So far, the structure of several gamones in water molds has been clarified (*8*). It is of great interest that a gamone in Protozoa structurally belongs to a novel type of compound which has general biochemical importance. Elucidation of the structure of blepharismin is expected to facilitate the molecular study of the conjugation mechanism in *Blepharisma*.

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Embryonic Chick Intestine in Organ Culture: Response to Vitamin D_3 and Its Metabolites

Abstract. *Embryonic chick intestine maintained in organ culture responded to vitamin D_3 and its metabolites 25-hydroxycholecalciferol and 1,25-dihydroxycholecalciferol by synthesis of calcium-binding protein and enchanced calcium-45 uptake. The dihydroxy metabolite was by far the most potent inducer of the protein and also acted more rapidly than vitamin D_3 to stimulate isotope uptake. Despite its lower potency, vitamin D_3 itself was effective.*

Vitamin D_3 induces de novo synthesis of a calcium-binding protein (Ca-BP) and enhances uptake and mucosal to serosal transport of Ca in embryonic chick intestine maintained in organ culture (*1*, *2*). Much correlative evidence supports a role for Ca-BP in the vitamin D–mediated intestinal transport of Ca in vivo (*3*). Also, direct evidence was obtained with the organ culture technique: absorption and transport of Ca was enhanced in intact, everted duodena maintained with purified Ca-BP but no vitamin D_3 in the culture medium (*2*).

With improvements in the culture technique, mucosal structure was well preserved after 48 hours of incubation in a defined, serum-free medium (*4*). The marked similarity between responses to vitamin D in vivo and in this system indicates that the metabolic fate and mode of action of the vitamin can validly be studied in the organ culture. This system may be of use in understanding the physiological significance of active metabolites of vitamin D_3 (cholecalciferol): 25-hydroxycholecalciferol (25-OHD_3), formed in the liver (*5*) and kidney (*6*) from vitamin D_3; and 1,25-dihydroxycholecalciferol [1,25-$(OH)_2D_3$], formed in the kidney from 25-OHD_3 (*7*). I report here the relative effectiveness of vitamin D_3 and its metabolites. Although 1,25-$(OH)_2D_3$ was extremely potent, vitamin D_3 itself was effective.

Entire duodena from four 20-day chick embryos (300 to 400 mg of tissue) were slit open and laid with mucosa upward on a 60-mesh stainless steel grid (51 by 63.5 mm on 6.35-mm legs) inside a petri dish. The dish contained McCoy's 5A medium without serum (*8*), with 1.25 m*M* Ca (except when noted otherwise) and nystatin (100 unit/ml). The guts on grids, cultured for 48 hours in a humidified incubator (37.5°C) continuously gassed with 5 percent CO_2 in air, produced up to ten times as much Ca-BP in response to 26 μM vitamin D_3 as did tissue in the original immersion culture method (*1*). After culture, the guts were either homogenized before Ca-BP measurement by radial immunoassay (*1*), or ^{45}Ca uptake was measured during a 30-minute incubation in a buffer solution (*1*). As measured here, ^{45}Ca uptake reflects the first step in the transport of Ca across the intestine, namely, accumulation of Ca by the cell. The validity of this method has been established (*9*, *10*) and the dependence of uptake on vitamin D was shown in vivo (*9*) and in this organ culture system (*1*).

The results of culturing duodena in the presence of vitamin D_3, 25-OHD_3, or 1,25-$(OH)_2D_3$ (*8*) are shown in Table 1. The minimum effective concentrations of either vitamin D_3 or 25-OHD_3 was only 6.5 n*M* for induction of detectable Ca-BP synthesis and 65 n*M* for enhancement of ^{45}Ca uptake. At 65 n*M*, 25-OHD_3 was about 1.4 times as effective as vitamin D_3 in inducing Ca-BP synthesis and twice as effective in stimulating ^{45}Ca uptake. Although the cultured duodena responded to relatively low concentrations of both

