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CALCIUM METABOLISM

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Calcium is one of the most abundant metal ions found in all living organisms. In the higher animal species its abundance can, for the most part, be attributed to its central role as a component of the structural elements or skeleton. Ninety-nine per cent of all the calcium in man is deposited in the skeleton as hydroxyapatite. However, the multiple non-structural functions of this divalent cation and its essentiality in some cases dictates that it must be very closely regulated. For example, calcium ions are essential for nerve conduction, for muscle contraction and relaxation, for the structural integrity of cell membranes and for the adhesion of one cell to another. It has other general functions, such as participating in the blood clotting mechanism and in certain enzymatic reactions. It is, therefore, reasonable that its concentration in the extracellular fluid and intracellular fluid must be closely regulated and that a sufficient and continuing external source of calcium must be assured. In terrestrial animals the infrequent intake of calcium has necessitated that the skeleton make available its calcium for the maintenance of extracellular fluid concentrations and that an efficient and regulated system of absorption be developed.

Although much had been known about calcium metabolism in higher animals, within the past decade a new wealth of information has become available in this area, primarily because of the discovery of calcitonin, a hormone believed to be involved in calcium regulation, and the discovery that vitamin D is converted to at least one hormone which functions basically in control of calcium metabolism. It will be the purpose of this review to bring into focus these new developments by

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presenting what is known concerning the mechanisms involved in calcium utilization and regulation.

Intestinal calcium absorption

Of basic importance to calcium physiology is the utilization of this important environmental element. Terrestrial animals which are infrequently exposed to calcium have developed efficient mechanisms for its absorption from the intestinal tract and efficient conservation mechanisms in the kidney. It is now known that calcium is absorbed throughout the small intestine and there has been some discussion as to which segment of the intestine accounts for the major portion of calcium utilized. It seems clear that the upper duodenum has the most efficient mechanism for calcium transport, but that the bulk of calcium is absorbed in the remainder of the small intestine, simply because of the length of time during which calcium is exposed to that portion of the intestine (Harrison & Harrison 1969, Schachter & Rosen 1959, Lengman 1963).

The role of vitamin D

The most important factor in determining the rate of calcium absorption in all segments of the intestine is vitamin D. Very recently, Harrison & Harrison have clearly shown that vitamin D stimulates intestinal calcium absorption in all segments of the small intestine and in the colon as well (Harrison & Harrison 1969). Although it had been believed for many years that vitamin D must function directly in the small intestine without metabolic change (Schachter et al. 1964, Kodicek 1960), recent evidence has clearly demonstrated that under physiologic circumstances it must be metabolically converted to an active form before it stimulates intestinal calcium absorption (DeLuca 1974). As shown in Figure 1, vitamin D, which can be derived either from the diet or from ultraviolet irradiation of skin, first progresses to the liver, where it undergoes hydroxylation on carbon 25 to produce 25-hydroxyvitamin D_3 (25–OH–D_3). This reaction occurs predominantly in the microsomal fraction of the endoplasmic reticulum in which NADPH + molecular oxygen is utilized to carry out this hydroxylation. Since this reaction is carbon monoxide insensitive, it is probably not a cytochrome P-450 dependent reaction (Horsting & DeLuca 1969). This reaction is feed-back regulated by the liver level of 25–OH–D_3 itself (Bhattacharyya & DeLuca 1973). Thus as the liver 25-OH-D_3 is utilized,

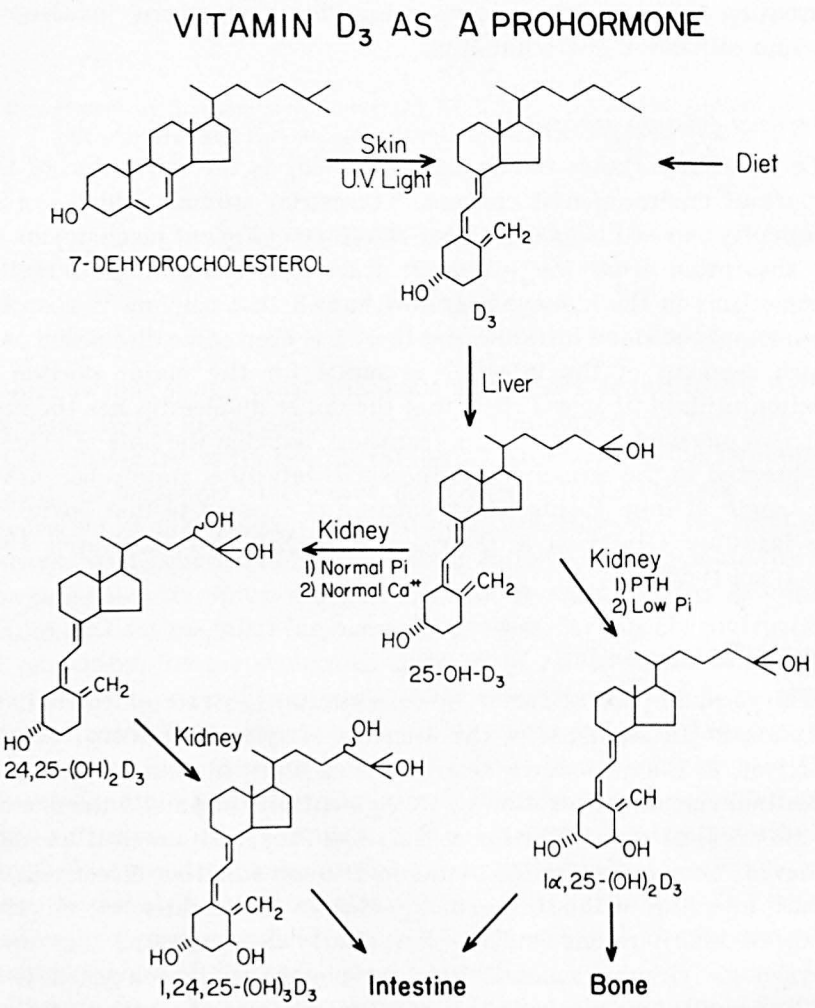

Figure 1. Diagrammatic representation of the biogenesis and metabolism of vitamin D_3.

its disappearance causes a release of the 25-hydroxylase to produce additional amounts of this important metabolite. The regulation is such that the circulating level of 25-OH-D_3 in the blood is of the order of 25–40 ng/ml. This level can be raised by the administration of large amounts of vitamin D to levels of as high as 1200 ng/ml (Haddad & Stamp 1974).

The 25-OH-D_3 is bound to an α^2-globulin of the plasma and is trans-

ported in this manner (Rikkers & DeLuca 1967, Edelstein et al. 1973, Belsey et al. 1974). The 25-OH-D_3 represents the most abundant metabolite of vitamin D found in the blood and may be considered the circulating form of vitamin D (Ponchon & DeLuca 1969). So far no direct function has been demonstrated for this metabolite and instead it progresses to the kidney where it undergoes hydroxylation on carbon 1 to form 1,25–dihydroxyvitamin D_3(1,25–$(OH)_2D_3$) (Holick et al. 1971, Fraser & Kodicek 1970). This hydroxylation occurs in the mitochondria of renal cells and requires molecular oxygen and internally generated NADPH (Gray et al. 1972, Ghazarian & DeLuca 1974). Very recently, clear evidence has been obtained that this reaction involves a specific cytochrome P-450 (Ghazarian et al. 1974). This hydroxylation reaction is strongly feed-back regulated, as will be described below.

