective parathyroid response at this time?

[4] FRASER, D.; KOOH, S.W. and PAUNIER, L.: unpublished data.

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Hypophosphatasia*

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WHEN Garrod [1] delivered his classic lectures on the inborn errors of metabolism, he grouped together with remarkable insight a number of entirely separate entities, each characterized by a permanent inherited biochemical disturbance as a common feature. In recent years it has become increasingly evident that many other metabolic diseases are genetically determined, and rightly belong to this fascinating category.

In 1948, when Rathbun [2] coined the term "hypophosphatasia," he was not aware that the syndrome he was describing would later prove to be one of the inborn errors of metabolism. However, subsequent studies have shown this to be the case. There is now good evidence that hypophosphatasia is a specific genetically determined metabolic disease characterized by three salient features: (1) abnormal mineralization of bone, (2) diminished alkaline phosphatase activity, and (3) increased urinary excretion of phosphorylethanolamine. Therefore, it is evident that the biochemical nature of the syndrome may be established not only by the demonstration of an enzyme deficiency but also by the detection of a chemical abnormality. Such a dual manifestation is at present unique within this category of diseases.

The syndrome is rare, and only after some nine years has sufficient information become available to justify a composite description. It has become apparent, for instance, that there may be marked variations from one patient to another in the form and intensity of the disease, a feature which is in keeping with the modern concept of genetically determined biochemical disorders. Further, there may be considerable variation in the severity of the lesions in a given patient with the passage of time; but again this is not contrary to modern teaching, for there have been no complete spontaneous cures recorded to date.

Since Rathbun's report, patients with the general features of hypophosphatasia have been described by a number of investigators [3–16]. Furthermore, although Rathbun is rightly given credit for establishing the syndrome as an entity, earlier authors [17–20] have described cases which, although incompletely investigated, are now believed to be examples of the disease. The present paper also includes information concerning three hitherto unpublished cases [21–23].

In all, the review includes thirty-five patients all of whom I consider to be typical examples of hypophosphatasia. I shall also comment briefly upon one atypical case [24]. In addition to the reports cited, the reader is referred to five reviews and discussions which have been published on the subject [25–29].

Certain cases which have sometimes been considered examples of hypophosphatasia have been excluded from the present review since in my opinion the recorded data do not establish the diagnosis [30–34].

It is not my intention in this review to deal exhaustively with the various case reports. I have summarized the available data in Table 1. In the paragraphs which follow I have attempted to set forth an integrated picture of this still mysterious syndrome.

INCIDENCE

In a disease as rare as hypophosphatasia any estimate concerning incidence is obviously pure speculation. With this understanding, our own experience will be cited. In ten years four confirmed cases of hypophosphatasia from three unrelated families have been diagnosed at The Hospital for Sick Children, Toronto. Under the prevailing circumstances almost all children with serious subacute illnesses are referred to this institution, which serves a local population estimated at 2,000,000 persons. On the basis of the present birth rate in Ontario this population

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is considered to have produced approximately 500,000 infants during the past ten years. If one accepts the assumptions mentioned, the incidence of hypophosphatasia during the latter period was approximately 1:100,000 live births in the Toronto area.

In the present series, twenty-one cases were females and fourteen were males.

GESTATION

Although hypophosphatasia is an inborn error of metabolism, and although skeletal lesions developed *in utero* in some cases, there have been no obvious disturbances in the course of pregnancy. Most of the reported pregnancies reached term, and the disturbances mentioned by Rathbun and Chown were minor in degree.

AGE OF ONSET

There is radiographic proof that the bone lesions of hypophosphatasia may occasionally develop *in utero* (Case 3). Certain circumstantial evidence suggests that in other cases lesions may begin to develop (or at least may become much more severe) at the time of birth (Cases 9 and 10). In addition, it has been shown that occasionally bone lesions may not become detectable until several months after birth. In the latter patients it is probable from theoretical considerations that a biochemical lesion is present at the time of birth in all cases. In this regard one patient is known to have had no clinical or radiologic evidence of hypophosphatasia until the age of nine months although at birth the serum alkaline phosphatase level had been found to be markedly reduced (Case 19, sib of Case 9).

THE CLINICAL AND RADIOLOGIC MANIFESTATIONS

In describing the clinical manifestations of hypophosphatasia it is practical to divide the cases arbitrarily into three groups based upon the estimated time of development of demonstrable bone lesions: Group I: Infants in whom lesions were present at birth or within the first six months of life (Cases 1 to 16). Group II: Children in whom lesions gradually became apparent after the age of six months (Cases 17 to 31). Group III: Patients in whom hypophosphatasia was first diagnosed in adult life (Cases 32 to 35).

In general, the patients in group I were severely affected and had a poor prognosis; those in group II and group III were less severely affected and the outlook was correspondingly better. It is not known what determines the severity of a given case. However, it should be noted that when sibs have been affected (Cases 2 and 3; 4 and 5; 6 and 7; 12 and 13; 22, 23 and 24; 30 and 31), the age of onset, severity and clinical course have usually been almost identical. As the only exception to this observation the marked dissimilarity in the clinical features of the sibs reported from this center (Cases 9 and 19) should be noted.

Group I

Infants in whom lesions were present at birth or within the first six months of life (Cases 1 to 16)

General. All the infants in group I were found upon examination to have extensive skeletal lesions. However, it was usually because the infant had acute generalized symptoms and signs that the parents sought medical advice. The infants presented with anorexia, irritability, persistent vomiting, mild pyrexia of undetermined cause, and occasionally mild anemia and dehydration. Although even the severely affected children had normal weights at birth, marked failure in linear growth and weight gain had become evident before six months of age. Blue scleras were observed in three cases (Cases 9, 10 and 33) and mild skin pigmentation was mentioned, without comment, in two cases (Cases 9 and 10). The more severely affected infants also suffered from unexplained convulsions (Cases 1, 2, 3, 4, 5, and 10) and repeated attacks of cyanosis and pneumonia (Cases 2, 3 and 8).

In seven cases acute symptoms were present within the first few days of life (Cases 1 to 7); in these there is no doubt that bone lesions must have been present before birth. In the others, symptoms did not make their appearance for three to six months, and it is more difficult to fix the date of onset of the skeletal lesions.

Skeletal Lesions. The bones of the skull and skeleton were extensively affected in this group of infants. Marked costochondral beading and prominence of the ends of the long bones similar to that seen in severe rickets were characteristically present (Cases 1, 2, 3, 4, 5, 7, 8, 9, 10 and 14). In some cases marked angulations and deformities, presumably due to previous fractures, were reported at a very early age. Frequently, kyphosis developed as the child grew older (Cases 2, 3, 8 9 and 15). Bone tenderness (Cases 1, 9 and 11) and hypotonia (Cases 8, 10, 11 and 18) were mentioned occasionally.

The radiographic signs of hypophosphatasia

TABLE I

PRINCIPAL FEATURES OF REPORTED CASES OF HYPOPHOSPHATASIA

Case No.	Author(s)	Sex	Birth Weight (gm.)	Age at Onset (estimated)	Age (when last reported)	Outcome	Histologic Study	Clinical Manifestations: Long Bone Lesions	Cranial Abnormalities	Head Circumference	Premature Dental Exfoliation	Skeletal Growth	Renal Lesions and/or Renal Insufficiency	Serum Chemistry: Serum Calcium mg./100 ml. (normal 9.5 to 11.5)	Alkaline Phosphatase: Method*	Alkaline Phosphatase: Value	Urinary Phosphorylethanolamine
								Group I									
1	Rathbun [2]	M	4,300	Fetal	2 mo.	Dead	Autopsy	+	+	↓	..	↓	+	10.9 to 13.5	BLB	0 to 0.7	..
2	McCance et al. [73] (Case 1)	F	2,600	Fetal	3 mo.	Dead	Autopsy	+	+	.....	..	↓	+	14.4	....	.....	..
3	McCance et al. [73] (Case 2)	M	3,500	Fetal	5 mo.	Dead	Autopsy	+	+	↓	..	↓	+	11.3	Bod	0.6 to 1.2	+
4	Chown [78] (Case 1)	F	3,200	Fetal	3 mo.	Dead	Autopsy	+	..	↓	..	.....	+	15.5 to 15.8	....	.....	..
5	Chown [78] (Case 2)	F	3,400	Fetal	6 mo.	Dead	Autopsy	+	+	↓	..	↓	+	14.3 to 18.4	....	.....	..
6	Kubatsch [79] (Case 1)	M	3,750	Fetal	7 mo.	Dead	.....	+	+	.....	..	.....		.....	....	.....	..
7	Kubatsch [79] (Case 2)	F	3,500	Fetal	6½ mo.	Living	.....	+	+	Normal	..	.....		11.6	....	.....	..
8	Engfeldt et al. [8]	F	4,200	Fetal	10 mo.	Dead	Autopsy	+	+	↓	..	↓	+	9.5 to 14.0	BB	3.3 to 4.0	..
9	Fraser et al. [76,40,57] (Case 1)	F	2,700	Perinatal	2½ yr.	Living	Biopsy	+	+	↓	..	↓	+	10.7 to 15.0	KA	3.0 to 5.6	+
10	Schlesinger et al. [9,80] (Case 1)	F	3,400	Perinatal	2 yr.	Living	Biopsy	+	+	↓	..	↓	+	10.0 or 14.9	KA	2.0 to 9.0	+
11	Anspach et al. [20] (Case 2)	F	.....	3 mo.	4 yr.	Living	.....	+	+	.....	..	↓	+	11.7 to 16.2	Bod	1.2, 1.5, 12.0	..
12	Sobel et al. [7] (Case 3)	M	.....	3 mo.	4 mo.	Dead	.....	+	..	.....	..	.....	..	↑	BLB	0.2 to 0.9	..
13	Sobel et al. [7] (Case 4)	F	.....	3 mo.	8 mo.	Dead	.....	+	..	.....	..	.....	..	↑	BLB	0.5 to 1.6	..
14	Landal [23]	F	.....	3 mo.	1 yr.	Dead	.....	+	+	.....	..	.....	..	Normal	Bod	3.3	+
15	Schlesinger et al. [70] (Case 2)	F	5,900	6 mo.	2 yr.	Living	.....	+	+	Normal	..	.....	+	10.7 to 10.8	KA	3.0 to 7.5	..
16	Scaglione et al. [75]	F	.....	.....	4 yr.	Living	Biopsy	+	+	.....	+	↓	..	Normal	Bod	0.2 to 1.1	+
								Group II									
17	Illingworth et al. [12]	M	4,100	5 mo.	4 yr.	Living	.....	+	0	.....	+	Normal	0	10.8 to 11.5	JK	4.0 to 5.5	..
18	Sobel et al. [7] (Case 5)	F	.....	6 mo.	9 mo.	Living	.....	+	..	.....	+	.....	..	Normal	BLB	0.7	..
19	Fraser et al. [76] (Case 2)	F	3,500	9 mo.	18 mo.	Living	.....	+	0	Normal	+	Normal	0	10.6 to 13.2	KA	3.2 to 5.2	+
20	Fraser et al. [76] (Case 3)	F	1,900	10 mo.	10 yr.	Living	.....	+	0	Normal	+	↓	0	10.0 to 11.5	KA	2.0	+
21	Felder et al. [77]	M	3,500	8 mo.	19 mo.	Living	.....	+	0	Normal	+	↓	0	11.2 to 11.9	Bod	0.1 to 0.4	..
22	Clausen [6] (Case 1)	F	.....	10 mo.	12 yr.	Living	.....	+	0	.....	+	↓	..	Normal	BLB	0.8	..
23	Clausen [6] (Case 2)	F	.....	10 mo.	10 yr.	Living	.....	+	0	.....	+	Normal	..	.....	.....	.....	..
24	Clausen [6] (Case 3)	M	.....	10 mo.	6 yr.	Living	.....	+	0	.....	+	Normal	..	.....	.....	.....	..
25	Sobel et al. [5,7] (Case 1)	F	3,000	16 mo.	3½ yr.	Living	Biopsy	+	0	.....	+	Normal	0	9.8 to 10.3	BLB	0.8 to 1.6	..

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26	Sobel et al. [7] Case 2	M	.....	20 mo.	1 yr.	Living	.....	+	..	.....	+	.....	..	Normal	BLB	0.5	..
27	Schlesinger et al. [10] (Case 3)	F	.....	2 yr.	3 yr.	Living	.....	+	+	.....	0	.....	+	12.1	KA	2.4	..
28	Hühne et al. [77]	M	3,000	3 yr.	5 yr.	Living	.....	+	0	.....	..	↓	0	10.0	.....	.....	..
29	Harper [27]	F	.....	Childhood	6 yr.	Living	.....	+	+	.....	+	↓	+	11.6	BLB	0.1	0
30	Schneider et al. [3] (Case 1)	M	.....	Childhood	15 yr.	Living	.....	+	..	.....	..	.....	0	12.0	Bod	<1	..
31	Schneider et al. [3] (Case 2)	M	.....	Childhood	18 yr.	Living	.....	+	..	.....	..	.....	0	12.0	Bod	<1	..
								Group III									
32	Dent [35,43]	F	.....	Childhood	40 yr.	Living	.....	Bone fragility	0	.....	..	↓	0	10.0	KA	2.9	+
33	Macey [4] (Case 1)	M	.....	Childhood	36 yr.	Living	.....	Bone fragility	+	.....	..	.....	..	10.3	?	0.4 to 0.7	..
34	Macey [4] (Case 2)	M	.....	Childhood	Adult	Living	.....	.....	..	.....	..	.....	..	.....	?	<1	..
35	Henneman [22]	M	.....	Adult	44 yr.	Living	Biopsy	Bone fragility	0	.....	..	↓	0	9.3 to 9.7	Bod	0.1 to 3.6	+

* BLB = Bessey, Lowry, Brock
KA = King and Armstrong
Bod = Bodansky
BB = Buch and Buch
JK = Jenner and Kay

are essentially the result of defective calcification in pre-osseous cartilage and in recently deposited bone matrix. Longitudinal growth of bone takes place, but at a reduced rate, and little or no calcium is deposited at the disrupted zone of provisional calcification or in the osteoid which accumulates in the metaphyses.

It is generally conceded that the resultant radiographic picture is characteristic. (Fig. 1.) There was nearly always complete disappearance of the zone of provisional calcification (Cases 1, 2, 3, 7, 8, 9, 10 and 11) and little or no bone mineral was visible in the region extending from the epiphyseal cartilage occasionally as far as 1 cm. into the diaphysis. In this region a shadow slightly less radiolucent than the surrounding soft tissues was frequently observed which conformed to the shape of what would normally have been metaphyseal bone. Occasionally, longitudinal tongues of completely uncalcified tissue were reported extending into this zone from the region of the epiphyseal plate (Cases 1 and 9).

In most radiographs a gradual transition was observed from the uncalcified metaphysis to relatively normal diaphysis, and in the latter location a frayed and frequently cupped zone of diaphyseal bone was visible (Cases 2, 3 and 11). However, as seen in Figure 1 (Case 9) and in the radiographs published by Schlesinger et al. [10] (Case 10), the transition was occasionally sharply demarcated and convex, beyond which, at each end of the bone, a zone of poorly calcified metaphysis projected. In Case 9 it was noted, at the age of five months, that the length of the relatively well calcified portion of the diaphyseal shadow in the tibia and the femur was approximately equal to the mean length of the corresponding diaphysis in newborn infants of comparable birth weight. Taken in conjunction with the history, the finding was thought by Fraser et al. [41] to suggest that a sudden metabolic alteration had occurred in the infant during the perinatal period.

In several severely affected children, radiographic evidence of irregular subperiosteal new bone formation was observed in the diaphyseal regions (Cases 1, 9 and 10). The calcification of the diaphyses appeared to be relatively normal but the diaphyses failed to grow normally in diameter. Definite defects in calcification were observed in the secondary epiphyseal centers in several cases (Cases 2, 3, 8, 10, 21, 30 and 31). The bone age was retarded

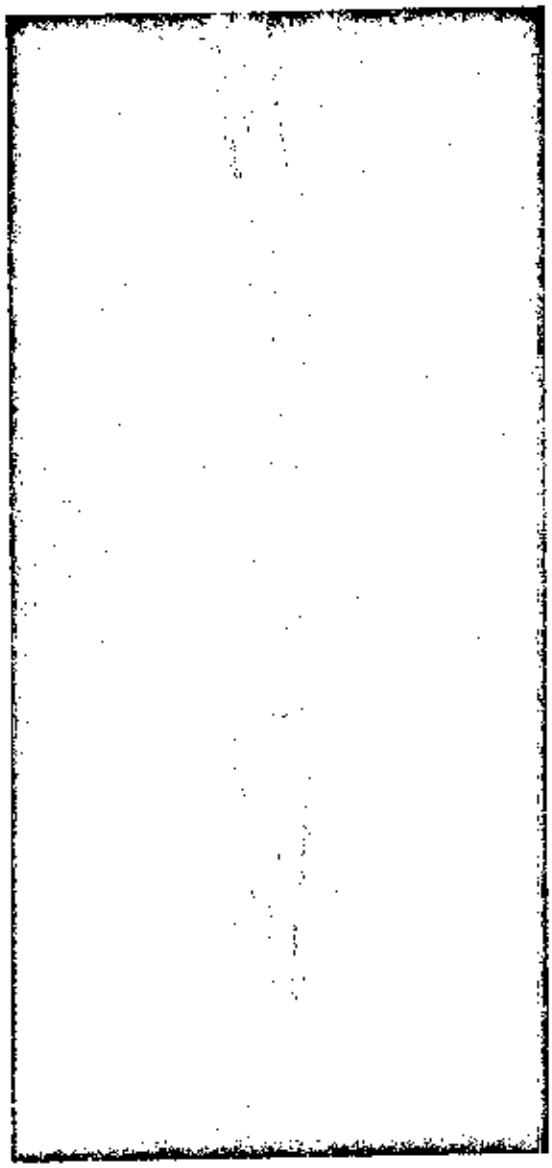

FIG. 1. Radiograph of the lower limb: Group I (Case 9, age five months). Note disappearance of the zones of provisional calcification, extreme defect in calcification of metaphyses and abrupt transition to relatively normal diaphysis. Subperiosteal calcification is visible. Epiphyseal centers are normal.

in some cases (Cases 2, 3, 10 and 21) but normal in others (Cases 1, 8 and 27).

Cranial Lesions. Cranial abnormalities were observed in thirteen of the cases in group I. It is probable that significant cranial lesions always occur when the infant is severely affected in early infancy. In Rathbun's patient, in whom the lesions undoubtedly developed *in utero*, the changes which occurred are representative of

FIG. 2. Radiograph of the skull: Group I (Case 9, age five months). Note wide bands of uncalcified osteoid between the poorly calcified membrane bones. Clinically, bulging fontanelle, reduced head circumference and craniostenosis were present.

the most extreme situation. The head was globular and the brain appeared to be enclosed in a soft fibrous membrane in which could be palpated four small plaques—the only vestiges of the frontal and parietal bones.

In no other reported case has there been so marked a defect in the cranial vault but practically all the infants in this group had conspicuous abnormalities in the development of the skull. In the more typical case the skull was described as soft, with a large bulging anterior fontanelle. In some cases the patient's eyes were unusually prominent (Cases 9, 10 and 11) and the scalp veins distended (Case 9).

The head circumference was recorded in nine cases in group I. Of these only two equalled the mean value for age (Cases 7 and 15). The remainder lay below the fifteenth percentile, and all but one below the third percentile.

The radiographic appearance of the skull in the severely affected patients with hypophosphatasia is both striking and characteristic. In Rathbun's case only four rudimentary plaques of calcified tissue were visible in the cranial vault. Frequently, reports of other severely affected infants (Cases 2, 3, 5, 8, 9, 10 and 15) have described between the cranial bones, wide bands which contained little or no visible bone mineral. (Fig. 2.) Because of their

latter were much less conspicuous than in the younger patients. Changes in the periosteum and the epiphyseal centers were minor in extent and frequently undetectable. Although bowing was often present in the long bones, the radiographic structure of the diaphyses appeared relatively normal.

In this group of fifteen patients craniostenosis was a relatively uncommon complication, and radiographs of the skull were usually normal. However, in two children many or all the cranial sutures had closed completely at an early age and the skull had acquired typical lacunar markings (Cases 27 and 29).

Group III

Patients in whom hypophosphatasia was first diagnosed in adult life (Cases 32 to 35)

Four patients have been designated as cases of adult hypophosphatasia. Cases 33 and 34 were sibs, and the sister of Case 31, although healthy, had typical chemical abnormalities and had had certain skeletal abnormalities as a child [*35*]. Three of the four patients presented after the age of twenty years because of undue fragility of the long bones. In each the serum alkaline phosphatase was found to be low. However, the only radiographic feature, apart from fractures or pseudo-fractures, was mild osteoporosis. In one patient the histologic appearance of the skull and ilium was normal (Case 35). The height was considerably below average in two cases (Cases 32 and 35) and was not recorded in the other two.

Two of the patients (Cases 34 and 35) had had no symptoms of bone disease during childhood and the question arises whether these patients form part of the general syndrome under discussion or whether they represent a separate entity. Further studies of the natural course of established childhood cases are required before the question can be answered. However, three features indicate that the manifestations described probably constitute the adult counterpart of the childhood illness. (1) In Cases 32 and 33 the patients had been considered "rachitic" as children. (2) In Case 33 the skull was dolicocephalic. (3) In Cases 32 and 35 phosphorylethanolamine was detected in the urine.

Group IV

"Atypical Hypophosphatasia"

In completing the description of the clinical manifestations of hypophosphatasia brief mention should be made of a case recently reported by Klein, Laron, Knapp and Sissman [*24*]. The patient, a male infant, presented with hypoglycemia, renal glycosuria and marked skin pigmentation, but without detectable bone lesions. The serum alkaline phosphatase levels were much reduced, and phosphorylethanolamine was present in the urine. A previous sib was said to have been similarly affected. These diversified biochemical disturbances might well be accounted for by a generalized deficiency of tissue alkaline phosphatase. However, in view of the absence of bone lesions it would seem unreasonable, in the present state of knowledge, to include this patient with the others.

DENTAL ASPECTS

The principal dental abnormality in hypophosphatasia is premature loss of primary teeth. The manifestation occurred in three-quarters of the children in group II and occasionally constituted the chief presenting feature (Cases 17 and 19). In Group I early loss of teeth was observed in only one case, but this was probably because few infants survived beyond the age of one year.

There appear to be two causes for premature exfoliation of the primary teeth in hypophosphatasia: (1) inadequate growth of alveolar bone [*36*], and (2) incomplete formation and early resorption of the roots of the teeth [*12*]. Other dental findings which have been reported include dental hypoplasia [*18*], enlarged pulp spaces [*7*], irregular calcification [*13*] and severe caries [*21,35*]. On the other hand, no radiologic or histologic abnormalities were observed in the primary teeth of our three patients despite the presence in each of lesions in the alveolar bone (Cases 9, 19 and 20). In the latter patients it is apparent that the metabolic derangement which resulted in an obvious defect in calcification of bone produced no demonstrable abnormality in calcification of the teeth. This anomaly suggests a difference in the genetic control of the cellular processes concerned with matrix formation or calcification in these two tissues. It deserves further investigation.

Little information has been recorded concerning the permanent dentition. The teeth appeared normal clinically and radiographically in Case 20. Dent reported severe caries in Case 32.

HISTOLOGY

Bone. A number of investigators have studied the histologic changes in hypophosphatasia, both

in the severely affected infants [2,8,13,15,16,18] and during the less acute phase [5,10]. In keeping with the radiographic changes the histologic defects were most marked at the growing regions of the bones. Both membrane and cartilage bone were involved.

It has been stressed that the lesions of hypophosphatasia are morphologically indistinguishable from those of simple rickets [13,37] unless special stains are performed to demonstrate phosphatase. In the epiphyseal plate the cartilage and the resting and proliferating cells near the epiphysis appeared normal. However, the hypertrophic chondrocytes close to the metaphysis had lost their normal columnar arrangement.

At the junction between cartilage and bone only occasional segments of the zone of provisional calcification could be distinguished. In this region tongues of apparently degenerating uncalcified cartilage projected toward the diaphysis, completely disrupting the normal architecture in this region [2,13,16,18]. In the metaphyseal regions islands of uncalcified cartilage were enveloped by uncalcified bone, and excessive amounts of uncalcified osteoid extended in a disorderly arrangement for several millimeters into the diaphysis. In the latter region the structure of the tissue approached that of relatively normal cortical bone, although several months after birth the shafts were abnormally small in diameter and cores of immature bone could still be seen surrounded by lamellar bone of mature type [10,16]. There was a considerable amount of irregularly calcified sub-periosteal osteoid which accounted for the radiographic changes seen in the shafts [2,8,13,16].

A number of special biophysical technics were employed by Engfeldt and Zetterström [8] in a study of undecalcified slices of affected bone. There was evidence of delay in the normal maturation of bone; linear growth and remodelling were defective; and there was a disturbance in the collagen structure and in the mineralization of the Haversian systems. *In vitro* uptake of Ca^{45} was reduced but mineral salts were deposited as hydroxyapatite crystals of normal form.

Similar defects in the growth and mineralization of the membrane bones have been described by several authors [2,10,13,18]. The widened "sutures" seen in radiographs of the skull were shown to consist of uncalcified osteoid tissue. Indeed, when the high incidence of craniostenosis is considered it may be of significance that the histological picture of a cranial suture has never been reported in this condition.

There are two fundamental matters upon which there is considerable disagreement. The first concerns the status of the osteoblasts and osteoclasts. Some authors [8,10,15] reported a paucity of both cell types at the costochondral junction and in trabecular bone. On the other hand, Park [37] believed that the osteoblasts were morphologically and numerically normal at the costochondral junction (Case 25).

The second point of contention concerns the morphologic appearance of the osteoid tissue. Some authors [16,37] have considered it normal whereas others [8,10] have suggested that the material appeared fibrous or hyalinized.

In essence, the histologic lesion in hypophosphatasia is characterized by two abnormalities, (1) a disturbance in the normal calcification process of cartilage and bone, and (2) excessive production and disordered arrangement of pre-osseous cartilage and of osteoid. If, as has been suggested, the osteoid is also qualitatively altered, then either abnormality theoretically could be the result of the other. It is not yet known which disturbance is primary in hypophosphatasia.

Kidneys. Autopsies have been reported in six of the ten fatal cases and in each, lesions were present in the kidneys. Deposits of calcium salts were observed in the renal tubular epithelium and in the surrounding interstitial tissues of five patients (Cases 2, 3, 4, 5 and 8) and calcium casts were sometimes present. In no case was nephrocalcinosis of sufficient degree to be visible radiographically. One is strongly inclined to attribute the nephrocalcinosis to hypercalcemia (*vide infra*) despite the reported absence of the latter feature in one case (Case 2).

In Case 1 no calcium deposits could be found in the kidneys. However, the collecting tubules were distended with casts and there was some necrosis of tubular epithelium.

It is not possible to assess the clinical importance of these lesions. Renal insufficiency, when present, was not great and it does not seem likely that the renal lesions were the primary cause of death in any of the children. Nevertheless, the potential danger of progressive renal damage in acutely affected infants with persistent hypercalcemia should not be underestimated.

Parathyroid Glands. A search was made for the parathyroid glands in each of the fatal cases cited. In three patients no glands could be located; in the others (Cases 4, 5 and 8) the parathyroid glands showed no significant lesions. Present evidence suggests that the parathyroid

glands do not play a primary role in the pathogenesis of hypophosphatasia.

Other Tissues. The adrenal glands were considered normal in four cases (Cases 1, 4, 5 and 8). The liver, thyroid, gonads and pancreas have shown no abnormalities. There have been no significant histologic changes reported in the pituitary.

CHEMICAL DETERMINATIONS

Serum Alkaline Phosphatase Activity. A marked reduction in the serum alkaline phosphatase activity is one of the cardinal features of hypophosphatasia. Values are not available for some of the patients but they have been recorded in twenty-five cases considered in the present review. (Table I.) The measurements have been made by a variety of methods, each with its specific substrate and conditions of reaction. Since the validity of the various conversion ratios is open to question, I have preferred to compare the figures obtained in a given case with the lowest value accepted as normal for the age of the patient and for the method employed.

Taking the group as a whole, the serum alkaline phosphatase activity in hypophosphatasia was reduced to approximately 25 per cent of the lower limit of normal, the mean levels varying widely from patient to patient, between almost no activity and 40 per cent of normal activity. There is no clear-cut relation between the degree of serum alkaline phosphatase depression and the severity of the clinical manifestations.

In following the alkaline phosphatase values of individual patients over a period of time, some showed remarkable constancy (Cases 1, 8 and 9). In others (Cases 10, 11, 15 and 25) a considerable variation occurred, without evident relation to the progress of the disease. Certain methods for determination of alkaline phosphatase are inaccurate at low enzyme concentrations, and it is possible that some of the variations noted are attributable to technical error.

Tissue Alkaline Phosphatase Activity. Several investigators have studied the alkaline phosphatase activity of various tissues in hypophosphatasia. Their findings regarding bone phosphatase have been [*2,7,8,10,13,15,16*] consistent and conclusive. They have demonstrated that the activity was markedly reduced in the metaphysis, the periosteum and the region of the costochondral junction during the active phase of the disease. For example, McCance et al. [*13*] found that the phosphatase activity at the costochondral junction of their patient was reduced to approximately 1 per cent of the amount present in normal infants, and the reductions recorded by other investigators, although not so marked, were definite nevertheless. To date, no tissue phosphatase studies have been carried out in bone from patients in whom optimal healing had already taken place.

Rathbun's discovery that phosphatase activity was reduced in bone, kidney and intestinal mucosa suggested the presence of a "generalized hypophosphatasia." His original concept has been confirmed by most subsequent studies. McCance et al. [*13*], employing different ester phosphate substrates, demonstrated an appreciable reduction in the phosphatase activity of liver, kidney and intestine. Engfeldt et al. [*8*] found the activity reduced to about 30 per cent in kidney, and Sobel et al. [*7*] noted a similar reduction in liver phosphatase activity.

To date, the only contradiction to the hypothesis of a generalized tissue phosphatase depression has been furnished by Scaglione and Lucey [*15*]. Their preliminary report indicated that the phosphatase level in the liver and the duodenal juice was normal in their patient. In view of the difficulties inherent in the measurement of tissue alkaline phosphatase activity [*38*], judgment upon this important contradictory finding should possibly be reserved until their data have been reported in detail.

Total Serum Calcium. Reference to Table I indicates that ten of the patients in group I had elevated serum calcium levels and that the value was normal or undetermined in six. However, when several determinations were carried out in a given patient wide variations usually occurred in the individual readings, and it is apparent that most of the hypercalcemic patients had normal serum calcium concentrations on occasion. The latter observation emphasizes the necessity of carrying out a number of determinations before eliminating the possibility of hypercalcemia in any given case. It is apparent that further study might well have uncovered additional cases of hypercalcemia among the less completely investigated infants in this group. Certainly, hypercalcemia is a common if not invariable occurrence in infants who are seriously affected. The degree of hypercalcemia was slight in some cases but in several, levels above 14.0 mg./100 ml. were recorded.

Hypercalcemia was a much less common finding in patients in group II, and when present was less severe. Five children were found to have elevated levels on occasion; the serum calcium values were normal or not reported for the remaining ten. Hypercalcemia was not observed in any of the adult cases (group III).

The cause of the hypercalcemia cannot be stated with finality. Since calcium balances have been invariably positive [*2,4,10,11,13*], since bone resorption would appear to be inhibited [*8*] and since there is no histologic or biochemical evidence of hyperparathyroidism, there is no indication that the excessive circulating calcium is of skeletal origin. Urinary calcium output was elevated in hypercalcemic patients (Cases 1, 10 and 21) and normal in normocalcemic patients (Cases 3, 25 and 35); therefore renal mechanisms are unlikely to be involved. One must conclude [*8,13*] that, for some unexplained reason, intestinal absorption of calcium takes place at a rate which exceeds the patients' ability to deposit the material in bone. On the basis of this conclusion it would seem logical to restrict calcium intake during the hypercalcemic phase of this disease.

Serum Inorganic Phosphorus. In general the serum inorganic phosphorus levels were normal. Individual determinations occasionally fell below or above the normal range for children but there were no consistent abnormalities. Terminal hyperphosphatemia was noted in a few cases.

Renal Function. As shown in Table I evidence of renal damage was present in ten of the infants in group I. In six the lesions were demonstrable at autopsy, and in four other patients (Cases 9, 10, 11 and 15) the presence of an elevated blood urea level and the demonstration of proteinuria and diminished concentrating power provided evidence of mild renal insufficiency. Elevation of the blood pressure was reported occasionally. No reference was made to renal function or urine in six cases.

In group II moderate renal insufficiency was present in only two cases. There was no nitrogen retention in eight other cases (Cases 17, 19, 20, 21, 25, 28, 30 and 31) and the urine was normal. No reference was made to renal function or urine in five cases.

Renal function was considered to be normal in two of the adult cases (Cases 32 and 35).

Other Determinations. A great many other determinations have been carried out on blood and urine but none have been significantly abnormal. The acid phosphatase, serum electrolytes, serum proteins, protein-bound iodine, Wassermann reaction and tests of adrenal cortical function have been normal. With the exception of the atypical case of Klein et al. both oral and intravenous glucose tolerance tests have been normal. On one occasion in Case 29 the fasting blood sugar was 30 mg. per 100 ml. Moderate anemia was present in some cases. When one considers the importance of phosphatases in biologic reactions it is surprising that evidences of metabolic disturbance have not been observed in other systems.

THE EXCRETION OF PHOSPHORYLETHANOLAMINE IN THE URINE

In the autumn of 1954 two independent groups discovered almost simultaneously the presence of a hitherto unreported ninhydrin-positive substance in the urine of infants with hypophosphatasia. Preliminary reports were made of these findings early in 1955 by McCance et al. [*39*] and by our group in Toronto [*40*]. At that time the evidence, based upon paper chromatographic technics, suggested that the material might be phosphorylethanolamine (amino ethyl phosphoric ester). Further evidence concerning the identity of the substance was presented by our group [*41*] in a paper delivered before the combined meetings of the American, British and Canadian Paediatric Societies at Quebec City, June 1955. We reported the isolation by ion exchange chromatography of a minute amount of crystalline material which behaved in a manner similar to synthetic amino ethyl phosphoric ester on ion exchange and paper chromatograms, and we showed that the material contained an ester-phosphate linkage as indicated by the technic of Hanes and Isherwood [*42*]. The chromatographic and electrophoretic properties of the substance were further discussed by Dent [*43*] at approximately the same time.

Recently, Cusworth [*44*] has succeeded in isolating a larger amount of the material from the urine of an adult patient with hypophosphatasia and has identified the substance as "phosphoethanolamine" by classic chemical methods.

Although phosphorylethanolamine had not been observed previously in human urine, it soon became evident that excretion of this compound was not limited specifically to patients with hypophosphatasia. Since the original reports detectable concentrations of what was

presumably the same material have been demonstrated on occasion in the urine of children with celiac disease [45], scurvy [46] and hypothyroidism [46], all of which diseases tend to have an associated suppression of the serum alkaline phosphatase level. On the other hand, the current studies of Dent and his associates [35] have suggested that phosphorylethanolamine is not detectable in the urine of randomly selected normal adults by their chromatographic procedure (1.25 mg. total nitrogen loading). The experience of our group has been similar, except that we believe small amounts of urinary phosphorylethanolamine may be detectable occasionally in randomly selected normal adults whose serum phosphatase levels are at the lower limit of normal.

Since its discovery, phosphorylethanolamine has been observed in the urine of several other cases of hypophosphatasia [10,13,22,35,43], and recently McCance et al. [13] have recorded for the first time the occurrence of a material which has been identified tentatively as adenosine monophosphate.

The question arises whether or not phosphorylethanolamine is invariably present in the urine of patients with hypophosphatasia. There is as yet little published information on this subject. Dent and Cusworth [43] found it in increased amounts in the urine of each of six established cases, and Harris and Robson [47] have identified it in each of fourteen. On the other hand, we have found it in only seven of eight patients. In the eighth, a typical case (Case 29), we were unable to detect even traces of phosphorylethanolamine on paper chromatograms loaded with 1.25 mg. total nitrogen—an amount five times the normal aliquot. It is unfortunate that it has not been possible to obtain another sample of urine from this patient, the only known non-excretor, since in studies of our other patients considerable differences have been observed in the rates of phosphorylethanolamine excretion.

As an explanation for the occurrence of phosphorylethanolamine in the blood and urine of patients with hypophosphatasia, it is tempting to suggest [39,40,43] that the substance accumulates because there is insufficient phosphatase to hydrolyse the ester phosphate linkage. This theory would suggest, in effect, that phosphorylethanolamine is one of the long-sought naturally-occurring substrates of alkaline phosphatase. In consonance with this concept is the recent demonstration by McCance et al. [13] that alkaline phosphatases derived from various normal tissues possessed the ability to hydrolyse synthetic phosphorylethanolamine *in vitro*. On the other hand, it is possible to propose other theories to explain the occurrence of phosphorylethanolamine in biologic systems. The latter cannot be excluded until more is known concerning the conditions which influence the excretion of this newly discovered substance.

GENETICS*

Schneider and Corcoran [3] were the first to propose a genetic factor in the etiology of hypophosphatasia. The evidence that has since accumulated fully supports their hypothesis. In early studies carriers were identified among the parents and relatives by the demonstration of low or borderline serum alkaline phosphatase levels. More recently the presence of increased amounts of urinary phosphorylethanolamine has also been considered an indication of heterozygosity. Unfortunately, in many papers on hypophosphatasia details which are of importance in evaluating the genetic aspects have been omitted, and for this reason the value of any conclusions is considerably reduced.

Fortunately, however, interpretation of the sex ratio is not affected by such shortcomings; although more females (twenty-one) than males (fourteen) were affected with hypophosphatasia, out of a total of thirty-five probands and affected sibs this deviation from a 1:1 sex ratio is not statistically significant (χ^2 of 1.0, 1 d.f., p is 0.30).

When more than one member in a family is affected with an uncommon anomaly the possibility of inheritance is suggested. Further, when both parents are apparently normal, and when, on the average, one-quarter of the sibs of the proband are affected, recessive inheritance of the disease and heterozygosity in both parents can be suspected. The parents were mentioned in fifteen of the families included in the present review; these parents were all considered normal as far as the bones were concerned, although to date no tissue biopsies have been studied. In twelve of these families (Cases 1, 2, 9, 10, 12, 18, 20, 21, 25, 26, 29 and 30) the serum of twenty-

* The genetic data have been interpreted by Professor N. Ford Walker, Department of Zoology, University of Toronto, and Director, Department of Genetics, The Research Institute, The Hospital for Sick Children, Toronto, Canada.

served to deteriorate during the first year of life. Those who survived usually began to improve sometime during the first or second years of life but wide variations were observed in the rate and extent of the healing process. For example, radiologic improvement in the patient studied by Anspach and Clifton [20] did not commence until the third year of life, after which time there was a gradual but incomplete deposition of calcium in the metaphyses. Scaglione and Lucey questioned whether or not their patient had yet reached the turning point at the age of four years.

In contrast, the patients reported by Schlesinger et al. [10] began to improve at about one year of age; fairly rapid although incomplete radiologic improvement took place during the subsequent twelve-month period. Concurrently, the wide defects in calcification between the bones of the skull gradually became mineralized and, as the latter process neared completion, radiographs indicated that a total cranial synostosis had resulted.

Anspach and Clifton attributed the progress of their patient to radiation of the parathyroid glands, and Schlesinger et al. attributed the improvement they had seen to the administration of large doses of vitamin D. Other experience would cast doubt upon the efficacy of these measures [6,7,11,16,35,48], and the author believes that the improvement observed in the foregoing patients was independent of the therapeutic measures employed.

The radiographic improvement observed by Currarino et al. [29] in an untreated, severely affected infant was even more dramatic. Skeletal healing commenced between eight and twelve months of age and proceeded rapidly at first, then more slowly. By the age of twenty months certain of the long bones had become normal in appearance and the others were much improved. This case illustrates the rapidity with which bone healing may occasionally take place in hypophosphatasia.

To date, no relapses have been reported once radiologic improvement has become established. Present evidence indicates that a corresponding spontaneous improvement has not been observed in the low serum levels of alkaline phosphatase [10,16,43,50] or in the phosphorylethanolaminuria [16], and rarely in the hypercalcemia [10]. As yet there is no information concerning the long term progress of such infants. The prognosis must remain guarded with respect to the effects of persistent hypercalcemia and untreated complete craniostenosis (Case 11).

Recently, observations have been made [16,49,50] upon the progress of four less severely affected children (group II: Cases 20, 22, 23 and 24). In each, gradual improvement in the skeletal lesions had occurred over a period of years but the bones had not become normal. A normal height was attained in two cases (Cases 23 and 24) but was much reduced in one (Case 20). Renal function was normal. Little or no improvement was observed in the serum alkaline phosphatase activity. As Dent has suggested [43], it is possible that in children who fall within this group increased bone fragility will develop in adult life.

Although the outlook for complete cure of hypophosphatasia seems remote, the life expectancy of the less severely affected children is probably normal.

THERAPY

In the past nine years many attempts have been made to influence the course of hypophosphatasia. For the most part they have been completely unsuccessful. Vitamin C [7], thyroid extract [2,7], testosterone [2], growth hormone [7], thiamin [7], oral citrate [16] and benemid® [3] have been administered to patients with hypophosphatasia without any significant effect upon either phosphatase activity or skeleton.

Several groups [4,6,7,10,11,16] have administered large doses of vitamin D to these patients, on the basis of those few features of hypophosphatasia which superficially resemble vitamin D deficiency rickets. Initially, the results of this treatment were thought to show promise [10]. However, considered in the light of more recent information regarding the natural course of untreated cases, the beneficial effects of vitamin D therapy have been unconvincing. Furthermore, severe hypercalcemia occurred rapidly on numerous occasions [6,7,10,11,16]. In my opinion there is no justification at the present time for trial of this precarious form of therapy.

In 1956 a preliminary report [57] was published of our experiences with the use of cortisone in a severe case of hypophosphatasia (Case 9). Within three weeks of commencing an oral dose of 37.5 mg. of cortisone per day we observed that mineralization had appeared in the long bones at the zones of provisional calcification. Within the next seven weeks considerable cal-

served to deteriorate during the first year of life. Those who survived usually began to improve sometime during the first or second years of life but wide variations were observed in the rate and extent of the healing process. For example, radiologic improvement in the patient studied by Anspach and Clifton [20] did not commence until the third year of life, after which time there was a gradual but incomplete deposition of calcium in the metaphyses. Scaglione and Lucey questioned whether or not their patient had yet reached the turning point at the age of four years.

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In 1956 a preliminary report [51] was published of our experiences with the use of cortisone in a severe case of hypophosphatasia (Case 9). Within three weeks of commencing an oral dose of 37.5 mg. of cortisone per day we observed that mineralization had appeared in the long bones at the zones of provisional calcification. Within the next seven weeks considerable cal-

cification had taken place in the metaphyseal and subperiosteal regions and the bones were approaching the normal. We were aware that fairly rapid healing of the bone lesions had occasionally been observed in untreated cases of hypophosphatasia; however, there were certain features of our case which convinced us that the dramatic skeletal improvement had not been entirely fortuitous. For instance, within a few days of initiating therapy we observed a definite and sustained rise in the serum alkaline phosphatase activity to approximately twice the pretreatment value, and a marked fall in the serum calcium concentration, features which have not been reported in natural remissions. When the hormone was withheld for two months skeletal lesions again became apparent, and the alkaline phosphatase activity fell to pretreatment levels; subsequently the bones healed rapidly when cortisone therapy was reinstituted.

At present, the child has been receiving 37.5 mg. of cortisone daily for one and one-half years. She is healthy and is growing slowly. Although certain of the epiphyseal plates remain distorted the metaphyses throughout the body appear to be completely and normally calcified. The serum calcium concentration is normal. The serum alkaline phosphatase level is slightly higher than during the pretreatment period but remains considerably less than normal, and phosphorylethanolamine is still present in the urine.

In support of our general findings Klein [*52*] has recently observed clinical improvement and a reversible increase in the alkaline phosphatase activity of his atypical patient during a period of cortisone therapy. On the other hand, preliminary trials of cortisone therapy in two affected children [*23,53*] and two adults [*22,35*] have failed so far to duplicate our findings. It is our present belief that cortisone has been of specific benefit to our patient. However, further confirmation of our observations is required in order to establish the efficacy of the treatment we have proposed.

The theoretical indications for early craniectomy and for restriction of calcium intake have been discussed in earlier sections.

FUNDAMENTAL NATURE OF HYPOPHOSPHATASIA

As the clinical features of hypophosphatasia have gradually unfolded during the past decade, so also the concepts regarding pathogenesis have become more clearly defined. Early investigators were struck by the markedly reduced alkaline phosphatase activity in the serum, and this observation has been extensively explored. Examination of urine, saliva and feces [*7,11*] failed to demonstrate excessive losses of enzyme. Sobel et al. [*7*], recognizing that the measurement of phosphatase activity did not necessarily represent the absolute concentration of the enzyme protein, searched in vain for evidence of possible phosphatase inhibitors. Further, it was shown [*16*] that magnesium, a known activator of certain phosphatases, was present in the serum in normal concentration. The incubation of serum from affected children with that of normal or rachitic persons has repeatedly failed to demonstrate the presence of non-specific inhibitors [*7,13,16*].

These findings lead to the conclusion that the low serum phosphatase activity reflects a true decrease in enzyme concentration, a decrease which theoretically could be caused either by an increased destruction or, more probably, by a decreased production of this group of enzymes in the tissues. In consonance with this hypothesis a marked reduction in tissue phosphatase concentration has invariably been demonstrated in bone, kidney and intestinal mucosa, and usually also in liver.

The possibility exists that the serum in hypophosphatasia might be unable to provide bone with an environment which could support mineralization. This suggestion seems unlikely, since it has been demonstrated [*40*] that adequate calcification took place *in vitro* when the cartilage of rachitic rats was incubated in the serum of a severely affected patient (Case 9). Similar *in vitro* experiments have provided further evidence that the seat of the disturbance lies within the bone itself. In contrast to the situation which obtains in cases of vitamin D resistant rickets [*54*], it has been shown [*47*] that no new deposition of bone mineral occurred when a slice of tissue from the patient's costochondral junction was incubated in the serum of healthy children.

All experimental data obtained to date have implicated the tissues, and for clinical purposes, principally the skeletal tissues, as the site of the pathologic process in hypophosphatasia. The lesion can be localized further to the proliferating osteoblasts [*2,8,10,15,16*] and the hypertrophic chondrocytes, since there is abundant evidence [*55*] that these cells normally constitute the principal source of bone phosphatase. On the basis of this cellular concept, the observed reduction in the phosphatase concentration must be

considered a secondary phenomenon. There is no doubt that studies of the phosphatase system in hypophosphatasia may help to answer some of the questions regarding the physiologic role of this enzyme in the formation of bone matrix and its calcification. Of more fundamental importance, however, is elucidation of the factors which give rise to the impaired function of the proliferating osteoblasts and the hypertrophic chondrocytes and, considering the disease in all its aspects, of phosphatase-producing cells throughout the body.

Whether the genetically determined disturbance in cellular function is caused by some deficiency, or by some inhibitor—exogenous or endogenous—remains a matter for speculation. In this connection the recent experiment of Scaglione and Lucey [*15*] is very stimulating, but the interpretation is open to argument. A block of bone was removed at operation from the skull of a patient who had a relatively severe hypophosphatasia (Case 16). The tissue contained no measurable phosphatase activity. A small fragment of the bone was placed in a tissue culture medium. During incubation there was a proliferation of cells which possessed the characteristics of osteoblasts, and it was observed that the new cells contained abundant phosphatase. It may be concluded that the culture medium had provided a more suitable environment for the proliferating cells than did the host. However, it is unjustifiable in the present state of knowledge to employ, without reservation, conclusions based upon *in vitro* results to explain observations made in the intact organism.

In a previous section of this review I have set forth in summary the reasons for our belief that cortisone exerts a specific effect upon the bone lesions in hypophosphatasia. In conformity with my concept of the fundamental nature of hypophosphatasia we postulated at one time that the disease process was caused by a derangement in adrenal function, and we explained the action of cortisone on the basis of its adrenal suppressive action. However, subsequent studies have not confirmed our original postulate, and we have put forward two additional explanations for the beneficial action of cortisone in our case. In the first, we postulate a direct pharmacologic action of cortisone on the disease process: in the second, we postulate that the beneficial action is exerted primarily in blocking some unknown toxic factor which, unopposed, acts as an osteoblast inhibitor. Both suggestions are in conformity with the basic concept of the pathogenesis of the disease but both are speculations which we cannot support by specific evidence. It is perhaps of interest that certain investigators [*16,43*] have tentatively suggested, each for his own reasons, the possible existence of vitamin D hypersensitivity as a factor in the pathogenesis of hypophosphatasia. Although there are a number of important drawbacks to such an hypothesis it fits in some respects the known facts concerning the toxic actions of vitamin D, and the postulated antagonistic effect of cortisone [*56,57*] upon the action of this vitamin.

In considering the present knowledge of hypophosphatasia it is obvious that little factual information is available and that much of the foregoing discussion is hypothetical. Perhaps this situation is inevitable for a condition so recently discovered and so rare. There is strong evidence that hypophosphatasia is genetically determined. Further, it is reasonable to suggest that the bone lesions are the result of a disturbance in the cellular processes concerned with either the elaboration of organic matrix, or its calcification, or both. Since the osteoblast is considered to play a key role in the control of both matrix formation and mineral deposition, and since the enzyme it elaborates, phosphatase, has been variously implicated in both processes, further elucidation of the primary disturbance in hypophosphatasia may contribute materially to the understanding of the fundamental physiology of bone.

TERMINOLOGY

In earlier papers on hypophosphatasia there was an obvious lack of agreement concerning nomenclature, and several designations have been applied. "Osteodysmetamorphosis fetalis" was suggested by the Swedish investigators [*8*] but the name is unwieldy and does not seem appropriate for those patients whose lesions are of postnatal onset. The term "rickets" has been used frequently in the past, perhaps with some justification. However, from almost every standpoint hypophosphatasia differs from the usually accepted connotation of rickets, and to use the latter term is to confuse the whole concept of hypophosphatasia.

Although future findings may suggest a more appropriate designation, the original term

"hypophosphatasia" would seem for the present to be the most satisfactory.

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RICKETS

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General Definition

Rickets is here defined as any condition in the growing person in which there is a generalized failure of bone salts to be deposited promptly in the bone matrix and in the preosseous cartilage at the zone of provisional calcification.

Types of Rickets

Apart from vitamin D deficiency rickets, there are 12 or more syndromes with bone lesions and clinical features that fit the above morphologic definition, but that occur despite intake of adequate prophylactic quantities of vitamin D. Each is discrete with respect to etiology, therapy, prognosis, and genetics. The diagnosis can usually be pinpointed by office procedures. It is practical to classify the various syndromes into the following four groups.

GROUP I. RICKETS DUE TO DEFICIENT INTAKE OR ABSORPTION OF VITAMIN D

(1) Vitamin D deficiency rickets is the prototype. (2) Celiac rickets and (3) rickets due to biliary obstruction are rare syndromes in group I; their pathogenesis is more complex.

Vitamin D Deficiency Rickets (Simple Rickets)

Vitamin D deficiency rickets is probably still the most common form in North America (United States, 1:2700 pediatric admissions; Canada, higher).

Three clinically and biochemically dissimilar syndromes of simple rickets are distinguishable. They apparently represent stages of progressing vitamin D depletion. The various combinations of physical and biochemical manifestations are indicated in Table 1. Sex ratio—two male: one female.

Biochemically, there may be hypocalcemia or hypophosphatemia, or both: generalized aminoaciduria may be present or absent. The physical manifestations may be (a) skeletal, and/or (b) neuromuscular. The classic skeletal lesions include craniotabes, costochondral beading, enlargement of wrists, knees, and ankles, Harrison's groove, frontal bossing, and sometimes long bone deformities. Neuromuscular signs are due to hypocalcemia and comprise (a) generalized convulsions, indistinguishable from idiopathic epilepsy, or (much less commonly) (b) tetany (carpopedal spasms, laryngeal stridor), or (c) latent tetany.

Differentiation of deficiency rickets from other syndromes (see Table 2) is aided by careful dietary history. Typically, patients have

not received vitamin D supplements (beware of misleading statements by the mother), or significant exposure to sunlight, and have received breast milk or dairy milk not enriched with vitamin D. Most processed milk and, in the United States, most brands of dairy milk, contain sufficient vitamin D for prophylaxis.

GROUP II. VITAMIN D-REFRACTORY RICKETS RESULTING FROM EXCESSIVE RENAL LOSS OF PHOSPHATE AND OTHER SUBSTANCES

Included are the seven hypophosphatemic vitamin D-refractory syndromes (including the Fanconi syndrome group) and rickets secondary to renal tubular acidosis.

Certain of this group (especially vitamin D-dependent rickets) may be indistinguishable from vitamin D deficiency rickets on history and physical examination. If any doubt exists regarding adequacy of previous vitamin D intake, it is essential, before embarking on high-dosage vitamin D therapy, to carry out a therapeutic test (vitamin D, 2000 I.U. per day orally for 3 months; observe serum calcium, phosphorus, alkaline phosphatase, and x-ray films of wrists and knees at monthly intervals). The test will always produce definite improvement in vitamin D deficiency rickets, but not in any form of vitamin D-refractory rickets.

The four commonest types of vitamin D-refractory rickets, and also the most difficult to differentiate, are compared in Table 2.

The other syndromes in group II are: *oculocerebrorenal syndrome (Lowe's syndrome)*: (i) onset in early infancy, (ii) severe mental defect, (iii) buphthalmos and congenital cataracts, (iv) generalized aminoaciduria, (v) usually hypophosphatemia and refractory rickets, (vi) absent deep tendon reflexes, (vii) almost always males.

Hypophosphatemic vitamin D-refractory rickets with hyperglycinuria: (i) onset of severe hypophosphatemic rickets at puberty, (ii) large quantities of glycine in urine, normal plasma glycine concentration.

Hypophosphatemic vitamin D-refractory rachitic syndromes secondary to chronic lead poisoning, Wilson's disease, neurofibromatosis, etc.

Rickets secondary to renal tubular acidosis (Lightwood's syndrome, Albright's syndrome): (i) failure to thrive, (ii) metabolic acidosis, along with acid urine, (iii) often nephrocalcinosis, (iv) rickets of variable degree.

Table 1. *Features of the Three Stages of Vitamin D Deficiency*

	STAGE I	STAGE II	STAGE III
Onset	3 to 7 months	5 to 12 months	6 to 15+ months
Season	January to April	January to April	Any season, commonly spring
Mode of presentation	Generalized convulsions 75% or Tetany 25%	Classic skeletal signs or As incidental finding in acute respiratory or other infection	Classic skeletal signs Incidental finding in acute infection General "malnutrition" Convulsions or tetany
Additional physical signs	General physical status good Minimal or no skeletal signs of rickets Trousseau test + or −* EEG + or − (epileptiform)	Clinical rickets, moderate to severe Trousseau test* negative	Clinical rickets usually severe May have deformities Often malnourished, anemic, rarely scorbutic May have positive Trousseau test*
X-rays	Minimal signs of rickets	Classic skeletal signs, variable degree	Classic skeletal signs, moderate to severe
Serum Ca	↓ (9.0 to 5.0 mg. per 100 ml.)	Normal	↓ (9.0 to 5.0 mg. per 100 ml.)
Serum P_i	Normal	↓ (below 4.0 mg. per 100 ml.)	↓ (below 4.0 mg. per 100 ml.)
Serum Alk. P'tase	Slightly or moderately ↑	+ to +++	+ to +++
Urine	Normal amino acids	Generalized aminoaciduria	Generalized aminoaciduria

* Trousseau test: Obstruct circulation above elbow with B.P. cuff inflated above systolic pressure for 3 minutes. Watch for carpal spasm.

Table 2. *Differential Diagnosis of the Four Commonest Types of Group II, Hypophosphatemic Vitamin D-Refractory Rickets*

		FANCONI SYNDROME GROUP		
TYPES	1. Hypophosphatemic vitamin D-refractory rickets (simple type) (Familial vitamin D resistant rickets)	2. Vitamin D-dependent rickets (Vitamin D-refractory rickets with aminoaciduria: Pseudo-vitamin D-deficiency rickets [Prader]).	3. Cystine storage disease (cystinosis)	4. Tyrosyluria (tyrosinemia)
Onset	12 mos. to 18 mos.	3 mos. to 15 mos.	Infancy or preschool	Infancy or preschool
Genetics	Sex-linked dominant, or sporadic	(?) Autosomal dominant	Autosomal recessive	Autosomal recessive
Mode of presentation	Onset of bow-legs when starting to walk Short stature	Weakness, failure to stand or walk Generalized convulsions or tetany	*Infancy:* irritability, anorexia, failure to thrive, pallor, polydipsia *Preschool:* bow-legs, or knock-knees, short stature, pallor, photophobia	Failure to thrive, weakness, failure to stand or walk, irritability, anorexia
Additional physical signs	Healthy, stocky, strong Slight to moderate rickets No urinary symptoms	Severe, rapidly increasing rickets → deformities May have + Trousseau No urinary symptoms	Mild to severe rickets Usually pale, irritable, sickly Hair characteristically gray-blonde Polyuria (Occasionally hypothyroid.)	Usually severe, rapidly increasing rickets → deformities Pathognomonic large, firm, nodular liver ± hepatoma
Cystine deposits*	No cystine deposits	No cystine deposits	Cystine deposits, cornea and bone marrow	No cystine deposits
X-rays	Epiphyseal plate and metaphyses show mild to moderate rickets Shafts usually sturdy, well mineralized, coarse trabecular pattern = "chronic rickets"	Acute severe, active rickets Thin, poorly mineralized shafts Osteoporosis Pathologic fractures and pseudo-fractures	Rickets usually moderate, varies from mild to severe Osteoporosis common	Rickets varies from 0 to severe, active, with osteoporosis
Serum Ca	Always normal	Slight ↓ to marked ↓	Normal to marked ↓	Normal to moderate ↓
Serum P_i	Marked ↓	Moderate ↓ to marked ↓	*Early:* marked ↓ *Late:* normal to ↑	Marked ↓
Serum Alk. P'tase	Slight ↑ to moderate ↑	Marked ↑	Moderate ↑	Marked ↑
Electrolytes and acid base	Normal	Normal	Normal or acidosis ± serum K ↓	Normal
BUN	Normal	Normal	Normal or marked ↑	Normal
Fasting blood sugar	Normal	Normal	Normal	Normal to ↓
Liver function tests	Normal	Normal	Normal	Abnormal
Plasma amino acids	Normal	Normal	Normal (cystine may be slightly ↑)	Tyrosine typically ↑ (± methionine ↑)
Urine				
Protein	0	0 → trace	+ to +++	0 to ++
Glucose	0	0 → trace	+ to +++	++ to +++
pH	Normal range	Normal range	Acid	Normal
Concentration	Normal range	Normal range	Normal to dilute	Normal to dilute
Urine aminoacids	Normal	Gross generalized aminoaciduria	Gross generalized aminoaciduria	Gross generalized aminoaciduria (especially tyrosine)
Prognosis	Life expectancy normal Usually remains hypophosphatemic, stunted and slightly deformed, despite therapy	Life expectancy normal "Cured" on high-dosage vitamin D therapy	Death from uremia at or before puberty	Death from cirrhosis or malignant hepatoma in infancy or childhood Dietary phenylalanine and tyrosine restriction may modify prognosis.

* Cystine visible on cornea with standard ophthalmoscope (+40 lens) or with slit lamp. Visible on unstained bone marrow smears with Nichol prisms.

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GROUP III. RENAL OSTEODYSTROPHY

The skeletal lesions (formerly termed "renal rickets") are secondary to chronic renal insufficiency (especially congenital renal hypoplasia or chronic pyelonephritis). Azotemia is always present.

Signs associated with the renal insufficiency are anemia, anorexia, elevated blood urea nitrogen, polydipsia, polyuria, and proteinuria. Serum alkaline phosphatase is increased, serum phosphorus is normal or elevated, serum calcium is normal or decreased, and acidosis is usually present.

The skeletal component, osteodystrophy, has radiographic and histologic features of both rickets and hyperparathyroidism, the latter consisting of erosions of the cortex of the phalanges and of the metaphyses of the long bones, loss of the lamina dura, and characteristic "fluffy" appearance of the skull bones.

Deformities (stunted growth, bow legs or, often, knock knees) tend to appear somewhat later (2 or 3 years of age).

GROUP IV. RICKETS WITH NORMAL HOMEOSTASIS OF SERUM CALCIUM AND INORGANIC PHOSPHORUS—PRIMARY MATRIX DEFECT

Metaphyseal Dysostosis

Clinical features of this rare condition are very similar to those of simple hypophosphatemic vitamin D-refractory rickets, but no biochemical abnormalities have been demonstrated to date. A matrix defect is postulated. Onset of bow legs, costochondral beading and stunted stature becomes apparent at about 1 year. The child is stocky, strong, and otherwise healthy. Radiographs show widening of the epiphyseal plates, but in contrast to other types of rickets, the limit of calcified tissue at the zone of provisional calcification is sharply defined, and the structure of trabeculae and cortex appears normal.

Serum calcium, phosphorus, and alkaline phosphatase are normal, as are acid-base metabolism and renal function.

Metaphyseal dysostosis has an autosomal recessive inheritance.

Hypophosphatasia

An uncommon autosomal recessive disturbance of bone matrix resulting in skeletal and cranial lesions of variable intensity. In the most severe cases, defective calcification of bone is present in utero, and at birth the bones are short, thin, fragile, and deformed. The calvarium may be virtually uncalcified and gives little support to the brain. Neonatal death occurs.

In the average patient, onset is during the first 6 months of life. Clinically prominent rickets-like skeletal lesions are present, and the skull tends to be small, to grow slowly, and to have a bulging anterior fontanelle. Failure to thrive is usual. X-ray films have the typical features of rickets, with, in addition, characteristic projections of defectively mineralized cartilage extending into the diaphyses. The prognosis is precarious until 18 months of age, after which partial remission is the rule.

In less severely affected patients, with onset at preschool age, premature loss of deciduous teeth, bow legs or knock knees, stunted growth, and symptomatic craniosynostosis may be presenting features.

Two pathognomonic biochemical features of the disease are (1) conspicuously low serum alkaline phosphatase activity (<4 King-Armstrong units) and (2) phosphorylethanolamine in the urine (paper chromatography). Urinary inorganic pyrophosphate is increased. The serum calcium, phosphorus, and other biochemical parameters are normal (except for a tendency for hypercalcemia in severely affected infants).

not useful

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1902

Conformational Similarities of Vitamin D and Cholesterol as Enzyme Substrates

INVESTIGATIONS of the synthesis of vitamin D long chain fatty acid esters found in rats have indicated that cholesterol-specific esterifying enzymes in the small intestine and plasma have vitamin D as substrate[1].

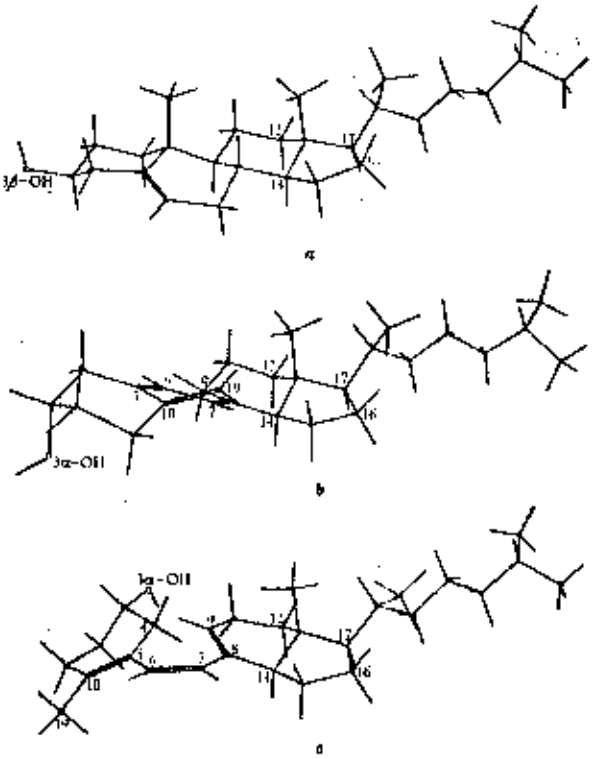

Fig. 1. Molecular structure of (a) cholesterol; (b) cholecalciferol (after Crowfoot Hodgkin, Webster and Dunitz[11]); and (c) precholecalciferol (after Vercloop, Koevoet and Havinga[6]).

Specificity studies show that the substrate conformation required by the pancreatic esterifying enzyme is a β configuration of a $C_{(3)}$-hydroxy group, an A/B-*trans*-decalin configuration and an intact, preferably saturated B-ring[2-5]. Vitamin D in the form of ergo or cholecalciferol satisfies none of these requirements, but it is a suitable substrate for this enzyme.

To help resolve this paradox, models of vitamin D and cholesterol were compared (Fig. 1*a* and *b*). Cholecalciferol has a side chain and rings C and D identical with those of cholesterol. Carbon 8 in ring C, through its ethylenic linkage, inclines the conjugated bond system and the A-ring to the β side of the molecule (Fig. 1*b*). If a horizontal plane is imagined passing through $C_{(12)}$, $C_{(13)}$, $C_{(16)}$ and $C_{(17)}$ then the oxygen of the 3β-hydroxyl of cholesterol (Fig. 1*a*) is about 2 Å above this level. Because of the molecular tilt, the 3α-hydroxyl of cholecalciferol is suspended downwards and its oxygen is also 2 Å above the $C_{(12)}$, $C_{(13)}$, $C_{(16)}$, $C_{(17)}$ plane (Fig. 2). Thus although the 3-hydroxyl is sterically on the opposite side of the molecule from cholesterol, it is in a similar horizontal plane with regard to the D-ring. If the molecule is viewed from the β side, cholecalciferol hydroxyl is seen to have moved to the left by about 4·8 Å from the cholesterol hydroxyl position.

These observations suggest two explanations for the esterification of cholecalciferol based on: (1) an enzyme binding site specific for a steroid substrate, and (2) substrate availability to the enzyme in a micellar solution.

The first explanation assumes that the enzyme binds cholesterol from the α side, for the angular methyl groups at $C_{(10)}$ and $C_{(13)}$ would tend to hinder a β approach. This view has been expressed with reference to other studies on substrate binding to steroid-active enzymes such as oestradiol-17β dehydrogenase[6] and 3-steroid sulphohydrolase[7]. Because alterations to the cholesterol side chain impair activity, it is also postulated that the

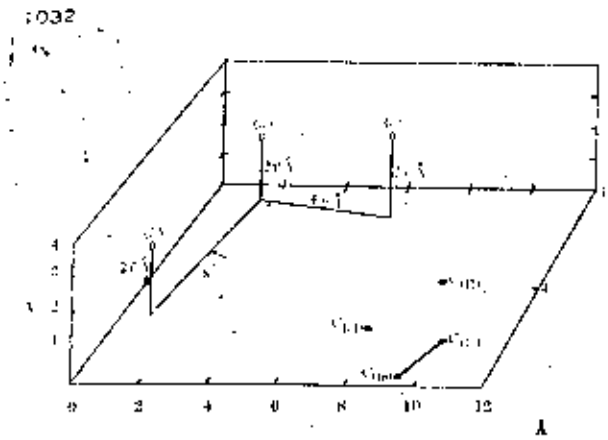

Fig. 2. Comparison of the position in three dimensions of the 3-hydroxy group oxygen of (a) cholesterol; (b) cholecalciferol; and (c) precholecalciferol, relative to a plane through carbon atoms $C_{(12)}$, $C_{(13)}$, $C_{(16)}$, $C_{(17)}$.

enzyme has a site which is specific for the full length of the molecule from the A-ring to the side chain. Both cholesterol and cholecalciferol would bind to the enzyme with the side chain and the C/D-rings. After this cholecalciferol bends away from the enzyme with the A-ring hanging free. The hydroxy group of cholecalciferol would, however, be positioned at a similar distance to that of cholesterol from the enzyme, although displaced laterally from the cholesterol hydroxyl position. If the carboxy group of the fatty acid can be held near this point then esterification might occur. The reaction rate with cholecalciferol was only about 10 per cent that of cholesterol[1] which could reflect greater difficulty in apposing the hydroxy and carboxy groups.

Precholecalciferol is esterified at about 25 per cent the rate of cholesterol[1], but it is difficult to fit the accepted formula[6] to a model because there would be repulsion between the hydrogen atoms of $C_{(4)}$ and that on $C_{(6)}$. Thus the A-ring double bond cannot be co-planar with the double bonds $C_{(6)}$:$C_{(7)}$ and $C_{(8)}$:$C_{(9)}$. Vercloop, Koevoet and Havinga[6] have suggested that the previtamin D molecules occur in two sets of rotational isomers with rotation around the $C_{(6)}$:$C_{(6)}$ and $C_{(7)}$:$C_{(8)}$ single bonds. A compromise position was therefore chosen for Fig. 1*c*. In this way the 3α-hydroxyl is about 2·6 Å above the $C_{(12)}$, $C_{(13)}$, $C_{(16)}$, $C_{(17)}$ plane and is displaced 4·6 Å towards the C-ring with reference to cholesterol (Fig. 2). With rotation of the A-ring possible, the most suitable conformation for esterification can be induced in precholecalciferol while bound to the enzyme, and thus a higher rate of reaction can be obtained.

By this hypothesis, cholecalciferol and precholecalciferol are not attached by A and B-rings and so the binding specificity depends on the rest of the molecule. This is in agreement with the elegant experiments of Ringold, Graves, Clark and Bellas[8], who demonstrated that the substrate recognition site of 3α-hydroxysteroid dehydrogenase was complementary to C and D steroid rings. It could thus be predicted that the ergosterol-type side chain would hinder the binding of ergocalciferol to the cholesterol-specific enzyme. Its reaction rate is only 2 per cent that of cholesterol, which is evidence for the further decrease in enzyme recognition.

The second hypothesis proposes that variation in the activity of the enzyme with a change in substrate structure may be a result of changed ability to form micelles. The enzyme may be specific only for the hydroxy and carboxy groups suitably positioned in micelles and not for the rest of the molecule. When it is difficult to form a substrate micelle, for example, ergosterol compared with cholesterol[10], there may be few hydroxy groups available for reaction, and so the position of the hydroxyl in the lipidic substrate complex, rather than its position on an enzyme-steroid reaction intermediate, would determine whether esterification occurs.

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Cholesterol esterifying enzyme from plasma, unlike that from pancreas, has a stable lipoprotein substrate and not a micellar dispersion of fatty acid and cholesterol. Yet both enzymes esterify vitamin D at rates much lower than they esterify cholesterol. It seems therefore that vitamin D esterification depends more on the steroid-type interaction with enzyme than on the physical properties of the substrate preparations.

If this interpretation is correct it is possible that vitamin D is a substrate for other cholesterol-specific enzymes, or indeed other enzymes requiring a 3β-hydroxy group on a steroid molecule. Thus the reported formation of vitamin D sulphate in liver, stomach and intestinal tissues of the rabbit[11] by a "vitamin D sulphokinase" could be regarded as a reaction by a cholesterol sulphokinase. Likewise if the "active site" for vitamin D function were a protein its specificity may be akin to that of other steroid sites with, in this case, the essential conjugated triene system positioned exactly for function. Here the steroid side chain need not be of prime importance for the site specificity, for in many species ergo and cholecalciferol are equally active.

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Unique Biosynthesis by Kidney of a Biologically Active Vitamin D Metabolite

Before the molecule of vitamin D acts in the control of calcium metabolism it is converted to a functional form in at least two metabolic steps. The first, a side chain hydroxylation at carbon 25, was shown by the elegant experiments of DeLuca and his co-workers[1-4]. This metabolite is synthesized in the liver from vitamin D[5,6] and is the major form of the circulating vitamin in blood plasma, not only of experimental rats[1] and chickens[7], but also of man[8]. A second more polar metabolite, localized in intestinal cell nuclei[9] and having at least three times the biological activity of an equivalent amount of cholecalciferol[10,11], is derived from 25-hydroxycholecalciferol (25-HCC)[12]. When it is obtained from chickens that have been given [4-^{14}C,1-^{3}H] cholecalciferol, there is a loss of tritium from carbon 1 (refs. 7 and 12). The tritium deficiency and the greater polarity are compatible with oxygen insertion at carbon 1 of 25-HCC.

The site of synthesis of this substance was not obvious. Although the small intestine mucosal cells contain the highest quantities of the tritium deficient metabolite (up to 1 ng/g), it was found in most tissues, with the lowest concentration in blood[13]. The time course of its

formation gave some indication that 25-HCC was converted to the more polar form in the intestinal mucosa, and indeed a recent article from the Wisconsin workers suggests this[11].

Attempts have been made in this laboratory to find the anatomical site of the enzyme and to study the mechanism of tritium removal from [4-^{14}C,1-^{3}H] 25-HCC. Conditions for *in vitro* experiments were based on the possible requirements of oxygen, NADPH and Mg^{2+} as in the hydroxylation of steroids by mixed function oxidases[11]. Homogenates, everted sacs, isolated cells and slices of small intestine from vitamin D deficient chickens were incubated with [4-^{14}C,1-^{3}H] 25-HCC in fourteen experiments; all failed to produce any trace of the tritium deficient metabolite. Incubations of 25-HCC with liver, adrenal, parathyroid, thyroid, ultimobranchial body, thymus, pituitary epiphysis, erythrocytes and blood plasma gave similar negative results. It was observed also that surgical removal from the rachitic chicken of the gastrointestinal tract, pancreas, spleen, thyroids, parathyroids, ultimobranchial bodies, adrenals and thymus did not prevent the formation of the metabolite *in vivo*. Kidney, a seemingly unlikely organ, was the tissue which in *in vitro* preparations eventually produced a reduction in the total ^{3}H : ^{14}C specific radioactivity ratio with [4-^{14}C, 1-^{3}H] 25-HCC. Chromatography of the lipid extract from such preparations showed that this ratio was reduced by the formation of a polar, tritium deficient metabolite, chromatographically identical to that found *in vivo* (Fig. 1). As kidney was the only tissue to convert 25-HCC in *vitro* in this way, the possibility of its unique role in vitamin D metabolism was investigated in bilaterally nephrectomized rachitic rats. These were each given 125 ng [4-^{14}C, 1-^{3}H] cholecalciferol in 0·1 ml. propylene glycol by intracardial injection immediately after surgery. Mucosa of the small intestine and blood plasma were collected 24 h later and the lipid extracts were chromatographed on silica gel G thin layers. The proportion of the metabolites is shown in Table 1. The nephrectomized rats did not produce any tritium deficient metabolite in contrast to intact controls which in mucosa and plasma contained about 30 per cent and 7 per cent respectively of total ^{14}C as this substance. Nephrectomy did not impair the synthesis of 25-HCC. These results indicate that the kidney is the only site of synthesis of a vitamin D metabolite postulated to be necessary for vitamin D function.

Table 1. PERCENTAGE DISTRIBUTION OF VITAMIN D AND ITS METABOLITES IN RACHITIC RATS *in vivo* 24 H AFTER INTRACARDIAL INJECTION OF 125 ng [4-^{14}C,1-^{3}H] CHOLECALCIFEROL

	Nephrectomized rats*		Intact rats*	
	Percentage ^{14}C	Ratio ^{3}H : ^{14}C	Percentage ^{14}C	Ratio ^{3}H : ^{14}C
Small intestine mucosa				
Cholecalciferol	33·8	4·2	16·8	1·2
25-HCC	33·7	4·0	37·1	3·8
^{3}H-deficient metabolite	0		29·4	0·7
Other polar metabolites	29·5	4·8	16·8	2·4
Blood plasma				
Cholecalciferol ester	0·8	†	0·4	–
Cholecalciferol	31·8	3·2	10·7	3·6
25-HCC	63·6	3·2	78·1	3·1
^{3}H-deficient metabolite	0		7·1	1·7
Other polar metabolites	3·7	5·0	3·6	4·2

* $CHCl_3$-methanol lipid extracts of pooled tissues from two bilaterally nephrectomized rats and four intact rats, chromatographed on silica gel G thin layers with ethyl acetate–chloroform (1 : 1).
† Radioactivity was too low to calculate reliable ratios.

The mechanism of the metabolic removal of tritium was studied further *in vitro* in kidney from rachitic chicken. Homogenates (12 per cent) of kidney in 0·32 M sucrose, 0·05 M Tris buffer (pH 7·4) were incubated in 25 ml. stoppered tubes which contained 2·8 µmoles Mg^{2+}, 30 µmoles nicotinamide, 0·6 µmoles NADP, 6 µmoles glucose-6-phosphate, 0·6 units of glucose-6-phosphate dehydrogenase and 15 µmoles α-oxalate together with 10–20 mg kidney protein, and 17 ng [4-^{14}C,1-^{3}H] 25-HCC were added in 1 drop of ethanol. The total volume was 1·5 ml. The tubes were gassed with oxygen for 1 min and

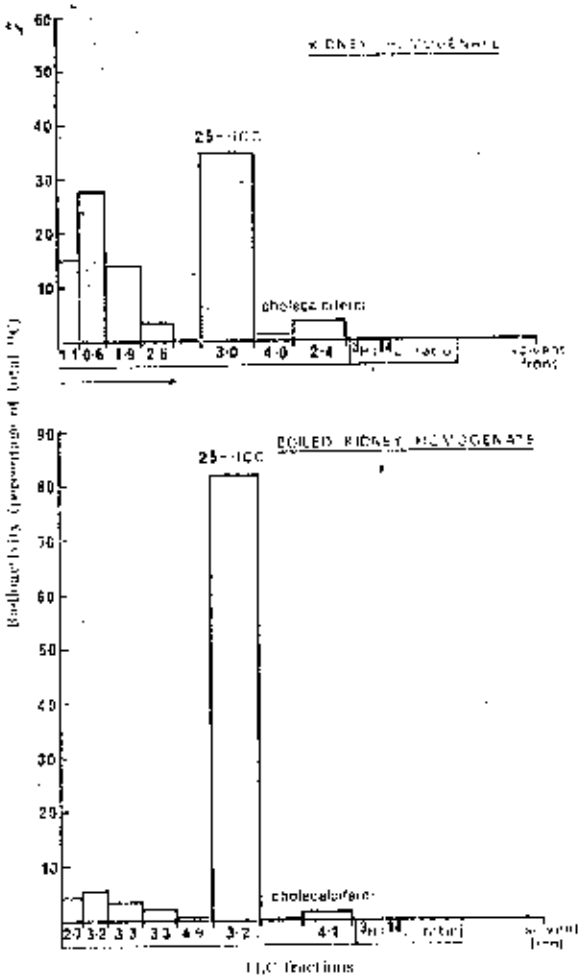

FIG. 1. Thin layer chromatogram of CHCl$_3$-methanol extract from incubation of [4-^{14}C,1-^{3}H] 25-HCC with vitamin D deficient chick kidney homogenate. Silica gel G thin layers were developed with ethyl acetate–benzene (1 : 1) to 17 cm solvent front. ^{3}H : ^{14}C specific radioactivity ratios are given for each fraction. The shaded peak represents the ^{14}C of the tritium deficient metabolite.

incubated with continuous gentle agitation at 37° C. The rate of synthesis of the tritium deficient metabolite was linear up to 1 h and had ceased by 2 h. Lipid was extracted with 10 ml. $CHCl_3$–methanol (2 : 1), and the $CHCl_3$ phase, which separated after adding 2 ml. water, was removed. The aqueous methanol residue was further extracted with 10 ml. $CHCl_3$. Recovery of ^{14}C radioactivity in the combined $CHCl_3$ extracts was 85–95 per cent. In boiled enzyme controls recovery was close to 100 per cent. Distribution of enzyme activity in subcellular fractions prepared by differential centrifugation revealed that most of the activity was present in the 7000 *g* nuclei-debris pellet (Table 2). The major component of this pellet was observed by phase contrast and electron microscopy to be mitochondria which when separated from nuclei, brush borders and erythrocytes by centrifugation on a sucrose gradient continued the enzyme activity. For enzymes involved loss of tritium to occur there was an absolute requirement for molecular oxygen. The heavy mitochondria had little of this activity in the absence of Mg^{2+} or nitrite, or in the presence of carbon monoxide. These properties are rather similar to those of steroid 11β-hydroxylase found in adrenal cortex heavy mitochondria[12] which requires oxygen, Mg^{2+} (ref. 16) and nitrite (ref. 17) (the latter being a generator of NADPH via intramitochondrial malic enzyme[18]) and which is inhibited by binding of CO to mitochondrial cytochrome P-450 (ref. 19). Such similarity implies again that the tritium is lost from [4-^{14}C,1-^{3}H]

25-HCC by oxygenation of carbon 1. The fate of the lost tritium was discovered to be in the formation of tritiated water, collected from the total incubation mixture by lyophilization. Recovery of tritium in water exactly matched the loss observed in the lipid extract and in fact proved to be a sensitive assay of the enzyme activity.

What now seems to be the functional intermediary metabolism of vitamin D is illustrated in Fig. 2. The formation of 25-HCC seems to be relatively independent of the supply of environmentally derived vitamin D. Its concentration in plasma of pigs rises as large doses of dietary vitamin are provided[1] and it probably constitutes the circulating reserve of vitamin D activity[1]. On the other hand the synthesis of the kidney metabolite cannot be increased by further administration of vitamin D or 25-HCC (ref. 12). Indeed the administration of 46.5 μg [1-^{3}H,1-^{14}C]-cholecalciferol to chickens given 3.5 μg unlabelled cholecalciferol per week for 5 weeks produces very little tritium deficient metabolite and the *in vitro* kidney enzyme activity of such birds was 0.25 per cent of that of vitamin D deficient chickens. We therefore suggest that there is some control over the synthesis of the metabolite by kidney which allows only sufficient quantities for target organ requirements.

Table 2. SUBCELLULAR DISTRIBUTION OF 25-HYDROXYLASE ENZYME ACTIVITY IN HOMOGENATES OF VITAMIN D DEFICIENT CHICK KIDNEY INCUBATED WITH [4-^{14}C]-25-HCC

	Percentage conversion of 25-HCC to 24,25-dihydroxycholecalciferol	Protein assay (mg)
(1) Subcellular fraction		
Whole homogenate	25·4	130
700 g – 10 min pellet	24·0	18·5
10,000 g – 10 min pellet	5·6	10·7
100,000 g – 60 min pellet	1·61	2·60
100,000 g – 60 min supernatant	1·01	10·6
(2) Subcellular fraction of 700 g pellet		
Whole homogenate	25·8	18·5
700 g – 10 min pellet	21·2	9·2
(3) Cell fraction including heavy mitochondria	16·6	16·1
Nuclei	1·3	3·4

The findings reported here could be relevant to the understanding of the pathogenesis of some diseases of calcium metabolism. For instance in chronic renal failure of man there is a variable resistance to the action of vitamin D (refs. 20, 21), and an osteosclerotic osteopathy develops resembling the osteomalacia of vitamin D deficiency[22]. Although previous attempts to identify changes in vitamin D metabolism in renal insufficiency have revealed very little[23], the synthesis of the polar biologically active metabolite was not examined and the possibility exists that in renal disease its production from 25-HCC is impaired or abolished. Similarly in familial vitamin D resistant rickets the genetic defect may result in the absence of the kidney enzyme described here. Finally, failure of the kidney vitamin D metabolism to supply sufficient metabolite for intestine and bone to maintain blood calcium levels could explain uncompensated hypocalcaemia such as is seen in dairy cows with parturient paresis.

We thank Dr D. E. M. Lawson for the supply of [4-^{14}C, 1-^{3}H] 25-HCC and Dr D. C. Barker for electron microscopic examination of subcellular fractions.

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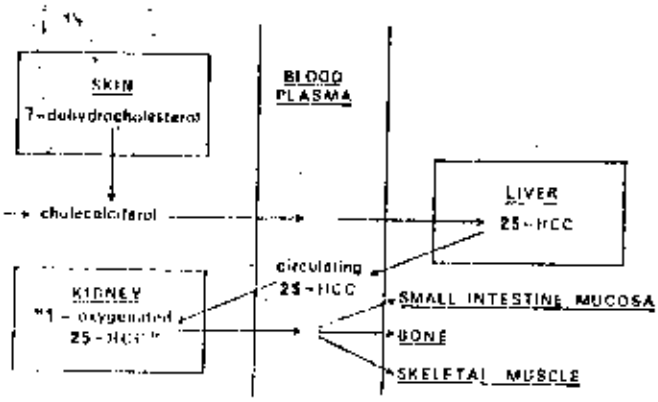

Fig. 2. Functional intermediary metabolism of vitamin D.

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The Synthesis of Vitamin D Esters in the Rat

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The discovery and identification of vitamin D fatty acid esters in liver and kidney of rats, 24hr. after the oral administration of [1-^{3}H]cholecalciferol has been reported (Fraser & Kodicek, 1965). To investigate the synthesis and fate of these esters, 1·5mg. [1-^{3}H]Cholecalciferol (specific activity 1·19mc/m-mole) was given orally to rachitic rats fitted with tailcups to prevent coprophagy. The animals were killed at 5, 10, 24, 48 and 72hr. after dosing and vitamin D and vitamin D ester were estimated on silica-gel thin-layer radiochromatograms of diethyl ether extracts of liver, kidney and small intestine, and of acetone–ethanol (1:1, v/v) extracts of plasma.

While the free vitamin D in liver decreased exponentially from 19% of the dose at 5hr. to 0·5% at 72hr. the ester remained at a relatively constant low level from 5hr. so that by 72hr. it represented 67% of the total vitamin D. The ester in kidney also remained at a low level but at 72hr. was only 10% of the kidney vitamin D. Free vitamin D in plasma fell exponentially from 0·26% of the dose per ml. at 5hr. while vitamin D ester rose from 11% at 5hr. to 46% of the total vitamin at 24hr. and then fell at the same rate as the free vitamin. This raised the possibility that the ester found in liver and kidney had been formed elsewhere and was transported to these organs in blood. The small intestine seemed the most likely site of synthesis as this was the only tissue where ester was found before 10hr. in higher amounts than afterwards.

It was postulated that during the process of absorption from the alimentary tract some esterification of vitamin D occurred. [1-^{3}H]Cholecalciferol (0·5mg.) was given orally to a 125g. rat and lymph from a thoracic duct cannula was collected at hourly intervals. Maximal absorption occurred between 2–3hr. in agreement with Schachter, Finkelstein & Kowarski (1964), and this decreased gradually so that after 12hr. 43% of the dose had passed into lymph. Up to 2·6% of vitamin D in each fraction was found to be esterified and the maximum appeared between 2–3hr. with a gradual decrease to 12hr. The ester composition determined by two-dimensional thin-layer radiochromatography showed stearate, 25%; oleate, 16%; linoleate, 16%; palmitate, 31%; others, 13% (each <5%). This pattern was similar to that found in liver but different from that in kidney (Fraser & Kodicek, 1965).

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Hypervitaminosis D, anaemia and renal failure

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A 53 year-old English lady was referred in January 1967 because of reduction in renal function. The only symptom disclosed was an invariable nocturia and daytime frequency of micturition every 2 to 3 hr during the previous year. The past history included an 18 month history of variable anaemia of 7–10 g/100 ml (Table 1), unexplained by barium meals, gastroscopy and laparotomy. Study of the bone marrow in June 1966 showed normal cellularity, erythropoiesis was normoblastic with normal haemoglobinization; 10% of the white cell precursors were developing eosinophils. There had been no sustained response to iron, vitamin B_{12} or folic acid.

On examination: no abnormality; BP 130/80; height 5 ft 4 in (160 cm); weight 47 kg.

Random and early morning urines were normal save for a specific gravity not exceeding 1010. Haemoglobin 8·8 g/100 ml; red blood count 3·1 m/mm³; MCHC 30%; MCH 28·7 γγo; MCV 96·7 cu μ; reticulocytes 3·5%; serum iron 105 μg/100 ml; total iron binding capacity 270 μg/100 ml; white

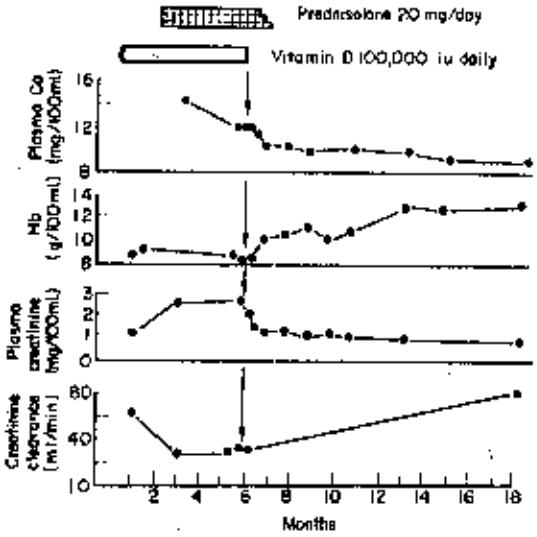

Fig. 1.

blood count 8000/mm³ with an eosinophilia of 850/mm³. Plasma creatinine 1·1 mg/100 ml and 24-hr creatinine clearance was 61 ml/min. Two weeks after the initial haematological measurements the eosinophilia was absent and the haemoglobin had risen to 9·4 g/100 ml with 0·8% reticulocytes. IVP: no anatomical abnormality of the renal tract. A percutaneous renal biopsy was performed: histology was compatible with either a focal and local glomerulonephritis with moderately severe nephron loss, or an interstitial nephritis with focal glomerular changes. Although positive serological evidence was absent, the combination of anaemia with reticulocytosis, transient eosinophilia and focal glomerular changes was suggestive of a systemic sensitivity phenomenon. On this basis prednisolone 20 mg/day was started.

Investigations were repeated after two months of corticosteroid therapy. The patient felt unwell, had headaches and anorexia. The haemoglobin was unchanged, the plasma creatinine had risen to 2·4 mg/100 ml and creatinine clearance fallen to 27 ml/min. Ampicillin (1 g/day) was added to the regimen on the basis that the renal interstitial changes might represent a chronic intrarenal infection. There was initially a short-lived improvement in well-being, but no change in renal function. Plasma calcium was 14·5 mg/100 ml, inorganic phosphate 3·6 mg/100 ml with a total protein of 6·7 g/100 ml. Serum alkaline phosphatase 5·2 KA units/100 ml. Urinary calcium 325 mg/day. (An 'average' diet was taken.)

The patient was re-admitted in mid-May of the same year for metabolic study. Renal function remained moderately severely impaired, plasma calcium was 12·8 mg/100 ml with a 24-hr urinary calcium excretion of 450–500 mg. Eventually the additional history was obtained that for the previous 7–8 years the patient had been regularly taking 100,000 i.u. of vitamin D daily, initially prescribed for finger nail splitting. From 31 May 1967, vit. min D was withdrawn and the prednisolone tapered off.

From being moderately unwell, the patient over a period of 2–3 weeks deteriorated markedly developing an agitated depression, requiring psychiatric advice as an in patient. Physical examination was negative, but as the plasma calcium at the time had fallen to 11·0 mg/100 ml, it was postulated that the symptoms were related to the changing plasma calcium. Accordingly 90 g of calcium gluconate were given intravenously. There was no change in condition. The depression yielded to trimipramine (Surmontil) over 1 month, during which the calcium fell to normal levels. Good health returned and was maintained after withdrawal of the antidepressant. The haemoglobin is now 13·6 g/100 ml, plasma creatinine 0·8 mg/100 ml, and creatinine clearance 8[illegible] ml/min (May 1970).

Discussion

The chart demonstrates the biochemical and haematological improvement following withdrawal of vitamin D. During the hypercalcaemia and hypercalcuria the skeleton was radiologically normal. Densitometry measurements by Dr F. Doyle of Hammersmith Hospital of the distal 8 cm of the left and right ulna bones were 434 mg/cm³ and 402 mg/cm³ respectively. These figures are close to the normal mean for age (Doyle, 1961). There was therefore no evidence of bone sclerosis, and the ratio of trabecular to cortical bone was normal. Absence of sclerotic or other bone changes after 7–8 years of vitamin D consumption is surprising.

In some patients being investigated for renal disease the presence of calcium in needle biopsy tissue of the kidneys has led to the diagnosis of a parathyroid adenoma (Evans & Zutshi, 1961; Joekes, 1963). In reviewing the tissue we obtained from this patient, which included twenty-two glomeruli and 5 cm strips of medulla, we were impressed by the absence of any calcium deposits.

The severe renal functional impairment, to approximately one quarter of normal, recovered following withdrawal of the vitamin D and return of the plasma calcium to normal. The glomerular and interstitial changes must presumably be interpreted as caused by either the hypercalcaemia or some other effect of hypervitaminosis D.

The anaemia is of particular interest. With both acute and chronic renal failure a marked diminution in red cell mass occurs with shortening of the red cell survival time. Acute renoprival anaemia

develops rapidly from the time of nephrectomy and does not appear to be related to the severity of the metabolic disturbance consequent upon the renal failure. In chronic renal failure, a clinically significant anaemia does not usually develop until the blood urea is persistently above 100 mg/ml or the creatinine clearance has fallen to less than 25 ml/min (Pryor & Joekes, 1969) although there is no direct relationship between the degree of nitrogen retention and the anaemia (Stewart, 1967). In the present case the serial blood ureas (Table 1) demonstrate that the

TABLE 1. Female aged 53 years

Date	Haemoglobin (g/100 ml)	Blood urea (mg/100 ml)
Jan 1964	13·0	...
Aug 1965	9·8	.
May 1966	9·8	30
Sept 1966	7·4	63
Oct 1966	14·0	46
Jan 1967	8·4	50
May 1967	8·2	94
Oct 1967	11·0	59
June 1968	13·6	43

anaemia is not dependent on the severity of the metabolic disturbance due to the renal involvement. It must be postulated that hypervitaminosis D is a direct or indirect cause of the anaemia, possibly acting by interfering with the renal production of some substance affecting erythropoiesis or red cell survival. Regrettably no observations were made of the red cell mass or red cell survival time. There is no evidence from patients with primary hyperparathyroidism that hypercalcaemia *per se* leads to an anaemia. We are unable to accept the view of Davies (1960) that with vitamin D poisoning 'an anaemia invariably occurs and is associated with the uraemia'. Schartman & Propp (1956) described four cases in which vitamin D intoxication presented with a normochromic, normocytic anaemia. One of these patients, whilst anaemic, had normal renal function and the anaemia resolved on withdrawal of the vitamin. The fact that vitamin D causes an anaemia directly or indirectly in the presence of normal renal function is not demonstrated in the papers of vitamin D poisoning (Kaufman, Beck & Wiseman, 1947; Howard & Meyer, 1948).

It should be emphasized that the recovery of the haemoglobin concentration in our patient, followed withdrawal of the vitamin and occurred without any haematinics.

Vitamin D intoxication may present as a neuropsychiatric problem (Lehrer & Levitt, 1960) or mental depression may complicate a pre-existing hypercalcaemia (Chaplin, Clark & Ropes, 1951) on withdrawal of the vitamin the mental abnormality usually resolves (Anderson, Cooper & Naylor, 1968). In our patient there was no mental ill health until the vitamin was stopped and this was short lived.

Acknowledgments

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IDIOPATHIC HYPERCALCEMIA AND SUPRAVALVULAR AORTIC STENOSIS*

Documentation of a New Syndrome

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A PECULIAR facies suggesting kinship has been found in some nonfamilial cases of supravalvular aortic stenosis in childhood.[1-6] In addition, it has been recognized that infants with idiopathic hypercalcemia have a rather characteristic "elfin" facial appearance. Recently described are a group of older children with "aortic stenosis" and an "elfin" facies. An illness during infancy in each patient was suggestive of idiopathic hypercalcemia.[3] Their facies appeared identical to those of patients with supravalvular aortic stenosis described by others.[1,3,4] It seems unlikely that this remarkable similarity is coincidental.

Although an association between idiopathic hypercalcemia and supravalvular aortic stenosis has been alluded to in the recent literature[2,6] no proved case has thus far been reported. The purpose of this paper is to present such a case and to establish this association as a clinically recognizable syndrome.

CASE REPORT

C.S. (J.H.H. 108-01-12), a 9-month-old boy, was admitted for the 2d time to the Harriet Lane Home for evaluation of psychomotor retardation and marked muscular hypotonia.

The family history was pertinent in that an 8-year-old male sibling had severe mental retardation. This sibling suffered primary and secondary apnea at birth, and a convulsive disorder subsequently developed. His facial appearance did not suggest hypercalcemia. He was normotensive and had no cardiac murmur. There was no renal dysfunction; calcium and phosphorus and cholesterol determinations yielded normal results. Roentgenograms of the bones gave no evidence of earlier increased mineralization.

The past history disclosed that the patient was the product of an uncomplicated 9-month gestation. Labor and delivery were normal. The birth weight was 3685 gm. (8 pounds, 2 ounces). The neonatal course was not unusual. He was fed a standard evaporated-milk formula (400 units of vitamin D per quart) and received 1000 units of vitamin D daily as a vitamin supplement. At the age of 5 months he was referred by Dr. A. C. Kolls to the Harriet Lane Home Cardiac Clinic for evaluation of cardiomegaly and cardiac murmur. A diagnosis of valvular pulmonary stenosis was entertained. At 6 months of age an uncomplicated bilateral inguinal herniorrhaphy was performed. At follow-up visits it became increasingly evident that the patient was failing to attain normal developmental milestones. At the age of 9 months he was unable to sit with support or to crawl, and he had a weak cry.

Physical examination showed him to be in the 10th percentile for height and in less than the 3d percentile for weight. The blood pressure was 150/62 mm. of mercury in the right, and 126/60 mm. in the left arm and 140/70 mm. in the lower extremities. The facies was elfin (Fig. 1). Slit-lamp examination disclosed bilateral lenticular opacities compatible with calcium deposition. Marked muscular hypotonia was evident. There was diminished muscle mass, poor motor control and symmetrically hyperactive deep tendon reflexes. Cardiac examination revealed a right precordial lift and a diffuse apical impulse. A systolic thrill was palpable over the upper sternal borders and in the suprasternal notch. The 2d heart sound split normally with respiration, and both aortic and pulmonary-valve closure sounds were easily distinguished. A Grade 4 (on the basis of 6) systolic ejection murmur was heard maximally over the right and left upper sternal borders and radiated over the entire precordium, into the neck and to the back. No manifestations of congestive cardiac failure were present.

The hemogram revealed a mild normochromic, normocytic anemia. Urinalyses showed acid pH, occasional pyuria and microscopical hematuria without bacilluria. Urine cultures were sterile. A 12-hour urine concentration test yielded a specific gravity of 1.015. Standard urea clearance, corrected for body-surface area, was 27 per cent of the normal value for the patient's age. The creatinine clearance was similarly reduced. The serum chemical findings were as follows: sodium, 140 milliequiv., chloride, 100 milliequiv., potassium 4.5 milliequiv., and carbon dioxide combining power, 21 milliequiv. per liter; urea nitrogen, 18 and 30 mg., total lipids, 1.9 gm., total cholesterol, 165 mg., calcium (method of Harrison and Harrison[7]), 13.1 and 13.3 mg., phosphorus, 3.8 and 3.2 mg., alkaline phosphatase, 2.8 Bodansky units, total protein, 6.1 gm., albumin, 3.5 gm., globulin, 2.6 gm., protein-bound hexosamine (method of Winzler[8]), 215 mg., and vitamin D assay (method of Bell et al.[9]), 1540 units per 100 ml. (normal, 60-400 units).

Roentgenologic examination revealed considerable sclerosis of the bony calvaria and base of the skull, as well as diffuse osteosclerosis of the long bones and vertebras (Fig. 2). An intravenous pyelogram was normal. X-ray study of the chest disclosed an increase in the cardiomegaly first noted at the age of 5 months.

An electrocardiogram showed a vertical electric position, right-axis deviation and combined ventricular hypertrophy.