Table 1. Relative potency of vitamin D_3 metabolites in stimulating Ca-BP synthesis and ^{45}Ca uptake in embryonic chick intestine in organ culture. Values are mean ± standard error. Duodenal tissue was cultured for 48 hours with sterol in the culture medium. The tissues were then analyzed for Ca-BP content or transferred to a ^{45}Ca-containing buffer solution and incubated for 30 minutes at 37°C (*1*). Uptake of ^{45}Ca was determined by liquid scintillation counting of duodena in Nuclear-Chicago solubilizer. Relative potency of Ca-BP synthesis is 100 times the average Ca-BP concentration induced by a particular sterol divided by the average Ca-BP concentration induced by vitamin D_3. Relative potency of ^{45}Ca uptake is 100 times the average net increase in ^{45}Ca uptake stimulated by a particular sterol divided by the average net increase in ^{45}Ca uptake stimulated by vitamin D_3.

Sterol (n*M*)	Ca-BP (μg per 100 mg of tissue)			^{45}Ca uptake (% of control)		
	D_3	25-OHD_3	1,25-$(OH)_2D_3$	D_3	25-OHD_3	1,25-$(OH)_2D_3$
0 (Control)	0	0	0	100	100	100
0.65			8.4			100 ± 12
6.5	1.5 ± 0.2	0.8 ± 0.1	21.0	103 ± 8	99 ± 8	101 ± 3
65	3.0 ± 0.5	4.3 ± 0.3	28.8	120 ± 12*	140 ± 8*	144 ± 6*
	*Relative potency of 65 n*M *sterol*					
	100	143	960	100	200	220

* $P < .01$ by Student's *t*-test applied to uptake data expressed as percentage of ^{45}Ca dose accumulated per 100 mg of tissue.

vitamin D_3 and 25-OHD_3, Ca-BP induction by 65 n*M* 1,25-$(OH)_2D_3$ in this system was about ten times greater than that by 65 n*M* vitamin D_3 and seven times greater than that by 65 n*M* 25-OHD_3. Indeed, the minimum effective concentration of 1,25-$(OH)_2D_3$ in the medium for induction of detectable Ca-BP synthesis was no more than 6.5 p*M*, one-thousandth of that of either vitamin D_3 or 25-OHD_3 (data not shown); this makes 1,25-$(OH)_2D_3$ one of the most potent biologically derived substances known. However, 65 n*M* 1,25-$(OH)_2D_3$ was no more potent than 65 n*M* OHD_3 in enhancing ^{45}Ca uptake (Table 1).

Induction of Ca-BP synthesis in this system was more sensitive to vitamin D_3 and its metabolites than was enhancement of ^{45}Ca uptake, especially in the case of 1,25-$(OH)_2D_3$. Also, large amounts of Ca-BP were induced by 1,25-$(OH)_2D_3$ concentrations insufficient to stimulate ^{45}Ca uptake. Thus, twice as much Ca-BP was induced after 48 hours of incubation of duodena with 0.65 n*M* 1,25-$(OH)_2D_3$ as compared to incubation for this period with 100 times as much 25-OHD_3, yet ^{45}Ca uptake was not stimulated by this low concentration of 1,25-$(OH)_2D_3$ (Table 1). However, merely raising the Ca concentration from 1.25 m*M* to between 2.5 and 5 m*M* resulted in significant enhancement of ^{45}Ca uptake after 48 hours of incubation with 0.65 n*M* 1,25-$(OH)_2D_3$. The responsiveness of the cultured duodenum to 0.65 n*M* 1,25-$(OH)_2D_3$ was shown to be a function of time as well as Ca concentration (Fig. 1). Thus, with 1.25 m*M* Ca maximal stimulation of ^{45}Ca uptake was reached after only 6 hours, persisted through 24 hours, but was no longer evident at 48 hours. With 2.5 m*M* Ca, however, enhancement of ^{45}Ca uptake was not seen until after 24 hours and persisted through 48 hours. When the effect of higher Ca concentration on 1,25-$(OH)_2D_3$ action was measured at 48 hours only, the higher Ca concentration appeared to stimulate ^{45}Ca uptake when, in fact, it was retarding the rapid response. (By comparison, 26 μM vitamin D_3 significantly stimulated ^{45}Ca uptake only after 12 hours.) At either Ca concentration, Ca-BP was induced either before or simultaneously with stimulation of ^{45}Ca uptake. This suggests that Ca-BP synthesis was the initial response to 1,25-$(OH)_2D_3$, and that enhancement of Ca uptake followed, whether or not as a direct consequence.

