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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

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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 8,600, 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[2] 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

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.[8] 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.[4] 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	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_3 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.[5] 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.*[6] 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[7] 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*		*Urine P*
	Ca	*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 ineffectivness 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

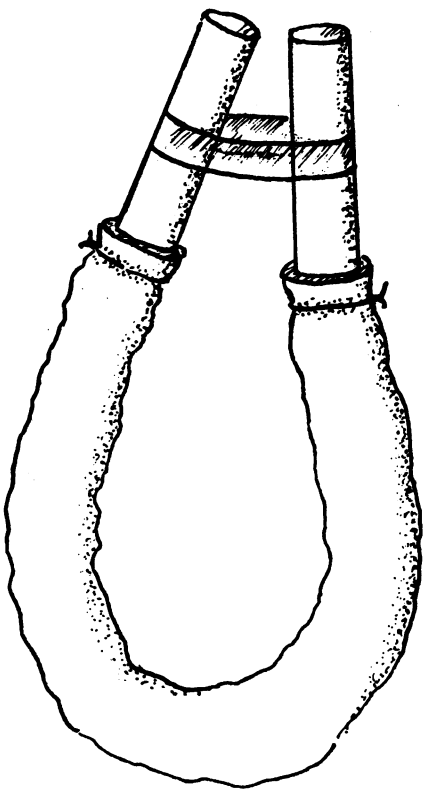

FIG. 1. Everted loop of rat small intestine preparatory to immersion in buffer solution. The mucosal surface is on the outside and solutes are transported across the mucosal layer and are collected in the fluid filling the serosal lined interior of the loop. (Taken from Harrison and Harrison.[13])

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.

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[12] 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[13] (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 mocosal 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

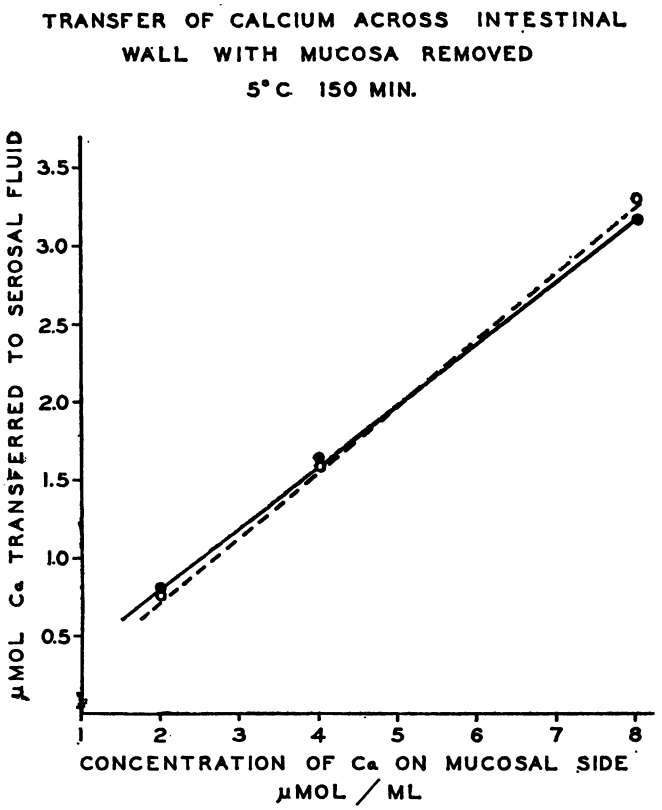

FIG. 2. Calcium transport in everted intestinal loops from which mucosa has been removed, incubated at 5° C. ●——● loops from vitamin D-treated rats, o- - - - o loops from vitamin D-deficient rats. Each point represents the average value of determinations on 4–8 loops. (Taken from Harrison and Harrison.[14])