Cardiac catheterization with selective cineangiocardiography was performed. The arterial catheter was advanced to the ascending aorta, but it could not be passed across the aortic valve. The venous catheter was advanced to the main pulmonary artery. The catheterization data are presented in Table 1. There was a systolic gradient of 21 mm. of mercury across the pulmonary valve. A cineangiogram in the left anterior oblique position after injection of contrast material into the main pulmonary artery suggested mild stenosis at the origin of the left pulmonary artery. A thickened, abnormal pulmonary valve was identified. A 2d cineangiogram with the catheter in the ascending aorta distal to the aortic valve (Fig. 3) demonstrated a supravalvular aortic narrowing, with a supravalvular aortic diaphragm, a dilated left coronary artery and mild aortic insufficiency. The wall of the left ventricle appeared strikingly thickened.

Surgical correction of the cardiovascular anomalies was not deemed feasible at this age. The patient was placed on a low-calcium diet with no added vitamin D and was

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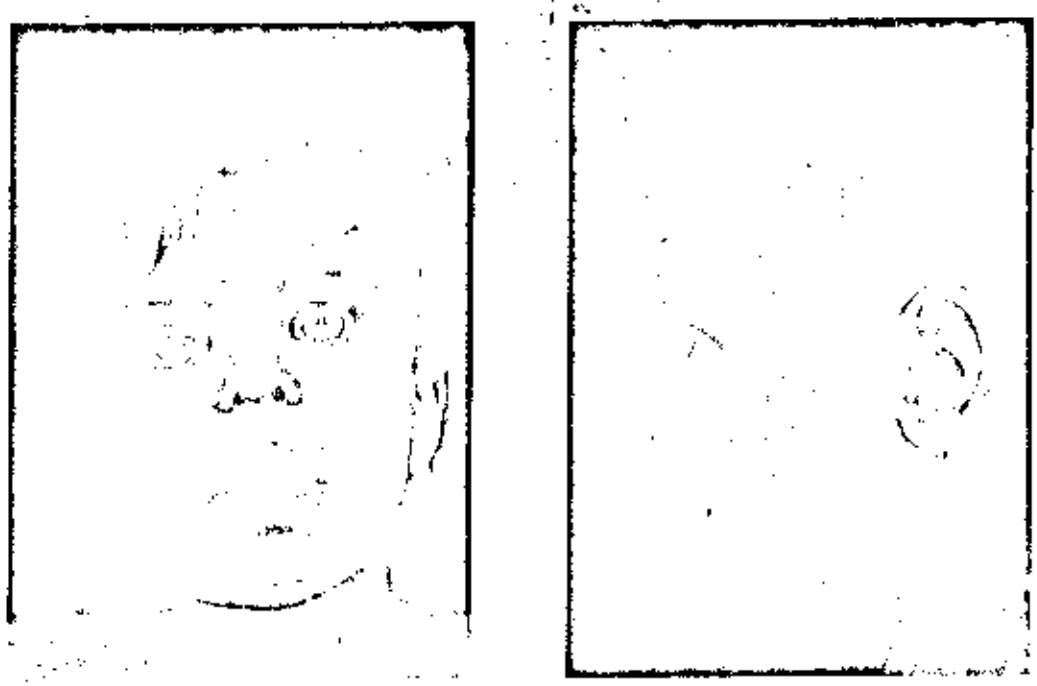


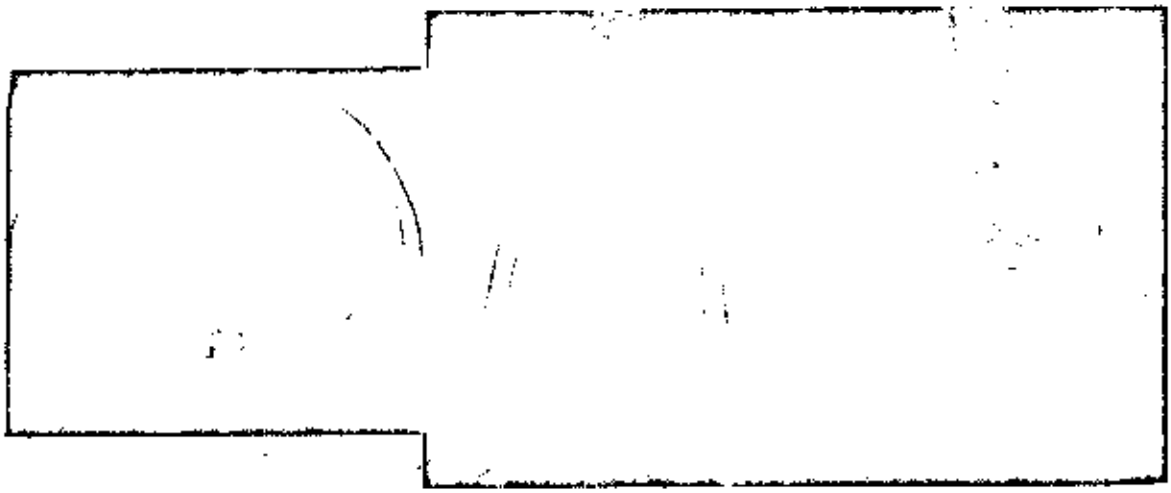
FIGURE 1. *Appearance of the Patient at Nine Months of Age.*
The physiognomy is characteristically "elfin." Note the high, prominent forehead, epicanthal folds, underdeveloped bridge of the nose and mandible and overhanging upper lip.

discharged to be followed in the Outpatient Department. The serum calcium determination 3 weeks after discharge was 11.4 mg. per 100 ml.

DISCUSSION

The syndrome of idiopathic hypercalcemia described by Fanconi et al.[11] consists of growth and mental retardation, irritability, anorexia, vomiting, muscular hypotonia, an elfin facies and generalized osteosclerosis. There is renal dysfunction, often associated with infection, and hypercalcemia, with normal serum phosphorus and normal or low serum alkaline phosphatase levels. Systemic hypertension and cardiac murmurs are common. This syndrome, as well as the milder form described by Lightwood,[12,13] is etiologically related to vitamin D administration and metabolism.[14-17]

Autopsies have not revealed that a congenital cardiac anomaly is the cause of the commonly heard cardiac murmur.[18] Black and Bonham Carter[9] reported a group of cases with the facies of hypercalcemia and the murmur of aortic stenosis. Documen-



A B C

FIGURE 2. *Roentgenograms of the Skull* (A), *Pelvis and Long Bones* (B) *and Spine* (C).
There is appreciable sclerosis of the calvaria and base of the skull, as well as diffuse osteosclerosis, with bands of increased density at the metaphyseal ends of the long bones and the epiphyseal margins of the vertebras.

TABLE 1. *Cardiac-Catheterization Data.*

Catheter Position	Blood Oxygen Saturation	Pressure
	%	mm. Hg
Aortic arch	95	140/77
Aorta just distal to aortic valve	—	154/77
Main pulmonary artery	64	29/15*
Right ventricle	57	50/2-7
Right atrium	63, 68, 68	(A wave, 4) (V wave, 1) (Mean, 2)
Superior vena cava	67	—
Inferior vena cava	67	—

*Main pulmonary-artery pressure tracing normal & thus compatible with unilateral stenosis of main branch of pulmonary artery.[16]

tation of hypercalcemia was absent in all their patients, and the cardiac lesion, supravalvular aortic stenosis, was described in only 1 patient. These authors postulated that the myxomatous thickening of the aorta found in their case and the similar pathological changes found in the aortic valve in the case reported by Rashkind et al.[10] were responsible for the cardiac murmur. The similarity between the cardiovascular abnormality seen in acquired vitamin D intoxication and idiopathic hypercalcemia has also been recognized. Excessive vitamin D administered to animals has been shown to produce changes in the wall of the aorta, coronary arteries and heart valves that range in severity from subendothelial edema to calcification.[19-22] It is interesting that the lesions on the rat and rabbit aorta seem to begin and predominate just above the aortic sinuses.

The triad of mental subnormality, unusual facial features and supravalvular aortic stenosis is now an established clinical syndrome.[1,3,5] It is probable that most, if not all, of the patients thus far described were born with supravalvular aortic stenosis.[9]

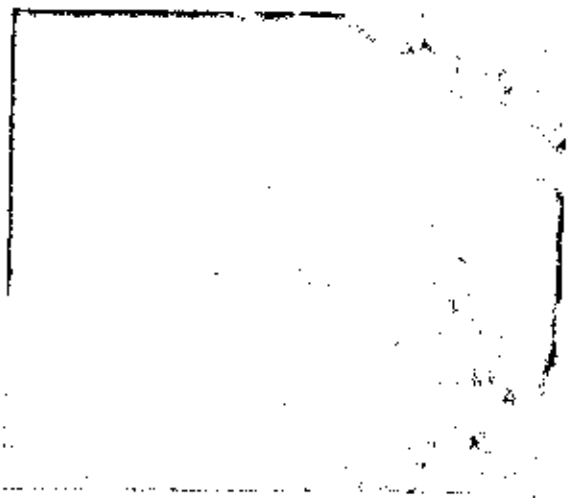


FIGURE 3. *Cineaortogram Taken at Nine Months of Age, Showing Supravalvular Aortic Stenosis (Arrow) a Short Distance above the Aortic Valve.*

Note the enlarged left coronary artery.

The findings in the patient described above suggest that idiopathic hypercalcemia is also a feature of this syndrome. It is not yet certain, however, whether the hypercalcemia is one component of a genetically determined multiple-system abnormality or whether the total syndrome stems from a derangement of vitamin D metabolism. The probability that the cardiac lesions in the present case are congenital does not preclude a metabolic disorder *in utero*. The implication is sufficient to warrant a major effort to obtain detailed studies of other patients and their families.

SUMMARY AND CONCLUSIONS

An infant with severe idiopathic hypercalcemia associated with supravalvular aortic stenosis and valvular and postvalvular pulmonary stenosis is described. To our knowledge, this represents the first proved association between the aortic lesion and idiopathic hypercalcemia. This case suggests the possibility that idiopathic hypercalcemia is part of the newly recognized syndrome of mental subnormality, a peculiar facies and supravalvular aortic stenosis. The characteristic facies seen in each of the two syndromes appears identical.

Attention is called to the similarity between the cardiovascular lesion seen in experimental vitamin D intoxication and idiopathic hypercalcemia.

Since this paper was submitted for publication complex dental malformations and peripheral pulmonary stenosis have been described in association with the syndrome of supravalvular aortic stenosis, mental retardation and peculiar facies.[24]

We are indebted to Dr. Harold E. Harrison for performing the serum calcium determinations, to Dr. Olga Baghdassarian for reviewing the roentgenograms and to Dr. Robert E. Cooke for his helpful suggestions. Mr. Walter Hooper and Mr. Glen Shue, of the United States Food and Drug Administration, Washington, D. C., kindly performed the vitamin D assay.

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USE OF PHOSPHATE AND VITAMIN D TO PREVENT DWARFISM AND RICKETS IN X-LINKED HYPOPHOSPHATEMIA

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Abstract Eight children with X-linked hypophosphatemia (three girls and five boys between three and 15 years of age) were treated for a total of 11,297 patient days with an inorganic phosphate salt supplement by mouth (1 to 4 g of inorganic phosphate per day in five divided doses given at four-hour intervals) and vitamin D_2 (10-50 × 10^3 U per day). On this regimen the mean value for serum phosphorus in the group was 4.0 mg per 100 ml, and 85 per cent of values were above 3.0 mg per 100 ml. Radiographic evidence of rickets completely resolved, and an accelerated growth rate was observed in all patients when phosphate supplements were provided continuously. Dwarfism was corrected in three female and two male subjects. The whole-blood oxygen pressure at 50 per cent oxygen saturation was low (24.5 mm Hg at pH 7.4) in untreated patients but was restored to normal during phosphate treatment. There were no serious episodes of hypercalcemia, and no ectopic calcification was found.

HYPOPHOSPHATEMIA in one of its familial forms is inherited as an X-linked dominant trait. This type of hypophosphatemia declares itself in early infancy,[3] with subsequent appearance of rickets, which may be responsive only to large and continuous doses of vitamin D, accounting for the term "familial vitamin-D-resistant rickets" often attached to the disease. Dwarfism is the third important clinical feature of this trait.[4] All aspects of the phenotype are generally more severe and uniform in male than in female patients, as expected of an X-linked trait.

Treatment of X-linked hypophosphatemia has always been a challenge. Although vitamin D alone may heal the rickets when given in very large amounts,[4,5] the effective dose is usually close to the toxic range, and episodes of hypercalcemia are a serious hazard of such treatment in this disease.[6-8] Furthermore, the dwarfism has almost never been corrected by vitamin D treatment alone. Nonetheless, it has long been assumed that a primary abnormality of vitamin-D-controlled mineral metabolism was responsible for this disease. We have re-examined this hypothesis,[9] and in the absence of any evidence that hypophosphatemia is parathyroid-dependent, and thus might be responsive to vitamin D, we have given more emphasis to a primary defect of phosphate conservation in the kidney.[10] At present there is no way to correct the renal loss of

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Abbreviations Used

IPTH: immunoreactive parathyroid hormone
P_{50}: whole-blood oxygen pressure at 50 per cent oxygen saturation
Pi: inorganic phosphate

phosphate. Therefore, we have adopted long-term, in-home phosphate replacement as the major component of a combined phosphate and vitamin D treatment regimen that will raise the concentration of inorganic phosphate (Pi) in serum almost to the normal range, will heal the rickets and, even more important, will increase linear-growth velocity, so that the dwarfism can be overcome in many of our patients. This paper reports our experience of 11,297 patient treatment days with eight children with proved X-linked hypophosphatemia. The supportive ambulatory program that makes this mode of treatment practical was previously described in detail.[11]

Material and Methods

The patients were eight subjects (three girls and five boys between three and 15 years of age) with X-linked hypophosphatemic rickets. The average age at diagnosis was 3 10/12 years (range, 8/12 to 7 10/12), and the height of all patients was below the third percentile. The criteria for diagnosis were as follows: a concentration of inorganic phosphate in serum consistently below 4 mg per 100 ml after an overnight fast; rickets appearing in infancy; no hypocalcemia; no renal loss of metabolites other than phosphate; and no male-to-male transmission of the hypophosphatemic trait in the families (there was mother-to-child transmission in all our patients). Each subject described in this report has been followed and treated for more than a year and, in one case, as long as eight years. The total period of treatment covered by the report is 11,297 patient days.

Follow-up Procedure

Each patient was visited at home regularly, to supervise medication, to draw venous blood samples and to describe the progress of therapy. Renal-clearance studies were performed every three months. The long bones, kidneys and skull were examined radiographically during half-yearly clinic visits (or more often if indicated) for signs of rickets, hyperparathyroidism, and complications of therapy. Slit-lamp examination of the eyes was performed at least once for evidence of ectopic calcification. Blood-pressure and blood pH measurements were obtained frequently.

Analytical Methods

Total calcium, Pi, alkaline phosphatase activity and creatinine were determined by Technicon Autoanalyzer methods. The amino acid and imino acid-containing oligopeptide content of urine was evaluated by two-dimensional partition and ion-exchange chromatography, and endogenous renal clearance of phosphate was measured, as previously described.[12,13] Serum immunoreactive parathyroid hormone (IPTH) was measured by Dr. Claude Arnaud with use of a radioimmunoassay[14] for which venous blood was collected in plastic syringes, transferred to plastic tubes, allowed to clot and, after removal of the cells, frozen at −20°C until shipment on dry ice. Whole-blood oxygen pressure, at pH 7.4 and 50 per cent oxygen saturation (P_{50}, in millimeters of mercury), was determined by the Severinghaus method.[15] Samples were obtained from patients during phosphate treatment, and again at the same time of day two weeks after the treatment had been stopped. The patients and their obligate heterozygous mothers, in whom P_{50} and Pi were also measured, were matched for age and sex by a paired control subject.

Measurements of linear growth were made in the standing position, the "lower segment" of total height being the distance from the top of the symphysis pubis to the heel. Measurements were plotted on copies of the charts drawn by Tanner et al.[16] and by McNair and Stickler.[17]

Bone density on standardized roentgenograms of the hands was determined by computerized photodensitometry of the bone image.[17]

Treatment Regimen

A phosphate mixture comprising dibasic sodium phosphate (136 g per volume) and phosphoric acid (NF85 per cent, 58.8 g per volume) in 1 liter of tap water (Joulie's solution) was found to be most acceptable for long-term treatment. The solution is pH 4.9 and 1725 mOsm, and provides 30.4 mg of Pi per milliliter. The mixture is given in multiples of 5 to 15 ml per dose until an average intake of 15 mg every four hours, five times daily, is achieved. The amount taken on this time schedule is that required to maintain the average phosphorus concentration above 3.0 mg per 100 ml and is equivalent to 1 to 4 g of Pi per day. Transient diarrhea may occur during the first days of this regimen, but careful explanation to parents and patient usually overcomes the inconvenience.

Vitamin D_2 (10,000 to 50,000 IU per day) was used to offset hypocalcemia during high-phosphate intake. If secondary hyperparathyroidism appeared, phosphate was discontinued temporarily, and calcium gluconate (2 to 9 g per day) was given in combination with vitamin D_2 (100,000 U per day) for two to three weeks, or until parathyroid-gland hyperactivity abated. Cycling of treatment in this manner prevented the clinical complications of hyperparathyroidism.

Results

Serum Pi

Serum Pi oscillates with intermittent dietary loading (Fig. 1). However, the four-hour Pi loading cycle maintained serum Pi above 3.0 mg per 100 ml in 8[illegible] per cent of venous-blood samples obtained from patients on this regimen. The serum Pi concentration (mean ± 1 S.D.) on 192 determinations obtained during 2725 in-home treatment days in 1970–71 was 4.0 ± 0.8 mg per 100 ml (range, 2.8 ± 0.4 to 4.9 ± 0.9). An average of 28 home visits per year

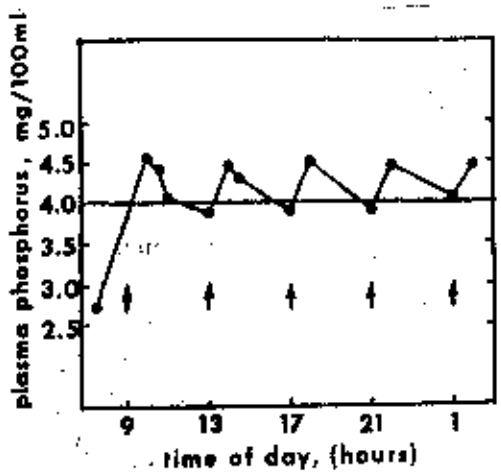

Figure 1. Effect of Repeated Loading by Mouth with a Pi Mixture on the Concentration of Pi in the Serum of a Male Patient with X-linked Hypophosphatemia.

The amount of phosphorus administered is 750 mg per dose (indicated by the arrows). The diurnal timing is shown on the abcissa.

per patient were made in support of treatment to monitor the response.

Bone

Active rickets was not found in any of our patients during regular treatment with phosphate. Coarsening of the trabecular pattern was sometimes evident, but mean radiographic density measured by photodensitometry of standardized middle phalangeal x-ray images was between the 15th and 87th percentiles, for bone density in normal subjects, matched for age.[17]

Linear Growth

The average linear-growth velocity for the patient group was below normal (63 ± 24 per cent of the normal rate on the 50th percentile) before the initiation of phosphate treatment. At the time, seven of the patients were receiving vitamin D_2 in the usual large doses for treatment of vitamin-D-resistant rickets. The linear-growth rate increased in each patient during phosphate treatment (Fig. 2). The mean value for the total patient group on phosphate treatment was double the pretreatment rate, or equivalent to 126 ± 22 per cent of the age-equivalent 50th percentile for normal children. After prolonged phosphate therapy "an accelerated" growth rate was still apparent in most of the patients (upper half of Fig. 2A and B). The dwarfism that characterized the pretreatment physique is no longer present in the three female patients in the group and in two of the male patients.

The impairment of linear growth before phosphate treatment was most obvious in the lower segment, as noted by others.[4] This is not surprising since the lower limbs are the fastest growing segment in the prepubertal period. The post-phosphate treatment acceleration of total linear growth was clearly expressed in the lower segment of our patients (Fig. 3).

Whole-Blood P_{50}

In the absence of phosphate treatment, patients with X-linked hypophosphatemia have lower values for whole-blood P_{50} and serum Pi than during treatment with phosphate (Fig. 4). The difference between their on-treatment and off-treatment P_{50} values is significant at the 0.05 level by the Wilcoxon signed-rank test.[18] The values for patients on treatment were not significantly different from the control group of age-matched healthy children. Heterozygous mothers not receiving phosphate had lower values for whole-blood P_{50} and serum Pi than the age-matched control group of women. The relation between the two groups of adult subjects parallels that for corresponding groups of children (Fig. 4). There were no significant differences between patients and controls in hemoglobin concentration, hematocrit or blood pH.

Complications of the Treatment Regimen

There were five episodes of hypercalcemia during 11,297 treatment days in patients on the combined phosphate and vitamin D regimen. None lasted for more than two weeks, and no serum calcium value exceeded 12.4 mg per 100 ml. There was no evidence of ectopic tissue calcification or of urinary calculus formation, and the glomerular filtration rate remained normal in all patients. The sodium phosphate salt supplement did not cause systemic vascular hypertension or acidemia.

There was no evidence of hyperparathyroidism in our patients before phosphate treatment.[9] However, phosphate administration will lower serum calcium,[19] and as expected, this reduction is associated with elevated serum IPTH levels in X-linked hypophosphatemia.[9] The relation is reversible with reduction of phosphate intake and with supplemental calcium nutrition (Table 1).

In the early phase of our treatment program, severe hyperparathyroidism developed in one patient and did not respond to medical management: subtotal parathyroidectomy with removal of about 7/8 of the four diffusely hyperplastic glands restored parathyroid activity to an acceptable range (serum IPTH, 14 to 52 μEq per milliliter — normal, less than 40), in the subsequent three years. Parathyroidectomy did not influence the phosphate transport defect in the patient.

A generalized hyperaminoaciduria was apparent, as expected,[12] when secondary hyperparathyroidism developed (Table 1). The hyperaminoaciduria abated with treatment to suppress hyperparathyroidism. An iminopeptiduria, which may reflect increased bone turnover,[13] was evident during reversible episodes of hyperparathyroidism.

Discussion

Until a decade ago, X-linked hypophosphatemia was usually included in a group of familial rickets classified as resistant to vitamin D,[20] even though the rickets in this disease can be healed when

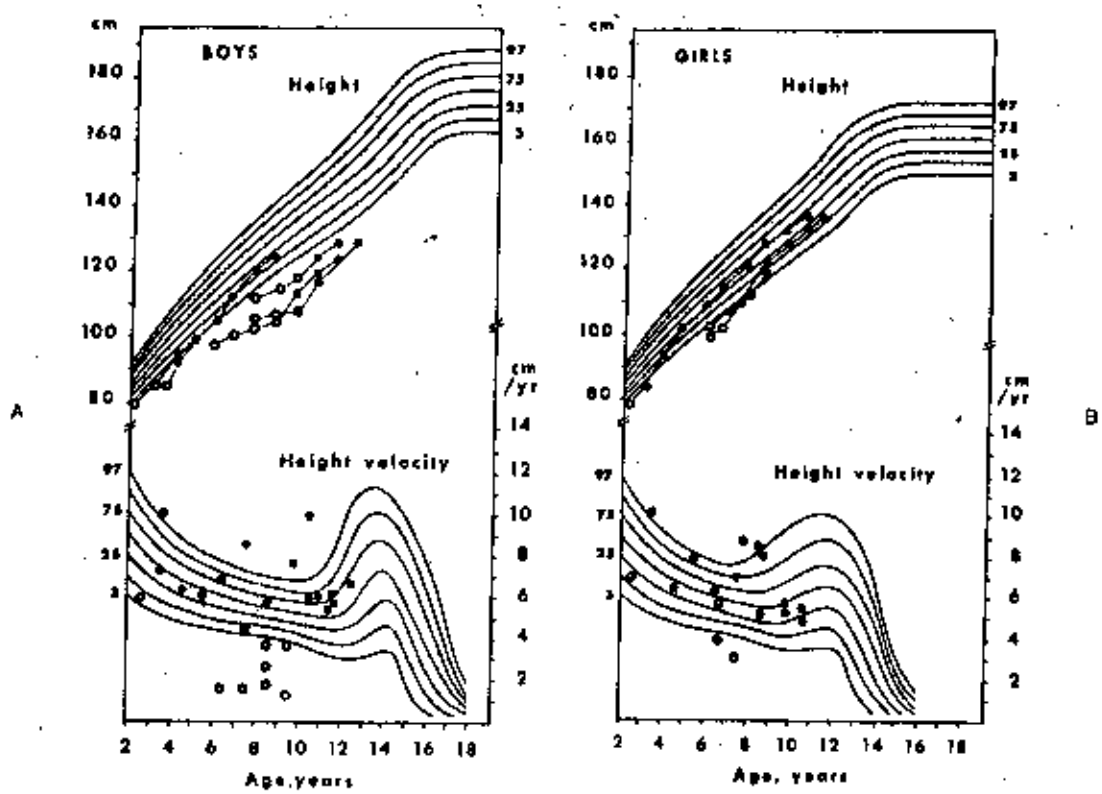

Figure 2. Growth Charts for Boys (A) and Girls (B) before (Open Circles) and during (Closed Circles) Phosphate Supplementation.

Vitamin D alone was the major form of treatment given before the combined regimen was begun. The upper graphs show growth as a linear measurement related to age. The lower graphs indicate the rate of growth (gain in height per year). Graphs show percentiles for a normal reference population as described by Tanner et al.[18]

sufficent doses of vitamin D are administered carefully.[4,5] The X-linked trait has also been classified as one of the hypophosphatemic diseases resulting from renal tubular dysfunction.[5,21-23] Several interpretations of the basic defect in phosphate metabolism have been advanced. A primary disorder of intestinal absorption of calcium was first suggested by Albright,[20,24] and more recently others have favored an abnormality of vitamin D metabolism.[5] Any hypothesis that proposes that vitamin-D-dependent calcium transport is compromised initially in X-linked hypophosphatemia should include secondary hyperparathyroidism as the cause for the renal loss of phosphate. We now know that serum levels of immunoreactive parathyroid hormone are normal in untreated patients with X-linked hypophosphatemia.[9] Moreover, hypophosphatemic patients can synthesize 25-hydroxycholecalciferol, the major polar metabolite of vitamin D,[20] and treatment with this form of the vitamin does not correct the abnormal phenotype.[27,28]

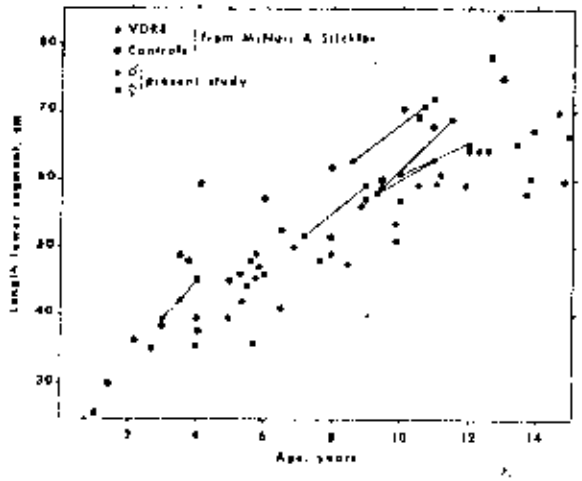

Figure 3. Growth in the Lower Segment of Patients with Vitamin-D-Resistant Rickets (VDRR) Treated with Phosphate and Vitamin D as Compared with Lower-Segment Growth of Patients Treated with Vitamin D Alone, as Reported by McNair and Stickler.[1]

There are at least two components of phosphate transport in human kidney, one of which is deficient in the X-linked disease,[10] and although the residual transport functions at its maximum capacity, it is not sufficient to sustain the endogenous phosphate pool. Sustained calcium infusion improves tubular transport of phosphate in X-linked hypophosphatemia,[29,30] and current evidence[10] indicates that it is the residual component of phosphate transport that is sensitive to calcium. We have shown

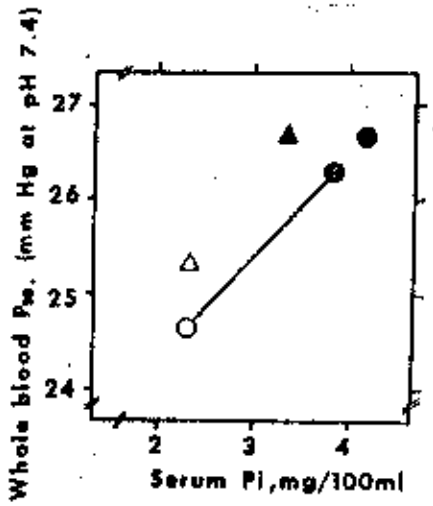

Figure 4. Relation between Serum Phosphorus (Pi) and Whole-Blood P_{50} Value in Eight Untreated Adult Female Heterozygotes (Δ) and Eight Untreated Children (O) with X-Linked Hypophosphatemia.

Controls for adults (▲) and children (●) were individually matched for age and sex. Phosphate treatment of children with the disease (⊘) raises the serum Pi and whole-blood P_{50}.

that the residual component of phosphate transport is not influenced by parathyroid hormone,[10] and we believe it may be equivalent to the parathyroid-independent, calcium-sensitive phosphate transport described earlier for mammalian kidney.[31] The therapeutic benefit of massive doses of vitamin D with associated hypercalcemia may derive in part from this calcium effect on phosphate conservation.

It has also been suggested[32] that a metabolite of vitamin D may be directly involved in proximal tubular transport of phosphate and that its synthesis is regulated by the ambient calcium level. There is also new evidence[33] that 25-hydroxylated vitamin D, or a derivative, enhances tubular reabsorption of phosphate by direct action on the kidney. Thus, a partial defect in the biosynthesis of the proposed phosphate transporting substance might explain vitamin D resistance and the deficit in tubular transport of phosphate in X-linked hypophosphatemia. However, until that substance can be identified, isolated and shown to be of therapeutic value, it is necessary to look elsewhere for an effective mode of treatment.

Lilly, Peirce and Grant observed bone healing induced by dietary phosphate in experimental rickets[34]; these authors also reviewed the literature as far back as 1872 reporting that elemental phosphorus increases bone formation in growing animals. The bone lesions of vitamin D deficiency, and various forms of vitamin-D-resistant rickets, can be healed by short-term phosphate supplements.[35-37] Long-term phosphate supplementation should therefore be a logical form of treatment for a condition in which there is specific chronic renal loss of phosphate amounting to 2 to 3 g per day in mutant hemizygotes. However, phosphate treatment yielded negative results in some studies,[38,39] probably because the administration of phosphate was inadequate. When the phosphate dose is sufficient, serum phosphorus is increased, and long standing vitamin-D-resistant rickets will heal.[2,5,40-44] Despite these reports, phosphate is still more often used as an adjunct to vitamin D therapy to avoid vitamin D intoxication than as a primary component of therapy.

Albright proposed[45] that hypophosphatemia stimulates bone resorption and that phosphate replacement merely prevents this event, but Rasmussen[46] challenged this interpretation on the grounds that Albright's data show stimulation of bone mineralization by phosphate replacement. Recent studies in the rat with severe hypophosphatemia[47] demonstrate that inhibition of bone mineralization and matrix formation is reversed by phosphate replacement. Similar mechanisms may account for improved bone mineralization with phosphate treatment in X-linked hypophosphatemia.

Phosphate loading causes serum calcium to fall and serum immunoreactive parathyroid hormone to rise.[29] We have observed a similar response in our patients. The appearances of a characteristic reversible hyperaminoaciduria[12] and aminopeptiduria[13] in association with elevated serum parathyroid hormone in the patients treated with phosphate indicates that kidney and bone are apparently both responsive to parathyroid hormone in X-linked hypophosphatemia. This effect on bone may actually benefit its turnover, remodeling and linear growth during phosphate treatment.

Stimulation of the linear-growth rate is an important finding in our study. With the exception of one patient,[48] we are not aware of any publication describing a similar response in patients treated primarily with vitamin D. The only other patients reported to have an accelerated growth response to treatment were treated with phosphate.[5,42,49] Harrison et al.[42] were the first to report a positive correlation between serum Pi and growth rate. We ob-

Table 1. Effect of Phosphate Treatment on Parathyroid Activity* in X-Linked Hypophosphatemia.

Case No.	High Phosphate Regimen†				Moderate Phosphate Regimen‡			
	Serum Pi	Serum Calcium	Serum IPTH	Amino Acids in Urine§	Serum Pi	Serum Calcium	Serum IPTH	Amino Acids in Urine§
	mg/100 ml		μEq/ml		mg/100 ml		μEq/ml	
1	5.4	8.9	290	General increase	3.9	10.4	40	Normal
2	5.0	9.3	100	General increase	3.8	11.4	67	Normal
3	4.4	7.4	750	General increase	3.9	10.6	43	Hyperglycinuria
4	3.4	9.4	90	Hyperglycinuria	3.4	10.0	36	Normal
Average	4.5	8.7	307	Increased	3.7	10.6	46.5	Normal

*Normal serum values: Pi, >4.0 mg, & calcium, 9-11 mg/100 ml; IPTH, <60 μ Eq/ml.
†Pi supplement, 3-4 g/day; vitamin D, <50,000 U/day.
‡Pi supplement, 1-3 g/day; vitamin D, 100,000 U/day for 3 wk or more.
§Aminoaciduria assessed by partition chromatography.[14]

served that chronic hypophosphatemia in our patients was associated with a low whole-blood P_{50}, indicating a shift to the left of the oxygen dissociation curve. If depletion of serum inorganic phosphate inhibits 2,3-diphosphoglycerate synthesis in red cells,[30-31] thus causing a fall in whole-blood P_{50}, release of oxygen to tissues may be inhibited sufficiently over long periods to impair growth in patients with the X-linked trait. This hypothesis has yet to be tested. The growth failure in hypophosphatemic rickets is clearly not the result of growth-hormone deficiency,[32] and it cannot be the direct result of rickets[33] since the bone disease can be healed with vitamin D while growth remains impaired.

The rise and fall of the Pi concentration in serum occurs in a four-hour cycle after phosphate loading by mouth (Fig. 1). Consequently, phosphate must be given frequently to maintain a near-normal serum Pi level. Several of our patients endured "failed-phosphate" treatment before enrollment in the current program, probably because they could not maintain the onerous dosage schedule. These patients responded satisfactorily after aggressive monitoring and supervision was initiated in our program.

There were few episodes of toxicity in the period of observation (11,297 patient-treatment days). Vitamin D alone, as a major means of treatment, is often accompanied by hypercalcemia and nephrotoxicity.[8,8] Phosphate supplementation can reduce these hazards,[34] and our program kept the total duration of the hypercalcemic episodes to less than 0.5 per cent of patient-treatment days. In no case was the hypercalcemia of serious magnitude, and we found no evidence of ectopic calcification or renal damage in our patients.

Phosphate supplementation has other real or potential inconveniences. It may cause transient diarrhea, and the taste of the mixture is unpleasant, although the acidic solution is preferable to the "neutral" phosphate salt mixture. The sodium intake is high in the phosphate mixture (up to 100 mEq per day), and the mixture also provides an acid load. Nevertheless, we have not detected hypertension or acidemia in any of our patients over the years; diarrhea has not been a problem, and some ingenious manipulations in the pharmacy and at home have improved the taste of treatment. We believe treatment with phosphate and vitamin D provides a useful way to neutralize the clinical effects of the mutation in X-linked hypophosphatemia, until a more basic approach is discovered to correct the defect in phosphate transport.

Note added in proof: Since this paper was submitted for publication, two reports have updated two of our citations. Balsan[35] amplified her earlier report[25] showing that a defect in the synthesis of 25-hydroxycalciferol is probably not the primary defect in X-linked hypophosphatemic rickets. West and his group[36] added to their earlier report[11] giving some indication that combined phosphate and vitamin D treatment had a positive effect on the growth rate of their patients.

We are indebted to Mrs. Carol Chow, Miss Joanna Bates and Mr. Peter Lamm for support and interest in these studies, to Drs. Donald Fraser, Gunnar Stickler and Claude Arnaud for numerous discussions over many years and to Mr. Charles Colbert, who performed the densitometry of bone x-rays.

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NATURE AND ETIOLOGY OF THE POLAND ANOMALY

TIMOTHY J. DAVID, M.B., CH.B.

Abstract The Poland anomaly consists of congenital unilateral absence of the sternocostal part of the pectoralis major muscle, with ipsilateral syndactyly. Dermatoglyphic evidence confirms the unilaterality of the Poland anomaly.

Of 10 patients with this anomaly five were adopted. The mothers of the other five had probably tried to obtain an abortion in early pregnancy, with four of these five living in the same city suburb. Thus, at least in these cases, there is a prevalence of potentially noxious social and physical ante-natal influence.

THE essential features of the Poland anomaly are unilateral absence of the sternocostal head of the pectoralis major muscle and ipsilateral syndactyly. Recognized complications include short fingers, absence of digits, rib and costal-cartilage defects, herniation of the lung, absence or hypoplasia of the nipple or breast and genitourinary anomalies.[1] The aim of this study was to identify the nature of both the defect and the possible etiologies in the Poland anomaly. The family and ante-natal histories were studied. Palmar dermatoglyphics were examined to see if there was any evidence of minor effects on the clinically normal hand.

PATIENTS AND METHODS

The first three patients with the Poland anomaly were obtained through review of hospital records. The other seven patients with the Poland anomaly were referred by other physicians.

Histories were obtained from the patients, from their general practitioners and from the parents. Ante-natal records and radiographs were examined when possible. Dermatoglyphics were recorded with black fingerprint ink.

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Gray

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Vitamin D Metabolism: The Role of Kidney Tissue[2]

Abstract. *The appearance of a polar metabolite of 25-hydroxycholecalciferol has been studied in the intestinal mucosas of nephrectomized rats and rats which have been made uremic by ureter ligation. In confirmation of previous work by Fraser and Kodicek, it was found that nephrectomy prevents the appearance of this metabolite in the intestine. On the other hand, equivalent amounts of the metabolite were found in uremic rats and in sham-operated controls, showing that the production of this metabolite is independent of the uremic state of the animals. In addition, it was shown that the metabolite produced in vitro by kidney homogenates is identical to that found in vivo in the intestine.*

Impaired renal function has for several years been known to be associated with alterations in vitamin D and calcium metabolism. Kessner and Epstein (*1*) showed the active transport of calcium by everted duodenal sacs to be reduced in the chronically uremic rat, and Avioli *et al.* (*2*) have in addition demonstrated decreased intestinal calcium binding protein activity in such animals. These situations are not reversed by physiological amounts of vitamin D, though they may be partially reversed by 25-hydroxycholecalciferol (25-HCC), the "circulating" or "hormonal" form of vitamin D (*3, 4*). There is, however, no evidence that the hepatic synthesis of 25-HCC from vitamin D is impaired in uremia.

Recently, Fraser and Kodicek (*5*) have shifted attention from the role of renal function to the role of renal tissue in vitamin D metabolism by their report that the conversion of 25-HCC to a more polar metabolite takes place exclusively in the kidney. This metabolite (designated "peak V" in this laboratory) has been shown to act even more rapidly than 25-HCC in the stimulation of calcium absorption and may very well be the active form of the vitamin in the intestine (*6*). The evidence for the role of kidney tissue in the production of this metabolite is based principally on the observation that the normal appearance of this metabolite in the intestinal mucosa after administration of vitamin D is not seen in nephrectomized rats (*5*). Furthermore, Fraser and Kodicek reported kidney to be the only tissue capable of producing this metabolite in vitro (*5*).

The present study was undertaken with two objectives in mind. The first was to determine whether the absence of the peak V metabolite in the intestine of nephrectomized rats is due to toxic effects of the uremia produced by nephrectomy or whether kidney tissue is required for the further metabolism of 25-HCC. The second objective was to determine whether the peak V produced in vitro by kidney homogenates is identical to that observed in the intestine after in vivo administration of 25-HCC or vitamin D_3.

Male weanling Holtzman rats were fed a vitamin D–deficient diet for 5 to 6 weeks prior to being used for experiments (*7*). The [26,27-^{3}H]-25-HCC used in these experiments was prepared in this laboratory and had a specific activity of 1.3 c/mmole (*8*). Groups of three rats were either bilaterally nephrectomized or sham-operated and were injected intrajugularly with 125 ng of ^{3}H-25-HCC in 95 percent ethanol immediately after surgery. A third group of rats was ureter-ligated 6 hours before being injected with 125 ng of ^{3}H-25-HCC, the 6-hour delay being to ensure a degree of uremia similar to that of the nephrectomized animals at the time of killing. The rats in all groups were killed 12 hours after the injection of ^{3}H-25-HCC, having also been fasted 16 hours prior to killing. The intestinal mucosas from the rats in each group were pooled, homogenized in 0.9 percent saline, and extracted with $CHCl_3$-methanol as previously described (*4*). Identification of ^{3}H metabolites was

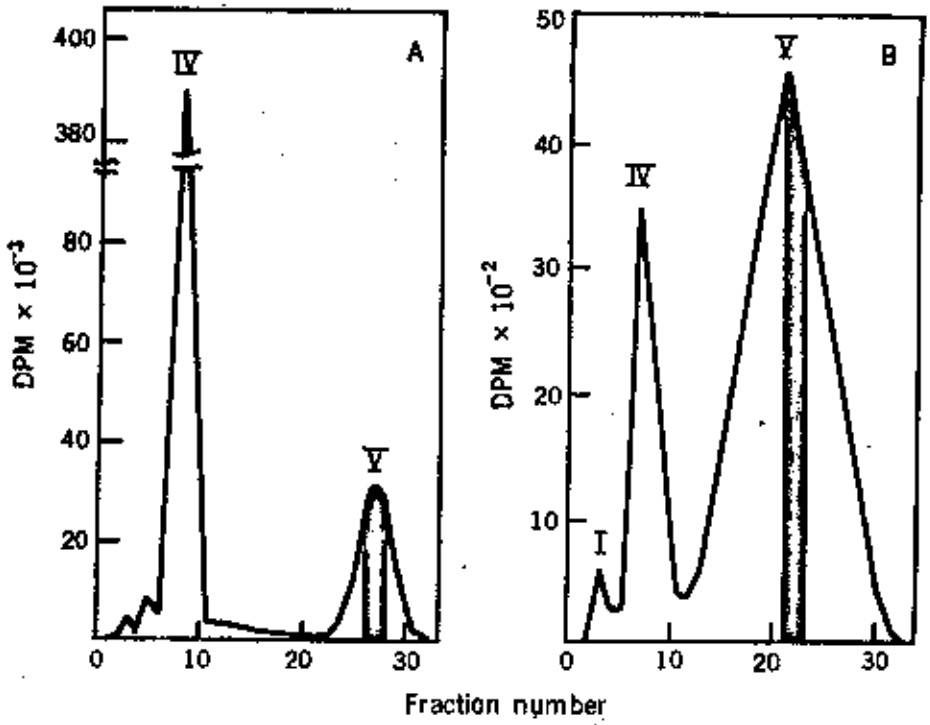

Fig. 1. (A) Sephadex LH-20 chromatography of the combined lipid extracts from six in vitro incubation mixtures. Incubation was for 2 hours at 37°C. A column containing 2 g of Sephadex LH-20 was used and 5.3-ml fractions were collected. The fractions in the shaded area were pooled and taken up in a known volume of solvent. (B) Sephadex LH-20 chromatography of the lipid extract of the mucosal scrapings from six vitamin D–deficient chicks each given 125 ng of [26,27-^{3}H]-25-HCC 12 hours prior to killing. A column containing 10 g of Sephadex LH-20 was used and 5.5-ml fractions were collected. The fractions in the shaded area were pooled and taken up in a known volume of solvent.

accomplished by Sephadex LH-20 chromatography (9). A 30 by 2 cm column containing 10 g of Sephadex LH-20 was used, and 5-ml fractions were collected. The samples were applied to the column in less than 1 ml of 65 percent chloroform in Skellysolve B (by volume) and were eluted with 175 ml of the same solvent. The column was stripped with 100 ml of 70 percent chloroform in Skellysolve B. Radioactivity in the chromatographic fractions was determined by liquid scintillation counting using a Packard Tri-Carb spectrometer No. 3375 equipped with external standardization. In vitro conversion of 25-HCC to the peak V metabolite was accomplished by using a 25 percent homogenate of kidney tissue from chicks fed a vitamin D–deficient diet for 4 to 5 weeks. Incubation conditions were the same as those used by Fraser and Kodicek except that 25 ng of [26,27-^{3}H]-25-HCC was used as substrate (5). Also, identification of the peak V metabolite was determined by chromatography on Sephadex LH-20 as described above.

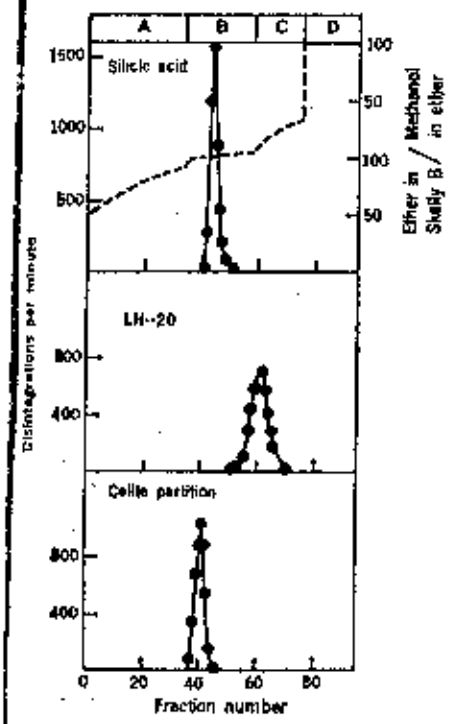

Fig. 2. Cochromatography of combined aliquots of the metabolites isolated in Fig. 1. Silicic acid chromatography was done as described by Suda *et al.*, using a multibore column containing 14 g of silicic acid with 11.5-ml fractions being collected (*11*). Total recovery was 90 percent. Sephadex LH-20 chromatography was done (*12*) using a 60- by 1-cm column containing 20 g of Sephadex LH-20 with 3.2-ml fractions being collected. Total recovery was 100 percent. Celite partition chromatography was done as described by Suda *et al.* with 90 percent methanol, 10 percent water as the stationary phase and 30 percent chloroform, 70 percent Skellysolve B as the mobile phase, and 5.3-ml fractions were collected (*11*). Total recovery was 87 percent.

The percentage distribution of ^{3}H metabolites in the lipid extracts of tissues from vitamin D–deficient rats subjected to various treatments is seen in Table 1. In the case of both intestinal mucosa and blood plasma, the nephrectomized rats failed to show any of the peak V metabolite, while equivalent amounts of this metabolite were seen in both the sham-operated controls and the ureter-ligated rats. On the other hand, the ureter-ligated rats had a blood urea nitrogen value similar to that for the nephrectomized rats, indicating that the state of uremia in these two groups of animals is about the same. From this data, we can conclude that the uremic state of the animals has little effect on the appearance of the peak V metabolite in the intestine and that the presence of kidney tissue is required for 25-HCC metabolism to peak V to occur.

In order to establish the identity of the peak V metabolite produced in vitro by kidney homogenates with the metabolite observed in the intestine in vivo after administration of ^{3}H-25-HCC, it was first necessary to isolate metabolites from these two sources. The in vitro metabolite was isolated by combining the lipid extracts from six of the incubation mixtures described above. The combined lipid extracts were chromatographed on Sephadex LH-20 and the metabolite was isolated as shown in Fig. 1A. The in vivo metabolite was isolated by injection of six vitamin D–deficient chicks with 125 ng each of ^{3}H-25-HCC 12 hours prior to killing, and isolating the metabolite from the lipid extract of the mucosal homogenate by Sephadex LH-20 chromatography as shown in Fig. 1B. The smaller elution volume obtained for this metabolite is due to the fact that this column contained 10 g of Sephadex, while the other contained about 12 g. Also, the large amount of lipid observed in the in vivo sample could have caused some peak broadening. Aliquots of each of the metabolites isolated in Fig. 1 were combined and chromatographed in three different chromatographic systems as shown in Fig. 2. It can be seen that in each case virtually all of the recovered radioactivity can be found in

Table 1. Effect of nephrectomy and ureter ligation on peak V accumulation in rat tissues. The rats were killed 12 hours after the intravenous administration of 125 ng of [26,27-^{3}H]-25-HCC. Urea nitrogen determinations were done on an aliquot of the pooled plasma from the rats in each group by the method of Marsh *et al.* (*10*) using a Technicon AutoAnalyzer.

Metabolite	Nephrectomized rats	Sham-operated rats	Ureter-ligated rats
*Small intestine mucosa**			
25-HCC ester	7.7	11.9	6.7
25-HCC	92.3	25.7	33.7
Peak V	0	62.4	59.6
*Blood plasma**			
25-HCC	100	89.9	91.6
Peak V	0	10.1	8.4
Blood urea nitrogen†			
	129	21	141

* These values are percentages of the recovered radioactivity, the recoveries usually being about 90 percent. † These values are milligrams per 100 milliliters.

a single, symmetrical peak. These results provide further evidence that the metabolite produced in vitro by kidney homogenates is, in fact, identical to that found in intestinal mucosa after in vivo administration of ^{3}H-25-HCC or vitamin D_3.

The present results clearly confirm the observation of Fraser and Kodicek (5) that kidney tissue is necessary for the conversion of 25-HCC to a more polar metabolite. It has also been shown that the acute elevation of blood urea nitrogen caused by nephrectomy does not by itself impair this conversion in the short term. We have furthermore provided convincing evidence that the metabolite produced in vitro by kidney homogenates is identical to that found in the intestine in vivo after administration of 25-HCC.

These findings and those of Fraser and Kodicek open the possibility that in azotemic chronic renal disease there is a failure to produce the intestinal metabolically active form of vitamin D (peak V), thus resulting in impaired calcium absorption.

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Parathyroid-dependent phosphaturia and aminoaciduria in the vitamin D-deficient rat[1]

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GROSE, JOHN H., AND CHARLES R. SCRIVER. *Parathyroid-dependent phosphaturia and aminoaciduria in the vitamin D-deficient rat.* Am. J. Physiol. 214(2): 370–377. 1968.—Long-Evans and Holtzman rats, deprived of dietary calcium and/or vitamin D, developed hypocalcemia. Hyperphosphaturia and generalized hyperaminoaciduria both appeared when the plasma calcium level fell below 6.5 mg/100 ml. The appearance of hyperphosphaturia preceded the hyperaminoaciduria by 2–4 weeks. The excretory abnormalities were attributed to renal tubular dysfunction. Calcium injection, causing temporary elevation of plasma calcium above 6.5 mg/100 ml, transiently suppressed hyperexcretion of phosphorus and amino acids within 6 hr of injection. Parathyroid extract enhanced the hyperexcretion even when also capable of raising the plasma calcium. Hyperexcretion of phosphorus and amino acids was suppressed within 6 hr after parathyroidectomy; the most hyperplastic parathyroid glands were found in the animals with the most severe hypocalcemia, and the greatest excretory abnormality.

endocrine regulation; kidney; transport; rickets; hypocalcemia; calcium

VITAMIN D DEFICIENCY in man is accompanied by impaired renal tubular absorption of phosphorus (3, 4), amino acids (4, 10, 21), and bicarbonate (27). Experimental vitamin D deficiency in the dog (16, 17) and the rat (1, 20) is also associated with hyperphosphaturia and acquired renal tubular dysfunction. Rather unexpectedly one can find little evidence for serious investigation of hyperaminoaciduria in vitamin D-deficient animals, with the exception of those performed in the rat by Engstrom and co-workers (10), and by Chisholm and Harrison (4), which did not demonstrate hyperaminoaciduria. Some degree of endogenous hyperparathyroidism would appear to accompany the tubular dysfunction affecting phosphorus absorption by the D-deficient animal (1, 2, 6, 16, 17, 20); a similar situation is believed to pertain for the complex tubular disorder of human vitamin D deficiency (3, 4, 21).

In the present study some of the effects of vitamin D deficiency on renal tubular function were evaluated in two strains of rat by traditional techniques to produce the deficiency. Hyperaminoaciduria was documented in both strains; the hyperaminoaciduria appeared later than the hyperphosphaturia. Impairment of renal absorption accounted for the changes in excretion of phosphorus and amino acids. The stimulus for tubular decompensation was severe hypocalcemia; vitamin D depletion, per se, was not necessary to provoke the excretory abnormality. Evidence was obtained indicating that endogenous hyperparathyroidism accounts for the impairment of tubular transport of phosphorus and amino acids in vitamin D deficiency.

METHODS

Long-Evans hooded rats (Venning stock) of both sexes were used in a preliminary study (*A*); weanling, male Holtzman rats were used in *study B*. The animals were housed in overhanging wire cages in an artificially illuminated room, protected from ultraviolet light, and maintained at constant temperature and humidity. All animals had unlimited access to the prepared diets and tap water. Both studies were conducted over a 12-week period.

Diets

Study A. The effect of feeding synthetic diets of varying calcium, phosphorus, and vitamin D contents, upon phosphorus and amino acid excretion was studied first in the Long-Evans rat. Three-month-old rats of both sexes were obtained[3] and mated. Pregnant female animals were then fed the "Rockland Vitamin D-free" diet[4] throughout pregnancy. Litters produced by these mothers were weaned and divided randomly into six groups of 14

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animals each; four diets were used to feed the six groups. Purina rat chow was fed to one group of animals. Rockland D-free pellets (calcium, 1.45%; phosphorus, 0.92%) were fed to a second group of animals. A low-calcium or a low-phosphorus powdered, synthetic diet (26) (Table I) was used in the third and fourth groups; the remaining two groups were fed one or other synthetic diet, to which vitamin D_2 (3 IU/gm diet) in propylene glycol was added daily.

Study B. Sixty male albino Holtzman rats were divided into four groups at weaning and fed pelleted synthetic diets (Table I), calculated to produce various forms of the low-calcium, D-deficiency syndrome, in the manner described by Steenbock and Herting (26). The vitamin D-supplemented animals were given 75 units of vitamin D_2 in 0.5 ml propylene glycol by gavage every 3 days. Low corn starch (5% w/w) and high dextrose (65% w/w) content were used in these diets to allow pelletizing. Protein was supplied as yeast (4% w/w), and fibrin (15% w/w); corn oil (114 ml/1,500 g diet) supplied fat and 100 units vitamin A.

Procedures

Urine collections were obtained at fortnightly intervals. Six to eight rats from each diet group were weighed and transferred in the morning into metabolic cages; 6-hr pooled urine samples, free of feces, were collected. The animals had access to water only during the collection period. Immediately after each collection, the pooled urine specimens were acidified (pH 4) and stored at −20 C until analysis. Individual or pooled heparinized blood samples were collected from the warmed tail vein or from the retroorbital venous plexus. The plasma was immediately separated from the cells and stored at −20 C until analysis.

Parathyroidectomy was performed under ether anesthesia, and with the aid of a dissecting microscope, by electrocautery in *study A*, and by excision in *study B*. Injections of calcium gluconate were given intraperitoneally; parathyroid extract (Lilly) was injected subcutaneously in the scapular region.

TABLE I. *Mineral content of experimental diets*

	Study A*		Study B†	
	Diet 1, Low-P	Diet 2, Low-Ca	Diet 1, N-Ca	Diet 2, L-Ca
Calcium content (%, w/w)				
Calculated	1.20	0.02	0.40	0.02
Analyzed			0.45	0.03
Phosphorus content (%, w/w)				
Calculated	0.02	0.30	0.30	0.30
Analyzed			0.37	0.30

Abbreviations: Low-P, low phosphorus; Low-Ca, low calcium; N-Ca, normal calcium; L-Ca, low calcium. * Diets prepared by authors and fed in powdered form. † Prepared by Nutritional Biochemical Corp. to authors' specifications, and fed in pellet form.

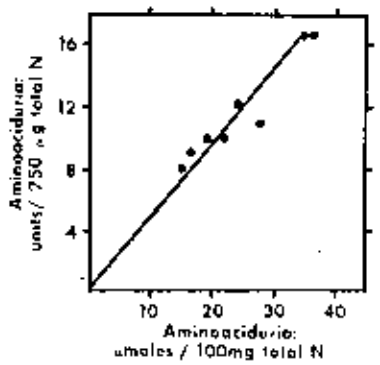

FIG. 1. Visual assessment of aminoaciduria displayed by partition chromatography (*ordinate*), plotted against results obtained on the same specimen by quantitative analysis using elution chromatography on ion exchange resin (*abscissa*).

Analytical Techniques

Aminoaciduria was screened by two-dimensional partition chromatography (7) on 10-inch-square Whatman no. 4 filter paper, to which aliquots of urine equivalent to 250 μg total nitrogen (Kjeldahl method) were applied. The chromatograms were developed first in ascending water-saturated phenol in an ammonia atmosphere, and then dried at 40 C overnight. They were then developed in a second dimension in ascending 2,6-lutidine:H_2O (2:2:1). After drying, they were stained with a ninhydrin-isatin mixture (22), heated at 80 C for 10 min, and inspected by transmitted light on a viewing screen. The intensity of each spot was scored in arbitrary units independently by two observers. The degree of aminoaciduria has been expressed as the sum of the "units" per 250 μg of total nitrogen. This method of assessment was sufficiently reliable for semiquantitative determination within the range of aminoaciduria encountered in the study (Fig. 1). Free amino acids in the appropriate pooled urine samples were subsequently analyzed by elution chromatography on spherical ion-exchange resins with a Spinco 120 amino acid analyzer according to the methods of Spackman, Stein, and Moore (25). Pooled plasma from several animals in each group was deproteinized with 1% picric acid (plasma:picric acid, 1:5) (24) and the amino acid content analyzed by elution chromatography.

Calcium was measured in plasma by the fluorometric method of Kepner and Hercules (14) as modified by Turner Associates. The titrimetric method of Clark and Collip (5) was used to confirm the calcium content of the diets. A micromodification of the molybdate method (9) was used for phosphorus determination. Statistical analyses were made according to standard statistical procedures (23).

TABLE 2. *Long-Evans (hooded) rats: comparison of response to dietary regimes at 12th week*

Regime	No. of animals	Avg Wt., g	Plasma: Calcium, mg/100 ml	Plasma: Phosphorus, mg/100 ml	Urine: Phosphorus, mg/24 hr per 100 g wt	Urine: Amino acids, U/24 hr per total nitrogen	Rickets
Purina rat chow	8	980	9.8	9.6	547	5	—
Rockland diet	8	570	10.1	9.0	770	5	—
Low P + D (high calcium)	6	200	11.3	5.2	14.1	8	—
Low P − D (high calcium)	6	178	10.5	4.8	15.0	8	++
Low Ca + D	6	185	7.1	9.2	682	8	—
Low Ca − D	8	155	3.6	6.8	900	12	+

TABLE 3. *Effect of parathyroidectomy at 12th week on phosphaturia and aminoaciduria in Long-Evans (hooded) rats*

	Control, N = 8, Purina Chow		Test, N = 8, Calcium- D Deprived	
	Pre PTX	Post PTX 6–12 hr	Pre-PTX	Post PTX 6–12 hr
Urinary phosphorus, μg/6 hr per 100 g	547	524	723	530
Aminoaciduria, μmoles/100 mg total nitrogen	16.9	16.8	33.4	23.7

Determinations performed on pooled urines.

RESULTS

Preliminary Study A

Long-Evans rats developed a progressive increase in phosphaturia and aminoaciduria when fed the low-calcium, D-deficient diet. Increased free amino acid excretion first became apparent in these animals at the 6th week of the study when the plasma calcium fell to 5.6 mg/100 ml. Data on plasma and urinary changes in the various groups of animals at the 12th and terminal week of the study are given in Table 2. Animals which were not severely hypocalcemic, did not develop hyperaminoaciduria. The presence of rickets was not an explanation for the occurrence of hyperaminoaciduria; animals fed a high-calcium, low-phosphorus, D-deficient diet had florid rickets, but did not have hyperexcretion of free amino acids.

Phosphaturia could not be compared between groups of Long-Evans rats because of the different phosphorus intakes. Intergroup differences in dietary composition probably also account for the difference in aminoaciduria noted between the animals fed on Purina chow and Rockland diets, and the animals on the powdered diets. The impact of vitamin D deprivation offered by the Rockland diet can be questioned since these animals grew well and had no hypocalcemia.

Parathyroidectomy by electrocautery was performed during the 12th week on control animals (Purina chow) and on animals fed the low-calcium, D-deprived diet. In the latter group, urinary excretion of phosphorus and amino acids decreased sharply in the postoperative period (Table 3), despite a further decline in the plasma calcium concentration. Five of the eight animals were dead 48 hr after the operation. Animals in the control group rendered hypocalcemic by parathyroidectomy showed no trend in urinary excretion in response to this procedure. The parathyroid glands were visibly enlarged in the hypocalcemic group of animals.

Study B

Growth. Holtzman rats grew poorly on the low-calcium and/or D-deficient regimens (Table 4). Growth impairment was not solely attributable to malnutrition since animals pair-fed the control diet (NCa + D) in parallel with the low-calcium, D-deficient group, grew better than the latter group.

Plasma calcium and phosphorus data. Animals with vitamin D deprivation and calcium deprivation alone or combined, developed progressive hypocalcemia by the 2nd week of the study; the double deprivation provoked the greatest fall in plasma calcium (Fig. 2, Table 4). From the 6th week of the study, hypocalcemia produced by calcium deprivation alone (Table 4) was more severe than that produced by vitamin D deprivation alone.

Plasma phosphorus concentration fell slightly in all animals with increasing age (Fig. 2, Table 4). However, plasma phosphorus fell to lower values ($P = 0.05$) in the group with the most severe hypocalcemia (LCa − D) as compared with age-matched control (NCa + D) animals.

Urinary phosphorus data. Phosphorus excretion was greater in two groups of hypocalcemic animals (LCa + D and LCa − D) compared to the control (NCa + D) animals at all times, from and including the 2nd week of the study (Figs. 2 and 3; Table 4); less striking, but similar changes were seen in the NCa − D animals from the 4th week. Doubly deprived animals had the greatest phosphaturia despite their relatively low plasma phosphorus values, implying an elevated renal clearance of phosphorus. Animals pair-fed the control (NCa + D) diet in parallel with the doubly deprived (LCa − D) animals (Table 4) handled calcium and phosphorus normally in spite of emaciation. Animals made hypocalcemic by the low-calcium diet alone had greater phosphaturia than animals in which hypocalcemia was produced by simple D deprivation (Figs. 2 and 3; Table 4).

Aminoaciduria. Free aminoaciduria increased with age in control animals (Fig. 2). However, in the calcium-deprived and doubly deprived animals, free aminoaciduria was greater than in the age-matched control (NCa + D) group; this occurred particularly when plasma calcium fell and remained below 6.5 mg/100 ml (Figs. 2 and 3; Table 4). The pair-fed animals, serving as

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TABLE 4. *Comparison of plasma and urine data at two intervals during study with Holtzman rats*

Dietary Group	Time, weeks	Avg Wt, g	Plasma Calcium, mg/100 ml, mean ± SD	Plasma Phosphorus, mg/100 ml, mean ± SD	Urine Phosphorus, mg/6 hr per 100 g wt	Aminoaciduria, U/250 μg Total Nitrogen	Rickets
NCa + D, N = 6	0	55	11.6±0.2	11.4±0.2	0.09	6	—
	6	149	10.2±0.2	11.1±0.6	0.15	8	—
	12	272	11.1±0.3	10.2±0.3	0.12	10	—
NCa − D, N = 6	6	130	6.3±0.3[a]	12.1±0.9	0.45	6	—
	12	236	6.8±0.4[a]	10.1±0.1	0.30	9	—
LCa + D, N = 6	6	102	6.2±0.5[a]	10.8±0.5	1.15	8	—
	12	208	6.1±0.2[a, b]	9.4±0.2[g]	1.44	12	—
LCa − D, N = 6	6	87	4.2±0.3[a]	9.9±0.5	1.40	11	+
	12	120	5.1±0.1[a, c]	9.0±0.2[c]	2.10	16	+
Pair-fed, N = 3	6	112	10.7	10.2	0.14	8	—
	12	195	10.4	10.6	0.17	10	—

Calcium content of diets: NCa = 0.45%; LCa = 0.03%. Vitamin D_2 content of diets: +D = 75 units twice a week; −D = no vitamin added to basic D_2-free diet. [a] Significantly lower ($P < 0.001$) than equivalent value for control (NCa + D) group. [b] Value significantly lower ($P < 0.01$) than 12th week value for NCa-D group. Thirty-two serum calcium values from 6th to 12th weeks in the NCa-D group were compared with the equivalent data in the LCa + D animals; the latter values as a group were also significantly lower ($P = 0.001$). [c] Value significantly lower than that for (NCa + D), (NCa − D), and (LCa + D) groups ($P < 0.001$ in each case). [d] Significantly lower ($P = 0.01$) than (NCa + D) group. [e] Rate of decrease in plasma phosphorus from 0 to 12th week of study in (LCa − D) group different from respective values in control group ($P = 0.05$). Level at 12th week significantly lower than control (NCa + D) group ($P < 0.01$).

additional controls for the doubly deprived group, did not manifest hyperaminoaciduria (Table 1).

The hyperaminoaciduria was generalized (Table 5), and was apparently related to altered tubular absorption since the plasma levels of amino acids (Table 5) were actually lower in hypocalcemic animals than in the age-matched controls, thus eliminating a prerenal basis for the hyperaminoaciduria.

Effect of calcium injection. This, and the subsequent PTE injection study, were performed on an additional group of 32 weanling rats kept separate from the animals used in the other studies. The rats were reared, fed, and divided into four equal groups as described above. Animals in NCa + D and LCa − D groups were given intraperitoneal injections of calcium gluconate at the 8th week; urine was collected before and after the injection in 6-hr periods; six animals were used in each group. Raising the plasma calcium modestly, suppressed phosphaturia and aminoaciduria to control levels in the hypocalcemic animals (Table 6), whereas control animals exhibited no important changes in excretion of phosphorus and amino acids in response to calcium injection.

Response to exogenous parathyroid extract. In the 6th week, the separate lot of animals was given 50 units of parathyroid extract (PTE) every 6 hr by injection for eight doses. Determinations were made on pooled plasma obtained from each group of animals, and on pooled urine collections obtained immediately before starting PTE, and again during the final period of PTE injection. PTE augmented phosphaturia most in control animals and least in the doubly deprived animals. The initial phosphaturic excretion was elevated in animals fed on the diets low in calcium, as noted in the main body of the investigation. The final phosphorus excretion rates were comparable in all four groups (Table 7). The phosphaturic response occurred in control animals, despite the induction of hypercalcemia. Plasma phosphorus fell slightly in all groups. Serum calcium rose sharply in animals re-

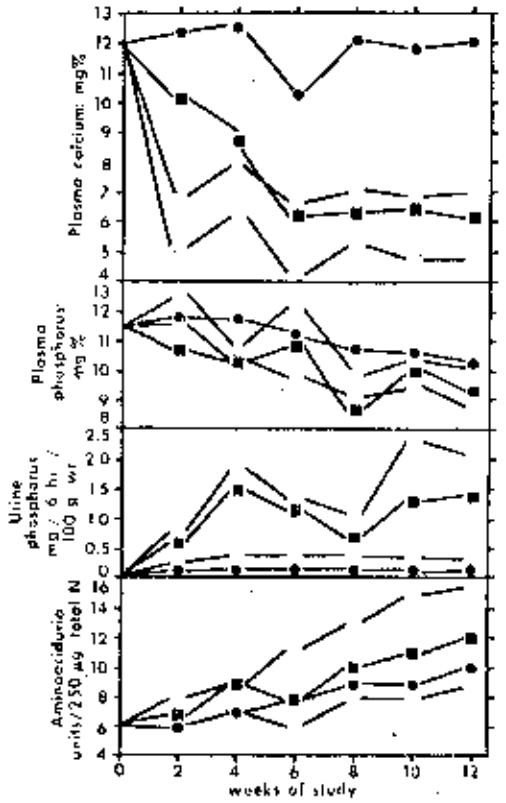

FIG. 2. Plasma and urine data obtained from Holtzman rats fed four different diets: ●, NCa + D; ○, NCa − D; ■, LCa + D; □, LCa − D. NCa and LCa diets contain 0.45 and 0.03% calcium, respectively; +D diets supplied 25 units vitamin D_2 daily; and −D diets were devoid of the vitamin.

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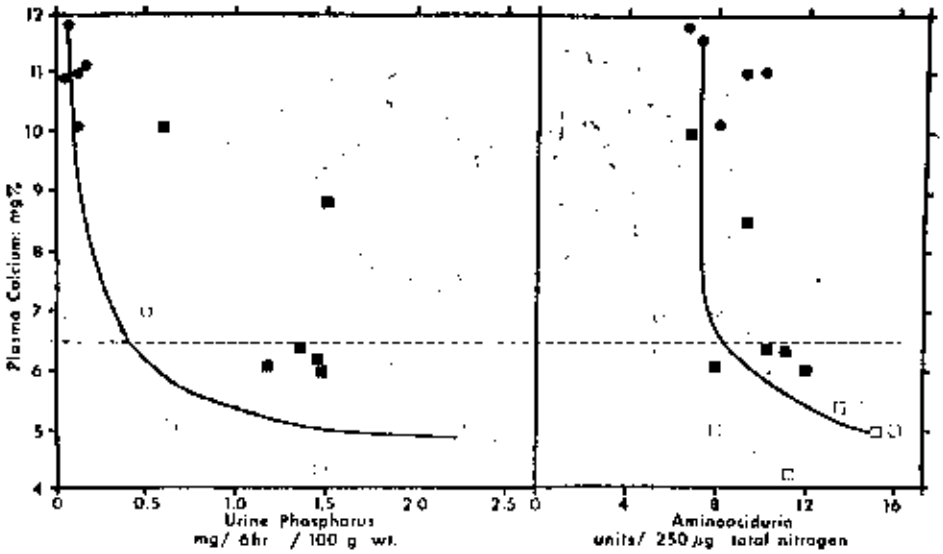

FIG. 3. Urinary excretion data plotted in relation to the equivalent plasma calcium concentration; data obtained at 2-week intervals from each group of animals. Symbols used as in Fig. 2. Starting point for all four groups of animals was: plasma calcium, 11.6 mg/100 ml; urine phosphorus, 0.09 mg/6 hr per 100 g body wt; aminoaciduria, 6 U/250 µg total nitrogen. The single discordant value for phosphorus excretion in the LCa + D group was obtained at the 4th week of the study (viz. Fig. 2).

TABLE 5. *Free amino acids in urine and plasma in the different groups of Holtzman rats at 12th week of the study*

Amino Acids	Urine, µmoles Amino Acid/100 mg Total N						Plasma µmoles Amino Acid/ml Plasma			
	Control, NCa + D		Test				Control, NCa + D	Test		
	(i)	(ii)	NCa − D	LCa + D	LCa − D (i)	LCa − D (ii)		NCa − D	LCa + D	LCa − D
Taurine	17.10	18.10	11.70	6.52	21.25	21.70	0.59	0.27	0.42	0.47
Acidics + neutrals										
Hydroxyproline				0.42	0.47	0.83	Trace	Trace	Trace	0.01
Aspartic acid		0.21	0.15	0.31	0.55	0.86	0.09	Trace	0.10	0.05
Threonine	3.57	3.26	3.05	4.69	4.10	4.30	0.40	0.45	0.29	0.30
Serine	0.99	1.20	1.45	1.74	1.70	1.96	0.43	0.58	0.26	0.27
Asp-NH_2 + Glu-NH_2	1.44	1.28	1.30	1.14	1.40	2.59	0.48	0.29	0.21	0.22
Proline		0.61		0.55	0.60	0.97	0.22	0.29	0.12	0.11
Glutamic acid	0.48	0.52	0.45	1.38	1.05	1.36	0.28	0.61	0.45	0.29
Glycine	2.52	2.78	3.60	4.16	4.05	4.96	0.62	0.83	0.34	0.39
Alanine	1.01	1.09	1.70	1.36	1.75	2.47	0.56	0.52	0.43	0.52
Valine	Trace	0.30	0.25	0.35	0.32	0.32	0.22	0.23	0.22	0.23
Methionine	0.34	0.52	0.75	0.53	1.18	1.20	0.09	0.07	0.09	0.10
Isoleucine	0.12	0.16	0.11	Trace	0.73	0.12	0.16	0.09	0.06	0.18
Leucine	0.51	0.18	0.15	0.24	0.72	0.06	0.23	0.17	0.24	0.26
Tyrosine	0.16	0.24	0.10	0.38	0.33	0.46	0.07	0.15	0.06	0.07
Phenylalanine	0.10	0.16	0.25	0.17	0.22	0.46	0.10	0.06	0.09	0.11
β-Alanine	0.27	0.30	0.54	0.33	0.73	0.59				
β-Aminoisobutyric a.	0.41	0.56	0.44	0.71	0.26	0.77				
Subtotal*	14.32	13.45	14.16	10.46	20.56	24.20	4.03	4.34	2.95	3.13
Basics										
Ornithine	0.45	0.44	0.22	0.27	0.37	0.89	0.22	0.31	0.13	0.12
Lysine	5.49	5.03	2.28	5.30	11.60	9.11	0.65	0.75	0.44	0.39
1-Methylhistidine	0.28	0.37	0.05	0.35	0.39	0.09				
Histidine	0.92	0.68	0.38	0.56	1.19	0.66	0.07	0.05	0.07	0.07
Arginine	0.67	0.17	0.19	0.23	0.81	0.53	0.15	0.11	0.13	0.17
Subtotal	7.81	6.71	3.12	6.71	14.36	11.28	1.09	1.22	0.77	0.75
Total	22.13	20.16	17.28	25.17	34.92	35.56	5.12	5.56	3.72	3.88

Determination made on pooled urine from eight animals. (NCa + D) and (LCa − D) groups studied twice on successive days.
* Exclusive of taurine.

TABLE 6. *Effect of calcium gluconate injection on urinary excretion in the Holtzman rat at 8th week of study*

	NCa + D (Control) Group			LCa − D (Test) Group		
	Plasma calcium*, mg/100 ml	Urine phosphorus,† µg/6 hr per 100 g wt	Aminoaciduria,† µmoles/100 mg total nitrogen	Plasma calcium,* mg/100 ml	Urine phosphorus,† µg/6 hr per 100 g wt	Aminoaciduria,† µmoles/100 mg total nitrogen
Preinjection 6-hr period	10.5	273	16.12	5.2	790	27.16
Postinjection 2nd-8th hr	11.0	243	18.17	6.5	263	16.05
Postinjection 20th-26th hr	10.2	292		5.1	867	29.79

* Obtained from pooled plasmas from six animals in each group. † Determination made on pooled urine obtained from six animals in each group.

ceiving vitamin D; this response was inhibited in D-deprived animals. Aminoaciduria was augmented by PTE administration in all four groups. Again, as noted earlier, the initial excretion rate in the doubly deprived group was already much greater than in control animals. This was slightly increased by PTE, and thus the calcium-deprived animals exhibited the greatest aminoaciduria after PTE.

Response to parathyroidectomy. Total parathyroidectomy (PTX) was performed in three groups of rats at the 12th week of the study. Urine collections were made prior to the operation, and again between the 6th and 12th hr after the operation. All 6 animals survived in the control (NCa + D) group; 2 of the 8 rats died in hypocalcemic tetany in the (LCa + D) group; 3 of the 10 animals in the (LCa − D) group died. The post-PTX data are derived from surviving animals (Table 8).

Parathyroidectomy provoked or aggravated hypocalcemia in all animals in the study. Phosphaturia and aminoaciduria were not significantly altered in the control group. Both groups of initially hypocalcemic animals responded to PTX with a marked decline of phosphaturia and aminoaciduria to the preoperative levels of the control group.

Morphological observations. Excised parathyroid glands from Holtzman rats were examined by light microscopy at the 6th and 12th weeks of the study.[6] Macroscopic and microscopic evidence of hypertrophy was evident at the 6th week of the study in the (LCa − D) group of animals; the (LCa + D) and (NCa − D) animals showed only slight cellular hypertrophy by microscopic examination. At the 12th week, macroscopic enlargement of the (LCa − D) glands was clearly evident at operation; the gland in the (LCa + D) animals were just visible to the naked eye. Microscopic examination revealed cytoplasmic hypertrophy typical of diffuse hyperparathyroidism in the three groups exhibiting hypocalcemia; the changes were most evident in the (LCa − D) group, and least prominent in the (NCa − D) animals.

Sections were made of femora and tibia taken at the 12th week from Holtzman rats in each group. Typical rachitic changes were present only in the (LCa − D) animals.

Kidneys taken from (LCa − D) Holtzman rats showed no morphological abnormalities on light-microscopic examination when compared with kidneys removed from age-matched control (NCa + D) rats.

DISCUSSION

Hyperaminoaciduria was observed in Long-Evans and Holtzman rats fed diets producing severe hypocalcemia. Hyperphosphaturia was also demonstrated in the Holtzman strain receiving low-calcium diets in which the phosphorus intake was also controlled. The abnormalities in urinary excretion could be attributed to impaired net tubular absorption of phosphorus and amino acids. Hyperphosphaturia became apparent in the Holtzman rat after the deficient diets provoked hypocalcemia (Fig. 2). Hyperaminoaciduria, on the other hand, developed unequivocally only around the 6th week of the study. This may account for the failure of others (4, 8) to observe aminoaciduria in vitamin D deficiency in the rat, since their studies were terminated after only 3 weeks of deprivation of calcium or vitamin D, or both.

The purpose of this investigation was to determine whether the hyperexcretion of phosphorus and amino acids in vitamin D deficiency could be attributed directly to deficiency of the vitamin itself, or to other factors. The appearance of hyperphosphaturia and hyperaminoaciduria in Holtzman rats fed a diet deficient in calcium, but adequate in vitamin D (LCa + D group; Table 4, Fig. 2), suggests that vitamin D-deficiency itself cannot be the sole mechanism through which the excretory abnormality occurs. Arnaud and colleagues have shown that the effect of parathyroid hormone on tubular function does not require vitamin D (1).

The predominant feature common to all animals exhibiting the disturbance of phosphorus and amino acid excretion is severe and persistent hypocalcemia (Figs. 2 and 3); a plasma calcium level less than 6.5 mg/100 ml was required to initiate the aminoaciduria in particular (Fig. 3). Animals who were hypocalcemic, but whose plasma calcium level exceeded 6.5 mg/100 ml (NCa + D group in Table 4, Figs. 2 and 3) manifested less phosphaturia and little hyperaminoaciduria. It seems therefore that the degree of hypocalcemia, rather than the presence of vitamin D deficiency, per se, is an important determinant of tubular dysfunction.

Temporary elevation of plasma calcium above 6.5 mg/100 ml subsequent to injection of calcium (Table 6) transiently suppressed the excretory abnormalities in affected animals. This beneficial response could be attributed either to a direct stimulatory effect of calcium on tubular transport of solute (15), or to the suppression of endogenous synthesis and release of parathyroid hor-

[6] Examination performed by Dr. John Fawcett, of the Department of Pathology, McGill University.

TABLE 7. *Effect of exogenous parathyroid extract (PTE) on plasma and urine values in the Holtzman rat at 6th week of study*

	NCa + D		NCa − D		LCa + D		LCa − D	
	C	PTE	C	PTE	C	PTE	C	PTE
Plasma calcium, mg/100 ml*	11.3	15.1	7.4	8.0	7.2	12.6	5.1	5.6
Plasma phosphorus, mg/100 ml*	8.7	7.9	10.2	8.5	9.7	8.4	9.6	8.4
Urine phosphorus, μg/6 hr per 100 g wt†	150	1,920	240	1,740	1,280	1,680	900	1,360
Aminoaciduria, μmoles/100 mg total nitrogen†	12.0	16.5	8.0	16.5	7.2	30.2	28.6	34.7

* Obtained on pooled plasma from six animals in each group. † Determination made on pooled urine collection obtained from six animals in each group.

TABLE 8. *Influence of parathyroidectomy (PTX) on plasma calcium and urinary excretion of phosphorus and amino acids in the Holtzman rat at 12th week of study*

	NCa + D		LCa + D		LCa − D	
	C, N = 6	PTX, N = 6	C, N = 8	PTX, N = 6	C, N = 10	PTX, N = 7
Plasma calcium, mg/100 ml*	10.2	6.7	6.3	4.9	5.0	4.3
Urinary phosphorus, μg/6 hr per 100 g wt†	110	120	1440	430	2050	320
Aminoaciduria, μmoles/100 mg total nitrogen†	21.1	10.2	25.2	13.7	35.2	20.1

N = number of animals in group from which samples were obtained and pooled. * Determinations made on pooled plasma. † Determinations made on pooled urine collections.

mone (18) and the subsequent effect of that upon urinary excretion of solute (19). The former interpretation seems less likely in the light of a subsequent experiment where repetitive administration of large doses of exogenous parathyroid extract (Table 7) not only produced marked elevation of the plasma calcium concentration, except in those animals deficient of vitamin D, but also augmented phosphaturia and aminoaciduria. This excretory response might, however, be attributed, in the opinion of some, to an increase in the filtered load of solute in the presence of exogenous parathyroid extract; measurement of glomerular filtration rate was not performed to eliminate this possibility unequivocally.

Evidence favoring hyperparathyroidism as the underlying cause of impaired tubular function lies particularly in those experiments where the effect of parathyroidectomy was observed. In both strains of rat, this procedure eliminated the excretory abnormality in the hypocalcemic animals (Tables 2 and 8), whereas it had no effect on urinary excretion in normocalcemic animals.

The parathyroid glands which were removed showed macroscopic and microscopic evidence of hyperplasia, which was inversely proportional to the serum calcium concentration, regardless of the status of vitamin D nutrition. Au and Raisz (2) have demonstrated conclusively that parathyroid reactivity to hypocalcemia in vivo in the rat is independent of the vitamin D nutritional status.

Certain anticipated relationships between vitamin D and parathyroid activity (19) were apparent in the study. Exogenous PTE failed to raise the plasma calcium concentration effectively in the two groups of D-deprived animals, in comparison to the response in the two D-supplemented groups (Table 7); this phenomenon has been well documented in the rat (13, 20). On the other hand, phosphaturia and aminoaciduria were augmented in D-supplemented and in D-deprived animals following PTE injection, as expected (1). The phosphaturic response can be attributed solely to suppression of tubular absorption (1).

Comparison of the findings discussed here with those obtained in other species, must be made with caution. For instance, the rat maintains high levels of phosphorus in plasma in the face of severe vitamin D depletion, hypocalcemia, and hyperparathyroidism (1, 2, 12, 20) unlike the dog (11, 17) and man (3, 4, 21). The persistent severe hypocalcemia which develops in the D-deficient rat may result partly from continuing mineral deposition in bone, abetted by the high plasma phosphorus level (12), and from the impaired release of mineral from bone (1). Rickets is thus found only in those animals with very low plasma calcium and slightly depressed plasma phosphorus levels, as noted in the present study ((LCa − D) group, Table 4). Hyperaminoaciduria is found in human vitamin D deficiency quite early in the development of the syndrome (4, 21); for reasons still unknown, it appears late in the rat, and apparently never in the dog (10).

The experimental observations found in the rat in the present and in other reports can be integrated in the following scheme, to explain the effect of vitamin D deficiency on urinary excretion of phosphorus and free amino acids:

vitamin D deficiency → deficient intestinal absorption of calcium and impaired release of calcium from bone → hypocalcemia → secondary hyperparathyroidism → inhibited tubular absorption of phosphorus and amino acids

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The mechanism by which parathyroid hormone impairs renal tubular transport of certain solutes is still unknown.

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Citrate and action of vitamin D on calcium and phosphorus metabolism[1]

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GUROFF, GORDON, HECTOR F. DELUCA, AND HARRY STEENBOCK. *Citrate and action of vitamin D on calcium and phosphorus metabolism.* Am. J. Physiol. 204(5): 833-836. 1963.—Pantothenic acid and pyridoxine deficiencies in rats greatly minimized the rise in serum citrate following vitamin D administration without affecting the rise in serum calcium, serum phosphorus, and femur ash. In addition, pyridoxine deficiency reduced the response of bone citrate to the administration of vitamin D. The administration of cortisone acetate to rats on a low-calcium diet completely prevented the response of serum and bone citrate to vitamin D administration while having no effect on the response of serum calcium to vitamin D. These findings make it unlikely that an elevated citrate content of extracellular fluid and bone mediates the basic effects of vitamin D on mineral metabolism.

THE DISCOVERY OF LARGE AMOUNTS of citric acid in bone and its relative decrease in rickets (1) opened the question of the relationship of citric acid to calcification. It is well established that both vitamin D and parathyroid hormone increase bone and serum citrate (2-10). Vitamin D administration raises the citrate content of urine and of certain soft tissues as well (2, 5, 11). Earlier studies in this laboratory revealed that the antirachitic action of dietary citrate was due to an increase in the availability of dietary phytic acid phosphorus and not through a metabolic action (12). However, the exact role, if any, played by metabolically produced citrate in calcium metabolism is as yet not understood.

The well-known ability of citrate to complex calcium ions has been the basis of many suggested mechanisms in calcium metabolism. For example, it has been suggested that vitamin D has a calcium-mobilizing effect on bone which is mediated by citric acid (8). On the other hand, it has been argued on the basis of evidence obtained with model systems that the occurrence of citric acid in bone is adventitious (13).

Recently it has been shown that cortisol prevents the elevation of citrate in bone and serum of rats given vitamin D (14, 15). This agent did not prevent the effect of vitamin D on serum calcium and phosphorus or on the deposition of calcium in bone (14, 15).

Previous work in this laboratory (16) revealed that deficiencies of pantothenic acid and pyridoxine but not of vitamins A, B_1, or biotin interfere with the effect of vitamin D on urine and tissue citrate. In this paper, it will be shown that, although pantothenic acid and pyridoxine deficiencies interfere with the action of vitamin D on citrate, they do not interfere with its action on serum calcium and phosphorus or on bone ash accretion. Furthermore, it will be shown that cortisone, added to a low-calcium diet, completely prevents the action of vitamin D on serum and bone citrate but has no effect on the vitamin D-induced elevation of serum calcium.

EXPERIMENTAL METHODS AND RESULTS

Male Sprague-Dawley weanling rats weighing 40-60 g were used in all experiments. They were housed in screen-bottomed cages and given food and water ad libitum.

The basal diet, as described by Bellin and Steenbock (11), was composed of the following in per cent: glucose monohydrate 67 (Cerelose), vitamin-free casein 18 (General Biochemicals, Inc.), cystine 0.2, cottonseed oil (Wesson) 10, roughage (Cellufloun) 3, calcium and phosphorus-free salts 2 (11), and vitamins. Each kilogram of diet supplied the following quantities (mg) of crystalline, water-soluble vitamins: thiamine hydrochloride 5, riboflavin 5, pyridoxine hydrochloride 5, calcium pantothenate 28, nicotinamide 20, inositol 200, folic acid 0.2, vitamin B_{12} 0.02, biotin 0.1, and choline chloride 500. In addition, the rats received a supplement of fat-soluble vitamins in cottonseed oil solution. The supplement provided the following quantities (μg) per week: β-carotene 70, α-tocopherol 875, and 2-methyl-1,4-naphthoquinone (Menadione) 105. Crys-

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TABLE 1. *Response of rachitic rats deficient in either pantothenic acid or pyridoxine to vitamin D administration*

Treatment	Femur			Serum	
	Dry wt., mg	Citrate, mg	Ash, mg	Citrate, mg/100 ml	Phosphorus, mg/100
			Pantothenic Acid-Deficient		
0-time	95.3	0.391 ± 0.008	29.4 ± 1.2	3.6 ± 0.4	2.8 ± 0.3
Plus vitamin D	123.8	0.630 ± 0.010	41.9 ± 2.2	4.1 ± 0.3	4.6 ± 0.1
Plus vitamin D and pantothenic acid	117.9	0.588 ± 0.015	39.9 ± 0.9	3.8 ± 0.5	4.4 ± 0.2
			Pyridoxine-Deficient		
0-time	102.4	0.290 ± 0.013	26.2 ± 1.4	3.1 ± 0.3	2.8 ± 0.2
Plus vitamin D	120.4	0.527 ± 0.006	42.9 ± 1.7	3.4 ± 0.4	4.2 ± 0.1
Plus vitamin D and pyridoxine	129.6	0.740 ± 0.017	40.9 ± 2.4	6.0 ± 0.3	4.3 ± 0.2

Values are means ± SEM. The 0-time values are taken from animals after 21 days on the doubly deficient, rachitogenic diet (no. 24). The response values are from animals given 75 IU of vitamin D_2 every 2 days during the 10-day period following 0-time. There were at least 4 rats in each group.

talline vitamin D_2 was administered orally, in the indicated amounts, in cottonseed oil solution. Cortisone acetate, where used, was incorporated in the diet at a level of 30 mg/100 g diet. The respective deficiencies were produced by omitting the appropriate vitamin from the diet.

The desired levels of calcium and phosphorus in the diet were obtained by the addition of $CaCO_3$ and an equimolar mixture of KH_2PO_4 and K_2HPO_4, respectively, at the expense of the Cerelose. The nonrachitogenic diet (no. 11) contained 0.47% calcium and 0.3% phosphorus; the high-calcium rachitogenic diet (no. 24) contained 1.5% calcium and 0.12% phosphorus; and the low-calcium diet (no. 11D) contained 0.016% calcium and 0.3% phosphorus.

In the experiments in which the nonrachitogenic diet (no. 11) was employed (Table 2), the animals were maintained for 3 weeks on diets deficient in either vitamin D or the appropriate water-soluble vitamin. The control group received the complete diet.

In the experiments in which the high-calcium rachitogenic diet (no. 24) (Table 1) was used, the animals were depleted of vitamin D and the appropriate water-soluble vitamin for 21 days. At the end of this depletion period one group was killed for 0-time values. A second group was given 75 IU of vitamin D every other day for 10 days. A third group was given identical doses of vitamin D and in addition received the water-soluble vitamin in question. The cortisone experiments (Table 3) were done in a similar manner except that the low-calcium diet (no. 11D) was used. The animals were kept on the diet without vitamin D for 15 days and then given cortisone or vitamin D or both.

At the conclusion of each experiment the rats were killed by decapitation and blood was collected. The serum was obtained by centrifugation, deproteinized with 20% trichloroacetic acid (TCA) and analyzed for citric acid. Tissues were quickly removed and frozen in liquid air. They were ground in cold 20% TCA and centrifuged. The precipitate was resuspended in TCA, centrifuged, and the combined supernatant solutions were analyzed for citric acid.

Femurs were removed from the carcasses and free of adhering tissue. For citric acid analysis they wer extracted with diethyl ether in a Soxhlet extractor f 24 hr, dried at 100 C, ground in a mortar, and weighe The bone powder was extracted for four 1-hr period with 1 N H_2SO_4 and the extracts combined for analysi For ash determination, the bones were extracted for 2 hr with 95% ethanol and 24 hr with diethyl ether. The were finally ashed at 1,200 F for 8–12 hr.

Citric acid analyses were by the method of Speck Moulder, and Evans (17). Serum phosphorus wa determined by the procedure of Fiske and SubbaRow (18), and serum calcium was estimated by the metho of Wang (19).

Since it was found previously that pantothenic acid and pyridoxine deficiencies interfered with the vitamin D-induced rise in citrate of serum, kidney, urine, and to a limited extent of bone, the first series of experiments were designed to determine whether these deficiencies would also interfere with the action of vitamin D on bone ash accretion and on serum P in rachitic rats. It was quite evident (Table 1) that neither deficiency interfered with femur ash accretion or the rise in serum phosphorus brought about by the administration of vitamin D. Nevertheless, it was evident from previous (16) and present experiments that they greatly interfered with the effect of vitamin D on serum citrate. In addition, pyridoxine deficiency reduced the rise in bone citrate following vitamin D as well. In agreement with previous results (16), pantothenic acid had little or no effect on bone citrate. It should be noted that prolonged deficiency of these B vitamins was avoided since it has been shown that calcification is adversely affected under these conditions, perhaps by interference with matrix synthesis (20, 21). The data from these experiments then indicated that the rise in serum citrate and to some degree of bone citrate was not necessary for the rise in serum P and bone ash deposition in response to vitamin D. These results agree closely with those of Harrison et al. (15) in which cortisol was used to prevent the vitamin D-induced rise in serum and bone citrate.

TABLE 2. *Effect of pantothenic acid and pyridoxine deficiency on serum calcium and citrate of rats given vitamin D*

	Citrate, mg/100 ml	Calcium, mg/100 ml
Pantothenic acid		
Minus vit. D, minus panto. acid	2.6 ± 0.2	8.2 ± 0.2
Minus vit. D, plus panto. acid	2.7 ± 0.3	8.0 ± 0.3
Plus vit. D, minus panto. acid	3.0 ± 0.1	12.4 ± 0.1
Plus vit. D, plus panto. acid	4.2 ± 0.1	12.7 ± 0.2
Pyridoxine		
Minus vit. D, minus pyridox.	2.4 ± 0.4	9.0 ± 0.2
Minus vit. D, plus pyridox.	2.5 ± 0.3	8.8 ± 0.2
Minus pyridox., plus vit. D	2.4 ± 0.2	11.3 ± 0.2
Plus vit. D, plus pyridox.	3.7 ± 0.3	11.4 ± 0.1

Values are means ± SEM. Four to six rats in each group; killed after 21 days on the indicated diets; 75 IU vitamin D given to the indicated animals every 3 days throughout experimental period.

TABLE 3. *Effect of vitamin D on serum calcium and serum and bone citrate of rats fed a low-calcium diet with and without cortisone*

	Serum Citrate, mg/100 ml	Bone Citrate, mg/g	Serum Calcium, mg/100 ml
Minus vitamin D	1.2 ± 0.1	1.5 ± 0.2	7.6 ± 0.1
Minus vitamin D Plus cortisone	0.9 ± 0.2	1.6 ± 0.1	8.1 ± 0.1
Plus vitamin D	3.3 ± 0.4	4.3 ± 0.5	11.8 ± 0.1
Plus vitamin D Plus cortisone	0.9 ± 0.1	1.9 ± 0.1	12.0 ± 0.2

Values are means ± SEM. The animals were fed the low Ca diet (1cD) for 25 days. Cortisone acetate was fed to the appropriate groups at a level of 30 mg/100 g of diet beginning on the 15th day. Vitamin D was given on the 17th day in a single dose of 4,000 IU, and in doses of 75 IU every other day thereafter to the rats in the designated group. There were 6 rats in each group.

DISCUSSION

It has been known for many years that vitamin D consistently increases serum calcium. This was again reemphasized by Steenbock and Herting (22), who showed that this effect of vitamin D is found regardless of the Ca and P content of the diet. When an adequate calcium and phosphorus diet was fed, vitamin D had a rather striking effect on the serum calcium (Table 2). It is apparent that pantothenic acid and pyridoxine deficiencies had no effect on serum calcium. Again it is clear that the vitamin B deficiencies depressed the serum citrate values in the vitamin D-fed rats.

Finally, the postulation of Carlsson and Hollunger (8) that the rise in bone and serum citrate mediates the mobilization of bone mineral induced by vitamin D received some attention. The effect of vitamin D on bone mineral mobilization is especially evident with diets low in calcium in which vitamin D increases serum calcium, presumably at the expense of bone mineral (23). An attempt was made to test the Carlsson proposal by preventing the vitamin D-induced rise in serum and bone citrate and studying the response of serum calcium to vitamin D when a low-calcium diet is fed. It is obvious that the vitamin B deficiencies were inadequate in this regard since they would not prevent the rise in bone citrate (Table 1). By utilizing the finding of Harrison et al. (15) that cortisol administration prevents the effect of vitamin D on bone citrate, it was possible to carry out such an experiment. The results (Table 3) show clearly, in agreement with Harrison et al. (14, 15), that cortisone completely prevents the action of vitamin D on bone and serum citrate. However, it had no effect on the vitamin D-induced rise in serum calcium. These results then do not support the hypothesis that bone citrate mediates the vitamin D-induced rise in serum calcium even when diets very low in calcium are fed.

It may be of interest to note the change in serum citrate noted in the three experiments presented. Serum citrate values were found low with low calcium diets (Table 3) and high with high calcium diets (Table 1). This is not unexpected and may provide some insight into the accumulation of citrate following vitamin D administration.

Our over-all results agree closely with those of Harrison et al. (14, 15). A clear separation of the effects of vitamin D on citrate and mineral metabolism can be achieved by means of pantothenic acid and pyridoxine deficiencies or cortisone, which indicates that citrate per se does not play a role in the basic actions of vitamin D on calcium and phosphate metabolism. However, the citrate phenomenon should not be discarded completely since it may nevertheless serve as an indicator or may be the direct result of the basic biochemical action of vitamin D. It is quite possible that citrate accumulation following vitamin D administration is a reflection of changes in cell membranes or subcellular particulate membranes. For example, the decreased oxidation of citrate by kidney mitochondria from rats fed vitamin D now appears related to structural changes of these particles (24). However, the elucidation of the exact mechanism of citrate accumulation following vitamin D administration must await further investigation.

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THE SMALL INTESTINE IN VITAMIN D DEPENDENT RICKETS

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ABSTRACT. We have demonstrated impaired intestinal absorption of calcium in a child with active vitamin D dependent rickets (hereditary pseudovitamin D deficiency rickets) at a time when the patient had normal anti-rachitic activity in her serum. Calcium absorption improved greatly in response to vitamin D, administered in the massive dosage that was necessary to heal the rachitic lesions. Phosphorus absorption may have been slightly impaired in the same patient, but no other absorptive defect was found. We studied intestinal function in five additional patients after they had been placed on vitamin D therapy. No abnormalities were found. In these treated patients, calcium absorption was not measured. Duodenal mucosa studied by light and electron microscopy was normal in all patients. Future investigation of intestinal transport of calcium in these patients should help to explain the pathogenesis of this disease. *Pediatrics*, 45:364, 1970, RICKETS, VITAMIN D DEPENDENT RICKETS, INTESTINAL ABSORPTION OF CALCIUM, HEREDITARY PSEUDOVITAMIN D DEFICIENCY RICKETS.

VITAMIN D dependent rickets* is characterized by a severe rachitic process, hypocalcemia, hypophosphatemia, and generalized amino-aciduria beginning in infancy in spite of an adequate intake of vitamin D.[1-3] Unlike other types of refractory rickets, it heals completely in response to continuous therapy with massive doses of the vitamin. The pathogenesis of this rare disease is unknown.

We undertook a detailed assessment of the small intestine in six patients with vitamin D dependent rickets. In one we had an opportunity to measure intestinal absorption of calcium before and after healing of the rickets in response to vitamin D therapy. Our finding of profound disturbance of intestinal absorption of calcium during the active phase of rickets in this instance, considered in relation to our other data on intestinal function and structure, provides new information concerning the pathogenesis of this disease.

PATIENTS

Of the six patients, five had been diagnosed previously and their rickets had healed completely in response to treatment; one was studied before treatment and the assessment was repeated after rickets was healed. The condition of each patient at the time of the present studies is summarized in Table I. Two of the five patients treated (Cases 1 and 5) showed evidence of reactivation of rickets at the time of our studies because they had failed to take vitamin D regularly for the previous several months.

The sixth child, a sister of Case 5, was the only child studied in both the active and the healed phase of the disease. We saw her first at the age of 18 months with florid rickets that had progressed over 3 months in spite of a prophylactic intake of vitamin D. She had profound biochemical (Table I) and radiological abnormalities (Fig. 1) typical of vitamin D dependent

* Alternate nomenclature: Hereditary pseudovitamin D deficiency rickets; Hypocalcemic, hypophosphatemic vitamin D refractory rickets with amino-aciduria.

