

EMBRYONIC CHICK INTESTINE IN ORGAN CULTURE

A Unique System for the Study of the Intestinal Calcium Absorptive Mechanism

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ABSTRACT

Duodena from 20-day-old chick embryos can be maintained in large scale organ culture on specially designed stainless-steel grids in contact with serum-free medium for 48 h with excellent preservation of mucosal structure at both the light and electron microscope levels. Although mitotic rate was subnormal, several other factors attest to the essential viability of the cultured intestine: L-leucine incorporation into protein, as well as the synthesis of a specific vitamin D₃-induced calcium-binding protein (CaBP), increased over a 48-h culture period, and the electropotential gradient across the intestine was maintained throughout the culture period as was a concentration gradient for calcium. The tissue responded to vitamin D₃ in the medium by synthesizing the calcium-binding protein within 6 h and by exhibiting enhanced ⁴⁵Ca uptake within 12–24 h. Concentrations of vitamin D₃, or its 25-hydroxylated derivative, higher than necessary for CaBP induction, also increased the activity of alkaline phosphatase. The 1,25-dihydroxylated derivative of vitamin D₃, at a level extremely potent in CaBP induction, did not stimulate alkaline phosphatase. Mucosal to serosal transport of ⁴⁵Ca could also be measured in everted duodenal sacs, subsequent to culture under similar conditions, and was also increased by vitamin D₃ in the medium. Other embryonic organs, esophagus, stomach, liver, pancreas, lung, skin, and muscle, did not produce CaBP in response to vitamin D₃ in the culture medium. However, CaBP-synthesizing capacity was present in the entire intestinal tract, exclusive of the rectum. ⁵⁹Fe and ³²P uptake by cultured duodenum were also stimulated by vitamin D₃. The system has proven quite useful in the study of the vitamin D-mediated calcium absorptive mechanism but should be applicable to the study of the absorption of other nutrients, drugs, hormones, etc., as well as other studies of intestinal function.

INTRODUCTION

Since the discovery of the action of vitamin D on the enhancement of intestinal calcium absorption by Nicolaysen in 1937 (1), a variety of *in vivo* and *in vitro* techniques have been employed to study the transport mechanism(s) involved. In the living animal, calcium absorption has been

measured using methods of oral intubation (2, 3), *in situ* perfusion (2, 4, 5), *in situ* ligated loops (1, 2, 6, 7), and Thiry-Vella fistulas (8, 9).

In vitro, the everted intestinal sac technique of Wilson and Wiseman (10) has been extensively used in calcium absorption studies (11–14). Others

have employed intestinal ringlets (15, 16) or squares (17) and intestinal tissue mounted in a variety of Ussing-type (18) chambers (14, 17, 19, 20). These and other studies, too numerous to mention, have provided considerable insight into the phenomenon of vitamin D-mediated calcium transport (21). However, a major difficulty existed in this research: it was never possible to be certain that perturbation of the system was a direct result of the physiological, nutritional, pathological, or pharmacological means employed. This was true of the *in vitro* as well as *in vivo* studies since the vitamin D status of the intestine, and any alterations thereof, had been established *in vivo* before the measurement of transport *in vitro*. An unrecognized technical problem of considerable importance also existed: survival of the structural integrity of the tissue *in vitro* is brief. Recent studies, for example, have shown that the mucosal structure of the everted rat intestine is completely lost within 60 min of being immersed in a buffer solution (22). The fine structural integrity of the everted hamster intestine is well preserved for at least 1 h (23) but, at best, this still puts a severe time limitation on the types of agents or other variables to which the intestine could be subjected *in vitro*.

An alternative approach, which would allow close control of a wider range of variables influencing calcium absorption and be more likely to allow separation of direct and indirect effects, is organ culture. However, this technique, as pioneered most notably by Strangeways and Fell (24), although extensively applied by them and many others in morphological, developmental, and biochemical studies of a number of tissues (25), had not been applied to the study of the vitamin D-mediated calcium transport mechanism of the intestine until recently (26). In that recent report was documented "the first demonstration of an *in vitro* physiological effect of vitamin D₃ on . . . the intestine."

Initial attempts to develop an intestinal organ culture system were motivated by the observation that the vitamin D-mediated, intestinal calcium-binding protein, CaBP¹ (27), was not present in

¹ *Abbreviations used in this paper:* Ca-ATPase, calcium-dependent ATPase; CaBP, calcium-binding protein; 25-OHD₃, 25-hydroxycholecalciferol; 1,25-(OH)₂D₃, 1,25-dihydroxycholecalciferol; TCA, trichloroacetic acid.

the chick duodenum during embryogenesis but only appeared at the time of hatching (28). The embryonic duodenum, a normal tissue presumably microbe-free, therefore, seemed a logical choice for organ culture. Upon incubation of this tissue in medium containing vitamin D₃, it was found that CaBP was induced, that the induction was *de novo* and that, accompanying CaBP induction, there was enhanced radiocalcium uptake by the intestine (26), all features of the *in vivo* calcium absorptive mechanism in the chick.

The technique employed in those early studies was to simply immerse the embryonic gut in the medium in a sealed flask. Gaseous exchange and pH control were, thus, severely limited. It is the purpose of the present report to describe a much improved organ culture technique, and some results obtained using it.

MATERIALS AND METHODS

Embryonated Eggs

Day-old White Leghorn embryonated eggs (Babcock Poultry Farm, Inc., Ithaca, N.Y.) were incubated at 37.5°C and 60% relative humidity (Petersime Incubator Co., Gettysburg, Ohio).

Culture Medium

McCoy's 5A (modified) medium without calcium (Grand Island Biological Co., Grand Island, N.Y.) was reconstituted from the dry powder within 1 mo of use and sterile filtered through a 0.22- μ m filter (Millipore Corp., Bedford, Mass.). The antibiotics, neomycin sulfate, streptomycin sulfate, and sodium penicillin G (Sigma Chemical Co., St. Louis, Mo.), were added on the day of an experiment at 100, 50, and 50 mg/liter (wt/vol), respectively. Nystatin (Mycostatin, E. R. Squibb & Sons, New York), a fungicide, was added at the same time at 100 U/ml medium. Crystalline vitamin D₃ (Mann Research Labs. Inc., New York) and other sterols, when used, were added as ethanol solutions such that the ethanol content of the medium was only 0.1%. Calcium was added to the medium, usually to a level of 1.25 mM, as a sterile water solution at the time of experiment.