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-

patible 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

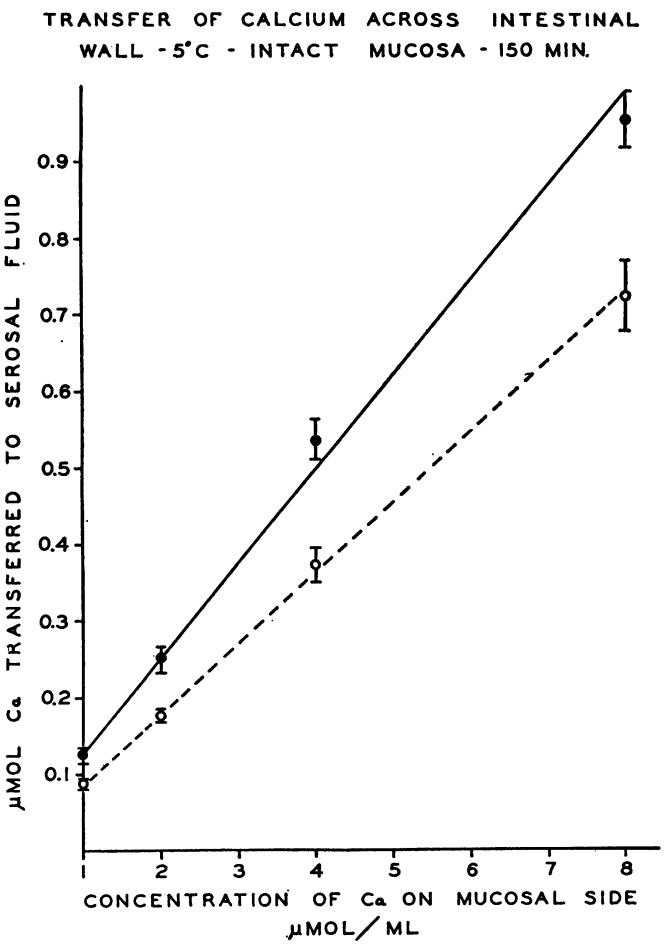

FIG. 3. Calcium transport in everted jejunal intestinal loops with intact mucosa incubated at 5° C. ●——● loops from vitamin D-treated rats, o- - - - o loops from vitamin D-deficient rats. The vertical lines indicate ± standard error of mean. (Taken from Harrison and Harrison.[14])

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-

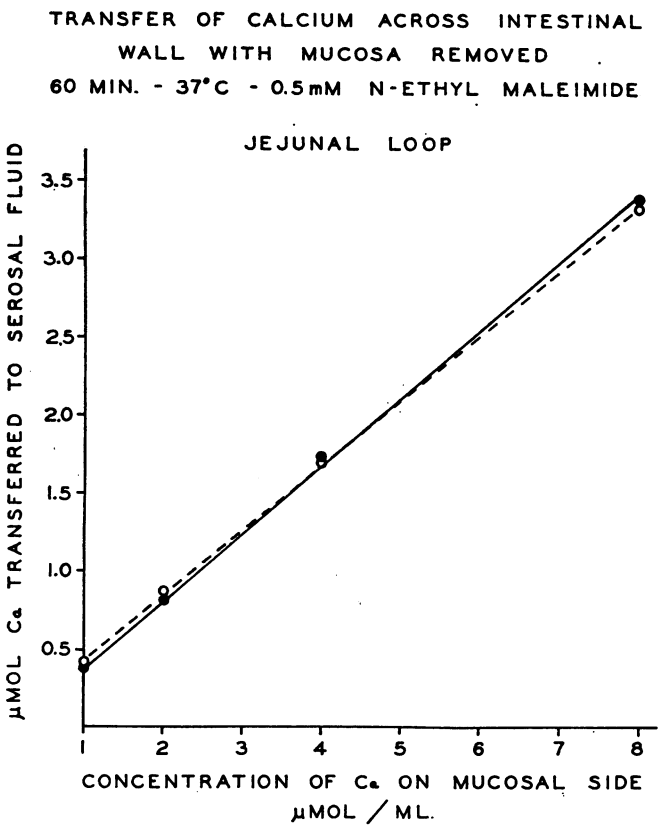

FIG. 4. Calcium transport in everted intestinal loops from which mucosa has been removed, incubated at 37° C, 0.5 mM/liter n-ethyl maleimide in medium. ●——● loops from vitamin D-treated rats, o----o loops from vitamin D-deficient rats. (Taken from Harrison and Harrison.[14])

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[15] 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[16] that certain hormones produce their effects through a genetic mechanism, i.e., the

