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 D$_3$-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 D$_3$ in the medium by synthesizing the calcium-binding protein within 6 h and by exhibiting enhanced $^{45}$Ca uptake within 12–24 h. Concentrations of vitamin D$_3$, 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 D$_3$, at a level extremely potent in CaBP induction, did not stimulate alkaline phosphatase. Mucosal to serosal transport of $^{45}$Ca could also be measured in everted duodenal sacs, subsequent to culture under similar conditions, and was also increased by vitamin D$_3$ in the medium. Other embryonic organs, esophagus, stomach, liver, pancreas, lung, skin, and muscle, did not produce CaBP in response to vitamin D$_3$ in the culture medium. However, CaBP-synthesizing capacity was present in the entire intestinal tract, exclusive of the rectum. $^{59}$Fe and $^{82}$P uptake by cultured duodenum were also stimulated by vitamin D$_3$. 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 Nicolaesen 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, CaBP (27), was not present in 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-µm 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 duodenal 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...
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 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 assayed 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 Disposo-Tray (Linbro Chemical Co., Inc., New Haven, Conn.) with 0.5 ml of a buffered agar solution (1.5% agar, 7.5% glycerol, 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, 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 viability 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 inactivation of 20 µl 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.

₄⁴Ca uptake and mucosal to serosal transport: At the end of the culture period, the everted duodenum was briefly rinsed with a low-so-
medium buffer solution (13) at room temperature and a ligature tied around one end. While injecting the open end of the sac with 25 μl 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-μl 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 μl 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 μg 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/mmol, was included in the culture medium (1 μCi/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/mmol, was included in the culture medium (1 μCi/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 KCI-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 radiocalcium 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 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 μM 3 h before the termination of a

**Figure 1** 39-day embryonic chick intestine both (A) uncultured 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-μm 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.
[3H]thymidine incorporation: Unlike radioleucine incorporation, the rate of [3H]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 D₃

Slit-open embryonic chick duodena were incubated mucosal side up in the presence of vitamin D₃ in the medium (26 μM) for varying periods of time. At each time interval, duodena were assayed for total protein, CaBP, and ⁴⁴Ca uptake as described above (Materials and Methods). CaBP was detected in duodena cultured in medium containing vitamin D₃ as early as 6 h after the start of incubation and increased steadily with increasing incubation time (Fig. 3 A). A significant increase in ⁴⁴Ca 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-

Dose-Response: Vitamin D₃, 25-OHD₃ and 1,25-(OH)₂D₃

Slit-open embryonic chick duodena were incubated mucosal side up in the presence of varying concentrations of vitamin D₃, 25-hydroxycholecalciferol (25-OHD₃) (provided courtesy of the Upjohn Co., Kalamazoo, Mich.) or 1,25-dihydroxycholecalciferol (1,25-(OH)₂D₃) (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 ⁴⁴Ca 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. Waserman. 1972. Embryonic chick intestine in organ culture: relative potency of vitamin D₃ analogues in stimulating CaBP synthesis and the calcium absorptive mechanism. Fed. Proc. 31:686. (Abstr.)
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
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 | Vitamin D3 | 25-OHD3 | 1,25-(OH)2D3 |
|------------------|------------|---------|--------------|
| nM               | % of zero control* | % of zero control* | % 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 ±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 D3 in medium | 0 µM | 26 µM |
|----------------------|------|-------|
| CaBP (µg/100 mg duodenum) | 0    | 7.2   |
| 45Ca uptake by tissue (% of zero control)* | 100  | 187.6 ± 13.6† |
| Mucosal to serosal transport of 45Ca (% of zero control)* | 100  | 274.0 ± 19.6§ |

* Values are ±SE; four guts per group, except the CaBP value which was from a pooled sample.
† Significantly higher than zero control (1% level).
§ Significantly higher than zero control (1% level).

