Possible Precursor of Vitamin D Stimulated Calcium Binding Protein in Rats*

D. Drescher and H. F. DeLuca†

ABSTRACT: The supernatant fraction of intestinal mucosa has been fractionated by means of urea disc gel electrophoresis. Seventy-two hours after administration of vitamin D_3 to vitamin D deficient rats, a new protein (band C) appears in the supernatant fraction. Concomitant with the appearance of the new protein, there is a depression of a preexisting protein (band A) of apparently higher molecular weight than the newly appearing one (band C). These changes are confirmed by experiments involving incorporation of radioactive amino acids into these proteins. The molecular weight range, time course of appearance, and effect of dietary strontium all suggested that the newly appearing protein (band C) might represent the calcium binding protein formed in response to vitamin D. Using chromatographically purified calcium binding protein from rat intestine, it was shown that this protein (band C) most probably is the calcium binding protein. The major contaminating protein in the final chromatographic purification of rat calcium binding protein has also been identified on gels as the preexisting protein (band A), which is probably the precursor of the calcium binding protein.

The molecular mechanism of calcium transport by the small intestine and the role of vitamin D in this system has been a subject of intense investigation in the past decade (Schachter and Rosen, 1959; Harrison and Harrison, 1960; Wasserman *et al.*, 1960; Martin and DeLuca, 1969). Of major significance is the observation that actinomycin D prevents the intestinal calcium transport response to vitamin D (Zull *et al.*, 1965, 1966; Norman, 1965). Furthermore a vitamin D stimulation of ribonucleic acid synthesis in intestine suggests that protein synthesis is involved in the vitamin D mediated transport process (Stohs *et al.*, 1967; Hallick and DeLuca, 1969). Several proteins have been isolated which are believed to be components of bacterial transport systems (Pardee, 1968). Analogously, at least two proteins have been described which may be involved in the vitamin D stimulated transport of calcium across the small intestine. The first of these is the calcium binding protein (Wasserman and Taylor, 1966; Kallfelz *et al.*, 1967; Wasserman, 1970a,b) which is a possible calcium carrier (Wasserman and Taylor, 1968). The second is a vitamin D stimulated, calcium-dependent adenosine triphosphatase (Martin *et al.*, 1969; Melancon and DeLuca, 1970).

The present paper demonstrates that for the rat, there may be still another specific protein involved in vitamin D stimulated calcium transport; a protein which is apparently depressed after administration of the vitamin. This protein appears to be of slightly higher molecular weight than the calcium binding protein, and electrophoretic gel patterns as well as studies of incorporation of labeled amino acids provide evidence for its possible role as a precursor of the calcium binding protein.

Materials and Methods

Animals. Male, 21-day-old albino rats were obtained from Holtzman Co., Madison, Wis. They were maintained in hanging wire cages and given food and water *ad libitum*. The rats were fed a purified, vitamin D free diet 11 (0.47% calcium–0.30% phosphorus) described by Guroff *et al.* (1963). Although diet 11 does not induce rickets, the animals became severely vitamin D deficient after several weeks on the diet, as revealed by lack of growth and low serum calcium values (Steenbock and Herting, 1955). The rats were used in the experiments after they had been fed the diet for 5.5 weeks.

Chemicals. Crystalline vitamin D_3 (cholecalciferol) was obtained from N. V. Philips-Duphar, Weesp, The Netherlands. The reagents used for disc gel electrophoresis were Eastman grade (Distillation Products Industries, Rochester, N. Y.). Coomassie brilliant blue stain was from Colab Laboratories, Chicago Heights, Ill. The [^{3}H]- and [^{14}C]amino acid mixtures were reconstituted protein hydrolysates (algal profile) obtained from Schwarz BioResearch, Orangeburg, N. Y. Crystalline bovine serum albumin was obtained from Nutritional Biochemicals, Corp., Cleveland, Ohio, and horse heart cytochrome *c* type II from Sigma Chemical Co., St. Louis, Mo. Rabbit carbonic anhydrase was prepared by one of the authors (D. D.). All other chemicals were of analytical reagent grade.

