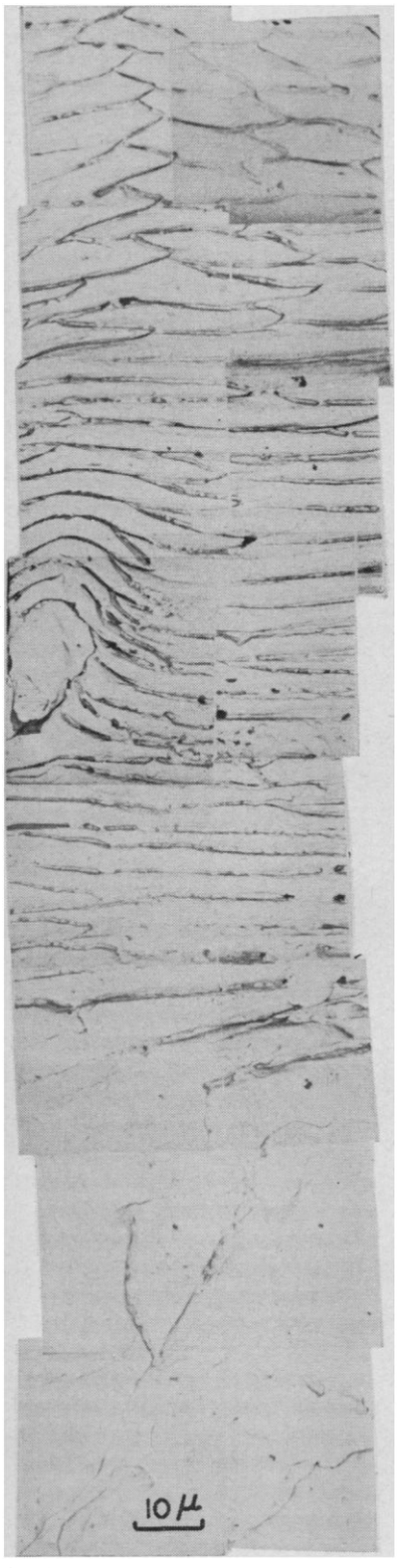

Fig. 2. A composite electron micrograph of a transverse section of the brachial valve of the species *Gryphus stearnsi*. The uppermost portion shows the outer extremity of the inner carbonate layer. The medial portion shows the remainder of the layer and curving of the calcite crystals around an obliquely transected puncta. The bottom shows the large crystals in the adventitious layer.

bratuloid, *Laqueus Californicus* (Koch). He found this inner layer sharply differentiated from the outer layer and composed of parallel prismatic crystals inclined to the outer layer.

Our electron-microscope observations reveal a somewhat different texture in this inner carbonate layer for both species studied. As one proceeds downward from the sharp boundary with the outer carbonate layer, the crystallites are slightly tabular in shape and oriented parallel to the shell layers. The orientation is modified, however, by the passage of the punctae. The crystals in proximity to these tubular structures tend to parallel and wrap about the cylindrical walls of the punctae (Fig. 1*A* and Fig. 2).

As one continues downward through this layer, the crystals become larger and more pronouncedly tabular. The flattening is still parallel to the shell layers except in the vicinity of the punctae. These more tabular crystals also accommodate the punctae by tending to parallel the cylindrical walls. The inner surface of a puncta has a corrugated ring appearance resulting from this unique crystal orientation (Fig. 1, *A* and *B*).

Finally, these flattened, parallel crystallites gradually change their character by becoming irregularly shaped. This change in texture marks the gradational boundary between the inner carbonate layer and the innermost layer, the adventitious (Fig. 1, *B* and *C*; Fig. 2).

The adventitious layer is composed of relatively large irregularly shaped crystals. The crystals do not offer the same special accommodation for the passage of the punctae as was found in the inner carbonate layer. This is to be expected since this layer of calcite is deposited subsequent to the primary shell development (Fig. 1*C*).