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 D3 in medium | % of zero control* |
|-----------|---------------------|--------------------|
|           | 0 µM                | 26 µM              |
| 65Zn      | 100                 | 95.7 ± 15.8        |
| 63Ni      | 100                 | 81.8 ± 13.4        |
| 60Co      | 100                 | 118.5 ± 10.2       |
| 59Fe      | 100                 | 162.3 ± 11.4†      |
| 54Mn      | 100                 | 94.4 ± 13.0        |
| 45Ca      | 100                 | 133.7 ± 10.4‡      |
| 32P(-calcium) | 100               | 129.6 ± 10.6‡      |
| 32P(+calcium) | 100               | 128.7 ± 8.1‡       |

* Values are x ±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 duodenal 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, 63Ni, 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, 4Ca 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. 30:467. (Abstr.)

DISCUSSION

The data reported here establish three important facts: first, duodenal 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 "reconstruction" 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 D₃ (Fig. 3 A). After 12-24 h, a significant increase in ⁴⁶Ca uptake was observed. In vivo work has shown a similar pattern of early synthesis of CaBP in response to vitamin D₃ 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 D₃ to more active metabolites, 25-OHD₃ formed in the liver (52) and, to some extent, in the kidney (53), and 1,25-(OH)₂D₃ formed in the kidney (54). It has been contended that metabolism of vitamin D₃ via 25-OHD₃ to 1,25-(OH)₂D₃ is a prerequisite of vitamin D₃ action (55). However, other work has shown that, at least for embryonic chick intestine maintained in organ culture, metabolism of vitamin D₃ is unnecessary (56).

Another significant parallel exists between in vivo and in vitro responses to vitamin D₃ metabolites. It has been shown that 25-OHD₃ and 1,25-(OH)₂D₃ are faster acting and more potent than vitamin D₃ in vivo (57, 58). While a time study was not conducted in the present case, it is evident that 25-OHD₃ and most especially, 1,25-(OH)₂D₃ are more potent than vitamin D₃ in vitro (Figs. 5 and 6). While ⁴⁶Ca uptake was stimulated by all vitamin D₃ 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 D₃ 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 D₃ itself, for the organ culture system.

In vivo studies have shown that vitamin D₃ 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 D₃ 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 D₃-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 D₃ or 25-OHD₃ in the culture medium (Table I) but at levels higher than required to demonstrate enhancement of ⁴⁶Ca uptake (Fig. 5 A). Also, at a level of 1,25-(OH)₂D₃ in the medium which stimulated maximal enhancement of ⁴⁶Ca 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 ⁴⁶Ca (and ⁴⁸Sr) 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. ⁴⁸Sr absorption, a well-established correlate of calcium absorption, was also reinstated to a normal and, finally, a supernormal 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 D₃-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 D₃ enhancement of ⁴⁶Ca 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 D₃ by exhibiting
enhanced ⁴⁰Ca 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 ³²P, ⁴⁰Fe, and, perhaps, a slight increase in ⁵⁷Co 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 ³²P uptakes were obtained in intestine cultured in the presence of vitamin D3, whether or not calcium was present in the incubation solution. Thus, ³²P absorption, at least in this system, would appear to be independent of calcium.⁴

Of course, at 26 μM vitamin D3 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 D3 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 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.

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REFERENCES

1. NICOLAYSSEN, R. 1937. Studies upon the mode of action of vitamin D. IV. The absorption of calcium chloride, xylose and sodium sulphate from isolated loops of the small intestine and of calcium chloride from the abdominal cavity in the rat. Biochem. J. 31:323.

2. COATES, M. E., and E. S. HOLDSWORTH. 1961. Vitamin D₃ and absorption of calcium in the chick. Br. J. Nutr. 15:131.

3. MACGREGOR, R. R., J. W. HAMILTON, and D. V. COHN. 1971. The induction of calcium-binding protein biosynthesis by vitamin D₃ and 25-hydroxycholecalciferol. Clin. Orthop. Related Res. 78:83.

4. WASSERMAN, R. H., and F. A. KALLFELZ. 1962. Vitamin D₃ and unidirectional fluxes across the rachitic chick duodenum. Am. J. Physiol. 203:221.