Administration of Vitamin D and Labeled Amino Acids. Vitamin D deficient rats maintained on diet 11 for 5.5 weeks were each given 12.5 μg of (500 IU) vitamin D_3 in 0.25 ml of vegetable (Wesson) oil orally at 0, 24, and 48 hr. Control rats received Wesson oil alone. The rats were fasted for 24 hr before sacrifice and sacrificed at 72 hr after the initial dose of vitamin D_3. If labeled amino acids were administered, each isotope was given in two identical doses, one at 6 hr and one at 3 hr prior to sacrifice. The radiochemicals were injected in amounts indicated in Figure 3. The rats were restrained and lightly anaesthetized with ether. Jugular veins were located by cutaneous incision and labeled amino acid mixtures were injected intrajugularly. The animals were sacrificed by carbon dioxide suffocation.

Preparation of Supernatant Fraction from Intestional Mucosa. After sacrifice, the first 10 cm of small intestine was immediately excised, slit lengthwise, and rinsed in ice-cold isotonic saline. When the intestine was quite clean, mucosa

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† To whom all inquiries should be addressed.

was scraped off by means of a glass slide. For dual-label experiments, mucosa from the vitamin D deficient (^{3}H) and the vitamin D dosed (^{14}C) rats were mixed before homogenization. The mucosa was homogenized in ice-cold 5 mM EDTA (pH 7.4) for 25 sec at high speed in a Waring blendor (Forstner *et al.*, 1968). Thirty milliliters of 5 mM EDTA per rat was used. The homogenate was centrifuged for 30 min at 40,000*g* in a Beckman Model L-2 refrigerated ultracentrifuge. Supernatant fluid was decanted, shell frozen in Dry Ice–acetone, and lyophilized overnight. Identical results could be obtained with respect to supernatant electrophoretic patterns if instead of the 5 mM EDTA solution–0.08 M NaCl–0.08 M Tris-HCl (pH 7.0) was used in the homogenization. The latter buffer was used in the purification of calcium binding protein from rats, as reported in the preceding paper (Drescher and DeLuca, 1971).

Disc Gel Electrophoresis. The 7% acrylamide–9 M urea gel system of Neville (1967) was used in these studies. The method of preparing the gels is described in detail in the preceding paper (Drescher and DeLuca, 1971). Samples were also prepared by the procedure of Neville, using lyophilized protein in the proportions of 10 mg of protein to 1 ml of water (final volume was 1.83 ml after addition of other components). Thus the samples had a protein concentration of about 5.5 mg/ml, and 1.1 mg of protein (0.2-ml sample) was applied per gel. The gels used for dual-label analysis could be stained, since the coomassie blue did not interfere in the liquid scintillation detection of either ^{3}H or ^{14}C.

Photographs and Densitometric Tracings of Electrophoretic Gels. Gels were photographed in the manner described in the preceding paper (Drescher and DeLuca, 1971). Densitometric tracings of gels were made on a Joyce-Loebl Chromoscan densitometer, using a red filter. The gels were mounted in 9 × 130 mm Pyrex tubes completely filled with 12.5% trichloroacetic acid and corked at the ends.

Dual-Label Analysis of Gels. Protein bands stained with Coomassie Blue were sketched, photographed, and traced on the densitometer. The bands were numbered, and the gels were sliced into small fractions with a razor blade. Four identical gels were fractionated simultaneously, and each set of four identical gel slices was placed in a counting vial. The gels were dried in open vials under a heat lamp at about 60° for 4–6 hr. Next, 0.1 ml of 30% hydrogen peroxide was added per vial (Tishler and Epstein, 1968) the vials were capped and heated for 15 hr at 40°. Soluene-100 solubilizer (1 ml; Packard Instrument Co., Downers Grove, Ill.) was added per vial, the contents of the vial mixed thoroughly, and 15 ml of scintillation counting solution added. Recoveries of sample radioactivity were routinely 80–90% by this method.

Liquid Scintillation Counting Solution. The counting solution used in the dual-label analysis consisted of 0.5% 2,5-diphenyloxazole and 0.025% 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene (Packard) dissolved in toluene.

Radioactivity Measurement. Dual-label analysis of the fractions was performed by means of a Packard Tri-Carb liquid scintillation spectrometer Model 3003, with automatic external standard for efficiency determinations. One channel was optimized for ^{3}H, another for ^{14}C, and the third for the external standard. Standard curves were constructed using standards prepared from ^{3}H- and ^{14}C-labeled toluene (Packard) serially quenched with methanol. Computation of net ^{14}C and ^{3}H disintegrations per minute was readily performed on a Monroe Epic 2000 calculator.

