J. Membrane Biol. 3, 43–53 (1970)

The Effect of Vitamin D on Enzyme Activities in the Mucosal Cells of the Chick Small Intestine

E. S. HOLDSWORTH

Biochemistry Department, University of Tasmania, Hobart, Tasmania, Australia

Received 16 March 1970

Summary. A search was made for enzyme activities that are increased after vitamin D treatment of rachitic chicks. Three enzyme activities located in the brush borders of the mucosal cells of the intestine – ATPase, p-nitrophenyl phosphatase, and pyrophosphatase – were found to approximately double in activity 48 hr after vitamin D was given. The ATPase and the p-nitrophenyl phosphatase required Mg^{++} for activity but could be further stimulated by addition of Ca^{++}. The three activities are probably caused by the same enzyme since 20 mM phenylalanine inhibited all three activities. It is unlikely that the Ca^{++}-stimulated ATPase is concerned with Ca^{++} translocation since phenylalanine, which inhibits this enzyme, had no effect on ^{45}Ca transport from mucosal to serosal fluids of everted sacs of intestine.

Vitamin D is known to be directly concerned with the translocation of Ca^{++} (reviewed by Norman, 1968) and indirectly concerned with the translocation of phosphate across the epithelium of the small intestine of the chick (Neville & Holdsworth, 1968). The effect of the vitamin can be prevented by actinomycin D (Zull, Czarnowska-Misztal & De Luca, 1965), and the suggestion is that vitamin D causes the formation of specific proteins that are concerned in Ca^{++} transport. One such protein appears to be a protein that specifically combines with Ca^{++}, and which is formed after vitamin D is given and at the same time as Ca^{++} translocation is increasing (Ebel, Taylor & Wasserman, 1969).

An increase in ion transport could be obtained if vitamin D increased cell metabolism, but no increased O_2 uptake or formation of more lactate from glucose by mucosal cells could be observed when vitamin D was given to rachitic chicks (Sallis & Holdsworth, 1962). By studying frozen sections of rat jejunum and using specific staining methods, Cheesman, Copping and Prebble (1964) found that the only significant change in rats receiving vitamin D, compared with rachitic rats, was in the alkaline phosphatase Since the assessment of stained sections is subjective, the authors kindly made the slides available for study and it was obvious that there was

increased alkaline phosphatase in the vitamin D-treated rats and that this activity was situated at or just below the mucosal brush border. More recently, it was reported that vitamin D stimulated the synthesis of a Ca^{++}-dependent ATPase in the brush borders of rat small intestine (Martin, Melancon & De Luca, 1969). Since alkaline phosphatase can cause the hydrolysis of ATP, it was of interest to see whether the enzyme stimulated by vitamin D was a specific ATPase or a nonspecific phosphatase. Observations on chick mucosal cells reported in this paper support the view of a nonspecific phosphatase.

Materials and Methods

Chickens

White crossbred cockerels were raised from hatching for 4 weeks on a diet deficient only in vitamin D as described in a previous publication (Sallis & Holdsworth, 1962.) To study the effect of vitamin D, chicks were given an intramuscular injection of 400 IU vitamin D_3 in 0.1 ml propylene glycol 8, 16, 48 or 72 hr before the experiment. When the action of vitamin D_3 was to be prevented by actinomycin D, the antibiotic was given by intraperitoneal injection (0.5 μg/g body weight) at the time of injection of vitamin D_3.

Homogenate of Mucosal Cells

Mucosal cells were scraped from washed duodena and homogenized in 30 mM Tris-HCl (pH 7.4), using 10 ml buffer per duodenum.

Preparation of Brush Borders

With the chicks under ether anethesia, the duodenum was washed out with warm saline, removed from the chick and everted onto a stainless steel rod. The rod was placed in a vibrator (Vibro-Mixer from A.G. fur Chemie-Apparatebau, Zurich) and the brush borders detached by vibrating for 4 min in ice-cold buffer. Two separate procedures were used.