Culture Technique

The duodenal loop was excised from the 20-day chick embryo and the pancreas removed. (Earlier studies had shown that duodena from chick embryos at least as early as day 15 can also be cultured and are responsive to vitamin D₃.) The duodenum was then either slit lengthwise with fine scissors or everted on a 25-gauge platinum-iridium (30%) wire (Engelhard

Industries Div., Englehard Minerals & Chemicals Corp., Murray Hill, N.J.). The gut was placed on a rectangular grid (51 × 63.5-mm surface with 6.35-mm legs formed by making 90° angle bends on the long dimension) of 60-mesh stainless steel (Newark Wire Cloth Co., Newark, N.J.) in a polystyrene petri dish containing approximately 37 ml culture medium (see above). In the case of slit-open duodena, the gut was actually slit while resting on the grid and arranged so that the mucosal surface was uppermost. A total of four duodena (300–400 mg fresh weight) were placed on each grid in bare contact with the medium.

The petri dishes were kept in an incubator at 37.5°C and approximately 90% relative humidity usually for 2 days. The incubator was continuously gassed with 5% CO₂ in air. The pH of the medium at the start of incubation was 7.4 and never dropped below 7.1. At the end of the culture period, the duodena were either homogenized in a motor-driven, ground glass, tapered homogenizer (Tri-R Instruments, Inc., Rockville Centre, N.Y.) in 2 vol of a Tris buffer (0.0137 M Tris-HCl, 0.12 M NaCl, and 4.74 mM KCl; pH 7.4) preparatory to various assays, or subjected to measurement of radiocalcium uptake and/or transport as described below.

Assay Procedures

TOTAL PROTEIN: Portions of duodenal homogenates were solubilized in 100 vol of 1 N NaOH (2 h at room temperature). Total protein assay was performed by the procedure of Lowry et al. (29) using an automatic analyzer (Technicon Instruments Corp., Tarrytown, N.Y.) and crystallized bovine albumin (Pentex Biochemical, Kankakee, Ill.) in 1 N NaOH as a standard.

ALKALINE PHOSPHATASE ACTIVITY: Portions of duodenal homogenates were solubilized in 50 vol of 1% sodium dodecyl sulfate (4 h at 37.5°C). Alkaline phosphatase activity was assessed by measurement of the hydrolysis of *p*-nitrophenylphosphate (Sigma Kit 104s).

CaBP ASSAY: Portions of duodenal homogenates were analyzed for CaBP concentration by radial immunodiffusion. The procedure consisted of filling the wells of a Model FB-20 Dispo-Tray (Linbro Chemical Co., Inc., New Haven, Conn.) with 0.5 ml of a buffered agar solution (1.5% agar, 7.5% glycine, and 0.02% thimerosal (all wt/vol) in barbital buffer, ionic strength 0.1 at pH 8.6) at 48°C containing 2% (vol/vol) rabbit antiserum to purified chick intestinal CaBP. After gelling, a sample hole (2 mm) was cut into the center of each agar well and 20 μl of duodenal homogenate dispensed into it. After a reaction period of 24 h in a humidified chamber, visible precipitin rings formed around the central sample hole. The diameters of these rings were measured microscopically.

A linear relationship was found between ring diameter and concentration of purified CaBP over a range of concentrations between 2.5 and 30 μg CaBP/ml. The visibility of precipitin rings could be enhanced by either soaking the trays in 0.02% (wt/vol) cadmium acetate solution (up to 24 h) or for 15 min in a solution consisting of 0.18% (wt/vol) of Buffalo Black NBR (Allied Chemical Corp., Specialty Chemicals Div., Morristown, N.J.) in methanol:H₂O:acetic acid (5:5:1) followed by destaining in the same solvent for up to 24 h.

DNA: Portions of duodenal homogenates were heated in a H₂O bath at 95°C in 50 vol of 5% trichloroacetic acid (TCA) for 30 min. After cooling, 2 ml of the diphenylamine reagent prepared exactly as described by Burton (30) were added, the solution mixed, and the tubes sealed and allowed to stand at room temperature for 18–24 h. Standards, over the range of 10–300 μg DNA/ml, were prepared from calf thymus DNA, Na salt, (Mann Research Labs.) in 5 mM NaOH and treated exactly as the homogenates. (Preliminary trials proved that initial nucleoprotein extraction, as described by Schneider (31), was unnecessary since values for duodenal homogenate DNA obtained by both methods were virtually identical.)

⁴⁵Ca UPTAKE: At the end of the culture period, duodena which had been slit open were briefly rinsed with a low-sodium buffer solution (13) at room temperature and transferred to 3 ml of the same buffer containing 0.5 μCi ⁴⁵Ca/ml in a 25-ml Erlenmeyer flask maintained at 37.5°C in a shaking water bath. The flasks were sealed with rubber stoppers. After a 30-min incubation period, the guts were removed from the flasks, rinsed thoroughly with ice-cold buffer, blotted lightly on tissue, and weighed. They were then transferred to counting vials, 1 ml of NCS solubilizer added (Amersham/Searle Corp., Arlington Heights, Ill.), the vials sealed and heated at 50°C in an oven overnight. After cooling the vials, 50 μl of concentrated acetic acid were added followed, after mixing, by 20 ml of a cocktail composed of 5 g 2,5-diphenyloxazole and 0.1 g 1,4-bis[2-(5-phenyloxazolyl)]benzene (Amersham/Searle Corp.) per liter of toluene. The samples were assayed for ⁴⁵Ca activity in a liquid scintillation counter (Nuclear-Chicago Mark I; Nuclear-Chicago Corp., Des Plaines, Ill.). Channels ratio measurement using a ¹³³Ba external standard was always monitored but quench correction was not necessary since there was minimal variable quench in the samples. ⁴⁵Ca uptake was calculated as percent of total ⁴⁵Ca in the 3 ml of incubation buffer/100 mg fresh weight of duodenum. The data were statistically analyzed in this form, but then converted to percent of zero control for presentation.

⁴⁵Ca UPTAKE AND MUCOSAL TO SEROSAL TRANSPORT: At the end of the culture period, the everted duodenum was briefly rinsed with a low-so-

dium buffer solution (13) at room temperature and a ligature tied around one end. While injecting the open end of the sac with 25 μ l of the same buffer at 37.5°C, a second ligature was tightened around the needle and finally tied securely after withdrawal of the needle. The sac was then incubated, rinsed, and weighed exactly as were the slit-open guts (see ⁴⁵Ca Uptake above). The sac was then transferred to a counting vial and cut open. As the serosal fluid drained out, a 5- μ l aliquot was taken and transferred to another counting vial. Guts and serosal fluid samples were processed for counting exactly as given above (see ⁴⁵Ca Uptake). From a knowledge of the count rate of the 5 μ l of serosal fluid, a correction of the tissue count rate for contained serosal fluid was made. The actual weight of the tissue was assumed to be 25 μ g less than the weight of the sac plus contained serosal fluid.