The retardation by Ca of the response to 1,25-$(OH)_2D_3$ suggests a possible role of Ca in the regulation of Ca-BP synthesis, localization (*11*), or function. Alternatively, Ca may have potentiated a more direct action of 1,25-$(OH)_2D_3$ on ^{45}Ca uptake. This substance stimulated in vivo intestinal Ca absorption in rats, even when protein synthesis had been inhibited by prior administration of actinomycin D (*12*). This observation leaves unexplained considerable in vivo evidence that vitamin D and its metabolites are preferentially localized in intestinal cell nuclei (*13*) and that the earliest known actions of vitamin D are stimulation of DNA template activity (*14*), incorporation of RNA precursors into RNA (*15*), and synthesis of Ca-BP (*16*). Perhaps most relevant are the results obtained when embryonic chick duodenum was cultured in medium containing 5 m*M* Ca and 0.65 n*M* 1,25-$(OH)_2D_3$—conditions producing enhanced ^{45}Ca uptake at 48 hours. When actinomycin D was included in the medium, at concentrations neither altering total protein concentration in the gut nor producing general cytotoxic effects, 1,25-$(OH)_2D_3$ induction of Ca-BP synthesis was inhibited and the enhancement of ^{45}Ca uptake was completely blocked (*4*). This seems compeling evidence against a direct action of 1,25-$(OH)_2D_3$ on Ca absorption.

Table 2. Action of 1,25-$(OH)_2D_3$ in the presence of a high concentration of vitamin D_3 in the medium. Details are given in text and legend to Table 1.

Sterol		Ca-BP (μg per 100 mg of tissue)	^{45}Ca uptake (% of control)
D_3 (n*M*)	1,25-$(OH)_2D_3$ (n*M*)		
0	0	0	100
6500	0	5.5	146 ± 2*
0	0.65	8.0	111 ± 9
6500	0.65	6.4	152 ± 7*

* $P < .01$, determined as for Table 1.

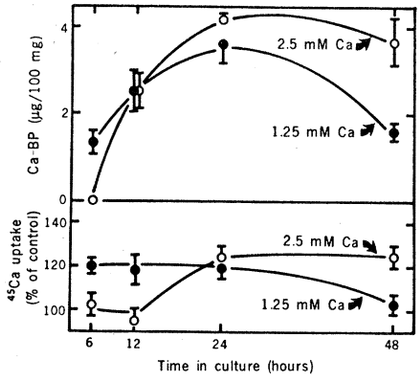

Fig. 1. Time course of 1,25-$(OH)_2D_3$ action as a function of Ca concentration in the medium. Values for ^{45}Ca uptake were calculated as percentage of total ^{45}Ca in the buffer accumulated per 100 mg of duodenum in the presence of 1,25-$(OH)_2D_3$ and divided by the corresponding value obtained for the same Ca concentration and incubation time in the absence of 1,25-$(OH)_2D_3$. Other details are given in the text and legend to Table 1.

It has been stated recently that 1,25-$(OH)_2D_3$ is the only biologically active form of vitamin D_3 in vivo; that is, unless this metabolite is formed, there is no vitamin D_3 action on the intestine (*12*). However, the present data indicate that both vitamin D_3 and 25-OHD_3, as well as 1,25$(OH)_2D_3$, are effective in stimulating the Ca absorptive mechanism in organ cultures of intestine. In addition, high-resolution chromatographic analyses have indicated that vitamin D_3 itself can induce Ca-BP synthesis and ^{45}Ca uptake in cultured intestine in the complete absence of either vitamin D metabolite. Duodena were cultured as described above. To the medium was added either ethanol alone or an ethanol solution of [4-^{14}C]vitamin D_3 (specific activity, 32.3 mc/mmole); final concentrations were 650 n*M* [4-^{14}C]vitamin D_3 (the only vitamin D_3 added) and 0.1 percent ethanol. After 48 hours, Ca-BP and ^{45}Ca uptake were measured on some of the guts. Other duodena, cultured in the presence of labeled vitamin D_3, were dissolved in Nuclear-Chicago solubilizer (*8*) and assayed for ^{14}C radioactivity. Still other duodena, and the medium after lyophilization, were homogenized and extracted with chloroform : methanol according to the procedure of Bligh and Dyer (*17*). The chloroform layers were evaporated and redissolved in small amounts of ethanol for application to chromatographic columns (legend to Fig. 2).