(1) This was essentially the method of Forstner, Sabesin and Isselbacher (1968) but using Tris ethylenediaminetetraacetate (EDTA), pH 7.4, rather than Na EDTA. The step of resuspending the crude brush borders in 90 mM NaCl:0.8 mM EDTA was repeated at least three times until the preparation was free of nuclei. (2) This method was the same procedure but no EDTA was used, the buffer consisting of 20 mM Tris-HCl, pH 7.4.

The processes were checked using observations by phase contrast microscopy, and the preparations obtained using EDTA showed sheets of brush borders with apparently intact microvilli. In the absence of EDTA during preparation, the brush borders did not have clearly defined microvilli which appeared to be swollen and the yields were much smaller. Both preparations of brush borders were washed once with 30 mM Tris-HCl, pH 7.4, and the protein concentration was adjusted to 0.5 mg/ml.

Estimation of Enzyme Activities

All estimations were made at 26 °C in suitable buffer solutions to which different combinations of metal ions were added in the following concentrations: 2 mM Mg^{++},

5 or 20 mM Ca^{++}, 15 mM K^+, and 50 mM Na^+. The enzyme reactions were linear over the time periods choosen. The results were calculated as the number of µmoles substrate hydrolyzed/min per mg protein. The effects of inhibitors on the enzyme reactions were studied by adding one of the following substances to the assay medium: 10^{-3} M ouabain, 2×10^{-2} M D-L-phenylalanine, 10^{-3} M phlorizin or 10^{-4} M dicyclohexylcarbodiimide (DCC). The effect of the inhibitor was expressed as the percent of activity obtained in the absence of inhibitor.

Acid p-nitrophenyl phosphatase (EC. 3.1.3.2). Suitable numbers of the preparations were incubated with 1 ml 10 mM p-nitrophenyl phosphate in 0.1 M sodium citrate buffer, pH 4.8, for 5 min. The reaction was stopped with 5 ml 0.02 N NaOH, and the p-nitrophenol released was measured at 405 nm.

Alkaline p-nitrophenyl phosphatase (EC. 3.1.3.1). Suitable numbers of the preparations were incubated with 3 ml 10 mM p-nitrophenyl phosphate in 0.1 M Tris-HCl buffers at pH values 8.0 to 9.5, and the p-nitrophenol released was followed continuously by measuring optical density at 405 nm.

Pyrophosphatase (EC. 3.6.1.1). Suitable numbers of the preparations were incubated with 1 ml 2 mM sodium pyrophosphate in 300 mM Tris-cacodylate buffer of pH values 6.2 to 7.4 or in 300 mM Tris-HCl of pH values 7.4 to 9.2. After 5-min incubation, 2 ml acid phosphate reagent and 3 ml n-butanol were added, and the liberated inorganic phosphate was measured at 310 nm as described by Parvin and Smith (1969).

Adenosine triphosphatase (EC. 3.6.1.3). Suitable numbers of the preparations were incubated in 1 ml 2 mM Tris-ATP in the same buffers as for pyrophosphate, and the inorganic phosphate released in 5 min was measured in the same manner.

In some experiments, γ^{32}P ATP was used in the same concentration and with the same buffers, but after 5-min incubation the reaction was stopped with 5 ml ice-cold 5 % w/v trichloroacetic acid. After centrifugation to remove protein, the supernatant was shaken with 100 mg acid-washed charcoal, centrifuged and treated with an additional 100 mg charcoal, which completely removed any unhydrolyzed ATP. The $^{32}P_i$ in the supernatant was measured by scintillation counting and the amount of hydrolysis calculated from the original counts of the ^{32}P ATP.

Phosphodiesterase (EC. 3.1.4.1). Suitable numbers of the preparations in 0.1-ml were incubated for 30 min with 0.1 ml 300 mM glycylglycine amounts buffer, pH 7.8, containing 2 mM Mg^{++}, 1 µmole ^{3}H adenosine 3′ 5′ cyclic monophosphate (1 µc) and 200 µg purified alkaline phosphatase (from chick intestine; Worthington). The reaction was stopped by heating to 100 °C for 3 min. The material was transferred to the top of a column, 1 × 5 cm of Ecteola-cellulose (Whatman ET 41) that had previously been equilibrated with 0.2 M triethylamine acetate, pH 7.5, and then washed with water. The material was washed on the column with 20 ml distilled water and the effluent collected. The phosphodiesterase converts ^{3}H cyclic AMP to ^{3}H AMP which is hydrolyzed by the added alkaline phosphatase to ^{3}H adenosine which passed through the column and was measured by scintillation counting. The unhydrolyzed ^{3}H cyclic AMP could be recovered by elution with 0.2 M triethylanine acetate, pH 6.0. The nmoles of cyclic AMP hydrolyzed were calculated from the nmoles ^{3}H adenosine formed.