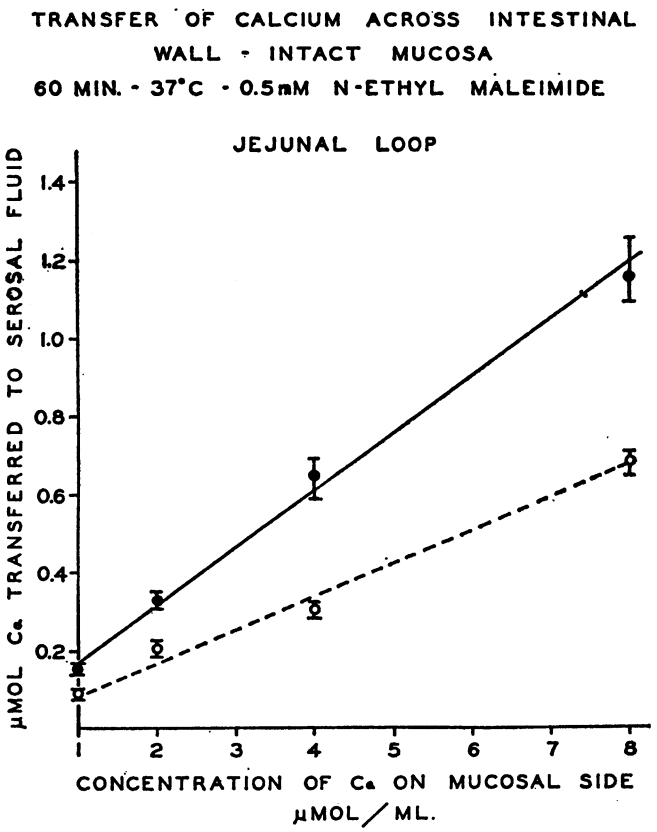

FIG. 5. Calcium transport in everted jejunal intestinal loops with intact mucosa incubated at 37° C. 0.5 mM/liter n-ethyl maleimide in medium, ●——● loops from vitamin D-treated rats, o- - - o loops from vitamin D-deficient rats. The vertical lines indicate ± standard error of mean. (Taken from Harrison and Harrison.[14])

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

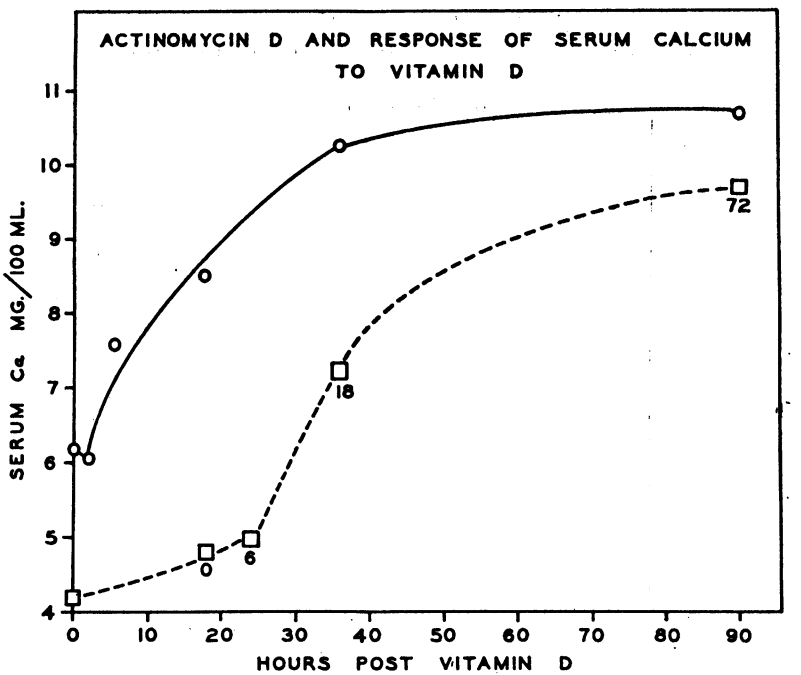

FIG. 6. The effect of actinomycin D in blocking the action of vitamin D on serum calcium concentrations of vitamin D-deficient rats. The solid line shows the increase in serum calcium level with time following a single oral dose of 50,000 units of vitamin D_3 to hypocalcemic vitamin D-deficient rats. 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-

hibits 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 sytem 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

