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PROGRESS REPORT

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Phase I, Part B

Alkaline Earth Transport and the
Calcium-Binding Protein

Submitted by

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Abstract

This section of the U.S.A.E.C. Contract continues to be concerned with various aspects of the transport of alkaline earth metals and phosphate across the gastrointestinal tract and other epithelial membranes. We report herein some of the accomplishments of the past year's work. Those segments that have now appeared in print are alluded to only briefly whereas relatively completed but unpublished investigations are given in more detail.

The main accomplishments are, as follows: (1) vitamin D-dependent calcium-binding proteins (CaBP) have been isolated in high purity from the intestinal mucosa of the bovine, pig, horse and guinea pig. Their amino acid composition and u.v. absorbancy patterns have been determined. Antisera against the bovine and guinea pig CaBPs have been produced; (2) the uterine CaBP from the laying hen has been isolated in high purity and, from its properties, appears to be identical to avian intestinal CaBP; (3) a brain CaBP in the chick has been identified and this protein also appears identical to chick intestinal CaBP. However, the vitamin D-dependency of the brain CaBP has not been shown unequivocally and this aspect requires further work; (4) preliminary analysis of the properties of CaBP's from various animals in terms of molecular evolution indicates some interesting patterns. For example, the reptilian intestinal CaBP was apparently not modified (or in a minor way) in progression into the avian species but was altered considerably during mammalian evolution. The brain CaBP and kidney CaBP appear, in

general, quite similar to that in reptiles, birds and mammals. Detailed amino acid analyses and sequencing of these various proteins should reveal significant relationships between species at the molecular level; (5) a radioimmunoassay for chick CaBP is being developed and considerable progress, as reported herein, has been made; (6) a factor in the South American plant, Solanum malacoxylon, has been uncovered which has properties analagous to the active form of vitamin D, 1,25-dihydroxy-cholecalciferol. The factor has been demonstrated to be active in embryonic chick intestine in organ culture. Some of its chemical properties have been determined and its isolation is in progress; (7) the investigation of the transport of phosphate across the intestine of chicks in vitro and in vivo continues. It has been shown that ^{32}P transport has a vitamin D-dependent component, is dependent on Na^+ , possibly involves the Na, K stimulated -Mg dependent ATPase, and is not inhibited by L-phenylalanine, a specific inhibitor of intestinal alkaline phosphatase. Alteration of the diet showed that a calcium-deficiency or phosphorus-deficiency has relatively little effect on ^{32}P absorption in vivo but greatly enhances ^{47}Ca absorption and CaBP synthesis. High strontium diets inhibited ^{47}Ca absorption and CaBP synthesis but also had little effect on ^{32}P absorption; (8) recent investigations with the embryonic chick intestine in organ culture revealed a possible involvement of the adenylate cyclase system and cyclic AMP in its response to vitamin D and vitamin D metabolites. The calcium regulating hormones, parathyroid hormone and calcitonin, were without effect on ^{45}Ca uptake or CaBP synthesis in the cultured intestine.

ALKALINE EARTH TRANSPORT AND CALCIUM BINDING PROTEIN

R. H. Wasserman, A. N. Taylor, R. A. Corradino, C. S. Fullmer,
L. Lippiello and M. Peterlik

A. Vitamin D-Induced Calcium-Binding Protein: Isolation and Properties from Various Epithelial Tissues

Intestinal vitamin D-dependent calcium-binding proteins (CaBP) have been previously isolated in high purity from the chick (Wasserman et al., J. Biol. Chem. 243: 3978, 1968) and bovine (Fullmer and Wasserman, Biochem. Biophys. Acta 317: 172, 1973). More recently, intestinal calcium-binding proteins were obtained in high purity from other species, including the pig, guinea pig and horse, and the details of this work follow (Appendix A), which include information on their amino acid composition and ultraviolet absorbancy characteristics.

The calcium-binding protein from laying hen uterus has also been isolated in high purity and found to be identical to chick intestinal protein. The details of this work also follow (Appendix B).

It might be mentioned that the bovine CaBP is undergoing sequencing in collaboration with others and is now about 90% complete. This information should be available shortly. Further, it was recently observed that the enzymatic hydrolysis of bovine CaBP by trypsin depends upon the degree of saturation of the calcium-binding sites by calcium. In the presence of calcium, CaBP is modified only slightly, retaining both its calcium-binding property and its immunological reactivity with anti-bovine CaBP anti-serum. Both of the above properties are lost when trypsinized in the absence of calcium.