Adenyl cyclase. Suitable numbers of the "membrane preparation" (Streeto & Reddy, 1967) or brush borders in 0.2-ml amounts were ncubated with 0.2 ml of the incubation mixture described by Neville and Holdsworth (1969), which contained 1 µmole [8 ^{14}C] adenosine-5′-triphosphate (2.5 µc) and a regenerating system. After 10 min, the reaction was stopped by heating to 100 °C for 3 min. All the adenine-containing compounds were isolated by chromatographing 0.1 ml supernatant on

Whatman 3 MC paper using the solvent ethanol-1 M ammonium acetate, 75:30, and developing for 20 hr. The radioactive zones were located by autoradiography, the zones cut out, eluted and measured by scintillation counting. From the known radioactivity of the ^{14}C ATP and of an internal standard of ^{3}H cyclic AMP (Streeto & Reddy, 1967), the nmoles of the substances formed were calculated.

The Transport of ^{45}Ca by Everted Sacs of Chick Ileum

Segments of the distal third (ileum) of chick small intestine were everted as described by Sallis and Holdsworth (1962). The medium was Krebs-Henseleit bicarbonate buffer containing 20 mM glucose. The everted sacs were filled with 1 ml of this buffer containing 1 mM Ca^{++} and immersed in 5 ml of the same buffer but containing 1 mM ^{45}Ca (2 μc/ml). After incubation at 35 °C in an atmosphere of 95 % O_2 − 5 % CO_2 for 30 min, the ^{45}Ca accumulated in the serosal fluid was estimated (Sallis & Holdsworth, 1962). When the effect of phenylalanine on Ca^{++} transport was to be studied, it was included in the mucosal fluid at 50 mM.

Results

Adenyl Cyclase

The "membrane fraction" prepared from mucosal cells has previously been shown to contain adenyl cyclase, and the activity was greater in vitamin D_3-replete than in rachitic chicks (Neville & Holdsworth, 1969). No adenyl cyclase could be detected in brush borders. Table 1 shows the products formed by incubating ATP with brush borders and the "membrane fraction". Although the assay system contained an ATP-regenerating system, the preparations made from vitamin D_3-replete chicks had enzymes present that caused the formation of more ADP, AMP and adenosine than when rachitic chicks were used.

Table 1. *Assay of preparations from mucosal cells for adenyl cyclase*[a]

Preparation	Type of chicks	Adenyl cyclase (nmoles/10 min)				
		ATP	ADP	AMP	Cyclic AMP	Adeno-sine
Membrane fraction	Rachitic	840	110	40	1.1	10
	Vitamin D_3-replete	620	190	130	1.7	28
Brush borders	Rachitic	870	100	none	none	10
	Vitamin D_3-replete	510	290	147	none	40

[a] "Membrane fraction" (2.3 mg) or brush borders (0.9 mg) prepared from mucosal cells of the duodena of rachitic or vitamin D_3-replete chicks were incubated 10 min at 26 °C with 1 μmole ^{14}C-ATP as described in Methods section. The adenine-containing materials were separated by chromatography and the number of nmoles present at 10 min calculated (mean values of duplicate assays). At zero time, 1 μmole ATP contained 860 nmoles ATP, 100 nmoles ADP, and 10 nmoles adenosine.

Phosphodiesterase

No phosphodiesterase could be detected in the brush borders of mucosal cells. Homogenates of the mucosal cells hydrolyzed cyclic AMP, and the same amount of activity was present in rachitic or vitamin D_3-replete cells (vitamin D_3, 400 IU for 16 or 48 hr) – 1.9 ± 0.3 nmole per mg protein/min (mean value of six rachitic and six vitamin D_3-replete chicks).