The 1,25–$(OH)_2D_3$ has now been shown to be at least one of the metabolically active forms of vitamin D in the intestine (Boyle et al. 1972 a, Wong et al. 1972). The kidney is the sole site of production of this important metabolite, and hence renal tissue is essential for vitamin O to exert its characteristic effects on the intestine, bone and elsewhere. Recent work with radioactive 1,25-$(OH)_2D_3$ has provided evidence that this compound is probably not further metabolized before it functions in the small intestine and in bone (Frolik & DeLuca 1971, 1972). Because its production is regulated in a negative manner by serum calcium concentration working through the parathyroid gland, one must consider 1,25-$(OH)_2D_3$ a hormone derived from vitamin D whose primary function is in the utilization or mobilization of calcium from intestine and bone (Boyle et al. 1972 b, Garabedan et al. 1972).

Receptor hypothesis of 1,25-$(OH)_2D_3$ function in intestine

The 1,25–$(OH)_2D_3$ progresses to the intestine, where presumably it enters the columnar epithelial cells of the villi and appears in the nuclear "fraction" (Figure 2) (Haussler et al. 1968, Chen et al. 1970). So far no clear evidence has been provided which would prove conclusively that all of the 1,25–$(OH)_2D_3$ of the nuclear fraction is located in the nucleus itself. Autoradiography has not been performed to demonstrate this and by subcellular fractionation techniques, only crude nuclei have been prepared. This fraction contains not only nuclei, but also brush border membranes, other cell fragments, plasma membrane and debris. Attempts to obtain highly purified intestinal nuclei

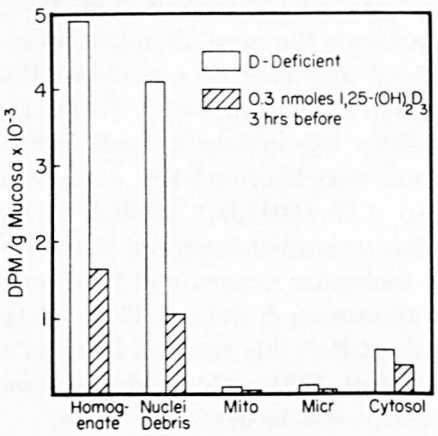

Figure 2. Subcellular location of ^{3}H–1,25–(OH)$_2$D$_3$ in intestinal mucosa. Vitamin D-deficient rats were divided into two groups. One group received 0.3 nmoles 1,25–(OH)$_2$D$_3$ intravenously in 0.02 ml ethanol 3 hours before receiving 0.3 nmoles ^{3}H–1,25–(OH)$_2$D$_3$ (▨) while the other group received the 0.02 ml of ethanol 3 hours before receiving the ^{3}H–1,25–(OH)$_2$D$_3$ (□). The rats were killed 3 hours after receiving the ^{3}H–1,25–(OH)$_2$D$_3$ and the cell fractions prepared. (Chen & DeLuca 1973, reproduced with the kind permission of the publisher.)

have been frustrated by technical difficulties. So far pure nuclei in yields of only 20 per cent can be obtained from intestinal mucosa and thus it is not possible to fully examine the question of whether the 1,25–(OH)$_2$D$_3$ which appears in the nuclear fraction is in fact associated with the nuclei. In any case, somewhere in the neighborhood of 80 per cent of administered 1,25–(OH)$_2$D$_3$ appears in the nuclear fraction. There have been attempts, primarily by Haussler and coworkers, to demonstrate that 1,25–(OH)$_2$D$_3$ acts in a fashion similar to that described for other steroid hormones; namely that it becomes associated with a 3.5 S cytoplasmic receptor which becomes converted to a larger receptor in becoming bound to chromatin of the nucleus (Brumbaugh & Haussler 1974 a, b). Although this concept is appealing, inasmuch as it would be consistent with existing dogma on the function of steroid hormones, proof that this mechanism actually occurs is far from complete. In rat intestine the only protein which has been shown to bind 1,25–(OH)$_2$D$_3$ is a 6S component (Knutson et al. 1975, Haddad & Birge 1971). This component prefers binding 25–OH–D$_3$ to 1,25–(OH)$_2$D$_3$. Recent work has demonstrated that the 6S protein does not bind 1,25–(OH)$_2$D$_3$ *in vivo* and hence cannot represent a receptor for 1,25–(OH)$_2$D$_3$ in the initiation of intestinal calcium transport (Knutson et al. 1975). The administration of saturating amounts of 1,25–(OH)$_2$D$_3$ *in vivo* will not prevent the binding of radioactive 25–OH–D$_3$ to the 6S component of cytoplasm incubated *in vitro*. On the other hand, administration of saturating amounts of 25–OH–D$_3$ will prevent the *in vitro* binding of radioactive 1,25–(OH)$_2$D$_3$ to the 6S component (Figure 3). Since 1,25–(OH)$_2$D$_3$ and not 25–OH–D$_3$ is the