Calculations. "Enhancement" values for the gel fractions (see Figure 3) were calculated by dividing the ^{14}C/^{3}H ratio of the fraction by the ^{14}C/^{3}H ratio of whole homogenate. In these experiments, an enhancement value of 1.0 (base line) indicates no measured response to vitamin D.

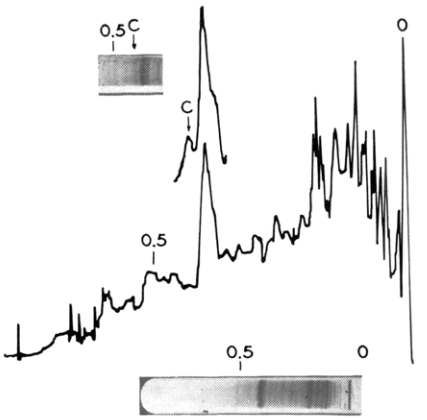

FIGURE 1: Densitometric tracing of urea disc gel electrophoretic pattern of the supernatant fraction from vitamin D deficient intestinal mucosa. Gel photos are included for comparison. The origin of the separation gel is marked O, and the position halfway to the marker dye front is marked 0.5. The position of the front itself is not shown in the tracing. The inset shows a protein (C, marked with arrow) which is present in the supernatant fraction 72 hr after administration of vitamin D and absent in the supernatant fraction from a deficient animal.

Results

Fractionation of Intestinal Mucosal Supernatant by Disc Electrophoresis. Figure 1 shows the fractionation of intestinal supernatant by urea–disc gel electrophoresis. Such fractionations generally yielded at least 25 sharp bands. The full densitometer trace shown in the figure corresponds to intestinal supernatant fraction of a vitamin D deficient rat. The inset in Figure 1 shows the position of a new protein (marked C) which is present 72 hr after a deficient rat has received an oral dose of from 12.5 μg (500 IU) to 250 μg (10,000 IU) of vitamin D_3 in 0.25 ml of cottonseed (Wesson) oil. Protein C is also absent from intestine when only the cottonseed oil vehicle is given to deficient rats.

Visible Changes in Electrophoretically Separated Proteins after Vitamin D. With the formation of protein C apparently in response to vitamin D_3, another change in the electrophoretic pattern of intestinal supernatant could be observed, as shown in Figure 2. Protein A (marked in the figure), diminished in staining intensity with the appearance of C. The darkly staining protein B appeared to be unaffected by vitamin D pretreatment. The appearance of C after vitamin D was observed repeatedly in many separate experiments, and in all cases a decrease in staining of A was also apparent. However, the amount of A was never reduced to zero by the prior treatment of rats with vitamin D.

Dual-Label Experiments and the Effect of Vitamin D. The data plotted in Figure 3 reveal that dual-label analysis of the intestinal supernatant proteins confirmed the appearance of C and a diminution of A as a result of vitamin D. (Similar dual-isotope techniques have been employed by Gorski and Barnea (1970) in their studies of estrogen response.) Part I of Figure 3 illustrates the effect of vitamin D on amino acid labeling of the proteins. Protein C shows a ^{14}C/^{3}H ratio (enhancement) which is considerably higher than the base line. In the same gel, protein A has a ^{14}C/^{3}H value considerably

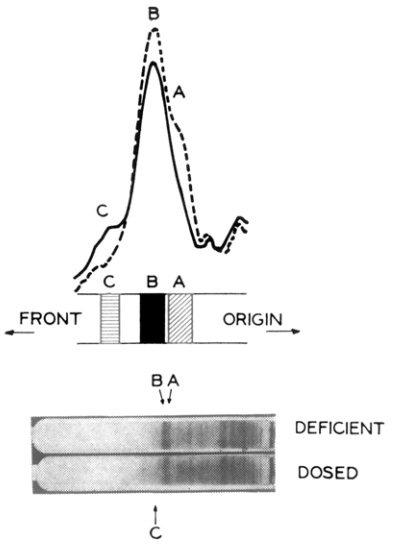

FIGURE 2: Effect of vitamin D on proteins of intestinal supernatant separated by disc gel electrophoresis. Where indicated, rats received 12.5 μg of (500 IU) vitamin D_3 in 0.1 ml of vegetable oil orally at each of the three time periods 0, 24, and 48 hr and were sacrificed at 72 hr. Deficient controls received vehicle alone. The densitometric tracings correspond to the region of the gel containing proteins A, B, and C. Directly below the tracings is an enlarged schematic diagram of the corresponding section of gel. Photos of the whole gel are also shown. The solid lines of the tracing represent the pattern in mucosa from rats given vitamin D while the dotted lines represent that from deficient rats.