³H]LEUCINE INCORPORATION: L[4,5-³H]-leucine (Amersham/Searle Corp.) of specific activity 38 Ci/mmmole, was included in the culture medium (1 μ Ci/ml) to assess its time-dependent incorporation into protein as one measure of the functional integrity of the duodenum in culture. At time intervals after the initiation of culture, duodena were homogenized and the protein precipitated from portions with 50 vol of 10% TCA. After centrifugation, the precipitates were washed with 200 vol of 10% TCA and recentrifuged. (Preliminary trials proved that the recovery of protein from the whole homogenates in the TCA precipitates was better than 92%.) The precipitates were then dissolved in 1-ml NCS solubilizer, processed and counted as above (see ⁴⁵Ca Uptake). Additional portions of whole homogenate were analyzed for total protein by the method outlined above (see Total Protein).

³H]THYMIDINE INCORPORATION: [*Methyl*-³H]thymidine (Amersham/Searle Corp.) of specific activity 23 Ci/mmmole, was included in the culture medium (1 μ Ci/ml) to assess its time-dependent incorporation into DNA in an attempt to estimate the degree of cell replication of the duodenum in culture. At time intervals after the initiation of culture, duodena were homogenized and the nucleoprotein precipitated from portions with 175 vol of ice-cold 7% TCA. After centrifugation, the precipitates were washed with 200 vol of ice-cold 7% TCA and recentrifuged. (Preliminary trials proved that the recovery of DNA from the whole homogenates in the TCA precipitates was better than 96%.) The precipitates were then dissolved in 1-ml NCS solubilizer, processed and counted as above (see ⁴⁵Ca Uptake). Additional portions of whole homogenate were analyzed for DNA by the method outlined above (see DNA).

MEASUREMENT OF ELECTROPOTENTIAL DIFFERENCE: As another indicator of the viability of

the cultured duodenum, the electropotential gradient across cultured everted sacs (see Culture Technique above) was measured as a function of time in culture. Agar bridges were prepared by filling thin-wall Teflon tubing (0.56-mm ID.; 0.25-mm wall) with a molten 3% (wt/vol) agar solution containing 20 g KCl. The bridges were connected to a DC voltmeter, model 412 A (Hewlett-Packard Co., Palo Alto, Calif.) via calomel reference electrodes, model 40250 (Beckman Instruments, Inc., Fullerton, Calif.) filled with a KCl-AgCl solution (Beckman no. 4787). At time intervals after initiation of culture, the everted duodenum was filled with the culture medium and tied into a sac around the end of one agar bridge, the whole remaining immersed in the culture medium. The end of the other agar bridge was also immersed in the culture medium. The potential gradient existing across the intestine was then measured. A correction was made for the potential existing between the agar bridge tips when both were immersed in the culture medium.

RESULTS

Assessments of the Organ Culture Technique

SUITABILITY OF SERUM-FREE MEDIUM: A series of preliminary experiments proved the suitability of serum-free medium for use in this culture technique. Of course the use of serum-free medium is an advantage since more clearly defined conditions are possible. Not only was serum found unnecessary, but several additives, proven useful as serum substitutes by others working with cell culture, were also found unnecessary. The following serum substitutes were tried: albumin (Pentex Biochemical) at 1 g/liter and/or dextran (Sigma Chemical Co., 100,000–200,000 daltons) at 10 g/liter; fraction VI glycoprotein (Pentex Biochemical) at 10 mg/liter and/or dextran at 10 g/liter (32). As will be seen, the embryonic chick duodenum responds to vitamin D₃ in the serum-free culture medium by synthesizing CaBP and exhibiting enhanced radio-calcium uptake and transport. The use of serum or serum substitutes did not increase this responsiveness. Histologically, at the light microscopic level, the tissue is quite well preserved even after 48 h of incubation at the surface of serum-free medium (Fig. 1). At the electron microscopic level, preliminary results with a few sections indicate no change in the fine structure of duodenal absorptive cells from either uncultured duodena or duodena cultured for as long as 48 h. These studies are continuing and being extended to include investigation of the

effects of various treatments on duodenal fine structure.

MITOSIS: Mitotic figures were less frequently seen in the cultured duodenum in agreement with

the observations of others (33). Preliminary studies have been undertaken in which colchicine was introduced into the culture medium to a level of $1.5 \mu\text{M}$ 3 h before the termination of a