ATPase

Brush borders contained a very active ATPase that had a broad pH optimum between pH 7.8 and 8.9. The enzyme required Mg^{++} or Ca^{++} for activity but was not affected by small concentrations of K^+ or Na^+. In a system comprised of 2 mM Mg-ATP, there was an increase in activity with 5 mM Ca^{++} and a further slight increase when the Ca^{++} was made 20 mM (Table 2). These results were obtained with $\gamma^{32}P$ ATP (three experiments), but approximately twice these values were obtained if the inorganic phosphate released was measured colorimetrically (four experiments), and in these experiments the simultaneous presence of Na^+ and K^+ was found to be without effect on activity. The presence or absence of EDTA in the buffer for the isolation of the brush border had no effect on the results, and there was always more ATPase present when the brush borders were prepared from vitamin D_3-replete chicks.

Pyrophosphatase

Brush borders contained a pyrophosphatase that had a broad pH optimum between pH 7.5 and 8.5 and which required Mg^{++} for activity.

Table 2. *The effect of cations on the ATPase of brush borders*[a]

Additions	ATP hydrolyzed (nmoles/min/mg protein)	
	Rachitic	Vitamin D_3-replete (400 IU, 16 hr)
Tris only	5	5
Tris + 2 mM Mg^{++}	47	85
Tris + 2 mM Mg^{++} + 50 mM Na^+	45	86
Tris + 2 mM Mg^{++} + 15 mM K^+	48	86
Tris + 5 mM Ca^{++}	22	45
Tris + 2 mM Mg^{++} + 5 mM Ca^{++}	84	127
Tris + 2 mM Mg^{++} + 20 mM Ca^{++}	96	142

[a] Brush borders, prepared by the EDTA method, were incubated with $\gamma^{32}P$ ATP as described in Methods section. Cations were added to the Tris buffer (pH 8.5) as indicated and the nmoles ATP hydrolyzed was calculated.

The effect of Ca^{++} could not be studied since it formed a precipitate under the conditions of the assay. Treatment of rachitic chicks with vitamin D_3 16 hr previous to the assay caused an increase in pyrophospatase 51 ± 9 and 65 ± 5 nmole hydrolyzed per min/mg protein for rachitic and vitamin D_3-replete chicks, respectively (mean of five experiments with a significant difference $P = 0.05$).

p-Nitrophenylphosphatase

Homogenates of mucosal cells contained p-nitrophenylphosphatase active in the range 4.2 to 5.8 ("acid phosphatase"), but there was no difference in the activity of homogenates from rachitic or vitamin D_3-replete chicks.

The homogenates also contained an "alkaline phosphatase" with no obvious pH optimum, activity gradually increasing from pH 8.0 to 10.2. The "alkaline phosphatase" required Mg^{++} for activity, and Ca^{++} would not replace Mg^{++}. Treatment of rachitic chicks with vitamin D_3 (400 IU, 16 hr) increased the activity of the "alkaline phosphatase", rachitic 410 ± 50, vitamin D_3-replete 600 ± 70 nmoles p-nitrophenylphosphate hydrolyzed per min/mg protein (mean of nine experiments at pH 8.5). When actinomycin D was given at the same time as the vitamin D, no increase in enzyme activity was found.

The major part of the "alkaline phosphatase" was present in the brush borders of the mucosal cell, and Table 3 shows the effect of cations and of vitamin D_3 treatment on the activity of this enzyme.

Table 3. *The effect of cations on the alkaline phosphatase of brush borders*[a]

Cation added	p-Nitrophenylphosphate hydrolyzed (nmole/min/mg protein)	
	Rachitic	Vitamin D_3-replete (400 IU, 16 hr)
2 mM Mg^{++}	608	834
+ 15 mM K^+	590	798
+ 50 mM Na^+	580	780
+ 15 mM K^+ + 50 mM Na^+	620	840
5 mM Ca^{++}	15	24
+ 15 mM K^+	38	60
+ 15 mM K^+ + 50 mM Na^+	17	28
2 mM Mg^{++} + 5 mM Ca^{++}	720	944
+ 20 mM Ca^{++}	902	1,166

[a] Brush borders were prepared by Tris buffer method and the p-nitrophenylphosphatase estimated at pH 8.9 as described in Methods section. Cations were added as indicated, and the nmoles substrate hydrolyzed were calculated.