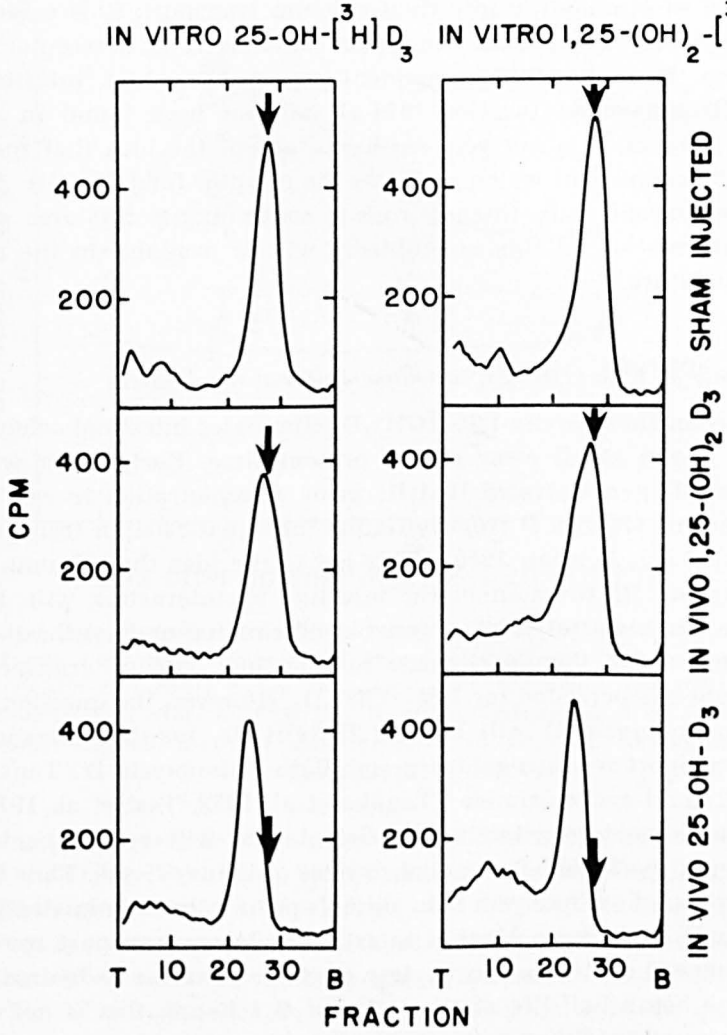

Figure 3. Intestinal mucosa cytosol binding of either ^3H–1,25–$(OH)_2D_3$ or ^3H–25–OH–D_3. Vitamin D–deficient rats were bilaterally nephrecotmized to prevent conversion of ^3H–25–OH–D_3 to ^3H–1,25–$(OH)_2D_3$. They were then injected with 0.05 ml ethanol, 0.05 ml ethanol containing 650 pmole 1,25–$(OH)_2D_3$ or the ethanol containing 650 pmole 25–OH–D_3. One hour later the intestinal cytosol was prepared and incubated with either 3 pmole ^3H–25–OH–D_3 or 3 pmole ^3H–1,25–$(OH)_2D_3$. Samples were then analyzed on a linear 10–30 per cent density gradient centrifugation system. The arrows indicate the position of sedimentation of ^3H–1,25–$(OH)_2D_3$ and ^3H–25–0H–D_3 binding protein (Knutzon et al. 1975). Note that predosing with 25–OH–D_3 but not with 1,25–$(OH)_2D_3$ prevents binding in vitro *to the 6S protein labeled with the arrow.*

active form in stimulating intestinal calcium transport, it is evident that this cytosolic component cannot be functioning as a receptor in this system. Since the 3.5S component reported in chick intestinal cytosol (Brumbagh & Haussler 1974 a) has not been found in rat intestinal mucosa, it is not yet possible to accept the idea that there exists a 3.5S component which serves as the receptor for 1,25-$(OH)_2D_3$, at least not in mammals. Intense work is continuing in this area and further information on this undoubtedly will be available in the not too distant future.

Possible role of 1,25-$(OH)_2D_3$ in transcriptional mechanism

The mechanism whereby 1,25-$(OH)_2D_3$ stimulates intestinal calcium transport is not at all clear at the present time. Early work with actinomycin D demonstrated that its prior administration to rats or chicks prevents vitamin D from initiating intestinal calcium transport (Zull et al. 1965, Norman 1965). This led to the idea that vitamin D must carry out its function in the intestine by interacting with the nucleus to stimulate retrieval of genetic information and synthesis of the proteins which then participate in intestinal calcium transport. This concept has persisted for 1,25-$(OH)_2D_3$. However, the question of whether actinomycin D will block 1,25-$(OH)_2D_3$ induced intestinal calcium transport remains controversial. Both actinomycin D (Tanaka et al. 1972) and cycloheximide (Tanaka et al. 1972, Tsai et al. 1973) when administered to vitamin D-deficient rats will bring about a diminution of the 25-0H-D_3-1α-hydroxylase of kidney tissue. Thus the administration of actinomycin D to animals prior to the administration of vitamin D could have blocked intestinal calcium transport by inhibiting renewal of the 1α-hydroxylase enzyme. Since the 1α-hydroxylase enzyme has a half-life of the order of 2–4 hours, this is not an unlikely possibility. When actinomycin D is given 2 hours prior to the administration of 1,25-$(OH)_2D_3$ to vitamin D-deficient rats, intestinal calcium transport is initiated virtually as well as in animals not given antibiotic (Tanaka et al. 1971) (Figure 4). To illustrate that the antibiotic was functional in these experiments, the action of 1,25-$(OH)_2D_3$ in mobilizing calcium from bone was completely blocked by that antibiotic in the same animals (Tanaka & DeLuca 1971). The question nevertheless remains as to whether actinomycin D does progress to the intestinal cells which are responsive to 1,25-$(OH)_2D_3$. Quite opposite results have been obtained in the chick in which actinomycin

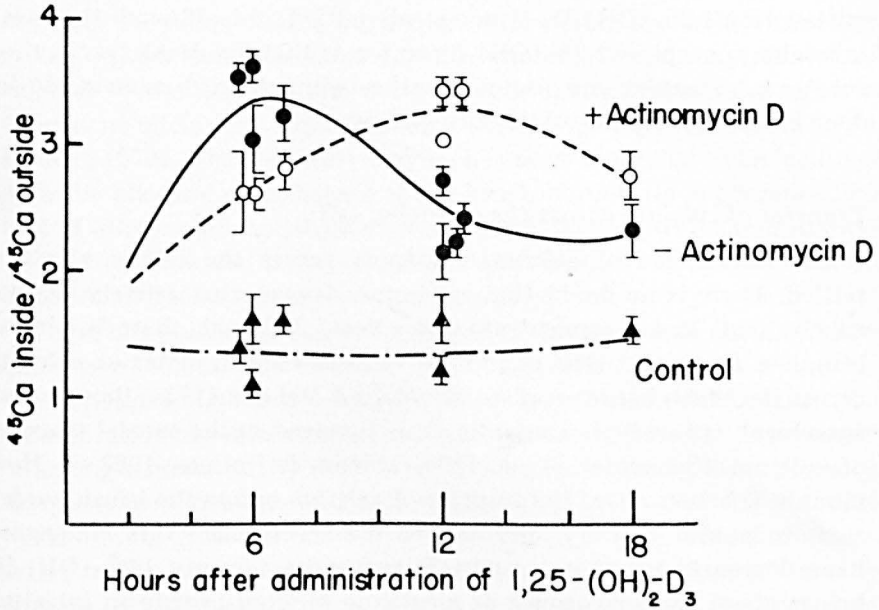

Figure 4. Intestinal calcium transport response to 1,25–$(OH)_2D_3$ of vitamin D-deficient rats given actinomycin D. Vitamin D-deficient rats were given 1 μg/g of actinomycin D 2 hours before they were given 650 pmole 1,25–$(OH)_2D_3$, while controls received either no 1,25–$(OH)_2D_3$ and actinomycin D or no actinomycin D. Intestinal calcium transport was measured in vitro at various times after 1,25–$(OH)_2D_3$ dosing.

D was given every 2 hours prior to and during the administration of 1,25–$(OH)_2D_3$ (Tsai et al. 1973). Although the intestinal calcium absorption response to 1,25–$(OH)_2D_3$ was blocked, such a protocol is extremely toxic, especially to chickens. The experience in our laboratory is that when actinomycin D is given at concentrations not toxic to chickens, the 1,25–$(OH)_2D_3$ stimulated intestinal calcium transport is not blocked (Omdahl & DeLuca 1973, and unpublished results). No matter which experiment proves to be correct, interpretation of mechanisms in whole animals with the use of toxic antibiotics is risky at best. Corradino has reported that the 1,25–$(OH)_2D_3$ stimulation of calcium binding protein in tissue culture is blocked by actinomycin D and α-aminitin (Corradino 1973). Likely induction is taking place in this system which uses embryonic intestine not yet developed, but it is a great extrapolation that this reflects the situation in a vitamin D-deficient chicken with a fully developed intestine. It must, therefore, remain that the mechanism whereby intestinal calcium transport is

initiated by 1,25-$(OH)_2D_3$ is not at all settled and although the most favorable concept of 1,25-$(OH)_2D_3$ action is that involving retrieval of genetic information and protein synthesis, much work remains to be done before this can be established clearly.