Authentic vitamin D_3 and 25-OHD_3 and biosynthetic 1,25-$(OH)_2D_3$ were chromatographed on Sephadex LH-20 (*18*) and Celite (*19*) columns to determine elution patterns (Fig. 2D). Although these three sterols were adequately resolved in both systems, resolution of 1,25-$(OH)_2D_3$ was much better by Celite chromatography. (The minor ^3H peak in both systems was present in the 1,25-$(OH)_2D_3$ preparation and was not identified.) The solution of labeled vitamin D_3 used was pure (Fig. 2C) in both systems, there being no trace of either metabolite (as little as 0.03 percent of either could

have been detected). After 48 hours of incubation, total lipid extracts of the duodenal tissue and the medium were chromatographed. No trace of either metabolite was formed in the cultured intestine (Fig. 2A) or was present in the medium (Fig. 2B); the only radioactivity peak was unchanged vitamin D_3.

The total [4-^{14}C]vitamin D_3 content of the cultured intestines (average of six) was 2.1 ± 0.2 pmole per milligram of tissue. This represents an average gut accumulation of 0.4 percent of the total labeled vitamin D_3 available in the medium. It is not known what portion of this activity was specifically bound. Approximately 50 percent of labeled vitamin D_3 was lost from the medium after 48 hours. The cause of the loss was not determined, but the radioactivity was probably either insoluble or adsorbed to surfaces. [4-^{14}C]Vitamin D_3 although not metabolized in the culture system (Fig. 2), stimulated Ca-BP synthesis (2.85 ± 0.31 μg per 100 mg of duodenum) and enhanced ^{45}Ca uptake (123 ± 7.1 percent of uptake in absence of vitamin D_3).

These observations leave little doubt that vitamin D_3 itself is effective in stimulating the Ca absorptive mechanism in organ cultures of intestine. There is also chromatographic evidence that 25-OHD$_3$, like vitamin D_3, is not metabolized in cultured intestine and yet stimulates the Ca absorptive mechanism (*20*). Perhaps the cultured intestine is unique in using vitamin D_3. However, an alternative suggestion, not yet ruled out by any in vivo results, is that there may be a family of sterols with vitamin D activity. Which sterol effects an intestinal response, in animals of varying nutritional or physiological status, may depend on relative sterol concentrations at a critical intestinal site.

Abundant in vivo evidence indicates that the vitamin D "receptor site" in the intestine is not absolutely specific (*21*). Additional evidence was obtained in the organ culture system. The approach was based on the hypothesis that, if 1,25-(OH)$_2$D$_3$ is the only biologically active form of vitamin D_3 in the intestine, then this sterol should be active even in the presence of a high concentration of the presumably inactive vitamin D_3. This was found not to be the case. After 48 hours of culture with 6.5 μ*M* vitamin D_3, there was the usual synthesis of Ca-BP and enhancement of ^{45}Ca uptake (Table 2). In contrast, 1,25-(OH)$_2$D$_3$ at 0.01 percent of this concentration was more effective in Ca-BP induction but did not significantly enhance ^{45}Ca uptake after 48 hours (only 1.25 m*M* Ca was in the medium). However, when the two sterols were combined in the medium at these concentrations, the effects on Ca-BP synthesis and ^{45}Ca uptake were essentially those of vitamin D_3 alone. The greater potency of 1,25-(OH)$_2$D$_3$ for Ca-BP induction was not manifested, nor were the effects of the two sterols additive. Also, if vitamin D_3 is inactive itself, one would not expect to see the enhanced ^{45}Ca uptake after 48 hours when both vitamin D_3 and 1,25-(OH)$_2$D$_3$ were present in medium containing 1.25 m*M* Ca, because the action of 1,25-(OH)$_2$D$_3$ on ^{45}Ca uptake is not seen after 48 hours at this Ca concentration (Fig. 1).