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TABLE I

CONDITION OF PATIENTS AT TIME OF STUDY

Case	History Number	Age (yr) At Time of Study	Age (yr) At Onset of Rickets	Sex	Vitamin D_2 Supplement (IU/day)	Skeletal X-rays	Serum Ca* (mg/100 ml)	Serum P* (mg/100 ml)	Serum Alkaline Phosphatase* (K.A. units)	Urinary Amino Acids
1	184171	[illegible]	[illegible]	M	60,000	rickets	8.4	9.1	60	generalized excess
2	847040	15 7/12	1	F	100,000	normal	8.5	3.4	18	normal
3	805252	8 4/12	10/12	M	50,000	normal	9.5	4.8	17	normal
4	808403	3 10/12	4/12	M	60,000	normal	9.0	3.5	19	normal
5	850076	4 0/12	2	F	82,500	rickets	8.3	3.3	14	generalized excess
6	924869 (a) active	1 8/12	1 5/12	F	0	rickets	8.0	5.4	190	generalized excess
	(b) healed	2 5/12	—	F	100,000	normal	9.2	5.0	19	normal

* Normal values: serum calcium—9-11 mg/100 ml; serum inorganic phosphorus—4-7.5 mg/100 ml; serum alkaline phosphatase—12-25 K.A. units.

rickets. The course of her disease, dating from the initiation of therapy, is summarized graphically in Figure 2. Initially, vitamin D_2 was administered in a dose of 1,000 IU per day, but after 8 weeks no healing had occurred and the dose was increased, first to 10,000 IU per day, then to 50,000 IU per day. Not until she had been receiving 100,000 IU per day for 4 weeks was satisfactory improvement observed. Within 3 months on that dose, serum concentrations of calcium and phosphorus, serum alkaline phosphatase activity, and urinary excretion of amino acids were normal; within 6 months radiological evidence of rachitic activity had disappeared (Fig. 3). She re-

TABLE II

DATA RELEVANT TO SMALL INTESTINAL FUNCTION

Case	Fecal Fat Excretion % of Fat Intake	Urinary Excretion of D-xylose in 6 Hours (% Intake)	Oral Glucose Tolerance Maximum Rise in 1 Hour (mg/100 ml)	Small Intestine X-ray	Oral Iron Tolerance Maximum Rise - Serum Iron (μg/100 ml)	Serum Concentration Vitamin B_{12} (μμg/100 ml)	Urinary FIGLU Excretion (mMol/hr)
(Normal Values)	<10	>15	>40		>100	>100*	<20
1	3.2	24	38	normal	128	200	2.0
2	2.6	30	45	normal	170	140	7.2
3	2.0	30	44	normal	98	600	0.8
4	1.4	41	52	normal	137	360	2.0
5	2.1	20	64	normal	138	640	—
6†	5.4	17	67	normal	301	500	4.9

* Normal for adults.

† Pre-treatment.

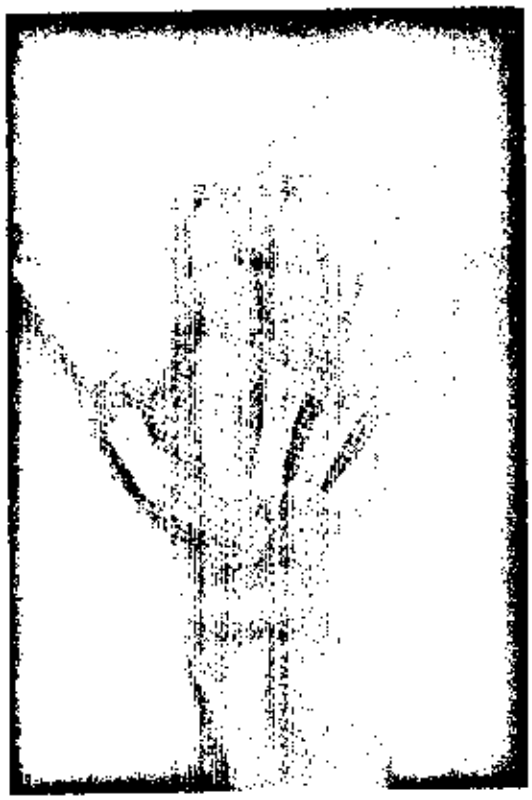

FIG. 1. Case 6. X-ray of left wrist, March 1, 1966, florid rickets. Patient receiving vitamin D_2 50,000 IU/day.

ceived a gluten restricted diet† for 12 weeks beginning 4 weeks after the start of vitamin D therapy, without beneficial effect.

METHODS

The children were studied in a clinical investigation unit where skilled nursing supervision, careful dietary control, and accurately timed collections of urine and feces were assured. Those studied while on vitamin D therapy received their prescribed oral dose of the vitamin‡ each morning throughout the study period.

Calcium, phosphorus, and magnesium balance studies were undertaken on Case 6 at times shown in Figure 2. During the first study (Balance Study I) her rickets was active, having failed to respond to vitamin D therapy (1000 IU per day for 6 weeks, followed by 10,000 IU per day for 4 weeks). The second study (Balance Study II) was undertaken after healing of the rickets was complete on 100,000 IU per day. Each 12-day balance study was conducted with the child on a metabolic frame and was preceded by a 4-day pre-balance period to permit stabilization of her activity and food intake on the frame. Duplicate diets on the first and last day of each balance and daily food rejects were analyzed. The diet as offered contained fixed amounts of calcium, phosphorus, and lactose. Caloric intake was

† Removal of all dietary wheat, rye, barley, and oat gluten.

‡ Radiostol (B.D.H.) 100,000 IU vitamin D_2 per milliliter.

TABLE III

CASE 6—INTAKE, EXCRETION OF CALCIUM AND PHOSPHORUS

Data	Balance Study I (Active Rickets)						Balance Study II (Healed Rickets)					
	Time—Days						Time—Days					
	0–4		4–8		8–12		0–4		4–8		8–12	
	Ca	P	Ca	P	Ca	P	Ca	P	Ca	P	Ca	P
Dietary intake (mg/day)	648	426	688	468	786	500	664	479	711	489	656	439
Excretion (mg/day)												
urine	1.8	854	2.0	328	1.6	816	171	397	210	424	178	429
stool	787	231	713	108	796	265	329	46	196	40	237	34

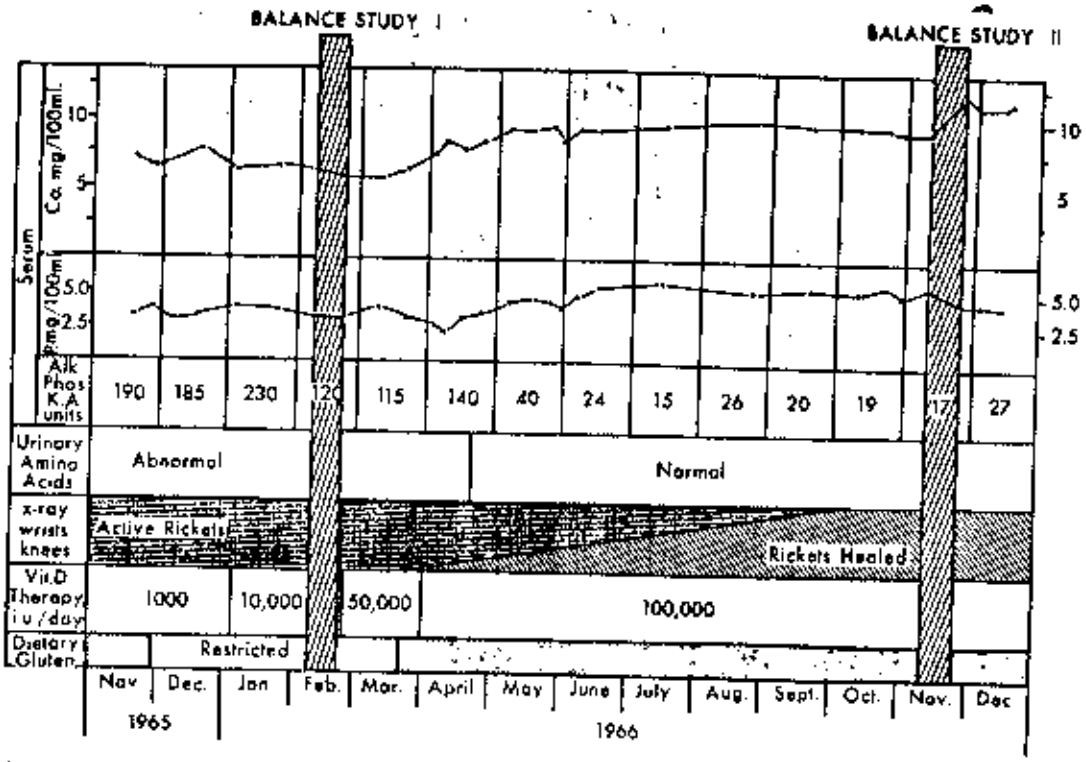

FIG. 2. Case 6. A summary of response to therapy with vitamin D in increasing dosage. Times of two balance studies are superimposed on graph.

constant during each study, but it was greater during the second study because her appetite increased. Measurements of calcium, phosphorus, and magnesium excretion in urine and stool were carried out on consecutive 96-hour pooled specimens.

At the start of each balance study, a single intravenous injection of Ca^{47} (2 μCi) of high specific activity was given. Radioactivity was determined in consecutive 24-hour urine samples and consecutive defecations.[4] Endogenous fecal calcium, the exchangeable calcium pool, and the accretion of calcium to bone were calculated using a two-term exponential model.[5]

The Crosby Kugler intestinal biopsy capsule[6] was used to obtain mucosal tissue just proximal to the ligament of Treitz, 6 to 7 hours after the last meal and daily dose of vitamin D. For electron microscopy, tissue was fixed in 1% osmium tetroxide[7] for 1 hour at 4°C and embedded in Epon[8] after rapid dehydration in increasing concentrations of ethanol. Blocks were cut on a Porter Blum ultramicrotome. One micron-thick sections were stained with toluidine blue[9] and examined with the light microscope. The tissue was oriented in such a way that a specific area could be chosen for thin sec-

TABLE IV

CASE 6—SUMMARY OF BALANCE DATA

Data	*Balance Study I (Active Rickets)*	*Balance Study II (Healed Rickets)*
Dietary calcium mean (mg/day)	707	677
Urinary calcium mean (mg/day)	1.8	186
Fecal calcium mean total (mg/day)	765	251
endogenous (mg/day)	50	40
Calcium absorption (percentage of intake)	nil	68

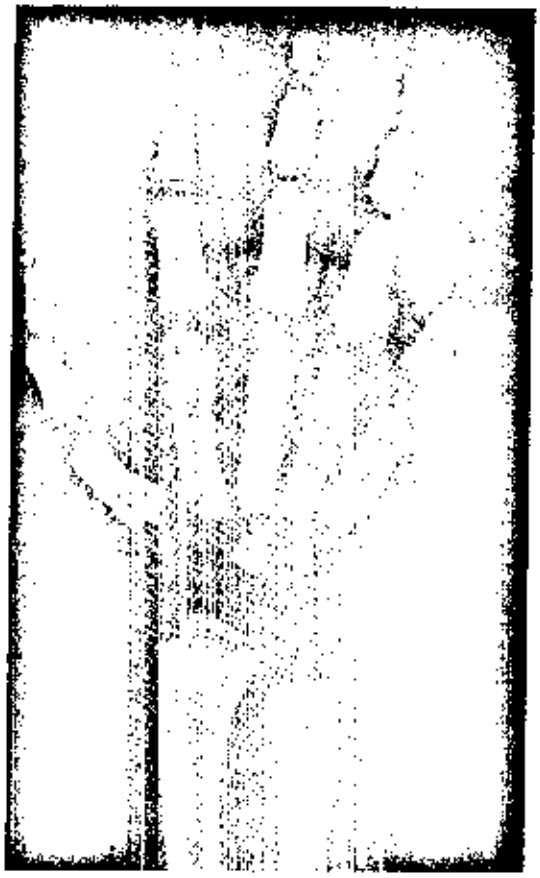

FIG. 3. Case 6. X-ray of left wrist after healing of rickets. October 6, 1966, healed rickets. Patient had been receiving vitamin D_2 100,000 IU/day for 6 months.

tions. These were stained with lead hydroxide[19] and examined in a Phillips 200 electron microscope.

Fecal fat was measured in 5-day pooled collections of stool,[11] and excretion was expressed as the percentage of the calculated dietary intake after the child had been on a controlled fat-containing diet for at least a week. Blood sugar,[12] urinary excretion of an oral load of d-xylose,[13] and urinary excretion of formiminoglutamic acid (FIGLU)[14] after oral administration of histidine were measured by standard methods. In addition to the usual hematological indices, we measured serum concentrations of vitamin B_{12}[15],§ and iron,[16] the latter in response to an oral load of iron (4 mg per kilogram body weight). Urinary excretion of amino acids was assessed on random specimens by two-dimensional paper chromatography[17] loaded on the basis of creatinine content.

For determinations of calcium and magnesium, flame spectrophotometry was used.[18] In urine, calcium was co-precipitated with strontium before flaming when low concentrations were anticipated.[19] Inorganic phosphorus was measured in serum by the Gomori procedure,[20] and alkaline phosphatase was measured by the method of King and Armstrong.[21] Anti-rachitic activity was measured in serum by a bio-assay technique.[22],‖ For these assays, blood was drawn in an opaque syringe 24 hours after the last dose of the vitamin, and serum was stored in the dark at −20°C until assayed.

RESULTS

Intestinal Function

GENERAL: We did not detect symptoms or signs of intestinal malabsorption in our six patients. Furthermore, no abnormality was found in the laboratory studies related to the assessment of the small intestine, either in the patients receiving treatment or the one case studied before treatment (Table II).

ABSORPTION OF CALCIUM, PHOSPHORUS AND MAGNESIUM, CASE 6: Intake and excretion of calcium and phosphorus for each 4-day period during the two balance studies on Case 6 are tabulated in Table III. The data are consistent for consecutive periods within each of the balance studies. During Balance Study I, serum calcium concentration remained normal; but, during Balance Study II, unexpectedly, it increased from normal (9.2 mg/100 ml) on the first day to a hypercalcemic level (12.2 mg/100 ml) at the end of the study. In Figure 4 specific activity of Ca^{47} in urine is plotted against time for each study. For Balance Study I, the quantity of calcium in

§ Bioscience Laboratories, Los Angeles, California.

‖ Wisconsin Alumni Research Foundation, Madison, Wisconsin.

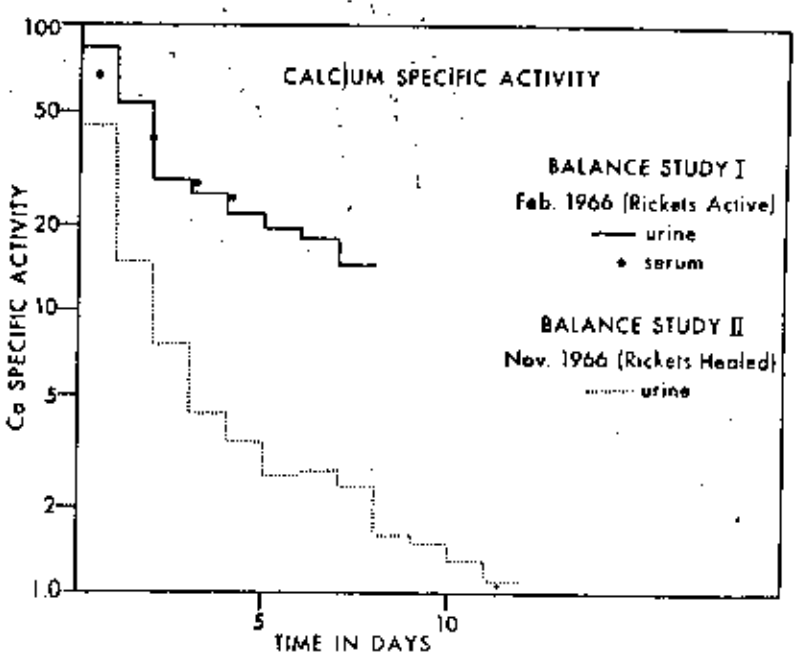

FIG. 4. Case 6. Specific activity of urinary calcium (% dose Ca^{47}/gm stable Ca) plotted against time comparing two balance studies before and after healing of rickets. Serum data plotted for first study show positive correlation with urine specific activity.

the exchangeable pool (E) was 2.0 gm and the calculated rate of calcium accretion to bone (A), was 0.22 gm per day. For Balance Study II, E was 6.5 gm and A was 0.84 gm per day. In Table IV data on intestinal absorption of calcium are compared. At the time she had active rickets (Balance Study I), the content of calcium in feces was massive compared with fecal calcium after healing was complete (Balance Study II), although endogenous fecal calcium excretion differed little between Balance Study I (50 mg/day) and Balance Study II (40 mg/day). Absorption of dietary calcium was not measurable at the time she had active rickets, but it was 68% of calcium intake after the rickets was healed.

Fecal excretion of phosphorus was greater during Balance Study I than during Balance Study II (Table III). Table V shows that magnesium intake was greater than the combined excretion in urine and stool during both studies. Serum magnesium concentration was normal at all times.

Intestinal Biopsy

The structure of the duodenal mucosa was normal in the one specimen taken before treatment and in the six studied after treatment. Examined by light microscopy, the cells covering the villi and in the lamina propria, the depth of the crypts, and the number of mitotic figures were normal. The ultrastructural findings were identical to those described by other investigators in normal adults.[23,24] Figure 5 illustrates a representative portion of the absorptive epithelium of Case 6. The electron micro-

TABLE V

CASE 6—MAGNESIUM INTAKE AND EXCRETION—MEAN FOR EACH 12-DAY BALANCE STUDY

Data	*Balance Study I*	*Balance Study II*
Dietary intake (mEq/day)	8.7	9.1
Excretion (mEq/day)		
urine	1.0	5.4
stool	5.5	3.0

TABLE VI

CASE 6—SERUM CONCENTRATIONS OF ANTI-RACHITIC ACTIVITY BEFORE AND DURING THERAPY

Date	Vitamin D_2 Therapy		Serum Concentration of Anti-rachitic Activity* (units/ml)
	Dose (IU/day)	Time on Dose (weeks)	
20/9/65	0	4	6
7/12/65	1,000	6	2
21/2/66	10,000	8	7
20/8/66	50,000	5	38
14/11/66	100,000	30	24

* Normal range: 2–5 units/ml.

scopic findings were evaluated on the basis of the experience of one of us (R.M.) with eight biopsies taken from infants and children who were normal or who had unrelated diseases and with 30 biopsies from normal adults.

Anti-Rachitic Activity in Serum

Results of bio-assays carried out on Case 6, before and after healing of her rickets are summarized in Table VI. Normal anti-rachitic activity was detected in her serum prior to therapy and rose to high levels on massive doses of the vitamin.

DISCUSSION

Our studies demonstrated impaired intestinal absorption of calcium in a child with active vitamin D dependent rickets. This conclusion is based on a comparison of balance and kinetic data in a child with active rickets with data from the same child after her rickets was healed and with published data on normal adults[25] and a few children.[26]

The calcium balance technique has some deficiencies, particularly in the short-term studies which are unavoidable in diaper-age children.[27] We minimized these deficiencies since we compared balances undertaken in the same child using the same procedures and under the same environmental conditions. The conclusion that intestinal absorption of calcium is grossly defective in active vitamin D dependent rickets cannot be challenged. Furthermore, the data from individual periods were consistent during both studies. During the second study the patient became hypercalcemic in spite of the fact that, during the previous 6 months on a constant dose of vitamin D_2, serum calcium concentration and other measurements of mineral metabolism had remained stable. Hypercalcemia probably occurred because she was immobilized. No mechanism is known by which hypercalcemia enhances intestinal absorption of calcium.

Recently, a selective absorptive defect of calcium[28] has been described in a case of celiac disease without steatorrhea. We can exclude the possibility that our patients had celiac disease since mucosal structure was normal[29] in all patients and one failed to improve on a gluten-free diet. Electron microscopic studies failed to demonstrate an abnormality, although as yet very few normal children have been studied with this technique. Apart from the defect observed in calcium absorption, no abnormalities were detected in any absorptive processes, although we assessed several pathways involving different regions of the intestine. Normal fat excretion and normal response to ingested glucose, iron, and d-xylose demonstrate that four absorptive pathways involving the proximal small intestine were intact.[30] Normal serum concentrations of vitamin B_{12} and normal rates of excretion of FIGLU in urine suggest that our patients did not have gross disturbances of vitamin B_{12} and folic acid absorption, processes which, in the human, take place in the distal ileum and jejunum, respectively.[30] Reports have linked the intestinal absorption of magnesium with that for calcium[31] and phosphorus.[32] In our patient, magnesium balance was normal both before and after therapy. Net intestinal absorption of phosphorus was greater in the treated state than in the rachitic state, although our data deviated only slightly from normal.[33] Vitamin D absorption was probably normal since serum anti-rachitic activity increased with

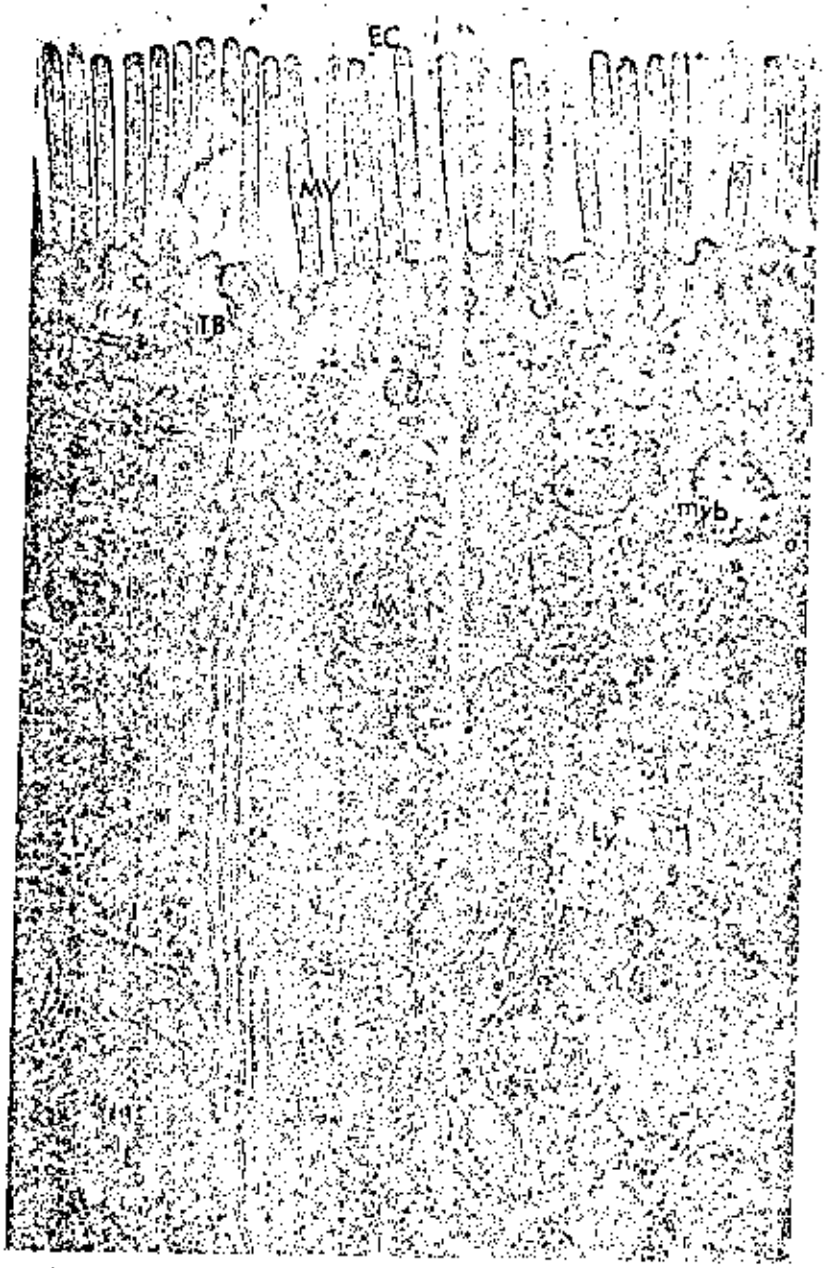

FIG. 5. Case 6. Electron micrograph of intestinal absorptive cells during active vitamin D dependent rickets. EC = extracellular coat; MV = microvilli; TB = terminal bar; mvb = multivesicular body; M = mitochondria; Ly = lysosome.

increasing oral dosage of the vitamin and since fat absorption was normal.[34]

Our observation of normal anti-rachitic activity in serum at a time when the patient had florid rickets raises some intriguing questions regarding pathogenesis of this disease. A major action of the vitamin is to enhance intestinal absorption of calcium.[35] Wasserman and Taylor[36] have shown that vitamin D stimulates formation of a protein in the intestinal mucosa which binds calcium, and suggest that this protein acts as a

carrier for calcium transport. Perhaps the synthesis of this protein is defective in patients with vitamin D dependent rickets. A biologically active metabolite of vitamin D_3, 25-hydroxycholecalciferol,[37] has been isolated from plasma, bone, intestine, and kidney of rats and from pig and human plasma.[38,39] This metabolite is a potent activator of calcium transport in everted gut sacs. Are patients with vitamin D dependent rickets unable to convert vitamin D_3 to 25-hydroxycholecalciferol? We are not suggesting that the intestine is the only organ impaired nor that intestinal malabsorption of calcium is necessarily the primary defect in vitamin D dependent rickets. Our clinical studies do suggest that, in the future, investigation of the intestinal transport of calcium in these patients will contribute to our understanding of the pathogenesis of this intriguing disease.

SUMMARY

Intestinal function and structure were studied in six children with vitamin D dependent rickets. In one of these children, intestinal absorption of calcium was assessed before and after the rickets healed. Calcium absorption was grossly impaired when the rickets was active, compared with data from the same patient in the healed state and compared with data from the literature. No additional defect of intestinal function or structure could be proved in any of the six children.

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Influence of Large Doses of Vitamin D on Magnesium Metabolism in Rats

By Samir Hanna

Two groups of 150–170 Gm. hooded female rats received a diet containing 288 μEq. of Mg and 2400 μEq. of Ca daily. One group was given intramuscularly 40,000 units of vitamin D_2 daily for 3 days, and urinary, fecal and plasma Ca, P and Mg compared with values for the control group. The known effects of vitamin D on Ca metabolism occurred. By the third day mean urinary Mg had increased significantly over control values, 182 as compared to 111 μEq./day. Mean fecal Mg decreased, 107.0 as compared to 183.7 μEq./day for controls. Mean plasma Mg was significantly lowered, 0.98 vs. 1.43 μEq./L. From other studies there is evidence that citrate may play a role in calcium metabolism, and it is possible that absorption of Ca and Mg may be citrate-dependent.

THE PROFOUND EFFECTS of vitamin D on calcium metabolism are widely recognized. Although the mechanism of its action is not understood, this vitamin has three cardinal effects on calcium metabolism:

1. It increases absorption of calcium from the gut in man,[2] in rats,[26] in everted gut-sacs.[30] In physiological doses the effect depends upon the requirements; thus in rats calcium absorption will be only affected in the first 21 weeks of life.[13] The effect of physiological doses apparently depends on an unidentified "endogenous factor" which helps absorption of calcium from the gut when required; e.g., after prolonged calcium deprivation.[32,37]

2. It raises plasma calcium level. Albright and Reifenstein[1] claimed that this is brought about by two physiological effects of vitamin D: (a) increased absorption of calcium from the gut, and (b) increased phosphorus excretion via the kidneys.

It does not seem that the rise in plasma calcium following vitamin D administration is dependent on increased absorption of calcium from the gut. Dancaster and Jackson[8] showed that plasma calcium level may rise under the influence of vitamin D without increased absorption of calcium from the gut.

Albright and Reifenstein's second proposition is also untenable. McJunkin et al.[23] showed that injection of vitamin D into nephrectomized rats produced rise in plasma calcium. However, it is impossible to understand how an increased phosphorus excretion via the kidneys will produce a rise in plasma calcium, since vitamin D usually raises the plasma phosphorus level.[1]

3. It increases urinary calcium. Albright and Reifenstein[1] proposed that this was secondary to the hypercalcemic effect of vitamin D. Dent[11] and Dancaster and Jackson[8] showed that the hypercalciuric effect of vitamin D is independent of its effect on plasma calcium.

Vitamin D has an effect on plasma citrate which parallels the plasma calcium

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level. Carlsson and Hellinger[6] injected vitamin D into calcium deficient rats. There was an initial drop in plasma citrate (6–8 h.) followed by a rise. The plasma calcium level paralleled the plasma citrate even in the initial drop.

Despite the recognition of these profound effects of vitamin D on calcium metabolism, very little is known regarding its effects on magnesium metabolism. McHargue and Roy[22] found that when they subjected rats to ultraviolet irradiation, the carcasses' content of magnesium was less than in control animals. Smith[33] failed to find any effect after irradiation or administration of vitamin D on the fecal magnesium in calves.

In view of this lack of information about the effects of vitamin D on magnesium metabolism, an experiment was undertaken to show whether vitamin D has any effect on plasma, urinary and/or fecal magnesium.

Material and Methods

Twenty-four female hooded rats, 150–170 Gm., were used. Rats were fed on a homogenized liquid diet by stomach-tube for an equilibration period of 5 days before the metabolic studies. This procedure was continued during the balance period (3 days). The homogenized diet was prepared daily by mixing diet, stock vitamin solution and chromium sesquioxide with distilled water; 10 ml. of diet mixture contained 8 Gm. of diet, 0.2 ml. of vitamin mixture and 50 mg. chromium sesquioxide. The diet and stock vitamin mixture were those described by MacIntyre and Davidson.[23] Each rat's daily intake of Mg and Ca on this diet was 288 and 2400, μEq. respectively. Chromium sesquioxide was added to the diet because this substance is not absorbed from the gut. By referring the fecal excretion of substances to that of chromium the need of accurate fecal collection is eliminated and the scatter of balance data by irregular colonic emptying is avoided.[14]

After equilibration rats were housed separately in perspex metabolic cages. Feces and urine were collected separately by using a separating device.

Twelve rats were used as a control group, the other 12 were given (starting from the first day on balance) 40,000 units of vitamin D_2 per day. The vitamin was given in single intramuscular doses each morning of the three days of the experiment. On the fourth day rats were anesthetized and samples of blood were collected from the aorta.

Magnesium and calcium were estimated using the flame-spectrophotometric techniques described by Alcock et al.[4] Plasma and urinary phosphorus were determined by the Auto-Analyser* using the technic recommended by the manufacturers.

Results

Urine: Urinary excretion of calcium, magnesium and phosphorus is given in table I and is expressed graphically in figure 1. From the first day of the experiment, urinary calcium was significantly higher in the vitamin D-treated animals than in the control group. The mean urinary magnesium in the vitamin D-treated group was also higher than in the control group, but the difference between the two groups was not significant. The difference increased until the third day when the mean urinary magnesium was 182 μ-Eq. per day in the vitamin D-treated group, compared with a mean of 111 μ-Eq. per day for the control group ($P < 0.01$). The mean urinary inorganic phosphate was slightly lower in the vitamin D treated group than in the control group on the

*Technicon Instruments Corporation, New York, N.Y.

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Table 1.—*Effect of Vitamin D on Urinary Ca, Mg and P*

	Urinary Ca*		Urinary Mg*		Urinary PO_4†	
	Control	Treated	Control	Treated	Control	Treated
Number of animals	12	9	12	9	12	9
1st day						
Mean	39	117‖	136	147	526	461
S.E.M.	5.8	18.9	6.5	6.6	41	30
2nd day						
Mean	52	245‖	139	164	539	571
S.E.M.	7.4	27.3	8.2	12.3	20.9	32
3rd day						
Mean	37	576‖	111	182‖	448	642‖
S.E.M.	4.9	65.5	4.8	10.4	15.9	42.7

*mEq./day.
†μmol./day.
‖$P < 0.01$.

first day. On the third day urinary inorganic phosphate was significantly higher in the vitamin D-treated rats than in the control animals.

Feces: Table 2 gives the fecal excretion of calcium and magnesium and figure 2 illustrates these results graphically. The fecal excretion of calcium was significantly lower in the vitamin D-treated rats (mean: 1603 μEq./day) than in the control group (mean: 2456 μEq./day). This was also true for magnesium, the mean fecal magnesium for the vitamin D-treated animals being 107.0 μEq./day compared with 183.7 μEq./day for the control group.

Plasma: (Fig. 2, table 3.) In the vitamin D-treated animals, plasma calcium and inorganic phosphorus were significantly higher than in the control group. Plasma magnesium was significantly lower in the vitamin D-treated animals.

Discussion

The results of this experiment confirm the known effects of vitamin D on calcium metabolism. However, the object of the experiment was to show whether this vitamin has any effect at all on magnesium metabolism. Large doses of vitamin D were deliberately given in the hope that any possible action of the vitamin on magnesium metabolism would be exaggerated. The effect of vitamin D on the intestinal absorption of magnesium is shown to equal in this respect its effect on calcium. The effect on urinary magnesium, however, is not marked until the third day of administration of the vitamin. Lowering of the plasma magnesium level under the influence of vitamin D raises two important points:

1. Obviously the hypermagnesiuria cannot be explained in terms of change in plasma magnesium. If changes in urinary magnesium and urinary calcium are produced by the same mechanism after administration of vitamin D (as can be expected from their chemical similarity), the explanation of hypercalciuria in association with treatment with vitamin D as due to hypercalcemia[1] would

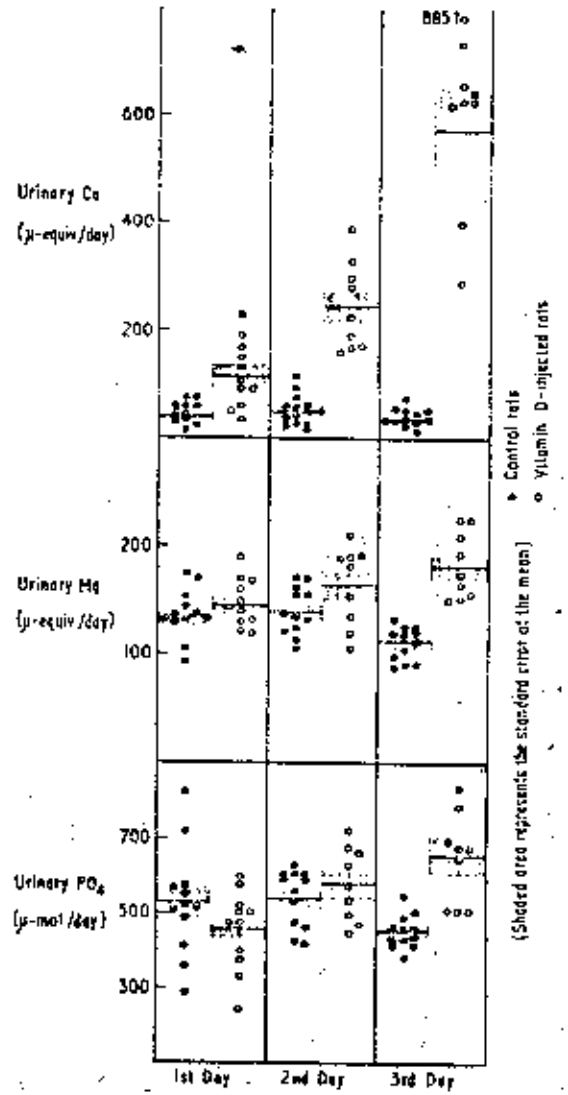

Fig. 1.—Effect of vitamin D on urinary calcium, magnesium and phosphate.

seem unlikely. This may add some evidence in favour of Dent's conclusion that increase in urinary calcium following the administration of vitamin D is independent of its effect on plasma calcium.[10]

2. Hypocalcemia is sometimes treated with large doses of vitamin D. Most of the pathologic conditions which produce hypocalcemia may lead as well to hypomagnesemia. This was shown to occur following parathyroidectomy,[11] and in association with steatorrhoea.[12,13] In view of this hypomagnesemic effect of vitamin D, it may be of importance to take this into consideration when treating such patients.

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Table 2.—*Effect of Vitamin D on Fecal Ca and Mg*

	Fecal Ca*		Fecal Mg*	
	Control	Treated	Control	Treated
Number of animals	12	9	12	9
Mean	2456	1603‖	185.7	107.0
S.E.M.	141	122	15.9	7.9

*mEq./day.
‖$P < 0.01$.

Because of the chemical similarity between magnesium and calcium and in view of the marked effect of vitamin D on urinary and fecal magnesium, a discussion of the mechanism of action of this substance on urinary and fecal magnesium and calcium is justified.

Very little is known concerning the renal handling of calcium and magnesium.[31] Albright's school tried to attribute changes in urinary calcium produced by vitamin D and parathyroid hormone to changes in plasma calcium level.[1] However, efforts to correlate urinary and plasma levels of calcium are frequently frustrated by nonconforming results. Patients suffering from idiopathic hypercalciuria usually have normal plasma calcium levels.[18,29] Injection of vitamin D may produce hypercalciuria despite a falling plasma calcium level.[10] Hypercalcemia in hyperparathyroidism may be accompanied by hypocalciuria.[9]

A similar lack of correlation is also observed in magnesium metabolism. In this experiment, vitamin D was shown to produce hypermagnesiuria despite the presence of hypomagnesemia. Hypermagnesiuria may also occur following injections of growth hormone and in cases of acromegaly or hyperparathyroidism, despite the presence of normal or low plasma magnesium.[16]

A promising approach to a better understanding of the renal handling of calcium is that adopted by Freeman and Chang,[11] Chen and Neumann[8] and B[illegible].[9] These authors proposed that the small but uniform urinary excretion of calcium consists predominantly of complexed calcium, mainly as citrate, presumably because ionized calcium is completely absorbed by the kidney tubules. Besides their experimental findings, there is ample evidence in favour of this suggestion. A large proportion of the conditions known to be associated with hypercalciuria show high plasma citrate level. This is true for hyperparathyroidism,[9] treatment with vitamin D,[6] administration of thyroxin,[25] injection of parathyroid hormone[1] and acromegaly.[18]

[illegible] acid chelates magnesium as well as calcium. Injection of neutralized [illegible] acid in human subjects was shown to produce a tremendous increase in urinary magnesium (Hanna, unpublished). Since vitamin D is known to increase the plasma citrate level this may be the explanation for the hypermagnesiuria in presence of hypomagnesemia under the influence of this vitamin.

Several factors are known to influence the fecal excretion of calcium. However, despite a wide variety of data, there is no broad understanding of the physiologic processes involved in the absorption of calcium and magnesium from the gut.

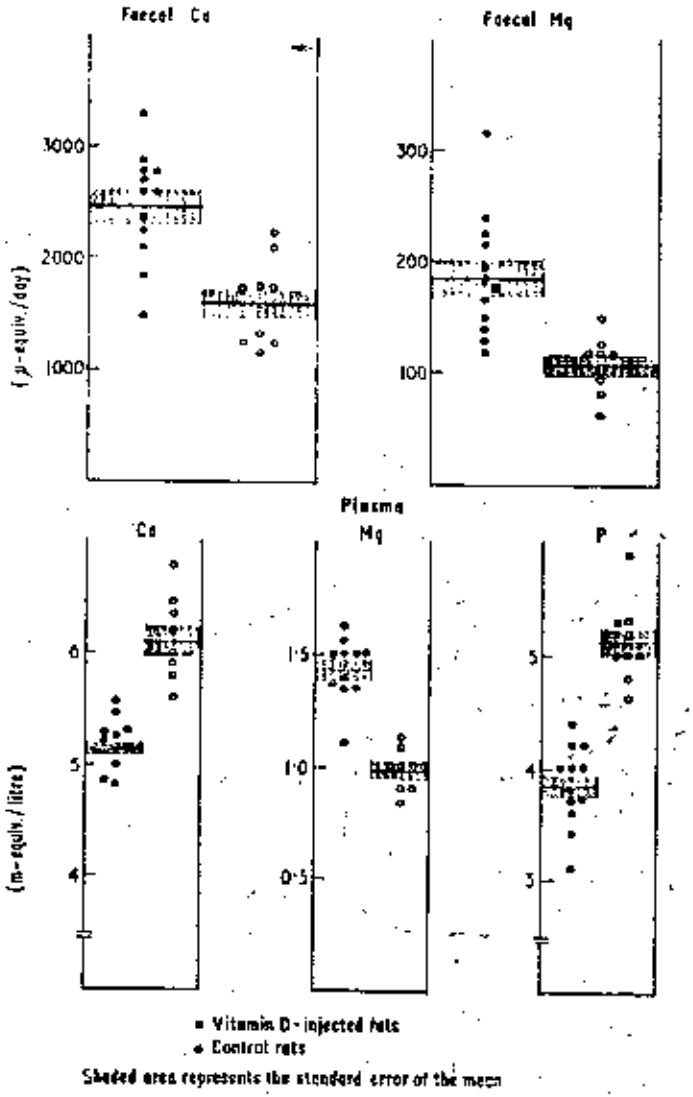

Fig. 2.—Effect of vitamin D on faecal excretion of calcium and magnesium and on plasma level of calcium, magnesium and phosphate.

Three important features revealed by studies in calcium and magnesium metabolism may provide a better understanding of the intestinal absorption of these substances:

1. Despite the wide range of urinary calcium output in normal adults, the daily urinary output of each subject is relatively constant.[21,27] Yet, whatever

Table 3.—*Effect of Vitamin D on Plasma Ca, Mg and P*

	Plasma Ca*		Plasma Mg*		Plasma PO4†	
	Control	Treated	Control	Treated	Control	Treated
Number of animals	12	9	12	9	12	9
Mean	5.17	6.11‡	1.13	0.98‡	3.84	3.12‡
S.E.M.	0.06	0.13	0.039	0.035	0.10	0.12

*mEq./l.
†mmol/l.
‡$P < 0.01$.

the daily variations of calcium intake may be, each normal adult maintains a net balance for calcium when considered over long periods. This means that intestinal absorption must be related in some way to urinary calcium.

2. Most of the conditions associated with hypercalciuria show an increased absorption of calcium from the gut.[18,19] In these conditions a negative calcium balance is sometimes produced because the urinary loss exceeds the high absorption, but the balance will be corrected if the intake is increased.[1,20]

3. It seems that calcium and magnesium are absorbed from the gut by the same mechanism. Alcock and MacIntyre[4] showed that deficiency of one of these two divalent cations produces increased absorption of the other. MacIntyre et al.[21] found that increased intake of calcium produces higher fecal loss of magnesium. The findings reported here show that vitamin D promotes the absorption of both calcium and magnesium from the gut. In acromegaly and in subjects receiving growth hormone the same effect was noticed.[16]

These three important features point to a common mechanism for absorption of calcium and magnesium from the gut, which is related in some way to the urinary loss of these substances. The increased urinary loss in hypercalciuric states cannot be explained by increased intestinal absorption since urinary calcium output exceeds greatly the total intake during periods of low calcium intake in these conditions. It is also difficult to visualize a mechanism by which the body will "know" how much calcium or magnesium is [illegible] via the kidneys and try to "correct" it by increasing absorption from the gut [illegible] plasma levels are not low in these subjects. A possible explanation is [illegible] the same mechanism involved in producing hypercalciuria enhances the absorption of calcium and magnesium from the gut.

[illegible]trate metabolism plays a role in the urinary excretion of calcium and magnesium, it could be that the absorption of these substances from the gut is [illegible] dependent. It is therefore suggested that the effects of vitamin D: (1) [illegible] citrate formation or diminish citrate metabolism in some tissues [illegible] bone?); which leads (2) to formation of citrate-gradients between these tissues and plasma; (3) citrate, chelating calcium and magnesium will [illegible] from these tissues to extracellular space; (4) part of the citrate will be metabolized by some tissues setting free calcium and magnesium ions; and (5) the rest of the citrate will pass into the urine, taking with it chelated calcium and magnesium.

This sequence of events needs further evidence and is now under investigation.

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Effect of Dietary Vitamin D and Protein on Free Amino Acids and Lipids in Selected Rat Tissues [1]

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ABSTRACT Weanling male rats were fed for 6 weeks vitamin D-deficient rations which contained 2 levels of protein with varying levels of supplemental calciferol. Vitamin D decreased the concentration of free lysine but had no apparent effect on free methionine or valine in plasma. Increasing the level of the vitamin significantly increased the concentration of cholesterol and total lipids in liver of rats fed 9% of casein but had no significant effect on that of animals fed 18% of casein. Increasing the protein intake decreased the level of lipids in liver of animals fed no vitamin D supplement or either level of the supplementary vitamin. The results suggested that amino acid-calciferol ratio may be important in lipid metabolism.

The work of Colovos et al. (1) showed that insufficient vitamin D in the ration of calves significantly decreased protein utilization and inhibited the growth rate. Since then, Jonxis and Huisman (2) observed that vitamin D_2 therapy reduced the amount of amino acids excreted by rachitic children. Steenbock and Herting (3) reported that vitamin D may be concerned with metabolism of soft tissue. Other workers have observed that addition of calciferol to the diet of experimental animals increases the concentration of cholesterol in body tissues (4, 5). On the basis of these observations, the present study was made to determine the effect of dietary vitamin D on the concentration of certain free amino acids in plasma and cholesterol and total lipids in liver tissue of rats fed at 2 levels of protein.

EXPERIMENTAL

Male rats weighing 50 to 70 g of the Sprague-Dawley strain, were housed in individual galvanized metal cages with wire bottoms and given food and water ad libitum during the 6-week experimental period. Three groups of 12 rats each, similar in weight and littermate distribution each received diets which contained 9% of protein and zero, 100 or 10,000 IU of vitamin D (calciferol) per week. Three more groups received 18% of protein with corresponding amounts of vitamin D. The vitamin D supplements, which were administered orally 3 times/week, were prepared by the addition of weighed quantities of calciferol to weighed amounts of cottonseed oil to give the desired concentrations of calciferol and to provide a comparable amount of carrier for each group of animals. The complete diets are shown in table 1.

Blood from non-fasted, decapitated rats was collected in 15-ml centrifuge tubes which contained 3 mg of sodium heparinate (6). Equal volumes of plasma from 4 rats in each dietary group were pooled. Protein-free filtrates were prepared (7) and assayed microbiologically for lysine, methionine and valine (8).

Liver tissue from each rat was analyzed for cholesterol (9) and total lipids of liver were measured by the method of Bloor (10) as modified by Okey and Lyman (11).

RESULTS

Free amino acids in plasma. The plasma levels of lysine, methionine, and valine of the animals receiving the different diets are shown in table 2. Supplemental feeding of 100 or 10,000 IU of vitamin D/week decreased the concentration of plasma lysine at both levels of protein intake, but had no significant effect on methionine or valine (F value for methionine = 0.88, for valine = 0.66, required

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TABLE 1

Composition of diets

	9% Protein	18% Protein
	%	%
Casein [1]	9	18
Sucrose	78	69
Cottonseed oil [2]	5	5
Salt mixture [3]	4	4
Cellulose [4]	4	4
		mg/kg
Thiamine·HCl		5.0
Pyridoxine·HCl		2.5
Riboflavin		8.0
Ca pantothenate		20.0
Niacin		10.0
Biotin		0.1
Folacin		2.0
Vitamin B_{12}		0.02
Inositol		100.0
p-Aminobenzoic acid		100.0
Menadione		0.5
Choline chloride		1300.0
Vitamins A, E [5]		

[1] Vitamin-Free Casein, General Biochemicals, Inc., Chagrin Falls, Ohio.
[2] Wesson Oil, Wesson Sales Company, Fullerton, California.
[3] U. S. Pharmacopoeia, ed. 14, p. 789, 1950.
[4] Solka Floc, Brown Company, San Francisco.
[5] Cottonseed oil-vitamin D preparation provided 1000 IU vitamin A acetate and 0.6 mg α-tocopherol/rat/week.

for $P < 0.05 = 3.88$). The effect of vitamin D on plasma lysine was significant at the 1% level. Increasing the casein level of the diet significantly increased ($P < 0.01$) the concentration of plasma methionine and valine but had no significant effect on plasma lysine ($F = 1.98$, required for $P < 0.05 = 4.75$).

Liver cholesterol and total lipids. Increasing the dietary protein from 9 to 18% significantly decreased ($P < 0.01$) the concentration of liver cholesterol and of total lipids (table 3). When 9% of casein was fed, the concentrations of liver cholesterol and of total lipids increased when vitamin D was added to the diet. The effect was highly significant ($P < 0.01$). Vitamin D had no effect on the concentration of lipids in liver of animals fed 18% of protein.

Growth. A summary of the growth data is shown in table 3. At the close of the experimental period the total weight gain of animals fed 9% of casein was not significantly affected by the level of dietary vitamin D. However, the mean body weight of animals fed no vitamin D supplement had been less than that of the other 2 groups until the last week of the

TABLE 2

Effect of vitamin D on free amino acids in plasma of rats fed at 2 levels of protein

Dietary protein level	Vitamin D (calciferol)	Free amino acids [1]		
		Lysine	Methionine	Valine
% of diet	*IU*	*μg/ml*	*μg/ml*	*μg/ml*
9	0	70.7 ± 0.4	7.9 ± 0.4	20.8 ± 2.1
9	100	66.6 ± 1.4	6.1 ± 0.1	21.5 ± 1.5
9	10,000	66.5 ± 1.4	6.8 ± 0.1	24.3 ± 3.4
18	0	77.0 ± 1.9	11.8 ± 0.4	33.7 ± 2.6
18	100	63.9 ± 3.9	12.4 ± 0.3	29.7 ± 3.6
18	10,000	70.5 ± 2.1	12.2 ± 0.5	33.1 ± 2.8

[1] Mean value of 3 pooled samples ± SE of mean.

TABLE 3

Influence of dietary vitamin D on weight gain, cholesterol and total lipids in liver of rats fed at 2 levels of protein

Dietary protein level	Vitamin D (calciferol)	Body wt gain [1]	Liver wt [1]	Total cholesterol [1]	Total lipids [1]
% of diet	*IU*	*g*	*g*	*mg/g*	*mg/g*
9	0	100 ± 3	6.5 ± 0.40	2.34 ± 0.2	43.2 ± 2.5
9	100	98 ± 4	6.8 ± 0.14	3.61 ± 0.3	60.5 ± 5.2
9	10,000	96 ± 4	7.1 ± 0.08	5.32 ± 0.7	81.6 ± 9.9
18	0	214 ± 8	11.0 ± 0.05	1.86 ± 0.04	36.6 ± 0.4
18	100	257 ± 5	13.3 ± 0.05	1.90 ± 0.05	36.1 ± 0.5
18	10,000	264 ± 5	13.5 ± 0.08	1.95 ± 0.04	37.9 ± 0.6

[1] Mean of 12 animals ± SE of mean.

experiment when it surpassed them. When 18% of casein was fed, the weight gain of animals fed 100 IU of the vitamin was significantly greater ($P < 0.01$) than that of animals receiving no vitamin supplement. The weight gain of animals fed 10,000 IU of the vitamin did not differ significantly from that of animals receiving the lower level of vitamin supplement. The weight gain of animals fed 18% of casein was more than twofold that of corresponding animals fed 9% of casein.

During the last 2 weeks of the experiment a marked change occurred in the appearance of the animals fed 9% of casein plus calciferol. The rats showed alopecia over parts of the body and the remaining hair was matted and coarse. The irregular appearance of the fur was most pronounced in 6 animals fed 100 IU of vitamin D and 4 animals fed 10,000 IU of the vitamin. The fur of all animals fed 9% of casein with no vitamin D supplement was smooth. At the end of the experimental period, the animals fed 18% of protein were all healthy in appearance.

DISCUSSION

Since previous work (12) indicated that the level of free lysine, methionine and valine in plasma of the rat may be affected by certain dietary nutrients, these amino acids were selected for the study of the relationship between dietary vitamin D and free amino acids in plasma. The results suggest that the elevated level of lipids in liver of animals fed 9% of protein compared with those fed 18% of casein were due, in part, to the decreased levels of valine and methionine in plasma. The effect of dietary methionine on lipid deposition in tissues has been well documented (12–15). Sidransky and Baba (16) reported that rats force-fed a diet deficient in valine developed fatty livers. The data for animals fed 9% of protein suggest further that the change in ratio of plasma lysine to other amino acids which resulted from the addition of vitamin D to the diet may have been the critical factor which precipitated the higher levels of liver lipids in the animals fed this supplement. Reports from other laboratories have shown a relationship between dietary lysine and the concentration of lipids in tissues (16–18). In the present study the decreased level of lysine in plasma of animals fed 18% of protein plus vitamin D had no effect on lipid tissues. This suggests that amino acids or other nutrients not included in the analyses may also be related to vitamin D and lipid metabolism at low levels of protein intake. In a recent evaluation of mineral mixtures used for experimental studies Williams and Briggs (19) pointed out that the USP XIV salt mixture used in this study is deficient in zinc and supplies less than the National Research Council (20) recommendation for certain other minerals. The apparent healthy condition of all animals fed 18% of protein indicated that zinc per se was not a contributing factor in observations of this study.

Whether the use of a higher level or a different type of fat in the present study would have shown a relationship between vitamin D and lipids in animals fed 18% of protein is unknown. Nath and Harper (5) obtained evidence that the addition of 1 mg of calciferol to 100 g of a diet which contained 25% of hydrogenated coconut oil and 20% of casein with added methionine increased deposition of cholesterol and total lipids in liver of rats. In the studies of Lee and Herrmann (4) the addition of vitamin D to a diet which contained 66% of sucrose, 18% of casein and 8% of hydrogenated oil increased serum and liver cholesterol of normal rats. With a diet of commercial laboratory chow[2] and added corn oil these workers showed that vitamin D had no effect on lipid concentration in tissues of the rat.

The use of pooled samples was necessary because of small amounts of blood but the data presented indicate that dietary vitamin D may be related to lipid and amino acid metabolism.

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EXPERIMENTAL ARTERIAL DISEASE PRODUCED BY CHOLESTEROL AND VITAMIN D.

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(Plate LI.)

Most authorities are agreed in including mechanical factors as possible agents in the ætiology of arterial disease, but hitherto experimental proof of the mechanical hypothesis has been difficult to furnish. Although experimental lesions, such as cholesterol sclerosis, can be readily produced in the rabbit, the mechanical factors concerned in their production have not been satisfactorily investigated. With the recent discovery of vitamin D sclerosis, a useful method of approach to the problem has presented itself, for two distinct types of experimental arterial disease are now readily available for such investigation, namely cholesterol sclerosis affecting the intima on the one hand, and vitamin D sclerosis consisting of calcification of the media, on the other. Both these processes interfere to some extent with the elasticity of the vessel wall, and must consequently interfere more or less with its mechanical functions. Thus an investigation of the mechanical hypothesis may be attempted by producing the two lesions successively in the one animal, and studying the effects of the mechanical disturbances resulting from the first lesion, on the development and distribution of the second. This was the procedure adopted in the following two experiments.

Experiment 1.

In this experiment cholesterol sclerosis was produced in the rabbit's aorta, and later vitamin D lesions were superimposed. The plan of the experiment is set out in the table (p. 448).

Twelve young rabbits (each about one year old) were given a course of 1 grm. of cholesterol daily by mouth, lasting until one of them died on the eighty-fourth day and another on the ninetieth day, both having developed severe aortic lesions. It was then concluded that the administration had been sufficient, and the treatment was accordingly stopped on the ninetieth day. After an interval of ten days, six of them (group A) were given a course of 100,000 M.R.C. units of irradiated ergosterol in olive oil daily by mouth, while the remaining four were

	Number.	Sex.	Initial weight in grms.	Age in months.	Treatment.	Duration of cholesterol treatment.	Duration of irradiated ergosterol treatment.	Killed or died.	Intimal aortic lesions.	Medial aortic lesions.	Intimal lesions in other vessels.	Medial lesions in other vessels.	Renal calcification.
					First Experiment.								
A.	1	F	1800	12	Cholesterol 1 grm. per day followed by irradiated ergosterol 100,000 units per day	90 days	62 days	Killed	+	+	+	...	+
	2	F	1590	14		90 "	62 "	"	+	+	+	+	+
	3	M	1720	13		90 "	14 "	Died	+	+	+	...	+
	4	M	1320	11		90 "	15 "	"	+	+	+	...	+
	5	M	1750	12		90 "	33 "	"	+	+	+	+	+
	6	M	1950	12		90 "	16 "	"	+	+	+	...	+
B. Control.	7	M	1930	15	Cholesterol 1 grm. per day	83 "	...	"	+	...	+	...	...
	8	F	2280	15		89 "	...	"	+	...	+	...	...
	9	M	1890	13		90 "	...	Killed	+	...	+	...	...
	10	M	1730	12		90 "	...	"	+	...	+	...	...
	11	M	1600	12		90 "	...	"	+	...	+	...	...
	12	M	1670	13		90 "	...	"	+	...	+	...	...
C. Control.	13	F	1930	11	Irradiated ergosterol 100,000 units per day	...	14 days	Died	...	+	...	+	+
	14	F	1530	12		...	62 "	Killed	...	+	...	+	+
	15	F	2190	17		...	16 "	Died	...	+	...	+	+
	16	F	1660	16		...	21 "	"	...	+	...	+	+
	17	M	1510	11		...	21 "	"	...	+	...	+	+
	18	M	1630	11		...	16 "	"	...	+	...	+	+
					Second Experiment.								
	19	M	1200	10	Irradiated ergosterol 100,000 units followed by cholesterol 1 grm. per day	83 days	11 days	Killed	+	+	+	+	+
	20	M	1550	12		83 "	11 "	Died	...	+	...	+	+
	21	F	2140	14		83 "	11 "	Killed	+	+	+	+	...
	22	F	1640	12		83 "	11 "	"	+	+	+	...	+
	23	F	1940	12		83 "	11 "	"	+	+	+	+	+
	24	M	1900	13		83 "	17 "	"	...	...	+	...	+

2440

allowed to live as controls for six months. These, together with the two already dead, constituted the control group B. A further control group of six rabbits (group C) were given a course of irradiated ergosterol treatment as above, but without any previous cholesterol treatment. In order to accentuate the calcifying effects (Schmidtmann 1929, Rabl 1929, Duguid 1930) 1 grm. of calcium lactate was added to the diet of all the animals receiving irradiated ergosterol. The irradiated ergosterol treatment was continued for sixty-two days, at the end of which time four of the rabbits of group A and five of the control group C were already dead. The remaining three rabbits were then killed. Throughout the experiment the animals were given a diet of bran and oats, varied by the addition of cabbage twice a week. The animals of group A, in spite of their previous treatment with cholesterol, bore the irradiated ergosterol treatment equally as well as those of group C, which were presumably healthy at the outset of the course of treatment.

In all cases, severe and extensive aortic lesions of the required types developed, and in the rabbits of group A, vitamin D sclerosis was successfully produced in aortas which were already the seat of severe cholesterol sclerosis. It is unnecessary to enter into a detailed account of the histology of these two lesions, which were always clearly distinct from one another. The plaques of intimal sclerosis produced by cholesterol conformed to the accepted descriptions of such lesions as given by Anitschkow and Chalatow 1913, Anitschkow 1914, Bailey 1916, Scarff 1927 and others; and similarly the medial calcification produced by irradiated ergosterol corresponded with that described by Kreitmair and Moll 1928, Kreitmair and Hintzelmann 1928, Huckel and Wenzel 1929, Duguid 1930, Varela Collazo Moreau and Rubino 1930 and other workers. Both types of lesion favoured the proximal half of the aorta including the arch, so that in the rabbits of group A both lesions were intermingled in this region of the vessel. In these latter animals, the influence of the one process on the other was clearly shown by the relative distribution of the two lesions. With striking uniformity, the vitamin D sclerosis was confined to the parts left unaffected by the pre-existing cholesterol sclerosis, so that practically invariably the media subjacent to the cholesterol plaques was free from calcification. The abruptness with which the medial calcification stopped short at the edges of the cholesterol plaques seemed strongly to suggest that the latter in some way protected the subjacent media from vitamin D calcification (fig. 1). So notably was this the case, that in the aortas which were most severely affected the two lesions, intimal and medial, alternated in an almost unbroken chain around the circumference of the vessel, the second lesion taking up all the space left unoccupied by the first (fig. 2). Apart from the familiar vitamin D lesions, granular masses of calcification were sometimes found in the fatty debris in the deeper parts of the cholesterol plaques; this was present even in the rabbits which had not received irradiated ergosterol, although in them it was never quite so marked as in those which had.

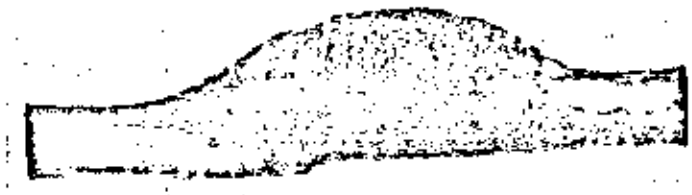

FIG. 1.—Frozen section. Haemalum and sudan III. × 42. Arch of aorta. The medial calcification stops short at the edges of the intimal cholesterol plaque.

FIG. 2.—Frozen section. Haemalum and sudan III. × 10. Arch of aorta. The calcified vitamin D lesions (which appear as black lines in the media) alternate with the intimal lesions.

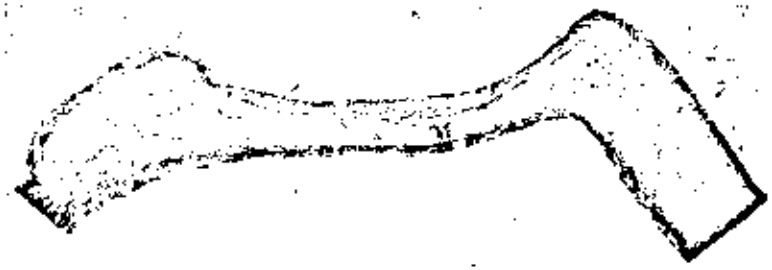

FIG. 3.—Frozen section. Haemalum and sudan III. × 20. Arch of aorta. Cholesterol lesions localised to the edge of a calcified area.

FIG. 4.—Frozen section. Haemalum and sudan III. × 43. First part of aorta. Cholesterol lesions localised over points of fracture of the calcified media.

Experiment 2.

The clear-cut results of the above experiment prompted an investigation of the problem from the reverse aspect, and accordingly in experiment 2, vitamin D sclerosis was produced as a preliminary lesion in order that its effects on the distribution of a subsequent cholesterol sclerosis might be studied.

Six young rabbits (about twelve months old) were given a daily dose of 100,000 M.R.C. units of irradiated ergosterol together with 1 grm. of calcium lactate, until one of them died on the eleventh day. This animal was found to have developed severe aortic calcification, and since the other five all showed signs of toxæmia, the treatment was stopped. After an interval of ten days, during which the animals appeared to recover, the remaining five were given a course of cholesterol feeding (1 grm. per day as in experiment 1) which lasted for eighty-three days. During this treatment all gained weight and improved in appearance. One month after the termination of the treatment they were killed.

All but one of the five showed the desired aortic lesions of both types intermingled (the one exception was free from arterial disease of any kind). The vitamin D sclerosis, as usual, took the form of irregular patches of rigid medial calcification, which in the severest cases occupied most of the wall of the ascending aorta and the arch. Again as in experiment 1, the distribution of the second lesion bore a well-defined relationship to that of the first. The cholesterol lesions were for the most part localised either to the non-calcified areas of the vessel or to the margins of the calcified patches (fig. 3). The only places where intimal lesions were found directly superimposed upon areas of calcified media were at points where the calcified tissue had fractured and become displaced (fig. 4). In two cases, where the calcification was so severe as to occupy practically the whole circumference of the vessel, the cholesterol lesions mapped out, in abrupt relief, the narrow interstices between the calcareous patches. Thus it appeared that the calcified parts of the vessel generally escaped the cholesterol sclerosis whilst the parts immediately surrounding them were especially susceptible.

Discussion.

Both these experiments show that the distribution of arterial lesions is affected by the presence of pre-existing lesions. In order to show how this bears on the mechanical hypothesis of arterial disease, the immediate effects of the respective pre-existing lesions must first be analysed. Cholesterol sclerosis, which was used as the preliminary lesion in the first experiment, can be shown to have a considerable effect on the mechanical working of the vessel. The blood vessel is an elastic tube, whose function is to expand and recoil with each pulse wave. The expansion is a passive movement determined by the distensile force of the blood, whilst the recoil is an active one, depending on the inherent elastic properties of the tissues. Hence

any lesion, such as cholesterol sclerosis, which imposes relatively inelastic fibrous tissue on the wall, will have the effect of lessening the elasticity of the part concerned. That this is the case can be appreciated from a histological examination. In severe cholesterol sclerosis, the media subjacent to the intimal plaques commonly appears thinned and its elastic laminæ less wavy than in other parts. Obviously, compared with the rest of the wall the tissue is held in a relatively stretched position, its recoil being hampered by the thickened intima. In short, the movements of the vessel wall tend to be diminished at the site of the cholesterol plaques. The fact that the subsequent medial calcification of hypervitaminosis D is clearly localised to the parts which are not so affected seems to indicate that the process of calcification is associated with the mechanical mobility of the vessel.

In the second experiment where vitamin D sclerosis provided the preliminary lesion, it was again found that the one lesion affected the distribution of the other. With certain exceptions, the cholesterol plaques were confined either to the unaffected parts of the wall or to the margins of the vitamin D lesions. Again, it is necessary to consider the effects of the preliminary lesion on the mechanical working of the vessel. The fully-calcified vitamin D lesions render the affected parts of the vessel wall firm and rigid (a point which I have confirmed by examining an animal dying under an anæsthetic). The tissues become fixed in the stretched or distended position because the calcification, in so far as it destroys the media, destroys its function of elastic recoil. This point is made obvious histologically by the thinning of the wall and straightening of the elastic laminæ in the affected areas. Thus vitamin D sclerosis, even more than cholesterol sclerosis, has the effect of lessening the elasticity and the mobility of the tissues of the aorta. The particular localisation of the cholesterol lesions therefore indicates that the more mobile parts of the wall are the more susceptible to the development of these lesions. To explain the special predilection of cholesterol sclerosis to the margins of the vitamin D lesions, this hypothesis must be pushed further, and an exaggerated or disordered mobility postulated as the exciting factor. It can hardly be doubted that in an elastic tube, such as a blood vessel, built of concentric layers of tissue, the introduction of rigid patches into some of the layers will introduce disharmony in the form of disordered and, in some regions, exaggerated movements. Such would occur chiefly at the junction of the rigid with the non-rigid tissues, where in fact the cholesterol lesions are especially common. This suggestion that cholesterol lesions are determined by disordered movements is supported by their occurrence also over points at which the calcified media is fractured. At these points, serious structural disorganisation can frequently be demonstrated and the accompanying intimal proliferation can, as was shown by Duguid in the rat's aorta, be interpreted as a process of repair — at least in so far as the initial stages are concerned.

Histologically the intimal lesions at the points of fracture are essentially similar to those occurring at the margins of the calcified areas, and there seems to be no obvious reason for doubting the possibility that repair of damaged tissues is the basis of the change in both cases, although the initial damage may not always be in evidence.

Whatever may be the exact sequence of events in the disease processes with which these two experiments are concerned, one general point seems to emerge, namely that mechanical factors play a highly important part in the localisation of the lesions. Both vitamin D sclerosis and cholesterol sclerosis show a tendency to fall upon the most mobile parts of the vessel wall and to avoid such parts as are rigid or less elastic. This is no new conception, but merely an experimental confirmation of the observations of many workers in this field. The localisation of cholesterol lesions in the rabbit's aorta to the arch and to the origins of the branches has frequently been interpreted as indicating that the more mobile parts of the wall suffer the most destruction in the course of the disease. In a similar way, the localisation of vitamin D sclerosis, especially in the first part of the aorta, has been interpreted as indicating mechanical factors in its ætiology also.

A further point arises in the first experiment which, though not bearing directly on the mechanical hypothesis, is of some interest; namely that the vitamin D calcification affects the apparently healthy media rather than the damaged intima. This has some bearing on the actual process of calcification in vitamin D sclerosis. Although several authorities consider that this is preceded by some degenerative change in the tissues, no such degeneration has yet been satisfactorily demonstrated. In experiment 1, a recognised tissue degeneration was provided in the form of intimal cholesteatosis. If vitamin D calcification were simply a consequence of tissue degeneration combined with the hypercalcæmia of hypervitaminosis D, then the intimal lesions ought to have formed the chief sites of calcification, which they did not do. Some deposition of calcium did occasionally occur in the cholesterol lesions, but it was never more than an exaggeration of what is normally found in such lesions when they are undergoing healing, and it was of an entirely different form from the true vitamin D sclerosis. The latter process would therefore appear to be governed by some entirely specific change in the medial tissues, and simple degeneration in the broad sense cannot explain it.

The bearing of these findings on human arterial disease need not be discussed in this paper. The relationship of cholesterol sclerosis in the rabbit to human atherosclerosis has been fully traced by Anitschkow, whilst that of vitamin D lesions in animals to Mönckeberg sclerosis in man has been discussed by Duguid. I believe that such interpretations as have been put upon the results of these two experiments can also be applied in the ætiology of human arterial disease; and that whatever be the exciting cause, the localisation of human atheroma is largely determined by the mechanical activity of the vessel wall.

Since this work was undertaken, combined experiments of the type described above have been published by Schmidtmann and Pfleiderer. Both these workers produced cholesterol and vitamin D sclerosis in the same animal, but neither considered the problem from the mechanical aspect, and they published no details of the topographical relationship of the one lesion to the other.

Summary.

1. In experiment 1, cholesterol sclerosis was induced in the rabbit's aorta as a preliminary lesion and vitamin D sclerosis was later superimposed. In experiment 2, vitamin D sclerosis constituted the preliminary lesion and cholesterol sclerosis was then added.

2. In both cases, the second lesion was confined to the parts of the vessel left unaffected by the first lesion.

3. Both cholesterol sclerosis and vitamin D sclerosis render the affected parts of the vessel wall relatively immobile, and this immobility appears to render them less susceptible to the subsequent lesion.

4. It is concluded that the movements of the vessel determine the localisation of both vitamin D and cholesterol lesions and probably also human arterial disease.

I should like to thank Professor J. B. Duguid, at whose suggestion this work was undertaken, for his kindly help and criticism. I should also like to thank H. Jephcott and A. L. Bacharach, who very kindly supplied the irradiated ergosterol.

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PARATHYROID HORMONE AND VITAMIN D**

Along with many others of my generation at the Yale School of Medicine and the New Haven Hospital, I owe a great debt to Grover Powers as an inspiring teacher, friend, and benefactor. The invitation to give the Grover Powers lecture at Yale had a special emotional significance, therefore, and it is with a sense of great privilege that I address you this afternoon. I do not consider the primary purpose of this lecture as an occasion to honor Grover Powers. He has received so many honors under such important auspices that anything that I could add today would be relatively minor. The major purpose is to remind ourselves and succeeding classes of students at Yale of the great heritage which Grover Powers has given us—his teachings, counseling, and personal example which have contributed so much to the development of pediatrics in this country and to the emergence of the Yale University School of Medicine as an important influence in medical education and practice.

In Osler's felicitous words, "The great possession of any university is its great names—not its wealth, nor the number of its schools but the men who have trodden in its service the thorny road through toil." Grover Powers is one of the great possessions of this school and his contributions continue to have relevance to the problems that we face today. One of the current preoccupations and concerns of members of clinical departments in our medical schools is the balancing of the importance of fundamental research against the responsibilities of the department for teaching and for the care of patients in the teaching hospital. This is not a new problem and I should like to remind you that within a relatively small department Grover Powers developed two major research laboratories: in electrolyte physiology under Daniel C. Darrow and in microbiology and immunology under James D. Trask. His department was noted for its devotion to the teaching of medical students and residents, and also of physicians in this State interested in maintaining continued excellence in the care of children. The extraordinary dedication of Grover Powers to the care of patients and the success with which he transmitted this dedication to his students is legendary.

Inasmuch as Grover Powers was directly responsible for my start in medical research, I have chosen to devote this Grover Powers lecture to a review of our research efforts in a single area. Exciting contributions in the biological sciences have been made in the elucidation of the structure of biologically important molecules and in the understanding of the mechanisms that link the structure of the genetic material, DNA, with the structure of specific proteins. As physiologists, however, we are interested in molecular function and in structure as it is expressed in function. As physicians we are hopeful that knowledge of molecular structure and function can be applied to the understanding, diagnosis, and treatment of the abnormalities of physiologic states which are present in our patients. Although studies of the action of molecules that regulate cell functions may seem of only theoretic interest, in the long run such information will contribute to our understanding of disease. For many years my wife and I have been interested in unraveling the role of two molecules that control the concentration of Ca^{++} ion in the body fluids: parathyroid hormone and vitamin D, and this report represents our joint research.

Aurbach and Rasmussen have both succeeded in purifying parathyroid hormone.[1,2] It is a single chain polypeptide of a molecular weight of about 8,000, although somewhat smaller peptides have been split off which still have biological activity. The amino acid composition is known but the exact sequences and the precise molecular configuration have not yet been determined. The purified peptide acts on the renal tubule to reduce the amount of filtered phosphate which is retrieved by the tubule cells thus increasing the excretion of phosphate in the urine. There are differences of opinion whether this action is through the blocking of phosphate transport from tubular lumen to cell to peritubular fluid or through increased transport from peritubular fluid to cell to lumen. At the moment this need not concern us. Another action of this peptide is to activate bone cells, osteoclasts and osteocytes, so that through their increased metabolic activity local changes result that effect the dissolution of the bone crystals in contact with the cell surfaces and the proteolysis of the demineralized collagen. The biochemical mechanism through which parathyroid hormone produces these alterations of phosphate transport and of bone cell activity is unknown. Rasmussen[3] has suggested that parathyroid increases inorganic phosphate uptake by mitochondria *in vitro* and that its primary action is to alter membrane properties with respect to divalent ion transport. The specificity of

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this mitochondrial reaction has been disputed since Aurbach has reported that peptides which are physiologically inert when given to the animal have the same action on mitochondrial systems.

In contrast to parathyroid hormone, vitamin D, the structure of which has been known for over 30 years, is a relatively simple organic molecule which is usually classified in the group of steroids. Ever since highly concentrated and purified preparations of vitamin D have been available it has been recognized that this steroid in pharmacologic doses can be used as substitution therapy in patients with hypoparathyroidism. In these doses, which are more than 100 times the physiologic requirement, vitamin D restores to normal the serum calcium and phosphorus concentrations of the hypoparathyroid subject and to all intents and purposes replaces the missing parathyroid hormone. Parathyroid hormone, on the other hand, cannot replace vitamin D since vitamin D deficiency occurs in patients with intact parathyroids. Although parathyroid hyperplasia occurs in response to deficiency of calcium ion, this parathyroid overactivity does not rectify the abnormal physiologic state in vitamin D depleted subjects. Indeed the parathyroid overactivity is thought by some to intensify the abnormality of bone mineralization in vitamin D deficiency rickets.

The hypocalcemia of vitamin D deficiency in the face of parathyroid hyperplasia is a familiar phenomenon and in the rachitic or osteomalacic subject had been ascribed to structural changes in the poorly mineralized bone which supposedly made the bone calcium less available. In order to reinvestigate this problem we produced vitamin D deficiency in rats without rickets.[7] This animal can be depleted of vitamin D and rickets prevented by feeding a suitable proportion of calcium and phosphate in the diet. The bones of such rats are histologically normal and bone ash content is not reduced. There is some reduction of body growth in comparison with vitamin D fed controls. The blood serum analyses show a striking hypocalcemia while serum phosphorus concentrations remain in the high normal range of young rats (Table 1). The parathyroid glands of these rats are enlarged. This suggested to us that the tissues of the vitamin D deficient rats were unresponsive to parathyroid hormone. As shown in Table 1, when doses of parathyroid extract that produce hypercalcemia and hypophosphatemia in normal rats were given to the Vitamin D depleted animals, no effect was seen. When the animals were given a small dose of vitamin D (500 units) 48 hours before the injection of parathyroid extract, the expected hypercalcemia and depression of serum phosphate followed the parathyroid hormone treatment. We concluded that vitamin D was required for the responsiveness of the end organs to physiologic amounts of parathyroid hormone. These studies were repeated with purified parathyroid

hormone including determinations of urine phosphate excretion.[7] Again the hypocalcemic vitamin D depleted rats failed to respond to parathyroid hormone either by a rise in serum calcium or by an increase in urine phosphate and decrease of serum phosphate. Repletion with vitamin D restored the normal response to parathyroid hormone (Table 2). These experiments have been repeated by Rasmussen with essentially similar results. Rasmussen has pointed out, however, that parathyroidectomy in the vitamin D deficient rat causes a further increase of serum phosphate but no change

TABLE 1. VITAMIN D AND RESPONSE TO PARATHYROID EXTRACT

Group	Serum Ca	Serum P
	mg/100 ml.	
Deficient	5.9	10.2
Deficient + P.T.E.*	5.7	9.7
Vitamin D**	10.9	13.2
Vitamin D** + P.T.E.*	13.3	8.2

* Parathyroid extract (Lilly). 100 units injected subcutaneously at 24 and 6 hours before blood sample.

** 500 units of vitamin D_2 orally 72 hours before blood sample (48 hours before first P.T.E. injection).

Underlined figures are significantly different from values in rats not receiving parathyroid extract.

in serum calcium.[8] His interpretation of the data is that vitamin D is required for the response of bone cells to parathyroid hormone but that the effect of parathyroid hormone on phosphate transport can occur to some degree in the absence of vitamin D. Studies of the interrelation of vitamin D and parathyroid hormone have been made by DeLuca, *et al.*[9] using separated kidney mitochondria. Under conditions in which ATP and oxidisable substrate are available, mitochondria accumulate calcium and inorganic phosphate and the accumulated ions are liberated when the high energy phosphate bonds are exhausted. The rate of liberation or mobilization of accumulated calcium from the mitochondria is greatly retarded if preparations are obtained from tissues of vitamin D deficient rats and the rate is accelerated by addition of calciferol or parathyroid hormone *in vitro*. The parathyroid hormone effect, however, is obtained only if vitamin D is present in the system either by addition or by virtue of the treatment with vitamin D of the animal from which the mitochondria were obtained.

Another type of *in vitro* study also indicates the role of vitamin D in sensitizing tissues to parathyroid hormone. Raisz[10] incubated embryonic rat bone in tissue culture in a medium containing rat serum to which para-

thyroid hormone was added. In the presence of this hormone, cell proliferation occurred to a much greater extent than in control preparations without parathyroid hormone. If the rat serum added to the culture medium was obtained from vitamin D deficient rats rather than normal animals, the addition of parathyroid hormone did not produce cell proliferation.

The uptake of inorganic phosphate by mitochondrial suspensions is accelerated by addition of parathyroid hormone to the incubation medium and this effect is obtained even though the tissues supplying the mitochondria

TABLE 2. VITAMIN D AND RESPONSE TO PURIFIED PARATHYROID HORMONE

Group	Serum Ca	Serum P	Urine P
	mg/100 ml.		mg/6 hrs.
Deficient	7.9	9.2	1.9
Deficient + P.T.H.*	7.9	8.8	2.5
Vit. D**	9.6	10.7	3.1
Vit. D** + P.T.H.*	12.5	5.5	9.2

* Parathyroid hormone equivalent to 100 units injected subcutaneously 6 hours before blood sample. Urine collected during this 6 hour interval.
** Vitamin D_2, 100 units 24 hours before blood sample.
Underlined figures are significantly different from values in rats not receiving parathyroid hormone.

are removed from vitamin D deficient rats. The physiologic significance of this reaction has been questioned, however, because of the high concentrations of parathyroid peptide used in this experiment and because peptides that are physiologically inert in the intact animal will also produce this effect on isolated mitochondria.

In vitamin D deficient infants with rickets, parathyroid extract does not raise the serum calcium nor induce phosphaturia until after vitamin D treatment has been given. It is probable, however, that the unresponsiveness of the vitamin D depleted subject to parathyroid hormone is not complete and is dose dependent. In dogs and in rats of a strain other than the ones used by Rasmussen and ourselves, Bartter[8] and Toverud[9] respectively have found a response to parathyroid extract despite apparent deficiency of vitamin D. However, the vitamin D deficient dogs and rats are hypocalcemic with concomitant parathyroid hyperplasia, and in a recent report Raisz and his co-workers have shown that the enlarged parathyroid glands of such hypocalcemic rats are physiologically active in demineralizing bone under *in vitro* conditions so that their ineffectiveness in the intact animal almost certainly represents relative unresponsiveness of the end organ

in the absence of vitamin D.[10] It is likely that vitamin D sensitizes specific target tissues to parathyroid hormone and the inhibitory effect of vitamin D deficiency may be overcome by increasing the hormone concentration greatly above the physiologic level. It is of interest that Stanbury[11] has extended

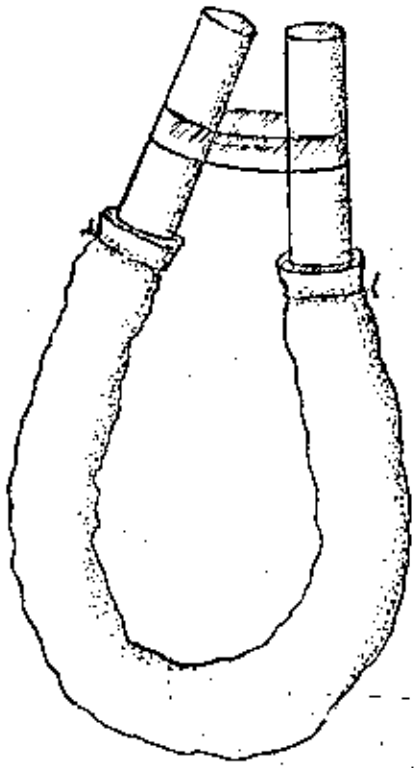

FIG. 1. Everted loop of rat small intestine preparatory to immersion in buffer solution. The mucosal surface is on the outside and solutes are transported across the mucosal layer and are collected in the fluid filling the serosal lined interior of the loop. (Taken from Harrison and Harrison.[14])

this concept to explain the hypocalcemia and the marked parathyroid hyperplasia of subjects with renal insufficiency. He has postulated that these patients are vitamin D resistant and are relatively unresponsive to parathyroid hormone unless given doses of vitamin D much in excess of ordinary requirements. It is well-known that large doses of vitamin D given to patients with chronic renal insufficiency will restore the serum calcium to normal and reverse the bone changes of secondary hyperparathyroidism.

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Further examination of the specific biochemical reactions influenced by vitamin D and parathyroid hormone is required. In the instance of vitamin D, studies making use of surviving segments of small intestine *in vitro* have been informative. In the technique developed by Wilson and Wiseman[11] these segments are everted so that the mucosa is on the outside and the ends tied off to form closed sacs. We have modified the method using open ended everted loops[16] (Fig. 1). The mucosal epithelium of these everted sacs or loops forms the outside surface and continues to pump solutes from the luminal surface across the serosal pole of the cell into the interstitial fluid from which the solutes diffuse into the space lined

TABLE 3. EFFECT OF VITAMIN D ON CONCENTRATIVE TRANSPORT OF CALCIUM BY RAT SMALL INTESTINE

	*Cs/Cm**	
Intestinal Segment	Vit. D Deficient	Vit. D.** Treated
Duodenum	1.06	3.39

* Ratio of concentrations of Ca^{45} in loop contents (Cs) and mucosal fluid (Cm) at end of period of incubation. Initial ratio = 1.
** 1,000 units of vitamin D given to rats 72 hours before intestine removed for preparation of loops.

by the serosal cell layer. The transported solutes thus accumulate in the serosal fluid within the loop at a higher concentration than in the mucosal solution and the efficiency of the transport system can be measured by the ratio of the concentration of the ion or molecule in serosal fluid to its concentration in the fluid bathing the mucosal surface. When the transport of calcium is studied by this method it is found that intestinal loops from vitamin D deficient rats have a greatly diminished capacity to transport calcium in comparison with loops from animals given vitamin D (Table 3). This transport of calcium against a concentration difference is an energy requiring process. However the movement of calcium across the intestinal mucosa can be separated into two stages: the first, which is not directly dependent on metabolic energy, is the diffusion of calcium into the mucosal cell at the luminal surface and the second is the energy requiring active transport of calcium against a concentration difference from cell into the interstitial fluid. To examine whether vitamin D influences the first step, everted loops of rat small intestine were studied[14] under conditions in which metabolic energy production was inhibited either by low temperature or addition of a metabolic inhibitor, N-ethyl maleimide. In these experiments the intestinal mucosa acted as a semi-permeable membrane rather than an

active transport system and the permeability of the membrane to calcium was being tested. Varying concentrations of calcium labeled with Ca^{45} were placed on the mucosal surface whereas the serosal fluid initially contained no calcium. At the end of the period of incubation the quantity of

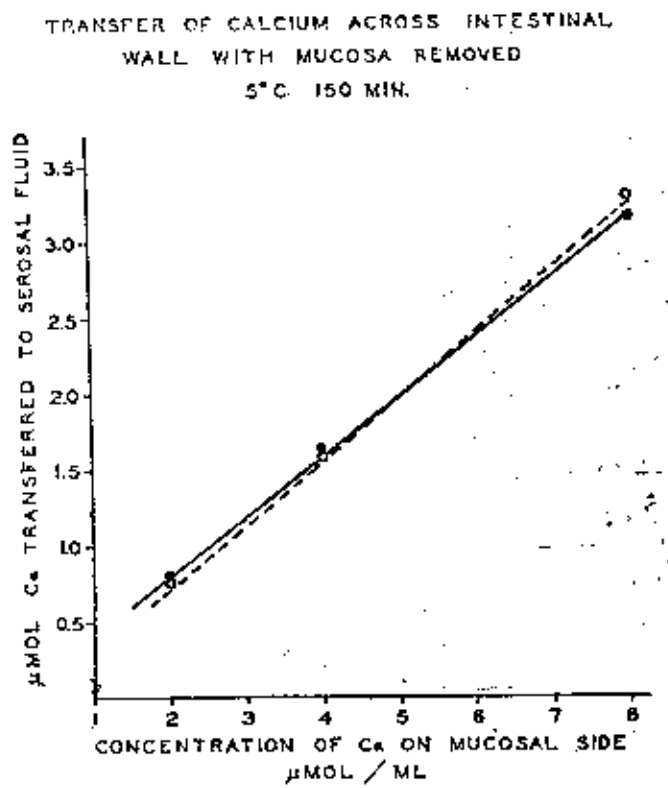

FIG. 2. Calcium transport in everted intestinal loops from which mucosa has been removed, incubated at 5° C. •——• loops from vitamin D-treated rats, o----o loops from vitamin D-deficient rats. Each point represents the average value of determinations on 4-8 loops. (Taken from Harrison and Harrison.[14])

calcium entering the serosal solution was determined. Intestinal loops from vitamin D deficient and vitamin D treated rats were studied and the importance of the mucosal cell layer was also examined by use of preparations from which the mucosa had been mechanically scraped off. If we first look at one of the latter preparations incubated at 5° C. (Fig. 2), there is a strict linear relation between the calcium entering the serosal phase and the initial concentration of calcium on the mucosal surface which is com-

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passible with diffusion. No difference between loops from vitamin D deficient and vitamin D treated rats is found in this preparation from which mucosal cells have been removed. Similar studies with loops with intact mucosa (Fig. 3) show the same linear relation between mucosal calcium concentration and calcium entering the serosal phase but there is now a definite effect of vitamin D treatment in increasing the quantity of calcium diffusing across the intestinal wall. The results at 37°C. with a metabolic inhibitor

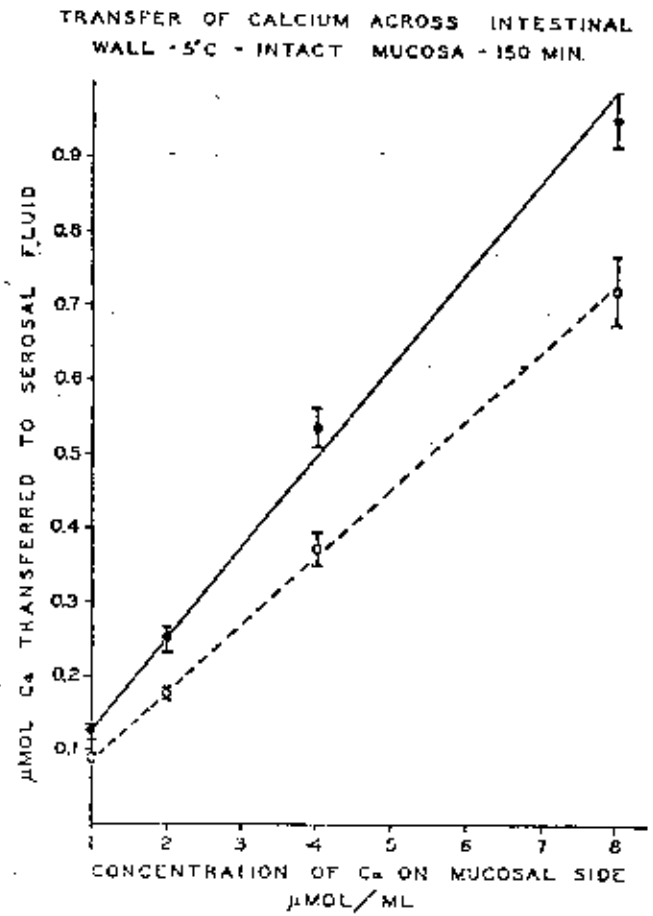

FIG. 3. Calcium transport in everted jejunal intestinal loops with intact mucosa incubated at 5° C. •——• loops from vitamin D-treated rats, o- - - - o loops from vitamin D-deficient rats. The vertical lines indicate ± standard error of mean. (Taken from Harrison and Harrison.[6])

present are similar (Fig. 4 and 5). We have concluded that the intestinal mucosal cells present a diffusion barrier to calcium, and that vitamin D, in effect, alters the permeability of the membrane to calcium so that the diffusion barrier is reduced. The net effect of vitamin D on calcium trans-

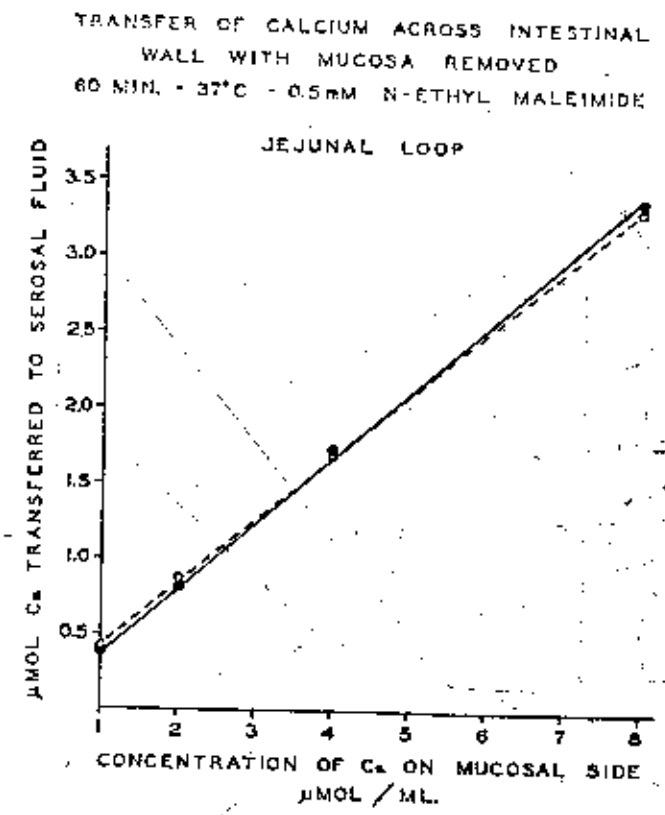

FIG. 4. Calcium transport in everted intestinal loops from which mucosa has been removed, incubated at 37° C, 0.5 mM/liter n-ethyl maleimide in medium. •——• loops from vitamin D-treated rats, o- - - - o loops from vitamin D-deficient rats. (Taken from Harrison and Harrison.[6])

port could depend on the more rapid penetration of calcium into the cell, which increases the amount of calcium available to the energy dependent transport system. It is interesting to compare this action of vitamin D on calcium transport with the action of d-aldosterone on sodium transport in an *in vitro* system. Sharp and Leaf[8] have suggested that the major action of aldosterone on the toad bladder is "manifest as an increase in the

permeability to sodium of the mucosal surface of the single layer of epithelial cells."

During the past few years the theory has been proposed[4] that certain hormones produce their effects through a genetic mechanism, i.e., the

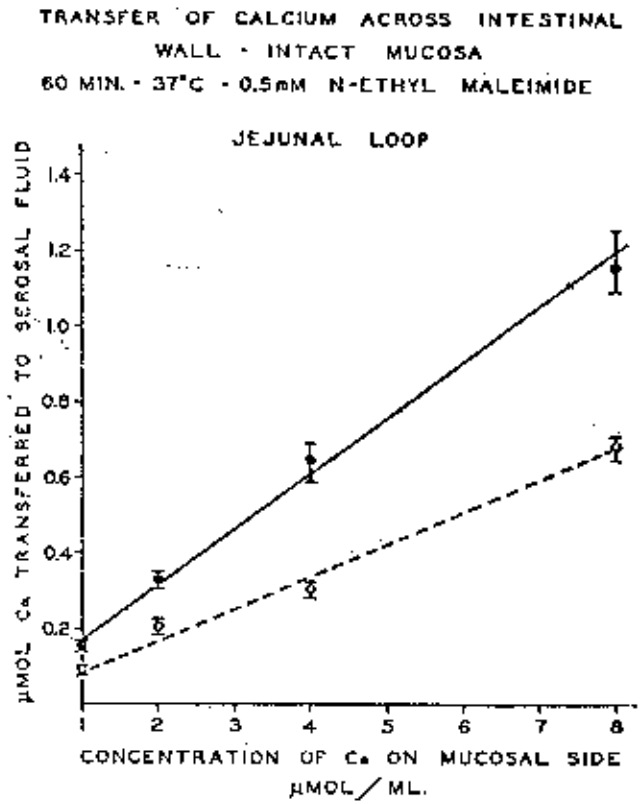

FIG. 5. Calcium transport in everted jejunal intestinal loops with intact mucosa incubated at 37° C. 0.5 mM/liter n-ethyl maleimide in medium, ●——● loops from vitamin D treated rats, o——o loops from vitamin D-deficient rats. The vertical lines indicate ± standard error of mean. (Taken from Harrison and Harrison.[5])

hormone activates specific gene loci in the DNA molecule which promotes the production of new messenger RNA and ultimately the synthesis of specific enzymes. One type of evidence used to support this theory is the inhibition of hormone effects by prior administration of actinomycin D. This antibiotic, which has some value as an antitumor agent, is a complex polypeptide which complexes with DNA at the guanine locus and thus

blocks the action of RNA polymerase in forming messenger RNA molecules on the DNA template. Actinomycin D does inhibit the physiologic activity of vitamin D, preventing both the increase of serum calcium and the increased transport of calcium across the intestinal wall. Figure 6 shows the inhibition of the serum calcium raising effect of vitamin D by

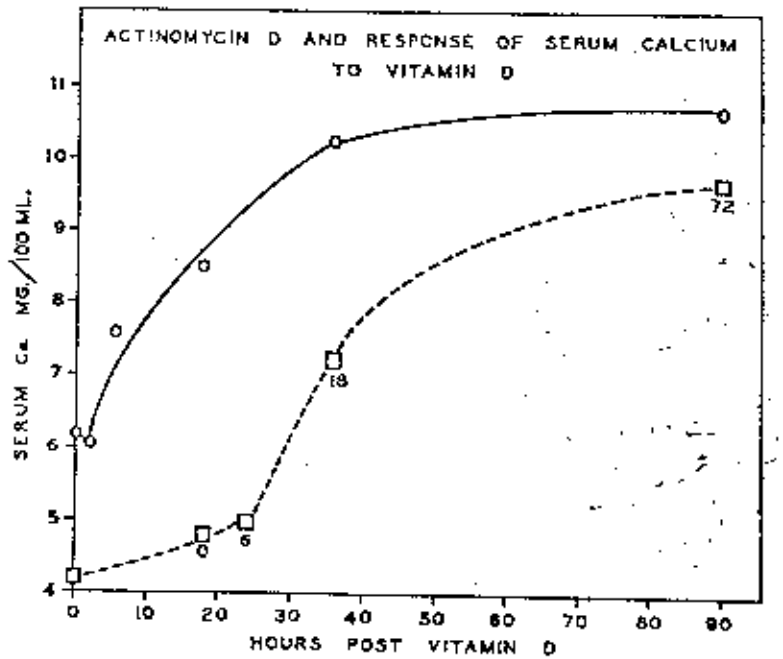

FIG. 6. The effect of actinomycin D in blocking the action of vitamin D on serum calcium concentrations of vitamin D-deficient rats. The solid line shows the increase in serum calcium level with time following a single oral dose of 50,000 units of vitamin D_2 to hypocalcemic vitamin D-deficient rats. The dashed line shows the serum calcium concentrations of rats given the same vitamin D treatment but also injected with actinomycin D 1 μg/gm body weight either simultaneously with the vitamin D or at the intervals after vitamin D, shown by the figures under the points which represent hours. The serum calcium concentration of the actinomycin D-treated rats was measured in every instance 18 hours after the injection of the compound. The initial points of both curves give the serum values of rats not given vitamin D. Each point represents the average of determinations on 4 to 8 rats.

actinomycin D treatment in experiments in which actinomycin was given at various intervals after a dose of vitamin D and serum calcium concentrations measured 18 hours after the actinomycin injection. Treatment of the vitamin D deficient rat with actinomycin D reduces the serum calcium levels below the abnormally low levels due to vitamin D deficiency and in-

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inhibits the vitamin D effect in raising the serum calcium concentrations. This inhibition is almost complete unless the vitamin D is given between 6 to 18 hours before the actinomycin D injection. It is also important to emphasize that actinomycin D decreases the active intestinal transport of calcium in the vitamin D deficient rat below the already low levels due to lack of vitamin D (Table 4). The Cs/Cm values of much less than one after actinomycin D treatment indicates that transfer of calcium across the mucosal surface is reduced to negligible amounts so that the mucosal concentration remains unchanged whereas the Ca^{45} concentration is serosal

TABLE 4. INHIBITION OF INTESTINAL TRANSPORT OF CALCIUM BY ACTINOMYCIN D (VITAMIN D DEFICIENT RATS)

Treatment	*Conc. transport of calcium (Cs/Cm)*
None	1.1 ± 0.11
Actinomycin D*—3 hrs.	0.69 ± 0.13
Actinomycin D*—7 hrs. (Vit. D** —4 hrs.)	0.54 ± 0.04
Vitamin D**—4 hrs.	2.1 ± 0.05

*Actinomycin D 1 μg/gm. body weight injected subcutaneously at stated time before intestine removed for *in vitro* incubation.
**Vitamin D, 50,000 units given orally at stated time before intestine removed.

fluid diminishes below the initial value owing to diffusion into the intestinal wall. The effect of actinomycin D is to inhibit the calcium transport system itself either by some direct toxic effect or by blocking the regeneration of a labile component. There is no evidence that vitamin D operates through a genetic mechanism by inducing mRNA and protein synthesis. Its mechanism of action may be its direct combination with some component of cell membranes.