5. OLSON, E. B., and H. F. DELUCA. 1969. 25-Hydroxycholecalciferol: direct effect on calcium transport. Science (Wash., D.C.). 163:405.

6. WASSERMAN, R. H. 1962. Studies on vitamin D₃ and the intestinal absorption of calcium and other ions in the rachitic chick. J. Nutr. 77:69.

7. OMDAHL, J., M. HOLICK, T. SUVA, Y. TANAKA, and H. F. DELUCA. 1971. Biological activity of 1,25-dihydroxycholecalciferol. Biochemistry. 10:2935.

8. CRAMER, C. F., and J. DUECK. 1962. In vivo transport of calcium from healed Thiry-vella fistulas in dogs. Am. J. Physiol. 202:161.

9. CRAMER, C. F., C. O. PARKES, and D. H. COPP. 1969. The effect of chicken and hog calcitonin

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on some parameters of Ca, P, and Mg metabolism in dogs. *Can. J. Physiol. Pharmacol.* 47:181.

10. Wilson, T. H., and G. Wiseman. 1954. The use of sacs of everted small intestine for the study of the transference of substances from the mucosal to the serosal surface. *J. Physiol. (Lond.)* 123:116.

11. Schachter, D., and S. M. Rosen. 1959. Active transport of $^{48}$Ca by the small intestine and its dependence on vitamin D. *Am. J. Physiol.* 196:357.

12. Wasserman, R. H., and A. N. Taylor. 1963. The non-essentiality of sodium ions for intestinal calcium transport. *Proc. Soc. Exp. Biol. Med.* 114:479.

13. Hurwitz, S., H. C. Harrison, and H. E. Harrison. 1967. Effect of vitamin D$_{3}$ on the in vitro transport of calcium by the chick intestine. *J. Nutr.* 91:319.

14. Kimberg, D. V., R. D. Baerg, E. Gershon, and R. T. Graulius. 1971. Effect of cortisone treatment on the active transport of calcium by slices of the small intestine. *Am. J. Physiol.* 198:275.

15. Bar, A., and S. Hurwitz. 1972. In vitro calcium transport in laying fowl intestine: effect of bile preparations. *Comp. Biochem. Physiol.* 41A:383.

16. Martin, D. L., and H. F. DeLuca. 1969. Calcium transport and the role of vitamin D. *Arch. Biochem. Biophys.* 134:139.

17. Ussing, H. H., and K. Zerahn. 1951. Active transport of sodium as the source of electric current in the short-circuited isolated frog skin. *Acta Physiol. Scand.* 23:110.

18. Schachter, D. 1963. Vitamin D and the active transport of calcium by the small intestine. In the Transfer of Calcium and Strontium across Biological Membranes. R. H. Wasserman, editor. Academic Press Inc., New York. 197.

19. Adams, T. H., and A. W. Norman. 1970. Studies on the mechanism of action of calciferol. I. Basic parameters of vitamin D-mediated calcium transport. *J. Biol. Chem.* 245:4421.

20. Wasserman, R. H., and A. N. Taylor. 1969. Some aspects of the intestinal absorption of calcium, with special reference to vitamin D. In Mineral Metabolism: an Advanced Treatise. Vol. 3. Calcium Physiology. C. L. Comar and F. Bronner, editors. Academic Press Inc., New York. 321.

21. Levine, R. R., W. F. McNary, P. J. Kornguth, and R. LeBlanc. 1970. Histological revaluation of everted gut technique for studying intestinal absorption. *Eur. J. Pharmacol.* 9:211.

22. Strauss, E. W. 1963. The absorption of fat by intestine of golden hamster in vitro. *J. Cell Biol.* 17:597.

23. Strange, W. 1926. Experimental studies on the differentiation of embryonic tissues growing in vivo and in vitro. I. The development of the undifferentiated limb bud. *Proc. R. Soc. Lond. B Biol. Sci.* 99:340.