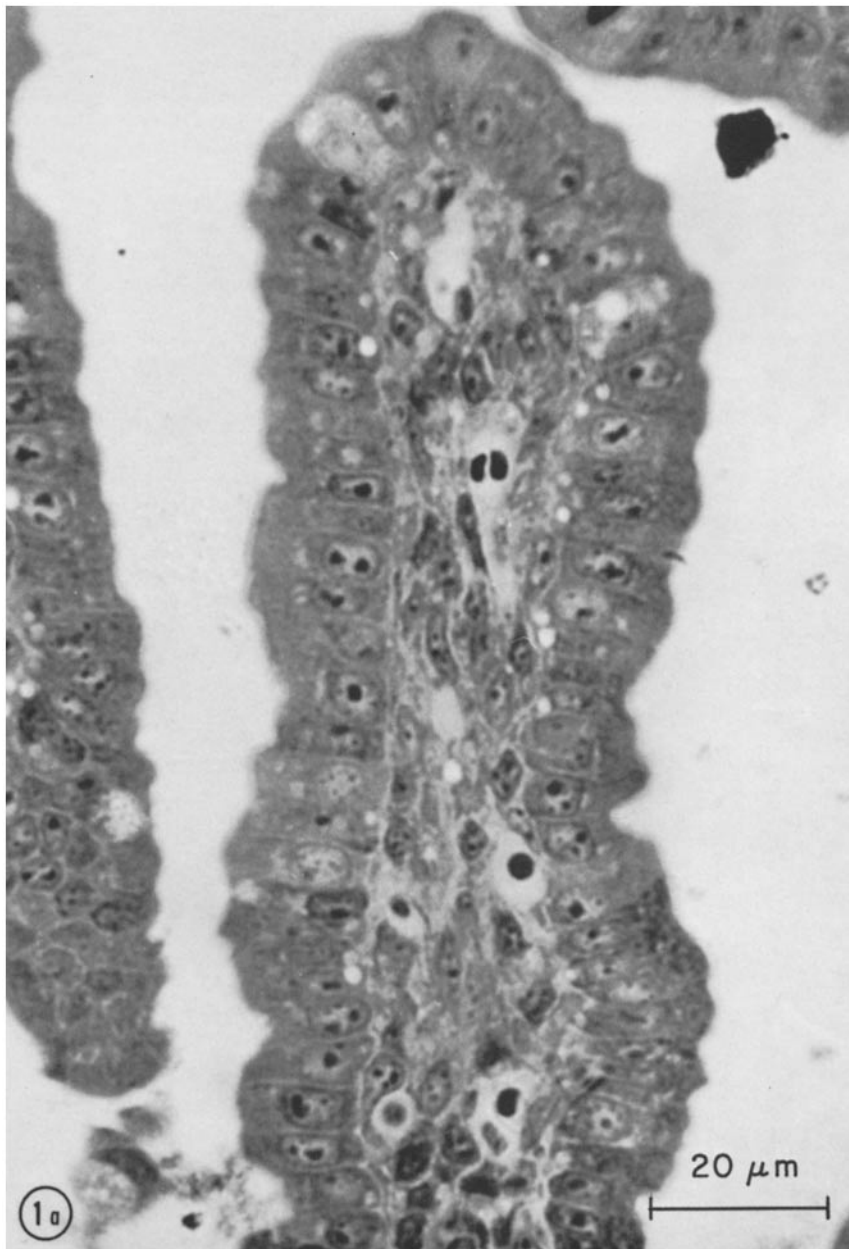
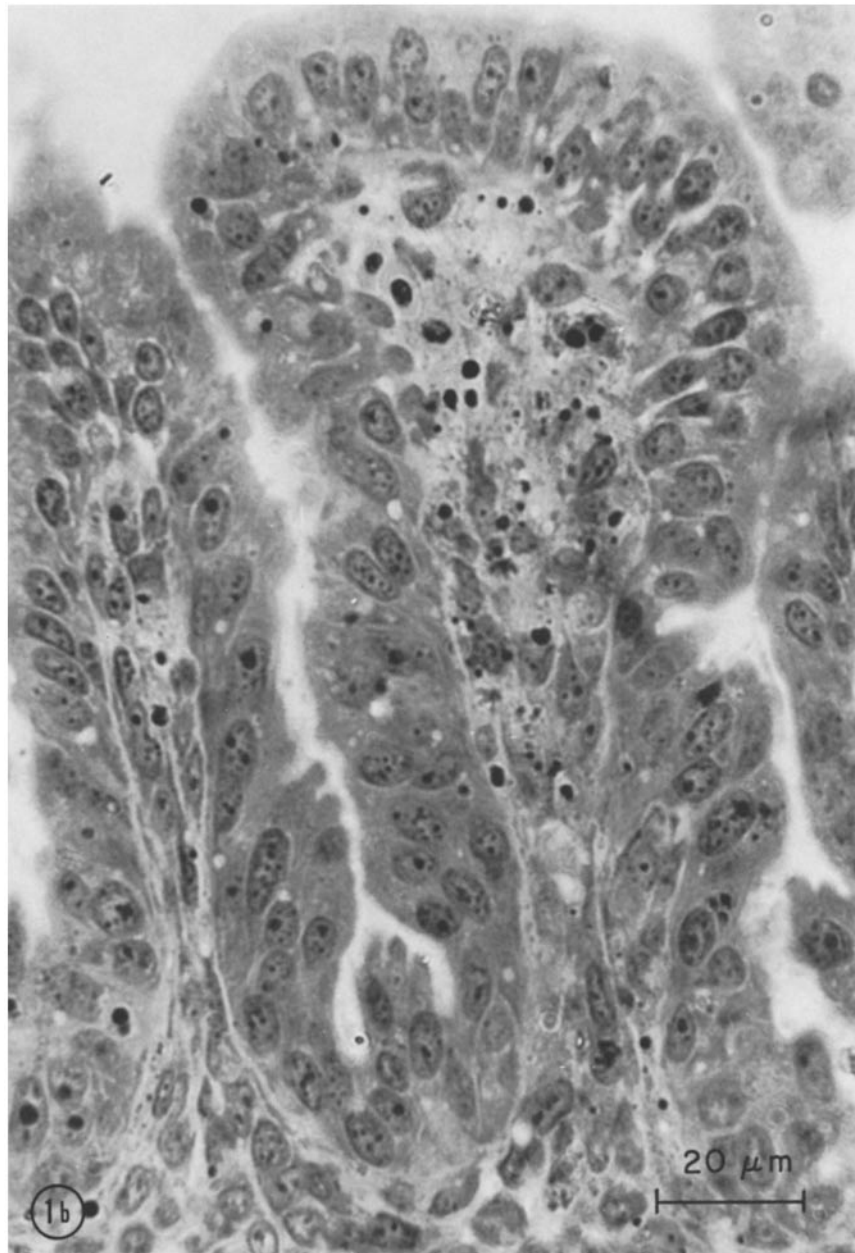


FIGURE 1 20-day embryonic chick intestine both (A) un cultured and (B) maintained in organ culture for 48 h in contact with serum-free medium. Tissues were fixed in a 0.1 M phosphate-buffered glutaraldehyde fixative, dehydrated in methanol, embedded in glycol methacrylate; $1\text{-}\mu\text{m}$ sections were prepared and stained with acid fuchsin-toluidin blue (48). The only definite change in mucosal structure at the light microscopic level was a possible shortening and thickening of the villi in the cultured intestine, probably induced by gravity as the tissue rested on the grid with the villi uppermost.

specific time interval of culture to force an accumulation of metaphase figures. Strictly quantitative data are not yet available, but initial histological examinations revealed the presence of many mitotic figures even after 48 h of organ culture (colchicine introduced at 45 h) clearly indicating that, while cell proliferation is reduced in organ-cultured duodenum, it does proceed. The [^3H]thymidine incorporation studies (see below) suggest a similar conclusion.

[^3H]LEUCINE INCORPORATION: As shown in Fig. 2, the incorporation of [^3H]leucine into duodenal protein increased directly as a function of time of incubation, but tended to taper off towards the end of the experiment. The protein synthetic mechanism was undoubtedly functioning, although not at peak efficiency, throughout the culture period. Synthesis of the specific vitamin D-induced CaBP followed a similar course as can be seen in Fig. 3.