Vitamin D_3 in this system, while considerably less potent than 1,25-(OH)$_2$D$_3$, is itself capable of stimulating the Ca absorptive mechanism. It is possible that 1,25-(OH)$_2$D$_3$ was ineffective in the presence of a high concentration of vitamin D_3 because of competition for a common site of activation.

The dihydroxy derivative is an extremely potent D vitamin both in vivo and in vitro. This compound, or even more potent synthetic derivatives, may find useful applications in certain disturbances of Ca metabolism (*22*). Nonetheless, it is possible that the concentration of 1,25-(OH)$_2$D$_3$ in normal blood is very low compared to that of vitamin D_3 and that, under ordinary circumstances, vitamin D_3 itself may stimulate the intestinal Ca absorptive mechanism, just as it does in vitro. It may be possible to utilize the extremely high potency of 1,25-(OH)$_2$D$_3$ in this culture system as the basis for a simple yet

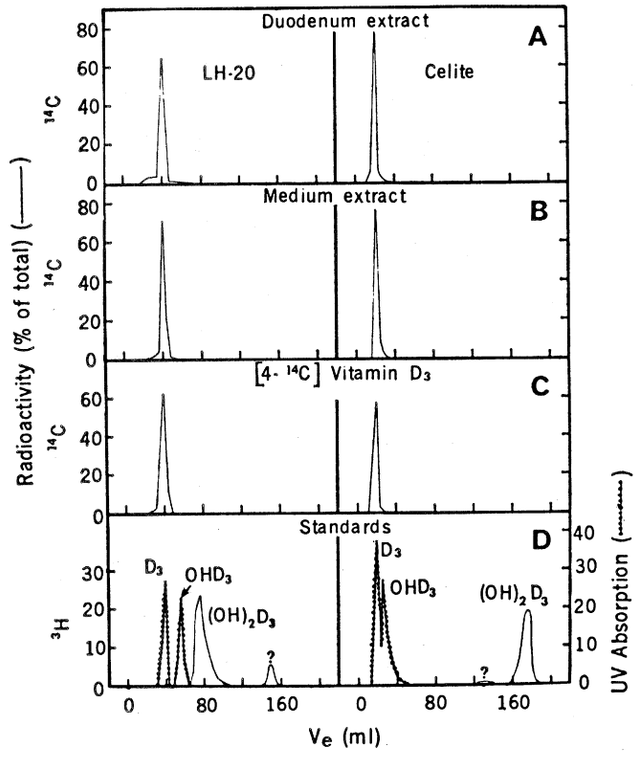

Fig. 2. Chromatographic profiles of vitamin D_3 metabolites on Sephadex LH-20 (gel filtration) and Celite (liquid : liquid partition). Twenty grams of Sephadex LH-20 were slurried in chloroform : hexane : methanol (75 : 23 : 2) (*18*), and a column (48 by 1.3 cm) was prepared. Chromatograms were developed with the same solvent delivered by a pump (CMP-2 metering pump, Chromatronix, Inc., Berkeley, California) at a flow rate of 0.4 ml/min; 4-ml fractions were collected. Celite [method modified from (*19*)] was washed three times with 6*N* HCl, rinsed extensively with water to approximate neutrality, and extracted with ethanol, ether, and hexane. The Celite was then dried, and 13 g were slurried in 45 percent water in ethanol (stationary phase) and applied to a column of 1.3-cm diameter. The mobile phase (10 percent ethyl acetate in hexane saturated with stationary phase fluid) was then forced through the column at high pressure with the aid of a pump (Chromatronix), the excess stationary phase being forced out ahead of the mobile phase. This resulted in an extremely well-packed column with excellent and reproducible resolving power. Mobile phase for development of the chromatogram was delivered by pump at a flow rate of 0.4 ml/min; 4-ml fractions were collected. Total lipid extracts of homogenates of (A) duodena cultured for 48 hours in the presence of [4-^{14}C]vitamin D_3 or of (B) the lyophilized culture medium itself; no trace was found of the metabolites 25-OHD$_3$ or 1,25-(OH)$_2$D$_3$, which chromatographed in these systems as shown in (D). Authentic crystalline vitamin D_3 and 25-OHD$_3$ were measured in the column effluent by an in-line ultraviolet monitor, and radioactivity of ^{3}H-labeled 1,25-(OH)$_2$D$_3$ was measured. The labeled vitamin D_3 added to the medium was quite pure, as shown in (C).

highly sensitive bioassay. This would be important in evaluating the role of $1,25\text{-}(OH)_2D_3$ in both normal and disordered states of Ca metabolism. Until circulating concentrations of $1,25\text{-}(OH)_2D_3$ can be measured, the proposal that it is the only biologically active D vitamin in vivo seems premature.