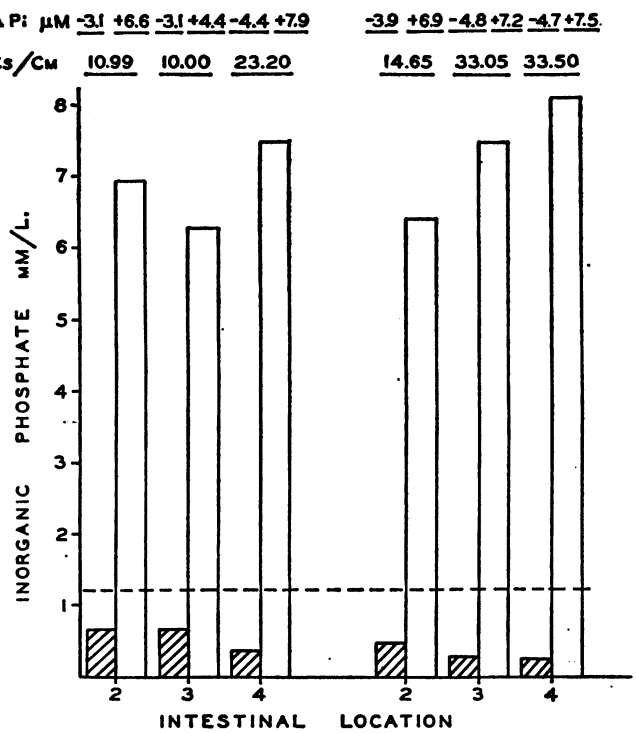

FIG. 7. Concentrative transport of inorganic phosphate across wall of everted intestinal loops *in vitro*. The horizontal dashed line indicates the initial concentration of phosphate in both mucosal and serosal fluids. Height of the hatched column indicates concentration of phosphate in mucosal fluid 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.

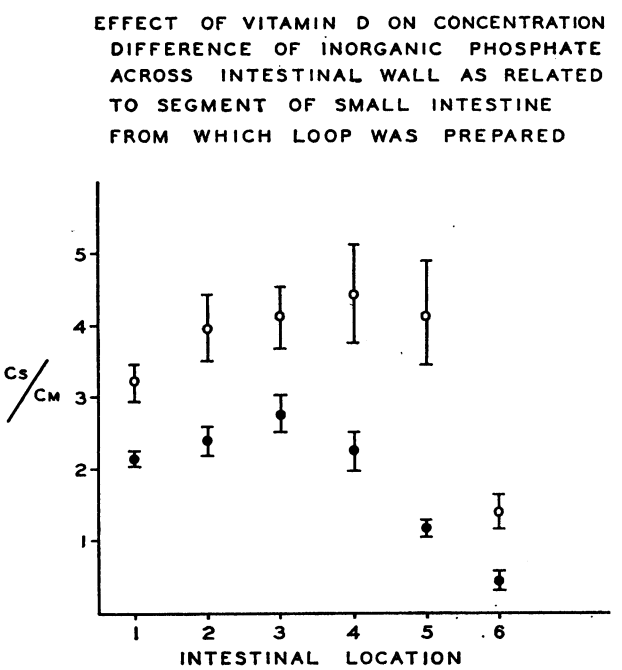

FIG. 8. Effect of Vitamin D treatment of rats upon the concentrative transport of inorganic phosphate across wall of everted intestinal loop *in vitro*. Cs/Cm ratios are averages of values of 4 to 15 loops. • loops from vitamin D-deficient rats, o loops from vitamin D-treated rats. The vertical lines indicate ± standard error of mean. (Taken from Harrison and Harrison.[37])

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

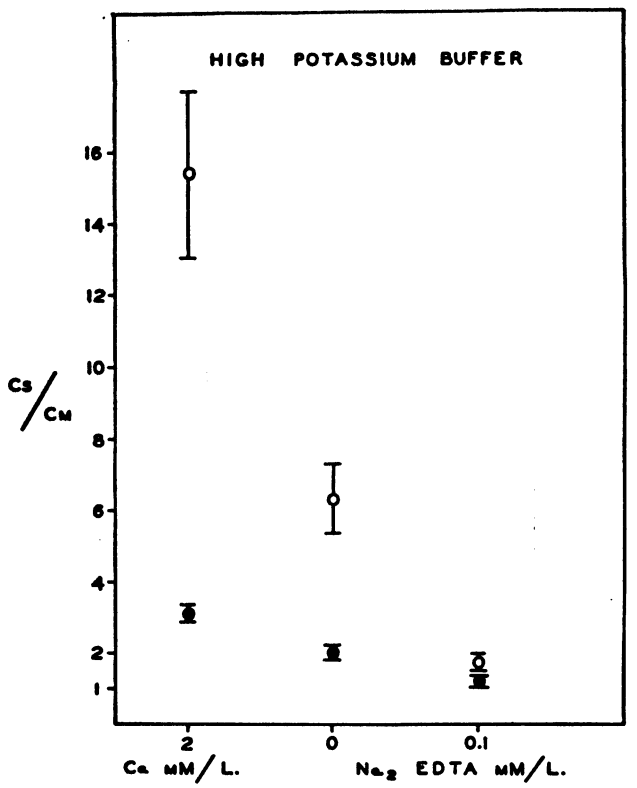

FIG. 9. Interrelation of calcium and vitamin D effect on concentrative transport of phosphate. Three series of experiments are diagrammed from left to right in which the medium contained 2mM/liter Ca, O Ca and 0.1 mM/liter Na_2EDTA respectively. Points are averages of Cs/Cm values from 7 to 39 everted 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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