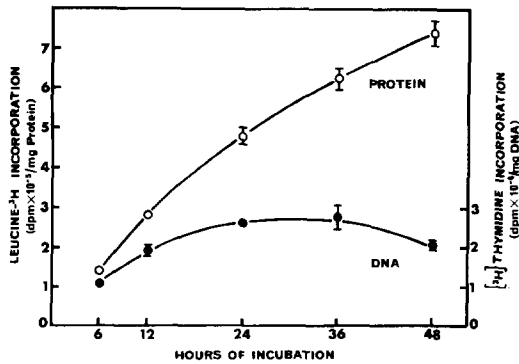


FIGURE 2 Embryonic chick intestine in organ culture: time-course of $[^3\text{H}]$ leucine and $[^3\text{H}]$ thymidine incorporation into protein and DNA, respectively. Values are the $\bar{x} \pm \text{SE}$; five guts per group.

$[^3\text{H}]$ THYMIDINE INCORPORATION: Unlike radioleucine incorporation, the rate of $[^3\text{H}]$ thymidine incorporation into DNA reached a peak at 24 h of incubation and fell thereafter (Fig. 2). This decreased DNA synthesis probably was a reflection of the decline in mitosis referred to earlier.

MEASUREMENT OF ELECTROPOTENTIAL DIFFERENCE: The electrical potential across the everted, uncultured 20-day chick embryo duodenum averaged 2.2 mV (four duodena) with the inside of the sac (serosal side) negative with respect to the outside. This value did not change appreciably over the entire 48-h period (measured at 6, 12, 24, 36, and 48 h) and was still averaging 2.4 mV (lumen negative) at 48 h (two duodena).

Time-Course of Response to Vitamin D_3

Slit-open embryonic chick duodena were incubated mucosal side up in the presence of vitamin D_3 in the medium ($26 \mu\text{M}$) for varying periods of time. At each time interval, duodena were assayed for total protein, CaBP, and ^{45}Ca uptake as described above (Materials and Methods). CaBP was detected in duodena cultured in medium containing vitamin D_3 as early as 6 h after the start of incubation and increased steadily with increasing incubation time (Fig. 3 A). A significant increase in ^{45}Ca uptake occurred within 24 h (within 12 h in an earlier experiment) after the start of incubation and a further increase was observed at 36 h after which there was no further rise (Fig. 3 B). There were no signifi-

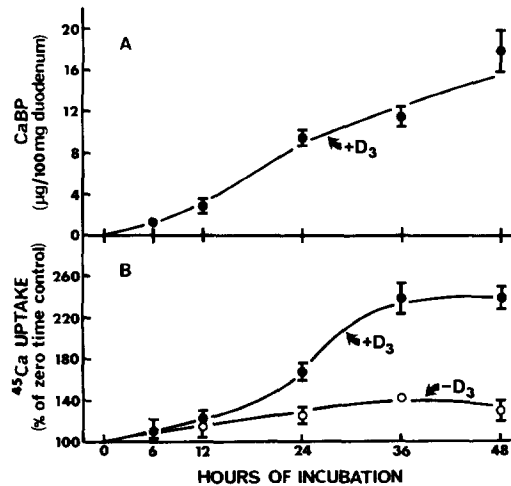


FIGURE 3 Embryonic chick intestine in organ culture: time-course of response to vitamin D_3 in the culture medium ($\pm 26 \mu\text{M}$) with respect to (A) CaBP synthesis and (B) uptake of ^{45}Ca . Closed circles, $+\text{D}_3$; open circles, $-\text{D}_3$. Values are the $\bar{x} \pm \text{SE}$; four to six guts per group.

cant changes in total protein or DNA concentrations of duodena either as a function of vitamin D_3 in the medium or time of incubation and values are, therefore, combined (Fig. 4). There was a slight decline in duodenal weight after 48 h of incubation.

Dose-Response: Vitamin D_3 , 25-OHD $_3$ and 1,25-(OH) $_2\text{D}_3$ ²

Slit-open embryonic chick duodena were incubated mucosal side up in the presence of varying concentrations of vitamin D_3 , 25-hydroxycholecalciferol (25-OHD $_3$) (provided courtesy of the Upjohn Co., Kalamazoo, Mich.) or 1,25-dihydroxycholecalciferol (1,25-(OH) $_2\text{D}_3$) (generously provided by Dr. A. W. Norman, University of California, Riverside) for 48 h. The latter compound, available only in minute quantities, was tested over a broad, but much lower, range of concentration. Duodena were assayed for total protein, CaBP, alkaline phosphatase, and ^{45}Ca uptake as described above (Materials and

² A preliminary report of this aspect of the work has already appeared: R. A. Corradino and R. H. Wasserman, 1972. Embryonic chick intestine in organ culture: relative potency of vitamin D_3 analogues in stimulating CaBP synthesis and the calcium absorptive mechanism. *Fed. Proc.* 31:686. (Abstr.)

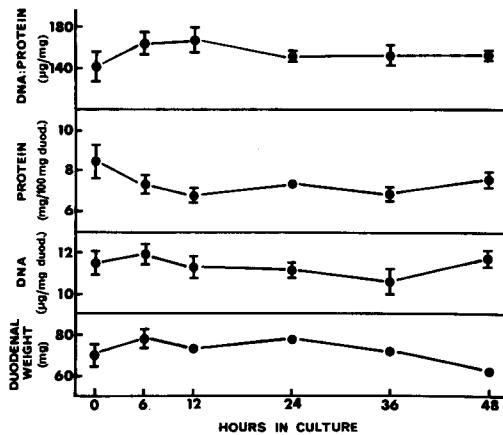


FIGURE 4 Embryonic chick intestine in organ culture: time-course of duodenal weight, total protein, and DNA concentrations. Values are the $\bar{x} \pm SE$; 10 guts per group.