These findings suggest that Ca-loaded CaBP assumes a configuration which masks the trypsin-susceptible groups whereas, in the absence of calcium, these susceptible groups become available for hydrolysis. The essential features of this latter study are being replicated and will soon be submitted for publication.

B. Identification and Partial Characterization of Brain CaBP, and Possible Relationship Between CaBP's from Different Tissues and From Different Species.

It was recently reported that a calcium-binding protein has been identified in brain tissue of the chick and this protein is apparently identical to the vitamin D-induced CaBP in chick intestine (Taylor, Arch. Biochem. Biophys. 161: 100-108, 1974) (Appendix C). Additional investigations have disclosed that the brain protein is present in brain tissue of severely vitamin D-deficient chicks, and its absolute dependency on vitamin D for synthesis has not yet been demonstrated. This points to the need to acquire information on the turnover and uptake of vitamin D and vitamin D metabolites by brain tissue and plans are being made to undertake such a study.

Brain tissue homogenates from the bovine, as studied here by C. S. Fullmer, did not show any immunoreactivity against anti-bovine CaBP anti-serum. This was unexpected in view of Taylor's earlier finding of a CaBP in avian brain. It is now apparent that bovine brain CaBP is different from the bovine intestinal protein and has properties more similar to the chick intestinal protein. Bovine brain CaBP has an apparent molecule weight of about 28,000 and cross-

reacts with anti-chick intestinal CaBP anti-serum but not anti-bovine CaBP antiserum. Additional studies indicate that the rat brain protein is also more similar to chick intestinal CaBP than to rat intestinal CaBP. The pattern is emerging, although not absolutely clarified, that the CaBP of brain tissue in the chick, rat, and bovine are more similar to each other than their respective intestinal proteins, and apparently the brain protein has not been altered in a major way during evolutionary development. This holds true, for the most part, with the kidney CaBP, since CaBP's from renal tissue of the chick, rat, and man show similarities (the bovine is an exception). With regard to the intestinal CaBP's, the reptilian and chick proteins are reasonably similar (e.g., a m.w. of 28,000; cross reactivity with anti-chick intestinal CaBP anti-serum) whereas, in the mammal, the intestinal CaBP's assume a molecular weight of about 11,000 and no cross-reactivity with anti-chick CaBP antiserum is observed. These and other points are summarized in the table below.

SOME EVOLUTIONARY ASPECTS OF CaBP

	<u>Approx. M.W.</u>	<u>Chick Antiserum</u>	<u>Bovine Antiserum</u>
Chick			
Intestine	28,000	+	-
Kidney	28,000	+	-
Brain	28,000	+	-
Uterus	28,000	+	-
<hr/>			
		↑	
Turtle			
Intestine	28,000	+	-
<hr/>			
		↓	
Rat			
Intestine	11,000	-	-
Kidney	28,000	+	-
Brain	(28,000)	+	-
Bovine			
Intestine	11,000	-	+
Kidney	11,000	-	+
Brain	(28,000)	+	-

C. Development of a Radioimmunoassay for CaBP

Quantitative assays presently in use for the vitamin D-induced Ca-binding protein (CaBP) consist of the following: Chelex-100 competitive ion exchange resin binding assay (Wasserman and Taylor, Science 152: 791, 1966), radialimmunoassay (Corradino & Wasserman, Science 172: 731, 1971) and electroimmunoassay (Taylor, Arch. Biochem. Biophys. 161: 100, 1974). With the ever increasing demand for more assays and also for quantitation of smaller amounts of CaBP, it became apparent that an assay was required which was at least as sensitive as the above immunoassays, yet was more conservative of specific antiserum. The technique of radioimmunoassay would seem to be suitable for that purpose and the following describes the preliminary experiments designed to establish a radioimmunoassay.