Some understanding of the possible link between the action of vitamin D in increasing the rate of penetration of calcium into cells and its sensitization of cells to parathyroid hormone action may be afforded by studies of intestinal transport of inorganic phosphate.[17] By use of the everted intestinal loop the transport of inorganic phosphate across the intestinal wall against a concentration difference can be demonstrated *in vitro* (Fig. 7). This transport, like other active transport systems, requires metabolic energy. The concentration differences developed by this system are much less in preparations from vitamin D deficient rats than in

those from vitamin D treated animals (Fig. 8). The effect of vitamin D on this transport function is apparently mediated through calcium ion since removal of calcium from the buffer solution in which the loops are incubated reduces the concentration ratio of phosphate between serosal

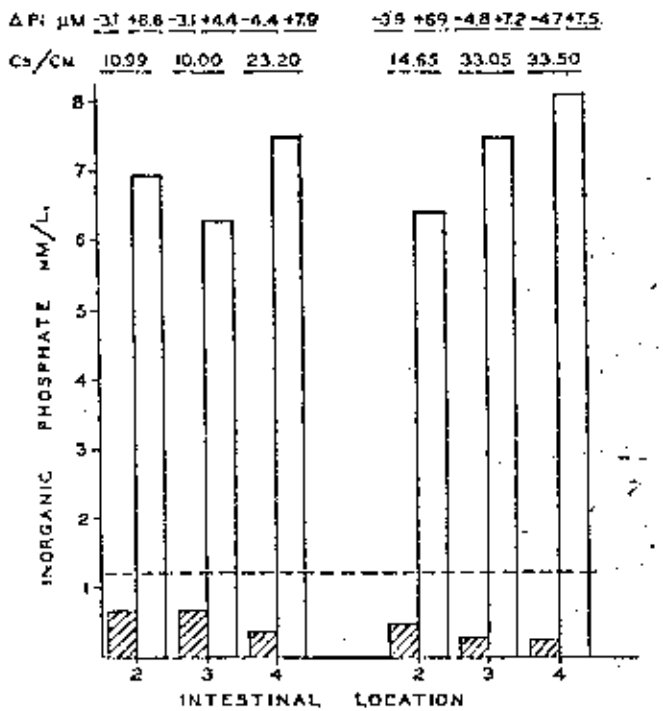

FIG. 7. Concentrative transport of inorganic phosphate across wall of everted intestinal loops *in vitro*. The horizontal dashed line indicates the initial concentration of phosphate in both mucosal and serosal fluids. Height of the inked column indicates concentration of phosphate in mucosal fluid at end of incubation, height of the hollow column indicates concentration of phosphate in serosal fluid at end of incubation. The ratio, Cs/Cm, is shown at the top of each experiment. (Taken from Harrison and Harrison.[17])

and mucosal fluids and if calcium ion is completely removed by addition of a low concentration of a chelating agent, EDTA, the transport of phosphate ceases and the effect of vitamin D is therefore abolished (Fig. 9).

There are, therefore, direct effects of vitamin D and secondary effects presumably mediated through the action of vitamin D in increasing the concentration of calcium in the cell surface or within the cell. We can speculate that the sequence of reactions in the physiologic response to

parathyroid hormone includes a step in which calcium or other divalent cation is a necessary cofactor. In the absence of vitamin D, the concentration of divalent cation in a cell compartment is too low to permit the parathyroid hormone initiated chain of reactions to occur except possibly to a limited extent in the presence of very high concentrations of hormone.

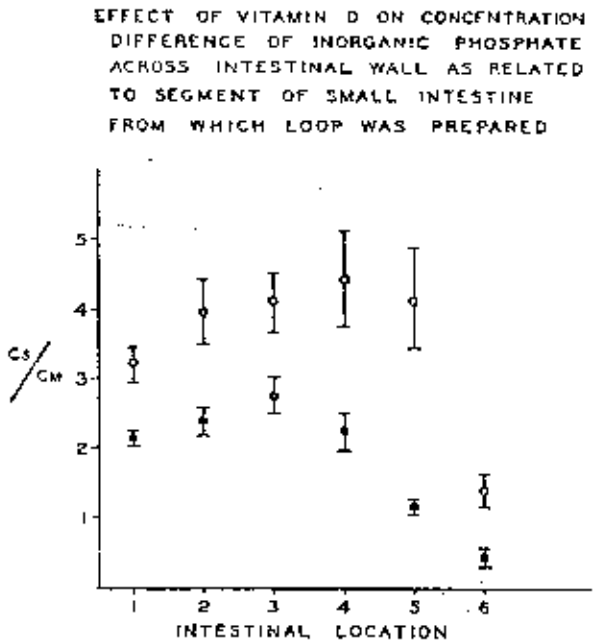

FIG. 8. Effect of Vitamin D treatment of rats upon the concentrative transport of inorganic phosphate across wall of everted intestinal loop *in vitro*. Cs/Cm ratios are averages of values of 4 to 15 loops. • loops from vitamin D-deficient rats, o loops from vitamin D-treated rats. The vertical lines indicate ± standard error of mean. (Taken from Harrison and Harrison.[17])

In the absence of hormone, high concentrations of vitamin D may trigger the cell response by increasing intracellular calcium above the usual physiologic concentration. This could explain the action of high doses of vitamin D in the hypoparathyroid subject. This, of course, is speculative. We do not as yet know the specific biochemical reaction that is responsible for the physiologic action of parathyroid hormone. Although the action of vitamin D appears to be at the cell surface with a resultant increase in the permeability of the cell membrane to calcium, the mechanism of this change in cell membrane properties is also undetermined. The data

presented do suggest that vitamin D acts as a regulatory agent in certain cell processes by governing the rate of movement of calcium and possibly other divalent cations across the plasma membrane or the membranes of cell organelles. Parathyroid hormone may also function by altering the

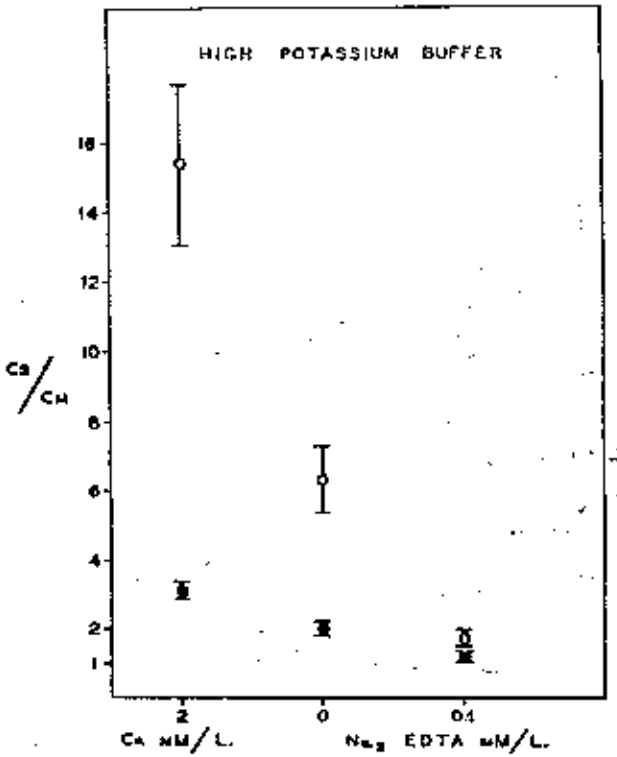

FIG. 9. Interrelation of calcium and vitamin D effect on concentrative transport of phosphate. Three series of experiments are diagrammed from left to right in which the medium contained 2mM/liter Ca, 0 Ca and 0.1 mM/liter Na_2EDTA respectively. Points are averages of Cs/Cm values from 7 to 39 everted intestinal loops from the jejunum of vitamin D-deficient rats (•) and vitamin D-treated rats (o). The vertical lines indicate ± standard error of mean. (Taken from Harrison and Harrison.[17])

properties of membranes that control the movement of divalent ions into specific compartments of the cell. It will remain for future investigators to determine whether these ideas will be fruitful in reaching the sought-after goal of determining the exact action of these specific molecules in controlling those cellular functions that regulate the flow of Ca^{++} into body fluids and into bone mineral.

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TURNOVER STUDIES OF 1,25-DIHYDROXYCHOLECALCIFEROL AND CHOLECALCIFEROL IN VITAMIN D DEFICIENT AND REPLETED CHICKS AND DOGS. D. L. Hartenbower*, H.C. Tsai*, H. L. Friedlert, J. H. Coburn and A. W. Norman. Wadsworth VA and Dept. Medicine UCLA and Dept. Biochem., Univ. Calif., Riverside, CA 92521.

A study was made of the plasma clearance and tissue uptake of cholecalciferol (CC) and 1,25-dihydroxycholecalciferol (1,25-(OH)$_2$-CC) in D-deficient and D-replete chicks and dogs. Mixed doses of ^{14}C-CC (1500 pmoles) and ^{3}H-1,25-(OH)$_2$-CC (250 pmoles) were given intracardially to 200g chicks, while a dose of 650 pmoles of ^{3}H-1,25-(OH)$_2$-CC was given i.v. to 5-15 kg dogs. Lipid extracts from plasma (chicks and dog) and tissues (chicks) were obtained from 10 min-36 hrs (dogs) or 72 hrs (chicks) following isotope administration. In the chick, prior vitamin D repletion resulted in lower circulating levels of isotopic CC and 1,25-(OH)$_2$-CC, diminished uptake of CC and particularly 1,25-(OH)$_2$-CC by the target organs the intestine, bone and kidney, and an enhanced uptake of CC and particularly 1,25-(OH)$_2$-CC by the liver. This suggests the liver as a possible site for breakdown or further metabolism of 1,25-(OH)$_2$-CC. The rate of plasma clearance in the chicks initially had a t 1/2 of 2-4 hrs, but once the normal circulating level of ca. 60 pg/ml had been achieved, there was relatively little further fall up to 72 hr. In the dog the rate of plasma disappearance in both the D-deficient and replete animals, did not fit a single exponential decay, suggesting multiple pathways of handling. (Supported by AM-5,012 and AM-14,750).

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Studies on the Effect of Vitamin D on Calcium Absorption and Transport

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1. Mucosal cells of the small intestine obtained from rats deprived of vitamin D or given excessive amounts of the vitamin accumulated significantly more calcium than did cells from control animals. 2. Mucosal cells from vitamin D-deficient rats released less calcium than did cells from normal or hypervitaminotic D animals. 3. Studies *in vivo* showed that the transfer of ^{45}Ca from the intestine to the blood was delayed in vitamin D deficiency, but was accelerated in hypervitaminosis D. 4. The findings support the thesis that vitamin D is involved in the release of calcium rather than in its uptake by mucosal cells. 5. Further evidence is presented suggesting that uptake of calcium by intestinal mucosal cells at 0° is primarily passive, whereas at 38° uptake and release are effected by an active process that depends on energy derived from glycolytic activity.

It is well established that absorption *in vivo* of calcium from the small intestine is dependent on vitamin D (Harris & Innes, 1931; Nicolaysen, 1937; Greenberg, 1945). Studies *in vitro* using the everted-gut-sac technique showed that previous treatment of the animals with vitamin D enhanced calcium transport against concentration gradients from fluids bathing the intestinal mucosa to those bathing the serosa (Schachter & Rosen, 1959; Harrison & Harrison, 1960; Schachter, Kimberg & Schenker, 1961). There is, however, disagreement whether this transport is the result of increased active transport (Schachter *et al.* 1961), increased permeability of the intestine to calcium (Harrison & Harrison, 1960, 1965; Wasserman & Kallfelz, 1962) or increased formation of a protein carrier for calcium (Wasserman & Taylor, 1963, 1966*a*; Taylor & Wasserman, 1966). In such preparations, the initial step in the transfer of calcium involves the uptake of calcium by mucosal cells, and this process is probably the same *in vivo*. However, the continued transfer of calcium through the other layers of the intestine, i.e. lamina propria, submucosa, muscularis externa and serosa, does not occur in the living animal, where calcium is transferred from mucosal cells into the vascular system. These layers of the intestine form diffusion barriers for calcium transport *in vitro* (Wasserman & Taylor, 1966*b*; Birnwitz, Harrison & Harrison, 1967) and Holdsworth (1966) showed that calcium binding by the muscle layers of the small intestine was greatly influenced by vitamin D. Therefore, interpretation of experiments designed to study the role of vitamin D or other substances in calcium absorption by measuring calcium transfer across the full thickness of the intestine may be hampered by effects of vitamin D on tissues underlying the mucosal cells, which are the functional absorbing units of the small intestine (Wilson, 1964).

Previous workers have attempted to isolate the absorptive cells from the remainder of the intestine, and have noted some of the conditions necessary for the survival of such preparations (Dickens & Weil-Malherbe, 1941). The uptake of formate into purines was successfully measured by using such a preparation (Paterson & Zbarsky, 1955, 1958), although after continued incubation as much as 95% of the DNA was sometimes lost. Also, villi preparations were used for studying the transport and release of ^{45}Ca (Rasmussen, Weldoff, Dziewiatkowski & DeLuca, 1963). More recently attempts were made to prepare epithelial cells free from contaminating mesenchymal cells by enzymic digestion (Harror, Stern & Reilly, 1961; Ferris, 1966), and Ferris (1966) showed that oxygen consumption was almost linear over a period of 1hr.

This paper describes the effect of vitamin D and other conditions on cell survival and uptake and release of calcium by mucosal cell suspensions of the small intestine of rats, which mainly contain absorptive cells. Such preparations permitted the

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study of calcium uptake and release, independent of underlying tissues that are present in intestinal slices or sacs. Further, the effects of vitamin D on the transfer of calcium *in vivo* from the intestine to the blood were also investigated.

MATERIALS AND METHODS

Preparation of rats. Male albino rats of the Holtzman strain were used in all experiments. In studies of the effects of vitamin D deficiency, weanling rats weighing 40–50g. were fed on a vitamin D-free diet (DeLuca, Gran & Stoenbock, 1957) until significant differences were obtained between their body weights and those of controls. This diet (DeLuca *et al.* 1957) was prepared in the laboratory and modified to contain 60% of glucose (Cerelose), 24% of protein (vitamin-free casein), 10% of fat (Mazola oil) and 4·14% of salt mixture. The complete diet contained 0·8% of Ca, 0·5% of P, 0·12% of Na, 0·30% of K, 0·05% of Mg, 0·10% of Cl and 0·11% of Fe. The diet also contained trace amounts of Co, Mn, Zn and I, in addition to all vitamins known to be required by the rat, except vitamin D. Vitamin D_2 (75 i.u.) in 0·1 ml. of sesame oil were given orally, three times per week, to control and pair-weighed rats. In the vitamin D-toxicity studies, rats weighing 200–250g. were used. Toxic doses of vitamin D (10000 i.u./100g. body wt./day) were also given orally to rats in 0·1 ml. of sesame oil. The rats were grouped as follows, so that the mean weight of each group was the same: control, pair-weighed and vitamin D deficient or hypervitaminotic-D. The pair-weighed group received restricted amounts of food daily to keep its body-weight gain about the same as that of the vitamin D-deprived or hypervitaminotic-D group. All rats were deprived of food overnight (16–20hr.) before they were decapitated. In the experiment designed to measure the release of calcium *in vitro*, the rats, after being kept without food overnight, received ^{45}Ca by stomach intubation and 30min. later were killed.

In studies of calcium uptake and release *in vivo*, the animals were killed at timed intervals after the oral administration of ^{45}Ca. Serum was collected and analyzed for calcium by a fluorometric procedure (Kapner & Hercules, 1963); ^{45}Ca was measured by liquid-scintillation counting (counting efficiency 80%) with Bray's (1960) solution, and phosphate by a modification of the method of Fiske & Subbarow (1925).

Preparation of mucosal cell suspensions. Immediately after decapitation, the small intestine (from the pylorus to the ileocaecal valve) was removed, flushed with 25 ml. of cold 0·9% NaCl and cut into 10cm. segments. A glass rod was then inserted into each segment and a longitudinal incision was made by passing the sharp edge of a scalpel over the intestine. The open strip was then laid flat over a large Petri dish inverted over ice. The mucosa was gently scraped with glass slides and the cell scrapings were suspended in cold 0·9% NaCl, filtered through nylon cloth to remove connective tissues and large masses of extraneous material and centrifuged at 500*g* for 5min. Histological examinations of the intestine before and after scraping showed that mainly the tips of the villi were removed. Examination of the cells after centrifugation revealed that they were mostly absorptive cells, although some small lymphocytes were also present. The sedimented cells were resuspended in Krebs–Ringer bicarbonate buffer (Krebs & Henseleit, 1932), previously gassed with O_2+CO_2 (95:5) to maintain pH 7·4. For anaerobic conditions the medium was gassed with N_2+CO_2 (95:5). The medium also contained tracer amounts of ^{45}Ca, which was used to follow the uptake of calcium by the cells, except in studies where ^{45}Ca was administered to the rats and its release from mucosal cells was measured.

Incubation of mucosal cell suspensions. Incubation of cell suspensions was carried out at 38° in a Dubnoff shaker in an atmosphere of O_2+CO_2 (95:5) or N_2+CO_2 (95:5) for the required times. After incubation, a sample of the suspension was centrifuged at 500*g* for 3–5min. and the pellet washed twice with ^{45}Ca-free cold buffer. The sedimented mucosal cells were extracted twice with 0·5M-$HClO_4$ at 70°. Portions of the extract were used for determination of DNA (Burton, 1956), calcium (DeLuca *et al.* 1957) and ^{45}Ca (Bray, 1960). Since there was the possibility of non-specific adsorption of ^{45}Ca on cell surfaces as well as its penetration into the cells, cells incubated at 0° were used as controls and the calcium uptake was defined as the difference between the ^{45}Ca uptakes by cells at 38° and at 0°. Also, since a variable amount of cells always survived to the end of each incubation period, the uptake was expressed as c.p.m./μg. of DNA of the cells sedimented at 500*g* for 5min., and calcium uptakes were expressed as the difference between the ratios of ^{45}Ca to DNA at 38° and at 0° of washed mucosal cells sedimented after incubation. Serum specific radioactivity was calculated by the following formula, which takes into account differences in body weight:

$$\text{Serum specific radioactivity} = \frac{\text{c.p.m./ml. of serum}}{\text{c.p.m./dose of } ^{45}Ca} \times \frac{1}{\mu\text{g. of Ca/ml.}} \times \frac{\text{Body wt. (g.)}}{100}$$

RESULTS

Stability of cell suspensions. Measurement of cellular DNA of sedimented cells before and after incubation (Table 1) in Krebs–Ringer bicarbonate buffer, pH 7·4, showed that about 75% of the cells disintegrated after 15min. of incubation at 38° when the medium contained 2·6mM-calcium. Increasing the calcium concentration to 5·0mM improved DNA recovery, and at a calcium concentration of 10mM the recoveries were about 75–80%; in some experiments recoveries were almost 100% (Table 2). At 0°, DNA recovery was practically complete, regardless of the calcium concentration in the medium. At 38° the uptake of calcium (c.p.m./μg. of DNA) rose with decreasing calcium concentration whereas at 0° the uptake of calcium (c.p.m./μg. of DNA) fell (Table 1). Although the uptake of ^{45}Ca at 38° rose with decreasing calcium concentration, it was considered that greater cell stability was more desirable and 10mM-calcium was used in all other experiments. It appeared that, as the concentration of calcium in the medium fell, the amount of calcium entering the cell increased, i.e. net ^{45}Ca uptake (Table 1, next to last column). In reality this was not so, since the

Table 1. *Effect of calcium concentration on DNA recovery and net calcium uptake by mucosal cells* in vitro

Mucosal cells were incubated at pH 7·4 in an atmosphere of O_2+CO_2 (95:5) in a complete medium that consisted of Krebs–Ringer bicarbonate buffer containing (final concentrations) 10mM-calcium, a tracer quantity of ^{45}Ca, 0·25M-sucrose and 0·02M-glucose, and were shaken for 15min. in a Dubnoff shaker. The medium was diluted with calcium-free medium to obtain the other calcium concentrations (5·0 and 2·5mM). The percentage of DNA recovered was calculated as 100× the amount of DNA that remained in the sedimented cells after incubation divided by the amount of DNA in the sedimented cells before incubation. Results are given as the mean ± S.D. of three determinations.

Expt. no.	Concn. of calcium (mM)	Temp. ...	DNA recovered (%) 38°	DNA recovered (%) 0°	Radioactivity of ^{45}Ca in cells (c.p.m./μg. of DNA) 38°	Radioactivity of ^{45}Ca in cells (c.p.m./μg. of DNA) 0°	Net ^{45}Ca uptake (c.p.m./μg. of DNA)	
1	10·0		76 ± 2	84 ± 1	1573 ± 28	1194 ± 13	379	379
	5·0		40 ± 1	102 ± 13	1705 ± 16	722 ± 16	983	491*
	2·5		20 ± 5	101 ± 2	2485 ± 262	594 ± 12	1791	448*
2	10·0		83 ± 3	89 ± 4	475 ± 11	312 ± 1	163	163
	5·0		54 ± 5	96 ± 4	591 ± 25	239 ± 39	352	176*
	2·5		28 ± 6	98 ± 3	947 ± 17	197 ± 7	750	188*

* These are the values for net ^{45}Ca uptake corrected for dilution (see the text).

Table 2. *Effect of* pH *on net calcium uptake by mucosal cells* in vitro

Mucosal cells were incubated in complete medium as in Table 1 for 15min.; pH 7·4 was attained by gassing the medium for 10min. with O_2+CO_2 (95:5), pH 7·9 by further gassing with 100% O_2 for 10min., and pH 5·0 and pH 6·1 by adjusting the pH of the medium with dil. HCl and then gassing with 100% O_2 for 10min. Two rats were used in each experiment.

pH	Temp. ...	DNA recovered (μg.) 38°	DNA recovered (μg.) 0°	Radioactivity of ^{45}Ca in cells (c.p.m./μg. of DNA) 38°	Radioactivity of ^{45}Ca in cells (c.p.m./μg. of DNA) 0°	Net ^{45}Ca uptake (c.p.m./μg. of DNA)
5·0		216	227	17	10	7
5·0		216	224	23	14	9
6·1		170	162	18	7	11
6·1		153	147	12	8	4
7·4		135	134	439	181	258
7·4		132	132	394	175	219
7·9		161	158	211	104	107
7·9		155	165	221	78	143

same amount of radioactive isotope was used in each suspension but the concentrations of unlabelled calcium were different, i.e. the specific radioactivity of the solution containing 2·5mM unlabelled calcium was four times that of the 10mM-calcium solution and twice that of the 5·0mM-calcium solution. Thus if the net uptake at 2·5mM was divided by 4 and that at 5·0mM by 2, the uptake of ^{45}Ca was about the same in each case when the dilution was taken into account (Table 1, last column). Incubation of cell suspensions for 15min. at different pH values had essentially no effect on the final pH or on the stability of the cell suspensions as measured by the amount of DNA recovered at 38° and at 0° (Table 2). However, calcium uptake was greatest at pH 7·4; at pH 7·9 it fell considerably and it was negligible at pH 5·0 and 6·1.

As the incubation time increased, the number of cells sedimented decreased, as measured by the DNA content of the extract, and at 120min. about 80% of the cells had disintegrated (Fig. 1). Further, the stability of the epithelial cell suspensions was unaffected by the vitamin D status of the animals.

Calcium uptake in vitro. Calcium uptake by cells incubated at 0° was considerably less than that by cells incubated at 38° at pH 7·4 (Tables 1 and 2). When glucose was omitted from the medium (Table 3) uptake was decreased by more than 50%. It was not affected when the incubation was done in an atmosphere of nitrogen. In the presence of cyanide (0·085mM) or arsenate (10mM), however, uptake was about twice that of the control. Although actinomycin D (10μg./100g. body wt., injected intraperitoneally) had no effect on calcium

uptake, it prevented the enhanced uptake usually seen after vitamin D treatment (Table 3). Calcium uptake by mucosal cells from rats treated with large oral doses of vitamin D was higher than that of control cells after 15min. incubation (Table 4), and rose with increasing doses of the vitamin until a maximum was reached with a dose of 5000 units. With larger doses (up to 25000 units), calcium uptake, although greater than that by control cells, was lower than that by cells from rats given 5000 units. Fig. 2 shows that at 30min. more calcium was taken up by cells from vitamin D-treated rats as compared with control cells, but at 60min. and particularly at 120min. cells from the vitamin D-treated rats took up significantly less calcium than did control cells.

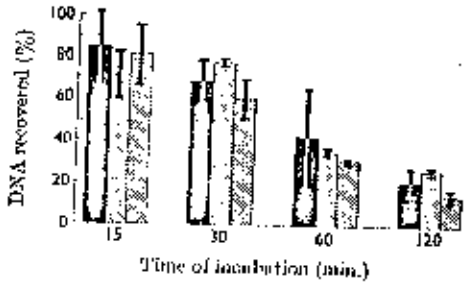

Fig. 1. Percentage recovery of DNA from mucosal cells incubated for different times in complete medium at pH 7·4 (see Table 1). The percentage of DNA recovered was determined by measuring DNA of sedimented cells before and after incubation. ■, Control; ▨, vitamin D-deficient; ▩, hypervitaminotic D. The bars show the S.D. from the means of four determinations.

Table 3. *Effect of varied incubating conditions on net ^{45}Ca uptake by mucosal cells*

Mucosal cell suspensions were incubated in complete medium at pH 7·4 (Table 1). Actinomycin D was given intraperitoneally (10μg./100g. body wt.) and vitamin D_2 orally (5000i.u. in 0·1ml. of sesame oil) 20hr. before mucosal cell suspensions were prepared. The control value was arbitrarily set at 100.

Incubation conditions	Net ^{45}Ca uptake (% of control)
Complete medium (control)	100
No glucose	41
Atmosphere of N_2	88
Potassium cyanide (0·085mM)	215
Sodium arsenate (10mM)	178
Actinomycin D (10μg./100g.)	98
Actinomycin D (10μg./100g.), vitamin D_2 (5000i.u.)	110

Calcium uptake by mucosal cells from vitamin D-deficient rats was also higher than that of control cells, whereas uptake by cells from rats given restricted amounts of food daily (pair-weighed control) was the same as that of controls (Table 5). As the incubation was continued (Fig. 3), cells from vitamin D-deficient rats accumulated more calcium than did those of controls, up to 2hr.

Calcium release in vitro. Table 6 shows the results of experiments designed to study calcium release from cells prelabelled *in vivo* with ^{45}Ca. Mucosal cells from vitamin D-deprived rats lost essentially no calcium, whereas those from hypervitaminotic D animals tended to lose more calcium than did cells from control or pair-weighed animals.

Calcium uptake and release in vivo. Table 7 shows the effects of vitamin D deprivation and toxicity on gains in body weight and on serum concentrations of calcium and phosphorus. Both deprivation and excess of vitamin D markedly decreased weight gains. Vitamin D deficiency lowered the serum calcium concentration and raised the serum phosphorus concentration, whereas hypervitaminosis D raised the serum calcium concentration significantly. After a single oral dose of ^{45}Ca, the specific radioactivity of the mucosal cells of the rats given large amounts of vitamin D was significantly less than that of control cells at 15min.; from 30min. on there was no difference between the

Table 4. *Effect of oral vitamin D on net calcium uptake by mucosal cells in vitro*

Control rats were given their last regular dose (75i.u.) of vitamin D_2 orally 16hr. before the mucosal cell suspensions were prepared in complete medium at pH 7·4 (Table 1). The calcium uptake of the control is set arbitrarily at 100%. The radioactivities are given as means ± S.D. for four determinations.

Vitamin D_2 (i.u.)	Radioactivity (c.p.m./μg. of DNA)		Net ^{45}Ca uptake	
Temp.	38°	0°	(c.p.m./μg. of DNA)	(% of control)
Control	426 ± 87	103 ± 17	323	100
2500	831 ± 31	145 ± 18	688	213
5000	1379 ± 58	188 ± 10	1191	369
10000	975 ± 82	186 ± 4	793	246
15000	605 ± 8	115 ± 1	490	152
25000	669 ± 6	154 ± 18	515	159

specific radioactivities for treated and control rats (Fig. 4). On the other hand, the serum specific radioactivity of the treated rats was the same as that of the controls at 15min., but was significantly greater at 30min.; there were no differences after this time (Fig. 5). In contrast, specific radioactivity in mucosal cells of vitamin D-deficient rats was significantly higher than that of controls (Fig. 6) at 15, 30 and 60min., whereas the ^{45}Ca specific radioactivity of the serum of vitamin D-deficient rats was significantly lower than that of controls at all times (Fig. 7).

DISCUSSION

Although uptake and release of calcium by isolated mucosal cells may not necessarily reflect calcium absorption as it occurs *in vivo* any more accurately than does calcium uptake by the everted gut sac, the isolated mucosal cell system does obviate some of the difficulties inherent in the use of everted gut sacs and intestinal rings, such as passage of ions through tissues not normally involved in calcium absorption (submucosal tissue layers and serosal membranes). Also, one can clearly separate the processes of uptake by the cell

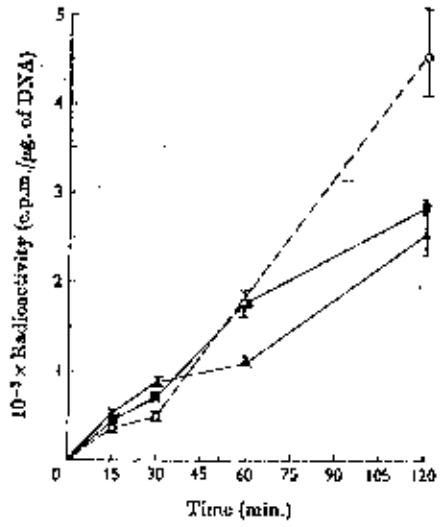

Fig. 2. Effect of vitamin D treatment on net ^{45}Ca uptake by mucosal cells incubated *in vitro* for different times. Groups of control rats were given the following amounts of vitamin D_2 10hr. before mucosal cells were isolated: ○, none (control); ■, 2500i.u.; ▲, 5000i.u. Mucosal-cell suspensions were incubated in complete medium at pH 7·4 (see Table 1). The bars show the S.D. from the means of three or four determinations.

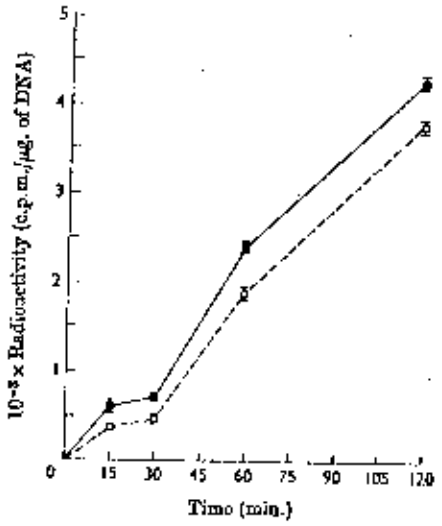

Fig. 3. Effect of vitamin D deprivation on the net ^{45}Ca uptake by mucosal cells incubated *in vitro* for different times. Vitamin D-deficient rats were given the vitamin D-free diet for 70 days. Mucosal-cell suspensions from vitamin D-deficient (●) and control (○) rats were incubated in complete medium at pH 7·4 (see Table 1). The bars show the S.D. from the means of four determinations.

Table 5. *Effect of vitamin D deficiency on net calcium uptake by mucosal cells* in vitro

Cell suspensions were prepared in complete medium at pH 7·4 (Table 1). The radioactivities are given as means ± S.D. for four determinations.

Rats ...	Control			Vitamin D-deficient			Pair-weighed		
	Radioactivity (c.p.m./μg. of DNA)		Net ^{45}Ca uptake (c.p.m./μg. of DNA)	Radioactivity (c.p.m./μg. of DNA)		Net ^{45}Ca uptake (c.p.m./μg. of DNA)	Radioactivity (c.p.m./μg. of DNA)		Net ^{45}Ca uptake (c.p.m./μg. of DNA)
Expt. no.	38°	0°		38°	0°		38°	0°	
1	1402 ± 182	701 ± 79	701	2473 ± 129	1029 ± 35	1444	1672 ± 102	948 ± 49	724
2	3254 ± 125	1145 ± 14	2109	4259 ± 156	1009 ± 209	3250	—	—	—
3	2119 ± 92	321 ± 20	1798	3706 ± 117	269 ± 37	3437	1882 ± 86	220 ± 8	1662
4	720 ± 6	345 ± 10	375	1042 ± 107	423 ± 23	619	—	—	—

and release by the cell, at least within short periods of time. Use of hyaluronidase to liberate the absorptive cells from underlying tissue, as reported by Perris (1966) and Hurrer *et al.* (1964), may result in less contamination by non-absorptive cells. Histological study of the cells in the present study, however, showed no gross contamination by underlying structures, but did reveal the presence of lymphocytes. However, most of the cells appeared to be epithelial. Thus it was considered that this preparation, which mainly contained absorptive cells, could serve as a useful model for measuring uptake and release of ^{45}Ca as affected by various treatments.

The effect of temperature on ^{45}Ca uptake in these studies is in contrast with that described by Rasmussen *et al.* (1963) and Holdsworth (1966). They reported that mucosal cells took up more ^{45}Ca at 0° than at 38°. This discrepancy can be accounted for by the method of expression of the results. In the studies of Rasmussen *et al.* (1963) the ^{45}Ca liberated into the medium was measured and used as an index of calcium release. However, in the period for which they incubated their preparations at 38°, most of the cells would have ruptured (Paterson & Zborsky, 1968; and Fig. 1)

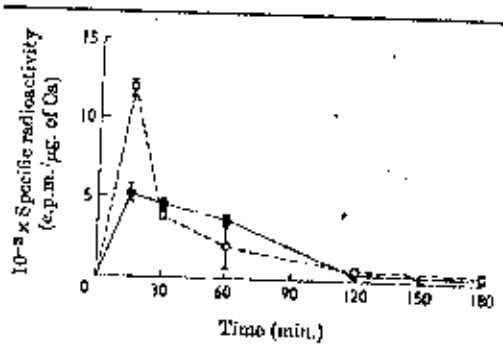

Fig. 4. Effect of hypervitaminosis D on the specific radioactivity of calcium in mucosal cells. Mucosal cells of hypervitaminotic D (●) and control (○) rats were isolated at the times indicated after a single oral dose of $^{45}CaCl_2$. Other details are described in the Materials and Methods section. The bars show the S.D. from the means of three determinations.

Table 6. *Percentage loss of ^{45}Ca from mucosal cells previously labelled in vivo*

The ^{45}Ca content of cell suspensions at zero time was arbitrarily set at 100%. The results are the percentages of ^{45}Ca lost from the cells after 5min. incubation in ^{45}Ca-free complete medium at pH 7·4 (Table 1). The results are the means of determinations on two animals.

Expt. no.	Loss of ^{45}Ca (%)			
	Controls	Pair-weighed controls	Vitamin D-deficient	Hypervitaminotic D
1	26	25	3	30
2	12	14	4	26
3	10	—	0	13
	16	—	3	29
4	10	—	0	26

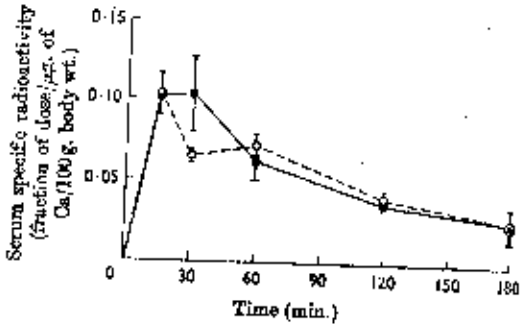

Fig. 5. Serum specific radioactivity from hypervitaminotic D (●) and control (○) rats at the times indicated after a single oral dose of $^{45}CaCl_2$. Serum specific radioactivity was calculated by the formula given in the Materials and Methods section. The bars show the S.D. from the means of three determinations.

Table 7. *Effect of vitamin D deficiency, hypervitaminosis D and food restriction on body weight, serum calcium concentration and serum phosphorus concentration*

Experimental details are given under 'Preparation of rats' in the Materials and Methods section. The results are given as means ± S.D. for 10–15 animals in each group.

	Control	Pair-weighed control	Vitamin D-deficient	Hypervitaminotic D
Body wt. (g.)	292 ± 18	154 ± 9	160 ± 30	182 ± 19
Concn. of serum calcium (mg./100ml.)	10·9 ± 0·3	10·2 ± 0·4	5·8 ± 0·2	16·0 ± 1·3
Concn. of serum phosphorus (mg./100ml.)	8·7 ± 0·9	7·4 ± 0·7	10·4 ± 2·2	8·5 ± 0·5

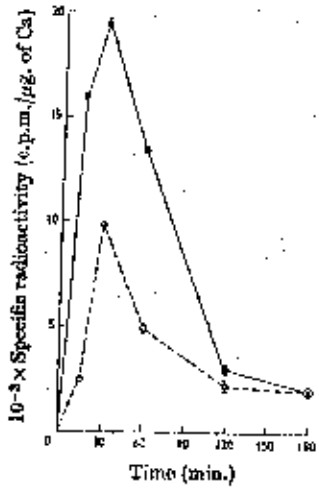

Fig. 6. Effect of vitamin D deficiency on specific radioactivity of calcium in mucosal cells. Mucosal cells from vitamin D-deficient (●) and control (○) rats were isolated at different times after a single oral dose of $^{45}CaCl_2$. Other details are described in the Materials and Methods section. The bars show the S.D. from the means of three determinations.

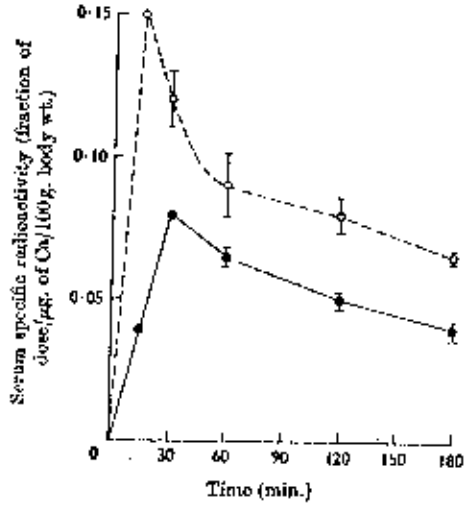

Fig. 7. Serum specific radioactivity from vitamin D-deficient (●) and control (○) rats at the times indicated after a single oral dose of $^{45}CaCl_2$. Serum specific radioactivity was calculated by the formula given in the Materials and Methods section. The bars show the S.D. from the means of three determinations.

and released their ^{45}Ca into the medium. This would account for the rise in the concentration of ^{45}Ca in the medium as the incubation was continued. Further, at the concentration of calcium in their buffer (0·1mM) very few cells would survive after incubation at 38° for 15min. and the radioactive isotope would be released into the medium. As shown in Table 1, after 15min. of incubation at 38° 75% of the cells incubated in 2·5mM-calcium disintegrated whereas at 0° they remained intact.

In the studies by Holdsworth (1965), the ^{45}Ca in the supernatant was measured after centrifugation of incubated mucosal cells at 30000g, and the results were expressed as μg. of Ca/mg. of protein in the original incubation mixture. Hence, there is no way to determine the extent of cell breakage, since the protein in the supernatant was not determined after incubation. The present study (Table 1) indicates that there was a greater amount of cell rupture at 38° than at 0°, even at a calcium concentration of 10mM. Further, it was observed that in the absence of 0·25M-sucrose there was a significant increase in cell breakage during incubation at 38° even at a calcium concentration of 10mM (G. Hashim & I. Clark, unpublished work). It is therefore possible that the greater amount of radioactive isotope found in the supernatant of mucosal cells at 38° in the studies of Holdsworth (1965) resulted from increased fragility of cells as a consequence of the temperature and the lack of sucrose in the medium. In the present work 0·25M-sucrose was used in all experiments and the ^{45}Ca and DNA of only the cells that sedimented at 500g were analysed, thus avoiding the complications arising from cell breakage. In every experiment, the uptake of ^{45}Ca/μg. of DNA was significantly greater at 38° than at 0°.

Although most of the cells did not survive an incubation for 2hr. at 38°, as measured by DNA recovery (Fig. 1), those that did survive were viable as measured by the difference in ^{45}Ca uptake at 38° and at 0° (Figs. 2 and 3). However, cell stability alone, as measured by DNA recovery, cannot be considered to be an adequate criterion for measuring active ^{45}Ca uptake since, at low pH (Table 2), ^{45}Ca uptake was negligible and yet DNA recovery was about 100% after 15min. incubation. At 0°, when metabolic reactions proceed at a minimal rate, the entry of ^{45}Ca into the cell is mostly by passive diffusion. Further evidence for passive diffusion is shown in Table 2, where the ^{45}Ca uptake by the cells at 0° increased with increasing concentration of calcium despite a significant decrease in the specific radioactivity of the incubation medium. It is well established that the rate of diffusion of ions varies with the concentration. Further evidence for passive diffusion comes from the studies with metabolic inhibitors. In the presence of nitrogen, no effect was observed on the uptake of calcium. The large differences between the concentration of ^{45}Ca/μg. of DNA at 0° and at 38° clearly show that entry of the radioactive isotope at 38° is not the

result of simple diffusion but rather the result of some active process. Also, studies of the effect of pH (Table 2) showed that cell stability is maintained for at least 15min., whereas ^{45}Ca uptake may be altered, indicating that active transfer occurred at pH7·4, whereas at acid pH only passive transfer occurred. When cyanide or arsenate was present ^{45}Ca uptake was approximately doubled, and in the absence of glucose it was significantly decreased (Table 3). These findings suggest that calcium uptake and release are associated with normal glycolytic activities. In the absence of sufficient oxidizable substrate calcium entry was impaired, and in the presence of respiratory inhibitors such as cyanide release from within the cells was inhibited. By using the everted-gut-sac technique it was demonstrated that, when oxidative phosphorylation was uncoupled with 2,4-dinitrophenol, there was a marked decrease in the transfer of ^{45}Ca from the mucosa to the underlying intestinal coats, although its entry into the mucosa was increased (Schachter, Kowarski, Finkelstein & Mo, 1966). These findings are in accord with the present study, which also shows an accumulation of ^{45}Ca in epithelial cells treated with respiratory poisons (Table 3).

The biphasic nature of the uptake curves shown in Fig. 2 and 3 can perhaps be accounted for by the time necessary for the transition from passive to active uptake. The preparation of cells was at 0°. The slow uptake for the first 30min. may be accounted for by the time necessary for the cells to reach 38°. At 0° transport would be mainly passive, but as the temperature rose active transport would begin, and very probably at temperatures below 38° there would be a combination of active and passive transport, and at 38° transport would be mainly active.

The present studies also show that vitamin D deficiency does not interfere with the entry *in vivo* of calcium from the incubation medium into isolated epithelial cells (Table 5). In fact, mucosal cells from the vitamin D-deficient rats contained more ^{45}Ca than did those of control rats after 15min. of incubation *in vitro* (Table 5 and Fig. 3) and also after 15, 30 and 60min. *in vivo* (Fig. 6). The increased amount of ^{45}Ca in the cells from vitamin D-deficient rats may be the result of more rapid uptake, slower release or a combination of both processes. From studies of the release of ^{45}Ca from intestinal cells prelabelled *in vivo* (Table 6), the results clearly show that after 5min. of incubation, significantly less radioactive isotope was released from cells of the vitamin D-deficient animals. Further evidence, albeit indirect, to support this thesis comes from the study *in vivo*, where comparison of the serum specific radioactivities (Fig. 7) with those of the mucosal cells (Fig. 6) shows that the radioactivity is greater in the vitamin D-deficient cells than in the control cells, but the radioactivity in the serum of the controls is higher than that in the serum of the vitamin D-deficient animals. This suggests delay in the transfer of ^{45}Ca from the intestine to the vascular sytem. Thus it appears that normal amounts of vitamin D are not essential for calcium uptake, but are involved in its release from mucosal cells. These findings and those of others (DeLuca & Engstrom, 1961; Engstrom & DeLuca, 1962, 1961) argue against the necessity for vitamin D in the initial steps of calcium absorption. Also, Schachter *et al.* (1966) showed that uptake from the mucosal medium to the mucosa was almost the same in the 'deficient' animals as in those given large amounts (200000i.u.) of vitamin D. However, large amounts of vitamin D increased uptake of calcium by the cells (Table 4). The decrease in uptake as the dosage of vitamin D rose from 5000i.u. to 25000i.u. may be the result of toxicity to the cells of this large dose of vitamin D, or of an enhancement of calcium release. Removal of calcium from the mucosal cells is enhanced by excessive doses of the vitamin, since radioactivity in mucosal cells of hypervitaminotic D rats was significantly lower than that in control cells after 15min. (Fig. 4), whereas serum specific radioactivity of the vitamin D-treated rats at 15min. was equal to, and at 30min. was higher than, that of controls (Fig. 5) despite the 40–50% increase in the serum concentration of calcium in the hypervitaminotic D rats (Table 6). If the same amount of ^{45}Ca or less had been transferred, the serum specific radioactivity would have been less. These findings indicate more rapid exit, as well as of entry, of calcium from mucosal cells as a result of excessive amounts of vitamin D. In addition, cells of animals given large amounts of vitamin D (Fig. 2), when incubated for times longer than 30min., contained significantly less ^{45}Ca than did cells from control rats despite the greater initial uptake by cells from hypervitaminotic D rats at 15 or 30min. This also indicates more rapid removal from the cells. Further, cells from hypervitaminotic D rats tended to release more ^{45}Ca after 5min. of incubation than did control cells (Table 5). This rapid removal of calcium from the cells is also in accord with the data of Schachter *et al.* (1966), who observed a significantly greater transfer of ^{45}Ca from the mucosa to the underlying layers of the intestine after the administration of a large amount of vitamin D.

The exact site of accumulation of ^{45}Ca within the epithelial cells is not apparent from these studies. Work with subcellular particles has shown that vitamin D influences the binding and release of calcium by mitochondria and microsomes. Vitamin D *in vitro* or *in vivo* enhances the release of calcium by mitochondria (DeLuca & Engstrom, 1961;

Engstrom & DeLuca, 1962, 1964). Also, cyanide increased the uptake of calcium by cells (Table 3), and a similar event has been shown to occur with mitochondria (Rasmussen, Chance & Ogata, 1965). Thus it is possible that the site of accumulation may be the mitochondria, since vitamin D and cyanide affect release and uptake by the whole cell in the same way that they affect them in mitochondria. Microsomal preparations from chick intestinal mucosa bound calcium *in vitro* (Snellgrove & DeLuca, 1965), and the binding was depressed by the addition of vitamin D. The apparently depressed binding could also have been a consequence of increased release. Recently a protein was isolated from the duodena of vitamin D-treated chicks (Wasserman & Taylor, 1963; Taylor & Wasserman, 1966) and rats (Kallfelz, Taylor & Wasserman, 1967), that depressed calcium binding to cellular debris. It is possible that the influence of vitamin D on calcium transfer may be the result of increased synthesis of such a carrier protein, which would prevent the accumulation of calcium in cells perhaps by rapidly removing it from the cells. It has been shown that actinomycin D suppresses the effect of vitamin D on calcium absorption *in vivo* (Norman, 1965, 1966*a*,*b*; Bull, Czarnowski-Misztal & DeLuca, 1965, 1966), and in the present study the antibiotic also prevented the enhancement of calcium uptake *in vitro* (Table 3). Since actinomycin inhibits protein synthesis, these findings suggest that the vitamin may be involved in the formation of a carrier protein, which could possibly prevent intracellular accumulation of calcium by enhancing its transport. However, increased permeability of cellular membranes as a consequence of excess of vitamin D cannot be ruled out in these studies.

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AN EXPERIMENTAL HISTOLOGIC STUDY OF HYPERVITAMINOSIS D.*

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Soon after the discovery of the similarity of Vitamin D to irradiated ergosterol, several investigators became concerned with the action of excessive doses of irradiated ergosterol.[1-5] It was generally agreed that the compound exerted its effects by its influence upon calcium and phosphorus metabolism, presumably through some intermediary hormonal mechanism.[5,7] It was proved that the compound had no serious toxic action except when given in doses far in excess of those required for prevention or cure of rickets due to a deficiency of Vitamin D.[8] Finally, it was established that the principal pathologic changes attributable to excessive doses in man or animal were in the form of abnormal deposits of calcium in many organs and tissues.[9-11] Having settled practical matters concerning the clinical use of the compound, interest in hypervitaminosis D subsided, and except for the studies of Follis, little attention has been given to the subject since 1935.[12,13]

Our interest in hypervitaminosis D arose as a result of the emphasis of Wolbach, Bessey and Fell upon the use of avitaminoses and hypervitaminoses as tools for study of pathologic sequences and their relations to the locus or nature of action of vitamins.[14-18] Sequences in human disease with which we have been concerned relate to so-called degenerative changes which are especially conspicuous in connective tissues and more common in arteriosclerosis than in any other human disorder.[12,19] The lesions described in hypervitaminosis D seemed to offer a promising source of information in further study of these noninflammatory degenerative sequences.[2-5,8] Hence, histologic studies of

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the distribution and evolution of lesions in rabbits given excessive doses of irradiated ergosterol were undertaken with special attention to changes in the vascular system.

METHODS

The viosterol used in the experiments was a solution of irradiated ergosterol (15 mg. per ml.) in peanut oil. (Abbott Laboratories, Chicago, Ill.) The potency was assayed as about 10^6 U.S.P. units per ml. This was given intramuscularly in doses of 0.1 ml. (10^5 U.S.P. units) at daily, biweekly or triweekly intervals for periods as long as 6 to 8 weeks. The regime was varied at lower dosages so that minimal and maximal pathologic effects could be defined and their evolution recognized over a period of several months.

Male albino rabbits from a single stock, about 5 pounds in weight and 3 months old, were used. Records of weight and analyses for serum calcium, phosphorus and cholesterol were made at intervals of about 2 weeks. The animals were fed a Purina Rabbit Pellet diet with fresh vegetables. For control purposes, complete microscopic studies were made of 75 normal rabbits from the same stock, of the same age and maintained on the same diet.

Since the initial plan was to produce maximal pathologic effects, the dose was regulated at levels just below the quick lethal range. Hence, most animals lost weight and died within 6 weeks. Later, the plan was to analyze minimal, sublethal and early pathologic effects, and animals were either sacrificed at any early stage or given viosterol at longer intervals and sacrificed at a later stage. Complete necropsies were done and a record of the gross findings made. All tissues and organs were fixed in formaldehyde (4 per cent U.S.P.) and studied microscopically. A series of 54 animals were suitable for the purposes of this report, with 49 additional animals on other dietary and viosterol regimes available for supplementary and confirmatory observations.

RESULTS

Gross Pathologic Changes

The pathologic processes attributable to hypervitaminosis D varied with the dosage of viosterol, the length of the period between doses, duration of the regime, and the occurrence of intercurrent infections.

Minimal conditions for production of significant generalized calcinosis were a duration of 8 days and a total of 300,000 units given in 3 equal doses at intervals of 2 days. These conditions were usually complicated by intercurrent chronic purulent pyelonephritis of a type occasionally encountered in control animals. Among animals free

from ext[illegible]s disease, except hepatic coccidiosis, the total minimal effective dose was 500,000 to 600,000 units. This dose produced generalized calcinosis of increasing severity as the time of administration in periodic equal doses was extended to at least 3 weeks. The severity of the disease was further increased as the biweekly administration of doses of 100,000 units was continued beyond 3 weeks. This regime generally led to anorexia, loss of weight and death within about 6 weeks. Accurate control of the rate of development of pathologic processes was also obtained, so that prolonged participation of tissue elements in mechanisms of resorption, repair and regeneration might be studied, but this required careful regulation of dosage over a period of about 6 months.

Significant gross anatomic changes were encountered only in animals with considerable disease, manifest by changes in bones or deposition of calcium in numerous extra-osseous tissues. These could not be correlated with any consistent changes in values for cholesterol, calcium, or phosphorus in the serum.

The changes in bones varied with the severity and duration of disease. In general, the bones were more brittle than normal (Fig. 9). The increased fragility was due largely to resorption of cortical bone, often sufficient to allow mechanical deformity of bodies of vertebrae so that partial collapse and abnormal cortical contours were occasionally conspicuous. In these cases, the trabecular structure was compact and the marrow spaces reduced so that the internal structure of the vertebral bodies resembled that found in human osteosclerosis and Paget's disease (Fig. 10).

The amount and distribution of abnormal deposits of calcium in extra-osseous tissues also varied with the duration and severity of the disease. In mildly affected animals there were no gross changes. With increasing severity of the disorder, calcium salts began to appear as white streaks which acquired an opaque brittle or granular character. The deposits conformed to anatomic planes and structural outlines of affected tissues. They were most conspicuous in the aorta and its major branches, being maximal proximally and diminishing distally in a consistent pattern.[2,20] The deposits were in the media, usually in the form of continuous sheets or as a series of discontinuous rings with aneurysmal deformities of the wall. Calcium deposits were less conspicuous in the kidney, where bandlike granular deposits, were noted, first in the inner cortex and later, as the calcified zone of the innermost cortex spread toward the capsule, as one or more convex parallel bands in the periphery of the medulla (Fig. 7).[4]

The next most common location of calcium deposits was in muscle (Fig. 2). The deposits coincided with white streaks which followed the pattern of orientation of muscle cells or distribution of arteries. They were less common in skeletal than cardiac muscle, but frequently were clearly distinguished in the diaphragm and smooth muscle of special parts of the alimentary tract. The esophagus was never affected. The walls of the ileum and colon were occasionally altered, but this never occurred unless there were extensive deposits in the muscular wall of the stomach subjacent to the acid-secreting mucosa (Fig. 5). The acid-secreting gastric mucosa also commonly contained deposits of calcium but less frequently than the subjacent musculature. Calcium deposits were not found in the mucosa or muscle of those parts of the stomach which have no acid-secreting function.

Another common location of calcium deposits was in the respiratory tract. The tracheal and bronchial cartilages were often rigid and calcified, and thin plaques of calcium were at times recognized in the mucosa of the larger respiratory passages. Calcium deposits were rarely found in the media of large pulmonary arteries but were fairly common in the walls of pulmonary veins. In connection with these changes, pulmonary emphysema and decreased elasticity of the alveolar structure were usually demonstrable.

Other tissues and organs occasionally contained gross traces of calcium salts, but in many locations the presence of the mineral deposits was established only by microscopic studies.

Microscopic Pathologic Changes

Heart

Deposition of calcium in the heart was usually associated with a peculiar inflammatory reaction similar to that described in rats (Fig. 1).[1,8] In general, the calcification potential of various cardiac structures in order of decreasing intensity and frequency was as follows: internal elastic membrane of coronary arteries, smooth muscle of coronary arteries, fibroelastic tissue of the endocardium, and cardiac muscle. Calcium deposition in cardiac muscle cells was confined initially to the "A" disks of the myofibrils. As the amount of calcium increased, the "I" disks were involved, and later the interfibrillar compartment was affected so that the muscle cell appeared to be impregnated uniformly with calcium. At times, as much as one third of the myocardium was involved, but as a rule the calcified muscle cells were distributed irregularly around the coronary arteries or in the subendocardial region (Fig. 2). This localization seemed to be determined partly by the

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comm. occurrence of a low-grade inflammatory reaction (Fig. 1). At times, the reaction was principally in the form of arteritis and periarteritis, characterized by swelling of collagen, perivascular proliferation of histiocytes, and formation of focal nodular lesions which resembled Aschoff bodies. Usually these lesions lay either adjacent to or around cardiac muscle cells in various stages of degeneration, calcification, and resorption. Their incidence increased with the level of dosage of viosterol and perhaps with the intrusion of intercurrent infections, though data concerning the role of infections were too meager to justify final conclusions. Suffice it to say that control animals dying of the same infections had no lesions of the type described.

The presence of an active periarteritis and myocarditis may have had something to do with the distribution of calcified cardiac muscle cells, but there seemed to be little relation between the inflammatory reactions and calcification of the media of coronary arteries. The main coronary arteries were free from inflammation, and calcium deposits in the internal elastic membrane or smooth muscle of the media were more conspicuous in these vessels than in the smaller intramyocardial branches which exhibited inflammatory reactions. In some instances, the deposition of calcium was so great that it was detectable in coronary arterial branches of all dimensions. This process was never uniform in arteries of equal dimensions but involved certain arterial branches more severely than others.

Calcification of the fibrous or fibroelastic stroma of the heart was never impressive in valves or in the myocardium. The endocardium, especially of the left atrium, was occasionally severely calcified, the process being more pronounced in elastic networks than collagenous fibrils.

Respiratory Tract

The sequences and pattern of calcification in the respiratory tract were of great interest. The earliest deposition, other than that occurring in the tracheobronchial cartilages and goblet cells, was in the fibroelastic stroma of the tracheal mucosa. The calcium deposits were distributed at regular intervals just below the basal layer of epithelial cells. As a rule, the basement membrane and underlying fibroelastic tissues were affected. In more advanced cases, the deposits involved the entire basement membrane and variable amounts of subjacent fibroelastic tissue, as deep as the plane of the mucosal vascular plexus (Fig. 3). Only rarely did the calcium deposits extend in continuity from basement membrane to the perichondrium of cartilaginous rings. Invariably, however, in these severe lesions there was excessive calcification of the cartilaginous matrix. When this occurred, the calcified zones of cartilage lay adjacent to those of the mucosa, and at times there was erosion and stromal penetration of the margins of calcified cartilage. This was not followed by osteogenesis. On the contrary, the stromal invasion stimulated resorption of mineralized matrix and regional proliferation of young cartilage cells.

Calcification of the subepithelial tracheobronchial stroma was often preceded or accompanied by swelling and distortion of collagenous fibrils, but customary signs of inflammation were usually absent. However, once calcification had occurred, reparative sequences were at times recognized. These seemed to be initiated by two factors. One was the fragmentation of calcified fibroelastic tissue and basement membranes, leading to local proliferation of histiocytes and encapsulation of displaced degenerated calcified tissue by histiocytes and multinucleated giant cells. As this occurred, the subepithelial basement membranes and adjacent mucosal stroma were usually slowly regenerated. When the reparative process was unduly active, the epithelium regressed to an undifferentiated type of columnar cells which regenerated as either a characteristic respiratory epithelium or a metaplastic epithelium of stratified squamous type. This form of epithelial behavior called to mind the sequence described by Wolbach in the course of deprivation and restoration of vitamin A.[10-13] The second factor initiating reparative reaction was the deterioration of epithelial cells which followed calcification of mucus in the secretory vacuoles of goblet cells (Fig. 3). These cells, in the absence of any signs of diminished viability, inflammation, and undue calcification of subepithelial basement membranes or deeper stroma, were occasionally transformed into spherical, concentrically laminated, calcified crystalline structures. This form of primary epithelial calcification usually stimulated a regenerative reaction in which proliferation of basal cells led to atypical orientation of the new epithelium in relation to rigid calcified basement membranes. Ordinarily this activated the regional stroma which produced an entirely new structure with characteristics of the original basement membrane. The new membrane was interposed between the discontinuous calcified original basement membrane and the proliferating epithelium. As this occurred, the displaced calcified membrane became encapsulated by histiocytes or incorporated in the cytoplasm of multinucleated giant cells.

Despite the apparent interference of these changes with tracheobronchial function, little associated inflammation was provoked. A low-grade chronic tracheobronchitis, characterized principally by an

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infiltrat. of plasma cells in the respiratory mucosa, was found in a few animals. This followed the most severe instances of stromal and epithelial calcification. It was never encountered in control animals and was regarded as a specific feature of hypervitaminosis D.

Not all parts of the tracheobronchial tree were equally affected by the calcific changes. The process developed more readily in the trachea and, as the disease became more severe, spread distally. Hence, in mild cases the principal changes were in the trachea. In moderately severe cases, the lesions were conspicuous as far distally as the terminal bronchi supported by cartilage. In the most severe cases, the process extended all the way into the walls of the alveolar ducts and alveoli. At times, the calcification of the terminal structures was very advanced, with involvement of pulmonary elastic tissue and the walls of interalveolar capillaries and pulmonary venules (Fig. 4). The alterations led to diminished elasticity with increased rigidity, relaxation, and fragility of alveolar structures. Thus, early stages of emphysema became conspicuous, and although peripheral pulmonary changes were usually associated with advanced tracheobronchial calcification, the relationship was not always encountered and was by no means quantitative. It was clear that factors which determined the locus and quantity of tracheobronchial calcification did not necessarily operate equally in determining the distribution and magnitude of calcification in alveolar walls and pulmonary vascular channels.

Alimentary Tract

The upper alimentary tract was studied more carefully than the lower tract so that conclusions concerning changes distal to the duodenum were only tentative.

The esophagus showed no evidence of disease.

The stomach was a common site of severe calcification but only in special locations; namely, the part of the gastric wall concerned with support, nutrition, and the specific performance of acid secretion (Fig. 5). This part encircled the cardiac orifice and occupied the principal part of the fundus and greater curvature. The mucosa of the lesser curvature and the pyloric region contain few if any parietal cells which are generally held to be responsible for secretion of hydrochloric acid. The parts of the gastric wall which were calcified are therefore spatially and presumably functionally integrated. Calcium in the muscular wall was usually most conspicuous, and there was concurrent calcification of the mucosa and arterial system. Deposits of calcium in the mucosa

usually appeared first in the *tunica propria* and basement membranes adjacent to the common junction of branches of the compound glands in the midmucosal regions. Occasionally, cells lining the glands were calcified, and at times calcified concretions lay in the lumens of glands. Later, stromal calcification also occurred in other locations, especially near the muscularis mucosae. In connection with the stromal deposits, calcified walls of small arterioles were conspicuous, and at the level of the muscularis mucosae and submucosa, calcification of walls of larger arterioles and small arteries was still more prominent. Walls of larger arteries deeper in the *tunica muscularis* and serosa were less severely calcified although calcium deposits in the internal elastic membrane and subjacent media were common. In these vessels, fibroelastic intimal proliferation was apparent, always overlying calcified or fragmented stretches of abnormal internal elastic membranes or subjacent media.

Calcium deposition in the *tunica muscularis* and the muscularis mucosae of the stomach occurred in a characteristic sequence and pattern. Major deposits lay in the cytoplasm of smooth muscle cells which seemed, initially at least, to be structurally normal. The smooth muscle cells were ordinarily affected in groups, and with increased calcification only the bare outlines of initial structure remained. In its place there were rows of curious calcified bodies which resembled strings of beads without recognizable origin in cytoplasmic structure (Fig. 6). Minor deposits of calcium appeared in tortuous curved structures which at times resembled fibrous or elastic elements and at other times minute canaliculi or autonomic nerve axons. Further study will be required for the identification of these calcified filaments, fibers and other structures.

The pattern of transmural calcium deposition in elective sites in the stomach disappeared with the transition of gastric mucosa to the type which secretes no acid. Indeed, the entire thickness of the gastric wall just beyond the level of mucosal transition was normal in all cases. In other words, all arteries, nerves, smooth muscle, mucosal glands and stroma in the non-acid-secreting part of the stomach were free from calcium deposition.

Immediately beyond the pyloric sphincter, which at times contained a few clusters of calcified smooth muscle cells, a different pattern of calcification was encountered. Here, the duodenal mucosa, *tunica muscularis* and muscularis mucosae were ordinarily spared while calcium deposits appeared conspicuously in the walls of small arteries and arterioles. Distal to the duodenum the only important calcium

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deposits were in the *tunica muscularis* of the ileum and especially the colon. The deposits varied greatly in amount and position. Mucosal and arteriolar calcification was indistinct or absent, except in the colon, in the reticulum of lymphoid follicles or in the basement membranes of interglandular surface epithelium. There was a peculiar concentration of calcium in and around ganglionic plexuses in the *tunica muscularis* of the colon. Other plexuses were uninvolved. Mucus-secreting goblet cells, commonly calcified in the respiratory mucosa, were unaffected throughout the ileum and colon.

Urinary Tract

The kidney was also a common site of severe disturbance and was affected in all cases in which there was significant calcification in other tissues.[3,4,8] The renal calcification was confined principally to aggregates of functionally related units. For instance, nephrons were not involved equally or in a random manner. Those first affected were nearest the medulla, and from this region the process spread to involve the more peripheral nephrons but seldom reached those in subcapsular areas (Fig. 7). In connection with this curious wave of calcification there was a similar spread in the medullary tubular areas corresponding to the distribution of loops of Henle.

The sequence of changes in nephrons was also of interest. The epithelium of the convoluted tubules seemed most susceptible; the cells were frequently solidly impregnated with calcium often disposed in a spherical, concentrically laminated pseudo-crystalline fashion, occupying and expanding the original volume of the cytoplasm and nucleus. Intraluminal calcified protein casts were also encountered in these tubules. The basement membranes of the convoluted tubules had a conspicuous affinity for calcium so that at times they were more heavily calcified than the epithelial cells. This affinity was prominent beyond the limits of the convoluted tubules, especially in the stroma of Bowman's capsule. Less often, the subendothelial membrane of glomerular arterioles was affected.

No conspicuous calcification of tubular epithelium and basement membranes of the kidney appeared without calcification of the related arterial system. The large extrarenal arteries were least involved, although calcification of internal elastic membranes and subjacent patches of medial structure was usual. Where the extrarenal arteries branched to enter the cortex, near the corticomedullary junction, pathologic changes became more prominent. The walls of arteries and arterioles supplying the nephrons undergoing mineralization were frequently solidly impregnated with calcium so that mural structure was hardly recognizable (Fig. 15). The process of arterial calcification ordinarily extended in continuity to the glomerular tuft (Fig. 8). Involvement of the glomerular arteriolar plexuses was uncommon except when calcium was deposited in spherical masses scattered throughout the tuft in swollen glomerular endothelial cells. The postglomerular arterioles and venous channels were spared, except in the most severe instances of diffuse calcification. This was equally true of all arterial and venous systems connected with the nephrons of the peripheral half of the renal cortex.

The most remarkable feature of the renal lesions was their distribution. Of interest also, however, was the absence of expected signs of inflammation. Nor was there ever more than a trace of the reparative reaction one would anticipate in response to the observed magnitude and duration of renal damage. The disease was essentially indolent, progressive and degenerative.

The remainder of the urinary tract was not studied carefully, but ureters and renal pelves were often included in the sections. The ureters were always normal. The pelves ordinarily contained subepithelial deposits of calcium, occurring at intervals in the fibroelastic connective tissue and among bundles of smooth muscle cells. There was no inflammatory reaction preceding calcium deposition although the stromal fibers were often swollen and nodular. Following calcium deposition, there was in some instances a proliferation of histiocytes resembling the reaction encountered in similar calcified tissues in several other locations.

Vascular System

Particular attention was given to a study of the vascular system.[2,6] The earliest evidence of hypervitaminosis D was in the proximal aorta and changes were always found here, whenever there were calcific deposits in other locations such as the tracheobronchial mucosa, gastric wall and kidneys. As the disease became more severe and prolonged, calcium deposits appeared in the midaorta, then in the distal aorta, and at about the same time, in various aortic branches.

Microscopically, the initial calcium deposits in the aorta were in and along the innermost elastic membranes and delicate intermembranous fibrils or matrix (Fig. 11). With increasing duration and severity of the disease, calcification increased in depth and magnitude. In advanced cases the inner third of the media of the proximal aorta was calcified in continuity, and there were discontinuous annular zones of calcification in the middle third of the media. The depth of pene-

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tration of calcification in continuity decreased slightly with increasing distance from the aortic valve but not in proportion to the decrease in thickness of the media. Hence, in advanced cases, the full thickness of the media of the distal aorta was calcified, while not more than the inner half of the media of the proximal aorta was similarly involved.

Each aortic branch had individual susceptibility to mineralization, and each had its own pattern of calcium deposition. In general, branches of similar size and structure had similar susceptibilities and patterns, but this was not an invariable rule. Without undue description of deviations, the trends in the different systems may be given as follows. As the elastic structure which dominated the composition of the upper aorta was gradually replaced by an increased amount of smooth muscle in the distal aorta and carotid, renal or iliac arteries, calcification of the internal elastic membrane became more conspicuous and local deposits of calcium appeared in and between the subjacent smooth muscle cells (Fig. 14). The deposits in the beginning were discontinuous and distributed in an annular manner (Fig. 12). As they increased in number and extent, they tended to fuse to form annular rings and to spread in the long axis of the media, eventually occupying it entirely. The process was especially conspicuous in the iliac and femoral arteries. A similar lesion but with less annular and axial discontinuity of calcification was common in the carotid, axillary and brachial arteries (Fig. 13).

Changes in the smaller and more peripheral branches of these arteries did not follow any uniform pattern which could be related to the dimension or structure of the vessel. For instance, the immediately extrinsic and intrinsic vessels of the cerebral vascular system were always normal. The main pulmonary artery and major branches occasionally contained discrete calcified lesions of the inner media, but the minor branches down to the level of the alveolar capillary network were usually normal. At the alveolar level, calcium deposition became conspicuous and extended in continuity to involve the media of vessels of the outflow venous system into the left atrium (Fig. 4). Arteries to special organs such as the submaxillary glands, kidney, spleen, thymus, duodenum, acid-secreting part of stomach and thyroid were often very severely affected, while those supplying the eye, testis, ileum, adrenal, pituitary and liver were spared from significant alteration. Arteries to skeletal muscle, pancreas, bone marrow and skin contained lesions to a variable degree among different animals and from place to place in the same animal. These general statements also pertain to arterioles and capillaries.

Veins, except those carrying blood from pulmonary alveolar spaces to the left atrium, were never electively involved, though venous channels in the kidneys, bone marrow and elsewhere were occasionally secondarily affected in connection with massive calcification of regional tissue.

Not only were there unexplained vagaries in localization of calcium in different arteries and arterial systems, but the patterns of reaction associated with the localization were equally variable. For instance, fibrous intimal proliferation was negligible in the proximal aorta. It increased in magnitude in the aorta with increasing distance from the aortic valve. It was maximal in the systemic arteries of large caliber which showed severe calcification and fragmentation of internal elastic membranes supported by medias composed largely of smooth muscle rather than layers of elastic lamellae. It was limited exclusively to areas covering abnormal internal elastic membranes and subjacent medial structures in most animals, and predominantly so in all animals. The fibrous intimal plaques formed sluggishly but at times within 6 weeks acquired a thickness equal to that of the arterial wall (Fig. 14). In smaller arteries, the intimal proliferation was less conspicuous so that in arterioles, despite extensive transmural calcification, intimal proliferation was scarcely recognizable, except in the presence of active or healed arteritis (Fig. 16).

The occurrence of arteritis was another unexplained variable. The evidence indicated that the arteritis was related to exceedingly high dosage levels and perhaps to acute intercurrent infection. Suffice it to say that arteritis of the type occurring in animals with hypervitaminosis D was never encountered in the control animals, many of which also died of acute intercurrent infections. Whatever the eventual explanation, the arteritis and periarteritis had no specific connection with the degenerative calcifying disease of arteries in general. There was good evidence that it was connected with the occurrence of calcified deposits in distal arterial systems lying within muscle and concerned principally with the nutrition of cardiac and skeletal muscle (Figs. 1, 16). Seldom was arteritis or periarteritis encountered elsewhere and then only in isolated vessels and never in the conspicuous generalized form noted in the muscles.

Not only was the anatomic distribution of the arteritis a matter of considerable significance, but the pattern of inflammation of arterial walls and perivascular tissues was equally interesting. The most severe forms were in the nature of an indolent panarteritis, which resembled reactions occurring in periarteritis nodosa, lupus erythematosus, rheumatic fever, and occasionally rheumatoid arthritis. In less severe cases,

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the sig inflammation were principally in the collagenous adventitial tissues. These signs varied from a mild nodose swelling of the fibrils to a more pronounced response characterized by aggregates of histiocytes, neutrophils and lymphocytes in and around foci of degeneration of perivascular interstitial tissues. At times, especially in the myocardium, several persons who have studied these sections have remarked the similarity of the lesions to Aschoff bodies. Similarity was all that could be claimed, however. The lesions were entirely unlike the random inflammatory foci encountered occasionally in the myocardium of rabbits in the control series.[1,8]

Muscular Systems

The preceding descriptions have disclosed that the muscular tissues of the body were usually affected. There was no evidence that this involvement was secondary to alterations of arteries, although arterial lesions customarily accompanied muscular abnormalities. Smooth muscle was more generally affected than cardiac or skeletal muscle. Changes in smooth muscle of the media of systemic arterial and pulmonary venous systems, the *tunica muscularis* of the alimentary tract and the tracheobronchial tree, and the mucosa of the renal pelvis have already been described. Similar changes were less common in such other locations as the corium of the skin and septa of the spleen. Though successive stages of development of the lesions in smooth muscle were similar in most locations, this was not true of all locations, and there were wide differences in the susceptibility of various smooth muscle cells. In general, cardiac and skeletal muscle were less susceptible than smooth muscle, whereas skeletal muscle in most locations was less susceptible than cardiac muscle. In some instances, manifestations of inflammation and cellular degeneration preceded the microscopic signs of calcification of muscle. In other instances, however, the intracellular deposition of calcium seemed to occur in muscle which showed no definite evidence of inflammation or degeneration (Fig. 6). Whether these occurred or not seemed to be related to the rapidity of development and severity of the disease. At times, the stromal elements around smooth muscle cells were affected before there was conspicuous calcification of the cells. At other times, the cells were initially altered, and the stromal elements resisted calcification. This resistance of the stroma was generally apparent when cardiac muscle was undergoing calcification, but less so when calcium was being deposited in skeletal muscle cells. In the latter instance, the sarcolemma of some fibers was calcified before the cells had acquired much calcium.

Skeletal and Hematopoietic Systems

The dominance of this disease in many tissues concerned with motion is well illustrated by the remarkable changes which occurred in the skeletal system.[12,13] The first effects occurred early and were characterized by a resorption of bone, a decrease in the prominence of osteoblasts, and an increased prominence of osteoclasts (Fig. 9). These changes were more conspicuous in cortical than trabecular structures. A significant amount of pathologic calcification of the viscera did not occur unless these alterations were demonstrable. The development of abnormal calcium deposits in the viscera was also accompanied or followed by abnormal basophilic deposits of osteoid tissue in the skeleton. These deposits occurred not only in locations notable for the degree of osseous resorption but also in the bone marrow, along the margins of persistent trabecular and cortical bone (Fig. 10). Though there was some local increase in stroma which either preceded or accompanied the abnormal massive osteoid deposits, any close resemblance to normal sequences of osteoblastic orientation, osteoid production or calcium deposition was lacking. The osteoid tissue seemed to engulf old stroma, reticulum cells and other structures in a spreading wave beginning at the margin of pre-existing bone and progressively obliterated the adjacent bone marrow. When this process ceased, the customary form of normal eosinophilic osteoid tissue with incorporated osteocytes began to appear as local islands in the midst of the widespread basophilic osteoid deposits. This indicated onset of repair and, with passage of time, the deposits were largely replaced by new bone. There resulted an osteosclerosis characterized by excessive reformation of bone along lines which did not reproduce the initial or normal pattern of bone growth. The final result was a deformed skeletal structure, usually with a porous cortex. This was accompanied by a thick layer of periosteal new bone, especially excessive at the margins of some articular surfaces, and an excess of trabecular bone which had encroached upon an abnormal bone marrow (Fig. 10). As a rule, the response of the bone marrow to calcification and osteogenesis was fairly consistent in the areas which remained free from fibrosis. The marrow which had not been replaced by fibrous tissue, abnormal osteoid tissue, or new bone, was depleted of fat cells and was densely cellular. Hematopoietic elements varied considerably in their relative proportions and stages of maturation in different animals. The commonest abnormalities were maturation arrest and diminished numbers of megakaryocytes associated with an increase of cells resembling megaloblasts or plasma cells. Conspicuous cytologic changes in the marrow were

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usually accompanied by calcification of the media of blood vessels which appeared to belong to the arterial system. As yet, no connection between the changes in hematopoietic elements and the severity of the disorder in other parts of the body has been established though a correlation with gastric disease is strongly suspected.

The spleen was usually not severely disturbed. The earliest deposits of calcium occurred in the interstices of the septa. Here, fibroelastic tissue and smooth [illegible] actively calcified, but the bulk of the calcium was deposited in collagen. There was no consistent evidence of any inflammatory or degenerative change prior to calcification, although collagen fibrils often were swollen and irregular in outline. In some instances the arterial system was more heavily calcified than the trabecular structure. Here, the main splenic artery and its major branches regularly showed calcification with fragmentation of the internal elastic membrane overlying foci of medial calcium deposition. Fibrous intimal proliferation was seldom conspicuous but, when found, always was superimposed on degeneration of the vascular wall. As the splenic arterial branches entered the spleen and decreased progressively in diameter, transmural calcification became increasingly conspicuous so that the walls of many follicular arterioles were uniformly calcified. Nor did the impregnation of vascular structure cease here, for in some cases it continued into the walls of the sinusoids and outlined the reticular framework of lymphoid follicles.

Endocrine and Other Glands

The principal calcium deposits in endocrine glands were in the thyroid and thymus. In the thyroid the small arteries were usually severely affected in animals with advanced generalized disease. The vascular process was associated with atrophic changes in glandular epithelium and diminution in the amount of colloid. This in turn was related to variable calcium deposits in basement membranes of follicles and interfollicular stroma.

The thymus gland showed conspicuous atrophy in severely diseased malnourished animals. Calcium deposits were restricted principally to Hassall's corpuscles although occasionally there were deposits in the media of small arteries and arterioles.

The pituitary never contained calcium deposits. There were unexplained variations in the ratios of cell types in the anterior lobe. The parathyroid glands were normal in size and in histologic characteristics. The adrenal glands were resistant to calcification with deposits occurring only in the most severe cases. These appeared only in occasional

swollen endothelial cells of the vascular sinuses in the cortex. Large vessels were spared.

The testes were seldom affected, except insofar as the illness led to reduced spermatogenesis. There were occasional calcified concretions in tubules and insignificant calcium deposits in the media of large arteries leading to the testis; these were minor late manifestations of severe generalized disease.

Other glandular structures had their individual changes. The liver was never affected, even though the arterial branches at the hilus showed the same types of calcium deposition as other branches of the celiac axis. Intrahepatic vessels were free from lesions.

The large branches of the splenic artery to the pancreas were affected to about the same extent as the arteries to the spleen. The vascular changes decreased with diminishing size of the arteries so that in contrast to splenic arterioles the arterioles in the pancreas were usually normal. In occasional lobules of the pancreas of severely affected animals, the process extended into the arterioles, and the extension was accompanied by atrophy of acinar cells with calcification of the delicate basement membranes of glands. The pancreatic islets were not affected.

The arteries and arterioles supplying the submaxillary glands were among the most susceptible peripheral vascular structures. The walls of these vessels were often densely calcified. The process varied from one lobule to another. Accompanying the most severe arteriolar lesions, there was a tendency for other degenerative and calcific changes to occur; these were epithelial atrophy, calcification of inspissated secretion in the acini of glands or small ducts, and heavy impregnation of basement membranes with calcium. The sequence of these changes was not clearly defined, but the evidence indicated that vascular and epithelial changes preceded changes in the intervening stroma.

Central Nervous System and Eye

The eye was regularly spared except for small deposits of calcium in the sclera. This comment excludes any consideration of the lens because it was not examined microscopically. Retinal arteries were never calcified.

There were no deposits of calcium in the brain or spinal cord. This was a consistent observation and applied to all structures ordinarily associated anatomically with the brain and spinal cord. The level to which calcified deposits in the carotid, vertebral and spinal arteries extended from their points of origin has not been determined, but no vascular alteration has yet been found in the intracranial or intraspinal

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division these vessels. Furthermore, the vascular system within the confines of the pia mater and gray or white matter was always spared from calcification even around foci indicative of chronic intercurrent meningo-encephalitis which was encountered in a few animals of our current stock.

Skin and Adipose Tissue

The skin showed changes which need not be described in detail. Beneath the epithelium and at a slightly deeper level in the corium, the lesions resembled those occurring in fibroelastic tissues elsewhere. In connection with these alterations in their advanced form, there was mild calcification of the tributary arteriolar and precapillary walls. Other histologic features resembled the early modifications in human cutaneous fibroelastic tissue designated "senile elastosis." In the deeper tissues, lesions were insignificant except where short stretches of fascia were lightly calcified and where skeletal muscle showed degeneration of the type described elsewhere.

Adipose tissue in most locations was unaffected by the disorder. However, in the retroperitoneal tissues, especially around the larger arteries and adrenals, severe atrophy of fat cells was encountered, usually accompanied by a deposition of calcium. The principal deposits were either adjacent to or in the cytoplasmic membranes of fat cells. No massive deposits were encountered in the interior of fat cells.

Discussion

A major problem in analysis of the pathogenesis of many generalized diseases is an inability to explain the vagaries of distribution of the anatomic or functional manifestations. In one patient the manifestations of a disease may be referable to a single system or some part thereof. In another, the manifestations of the same disease may be referable to more than one system or more than one part of any system. In still others, the manifestations may be dissociated so that the expected quantitative relations between the anatomic and functional changes are not found. Concepts which guide thinking in these matters have no solid foundation in structural or functional pathology.

It was our belief that an experimental study of an easily controlled metabolic derangement which produced widespread structural changes of a simple degenerative nature rather than a complex inflammatory reaction might lead to formulation of more suitable concepts.[19] Wolbach demonstrated with well-planned experiments and great interpretive insight that the use of deficiencies and excesses of vitamins was an elegant method for approaching problems of this kind.[14-18] After surveying this subject, it was decided that a systematic experimental

study of hypervitaminosis D might be helpful for our purposes, and that this eventually might be compared with experimental hyperparathyroidism.[11,21,22]

The widespread changes disclosed by this study may be summarized as follows. First, degenerative changes with calcium deposition occurred in many organs and tissues. Second, the pattern of distribution of the degenerative changes was not specifically related to any common physical, chemical, or other characteristic of the affected elementary tissues or their combinations. Third, the sequences in development of these changes were not the same everywhere but were determined in part by unknown factors characteristic of the affected organ or tissue. Finally, the pattern and sequences of degeneration occurred in a reproducible fashion which could not have been predicted from a knowledge of factors which ordinarily determine the local characteristics of generalized disease processes.[19]

The widespread distribution of pathologic changes was largely dependent upon the amount of irradiated ergosterol given, the interval between doses, and the duration of the experiment. The data indicated that a sufficient quantity of the vitamin would produce minimal changes if it were given in a single dose or in a series of small daily doses over a period of several days. Maximal subacute changes due to the same quantity of the vitamin occurred when it was given in equal divided doses at intervals of 2 or 3 days. The best regime for obtaining maximal, chronic, slowly progressive changes has not been established, but good results followed the interposition of prolonged rest periods between brief dosage schedules designed to produce bursts of maximal active changes.

The pattern of distribution of pathologic alterations was constant under a given regime but varied somewhat with variation of the experimental conditions. In general, however, the earliest evidence of degeneration appeared in a particular location in each affected tissue or organ. Then, the process spread to involve other tissues or the same tissue in a succession of locations, each of which was resistant to change until the antecedent pattern of degeneration was set. Each affected type of tissue in each affected location in each affected organ had a definite level of susceptibility to the degenerative changes. This curious order of susceptibility to degeneration with calcification led to the conclusion that each type of cell or tissue in a functionally integrated system had a characteristic degeneration-calcification potential and that the level of the potential varied among different integrated systems. Hence, the succession of tissue alterations might be regarded as a result of the operation of four mechanisms. The first mechanism

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raised th ification potential of the tissue. The second raised the calcifying potential of the environmental fluids. The third was concerned with adaptation, and the fourth with restoration.

It is generally accepted that the best way to increase the calcification potential of a cell or tissue which does not normally calcify is to reduce its viability without bringing about rapid structural disintegration.[11] In the present experiments, it was clear that there was a reduction in viability of certain cells and stromal elements. It was not clear whether the reduction preceded or followed the deposition of calcium salts.[8] If reduction in viability preceded calcification, there was at this stage little evidence of customary findings of lessened viability. In other words, the earliest evidence of modification of stroma or cells did not definitely precede evidence of calcification in many locations. Furthermore, in most pathologic conditions, the reduction of viability of stroma and cells ordinarily initiates a sequence of inflammatory and regenerative reactions before calcium deposition becomes conspicuous. In the present study, this was not encountered as an early reaction in any location, except perhaps in certain muscular tissues, and then only in connection either with intolerably high dosage or intercurrent infection. However, under these conditions, conspicuous inflammatory reactions did not occur prior to degenerative calcifying changes, and the reactions were restricted principally to the arterial systems in cardiac and skeletal muscle. Usually, the reactions were confined to the media and adventitia of arteries, but in the severe cases there was a spread into neighboring muscle. The distribution of the inflammatory reaction, when it occurred, coincided with the distribution of the degenerative calcifying changes which developed without signs of inflammation in muscle of other experimental animals. From this it may be inferred that some factor increased the degeneration-calcification potential in all instances, but an inflammatory reaction was not elicited except when it was excessive or when other factors, perhaps related to intercurrent infection, were introduced. In any event we must admit the possibility of a more complex form of local tissue potential which might be called an inflammation-degeneration-calcification potential. Thus, under the same systemic conditions local factors may induce various combinations of inflammation, degeneration and calcification to occur at the same time in different organs or in the same tissue types in different organs.

The second mechanism seemed to be related to the composition of oxygenated or arterial blood and to special units of tissue integrated together in the performance of some particular function. For instance, emphasis has often been placed upon the deposition of calcium in tis-

sues concerned with acid secretion.[3,5,11] The explanation ha.. been that the secretion of acid led to a local alkalosis which in turn enhanced the likelihood of local precipitation of calcium phosphate and carbonate. Inasmuch as arterial blood is ordinarily more alkaline than venous blood, the view may be taken that through diffusion, systemic arterial walls may be more alkaline than venous walls, the reverse being true in the pulmonary system. The view may also be taken that the alkalinity of oxygenated systemic arterial blood decreases progressively with increasing distance from the alveolar capillaries and venules. Hence, if the interstitial fluids of the vascular wall reflect the local alkalinity of the blood, the deposition of calcium and associated degenerative changes of constituent tissues might be expected to fall off progressively in the direction of flow of the arterial blood. The microscopic observations disclosed that this was not always so. In a further analysis of this matter, the lung, kidney and stomach may be considered separately as the three principal acid-secreting organs.

Theoretically, it might be predicted that the region of maximal alkalinity in the lung would be in connection with the blood in the postalveolar pulmonary venules, and that maximal degenerative calcifying changes would occur in the walls of these vessels. In general, this was the case (Fig. 4). There were negligible lesions in the pulmonary arterial system, and the conspicuous changes were initially found in the walls of interalveolar capillaries and adjacent interalveolar stroma. Later, the venules and small pulmonary veins were affected. Insofar as the respiratory passages are concerned, if we accept free diffusion of gases into tissues, the level of maximum alkalinity would be in the upper trachea (Fig. 3). Hence, it might be predicted that the changes would be more conspicuous here than in the terminal bronchi. In general, this was found. The tracheal structures showed the earliest changes, and as a rule calcification along the respiratory passages decreased progressively in identical tissue types in identical locations with increasing distance from the larynx. In a consideration of this rule, however, other factors have to be considered. First, the lesions around the circumference of any respiratory passage were not uniform at a given level. The earliest changes were either in goblet cells of the epithelium or in the subjacent stroma. With increasing time and severity of the disease, the changes spread circumferentially and also in a very specific way in depth. The circumferential spread finally led to involvement of the entire basement membrane zone and an increasing span of epithelium. The spread in depth tended to skip the loose collagenous tissues of the mucosa and to appear in the walls of mucosal vessels nearest the subepithelial zone of calcification, the smooth

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muscle neighborhood, and the part of bronchial cartilages contiguous to the maximal mucosal changes. Thus, it seemed that the distribution, the sequences, and the magnitude of lesions were determined by the operation of local influences, as follows: alkalinity of environmental gases and fluids, type of tissue, type of cell, and the locally integrated functions of susceptible types of tissues or cells.

An analysis of alterations in the stomach may also be attempted in terms of relative alkalinity of parts of an acid-secreting organ. One might postulate that maximal alkalinity would be in relation to the venous blood flowing from the acid-secreting mucosa and that maximal calcification would occur in the tissues supporting the acid-secreting glands and in the walls of vessels leading from these tissues. This was not found even though, when mucosal changes did occur, they were restricted to the acid-secreting mucosa and to the *tunica propria* or cells of glands responsible for this function. Such changes, however, were inconspicuous in the early stages and often followed the development of prominent changes in the walls of arteries, the *tunica muscularis*, and muscularis mucosae (Fig. 5). Veins or venules were seldom affected. It might be assumed, therefore, that if relative alkalinity governed the distribution of the lesions in the stomach, it would have to operate in a curious way throughout the gastric wall. Consequently, it seemed that an inquiry should be made into the role of locally integrated functions of susceptible types of tissues or cells. It was apparent that the calcification was not a disorder of the acid-secreting gastric mucosa alone but a disturbance of the full thickness of the gastric wall concerned with acid secretion. For present purposes, the acid-secreting part of the stomach should be considered transmurally as a functional unit, separate and distinct, pathologically, from the remainder of the stomach. We have no explanation as to why the arterial system, the *tunica muscularis*, the muscularis mucosae and, to a lesser degree, the mucosa of only this part of the stomach were so severely affected by this degenerative calcifying disease. It offered a puzzling example of localization of pathologic changes in all susceptible tissue types in a functionally integrated system in a special location. The remainder of the intestinal tract offered other problems in this connection; namely, the variable pattern of involvement of the *tunica muscularis* at different levels, the variable resistance of different mucosal types to the disorder, and the restriction of severe arteriolar disease to the duodenal and, to a lesser extent, colonic segment of the intestinal tract.

Changes of the type found in the kidney have also been attributed to a local alkalosis, secondary to secretion of acids by the kidney.[10,11] It might be predicted therefore that changes would occur in that part

of the kidney just distal to the postabsorptive tubulovascular structure or in some special region concerned with selective absorption of alkaline elements or selective secretion of acidic elements. Actually, the earliest alterations occurred in the cells of the proximal convoluted tubules, soon thereafter in the basement membranes of these tubules, and somewhat later in the arterial supply to the affected nephron rather than the venous return from the nephron (Fig. 7). Thereafter, the arterial tree was usually progressively involved with increasing severity (Figs. 8, 15). It was concluded that within the limits of present knowledge of renal function, other factors than local alkalinity determined the distribution and sequence of the changes. As in the instance of the lungs and stomach, these factors were concerned with the interrelationships between susceptible tissue types in a functionally integrated system in a given location. The spread of changes in the kidney demonstrated this very well. In the early stages only a scattering of total nephrons as complete units showed lesions, but these were the nephrons with short arterial connections close to the medullo-cortical junction. As more lesions developed, the intervening nephrons with similar arterial connections were affected. With increasing time and severity of the disease, there was a spread of the changes distally toward the capsule, but seldom did the animal live long enough to show much alteration in the nephrons of the peripheral third of the cortex. As cortical spread developed, there was a similar spread of changes in an ever-broadening medullary zone within which the medullary loops of the successively affected nephrons were presumably located. These sequences are presumably related to the distribution of functional load rather than of a specific secretory function among a system of nephrons.

The preceding comments have touched upon local factors such as alkalinity, type of tissue, type of cell and locally integrated functions of susceptible tissues or cells. Muscle cells were among the least resistant, the order of decreasing susceptibility being smooth, cardiac, and skeletal muscle. Furthermore, the most readily affected component of these cells was the contractile protein, especially the "A" disk. Among the stromal elements, the severest lesions appeared in elastic tissue, basement membranes, the matrix of cartilage and the sarcolemmal investiture of muscle cells. Cellular and stromal elements were not equally susceptible in all locations. Hence, the development of this systemic metabolic disorder did not result in a generalized modification of a given element, and this is analogous to the effects of certain "toxins."[12,13] On the contrary, it led to conspicuous modifications of these elements in specific locations without changes in identical struc-

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tures in other locations. The extent to which this selectivity may be attributed to unrecognized differences in elements assumed to be identical, remains an intriguing problem. Certainly there are good reasons for believing that the composition of structures such as smooth muscle cells, collagen, elastic tissue, basement membranes and cartilaginous matrices is not the same everywhere. By the same token, the functional burden to which these elements are subjected is not the same everywhere.

The third mechanism was concerned with adaptation. Three adaptive activities deserve mention: the intimal proliferative reactions in arteries; the excessive production of atypical osteoid matrix following extensive resorption of bone; and the regression of the disease despite continuation of the same dosage regime which produced it. The discussion of these and other less obvious adaptive reactions awaits the completion of more prolonged experiments.

The fourth mechanism was concerned with restoration, principally the resorption or elimination of abnormal mineralized tissue, and the regeneration of tissues to replace those attacked by the disease. The salient feature of this mechanism was its indolence. In the respiratory mucosa, desquamation of calcified goblet cells and regeneration of basal cells to replace them were sluggish processes. At times, the regenerative sequences took a metaplastic trend so that the degenerated epithelial layer was replaced by one or more layers of stratified squamous epithelium. Wherever this occurred, there were local defects in the basement membrane and chronic inflammation in the subjacent mucosa. The mineralized basement membranes also tended to persist, being very slowly resorbed (Fig. 3). With increasing mineralization, there was increased fragility of these structures. Fractures developed and discontinuities thereby created seemed to stimulate local proliferation and accumulation of histiocytes. These commonly formed multinucleated giant cells which slowly resorbed the mineralized stromal structure, often encapsulating it completely. As this proceeded, the mineralized basement membrane seemed to migrate to a deeper level, and a regenerated stromal support appeared as a new structure between it and the respiratory epithelium. Similar mechanisms of encapsulation and resorption were encountered as far distally as the alveolar ducts but were seldom detected in connection with the calcified components of alveolar walls or pulmonary vascular channels. Alveolar walls seemed to break down spontaneously with the production of emphysema, but it was not clear that these mechanisms participated in the rupture (Fig. 4).

The behavior of the mesenchyme adjacent to abnormally mineralized

cartilages along respiratory passages was also interesting. Intense mineralization was usually conspicuous on the mucosal side of the cartilage. The perichondrium became less distinct, and the margin of the calcified matrix acquired a moth-eaten appearance. Capillaries occasionally invaded the marginal lacunae, and in some instances there was active formation of new cartilaginous matrix by proliferating chondrocytes. These activities resembled those which normally occur at provisional zones of endochondral bone formation. The overall result here, however, was the slow resorption of the heavily calcified bronchial and tracheal cartilages without osteogenesis.

Resorption and regeneration were still more sluggish in the kidney. Calcified tubulovascular components of the nephron seemed to endure without resorption or regeneration for at least several weeks. Evidence of regeneration of tubular epithelial cells of affected nephrons, compensatory hyperplasia of tubules of spared nephrons, interstitial fibrosis, inflammation, arteritis, glomerulosclerosis or other responses were never significant. The principal reaction occurred in the precortical and large cortical arteries. Here, there was intimal proliferation, excited less by degeneration and calcification of the media than by discontinuities due to fractures of calcified internal elastic membranes (Fig. 15). Whatever lesser reactions occurred in the subepithelial stroma of the renal pelvis were similar in most respects to those described in connection with the basement membranes of the respiratory tract.

Resorption and regeneration in relation to the acid-secreting part of the gastric wall were also very sluggish. The mucosal structures were not very responsive to the degenerative changes. The intimal reactions in the arterial tree were more active than in other organ systems, and the proliferative reactions also extended further into the smaller arterioles. The muscularis mucosae and the *tunica muscularis* showed the most conspicuous evidence of resorption and regeneration. In both locations, the degenerated calcified smooth muscle and intervening stroma often stimulated local histiocytic and giant cell reactions. These cells assisted in the resorptive process. Also, at times, there were many large pale elongated immature elements which appeared to be regenerating smooth muscle cells. In no case, however, was there significant leukocytic infiltration, local vascularizing reaction or fibrosis.

The complexities of resorption and regeneration in the skeletal system were difficult to interpret. Initially, the resorption of bone was accelerated, the number of osteoclasts was increased, and the number

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of oste[illegible]ts was decreased (Fig. 9). A curious proliferative process followed, resulting in the formation of a large amount of abnormal osteoid matrix beneath the periosteum and in the marrow around the residual cortical and trabecular bone (Fig. 10).[12,13] Though this matrix may be called "osteoid," it was more fibrillar, less homogeneous and much more heavily stained with hematoxylin than normal osteoid tissue. Furthermore, there was no relation between the voluminous deposits of matrix and any particular array of cells which resembled osteoblasts. The tissue looked more like collagen which had as much affinity for hematoxylin as any of the extra-osseous calcified stromal or cellular elements. With onset of repair, normal eosinophilic osteoid tissue and bone appeared in the midst of this voluminous basophilic fibrillary material which was resorbed. It was conspicuous only when extra-osseous calcium deposition had essentially ceased or in the period following termination of ergosterol administration. The prompt and rapid formation of bone was more pronounced in connection with the peritrabecular matrix and in the subperiosteal regions adjacent to the margins of articular cartilages. This led to a deformed osseous framework with depleted porous cortical bone, an excess of periosteal new bone, an excess of endosteal bone, and a coarse dense trabecular structure displacing the marrow. The final result had some resemblance to osteosclerosis of a pagetoid type with myelofibrosis (Fig. 10). The development of atypical hematopoietic elements as these changes encroached upon the bone marrow is beyond the limits of this discussion.

Finally, it was clear that a moderate dosage regime was most effective in the first few weeks. As the regime was continued, the expected progression of pathologic changes failed to occur, and evidence of the disease slowly disappeared. This was attributed to the activation of obscure mechanisms which provided increasing tolerance, resistance or immunity to the action of excessive vitamin D. In the light of certain degenerative calcifying human diseases, an inquiry into the nature of these adaptive and restorative mechanisms would seem to be of first importance.

Summary

A microscopic study of the evolution of hypervitaminosis D in rabbits disclosed generalized pathologic sequences which were due to partly reversible inflammatory, degenerative and mineralizing processes. The participation of these processes in the pathogenesis of the disease in a given location was governed principally by the level and duration of dosage with the vitamin. Administration of the vitamin in amounts just sufficient to produce pathologic changes in 2 or 3 weeks

led to mineralization of certain tissues which do not normally calcify. Under these conditions the deposition of calcium occurred without significant preliminary local structural modifications attributable to inflammatory or degenerative processes. When somewhat larger doses of the vitamin were used, abnormal mineralization of tissues was often preceded and accompanied by structural changes of a degenerative type. In order to excite significant early inflammatory reactions, still larger doses of the vitamin and perhaps other factors were required. These reactions occurred only in cardiac and skeletal muscle. They were localized to the walls of arteries, periarterial tissues and adjacent muscle, in which there were signs of progression of degeneration and mineralization.

Studies of restorative phases of the disorder disclosed evidence of reversibility and resistance to progression of these processes. The inflammatory reactions in muscle subsided, and mild indolent reparative reactions appeared in many degenerated mineralized tissues. Furthermore, additional degenerative changes seemed to progress very slowly, if at all, while, at least in some locations, the process of mineralization was slowly reversed even though the dosage was maintained. The reversal was characterized by the resorption of abnormally calcified matrices. At times, this occurred spontaneously, but as a rule the resorption was associated either with mobilization of macrophages and giant cells or penetration of the calcium deposits by vascularized stroma. The signs of reversal were usually preceded or accompanied by resumption of osteogenesis in the abnormal osteoid matrices in bone.

The same type of tissue or cell in different locations was not equally affected by the inflammatory-degenerative-mineralization mechanisms, presumably because of a difference not only in the composition of tissue gases and fluids from place to place but also in the susceptibility of a particular tissue in different locations. The latter was governed primarily by participation of a cell or tissue in a functionally integrated and spatially related system which had in one or more of its parts a high calcification potential.

The designation of function as adding to or detracting from the inflammatory-degenerative-calcification potential of a given structure led to a better understanding of patterns of the disease, especially in the respiratory tract, kidney, stomach, muscles and arteries. It may also assist in the future analysis of the reasons for the resemblance between certain structural changes in this experimental disease and those encountered in some presumably unrelated human disorders which are indolent, fundamentally degenerative, and ordinarily attributed to ill-defined metabolic derangements of advancing age.

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Among [illegible] senile osteoporosis, hypertrophic arthritis, pulmonary emphysema and arteriosclerosis deserve more than fleeting consideration, so long as it is recognized that identity of structural change does not imply identity of pathogenesis.

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LEGENDS FOR FIGURES

FIG. 1. Multiple, focal, inflammatory-degenerative-calcification changes in the myocardium of a rabbit given 600,000 units of viosterol in 2 weeks. The inflammatory reaction is located principally in and around the walls of small arteries and arterioles which in turn are surrounded by cardiac muscle showing degeneration with early calcification. Hematoxylin and eosin stain. × 200.

FIG. 2. Massive calcification of cardiac muscle in a rabbit given 1,200,000 units of viosterol in 7 weeks. The degenerated calcified muscle cells have multiple transverse fractures through which proliferating stromal elements have grown. The isolated calcified fragments of cells have undergone partial resorption. There is no inflammatory reaction. Hematoxylin and eosin stain. × 150.

FIG. 3. The tracheal mucosa of a rabbit given 1,200,000 units of viosterol in 7 weeks. Intra-epithelial calcification is in the form of concentrically laminated pseudocrystals which seem to develop exclusively in the goblet cells. The subepithelial calcification is concentrated in the darkly stained fragmented basement membrane. Note the absence of inflammation. Hematoxylin and eosin stain. × 500.