Transfer of calcium across the epithelial cell

The mechanism of calcium transport across the cell is also not settled. There is no doubt that calcium is transported actively against an electrical and a concentration gradient. Although there is almost complete agreement that vitamin D increases the transfer of calcium across the brush border surface (Omdahl & DeLuca 1973), there is less agreement as to whether vitamin D_3 is involved in the serosal transfer of calcium (Schachter et al. 1966, Martin & DeLuca 1969 a). How vitamin D brings about the transfer of calcium across the brush border surface is also entirely speculative. Wasserman and his colleagues have demonstrated that vitamin D and more recently 1,25-$(OH)_2D_3$ brings about the appearance of a calcium binding protein in intestine (Wasserman & Taylor 1966, Wasserman 1970). This protein, which has a molecular weight in the neighborhood of 24,000 in chicks (Wasserman 1975 a) and in mammals in the neighborhood of 8–12,000 (Drescher & DeLuca 1971 a), binds aproximately 4 calcium ions per molecule of protein. Although there is evidence that this protein is formed in the goblet cells (Taylor &Wasserman 1970), there is some reason to question that information. In any case, its participation in calcium transport is supported by the idea that calcium absorption rate is in a very rough way correlated with the amount of calcium binding protein present (Ebel et al. 1969, Wasserman 1970). Additionally the fact that the protein binds calcium and appears only after vitamin D also gives support to the idea of its participation in calcium transport. However, no experiment has satisfactorily demonstrated its clear involvement in the transfer process. Furthermore, there is no quantitative relationship between the amount of this protein and the calcium transport rate (Harmeyer & DeLuca 1969, Wasserman 1975 a). A most striking discrepancy is the time course of calcium binding protein levels in the intestine of chick after 1,25-$(OH)_2D_3$ administration. Twenty-four hours after 1,25-$(OH)_2D_3$, intestinal calcium absorption has diminished to rachitic levels while the calcium binding protein level remains high (Wasserman 1957 a, Norman 1974), suggesting some other controlling factor or that the calcium binding protein is a con-

sequence of, rather than a participant in, calcium absorption. Another system which could account for the vitamin D-induced transfer of calcium across the brush border surface is the activation of a calcium dependent adenosine triphosphatase (Martin et al. 1969, Melancon & DeLuca 1970). Although this enzyme has been suggested to be identical with the alkaline phosphatase of the brush border, its appearance and that of alkaline phosphatase does not correlate well with the initiation of intestinal calcium transport. Whether it is involved then in calcium transfer, therefore, remains controversial.

There has been much work regarding the biogenesis of chick calcium binding protein and the possible role of $1,25\text{-}(OH)_2D_3$ in initiating this synthesis. Although many attempts have been made to demonstrate the incorporation of radioactive amino acids in the calcium binding protein following stimulation by some form of vitamin D, the results have been disappointing (Drescher & DeLuca 1971 b, Bruns & Avioli 1975). Some work by MacGregor et al. (1970) has shown a stimulation of radioactive amino acid incorporation into the calcium binding protein by vitamin D, although it was necessary to manipulate the deficient chickens in such a way as to make interpretation of the experiment unclear. More recently Emtage et al. (1973) and Lawson & Emtage (1974) have studied the synthesis of calcium binding protein by polysomes isolated from the intestines of chicks given vitamin D as compared to rachitic chickens. They found that the polysomes from the vitamin D treated chickens synthesize calcium binding protein *in vitro*, whereas those from the deficient chicks do not. Calcium binding protein was detected by immunological methods and hence there seems little doubt that they are in fact measuring synthesis of the calcium binding protein or a related protein. Since chicks given vitamin D must synthesize the calcium binding protein backbone, it is not surprising to find that polysomes from vitamin D-treated chicks synthesize calcium binding protein. The key question must, therefore, remain: what is the sequence of events leading to the appearance of the calcium binding protein and what is its relationship to calcium transport?

The role of mitochondria in the transfer of calcium has also received a good deal of attention. There is no doubt that at calcium concentrations about 10^{-7} M, mitochondria will actively take up calcium (DeLuca & Engstrom 1961, Vasington & Murphy 1962, Rossi & Lehninger 1964). This is believed to play an important role in calcification and may well be an important mechanism to prevent intracellular damage by high calcium concentrations. It may also be a mechanism whereby calcium

can be transferred through the cell without damaging the enzymatic and metabolic machinery. Whether mitochondria actually participates as a calcium shuttle remains unknown, although it represents an interesting idea (Omdahl & DeLuca 1973).

Sodium ions are required for intestinal calcium transport (Martin & DeLuca 1969 b, Harrison & Harrison 1963). In contrast to the role of sodium in glucose and amino acid transport, sodium is required for the serosal transfer of calcium. Its participation is not defined, although a suggestion that it provides a concentration driven exchange mechanism for calcium at the basal-lateral membrane has been made (Omdahl & DeLuca 1973).

A new compound which has a marked effect on calcium absorption is found in extracts of a toxic plant: *Solanum malacoxylon* (Wasserman 1975 b). This substance, which apparently has a molecular weight of over 2000, initiates calcium transport (Wasserman 1975 b, Uribe et al. 1974) and stimulates the appearance of calcium binding protein (Wasserman 1975 b). This has led to the suggestion that it represents a plant source of $1,25\text{-}(OH)_2D_3$ (Wasserman 1974). However, unlike $1,25\text{-}(OH)_2D_3$, it does not mobilize calcium from bone (Uribe et al. 1974), is aqueous soluble and is stable in oxygenated aqueous solutions, all of which make it unlikely that it is $1,25\text{-}(OH)_2D_3$. Rather it may illustrate the lack of specificity of the intestinal calcium transport system.