24. Thomas, J. A., editor. 1970. Organ Culture. Academic Press Inc., New York.

25. Corradino, R. A., and R. H. Wasserman. 1971. Vitamin D$_{3}$: Induction of calcium-binding protein in embryonic chick intestine in vitro. *Science (Wash. D. C.)* 172:731.

26. Wasserman, R. H., and A. N. Taylor. 1966. Vitamin D$_{3}$-induced calcium-binding protein in chick intestinal mucosa. *Science (Wash. D. C.)* 152:791.

27. Strauss, E. W. 1969. Appearance of vitamin D$_{3}$-induced calcium-binding protein (CaBP) in chick intestine during development. *Fed. Proc.* 28:1834.

28. Lowry, O. H., M. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* 193:265.

29. Burton, K. 1956. A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. *Biochemistry.* 62:315.

30. Schneider, W. C. 1945. Phosphorus compounds in animal tissues. I. Extraction and estimation of desoxypentose nucleic acid and of pentose nucleic acid. *Proc. Natl. Acad. Sci.* 31:295.
37. STOHS, S. J., and H. F. DELUCA. 1967. Subcellular location of vitamin D and its metabolites in intestinal mucosa after a 10-I.U. dose. Biochemistry. 6:3338.

38. HAUSLIER, M. R., J. F. MYRTLE, and A. W. NORMAN. 1968. The association of a metabolite of vitamin D with intestinal mucosa chromatin in vivo. J. Biol. Chem. 243:4055.

39. HAUSLIER, M. R., and A. W. NORMAN. 1969. Chromosomal receptor for a vitamin D metabolite. Biochemistry. 6:2155.

40. COUSINS, R. J., H. F. DELUCA, T. SUĐA, T. CHEN, and Y. TANAKA. 1970. Metabolism and subcellular location of 25-hydroxycholecalciferol in intestinal mucosa. Biochemistry. 9:1453.

41. HALLICK, R. B., and H. F. DELUCA. 1969. Vitamin D3-stimulated template activity of chromatin from rat intestine. Proc. Natl. Acad. Sci. U. S. A. 63:528.

42. NORMAN, A. W. 1966. Vitamin D mediated synthesis of rapidly labeled RNA from intestinal mucosa. Biochem. Biophys. Res. Commun. 23:335.

43. STOHS, S. J., J. E. ZULL, and H. F. DELUCA. 1967. Vitamin D stimulation of [1H] orotic acid incorporation into ribonucleic acid of rat intestinal mucosa. Biochemistry. 6:1304.

44. MACGREGOR, R. R., J. W. HAMILTON, and D. V. COHN. 1970. Metabolism and subcellular location of 25-hydroxycholecalciferol. J. Biol. Chem. 243:4055.

45. CORRADINO, R. A., and R. H. WASSERMAN. 1968. Actinomycin D inhibition of vitamin D3-induced calcium-binding protein (CaBP) formation in chick duodenal mucosa. Arch. Biochem. Biophys. 126:957.

46. TAYLOR, A. N., and R. H. WASSERMAN. 1969. Correlations between the vitamin D-induced calcium binding protein and intestinal absorption of calcium. Fed. Proc. 28:1834.

47. EBEL, J. G., A. N. TAYLOR, and R. H. WASSERMAN. 1969. Vitamin D-induced calcium-binding protein of intestinal mucosa: Relation to vitamin D dose level and lag period. Am. J. Clin. Nutr. 22:431.

48. CORRADINO, R. A., J. G. EBEL, P. H. CRAIG, A. N. TAYLOR, and R. H. WASSERMAN. 1971. Calcium absorption and the vitamin D3-dependent calcium-binding protein. I. Inhibition by dietary strontium. Calcif. Tissue Res. 7:81.

49. CORRADINO, R. A., J. G. EBEL, P. H. CRAIG, A. N. TAYLOR, and R. H. WASSERMAN. 1971. Calcium absorption and the vitamin D3-dependent calcium-binding protein. II. Recovery from dietary strontium inhibition. Calcif. Tissue Res. 7:93.