FIG. 4. A peripheral field of the lung of a rabbit given 1,200,000 units of viosterol in 6 weeks. Hematoxylin has a strong affinity for calcified tissue, demonstrated here as deeply-stained black segments of an emphysematous alveolar wall and the entire thickness of the wall of a small pulmonary vein. These changes in alveolar and venous walls regularly occurred together and were independent of inflammation. Hematoxylin and eosin stain. × 500.

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FIG. 5. A cross-section of the wall of a part of the stomach concerned with secretion of hydrochloric acid. The rabbit from which this section was made was given 1,600,000 units of viosterol in 14 weeks. Hematoxylin has an affinity for calcified tissues shown as irregular small dark areas in the mucosa, in the walls of submucosal arteries, in the bundles of smooth muscle of the inner half of the *tunica muscularis* and in the wall of a small serosal gastric artery. Hematoxylin and eosin stain. × 40.

FIG. 6. A high-power photomicrograph of one of the small darkly stained calcified areas in the *tunica muscularis* of the wall of the stomach shown in Figure 5, showing degenerated smooth muscle cells in the midst of granules and filaments of calcified protoplasm demonstrated by its strong affinity for hematoxylin. The small calcified granules are in the cytoplasm of the muscle cells. The tortuous calcified filaments are external to smooth muscle cells and seem to consist largely of interstitial fibrous or reticular elements. Hematoxylin and eosin stain. × 600.

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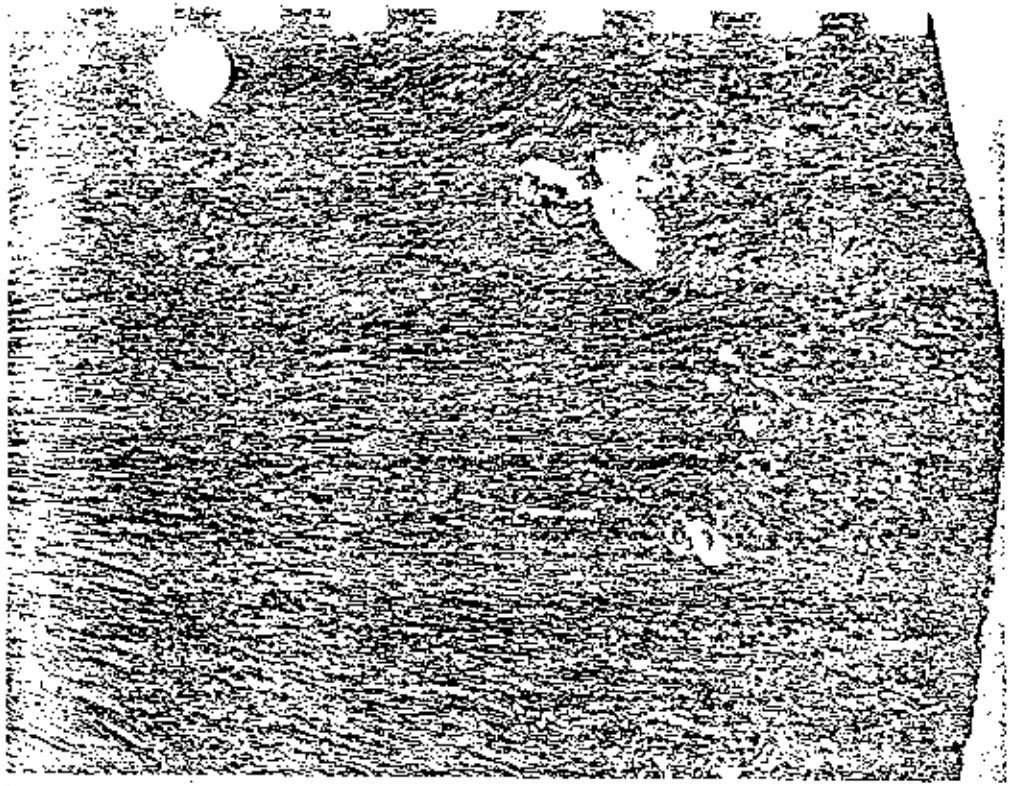

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FIG. 7. The cortex and medulla of a kidney from a rabbit given 1,000,000 units of viosterol in 5 weeks. Six weeks later the animal was sacrificed. The loss of normal structural detail is apparent in the inner half of the cortex and in a narrow transverse zone in the middle of the medulla. The small dark spots represent calcified structures in these 2 locations. The intervening lightly stained areas which are more conspicuous in the cortex are places where proliferating connective tissue has replaced degenerated tubulovascular units of nephrons. This represents an early stage of resorption of mineralized tissue with repair and regeneration. Inflammation is negligible in the kidney at all times during the development or regression of the disease. Hematoxylin and eosin stain. × 23.

FIG. 8. A field in the inner third of the cortex of a kidney of a rabbit given 800,000 units of viosterol in 4 weeks. This shows early calcification of glomerular structure with minimal associated alterations in tubular structure. The calcified elements have an affinity for hematoxylin. There is a tendency for calcium to accumulate in isolated glomerular loops. In one glomerulus the accumulation is in continuity with the calcified wall of the arteriole entering the glomerulus. Hematoxylin and eosin stain. × 220.

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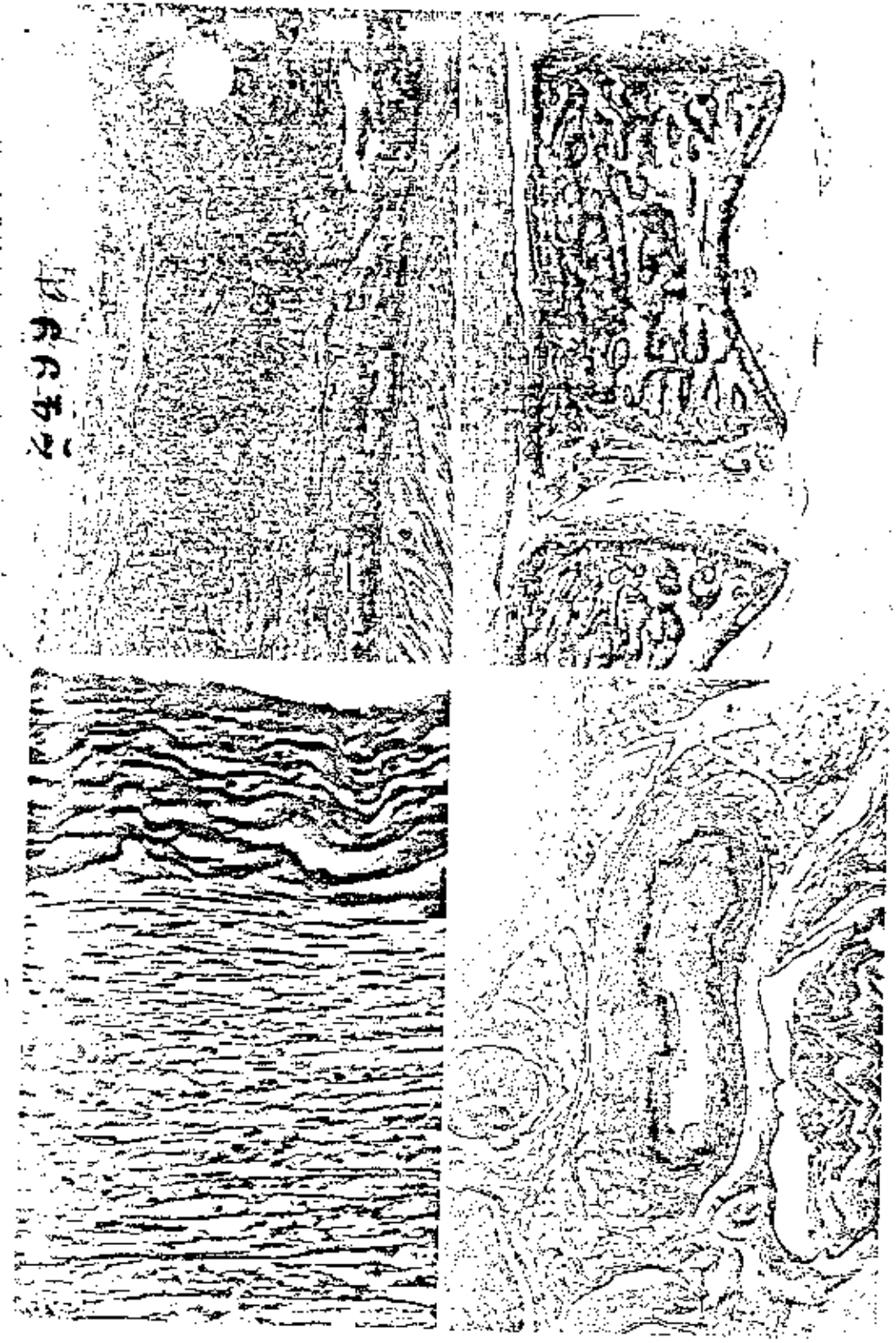

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FIG. 9. A longitudinal section of a rib from a rabbit given 1,200,000 units of viosterol in 4 weeks, showing widespread resorption of bone with conspicuous porosity of cortical bone, fibrosis of the marrow, and early subperiosteal deposition of osteoid tissue. The changes are characteristic of the disorder between the third and fifth weeks. After about the fifth week, the changes illustrated in Figure 10 become increasingly dominant. Hematoxylin and eosin stain. × 60.

FIG. 10. A longitudinal section of the vertebral column of a rabbit given 1,200,000 units of viosterol in 12 weeks. The resorption of bone, typical of early stages of the disease (see Fig. 9), has led to partial collapse of a vertebral body which is at this later stage undergoing an osteosclerotic pagetoid change. This is characterized by excessive deposition of osteoid tissue which has a conspicuous dark color due to its affinity for hematoxylin. Much of this abnormal osteoid tissue has been converted to bone, producing a dense trabecular structure which has seriously encroached upon the marrow. Also, note the proliferative changes which have produced "spurs" of new bone at the margins of the intervertebral disks. Hematoxylin and eosin stain. × 10.

FIG. 11. A cross-section of the wall of the thoracic aorta of a rabbit given 600,000 units of viosterol in 2 weeks. The inner third of the elastic tissue framework, stained deeply with hematoxylin, is uniformly impregnated with calcium. Note the absence of any inflammatory reaction though only a trace of residual cellular structure remains between the calcified elastic lamellae. Hematoxylin and eosin stain. × 250.

FIG. 12. A cross-section of an iliac artery of a rabbit given 1,000,000 units of viosterol in 5 weeks. The segmental darkly stained areas represent subintimal bands of calcific deposits in the inner half of the media. These discontinuous deposits tend to spread in all directions until they occupy the entire media. Meanwhile, there is a reactive proliferation of the intima which in time tends, as shown, to be equal in thickness to the part of the subjacent media inactivated by calcification. Hematoxylin and eosin stain. × 30.

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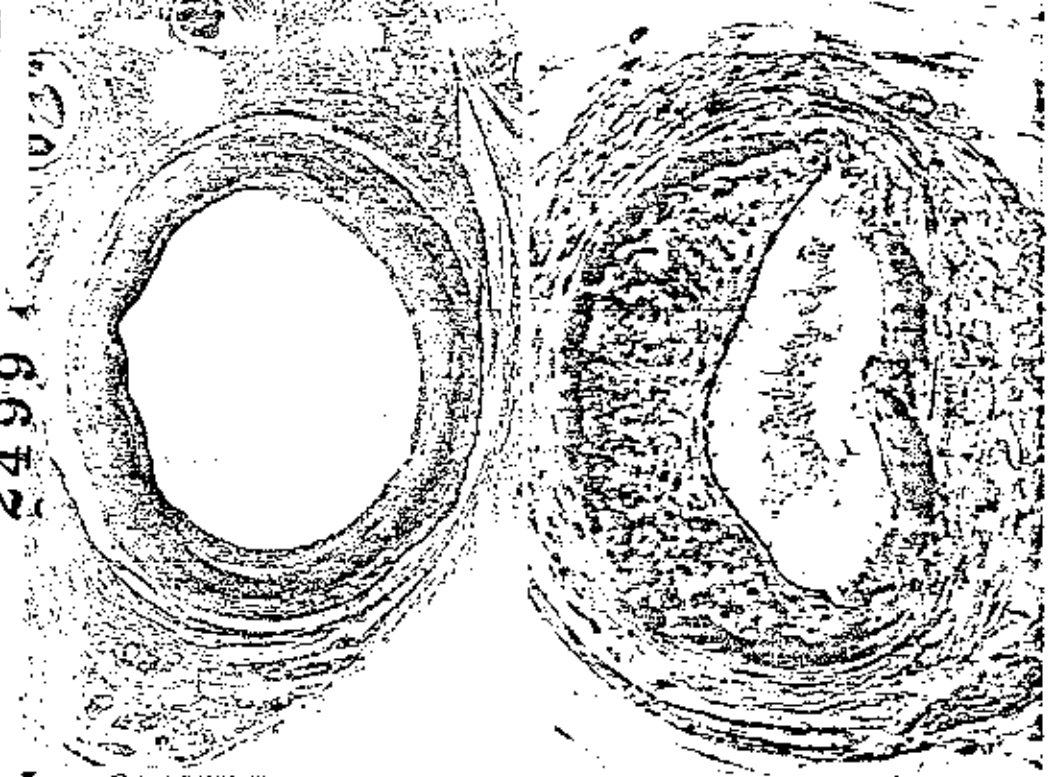

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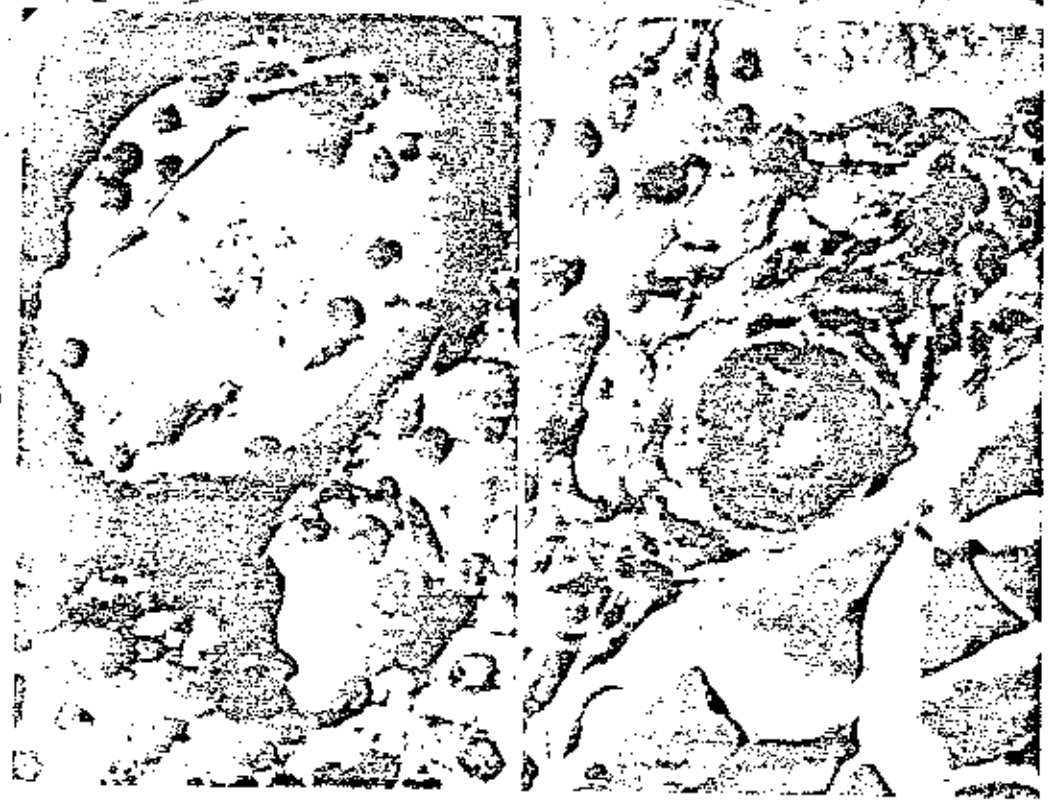

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FIG. 13. A cross-section of a carotid artery of a rabbit given 1,000,000 units of viosterol in 15 weeks. From the lumen outwards 3 distinct zones are shown. They are of almost equal thickness. The inner zone is composed of proliferating fibrocellular intimal tissue. The middle zone, deeply stained with hematoxylin, is the inner half of the media which has been uniformly impregnated with calcium. The outermost zone is the external half of the media which has been unaffected by the disease. Hematoxylin and eosin stain. × 32.

FIG. 14. A pancreatic artery of a rabbit given 1,600,000 units of viosterol in 14 weeks. The darkly-stained material in the vascular wall is calcified tissue with a strong affinity for hematoxylin. The deposits of calcium involve most of the internal elastic membrane and a modest amount of the media subjacent to the calcified parts of the internal elastic membrane. Three short stretches of the internal elastic membrane and adjacent media are normal. The loose-textured fibrocellular tissue internal to the calcific deposits is proliferating intima. Note that intimal proliferation did not occur over the 3 stretches of internal elastic membrane which were unaffected by the calcific disease. Hematoxylin and eosin stain. × 225.

FIG. 15. A small artery and adjacent arteriole in the renal cortex of a rabbit given 1,500,000 units of viosterol in 2 weeks. The walls of these vessels are heavily calcified as indicated by the strong affinity of the media for hematoxylin. Swollen intimal cells with their nuclei projected away from the calcified media toward the lumen of the larger artery represent the earliest intimal reaction to degenerative calcifying medial disease. Hematoxylin and eosin stain. × 600.

FIG. 16. A tiny arteriole in skeletal muscle of a rabbit given 1,200,000 units of viosterol in 7 weeks. The media of the arteriole, stained deeply with hematoxylin, is uniformly calcified. The swelling of the intima has reduced the size of the lumen. In the adventitia there are a few inflammatory cells representing a mild form of periarteritis often found in cardiac and skeletal muscle of rabbits dying of pneumonia during the course of a high-dosage viosterol regime. Hematoxylin and eosin stain. × 500.

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Induction of Intestinal Brush Border Alkaline Phosphatase by Vitamin D and Identity with Ca-ATPase

VITAMIN D may increase the rate of intestinal transport of calcium through a primary action on nucleic acid and protein synthesis[1,2]. Wasserman and Taylor have detected a calcium binding protein in chick intestine which is induced by vitamin D[3]. Harmeyer and DeLuca, however, reported a discrepancy between the time course of change in calcium transport and of the increase in calcium binding protein[4]. In addition, calcium binding protein appears in the soluble portion of the cell[3], whereas the major site of vitamin D action may be at the level of microvillar membrane[1,5]. Thus, a comparison of isolated intestinal microvilli from vitamin D deficient and repleted animals might reveal other specific alterations which could be related to the action of vitamin D.

One such alteration involves the enzyme alkaline phosphatase. Rachitic animals have been reported to have lower intestinal alkaline phosphatase activities than normal[7], but alkaline phosphatase has not been considered important to the effect of vitamin D on calcium transport[8]. Nevertheless, this enzyme is particularly abundant in intestine, kidney and bone[9], the tissues involved in calcium transport, and most of the intestinal alkaline phosphatase is localized in the microvilli[10,11].

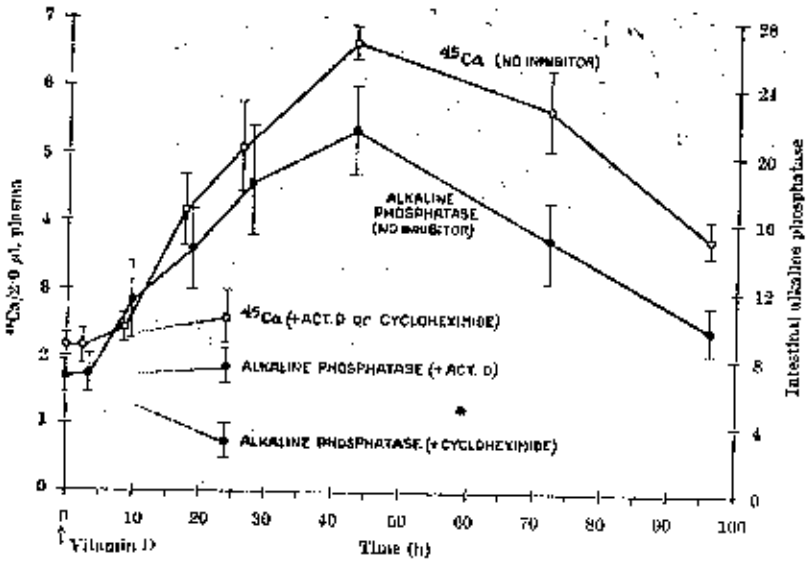

Fig. 1. Time course of the induction of calcium absorption and intestinal alkaline phosphatase by vitamin D. Rachitic chicks received an oral dose of 50 IU of vitamin D_3 and calcium absorption was assayed at the appropriate time period, *in vivo*, by administering an oral dose of ^{45}Ca and counting an aliquot of plasma 1 h later. The same animals were then killed and 20 per cent butanol extracts of intestinal mucosa homogenates were prepared. After removal of the butanol, the aqueous extracts were assayed for protein by the method of Lowry *et al.*[14] and alkaline phosphatase activity was measured by the procedure of Hansonen *et al.*[15]. The enzyme assay was done with p-nitrophenyl phosphate at pH 9·3 in the presence of 5 mM Mg^{2+}. Actinomycin D (20 μg/100 g chick) was administered intraperitoneally 2 h before vitamin D; cycloheximide (300 μg/100 g chick) was injected in two portions, one simultaneously with the vitamin and one 12 h later. Studies using these inhibitors were carried out only at 24 h. Each point represents the average value obtained from five animals ± s.e.

For these reasons, we examined the activity of this enzyme in relation to the physiological effects of vitamin D.

Four week old vitamin D deficient chicks[13] were used for these studies. Calcium transport and enzyme activity were assayed by standard methods[13,14]. A significant increase in both intestinal calcium absorption and intestinal alkaline phosphatase activity occurred in rachitic chicks following a single physiological dose of 50 IU of vitamin D_3 (Fig. 1). The change in calcium absorption and in enzyme activity followed identical time courses after vitamin D treatment. The increase in alkaline phosphatase was apparent when measured as either units of enzyme per mg protein or units of enzyme per mg DNA. Pretreatment of the chicks with actinomycin D or cycloheximide blocked both the increases in enzyme activity and the calcium transport measured 24 h after vitamin D (Fig. 1).

Because of the relationship between both alkaline phosphatase and vitamin D action and the intestinal microvilli or brush border, our attention was directed to this subcellular fraction. Intestinal microvilli were isolated according to a modification of the procedure of Forstner and Isselbacher[12]. Because alkaline phosphatase is a Zn^{2+}-metalloenzyme[16], known to be inactivated by EDTA[16], ethylene-*bis*(oxyethylenenitrilo)-tetraacetic acid (EGTA)—a less efficient chelator of Zn^{2+} (ref. 17)—was substituted in the preparation. Electron microscopic examination of the isolated microvilli revealed a preparation similar to that of Forstner and Isselbacher. Vitamin D caused a striking increase in the alkaline phosphatase activity of microvilli (Table 1). The alkaline phosphatase increase was not abolished by treatment with the detergent 'Triton X-100', and the activity therefore increased as a result of additional enzyme molecules (or inherently more active molecules) rather than as an alteration in enzyme environment in the membrane.

Recently, Martin *et al.*[18] reported a vitamin D mediated increase in Ca^{2+}-activated ATPase in isolated microvilli. We also found an augmentation of both the Mg^{2+}-activated and Ca^{2+}-activated ATPase activities in brush borders from vitamin D treated chicks (Table 1). By contrast, no change in the activity of the brush border enzyme, invertase, was seen in either intestinal homogenates or isolated microvilli. This finding emphasized the selectivity of the induction of alkaline phosphatase and ATPase and eliminated the possibility that a proliferation of microvilli was responsible for our observations.

Extraction of microvilli with 20 per cent butanol selectively released alkaline phosphatase from the membrane and raised its specific activity. Similarly, the Mg^{2+}-ATPase and especially the Ca^{2+}-ATPase activities were enhanced by 20 per cent butanol extraction. The corresponding enhancement of the ATPases and alkaline phosphatase by butanol extraction (Table 1) and the similarity in the magnitude of their induction after vitamin D repletion, coupled with a recent report that alkaline phosphatase has ATPase activity[20], led us to consider that the Mg^{2+}-ATPase and Ca^{2+}-ATPase activities and alkaline phosphatase represented different measures of the activity of a single enzyme. To test this hypothesis, we carried out parallel inhibition studies on the three activities in question: alkaline phosphatase, Mg^{2+}-ATPase, and Ca^{2+}-ATPase. L-Phenylalanine (10 mM), a stereospecific inhibitor of intestinal alkaline phosphatase[21], was a potent inhibitor of Ca^{2+}-ATPase, and blocked the Mg^{2+}-ATPase to a lesser degree. The D-stereoisomer of phenylalanine served as a control and had no effect on any of the three activities. Be^{2+} and Zn^{2+}, known to be inhibitors of alkaline phosphatase[16,22], blocked the Ca^{2+}-ATPase and Mg^{2+}-ATPase activities in a fashion analogous to L-phenylalanine. Co^{2+}, Fe^{3+}, Sr^{2+} and Ba^{2+} had little effect on any of the tested activities aside from a small stimulation of the Mg^{2+}-ATPase produced by Sr^{2+} and Ba^{2+}. Most likely, Sr^{2+} and Ba^{2+} mimic Ca^{2+} to create a pseudo Ca^{2+}-ATPase activity. All metals were used at a concentration of 4 mM. These results indicate that alkaline phosphatase, Ca^{2+}-ATPase and much of

Table 1. EFFECTS OF VITAMIN D TREATMENT ON THE ACTIVITY OF INTESTINAL MICROVILLEAR ALKALINE PHOSPHATASE AND Mg^{2+}-ATPase AND Ca^{2+}-ATPase

Preparation	Alkaline phosphatase specific activity (μ/mg protein ± s.e.)			Metal ion activator	ATPase specific activity (μ/g protein ± s.e.)		
	−Vit. D chicks	+Vit. D chicks	+D/−D		−Vit. D chicks	+Vit. D chicks	+D/−D
Intact microvilli	9 ± 2	29 ± 6*	3·2	Mg^{2+}	240 ± 51	278 ± 36†	1·3
				Ca^{2+}	33 ± 13	62 ± 21†	1·9
1 per cent 'Triton X-100' treated microvilli	27 ± 5	71 ± 7*	2·6	Mg^{2+}	—	—	—
				Ca^{2+}	—	—	—
20 per cent butanol extract of microvilli	44 ± 10	121 ± 28*	2·8	Mg^{2+}	292 ± 30	403 ± 80*	2·1
				Ca^{2+}	285 ± 58	503 ± 116*	1·8

ATPase activity was measured at pH 7·4 with 5 mM Tris-ATP and either 20 mM Mg^{2+} alone or 20 mM Mg^{2+} plus 40 mM Ca^{2+}. The Ca^{2+}-ATPase activity is computed by subtracting the Mg^{2+}-ATPase value. Phosphate was assayed by the method of Baginski and Zak[19]. ATPase assays were not carried out on 'Triton X-100' treated microvilli because the detergent was found to interfere with colour development in the phosphate determination. Vitamin D treated (+vit. D) chicks received 50 IU of vitamin D_3 orally 40 h before killing. Each number is the average of triplicate assays carried out on each of four separate animals. Significant differences between +vit. D and −vit. D are indicated * when $P < 0·01$ and † when $P < 0·05$.

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the Mg^{++}ATPase activity of intestinal microvilli are properties of the same enzyme protein.

We conclude that vitamin D increases the activity and probably the amount of intestinal brush border alkaline phosphatase in close temporal correspondence with vitamin D mediated calcium absorption. Both effects are inhibited by antibiotics which interfere with protein synthesis. These findings and previous studies implicating the microvillus as the site of vitamin D action lead us to believe that the change in microvillar phosphatase is related to the change in calcium transport in some fundamental fashion.

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Cholesterol as a carcinogen

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[Plate 2]

In confirmation of previous reports from this Institute, cholesterol has been shown to be carcinogenic. Highly purified cholesterol (Schwenk process), dissolved in olive oil by heating on the water-bath, was injected subcutaneously into 224 stock mice. Fourteen sarcomas were induced at the site of injection. In one group of these mice, eleven sarcomas arose in 115 mice, the percentage when calculated on the number of mice which survived to at least the minimal latent period being 14 %. There is no evidence that purification impairs the carcinogenic potency of cholesterol.

Introduction

The evidence that purified cholesterol is a carcinogen was put forward in earlier reports from this Institute (Hieger 1947, 1949; Hieger & Orr 1954).

Further experiments have now been completed which reinforce the statements presented there. Cholesterol purified by the bromination technique of Schwenk (1952) was found to give a product which, after one further crystallization from acetone, had no absorption in ultra-violet light above about 230 to 237 mμ and indeed one batch had no absorption above 225 mμ. The purification process used for the earlier preparations (described in Hieger & Orr 1954) consisted of six stages, viz. acetylation, bromination, de-bromination, chromatography, de-acetylation and crystallization. The u.v. absorption curve of the resulting purified cholesterol fell rapidly in the 225 mμ region and flattened out between about 230 and 260 mμ.

Method

If the u.v. absorption curve is accepted as the principal criterion of the purity of the cholesterol, then it would appear that the simpler purification process of Schwenk gives a purer end-product. In explanation, it is possible that as the purification processes are multiplied, traces of undesirable substances are introduced increasingly into the material. For the detailed description of the injection technique, the characteristics (histology and transplantation) of the tumours induced, and of the results of control tests on solvents in a large series of mice (700 animals, see below), see the previous paper in this series (Hieger & Orr 1954).

The cholesterol purified by the Schwenk process (which will now be referred to simply as 'Schwenk cholesterol') has been tested for carcinogenic activity upon 224 mice. These were divided into two groups: (*a*) a series housed in the 'hydrocarbon-carcinogen-free room' (where no such compounds were in use), and (*b*) a series kept in the 'carcinogen room' where some other experiments were being carried out which involved the use of potent carcinogens, chiefly 3:4-benzpyrene.

Results

Unfortunately, an epidemic broke out in the non-carcinogen room and some hundreds of mice were lost, including those injected with Schwenk cholesterol, but not before sarcomas had developed in three of them, and had been proved histologically (see figures 1 to 4, plate 2). The latent periods of these three (8½, 9 and 10 months) were unusually short for cholesterol-induced tumours. The epidemic did not spread to the 'carcinogen room' where the series of mice injected with Schwenk cholesterol had a reasonably high survival rate (although not as high as in some other series in this room). Altogether eleven sarcomas were obtained in this series, the first three of which appeared after short latent periods (9½, 11 and 11 months).

Table 1. Sarcoma induction in stock mice by highly purified cholesterol (Schwenk process) in olive oil

expt.	location	survival rate (months) at start	6	9	12	15	18	21	24	27	no. of sarcomas	latent period (months)	% incidence of sarcomas per 100 mice calculated on no. of mice at start	at minimal latent period
1	'non-carcinogen room'	109	107	102	(all mice killed: epidemic)						3	8½, 9, 10	—	—
2	'carcinogen room'*	115	90	84	66	33	22	10	—	—	11	9½, 11, 11, 16, 17, 17, 18, 20, 22, 22, 23	9·5	14
For comparison: a test† on stock mice in the 'carcinogen room' using commercial cholesterol in olive oil														
3	'carcinogen room'	100	—	—	75	64	45	32	10	2	8	11, 12, 16, 16, 17, 20, 20, 24	8	10·7

* For a detailed description of this term see Hieger & Orr (1954).

† The figures for this experiment have been reported previously (Hieger & Orr 1954).

The close similarity of the latent periods of the two sets of three sarcomas (i.e. the series in the carcinogen room and in the non-carcinogen room) leaves no doubt at all that had the epidemic been avoided, the subsequent history of tumour development in the *non*-carcinogen room in mice injected with Schwenk cholesterol would have resembled closely that of the parallel series in the carcinogen room. The data in table 1 show the yield of sarcomas and the survival rate for the two series, and for comparison the corresponding figures for a test of unpurified commercial cholesterol.

The similarity of the results of experiments 2 and 3 (table 1) shows that the solutions of commercial and of highly purified cholesterol have much the same carcinogenic potency, and suggests strongly that cholesterol *per se* is the carcinogen in commercial cholesterol, for here the kind of solvent, strain of mice and environment were kept constant in these two experiments. Alternatively, if the immediate carcinogen is produced *in vivo* from cholesterol, it is cholesterol which is the parent compound and not some associated substance present as impurity in commercial cholesterol.

The high incidence of sarcomas in experiments 2 and 3 in table 1—high, that is, for this type of carcinogen (cholesterol-rich preparations have in some of our previous experiments shown a much lower production of sarcomas)—cannot be easily explained away on the grounds of contamination by 3:4-benzpyrene conveyed from other mice in the same room ('carcinogen room') which were being treated with these compounds, for the following reasons:

TABLE 2. SARCOMA INDUCTION IN MICE BY INJECTION OF COMMERCIAL CHOLESTEROL IN OLIVE OIL

expt.	mouse strain	location	no. of mice at start	no. of mice surviving (months) 6	9	12	15	18	21	24	27	30	no. of sarcomas	sarcoma induction calculated per 100 mice at start (%)	at 1 yr. (%)	latency period (months)
1*	C_{57}	non-carcinogen room	60	34	30	28	21	14	9	3	1	—	1	1·7	3·6	22
2	C_{57}	non-carcinogen room	30	10	10	10	9	7	7	5	(3 at 25 m.) epidemic (all killed)		1	3·3	10	23
3	C_{57}	non-carcinogen room	39	28	26	24	20	16	11†		epidemic (all killed)		3	7·7	13	12, 14, 18
4	C_{57}	carcinogen room	75	62	48	46	40	26	15	3	0	—	4	5·5	8·7	21, 22, 22, [illegible]

* This experiment was reported (Experiment A, Table V) by Hieger & Orr (1954).

† Eleven mice were still alive in the 21st month of this experiment (no. 3). There can hardly be any doubt that but for the epidemic the percentage yield of sarcomas would have been still higher.

Hieger & Orr (1954) stated that the percentage sarcoma induction (stock mice) was 8% in the carcinogen room (CR) and 4·5% in the non-carcinogen room (non-CR). If the calculation is made to exclude the mice which did not survive 1 year and were therefore not at risk, the percentages then rise to 10·7% for the CR and 7·9 and 5·4% (two experiments; mean 6·7%) for the non-CR. Taking into account the difference between the two percentage yields of tumours, i.e. 10·7 and 6·7%, and the total number of mice involved in the experiment, i.e. 235, comprising 100 in CR and 70 and 65 (two experiments) in non-CR, this difference could not be considered a very serious divergence; at any rate a difference of such magnitude might well be incurred by variations in the 'sensitiveness' of the mice, a factor about which we know nothing as yet. For example, several experiments have shown that sarcoma development by cholesterol is more frequent in the fitter mice than in those groups which are obviously less healthy. Four experiments are detailed in table 2 to show the differences in sarcoma yield obtained when conditions are very similar, i.e. when the same material is injected in four separate groups of mice of the same strain. This table illustrates the order of similarities and differences which can be expected when a test on the carcinogenic potency of cholesterol is repeated a number of times.

The author is inclined to the explanation that some unidentified factor linked with the health of the mice, or some minor genetic factor, would account for the

resistance of certain groups of mice to the carcinogenic stimulus of cholesterol, and for the pronounced response of other groups. The foregoing results support the following conclusions:

(1) Approximately 10% of mice (Stock or C_{57}) surviving a minimum of one year, ultimately develop sarcoma at the site of injection when injected subcutaneously with a solution of cholesterol in olive oil prepared by heating on the water-bath.

(2) Purification of cholesterol (as judged by u.v. absorption) does not impair its carcinogenic activity.

(3) Housing mice in the same room with others which are being treated with 3:4-benzpyrene does not introduce a serious risk of contamination.

Tests on compounds related to cholesterol

In the earlier stages of this investigation, before solutions of purified cholesterol had been shown to have carcinogenic activity, and even before commercial cholesterol had been tested, sarcoma induction by the unsaponifiable fraction of human organs (liver, lung, kidney, muscle) suggested experiments carried out between 1941 and 1946, to test some members of the steroid group of compounds. Later, when such tests had given negative results, one was led to infer that cholesterol *per se* might be the essential carcinogen in the unsaponifiable fraction.

In table 3 are shown the results of the tests on compounds related to cholesterol. None of the steroids tested showed appreciable activity as carcinogens, but of course the number tested represents only a small fraction of all those which are now available. With regard to experiments 10 and 11 (deoxycholic acid and sodium deoxycholate), the single sarcomas obtained in each test show that the activity of these preparations is of the same order as that of the solvents. Fieser, Bischoff and co-workers (1946, 1954, 1955, 1955) have prepared a number of oxidized derivatives of cholesterol which are being tested.

The role of solvent

Only oily solutions of cholesterol have shown carcinogenic activity; no tumours were obtained in an experiment where a suspension of cholesterol in a 4% gelatin gel was used. As far as these tests go it would appear that the oil is a necessary part of the injection material. The control tests totalled about 700 mice treated with olive oil or lard which had been heated on the boiling water-bath; two sarcomas were obtained with these oils. Similar low orders of activity have been reported by Bischoff (1955), by Steiner (1943, 1947) and by other workers. Burrows, Hieger & Kennaway (1936) found that lard gave seven sarcomas and olive oil one sarcoma in 198 rats, but no tumours in 236 mice. Hartwell's bibliography (1951) of compounds which have been tested for carcinogenic activity describes some hundreds of tests with the female sex hormones. The great majority of these were carried out with olive oil or sesame oil as solvents, yet in only one or two cases did the hormones dissolved in oil induce sarcoma. The above facts show that olive

oil, lard and sesame oil* have very low potency as carcinogens; less than 1 % of injected mice develop sarcoma. Most probably the oil is necessary in the process of carcinogenesis by cholesterol to ensure solution.

TABLE 3. TESTS FOR CARCINOGENIC ACTIVITY OF COMPOUNDS RELATED TO CHOLESTEROL. INJECTIONS WERE MADE SUBCUTANEOUSLY, REPEATEDLY

expt.	compound	how administered	no. of mice at start, and surviving during expt.							no. of sarcomas
			at start	after 1 yr.	18 m.	24 m.	27 m.	30 m.	33 m.	
1	isocholesterol	15 % solution in lard	10	9	5	4	0	—	—	0
2	isolumisterol	15 % solution in lard	10	9	4	0	—	—	—	0
3	cholesterylene	(15 %) saturated solution in lard	10	5	5	3	2	1	—	0
	cholesterylene not purified	10 % solution in lard	10	9	7	2	1	0	—	0
4	ergosterol	15 % solution in lard	10	10	3	0	—	—	—	0
5	Δ^{5}-cholestene	15 % solution in lard	10	9	4	3	2	0	—	0
6	7-dehydrocholesterol	15 % solution in lard	10	10	7	2	2	0	—	0
	7-dehydrocholesterol	15 % solution in lard	10	9	4	0	—	—	—	0
7	epicholesterol + allocholesterol	15 % solution in lard	10	9	8	3	3	2	0	0
8	7-hydroxycholesterol (mixture of 7 (α) and 7 (β))	15 % solution in lard	20	14	6	2	1	1	1	0
9	lathosterol	10 % solution in olive oil	50	37	23	5	2	0	—	0
10	deoxycholic acid	1 % suspension in olive oil + tristearin (5:1)	73	34	21	6	1	0	—	1*
11	Na-deoxycholate	1·2 g cholesterol + 60 mg deoxycholate + 12 g olive oil + tristearin	62	44	26	6	0	—	—	1†

* C_{57} mouse ♂, latent period; 21 months.
† C_{57} mouse ♂, latent period; 18 months.

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* Such oils are liable to chemical changes on keeping, especially if they have been heated and exposed to atmospheric oxidation. These changes are accompanied by an increase in absorption in the u.v. in the region 220 to 250 mμ. There is no evidence, however, that any such change which might develop after the cholesterol is heated on the water-bath with the oily solvent contributes to the carcinogenic activity of the resulting solution.

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Science, 180: 190-191, 1973

1α-Hydroxy Derivative of Vitamin D_3: A Highly Potent Analog of 1α,25-Dihydroxyvitamin D_3

Abstract. *The 1α-hydroxy derivative of vitamin D_3 has been chemically synthesized and tested for its biological activity. This analog has comparable biological activity on a weight basis to 1,25-dihydroxyvitamin D_3 in the stimulation of intestinal calcium transport and bone calcium mobilization in normal and anephric rats. Because the 1α-hydroxy derivative is synthesized from cholesterol, it is easier and less expensive to prepare than 1α,25-dihydroxy derivative, making it attractive as a drug in the treatment of renal osteodystrophy and hypoparathyroidism.*

For vitamin D to carry out its biological functions in stimulating intestinal calcium transport and mobilizing bone calcium, it must be hydroxylated on C-25 in the liver (*1*) and subsequently on C-1 by the kidney (*2*). The resulting metabolite, 1α,25-dihydroxyvitamin D_3 [1α,25-$(OH)_2D_3$] (Fig. 1) was isolated in pure form, identified (*3*), and recently synthesized (*4*). This metabolite is the most potent form of vitamin D known (*5, 6*), and it is the only known

Table 1. Intestinal calcium transport and bone calcium mobilization response of rats to 1α-OH-D_3. Results are means of six rats; S.E., standard error.

1α-OH-D_3 (pmole)	^{45}Ca serosal/ ^{45}Ca mucosal (mean ± S.E.)	Serum Ca (mg/ml) (mean ± S.E.)
	Normal	
None*	1.5 ± 0.2	4.3 ± 0.1
6.2	1.8 ± 0.2	4.8 ± 0.1
62.5	2.8 ± 0.3	5.8 ± 0.1
625	2.9 ± 0.2	6.7 ± 0.1
1250	2.9 ± 0.2	6.9 ± 0.1
	Anephric	
None*	1.5 ± 0.2	4.1 ± 0.1
312	2.3 ± 0.2	5.2 ± 0.1

* The control dose consisted of 50 μl of 95 percent ethanol alone.

...D metabolite active in anephric ...(7).

During the course of the 1α,25-(OH)$_2$D$_3$ synthesis we explored the ...rious reactions utilizing a less expensive starting material, namely cholesterol instead of homocholenic acid. As a result, 1α-hydroxyvitamin D$_3$ (1α-OH-D$_3$) (Fig. 1) was prepared.

The possibility that such an analog of 1α,25-(OH)$_2$D$_3$ might be biologically active was of obvious interest and consequently was examined. The analog proved to be approximately equal to 1α,25-(OH)$_2$D$_3$ in inducing intestinal calcium transport and bone calcium mobilization in normal and anephric rats.

The synthesis of 1α-OH-D$_3$ was accomplished by the methods described for 1α,25-(OH)$_2$D$_3$, except that cholesterol was used as starting material (4). The resulting product showed the expected ultraviolet absorption spectrum (λ_{max} 265 nm, λ_{min} 228 nm) for the 5,6-*cis* triene system and mass spectrum [*m/e* 400 (M+), 287, 152, and 134].

Furthermore, the compound chromatographed as one peak on gas-liquid chromatography, demonstrating its pur- (it is interesting that, although this ...log has a 5,6-*cis* triene system, it ...oes not cyclize in the gas chromatograph to the pyro and isopyro forms as do other vitamin D metabolites, probably because of the close proximity of 1α-OH to the triene system).

Weanling male rats (Holtzman) were fed for 2 weeks a vitamin D–deficient diet containing adequate calcium and phosphorus (8) and then fed a low calcium (0.02 percent), vitamin D–deficient diet for another week (9). Groups of rats, either bilaterally nephrectomized or normal, received the appropriate dose via the jugular vein in 0.05 ml of 95 percent ethanol (Fig. 2 and Table 1). Intestinal calcium transport and bone mobilization assays were performed as described (9).

The results shown in Fig. 2 demonstrate that 1α-OH-D$_3$ is capable of stimulating intestinal calcium transport and bone calcium mobilization. This analog elicits an intestinal calcium transport response as early as 3 hours after its administration and shows a ...ximum response at 6 hours. 1,25-...D)$_2$D$_3$ also gives a maximum response 6 hours after intravenous administration (6). Although 1α-OH-D$_3$ does not show as large a transport response as 1,25-(OH)$_2$D$_3$ at 6 hours, it sustains the response for at least 24 hours, while the response to 1,25-(OH)$_2$D$_3$ decreases after 6 hours (6). The 1α-OH-D$_3$ is capable of mobilizing calcium from bone as early as 3 hours after its administration and shows a maximum response at 14 hours.

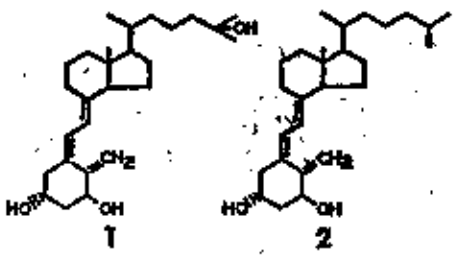

Fig. 1. Structures of 1α,25-(OH)$_2$D$_3$ (1) and 1α-OH-D$_3$ (2).

Table 1 shows that as little as 62.5 pmole of 1α-OH-D$_3$ induces intestinal calcium transport and bone mineral mobilization at 14 hours after administration. A similar response to 62.5 pmole of 1,25-(OH)$_2$D$_3$ has been demonstrated (10). Of great importance is the fact that 1α-OH-D$_3$ stimulates both intestinal calcium transport and bone mineral mobilization in anephric rats (Table 1). However, as the dose of 1α-OH-D$_3$ is increased from 625 pmole to 1250 pmole, there is no significant increase in the intestinal calcium transport response.

Because the 1α-OH-D$_3$ is less expensive and less difficult to prepare chemically as compared to 1α25-(OH)$_2$D$_3$, but has similar activity in both anephric and intact vitamin D-deficient rats, it is potentially attractive for the treatment of renal osteodystrophy and hypoparathyroidism. It has been suggested that the 5,6-*trans* form of vitamin D$_3$ may be a suitable substitute for 1,25-(OH)$_2$D$_3$ [illegible] induce both intestinal calcium transport and bone calcium mobilization in anephric rats (9). Although this analog is easy and inexpensive to make it is about 50 to 100 times less active than either the 1,25-(OH)$_2$D$_3$ or 1α-OH-D$_3$.

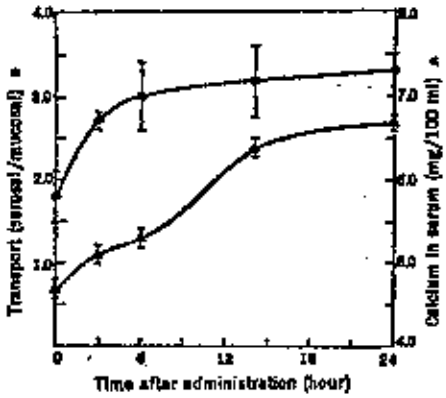

Fig. 2. Intestinal calcium transport (——) and bone calcium mobilization (- - - - -) response of vitamin D–deficient rats on a low calcium diet to 625 pmole of 1α-OH-D$_3$. The vertical bars represent the standard error of the means; six animals were used for each measurement.

It is not possible to predict at this time whether 1α-OH-D$_3$ is hydroxylated on C-25 in the liver before it is active. It is interesting that the time it takes to produce a maximum response in the intestine parallels that for 1α,25-(OH)$_2$D$_3$, which would argue against further hydroxylation. On the other hand the 1α-OH-D$_3$ does not attain the high intestinal calcium transport response seen for 1α,25-(OH)$_2$D$_3$ but can sustain its response for longer periods, which could be consistent with the idea that the 1α-OH-D$_3$ is further hydroxylated presumably on C-25. Additional investigation will be necessary to answer these questions, but the possible utility of this compound in medicine seems clear.

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Isotachysterol$_3$ and 25-Hydroxyisotachysterol$_3$: Analogs of 1,25-Dihydroxyvitamin D_3

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Abstract. *Isotachysterol$_3$, 25-hydroxyisotachysterol$_3$, and isovitamin D_3 have been synthesized and tested for biological activity. Like 1,25-dihydroxyvitamin D_3, isotachysterol$_3$ stimulates intestinal calcium transport and bone calcium mobilization in anephric rats, whereas 25-hydroxyvitamin D_3 does not. Although isovitamin D_3 is biologically active in normal rats it is inactive in anephric rats.*

Vitamin D must first be hydroxylated on carbon-25 (C-25) in the liver (*1, 2*) and then on carbon-1 (C-1) in the kidney (*3*) before it can carry out its physiologic functions. Unlike vitamin D or its 25-hydroxy derivative, 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) is capable of stimulating both intestinal calcium transport and bone calcium mobilization in anephric rats (*4*). These results make clear the potential use of 1,25-$(OH)_2D_3$ in the treatment of patients with hypocalcemia, impaired intestinal calcium transport, secondary hyperparathyroidism, and osteodystrophy due to renal dysfunction. However, be-

Table 1. Intestinal calcium transport and bone calcium mobilization response to isotachysterol$_3$, isovitamin D_3, 5,6-*trans*-vitamin D_3, 25-hydroxyisotachysterol$_3$, and 25-OH-D_3 in normal and anephric rats.

Compound	Amount (μg)	Animals (No.)	Ca in serum (mg/100 ml) (mean ± S.E.)	^{45}Ca serosal/ ^{45}Ca mucosal (mean ± S.E.)
		Normal		
None	0	6	4.3 ± 0.1	1.8 ± 0.2
Isovitamin D_3	5	6	6.5 ± .1	3.5 ± .3
Isotachysterol$_3$	5	6	6.3 ± .1	3.2 ± .3
25-Hydroxyisotachysterol$_3$	5	6	6.4 ± .1	3.0 ± .2
25-OH-D_3	0.25	6	6.4 ± .1	3.2 ± .2
5,6-*trans*-vitamin D_3	5	6	6.0 ± .1	3.5 ± .1
		Anephric		
None	0	6	3.7 ± 0.1	1.2 ± 0.2
Isovitamin D_3	5	6	3.8 ± .1	1.2 ± .1
Isotachysterol$_3$	5	6	5.0 ± .1	3.2 ± .2
5,6-*trans*-vitamin D_3	5	6	4.9 ± .1	3.5 ± .2
25-OH-D_3	0.25	6	3.9 ± .1	1.4 ± .2

cause of the limited amount of 1,25-$(OH)_2D_3$ available to the clinician, only a few patients with advanced renal diseases have been treated with the metabolite. Brickman *et al.* have reported that 1,25-$(OH)_2D_3$ is effective in stimulating intestinal calcium transport and raising serum calcium and phosphorus concentrations in three uremic patients (*5*).

Our 21-step chemical synthesis of 1α,25-$(OH)_2D_3$ provides the possibility that large quantities of this hormone may become available (*6*). However, the length and expense of this procedure prompted an examination of analogs of 1,25-$(OH)_2D_3$ which might be less expensive and less difficult to prepare. One approach taken has been the 180° rotation of the A ring of vitamin D_3 by isomerization, thus placing the 3β-hydroxyl in the position occupied by the 1α-hydroxyl of 1,25-$(OH)_2D_3$ (*7, 8*). Thus 5,6-*trans*-vitamin D_3 (Fig. 1) is capable of stimulating both intestinal calcium transport and bone calcium mobilization in anephric rats, while its 25-hydroxy derivative appears only to stimulate intestinal calcium transport (*7, 8*).

Because of the possible usefulness of 1,25-$(OH)_2D_3$ analogs in medicine, we tested the biological activity of two other isomers of vitamin D_3, which also have the 3β-hydroxyl function in the geometric position occupied by the 1α-hydroxyl of 1,25-$(OH)_2D_3$ (Fig. 1). Isotachysterol$_3$, 25-hydroxyisotachysterol$_3$, and isovitamin D_3 were synthesized and shown to be capable of inducing intestinal calcium transport and bone calcium mobilization in normal animals. It was surprising, however, to find that isotachysterol$_3$ but not isovitamin D_3 is biologically active in anephric rats.

Isotachysterol$_3$ and 25-hydroxyisotachysterol$_3$ were prepared from vitamin D_3 and 25-hydroxyvitamin D_3 (25-OH-D_3), respectively, according to the procedure of Inhoffen *et al.* (*9*). Fifty milligrams of vitamin D_3 or 25-OH-D_3 were dissolved in diethyl ether and treated with boron trifloride etherate for 24 hours. The reaction mixture was extracted with water and diethyl ether, and the product in the ether layer was chromatographed as described for 5,6-*trans* vitamin D_3 and its 25-hydroxy derivative (*7*). Both isotachysterol$_3$ and 25-hydroxyisotachysterol$_3$ exhibited a characteristic ultraviolet absorption spectrum, with maxima at 302, 290, and 280 nm (*9*), and each appeared as one component on gas-liquid chromatography and on thin-layer chromatog-

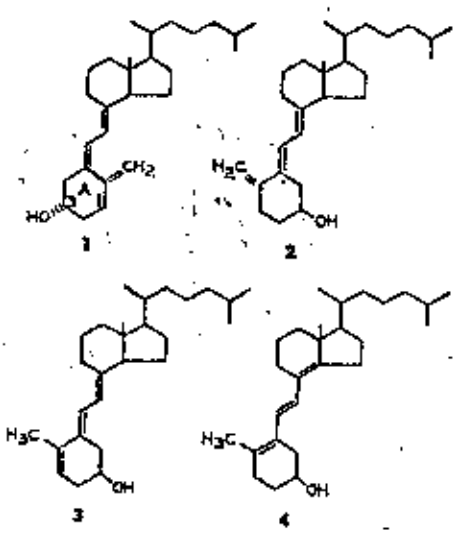

Fig. 1. Structures of (1) vitamin D_3, (2) 5,6-*trans*-D_3, (3) isovitamin D_3, and (4) isotachysterol$_3$.

raphy. The mass spectrum of isotachysterol$_3$ showed a molecular ion *m/e* at 384, and fragments at 369, 271, 259, 253, and 230 (Fig. 2). Isovitamin D_3 was prepared as reported (*10*), and the product was chromatographed as described above for isotachysterol$_3$. This product had an ultraviolet absorption spectrum with maxima at 300, 288, and 278 nm (*9, 10*) and chromatographed as a single homogeneous peak on gas-liquid chromatography and thin-layer chromatography. The mass spectrum displayed a molecular ion *m/e* at 384 and fragments at 369, 271, and 253 (Fig. 2). Because the ultraviolet absorption spectra for isotachysterol$_3$ and isovitamin D_3 are so similar it is very difficult to tell them apart. However, it is possible to distinguish one isomer from the other by mass spectrometry because the fragmentation pattern for each of these isomers is quite distinct.

For intestinal calcium transport and bone calcium mobilization measurements, weanling, male albino rats (Holtzman) were fed for 2 weeks a diet adequate in calcium and phosphorus but deficient in vitamin D, and then fed for an additional week on a diet low in calcum (0.02 percent) and deficient in vitamin D (*7*). At the end of the third week, groups of six rats, which were either bilaterally nephrectomized or sham-operated, received either isotachysterol$_3$, 25-hydroxyisotachysterol$_3$, isovitamin D_3, 5,6-*trans*-vitamin D_3, or 25-OH-D_3 intrajugularly in 0.05 ml of 95 percent ethanol. The animals were decapitated 20 hours later, and blood was collected. Small intestines were used immediately for measurements of intestinal calcium transport by the everted gut sac technique (*7*). The blood was centrifuged, and 0.1 ml of serum was mixed with 1.9 ml of a 0.1 percent lanthanum chloride solution. Serum calcium was determined with a Perkin-Elmer atomic absorption spectrophotometer (model 403).

Isotachysterol$_3$, isovitamin D_3, and 25-hydroxyisotachysterol$_3$ stimulate intestinal calcium transport and elicit a rise in serum calcium presumably at the expense of bone calcium (Table 1). Like 1,25-$(OH)_2D_3$ and 5,6-*trans*-vitamin D_3, isotachysterol$_3$ is able to stimulate both intestinal calcium transport and bone calcium mobilization in anephric rats (*4, 7*). Unlike isotachysterol$_3$, isovitamin D_3 and 25-OH-D_3 are incapable of inducing either intestinal calcium transport or bone calcium mobilization in anephric rats. Surprisingly, the 25-hydroxy derivative of isotachysterol$_3$ is

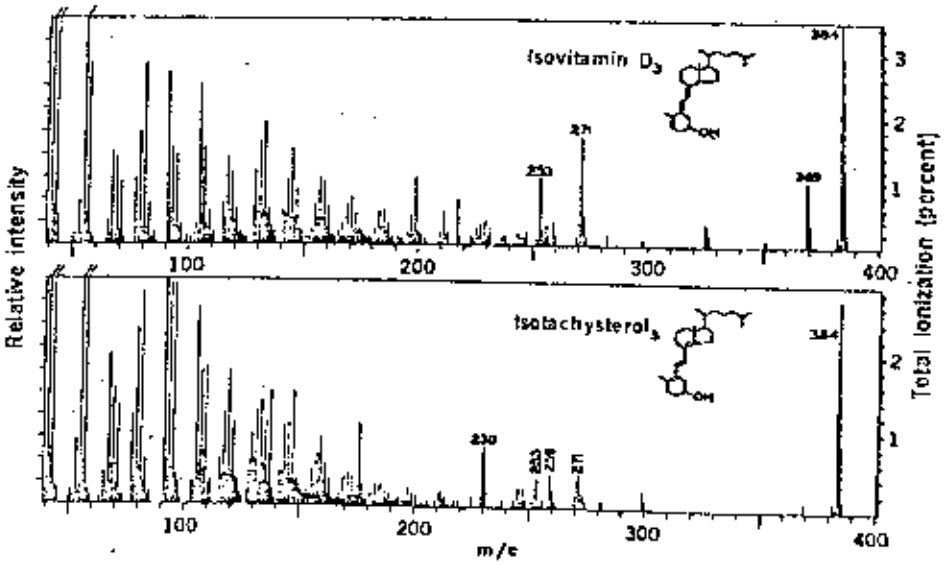

Fig. 2. Mass spectra of isovitamin D_3 and isotachysterol$_3$.

capable of inducing both intestinal calcium transport and bone calcium mobilization. The 25-hydroxy derivative of 5, 6-*trans*-vitamin D_3 is unable to stimulate bone calcium mobilization while stimulating intestinal calcium transport (*7*).

It is also surprising that isovitamin D_3 is biologically active in normal rats but not in anephric rats. Although there are many possible explanations, it seems likely that a small amount of vitamin D_3 may have remained in the preparation. This small amount, which could go undetected by either thin-layer chromatography or gas-liquid chromatography, would be metabolized only in normal animals to 1,25-$(OH)_2D_3$ and elicit a response in both the bone and intestine. Another possible explanation is that in isovitamin D_3 the A ring may not be rotated 180°, and therefore the kidney is required for a hydroxylation on C-1 to provide an analog of 1,25-$(OH)_2D_3$ which is biologically active in the intestine and bone. This problem remains to be solved.

In any case, our results demonstrate that, in addition to the 5,6-*trans* isomers, another isomer of vitamin D_3—isotachysterol$_3$—can function in bone and intestine of anephric animals. The conversion of vitamin D_3 to the isotachysterol$_3$ is as simple to carry out as the 5,6-*trans* isomerization. However, it has the added advantage that the procedure results in virtually quantitative conversion of vitamin D_3 to the isotachysterol. Thus its preparation would be less expensive than that of 5,6-*trans*-vitamin D_3, and its purification before use would be considerably simpler. Its possible utility in cases of renal osteodystrophy, therefore, seems evident.

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Identification of 1,25-Dihydroxycholecalciferol, a Form of Vitamin D_3 Metabolically Active in the Intestine

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ABSTRACT **A biologically active vitamin D_3 metabolite ("peak V metabolite") more polar than 25-hydroxycholecalciferol has been isolated from chicken intestines in pure form as a mono(trimethylsilyl)ether derivative. The molecule has been identified as 1,25-dihydroxycholecalciferol by means of mass spectrometry, ultraviolet absorption spectrophotometry, and specific chemical reactions.**

It has been demonstrated (1) with tritiated vitamin D_3 of high specific activity that vitamin D_3 is converted to metabolites more potent and rapidly acting than the parent vitamin. The most abundant of these, 25-hydroxycholecalciferol (25-HCC) (2), acts more rapidly than vitamin D_3 in enhancing both intestinal calcium transport and bone mineral mobilization (3). The existence in the intestine of additional polar metabolite(s) active in promoting calcium transport is evident from work in a number of laboratories (4–8). In agreement with Haussler *et al.* (8) and Myrtle *et al.* (9), we have observed that one of these metabolite(s), the peak V metabolite, induces intestinal calcium transport in deficient chickens more rapidly than vitamin D_3 or 25-HCC, although it is less effective than 25-HCC in curing rickets or inducing bone mineral mobilization.

By blocking the conversion of 25-HCC to peak V metabolite by prior administration of actinomycin D, we have shown (10) that peak V, or a further metabolite of peak V, is the metabolically active form of vitamin D in the intestine. In the presence of actinomycin D, the peak V compound stimulates intestinal calcium transport, whereas 25-HCC does not. This polar metabolite has now been isolated in pure form and identified as 1,25-dihydroxycholecalciferol (1,25-DHCC).

ISOLATION

Five vitamin D-deficient chicks were each given 2.5 μg of [1,2-^{3}H]vitamin D_3 (specific activity, 3.2×10^6 dpm/μg), orally, in vegetable oil (Wesson). 24 hr later the chicks were killed. Methanol–chloroform extracts of the small intestines were prepared, and the chloroform extract was subjected to a sequence of chromatographic separations on Sephadex LH-20 and BioRad (beads) S-X8 (BioRad Labs., Richmond, Calif.). 11 μg of highly purified peak V metabolite was obtained. 8 μg of the metabolite was then reacted with 5 μl of TBT (a combination of TMS-imidazole, bis-TMS-acetamide, and trimethylchlorosilane, Pierce Chemical Co., Rockford, Ill.) and 10 μl of pyridine for 10 min. The resulting tri(trimethylsilyl)ether derivative was chromatographed on a 1 × 60 cm column of Sephadex LH-20 in 50% chloroform in petroleum ether (bp 67–69°C). The tri(trimethylsilyl) derivative was partially desilylated in 100 μl of 3.6×10^{-4} M HCl in MeOH + 50 μl pyridine at 60°C for 4 hr. This gave a mixture of di(trimethylsilyl), monotrimethylsilyl, and desilylated products, which were separated by chromatography on Sephadex LH-20. The metabolite and the mono(trimethylsilylated) metabolite were separately chromatographed in methanol on Sephadex LH-20 (11) before being used for structural identification.

IDENTIFICATION OF THE METABOLITE AS 1,25-DHCC

Several lines of evidence established the structure of the active metabolite. The mass spectrum of the metabolite exhibited a molecular ion at m/e 416; this requires the incorporation of two additional oxygen atoms into the basic carbon skeleton of vitamin D_3 (cholecalciferol). The presence of three hydroxyl

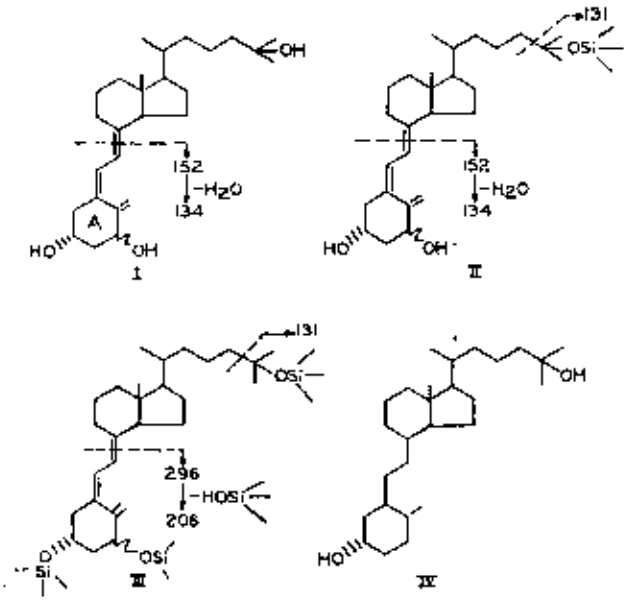

FIG. 1. Structures of 1,25-DHCC and its derivatives. (The numbers indicate schematically the origin of prominent peaks in the mass spectra of these compounds.) I = 1,25-DHCC; for discussion of II–IV, see text.

Abbreviations: 1,25-DHCC, 1,25-dihydroxycholecalciferol; 25-HCC, 25-hydroxycholecalciferol; TMS, trimethylsilyl.

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functions in the metabolite was demonstrated by the formation of a tri(trimethylsilyl)ether derivative (structure III, MW = 632, Fig. 1) and a mono(trimethylsilyl)diacetate derivative (MW = 572).

The mass spectrum of the tri(trimethylsilyl)derivative (III) and the mono(trimethylsilyl) derivative [MW = 488; structure II, produced from the tri(trimethylsilylated) compound by partial desdylation] and of the trimethylsilylether-diacetate exhibited an intense peak at m/e 131, which established the presence of a hydroxyl function at C-25 (2, 11, 12). In addition, the mass spectrum of the mono(trimethylsilyl) compound (II, Fig. 1) showed peaks at m/e 152 and 134 (152 − 18), which correspond to the characteristic ions at m/e 136 and 118 in the spectra of cholecalciferol, 25-HCC, and other cholecalciferol metabolites (2, 11, 12).

Since the ions at m/e 136 and 118 (136 − 18) are known to derive from ring A of the basic skeleton of vitamin D, the shift of these ions by 16 mass units (i.e., 136 + 16 = 152; 118 + 16 = 134) in the mass spectrum of the metabolite mono(trimethylsilyl)ether established the presence of an additional hydroxyl function in ring A. The same peaks (152, 134) are also observed in the mass spectrum of the metabolite itself, whereas in the mass spectrum of the tri(trimethylsilyl)ether derivative (structure III) these peaks are shifted to m/e 296 (152 + 2 silyl groups) and 206 [296 − $HOSi(CH_3)_3$]. In addition to providing conclusive proof for the presence of two hydroxyl functions in ring A, the peaks at m/e 152 and 134 (and 296 and 206 in the case of structure III) also establish the existence of the triene double-bond system in the metabolite, since these mass spectral peaks are very characteristic for the vitamin D-type triene structure (2, 12, 13). Ultraviolet absorption at 265 nm provided further strong evidence for a vitamin D-triene system. For the position of the additional hydroxyl function in ring A, carbons 1, 2, or 4 are thus the only possibilities. The presence of a C-1 hydroxy group was established by the following experiment: catalytic reduction (PtO_2/H_2) of the metabolite yielded a product that was shown to be identical (by cochromatography) with 25-hydroxyhexahydrocholecalciferol (structure IV). Since formation of compound IV from the metabolite requires hydrogenolysis of a hydroxyl group, the hydroxyl function lost in this process must have been located at either C-1 or C-4 (i.e., allylic to the double bond system). The failure of the metabolite to react with periodate eliminates a C-4 (as well as a C-2) hydroxyl group; this observation requires that the additional hydroxyl group be a carbon 1. The observation that the biosynthesis of the metabolite from [1-^{3}H]cholecalciferol results in the loss of the tritium provides corroborative evidence for a C-1 hydroxy compound (6). The data above thus establish structure I, 1,25-DHCC, for this new metabolite.

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Isolation and Identification of 1,25-Dihydroxycholecalciferol. A Metabolite of Vitamin D Active in Intestine*

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ABSTRACT: A metabolite of vitamin D_3, thought to be the "tissue-active" form of the vitamin in the intestine, has been isolated from chicken intestines in pure form as the monotrimethylsilyl ether derivative.

The structure of this metabolite has been identified as 1,25-dihydroxycholecalciferol by means of mass spectrometry, ultraviolet absorption spectrophotometry, and specific chemical reactions.

It is now generally accepted that vitamin D must be metabolically "activated" before it can produce its characteristic physiologic effects (Lund and DeLuca, 1966; Morii *et al.*, 1967; DeLuca, 1967; Ponchon *et al.*, 1969). As a direct consequence of this concept Blunt *et al.* (1968a,b) isolated and identified 25-hydroxycholecalciferol (25-HCC),[1] a metabolite of vitamin D which is more active in all respects than the parent vitamin. Continued experiments suggested that 25 hydroxylation of vitamin D is the initial reaction in the functional metabolism of vitamin D (Ponchon and DeLuca, 1969a; Ponchon *et al.*, 1969). This reaction takes place in the liver (Horsting and DeLuca, 1969) and is strongly controlled by product inhibition suggesting it to be of importance in the regulation of vitamin D metabolism and function.

The synthesis of [^{3}H]25-HCC (Blunt and DeLuca, 1969) made possible an examination of the question of whether 25-HCC is further metabolized before it functions. Cousins *et al.* (1970a,b) demonstrated clearly that 25-HCC is metabolized to more polar metabolites which appear in the target tissues before the physiological responses to 25-HCC ensue. Suda *et al.* (1970a,b) isolated two such polar metabolites, *i.e.*, 21,25-DHCC and 25,26-DHCC from the plasma of pigs. The former preferentially stimulates bone mineral mobilization while the latter has preferential activity on intestinal calcium transport. A metabolite derived from vitamin D_3 which is more polar than 25-HCC has also been found in the intestine (Haussler *et al.*, 1968; Lawson *et al.*, 1969; Ponchon and DeLuca, 1969b). This metabolite acts more rapidly than 25-HCC in initiating intestinal calcium transport (Haussler *et al.*, 1971; Kodicek *et al.*, 1970; Myrtle and Norman, 1971; Omdahl *et al.*, 1971) but is less active in the cure of rickets and does not appear to be more active than 25-HCC in the mobilization of bone mineral (Omdahl *et al.*, 1971) suggesting it to be the metabolically active form in the intestine. Actinomycin D inhibits the conversion of 25-HCC to this metabolite (Tanaka and DeLuca, 1971; Gray and DeLuca, 1971). Of great interest is that this metabolite but not 25-HCC will initiate intestinal calcium transport in the presence of actinomycin D providing strong evidence that it represents the metabolically active form in the intestine (Tanaka *et al.*, 1971).

This metabolite has now been isolated from chicken intestines in pure form as its monotrimethylsilyl ether derivative and identified as 1,25-DHCC. A preliminary report of this work has appeared (Holick *et al.*, 1971).

General Procedures

Radioactive determinations were carried out with a Packard Tri-Carb Model 3375 liquid scintillation counter equipped with an automatic external standardization system. Samples were dried in counting vials with a stream of air and dissolved in toluene-counting solution (2 g of 2,5-diphenyloxazole and 0.1 g of 1,4-bis[2-(4-methyl-5-phenyloxazolyl]benzene per l. of toluene).

Ultraviolet spectra were recorded with a Beckman DB-G recording spectrophotometer, while mass spectrometric determinations were carried out with an A.E.I. MS-9 mass spectrometer, using a direct probe inlet at temperatures of 118-130° above ambient. All solvents used were of reagent grade and those utilized for chromatography in the later stages of the isolation of the metabolite were triply distilled before use.

Isolation. Chickens (1450, 1-day old, White Leghorn cockerel chicks obtained from Northern Hatcheries of Beaver Dam, Wis.) were kept in cages at 38° and fed *ad libitum* for 4 weeks as previously described (Omdahl *et al.*, 1971). At the end of week 4 the chicks were starved for 12 hr and then each was given orally 2.5 μg of [1,2-^{3}H]vitamin D_3 (specific activity 3.2×10^5 dpm/μg) in 0.2 ml of vegetable oil (Wesson oil, a mixture of cottonseed and soybean oil). Twenty-four hours later they were killed by decapitation and the whole small intestine was excised, flushed with chilled distilled H_2O, and immediately frozen on Dry Ice. The chick intestines (12.5 kg) were divided into three batches and each was processed in a similar fashion. The first batch (5.5 kg) was homogenized in two parts MeOH(w/v) for 1 min. One part $CHCl_3$ (5.5 l.) was then added to the homogenate and the mixture allowed to stand at 4° for 3 days. The mixture was filtered and an additional 5.3 l. of $CHCl_3$, 0.5 l. of distilled H_2O, and 0.2 l. of saturated NaCl was added to the filtrate to effect a phase separation. The lower phase was separated from the aqueous phase. The aqueous phase was reextracted with an additional

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[1] Abbreviations used are: 25-HCC, 25-hydroxycholecalciferol; 21,25-DHCC, 21,25-dihydroxycholecalciferol; 25,26-DHCC, 25,26-dihydroxycholecalciferol; 1,25-DHCC, 1,25-dihydroxycholecalciferol.

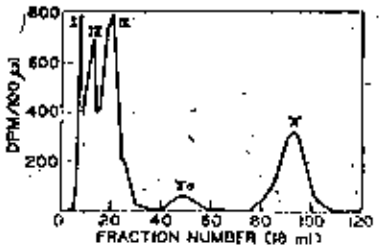


FIGURE 1: Sephadex LH-20 column (3 × 70 cm packed in 65:35 $CHCl_3$-Skellysolve B) profile of intestinal lipid extract from chicks given [1,2-^{3}H]vitamin D_3 orally 24 hr earlier.

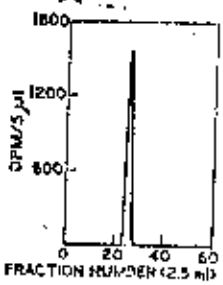


FIGURE 3: Bio-Beads S-X8 column (1 × 150 cm packed in 65:35 $CHCl_3$-Skellysolve B) profile of intestinal peak V isolated from Sephadex LH-20 (MeOH) column (Figure 2).

4 l. of $CHCl_3$-0.5 l. of MeOH. The residue from the initial extraction was reextracted with 6 l. of MeOH and 3 l. of $CHCl_3$. The combined $CHCl_3$ phases (25 l.) was mixed with 16 l. of tap H_2O, 1.5 l. of MeOH, and 0.4 l. of saturated NaCl. This mixture was allowed to stand at 4° for 24 hr. The $CHCl_3$ phase was concentrated to 210 ml on a rotary flash evaporator. This yellow oily residue was partitioned between 1.79 l. of Skellysolve B (predominantly *n*-hexane, bp 67–69°) and 2 l. of 9:1 MeOH-H_2O (v/v) according to the procedure of Suda *et al.* (1970a). The aqueous methanol phase (lower phase) was removed and extracted twice with a mixture of 1.5 l. of $CHCl_3$-0.5 l. of tap H_2O. The $CHCl_3$ phases were combined and evaporated to yield 10 g of a yellow residue. The other two batches 4 and 3.0 kg of chicken intestines were similarly extracted to yield 12 g of lipid residue.

Chromatography. The yellow residue (10 g) was dissolved in 15 ml of 65:35 $CHCl_3$-Skellysolve B and applied to a 3 × 70 cm glass column packed with 110 g of Sephadex LH-20 (an hydroxypropyl ether derivative of Sephadex G-25, Pharmacia Corp., Piscataway, N. J.) with 3 cm of Celite on the top of the bed in the same solvent according to the procedure of Holick and DeLuca (1971). Fractions (100 18 ml) were collected and 100 µl of each fraction used for tritium determinations (Figure 1). The remaining two batches of chicken intestinal extract were chromatographed in a similar fashion. The peak V region from all three batches was combined and dried on a flash evaporator to yield 11 µg of the metabolite (based on specific activity of vitamin D, and assuming 50% loss of tritium (Kodicek *et al.*, 1969; Omdahl *et al.*, 1971)) in 319 mg of yellow lipid. This residue was dissolved in 1 ml of MeOH and applied to a 1 × 150 cm glass column packed to a height of 148 cm with Sephadex LH-20 in MeOH (Suda *et al.*, 1970a). Fractions (80 2.5 ml) were collected and 5 µl of each fraction was used for tritium determination to reveal the elution position of the metabolite (see Figure 2). The peak tubes (39–41) were combined and dried under N_2 to give 11 µg of the metabolite in 68 mg of yellow lipid. The residue was dissolved in 0.1 ml of MeOH and reapplied to the same column. Peak V (11 µg) was recovered in 31 mg of lipid. This sample was dissolved in 0.15 ml of 65:35 $CHCl_3$-Skellysolve B and applied to a 1 × 150 cm glass column packed with Bio-Beads S-X8 (polystyrene resin produced by Bio-Rad Corp., Richmond, Cal.) to a height of 148 cm in the same mixed solvent. The fractions containing the metabolite were combined (see Figure 3), and the same chromatography was repeated three more times. This time only the peak tubes which contained the less amount of lipid were combined to provide 8 µg of the metabolite in less than 1 mg of lipid residue. This residue was dissolved in 0.1 ml of 65:35 $CHCl_3$-Skellysolve B and applied to a 1 × 100 cm Sephadex LH-20 column packed in the same mixed solvent.

The peak fractions were collected and dried under N_2. The metabolite (8 µg) was dissolved in 10 µl of pyridine and reacted with 10 µl of TBT (a special combination TMS-imidazole, bis-TMS-acetamide, and trimethylchlorosilane, Pierce Chemical Co., Rockford, Ill.) at 22° for 10 min. The reaction mixture was evaporated under N_2, dissolved in 0.1 ml of 1:1 $CHCl_3$-Skellysolve B, and applied to a 1 × 60 cm Sephadex LH-20 column packed and developed in the same mixed solvent as previously described (Holick and DeLuca, 1971). The trimethylsilyl ether derivative of the metabolite which was recovered from this column was dissolved in 50 µl of pyridine and desilylated by adding 100 µl of 3.6 × 10^{-4}% HCl-MeOH (v/v) and reacting it for 3 hr at 50° then an additional 1.5 hr at 60°. The solvent was removed under N_2, dissolved in 0.1 ml of 65:35 $CHCl_3$–Skellysolve B, and applied to a 1 × 60 cm Sephadex LH-20 column packed in and developed with a solvent of 65:35 $CHCl_3$-Skellysolve B. Fractions (27 3.4 ml) were collected followed by an additional 33 5.2-ml fractions as shown in Figure 4. The ditrimethylsilyl ether, monotrimethylsilyl ether, and the unsilylated products were chromatographed separately on a 1 × 60 cm MeOH Sephadex LH-20

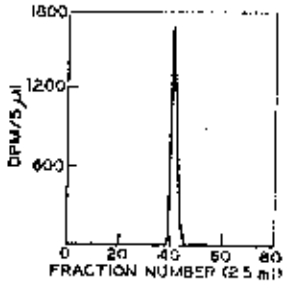


FIGURE 2: Sephadex LH-20 column (1 × 150 cm packed in MeOH) profile of intestinal peak V isolated from Sephadex LH-20 (65:35 $CHCl_3$-Skellysolve B) columns (Figure 1).

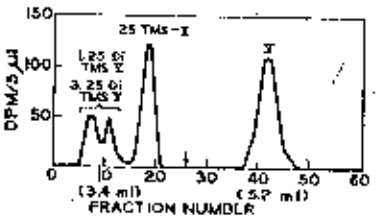


FIGURE 4: Sephadex LH-20 column (1 × 60 cm packed in 65:35 $CHCl_3$-Skellysolve B) profile of the products obtained from the partial desilylation reaction.

TABLE I: Stimulation of Intestinal Calcium Absorption in Rachitic Chicks by 25-HCC and 1,25-DHCC.

Compound	Dosage (μg)	t_a (hr)[a]	% ^{45}Ca Absorption[b]
25-HCC	0.125	24	54.5 ± 6.8[c] (4)
1,25-DHCC	0.065	8.5	54.6 ± 3.9 (5)
Control (0.2 ml of oil)		8.5	18.2 ± 2.3 (7)

[a] t_a represents: time of assay following dose. [b] Net per cent ^{45}Ca absorption for compound at time t_a. [c] Plus or minus the standard error of the mean. Numbers in parentheses show the number of animals in each group. The intestinal calcium absorption measurement was performed as described by (Omdahl *et al.*, 1971).

column as the last purification step before mass spectrometry and ultraviolet spectrophotometry.

Chemical Modifications of the Metabolite. CATALYTIC REDUCTION OF 25-HCC AND OF THE METABOLITE. To 25-HCC (*ca.* 10 μg, specific activity 1.8×10^6 dpm/0.25 μg) in 2 ml of methanol a large excess of PtO_2 catalyst was added and the mixture shaken under a hydrogen atmosphere (3 atm) for 3 hr at room temperature. The solution was decanted from the catalyst and solvent removed under a stream of N_2. The residue was dissolved in 1:1 $CHCl_3$-Skellysolve B and applied to a 1 × 60 cm Sephadex LH-20 column packed in the same mixed solvent. The identity of the material as 25-hydroxyhexahydrocholecalciferol was confirmed by mass spectrometry (mol wt 416). The metabolite (8000 dpm *ca.* 25 ng) was reduced under the same conditions as above. The major component was recovered after Sephadex LH-20 chromatography. A sample of the 25-hydroxyhexahydrocholecalciferol (3200 dpm) and the reduced metabolite (1600 dpm) were combined and cochromatographed on a 1 × 60 cm 65:35 $CHCl_3$-Skellysolve B Sephadex LH-20 column. All radioactivity (4780 dpm) was recovered as a single peak (Figure 9).

PERIODATE REACTION OF THE METABOLITE AND MODEL COMPOUNDS. The metabolite (8000 dpm) dissolved 20 μl of methanol was treated with 10 μl of a 5% aqueous solution of $NaIO_4$. After 12 hr the solution was evaporated to dryness under N_2 and the residue redissolved in 0.2 ml of 65:35 $CHCl_3$-Skellysolve B. Original metabolite (4000 dpm) not treated with $NaIO_4$ was added and the mixture applied to a 1 × 60 cm Sephadex LH-20 column in the same mixed solvent. All radioactivity (12,000 dpm) was recovered as a single peak corresponding to unchanged metabolite.

Periodate cleavage of model compounds (such as 24,25-dihydroxylanosterol and 5β-cholestane-3β,4α,7α-triol) under the same conditions (*i.e.*, 1 μg of compound in 20 μl of MeOH + 10 μl of 5% $NaIO_4$) went to completion within 4 hr at room temperature, as determined by mass spectrometry.

ACETYLATION OF 1,25-DIHYDROXYCHOLECALCIFEROL-25-TRIMETHYLSILYL ETHER. The monosilyl ether derivative of the metabolite (*ca.* 0.2 μg) was allowed to stand at room temperature in a mixture of pyridine (5 μl) and acetic anhydride (5 μl) for 2 hr. The mass spectrum of the product gave the expected molecular weight (mol wt 572) and fragmentation pattern (*m/e* 512, 452, 131) for the diacetylmonotrimethylsilyl ether derivative of the metabolite.

Biological Activity of 1,25-DHCC. Intestinal calcium transport response of the metabolite was measured by the *in situ* gut sac technique as previously described (Omdahl *et al.*, 1971). The results shown in Table I reveal that the isolated metabolite is fully effective in inducing intestinal calcium absorption in chicks 8.5 hr after an oral dose in agreement with previous data (Omdahl *et al.*, 1971).

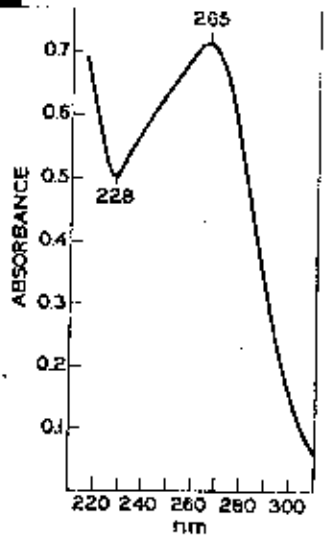


FIGURE 5: Ultraviolet absorption spectrum of the intestinal peak V metabolite.

Results

Identification of the Metabolite as 1,25-DHCC. Ultraviolet absorption maximum (265 nm) and trough at 228 nm (Figure 5), characteristic for the 5,6-*cis*-triene chromophore of the D vitamins, and the molecular ion peak at *m/e* 416 in its mass spectrum (Figure 6); suggested a dihydroxycholecalciferol structure for this compound. The presence of three hydroxyl functions was demonstrated by the formation of a tritrimethylsilyl ether derivative of mol wt 632 (structure III, Figure 7) and of a trimethylsilyl-ether diacetate of mol wt 572 (structure I, Figure 7). A mass fragment of *m/e* 131 in the case of the tritrimethylsilyl derivative (Figure 8), the monotrimethylsilyl derivative (II, Figure 7; Figure 6), and the trimethylsilyl ether diacetate (I, Figure 7) established the presence of a hydroxyl function at C-25 (Blunt *et al.*, 1968a; Suda *et al.*, 1969, 1970a). Small peaks at *m/e* 287, 269 (287 − H_2O), and 251 (287 − $2H_2O$) which arise by loss of the entire side chain (C-17–C-20 cleavage) confirm the lack of additional oxygen substituents on the side chain. The other two hydroxyl groups must be located in ring A, since the mass spectra of the metabolite (Figure 6) and of its 25-trimethylsilyl ether derivative (Figure 6; II, Figure 7) exhibited prominent ions at *m/e* 152 and 134 (152 − H_2O) which can only be interpreted as the oxygen analogs of the characteristic ions at *m/e* 136 and 118 (136 − H_2O) observed in the spectra of cholecalciferol, 25-HCC, and other cholecalciferol metabolites (Blunt *et al.*, 1968a; Suda *et al.*, 1969, 1970a,b). The origin of these peaks is graphically illustrated in Figure 6. In the mass spectrum of the tritrimethylsilyl ether compound (III, Figure 7) these peaks appear at *m/e* 296 (152 + 2 silyl groups) and 206 (296 − $HOSi(CH_3)_3$) (Figure 8). In addition to providing conclusive proof of the presence of two hydroxyl functions in ring A, the peaks at *m/e* 152 and 134 also corroborate the ultraviolet spectral

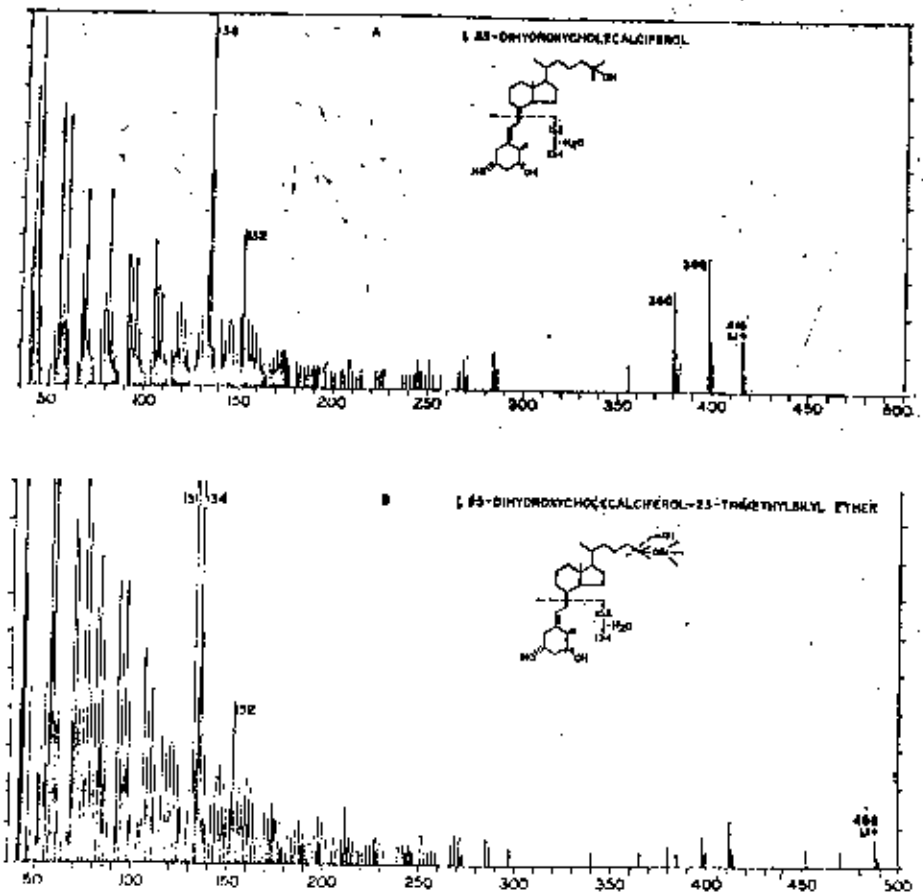


FIGURE 6: Mass spectrum of (A) 1,25-DHCC and (B) 25-monotrimethylsilyl ether derivative of 1,25-DHCC.

data (Figure 5) for the existence of a vitamin D type triene system (Blunt *et al.*, 1968a; Suda *et al.*, 1969, 1970a,b). Since 25-HCC is the immediate precursor of this new metabolite a hydroxyl group at C-3 can be assumed with confidence, and the foregoing results thus allowed only C-1, -2, and -4 as positions for the additional hydroxyl function in ring A. Upon catalytic hydrogenation (PtO_2-H_2) the metabolite yielded a product identical (by cochromatography) (Figure 9) with 25-hydroxyhexahydrocholecalciferol (IV, Figure 7). Since formation of IV (Figure 7) from the metabolite requires hydrogenolysis of an hydroxyl group, the hydroxyl function lost in the process must have been located at either C-1 or C-4 (i.e., allylic to the double-bond system). The failure of the metabolite to react with periodate (under conditions which oxidize model compounds readily, as, for example, 24,25-dihydroxyanosterol and 5β-cholestane-3β,4α,7α-triol) eliminated a C-4 (as well as C-2) hydroxyl group and thus required that the additional hydroxyl group be placed at C-1. The observation that the biosynthesis of this metabolite from 1α-tritiocholecalciferol results in almost complete loss of tritium (Lawson *et al.*, 1969) and in 50% loss of label with 1,2-ditritiocholecalciferol as precursor (Omdahl *et al.*, 1971) provides corroborative evidence for a C-1 hydroxy compound. The structure of the intestinal polar vitamin D_3 metabolite is therefore 1,25-DHCC (Figure 10).

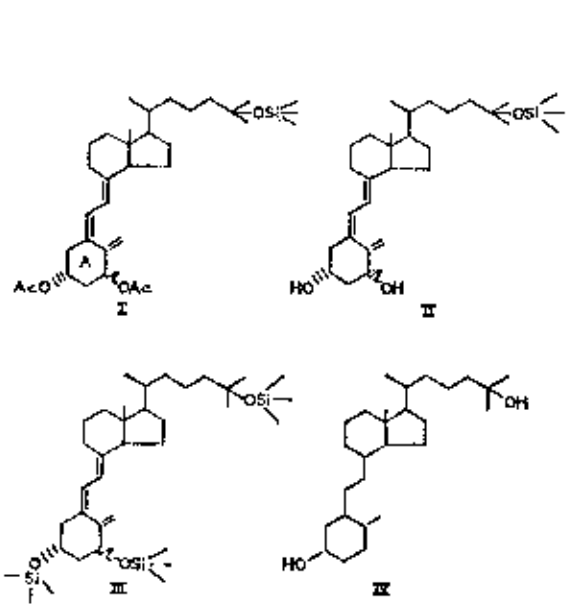


FIGURE 7: Structures of (I) 1,25-DHCC-1,3-diacetyl-25-trimethylsilyl ether, (II) 1,25-DHCC-25-trimethylsilyl ether, (III) 1,25-DHCC-1,3-25-tritrimethylsilyl ether, and (IV) 25-hydroxyhexahydrocholecalciferol.

The stereochemistry of the 1-hydroxy function cannot be specified as yet. Treatment of 1,25-DHCC with triphenylboroxine did not result in a cyclic boronate, suggesting a 1α,3β configuration for the two hydroxyl functions. On the other hand, Bell and Kodicek (1970) using 1-tritiocholecalciferol (83–85% 1α-^{3}H, 15–17% 1β-^{3}H) report at least 96% loss of label suggesting that epimerization at C-1 may have occurred. This stereochemical problem, therefore, will only be resolved by synthesis or by definitive degradation experiments.

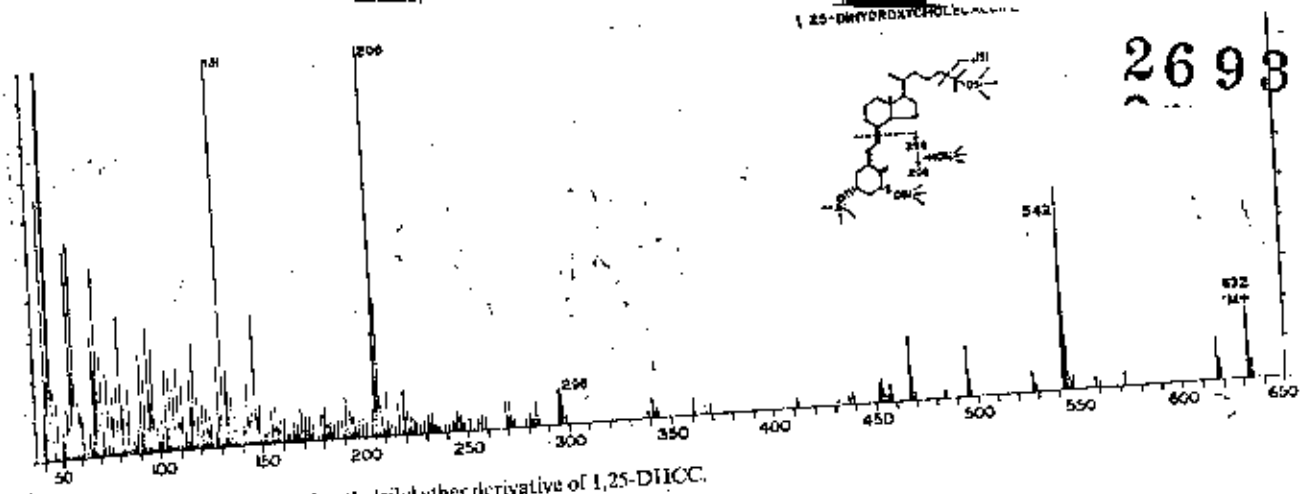


FIGURE 8: Mass spectrum of tritrimethylsilyl ether derivative of 1,25-DHCC.

Discussion

The present report establishes conclusively 1,25-DHCC (Figure 10) as the chemical structure of the polar vitamin D metabolite (designated by different laboratories as peak V, IB, or P) from intestine which is biologically active in intestine. This structure, as well as 1-oxo-25-HCC, were suspected structures when Lawson *et al.* (1969) and Bell and Kodicek (1970) demonstrated that during its biosynthesis from [1-^{3}H]-vitamin D_3 (85% 1α) the 1-^{3}H is lost. More recently attention was focused on the 1,25-dihydroxy compound by the work of Fraser and Kodicek (1970) which demonstrated that kidney homogenates are able to synthesize the metabolite from 25-HCC by a reaction requiring reduced pyridine nucleotide and oxygen. The 1,25-DHCC structure has now been established by unequivocal physical measurements and known chemical reactions on the metabolite isolated from the intestines of chicks given [1,2-^{3}H]vitamin D_3.

The isolation of this metabolite proved to be the most difficult yet performed in this laboratory. Chemical purifications such as saponification were avoided because of fear of losses and isomerization. Even silicic acid column methods were not used nor were the powerful partition methods previously developed for polar vitamin D metabolites (Suda *et al.*, 1970-a,b) because of 10–20% losses suffered during the use of those methods. Total purification depended heavily on liquid gel partition chromatography (Holick and DeLuca, 1971) and gel filtration using Sephadex LH-20 and Bio-Beads S-X8. However, complete purification was not achieved until the chromatographic properties of the metabolite were changed in a specific fashion. The relative difficulty of removing the silyl group from the tertiary hydroxyl (C-25) of the metabolite was used. The resulting 25-trimethylsilyl ether derivative chromatographed sufficiently different to separate it from the contaminating lipids.

The ability of the isolated metabolite (Table I) to stimulate intestinal calcium transport leaves no doubt that it represents the polar metabolite (peak V in our laboratory, 4B in that of Haussler and Norman, and P in that of Kodicek) believed to be the metabolically active form of vitamin D in the intestine. It is now clear that the 1,25-DHCC acts more rapidly than does 25-HCC in initiating calcium transport (Haussler *et al.*, 1971; Myrtle and Norman, 1971; Omdahl *et al.*, 1971). Of great importance is the fact that the 1,25-DHCC can initiate intestinal calcium transport in the presence of actinomycin D while 25-HCC cannot (Tanaka *et al.*, 1971). Actinomycin D and cycloheximide both block the conversion of 25-HCC into 1,25-DHCC illustrating that 25-HCC interacts with the genetic machinery to induce the formation of an enzyme or factor necessary for the appearance of 1,25-DHCC in the intestine. The 1,25-DHCC then probably acts by a mechanism not involving transcription of DNA. The data further show that the 1,25-DHCC or a further metabolite thereof must be the metabolically active form of vitamin D in the intestine.

It is not known whether 1,25-DHCC is the metabolically active form of vitamin D in inducing bone mineral mobilization. It is active in this system but does not appear to act faster nor does it appear more active than 25-HCC (Omdahl *et al.*, 1971). Furthermore the 25-HCC does induce bone mineral mobilization in isolated cultures of embryonic bone (Trummel *et al.*, 1969) which appear unable to produce the 1,25-DHCC. Only additional experiments can clarify this point, however.

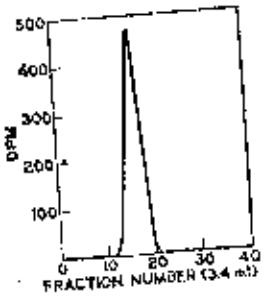
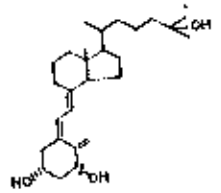


FIGURE 9: Sephadex LH-20 column (1 × 60 cm packed in 65:35 $CHCl_3$, Skellysolve B) profile for the cochromatography of 25-hydroxyhexahydrocholecalciferol with reduction product of intestinal peak V metabolite.



1,25 DIHYDROXYCHOLECALCIFEROL

FIGURE 10: 1,25-Dihydroxycholecalciferol (1,25-DHCC).

It must be emphasized that the exact stereochemical position of the hydroxyl at C-1 has not been established. It is likely that the position is α since 1-α-^{3}H is totally lost in its biosynthesis and since our attempt to form a boronate ester of the isolated metabolite failed. Experiments are now in progress to delineate the exact stereochemical position of the 1-hydroxy-function of 1,25-DHCC.

The elucidation of the structure of this important metabolite opens many possible avenues of investigation. The exact metabolic sequence involved in the vitamin D initiation of intestinal calcium transport can now be approached more directly. Furthermore, if synthesis is successful, this compound could be useful in some types of vitamin D resistant diseases such as renal osteodystrophy and familial hypophosphatemia.

Acknowledgment

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Vitamins D_2 and D_3 in New World Primates: Influence on Calcium Absorption

Abstract. *In* Cebus albifrons *monkeys it was demonstrated that vitamin D_3 promotes the intestinal absorption of calcium-47 and that vitamin D_2 does not increase absorption above that seen in monkeys deficient in vitamin D. These data support previous observations that vitamin D_2 is not effective in preventing metabolic bone disease in this species.*

The relative ineffectiveness of vitamin D_2 in the chick has been well established. It was demonstrated in 1930 that in this species vitamin D_2 has 10 percent (or less) of the activity of vitamin D_3 in the prevention and cure of rickets, promotion of body growth, egg production, and other parameters (*1, 2*). Approximately 30 years ago several reports presented clinical evidence indicating a similar but quantitatively smaller difference in the activity of vitamin D_2 and D_3 in human infants (*1*). These reports have been questioned, and whether or not a significant difference in activity does exist in man has been unresolved.

We have reported evidence (*3, 4*) which indicates that vitamin D_2 is not utilized or is significantly less effective than vitamin D_3 in the prevention of rickets, osteomalacia, and osteodystrophia fibrosa in several species of South American primates. These data were based on the development of bone disease in *Cebus albifrons* and several species of tamarins (*Saguinus oedipus, S. nigricollis, S. mystax*) maintained on a purified diet containing vitamin D_2 and the reversal of the disease process by substituting vitamin D_3 for D_2. We now report the influence of vitamin D_2 and vitamin D_3 on calcium absorption, which provides additional evidence that vitamin D_3 is more active than D_2 in the Cebus monkey.