Iodination of CaBP. An electrolytic procedure for iodination of CaBP was chosen because of the much milder oxidizing conditions as compared to the chloramine-T and iodine monochloride methods. The technique of Sammon et al. (Endocrinology, 92: 1596, 1973) as adapted from Rosa et al. (Biochim. Biophys. Acta, 133: 486, 1967; 86: 519, 1964) was employed as the basis for the radioiodination. Purified CaBP from chick intestinal mucosa in an appropriate buffer was placed in a small platinum crucible along with KI and ^{125}I . The crucible served as the anode when a DC potential of 780 ± 10 mvolts was applied. The I^- was oxidized at the anode, thus forming the reactive I and the iodination of the protein proceeded at the interface between the crucible and the solution. Rosa et al. (Biochim. Biophys. Acta, 133: 486, 1967)

had shown that free I does not exist in solution when these electrical conditions are employed.

The initial experiments indicated that near maximal iodination of CaBP took place within 40-60 minutes. Longer periods of electrolysis produced radioactive products with a mobility different from CaBP as judged by acrylamide gel disc electrophoresis. Chick CaBP has 8 tyrosine residues (Wasserman et al., J. Biol. Chem., 243: 3978, 1968) and if one assumes that only mono-iodotyrosine is formed, the preliminary experiments indicate that about 80% of the residues are available for iodination.

^{125}I -CaBP was separated from $^{125}\text{I}^-$ by gel filtration chromatography (Sephadex G-25) and frozen in small aliquots for later use.

Separation of free and bound ^{125}I -CaBP. A successful radioimmunoassay procedure requires the rapid, efficient and reproducible separation of free from bound antigen (CaBP). The technique of solid-phase radioimmunoassay in which specific antibodies are first bound to polystyrene test tubes (Catt and Tregear, Science 158: 1570, 1967) allows the subsequent separation of unbound antigen to be accomplished by a simple aspiration step. That technique was employed, using specific anti-CaBP antibodies. Unfortunately, the antiserum did not have a high enough affinity for the polystyrene matrix and that approach could not be used with the presently available antiserum. Two other separation methods have been tried in preliminary experiments. The double antibody method employs an antiserum directed against the original antibodies, thus allowing specific precipitation of the antigen-antibody complex. This procedure leaves the unbound ^{125}I -CaBP in solution and

either the unbound form or the immunoprecipitate can be counted for ^{125}I content. The second approach utilizes serum albumin coated charcoal. The reaction mixture was mixed with the coated charcoal and the free ^{125}I -CaBP was able to penetrate the charcoal particles while the much larger CaBP-antibody complex could not and was therefore left in solution. Not enough data are yet available using these methods, but the preliminary results suggest they should prove suitable as part of the radioimmunoassay procedure.

D. Factor in *Solanum malacoxylon* that Mimics 1,25-Dihydroxycholecalciferol ($1,25-(\text{OH})_2\text{D}_3$).

The disease "Enteque seco" in the Argentine is characterized by extensive soft tissue calcification and has been shown to be caused by the ingestion of a specific plant, *Solanum malacoxylon*. Recent studies by us (Wasserman, Science 183: 1092, 1974) (Appendix D) demonstrated that *S. malacoxylon* contains a factor that mimics the action of an active form of vitamin D, $1,25-(\text{OH})_2\text{D}_3$. The evidence was, as follows. Chicks were fed a complete diet (including vitamin D_3) for 3 weeks and then changed to the same diet except that the calcium was replaced by stable strontium. Earlier studies by our group and the DeLuca group at Wisconsin (Omdahl and DeLuca, J. Biol. Chem. 247: 5520, 1972; Science 174: 949, 1971) demonstrated that such a regime reduces calcium absorption and CaBP synthesis, and apparently does so by inhibiting the formation of $1,25(\text{OH})_2\text{D}_3$ from 25-hydroxycholecalciferol by kidney enzymes. When the strontium-fed chicks were given *S. malacoxylon* as part of the diet, the inhibitory effect

of stable strontium was reversed and thereby suggested a $1,25-(OH)_2D_3$ -like action of the botanical factor.

Additional studies showed that the S. malacoxylon factor, in confirmation of the work by others, is highly polar, being soluble in water and 75%-80% aqueous ethanol but not in acetone, chloroform, ether, or methanol: chloroform mixture (2:1). The molecular weight has not been established with precision. By gel chromatography, it was eluted from a Sephadex G-25 column in the region consonant with a molecular weight of 2000-3000 but this estimate assumes that the factor acts like a spheroid protein. The factor is destroyed by periodic acid, acid hydrolysis, is resistant to alkaline hydrolysis, adsorbed by charcoal and not adsorbed by Dowex 1 and Dowex 50.