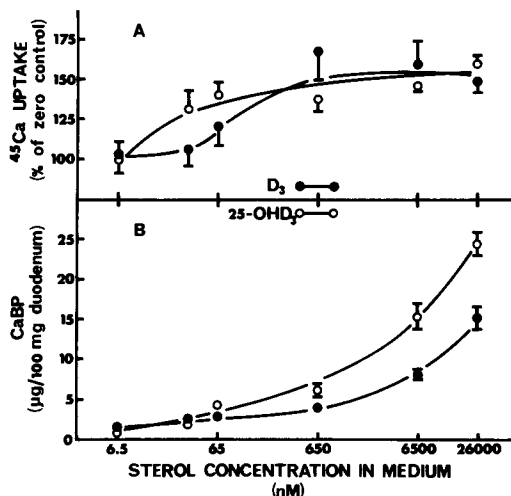


FIGURE 5 Embryonic chick intestine in organ culture: response to increasing concentrations of vitamin D₃ (closed circles) and 25-OHD₃ (open circles) in the culture medium with respect to (A) uptake of ⁴⁵Ca and (B) CaBP synthesis. Values are the $\bar{x} \pm SE$; four to six guts per group.

Methods). CaBP could be detected in duodena cultured in the presence of as little as 6.5 nM vitamin D₃ or 25-OHD₃ in the medium (Fig. 5 B). With increasing sterol concentration, CaBP increased but 25-OHD₃ was up to twice as potent as vitamin D₃. There was a significant increase in ⁴⁵Ca uptake in duodena incubated in medium containing 32.5 nM 25-OHD₃ or 65 nM vitamin D₃ (Fig. 5 A). Maximal stimulation of ⁴⁵Ca

uptake was observed at 650 nM of either sterol; there was a hint of a slightly higher response to vitamin D₃ than to 25-OHD₃ at this level but in the present experiment it was not statistically significant. There was no further increase in radiocalcium uptake with increasing sterol concentration.

The extraordinary potency of 1,25-(OH)₂D₃ in this system is shown in Fig. 6. CaBP was detected in intestine cultured in medium containing as little as 6.5 pM 1,25-(OH)₂D₃ (Fig. 6 B). Increasing 1,25-(OH)₂D₃ concentration resulted in increasing CaBP production. At the highest level of 1,25-(OH)₂D₃ that could be tested (65 nM), intestinal CaBP content was slightly greater than that found in intestine cultured in 26 µM 25-OHD₃, a 4,000-fold higher sterol concentration. In spite of this high potency for CaBP induction, stimulation of ⁴⁵Ca uptake, approximately equivalent to the maximum achieved by either vitamin D₃ or 25-OHD₃ (Fig. 5 A), was achieved only at 65 nM 1,25-(OH)₂D₃ (Fig. 6 A). There may have been a slight increase at the lowest level of 1,25-(OH)₂D₃ tested, 6.5 pM, but this has not yet been verified.

In several other experiments, alkaline phosphatase activity was also measured in organ-cultured intestine as a function of medium concentration of vitamin D₃, 25-OHD₃, and 1,25-(OH)₂D₃. Both vitamin D₃ and 25-OHD₃

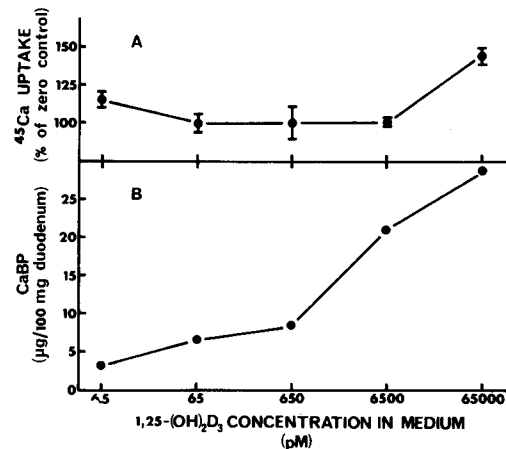


FIGURE 6 Embryonic chick intestine in organ culture: response to increasing concentrations of 1,25(OH)₂D₃ in the culture medium with respect to (A) uptake of ⁴⁵Ca and (B) CaBP synthesis. Values are the $\bar{x} \pm SE$; four guts per group, except CaBP values are from pooled samples.

TABLE I
Alkaline Phosphatase Activity of Embryonic Chick Duodena Cultured in Medium Containing Vitamin D₃, 25-OHD₃, and 1,25-(OH)₂D₃

Sterol in medium	Enzyme activity		
	Vitamin D ₃	25-OHD ₃	1,25-(OH) ₂ D ₃
<i>nM</i>		% of zero control*	
0	100	100	100
65	98.3 ± 6.5 †	104.0 ± 12.3	88
325	145.5 ± 15.3 ‡	—	—
650	—	122.5 ± 10.6 ‡	—
1,625	136.5 ± 16.7 ‡	—	—
6,500	—	132.8 ± 16.2 ‡	—
26,000	159.5 ± 13.5 ‡	136.2 ± 8.0 ‡	—

* Enzyme activity was measured as micromoles *p*-nitrophenol liberated per hour per milligram of homogenate total protein. These values were then converted to percentages of the zero control value.

† Values are $\bar{x} \pm SE$; four samples per group, except for 1,25-(OH)₂D₃ which were obtained on pooled samples.

‡ Significantly higher than zero control (1% level).

TABLE II
Vitamin D₃ Induction of CaBP and Enhancement of Mucosal-to-Serosal Transport of ⁴⁵Ca in Everted Duodena Maintained in Organ Culture

	Vitamin D ₃ in medium	
	0 μM	26 μM
CaBP (μg/100 mg duodenum)	0	7.2
⁴⁵ Ca uptake by tissue (% of zero control)*	100	187.6 ± 13.6 †
Mucosal to serosal transport of ⁴⁵ Ca (% of zero control)*	100	274.0 ± 19.6 †

* Values are $\bar{x} \pm SE$; four guts per group, except the CaBP value which was from a pooled sample.

† Significantly higher than zero control (1% level).

stimulated alkaline phosphatase activity, significant increases being observed at 325 nM vitamin D₃ and 650 nM 25-OHD₃ (Table I). (It is possible that 25-OHD₃ may stimulate alkaline phosphatase activity at 325 nM, but this was not tested.) This represents, perhaps, a 50–100 times greater sterol concentration than required for detectable CaBP induction (Fig. 5 B). At 65 nM 1,25-(OH)₂D₃ there was no stimulation of alkaline phosphatase activity (Table I). This is the same level of 1,25-(OH)₂D₃ which stimulated CaBP synthesis to a level in

excess of that induced by 4,000 times as much 25-OHD₃ (Figs. 5 B and 6 B).