Six *Cebus albifrons* monkeys were maintained on a purified diet containing 2000 international units (IU) of vitamin D_2 per kilogram of diet for 24 months (*3*). The diet was composed of (in percentages): sucrose, 62.00; casein, 25.00; corn oil, 8.00; salt mix number IV (Hegsted *et al.*, 1941), 4.00; choline chloride, 0.50; dry vitamin mix, 0.40; and inositol, 0.10. The dry vitamin mix contained (in percentages): thiamine, 0.50; riboflavin, 0.50; pyridoxine 0.50; niacin, 2.45; calcium pantothenate, 1.50; folic acid, 0.05; biotin, 0.01; and dextrose, 94.44. Vitamin A acetate and the vitamin D_2 in absolute alcohol were added to the diet in amounts of 12,500 IU/kg and 2000 IU/kg, respectively. Ascorbic acid in dilute alcohol was added to the ration daily to supply 25 mg per monkey per day.

After the development of severe osteodystrophia fibrosa, vitamin D_3 (2000 IU/kg of diet) was substituted for vitamin D_2 for a period of 5 months. After observations on the reversal of the disease process, all vitamin D was removed from the diet. After the monkeys had been on the diet lacking vitamin D for 12 months, the clinical findings, roentgenograms, and serum alkaline phosphatase signified the exacerbation of osteodystrophia fibrosa. At this point two animals (Nos. 201 and 202) were provided a daily oral supplement of 500 IU of vitamin D_3; two (Nos. 209 and 214) with 500 IU of vitamin D_2; and two (Nos. 221 and 222) were left untreated. Twelve days after the supplements were initiated radio-calcium uptake studies were conducted on each animal. After the monkeys were fasted for 24 hours, 7 μc of calcium-47 were administered via stomach tube and the animals were provided with 50 g of purified diet. Serum samples were obtained at 2, 4, 6, and 24 hours, and urine and feces were collected at 24-hour intervals for 5 and 7 days, respectively. The skull was monitored at 48 hours by holding the head beneath the probe of a scintillation counter. One milliliter of serum was counted for each time interval, in a gamma counter. The urine was heated for 1 hour with 10 ml of HCl, cooled, and made up to 200 ml with distilled water, and 2-ml aliquots were counted. The feces were mixed with a small amount of water and heated with 25 ml of HCl, cooled, and brought up to 200 ml with water, and 2-ml aliquots were counted.

To determine whether, in the Cebus monkey, vitamin D has any direct influence on the mineralization of bone, or on urinary excretion of calcium, the animals were maintained on the oral supplement of vitamin D for an additional 42 days and then injected intravenously with 3 μc of Ca^{47} (experiment 2). Serum samples were collected at 5, 10, 30, 60, 180, and 360 minutes. Urine and feces were collected and processed as in the first study and the skull was monitored at 48 hours. After the monkeys had been an additional 70 days on the vitamin D supplements, a second oral uptake study was performed (experiment 3), essentially as described above. However, a dose of 5 rather than 7 μc of Ca^{47} was given, and the animals were not fed for 6 hours.

Table 1. Thousands of counts per minute over the skull of monkeys at 48 hours.

Vitamin supplement	Thousands of counts		
	Exp. 1 (oral)	Exp. 2 (i.v.)	Exp. 3 (oral)
Vitamin D_3			
Monkey 201	17.1	6.8	12.2
Monkey 202	23.2	7.0	15.3
Vitamin D_2			
Monkey 209	2.5	6.8	9.1
Monkey 214	1.5	5.2	9.1
None			
Monkey 221	2.0	7.1	6.8
Monkey 222	0.7	5.9	6.6

The statistical comparison (*3*) of the means of the various determinations for the three groups was made with one-way analysis of variance. For analysis purposes the original data were verted as follows: serum peak spe- activity to log values; total urinary and fecal percent excretion to 2 arc sin $\sqrt{X}$; and counts per minute (cpm) over the skull to $\sqrt{}$ cpm. These transformations were used to obtain the homogeneity of variance necessary for comparison of means by analysis of variance. The resulting analysis of variance compared the three means simultaneously. In addition, *t*-tests were calculated to compare the means of the three groups.

The results of the first study are presented in Figs. 1 and 2. The specific activity of the serum (Fig. 1) clearly indicates a significantly greater absorption in the two animals given vitamin D_3 supplements ($P < .01$). There is no significant difference between the D_2 group and the animals given no supplement. The difference in absorption is reflected in fecal excretion (Fig. 2), which is significantly lower in the group given vitamin D_3 supplement ($P < .01$). The animals given vitamin D_2 supplement excreted more Ca^{47} than the nonsupplemented group although the difference was of lower significance ($P < .05$). There were no significant differences between the three groups in urinary excretion. The skull counts were significantly higher in the D_3 animals ($P < .01$) and without significant difference between the two other groups (Table 1).

Following intravenous injection of Ca^{47} the serum cleared at approximately the same rate in all six animals; however, differences were present in the pattern of excretion. The monkeys given vitamin D_3 supplement excreted significantly less Ca^{47} in the feces (Fig. 3) than the other two groups ($P < .01$). There was no significant difference between the D_2-supplemented and the nonsupplemented animals. Also in the urine (Fig. 4) significantly less Ca^{47} was excreted by the D_3 group ($P < .01$), but in addition the D_2 monkeys excreted significantly less than the nonsupplemented group ($P < .01$). Based on percent of original dose, combined excretion in the urine and feces in the three groups at 96 hours was 1.81 percent in the D_3 group, 8.88 percent in the D_2 group, and 15.31 percent in the nonsupplemented group. Most of this loss was in the feces. This resulted in a retention at 96 hours of 98.19 percent, 91.12 percent, and 84.69 percent, respectively, in the three groups. There were no significant differences in the head counts between the groups (Table 1).

The results of the second study of oral uptake were similar to those of the first study. The serum uptake was significantly higher in the monkeys given vitamin D_3 supplement ($P < .01$), with no difference between the D_2 and the nonsupplemented groups. Fecal excretion was significantly lower in the D_3 group ($P < .05$), with no significant difference between the two other groups. There was no significant difference in urinary excretion of Ca^{47} between the three groups. The head counts (Table 1) of the D_3 group were significantly higher ($P < .01$) than in the two other groups, and with no significant difference between the D_2 and nonsupplemented animals.

The results of the two oral uptake studies indicate that in the Cebus monkey vitamin D_3 promotes the absorption of Ca^{47} and that vitamin D_2 at the levels employed does not significantly increase absorption of Ca^{47} in comparison with animals deficient in vitamin D. These facts are reflected in greater deposition of Ca^{47} in bone and decreased fecal loss of Ca^{47} in the D_3-supplemented animals.

The results following intravenous injection of Ca^{47} indicated similar clearance from the serum with retention of 98.2 percent, 91.1 percent, and 84.7 percent of the original dose at 96 hours in the D_3, D_2, and nonsupplemented animals, respectively. These differences in retention are principally accounted

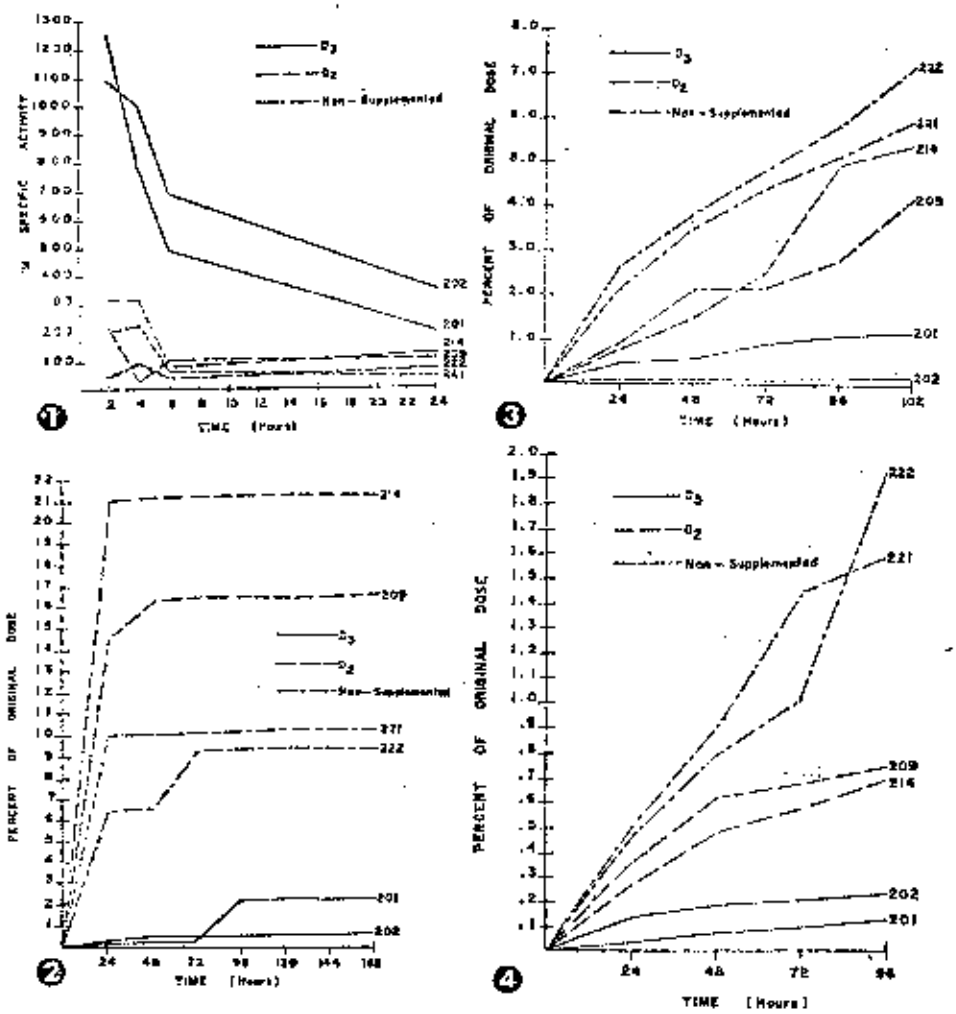

Fig. 1. Specific activity of the serum (counts per minute per milligram serum calcium) following an oral dose of 7 μc of Ca^{47} in monkeys deficient in vitamin D and monkeys ...en D_2 and D_3 supplements, respectively. Fig. 2. Cumulative excretion of Ca^{47} ...he feces, expressed as percent of original dose following an oral dose of 7 μc ... monkeys deficient in vitamin D and monkeys given D_2 and D_3 supplements, respectively. Fig. 3. Cumulative excretion of Ca^{47} in the feces, expressed as percent of original dose following an intravenous dose of 3 μc in vitamin-D deficient and D_2- and D_3-supplemented monkeys. Fig. 4. Cumulative excretion of Ca^{47} in the urine, expressed as percent of original dose following an intravenous dose of 3 μc in vitamin-D deficient and D_2- and D_3-supplemented monkeys.

for by the greater fecal loss in the D_2 and nonsupplemented animals. This is most likely the result of the failure to reabsorb calcium excreted via the intestine in these two groups. Urinary loss of Ca^{47} was significantly different in the three groups but the levels were so low in all three groups (0.31 percent in the D_3, 1.38 percent in the D_2, and 3.41 percent in the nonsupplemented) that it appears that if this reflects any action of vitamin D_3 or D_2 or both at the level of the kidney it is not of great importance in maintaining calcium balance. The differences in retention clearly indicate differences in deposition in the bone. However, neither the estimation of activity of the serum nor the external counting over the head are sufficiently accurate to demonstrate the differences in the degree of retention observed after intravenous injection. Whether vitamin D_2 or D_3 has an active role at the level of the bone was not demonstrated by this study and warrants additional experimentation.

The data for both oral and intravenous administration of Ca^{47} demonstrate a primary effect of vitamin D_3 on intestinal absorption of calcium, rather than at the level of the bone or kidney, and clearly indicate that vitamin D_3 is more active than vitamin D_2 in promoting calcium absorption in Cebus monkeys. Further investigation of the effectiveness of various forms of vitamin D in man is warranted in light of this evidence in a primate species.

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Was Virchow Right about Neandertal?

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Neandertals living in early Würm times may have suffered from a vitamin D deficiency.

NEARLY a hundred years ago Virchow diagnosed rickets in the Neandertal bones, accounting so for their peculiar brain case[1]. Though this was not the first time such an opinion had been published[2], it was the first authoritative statement by one expertly acquainted with the disease who was also personally familiar with the fossil material. Another diluvial hominids of the same type turned up in Belgium and France and the day was carried for Darwinism, however, Virchow's carefully argued and factual diagnosis concerning the earlier finds became discredited—association, if never objectively. But the growth of knowledge since, anthropological as well as medical, suggests that Virchow's view may have been essentially correct.

Variation in Neandertals

It has been clear for several decades, at least since the discovery of the Rhodesian[3] and Solo skulls[4], that the Neandertal phenomenon is not limited to Europe[5]. More recently, since the advent of isotopic dating methods, it has also become possible to situate each Neandertal sample quite accurately—give or take a few millennia or centuries—in the 35,000 years frame extending from the onset of the Würm glaciation (70,000 BP), when Neandertal enters, to the middle of the Pandorf interstadial (35,000 BP), when he vanishes[5,6]. If the general environment at each dated site is considered, it is found that the marked variability evident in the Neandertal populations can be related to two fairly straightforward conditions: latitude and palaeoclimate. Thus, if samples from the early Würm at Spy, La Chapelle-aux-Saints, and Monte Circeo, lying above 40° N latitude are compared with the more contemporaneous samples from Gibraltar, Tabun and Mapa, lying below it, it can be seen that a cline exists, the typical Neandertal traits decreasing towards the equator[6], Solo Man at 7° S being the least affected[7,8]. (It should be noted, however, that Shanidar does not fit the gradient; the discrepancy may perhaps be accounted for on the basis of altitude.) In other comparisons drawn from the same latitude belt but not contemporaneous, from the same latitude belt but not contemporaneous, and differing markedly in terms of palaeoclimate but not of culture, an attenuation of the Neandertal traits is also discernible in the interstadial samples, for example, in Krapina[9] as related to La Ferrassie, in Skhul as related to Tabun[10]. (Similarly the Niah skull from Borneo, dated 40,000 BP may be related to the Solo population from nearby Java; but the cultural context in the comparison is less well defined.) The radiocarbon method of dating has also made it possible to assign the transition between Mousterian flake cultures and Upper Palaeolithic blade-burin-bone cultures, on something like a worldwide basis, to the end of the Pandorf interstadial[11]. From the standpoint of human ecology, the most important element of the more recent cultures, in their fully evolved form associated with the Cro-Magnon type of modern man (30,000 BP and later), was the development of fishing by means of an expressly developed tool assemblage, and contributing substantially and routinely to the diet[12-15]. The archaeological record demonstrates that this revolutionary advance evolved from the Mousterian industries of the Neandertals[16,17].

Aetiology of Rickets

Virchow was of course ignorant of the aetiology of rickets. Though certain fatty fish oils had been used in Northern Europe since the Middle Ages as a home remedy for a variety of ailments, and the relation between a sunless climate and a high incidence of rickets had been realized by medical practitioners for some time, vitamin D, the antirachitic principle, was not definitely implicated and identified until after World War I[18]. Of particular interest to the palaeoanthropologist is the restricted distribution of vitamin D: fatty fish and egg yolk are rich sources of these sterols, but fats in general, offal, and milk contain but small amounts, while meat, shellfish, and vegetable foods have, for practical purposes, none[19]. In man, vitamin D is also produced endogenously, through the irradiation by solar or artificial ultraviolet light of the provitamin 7-dehydrocholesterol in the deep layers of the skin. At lower latitudes, even during temperate interglacial epochs like the present, it is possible to get by largely on this endogenous vitamin D alone, provided sociocultural factors such as crowding and parandi do not impede direct access to sunlight.

Above 40° N. except presumably during warm interglacials like the last, an exogenous source of vitamin D is also invariably required for normal growth in children and adequate bone maintenance in childbearing women: the alternative is rickets and osteomalacia. In a recent paper, Loomis has shown[20] that the photosynthesis of endogenous vitamin D in man is kept at a constant rate the world over by the intensity of skin pigmentation, which on the whole can be seen to vary directly with the general availability of solar ultraviolet at different latitudes: as a rule, populations in the equatorial belt are the darkest, and marked depigmentation of the skin begins at 40° N and S.

Palaeoclimate of the Early Würm

It is presumed that Neanderthal had limited access to ultraviolet. The palaeoclimate of the early Würm was characterized by cold and a marked increase in atmospheric turbulence and precipitation, which was worldwide but more intense in the higher latitudes above 40°[21,22]. Because the rays at the lower end of the sunlight spectrum are quite liable to any sort of atmospheric interference, dust laden high winds, rains, snow, or overcast through the greater part of the year would filter out the ultraviolet reaching the surface of the planet, again very drastically at higher latitudes but less so around the equator. The cold itself contributed further to the reduced availability of ultraviolet by driving Neanderthal out of the open to seek shelter in caves or tents, and perhaps to the wearing of thick furs. It has also been suggested that Neanderthal had little access to dietary vitamin D, because his basic hunter's food list, relatively adequate in terms of calories, protein, calcium and phosphorus and vitamin B, included only negligible amounts of fatty fish and eggs[12,13,23]. The evidence here is mostly of a negative nature: absence of specialized fishing implements, absence of the type of tool kit needed to make them, and absence of fish remains at Mousterian sites—all the opposite of the situation encountered in Aurignacian and Magdalenian times[13]. It would be more accurate to say that most Mousterian sites show no archaeological or osteological evidence of fish consumption: that the scant unidentified fish remains from Gibraltar are of doubtful association[13]; and that the three individual fish (one pike, one perch, one unidentified) excavated in the evolved Acheulian Mousterian site of Salzgitter-Lebenstedt[24] alongside the remains of eighty reindeer, sixteen mammoths, six bison, four horses and two rhinoceroses, only go to confirm the general impression that Neanderthal made very little use of fatty fish—which it may be surmised were come by sporadically in the course of food gathering. Eggs, also obtained by simple gathering, must have been rare for most Neanderthals.

Evidence of Rickets in Neanderthals

That an endemic deficiency of vitamin D prevailed for man during Mousterian times is certainly not contradicted by the direct evidence of the fossils themselves. It has long been held that the Neanderthal child is a small replica of the adult[25]—a situation unique among Primates, and something of an oversimplification as well, because in some respects, for instance frontal height, the Neanderthal child may fall within or even surpass the modern range of variation[26]. But the crucial point which has not been emphasized is that every Neanderthal child skull studied so far shows signs compatible with severe rickets*. Clinically, the disease is most active between 6 and 24 months of age, and as far as the skull is concerned is characterized chiefly by a large head, late closure of the sutures and fontanels, a high bulbous forehead ("olympian front"), bulging at the four corners ("caput quadratum"), localized round patches of weakened or defective bone ("craniotabes"), and faulty dentition[18,27]. The 2 year old from Pech-de-l'Aze[28] confirms the diagnosis for each of these criteria: estimated cranial capacity is a very substantial 1,200 cm³; bregma and the metopic suture are wide open; the ossification centres of the lofty brow are displaced inferolaterally on the frontal, which lacks the protruding tubera of thickened bone so characteristic of infant skulls; there is a smooth bagging out at the sides of the frontal and at the low posterior parietal regions; there are several large areas of thin, pitted bone in the frontal, occipital and right parietal, which in life must have been associated with focal softening; the grossly malocluded teeth show a number of crown anomalies and enamel irregularities. A subtle face change which may escape the clinician is the increase in the size of the orbits, especially in the vertical dimension: the latter is another feature of rickets to be seen in the Pech-de-l'Aze child. Also from the Würm I above 40° N latitude, the skull of the 1 year old from Starosel'e[29] shows obvious signs of severe rickets, and the 6 to 8 year olds from Engis[30], La Quina[31] and Subalyuk[31] indicate that the disease was allowed to run its course unchecked, and the bone to heal in the grossly deformed condition. On the other hand, Teshik-Tash, Gibraltar II[24], the Shanidar baby[25] and the Lebanese "Egbert"[32], as a rule show less extreme evidence of rickets, as one might expect from the greater general availability of vitamin D at these lower latitudes or temperate interstadial sites. And although much less has been published on the long bones of Neanderthal children, what little is available shows that the gross bowing of these, so characteristic of present day rickets, was not restricted to the adult members of these populations in whom, especially among Neanderthal women of child-bearing age, an element of osteomalacia cannot entirely be ruled out.

Vitamin D Deficiency and Neandertal Evolution

Two lines of evidence therefore, the ecological and the ontogenetical, point in the direction of vitamin D deficiency as a significant element in the determination of the Neandertal facies. At the onset of Würm I we appear to be dealing with an advanced *Homo erectus* (=*p. sapiens*) of the Ehringsdorf/Fontechevade/Saccopastore-Rhodes or similar stock whose thick bones have been deformed by severe rickets in childhood and perhaps by various grades of osteomalacia in adulthood; 30,000 years later, as Neandertal continued to evolve culturally and biologically in the direction of modern man, and as the milder climate of the Paudorf interstadial increased the general availability of endogenous vitamin D, he did not differ very much from the more archaic types[15] of Upper Palaeolithic *H. sapiens* 6,000–10,000 years ahead. The transition from Middle Pleistocene to Late Pleistocene fossil man may therefore be conceived of as a gradual worldwide one, and the vexing problem of where to place the Neandertal throwback is largely taken care of.

One of the strengths of this extended and revised version of Virchow's original hypothesis is that it can be tested empirically. The ordering of the world Neandertal sample in terms of degrees of inferred vitamin D deficiency does constitute an indirect kind of controlled observation. But two promising quantitative techniques are also available now for the direct testing of vitamin D deficiency in Neandertal fossil material: (1) the teeth may be sectioned and examined under the microscope for the presence of interglobular spaces in the dentine, a lesion practically specific of rickets[33,37]; and (2) the X-ray diffraction pattern of bone may be studied for the presence of rachitic matrix, which projects its own pattern. Sognnaes, using a small sample from lower latitude Würm I and the following interstadial, has demonstrated that the teeth provide unequivocal evidence of severe vitamin D deficiency in Neandertal man; similarly Bond *et al.*[34], using well dated high latitude bone samples were able to identify what is arguably a rickets ring

* I have examined the following Neandertal individual remains: La Chapelle-aux-Saints adult, Engis child, La Ferrassie I to VI adults and children, Gibraltar adult and child, Neandertal, Pech-de-l'Aze, La Quina adult and child, Skhul IX adult, Tabun I adult.

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man fragment from a Würm I Mousterian site which not turn up in their Magdalenian or Holocene material.

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Estrogen-Receptor Interaction

Estrogenic hormones effect transformation of specific receptor proteins to a biochemically functional form.

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One of the challenging problems confronting biological scientists has been the manner in which hormones serve as regulators of biochemical processes in tissues of higher animals. For most types of endocrine agents the physiologic effects have been known for many years, but only recently has insight been gained concerning the detailed biochemical mechanisms by which some of these actions are mediated.

Two general patterns of hormone-cell interaction have so far been recognized. In the first, illustrated by epinephrine and by many types of peptide hormones, the hormone reacts with membrane-bound nucleotide cyclase systems to stimulate the conversion of a nucleoside triphosphate, such as adenosine triphosphate (ATP), to the corresponding 3′5′-monophosphate, for example, cyclic adenosine monophosphate (cAMP) (*1*). The cyclic nucleotide then serves as a "second messenger," reacting with appropriate cellular entities to deliver and amplify the regulatory signal. In the second pattern of interaction, which appears operative with the various types of steroid hormones (*2*), the hormone enters the cell and binds to a specific extranuclear "receptor" protein, characteristic of the responsive or "target" cell. The resulting steroid-protein complex then migrates to the nucleus where specific RNA synthesis is initiated or accelerated, leading in the case of the estrogenic hormones to eventual tissue growth. With the estrogens, and probably with other steroid hormones as well, hormone induced translocation to the nucleus involves an alteration of the receptor protein, a phenomenon we have called "receptor transformation" (*2*).

In this article we summarize current knowledge about the interaction of estrogens with receptor proteins in hormone responsive organs, such as uterus, and the relation of receptor transformation to early biochemical responses elicited in the cell. More detailed information with original references is provided in recent reviews (*3*).

Estrogen Binding in Target Tissues

The presence in estrogen responsive tissues of characteristic hormone-binding components, now called estrogen receptors or estrophiles, was first indicated by the striking affinity of these tissues for the hormone. After administration of physiologic amounts of tritiated estradiol (Fig. 1) to immature rats (*4*) or tritiated hexestrol to young goats and sheep (*5*), the uterus, vagina, and anterior pituitary take up and retain radioactive hormone from the blood, against a large concentration gradient (Fig. 2). Despite extensive metabolism of estradiol in the animal giving rise to a variety of metabolites in the blood, only unchanged estradiol is taken up by the uterus of the immature rat (*6*) or mouse (*7*). The inference that estradiol binds to receptor substances and initiates uterine growth without itself undergoing chemical conversion has proved consistent with much subsequent evidence and served to direct investigative attention away from earlier considerations that had linked estrogen action to steroid metabolism.

Evidence that the uterotrophic action of estradiol depends on its binding to ments with specific binding inhibitors. Certain substances, such as ethamoxytriphetol (MER-25, Richardson-Merrell), clomiphene, nafoxidine (Upjohn-11,100), and CI-628 (Parke-Davis) (Fig. 1), which had been known (*8*) to inhibit the uterotrophic action of estradiol, were shown to prevent the characteristic uptake of estrogens by target tissues in vivo (*9, 10*). When varying doses of nafoxidine are administered to immature rats receiving tritiated estradiol, there is a quantitative correlation between reduction of hormone uptake by the uterus and inhibition of its growth response (*10*). In contrast to the foregoing agents, actinomycin D and puromycin, which also block the uterotrophic action of estradiol (*11*), do not decrease the uptake and binding of the hormone (*10*), suggesting that these antagonists act at later stages in a sequence of biochemical events in which the hormone-receptor interaction is an early if not the initial step.

When excised uterine tissue is exposed to dilute solutions of tritiated estradiol at physiologic temperatures in vitro, a hormone-receptor interaction occurs that shows the principal characteristics of that observed in the whole animal (*12, 13*). Comparison of hormone uptake in the presence and absence of those antagonists that prevent hormone-receptor association in vivo (*13, 14*) permits distinction between specific interaction and the nonspecific binding that estradiol shows with target and nontarget tissues alike (Fig. 3). The system in vitro has provided a valuable tool for studying various features of the hormone-receptor interaction in whole tissues, including its dependence on sulfhydryl groups (*15*) and the influence of temperature to be discussed later.

The foregoing studies of the fate of physiologic amounts of estrogenic hormones with intact mammalian tissues in vivo and in vitro, confirmed and extended by many other careful investigations (*3*), served to establish the principal characteristics of the interaction of estrogens with specific receptors in target cells and provided a basis for evaluating the significance of estrogen binding phenomena observed with broken cell systems where artifacts of nonspecific binding can occur. As more detailed investigations were conducted, it became evident that the interaction of estradiol with uterine cells is not a simple association of the

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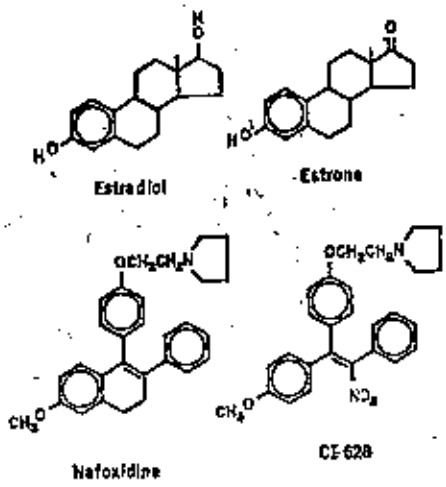
Fig. 1. Structures of the estrogens, estradiol and estrone, and two anti-estrogens, nafoxidine (Upjohn-11,000) and CI-628 (Parke-Davis).



hormone with a single binding site. As described below, radioactive hormone was found to be localized in two separate regions of the uterine cell. Moreover, determination of estradiol incorporation as a function of time and administered dose indicated two distinct binding phenomena in target but not in nontarget tissues: initial uptake, not saturable even at considerably hyperphysiologic doses of hormone, and retention, saturable at physiologic hormone doses (*16*). Study of the relation between these two processes and two binding sites led to the formulation of a two-step mechanism which has come to provide a model for the interaction of other classes of steroid hormones with their respective target tissues.

Estrogen-Receptor Complexes

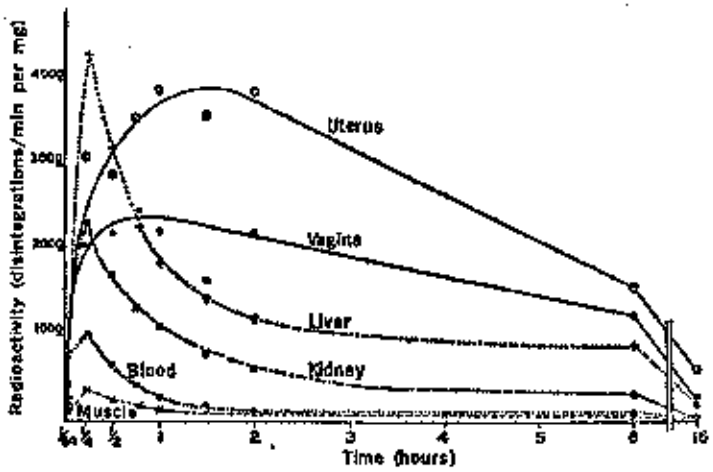
When uterine homogenates from estradiol-treated rats are subjected to differential centrifugation, the incorporated steroid appears in two cellular fractions (*16, 17*). Most of the hormone is found in the nuclei, with a smaller amount (20 to 30 percent) present in the high-speed supernatant or cytosol fraction. The predominance of nuclear binding, controversial in early reports (*17*), is confirmed by autoradiographic studies (*13, 18*), in which a dry-mount procedure is used (*19, 20*) that minimizes steroid translocation during tissue processing (Fig. 4).

The estradiol taken up by rat uterus is associated with a different form of the receptor substance in the cytosol than in the nucleus. The application (*21*) of ultracentrifugation in sucrose density gradients for characterizing estrogen-receptor complexes provided a valuable means for distinguishing between different forms of the receptor. By this technique the radioactive hormone in the cytosol was found to sediment as a discrete band (*21*) with a coefficient originally reported as 9.5*S* but later shown to be close to 8*S* (Fig. 5A). In sodium or potassium chloride of concentration above 0.2*M*, the 8*S* complex is reversibly dissociated into subunits (*22*), originally called 5*S* but later shown (*23*) to sediment at about 4*S*, just behind bovine plasma albumin (Fig. 5B).

The estradiol bound in the nucleus appears to be associated with chromatin (*24*). The nuclear hormone can be solubilized, unaccompanied by DNA, by extraction with 0.3*M* KCl at *p*H 7.5 (*13*) or, more effectively, by 0.4*M* KCl at *p*H 8.5 (*25*), to yield an estradiol-receptor complex which, in the presence of salt, sediments at about 5*S*, that is, slightly faster than bovine plasma albumin. On removal of salt, the nuclear complex usually aggregates to an 8*S* to 9*S* form but can lose this property on aging or partial purification. As shown in Fig. 5B, the nuclear complex is readily distinguished from the cytosol complex by careful ultracentrifugation in salt-containing sucrose gradients (*23*); this difference in sedimentation rate provided the first criterion for recognizing the phenomenon of receptor transformation.

Recent experiments described below indicate that the sedimentation coefficients of the cytosol and nuclear complexes, determined in salt-containing sucrose gradients, are close to 3.8*S* and 5.2*S*, respectively. For simplicity, the approximate values of 4*S* and 5*S* are used in this article.

The 8*S* estradiol-receptor complex, or its 4*S* subunit, forms directly in the cold when estradiol is added to the cytosol fraction of uteri not previously exposed to hormone (*13, 26*). Thus, the receptor content of the cytosol is easily estimated by adding sufficient



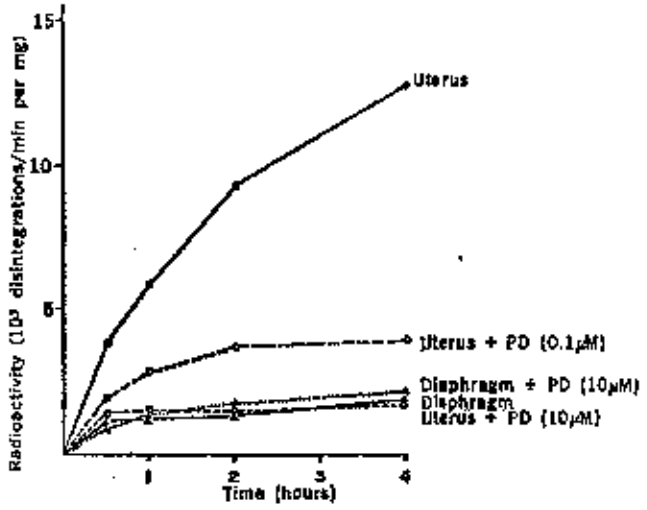


Fig. 2 (left). Uptake of tritiated estradiol by various tissues of immature rats receiving a single subcutaneous injection of 0.098 g (11.5 μc) of [6,7-³H]estradiol in 0.5 ml of saline. Total radioactivity expressed as disintegrations per minute per milligram of ry tissue or per 5 μl of blood. Because blood contains a mixture of radioactive metabolites but uterus and vagina incorporate only estradiol, the ratio of estradiol concentration between uterus and blood is about 500 to 1. Details in (*4*). Fig. 3 (right). Uptake of [³H]estradiol by uterine and diaphragm tissue of immature rats after incubation in 0.12 n*M* [³H]estradiol (57 c/mmole) at 37°C in Krebs-Ringer-Henseleit buffer, *p*H 7.3, in the presence and absence of different concentrations of an estrogen antagonist, CI-628 (PD) [from Jensen *et al.* (*56*)].

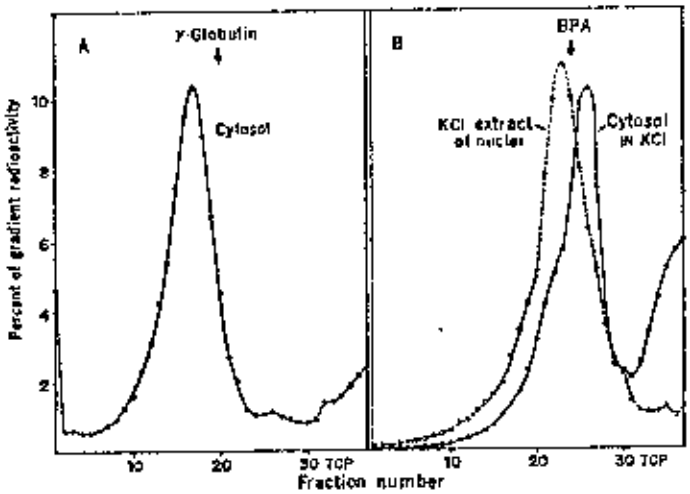
Fig. 4. Autoradiograph of frozen section of uterus of immature rat 2 hours after subcutaneous injection of 0.63 μg (131 μc) tritiated estradiol in saline. The 1-micrometer section showing epithelial gland and lamina propria was exposed to the emulsion 42 days and stained with methyl green-pyronin. Predominantly nuclear localization is also seen in myometrium. Reproduced from Stumpf and Roth (*20*), courtesy of Plenum Press.

tritiated estradiol to saturate the binding sites and determining the radioactivity present in the 8*S* sedimentation peak. This interaction of estradiol with the cytosol receptor is prevented by the presence of nafoxidine or CI-628.

From the sensitivity of their complexes to proteases but not to nucleases, the estrogen binding substances of both cytosol (*21*) and nucleus (*2, 25*) appear to be mainly protein in composition. They are somewhat unstable in crude extracts, tending to aggregate and to decompose during storage or attempted purification. Addition of calcium ions to the salt-dissociated complex of uterine cytosol, prepared in the presence of ethylenediaminetetraacetic acid (EDTA), yields a stabilized 4.5*S* form of the binding unit that is resistant to aggregation and does not revert to the 8*S* state on removal of salt (*27, 28*). This stabilization appears to result from the activation by calcium of an enzyme, present in uterine cytosol and rather unfortunately called "receptor transforming factor," that acts on the receptor protein to destroy its ability to aggregate in low salt concentrations (*28*).

Though noncovalent, the binding of estradiol to receptor proteins of uterine tissue is remarkably strong; association constants varying from 10^9 to $10^{12} M^{-1}$ have been reported for the cytosol complex (*3*). This tight binding appears to result from a slow rate of dissociation (*29*); once formed, the complex does not readily lose estradiol in the cold except by receptor decomposition or denaturation.

By a combination of salt precipitation, gel filtration, ion-exchange chromatography, and disc gel electrophoresis, microgram quantities of both the calcium-stabilized complex from calf uterine cytosol and the 5*S* complex extracted from nuclei have been obtained in apparently pure form; these purified products show clear differences in mobility on gel electro-



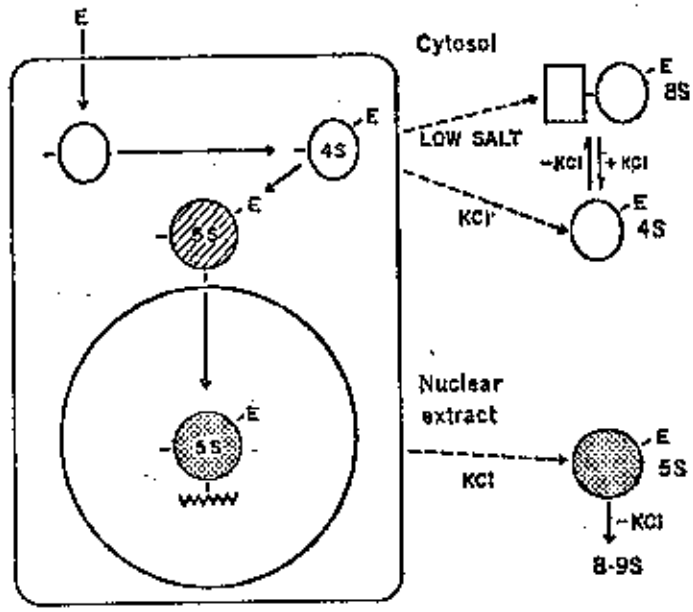


Fig. 5 (left). Sedimentation patterns of radioactive estradiol-receptor complexes of cytosol in tris-EDTA buffer [10 m*M* tris-(hydroxymethyl)aminomethane, 1.5 m*M* EDTA, *p*H 7.4] and nuclear extract (0.4*M* KCl in tris-EDTA, *p*H 8.5) from uteri of immature rats 1 hour after subcutaneous injection of 0.1 μg (20.8 μc) [^{3}H]estradiol. To saturate its receptor capacity, the cytosol fraction was made 5 n*M* with additional tritiated estradiol. Centrifugation was carried out at 2°C for 12 hours; (A) at 308,000*g* in 10 to 30 percent sucrose containing *p*H 7.4 tris-EDTA; (B) at 284,500*g* in 5 to 20 percent sucrose containing 0.4 m*M* KCl in tris-EDTA, *p*H 8.5. Globulin and BPA indicate positions of markers for 7.0*S* (bovine γ-globulin) and 4.6*S* (bovine plasma albumin) fractions. Fig. 6 (right). Schematic representation of interaction pathway of estradiol (*E*) in uterine cell. Diagram at left indicates uterine cell with extranuclear estradiol-receptor complex undergoing transformation and entering nucleus to bind to chromatin. Diagrams at right indicate sedimentation properties of complexes extracted from the cell.

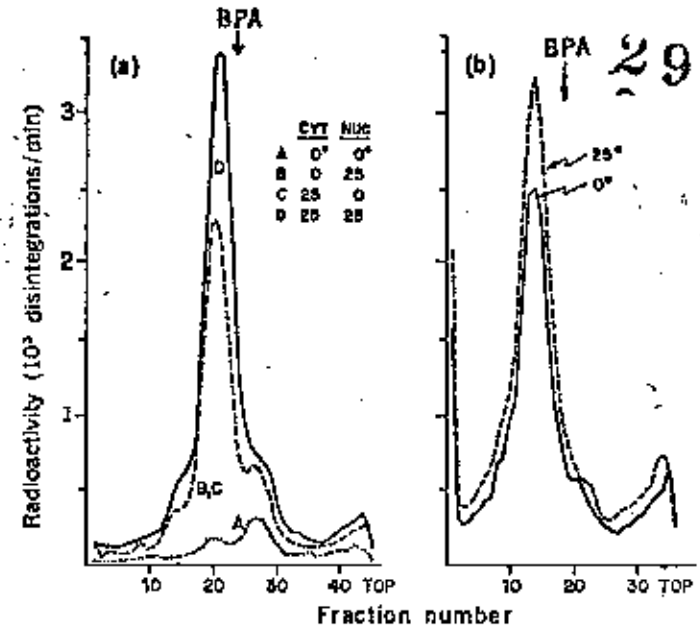


Fig. 7. Comparison of native and transformed complexes from calf endometrium cytosol in their binding to sucrose-purified nuclei from calf endometrium. Nuclei were incubated for 60 minutes at either 0° or 25°C: (a) with cytosol that had previously been incubated with 5.6 n*M* [^{3}H]estradiol for 45 minutes at either 0° (A, B) or 25°C (C, D); or (b) with redissolved ammonium sulfate precipitate from an unheated mixture of [^{3}H]estradiol and cytosol (final concentration of [^{3}H]estradiol, 5.6 n*M*). The separated nuclei were extracted with 0.4*M* KCl, and the extracts centrifuged on salt-containing sucrose gradients. Successive 100 microliter fractions of the gradient were counted. BPA indicates the position of bovine plasma albumin marker. Details in (*30*) and (*42*).

phoresis as well as in sedimentation properties (*30*). With partially purified complexes of calf uterine cytosol, estimates of 200,000 and 5.8 for molecular weight and isoelectric point, respectively, were found for the 8*S* complex, compared to 75,000 and 6.4 for the calcium-stabilized 4.5*S* unit (*27*); in another study, respective values of 238,000 and 6.2 for the 8*S* complex and 61,000 and 6.6 plus 6.8 for the 4.5*S* unit were obtained (*28*).

Two-Step Interaction Mechanism

A major advance in the understanding of the interaction of steroid hormones with target cells came with the recognition that the estradiol-receptor complex of the uterine nucleus is derived from the cytosol by a temperature-dependent process in which association with the hormone activates the cytosol receptor protein to migrate to the nucleus (Fig. 6). This two-step interaction pathway is supported by evidence from a variety of experiments.

A relation between the two intracellular sites of estrogen localization was first indicated by observations that a given dose of nafoxidine in vivo inhibits cytosol and nuclear binding in the rat uterus to the same degree and that there is a difference in saturability between estradiol uptake and its retention (*16*). These findings led to the suggestion that the cytosol protein, which is present in reserve amounts and binds spontaneously with estradiol, might serve as an "uptake receptor," that delivers the hormone to the nucleus for retention (*13*). In the autumn of 1967, the concept of a two-step mechanism, in which the cytosol complex is translocated to the nucleus, was put forth independently at the Laurentian Hormone Conference and at a meeting of the National Academy of Sciences. One proposal (*31*) was based on the temperature dependent shift of extranuclear to nuclear radioactivity with accompanying loss of cytosol binding in rat uteri previously exposed to tritiated estradiol at 0°C in vitro, while the other (*32*) arose from similar observations of temperature-induced redistribution of estradiol within the uterine cell, as well as the absolute requirement for cytosol receptor in the formation of the nuclear complex and the temporary depletion of cytosol receptor that follows administration of estradiol in vivo. These mutually supportive pieces of experimental evidence are elaborated below. Participation of uterine cytosol in nuclear binding was suggested independently (*33*) by the observation that, on incubation with tritiated estradiol, uterine nuclei take up more radioactivity when the incubation is with uterine cytosol rather than with buffer alone.

Subsequent to the first proposals that the 5*S* nuclear complex is derived from the 8*S* cytosol complex by a temperature-dependent process, it was recognized that the cytosol receptor contains 4*S* hormone-binding subunits and that these can undergo estrogen-induced, temperature-dependent conversion to a 5*S* form, as described in the next section. The sequence in Fig. 6 represents a modification of the original scheme in accordance with this additional knowledge.

More recent studies by various investigators, summarized with specific references in (*2*) and (*3*), have demonstrated that target tissues for other classes of steroid hormones contain specific extranuclear receptor proteins that bind with the hormone and then migrate to the nucleus. Thus, a two-step or perhaps a three-step (*34*) mechanism, similar to that elucidated for the estrogens, appears to provide a general model for the interaction of steroid hormones with their respective target cells.

Probably the most significant evidence supporting the two-step interaction mechanism is the dependence of nuclear binding on the presence of the cytosol receptor. In contrast to the 8*S* protein, which is present in uterine cytosol and reacts directly with estradiol to form a complex (*13, 26*), there is no detectable 5*S* binding protein in nuclei of uteri that have not been exposed to hormone (*13, 32*). No 5*S* complex is obtained either by addition of estradiol to an extract of uterine nuclei or by direct treatment of the nuclei themselves, although it is readily produced (Fig. 7a) when estradiol is incubated either with uterine homogenates or with isolated nuclei in the presence of the cytosol fraction (*23, 32, 35*). Formation of the nuclear complex is a temperature dependent phenomenon that proceeds at an appreciable rate between 25° and 37°C. Prior heating of the cytosol to 45°C destroys estradiol binding by the 8*S* receptor and eliminates its ability to

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produce 5S complex on subsequent incubation with nuclei (32).

In whole uterine tissue, nuclear binding is likewise temperature dependent. After immature rat or calf uteri are exposed to dilute solutions of tritiated estradiol at 37°C, the intracellular distribution pattern of uterine radioactivity, as determined by fractionation and by autoradiography, is similar to that seen after hormone administration in vivo (13, 18). But if the tissue is treated with hormone at 2°C, the major portion (70 to 75 percent) of the radioactive steroid appears as 8S complex in the cytosol fraction (31, 32, 36) and is seen in the extranuclear region on autoradiography (32). When such uteri, rich in extranuclear 8S complex, are warmed briefly to 37°C, redistribution of the steroid takes place within the tissue to yield predominantly nuclear bound steroid, extractable as 5S complex. These observations imply that radioactive estradiol, which can associate with the extranuclear receptor in the cold, is transferred to the nuclear binding site by a second process that does not proceed readily at low temperature.

As estradiol reacts with uterine cells to become localized in the nucleus, the extranuclear receptor protein temporarily disappears. It was found that the total receptor content of rat uterine cytosol is less after a large dose of estradiol than after a smaller one (32) and that after administration of physiologic amounts of hormone there is a progressive fall in cytosol receptor content for about 4 hours (Fig. 8), after which the 8S protein is gradually replenished (23, 37). This restoration appears to involve new synthesis of receptor protein, inasmuch as it can be blocked by the administration of cycloheximide at the proper time. With a massive dose of estradiol (5 μg), 90 percent of the receptor disappears within 15 minutes (2). Similar depletion of extranuclear receptor accompanies interaction of uteri with estradiol in vitro (35, 36, 38), consistent with its utilization to generate the nuclear complex.

The depletion of extranuclear receptor is four to five times greater than can be accounted for by the estradiol present in the nucleus at 4 hours (2). This observation indicates that nuclear turnover of estradiol in vivo must be fairly rapid. It also suggests the possibility that on leaving the nucleus estradiol may encounter more cytosol receptor and repeat the interaction cycle, so that each hormone molecule may induce the translocation of several receptor molecules to the nucleus.

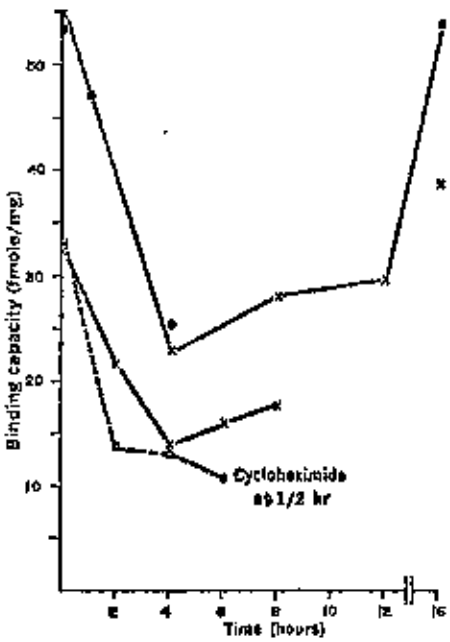

Fig. 8. Estradiol binding capacity (determined from the radioactivity of the 8S sedimentation peak and expressed as femtomoles per milligram fresh tissue) of cytosol fractions of immature rat uteri excised at different times after subcutaneous injection of 0.1 μg [^{3}H]estradiol in saline. Upper curve is a composite of two experiments; lower curves a third experiment in which some animals (○ - - - ○) received 0.2 mg cycloheximide intraperitoneally in 0.2 ml of saline 30 minutes prior to the injection of estradiol. Cytosols for each time point were prepared from pooled uteri from ten rats; before they were layered on the sucrose gradient, excess [^{3}H]estradiol (10^4 to 20 nM) was added to saturate the binding capacity. Details in (23).

Receptor Transformation

After the demonstration that incubation of uterine nuclei with an estradiol-cytosol mixture gives rise to an extractable 5S hormone-receptor complex, indistinguishable from that obtained in whole tissue, it was first assumed that the new complex was formed in the nucleus (23). But subsequent observations that conversion of the 4S receptor to a 5S form does not require the presence of nuclei (2, 39) and that only the transformed form of the complex is taken up by isolated uterine nuclei to stimulate their RNA polymerase activity (30, 40) suggest that the estrogen-induced alteration of the cytosol complex accompanies or even precedes its migration to the nucleus and may be a prerequisite for receptor translocation as well as for biological action.

Uterine cytosol warmed in the presence but not the absence of estradiol yields a hormone-receptor complex that sediments in sucrose density gradients containing salt at approximately the same rate as the nuclear complex (Fig. 9). This transformation in cytosol shows the same characteristics as those associated with incubations carried out in the presence of nuclei (2, 39). The reaction, which takes place only slowly in the cold, proceeds readily at 25° to 37°C and is accelerated with increasing pH over the range 6.5 to 8.5, as well as by the presence of salt. It is retarded slightly by calcium, magnesium, or manganese ions, and more strongly by EDTA. Under the same conditions that estradiol is effective, estrone does not induce the formation

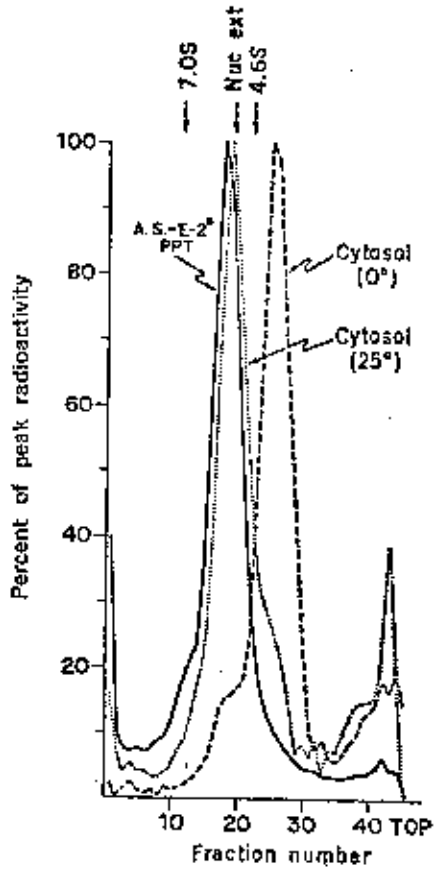

Fig. 9. Sedimentation patterns, in salt-containing sucrose gradients, of radioactive estradiol-receptor complexes of calf uterine cytosol, incubated with 3.8 nM [^{3}H]estradiol for 90 minutes at either 0° or 25°C, and of redissolved ammonium sulfate precipitate (*A.S.-E-2* PPT*) from cytosol containing 10 nM [^{3}H]estradiol. 4.6S and 7.0S indicate respective positions of bovine plasma albumin and γ-globulin markers; *Nuc. ext.*, that of complex extracted by 0.4M KCl from nuclei of calf uteri previously incubated with [^{3}H]estradiol in vitro. Details in (42).

of the 5S complex, either in the presence or absence of nuclei (*2, 30*), although it has been found to do so when it is present in higher concentrations (*41*).

Recently it was observed that ammonium sulfate precipitation of the estradiol-receptor complex of calf uterine cytosol, prepared in the absence of EDTA, is accompanied by conversion of the complex to a more rapidly sedimenting form suggestive of the transformed state (*42*). The complex from the redissolved precipitate sediments in sucrose density gradients containing salt slightly faster than that obtained by warming uterine cytosol with estradiol (Fig. 9). By careful comparison with 4.6S (bovine plasma albumin) and 7.0S (commercial bovine γ-globulin) markers, the following sedimentation coefficients can be calculated (*42*) for the receptor proteins of calf uterus: native cytosol, 3.8S; transformed (25°C) cytosol, 5.3S; nuclear extract, 5.2S; and ammonium sulfate precipitate, 5.5S. Whether these small but reproducible differences in sedimentation behavior among the various preparations are due to variations in the molecular milieu or whether they represent subtle differences in the hormone-receptor complexes themselves is uncertain.

In contrast to the temperature-dependent transformation in uterine cytosol, which takes place only if the receptor protein is complexed with the estrogenic hormone, the alteration that accompanies ammonium sulfate precipitation proceeds rapidly in the cold and does not require estrogen. When the uncomplexed receptor of calf uterine cytosol is precipitated with ammonium sulfate, and tritiated estradiol is then added to the redissolved precipitate, the same 5.5S complex is produced as when the receptor is precipitated in the presence of estradiol. Similar production of a more rapidly sedimenting form is observed when the native cytosol complex is subjected to other processes of partial purification, such as filtration through Sephadex G-25, preparative ultracentrifugation in sucrose density gradients, or dialysis, suggesting that uterine cytosol may contain a substance of small molecular weight that maintains the receptor protein in its native form. Although the significance of these observations is not yet clear, they provide clues for further investigation relative to the chemical basis of the transformation phenomenon.

Binding to Nuclei

The transformed and native forms of the estradiol-receptor complex of uterine cytosol differ markedly in their affinities for uterine nuclei (Fig. 7a). When incubated with estradiol-cytosol mixtures, sucrose-purified uterine nuclei show little incorporation of the native complex at 0°C, in contrast to a large uptake at 25°C where transformation to the 5S form accompanies the nuclear incorporation. But if the estradiol-cytosol mixture is first warmed to 25°C to effect receptor transformation, subsequent incubation with nuclei at either 0° or 25°C results in a substantial uptake of the transformed complex.

Thus, receptor transformation appears to be a prerequisite for binding of the hormone-receptor complex to isolated uterine nuclei and probably represents the temperature-dependent process associated with nuclear incorporation in whole uterine tissue. Similar conclusions have been reached in recent studies of the effect of temperature on the binding of the estradiol-receptor complex to isolated uterine chromatin (*42a*).

The estradiol-receptor complex obtained by ammonium sulfate precipitation resembles the transformed cytosol complex in its ability to bind to purified uterine nuclei at either 0° or 25°C (Fig. 7b). It is evident that the alteration that leads to increased sedimentation rate, whether induced by warming with estradiol or by salt precipitation, also results in enhanced affinity for nuclei.

Rat uterine chromatin has been reported to bind more uterine cytosol complex in the cold than does chromatin from liver, spleen, or lung (*43*), and earlier studies have indicated somewhat greater uptake of complex at 25°C by crude nuclear preparations from uterus than by those from diaphragm, liver, or kidney (*2, 23*). These observations suggest that uterine nuclei contain specific "acceptor" sites with affinity for the estrogen-receptor complex. In more recent experiments, however, we find that sucrose-purified nuclei from calf uterus, liver, and thymus do not show any marked difference in their incorporation of transformed estrogen-receptor complex when compared in terms of their DNA contents. However, the effect of the bound complex on nuclear RNA synthesis is highly tissue-specific, as discussed later.

Transformed Receptor and Nuclear RNA Synthesis

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The fact that estradiol moves to the nucleus together with the receptor protein might imply that the receptor system is simply a transport mechanism to deliver the hormone to its eventual site of action. On the other hand, it has been suggested (*2*) that the steroid-protein complex, or even the receptor protein itself, may be an important factor in some nuclear process and that the function of the hormone is to effect conversion of the protein to an active form that can enter the nucleus. Evidence supporting this concept is provided by the ability of the transformed but not the native estradiol-receptor complex to enhance RNA synthesis in isolated uterine nuclei.

Numerous investigations (*44*) have demonstrated that increased biosynthesis of RNA in the rat uterus is an early response to the administration of estrogen, although there has been some controversy as to how soon a bona fide effect on overall RNA synthesis can be detected. Indirect evidence suggests that production of messenger RNA for a specific soluble protein in the rat uterus is an especially early event (*45*). It was also found (*46*) that the RNA polymerase activity of nuclei isolated from uteri of immature or castrated rats is increased two- to threefold by administration of estradiol to the animal 1 to 4 hours before the uteri are excised, but that direct treatment of the nuclei with hormone has no effect. An important advance in our knowledge was provided by the discovery (*47*) that direct stimulation of nuclei from heifer endometrium is possible if treatment with estradiol is conducted in the presence of endometrial cytosol containing the receptor protein. It was also observed that the RNA polymerase activity of heifer endometrium nuclei or of the enzyme prepared from these nuclei could be enhanced by adding a mixture of estradiol and certain uterine fractions directly to the polymerase assay system (*48*).

We have found that susceptibility of RNA synthesis to stimulation by the estradiol-receptor complex is a specific characteristic of uterine nuclei and that to produce this activating effect the receptor must be in the transformed state (*40*). Purified nuclei from immature rat uteri incorporate less labeled nucleotide into RNA than do kidney

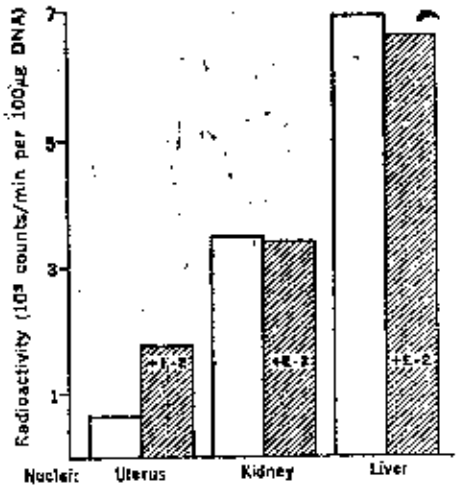

Fig. 10. Synthesis of RNA in nuclei, isolated in 2.2*M* sucrose from various rat tissues, after incubation at 25°C for 30 minutes with rat uterine cytosol (in 2.2*M* sucrose, 1 m*M* $MgCl_2$) in the presence and absence of 10 n*M* estradiol (E-2). After incubation, the nuclei were separated and resuspended in 0.32*M* sucrose for assay of magnesium-dependent RNA polymerase. Results expressed as counts per minute of radioactive nucleotide incorporated per 100 μg of nuclear DNA. Details in (*40*).

or liver nuclei (Fig. 10). After incubation at 25°C for 30 minutes with uterine cytosol containing estradiol, subsequent RNA synthesis is increased nearly threefold in uterine nuclei, whereas there is no enhancement of the already high synthetic capacity of kidney or liver nuclei after incubation with the hormone, either in uterine cytosol or in their own cytosols. Thus, nuclei from the hormone-dependent tissue appear to possess a specific deficiency in RNA synthetic capacity that can be alleviated by the estradiol-receptor complex of uterine cytosol.

Stimulation of uterine nuclei requires that the estradiol-receptor complex be converted from the 4*S* to the 5*S* form (Table 1). Nuclei from calf endometrium are activated by incubation with estradiol and endometrium cytosol at 25°C, where receptor transformation takes place readily, but not at 0°C, where it does not. However, if the estradiol-cytosol mixture is first warmed to effect transformation of the receptor, the resulting 5*S* complex can stimulate nuclei on incubation at either 0° or 25°C. Estrone, which binds to the native receptor protein but does not induce its transformation at the concentration used, does not cause nuclear stimulation under conditions where estradiol is effective. The 5*S* estradiol-receptor complex, extracted from calf uterine nuclei previously incubated with estradiol and uterine cytosol, resembles the transformed cytosol complex in stimulating uterine nuclei on incubation at either 0° or 25°C.

The redissolved dialyzed ammonium sulfate precipitate of uterine cytosol, containing the 5.5*S* form of the receptor, likewise can enhance the RNA polymerase activity of uterine nuclei, whether or not the receptor protein is complexed with estradiol (*42*). Because similar stimulation is seen with ammonium sulfate precipitates from cytosols of certain nontarget tissues, as well as from uterine cytosol previously heated to 50°C to destroy specific estradiol binding, the earlier conclusion that the uncomplexed 5.5*S* receptor protein can itself influence RNA synthesis must be held in abeyance until the action of purified preparations of this protein can be evaluated.

The enhancement of RNA synthetic capacity of uterine nuclei when they interact with transformed estradiol-receptor complex is considerably greater than that which would correspond to new messenger RNA for a limited number of specific protein species. Thus, the relation of this polymerase activation to early uterine responses to estradiol, such as the formation of "induced protein" (*45, 49*), is not clear. But the fact that a similar increase in nuclear RNA polymerase activity results from the administration of estradiol to the whole animal (*46*) provides reassurance that the stimulation of nuclei in vitro is of physiologic relevance.

How the hormone-receptor complex acts to enhance nuclear RNA synthesis needs elucidation. At least part of the effect appears to involve some action other than that on chromatin template activity. It is known (*50*) that the magnesium-dependent RNA polymerase activity of mammalian nuclei prepared in 2.2*M* sucrose can be separated into two fractions: bound enzyme, firmly associated with chromatin, and soluble

Table 1. Effect of transformed as opposed to native estradiol-receptor complex on RNA synthesis in calf endometrium nuclei. Purified nuclei from calf endometrium, prepared in 2.2*M* sucrose containing 1 m*M* $MgCl_2$, were incubated for 30 minutes (series A and B) or 45 minutes (series C) with endometrial cytosol in sucrose containing 10 n*M* estradiol or estrone, or with an extract of crude calf uterine nuclei previously exposed for 1 hour at 25°C to uterine cytosol containing 10 n*M* estradiol. Before incubation the nuclear extract in 0.4*M* KCl was concentrated (Diaflo XM-50 membrane) and then diluted with sucrose to a receptor concentration equivalent to that of the cytosol giving a KCl concentration of 35 m*M*. In series A, experiments 5 and 6, receptor transformation was effected prior to incubation with nuclei by warming the estradiol-cytosol mixture at 25°C for 30 minutes. After incubation, the nuclei were separated and resuspended in 0.32*M* sucrose for assay of magnesium dependent RNA polymerase by measuring the incorporation of uridine monophosphate, [^{3}H]UMP, from tritiated uridine triphosphate as described (*40*). Results are mean values of seven replicate determinations after subtraction of blanks from similar assays with 0.12*M* EDTA; estimated standard deviations of the mean are indicated. Control values were obtained with cytosol without steroid, except in series C, experiments 3 to 6, where heat-inactivated (50°C, 15 minutes) nuclear extract was used for the controls.

Exp.	Incubation: Addition to nuclei	Incubation: Temperature (°C)	[^{3}H]UMP into RNA: Picomoles per 100 μg of DNA	[^{3}H]UMP into RNA: Percent of control
		Series A		
1	Cytosol	0	4.63 ± 0.24	Control
2	Cytosol + estradiol	0	4.42 ± 0.22	95
3	Cytosol + estradiol	25	10.23 ± 0.87	220
4	Heated cytosol*	0	3.69 ± 0.37	Control
5	Heated cytosol + estradiol*	0	10.04 ± 0.54	270
6	Heated cytosol + estradiol*	25	8.80 ± 1.33	240
		Series B		
1	Cytosol	25	2.06 ± 0.13	Control
2	Sucrose† + estradiol	25	1.76 ± 0.15	85
3	Cytosol + estradiol	25	3.46 ± 0.11	170
4	Cytosol + estrone	25	2.16 ± 0.13	105
5	Cytosol + estradiol	0	2.15 ± 0.17	105
		Series C		
1	Cytosol	25	2.84 ± 0.26	Control
2	Cytosol + estradiol	25	5.57 ± 0.89	195
3	Heated nuclear extract‡	25	2.56 ± 0.34	Control
4	Nuclear extract	25	5.95 ± 0.87	235
5	Heated nuclear extract‡	0	2.67 ± 0.41	Control
6	Nuclear extract	0	4.72 ± 0.25	175

* Heated at 25°C for 30 minutes to transform receptor. † Homogenization medium: 2.2*M* sucrose containing 1 m*M* $MgCl_2$. ‡ Heated at 50°C for 15 minutes to destroy receptor.

enzyme, which is extracted from the nuclei by 0.32*M* sucrose. Both types of RNA polymerase activity are enhanced after incubation of endometrial nuclei with the estradiol-cytosol mixture (*40*). Inasmuch as the soluble enzyme is assayed with exogenous template, its increased activity is independent of uterine chromatin and appears to reflect an influence on the enzyme system itself. This conclusion is in agreement with the estradiol-receptor complex being able to cause stimulation when added directly to the polymerase assay mixture (*45*). It is also consistent with the observation (*51*) that the increased incorporation of precursor into RNA in uterine nuclei isolated from estrogen-treated rats results from the synthesis of longer RNA chains, rather than more chains as would be the case if the hormone acted to make new template sites available.

General Significance of Receptor Transformation

As previously noted, all classes of steroid hormones appear to interact with their target cells by similar mechanisms involving temperature dependent translocation to the nucleus of an initially formed extranuclear hormone-receptor complex. For other steroids, hormone mediated transformation of the cytosol receptor in the absence of nuclei has been demonstrated by a physical criterion (isoelectric point) only for the dihydrotestosterone receptor of rat prostate (*52*). However, changes in biochemical and nuclear binding properties suggest that glucocorticoid (*53, 54*) and progesterone (*55*) receptors undergo hormone induced, temperature dependent activation prior to their action in the target cell nucleus. A similar requirement for transformation has been postulated for aldosterone receptors in the kidney cell (*34*). It appears likely that receptor transformation will prove to be an important step in the biological action of all types of steroid hormones.

Recognition of the significance of receptor transformation serves to focus investigative attention on two important questions: the physicochemical basis of the transformation process, and the biochemical mechanism by which the transformed complex influences RNA synthesis in uterine nuclei. The eventual isolation of the native and transformed receptor proteins in amounts sufficient to permit comparison of their compositions and structures should provide insight into the nature of the transformation phenomenon, leading to a clearer understanding of the role of the hormone in this key step in estrogen action.

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Summary

The interaction of estradiol with uterine cells involves the association of the hormone with an extranuclear receptor protein, followed by temperature dependent translocation of the resulting complex to the nucleus. During this process, the steroid binding unit of the protein undergoes an alteration, called "receptor transformation," that can be recognized by an increase in its sedimentation rate from 3.8*S* to 5.2*S*, and by its acquisition of the ability to bind to isolated uterine nuclei and to alleviate a tissue specific deficiency in the RNA synthesizing capacity of such nuclei.

Receptor transformation can be effected in the absence of nuclei by warming uterine cytosol with estradiol. This preparation of transformed complex resembles that extracted from nuclei both in its sedimentation rate (5.3*S*) and in its ability to bind to uterine nuclei and augment RNA synthesis, properties that are not shown by the native complex. It is proposed that receptor transformation is an important step in estrogen action and that a principal role of the hormone is to induce conversion of the receptor protein to a biochemically functional form.

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FACTORS INFLUENCING RETENTION OF NITROGEN AND CALCIUM IN PERIOD OF GROWTH

VI. THE CALCIUM AND VITAMIN D REQUIREMENTS OF THE OLDER CHILD

JOSEPH A. JOHNSTON, M.D.

DETROIT

Several reviews[1] stressing the paucity of data on the calcium and vitamin D requirements of older children suggested the desirability of bringing together a number of determinations over a significantly long term of the retention of these two items by older girls with varying intakes. Some of these data have been reported in connection with other problems, such as the influence of puberty[2] and the effect of estrogen,[3] and are briefly summarized in table 1 as typical of the usual balance at puberty, without ingestion of additional vitamin D and without variation in the intake of calcium. Another group of cases in this age group is reported in detail to illustrate the effect of variation in the intake of vitamin D.

The comparative rarity of clinically recognizable rickets beyond infancy may account for the casual attitude of the average pediatrician toward adequate provision of calcium and vitamin D in the diets of older children, though it is logical to assume that other types of abnormality may be related to a deficiency of one or both of these substances in that age group. We have noted[4] that in the reinfection type of tuberculosis seen in adolescent children there is poor retention or no retention of nitrogen or calcium or both, though whether or not this condition is a cause or an effect is not wholly proved. Improved retentions and healing parallel each other, and the effecting of these increased retentions by high intakes of calcium and vitamin D has seemed to be a factor favorably influencing the healing. Data will be presented for the nitrogen and calcium balances of two girls who had the slipped upper femoral epiphysis of puberty, the underlying cause of which is unknown. It is significant that the highest incidence of this abnormality in girls is in the year preceding sexual maturity, when the speed of growth has become greatly accelerated. Whether or not hormonal effect is involved, the fact remains that one of these girls on what is considered an adequate intake had a negative balance for calcium, which could be converted into a positive balance by the use of supplemental vitamin D. The other girl had a calcium retention which was only a fraction of the usual amount and which was improved with vitamin D. Both of these patients were observed a year after the appearance of the slipping.

The daily allowances of calcium recommended by the Committee on Foods and Nutrition of the National Research Council are as follows:

	Age, Yr.	Calcium, Gm.
Girls	1 to 9	1.0
	10 to 12	1.2
	13 to 15	1.3
	16 to 20	1.0
Boys	13 to 15	1.4
	16 to 20	1.4

While superficially the relating of desirable intake to chronologic age would seem reasonable, a more logical approach would be to consider it a function of the rate of skeletal growth. There are a definite acceleration in the rate of skeletal growth in girls preceding menarche and an almost abrupt deceleration following menarche, indicating that the amount of calcium needed is thus becoming a function of physiologic rather than of chronologic age. The postmenarcheal deceleration is reflected in the diminished percentage utilization of calcium immediately following the menarche noted in the study on the influence of puberty.[3]

The amounts suggested by Leitch,[5] "assuming the maximum probable retention of fifty per cent

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of the intake in grams calcium daily: from 6 months to 2 years 0.8 grams, from 2 to 9 years 0.9 grams, and from 9 years 1 gram increasing to 2 grams between 15 and 16 years,[6] provide a generous allowance for the adolescent years and an extremely wide range. With an average American diet for this age group, deriving 15 per cent of the total calories from protein, 35 per cent from fat and 50 per cent from carbohydrate at a level of 2,400 calories with milk at each meal and an extra glass after school, a daily intake of 2 Gm. is seldom approached; and in the observations here cited retention was never greater than 40 per cent of the intake, after menarche it was considerably less. Leitch's recommendation for daily minimal intake at ages from 10 to 15 years is 1.37 Gm. Her method of arriving at these figures is criticized by McGowan[6] on statistical grounds, and indeed it would seem extremely difficult to plan an observation in this field that would be free from criticism, so many are the variables which affect retention and so ignorant are we of the desired goal; but if it is granted that retentions commensurate with good skeletal growth are to be sought, her approach has something to recommend it. The basic raw data, however, contain too many observations of a duration of questionable significance, and variations in amount of calcium ingested were seldom introduced. The recommended minimal intake, however, 1.37 Gm., does agree in general with the requirement based on the observations reported in this study.

More recently Duckworth and Warnock[7] offered a compilation of the balances listed in the literature, and their data for girls in the age group under discussion are as follows:

Age, Yr.	Intake, Mg.	Retention, Mg.
10 to 11	1,360	208
11 to 12	1,495	356
12 to 13	1,660	420
12 to 14	935	202

The relationship to vitamin D was not considered.

The value listed for girls 12 to 13 years old would be low except for those girls in whom menarche had been experienced and the demands of accelerated growth in length were no longer being made.

In compilations of data such as those made by Leitch and by Duckworth and Warnock care seems to have been exercised in the selection of data from some standpoints, for example the health of the patient, but little consideration seems accorded to what is probably the most important point, the duration of the uninterrupted observation. Any one conscious of the fluctuations in fecal calcium recognizes the truth of the statement by Porter-Levin[8] that a single statistically significant balance figure requires observation for fifteen to twenty days. In Duckworth's data, the standard for girls 13 years old was based on observation of a single patient for twenty-seven days and two other series of observations of five and eleven days' duration. Four series of observations on 12 year old girls were fifteen days or longer; these observations show a range of intake of calcium from 735 to 1,249 mg. with retention of 226 to 519 mg. My data[9] appear not to be included, presumably because it was assumed that the observations were made on abnormal children. The subjects used for the study of the effect of estrogen were normal girls who were in the Henry Ford Hospital because they were orphans; this hospital serves as a receiving home for such children until placement in suitable foster homes can be arranged. The ages of these girls ranged from 9 to 15 years, but all of them were physiologically close to the menarche. The subjects used for the study of the influence of puberty included 3 children who were normal to the extent that a clinician considers a child normal whose sole abnormality is a healed tuberculous first infection, something present in approximately 15 per cent of the entire population in this age group. Their stay in the hospital can likewise be considered as in lieu of residence in a home. Exercise, garden work and school sessions were part of their programs. The data obtained during the control periods in the study of the effect of estrogen are included in this summary.

CALCIUM

Relation of Intake to Retention.—Unlike reduced intakes of nitrogen, which will result in a positive balance ultimately, with a fall in the basal metabolism, there are intakes of calcium below which no retention occurs. Increased intakes are paralleled by increased retentions only up to a physiologic ceiling. Whether this maximum effective intake is also optimal is not known. There is much individual variation in the ability to retain calcium, influenced by a variety of factors, some of them hormonal, but chiefly by the state of the previous stores of this element. Because this last factor is so important studies of

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variables would seem more profitably made by intensive prolonged observation on a few subjects than by short term determinations of balance for many. The report on the influence of puberty is more significant because I had definite knowledge of the patients' intake of calcium for a year prior to the beginning of the observations, which then continued for two hundred and twenty-eight, one hundred and fifty and ninety-six days. With intake constant, information can be obtained on what can be stored in a given case, not on what might be stored with greater or smaller intakes.

If the intake of calcium is adequate and constant, utilization will vary with the rate of growth, and in particular this will be notable in the rapid acceleration preceding and the abrupt deceleration following menarche.

The retention of nitrogen may be unrelated to that of calcium and unaffected by variations in the intake of vitamin D. The synthesis of muscle tissue may proceed parallel with or bear a reciprocal relation to the formation of bone and we have no basis here for considering that there is any quantitative relation between the nitrogen balance and the intake of vitamin D. Determination of the nitrogen balance however, is essential in studies on utilization of calcium, since a negative nitrogen balance is known to produce a diminished retention of calcium.[10]

In all of my studies, a series of rotating diets was used the caloric level of which was dictated by the child's appetite. The composition of each was such that 15 per cent of the calories were derived from protein, 35 per cent from fat and 50 per cent from carbohydrate. The amount of calcium varied directly with the caloric intake, averaging 58.4 mg. per hundred calories of diet.

Source of Calcium in the Average Older Child's Diet.—The chief source of calcium in the diet of the average American child is milk. Sources other than milk in the 2,200 calory diet used in this study provided 328 to 466 mg. Analysis of milk continuously through the year showed a significant seasonal difference, the average calcium content in the winter months being 973 mg. per quart and in the spring and summer 1,331 mg. Accordingly, in studies made in the last eight years I have used a powdered preparation of milk, one lot of which would have a constant content of calcium for several months. When it is noted in some of the cases that a difference in intake of 0.2 mg. may mean the difference between a positive and a negative balance, the importance of a more exact knowledge of and provision for the optimal intake as well as the securing of its most efficient utilization by added vitamin D becomes evident.

The terms minimal and optimal are used here only in relation to retention, but whether or not the highest possible retentions are the most desirable is not known. In the metabolism of calcium there is definite evidence of the "physiologic ceiling" phenomenon noted with nitrogen; increased intake beyond a certain point results in no further retention. In the study of vitamin D, no attempt was made to explore the effects of massive doses, the range of daily doses investigated being from zero to 5,000 units.

The following study of a case bears on the matter of the relation of intake of calcium to retention. The subject was three years past menarche, and growth in height had apparently ceased.

CASE 1.—B. DeP., a girl, born Dec. 7, 1924, was observed to have a positive reaction to tuberculin at the age of 4 years (April 1929). Regular roentgen examinations showed nothing significant until April 1939, when a questionable patch of infiltration appeared in the upper lobe of the right lung. The child was admitted to the hospital for observation for tuberculosis of the reinfection type. The shadow persisted for several months but completely disappeared by August, so that for at least two months prior to the initiation of the studies of calcium balance there was no evidence of disease either roentgenographically or clinically, and there was no fever. Menarche occurred in November 1936. Between April and the beginning of the study, in October, the patient's weight increased from 104 pounds (47.2 Kg.) to 115 pounds (52.1 Kg.). The course of her changes in weight and height over the years preceding the observations is pertinent, her gain having been consistent until 1935, when, after a transfer from a good foster home to her own poor home, progress was interrupted; for a year she gained nearly 4 inches in height but actually lost ½ pound (0.5 Kg.) of weight. Between that time and the time of the detection of the suspicious lesion, however, the patient recovered weight and she seemed about average at the time of the study.

Date	Age, Yr.	Weight, Lb.	Weight, Kg.	Height, Inches
1/33	8	83	34	47
2/34	9	64	29	49.5
1/35	10	62.5	28.7	53.25
1/36	11	73	33.7	55.5
11/36	Menarche			
2/37	12	90	40.8	55
3/38	13.25	99.5	45.1	59.3
1/39	14.3	103	46.7	60
10/39 to 7/40	Study			
8/41	"	110	54	60

The observations on calcium balance covered two hundred and eighty-eight days (table 2 and chart 1), and the study was concerned with the amount of calcium to be added to a soybean preparation to promote retention of that element. Three variables were studied: caloric intake, source of calcium and amount of calcium. Two levels of caloric intake, 2,200 and 1,900 calories, were used; the higher intake produced gains in weight and a positive nitrogen balance; the lower intake produced loss of weight and a negative nitrogen balance. In the first period of eighteen days the calcium was derived largely from cow's milk; thereafter it was added to the soy-

10. Kinnetth, E. L., and Pittman, M. S.: A Long Time Study of Nitrogen, Calcium and Phosphorus Metabolism on a Low Protein Diet. J. Nutrition 17: 161-173 (Feb.) 1939.

TABLE 1.—*Calcium Balances of Nine Girls at Puberty*

Patient	Age, Yr.	Menarche*	Weight Lb.	Weight Kg.	Estimated Weight for Height and Age Lb.	Estimated Weight for Height and Age Kg.	Calcium Intake Gm.	Calcium Intake Gm./Kg.	Calcium Retention Gm.	Calcium Retention Gm./Kg.	Calcium Retention % of Intake	Vitamin D, U. S. P. Units	No. of Days	Basal Metabolic Rate Boothby	Basal Metabolic Rate Wetzel	Basal Calories	Comments
F. G.	13	+⅝	117	53.1	102	46.1	1.438	0.026	0.570	0.012	38	0	18	−20	−9	1,341	
B. P.	12	+¼	98	44.5	82	37.0	1.374	0.030	0.508	0.011	37	0	18	+ 2	+9	1,255	
O. B.	16	+1	122	55.3	116	52.6	1.155	0.021	0.219	0.006	19	0	17	−16	−5	1,217	
J. S.	13 9/12	+1⅞	119	54.0	96	43.5	1.387	0.026	0.354	0.006	25	1,000	18	−10	−1	1,340	
N. M.	13 7/12	+⅞	110	49.9	100	45.4	1.298	0.030	0.316	0.006	24	0	18	−20	−3	1,203	
F. S.	9¾	.....	70	31.8	74	33.6	1.125	0.035	0.315	0.010	28	..	15	− 9	−1	1,122	
V. G.	9 2/12	−½	80	36.2	88	39.9	1.298	0.034	0.495	0.014	40	0	77	− 7	+6	1,187	Before menarche
V. G.	9¾	+½	85	38.6	92	41.7	1.194	0.030	0.330	0.009	30	0	102	− 9	−1	1,298	After menarche
M. W.	10⅝	−1 7/12	114	51.7	104	47.2	1.173	0.028	0.310	0.006	29	0	288	−17	−4	1,304	Before menarche
M. W.	11	+⅞	110	49.9	104	47.2	1.104	0.022	0.237	0.005	21	0	68	−23	−8	1,253	After menarche
D. W.	11⅝	−1/12	98	44.5	102	46.3	1.201	0.031	0.649	0.016	47	0	21	−11	+6	1,365	Before menarche
D. W.	12 7/12	+⅞	96	43.5	102	46.0	1.250	0.031	0.460	0.011	34	0	60	−14	+2	1,306	After menarche

* Years after (+) or years before (−) menarche.

TABLE 2.—*Data for B. deP.**

No. of Period (Each 15 Days)	Date	Nitrogen Output, Gm.	Nitrogen Intake, Gm.	Nitrogen Balance, Gm.	Calcium Urine, Gm.	Calcium Stool, Gm.	Calcium Total Output, Gm.	Calcium Intake, Gm.	Calcium Balance, Gm.	Basal Calories	Weight Lb.	Weight Kg.	Basal Metabolic Rate Boothby	Basal Metabolic Rate Wetzel	Calories in Diet	Comments
1	11/ 7 to 24	13.89	14.93	+1.03	0.153	1.246	1.399	1.478	+0.160	1,227	115	52.1	− 7	+2	2,266	
2	11/25 to 12/13	13.46	14.57	+1.11	0.129	0.683	0.812	0.825	+0.013	1,356	116	52.6	− 4	+9	2,200	Soybean milk plus dicalcium phosphate
3	12/13 to 30	13.89	14.80	+0.91	0.122	0.680	0.816	0.886	+0.076	1,376	116¾	52.9	− 4	+1		
4	12/31 to 1/17	13.29	14.49	+1.10	0.123	0.774	0.812	1.007	+0.095	1,992	117¼	53.2	−10	−5		
5	1/18 to 2/4	13.09	14.02	+0.93	0.180	0.998	1.058	1.169	+0.111	1,316	118½	53.8	− 3	−4		
6	2/ 5 to 2/22	14.27	15.09	+0.81	0.117	1.085	1.202	1.231	+0.029	1,349	117¾	53.3	− 6	−1		
	Averages	13.64	14.71	+1.07	0.139	0.930	0.958	1.029	+0.065	1,336						
7	2/23 to 3/11	14.50	15.18	+0.68	0.097	0.455	0.552	0.466	−0.090	1,344	119	54.0	− 7	−2	2,300	Soybean milk without dicalcium phosphate
8	3/12 to 29	12.55	11.75	−0.80	0.061	0.251	0.312	0.328	−0.124	1,266	118	53.5	−12	−7	1,500	
9	3/30 to 4/16	12.13	11.39	−0.74	0.101	0.755	0.856	0.761	−0.095	1,203	116¾	52.9	− 9	− 5	.....	Dicalcium phosphate 0.446 mg.
10	4/17 to 5/4	12.36	12.22	−0.14	0.094	1.100	1.194	1.241	+0.047	1,278	116½	52.8	−11	−6	.....	Dicalcium phosphate 0.844 mg.
11	5/ 5 to 22	11.81	11.97	+0.16	0.074	1.647	1.721	1.641	−0.080	1,271	117	53.1	−10	−6	.....	Dicalcium phosphate 1.265 mg.
12	5/23 to 6/9	12.74	12.06	−0.68	0.090	1.803	1.893	2.115	+0.222	1,251	116¾	52.7	−12	−5	.....	Dicalcium phosphate 1.861 mg.
13	6/10 to 27	12.33	11.85	−0.48	0.114	2.624	2.738	2.542	+0.404	1,208	116	52.6	−12	− 6	.....	Dicalcium phosphate 2.295 mg.
14	6/28 to 7/15	12.22	12.33	+0.01	0.107	2.795	2.902	3.005	+0.103	1,268	116	52.0	−12	−0	.....	Dicalcium phosphate 2.795 mg.
15	7/16 to 8.1	12.18	11.23	−0.95	0.101	2.989	3.090	3.346	+0.256	1,197	116	52.6	−10	−12	.....	Dicalcium phosphate 2.[illegible] mg.

* Born Dec. 7, 1934; menarche Nov. 1935; height 59¾ inches.

bean preparation in the form of dicalcium phosphate. While the patient was receiving the higher caloric intake with the preparation of soybean and dicalcium

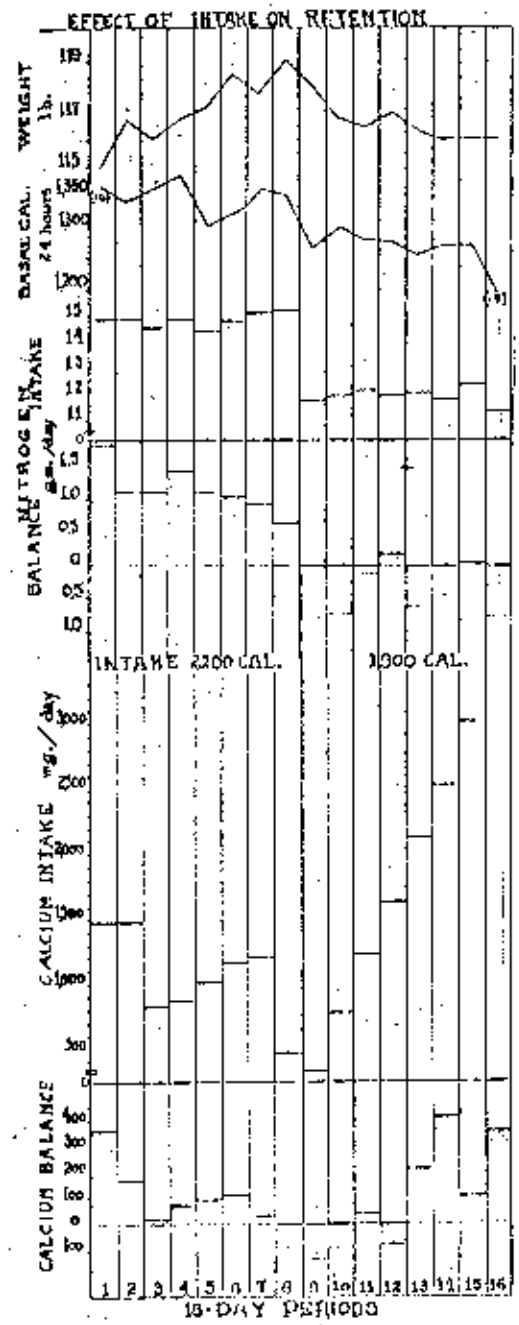

Chart 1 (B. deP.).—During the first period of eighteen days the calcium was derived largely from cow's milk; thereafter it was added to the soybean preparation in the form of dicalcium phosphate.

phosphate, daily intakes of calcium ranged from 466 to 1,231 mg. On all levels of calcium intake except the lowest positive balances prevailed. The highest retention occurred with an intake of 1,169 mg. The negative output which occurred in the following period, when the intake was 1,231 mg., may represent a "spill" of transiently held calcium. With the lower caloric level the intake of calcium ranged from 328 to 3,446 mg. per day and the balances from minus 134 to plus 404 mg. per day. With this highest level of retention (404 mg.), the "physiologic ceiling" seemed to have been reached and passed, for in the next period, with even higher intake, retention fell to 102 mg. per day. No added source of vitamin D was used. The effect of a negative nitrogen balance in rendering insufficient an otherwise adequate intake of calcium is described by Kuperth and Pittman.[19]

While exactly equivalent amounts of calcium were not given in the diets of cow's milk and of soybean with added dicalcium phosphate, comparable retention occurred: 12 per cent of a daily intake of 1,478 mg. was retained when the calcium was derived chiefly from cow's milk and 10 per cent of a daily intake of 1,169 mg. when it was derived from dicalcium phosphate in the soybean preparation. Considering only the observations made while the diet consisted of soybean with added calcium, during the period of positive nitrogen balance and gain in weight there was a steady increase in retention paralleling the increases in daily intake of calcium from 825 to 1,169 mg.; beyond this limit retention diminished. In the period when weight was being lost and the nitrogen balance was negative, twice as great an intake of calcium was required to produce retention of calcium and the ceiling was not reached until about 2,500 mg. were taken daily. Part of this increase in the requirement may be referable to the fall in the basal metabolic rate.

VITAMIN D

The function of vitamin D in the older child would superficially seem to be no different from that which has been shown for the infant. Its stabilizing effect has been noted, but an actual need for the vitamin seems questioned. No supplementary vitamin D was given the 9 children for whom data are listed in table 1. Its use will reduce the negative calcium balance noted with an inadequate intake of calcium, but unless the latter is marginal vitamin D will not promote a positive balance. Yet the acceleration of growth in height which precedes puberty would seem to make the desirability of supplementary vitamin D in this period comparable to that in infancy. The increased activity and greater exposure to light of older children compared with infants should quantitatively influence the requirement; but aside from the suggestion of Jeans and Stearns[16] that larger amounts were less effective than smaller and the repeated demonstration of the toxicity of extremely large doses, there is little information comparing the effects of various levels of vitamin D intake. A few studies are included on this point. The source of vitamin D used in these observations was an especially assayed viosterol in oil furnished by Mead Johnson and Company, one drop of which contained 200 U. S. P. units of vitamin D.

TABLE 3.—*Data for A. S.*

No. of Period (Each 7 Days)	Date	Nitrogen: Output, Gm.	Nitrogen: Intake, Gm.	Nitrogen: Balance, Gm.	Calcium: Urine, Gm.	Calcium: Stool, Gm.	Calcium: Total Output, Gm.	Calcium: Intake, Gm.	Calcium: Balance, Gm.	Total Calories	Weight: Lb.	Weight: Kg.	Height, In.	Calories in Diet	Comments
1	9/8 to 22	10.08	11.90	+1.82	0.185	1.031	1.216	1.071	—0.145	1,258	119	54	55¼	1,900	
2	9/23 to 10/10	10.60	12.03	+1.01	0.164	1.253	1.307	1.314	—0.102	1,162					
	Averages	10.34	11.77	+1.43	0.155	1.092	1.261	1.193	—0.123	1,210					
3	10/11 to 28	10.82	12.66	+1.84	0.202	1.118	1.320	1.140	—0.180	1,280	...	..	....	.....	1,850 U. S. P. units vitamin D
4	10/29 to 11/15	10.53	12.05	+1.52	0.012	0.996	1.030	1.343	+0.313	1,262	...	..	....	.....	2,050 U. S. P. units vitamin D and dicalcium phosphate, 232 Gm.
5	11/16 to 12/3	11.20	12.15	+0.95	0.023	1.262	1.285	1.578	+0.293	1,270	108	49	57¼	.....	1,550 U. S. P. units vitamin D and dicalcium phosphate 400 Gm.
6	12/4 to 21	11.14	12.64	+1.50	0.014	1.192	1.206	1.550	+0.250	1,210	...	..	....	.....	650 U. S. P. units vitamin D and dicalcium phosphate 400 Gm.

TABLE 4.—*Data for E. S.*

No. of Period (Each 15 Days)	Date	Nitrogen: Output, Gm.	Nitrogen: Intake, Gm.	Nitrogen: Balance, Gm.	Calcium: Urine, Gm.	Calcium: Stool, Gm.	Calcium: Total Output, Gm.	Calcium: Intake, Gm.	Calcium: Balance, Gm.	Total Calories	Weight: Lb.	Weight: Kg.	Height, In.	Calories in Diet	Comments
1	12/13 to 29	11.49	11.97	+0.50	0.015	0.871	1.097	1.103	+0.217	1,125	69½	31.6	55	1,900	
2	12/30 to 1/16	11.00	12.21	+1.21	0.012	0.839	0.851	1.110	+0.259	1,122	70½	32.0	55½	.....	
3	1/17 to 2/3	10.84	12.00	+1.16	0.028	0.781	0.809	1.125	+0.316	1,098	71	32.2	....	.....	
4	2/25 to 3/14	11.11	12.38	+1.27	0.008	0.795	0.821	1.135	+0.302	1,123	73¾	33.5	....	.....	
	Averages	11.11	12.14	+1.03	0.010	0.822	0.841	1.115	+0.274	1,117					
5	3/15 to 4/1	11.06	12.06	+1.38	0.009	0.749	0.768	1.094	+0.326	1,075	72	32.1	56¼	.....	Viosterol (1,500 U. S. P. units of vitamin D)
6	4/2 to 19	11.50	12.22	+0.72	0.010	0.780	0.790	1.063	+0.273	1,119	72	32.1	....	.....	
7	4/20 to 5/7	11.23	12.16	+0.93	0.010	0.810	0.823	1.090	+0.270	1,090	73	33.1	....	.....	
8	5/8 to 25	11.22	12.07	+0.85	0.007	0.832	0.839	1.160	+0.321	1,113	73¾	33.5	....	.....	
	Averages	11.35	12.15	+0.80	0.009	0.807	0.816	1.111	+0.295	1,107					
9	5/26 to 6/12	11.08	12.29	+1.26	0.010	0.773	0.783	1.150	+0.367	1,075	73	33.1	56½	.....	Viosterol (2,000 U. S. P. units of vitamin D)
10	6/13 to 30	11.57	12.32	+0.75	0.055	0.695	0.704	1.087	+0.383	1,017	72½	32.9	.....	.....	Viosterol (2,000 U. S. P. units of vitamin D)
	Averages	11.33	12.35	+1.05	0.033	0.734	0.743	1.118	+0.375	1,046					
11	7/1 to 7/18	11.74	12.37	+0.63	0.006	0.788	0.794	1.050	+0.256	1,065	72	32.7	57	.....	
12	7/19 to 8/5	11.52	12.44	+0.92	0.016	0.843	0.859	1.080	+0.221	1,032	72½	32.9	....	.....	
	Averages	11.63	12.40	+0.77	0.011	0.816	0.826	1.080	+0.254	1,038					

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In the study reviewed in the following report on a case there are two variables, the intake of calcium and the intake of vitamin D. With the intake of calcium practically constant the ingestion of 1,950 U. S. P. units of vitamin D as viosterol daily for a period of eighteen days (table 3, period 3) reduced the negative calcium balance from 168 to 70. The urinary excretion of calcium dropped to 77 mg. per day but this decrease did not promote a positive calcium balance.

Case 2.—*History.*—A. S., a girl, born prematurely and weighing 1,532 Gm. at birth, was fed at the breast and received cod liver oil during the first year. Her second winter was spent in Florida. When she was 5 years old a scoliosis was noted, which steadily progressed, and when she was 10 a Hibbs fusion operation was done, and she was confined in a body cast. It was after this operation that the metabolic observations were made. The child's diet during the year preceding this study had included a quart of milk a day but no added source of vitamin D. During the first period of these studies (September 6 to September 22) the patient was at rest in bed; during the remainder of the period of observation she was walking about the ward.

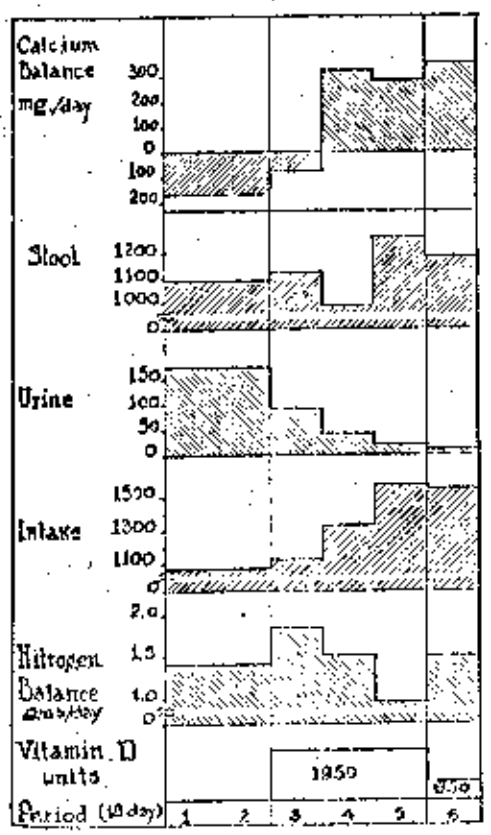

Chart 2 (A. S.).—No retention of calcium occurred until the intake reached 1,343 mg. per day, though the range between 1,340 mg. and this figure was not explored. Ingestion of vitamin D reduced the extent of the negative balance with a calcium intake of 1,140 mg. but did not make it positive. Increases in the intake of calcium beyond 1,343 mg. resulted in no significant increase in retention.

Study of Calcium Balance.—The study of the nitrogen and calcium balances covered one hundred and eight consecutive days, during which time there were three levels of calcium intake and two levels of vitamin D intake.

With a daily intake of approximately 1,100 mg. of calcium the metabolic balance for this element was negative; the negative balance was reduced but not made positive by the administration of 1,950 U. S. P. units of

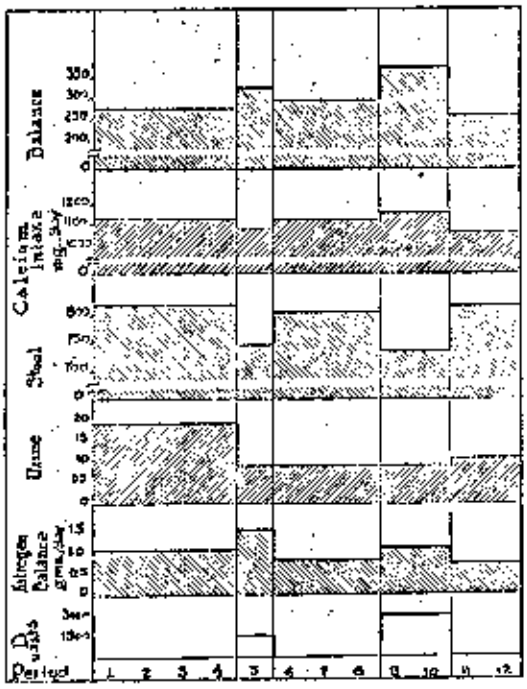

Chart 3 (E. S.).—With the intake of calcium constant at approximately 1,100 mg. per day, retention increased with added vitamin D; 3,000 U. S. P. units of vitamin D was more effective than 1,500. Both fecal and urinary losses diminished with the ingestion of additional amounts of the vitamin.

vitamin D. On a daily intake of 1,343 mg. of calcium the patient stored 313 mg. daily. The storage was not increased by the addition of dicalcium phosphate to bring the intake up to 1,578 mg. In the final period of observation the intake of vitamin D was reduced to 650 U. S. P. units daily and the highest retentions of calcium were noted. Since this period was continuous with two previous ones during which the patient received 1,950 U. S. P. units daily, the effect of which would still be present to a degree, comparisons of differences in the level of vitamin D are not possible here. The observation brings out the following facts: (1) that vitamin D does not affect the storage of calcium, though with it urinary loss is diminished when the intake of calcium is below a minimal level; (2) that in this girl the minimal level was approximately 1,300 mg. and (3) that there is probably a physiologic ceiling beyond which retention of calcium does not increase with increasing intake, which in this case was 1,300 mg., or 26 mg. per kilogram of body weight, per day.

Nitrogen retention increased transiently following the administration of vitamin D. The basal metabolic rate was minus 3 (Wetzel).

Case 3.—E. S., a child 8 years old, was hospitalized for the treatment of phlyctenular conjunctivitis. She

reacted to tuberculin and showed a healed first infection in the lung. She is included here rather than with the patients with tuberculosis because it is felt that her condition was local and unaccompanied by systemic disturbance. Her history showed an apparently adequate diet, though she had had no cod liver oil for several years.