The mechanism of intestinal calcium transport then remains relatively undefined and must be regarded as unsettled. Figure 5 demonstrates the variety of possible mechanisms involving the active form of vitamin D. The $1,25\text{-}(OH)_2D_3$ may or may not interact with a receptor in the cytosol and may or may not be transferred to the nucleus where it may initiate the transcription of specific genes coding for calcium transport proteins. The messenger then can be read out by the polysomes yielding the proteins which participate in intestinal calcium absorption. Alternatively, $1,25\text{-}(OH)_2D_3$ may function at the polysome level to initiate translation of functional proteins and calcium transport or it may function directly at the brush border membrane. It may also participate in the conversion of precursor protein to calcium transport proteins. The true transport protein may or may not be the calcium binding protein. In any case the brush border membrane is rendered permeable to calcium which upon entering the cell is sequestered perhaps by the mitochondria. The mitochondria can shuttle calcium to the basal-lateral membrane, where a deficient level of

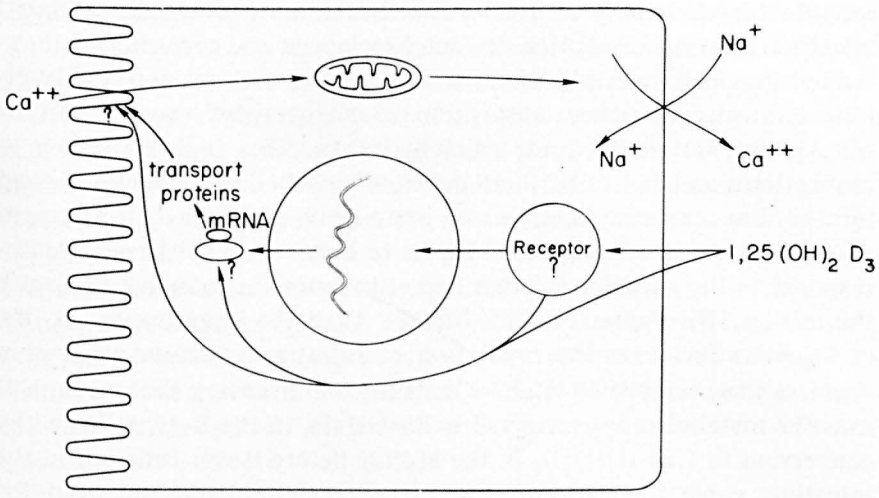

Figure 5. Diagrammatic sketch of known and hypothesized events in the 1,25-$(OH)_2D_3$ stimulation of intestinal calcium transport.

calcium brings about release of the intramitochondrial calcium. Calcium is then expelled through the basal-lateral membrane by a downhill sodium gradient which exchanges for the calcium. Alternatively, studies with lanthanum suggest that calcium may not even enter the cell during transfer and may be transferred from the brush border directly to the basal lateral membrane (Wasserman 1975 a). It is obvious that much remains to be learned concerning the mechanism of intestinal calcium transport and the role of vitamin D in initiating this process.

Regulation of intestinal calcium absorption by dietary calcium and phosphorus

A great deal of progress has been made in understanding the mechanism of regulation of intestinal calcium transport by dietary calcium and by the skeletal needs for calcium. Early work by Rottensten (1938), by Fairbanks & Mitchell (1936) and the definitive work of Nicolaysen et al. (1953) clearly demonstrated that animals or man placed on a low calcium diet have a markedly elevated rate of intestinal calcium absorption. On the other hand, animals or humans placed on a high calcium diet have markedly reduced rates of intestinal calcium absorption. Furthermore, it can be shown that animals or man requiring

calcium for skeletal growth or calcification have markedly elevated intestinal calcium absorption. In fact, Nicolaysen and coworkers demonstrated that prisoners placed on a low calcium diet develop highly efficient intestinal calcium absorption which persisted even after they were placed on an adequate calcium intake. This high efficiency of absorption persisted until their bones, which had been demineralized by the low calcium diet, were completely recalcified. Nicolaysen reasoned that some endogenous factor or hormone must be secreted in response to the need for calcium and stimulates the intestine to absorb the cation. Nicolaysen found further that the appearance of the endogenous factor in the regulation of intestinal calcium absorption requires the presenec of vitamin D. With the discovery that vitamin D must be metabolically converted to 25–OH–D_3 in the liver, followed by conversion to 1,25-$(OH)_2D_3$ in the kidney before it can function in the intestine, came the concept that dietary calcium and the need for calcium might well regulate the synthesis of the active form of vitamin D. The first clear evidence for this idea was provided by the work of Boyle et al. (1971) in which the effect of dietary calcium in rats both deficient and given vitamin D on the *in vivo* generation of 1,25-$(OH)_2D_3$ was studied. They demonstrated quite clearly that rats maintained on a low calcium diet synthesize large amounts of 1,25–$(OH)_2D_3$. As dietary calcium is increased, the synthesis of 1,25–$(OH)_2D_3$ is shut down. However, in the vitamin D-deficient animals, it is clear that the regulation of 1,25-$(OH)_2D_3$ synthesis from a single dose of ^3H 25-OH-D_3 by dietary calcium does not take place. It was discovered at the same time that as the synthesis of 1,25-$(OH)_2D_3$ is shut down by dietary calcium, another metabolite, 24,25-dihydroxy-vitamin D_3 (24,25-$(OH)_2D_3$) (Holick et al. 1972 b), is synthesized. The exact role of the 24,25–$(OH)_2D_3$ in calcium metabolism, if any, has not yet been established.

Utilizing the chicken, additional work has shown that kidney mitochondria will synthesize 24,25–$(OH)_2D_3$ when they are isolated from chickens maintained on a diet high in calcium plus vitamin D_3, whereas they will synthesize 1,25–$(OH)_2D_3$ when they are maintained on a diet low in calcium supplemented with vitamin D_3 (Omdahl et al. 1972, Knutson & DeLuca 1974). Furthermore, Omdahl & DeLuca (1971, 1972) have demonstrated that strontium inhibits intestinal calcium absorption by blocking the synthesis of 1,25–$(OH)_2D_3$. These results led to the concept that 1,25–$(OH)_2D_3$ might in part represent Nicolaysen's endogenous factor and might well be the message that the intestine receives in response to a need for calcium. Proof that this is the case

has been obtained in animals maintained on either 25–OH–D_3 or 1,25–$(OH)_2D_3$ as their sole source of vitamin D (Omdahl & DeLuca 1973, Ribovich & DeLuca, unpublished results). Chicks given the 25–OH–D_3 show a markedly elevated intestinal calcium absorption rate when a diet low in calcium is fed and a diminished rate when high calcium or strontium diet is fed. On the other hand, animals given 1,25–$(OH)_2D_3$ show a high rate of intestinal calcium absorption quite independent of dietary calcium and strontium (Omdahl & DeLuca 1973). Similar results have been obtained with the rat (Ribovich and DeLuca, unpublished results) (Table 1). Thus it seems that regulation of intestinal calcium transport by dietary calcium or the need for calcium is mediated by the regulation of 1,25–$(OH)_2D_3$ synthesis and that 1,25–$(OH)_2D_3$ itself may represent the endogenous factor Nicolaysen described (Boyle et al. 1972 b).

Table 1. Stimulation of intestinal calcium transport by low calcium and low phosphorus diets.

	Transport ratio ^{45}Ca serosal medium/^{45}Ca mucosal medium	
	Vitamin D_3	1,25–$(OH)_2D_3$
High calcium, normal phosphorus	2.3 ± 0.3	3.7 ± 0.5
Low calcium normal phosphorus	3.4 ± 0.1	3.8 ± 0.3
High calcium, low phosphorus	6.5 ± 0.5	6.7 ± 0.3

Rats were fed the various diets for 3 weeks. At 2 weeks they received a daily oral dose of either vitamin D_3 (25 ng) or 1,25–$(OH)_2D_3$ (12.5 ng) in 0.1 ml propylene glycol for 1 week. They were killed 24 hours after the last dose and intestinal calcium transport determined (Martin & DeLuca 1969 a).