Time Course of Increase in Enzyme Activities

Vitamin D_3 (400 IU) was injected into rachitic chicks and the increase in ATPase, p-nitrophenylphosphatase and pyrophosphatase of brush borders measured at pH 8.5 was determined at 8, 16, 48 and 72 hr. No significant differences could be observed at 8 hr but all three activities gradually increased, compared to the rachitic group, until at 72 hr the activities had approximately doubled (Table 4).

Table 4. *Effect of time after giving vitamin D_3, on enzyme activities*[a]

Enzyme	Ratio of activities (Vitamin D_3-replete/Rachitic)		
	16 hr	48 hr	72 hr
ATPase	1.51 ± 0.15	2.1 ± 0.22	2.0 ± 0.24
p-Nitrophenylphosphatase	1.43 ± 0.24	1.89 ± 0.19	2.2 ± 0.15
Pyrophosphatase	1.36 ± 0.14	1.66 ± 0.31	2.1 ± 0.17

[a] Brush borders were prepared by the EDTA method and tested at pH 8.5 for ATPase, p-nitrophenylphosphatase (both with 2 mM Mg^{++} + 5 mM Ca^{++}) and for pyrophosphatase (2 mM Mg^{++}). The ratio of activities of the vitamin D_3-replete chicks after giving 400 IU for 16, 48 or 72 hr to that of rachitic brush borders was calculated. (The results are the mean of five experiments.)

The Effect of Inhibitors on Enzyme Activities

The effect of four inhibitors on the ATPase, p-nitrophenylphosphatase and pyrophosphatase of brush borders is shown in Table 5. Ouabain and

Table 5. *Effect of inhibitors on enzyme activities of brush borders*[a]

Inhibitor	% Activity in presence of inhibitor					
	ATPase		p-Nitrophenyl-phosphatase		Pyrophosphatase	
	Rachitic	D_3	Rachitic	D_3	Rachitic	D_3
Ouabein (10^{-3} M)	98	105	95	95	96	98
DCC (10^{-4} M)	92	95	94	105	102	91
Phlorizin (10^{-3} M)	115	120	125	132	108	111
D-L-Phenylalanine (2×10^{-2} M)	27	19	8	18	22	26

[a] Brush borders were prepared by the EDTA method and tested at pH 8.5 for ATPase, p-nitrophenylphosphatase (both tested with 2 mM Mg^{++} + 5 mM Ca^{++}) and for pyrophosphatase (2 mM Mg^{++}). The enzyme activity in the presence of inhibitor was expressed as % of that of the activity without inhibitor.

DCC were without effect, phlorizin was slightly stimulatory, and phenylalanine inhibited all three activities approximately 80%.

The Effect of Phenylalanine on Ca^{++} Transport

Treatment of rachitic chicks with vitamin D_3 (400 IU, 16 hr) increased the translocation of ^{45}Ca to the serosal fluid approximately threefold (Table 6). The presence of 5×10^{-2} M D-L-phenylalanine in the mucosal fluid did not interfere with ^{45}Ca transport and may have slightly enhanced it (Table 6). During the 30-min period, 6.5 and 6.1 µmoles of phenylalanine were transported into the serosal fluid by rachitic and vitamin D_3-replete sacs, respectively.

Table 6. *Effect of phenylalanine on Ca^{++} transport by everted chick ileum*[a]

Type of chick	Presence of phenylalanine	^{45}Ca (counts/min)	
		Serosal fluid	Gut tissue
Rachitic	−	68,310[b]	425,400[b]
Rachitic	+	74,970[b]	472,650[b]
Vitamin D_3-replete	−	196,680[c]	595,300[c]
Vitamin D_3-replete	+	295,770[c]	860,550[c]

[a] Everted sacs of rachitic or vitamin D_3-replete chick ileum were prepared as in Methods section. The 5 ml of mucosal solution was Krebs-Henseleit buffer with 20 mM glucose and 1 mM ^{45}Ca (total 13,216,000 counts/min). When D-L-phenylalanine was present, it was 5×10^{-2} M. The 1 ml serosal solution initially contained 1 mM non-radioactive Ca^{++}. Incubation was for 30 min at 35 °C. The ^{45}Ca in the serosal fluid and in the washed sac was determined. Each figure is the mean of four sacs.