The patient was readmitted to the hospital because of an exacerbation of the conjunctivitis at the age of

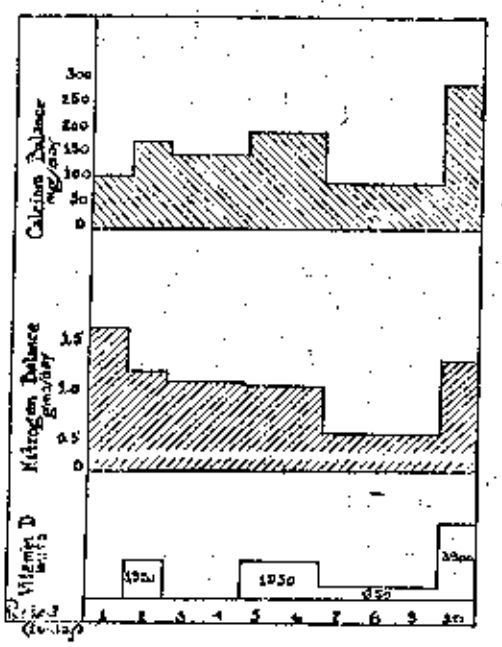

Chart 4 (H. S.).—With the intake of calcium constant at approximately 1,100 mg. per day, increasing retentions of calcium paralleled increasing intakes of vitamin D in amounts from zero to 3,500 U. S. P. units.

10 years. No secondary sexual characteristics had yet appeared. She was afebrile throughout the period of observation and was active around the ward, but she was receiving minute desensitizing doses of tuberculin.

Nitrogen and calcium balances were determined uninterruptedly from Dec. 13, 1940 to Aug. 5, 1941. The intake of both calcium and nitrogen was practically constant throughout the observations which covered three periods during which she received no vitamin D, one in which she received 1,500 U. S. P. units daily and another in which she received 3,000 U. S. P. units daily.

The patient at all times had a positive calcium balance, but the peak retention occurred while she was receiving 3,000 U. S. P. units of vitamin D daily. The increased retention is referable to a diminution in both fecal and urinary losses. Nitrogen retention showed a significant increase also during each period in which viosterol was administered. The basal metabolic rate was not significantly changed.

Case 4.—*History.*—H. S., a girl, was born in September 1926. Menarche occurred in March 1938, when she was 12 years old. The patient was hospitalized with tuberculous pleural effusion in January 1941 and became afebrile in May. Observations on calcium retention were begun in August 1941, and between May and August her weight had increased from 118 to 140 pounds (53.5 to 63.5 Kg.) on a "free" hospital diet containing more than 1 Gm. of calcium daily but not accurately measured. Her data are included here rather than with those of patients with tuberculosis since she had shown no evidence of illness for three months; she had presumably effected a replenishment of stores during that time, gaining 22 pounds (10 Kg.) on a diet containing a liberal amount of calcium.

Study of Calcium Balance.—Uninterrupted observations were made for one hundred and eighty days on this 15 year old girl who was three and a half years past the menarche and no longer growing in height. The intake of calcium was approximately constant at 1,100 mg. daily. During three periods of eighteen days each she received no supplementary vitamin D; during seven periods she received amounts varying from 650 to 3,900 U. S. P. units daily. Retention of calcium roughly paralleled these levels and was highest with the highest intake of the vitamin.

Two patients with slipped upper femoral epiphysis showed an abnormality of calcium retention which was improved with administration of vitamin D. In one patient (P. A.) a slightly negative balance became positive. The other patient (D. M.) showed a much lower initial calcium retention than would be expected for this age, and retention was doubled when additional vitamin D was given. Nitrogen retention in both of these instances increased greatly. The urinary output of calcium of P. A. was conspicuously large.

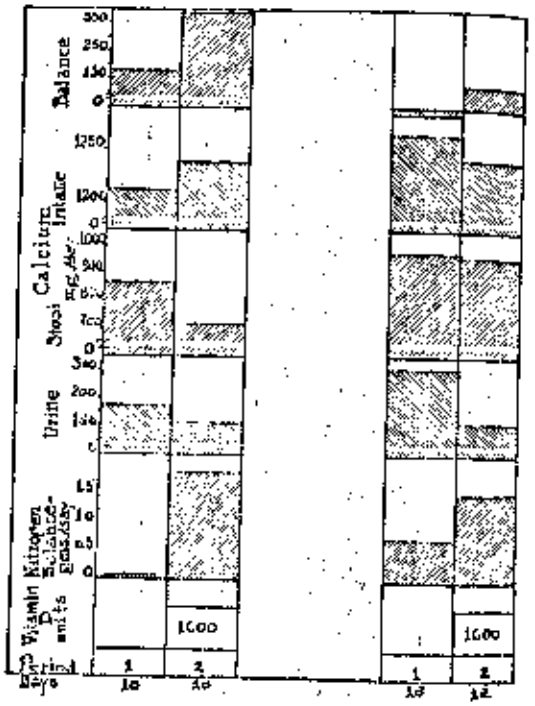

Chart 5 (D. M. and P. A.).—On daily intakes of calcium averaging 1,237 mg., the administration of 1,000 U. S. P. units of vitamin D resulted in the conversion of a negative calcium balance to a positive balance in one patient and the doubling of a low positive balance in the other. Both patients had epiphysiolysis of the upper end of the femur. The high urinary loss of calcium is notable in one (P. A.).

TABLE 5.—*Data for H. S.*

No. of Period (Each 18 Days)	Date	Nitrogen Output, Gm.	Nitrogen Intake, Gm.	Nitrogen Balance, Gm.	Calcium Urine, Gm.	Calcium Stool, Gm.	Calcium Total Output, Gm.	Calcium Intake, Gm.	Calcium Balance, Gm.	Total Calories	Weight Lb.	Weight Kg.	Height, In.	Calories in Diet	Comments
1	5/22 to 6/7	10.47	12.16	+1.69	0.102	0.909	1.001	1.106	+0.104	1,494	141.5	64.2	61¾	1,900	
2	9/5 to 21	10.50	12.14	+1.24	0.089	0.833	0.922	1.098	+0.175	1,507	140	63.5	....	.....	1,950 U. S. P. units vitamin D
3	9/28 to 10/13	10.93	12.02	+1.08	0.084	0.878	0.962	1.130	+0.171	1,421	138.5	62.8	....	.....	Vitamin D discontinued
4	10/14 to 31	11.25	12.44	+1.19	0.084	0.916	1.000	1.132	+0.132	1,390	137.5	62.3	....	.....	
	Averages	11.00	12.22	+1.13	0.084	0.897	0.981	1.124	+0.163	1,405					
5	11/1 to 12	11.19	12.43	+1.30	0.095	0.792	0.887	1.133	+0.246	1,329	135	61.2	....	.....	1,950 U. S. P. units vitamin D
6	11/15 to 12/6	11.31	12.22	+0.91	0.087	0.875	0.962	1.108	+0.110	1,396	134	60.8	....	.....	
	Averages	11.24	12.34	+1.10	0.091	0.833	0.924	1.120	+0.196	1,381					
7	12/7 to 21	11.26	12.30	+1.01	0.085	0.976	1.061	1.090	+0.029	1,368	133	59.9	....	.....	650 U. S. P. units vitamin D
8	12/22 to 1/13	12.21	12.59	+0.06	0.079	0.870	0.954	1.108	+0.154	1,322	132	59.9	....	.....	
9	2/1 to 21	11.44	12.22	+0.78	0.079	0.921	0.991	1.094	+0.103	1,348	131	59.1	....	.....	
	Averages	11.75	12.37	+0.62	0.078	0.921	1.002	1.097	+0.095	1,345					
10	2/1 to 18	11.29	12.51	+1.22	0.055	0.775	0.830	1.122	+0.293	1,397	...	....	....	.....	3,000 U. S. P. units vitamin D

TABLE 6.—*Data for D. M. and P. A.**

D. M.		Dates	Nitrogen Output, Gm.	Nitrogen Intake, Gm.	Nitrogen Balance, Gm.	Calcium Urine, Gm.	Calcium Stool, Gm.	Calcium Total Output, Gm.	Calcium Intake, Gm.	Calcium Balance, Gm.	Total Calories	Weight Lb.	Weight Kg.	Height, In.	Calories in Diet	Comments
18 day period	No. 1	7/1 to 17	13.73	13.82	+0.04	0.184	0.851	1.035	1.210	+0.175	1,350	104	47.2	61¾	2,200	
13 day period	No. 2	7/18 to 8/4	12.26	11.15	+1.00	0.153	0.711	0.864	1.232	+0.368	1,407	105	47.6	61¾	.....	Viosterol in oil, 2 drops, 1,600 U. S. P. units
P. A.																
18 day period	No. 1	11/15 to 12/2	11.68	12.74	+0.78	0.218	0.987	1.305	1.266	—0.039	1,317	87	39.5	60½	1,900	
12 day period	No. 2	12/3 to 14	10.91	12.46	+1.55	0.159	0.960	1.119	1.240	+0.121	1,324	86½	39.3	....	.....	Viosterol in oil, 2 drops, 1,600 U. S. P. units

* Observations on D. M. began three days and on P. A. seven days after ether anesthesia.

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CASE 5.—D. M., a girl born in July 1927, was first seen at 8 months of age, with bronchopneumonia and mild rickets. She had a history of adequate feeding save for the omission of any added source of vitamin D. She weighed 19 pounds (8.6 Kg.) at this time. At 3 years of age she had a tonsillectomy and adenoidectomy because of frequent infections. Her progress in height and weight from then on was as follows:

Date	Age, Yr.	Weight Lb.	Weight Kg.	Height, Inches
11/30	3½	33	16	39
9/35	8	65	24.0	49½
5/38	11	79	35.1	55½
6/39	12	91	41.3	59
7/40	13	101	47.2	61¾

A limp developed during the summer of 1938, and in October of that year there was roentgenographic evidence of a slipped upper femoral epiphysis on the left. The epiphysis was nailed. Preoperatively it was possible to determine the calcium balance for four days. This period was not of sufficient length to be significant, but in the light of the data from later observations over a longer period the results are interesting, particularly because of the high urinary output. The daily intake of calcium was 1,518 mg., and the output was 1,582 mg. in the stool and 392 mg. in the urine, giving a balance of minus 0.457 mg. The nitrogen balance was minus 0.57 mg. per day.

In July and August 1940 continuous observation for thirty-five days showed in the first period of eighteen days a retention of 175 mg. a day with an intake of 1,210 mg. of calcium; the retention increased to 368 mg. a day with the administration of 1,600 U. S. P. units of vitamin D as viosterol. The nitrogen balance also increased significantly. This balance is representative of the year following the period of sharp increase in the rate of growth preceding menarche, and it cannot be considered characteristic of the situation at the time the femoral epiphysis slipped. She had, in the year preceding this balance been on an adequate intake of vitamin D and calcium, started when the condition was recognized.

While the earliest balance cannot be considered conclusive because of the shortness of the period of observation, the urinary output, a factor which is trustworthy in shorter periods, was unusually high and correspondent to that observed in a similar case here reported (P. A.).

CASE 6.—P. A., a girl born in May 1927, was found to have a slipped upper epiphysis of the left femur in November 1938. Her symptoms dated from a fall which she had had four months previous to that time. The epiphysis was nailed in place. One year later, when the child was 12½ years old, the same condition appeared in the right hip. Our observations on the calcium and nitrogen balances were made a year later, during her stay in the hospital for the removal of both nails. During the two years which preceded the observations she had an average diet which included three glasses of milk and 500 U. S. P. units of vitamin D daily, with adequate meat, vegetables, fruit and eggs.

Study of the intake and output of calcium for thirty days showed a negative balance of 39 mg. per day for a control period of eighteen days on a daily intake of 1,266 mg. of calcium and a positive balance of 121 mg. per day during the remaining twelve days on an intake of 1,240 mg. per day (there had been some refusal) with 1,600 U. S. P. units of vitamin D. This is not descriptive of the condition at the time the epiphysis slipped, but, as in the case previously described, the retention of nitrogen was doubled when vitamin D was administered.

SUMMARY

An average daily intake of 1,280 mg. of calcium (range 1,100 to 1,500) resulted in an average retention of 410 mg. (range 237 to 649), on an intake of 28 mg. per kilogram of body weight (range 21 to 35) resulted in a retention of 9 mg. per kilogram of body weight (range 4 to 14) without ingestion of supplementary vitamin D. Vitamin D (as viosterol) increased the retention of calcium and the increase paralleled the amount of vitamin D ingested up to 3,900 U. S. P. units. In 1 patient, a girl who had a slipped femoral epiphysis, the ingestion of supplementary vitamin D resulted in changing a negative to a positive balance when the intake of calcium was 1,250 mg. per day. In a second instance of the same condition, a small balance (175 mg.) was more than doubled (368 mg.) by the administration of 1,600 U. S. P. units of vitamin D daily.

The amounts of vitamin D given to these older children when considered in terms of units of the vitamin per unit of body weight are comparable to the smaller amounts used for infants though there is no definite proof beyond these increased retentions that the requirement for this vitamin is related to body size. It seems more likely that the amount required may be related to the variations in the speed of increase in body length; the requirement would therefore be increased during the period of accelerated growth in height preceding sexual maturation.

There is a threshold a little below the average intake, below which no retention occurs, and there is a physiologic ceiling beyond which increased intake does not promote further retention. The margin of safety to insure storage in girls at puberty is small, and the data obtained from this study confirm the need for a full quart of milk per day in the diet during the pubertal period. A liberal intake of vitamin D at this period is desirable. The administration of amounts varying from 650 to 3,900 U. S. P. units of vitamin D in the form of a concentrate was paralleled by increases in the amounts of calcium retained; within this range no evidence of a depressing effect with the higher doses was noted.

Dr. Frank Hartman supervised the determinations of basal metabolic rate; Dr. O. H. Gaebler supervised the determinations of calcium and nitrogen balance. Dr. Philip Jeans and Dr. Genevieve Stearns reviewed and criticized the manuscript. The assayed preparation of viosterol was provided by the Mead Johnson Company.

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NUTRITION

ON THE SITE OF DISCRIMINATION AGAINST VITAMIN D_2 IN CHICKS.

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Previous work has shown that the chick prefers vitamin D_3 (D_3) to vitamin D_2 (D_2) and suggested that the discriminating step in vitamin D_2 metabolism occurs after 25-hydroxylation. Synthesis of a D_2 of high specific activity (1.2 Ci/mMole) was accomplished which enabled us to study the rate of 25-hydroxylation in a vitamin D-deficient chick liver system *in vitro*. It was found that chick liver homogenates converted D_2 and D_3 to their 25-hydroxy forms at the same rate. This suggests that 25-hydroxyvitamin D_2 (25-OH-D_2) is made normally in chicks. 25-OH-D_2 isolated from this liver *in vitro* system was later used as a substrate in two chick kidney *in vitro* hydroxylations to determine if there is discrimination against D_2 at the kidney level. A vitamin D-deficient chick kidney mitochondrial preparation metabolized 25-OH-D_2 to a more polar compound, probably 1,25-dihydroxyvitamin D_2, at the same rate as it 1-hydroxylated 25-hydroxyvitamin D_3 (25-OH-D_3). Similarly a chick kidney mitochondrial preparation from animals fed a high calcium diet (3%) supplemented with D_3 metabolized 25-OH-D_2 to a more polar compound, probably 24,25-dihydroxyvitamin D_2, at the same rate as it 24-hydroxylated 25-OH-D_3. These results suggest that the discrimination against D_2 is not at the kidney hydroxylation stage. (Supported by a grant from the NIH, No. AM-14881.)

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NOTES

Note on Chemical Determination of Vitamin D in Evaporated Milk

By S. W. JONES and D. A. LIBBY (Division of Nutrition, Food and Drug Administration, Washington, D.C. 20201)

Since the publication of a chemical method for determining Vitamin D in evaporated milk (1), we have encountered some difficulty with this method in certain unusual brands of evaporated milk. This difficulty is caused by an insufficient amount of interference being removed by column chromatography. Consequently, the 15 second absorbance reading gives a deceptively high value because of the interference on background material.

However, by using a third column of aluminum oxide (2), we can eliminate the excessive interference. With the use of this third column, our chemical values on these difficult samples then agree very well with the rat bioassays.

Since there is no way to predict which evaporated milk samples may have this excessive interference, consideration should be given to the routine use of the third column.

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J. Pediatrics 62:531-7, (1963)

Metabolic studies in a patient with idiopathic hypercalcemia of infancy

An asymptomatic 3½-month-old female infant who was receiving 1,000 units of supplemental vitamin D per day and whose serum calcium concentration was 14.5 mg. per cent was studied. While ingesting a diet containing less than 50 I.U. of vitamin D per day, the serum calcium fell to normal and the vitamin D concentration in the serum was normal. Subsequent ingestion of 400 I.U. of vitamin D for a 10 day period induced hypercalcemia, probable elevation of the serum vitamin D concentration and hypercalcuria. Sixteen days after discontinuing the vitamin D the serum calcium concentration was normal. There appeared to be a direct correlation between the amount of vitamin D ingested and the serum calcium and vitamin D concentrations. Severe idiopathic hypercalcemia had previously been noted in a sibling who had an elevated vitamin D concentration. The familial incidence of idiopathic hypercalcemia of infancy has rarely been recognized. There are no previous reports in which hypercalcemia and rise in plasma vitamin D were produced by a physiologic dose of vitamin D during the inactive phase. It is suggested that idiopathic hypercalcemia may, in some cases, be an inborn error of metabolism with defective inactivation of vitamin D, and that asymptomatic siblings of known cases of idiopathic hypercalcemia should be investigated for the mild form of the disease.

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PITTSBURGH, PA.

THE unusual aspects of a case of idiopathic hypercalcemia of infancy are being documented. First, the patient had asymptomatic and transient hypercalcemia although an older sister previously had severe idiopathic hypercalcemia.[1] A familial incidence of idiopathic hypercalcemia has only rarely been recognized. Second, this is the first recorded case of idiopathic hypercalcemia of infancy in which elevation of the serum calcium and probable elevation of the serum vitamin D level have been produced during the recovery phase by administering a physiologic dose of 400 units of vitamin D per day.

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CASE REPORT

K. H., born Oct. 26, 1960, was admitted to the Harriet Lane Home on Feb. 14, 1961, at 3½ months of age for investigation of asymptomatic hypercalcemia.

The family history was significant because an older sister, S. H., aged 11½ years, was treated for hypercalcemia at 5 years of age.[1] She continues to have mental retardation, impaired renal function, azotemia, and seizures even though the serum calcium values have been normal since 1956. An older brother, 11 years of age, had an unremarkable history. The mother, father, brother, and sister are nonhypercalcemic at this time.

Inquiry regarding the prenatal history revealed that Mrs. H. took 1 Natilet tablet per day from the third month of gestation until the time of delivery. Each tablet contained 250 mg. of calcium and 400 units of vitamin D. She drank only 1 glass of homogenized milk (400 units D per quart) per week, ate 1 or 2 eggs per day (25 to 70 units of vitamin D per egg), and ate no fish.

The infant weighed 7 pounds, 12 ounces when born at 38 weeks' gestation. Except for firm or hard bowel movements from the time of birth, the history was unremarkable, and the baby was described as being happy. This was in contrast to her older sister, who was an abnormally irritable child from birth until the hypercalcemia was eventually corrected.

A formula of evaporated milk, diluted with an equal volume of water, was given from birth until the age of 3 months. This contained 100 U.S.P. units of vitamin D per quart. By the age of 3 months the infant was taking almost a quart each day. She also received 0.6 c.c. of Tri-vi-sol per day, which contained 1,000 units of calciferol.[2] From the age of 3 months until the time of admission at 3½ months, she received whole milk containing less than 50 units of vitamin D per quart.[2]

On Jan. 28, 1961, at the age of 3 months, K. was seen by us only because the older sister had had idiopathic hypercalcemia. Although the baby was clinically normal, the serum calcium determined in the hospital laboratory was 14.5 mg. per cent. Three days later the serum calcium was performed in our laboratory and was 13.5 mg. per cent. All subsequent calcium determinations were performed in our laboratory, unless otherwise specified. On February 8 the value was 12.6 mg. per cent, and on February 10, 12.7 mg. per cent. She was then admitted to the hospital for further study.

At the time of admission the blood pressure was 75/50 mm. Hg and the pulse was 100 per minute. The height was 23.5 inches, and the weight was 10 pounds, 1 ounce. The general appearance was not "elfin" and the physical examination was perfectly normal. There was no cardiac murmur. The hemoglobin was 12.1 Gm. per 100 ml. and the hematocrit 36 per cent. The white blood count was 10,800 per cubic millimeter. The urine was clear; the specific gravity was 1.014; the pH, 5.5; and the tests for protein, sugar, and diacetic acid were negative. The Sulkowitch test was 3-plus. By microscopic examination, 5 to 10 white blood cells per high-power field were seen. The urinary specific gravity was 1.020 after a 9 hour thirst.

The radiologists found that the ends of the long bones and the base of the skull were suggestively increased in density, but this was not definite.

After a 9 hour thirst the serum urea nitrogen was 35 mg. per cent; carbon dioxide–combining power, 14.9 mM per liter; sodium, 147 mEq. per liter; chloride, 101 mEq. per liter; potassium, 5.1 mEq. per liter; and the cholesterol, 270 mg. per cent. One week later without a preceding thirst, a serum urea nitrogen was 24 mg. per cent and a CO_2-combining power was 21.6 mM per liter.

METHODS

Vitamin D activity in the serum was assayed by one of us (M. P.) who utilized the method of Bills.[3] The levels of serum calcium were determined by the method of Harrison and Harrison,[4] serum phosphorus by Fiske and Subbarow's method,[5] and serum citrate by the method of Natelson, Pincus, and Lugovoy.[6] Dr. Mackenzie Walser measured the serum magnesium[7] and Dr. Harold Nitowsky determined the serum vitamin E levels by the method of Quaife, Scrimshaw, and Lowry.[8]

Urinary calcium was determined by the method of Clark and Collip.[9] Stool calcium

[2] In January, 1961, the manufacturer changed the dosage of vitamin D in Tri-vi-sol from 1,000 to 400 units per 0.6 c.c. The baby did not receive the new product until vitamin D was administered during the studies which are described later.

was determined after dry ashing by the same method.

During the period when the calcium balance data were accumulated, Alacta of the same stock number, which contained less than 40 units of vitamin D per 100 Gm. of dry powder, was used as the only source of food. Analysis of this revealed a calcium content of 1,110 mg. per 100 Gm. of dry powder. Between February 14 and March 11, 105 Gm. per day was given and between March 12 and April 3, 125 Gm. per day. The intake was accurately determined by weighing each formula bottle before and after the feeding. Complete 24 hour urine and 72 hour stool specimens, the latter marked by carmine red, were collected while the patient was on a metabolic bed designed specifically for infants.

RESULTS OF EXPERIMENTAL STUDIES

See Fig. 1 on which are charted serum vitamin D levels, serum, urine, and stool calcium concentrations, and calcium balance on the regimens described below.

Hypercalcemia, plus recovery phases. Since the normal mean serum calcium value in our laboratory is 10.1 mg. per cent with a standard deviation of ± 0.7 mg. per cent, 5 elevated values for serum calcium were recorded before the value of 10.4 mg. per cent* was obtained 21 days after discontinuing the vitamin D supplement and vitamin D–enriched milk. At this time the serum magnesium was 3.3 mg. per cent, which is within normal adult limits. Subsequent calcium determinations varied between 11.0 and 11.9 mg. per cent until 400 international units (I.U.) of vitamin D were added to the diet. The serum vitamin D level on February 16 was 2 I.U. per milliliter of serum, and less than 1 unit on February 21. Three determinations of serum citrate were also normal; these were 2.3, 1.7, and 2.8 mg. per cent between February 2 and February 24. A fourth value for serum citrate obtained on serum drawn February 17 was elevated to 4.0 mg. per cent. On February 26 and February 28 the serum vitamin E levels were normal (0.31 mg. per cent and 0.44 mg. per cent). A vitamin E absorption test was also normal. The vitamin E concentration was 2.66 mg. per cent 9 hours after a dose of 20 mg. per kilogram of tocopherol and the vitamin E absorption index was 13.0 units.

Study with vitamin D. 400 units of vitamin D per day were given from March 3 through March 12. Prior to starting vitamin D, the serum calcium was 11.0 mg. per cent. On the third day of vitamin D administration, it was 11.1 mg. per cent, and on the seventh day, 11.5 mg. per cent. Seventy-two hours after the last dose, the calcium was 12.9 mg. per cent, and 90 hours after that dose, 12.4 mg. per cent. One week after discontinuing vitamin D, the serum calcium was 12.4 mg. per cent. All three of these values are more than 3 standard deviations above the normal mean of serum calcium as determined in our laboratory. Sixteen days later the value had decreased to normal levels.

Serum vitamin D levels increased concomitantly with the serum calcium. The concentration of vitamin D was 3 units per milliliter on the day prior to vitamin D administration; on the third day, the level was 4 units or more; on the thirteenth day, 90 hours after the last dose of vitamin D, the level was probably 8 units or more. Unfortunately only 2 rats were used for this assay which precludes a dogmatic statement regarding the concentration. It should be noted that the initial serum vitamin D level was normal, at a time when the patient's serum calcium was decreasing to normal levels. Had this test been performed earlier in the course of the disease, it is possible that an elevated level would have been found.

Hypercalciuria was observed during and just after the 10 day course of vitamin D. The average urinary calcium excretion was 47 mg. per day during the precontrol period. During vitamin D administration, the average urinary value was 87 mg. per day. In the

*This value was determined in Dr. Mackenzie Walser's laboratory at the same time as the magnesium determination.

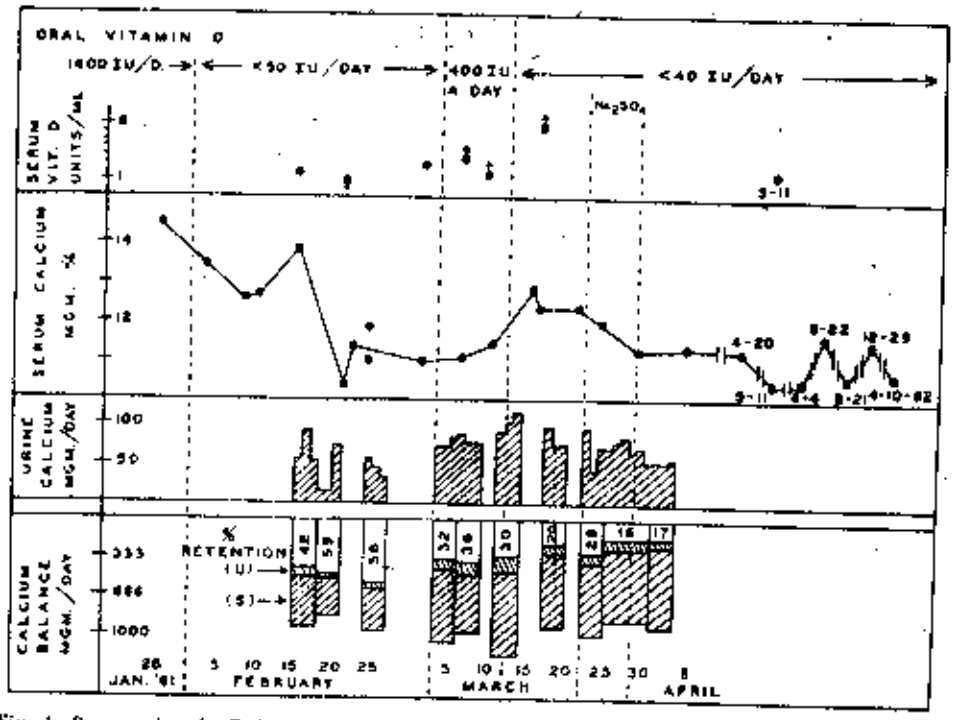

Fig. 1. Serum vitamin D levels, serum, urine, and stool calcium concentrations, and calcium balance on regimens described in text.

period from 24 to 48 hours after the last day of vitamin D administration, a peak excretion of 118 mg. of calcium was obtained.

There was no elevation of serum citrate 90 hours after discontinuing the vitamin D.

Study with sodium sulfate without added vitamin D. On March 22, 10 per cent sodium sulfate was begun in a dosage of 25 c.c. per liter of formula. This was given for 6⅔ days. The average intake of sulfate on this regimen was 7 mEq. per kilogram per day. Prior to starting the sulfate, the serum calcium was 12.4 mg. per cent; on the third day of sulfate administration the value was 12.0 mg. per cent, and on the day when sulfate was discontinued, 11.3 mg. per cent. This was 16 days after the last dose of vitamin D and may merely represent a slow fall in serum calcium, such as that observed the first time vitamin D was discontinued.

Low vitamin D diet. A low vitamin D diet, without fish or eggs and with nonirradiated milk, was then begun and has been continued to the present. Subsequent serum calcium determinations were 11.4 mg. per cent on April 4, 11.3 mg. per cent on April 20, 10.5 mg. per cent on May 11, and 10.4 mg. on June 5.

At the age of 1 year the height and weight were 28.5 inches and 15.8 pounds. The serum calcium was 11 mg. per cent. Six months later the height was 30 inches and the weight 20.3 pounds, and the mental development was normal. The serum calcium was 10.7 mg. per cent.

Balance data. An attempt was made to obtain calcium balance data. Reliance cannot be placed on precise quantitative interpretation of balance data because of errors inherent in the technique.[14] However, the *relative* changes in calcium balance reflect changes in regimen.

During the first time that the serum calcium was falling to normal levels, urine and fecal calcium were measured. During 3 successive 72 hour periods, 42.5, 59, and 58 per cent of the calcium ingested were retained. The average retention was 476 mg. per day. During the ten day period when 400 units per day of vitamin D were

administered the calcium retention was 32, 38, and 30 per cent for 3 successive 72 hour periods, or an average retention per day of 331 mg. Forty milligrams of this reduced retention of calcium were attributable to an increased urinary loss. After vitamin D administration and before starting sodium sulfate, 19.6 per cent of ingested calcium was retained for the one 72 hour period measured. The mean retention was 172 mg. of calcium per day.

No definite effect of sodium sulfate administration was demonstrated. During the period of 6⅔ days when the patient received sodium sulfate, the effect of vitamin D was waning. In the initial three day period on sulfate, the per cent of calcium retained was 29.5 per cent and the actual value was 295 mg. per day. Although this represents an increased calcium retention when compared to the control period immediately preceding, the period is too short for one to state unequivocally that calcium retention was augmented by sodium sulfate. Sulfate was given for the first 3⅔ of the next 6 days, and during this six day period the percentage retained was 16 per cent; the actual amount was 130 mg. per day. During the subsequent three day control there was essentially no change as 17.5 per cent or 156 mg. of calcium were retained per day.

DISCUSSION

One of the unusual aspects of this case is the familial incidence. This occurrence may indicate that some cases of idiopathic hypercalcemia result from an inborn error of metabolism.

In the literature there are only a few reports of idiopathic hypercalcemia occurring in more than one member of a family. Illig and Prader[10] reported idiopathic hypercalcemia in binovular twins whose sister had an elevated serum calcium. Forfar[11] has seen 2 and probably 3 sets of affected siblings among 11 families in which he has observed idiopathic hypercalcemia. Since K. H. had such a mild form of idiopathic hypercalcemia that it could have been overlooked, we would suggest that this entity may occur more frequently in siblings than has been documented.

Although abnormal metabolism of vitamin D has been suspected as causing this disease, Fellers and Schwartz[12] and Smith, Blizzard, and Harrison[1] who reported S. H., K. H.'s older sister, are the only authors who have recorded elevated serum levels of vitamin D during the active phase. Warkany and Mahon[13] noted the normal value for children to be 0.66 to 1.65 I.U. of vitamin D per milliliter of plasma. Fellers and Schwartz[12] found levels between 1.72 and 1.95 I.U. per liter of serum when studying normal infants. In the present case, the rise in the serum calcium to 12.9 mg. per cent and the probable rise in serum level of vitamin D to 8 units or more per milliliter, shortly after giving a test supplement of 400 units per day for 10 days, suggests that this disease sometimes is caused by defective inactivation or removal of vitamin D.

Short-term calcium balance studies on small infants are subject to error and the results are not precisely quantitative. Fomon[14] stresses the usefulness of balance technique in obtaining relative results, i.e., as a comparison of two regimens of management in a single subject. He cautions, "The successful use of metabolic balance studies [in obtaining comparative data] has suggested to some individuals that results are precisely quantitative. . . . Were this true . . . the change in nitrogen or calcium content of the body could be calculated." He discusses the reasons why "such calculations fail to provide valid estimates." With this caution in mind, and realizing that any losses in collection of excreta will falsely elevate apparent retention, the following observations are made. K. H. retained much calcium early during the recovery phase. Similar retentions are recorded during the active phase of idiopathic hypercalcemia by several authors who studied babies of comparable size. Morgan and his co-authors[2] reported 517 mg. per day retention in a 5.5 kilogram baby; Stapleton, McDonald, and Lightwood[15] found 500 mg. per day retained by an 8-month-old infant; Forfar and his associates[16]

recorded retentions of 445, 470, and 429 mg. per day in 3 infants with the active phase of idiopathic hypercalcemia.

The slight fall in calcium retention when 400 units of vitamin D was given daily was unexpected. This response was in contrast to those observed by Forfar[16] who gave 100,000 units of vitamin D per day to 3 patients. When administered to a patient during the active phase, this dosage increased the retention from 48 to 73 per cent; when given to a patient in the early recovery phase, the calcium retention rose from 16 to 40 per cent; and when given to a patient in the inactive phase, the retention increased from 16 to 42 per cent. A possible explanation for the unexpected results in our patient may be that the intestine was maximally absorbing calcium prior to the administration of the vitamin D, but the test dose still may have acted upon the bone with mobilization of calcium. The resulting increased urinary excretion of calcium then could partially account for the decreased positive calcium balance. This hypercalcuria (118 mg. per day) which occurred with the test dose of vitamin D was not unexpected since hypercalcuria has been reported consistently during the acute phase of the disease.[1-16] The hypercalcuria probably resulted from the increased concentration of calcium in the blood and glomerular filtrate because of the action of vitamin D on bone. However, a direct effect of vitamin D on the renal tubules cannot be excluded. It is of interest that Forfar[16] did not consistently produce hypercalcuria when he administered vitamin D during the recovery phase.

The balance study with sodium sulfate was undertaken since oral administration of this salt was found by Kowarsky[17] to lower the serum calcium in an infant with idiopathic hypercalcemia. In his patient the serum calcium returned to normal levels within a 7 day period after administration was begun. No balance data was obtained during his study. In the present study, oral administration of sodium sulfate produced no significant change in the urinary excretion of calcium or in the calcium balance.

During the entire period while balance data was collected, there was a steady decrease in calcium retention. This steady decrease appears to have been relatively unaffected by the test dose of vitamin D or by sulfate administration, except for the increase in urine output of calcium during and immediately after the vitamin D test period. This increase associated with the rise in serum calcium is indicative of a response to vitamin D with respect to mobilization of calcium from bone, even though intestinal absorption of calcium remained unaltered.

During the inactive phase of the disease, the retention of 130 to 150 mg. per day of K. H. is comparable with retention of calcium by normal infants, discussed by Jeans and co-workers,[18] Daniels and Stearns,[19] Swanson,[20] and Harrison.[21]

Idiopathic hypercalcemia occurs in both a mild and a severe form. Forfar, Tompsett, and Forshall[22] have suggested that the severe type of the disease ensues when the diagnosis and treatment of the mild form are delayed. In support of this contention Daeschner and Daeschner[23] noted that the interval between the onset and the diagnosis in the severe form averaged 13 months, while the interval was 6 months in 52 mild cases. After evaluating the renal histology from routine autopsies of children, Shanks and MacDonald[24] suggested that idiopathic hypercalcemia of the mild form occurs much more frequently than it is diagnosed. It is impossible to know whether K. H. would have developed severe hypercalcemia if she had continued to receive normal or excessive amounts of vitamin D, or whether the hypercalcemia would have been transient. Yet the occurrence of both a mild and a severe form of the disease in the same family supports the thesis that both forms are manifestations of the same disease, but differ only in their severity.

SUMMARY

A 3½-month-old infant, the sister of a patient previously recorded with severe idiopathic hypercalcemia, who presented with asymptomatic and transient hypercalcemia in

infancy has been described. Physical examination revealed a normal baby without "elfin" appearance. The serum calcium and urea nitrogen were elevated, but the serum phosphorus, alkaline phosphatase, and serum citrate were normal. The serum vitamin D concentration was 3 I.U. or less during the recovery phase, but became elevated to 8 units or more when 400 units of vitamin D per day was given for 10 days. At this time hypercalcemia and hypercalcuria occurred, although there was no increase in calcium retention during the period of vitamin D administration. At a time when the serum calcium was still elevated, administration of sodium sulfate had no appreciable effect on the serum calcium or the calcium balance.

It is suggested that "idiopathic hypercalcemia" may, in some cases, be an inborn error of metabolism with defective inactivation of vitamin D, and also that the disease may be found more commonly among asymptomatic siblings of known cases than has previously been reported.

We express our gratitude to Dr. John Peck, who permitted us to study this child; to Manuel DeGuzman for technical assistance; and to Mrs. Mary Westervelt for secretarial assistance.

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Vitamin D

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PHARMACOLOGY

REGULATION OF VITAMIN D METABOLISM IN EGG LAYING BIRDS.

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We are studying the metabolic fate of 25-[26,27-^{3}H]hydroxycholecalciferol (25-HCC) added to kidney homogenates prepared from Japanese quail representing different physiological states. The kidneys were removed from immature and mature males and from mature females with and without an egg in the oviduct. The ^{3}H-labelled substrate and metabolites, 1,25-dihydroxycholecalciferol (1,25-DHCC) and 24,25-dihydroxycholecalciferol (24,25-DHCC), were separated and identified using either Sephadex LH-20 column chromatography or silicic acid (ChromAR) thin layer chromatography. Kidneys from all birds with an egg in the oviduct exhibited enhanced production of 1,25-DHCC. In mature females with no egg in the oviduct and in immature and mature males, 24,25-DHCC was the predominant metabolite. These data indicate that ovulation results in enhanced 1,25-DHCC production.

Reproductive Status	No. Birds	1,25-DHCC*	
Immature male	1	0%	
Mature male	2	0%	
Mature female without egg	12	37%	$p < 0.005$
Mature female with egg	10	72%	

*Expressed as a % of total 1,25-DHCC + 24,25-DHCC

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Alkaline Phosphatase Activity in Developing Molars of Vitamin-D–Deficient Rats

II. Normal Calcium-Phosphorus Ratio Diet

ENRIQUE KIGUEL

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In the human infant, deprivation of vitamin D alone produces rickets. In order to produce a condition similar to rickets in young rats, a distortion of the calcium-phosphorus ratio in the diet seems to be necessary in addition to deprivation of vitamin D (*Acta Paediat.*, 23:405–33, 1939; *Lit. and Horm.*, 11:29–60, 1953). The primary purpose of the present investigation was to enlarge our knowledge of the role of alkaline phosphatase and vitamin D in the formation and mineralization of rodent's teeth. The diet used was the "Chick Basal" diet. This diet is free of vitamin D and has a calcium-phosphorus ratio adjusted to 1-2:1. Technical details of the experimental procedures, processing, and staining for alkaline phosphatase can be found in a previous article by this author (*J. dent. Res.*, 43:71–77, 1964). The microscopic observations of the developing rat molars of the vitamin-D–deficient rats showed reduced alkaline phosphatase activity as compared with homologous teeth in control rats of the same age. However, this decrease in alkaline phosphatase activity was not as dramatic as that found in vitamin-D deficiency with a high calcium-phosphorus ratio diet (*Ibid.*, 43:71–77, 1964). The enzymatic reaction in a group of rats receiving a supplement of vitamin D was similar to that in the control group. The sequential appearance of alkaline phosphatase in the tissues of developing rat molars, and the prevalence of this enzymatic activity in certain cell layers was previously described and discussed (*Ibid.*, 43:71–77, 1964). The present findings were correlated with alterations in the mucopolysaccharide and mineral content of developing dental matrixes observed previously in the offspring of rats fed the "Chick Basal" diet (*J. dent. Res.*, 39:672–73, 1960; *J. dent. Res.*, 40:685, 1961; Riggs Memorial Library, Georgetown University, Washington, D.C., thesis, 1962; Abstract 227, 40th General Meeting of the IADR, 1962). The organic and mineral changes were less severe than those observed in the offspring of rats fed the Steenbock diet (high Ca:P ratio). The results of the present study showed that although clinical symptoms were absent, the young rats were affected by a vitamin-D–deficient diet with a normal calcium-phosphorus ratio. Changes in the vitamic factor may develop a chain reaction affecting alkaline phosphatase before and during the formation of the dental matrixes. Alkaline phosphatase may influence mucopolysaccharide synthesis. This view is supported by the findings of Kroon (*Acta Anat.*, 15:317–28, 1952); Moog and Wenger (*Amer. J. Anat.*, 90:339–78, 1952); Ten Cate (*Proc. Nutr. Soc.*, 18:65–70, 1959) and Kiguel (*J. dent. Res.*, 43:71–77, 1964). It has also been suggested that mucopolysaccharides may influence collagen synthesis or the conversion of procollagen into mature collagen (*Proc. Soc. exp. Biol. [N.Y.]*, 98:843–48, 1958). Therefore, the chain reaction proposed above may ultimately lead to alterations in the normal process of mineral deposition in calcifiable matrixes. This view is supported by a previous study of this author (*J. dent. Res.*, 43:71–77, 1964). The mechanism by which absence of vitamin D alters alkaline phosphatase activity in the dental tissues of rats is uncertain. The concepts suggested in the present study need further investigation.

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Additional information is available on request to the author.

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PARATHYROID HORMONE RESPONSE AND EFFECTS OF VITAMIN D IN HYPOPARATHYROIDISM AND PSEUDOHYPOPARATHYROIDISM. H. Peter Kind, David, K. Parkinson, Se Mo Suh, Donald Fraser, Sang Whey Kooh. (Intro. by Julio M. Martin). Department of Paediatrics, University of Toronto, and Research Institute, The Hospital for Sick Children, Toronto, Canada.

Response to exogenous parathyroid extract was used to differentiate hypoparathyroidism (HP) and pseudohypoparathyroidism (PHP). Three parameters of PTH action were measured before and during vit D treatment - decrease in tubular reabsorption of phosphate after i.v. PTH (Δ%TRP), increase in urinary cyclic-AMP (Δ c-AMP) after i.v. PTH and increase in serum calcium (Δ Ca) after repeated i.m. PTH.

	Δ c-AMP (nmoles/min)			Δ%TRP			Δ Ca (mg%/day)		
	- D	+ D	p	- D	+ D	p	- D	+ D	p
Norm	284±38(13)*			15.7±1.1					
HP	330±84(3)	296±20(6)	ns	15.3±3.4(3)	34.8±3.3(6)	<.01	2.4±0.5(2)	4.6±0.3(3)	<.02
PHP	24± 6(5)	18± 7(4)	ns	6.6±2.0(5)	15.0±3.2(4)	<.02	0.6±0.2(4)	2.1±0.6(4)	=.05
P 1	0	0		10	8		0.5	0.6	
HP:PHP	p<0.001	p<0.001		p<0.05	p<0.005		p<0.01	p<0.025	

* Mean ± SE(n)

HP patients and normal persons were similar in their renal responses to PTH and HP patients showed prompt hypercalcemia with i.m. PTH. All parameters of response to PTH were significantly less in PHP than in HP. Vit D caused significant improvement in the phosphaturic and calcemic actions of PTH in both HP and PHP but had no affect on Δ c-AMP. Patient 1 had the same amount of phosphaturia and calcemia as PHP patients but had no Δ c-AMP on repeated testings. In this patient vit D did not alter PTH responsiveness. These studies demonstrate that vit D enhances the effects of PTH on renal handling of phosphate and calcium mobilization in both HP and PHP but does not alter Δ c-AMP. Patient 1 provides evidence for the hypothesis that the action of PTH on the renal handling of phosphate is not entirely mediated by cyclic-AMP.

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INTERACTION OF PARATHYROID HORMONE AND VITAMIN D ON THE RENAL EXCRETION OF PHOSPHATE*

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THE interrelationships of parathyroid hormone and vitamin D are important for the understanding of the pathogenesis and treatment of rickets and also to explain the apparently paradoxical action of vitamin D in hypoparathyroidism. This study attempts to explain their interaction on the kidney.

Vitamin D has been shown under certain conditions to increase tubular reabsorption of phosphate, and parathyroid hormone has been shown to decrease this reabsorption (1, 2). On the other hand patients with hypoparathyroidism may be treated with large doses of vitamin D with a resultant increased urinary excretion of phosphate and reduction of the concentration of phosphorus in the serum. More recently it has been demonstrated that parathyroid extract given intravenously increases the glomerular filtration rate and renal blood flow. (3) Crawford has recently suggested that elevation of serum phosphate stimulates parathyroid secretion (4).

METHODS

Inulin clearance was used as a measure of glomerular filtration in all but one of these experiments in which endogenous creatinine clearance was used. Inulin was measured by the method of Harrison (5), and creatinine by the Peters' modification of the Folin method (6). Phosphate was measured by the method of Fiske and Subbarow (7).

Fluid was infused intravenously at as close to a constant rate as possible, by a gravity drip which flowed from a 300-cc. dispensing burrette; it was adjusted by means of a tunnel clamp. A priming dose of inulin was given, followed by a maintenance dose dissolved in 0.57 or 0.85 per cent saline solution during the equilibration period of thirty to sixty minutes and during the control periods. If a phosphate load was to be given, the maintenance inulin for those periods was dissolved in a solution containing 11.36 Gm. of disodium phosphate and 2.76 Gm. of monosodium phosphate per liter. In a few clearances there was an equilibration period after the start of the infusion of the phosphate solution. When parathyroid hormone was given, it was injected rapidly intra-

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venously through the same needle by means of a three-way stopcock attached to the hub of the needle. The parathyroid extract contained 100 USP units per cc. Vitamin D was given intramuscularly in doses of 600,000 units (1 cc.) or 4,200,000 units in oil, or as oily concentrates by mouth.

Blood was drawn for measurement of phosphate and inulin content at the midpoint of each period. The periods ranged from ten to thirty minutes in length. At the end of each period the bladder was washed out with both saline and air. An attempt was made to produce a water diuresis in the patients during the clearances. This was usually successful in the smaller children, and only one third of the clearances were carried out with urine flows of greater than 1 cc. per minute. All clearances were performed in the postabsorptive state. Sedation with barbiturates was usually employed.

The subjects were children between the ages of 2 weeks and 12 years on the wards of the Children's Hospital of Pittsburgh, with 1 exception noted subsequently. (Newborn infants may suffer a period of relative hypoparathyroidism (8, 9) and hence are not included in this group.)

The patients without demonstrable vitamin D deficiency were for the most part in the hospital for investigation of convulsive disorders, but others were convalescent from acute infections of the respiratory system. They had no evidences of renal dysfunction. In addition, studies were carried out on 2 patients with rickets due to vitamin D deficiency and on 1 patient with familial resistant rickets. One of the investigators served as a subject for the study on a normal adult.

RESULTS

The intravenous injection of parathyroid extract in doses of from 2 to 10 cc. produced an increase in phosphorus excreted per minute as expected. In addition, there was an increase in glomerular filtration. This finding confirms the work published by Handler *et al.* while this study was under way (3). The increase in glomerular filtration was often preceded by a short period of depression. This lowering of inulin clearance could be entirely masked by increasing the length of the initial period after parathyroid injection. Because of the increase in filtration the phosphorus re-

TABLE 1. THE EFFECT OF PARATHYROID EXTRACT ON GLOMERULAR FILTRATION AND TUBULAR REABSORPTION OF PHOSPHATE IN A NORMAL INFANT

Patient, age & wt.	Time (min.)	Inulin clearance (cc./min.)	Serum PO_4 (mg. %)	Phosphate			
				Filtered (mg./min.)	Excreted (mg./min.)	Reabsorbed (mg./min.)	Reabsorbed (mg./100 cc. GF)
Co	10	23.4	5.5	1.29	0.26	1.03	4.4
6½ mos.	13	23.4	5.4	1.27	0.28	0.99	4.2
6.7 Kg.			*5 cc. parathyroid extract, i.v.*				
	10	21.1	—	—	0.23	—	—
	16	28.8	4.0	1.32	0.45	0.87	3.3
	14	31.5	4.7	1.40	0.68	0.81	2.6

TABLE 2. THE EFFECT OF PARATHYROID EXTRACT BEFORE AND AFTER THE ADMINISTRATION OF VITAMIN D IN NORMAL INFANTS AND CHILDREN

Patient, age & wt.	Time (min.)	Inulin clearance (cc./min.)	Serum PO_4 (mg. %)	Phosphate: Filtered (mg./min.)	Phosphate: Excreted (mg./min.)	Phosphate: Reabsorbed (mg./min.)	Phosphate: Reabsorbed (mg./100 cc. GF)
Gi	13	65.0	5.4	3.51	0.68	2.83	4.4
6 yrs.	10	64.0	5.4	3.47	0.69	2.78	4.3
17 Kg.			*5 cc. parathyroid extract, i.v.*				
	20	76.0	4.5	3.43	0.94	2.49	3.3
	16	80.0	4.3	3.45	0.99	2.46	3.1
			Repeated 25 hours after 1,200,000 units vitamin D, i.m.				
	22	60.0	5.2	3.12	0.43	2.69	4.5
			5.5 cc. parathyroid extract, i.v.				
	20	68.0	4.9	3.19	0.76	2.43	3.7
	16	80.0	4.6	3.18	1.03	2.15	3.4
Kr	21	11.0	6.5	0.72	0.17	0.55	5.0
2 mos.			*8 cc. parathyroid extract, i.v.*				
4.4 Kg.	18	13.8	6.4	0.88	0.20	0.68	4.9
	18	17.8	6.3	1.11	0.27	0.84	4.8
			Repeated 23 hours after 900,000 units vitamin D, i.m.				
	19	13.2	6.1	0.81	0.16	0.65	4.9
			8 cc. parathyroid extract i.v.				
	15	17.0	5.7	1.02	0.35	0.67	3.8
	15	15.9	5.7	0.90	0.24	0.66	4.2
Sh	15	20.2	5.4	1.58	0.32	1.26	4.3
11 mos.	23	20.8	5.4	1.45	0.25	1.24	4.6
8 Kg.			*5 cc. parathyroid extract, i.v.*				
	10	28.3	5.4	1.52	0.30	1.22	4.3
	15	28.0	4.9	1.38	0.29	1.09	3.9
			Repeated 60 hours after 1,200,000 units vitamin D, i.m.				
	16	21.0	5.3	1.27	0.36	0.91	3.8
		26.2	5.6	1.47	0.31	1.16	4.4
	20		*5 cc. parathyroid extract, i.v.*				
	18	18.5	4.9	0.90	0.20	0.70	3.8
	15	44.3	4.5	1.97	.63	1.32	3.0

absorbed by the tubules per minute may not always diminish and in Handler's dogs it sometimes actually rose (3). Because of this change in the filtration rates, the rate of phosphorus reabsorption has been calculated in terms of 100 cc. of glomerular filtrate. This method of expressing the data also permits a comparison of tubular reabsorption in a 3-Kg. child with that in a 30-Kg. child or an adult. Table 1 gives the results of a typical study made on a 6½-month-old boy. The average increase in glomerular filtration rate in 6 cases was 31 per cent compared to 37 per cent found by Handler *et al.* in dogs (3).

When vitamin D was given intramuscularly in large doses before such a study, the effect of exogenous parathyroid hormone was as great or greater than previously. In illustration, Table 2 shows the results of clearance studies made with the administration of parathyroid hormone to 2

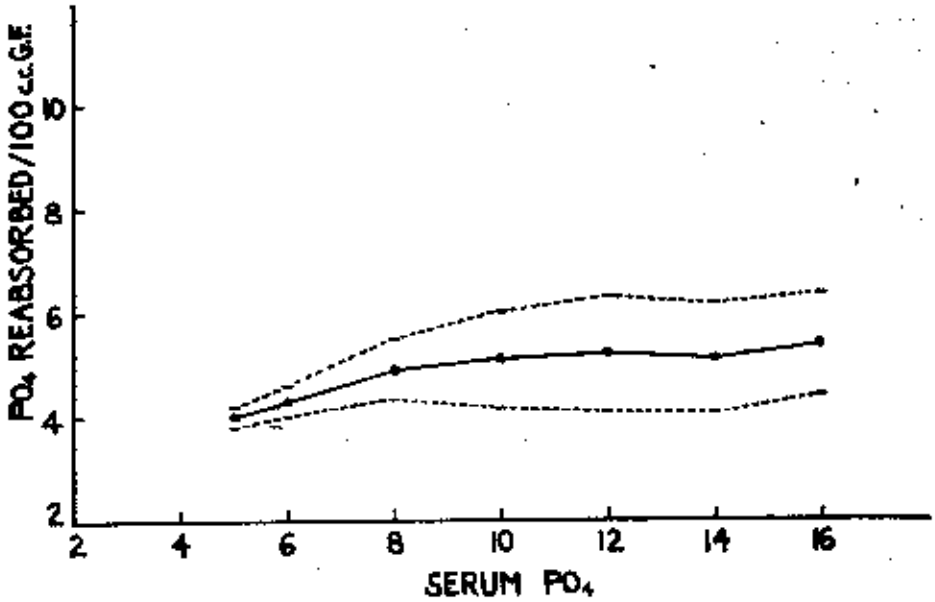

FIG. 1. *Tubular Reabsorption of Phosphate in Normal Children.*—Mean phosphate reabsorption per 100 cc. of glomerular filtrate is represented by solid line. Dotted lines represent one standard deviation from the mean. Serum phosphate is in mg. per cent.

patients both before and twenty-three hours after the injection of 600,000 and 1,200,000 units of vitamin D, respectively, and in 1 case both before and sixty hours after 1,200,000 units of vitamin D intramuscularly.

The results of 14 clearances in normal subjects, carried out with a rising level of serum phosphate produced by intravenous infusion of phosphate, are shown graphically in Figure 1. The reabsorption of phosphorus by the tubules rose slightly and then levelled off. This is similar to the results found in similar tests on dogs by Pitts and by Harrison (1, 2).

With the infusion of phosphate, glomerular filtration increased by an

TABLE 3. NORMAL PHOSPHATE CLEARANCE WITH A RISING LEVEL OF SERUM PHOSPHORUS IN A 10-YEAR-OLD BOY

Patient, age & wt.	Time (min.)	Inulin clearance (cc./min.)	Serum PO_4 (mg.%)	Phosphate			
				Filtered (mg./min.)	Excreted (mg./min.)	Reabsorbed (mg./min.)	Reabsorbed (mg./100 cc. GF)
Pa 10 yrs. 28 Kg	13	57.2	5.4	3.08	0.92	2.08	3.6
				Phosphate infusion started			
	15	53.0	8.1	4.30	1.87	2.43	4.6
	14.5	77.6	10.1	7.82	4.62	3.20	4.1
	15	50.3	11.2	6.62	4.20	2.42	4.1
	15	58.5	13.0	7.60	5.39	2.21	3.8

TABLE 4. THE EFFECT OF VITAMIN D ON GLOMERULAR FILTRATION AND PHOSPHATE REABSORPTION IN 2 NORMAL CHILDREN, 2 CHILDREN WITH VITAMIN-DEFICIENCY RICKETS AND 1 WITH FAMILIAL RESISTANT RICKETS

Patient, age & wt.	Time (min.)	Inulin clearance (cc./min.)	Serum PO_4 (mg.%)	Phosphate: Filtered (mg./min.)	Phosphate: Excreted (mg./min.)	Phosphate: Reabsorbed (mg./min.)	Phosphate: Reabsorbed (mg./100 cc. GFR)
Po (normal) 5 mos. 4 Kg.	19	7.0	6.6	0.46	0.12	0.34	4.9
	17	9.6	6.7	0.65	0.17	0.48	5.0
	15	11.5	12.5	1.44	0.66	0.78	6.8
	16	12.7	18.0	2.29	1.39	0.90	7.1
	Repeated 5 days after 600,000 units vitamin D, i.m.						
	10	8.2	5.6	0.46	0.13	0.33	4.0
	9	8.8	5.6	0.49	0.14	0.35	4.0
	19	9.3	6.4	0.60	0.11	0.49	5.2
	20	10.0	15.9	1.59	0.64	0.95	9.5
	5 cc. Parathyroid extract, i.v.						
	10	9.4	--	--	1.25	--	—
	15	10.6	20.7	2.20	1.57	0.63	5.9
St (normal) 16 mos. 11.5 Kg.	12	29.9	5.6	1.68	0.42	1.26	4.2
	10	30.2	5.6	1.70	0.42	1.28	4.2
	19	42.6	6.7	2.82	0.67	2.15	5.1
	14	37.6	10.0	3.76	1.91	1.85	4.8
	15	38.0	12.5	4.75	2.74	2.02	5.3
	Repeated 29 hours after 600,000 units vitamin D, i.m.						
	10	33.3	5.7	1.90	0.44	1.46	4.4
	17	33.2	6.1	2.03	0.54	1.49	4.5
	13	34.2	10.4	3.56	1.31	2.25	6.6
	15	34.7	13.9	4.82	2.54	2.28	6.6
Ger (resistant rickets) 6 yrs. 20 Kg.	10	58.5	3.3	1.94	0.37	1.57	2.7
	19	52.5	4.5	2.37	0.71	1.66	3.2
	13	58.2	6.7	3.91	2.08	1.83	3.1
	13	52.3	7.9	4.13	2.49	1.65	3.2
	Repeated 27 hours after 600,000 units vitamin D, i.m.						
	10	109.5	3.3	3.61	0.71	2.91	2.7
	10	101.0	3.3	3.34	0.71	2.63	2.6
	25	84.5	4.9	4.14	1.21	2.93	3.5
	12	75.0	7.4	5.56	2.79	2.77	3.7
	15	74.0	8.3	6.16	3.67	2.49	3.4
Repeated 8 months later, after having taken 100,000 units vitamin D per day by mouth for this period							
	10	59.0	2.9	1.72	0.03	1.69	2.9
	10	52.0	2.9	1.52	0.07	1.45	2.8
	17	51.5	3.6	1.86	0.35	1.51	2.9
	20	50.5	7.2	3.64	0.16	3.38	6.7
	13	53.5	8.5	4.55	0.26	4.29	8.0
Co (vitamin-D-deficiency rickets) 18 mos. 10 Kg.	21	34.7	3.3	1.15	0.19	0.96	2.8
	16	45.8	4.8	2.20	0.46	1.74	3.8
	12	30.0	10.2	3.07	1.58	1.49	5.0
	11	34.8	11.9	4.10	2.51	1.59	4.6
	Repeated 23 hours after 600,000 units vitamin D, i.m.						
	19	30.3	3.4	1.01	0.16	0.86	2.9
	16	38.6	4.8	1.86	0.39	1.47	3.8
	16	38.2	8.8	3.37	1.51	1.86	4.9
	11	32.8	11.8	3.87	2.50	1.37	4.2
	Repeated 55 hours after original vitamin D. No further Rx.						
	10	50.0	3.6	1.80	0.19	1.61	3.2
	11	59.0	3.6	2.13	0.25	1.88	3.2
	18	51.0	4.8	2.45	0.39	2.06	4.0
	10	53.0	8.8	4.67	1.34	3.33	6.3
	18	63.0	11.0	6.93	2.14	6.06	10.6

TABLE 4—*Continued*

Patient, age & wt.	Time (min.)	Inulin clearance (cc./min.)	Serum PO_4 (mg./%)	Phosphate: Filtered (mg./min.)	Phosphate: Excreted (mg./min.)	Phosphate: Reabsorbed (mg./min.)	Phosphate: Reabsorbed (mg./100 cc. GFR)
Repeated 18 days after original vitamin D. Had received 1,000 units vitamin D per day by mouth for two weeks							
	10	22.4	3.5	0.78	0.07	0.71	3.2
	16	28.2	4.0	1.13	0.19	0.91	3.5
	12	30.8	7.1	2.19	0.66	1.53	5.0
	11	35.2	10.4	3.66	1.53	2.13	6.0
Vo (vitamin-D-deficiency rickets) 10 mos. 7.2 Kg.	14	28.5	2.0	0.71	0.11	0.63	2.[illegible]
	14	24.0	2.6	0.62	0.09	0.53	2.[illegible]
	16	28.5	3.7	1.06	0.28	0.78	2.8
	13	25.3	7.1	1.79	1.09	0.70	2.[illegible]
	13	28.3	7.6	2.15	1.34	0.81	2.7
Repeated 23 hours after 600,000 units vitamin D, i.m.							
	9	19.4	2.5	0.49	0.09	0.40	2.1
	9	19.0	2.5	0.48	0.09	0.39	2.1
	17	Lost	—	—	—	—	—
	13	26.5	11.1	2.94	1.68	1.26	4.8
	13	24.6	11.9	2.93	1.88	1.05	4.3
Repeated 71 hours after original Rx. No further Rx.							
	14	27.6	3.7	1.03	0.22	0.81	2.9
	12	29.7	3.7	1.10	0.25	0.85	2.9
		Lost	—	—	—	—	—

average of 36 per cent in 14 patients. This rise was of brief duration and did not persist throughout the infusion. Table 3 gives the results of one such clearance performed on a 10-year-old boy.

The results of phosphate-loading experiments before and after the administration of vitamin D in 2 normal infants (Po and St), in 2 infants with rickets due to deficient vitamin D intake (Co and Vo) and in 1 boy with familial resistant rickets (Ger), are given in Table 4. Figure 2 records the reabsorption curves of the 2 normal infants.

The administration of exogenous parathyroid hormone to one of the normal patients (Po) near the end of his second study was followed by increased phosphate excretion. There was no more increase in glomerular filtration rate. The effect of the exogenous hormone here is confused by the increasing phosphate load.

The patient with resistant rickets (Ger) had been admitted to the hospital for an osteotomy and had had no vitamin D intake for one month following his osteotomy and preceding his study. His rickets had formerly and has since been controlled with 100,000 to 150,000 units of vitamin D by mouth per day. A virtual doubling of the filtration rate took place twenty-three hours after the administration of 600,000 units of vitamin D to the boy with resistant rickets. An 80 per cent increase in glomerular filtration rate occurred four days after the same dose in 1 boy (Co) with vitamin-D-deficiency rickets. In the other children and in the latter boy,

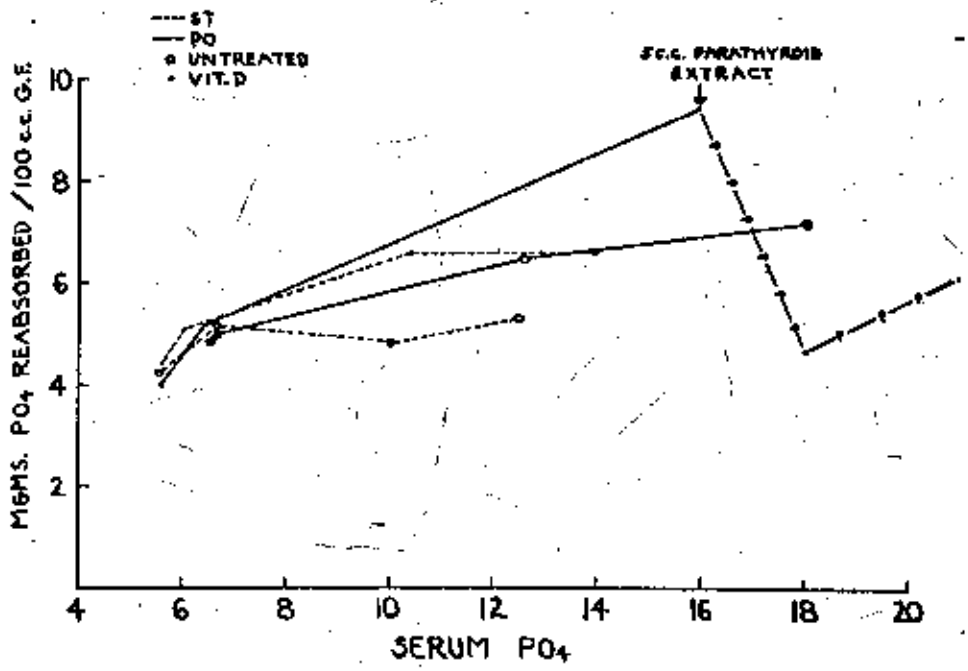

FIG. 2. *Tubular Reabsorption of Phosphate Before and After Vitamin D Administration in Normal Subjects.* The reabsorption of phosphate per 100 cc. of glomerular filtrate is plotted as before. The results in Patient Po are plotted with the solid lines, and in Patient St with the dotted lines. In both, the open circles represent reabsorption before vitamin D, the closed circles after administration of vitamin D.

twenty-three hours after the administration of vitamin D, the glomerular filtration dropped slightly or stayed the same in the control period or periods. This was also the case in those who did not receive a phosphate load (see Table 2). In the boy with resistant rickets the increase in glomerular filtration was not compensated for by an increased tubular reabsorption of phosphorus and as a result he excreted more phosphate. In contrast, in the case of the boy with vitamin-D-deficiency rickets, this increase in glomerular filtration rate was completely offset by the increased reabsorption of phosphate by the tubules and as a result he did not excrete more phosphate. The clearances were repeated 1) two months later in the boy with resistant rickets, while he was receiving 100,000 units of vitamin D per day by mouth, and 2) eighteen days later in the boy with vitamin-deficiency rickets, while he was receiving 1,000 units of vitamin D by mouth. At this time the situation had changed. The glomerular filtration rates were back to pretreatment levels, but the tubular reabsorption of phosphate had gone up in the boy with resistant rickets and stayed up

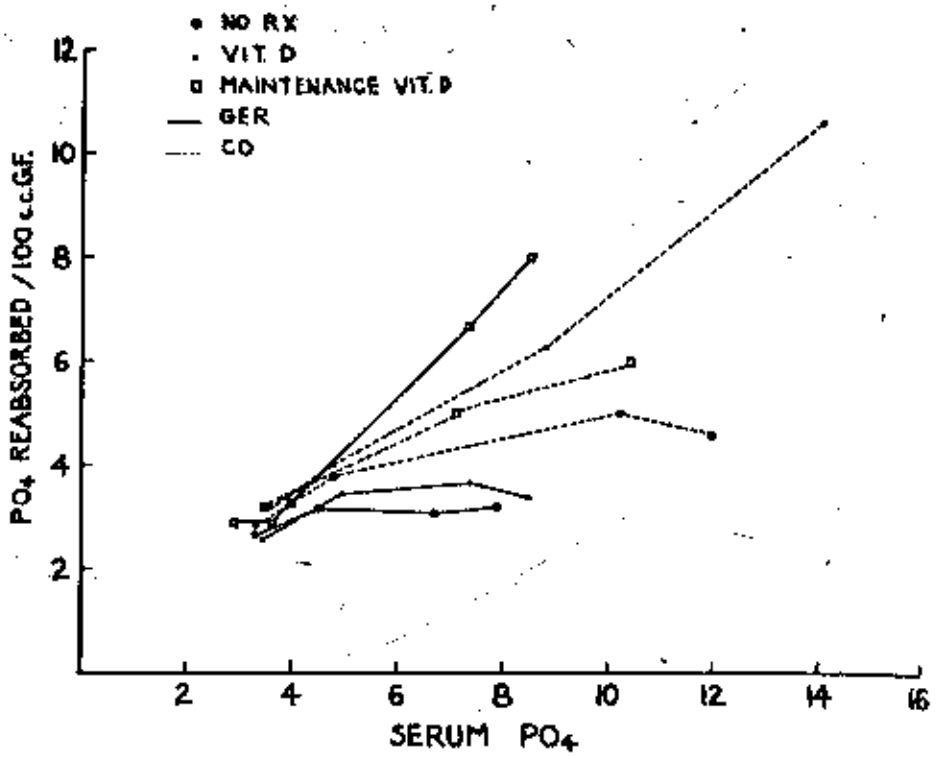

FIG. 3. *Tubular Reabsorption of Phosphate Before and After Vitamin D Administration in a Child with Vitamin-Deficiency Rickets and a Child with Familial Resistant Rickets.*—Reabsorption curves for Patient Ger with resistant rickets are plotted with solid lines; those for Patient Co, in dotted lines. Pretreatment curves in both are marked by the open circles; curves (one day for Ger and four days for Co) after intramuscular administration of 600,000 units of vitamin D are marked by closed circles; and the squares mark the results from the third clearances in both. These are two months later for Ger (while he was receiving 100,000 units of vitamin D a day by mouth) and eighteen days later for Co (while he was receiving 1,000 units of vitamin D per day by mouth).

in the other boy. X-ray examinations at this time showed healing of the rickets, though the serum phosphate level in both boys was still slightly low. When phosphate reabsorption was increased after administration of vitamin D in 2 patients (Ger and Co) maximal rates of reabsorption were not established, although previous loading tests had demonstrated apparent maxima for phosphate reabsorption. The reabsorption curves are plotted in Figure 3.

Similar studies on a 16-year-old boy with hypoparathyroidism are to be reported (10). These studies also show an increase in glomerular filtration rate of approximately 64 per cent following the administration of

100,000 units or more of vitamin D per day by mouth for fifteen days. At this time there was no increase in tubular reabsorption of phosphate. The net effect was a marked increase in phosphate excretion.

When parathyroid extract was given to this last patient in doses of from 2 to 5 cc. per day intramuscularly there was also an increased phosphate excretion. This however, was produced by a lowering of tubular reabsorption of phosphate at all levels of serum phosphate and was not accompanied by any change in the rate of glomerular filtration.

DISCUSSION

This study is complicated by the necessity of giving the vitamin D as an oily liquid with the possibility of variable absorption. Since the dose of vitamin D acting at any one time in large part decides the action of vitamin D, this is important. None the less, there are certain hypotheses that can be substantiated by the facts that emerge from this study.

1. Parathyroid extract has two modes of action on the kidney. It increases glomerular filtration and it reduces the tubular reabsorption of phosphate. These actions have been demonstrated in animals (1, 2, 3). In the usual therapeutic dosage, the second mode of action is the significant one.

2. Vitamin D apparently also increases glomerular filtration under certain circumstances. In addition, it increases the tubular reabsorption of phosphate. At present we believe it does the latter by inhibiting parathyroid secretion. It is possible that a more correct statement is, that it makes a higher level of serum phosphorus necessary in order to stimulate parathyroid secretion. It has been shown previously that tubular reabsorption of phosphate increases when rachitic dogs are given vitamin D (2). This increase in tubular reabsorption accompanies a rise in the level of serum phosphate. In this study phosphate reabsorption increased after vitamin D administration also, and a maximal rate of reabsorption could not be demonstrated in 2 cases (Ger and Co), whereas it had been demonstrated before vitamin D administration. In addition, the secretion of parathyroid hormone apparently establishes the maximal rate of tubular reabsorption of phosphate when a phosphate load is given (10).

Since vitamin D did not prevent the usual action of parathyroid extract when the extract was given after the administration of vitamin D, it does not appear to affect phosphate reabsorption by blocking the action of parathyroid hormone in any way. Since vitamin D in the absence of adequate parathyroid hormone in the patient with hypoparathyroidism did not increase tubular reabsorption of phosphate, we do not feel that it affects reabsorption by acting on another locus in the kidney, increasing

reabsorption more than parathyroid hormone lowers it. There is still the objection that the doses of vitamin D here used were too small or those of parathyroid extract too large. The doses of vitamin D were at least large enough to exert its effect in opposition to endogenous parathyroid hormone. Amounts of parathyroid extract as small as 2 cc. were administered and the doses used produced an average change in glomerular filtration rate which was less than that produced presumably by endogenous parathyroid hormone secreted in response to the stimulus of a rising level of serum phosphate. It is, therefore, believed that vitamin D directly or indirectly inhibits the secretion of parathyroid hormone.

It is possible now to explain speculatively the action of vitamin D in hypoparathyroidism, rickets and perhaps in resistant rickets.

In hypoparathyroidism, parathyroid hormone can increase phosphate excretion by its two actions on the kidney. It can both decrease phosphate reabsorption by the tubules and increase phosphate filtration, although we do not believe the latter mode is clinically significant. Vitamin D increases phosphate excretion in hypoparathyroidism, since in the massive doses used it increases the filtration of phosphate. Since in this disease there is no parathyroid effect to negate, the vitamin D has no effect on phosphate reabsorption. (There is an alternative explanation which is valid for this one situation. Tubular reabsorption of phosphate may be considered to be maximal in the absence of parathyroid hormone. Hence, vitamin D cannot increase phosphate reabsorption.) The action of large doses of vitamin D given over a long time on glomerular filtration has not been studied.

In cases of rickets, vitamin D inhibits parathyroid hormone secretion. Phosphate reabsorption is thus permitted to increase. The doses of vitamin D usually used to treat rickets do not elevate the glomerular filtration rate.

Vitamin D and parathyroid interaction may be the same in resistant rickets as in vitamin-deficiency rickets. Nevertheless, the alternative possibility must be considered that the amount of vitamin D required to inhibit parathyroid effect, relative to that required to raise glomerular filtration rate, is greater than in normal individuals or in individuals with vitamin-deficiency rickets.

These findings also permit the speculation that small doses of vitamin D may produce tetany in the rachitic child by causing increased phosphate reabsorption before calcium absorption from the alimentary tract is increased. On the other hand, massive doses of vitamin D may not cause tetany because the increased tubular reabsorption of phosphate is offset by increased filtration.

The absolute amounts of phosphate reabsorbed as reported herein, may be inaccurate for two reasons. The rapidity of the rise in the level of serum phosphate may be associated with significant amounts of nonfilterable phosphate. Secondly, there may be a lag in response to the rapidly rising loads and an error in assuming the average level of serum phosphate. For instance, we believe the slight rise in the rate of tubular reabsorption of phosphate before it levels off may be an artefact. There was, however, no noticeable difference in those cases in which the serum phosphate level was elevated more slowly. In any event the possible errors should not affect the validity of the changes in reabsorption with which this study is concerned.

SUMMARY

Parathyroid extract when given to humans has been shown to increase the glomerular filtration rate as well as to decrease tubular reabsorption of phosphate. The former may represent a true hormonal effect and not that of a contaminant, as this effect is seen when an intravenous infusion of phosphate raises the level of serum phosphate.

Vitamin D has been shown under certain circumstances to increase the glomerular filtration rate. Vitamin D has been shown not to inhibit the effect of exogenous parathyroid extract.

It has been suggested that vitamin D inhibits the secretion of parathyroid hormone.

Hypotheses have been suggested to explain the apparently paradoxical actions of vitamin D in rickets and in hypoparathyroidism.

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FAT-SOLUBLE VITAMINS[1,2]

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The biochemical function of fat-soluble vitamins at a molecular level is still unknown, but significant advances have been made this year which promise well for the future. A possible systemic effect of vitamin A on polycyclic compounds of sterolic nature; synthesis and metabolic studies of C^{14}-labelled carotene, vitamin A, and vitamin D; a new scheme of photochemical formation of vitamin D; the possible involvement of vitamin E in the respiratory chain and of vitamin K in photosynthetic processes; and the relationship of essential fatty acids with cholesterol metabolism are a few samples of the progress made.

Vitamin A

A number of reviews have appeared, discussing the mode of action of vitamin A [Morton (1)], its biochemistry, hypervitaminosis, and requirements (2, 3, 4).

Carotenoids have been considered only so far as they relate to vitamin A metabolism, and the interesting developments on biosynthesis of carotenoids have been left for future reviewers of this particular field.

Chemistry.[3]—A full account of the synthesis of various isomers of vitamin A, referred to in detail in last year's review, has been published [Robeson *et al.* (5 to 8)]. Beutel *et al.* (9) have been able to transform vitamin A acetate to *retro*-vitamin A acetate in good yield by treatment with aqueous hydrobromic acid. The resulting red-yellow oil had the typical absorption spectrum of *trans-retro*-vitamin A acetate. In this connection Henbest *et al.* (10) have shown that anhydrovitamin A_2, obtained from vitamin A_2 by treatment with ethanolic hydrochloric acid, is really 4′-ethoxyanhydrovitamin A_1, the latter being a hydrocarbon with a "*retro*"-structure. A suitable method of converting vitamin A_1 into the rarer vitamin A_2 has been described, via conversion of retinene$_1$ to retinene$_2$ by treatment with N-bromosuccinimide followed by dehydrobromination (11).

[1] The survey of the literature pertaining to this review was completed in November, 1955.

[2] The following abbreviations are used in this chapter: ADP for adenosine-5′-diphosphate; AMP for adenosine-5′-monophosphate; ATP for adenosine-5′-triphosphate; BAL for 2,3-dimercaptopropanol; DNA for deoxyribonucleic acid; DPN for diphosphopyridine nucleotide; DPNH for diphosphopyridine nucleotide (reduced form); FMN for flavin mononucleotide; RNA for ribonucleic acid.

[3] In the numbering system for vitamin A the terminal alcoholic carbon of the side chain is assigned number 1; the term *retro* is used for a change in the conjugated system one carbon back towards the ring, see Quaife, M. L., *Ann. Rev. Biochem.*, 23, 215 (1954). For the numbering system of carotenoids, see Goodwin, T. W., *Ann. Rev. Biochem.*, 24, 497 (1955).

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A valuable addition to the tools available for studying the pathway of the vitamin, is the synthesis of the radioactive vitamin A-2-C^{14} by Wolf *et al.* (12). A useful procedure of purification of milligram quantities of vitamin A from tissue extracts has been described [Powell *et al.* (13)]. It involves removal of sterols by digitonin and chromatography on aluminum oxide columns; the recovery is reported to be 68 to 89 per cent. Cormier (14) reports studies on the conversion of vitamin A alcohol into vitamin A epoxide.

The important syntheses in the carotenoid series by Inhoffen and collaborators (15, 16, 17) have been continued. They include the synthesis of 10,10'-*cis*-bixinmethylester (15) and the total synthesis of 3,4-3',4'-bisdehydro-β-carotene (16). The latter was obtained by condensation of a newly prepared *retro*-dehydro-C_{10}-aldehyde to a C_{40}-diol by treatment with acetylene-dimagnesiumbromide. The diol was converted to 3,4-15,15'-3',4'-trisdehydro-β-carotene which after hydrogenation and isomerization gave the crystalline "all"-*trans*-3,4-3',4'-bisdehydro-β-carotene. This synthetic pathway has been utilized to obtain a centrally C^{14}-labelled (15,15')-β-carotene (17).

Analysis.—The precision of the Carr-Price test for the estimation of vitamin A in margarine has been markedly improved by chromatographic purification of the unsaponifiable fraction on a column of defatted bone meal of appropriate particle size [Lord & Bradley (18)]. Bro-Rasmussen and associates (19) describe a satisfactory method for the separate estimation of neovitamin A_1 all-*trans* vitamin A_1, and vitamin A_2 in fish liver oils. The compounds were separated by chromatography on dicalcium phosphate columns with a mixture of light petroleum. Morton & Bro-Rasmussen (20) have compared the above procedure with that used in Liverpool which employs conversion factors to give aggregate vitamin A activity for mixtures of the three compounds. Both methods lead to much the same estimate of total vitamin A_1 potency. Partition chromatography using paraffin oil as a stationary phase and various aliphatic alcohols as the mobile phase have been used to separate vitamin A from some of the substances interfering with its spectrophotometric assay (21). Caster & Mickelsen (22) evaluated critically the vitamin A assay in serum. The time relation to intensity of coloured vitamin A-complexes has been studied photometrically (23). The Carr-Price reaction for vitamin A in the presence of carotene has been re-evaluated (24).

Lora-Tamayo & Leal (25) prepared the addition product of β-carotene-*p*-benzoquinone in which the ratio of compounds is 1:5 as expected from the existence of five conjugated double bonds.

Biopotency of vitamin A isomers and related compounds.—Ames, Swanson & Harris (26, 27) have published in detail their studies on the biopotency of five isomers of vitamin A acetate and of vitamin A aldehyde; since a full account of their work had been reported in last year's *Annual Review of Biochemistry* by Boyer (27a) no further comments appear to be needed. Karrer & Eugster (28) compared the biological activity of synthetic *dl*-α-carotene with that of the natural *d*-α-carotene and synthetic β-carotene.

While the *d*-α-carotene had, as expected, half the activity of the synthetic β-carotene, the synthetic *dl*-α-carotene had less than half the activity of the natural *d*-isomer. In fact, after four weeks of dosing, all animals showed deficiency symptoms. The authors suggest that the *l*-form acts as an antagonist of the active natural *d*-form, possibly by interfering with the enzyme system responsible for the conversion of carotenes to vitamin A. Eugster, Trivedi & Karrer (29) synthesized 2,2'-dimethyl-β-carotene which proved to be only about half as active as β-carotene.

Astaxanthin esters isolated from the integument of the shrimp, *Aristeomorpha foliacea*, and freed of carotene and vitamin A, cured the xerophthalmia of rats, but did not enable rats to gain weight [Grangaud *et al.* (30, 31)]. In this connection it may be mentioned that the carotenoid pigment astaxanthin has been found in the feather barbs and vermilion skin of the American flamingo and may be responsible for the striking pink colour of the bird [Fox (32)].

Occurrence and distribution.—Kon and associates (33, 34) have continued their interesting studies on carotenoids and vitamin A which occur in certain marine invertebrates and seem to be an important source of vitamin A for marine vertebrates. In 9 out of 11 species of *Euphausiid* crustaceans, vitamin A was found in high concentration, located principally in the eyes. The only carotenoid, in all species, was astaxanthin or its esters (33). From the eyes of the crustacean, *Meganictiphanes norvegica*, two vitamin A components were isolated by chromatography on alumina; one of the components absorbing maximally at 325 mμ was identified as all-*trans* vitamin A. The other component, having a peak at 311 to 312 mμ had properties consistent with its being a *cis* isomer of vitamin A (34). Wald & Burg (35) identified the vitamin A of the lobster eye as the *cis* isomer, neovitamin Ab, the specific precursor of the visual pigment, rhodopsin. The full papers will be awaited with interest to see whether the *cis* isomers found by the two groups are identical (35a). The *neo-b* isomer is evidently the 7-*cis*-vitamin A (35b). Lambertsen & Braekkan (36) also report the isolation of a new vitamin A_1 isomer from the eyes of the deep-sea prawn, *Pandalus borealis;* they claim that it could be the Δ-3,5-di-*cis*-isomer. The results of Dartnall (37) on the visual pigments of the clawed toad, *Xenopus laevis*, have been reinvestigated by Wald (38). He concludes that the retinal pigment is mainly porphyropsin accompanied by a small amount of rhodopsin.

In the search of intermediates participating in the visual cycle, particularly in the change of rhodopsin to "indicator yellow," Morton's group (39, 40) prepared from retinene and methylamine a crystalline indicator-yellow analogue, which is shown to be a Schiffs' base, retinylidene methylamine (39); its methylammonium ions are stable, but the uncharged compound hydrolyzes to retinene. This behaviour may explain the influence of pH on the stability of "indicator yellow" solutions obtained from eyes (40).

The conversion of all-*trans*-retinene to neoretinene b is catalyzed by an enzyme found in retinas of cattle, retinene isomerase, which is activated by

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light [Hubbard (41)]. Rushton & Campbell (42) developed a photocell system which permits direct estimation of rhodopsin in the living human eye.

A number of paper report the content of vitamin A of some Egyptian and Indian fishes (43, 44) and of Indian dairy products (45, 46).

Stability.—The preservation of carotene and vitamin A continues to be investigated. Booth published an excellent account of the problem of preventing loss of carotene during storage of dried green crops (47).

The average loss of carotene in chick mash containing 5 per cent of dried grass was, after 16 weeks, 13.5 per cent, while dried grass alone lost only 8 per cent. With dried grass of good quality, the stored chick mash supplied the needs of chicks [Booth *et al.* (48)]. Addition of 5 per cent of animal fats or animal oils to dehydrated alfalfa meal decreased the loss of carotene during storage, despite the content of animal fats of 5 to 40 per cent of free fatty acids [Bickoff *et al.* (49)]. Mitchell & Silker reported that various vegetable or animal oils, applied as antidust agents in concentrations of 0.8 per cent, do not protect carotene in alfalfa meal unless an antioxidant, 6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline, has been added (50). Similar experiences in mixed feeds are reported by Livingston *et al.* (51). Evidently the protective effect of fats depends on the amount applied and on type of fat used, since fats in some conditions may even accelerate destruction of carotene. The relation between structure of quinoline derivatives and their antioxidant activity has been investigated (52); the 6-alkoxy derivatives were most effective for alfalfa meal, while the 6-hydroxy derivative best protected the carotene in mineral oil (53).

The results of Scandinavian workers on the preservation, formation, and utilization of carotene were summarized in an English edition (54).

The stability of vitamin A was investigated in various mixed feeds (55); in stored poultry diets it was adversely affected by the presence of Fe, Cu, and Co salts (56). Extensive studies were made on the loss of vitamin A potency in Indian foods during preparation and cooking (57 to 61).

The administration in water suspension of preparations of vitamin A palmitate protected with gelatin or pectin (dry vitamin A) is advocated by Bernhard & Heinzelmann (62), but Høie & Sandvik (63) did not find any difference betwen dry preparations of vitamin A and medicinal cod liver oil given to chicks.

Studies of vitamin A deficiency.—An interesting development in vitamin A metabolism is the extensive study of Morton and associates (1, 64 to 67) on the constitutents of the unsaponifiable portion of animal tissue lipides. It appears that a new and abnormal metabolite, a polycyclic compound not derived from vitamin A and designated compound SC, accumulates in vitamin A deficiency (64). It is normally present in small amounts in the liver, kidneys, and intestines of the pig, sheep, dog, and pullets (65) and is considered to be derived from a sterol by dehydrogenation, but its chromophoric grouping has not been identified (1). Less clear is the connection

with vitamin A metabolism of other minor lipide unsaponifiable constituents in liver, namely cholesta-3,5-diene-7-one (66), compound SA (possibly cholesta-8(9)-ene-7,11-dione (66)), and the more polar compound SB, which are also found in small amounts in tissues of normal animals (1). The task of identification is made difficult by the presence of a variety of vitamin A artifacts (65, 68).

The accumulation of the abnormal metabolite is not associated with a marked derangement of the endogenous cholesterol metabolism, since the levels of plasma and liver cholesterol were not changed by vitamin A deficiency [Green, Lowe & Morton (69)]. On the other hand, cholesterol feeding substantially reduced the liver stores of vitamin A in rats and cockerels (70). Migicovsky reports that liver homogenates from vitamin A-deficient rats are unable to synthesize cholesterol from C^{14}-acetate; since starvation produces similar results, it is possible that these effects are nonspecific (71).

Balakhovskii *et al.* report that injection of S^{35}-labelled methionine was followed after 48 hr. by a greater rise of S^{35} in the liver and kidneys of the deficient rats than in the controls; it is suggested that deficiency may inhibit intracellular oxidation and excretion of sulphur (72).

The oxygen consumption of deficient rats was 10 to 25 per cent greater than in the controls (73); the rate of respiration of the isolated diaphragm of deficient rats was also somewhat higher than that of normals (74).

Reduced quantities of deoxyribonucleic and ribonucleic acid were found in a number of organs of young, vitamin A-deficient rats, but not in their kidneys; increased amounts of both were observed in their livers and spleen. Vitamin A therapy restored the DNA and RNA to normal [Nicsar (75)].

The dental depigmentation in rats deficient in vitamin A or vitamin E is accompanied by a diminution of the iron content in the depigmented enamel of the incisor teeth [Moore & Mitchell (76)].

Hedenberg investigated the macroscopic and microscopic changes and stone formation in the urinary tract of vitamin A-deficient rats. She finds a localized and marked hyperkeratosis at the base of the bladder, which gives rise to a marked stasis with dilation of the bladder and ureters; bacterial infection and alkaline urine appear to be responsible for the formation of the urinary calculi (77). The epithelium of the oesophagus, stomach, and sometimes of parts of the small intestine of deficient rats shows degenerative changes, but no damage occurs to the epithelium of the tongue or the large intestine [Planel *et al.* (78)].

The development of hydrocephalus with an accompanying stenosis of the cerebral aqueduct has been further studied in foetuses and young animals reared from vitamin A-deficient rabbits (79, 80) and rats (81). The papilloedema and compression of the optic nerves may be a direct result of increased pressure in the subarachnoid spaces in the region of the optic foramina (79). One of the earliest signs of vitamin A deficiency in the chick is an increased pressure of the cerebrospinal fluid (82). Similar findings are reported in

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deficient pigs (83). Field cases of blindness in beef cattle were observed, with constriction of the optic nerves, which might be associated with vitamin A deficiency (84, 85).

Effect of excess of vitamin A.—The increased pressure of cerebrospinal fluid in deficiency is difficult to reconcile with the reports that administration to infants of large doses of vitamin A (350,000 I.U.) produces symptoms of transient acute intracranial hypertension, which subside within 48 hr. [Marie *et al.* (86)].

Hedenberg observed that on administration of large doses of vitamin A to rats, the normal epithelium in the vagina and uterus was replaced by a noncornifying epithelium (77), which in appearance simulated the changes obtained *in vitro* with explants from chicken embryos [Fell (87)]. Locally applied vitamin A on the vagina of ovariectomised rats prevented the appearance in the vaginal smear of cornified cells which are a sign of the estrogenic effect of locally applied estradiol. Vitamin A decreased the concentration of protein-bound sulphhydryl groups [Kahn (88)]. Bern *et al.* observed no such reduction in protein-SH groups on local treatment of the male rat epidermis with vitamin A, nor was there any definite evidence of interference with the keratinization process. Vitamin A, locally applied on the male guinea pig epidermis in the nipple region, did not interfere with the hyperkeratotic response to estradiol benzoate. While no definite effects of vitamin A on the stratum corneum could be discerned, the number and size of cells of the stratum germinativum and granulosum greatly increased (acanthosis) after topical application, but not on subcutaneous administration of vitamin A (89).

Excess of vitamin A appears to increase the rate of mitoses in chick heart fibroblasts *in vitro* [Lasnitzki (90)]. Removal of parathyroids in rats had no influence on the changes of bone formation caused by hypervitaminosis A; Cohen *et al.* conclude that the pathological effect of excess vitamin cannot be explained by stimulation of parathyroids. The proteins in the blood of hypervitaminotic rats were reduced and mucoproteins much increased, but the calcium and phosphorus levels were not affected (91). The cholesterol content in rat brain was not changed in hypervitaminosis (92).

Administration of vitamin A (20,000 to 35,000 I.U. daily) to pregnant rats caused in most instances the death and resorption of foetuses. In the survivors there was a high incidence of malformations, which included cleft palate and ocular abnormalities [Giroud & Martinet (93, 94)].

Fully grown foxes and mink tolerate from 200 to 300 I.U. vitamin A per gm. body weight daily for 6 to 8 weeks, but young animals soon show signs of intoxication, such as anorexia, bone changes, loss of fur, exophthalmos, cramp, and local hyperesthesia of the skin [Helgebostad (95)].

Metabolism of carotene and vitamin A.—Instead of the oxidative fission at the central double bond of β-carotene, resulting in the formation of vitamin A aldehyde, which is later reduced to the alcohol, Glover & Redfearn suggest a step-wise oxidative fission from one end of the carotene molecule by a type of

β-oxidation of the α-methyl branched fatty acid chain. Possible intermediates such as β-apo-8′-, β-apo-10′-, and β-apo-12′-carotenals were prepared and found to be converted to vitamin A by rats; β-apo-10′-carotenal underwent β-oxidation to β-apo-12′-carotenol. Vitamin A aldehyde has methyl groups in β-position, hence it would not be oxidized from the open chain but reduced by alcohol dehydrogenase (96). Lycopene undergoes a similar stepwise oxidation, but the resulting lycopenals have no vitamin A activity [Painter & Glover (97)]. The "nibble" theory, as opposed to the fission hypothesis, would explain the fact that one molecule of β-carotene forms only one molecule of vitamin A and that the α- and γ-carotenes have only half the activity of the β-isomer (98).

Fluorescent microscopy has been used to demonstrate the conversion to vitamin A of carotene, given intravenously to vitamin A-deficient rats; vitamin A was detected both in liver cells and to a greater extent in Kupfer cells [Greenberg *et al.* (99)]. Krinsky (100) succeeded in bringing into cell-free preparations the enzyme system of cattle retinas and rat liver that esterifies all-*trans* vitamin A alcohol to its ester. Removal of the supernatant from the particulate fraction of the retinal suspension reduced the esterifying potency; triglycerides, lecithin, fatty acids, ATP, and coenzyme A did not restore the activity.

Laughland found 38 per cent of C^{14} in the expired CO_2 of a chick and similar amounts in a rat, when a single dose of uniformly labeled C^{14}-β-carotene (about 100 μg.) dissolved in cottonseed oil had been administered orally. The peak of $C^{14}O_2$ excretion occurred in the chick 5 hr. after dosing and at 10 hr. in the rat. Surprisingly, only 1 per cent of the administered radioactivity was found in body tissues of the chick, mainly in the liver. The excreta and gut content contained 36 per cent of C^{14} (101). Different results are reported by Krause *et al.* (102) who administered to rats C^{14}-β-carotene dispersed in water; there was apparently no label excreted in the respired CO_2, since the entire administered dose could be accounted for, partly in the nonsaponifiable matter of feces (70 per cent) and the rest in body tissues as nonsaponifiable substances not identical with carotene.

Wolf *et al.* injected intraperitoneally into rats 2.65 mg. of vitamin A-2-C^{14} in a watery emulsion. In the expired CO_2, 5 per cent of the radioactivity appeared; the rate of the appearance of $C^{14}O_2$ over a period of 24 hr. was almost constant. In the excreta 34 per cent C^{14} was found, of which ⅓ was in urine in the form of water-soluble, light petroleum-insoluble metabolites. Liver contained 14 per cent of the radioactivity, skin 0.5 per cent, eyes 0.01 per cent, and 24 per cent was found in the remaining carcass (12, 103). Solubilized β-carotene, in Tween dispersion, and injected intravenously into deficient chicks, was rapidly converted to vitamin A which appeared in the liver and serum. Injected cryptoxanthin did not form significant amounts of vitamin A. Rabbits injected with carotene also formed vitamin A which was about equally distributed between the liver and kidneys and which amounted to about 1 to 2 per cent of the dose on equimolar basis [Bieri (104)]. While

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Bieri found appreciable amounts of carotenoids circulating in rabbit blood even after 24 hr., Kon *et al.* report that carotene, injected in Tween dispersion, disappeared rapidly from the blood of rabbits and was apparently destroyed. The fast appearance of vitamin A alcohol in blood of rabbits and rats suggests that the intestinal wall was not the site of conversion of the injected carotene. In calves little or no vitamin A was formed from injected carotene, and the circulating carotenoids amounted to only half of the injected dose (105). The conversion of injected carotene to vitamin A was not influenced by administration of up to 20 mg. tocopherol daily [Bieri (106)]. Normal dogs appear to excrete in their urine 14 to 235 I.U. of vitamin A daily (107).

Nutritional studies.—Vavish *et al.* claim that fat-free milk has the property of increasing carotene utilization in rats (108). In human studies, however, no such effect was observed; the fecal excretion of carotene from sweet potatoes remained at a level of 71 per cent; the blood vitamin A and carotene did not increase on supplementation with sweet potatoes together with or without fat-free milk (109).

In dairy cows (110) and in their calves (111) carotene in doses of up to 300 mg. per head did not increase their blood or liver content of vitamin A or carotene.

The plasma vitamin A values of range sheep in Montana varied from 21 to 38 μg. per 100 ml. (112). Merino sheep, given maintenance rations of wheat and wheaten chaff, had low levels of vitamin A in the plasma and showed a high death rate. A single initial massive dose of 500,000 I.U. vitamin A prevented the deficiency (113).

Gillum *et al.* (114) determined the serum vitamin A levels in 514 healthy adults of both sexes over 50 years of age; the mean values were about 60 μg. per 100 ml. irrespective of sex. The serum carotene levels varied widely. Adam & Gutheil (115) estimated vitamin A in infants suffering from various diseases. Serum vitamin A and carotenoid levels were also investigated in chronic hospital patients (116), in liver diseases (117), and in rheumatic fever (118, 119).

One of the clinical aspects of use of vitamin A is its beneficial effect in diseases of the inner ear (120, 121). Tschirren (122) was able to improve with vitamin A therapy the auditory lesions produced in guinea pigs by streptomycin and neomycin intoxication.

Effect of antioxidants and antibiotics.—Addition of 0.01 per cent N,N'-diphenyl-*p*-phenylenediamine to rations appeared to increase the utilization of carotene in cows (123). Chlorinated naphthalenes given to rats or hamsters caused a decrease in liver vitamin A (124) but had no effect on blood vitamin A (125). Octachloronaphthalene decreased markedly the utilization of carotene when given orally to rats, but not when injected (125).

Chlortetracycline (aureomycin) and dianine penicillin increased in chicks the conversion of carotene to vitamin A, as measured by the vitamin content of livers (126). In rats, chlortetracycline increased the response to

vitamin A when tested by the vaginal smear assay in ovariectomized animals (127), but the liver and kidney stores were not affected (128, 129). When carotene was given to rats, chlortetracycline or vitamin B_{12} increased the deposition of vitamin A in liver and kidneys, but penicillin with vitamin B_{12} was ineffective. It is suggested that the two antibiotics have different effects on the microbial synthesis of vitamin B_{12} [High (129)].

The administration of a carcinogen, 3'-methyl-4-dimethylaminoazobenzene, to rats appeared to have the surprising effect of raising the concentration of liver vitamin A [Reiss *et al.* (130)].

Endocrines and vitamin A.—Clark & Colburn (131) report that administration of large doses of cortisone to normal or adrenalectomized rats on stock or vitamin A free diets resulted in a rapid loss of vitamin A from livers and kidneys. Comparison with effects produced by starvation indicate that losses of vitamin A from liver stores during inanition may be mediated through the adrenal gland. Similar conclusions are put forward by Kagan & Kaiser (132) who observed a marked lowering of serum and liver vitamin A of rats subjected to stress by the production of sterile abscesses.

Injections of an anterior pituitary preparation to cows appear to accelerate the mobilization of hepatic reserves of vitamin A into the blood as the alcohol and hence into the milk as both the alcohol and ester (133).

Extending previous studies on hypothyroidism, Arnrich (134) finds that thiouracil-treated dogs absorb and utilize carotene normally, but have increased blood levels of vitamin A, carotene, as well as of cholesterol. This finding may be a nonspecific, general effect on lipids levels. In humans no correlation was observed between the plasma levels of vitamin A or carotene and the basal metabolic rate (135). Vitamin A deficiency in rats results in cystic degeneration or atrophy of the parenchyma of the thyroid gland in the centres of the lobes (135a). Danowski *et al.* (136) suggest that administration to rats of large doses of vitamin A (150,000 I.U./kg.) causes alterations in production, release or transportation of the thyroid hormone as indicated by lowering of the circulating protein-bound iodine.

Bo (137) points out that, in contrast to the vaginal cornification [Murray & Campbell (127)], the metaplastic changes occurring in the uteri of vitamin A-deficient rats do not develop when the ovaries have been removed. Contrary to previous conceptions, the squamous metaplasia and keratinization of the uterus is not only influenced by the vitamin A status but depends on the normal functioning of the ovaries.

VITAMIN D

An excellent review on vitamin D has been published [Dam (2)].

Chemistry.[4]—In a series of papers Inhoffen *et al.* (138, 139, 140) and Braude & Wheeler (141, 142) describe the synthesis of model substances rela-

[4] In view of the increasing number of vitamin D isomers the following nomenclature of sterols with an open ring is adopted: *seco* denotes the opened bond; thus vitamin D_2 (III) would be 9,10-*seco*-ergosta-10(19), 5-*cis*, 7-*trans*, 22-*trans*-tetraen-3β-ol.

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ted to the conjugated semicyclic triene system of tachysterol. Several *cis* and *trans* isomers of substituted bis-cyclohexenyl-ethylene were prepared and spectroscopically investigated (138, 141, 142). Several new compounds for ring A synthesis were prepared (139) and condensed with Windaus' $C_{21}H_{34}O$-aldehyde or the $C_{16}H_{28}O$-ketone to give triconjugated tachysterol analogues of 9,10-*seco*-ergostatetraene structure, such as the "all"-*trans*-10(5),6,8(14),22-tetraene (VI), "all"-*trans*-1(10),5,7,22-tetraene (VII), and possibly the 6,7-*cis* analogue of tachysterol (140).