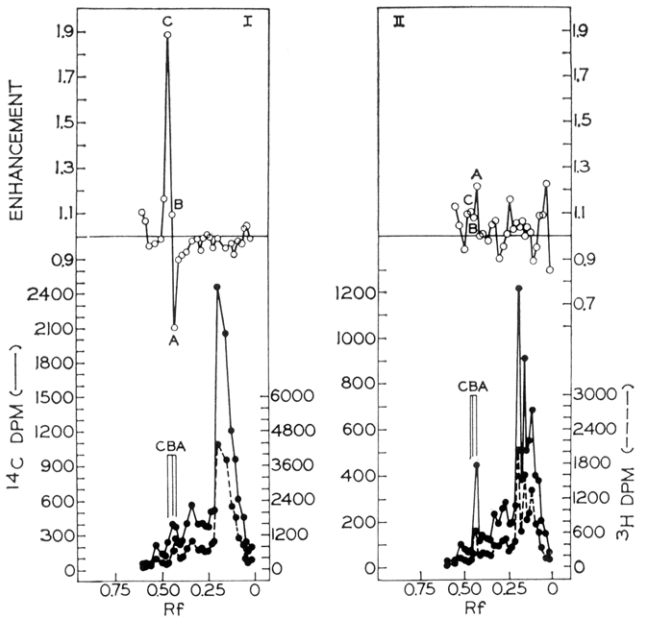

FIGURE 3: Dual-label electrophoretic analysis of intestinal supernatant proteins; (I) comparison of fractions from rats given vitamin D *vs.* those from vitamin D deficient rats and (II) comparison of fractions from deficient rats given [^{14}C]amino acids *vs.* other deficient rats given [^{3}H]amino acids. Preparation and electrophoretic analysis of the supernatant fractions was performed as described in the text. In part I, the rats were given 12.5 μg of (500 IU) vitamin D_3 orally in 0.25 ml of cottonseed oil three times, once at each of the times 0, 24, and 48 hr. The deficient rats in I were given only the oil vehicle. Each rat was injected with labeled amino acids in two identical doses before sacrifice, the first at 66 hr and the second at 69 hr. Each rat given vitamin D in I received two doses of 20 μCi each of ^{14}C-labeled hydrolysate of algal protein, and each deficient control rat received two doses of 50 μCi of ^{3}H-labeled hydrolysate of algal protein. Animals were sacrificed at 72 hr. In part II, the same labeling schedule was used, but neither the rats receiving the ^{14}C nor those receiving the ^{3}H were given vitamin D. The ^{14}C/^{3}H ratio or "enhancement," the ^{14}C disintegrations per minute, and the ^{3}H disintegrations per minute are plotted *vs.* R_F, and the positions of proteins A, B, and C referred to in the text are marked.

below the base-line level. As observed previously in the staining, the labeling pattern of all other proteins measured including B is not affected by vitamin D.

To be sure that these results were not an artifact of method, an experiment was performed identical with that of part I, Figure 3, except that no vitamin D was given to either group of animals. Thus one group of vitamin D deficient rats received ^{14}C while another received the [^{3}H]amino acids. The results in Figure 3, part II, show the "enhancement" values to be at base-line levels for all proteins. Clearly, the increased "enhancement" value for protein C as well as the decrease for protein A shown in Figure 3, part I, is the result of vitamin D administration.

TABLE I: The Effect of Vitamin D on [^{14}C]Amino Acid Content of Intestinal Supernatant Proteins Following the Dual-Label Experiment.[a]

Protein	Per Cent of ^{14}C or ^{3}H dpm in the Five-Band Section		Rel Intensity of Stain in the Band
	^{14}C (Vitamin D Dosed)	^{3}H (Vitamin D Deficient)	
X	16.4 ± 0.9	15.5 ± 0.5	Light
A	25.0 ± 2.8	44.6 ± 3.4	Medium
B	28.6 ± 2.4	22.1 ± 1.5	Dark
C	20.0 ± 2.3	9.9 ± 1.3	Light
Y	10.0 ± 2.0	7.8 ± 0.9	Light

[a] In the calculations, the total dpm of either ^{14}C or ^{3}H in the five-band section is taken as 100%. The letters X, A, B, C, and Y refer to the proteins described in Figure 4. The above percentages are listed with their standard errors, where $n = 3$ for ^{14}C values and $n = 5$ for the ^{3}H values. The dpm values used for either ^{14}C or ^{3}H averaged at least 75 dpm above background per protein and generally much higher (see radioactivity values in Figure 3). The relative intensity of stain in each band is obtained from photos and densitometric traces of the gels.