[b] No significant difference due to phenylalanine.

[c] Phenylalanine increase significant only at $P = 0.2$.

Discussion

The treatment of four-week-old rachitic chicks with 400 IU vitamin D_3 16 hr prior to the test caused a three- to sixfold increase in Ca^{++} translocation either *in vivo* or *in vitro* (Sallis & Holdsworth, 1962, & Table 6). Evidence was given earlier (Neville & Holdsworth, 1969) that cyclic AMP may be a "second messenger" in bringing about the effects of vitamin D. Certainly a significant increase in adenyl cyclase was found, but the activity of this enzyme observed in mucosal cells was low, particularly when compared with a system present in the "membrane fraction" that broke down ATP to ADP, AMP and adenosine (Table 1). No adenyl cyclase could be detected in brush borders of the mucosal cells, but very active ATPase,

alkaline p-nitrophenylphosphatase and pyrophosphatase activities were found. Vitamin D_3 treatment increased these three enzymic activities which reached a maximum 48 to 72 hr after the vitamin was given and at a time when the stimulation of Ca^{++} transport is also a maximum. Martin *et al.* (1969) found that 40 hr after giving 500 IU vitamin D_3 to rachitic rats, the ATPase of brush borders had increased $2\frac{1}{2}$ times; they stated that the ATPase was Ca^{++}-dependent. The concentration of Ca^{++} they used to show this dependence was high, 40 mM. In this paper, the release of the terminal $^{32}PO_4$ of ATP was used as a measure of ATPase and was found to require either Mg^{++} or Ca^{++} for activity, but Mg^{++} was the more active ion. This ATPase of brush borders did not require the presence of $Na^{+}+K^{+}$ ions. When activity was measured in the presence of 2 mM Mg^{++}, then further addition of Ca^{++} increased the activity. The "alkaline phosphatase" required Mg^{++}, and Ca^{++} was a very ineffectual substitute, but with optimal amounts of Mg^{++} present, the addition of Ca^{++} caused a further increase in activity (Table 3).

Alkaline phosphatase has been shown to possess pyrophosphatase activity (Moss, Eaton, Smith & Whitby, 1967); therefore it was not surprising to find that there was pyrophosphatase in intestinal brush borders and that the activity increased after dosing with vitamin D_3. The effect of Ca^{++} on this enzyme could not be tested due to the insolubility of Ca pyrophosphate. The results of these various enzyme measurements suggest that vitamin D_3 causes an increased synthesis of a nonspecific alkaline phosphatase that is stimulated by Ca^{++} ions.

An attempt was made to differentiate between the three phosphate-hydrolyzing activities by means of inhibitors. Ouabain, which inhibits $(Na^{+}+K^{+})$-stimulated ATPase, was not inhibitory. DCC inhibited a membrane-bound ATPase and cation transport in *Streptococcus faecalis* (Harold & Baarda, 1969) and inhibited the $(Na^{+}+K^{+})$-dependent ATPase of brain (Schoner & Schmidt, 1969), but it had no effect on the enzyme activities of brush borders. Phlorizin caused a slight stimulation of all three enzyme activities although it inhibits Ca^{++} transport by everted sacs (Sallis, 1962). Fishman, Green and Inglis (1962) evaluated a large number of substances to find a specific inhibitor of intestinal alkaline phosphatase and found 10 mM D-L-phenylalanine the most suitable. Brush border ATPase, p-nitrophenylphosphatase and pyrophosphatase were all inhibited approximately 80% by 20 mM D-L-phenylalanine. Thus the inhibitor studies support the idea that the three activities are properties of the same enzyme.