Vitamin D₃ Enhancement of Mucosal to Serosal Transport of Radiocalcium

20-day chick embryonic intestine can easily be everted on a thin wire and cultured on grids (see Materials and Methods; Culture Techniques). In the presence of vitamin D₃ in the culture medium (26 μM), not only was tissue ⁴⁵Ca uptake increased significantly in everted duodenal sacs, but mucosal to serosal transport was increased as well (Table II). The portion of the everted gut mucosa in contact with the medium had lost some of its structural integrity but, on the basis of the transport data obtained, this did not affect the responsiveness of the preparation to vitamin D₃.

Specificity of Tissue Response to Vitamin D₃

Small pieces of other embryonic chick organs were explanted on grids in contact with the medium. Esophagus, stomach, liver, pancreas, lung, skin, and muscle did not contain CaBP either before or after incubation in the presence of vitamin D₃ in the medium (26 μM). On the other hand, CaBP was present in the entire intestinal tract, exclusive of the rectum, when incubated as small segments mucosal side up, only in the presence of vitamin D₃ in the medium

TABLE III
*Effect of Vitamin D₃ in the Medium on the Uptake
of Various Nutrients by Embryonic Chick
Duodenum Maintained in Organ Culture*

Isotope	Vitamin D ₃ in medium	
	0 μM	26 μM
	% of zero control*	
⁶⁵ Zn	100	95.7 ± 15.8
⁶³ Ni	100	81.8 ± 13.4
⁶⁰ Co	100	118.5 ± 10.2
⁵⁹ Fe	100	162.3 ± 11.4 ‡
⁵⁴ Mn	100	94.4 ± 13.0
⁴⁵ Ca	100	133.7 ± 10.4 ‡
³² P(-calcium)	100	129.6 ± 10.6 ‡
³² P(+calcium)	100	128.7 ± 8.1 ‡

* Values are $\bar{x} \pm SE$; six guts per group.

‡ Significantly higher than zero control (1% level).

(26 μM). Embryonic chick kidney contains CaBP as early as day 11³ and, therefore, its induction by vitamin D₃ in vitro was not evaluated.

Absorption of Nutrients other than Calcium

Embryonic chick duodena were cultured mucosal side up in the presence of vitamin D₃ in the medium (26 μM) for 48 h. They were then transferred to Erlenmeyer flasks containing 3 ml of low-sodium buffer and tracer amounts of either ⁶⁵Zn, ⁶⁸Ni, ⁶⁰Co, ⁵⁹Fe, ⁵⁴Mn, ⁴⁵Ca, or ³²P. Duodena incubated in the presence of beta-emitting isotopes (⁶³Ni, ⁴⁵Ca, ³²P) were processed and counted at optimal counter settings as described above (Materials and Methods; ⁴⁵Ca Uptake). Tissues containing gamma emitters (⁶⁵Zn, ⁶⁰Co, ⁵⁹Fe, and ⁵⁴Mn) were dissolved in 10% NaOH and assayed at optimal counter settings in a Nuclear-Chicago Auto Gamma Counter (Nuclear-Chicago Corp.). As expected, ⁴⁵Ca uptake was enhanced by vitamin D₃ (Table III). In addition, ⁵⁹Fe uptake was also stimulated by vitamin D₃. ³²P uptake was increased in tissue cultured in the presence of vitamin D₃, and equally as much in the presence or absence of calcium in the buffer solution (Table III).

³ A preliminary report of this finding has already appeared: A. N. Taylor and R. H. Wasserman. 1971. Vitamin D-induced calcium-binding protein (CaBP) in chick kidney during embryonic development and growth. *Fed. Proc.* 30:407. (Abstr.)

DISCUSSION

The data reported here establish three important facts: first, duodena from 20-day chick embryos can be cultured on specially designed grids in fairly large quantities (at least 300–400 mg tissue); second, over a 48-h culture period, mucosal structure is well preserved even in serum-free medium; and, third, the cultured tissue responds to the presence of vitamin D₃, and two of its metabolites, in the medium by synthesizing CaBP and exhibiting enhanced uptake and transport of radiocalcium. From all available evidence, the system is one of tissue maintenance rather than tissue proliferation. Nevertheless, the system described is a valuable adjunct to other methods for the study of the intestinal calcium absorptive mechanism.

In a number of significant respects, the culture system mimics in vivo conditions. For example, not only does the tissue respond to vitamin D₃ but it does so with a characteristic lag period (Fig. 3). The first clue to the reason for this lag in responsiveness to vitamin D was obtained when it was shown that actinomycin D inhibited the action of vitamin D (34, 35). This suggested that the initial action of vitamin D was to induce protein synthesis necessary for stimulation of calcium absorption. Subcellular distribution studies lent credence to this concept since vitamin D₃ and its metabolites were preferentially localized in intestinal cell nuclei (36–40). Other work showed that early actions of vitamin D₃ in the intestine were to stimulate DNA template activity (41) and RNA synthesis (42, 43), presumably, preparatory to synthesis of new protein. The discovery of the intestinal, vitamin D-induced calcium-binding protein (27) seemed to confirm the predictions of these studies. CaBP was found to be synthesized *de novo* on the basis of amino acid-labeling studies (3, 44) and studies which showed that CaBP synthesis in rachitic chicks injected with vitamin D₃ was prevented by prior injection of actinomycin D (45). Substantial correlative evidence has been accumulated linking CaBP to the vitamin D₃ stimulation of calcium transport (46–50). Most recently, direct evidence was obtained in preliminary “reconstitution” experiments using the organ culture technique. When intact, everted duodena from embryonic chicks were cultured in medium containing purified chick intestinal CaBP but no vitamin D₃, increased absorption and mucosal-

to-serosal transport of radiocalcium was observed (51). On the basis of such information, it seems likely that at least a portion of the lag period is a result of induction of CaBP synthesis. In the present experiments, CaBP was first barely detectable in duodena after 6 h of culture in the presence of 26 μM vitamin D_3 (Fig. 3 A). After 12–24 h, a significant increase in ^{45}Ca uptake was observed. In vivo work has shown a similar pattern of early synthesis of CaBP in response to vitamin D_3 after an initial lag period, followed by enhancement of calcium absorption (3, 44).

A portion of the lag period in vivo may also be the result of conversion of vitamin D_3 to more active metabolites, 25-OHD $_3$ formed in the liver (52) and, to some extent, in the kidney (53), and 1,25-(OH) $_2\text{D}_3$ formed in the kidney (54). It has been contended that metabolism of vitamin D_3 , via 25-OHD $_3$ to 1,25-(OH) $_2\text{D}_3$, is a prerequisite of vitamin D_3 action (55). However, other work has shown that, at least for embryonic chick intestine maintained in organ culture, metabolism of vitamin D_3 is unnecessary (56).