50. MORRISSEY, R. L., and R. H. WASSERMAN. 1971. Calcium absorption and calcium-binding protein in chicks on differing calcium and phosphorus intakes. Am. J. Physiol. 220:1509.

51. CORRADINO, R. A., and R. H. WASSERMAN. 1971. Stimulation of calcium transport in embryonic chick intestine by incubation in medium containing vitamin D3-induced calcium-binding protein. Biophys. Soc. Annu. Meet. Abstr. 11:276a. (Abstr.)

52. PONGCH, G., A. A. KENNAN, and H. F. DELUCA. 1969. "Activation" of vitamin D by the liver. J. Clin. Invest. 48:2032.

53. HAUSLIER, M. R. 1972. Studies on the production of 25-hydroxy-vitamin D3 and 1,25-dihydroxy-vitamin D3 in vitro. Fed. Proc. 31:693. (Abstr.)

54. LAWSON, D. E. M., D. R. FRASER, E. KODICEK, H. R. MORRIS, and D. H. WILLIAMS. 1971. Identification of 1,25-dihydroxycholecalciferol, a new kidney hormone controlling calcium metabolism. Nature (Lond.). 230:228.

55. TANAKA, Y., H. F. DELUCA, J. OMDAHL, and M. F. HOLICK. 1971. Mechanism of action of I,25-dihydroxycholecalciferol on intestinal calcium transport. Proc. Natl. Acad. Sci. U. S. A. 68:1286.

56. CORRADINO, R. A. 1973. Embryonic chick intestine in organ culture: Response to vitamin D3 and its metabolites. Science (Wash. D.C.). 179:402.

57. BLUNT, J. W., Y. TANAKA, and H. F. DELUCA. 1968. The biological activity of 25-hydroxycholecalciferol. Proc. Natl. Acad. Sci. U. S. A. 61:1503.

58. MYRTLE, J. F., and A. W. NORMAN. 1971. Vitamin D: a cholecalciferol metabolite highly active in promoting intestinal calcium transport. Science (Wash. D.C.). 171:79.

59. MELANCON, M. J., JR., and H. F. DELUCA. 1970. Vitamin D stimulation of calcium-dependent adenosine triphosphatase in chick intestinal brush borders. Biochemistry. 9:1658.

60. NORMAN, A. W., A. K. MIRCHEFF, T. H. ADAMS, and A. SPIELVOGEL. 1970. Studies on the mechanism of action of calciferol. III. Vitamin D-mediated increase of intestinal brush border alkaline phosphatase activity. Biochem. Biophys. Acta. 213:548.

61. HAUSLIER, M. R., L. A. NAGODE, and H. RASMUSEN. 1970. Induction of intestinal brush border alkaline phosphatase by vitamin D and identity with Ca-ATPase. Nature (Lond.). 228:1199.

62. HOLDSWORTH, E. W. 1970. The effect of vitamin D on enzyme activities in mucosal cells of chick small intestine. J. Membrane Biol. 3:43.

R. A. CORRADINO Intestinal Calcium Absorptive Mechanism in Embryonic Chick 77
63. Nicolaysen, R. 1937. Studies upon the mode of action of vitamin D. III. The influence of vitamin D on absorption of calcium and phosphorus. *Biochem. J.* 31:122.

64. Kowarski, S., and D. Schachter. 1969. Effects of vitamin D on phosphate transport and incorporation into mucosal constituents of rat intestinal mucosa. *J. Biol. Chem.* 244:211.

65. Hurwitz, S., and A. Bar. 1972. Site of action of vitamin D. *Am. J. Physiol.* 222:761.

66. Wasser, R. H. 1962. Studies on vitamin D and the intestinal absorption of calcium and other ions in the rachitic chick. *J. Nutr.* 77:59.

67. Thomson, A. B. R., C. Shaver, D. J. Lee, B. L. Jones, and L. S. Volberg. 1971. Effect of varying iron stores on site of intestinal absorption of cobalt and iron. *Am. J. Physiol.* 220:874.