In experiments in collaboration with R. A. Corradino, it was shown that the S. malacoxylon factor is active in tissue culture, stimulating the synthesis of CaBP and ^{45}Ca uptake by embryonic chick intestine. This is illustrated in the data given in the following table.

<u>Embryonic Chick Intestine in Organ Culture: S. malacoxylon</u> <u>Extract - Action of Actinomycin D¹</u>				
<u>S.m. Extract(6x)-1%</u>	<u>Act.D (2 γ/ml)</u>	<u>CaBP (γ/100 mg duodenum)</u>	<u>^{45}Ca (% of -Sm control) Uptake</u>	<u>M $\rightarrow$ S transport</u>
-	-	0	100	100
+	-	2.9 $\pm$ 0.3	145 $\pm$ 5 ²	176 $\pm$ 12 ²
-	+	0	100	100
+	+	0	97 $\pm$ 4 ³	107 $\pm$ 12 ³

¹ Duodena cultured everted on grid for 48 hr.

² Sig > -S.m. control; 0.005% level - Students' t test

³ Not sig. diff. than - S.m. control

The data also indicate that the stimulatory effect is actinomycin D-sensitive, a property shared by vitamin D_3 , $25-(OH)D_3$ and $1,25-(OH)_2D_3$ in tissue culture. Two conclusions can be drawn from these data. First, the S. malacoxyton factor is effective without modification by either kidney, liver or other non-intestinal organs nor by intestinal micro-organisms. If modification of the factor is required, the necessary reaction must occur in the intestinal tissue itself. The second point is that the actinomycin D-sensitivity of the response to the S. malacoxyton factor suggests that de novo synthesis is involved in its action. This latter aspect again indicates that the factor acts like vitamin D_3 or a vitamin D_3 metabolite and that the factor does not modify the calcium transport directly but via a mechanism requiring protein synthesis, presumably CaBP, and perhaps other macromolecules.

E. Studies on the In Vitro Transfer of Phosphate

In the last Progress Report, in vitro experiments on the transfer of phosphate by everted ileal sacs of the rachitic chick were reported and since published (A. N. Taylor, J. Nutrition 104: 489-494, 1974) (Appendix E). Firstly, the data confirmed previous observations made in vivo in the chick (Wasserman and Taylor, J. Nutrition 103: 586-599, 1973; Hurwitz and Bar, Am. J. Physiol. 222: 761, 1972) and in vitro with rat intestine (Harrison and Harrison, Am. J. Physiol. 201: 1007, 1961; Kowarski and Schachter, J. Biol. Chem. 244: 211, 1969) that vitamin D accelerates phosphate transport. Secondly, it was shown that calcium was not required for phosphate transport, at least not as a co-ion as was stipulated by others over the years.

Thirdly, there was evidence given of a possible involvement of the Na-K ATPase and Na^+ in phosphate transport and, lastly, that L-phenylalanine, an inhibitor of intestinal alkaline phosphatase, did not depress phosphate transport in this in vitro preparation.

These studies on the mechanism of vitamin D action on phosphate transport are being continued through the efforts of Dr. Meinrad Peterlik, Institute of General and Experimental Pathology, Vienna, who is spending the year with us as a Max Kade Foundation Fellow. As an extension of the studies referred to above, it has already been shown that phosphate is actively transported by the various segments of the chick intestine in vitro, with the degree of uphill transport following the sequence: jejunum > duodenum > ileum. The ability of each segment to transport calcium against an apparent electrochemical gradient was highly dependent upon vitamin D. Net transport of phosphate by gut segments from rachitic animals was virtually nil. The development of this new procedure with everted gut sacs from the chick now allows more definitive studies to be done on the intestinal phosphate transport system and the effect of vitamin D thereon.

F. Effect of Low Calcium, Low Phosphorus and High Strontium Diets on Phosphate Absorption.

Previous studies (Wasserman and Taylor, J. Nutrition 103: 586-599, 1973; Hurwitz and Bar, Am. J. Physiol. 222: 761, 1972; Kowarski and Schachter, J. Biol. Chem. 244: 211, 1969) suggested that calcium is not required as a co-ion in the transport of the phosphate anion, and that the phosphate transport system and the calcium transport

system are separate processes. However, vitamin D appears to accelerate both.