These new details of shell morphology indicate that a reexamination of brachiopod shells is needed and that studies with the electron microscope may prove helpful in the problems of taxonomy.

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References and Notes

1. H. M. Muir-Wood, *A History of the Classification of the Phylum Brachiopoda* (British Museum of Natural History, London, 1955).
2. G. A. Cooper, in *Index Fossils of North America*, H. W. Shimer, R. R. Shrock, Eds. (Wiley, New York, 1944), p. 281.
3. B. F. Glenister, *J. Roy. Soc. W. Australia* **39**, 46 (1955).
4. A. Williams, *Biol. Rev. Cambridge Phil. Soc.* **31**, 243 (1956).
5. P. E. Cloud, Jr., *Geol. Soc. Am., Spec. Papers* **38**, 23 (1942).
6. We thank the staffs of the New York State College of Ceramics and Lamont Geological Observatory for their help and encouragement. Supported by grants from the College Center of the Finger Lakes and the Research Foundation of Alfred University.

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Actinomycin D Inhibition of Vitamin D Action

Abstract. *Injection of actinomycin D into rats completely prevents both the rise in serum calcium normally induced by vitamin D and the increased transport of calcium by everted intestinal sacs. Injection of excess parathyroid hormone did not alter this result; this eliminates the possibility that the inhibition of vitamin action was due to blocked hormone synthesis. As a result of these findings, a hypothesis concerning the mechanism of action of vitamin D is presented.*

It is now generally accepted that vitamin D acts by stimulating the transport of calcium and secondarily that of phosphate from the bone, intestinal lumen, and perhaps the renal tubule into the blood stream. The most recent concept is that it functions directly in the cellular or subcellular membranes (*1*). This is supported primarily by the demonstration of effects in vitro of the vitamin on calcium translocation in mitochondria (*2*) and on calcium-stimulated phospholipid labeling (*3*), as well as the demonstration that radioactive vitamin D accumulates in the membrane fractions of kidney and intestinal cells (*4*).

On the other hand, certain observations are not consistent with a direct action of the vitamin. Depending upon the amount given, a lag of 4 to 16 hours is required to observe the earliest physiological response to vitamin D whether it is given orally or intravenously (*5*). In addition, vitamin D added in vitro to intestinal preparations has no effect on calcium translocation, whereas it is markedly effective in identical experiments when given to

the animal prior to the incubation in vitro (*6*). Furthermore, the fact that very large amounts of vitamin D are required for the experiments in vitro relative to the very small amounts (0.1 to 0.025 μg) required for a response in vivo has yet to be adequately explained. Clearly, another hypothesis or at least a modification of the current one would be more satisfactory.

There has been much recent interest in the concept that certain hormones and vitamins may act by controlling the synthesis of specific proteins. Evidence that this may be true for agents controlling calcium homeostasis has come from experiments conducted by Rasmussen *et al.* (*7*), and Eisenstein and Passavoy (*8*). These investigators showed respectively that actinomycin D, an antibiotic which blocks messenger RNA and thus protein biosynthesis, inhibits both the serum-calcium response to parathyroid hormone and the hypercalcemia produced by very high doses of both vitamin D and parathyroid hormone. The interpretation in each case was that these agents act by inducing protein biosynthesis. Since it is not known whether the pharmacological effects of vitamin D have the same biochemical basis as its physiological action, and since the action of the vitamin and parathyroid hormone are intimately associated, it appeared important to find out whether control of protein synthesis by vitamin D could play a major role in its physiologic action. Therefore, experiments were carried out to determine whether actinomycin inhibits vitamin-D stimulation of calcium transport by the intestine and vitamin-D induced rise in serum calcium concentration.