Another important factor in the regulation of intestinal calcium absorption is the dietary phosphorus level. Carlsson (1953) initially demonstrated that rats on a low phosphorus diet have a markedly elevated intestinal calcium absorption rate. This was further established by the work of Morrissey & Wasserman (1971) and was confirmed by the work of Tanaka et al. (1973). Of great interest is that phosphate deprivation, even in the absence of parathyroid glands, brings about a stimulation of 1,25–$(OH)_2D_3$ synthesis (Tanaka & DeLuca 1973). Furthermore it could be shown that the intestines of

rats maintained on low phosphorus diets which develop high rates of intestinal calcium absorption also accumulate large amounts of 1,25-$(OH)_2D_3$ (Tanaka & DeLuca 1973). It seemed possible, therefore, that the regulation of intestinal calcium transport by phosphate deprivation might well be through the stimulation of 1,25-$(OH)_2D_3$ synthesis. However, in experiments in which rats were given 1,25-$(OH)_2D_3$ from exogenous sources, it is evident that they still show an elevated intestinal calcium absorption when they are given phosphate deficient diets (see Table 1). Thus the stimulation of intestinal calcium absorption by phosphate deprivation is not solely due to the regulation of 1,25-$(OH)_2D_3$ synthesis, but some other unknown factor or metabolic event must be involved. This has not as yet been determined.

Regulation of intestinal absorption by parathyroid hormone, calcitonin and glucocorticoids

The question of whether there are other hormones that might affect vitamin D metabolism can obviously be raised. Work by Garabedian et al. (1974) has shown that the 1,25-$(OH)_2D_3$ stimulated intestinal calcium transport does not require the presence of parathyroid hormone nor its activity enhanced by the presence of this peptide hormone. Furthermore, work being carried out by others with radioactive parathyroid hormone has shown that the intestine is not a target of parathyroid hormone action and the parathyroid hormone is not bound to that tissue (Zull & Repke 1972, Neuman, unpublished results). It therefore seems clear that the parathyroid hormone does not play a role directly on intestinal calcium transport. As will be shown in a later section, in response to the need for calcium, it is the parathyroid glands which sense the serum calcium concentration and secrete the parathyroid hormone. Parathyroid hormone in some unknown way proceeds to the kidney, where it stimulates the synthesis of 1,25-$(OH)_2D_3$. It seems clear then that the reported effects of parathyroid hormone on intestinal calcium transport are mediated by its role in the stimulation of the synthesis of the active form of vitamin D for that process. Therefore, chronic hypoparathyroidism is probably associated with reduced intestinal calcium absorption and an inappropriate response of the intestine to calcium deprivation (Avioli et al. 1974).

It is not at all certain to what extent calcitonin plays a role in the regulation of intestinal calcium transport. *In vitro* experiments using vascularly perfused intestine have shown that calcitonin does inhibit

intestinal calcium absorption (Olson et al. 1972). Work in intact animals, however, has not been uniform in this regard and it is uncertain to what extent calcitonin inhibits intestinal calcium transport *in vivo*. It seems likely that the major effect of calcitonin is on the bone rather than the gastrointestinal tract as far as calcium is concerned.

Finally some mention should be made of the glucocorticoids and their effect on intestinal calcium absorption. Harrison & Harrison (1960) demonstrated that cortisone could reduce intestinal calcium transport. This was taken as a mechanism which might reflect the glucocorticoids' ability to counteract vitamin D toxicity. Although this is probably not the case, the mechanism whereby the glucocorticoids can reduce intestinal calcium absorption is of some interest. It has been postulated that the glucocorticoids might interfere with vitamin D metabolism, thereby reducing the amount of $1,25\text{–}(OH)_2D_3$ reaching the intestine (Avioli et al. 1968). However, Kimberg and associates have provided data which indicate that corticoids reduce intestinal calcium transport in animals given exogenous $1,25\text{–}(OH)_2D_3$ (Kimberg et al. 1071). Furthermore, they failed to demonstrate any effect of the glucocorticoids on the metabolism of vitamin D to the $1,25\text{–}(OH)_2D_3$ (Favus et al. 1973 a, b). On the other hand, very recent work from Rasmussen's laboratory has shown that the glucocorticoids might well induce the conversion of $1,25\text{–}(OH)_2D_3$ in the intestine to a more polar but inactive metabolite (Carre et al. 1974). It seems strange that this was not observed by Kimberg and associates, but nevertheless might account for the glucocorticoid reduced intestinal calcium absorption. Only further investigation will provide the answer to this important question.

MOBILIZATION OF CALCIUM FROM BONE

Role of parathyroid hormone

Another important site is the mobilization of calcium from bone. There is a massive amount of literature which clearly illustrates that the parathyroid hormone, both *in vivo* and *in vitro,* will mobilize calcium from bone. The mechanism of this mobilization of calcium remains unknown in spite of a great deal of intensive investigation. Although attempts have been made to demonstrate that the parathyroid hormone brings about these changes by stimulating RNA and protein synthesis, these results are inconclusive (Park & Talmage 1968). In fact, it appears that the parathyroid hormone at least initially does not

require this mechanism to initiate mobilization of calcium from bone (Rasmussen et al. 1964). Although it seems clear that the parathyroid hormone stimulates osteoblast resorption, there is also evidence that resorption of bone takes place by osteocytes and also possibly by osteoblasts in response to this hormone (Belanger 1965). There is some evidence that this hormone stimulates mobilization of calcium from bone via activation of adenyl cyclase and the cyclic AMP mechanism (Wells & Lloyd 1967, Chase & Aurbach 1967). Although the parathyroid hormone is believed to function in the kidney in this fashion, the evidence in the bone is not as clear. The most likely possibility is that the parathyroid hormone in some way stimulates a calcium transport system which does not involve the synthesis of new protein.

Role of vitamin D

Another equally if not more important agent in the mobilization of calcium from bone is vitamin D and its metabolites. Carlsson and coworkers demonstrated quite early that vitamin D, even at physiologic doses, induces the mobilization of calcium from previously formed bone (Carlsson 1952). This has been confirmed by Nicolaysen & Eeg-Larsen (1956) and by work carried out in our own laboratory (Blunt et al. 1968). It is now clear that it is not vitamin D itself, but rather its active metabolite, 1,25–$(OH)_2D_3$, which brings about this mobilization (Holick et al. 1972, Reynolds et al. 1973, Raisz et al. 1972). The mobilization of calcium from bone in response to 1,25–$(OH)_2D_3$ is blocked entirely by the previous administration of actinomycin D, whereas if that antibiotic is given after the 1,25-$(OH)_2D_3$, the process is not inhibited (Tanaka & DeLuca 1971). It seems that there is more evidence to support the idea that in this system 1,25–$(OH)_2D_3$ may well initiate the process by stimulating the synthesis of specific messenger and specific proteins involved in the transport process. Work by Weber et al. (1971) has demonstrated that the bone may well accumulate 1,25–$(OH)_2D_3$ in the nucleus of the cells, which would be in support of such a mechanism.