In order to examine in more detail the independence of the calcium and phosphate absorption processes in chick intestine, the responsiveness of the phosphate absorptive system to dietary alterations was investigated. These included feeding to chicks diets containing adequate vitamin D₃ but deficient in either calcium (~0.1%) or phosphorus (~0.25%), or the replacement of calcium by stable strontium (2.3%). It has been shown that calcium absorption and CaBP synthesis are enhanced by adapting an animal to a low calcium or low phosphorus diet (Morrissey and Wasserman, Am. J. Physiol. 220: 1509-1519, 1971) and that a high strontium diet inhibits both calcium absorption and CaBP formation (Corradino and Wasserman, Proc. Soc. Expt. Biol. Med. 133: 960-963, 1970). The studies by Omdahl and DeLuca (Science 174: 949, 1971; J. Biol. Chem. 247: 5520, 1972) had further shown that the high strontium diet inhibits the formation of 1,25-(OH)₂D₃ from 25-(OH)D₃ by the kidney hydroxylase enzymes.

In brief, 1-day-old chicks were raised on commercial chick starter diet for 3 weeks at which time they were transferred to the semi-synthetic diets containing either: (A) normal calcium and phosphorus levels, (b) low calcium-normal phosphorus levels, (c) normal calcium-low phosphorus levels, or (d) high strontium-low calcium-normal phosphorus levels for 8 days.

The data from this study are given in accompanying table 2. In confirmation of previous work, it was shown that a calcium deficiency

or a phosphorus deficiency considerably increases the duodenal absorption of calcium and the duodenal concentration of CaBP; the CaBP content of the ileum was similarly enhanced. In a parallel study with another group of chicks, it was demonstrated that the ^{47}Ca absorption from the ileum was also enhanced by the calcium or phosphorus deficiency (not shown). The high strontium diet, as expected, completely inhibited CaBP synthesis and considerably reduced ^{47}Ca absorption in both segments (ileum ^{47}Ca data not shown). With regards ^{32}P -phosphate absorption, the low calcium or phosphorus intake tended to increase phosphate absorption and translocation, but the values were not significantly different from the control values. The high strontium diet also did not significantly affect ^{32}P absorption or ^{32}P accumulation by the tissue. However, there was a significant increase in the amount of ^{32}P -phosphate that was transferred from intestinal region to body.

A conclusion reached from these and other related experiments is that ^{32}P absorption is not greatly (or significantly) influenced by the calcium or phosphorus content of the diet whereas the absorption of ^{47}Ca from both the duodenum and ileum are significantly enhanced under the present conditions. The increase in ^{47}Ca absorption is accompanied similarly by an increase in duodenal and ileal CaBP concentrations. Thus, this constitutes additional evidence that the calcium and phosphate absorptive systems can be disassociated on the basis of their reactivity to dietary alterations.

The lack of responsiveness of ^{32}P -phosphate absorption to the high strontium diet adds another feature. Clearly, ^{47}Ca absorption and

Effect of Low Ca, Low Phosphorus or High Strontium Intake on Ileal ^{32}P and Duodenal ^{47}Ca Absorption, and CaBP Levels in Duodenum and Ileum¹.

Diet	Dietary Levels			Duodenal ^{47}Ca Absorption ² (% of dose)	^{32}P Transfer ²			CaBP Conc. ²	
	Ca	P	Sr		Absorption	Accumulation by mucosa (% of dose)	Transfer to body	Duodenum	Ileum
	(%)	(%)	(%)					($\mu\text{g CaBP/mg protein}$)	
1	0.94	0.58	-	15.3 $\pm$ 3.1 ^a	44.7 $\pm$ 4.5 ^a	26.1 $\pm$ 2.9 ^a	18.6 $\pm$ 2.7 ^a	8.9 $\pm$ 0.8 ^a	5.2 $\pm$ 0.5 ^a
2	0.09	0.62	-	60.4 $\pm$ 5.2 ^b	59.1 $\pm$ 6.9 ^a	34.6 $\pm$ 5.7 ^a	24.5 $\pm$ 1.9 ^a	30.0 $\pm$ 0.8 ^b	10.8 $\pm$ 0.7 ^b
3	0.94	0.25	-	55.4 $\pm$ 2.9 ^b	57.6 $\pm$ 4.4 ^a	31.0 $\pm$ 2.6 ^a	25.6 $\pm$ 3.5 ^a	47.4 $\pm$ 1.9 ^c	16.6 $\pm$ 2.0 ^c
4	0.05	0.57	2.3	4.3 $\pm$ 1.1 ^c	53.3 $\pm$ 6.8 ^a	25.0 $\pm$ 5.7 ^a	28.3 $\pm$ 2.1 ^b	None detected ^d	None detected ^d