Male, weanling rats (Holtzman, Madison, Wis.) were maintained in individual wire cages and fed a normal diet (0.47 percent Ca, 0.3 percent P) as described (*9*). After 3 to 4 weeks, the animals were vitamin-D deficient as evidenced by reduced growth and lowered serum calcium. The deficient rats were given either 2000 units of vitamin D in 0.1 ml of cottonseed oil orally, or a similar dose of the vitamin plus an intraperitoneal injection of actinomycin D. The antibiotic was given concomitantly with, or in some cases 1 hour before, the vitamin. The relatively large dose of vitamin D was used in order to obtain significant responses within a short time so that the length of time of exposure to actinomycin would be minimized.

Everted gut sacs were used to measure vitamin-D stimulation of calcium transport. At the indicated times after administration of the vitamin, the animals were killed, and everted gut sacs were quickly prepared (*10*), filled with Krebs-Ringer bicarbonate containing Ca^{45}, and incubated in the same medium. The method of Webster (*11*) was used to determine serum calcium.

Actinomycin D completely blocks the action of the vitamin as measured by these responses (Table 1). In control experiments, injection of actinomycin D alone to rats deficient in vitamin D had no effect on either serum calcium or gut-sac transport of calcium. Also, neither injection of the antibiotic 10 hours after vitamin administration nor its addition in vitro to the intestinal preparations (2 μg/ml) had any effect on the parameters measured, an indication that in these experiments the antibiotic was not affecting calcium metabolism directly.

The possibility that inhibition of the responses to vitamin D was due to blocked parathyroid-hormone synthesis is eliminated by the data (Table 2). An excess of injected hormone had no influence on the blocking of vitamin-D action by actinomycin, while eliciting the expected response in parathyroid-ectomized animals.

At no time, up to 15 hours, was there an intestinal response to the vitamin in actinomycin-treated animals (Fig.1). This is also the case for the serum-calcium response, and is in contrast to the results obtained by Rasmussen *et al.* (*7*) on the inhibition of parathyroid-hormone action by this antibiotic, where an initial serum-calcium response to the hormone was observed, and a complete block was not observed until nearly 24 hours after administration of the hormone.

Although our data demonstrate that actinomycin completely eliminates two responses to vitamin D, proof that the vitamin acts by controlling protein biosynthesis must await much more direct examination. The general toxic effects of actinomycin, and the possibility that it may inhibit or alter calcium-metabolism reactions completely unrelated to the action of vitamin D, require that certain reservations be held in regard to conclusions made from our experiments. Even if one accepts the assumption that the antibiotic is acting as expected, that is, by blocking protein synthesis, there are several possible explanations for our results. Possibly actinomycin does not influence directly the action of vitamin D, but only blocks some unknown processes necessary for its transport or incorporation into its active site. Alternatively, the metabolism of vitamin D to an active form may be blocked in some fashion. However, a more direct interpretation would be that vitamin D exerts direct control of the biosynthesis of some protein component of a calcium-transport system. Therefore, with the preceding reservations, it may be instructive to discuss this possibility briefly, especially with regard to the relationship of vitamin D and parathyroid hormone.

Table 1. Inhibition of effect of vitamin D (V) on intestinal calcium transport and serum calcium concentration by actinomycin D (A) (0.66 μg per gram of body weight). The antibiotic was given at the same time as vitamin D. Everted intestinal sacs were incubated for 1.5 hours in Krebs-Ringer bicarbonate buffer, *p*H 7.4, containing 2×10^{-4} *M* $CaCl_2$ labeled with Ca^{45}. The flasks were gassed continuously with a mixture of 95 percent O_2 and 5 percent CO_2. Portions were taken from inside the sac and the surrounding medium and plated as infinitely thin samples, and the concentration of Ca^{45} was determined with a thin-end window counter (Nuclear Chicago). All measurements were made 15 hours after administration of the vitamin D. Numbers in parentheses represent the number of rats used to determine the mean and standard deviation.