The mobilization of calcium from bone has also been studied *in vitro* using isolated bone cultures by both Raisz et al. (1972) and Reynolds et al. (1973). In this system vitamin D_3 itself is totally inactive, whereas 25–OH–D_3 functions at concentrations of the order of 10^{-7} M (Figure 6) (Stern, DeLuca, Tanaka and Schnoes, unpublished results). However, 1,25–$(OH)_2D_3$ is the most potent form functioning as low as 10^{-12} M. The mechanism whereby 1,25–$(OH)_2D_3$ initaites the mobilization of

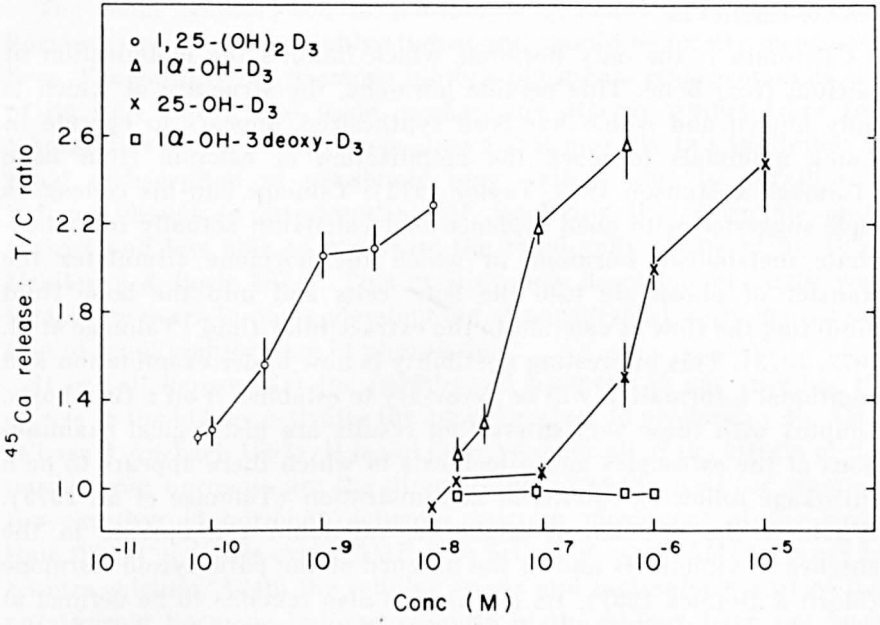

Figure 6. Resorption of embryonic bone by vitamin D metabolites and analogs in vitro. *Pregnant rats were given 250 μCi ^{45}Ca. Nineteen-day embryonic radii and ulnae from the fetuses were cultured such that one radius served as a control for the other radius from a single embryo. To one of these a metabolite at the given concentration was added while the control received the vehicle. The ratio of ^{45}Ca in the medium of experimental bone to that in the control was calculated and plotted (Stern, Tanaka, DeLuca, and Schnoes, unpublished results).*

bone in culture has not been closely studied. It is of some interest that in culture the 1,25–$(OH)_2D_3$ and the parathyroid hormone will initiate the mobilization of calcium independent of each other. On the other hand, *in vivo* it is well known that physiologic doses of either 1,25–$(OH)_2D_3$ or parathyroid hormone are required for this system to operate (DeLuca 1974, Rasmussen et al. 1963, Harrison et al. 1958). In the absence of either 1,25–$(OH)_2D_3$ or the parathyroid hormone, mobilization of calcium from bone is markedly diminished. The two agents, therefore, seem to operate in concert and the absence of either interferes with the process. The discrepancy between the results *in vivo* and those obtained *in vitro* are, therefore, of some concern. However, *in vitro* the mobilization of calcium is carried out in low phosphate medium. It may be that if the culture experiments are carried out at phosphate concentrations approaching normal, both agents must be present to elicit mobilization of calcium from bone.

Role of calcitonin

Calcitonin is the only hormone which inhibits the mobilization of calcium from bone. This peptide hormone, the structure of which is fully known and which has been synthesized, appears to operate in young mammals to block the mobilization of calcium from bone (Talmage & Munson 1972, Taylor 1972). Talmage and his colleagues have suggested with good evidence that calcitonin actually is a phosphate metabolism hormone in which the hormone stimulates the transfer of phosphate into the bone cells and into the bone fluid inhibiting the flow of calcium to the extracellular fluid (Talmage et al. 1972, 1973). This interesting possibility is now under examination and additional information will be necessary to establish it on a firm basis. Coupled with these very interesting results are histological examinations of the osteocytes and osteoblasts in which there appears to be a shrinkage following calcitonin administration (Talmage et al. 1975). Whatever the mechanism might be, calcitonin can operate in the absence of vitamin D and in the absence of the parathyroid hormone (Morii & DeLuca 1967). Its mechanism also remains to be defined at the cellular and molecular level.

RENAL REABSORPTION

A third and very important site of regulation of calcium in the blood is the kidney. It has been estimated that the kidney filters and reabsorbs on the order of 7 g of calcium/day. Virtually all of the calcium which is filtered is reabsorbed even in the absence of vitamin D and of parathyroid hormone (Bernstein et al. 1963, Gran 1960). However, there is clear evidence that parathyroid hormone does increase the renal reabsorption of calcium and there is some evidence that 1,25–$(OH)_2D_3$ also stimulates reabsorption of calcium (Steele et al. 1975). However, the effects of the 1,25–$(OH)_2D_3$ on this parameter have not been thoroughly studied. In any case, the existence of a calcium binding protein which appears in response to vitamin D has been demonstrated by Wasserman and his colleagues both by direct measurement and by fluorescent antibody methods (Taylor & Wasserman 1972). Thus although 99 per cent of the filtered calcium is reabsorbed in the absence of these agents it is evident that the remaining 1 per cent is reabsorbed much better when these agents are present. Much work remains to be done in this area and in regard to the well known effects of calcium and phosphate on their respective reabsorption mechanisms.

The renal reabsorption of phosphate in response to parathyroid hormone has been thoroughly studied and should be briefly mentioned here. The parathyroid hormone inhibits phosphate reabsorption as one of its well known and basic mechanisms. Recent results from two laboratories independently have shown that possibly this inhibition of renal reabsorption of phosphate may well be due to parathyroid induced change in intralumenal pH, rendering the phosphate more ionized and less able to penetrate the renal cells (Bank et al. 1974, Uchikawa & Borle 1974). This exciting new development might well signal a new era in our understanding of parathyroid hormone regulation of renal reabsorption of phosphate.

It is well known that the parathyroid hormone in carrying out its effects in the kidney activates the adenyl cyclase to produce cyclic AMP (Chase & Aurbach 1967). It is not clear whether all of the effects of the parathyroid hormone are the direct result of cyclic AMP or whether the parathyroid hormone activates calcium movement at the same time that it activates cyclic AMP. The action of cyclic AMP appears to require calcium. Again the cellular events and molecular events of the parathyroid hormone induced changes in the kidney have not been thoroughly studied. In any case, the kidney must be regarded as a major organ involved in the regulation of serum calcium and in fact is thought by many to be the major organ determining serum calcium concentration, a position which probably is extreme.