Another significant parallel exists between in vivo and in vitro responses to vitamin D_3 metabolites. It has been shown that 25-OHD $_3$ and 1,25-(OH) $_2\text{D}_3$ are faster acting and more potent than vitamin D_3 in vivo (57, 58). While a time study was not conducted in the present case, it is evident that 25-OHD $_3$ and most especially, 1,25-(OH) $_2\text{D}_3$ are more potent than vitamin D_3 in vitro (Figs. 5 and 6). While ^{45}Ca uptake was stimulated by all vitamin D_3 analogues tested at a minimum of 65 nM in the culture medium, it was clear that induction of CaBP synthesis was the more sensitive response. This provides support for in vivo observations showing that CaBP synthesis is a preliminary response to vitamin D_3 analogues, after which there is enhancement of calcium absorption (3, 44). The data in Fig. 3 bear this out directly, in the case of vitamin D_3 itself, for the organ culture system.

In vivo studies have shown that vitamin D_3 stimulates the activity of a calcium-dependent ATPase (Ca-ATPase) in brush borders isolated from chick intestinal mucosal cells (59). The time-course of stimulation of Ca-ATPase activity correlated well with vitamin D_3 enhancement of calcium transport. A similar correlation between brush border alkaline phosphatase activity and calcium transport was also shown (60). Later,

it was shown that intestinal Ca-ATPase and alkaline phosphatase were probably the same enzyme although, again, a good correlation between vitamin D-enhanced enzyme activity and calcium transport was observed (61, 62). In the in vitro studies reported herein intestinal alkaline phosphatase activity was increased by the presence of vitamin D_3 or 25-OHD $_3$ in the culture medium (Table I) but at levels higher than required to demonstrate enhancement of ^{45}Ca uptake (Fig. 5 A). Also, at a level of 1,25-(OH) $_2\text{D}_3$ in the medium which stimulated maximal enhancement of ^{45}Ca uptake, 65 nM (Fig. 6 A), alkaline phosphatase was not stimulated (Table I). Thus, the correlation between alkaline phosphatase (and/or Ca-ATPase) and calcium absorption may not, in fact, reflect an involvement of the enzyme(s) in calcium absorption. There are in vivo results which support this conclusion. When a high strontium diet was fed to previously normal chicks, there was a rapid and complete inhibition of CaBP and a parallel inhibition of ^{47}Ca (and ^{85}Sr) absorption (48, 49). Alkaline phosphatase activity also declined at a similar rate. However, if the chicks were returned to a normal diet, after 7 days on the high strontium diet, CaBP was rapidly restored to normal (49). By the end of a week, CaBP had, in fact, risen to well above normal. ^{85}Sr absorption, a well-established correlate of calcium absorption, was also reinstated to a normal and, finally, a supranormal level in very close correlation with CaBP synthesis. On the other hand, alkaline phosphatase activity rose to normal within 2 days and merely stayed at this level through the week. In another study, the vitamin D_3 -stimulated increase in intestinal brush border alkaline phosphatase (or Ca-ATPase) activity was inhibitable by inclusion of phenylalanine in the assay medium (62). However, the corresponding vitamin D_3 enhancement of ^{45}Ca uptake and mucosal-to-serosal transport by everted ileal sacs was not inhibited by the inclusion of phenylalanine in the mucosal bathing solution during incubation. On the basis of these in vivo results, and the present in vitro data, it must be concluded that the correlations observed between alkaline phosphatase (or Ca-ATPase) and the functioning of the calcium absorptive mechanism may be only coincidental.

The data of Table II are of interest because they established that the organ-cultured intestine not only responded to vitamin D_3 by exhibiting

enhanced ^{45}Ca uptake, but also maintained a mucosal-to-serosal transport system for calcium, mimicking precisely the in vivo situation. The relative simplicity of the culture procedure, along with the ability to rigorously control the environment of the tissue, makes the use of cultured, everted intestine an almost ideal system for the study of intestinal function. It is certainly well suited to investigation of the vitamin D-mediated calcium absorptive mechanism, but it should also be adaptable to studies of the absorption of other nutrients, drugs, hormones, etc.

As one indication of the culture system's adaptability, the data of Table III are relevant. Not only does the cultured intestine exhibit a vitamin D-mediated increase in calcium uptake, but also an increase in ^{32}P , ^{59}Fe , and, perhaps, a slight increase in ^{60}Co uptake. For years there have been indications that vitamin D stimulates phosphorus absorption (63). One important question has been whether vitamin D controls distinct calcium and phosphorus pumps, or a single pump with one ion following the other passively. Recent evidence favors the former possibility (64, 65). The data presented here also support that conclusion. Reference to Table III shows that essentially similar ^{32}P uptakes were obtained in intestine cultured in the presence of vitamin D_3 , whether or not calcium was present in the incubation solution. Thus, ^{32}P absorption, at least in this system, would appear to be independent of calcium.⁴

Of course, at 26 μM vitamin D_3 in the culture medium, alkaline phosphatase activity was stimulated, and CaBP induced, but there is no unequivocal evidence to conclude that this enzyme, or for that matter, CaBP, is involved in phosphate transport. Iron and cobalt absorption have been shown to be enhanced by vitamin D_3 in vivo (66) but no follow-up work has been done. There is, however, interest in cobalt and iron absorption per se (67) and the organ culture technique may provide a useful addition to existing methodology, for example, in testing intestinal luminal factors and/or serum extracts from animals in various iron nutritive states. In conclusion, it seems

⁴ Simultaneous work in vivo, a preliminary report of which has already appeared, supports this conclusion: A. N. Taylor and R. H. Wasserman. 1972. Intestinal phosphate (^{32}P) absorption: effect of vitamin D and other variables. *Fed. Proc.* 31:686. (Abstr.)

likely that the organ culture system described will prove useful not only in the study of the vitamin D-mediated, intestinal calcium absorptive mechanism but in other studies of intestinal function as well.

Thanks are due Mrs. Susan Travis and Karen Bucher Ni for their technical assistance, to Mr. Michael Dougherty for the histological preparations, to Mr. Louis Lippiello for initial EM work, and to Dr. R. H. Wasserman for his continuing interest and encouragement and for his critical evaluation of the manuscript.

This work was supported by National Institutes of Health Research Grants AM-15355 and AM-04652 and AEC Contract AT(11-1)-3167.

Received for publication 9 August 1972, and in revised form 27 March 1973.

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