If the Ca^{++}-stimulated ATPase of the brush border were part of a Ca^{++}-translocating system, then phenylalanine would be expected to

interfere with Ca^{++} transport. There was no interference with the passage of ^{45}Ca from the mucosal to serosal fluids when using everted sacs with 50 mM D-L-phenylalanine in the mucosal fluid; therefore, the presence of a "Ca pump" at the brush border is unlikely. Evidence for a metabolically operated pump at the serosal surface will be published elsewhere (Hamilton & Holdsworth, *in preparation*).

In the search for enzymes that are induced by vitamin D, the following do not appear to be affected: phosphodiesterase and acid p-nitrophenylphosphatase (this paper), the enzymes of glycolysis (Sallis & Holdsworth, 1962), cytochrome oxidase and 5′ nucleotidase (Hamilton & Holdsworth, *in preparation*). Three related enzymic activities of the brush border are increased by vitamin D; these activities appear to be due to an alkaline phosphatase whose function is unknown and may not be directly related to Ca^{++} transport. To date, the only protein observed to be concerned with Ca^{++} transport and induced by vitamin D is the Ca^{++}-binding protein of Ebel *et al.* (1969). The induction of increased transport may be brought about via the "second messenger", since we found an increase in adenyl cyclase of the crude nuclear fraction of chick intestinal mucosa after dosing the chicks with vitamin D.

References

Cheesman, E. M., Copping, A. M., Prebble, P. M. 1964. The effect of vitamin D on enzyme activities in the rat. *Brit. J. Nutr.* **18**:147.

Ebel, J. G., Taylor, A. N., Wasserman, R. H. 1969. Vitamin D-induced calcium-binding protein of intestinal mucosa. *Amer. J. Clin. Nutr.* **22**:431.

Fishman, W. H., Green, S., Inglis, N. I. 1962. Organ specific behaviour exhibited by rat intestinal and liver alkaline phosphatase. *Biochim. Biophys. Acta* **62**:363.

Forstner, G. G., Sabesin, S. M., Isselbacher, K. J. 1968. Rat intestinal microvillous membranes. *Biochem. J.* **106**:381.

Harold, F. M., Baarda, J. R. 1969. Inhibition of membrane-bound ATPase and cation transport in *Streptococcus faecalis* by N.N′ dicyclohexylcarbodiimide. *J. Biol. Chem.* **244**:2261.

Martin, D. L., Melancon, M. J., De Luca, H. F. 1969. Vitamin D-stimulated, calcium-dependent adenosine triphosphate from brush borders of rat small intestine. *Biochem. Biophys. Res. Commun.* **35**:819.

Moss, D. W., Eaton, R. H., Smith, J. K., Whitby, L. G. 1967. Association of inorganic pyrophosphatase activity with human alkaline phosphatase preparations. *Biochem. J.* **102**:53.

Neville, E., Holdsworth, E. S. 1968. Phosphorus metabolism during transport of calcium. *Biochim. Biophys. Acta* **163**:362.

— — 1969. A "second messenger" for vitamin D_3. *Fed. Europ. Biochem. Socs. Letters* **2**:313.

Norman, A. W. 1968. The mode of action of vitamin D. *Biol. Rev.* **43**:97.

Parvin, R., Smith, R. A. 1969. Determination of inorganic phosphate in the presence of labile phosphates. *Anal. Biochem.* **27**:65.

Sallis, J. D. 1962. Calcium metabolism of the chick with special reference to the action of vitamin D_3. Ph. D. Thesis. University of Adelaide, Australia.

– Holdsworth, E. S. 1962. The influence of vitamin D on calcium absorption. *Amer. J. Physiol.* **203**:497.

Schoner, W., Schmidt, H. 1969. Inhibition of Na + K activated ATPase by N.N′ dicyclohexylcarbodiimide. *Fed. Europ. Biochem. Socs. Letters* **5**:285.

Streeto, J. M., Reddy, W. J. 1967. An assay for adenyl cyclase. *Anal. Biochem.* **21**:416.

Zull, J. E., Czarnowska-Mistral, E., De Luca, H. F. 1965. Actinomycin D inhibition of vitamin D action. *Science* **149**:182.