CALCIFICATION

Much new information has become available in the area of calcification. Although the mechanism which has been favored for many years, namely the catalyzed crystallization by nucleated collagen fibrils, has not been disproved, there has been, in addition, new evidence which suggests that cells which govern calcification accumulate calcium in vesicles and that the vesicles may be either of mitochondrial origin or independent of mitochondria. The vesicles are supposedly secreted into the matrix area and are involved directly in transporting calcium to the calcification sites (Anderson 1969, Anderson & Reynolds 1973, Slavkin et al. 1972). In fact there may even be hydroxy apatite crystals forming in the vesicles. The vesicles also contain enzymes which may well prepare the collagen surface for mineralization. This important new development in vesicles may also usher in a large number of new considerations regarding the mechanism and regulation of calcification.

Exactly how calcification is regulated by humoral agents, if at all, is unknown. However, this important mechanism must be considered in any integrated evaluation of calcium metabolism.

CALCIUM HOMEOSTASIS (Figure 7)

Having discussed the sites at which there is humoral regulation of calcium metabolism it seems important now to bring together these various sites in an integrated concept of the regulation of calcium metabolism. It is important to realize that there are two cell types which continually monitor serum calcium concentration; namely cells of the parathyroid glands and the c-cells of the thyroid or, in the case of lower organisms, the ultimobranchial bodies. In response to hypocalcemia, the parathyroid hormone is secreted. This hormone, which is 84 amino acids long, has two basic sites of action. The first is the kidney, where it stimulates renal reabsorption of calcium and phosphate diuresis. At the same time, however, it stimulates production of $1,25\text{–}(OH)_2D_3$ from $25\text{–}OH\text{–}D_3$ (Garabedian et al. 1972, Fraser & Kodicek 1973). The parathyroid hormone also goes to bone where, together with the secreted $1,25\text{–}(OH)_2D_3$ it functions to mobilize calcium from previously formed bone. This then returns calcium to the extracellular fluid. The $1,25\text{–}(OH)_2D_3$ which is formed in response to parathyroid hormone, in addition to mobilizing calcium from bone, stimulates renal reabsorption of calcium and most important, stimulates intestinal calcium absorption. This mechanism also restores serum calcium to normal, which then shuts off secretion of parathyroid hormone. When the serum calcium rises above the normal level of 10 mg/100 ml, the c-cells react by secreting calcitonin. Calcitonin in an ill-defined manner proceeds to the bone and blocks mobilization of calcium, thus reducing serum calcium levels. It may also proceed to the kidney and to the intestine, where it may also block calcium utilization from the sources, although this is not established. Finally, phosphate deprivation will stimulate $1,25\text{–}(OH)_2D_3$ synthesis guite independently of any parathyroid hormone (DeLuca 1974) and, in addition, phosphate deprivation stimulates mobilization of bone mineral and stimulates intestinal calcium absorption (Baylink et al. 1971, Tanaka et al. 1972, Morrissey & Wasserman 1971). Thus phosphate deprivation by more than one mechanism will also elevate serum calcium concentration.

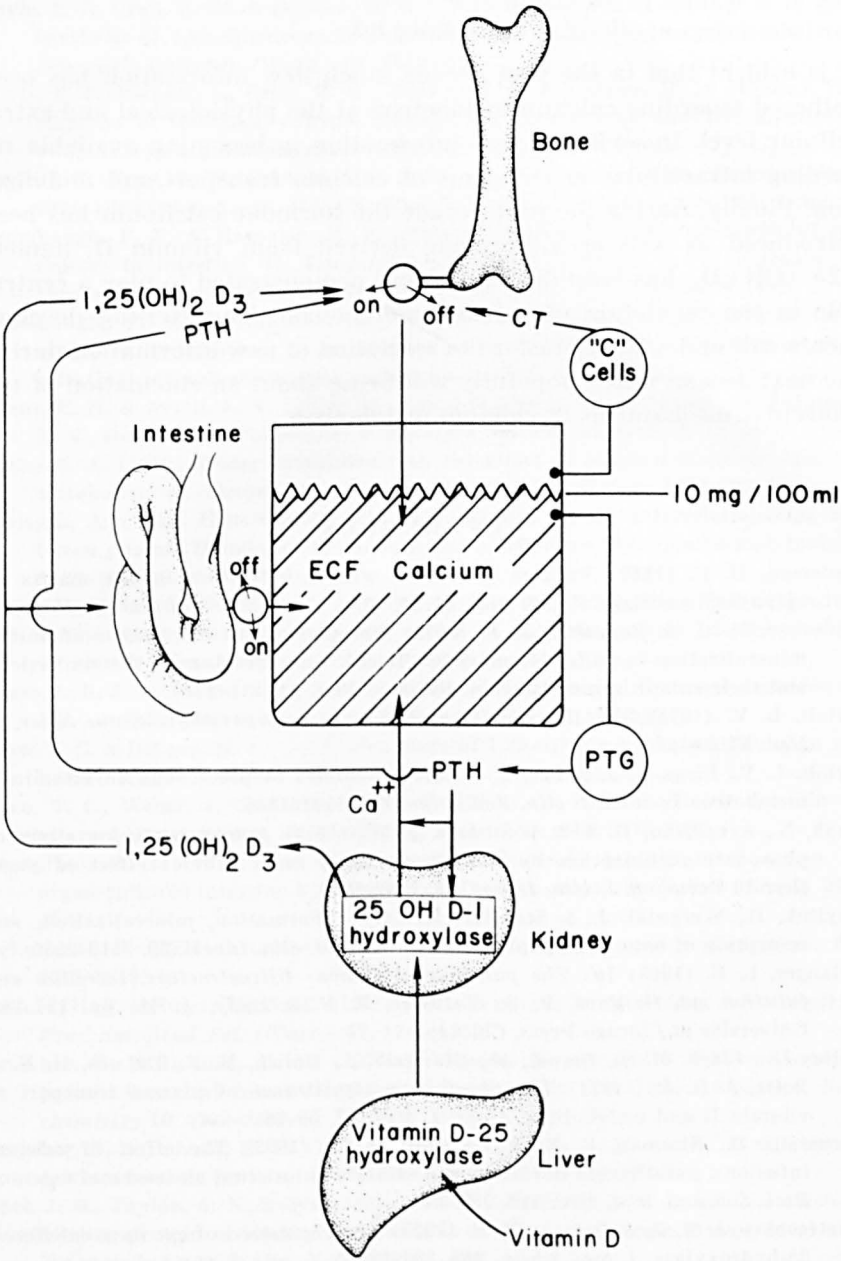

Figure 7. Diagrammatic representation of the humoral control of serum calcium concentration, taking into account the vitamin D system.

SUMMARY

It is evident that in the past decade much new information has been gathered regarding calcium metabolism at the physiological and extracellular level. In addition, new information is becoming available regarding intracellular mechanisms of calcium transport and mobilization. Finally, during the past decade the hormone calcitonin has been introduced as well as a hormone derived from vitamin D, namely 1,25-$(OH)_2D_3$, has been discovered and demonstrated to play a central role in the regulation of calcium metabolism. The exciting developments will undoubtedly foster the revelation of new information during the next decade which hopefully will bring about an elucidation of the molecular mechanisms of calcium metabolism.

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