^a Values represent mean $\pm$ standard error of the mean of 5-6 chicks.

^b Parameters designated by different letters are significantly different at $p < .05$ or less.

intestinal CaBP levels were significantly reduced by this regime. However, ^{32}P -phosphate absorption was relatively unaffected. Since ingestion of the high strontium diet presumably inhibits the conversion of $1,25-(\text{OH})_2\text{D}_3$ from $25-(\text{OH})\text{D}_3$ by the kidney hydroxylase system, one might conclude that vitamin D-dependent phosphate absorption does not require $1,25-(\text{OH})_2\text{D}_3$ but is responsive to $25-(\text{OH})\text{D}_3$ or vitamin D_3 or, as an alternate explanation, that the components of the phosphate transport system or the RNA messenger for its synthesis are relatively long-lived. The former seems more reasonable since intestinal cells in chick have a rapid turnover of about 2-3 days and any messenger or residual transport components should have disappeared during the 8-day experimental feeding period. Support for the contention that the intestinal system is responsive to $25-(\text{OH})\text{D}_3$ comes from studies by others on the reabsorption of phosphate by the kidney tubule. The administration of either $1,25-(\text{OH})_2\text{D}_3$ or $25-(\text{OH})\text{D}_3$ decreased phosphorus excretion in thyroparathyroidectomized dogs but the $25-(\text{OH})\text{D}_3$ elicited a greater effect than the $1,25-(\text{OH})_2\text{D}_3$ derivative (Puschett et al, Proc. Soc. Expt. Biol. Med. 141: 379, 1972).

G. Studies with the Embryonic Intestinal Organ Culture System.

The embryonic intestinal organ culture system has been described in previous Progress Reports. In brief, intestine from a 20 day old chick embryo is cultured in the appropriate medium for up to 48 hr under a gaseous phase of 5% CO_2 :95% O_2 . When obtained from the embryo, the gut tissue is devoid of the vitamin D-induced calcium-binding protein (CaBP) but is synthesized in culture if vitamin D_3 or one of

the active cholecalciferol metabolites is included in the medium (Corradino and Wasserman, Science 172: 731, 1971; Corradino, J. Cell Biol. 58: 64, 1973; Corradino, Science 179: 402, 1973). It was also demonstrated that α -amanitin, a cyclic octopeptide that specifically inhibits nucleoplasmic RNA-polymerase activity in various tissues, and actinomycin D, which inhibits DNA-directed RNA synthesis, inhibited the synthesis of CaBP and the vitamin D-enhanced ^{45}Ca uptake by the tissue. These results indicated that the vitamin D-mediated mechanism in tissue culture is dependent on de novo protein synthesis.

More recent investigations (Corradino, Endocrinology 94: 497, 1974) (Appendix F) reports the stimulation of the adenylate cyclase system in organ culture by vitamin D and cholecalciferol metabolites. In a time series, it was observed that vitamin D caused an increase in adenosine-3', 5'-cyclic monophosphoric acid (cAMP) at 3 hr., a return to baseline at 6 hr. and a subsequent increase at 12 hr. and thereafter. CaBP formation and a significant enhancement of ^{45}Ca uptake were seen at 12 hr but not before. Thus, cAMP appeared prior to any alteration in the latter parameters. In this same study, dibutyryl cAMP and theophylline increased ^{45}Ca uptake by the tissue but were incapable of inducing CaBP synthesis. Dibutyryl cAMP (dbc AMP) and theophylline were additive with each other and with vitamin D_3 on ^{45}Ca uptake, and both dbc AMP and theophylline depressed CaBP concentrations in the tissue at later times. Taken together, these results indicate that adenylate cyclase is responsive to vitamin D_3 and that there might be an interaction between vitamin D_3 , cAMP and CaBP synthesis.

additional

Following are two/detailed reports (Appendix G and H), the first describing additional studies on the enhancement of ^{45}Ca uptake and mucosal-to-serosal transport of ^{45}Ca by the direct addition of purified CaBP to the culture system (i.e., reconstitution) and, the second, on the response of the embryonic intestine in organ culture to parathyroid hormone and calcitonin. In the latter report, it is shown that neither hormone significantly altered ^{45}Ca uptake or CaBP synthesis.