Treatment		Transport (Ca^{45} inside/ Ca^{45} outside)	Serum calcium (mg/100 ml)
V	A		
−	−	1.97 ± 0.34 (13)	4.9 ± 0.8 (10)
+	−	3.14 ± 0.47 (11)	7.6 ± 0.2 (11)
+	+	1.96 ± 0.09 (7)	4.4 ± 0.8 (9)

Table 2. Influence of subcutaneous injection of parathyroid hormone (H) (*14*) on actinomycin blockage of vitamin-D responses. One hundred fifty units of hormone were given at the same time as vitamin D (V) and actinomycin (A) and again 8 hours later. All measurements were made after 15 hours. Numbers in parentheses are the numbers of animals in the experiment.

Treatment			Transport ratio	Serum calcium (mg/100 ml)
V	A	H		
+	−	−	3.52 (3)	7.9 (3)
−	−	−	1.76 (3)	6.1 (3)
+	+	−	2.17 (3)	5.7 (3)
+	+	+	2.05 and 1.95 (2)	5.2 (4)

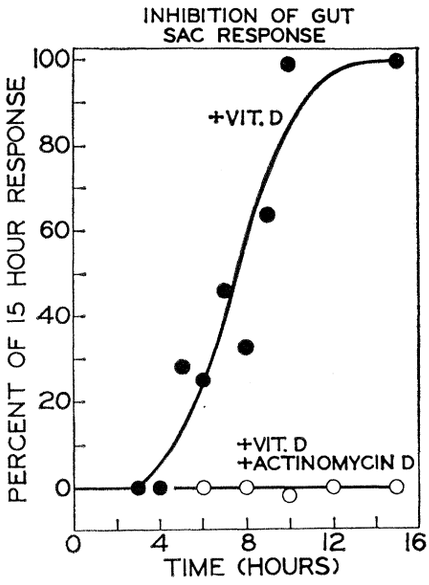

Fig. 1. Time course of the response of intestinal calcium transport to vitamin D in the presence and absence of actinomycin. The antibiotic was administered 1 hour before the vitamin. Each point on the graph represents the mean of values from five animals.

Conceivably, the vitamin may induce the synthesis of a calcium-translocating enzyme with which parathyroid hormone interacts to further stimulate transport of this ion. From this suggestion it would follow that without the vitamin-induced protein, as in extreme vitamin-D deficiency, no effect of parathyroid hormone on calcium metabolism would occur. That this is, in fact, the case has already been demonstrated (*12*). The blockage of hormone action by actinomycin observed by Rasmussen *et al.* might then be explained as an actual inhibition of vitamin-D function which renders these animals essentially vitamin-D deficient and thus insensitive to parathyroid hormone. This explanation would also account for the initial response to the hormone in actinomycin-treated animals, since loss of hormone action would only be seen once the protein synthesized by vitamin D and the messenger RNA for this protein were degraded.

This hypothesis would also explain the time lag between administration of the vitamin and the characteristic responses, the lack of intestinal-sac response to vitamin D added in vitro, the very low dose requirement, and the well-known inborn refractoriness to vitamin-D administration.

To explain the responses in vitro of subcellular systems to vitamin D, it may be necessary to suggest that the vitamin molecule does have a secondary effect on membrane permeability to calcium ions. This is consistent with data concerning the influence of vitamin D on calcium flux in the small intestine (*13*). However, this effect may be of a general nature, due mainly to hydrophobic interactions of the nonpolar vitamin with lipoid membrane systems, and may require large, nonphysiological amounts of the vitamin. In any case, this suggestion alone cannot account for all of the actions of vitamin D.

Finally, while much information must be forthcoming before a final answer concerning the mechanism of action of vitamin D is obtained, the suggestions presented here may stimulate investigations which can lead to this answer. Certainly, these ideas are all amenable to continued experimental examination, and it is in this spirit that they are now proposed.