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Phenothiazine Dosage Levels and Auditory Signal Detection in Schizophrenia

Rappaport, Silverman, Hopkins, and Hall (*1*) suggest that in an auditory signal-detection situation nonparanoid schizophrenics are hypersensitive (presumably in comparison with normals) at low and moderate stimulus intensities, but have an attenuated response at high stimulus intensities. They hypothesize that paranoid schizophrenics have a primary difficulty of an attentional nature such that they scan the environment rather than focus on relevant stimuli, and that phenothiazines have differential effects on the auditory signal-detection performance of paranoid and nonparanoid schizophrenics. The design of their experiment and the results presented warrant questioning of their conclusions.

Determinations of drug dosages in clinical populations often conflict with the requirements of experimental design. Since the assignment of drug dosage in the study by Rappaport *et al.* was not random, but was "determined by the ward physician, and was based on clinical impressions of the patient's condition" (*1*, p. 724), the patients who received different dosages also differed on whatever clinical variables the ward physician used to determine dosages. Such a confounding of clinical state with dosage makes it impossible to attribute any observed performance differences to dosage alone. That Rappaport *et al.* state "there were no significant relations between severity of mental pathology in either group of schizophrenics and d'" (*1*, p. 725), is not an assurance that the groups were clinically equivalent in other respects.

Rappaport *et al.* do not present evidence that phenothiazines have a statistically significant effect on signal-detection performance within either the paranoid or nonparanoid schizophrenic groups. They state (*1*, p. 725), "As was predicted, nonparanoid schizophrenics showed a decrease in d' with each increase of phenothiazine medication. In contrast, paranoid schizophrenics showed an increase in d' with each increase in phenothiazine medication." However, our calculations based on the data in their table 1 suggest that there were no statistically significant effects within either group related to phenothiazine dosage. In the absence of significant differences, the data would conventionally be taken as support for the lack of a drug effect on performance on their experimental task, rather than for the presence of such an effect. Rappaport *et al.* do state (*1*, p. 725) that "among normal subjects no consistent overall drug effect was observed." The mean drug effects exhibited by normals (table 1) appear to be of the same order of magnitude as those presented for the schizophrenics. None of the mean differences between drug conditions in any of the subject groups appears to be very large. Our calculations indicate that the largest of all of these differences is approximately equivalent to the change in mean performance, which would be expected as a result of a 1-db change in signal (*2*). Such a small effect is presumably of little practical significance.

There are data provided by Rappaport *et al.* that may be interpreted as indicating a significant drug effect. In the difficult signal-detection condition the nonmedicated normals differed significantly from the nonparanoids receiving the highest drug dosage, but not from the other nonparanoids. Since nonparanoids receiving the high drug dosage may have differed clinically from the nonparanoids receiving lesser amounts of phenothiazines, it would seem unwarranted to conclude that a drug effect is indicated.

It should be pointed out that, from the results presented by Rappaport *et al.*, it does not seem possible to tell whether any given significant difference in performance was due to a difference in sensitivity, or to a difference in the ability to focus attention, or to both. Thus, even if significant drug-related changes in performance on the auditory task had been demonstrated, additional evidence would be required to establish that such changes were due to attentional factors in paranoid subjects, but were due to variations in sensitivity in nonparanoid subjects. In their experiment the subject had only a single response button. A press on this button was taken to be a "yes" response, and a failure to press the button was taken to be a "no" response. If the subject in such a situation fails to respond as a result of a lapse of attention, this failure to respond will be treated as a "no" response. If the subject had been given both a "yes" button and a "no" button, then failures to respond could have been distinguished from intentional "no" responses. This proce-