H. Additional accomplishments.

1. A vitamin D bioassay based on the calcium-binding protein as end point has been investigated and the results of this study, done in collaboration with A. Bar, are in press in the Journal of Nutrition.

This manuscript is attached as Appendix I.

2. The detailed studies on the properties of chick CaBP have now been published and this is attached as Appendix J.

3. A study, done in collaboration with Dr. William Bergstrom and his group at the Upstate Medical Center, Syracuse, on the effect of the anti-convulsant, diphenylhydantoin, on calcium absorption and CaBP has been published and this appears as Appendix K.

4. A recently completed manuscript on the localization of CaBP in the shell gland of the laying hen has been submitted for publication and this is Appendix L.

5. A study concerned with mechanisms related to the control of calcium absorption and CaBP synthesis, involving the use of dihydro-tachysterol₃, has been published and is given as Appendix M.

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Appendices

1. Appendix A: Isolation and partial characterization of intestinal calcium-binding proteins from the cow, pig, horse, guinea pig and chick. C. S. Fullmer and R. H. Wasserman (to be submitted).

2. Appendix B: Isolation and purification of calcium-binding protein from the uterus of the laying hen. C. S. Fullmer, A. Bar and R. H. Wasserman (to be submitted).

3. Appendix C: Chick brain calcium-binding protein: comparison with intestinal vitamin D-induced calcium-binding protein. A. N. Taylor Arch. Biochem. Biophys. 161: 100-108, 1974.

4. Appendix D: Calcium absorption and calcium-binding protein synthesis: Solanum malacoxylon reverses strontium inhibition.

R. H. Wasserman, Science 183: 1092-1094, 1974.

5. Appendix E: In vitro phosphate transport in chick ileum: Effect of cholecalciferol, calcium, sodium and metabolic inhibitors. A. N. Taylor, J. Nutr. 104: 4, 489-494, 1974.

6. Appendix F: Embryonic chick intestine in organ culture: Interaction of adenylate cyclase system and vitamin D₃-mediated calcium absorptive mechanism. R. A. Corradino, Endocrinology 94: 6, 497-504, 1974.

7. Appendix G: Vitamin D-dependent calcium-binding protein: stimulation of calcium transport by addition to embryonic chick intestine in organ culture. R. A. Corradino, C. S. Fullmer and R. H. Wasserman (to be submitted).

8. Appendix H: Parathyroid hormone and calcitonin: no direct effect of vitamin D₃-mediated intestinal calcium absorptive mechanism. R. A. Corradino (to be submitted).

9. Appendix I: Duodenal vitamin D-induced calcium-binding protein in the chick: A new end-point for the bioassay of vitamin D.

A. Bar and R. H. Wasserman (in press) J. Nutr., 1974.

10. Appendix J: Chemical composition, affinity for calcium, and some related properties of the vitamin D dependent calcium-binding protein. P. J. Bredderman and R. H. Wasserman, Biochemistry 13: 8, 1687-1694, 1974.

11. Appendix K: Diphenylhydantoin: Effects on calcium metabolism in the chick. M. Villareale, L. V. Gould, R. H. Wasserman, A. Bar, R. T. Chiroff and W. H. Bergstrom. Science 183: 671-673, 1974.

12. Appendix L: Fluorescent antibody localization of the vitamin D-dependent calcium-binding protein in the oviduct of the laying hen.

L. Lippiello and R. H. Wasserman. J. Histochem. Cytochem. (submitted).

13. Appendix M: Control of calcium absorption and intestinal calcium-binding protein synthesis. A. Bar and R. H. Wasserman. Biochem. Biophys. Res. Comm. 54: 1, 191-196, 1973.