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References and Notes

1. H. Rasmussen and H. F. DeLuca, *Ergeb. Physiol. Biolog. Chem. Exptl. Pharmakol.* **33**, 109 (1963).
2. G. Engstrom and H. F. DeLuca, *J. Biol. Chem.* **237**, 974PC (1962).
3. N. Hosoya, T. Watanabe, A. Fujimori, *Biochim. Biophys. Acta* **84**, 770 (1964).
4. A. W. Norman and H. F. DeLuca, *Arch. Biochem. Biophys.* **107**, 69 (1964).
5. D. Schachter, D. V. Kimberg, H. Schenker, *Am. J. Physiol.* **200**, 1263 (1961).
6. E. B. Dowdle, D. Schachter, H. Schenker, *ibid.* **198**, 609 (1960).
7. H. Rasmussen, C. Arnaud, C. Hawker, *Science* **144**, 1019 (1964).
8. R. Eisenstein and M. Passavoy, *Proc. Soc. Exptl. Biol. Med.* **117**, 77 (1964).
9. H. Steenbock and D. C. Herting, *J. Nutrition* **57**, 449 (1955).
10. T. H. Wilson and G. Wiseman, *J. Physiol.* **123**, 116 (1954).
11. W. W. Webster, *Am. J. Clin. Pathol.* **131**, 330 (1962).
12. H. Rasmussen, H. DeLuca, C. Arnaud, C. Hawker, Marit von Stedingk, *J. Clin. Invest.* **42**, 1940 (1963); H. E. Harrison and H. C. Harrison, *Metabolism* **13**, 952 (1964).
13. H. E. Harrison and H. C. Harrison, *Am. J. Physiol.* **199**, 265 (1960).
14. The hormone was assayed for calcium-mobilizing action as described by P. L. Munson, *Ann. N. Y. Acad. Sci.* **60**, 776 (1955). Average serum calcium for rats given 0.5 ml (150 units) of hormone extract was 9.8 mg/100 ml, and for rats given no hormone 5.2 mg/100 ml.
15. Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported by a research grant No. AMO-5800-04 from NIH. We thank the Cancer Chemotherapy Center of the National Institutes of Health, U.S. Public Health Service, for the actinomycin, and Eli Lilly and Company for its supply of parathyroid extract.

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Actinomycin D and the Response to Vitamin D

Abstract. *The administration of low doses of actinomycin D to rachitic chicks inhibits the action of a subsequent dose of vitamin D_3 in promoting calcium absorption from the intestine.*

Many studies have demonstrated the essential role of vitamin D in mediating the absorption of calcium in the intact animal and in everted intestinal sacs or intestinal slices from the rat and chick (*1*, *2*). But despite intensive efforts the exact biochemical mechanism and loci of action of the vitamin remain unknown.

One of the characteristic features of the physiological expression of vitamin-D activity has been the time lag between the administration of vitamin D and the enhancement of calcium absorption across the intestinal mucosa. Although suboptimum enhancement of calcium absorption occurs in the rat in 3 to 5 hours after a massive dose [50,000 international units (I.U.)] of vitamin D, maximum expression of vitamin-D activity is not apparent until after 12 to 15 hours (*3*). In the chick very little calcium absorption enhanced by vitamin D_3 occurs until 12 to 16 hours after either an oral, intracardial, or intraperitoneal injection of 100 I.U. of vitamin D_3 (*2*, *4*).

It has been suggested (*5*) that the vitamin must be converted by the adrenals to a metabolically active form before the increased absorption of calcium can be observed. However, little direct evidence for this hypothesis has been found. Experiments with tritium-labeled vitamins D_2 and D_3 revealed that, in both the chick and rat, the adrenals accumulated an insignificant amount of vitamin D in any time interval after the administration of the ^{3}H-vitamin D. Further experiments with the chick revealed that the intestinal mucosa accumulated 70 percent of the radioactivity found at 30 hours within 3 to 5 hours after either an intracardial or intraperitoneal dose of 500 I.U. of ^{3}H-vitamin D (*6*). Although vitamin D was present at 5 hours, the manifestation of this vitamin did not become apparent until after 12 to 16 hours.

A possible hypothesis for the delay in biological response to vitamin D may be that delay reflects some as yet undefined induction process. Perhaps one or several enzymes necessary for the mediation of calcium absorption are synthesized by the mucosa cells only