Considerably greater difficulties were experienced when the introduction of a methylene group was attempted to form a triconjugated exocyclic triene related to vitamin D. Three laboratories accomplished it this year by application of the remarkable reaction of Wittig & Schöllkopf (143), in which the carbonyl oxygen of a ketone is directly replaced by an alkylidene group; Harrison *et al.* (144, 145) and Inhoffen *et al.* (146) prepared the model triene, *trans*-1-2′-cyclohexylidene-ethylidene-2-methylene-*cyclo*-hexane. Its unsaturated system differs from that of calciferol only in the transconfiguration of the central double bond. Milas *et al.* prepared the 5-methoxy-2-methylene-cyclohexane derivative (147).

An outstanding achievement is the partial synthesis of the *trans*-vitamin D_2, 9,10-*seco*-10(19), 5-*trans*, 7-*cis*, 22-*trans*-ergostatetraene [Inhoffen *et al.* (148)]; this was accomplished by allowing to react with triphenylphosphine-methylidene the C_{21}-ketone obtained through an aldol condensation of *p*-acetoxy-cyclohexanone with Windaus' $C_{18}H_{34}O$-aldehyde. Verloop *et al.* (148a) also report the preparation of *trans*-vitamin D_2 in the form of a crystalline phenylazobenzoate by isomerization of vitamin D_2 in apolar solvents at room temperature in diffuse daylight by the action of iodine. The preparation of new decalins for use in model syntheses in the 9,10-*seco*-D-homo-steroid series is reported [Inhoffen *et al.* (149)].

In a series of papers on ozonolysis, epoxidation, and the infrared spectrum of precalciferol, Velluz and collaborators reinvestigated its possible structure (150, 151); they conclude that precalciferol is a 6,7-*cis* isomer of tachysterol (152) and has not a spirosteroid configuration as previously proposed (150). Independently, Koevoet *et al.* (153a) arrived at the same conclusion. In support of the 6,7-*cis* structure Velluz *et al.* (152) cite also the similarity of the spectrum of a triol, 9,10-dihydroxy, 9,10-dihydro-precalciferol, with the ultraviolet absorption of 10,19-dihydro-vitamin D_2 II obtained by Schubert (153) through partial catalytic dehydrogenation of vitamin D_2 by Raney nickel. Precalciferol would thus be identical with the synthetic 6,7-*cis* isomer of tachysterol of Inhoffen (140).

Velluz *et al.* (154), and Koevoet *et al.* (153a) by the use of small amounts of I_2, were able, in spite of the accepted view of the irreversibility of photochemical changes, to transform precalciferol into tachysterol. Velluz *et al.* also converted precalciferol photochemically into ergosterol and lumisterol with a 3 per cent yield (155).

In view of these findings Velluz *et al.* (152, 155) proposed a new scheme of

photochemical conversion of conjugated dienes into triconjugated 9,10-*seco*-compounds in which precalciferol would be the pivot steroid, so that ergosterol would be converted directly into precalciferol, which would change spontaneously into vitamin D_2; formation of $lumisterol_2$ and $tachysterol_2$ would thus be a side reaction.

Independently, Havinga *et al.* (155a) suggested, from studies with C^{14}-labeled vitamin D_2 and D_3, an essentially similar scheme in which lumisterol and tachysterol are not essential intermediates in the formation of vitamin D; their scheme differs from that of Velluz in certain details which will have to be settled by further experiments; they postulate an unstable intermediate as the pivot steroid.

This new photochemical conversion chain opens interesting possibilities for the *in vivo* conversion of 7-dehydrocholesterol directly into $precalciferol_2$ without an obligatory formation of $lumisterol_2$ and $tachysterol_2$.

Inhoffen *et al.* (156, 157) confirmed the all-*trans* configuration of the crystalline isovitamin D_2 [identical with pyrotachysterol] and isotachysterol; the *u*-tachysterol, 4,6-*trans*, 8(14), 22-tetraene, is also a 6,7-*trans* isomer. Contrary to the suggestion of Braude & Wheeler (141), Inhoffen *et al.* (157) supports, with good evidence, the 6,7-*trans* configuration of tachysterol. The proposal of Sondheimer & Wheeler (158) to assign to vitamin D_2 the configuration of Inhoffen's *trans* vitamin D is not in accord with the results of other authors [Harrison *et al.* (145); Inhoffern *et al.* (148, 156, 157)].

Uniformly labeled C^{14}-vitamin D_2 in the form of the crystalline dinitrobenzoate ester has been prepared by photochemical conversion of C^{14}-ergosterol; the latter was obtained biosynthetically from yeast made, first, sterol deficient and then incubated with acetate-1-C^{14}. Small amounts of C^{14}-lumisterol were also prepared [Kodicek (159)]. Recently, Havinga *et al.* (155a) employed the biosynthetic approach to study the formation of C^{14}-vitamin D_2 from C^{14}-ergosterol in the presence of nonlabeled $lumisterol_2$ and $tachysterol_2$; the C^{14}-vitamin D_2 was isolated as the maleic anhydride adduct.

Milas *et al.* (160) report the preparation and physical properties of four different salts of vitamin D_2 phosphates; the lithium vitaminate, obtained on treatment with phenyllithium, was reacted with phosphorus oxychloride or di-tert-butyl chlorophosphate. Vitamin D_2 was changed by Openauer oxidation irreversibly into a crystalline conjugated ketone, the 9,10-*seco*-4,10(19), 7,22-ergostatetraene-3-one [Trippett (161)]. The 4,4-dimethyl calciferol has been obtained by photochemical conversion of 4,4-dimethyl ergosterol [Cooley *et al.* (162)]. The nature of the complexes of vitamin D_2, as well as of carotene and vitamin A, with γ-globulin was studied by Troitskii & Tarasova (163).

Analysis.—The measurement of radioactivity in the paw of rats given P^{32} for the bioassay of vitamin D has many advantages over the line test (164). The radioactivity of the bone ash of chicks given Ca^{45} has also been used for vitamin assay (165, 166).

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Vitamin D_2 can be estimated in pure solution by the formation of adducts with *p*-benzoquinone; the uncondensed quinone is then titrated (167). Ergosterol, tachysterol, and suprasterol II form similar condensation products (168).

Pharmaceutical preparations of vitamin D were estimated spectrophotometrically (169). The vitamin D_3 content of irradiated milk was about 500 I.U. per kg. according to rat and chick bioassays (170).

A critical evaluation of vitamin D assays has been published [Almquist (171)]. Oral doses of vitamins D_2 and D_3 and of tachysterol have a less rapid and less lasting effect on patients with parathyroid insufficiency than when given intravenously (172).

Corbisterol, the 7-dehydrosterol from the bivalve, *Corbicula leana*, had on irradiation about the same potency for rats as irradiated 7-dehydrocholesterol, but had only 1/25 of the potency for the chick [Rosenberg (173)]. Festenstein & Morton (174) studied the distribution of 7-dehydrosteroids in the skin of the pig, in the intestines of ox, pig, horse, and sheep, in herring roe and milt; it is emphasized that the significance of the 5,7-dienes may not only reside in their capacity to act as provitamins.

Relation to Ca and P metabolism.—Engfeldt & Zetterström (175) conclude from biophysical studies on bone tissue of rachitic dogs that in rickets there is not only a disturbance in mineralization but also in the submicroscopic and microscopic organization of the organic matrix; x-ray diffraction studies show that hydroxyapatite crystallites of normal dimensions have an atypical radial orientation in tranverse bone sections, thus following the abnormal radial disposition of collagen bundles in osteoid tissue. Zetterström & Winberg (176) studying the mineral metabolism in primary, vitamin D-refractory rickets, indicate that vitamin D appears to have also a more direct action on bone tissues than the indirect one of increasing the intestinal absorption of calcium and phosphate, since increased mineralization started despite an initially low Ca×P quotient. Similar conclusions on the basis of rat experiments are put forward by Nicolaysen (177); in support of a concurrent local action on bones, he cites the abnormal histological structure of "calcified" bones of deficient rats injected with calcium salts and their low citric acid content; after vitamin D administration to deficient rats, increased blood calcium levels can be demonstrated which are not caused by increased absorption of the mineral. In contrast Migicovsky & Jamieson (178) emphasize the increased intestinal absorption of calcium as the primary and sole effect of vitamin D, because the vitamin causes an increased Ca^{45} deposition in chick bones when the mineral is given orally but fails to do so when injected. The interpretation of net deposition of Ca^{45} is, however, difficult since it does not take into account the three factors, the sum of which gives the net result; the physical exchange of Ca, true physiological accretion, and resorption of bone salts. Bauer, Carlsson & Lindquist (179) propose an ingenious formula for calculating separately these three factors; they show that vitamin D has a local stimulating influence on bone resorption involving

possibly citric acid metabolism but have no convincing evidence of a local effect on the accretion of bone salts. In a later report Carlsson & Lindquist (179a) cite in support of a local action on bone the indirect evidence that 10 I.U. of vitamin D daily will cause the maximal absorption of Ca^{45} by the intestines; though a one hundredfold increase in vitamin dosage will further increase the blood level of Ca^{45} that is evidently derived from bones. Engfeldt & Zetterström (180), while studying the deposition of P^{32} injected into deficient and vitamin D-treated rats, note a local effect of vitamin D; the rate of uptake of injected P^{32} in the bone fraction, insoluble in saturated ammonium sulfate and apparently derived from true accretion of mineral salts, was markedly increased on administration of vitamin D.

Sobel & Burger (181), in agreement with previous results by Barrett (182), observe that vitamin D increases the level of circulating lead in rats to which lead is being administered. After lead dosing has ceased the vitamin depresses the Pb blood level proportionally to the rise of serum phosphate. This vitamin effect appears to support the intestinal absorption theory as the primary and sole factor in vitamin D action, but lead salts may not be deposited in bones at the same site as the hydroxyapatite crystallites and may thus not be influenced by vitamin D as bone salts are.

The phytic acid in high-calcium cereal diets is not completely hydrolyzed by the intestinal phytase of rachitic rats unless vitamin D is administered. The resulting increased activity of phytase, however, does not liberate enough inorganic P to account for the curative effect of the vitamin; its antirachitic action may be attributable to an improved utilization of inorganic P in the body [Pileggi *et al.* (183)]. Calcium complexed with phosphopeptides from casein is utilized well by chicks, even when vitamin D is not given [Mellander (184)]. Vitamin D causes a slight rise in the Mg absorption of rats [Meintzer & Steenbock (185)].

Relation to citrate metabolism.—The decrease of citric acid content of serum in infants during the first five days of life is not related to the vitamin D intake, Ca/P ratio, or citric acid content of the diet, but there appears to be some correlation between citric acid and Ca levels in serum (186). Citric acid appeared to have an effect on the calcification *in vitro* of epiphyseal cartilage slices of deficient rats only if it was given orally; subcutaneous injection or addition of citrate to calcifying solutions had no beneficial effect. It is possible that increased serum phosphate levels following citrate therapy are responsible for the healing process [Yendt & Howard (187)].

Studies in vitro.—The *in vitro* calcification of rachitic rat cartilage incubated in human serum was decreased when blood of patients suffering from renal insufficiency was used, despite a high Ca×P product in their serum; the raised Mg blood level may be the inhibitory factor [Yendt *et al.* (188)]. Sections of rachitic human cartilage, demineralized with ethylenediaminetetraacetate, were incubated in $Ca^{45}Cl_2$ solution; the uptake of Ca^{45} was lowered in the young cartilage adjacent to the abnormal hypertrophic region and appeared to be related to the distribution of sulphated mucopoly-

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saccharides (189). A calcium-collagen-chondroitin sulfate complex has been implicated in initiating nuclei of crystallization which then may undergo crystal growth to form hydroxyapatite [Sobel (189a, 190)]. A histochemical study of rachitic epiphyseal cartilage during healing has been published (191).

Picard & Cartier (192) claim that cartilage from rachitic rats fixes *in vitro* more P^{32} from inorganic phosphate and hexosemonophosphates than normal tissue but is unable to fix P^{32} of the pyrophosphate groups of ATP; similar results are obtained with intact rats (193). Rachitic cartilage respires only feebly but has an increased glycolytic and glycogenolytic activity (192). Addition of vitamin D_2 or ergosterol in aqueous lecithin causes an inhibition of the anaerobic glycolysis rate of rat liver slices; the inhibition increases with concentration of the steroid for the range of 25×10^{-4}–10^{2} μM (194).

The growth-promoting effect of vitamin D on *Lactobacillus casei*, the growth of which was inhibited by linoleic acid or other essential fatty acids, has been further studied (195, 196, 197). The most potent of reversing agents among the sterols tested were vitamin D_2, vitamin D_3, and dihydrotachysterol, all of which have in common a 9,10-*seco*-5,7-diene structure; Ca ions had a similar effect, and the reversing activities were inversely related to the phosphate concentration of the medium. A physico-chemical mechanism affecting the permeability of the cell membrane is proposed, and it is suggested that these findings provide model systems for the study of vitamin D action.

Metabolism of vitamin D.—Vitamin D_2, administered by mouth to deficient rats in doses of 0.25 to 8 mg., was estimated by reversed-phase paper-chromatography in liver, kidneys, and feces; irrespective of the amount given, about 10 per cent of the dose was found in the liver and about 20 per cent in the feces on the first day. The liver vitamin decreased to about half the value on the second day; since the vitamin D content of other tissues did not simultaneously increase, it is thought that liver tissue may be involved in the destruction of vitamin D (198). Uniformly labeled vitamin D_2-C^{14} (159) was given to rats in a single dose of 1 mg.; all the C^{14} was recovered: 70 per cent as breakdown products, mainly in feces and liver, and 30 per cent as vitamin D itself, two-thirds of which was in the feces and the rest in the body. The bulk of the body vitamin was in liver, bones, blood, intestinal wall, and kidneys, in descending order; the occurrence of vitamin D in intestinal wall, bones, and kidneys may be connected with the P and Ca metabolism of these tissues (197).

Since up to 70 per cent of a massive dose of vitamin was converted by rats into inactive breakdown products, it was possible that intestinal microflora might be partially responsible for the destruction. However, feeding of 1 per cent sulfasuxidine had no effect on the recovery of vitamin D in liver and feces [Cruickshank *et al.* (199)]. Experiments with antibiotics also provide no unequivocal answer; chlortetracycline (200) and penicillin (171) in diets appeared to increase the response of rats to small doses of vitamin D, while

Horn & Eder (201) report that penicillin decreased the response in chicks.

In contrast to the relatively large proportion (20 per cent) of the dose of vitamin D recovered in the feces of the rat, the feces of normal subjects and patients with osteomalacia, who received 20 mg. vitamin D_2 by mouth, contained only 0.1 per cent of the dose as biologically active vitamin D (202).

The amount of vitamin D formed on ultraviolet irradiation in the skin and liver of rachitic rats was determined; fur impaired, but did not prevent the effect of ultraviolet light. It is assumed that vitamin D formed in the deeper epithelial layers of the epidermis is directly transferred to other parts of the body (203). Efficient absorption of topically applied vitamin D through the skin of the rat has been confirmed (204).

Other metabolic relationships.—Raiha & Forsander (205) noted that rachitic rats phosphorylated thiamine to cocarboxylase to a lesser degree than did normal controls, and administration of vitamin D restored the cocarboxylase level in blood to normal. It is at present impossible to decide whether this effect is only a nonspecific result of deficiency (205). Any direct participation of the vitamin in enzyme systems presupposes its presence in significant quantities; in heart sarcosomal (mitochondrial) fragments no vitamin D could be detected, and it was concluded that there was less than 0.03 μM vitamin D/mg. sarcosomal protein, which is small compared with the content of other respiratory catalysts (206).

Vitamin D and parathyroids.—Crawford *et al.* (207, 225) noted that in totally aparathyroid rats, a thousandfold increase in vitamin D dosage caused the kidney tubules to reabsorb a smaller percentage of the glomerular filtrate phosphate. This effect was qualitatively similar to that observed previously for parathyroid hormone (206). In intact animals administration of vitamin D may cause a seemingly paradoxical rise in the tubular reabsorption rate; this rise is supposed to be an indirect effect caused by suppression of parathyroid activity (225). Dent *et al.* (208) made the interesting observation that patients with postoperative tetany who had become insensitive to vitamin D_2 therapy responded promptly to doses of vitamin D_3 or dihydrotachysterol.

The difference between vitamin D and dihydrotachysterol has been narrowed down, since the latter has not only a catabolic effect on bone calcium, but can also increase the intestinal absorption and retention of Ca in the body of patients with hypoparathyroidism (208) and of patients with rickets resistant to vitamin D (209). Carlsson & Lindquist (209a) reached similar conclusions from experiments on rats.

With particular interest will be awaited the full account of the short report of Raoul *et al.* (210, 211) that the lipide, nonnitrogenous fraction of parathyroids contains the active Ca-raising principle which appears to be a sterol possibly related to neo-vitamin D_2 (212); to the latter has been recently assigned the remarkable structure of 9,10-*seco*-5-cholestene-3,10-diol-7-one [Raoul *et al.* (212a)].

Effect of excess of vitamin D.—Hypervitaminosis D in rats and rabbits

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causes kidney lesions which in the glomeruli resemble those of intercapillary glomerulosclerosis in man; the blood vessels were mostly dilated, with spasms in the efferent arterioles (213). Overdosing with vitamin D provoked hyperchloremic renal acidosis (Butler-Lightwood-Albright syndrome); treatment with adrenocorticotropic hormone improved the condition (214). The symptoms of vitamin D intoxication in infants have been described (215).

Clinical studies.—The aminoaciduria in rachitic children involves the excretion of histidine, threonine, serine, glycine, lysine, and glutamic acid, caused probably by the failure of kidney tubules to reabsorb the amino acids which may normally be absorbed in the form of phosphate esters. However, a similar defect is observed in scurvy (216, 217, 218). Methionine administration greatly increased the aminoaciduria in vitamin D-resistant rickets (219).

A new syndrome of "idiopathic hypercalcemia of infancy with failure to thrive" is ascribed to oversensitivity to vitamin D or chronic overfeeding with vitamin D (1000 I.U. or more daily) (220, 221). Another situation which appears to be caused by oversensitivity to vitamin D develops in some patients with sarcoidosis; the dramatic effect of cortisone on the raised blood Ca levels, increased Ca absorption, hypercalcemic symptoms, and renal function led Dent *et al.* to postulate a direct metabolic antagonism between cortisone and vitamin D (222). However, cortisone treatment of rats with hypervitaminosis D did not ameliorate the weight loss, clinical appearance, histological lesions, or the increased urinary phosphate excretion [Cruickshank & Kodicek (223)]. The beneficial action of cortisone in man may be caused, at least in part, by its effect on the renal tubular reabsorption of phosphate as observed in dogs [Roberts & Pitts (224)]. In support of the suggestion of an interaction between vitamin D and corticosteroids may be cited the observation that patients suffering from vitamin D-resistant osteomalacia excrete in urine two abnormal substances which appear to be Δ^4-3-ketosteroids, not identical with cortisone, cortisol, corticosterone or its 11-deoxy,11-dehydro, 11-deoxy-17α-hydroxy derivatives, progesterone or testosterone (197). An interaction is also implied in the findings of Bazzi & Nicolosi (226) that administration of large doses of vitamin D_2 or vitamin D_3 decrease markedly the urinary excretion of 11-hydroxy corticosteroids.

A newly recognized disease, infantile and adult hypophosphatasia, resembles rickets except that hypercalcemia and low alkaline serum phosphatase are present (227, 228); an abnormal metabolite, phosphoethanolamine, has been found in the plasma and urine of patients with the disease (229, 230). The abnormal phosphate monoester may be the physiological substrate for bone phosphatase, unless other phosphate esters are accumulating in the same manner (231). In this connection, it may be recalled that Picard & Cartier (192) reported a failure by rachitic cartilage to utilize pyrophosphate groupings of ATP, while normal ossifiable embryonic cartilage metabolized ATP readily, but not orthophosphates nor hexose-, mono-, and di-phosphates (232).

Vitamin E

Almost 200 interesting communications given at the 3rd International Congress on Vitamin E in Venice, Italy, added to the difficult task of selecting, within the limitations of space, studies of biochemical interest. Several reviews on vitamin E have appeared (2, 233).

Chemistry, analysis.—Green *et al.* (234) used an effective two-dimensional technique which combined partition and reversed-phase paper-chromatography, to confirm the presence of ε-tocopherol (5-methyl-tocol) in wheat products; they discovered in cereal grains a new tocopherol analogue, ζ-tocopherol which is a 5,7-dimethyl-tocol, isomeric with β- and γ-tocopherols. As much as 68 per cent of ζ-tocopherol and 16 per cent of ε-tocopherol are present in wheat bran; about equal amounts of each, 30 per cent, are found in barley and rye (234, 235). The remaining possible analogue, the 7-methyl-tocol or η-tocopherol, has now been discovered in rice (235a). A revaluation of biopotencies of tocopherols in food stuffs is clearly needed. The biopotency of DL-α-tocopherol acetate is 75 per cent of that of the natural D-α-tocopherol acetate (233, 235b). According to rat resorption tests, the ζ-analogue is almost as active as α-tocopherol (236); according to liver storage tests, the biopotency of DL-ζ-tocopherol is half that of DL-α-tocopherol (237).

The biological response of animals to vitamin E is adversely influenced by the presence in the diet of "stress factors," such as unsaturated fat, o-cresylesters, CCl_4, and sulphonamides which may act as pro-oxidants, promote the production of lipide peroxides in body tissues and thus increase the need for vitamin E (2, 238). Similar experiences are recorded by Miller *et al.* (239) when sodium bisulphite was used in the diet of chicks. In contrast anti-oxidants such as diphenyl-p-phenylene diamine (DPPD) have a sparing action on vitamin E, as Singsen *et al.* (240) showed in studies on the prevention of encephalomalacia in chicks; Johnson & Goodyear (241) even claim that DPPD can completely replace vitamin E for the rat. Scott *et al.* (242) report the composition of a basal diet for chicks on which the birds develop a vitamin E deficiency apparently not influenced by the presence of anti-oxidants or pro-oxidants.

The disappearance of degenerating muscles fibres of vitamin E-deficient hamsters was used for bioassay of tocopherols; tocopheroxide had the same antidystrophic activity as α-tocopherol, β-tocopherol had one-half, while α-tocopherylhydroquinone and α-tocopherylquinone about one-third activity [West & Mason (243, 244)]. The average tocopherol levels in human and canine blood (245) and of summer and winter butterfat in Canada (246) were reported.

Metabolism of vitamin E.—No fission of the $C^{14}H_3$ group in 5 position of α-tocopherol occurred, when rats were given labeled α-tocopheryl succinate by interperitoneal or oral route; the urinary excretion of C^{14} amounted to less than 1 per cent, and 40 to 75 per cent C^{14} appeared in the feces [Niedner & Johnson (247)]. In rabbits about two-thirds of an intravenous dose of

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C^{14}-α-tocopheryl succinate was excreted in the feces, one-third in the urine; about 50 per cent of the fecal radioactivity was α-tocopherol; the urinary metabolite may be a glucuronic acid-conjugated lactone of 2-(3-hydroxy-3-methyl-5-carboxypentyl)-3,5,6-trimethylbenzoquinone which has also been isolated from human urine (248, 249).

An oral dose of β- and γ-tocopherols is deposited in the ingested form in livers of rats; apparently no methylation to α-tocopherol occurs (237). Oral or intramuscular, but not cutaneous application of DL-α-tocopherol produces a rise in plasma vitamin E (250).

During the later stages of pregnancy the tocopherol levels in human blood tend to rise simultaneously with the concentration of carotene (251, 252, 253). Human milk has a higher concentration of vitamin E than cow's milk, and a marked increase occurs after oral administration of vitamin E (254). Despite low plasma and milk tocopherol levels, cows whose offsprings died from muscular dystrophy reared normal foster calves (233, 255).

Effects of deficiency.—Hove & Scibold produced a fatal liver necrosis in pigs given a low-protein, vitamin E deficient diet containing cod liver oil (256). No liver necrosis developed but microcytic anemia was seen in chicks fed on a vitamin E deficient, liver-necrogenic diet containing American *Torula;* vitamin E or brewer's yeast cured the symptoms (242).

Increased autolysis *in vitro* of kidney tissue of deficient rats was observed by following the release of free amino nitrogen (257). The proteolytic activity of muscles, but not that of kidneys, liver, and spleen, was increased in vitamin E-deficient rabbits (258).

Methylene blue treatment of vitamin E-deficient rats prevented "brown uterus," kidney lesions, and, partially, paralysis but afforded no protection against resorption gestations or hemolysis by dialuric acid [Moore *et al.* (245, 259)]. However, Markees (259a) and Dam (2) observed a protective effect even in the last mentioned lesions. An excellent tabulation of various symptoms and curative effects of various agents can be found in the two recent reviews (2, 233).

Hemolysis of human blood, caused *in vitro* by oxygen, was reduced by prior administration of vitamin E (260). The degree of hemolysis with hydrogen peroxide was related to the plasma tocopherol content (261); a high proportion of infants fed skimmed cow's milk or suffering from steatorrhea showed an increased hemolysis titre (262). Erythrocytes of vitamin E-deficient rats show a strong hemolysis, without dialuric acid, on incubation at 37°C.; feeding of vitamin E or methylene blue provides partial protection. Deficient chick erythrocytes are resistant even to dialuric acid [Christensen & Dam (262a)].

The histological changes in skeletal muscle of deficient rabbits (244, 263), in heart muscle (264), and in tissues of the rat were examined (265). In deficient chicks muscle degeneration developed without additional dietary fat and could be prevented by tocopherol or cystine but not by methylene blue

or antabuse [Dam *et al.* (266)]. Vitamin E-deficient monkeys showed muscle dystrophy and anemia with leucocytosis (267). A combination of vitamin E and vitamin K deficiencies produced heart muscle lesions in mice and rats [Dessau *et al.* (268)]. Deficient guinea pigs and rabbits, but not rats, show nonatheromatous necrotic and fibrotic lesions in the media of the aorta (269); both species have a marked elevation of plasma cholesterol esters and of free cholesterol in muscles, but not in liver, while rats show increased amounts of esterified cholesterol in their livers [Deuel *et al.* (270)].

The serum of deficient rabbits had a lowered albumin/globulin ratio and considerably increased β-globulin (271), and the dystrophic muscles had an increased content of free amino acids with the exception of glycine which was markedly lowered (272). However, no such decrease of free glycine in deficient rabbit tissues was noticed by Dinning *et al.* (273). Azzone & Aloisi report that dystrophic myosin shows changes in solubility and lowered viscosity (274). Of the muscle protein fractions, prepared according to Herrmann & Nicholas (274a), in late-lactation paralysis, actomyosin rapidly decreases, the insoluble fraction increases, and the soluble fraction remains constant [Rumery *et al.* (275)]. Electron microscope studies are interpreted to show the appearance of the brown fluorescent pigment in smooth muscles (276) and the disappearance of myosin and depolymerization of actin in striated muscle (277).

Dinning (278) considers the increased incorporation of C^{14}-formate into the nucleic acids of liver and skeletal muscle of deficient rats an indication of an increased turnover rate of nucleic acids in these tissues. However, in further experiments Dinning *et al.* (273) note that in contrast to C^{14}-formate, C^{14}-glycine is incorporated *in vivo* into nucleic acid purines of a number of tissues of deficient rabbits to a lesser extent than in controls; they postulate that the differential effect of deficiency cannot be the result of pool sizes, but that vitamin E may be more specifically concerned with formate metabolism. It would affect the exchange reaction of the 2 position of the purine ring without complete degradation of the molecule. This specific effect on formate should stimulate further research. It may be mentioned that Keilin & Hartree (279) found that formate can undergo coupled peroxidatic oxidation specifically in the presence of catalase; investigations on vitamin E deficient tissues of coupled peroxidatic reactions in general, and of catalase in particular, may give interesting results, thus extending previous work on lipoxidase (280, 281) and on hematin-catalyzed oxidation of fats (282).

The increased creatinuria and the elevated specific activity of urinary C^{14}-creatine, following injection of C^{14}-methyl labeled choline or C^{14}-formate to rats deficient in both vitamin E and B_6, seems to be the result of an inability of the dystrophic muscles to retain formed creatine (283). The loss in creatine phosphate of heart muscle from deficient rabbits, without an accompanying decrease in inorganic phosphates and adenosinepolyphosphates, is interpreted in the same way [Mulder *et al.* (284)].

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Despite certain similarities of the symptoms of muscular dystrophy in rabbits suffering from various deficiencies, the acetylcholine content of potassium deficient muscles was elevated, while that in vitamin E- or choline-deficient muscles was lowered [Hove & Herndon (285)].

King *et al.* (286) report on unexplained relationship between vitamin E, ferrous or ferric salts, and trace elements in the diet for producing the paralytic syndrome of mice. Tentori *et al.* (287) note that thyroid intoxication in deficient rats accelerates the appearance of muscular dystrophy. However, no correlation appears to exist between plasma tocopherol levels and the basal metabolic rate of hyperthyrotoxic patients (135a); nor does methylene blue protect rats from the symptoms of thyroxine intoxication, but on the contrary aggravates them (288).

In human muscular dystrophy the urinary excretion of arginine, threonine, valine, leucine, arginine, and taurine was increased, while that of creatinine and phosphate was decreased; Hurley & Williams (289) suggest that a system involving possibly vitamins E, B_{12} and folic acid, the sulfur-containing amino acids, as well as arginine and glycine, may be functioning inadequately.

In vitro studies of the carbohydrate metabolism of alloxan-washed diaphragm of normal and vitamin E-deficient rats are indicative of an antagonism, as in the hemolysis test, between vitamin E and alloxan (290); less clear results have been obtained regarding the interaction of vitamin E and insulin on the *in vitro* glycogen metabolism of rabbit liver and muscles (291). The carbohydrate metabolism of normal and diabetic subjects, rats, and rabbits is reported to be affected (292, 293) and not affected (294) by administration of vitamin E.

Enzyme relationships.—An important contribution has been made by Nason & Lehman (295) who demonstrated with purified particulate and solubilized mitochondrial preparations of skeletal muscle of the rat and other species that α-, β-, γ-tocopherols and tocopheroxide can specifically activate the decreased DPN-cytochrome-*c* reductase activity of an aged preparation, especially after it has been extracted with isooctane. Similar results were obtained for the reduction of cytochrome-*c* by succinate. Tocopherylquinone and its hydroquinone were less effective, while the following were inactive: vitamins D and K_1, tocopherol esters, the tocopherol nucleus (penta-methyl-6-hydroxy chroman), lipoic acid, cystine, and a number of antioxidants.

Although the lipide residue from the isooctane extraction could completely restore the enzyme activity, no vitamin E could be detected in the lipide material. Antimycin A inhibited competitively the activating effect on tocopherol of the solubilized unextracted DPN-cytochrome-*c* reductase. Cytochrome-*c* oxidase was not affected by tocopherol. The Baltimore workers suggest that tocopherol (its free radicle) or a conjugated form or an unknown lipide cofactor are part of the respiratory chain occupying a place between $DPNH_2$, or flavin enzymes, and cytochrome-*c*.

That vitamin E is specifically involved in the respiratory chain is further supported by the interesting finding of Bouman & Slater (296) that heart sarcosomal (mitochondrial) fragments contain vitamin E in stoichiometric amounts comparable to those of the known components of the respiratory chain.

It is interesting to note that the place occupied by "tocopherol" has been also assigned to the BAL- and antimycin A-sensitive "intermediary factor" of Slater; he, however, recently suggested that his factor may be directly involved in the over-all catalytic reaction, although it may not be directly concerned in the acceptance of hydrogen atoms or electrons [Slater (297)]. This may also be true of the "tocopherol." If it were not so, one would have to postulate two alternative chains (*a*) for tissues containing vitamin E and (*b*) for those, like bacteria, which do not appear to contain it, as indeed has been postulated for the intermediary factor (297a); moreover, yeast and *Neurospora*, whose cytochrome-*c* reductases show also a tocopherol dependence (295), do not seem to have, as far as the reviewer is aware, any vitamin E. They may, however, contain substances functionally related [compare Scott *et al.* (242, p. 53)]. An indirect catalyzing effect, without it being in the direct hydrogen pathway, of "tocopherol" or "the isooctane-extractable factor," possibly in the manner suggested by Bücher (298) for action of vitamin E or K in lipide-water interfaces, would remove the need for additional hypotheses. The participation of vitamin E in exchange reactions in interfaces would fit in with the concept of the mode of action of fat-soluble vitamins as interface catalysts in contrast to prosthetins [Kodicek (299)].

In any case the above findings will have to be correlated with the concept of action of metalloflavoproteins in which at present no place is left for lipide constituents such as vitamin E [Mahler (300)]. While vitamin E is thus implicated in the respiratory chain system, the evidence for the involvement of vitamin E in coupled oxidative phosphorylation has still to come; the findings of Nason & Lehman (295) and of Bouman & Slater (296) give only circumstantial support. Previous work is contradictory as has been discussed in previous reviews and particularly by Boyer (301).

Recently, Chernick *et al.* (302) suggest, albeit without direct experimental evidence, that uncoupling of oxidative phosphorylation may be responsible for the biochemical lesion in livers of rats given a necrogenic diet containing vitamin E-free *Torula* yeast; the oxygen uptake of liver slices of these rats declined after 30 min. and could be prevented by prior feeding of tocopherol, cystine, or factor 3 of Schwarz. Weinstock *et al.* (303) found the oxygen uptake of intermediates of the tricarboxylic cycle greatly increased in homogenates of vitamin E-deficient rabbits; replacement of ATP by ADP and AMP eliminated the differences in oxygen consumption between the deficient and control livers by increasing the O_2 uptake of the latter. An increased oxygen consumption of liver, skeletal muscle, and adrenal slices, but not of heart and kidney preparations, of deficient rabbits has been noted

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by Rosenkrantz (304); the effect in adrenals was also elicited in other deficiencies and in stress reactions. An interdependence with calcium of the enhanced respiration was noted.

The pathological metabolic pattern in dietary necrotic liver degeneration still remains complex. The findings of Chernick *et al.* have been mentioned already (302); the decline in oxygen consumption of liver tissue of rats prior to the onset of necrosis was accompanied by a progressive failure of ketogenesis, lipogenesis, and oxidation of C^{14}-acetate to $C^{14}O_2$ (305). Olson *et al.* (306) note a greatly depressed rate of incorporation of S^{35}-cystine in liver coenzyme A of rats given necrogenic diets with a low vitamin E and sulphur-containing amino acid content; the incorporation, however, into liver protein was normal and so was the uptake of C^{14}-methyl labeled methionine into the protein and choline of liver tissue. An impairment of the function of coenzyme A is also implicated in the decreased synthesis *in vitro* of long-chain fatty acids from C^{14}-acetate by liver slices of diseased rats [Artom (307)].

Other metabolic relationships.—The interrelationship between vitamin B_6 and vitamin E has been further studied in rats deficient in both vitamins; administration of either vitamin E or vitamin B_6 reduced the elevated excretion of allantoin and creatine [Young *et al.* (308)].

Tocopherol supplementation increased the hatchability of the eggs of turkeys given an all-vegetable protein ration but had no effect on egg production and fertility; the administered vitamin was stored in the egg yolk (309). The incidence and severity of perosis in turkeys receiving various levels of phosphate was reduced by vitamin E (310). The rate of conversion of C^{14}-glycine to uric acid in gouty subjects was beneficially affected by long-term vitamin E therapy (311).

The ineffectiveness of vitamin E on the blood clotting mechanism has been confirmed (312, 313). Vitamin E, ascorbic acid, and methylene blue, acting possibly as antioxidants, interfere with the formation of anti-streptolysin antibodies in guinea pigs (314). Of potentially practical importance is the observation of Mason & Bergel (315) that an infection of *Mycobacterium leprae* can be established in rats and hamsters when the animals become vitamin E deficient. Deficient mice show after six months a lipide depletion of the adrenal cortex, subcapsular adenomas, and large masses of brown degeneration (316). The significance of the decrease of vitamin E content of blood in liver diseases has been discussed (317). Unlike phytol administration, feeding of tocopherol to rats does not result in an decreased incorporation *in vitro* of C^{14}-acetate in fatty acids of liver slices; evidently no splitting of the intact phytol side chain occurs in the body (317a).

Vitamin K

Chemistry and assay.—The adsorptiochromism of derivatives of 1,4-naphthoquinone, when chromatographed on alumina, has been studied (318). Reversed-phase chromatography on silicone-coated paper has been used to differentiate vitamin K_1 and related compounds by utilizing their fluorescence before and after exposure to ultraviolet light or KOH [Greeil & Dam (319)]. In a series of papers Yamagishi (320, 321, 322) describes the estimation of vitamin K_1 and its derivatives by Scudi's cysteine-HCl procedure or by colour formation of some derivatives with dimethylaniline.

Metabolic studies.—Dam and associates (323, 324) reported interesting studies on the fate of vitamin K_1 injected in aqueous dispersion into chicks and rats; about 70 per cent of a massive dose was found as vitamin K (estimated colorimetrically) in liver and spleen and to a small extent (1 per cent) in kidneys and lungs, none was found in the bile. Only traces of the vitamin were still in the blood 14 hr. after intravenous injection. Ingestion of an anticoagulant, coumachlor, did not appear to affect the vitamin deposition. After oral or intramuscular administration, only small amounts were found in the organs (323). Partial blockage by India ink of the reticulo-endothelial system decreased in chicks the deposition of injected vitamin K_1 in the liver, with a concurrent increase in the spleen. No vitamin K was found in the placenta, foetus, or newborn young when a massive dose had been injected intravenously to pregnant rats (324). Apparently the action of the microflora on vitamin K metabolism in chicks has been overestimated, since germ-free animals have been found to recover spontaneously from a vitamin K deficiency [Luckey, Pleasants & Reyniers (325)].

The importance of bile for the absorption of vitamins K, A, and possibly D has been demonstrated on growing puppies deprived of bile (326).

Feeding of menadione or its diphosphate to cows did not affect the lactic acid development in their milk; addition of menadione to milk reduced the rate of lactic acid formation but resulted in discoloration and off-flavors (327).

Action in blood coagulation.—The beneficial effect of vitamin K on the blood concentration of factor VII (or proconvertin) in congenital deficiency of the factor, in the newborn, and in patients with obstructive jaundice has been restated (328 to 331). Synthetic vitamin K_1 appears to be more efficient than the water-soluble 2-methyl-1,4-naphthohydroquinone dicalcium phosphate (synkavite) (330, 331, 332). The decreased prothrombin level in thyrotoxicosis was temporarily improved by injection or oral administration of synthetic vitamin K_1 or menadione; the failure in the blood clotting mechanism is caused by the impaired liver function [Gordin & Lamberg (333)]. Frost & Spruth (334) report that the hemorrhagic condition in chickens occurring in the field can be controlled by menadione sodium bisulphite; when a hemorrhagic condition was induced experimentally by addition of 0.1 per cent sulphaquinoxaline to the chick ration, the requirement for vitamin K was greatly enhanced, and the sodium bisulphite derivative was at least four times more efficient than menadione. This observation may explain the failure of menadione treatment as reported by Yacowitz *et al.* (335). From comparison of field cases of hemorrhagic disease in chicks with experimental vitamin K deficiency, Cover *et al.* (336) conclude that the two syndromes are definitely distinct.

Unspecified vitamin B_{12}-antibiotic supplements restored to normal the

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blood clotting time of vitamin K deficient chicks (337). The *ante partum* administration of vitamin K significantly decreased the incidence of hemorrhagic disease of the newborn (338). However, an important warning was given by Allison (339) of danger of overdosing and appearance of kernicterus and hemolytic anemia in premature infants when more than 5 mg. synkavite per day was injected. These findings were confirmed in studies on vitamin E-deficient rats; while injections of vitamin K_1 or oral administration of water-soluble vitamin K substitutes were harmless, injections of some of the water-soluble derivatives caused intense hemolysis [Moore & Sharman (340)].

The success of intravenous injection of vitamin K_1 in raising the low prothrombin-proconvertin levels of patients under treatment with anticoagulants has been well substantiated by a number of authors (332, 341 to 346).

Enzyme relationships.—In continuation of their interesting studies, Martius & Nitz-Litzow (347) investigated an additional number of anticoagulants for their ability to uncouple oxidation and phosphorylation; in general the uncoupling effect ran parallel with the anticoagulant potency. Vitamin K derivatives with a 5 and 11 carbon side chain in position 3, but not menadione, increased the oxidative phosphorylation of mitochondrial preparations from vitamin K deficient chicks, the vitamin analogue with the shorter chain being less effective. As to the interpretation of these data with regard to the concept that vitamin K acts as a member of the respiratory chain, the present reviewer concurs with the excellent analysis of the situation as presented by Boyer (301) in last year's Review. Of interest is the finding of Kruse (348) that sarcosomal (mitochrondrial) fragments, the carriers of the nonphosphorylating respiratory chain system, contain little or no vitamin K (less than 0.01 μM/gm. protein). Uchara *et al.* (349) report that menadione and some other derivatives, but not *p*-benzoquinone and catechol, increase markedly the oxidation of hexosemonophosphates in the presence of yeast autolyzate.

In line with the hypothesis of Wessels (350), Arnon *et al.* (350a) suggest that vitamin K is involved in photosynthesis by participating together with ascorbate and FMN in anaerobic photosynthetic phosphorylation, a process considered to be different from oxidative phosphorylation [Arnon (351)]; the circumstantial evidence rests on the presence of vitamin K_1 in chloroplasts (2) and the fact that the P^{32} incorporation by isolated chloroplasts into ATP is increased, on irradiation and in absence of oxygen, when various naphthoquinone derivatives are present. Some of the quinoid compounds have vitamin K activity, but some have the biologically important methyl group in position 2 replaced by a side chain containing an aromatic ring (350).

Of interest are the paramagnetic resonance studies on vitamin K and other quinones by Woernley (352) and Blois (353).

Other metabolic effects.—In an excellent review Mitchell (354) summarizes his laboratory studies and clinical radiotherapeutic trials of some

chemical radiosensitizers, particularly of water-soluble vitamin K derivatives. A negative effect of synkavite on survival of *Escherichia coli* and *Saccharomyces cerevisiae* exposed to x-rays has been reported (355). Synkavite produced morphological anomalies in early chick and amphibian embryos (356) and inhibited the proliferation of mouse fibroblasts (357, 358). The mechanism of the antibiotic action on bacteria of vitamin K derivatives has been studied (359, 360).

Essential Fatty Acids

An excellent account of newer concepts on essential fatty acids has been presented by Deuel & Reiser (361, 362, 362a).

Chemistry and assay.—Weedon and associates report the syntheses of oleic, *cis*-, and *trans*-octadec-11-enoic acids with the use of acetylenic acids in anodic "crossed" coupling reactions (363, 364). The synthesis of α-linolenic acid, starting from the condensation of 1-bromopent-2-yne with 2-prop-2-ynyloxy-tetrahydropyran, has been accomplished (365).

The separation and estimation of a number of unsaturated octadecanoic acids by a standard reversed-phase column has been reported by Crombie *et al.* (366); the elution rate increased with the number of double bonds. The electrophoretic movement of higher fatty acids (367) and their behaviour on reversed-phase paper-chromatography have been studied (368, 369, 370). Hofmann *et al.* (371) developed a quantitative procedure for the estimation of saturated, monoethenoid, and branched chain fatty acids in small samples of bacterial lipides. Kenaston *et al.* (372) compared the various methods for determining fatty acid oxidation produced by ultraviolet irradiation. A modified peracid process for making epoxy compounds from polyenic acid esters has been published (373). Schlenk *et al.* reported studies on protection of autooxidizable lipide substances by formation of adducts with urea, thiourea, deoxycholic acid, α-cyclodextrin, and starch (374, 375). The distribution of mono- to hexaenoic fatty acids in human plasma and blood cells has been determined by spectrographic methods [Evans *et al.* (376)].

Metabolic studies.—In a series of important studies Klenk and his collaborators (377 to 380) separated by counter-current distribution and identified a number of C_{20}- and C_{22}-polyene fatty acids from the glycerophosphatides of human and animal brain (377, 378) and liver (379). The type of the trienoic, tetraenoic, pentaenoic, and hexaenoic acids was in agreement with the hypothesis that all C_{20}- and C_{22}-polyene acids of liver and brain phosphatides possess the di-vinyl-methane structure and that they are predominantly of the linoleic or linolenic acid type and formed from them by addition of 2 carbon units (380). Similar conclusions were reached by Steinberg *et al.* (381) who fed to mice and rats methyl linoleate-1-C^{14}, while Klenk (380) studied the uptake of acetate-1-C^{14} by rats and rat liver slices.

Iongh (382) reports that the C_{22}-polyenic acids from human brain phosphatides are biologically active in protecting rats from fatty acid deficiency, thus providing confirmatory evidence for the "6,9 (term.)-hypothesis" of

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Thomasson (383, 384) and indicating that the central nervous system should also be affected by deficiency. The previously reported biological activity of the triple unsaturated stearolic acid, Δ^9-octadecanoic acid, could not be confirmed (385). The importance of configuration for biological activity is again re-emphasized by the finding of Privett, Pusch & Holman (386) that *cis*-9,*trans*-12-linoleate has no curative effect on fat-deficient rats; however, the *trans* double bonds derived from the acid are stored in tissue lipides.

Tove *et al.* (387) confirmed that the unsaturated fatty acids of the dietary fat exert a major effect on the fatty acid composition of the depot fat of the rat, but they claim that the nature of the dietary protein is capable of altering, to some extent, the depot fat composition. Blomstrand (388) confirmed, on feeding linoleic-1-C^{14} acid to rats, that the major part of the absorbed acid could be recovered in the thoracic lymph; no difference in the route of absorption of saturated and unsaturated fatty acid was noticed. From liver deposition studies with C^{14}-glycerol in fat-deficient rats, Cider (389) concluded that essential fatty acids appear to have a lipotropic effect and influence the normal liver glyceride turnover.

Diets low in fat result in significant decreases in the di- and tetraenoic fatty acids and an increase of trienoic acid in the serum of infants (390) and of dogs (391). Basnayke & Sinclair (392) describe the increased permeability and the effect of vitamin B_6 deficiency in rats suffering from an essential fatty acid deprivation. Hydrogenated peanut oil, coconut oil, or lard had a deleterious effect on growth of deficient rats (393, 394) and of guinea pigs (395). Deuel *et al.* (396) and Privett *et al.* (386) reaffirm that linolenic acid is considerably less effective biologically than linoleic acid, but addition of linoleic to linolenic acid has a "sparking" effect on the latter by increasing its potency above that of linoleic acid. A similar synergistic effect is observed for the protective action of unsaturated acids against x-irradiation (397). Male rats deficient in essential fatty acids showed early symptoms of degeneration of the seminiferous tubules of the spermatogenic tissue (398), particularly when hydrogenated peanut oil had been included in the diet (399). No correlation between the polyenic acid level in serum with parakeratosis in pigs was observed (400).

There is growing evidence that the intake of unsaturated fatty acids influences profoundly the cholesterol level in blood and liver; deficient rats showed an increased cholesterol content in the liver and a decrease in plasma (394). The hypercholesterolemic response to cholesterol feeding in rats on a sulfur-deficient diet was largely prevented by dietary fat containing a high level of essential fatty acids and by supplementation with methionine (401). In chicks addition of 10 per cent peanut oil resulted in a decreased cholesterol content in liver, but feeding of large amounts of cholesterol caused a reversed effect, namely the fat-deficient chicks showed a lower liver cholesterol than the supplemented ones (402). These differences may be partly attributable to the effect of fats on absorption and on the esterase activity of the intestine (403, 404). The most convincing proof of an interrelationship between cholesterol and essential fatty acids is provided by the observation of Peifer & Holman (405) that inclusion of 1 per cent cholesterol in the diet precipitated the fat deficiency within two to four weeks, while comparable deficiency symptoms on fat-free diets without cholesterol were observed only after three months. In a preliminary report Hammond & Lundberg (406) observed somewhat increased values of tri- and hexaenoic fatty acids in blood plasma of atherosclerotic subjects; the significance of these findings is partially marred by the fact that few samples were examined and because of the inherent inaccuracy of the method, but the results would point to a metabolic error common with situations when a diet low in essential fatty acids had been given (391, 392).

Enzyme relationships.—Unsaturated, more than saturated fatty acids, increased the level of alkaline phosphatase in the intestinal mucosa (407). The lipoxidase-catalyzed oxidation of polyunsaturated fatty acids differs to a certain extent from the autooxidation of these acids in that the enzymic process produces, apart from hydroperoxides, significant amounts of dimeric products with conjugated diene systems which are optically active [Lundberg (408)]. The hemin- and plant lipoxidase-catalyzed oxidation of linoleate and its inhibition by antioxidants has been further investigated (409, 410). A cortisone-copper complex catalyzed the autooxidation of linoleic acid; other 17-hydroxycorticosteroids were inactive (411). Ultraviolet-irradiated methyl esters of linoleate and linolenate inhibited a number of enzyme systems of rat liver mitochondria (412).

Bacterial metabolism.—In continuation of previous studies on the bacteriostatic effect of polyunsaturated fatty acids on Gram-positive bacteria, it was noted (196) that the inhibitory activity increased proportionally to the number of double bonds; the "all"-*cis* isomers were active, but *trans* isomers were not, thus simulating effects found in mammals by Privett *et al.* (386). Linoleate-adapted strains of *Lactobacillus casei* were also insensitive to oleic acid, partially to arachidonic, but not to linolenic acid. A physicochemical mechanism altering the permeability of the bacterial cell membrane has been proposed [Kodicek (196)]. A major constituent of the bacterial lipides appears to be the monounsaturated C_{18} component, *cis*-vaccenic acid, which is present in "bound" form [Hofman & Tausig (413, 414)].

Miscellaneous.—Tria (415) restated that the lipide-soluble unknown factor from liver which has a variety of metabolic effects is not identical with any of the known vitamins.

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The absorption of calcium from the intestine

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From studies of loss of radiocalcium from the intestinal lumen of rats it was well known that the absorption of calcium occurs mainly in the proximal small intestine (Harrison & Harrison, 1951; Nicolaysen, 1951). A similar observation was made in the pig (Moore & Tyler, 1955). In the chick, according to Coates & Holdsworth (1961), however, calcium absorption appeared to occur over the whole length of the small intestine.

The recent advances in the use of everted sacs provided a useful tool to study the absorption of calcium. Thus Schachter & Rosen (1959), Rasmussen (1959) and others could show that active transport of calcium against a concentration gradient can be achieved in everted sacs of the rat duodenum, but not of ileum, and the capacity of this transport mechanism decreased with distance from the pylorus. Anaerobic conditions, low temperature, dinitrophenol or other enzyme poisons effectively inhibited the active transport (Schachter & Rosen, 1959; Schachter, Dowdle & Schenker, 1960; Wassermann, 1960). Additions of glucose or fructose improved greatly the active absorption, while mannose, galactose, amino acids or some fatty acids had no marked effect (Schachter & Rosen, 1959).

The age of the animals influenced the active transport in everted sacs; younger rats had a more efficient transport mechanism than older animals (Kimberg, Schachter & Schenker, 1961). These effects were more marked when a low-calcium diet was given (Kimberg *et al.* 1961).

When rats were given a vitamin D-deficient diet the active transport in everted sacs was significantly diminished and could be restored by in vivo dosing with vitamin D (Schachter & Rosen, 1959; Dowdle, Schachter & Schenker, 1960; Kimberg *et al.* 1961). The response to vitamin D had a certain lag period of about 8–10 h. A biological assay based on the technique of everted sacs has been suggested by Schachter, Kimberg & Schenker (1961). Addition of vitamin D in vitro did not restore the impaired absorption, but recently Schachter, Kowarski & Finklestein (1964) reported that cholecalciferol placed directly into in vivo loops of rat duodenum in vitamin D-deficient animals increased markedly the subsequent (5 h later) uptake

of calcium by slices of the duodenal loop in vitro. This finding is interpreted that vitamin D acts directly on the intestinal tissues without prior activation in another organ. The avid uptake of [^{14}C]vitamin D_3 in intestinal tissue of deficient rats after intracardial injection would support the same conclusion (Kodicek, Cruickshank & Ashby, 1960; Kodicek, 1965).

While the effect of vitamin D on the active transport mechanism is generally accepted, Harrison & Harrison (1965) have postulated that the vitamin has also an influence on the permeability of the intestinal mucosa to calcium, as measured with in vitro preparations. Another interesting possibility has been advanced recently by Holdsworth (1965), namely that calcium is normally pumped out of the cell into the intestinal lumen and that vitamin D inhibits this process.

Parathyroid hormone appears to enhance the active transport of calcium across the intestinal mucosa. Rasmussen (1959) reported that after parathyroidectomy the ability of everted intestinal sacs to transfer calcium from the mucosal to the serosal side was somewhat reduced. Studies on plasma calcium concentrations following parathyroidectomy or administration of parathyroid hormone in absence or presence of vitamin D led several authors to the conclusion that a satisfactory vitamin D status was necessary to obtain the beneficial influence of parathyroid hormone on active calcium transfer (Rasmussen, DeLuca, Arnaud, Hawker & von Stedingk, 1963; Harrison & Harrison, 1964). On the other hand, Toverud (1964*a*,*b*) concluded that the effect of parathyroid hormone in the rat appeared to be independent of vitamin D.

Effect of vitamin D on absorption of ^{45}Ca from ligated loops of rat small intestine

We have been interested in studying the transport of calcium across the mucosa of the rat small intestine by using ligated loops in vivo. Although many variable factors are introduced by this technique, the effects observed may be considered more physiological than those studied in vitro by the everted sac technique.

Solutions of ^{45}Ca, from 0·31 to 125 mM, together with 50 mg glucose, were introduced into ligated loops of the proximal or distal half of the small intestine of rats, starved overnight (Kodicek, Cruickshank, DeLuca & Raisz, 1964). The transport of ^{45}Ca from the proximal or distal portions of the small intestine was measured

Table 1. *Absorption of ^{45}Ca from ligated loops of small intestine of rachitic rats*

Initial Ca concentration in loop		Percentage absorption from proximal loop		Percentage absorption from distal loop	
µg/ml	mM	Rachitic rats (%)	Rats given vitamin D supplement* (%)	Rachitic rats (%)	Rats given vitamin D supplement* (%)
12·5	0·313	57	85	34	58
25	0·625	60	72	29	58
50	1·25	62	74	27	56
500	12·5	36	62	20	36
5000	125	7	8	6	6

*600 i.u. vitamin D_3, twice by mouth in previous 48 h to rachitic rats; absorption time: 60 min (Kodicek *et al.* 1964).

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by determining the remaining ^{45}Ca in the loop after a suitable time interval and at the same time the appearance of radioactivity in the ash of the tibia was followed.

It was found (Table 1) that, after 1 h, in the rats given vitamin D supplement, 74–85% in the proximal loop and 56–58% in the distal loop had been transported, when the concentration of calcium in the loop was below that of ionized calcium in the blood (about 1·25 mM). At higher concentrations the percentage absorption was progressively reduced, until the absorptions were only 8 and 6%, respectively, when 125 mM ^{45}Ca (5 mg/ml) had been instilled. At the lowest concentration of ^{45}Ca (0·31 mM) the movement of the ions was against a concentration gradient, in both intestinal segments, suggesting an active transport mechanism. These findings are in agreement with those reported by Schachter & Rosen (1959) except that the latter authors found the activity mainly in the duodenal and jejunal portions.

The rate of absorption of 500 μg ^{45}Ca after 1 h was greater in the proximal segment than in the distal segment (Fig. 1), but after 4 h the same proportion of calcium had been transported from both segments of the small intestine.

When similar investigations were made on young rachitic animals, which were given for 2–3 weeks the high-calcium, low-phosphorus diet of Steenbock & Black (1925), the absorption of calcium was significantly lowered at calcium concentrations at or below 12·5 mM. At the highest concentration (125 mM) the deficiency of vitamin D had no effect on the calcium absorption (Table 1). Thus at high concentrations of calcium in the intestinal lumen the transfer was not vitamin D-dependent.

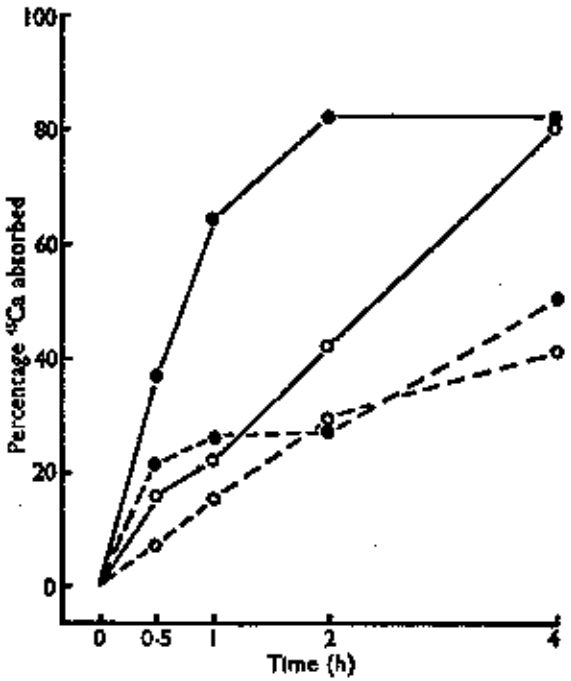

Fig. 1. Time course of absorption of 500 μg ^{45}Ca from loops of upper or lower parts of small intestine of rachitic rats (— — —) and rats given vitamin D_2 supplement (———) (Kodicek *et al.* 1964). ●, loop of upper half of small intestine; ○, loop of lower half of small intestine.

When the transport of ^{45}Ca was followed for 4 h, the rachitic animals showed a significantly lowered transport over the whole period, irrespective of the intestinal segment used.

The decreased transport efficiency of the deficient loops could be restored by oral administration of vitamin D_2 if the dose had been given at least 12 h before the loop experiment. A shorter period of 5 h had no effect nor had the instillation of the vitamin directly into the loop.

In previous experiments (Kodicek, 1963) it has been shown that up to about 5 h are required for the maximum accumulation of [^{14}C]vitamin D_3 in the mucosal cells of the small intestine, so that an appreciable lag period still remains during which there is no apparent effect of the vitamin. It might be presumed that during this period biochemical changes occur, such as elaboration or induction of a carrier system, which eventually increase the transport of calcium.

Effect of parathyroidectomy on ^{45}Ca absorption from ligated loops of rat small intestine

In young stock rats, parathyroidectomy, performed 7 days previously, decreased the ^{45}Ca absorption in both proximal and distal segments of the small intestine (Kodicek *et al.* 1964). This was particularly marked when the concentration of ^{45}Ca in the loop was either below or above that of the presumed ionized calcium level in blood (Table 2). The effect of the removal of parathyroid on lowering the serum calcium was already marked after 11 h, but the decrease in calcium absorption from the intestinal loops occurred later, appearing consistently after 72 h.

Table 2. *Absorption of ^{45}Ca from ligated loops of small intestine of stock rats after parathyroidectomy* (*Kodicek* et al. 1964)

Initial Ca concentration in loop		Percentage absorption from proximal loop		Percentage absorption from distal loop	
		PTX-ed* rats	Sham-operated rats	PTX-ed* rats	Sham-operated rats
μg/ml	mM	(%)	(%)	(%)	(%)
5	0·125	59	84	30	59
50	1·25	71	71	40	70
500	12·50	38	47	10	36

*Parathyroidectomized: the animals were operated on 7 days before the loop experiment; absorption time, 60 min.

The results cited above are in agreement with the findings of Cramer (1963) who investigated the absorption of calcium from Thiry-Vella intestinal loops in the dog.

These results are consistent with the conclusions that vitamin D and possibly parathyroid hormone have an effect on the active transport of calcium. Recently, Thompson & DeLuca (1964) and Hosoya, Fujimori & Watanabe (1964) suggested that phospholipids might be involved in the calcium transfer in the intestine that is vitamin D-dependent. In our laboratory, no such direct connexion between phospholipids and vitamin D-dependent calcium transfer could be found (Lawson & Kodicek, 1966, in preparation).

The recent reports on the effect of actinomycin D inhibition on calcium transport (Eisenstein & Passavoy, 1964; Zull, Czarnowska-Misztal & DeLuca, 1965, 1966; Schachter & Kowarski, 1965; Norman, 1965) have focussed the attention on a possible involvement of a DNA- or RNA-linked protein synthesis which might be affected by vitamin D. The effect of vitamin D on the incorporation of amino acids into bacterial proteins (McQuillen & Kodicek, cited by McQuillen, 1958) after the incorporation had been inhibited by linoleic acid might be a model system simulating a similar reaction (Kodicek, 1965).

How far mitochondria or microsomes and calcium release from these subcellular particles, as postulated by Engström & DeLuca (1962), De Luca, Engström & Rasmussen (1962) and Sneligrove & DeLuca (1965), is involved in the active transport of calcium across the cell has to await further research. The recent discovery of a protein in chick intestinal mucosa, which binds calcium and appears to be induced by administration of vitamin D, might possibly be connected with the carrier system for calcium (Wassermann & Taylor, 1963, 1966; Taylor & Wasserman, 1965).

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Chem. + Eng. News, Oct. 21. 1968

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Vitamin D efficacy against rickets

DEAR SIR:

While as primary producers of vitamin D for use by the food, feed, and pharmaceutical industries of Dr. De Luca and his associates, as reported in C&EN Aug. 5 (page 21), we are concerned about misunderstandings concerning the significance of his discoveries.

The compound 25-hydroxycholecalciferol may indeed prove to be useful in the treatment of refractory rickets, and we hope that it is. But we are confident that vitamin D as we know it will continue to be the preferred form of material for ordinary uses. Remember that all official bodies have defined vitamin D by its effects, not by the chemical structures of various forms of active materials. So it is grossly incorrect to write "Vitamin D will not cure rickets," as you have done in the table of contents for the issue containing the account of the discovery of the Madison investigators. If the editorial staff of C&EN can be misled so easily, think how the common person may well get the idea that vitamin D has no value. The industry is plagued by enough misinformation and attempts to regulate us in a manner that seems designed to cast aspersions on what we have been doing in making vitamin D preparations available. I do not think that your readers would be misled; they are too well informed to permit this interpretation. However, it would be appreciated, and might help us, if you could manage to print a simple statement to emphasize that "Vitamin D does cure rickets."

L. E. KOVACS

President, Vitamins, Inc.
Chicago, Ill.

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Effects of Vitamin D on Phosphate Transport and Incorporation into Mucosal Constituents of Rat Intestinal Mucosa*

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SUMMARY

The effects of vitamin D on transport of inorganic phosphate into and across rat intestinal mucosa *in vitro* were studied, and the results used to interpret observations on incorporation of ^{32}P-labeled phosphate into mucosal lipid, protein, and nucleic acids. Vitamin D given to intact rats increases phosphate transport *in vitro* selectively in the direction mucosa to serosa. The sterol *in vivo* acts directly in the intestine with no prior activation in other organs. $^{32}P_i$ transport is via restricted channels in the mucosa, as indicated by the observation that the specific radioactivity of transported ^{32}P-labeled phosphate is miscible with approximately one-third or less of the mucosal P_i pool. Because of this compartmentalization specific radioactivities of individual mucosal precursor pools of $^{32}P_i$ are difficult or impossible to estimate. Consequently, effects of vitamin D on incorporation of ^{32}P-labeled phosphate into mucosal constituents cannot be interpreted definitively in terms of rates of synthesis. The relative effects of vitamin D on calcium as compared to phosphate transport in various segments of intestine, and other evidence, suggests that the sterol influences separately these two intestinal transport mechanisms.

Vitamin D is required for normal intestinal absorption of calcium (1), and in recent years a number of investigators have obtained evidence that the sterol is required to maintain a specific calcium transport mechanism in the small intestine (2-5). The calcium transport is via an active cation pump which can be shown directly *in vitro* with a specific calcium electrode (6) or inferred from experiments *in vivo* (7). Vitamin D is also required for optimal transport of inorganic phosphate by rat intestinal segments *in vitro* (8, 9).

Inasmuch as the biochemical basis for the preceding effects remains unknown, a report by Thompson and DeLuca (10) that prior treatment with vitamin D markedly increases incorporation of $^{32}P_i$ into the phospholipids of rat intestinal mucosa *in vitro* was of considerable interest. Unfortunately, the authors did not estimate the specific radioactivity of $^{32}P_i$ in the mucosal precursor pool. Hence their results do not distinguish between absolute increases in rates of biosynthesis of phospholipid-phosphate *versus* increased transport of $^{32}P_i$ into the mucosa and higher specific radioactivity of the precursor pool. The present experiments were designed, therefore, to examine these alternatives. In addition, a number of features of the phosphate transport mechanism were studied further in relation to vitamin D and to calcium transport. It was established that vitamin D acted directly in the intestine to increase phosphate absorption, and biochemical evidence was obtained to show that the transport occurs across restricted channels in the mucosa.

MATERIALS AND METHODS

Animals—To induce vitamin D deficiency, weanling albino male rats, 40 to 50 g, of the Sherman strain, were maintained on the U.S.P. Rachitogenic diet No. 2 (Nutritional Biochemicals) for 4 to 5 weeks. Cages were shielded from light. Vitamin D repletion was with 20,000 i.u. of vitamin D_3 given subcutaneously in ethanol-propylene glycol (1:5) 18 hours before experiments. Rats on the diet above, as well as normal males, 100 to 200 g, maintained on a stock laboratory diet (*ad lib.*), were fasted for 18 hours in metabolism cages with free access to water before experiments.

Gut Sac Experiments—In typical experiments from five to seven rats were killed and a single everted sac, approximately 5 cm long, was prepared from the proximal duodenum or the midjejunum (or both) of each rat as previously described (2). The serosal compartments were filled with 0.5 ml of incubation medium composed of Krebs-Ringer bicarbonate (12) containing 20 mM fructose and no phosphate or $CaCl_2$. Groups of five to seven sacs were suspended together in flasks containing 20.0 ml

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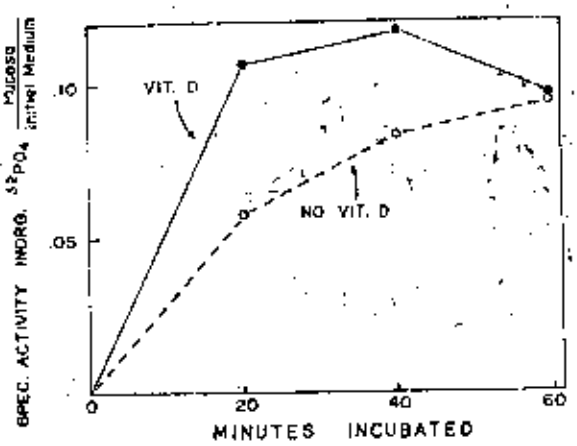

FIG. 1. Effect of vitamin D on specific radioactivity of $^{32}P_i$ in duodenal mucosa. Groups of five to seven duodenal sacs were incubated as described in the text with ^{32}P-labeled phosphate added to the mucosal medium alone initially. Mucosa was subsequently scraped from the underlying coats, homogenized, and P_i precipitated with magnesia mixture. Values plotted are means for three experiments and are expressed relative to the specific radioactivity of the initial mucosal medium. Range of values was ±12.5% or less.

of the incubation medium. Potassium phosphate, pH 7.4 (final concentration 1.2 mM), trace quantities of $NaH_2{}^{32}PO_4$, and $CaCl_2$ (final concentration 1.6 mM) were added to the medium bathing the mucosal surfaces, and the sacs were then agitated gently at 37° in an atmosphere of 95% O_2-5% CO_2 with constant gassing. At appropriate times sacs were removed, drained, and the mucosal tissue was scraped from the underlying coats with a spatula (11). Mucosal scrapings were weighed and homogenized in 5 volumes of ice-cold 145 mM NaCl-4 mM KCl.

Chemical Estimations—To estimate the specific radioactivity of $^{32}P_i$, samples of medium or homogenate were treated with 3% TCA[1] for 5 min at 2–5° and centrifuged for 5 min in a clinical centrifuge. The supernatant solutions were immediately neutralized with 1.0 N NaOH and samples were taken for precipitation of inorganic phosphate by magnesia mixture (13). The final precipitates were washed, dissolved in 10 N H_2SO_4, and aliquots were taken for estimation of phosphate by the method of Fiske and Subbarrow (14). Samples were also added to Bray's solution (15) and the ^{32}P counted in a liquid scintillation spectrometer. (Corrections for quenching, which amounted to less than 5%, were not necessary.) For transport studies, 5% TCA extracts of the media were prepared and phosphate was estimated as above (14); calcium was estimated by titration with EDTA in the presence of murexide (3). For incorporation studies, phospholipids were extracted from samples of mucosal homogenates with chloroform-methanol by the method of Folch, Lees, and Sloane Stanley (16). Extracted phospholipids were digested by heating in 11 N H_2SO_4, with addition of 30% hydrogen peroxide, and samples of the digest were taken to estimate ^{32}P, as described above, and total P_i (14). Mucosal homogenate was also fractionated into TCA-precipitable, nucleic acid, and protein fractions as described by Schneider (17). The fractions were finally digested, and ^{32}P and P_i were estimated as described above.

Transport Parameters—Transfer of phosphate or calcium was expressed, as described previously (18), as net uptake (micromoles per gut sac) at the mucosal surface (M_{net}) and net transfer to the serosal surface (S_{net}), positive in sign when net transfer was toward the serosa and negative when net transfer was in the opposite direction. In addition, M_{net} and S_{net} were each resolved into two unidirectional transfers of opposite sign (18), M_{net} into m_+ (transfer from mucosal medium to mucosa) and m_- (the reverse), and S_{net} into s_+ (transfer from gut wall to serosal medium) and s_- (the reverse). To estimate m_+, sacs were incubated with $^{32}P_i$ and stable P_i in the mucosal medium alone initially, and m_+ over a time interval was calculated as (total $^{32}P_i$ uptake at the mucosal surface)/(mean specific radioactivity of $^{32}P_i$ in mucosal medium over the time interval); m_- was calculated from the relation, $M_{net} = m_+ - m_-$. To estimate s_-, gut sacs were incubated with radioactive and stable phosphate in the serosal medium alone initially, and s_- was calculated as (uptake of $^{32}P_i$ at the serosal surface)/(mean specific radioactivity in the serosal medium); s_+ was calculated from the relation, $S_{net} = s_+ - s_-$. Phosphate transport was also expressed as a final concentration ratio for $^{32}P_i$ or stable P_i serosal medium to mucosal medium.

[1] The abbreviation used is: TCA, trichloracetic acid.

RESULTS

Incorporation of Inorganic Phosphate—In initial experiments the effects of vitamin D on the specific radioactivity of mucosal inorganic phosphate, the precursor pool for incorporation into phospholipid and other constituents, was examined. Everted sacs of duodenum prepared from vitamin D-deficient and -repleted rats were incubated in Krebs-Ringer bicarbonate (see "Materials and Methods") with $^{32}P_i$ in the mucosal medium alone initially. At various intervals five or more sacs were removed, drained, and the mucosa was scraped off and homogenized. Specific radioactivity of the mucosal $^{32}P_i$ was estimated after precipitation with magnesia mixture (13) and expressed relative to the initial values in the mucosal medium. Results were similar in each of three experiments, and the mean values are plotted in Fig. 1. After 20 min of incubation, the specific radioactivity in the vitamin-treated segments was approximately twice that in the depleted ones. Thereafter, values for the deficient sacs approached and finally equalled those for repleted intestine at approximately 60 min. The mean values for specific radioactivity in the 0- to 60-min interval were equal to the final values at 60 min times 0.93 for vitamin-repleted or 0.64 for vitamin-deficient segments. These factors, estimated from the curves in Fig. 1 and reproducible within 12%, were subsequently used to calculate the mean specific radioactivity of the precursor pools in the following experiments.

To determine the incorporation of ^{32}P-labeled phosphate into mucosal phospholipids, duodenal sacs prepared from depleted and treated rats were incubated for 1 hour as described above, the mucosa was scraped, homogenized, and samples were taken for extraction of phospholipids and for precipitation of inorganic phosphate. Both ^{32}P and total phosphate were estimated in these fractions, and the results of four experiments are summarized in Fig. 2. The total pool of mucosal phospholipid phosphate was identical, within experimental error, for vitamin-depleted and -treated segments. In agreement with Thompson and DeLuca (10), incorporation of ^{32}P-labeled phosphate into phospholipid, not corrected for specific radioactivity of the precursor pool, was increased by vitamin D in each experiment, with a mean increment of 32.0%. (In eight further observations similar

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results were obtained for an over-all significance of $p < 0.01$.) However, the incorporation of total phosphate into phospholipid, calculated as ($^{32}P_i$ incorporated)/(mean specific radioactivity of mucosal $^{32}P_i$), was in each experiment decreased by vitamin D ($p < 0.01$). When expressed in absolute units (micromoles of phosphate incorporated per g of mucosa per hour), vitamin D decreased the incorporation by 31.9% (range, 24.5 to 40.2%), and when expressed as a fraction of the mucosal lipid phosphate pool, vitamin D decreased incorporation by 31.2% (range, 18.7 to 42.7%). Very similar results were obtained in parallel observations with everted sacs of jejunum. Jejunum differed from duodenum only in more rapid uptake of $^{32}P_i$ from the ambient mucosal medium, both by vitamin D-deficient and -repleted segments (see below).

Effects of vitamin D on incorporation of ^{32}P-labeled phosphate into mucosal constituents other than phospholipid were studied with duodenal sacs incubated for 1 hour as described above. Mucosal homogenates were then prepared and phospholipid, total TCA-precipitable material, total nucleic acid, and protein fractions were obtained (see "Materials and Methods"). The incorporation for each fraction was expressed as the ratio (specific radioactivity ^{32}P-labeled phosphate in fraction)/(mean specific radioactivity of $^{32}P_i$ in mucosa). Vitamin D consistently lowered the ratio for all fractions studied, with mean decreases of 31.8% (range, 23.8 to 42.8, four experiments) for lipid phosphorus, 51.1% (range, 49.8 to 57.1, five experiments) for TCA-precipitable material, 44.5% (range, 19.5 to 66.6, four experiments) for nucleic acids, and 44.8% (range 26.7 to 62.7, four experiments) for proteins. The effect of vitamin D in lowering these ratios was significantly greater for TCA-precipitable material than for lipid phosphorus ($p < 0.01$). Although the results could be interpreted to mean that vitamin D decreases the rate of incorporation of phosphate into major constituents of intestinal mucosa, two further lines of evidence suggest an alternative explanation. Incorporation of ^{14}C-uridine into mucosal nucleic acids and of ^{14}C-L-leucine into mucosal proteins were examined with duodenal gut sacs from vitamin-deficient and -repleted rats incubated with the ^{14}C-precursors added to the mucosal medium. No significant effect of the vitamin either on mucosal uptake or incorporation of ^{14}C-uridine or ^{14}C-leucine was found in three experiments. Evidence will be described below which indicates that $^{32}P_i$ transport dependent on vitamin D occurs via restricted channels in the intestinal mucosa. If nonequilibrating or partially equilibrating pools of P_i exist in the mucosa, values for the over-all specific radioactivity of mucosal $^{32}P_i$ are clearly not valid for specific precursor pools. No conclusions concerning effects of vitamin D on rates of incorporation of phosphate seem justified, therefore, until methods are available to assay the specific precursor pools.

Vitamin D and Phosphate Transport.—Prior investigations have shown that optimal intestinal transport of phosphate in vitro is dependent on vitamin D, occurs maximally in jejunal segments, and has the features of an active transport (8, 9). Inasmuch as vitamin D also influences calcium transport, it was pertinent to ask whether the vitamin has two separate actions or only one, and whether the phosphate and calcium transfer mechanisms are coupled to each other. Accordingly, initial experiments were designed to explore similarities or differences in the action of vitamin D on calcium as compared to phosphate transport. A proximal duodenal and midjejunal everted sac was prepared from each of five to seven vitamin D-deficient or

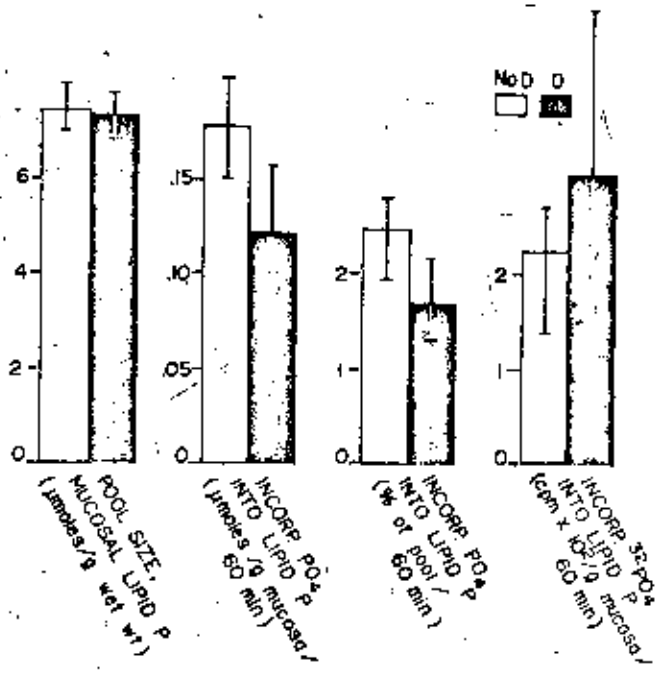

FIG. 2. Effects of vitamin D on incorporation of $^{32}P_i$ into duodenal mucosal phospholipids. *Heights of bars* indicate mean values for four experiments and *vertical lines* indicate range of values. Groups of five duodenal sacs (vitamin-deficient or -repleted) were incubated in each experiment and lipid phosphate and ^{32}P were estimated as described in the text. Incorporation of $^{32}P_i$ is shown by *bars on the right;* incorporation of phosphate corrected for the mean specific radioactivity of mucosal $^{32}P_i$ is shown by the *middle set of bars.*

-repleted rats. In six experiments, groups of sacs were incubated with potassium phosphate, $^{32}P_i$, and $CaCl_2$ in the mucosal medium alone initially; net transfer of phosphate and calcium to the serosal surface (S_{net}) and uptake and unidirectional transfer of phosphate at the mucosal surface (M_{net}, m_+, m_-) were estimated as described under "Materials and Methods." In three additional experiments, groups of duodenal sacs from deficient and repleted rats were incubated with potassium phosphate and ^{32}P-labeled phosphate added initially to the serosal medium alone. S_{net}, s_+, and s_- for phosphate were determined, and the results of all nine experiments are summarized in Table I. Similar to its effects on calcium transport (19) vitamin D increased net uptake of phosphate at the mucosal surface and net transport to the serosal surface, and the effects resulted entirely from increases in m_+ and s_+ with no significant change in m_- or s_-. Thus, as for calcium, the vitamin acted specifically on transfers in the direction mucosa to serosa. (Net movement of phosphate at the mucosal surface was out of the tissue, as previously reported (20), in both vitamin-deficient and -repleted segments. Net uptake into mucosa has, however, been observed in the presence of elevated concentrations of K^+ (5).) The effects of the vitamin on calcium *versus* phosphate transport differed markedly, however, in jejunal as compared to duodenal segments. Thus the vitamin-dependent increments in S_{net} for phosphate and calcium, respectively, were +0.60 and +0.50 μmole per gut sac for duodenum (ratio Δphosphate to Δcalcium = 1.2), whereas the corresponding values for jejunum were +0.84 and +0.12 (ratio Δphosphate to Δcalcium = 7.0).

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TABLE I

Effects of vitamin D on phosphate and calcium transport across duodenal and jejunal gut sacs

Gut sacs were prepared and incubated as described in the text. Transport parameters are defined under "Materials and Methods."

Transport mechanism	Transfer across duodenal sacs				Transfer across jejunal sacs			
	Vitamin D-deficient (a)	Vitamin D-repleted (b)	(b) − (a)	p	Vitamin D-deficient (a)	Vitamin D-repleted (b)	(b) − (a)	p
	moles/sac/hr				*moles/sac/hr*			
For phosphate								
m_+	0.73	2.06	+1.33	<0.01	1.86	2.88	+1.02	<0.05
m_-	3.78	4.03	+0.25		3.46	3.46	0	
M_{net}	−3.05	−1.97	+1.08	<0.01	−1.60	−0.58	+1.02	<0.05
S_{net}	+1.21	+1.81	+0.60	<0.01	+1.44	+2.25	+0.81	<0.05
For calcium								
S_{net}	+0.43	+0.93	+0.50	<0.001	+0.29	+0.41	+0.12	<0.05
For phosphate								
s_+	1.16	1.47	+0.29	<0.001				
s_-	0.68	0.73	+0.05					
S_{net}	+0.48	+0.74	+0.24	<0.01				

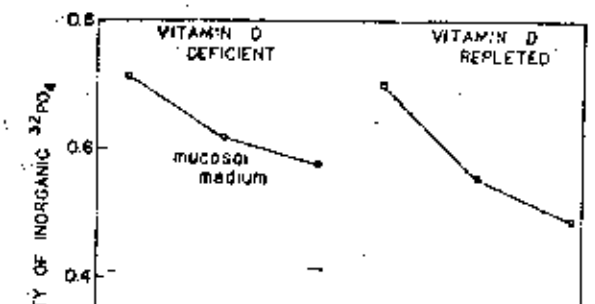

FIG. 3. Effects of vitamin D on specific radioactivity of $^{32}P_i$ in three compartments across duodenal segments *in vitro*: mucosal medium (○—○), mucosa (○- - -○), and serosal medium (●- - -●). Values are means of the three experiments described in the text for Fig. 1 and are expressed relative to the initial specific radioactivity in the mucosal medium in each experiment. Range of values was ±11.0% or less.

The results seem best explained by separate actions of the vitamin on two distinct transport mechanisms. Additional evidence that the mechanisms are distinct includes the following. Phosphate transport was greater in jejunum as compared to duodenum (Table I), both in vitamin-deficient and -repleted rats, and significant differences were noted in m_+ ($p < 0.001$), M_{net} ($p < 0.001$), and S_{net} ($p < 0.02$); calcium transport, in contrast, is consistently greater in duodenum (Table I and References 2 and 3). Under standard incubation conditions, *e.g.* Table I, M_{net} in duodenum is negative for phosphate but positive for calcium (3). Prior studies have shown that marked variations in phosphate concentration influence calcium transport across duodenal sacs *in vitro* very little (20).[2] Finally, the effects of calcium on phosphate transport were studied by incubating groups of duodenal sacs from vitamin-deficient and -repleted rats with 1.2 mM potassium phosphate, trace $^{32}P_i$ and with or without 1.6 mM $CaCl_2$ added to the mucosal medium alone. In these experiments no significant effect of added calcium on either M_{net}, m_+, or S_{net} for phosphate was observed with either vitamin-depleted or -repleted segments.

Channelized Phosphate Transport.—In the experiments illustrated in Fig. 1 the specific radioactivity of $^{32}P_i$ was estimated at various times in the mucosa and in the media bathing the mucosal and serosal surfaces of the everted duodenal sacs. Sacs from vitamin-deficient and -repleted rats were incubated with P_i and ^{32}P-labeled phosphate added initially to the mucosal medium alone. The values for relative specific radioactivity in all three compartments are illustrated in Fig. 3. Throughout 60 min of incubation, values for the mucosal medium, although declining, remained considerably above those in the other compartments. In vitamin-repleted sacs values for mucosal tissue were approximately constant in the 20- to 60-min intervals, whereas those for the serosal medium increased progressively to exceed the mucosal tissue values by approximately 80% at 1 hour. The results indicate that $^{32}P_i$ transported across the mucosa was miscible with only a portion of the tissue P_i, i.e. phosphate transport was via restricted channels. Results with the vitamin-deficient sacs were similar except that the specific radioactivity of $^{32}P_i$ in the serosal medium exceeded that in the mucosal tissue by only 24% at 1 hour. The fraction of mucosal P_i which is miscible with $^{32}P_i$ transported across the mucosa can be calculated as A_m/A, where A_m is the mean specific radioactivity of $^{32}P_i$ (counts per min per gmole) over a given time

[2] An increase in calcium transport across duodenal sacs *in vitro* on addition of P_i to the medium has been described by Bellows, Forte, and Salzman (21). Their results are not directly comparable to the present studies inasmuch as they used older rats weighing 400 g. We have demonstrated (3) that calcium transport is quite depressed in these older animals, and the active transport is often not detectable. The effects of phosphate might then result from complexing of calcium ion with resultant changes in passive permeability and over-all flux rate.

mucosal tissue, and A is the corresponding value for specific radioactivity of $^{32}P_i$ transported across the mucosa. Whereas A_m can be determined directly, A can be estimated only indirectly by sampling the serosal medium. The specific radioactivity in the serosal medium is less than true A, because endogenous, nonradioactive P_i is transferred from mucosal cells, particularly early in the course of incubation, and probably from other cells of the gut wall. Moreover, some equilibration of transported ^{32}P-labeled phosphate with pools in the submucosal gut layers seems probable. Accordingly, two methods of approximating A were used. In the first, A was defined for the increment in $^{32}P_i$ transport dependent on vitamin D, according to the relation

$$A = (P_d - P_e) \Big/ \left(\frac{P_d}{A_d} - \frac{P_e}{A_e}\right)$$

Here P_d and P_e equal the $^{32}P_i$ (counts per min) transferred to the serosal surfaces of vitamin-repleted and -deficient sacs, respectively, incubated in parallel. (P_d and P_e were found to equal total $^{32}P_i$ in the serosal media by isotope dilution experiments. Aliquots of the media were mixed with large quantities of carrier inorganic phosphate and precipitated with magnesia mixture. The final specific radioactivity was estimated and indicated that essentially all the radioactivity was precipitable.) A_d and A_e equal the specific radioactivity of $^{32}P_i$ (counts per min per μmole) in the serosal media of the vitamin-repleted and deficient sacs, respectively. These calculated values of A should be independent of possible errors, *e.g.* equilibration with submucosal gut layers, common to vitamin-deficient and repleted segments. Values for the fraction of mucosal P_i miscible with transported $^{32}P_i$, *i.e.* A_m/A, where A_m was determined for vitamin-repleted sacs and A was calculated as above, ranged from 0.17 to 0.29 in four experiments (Table II). A second method of calculating A was based on the observation (Fig. 3) that the specific radioactivity of $^{32}P_i$ in serosal fluid increased progressively with time of incubation, indicating that dilution of transported $^{32}P_i$ was greatest in the early time periods. Consequently A was calculated for the increment in transport of $^{32}P_i$ with time, *i.e.*

$$A = (P_2 - P_1) \Big/ \left(\frac{P_2}{A_2} - \frac{P_1}{A_1}\right)$$

where subscripts 1 and 2 refer to serosal media obtained at successive time points, and P and A are defined in the preceding calculation. Values for A_m/A calculated for vitamin-depleted and -repleted segments ranged, respectively, from 0.30 to 0.68 and 0.22 to 0.33 in three experiments. Finally, Table II also lists values for A_m/A calculated for experiments in which net transport of ^{32}P-labeled phosphate was from serosa to mucosa. Thick sacs from vitamin-deficient and repleted rats were incubated with P_i and ^{32}P labeled phosphate added initially to the serosal medium alone. Total radioactivity transferred and the specific radioactivity of $^{32}P_i$ in the mucosal medium were estimated at several time intervals, and A was calculated by the latter method described above. In three experiments, the values for A_m/A ranged from 0.27 to 0.36. In summary, the foregoing results show that $^{32}P_i$ transported across the intestinal mucosa in either direction is miscible with only approximately 32% (mean of all values in Table II) of the mucosal pool of P_i.

Direct Action of Vitamin D on Phosphate Transport.—A direct effect of vitamin D added *in vitro* on calcium transport across vitamin-deficient intestinal segments has not been established. Nonetheless, an experimental method was devised (22) to show that the sterol acts directly in the duodenum to affect calcium transport, without prior activation in other organs. In the following experiments a similar method was used to show a direct intestinal action on phosphate transport. Rats depleted of vitamin D were anesthetized with ether, and two midjejunal segments, separated by 3 cm of gut, were used to prepare closed loops, each about 5 cm long. Loops were washed clean with isotonic NaCl and in half the animals adjacent loops were filled with 0.25 ml of a control solution, 0.001 M sodium taurocholate in 0.85% NaCl. In the remaining rats one loop was filled with the control solution and the other with the same containing 400 i.u. of vitamin D_3; vitamin was instilled into the proximal or distal loop alternately in successive animals. (Vitamin D_3 was added to the control solution in a minimal amount of ethanol, and the final preparation was a clear micellar solution. Identical amounts of ethanol were added to the solution for control loops.) Abdominal incisions were closed and rats awoke approximately 10 min after onset of anesthesia. After 5 hours the loops were removed, washed, everted, a single sac prepared from each, and groups of seven sacs were incubated *in vitro* to study $^{32}P_i$ transport from mucosa to serosa ("Materials and Methods").

If vitamin D acts directly in the intestine, a greater increment in phosphate transport should be observed in the jejunal loop filled with the sterol as compared to the adjacent loop in the same rat. If the vitamin must first be absorbed and its action initiated in another organ, both loops, where one has been filled with vitamin D, should show similar increments in transport

TABLE II

Fraction of mucosal pool of inorganic phosphate miscible with inorganic $^{32}P_i$ transported across duodenal segments in vitro

Values listed are for A_m/A, which is calculated and defined in the text. Values calculated from the increment in phosphate transport with vitamin D are listed as Δ vitamin D, and those calculated from the increment in net transport with time are listed as Δ time.

Direction of transfer	Mode of calculation	Time interval	Mucosal P_i miscible with transported $^{32}P_i$ (A_m/A)
		min	
Mucosa to serosa	Δ vitamin D	0–60	0.22
		0–60	0.20
		0–80	0.29
		0–80	0.17
	Δ time		
	Vitamin-deficient	40–80	0.46
		40–80	0.30
		20–60	0.68
	Vitamin-repleted	40–80	0.22
		40–80	0.29
		20–60	0.33
Serosa to mucosa	Δ time		
	Vitamin-deficient	30–60	0.27
		30–60	0.29
	Vitamin-repleted	30–60	0.36
		30–60	0.35

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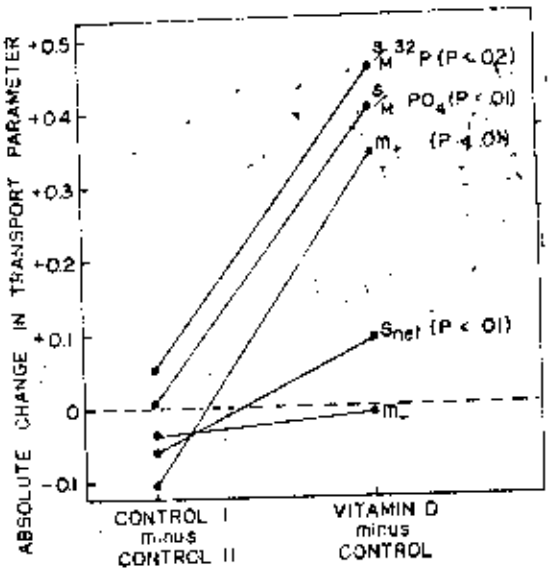

FIG. 4. Direct effect of vitamin D on phosphate transport across rat duodenum. Rats depleted of vitamin D were used to prepare adjacent jejunal loops as described in the text. In half the animals, both loops were filled with a vitamin D-free control vehicle. Subsequently, the loops were removed, tested for phosphate transport *in vitro*, and results are plotted to the *left (Control I minus Control II)* as absolute differences. In the remaining half of the animals, one loop was filled with vehicle alone and one with vehicle plus 400 i.u. of vitamin D_3. Absolute differences in phosphate transport between these loops *(Vitamin D minus Control)* are plotted on the *right*. Five rats were used in each group and results above are means for four experiments. *p* values for differences between *left* and *right-hand* values are indicated. Units for S_{net}, m_+, and m_- are micromoles per gut sac per hour. *S/M* refers to the final concentration ratio: serosal medium to mucosal medium.

as compared to rats receiving control solution alone. Fig. 4 illustrates the results of four experiments in which the effects on five parameters of phosphate transport were expressed as absolute differences between adjacent loops. Direct instillation of vitamin D significantly increased phosphate transport as compared with the adjacent control loop. The final concentration ratios serosal medium to mucosal medium for ^{32}P ($p < 0.02$) and phosphate ($p < 0.01$), as well as m_+ ($p < 0.01$) and S_{net} ($p < 0.01$) were increased, whereas m_- was not significantly affected, in accord with results above. Moreover, the control loops adjacent to vitamin D-filled segments showed no significant increase in phosphate transport as compared to control loops in rats given no vitamin D.

DISCUSSION

The experimental results indicate that $^{32}P_i$ transported across rat intestinal mucosa is miscible with only one-fifth to one-third of the mucosal pool of P_i, i.e. phosphate transport is via restricted channels or compartments. Before considering the implications for studies of incorporation of ^{32}P-labeled phosphate, one possible objection to this conclusion should be discussed. It might be argued that the relatively low values observed for specific radioactivity of $^{32}P_i$ in mucosal tissue, as estimated in homogenates by precipitation with magnesia mixture, results from breakdown of very labile esterified phosphate in the course of the chemical estimation. Although efforts were made to avoid this by using only 3% TCA, maintaining the acid in the mixtures at 2–5°, and working as rapidly as possible, the possibility of breakdown exists. However, the following quantitative considerations indicate that the results cannot be explained wholly in this way. In the experiments with vitamin D in the duodenal sac illustrated in Figs. 1 and 3, the average [illegible] over 1 hour of incubation for total ^{32}P-labeled phosphate in mucosal tissue (counts per min per g of mucosa) and the specific radioactivity of $^{32}P_i$ in serosal fluid and in mucosal tissue (counts per min per μmole of phosphate), respectively, are 25×10^3, 5×10^3, and 0.9×10^3. Thus the quantity of hypothetical labile ester phosphate in mucosa which would have to break down to dilute the specific radioactivity of serosal fluid to the value observed for mucosa is: $(25 \times 10^3 - 5 \times 10^3) - (25 \times 10^3 - 0.9 \times 10^3) = 23$ μmoles per g of mucosa. The small intestine contains a total of approximately 32 μmoles of phosphorus per g (23), of which 6.5 is nucleic acid phosphorus (23), 7.0 is lipid phosphorus (see above), and at least 5.0 is present as P_i in the experiments under discussion. The last value is calculated from the total counts per min of ^{32}P-labeled phosphate in the mucosa and the mean specific radioactivity of $^{32}P_i$ in the mucosal medium during incubation. Isotope dilution studies with magnesia mixture indicated that >90% of the mucosal ^{32}P-labeled phosphate was precipitable in these studies. Therefore, the mucosal phosphate available as hypothetical labile ester would not exceed $32 - (6.5 + 7.0 + 5.0) = 13.5$ μmoles per g, clearly inadequate to explain the results. Moreover, it seems improbable that all the mucosal phosphate not accounted for in the three pools listed above would be in the labile pool. We conclude, therefore, that the possibility of breakdown of tissue ester phosphate does not invalidate the general conclusion of channelization of phosphate transport, although it might influence the size of the channels as estimated above.

The present studies do not identify the site of the phosphate channels. They could presumably be within or between mucosal cells generally, or they might represent a specialized subpopulation of mucosal cells along the villus. Inasmuch as the tips of the villi might have preferential access to materials in the luminal bathing media, it is noteworthy that channelization of phosphate transport was also observed when the transport was from serosal surface to mucosal surface.

The demonstration that P_i is compartmentalized in the mucosa implies that the specific radioactivity of $^{32}P_i$ estimated in the mucosa as a whole need not be representative of individual precursor pools for incorporation into specific cellular constituents. We are unable, therefore, to conclude that vitamin D influences directly the rates of incorporation of ^{32}P-labeled phosphate into mucosal constituents. Although we can confirm the observation of Thompson and DeLuca (10) that vitamin D increases total incorporation of ^{32}P-labeled phosphate into phospholipid, our results indicate this could be secondary to increased mucosal uptake and a greater specific radioactivity of precursor ^{32}P-labeled phosphate. Indeed, vitamin D decreases the ratio of specific radioactivity in lipid-phosphorus to specific radioactivity in mucosal $^{32}P_i$ as well as the corresponding ratios for total TCA-precipitable material, nucleic acids, and proteins. The decreases can be ascribed to compartmentalized phosphate transport, i.e. the increment in mucosal ^{32}P-labeled phosphate with vitamin D is not necessarily available as precursor for incorporation. This seems reasonable, particularly for one of

that incorporation of ^{32}P-labeled phosphate into nucleic acid, protein, or total TCA-precipitable material was not influenced by the vitamin when expressed relative to tissue weight, and no differences in incorporation of ^{14}C-uridine or ^{14}C-leucine were noted. However, total incorporation of ^{32}P-labeled phosphate into lipid phosphate was increased by vitamin D when expressed relative to either tissue weight or incorporation into total TCA-precipitable material. Although in accord with an increased rate of synthesis of lipid phosphate, this observation, too, could result from channelized transport, on the reasonable assumption that the phosphate channels are lined with membrane containing phospholipid, *e.g.* the endoplasmic reticulum. Hence the increased mucosal ^{32}P, resulting from the action of vitamin D might yield additional precursor for phospholipid but not for nucleic acid synthesis.

Although the biochemical mechanism by which vitamin D increases intestinal transport of phosphate remains unknown, the effect seems to be distinct from that on calcium transport. Calcium transfer is a cation, active transport (3, 6) is maximal in the duodenum (3), and relatively independent of phosphate added *in vitro* (20). Phosphate transport is maximal in the jejunum (8) and relatively independent of calcium added to the lumen, as demonstrated above. Moreover, the effects of vitamin D on calcium as compared to phosphate transfer are approximately equal in duodenum, but much greater on phosphate in jejunum. Finally, recent studies have shown that the effect of vitamin D on calcium transport is associated with the appearance of a calcium-binding protein in intestinal mucosa of chickens (21) and rats (6). In rats the calcium-binding protein activity also correlates with the activity of the calcium transport mechanism as a function of distribution in the intestine (*i.e.* mainly in duodenum and quite low in more distal intestine), age of the rat, pregnancy, and level of dietary calcium (6). If the effect of vitamin D on phosphate transport were secondary to its effect on calcium transfer, as proposed by Harrison and Harrison (8), one might predict, on the basis of the distribution of the calcium-binding protein, rather little influence on phosphate transport in jejunum as compared to duodenum. Yet the observed effects are approximately equal in the two segments. The evidence of the prior authors (8) that EDTA decreases the increment in phosphate transport *in vitro* observed with vitamin D seems inconclusive, because the chelating agent is known to inhibit other intestinal transport mechanisms, *e.g.* uptake of the vitamin B_{12}-intrinsic factor complex (25), and may act unspecifically.

The present results demonstrate that the actions of vitamin D on calcium and phosphate transport are similar in at least two ways. In both mechanisms the sterol increases selectively the unidirectional transfer from mucosa to serosa, and the vitamin acts directly in the intestine without prior activation in another organ. Further studies are needed to determine if the action on phosphate transport, like that on calcium transfer, is associated with the biosynthesis or maintenance of a specific protein (or proteins) which may play an essential role in the mechanism.

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EXPERIMENTAL STUDIES ON THE INFLUENCE OF VITAMIN D UPON THE FEMALE SEXUAL FUNCTION

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Since Vitamin D resembles the steroid hormone, there is a conjecture that there is a relation between Vitamin D and the female sexual function. Based on this assumption, there are these reports: "the Estrongenic effect of Vitamin D", "the suspension of sexual cycle and the transformation of cells of pituitary gland caused by the lack of Vitamin D," and also "the symptoms resulting from cutting off the pituitary gland." Mr. Chinone of the same laboratory reported on both the atrophy of genitals and the lessening of pregnancy potential because of Vitamin D deficiency, and on the effect of Vitamin D on sexual functions. And Mr. Oniwa, who also belongs to the same laboratory, reported on the estrogen reinforcement effect of Vitamin D.

I have done studies on the effect of Vitamin D and will present my findings on the effect in white rats of the lack of Vitamin D on the sexual cycle and the morphological transformation of the pituitary gland, the adrenal glands, the ovaries, and the uterus.