A More Detailed Examination of the Supernatant Proteins Affected by Vitamin D. The effect of vitamin D in the A, B, and C regions of the disc gels is more clearly illustrated in Figure 4. When the radioactivity in each protein is divided by the total radioactivity in the gel, it is even more evident that the effect of vitamin D is to decrease the ^{14}C in protein A and increase it in protein C.

The numerical values for radioactivity in the experiments shown in Figures 3 and 4 are given in Table I. There is approximately a twofold increase of radioactivity in C for the vitamin D treated animals as compared to the deficient controls. Conversely, there is almost a halving of radioactivity in protein A as a result of vitamin D. As a further control it is evident that there is no significant effect of vitamin D in the cases of X and Y.

Molecular Weight Calibration of Urea Electrophoretic Disc Gels. Figure 5 is a molecular weight calibration curve for the

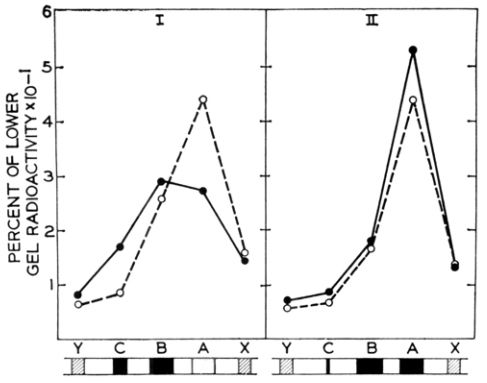

FIGURE 4: The effect of vitamin D on radioactive amino acid incorporation into intestinal supernatant fractions. (I) comparison of proteins from rats given vitamin D *vs.* those from rats deficient in vitamin D; (II) comparison of proteins from vitamin D deficient rats given [^{14}C]amino acids *vs.* those given [^{3}H]amino acids. Solid lines represent ^{14}C, while dotted lines represent ^{3}H. This figure is an enlargement of the gel regions of proteins A, B, and C shown in Figure 7. Per cent of total radioactivity in each is plotted for proteins A, B, C, and two bordering proteins X and Y. Schematic diagrams of the corresponding portion of gel are included. The experiment is that described in the legend for Figure 3.

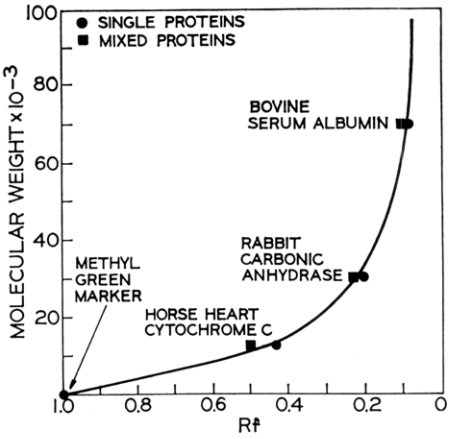

FIGURE 5: Molecular weight calibration curve for 9 M urea, 7% acrylamide gels. Molecular weight is plotted *vs.* R_F as measured relative to the methyl green front. The indicated known pure proteins were applied to the gels both individually and as a mixture of three. The gel system and conditions of electrophoresis are described in detail under Materials and Methods.

urea gels employed in this work. The calibration indicates that proteins much over 100,000 molecular weight remain near the origin of the gel or in the spacer gel.[1] The log plot for these urea gels is not linear, as opposed to the log plot for sodium dodecyl sulfate gels (Shapiro *et al.*, 1967), so actual molecular weight values and not their logs are plotted on the ordinate. The plot indicates that the region of A, B, and C corresponds to molecular weights between 10,000 and 20,000.

Identification of Calcium Binding Protein (C). The protein C described in the dual-label experiments of this paper appears to have an R_F similar to C′, the calcium binding protein isolated as described in the previous paper (Drescher and DeLuca, 1971). When the purified calcium binding protein C′ (Drescher and DeLuca, 1971) is added to a supernatant fraction from the intestine of a vitamin D deficient rat and disc gel electrophoresis is carried out, it migrates to a region very near if not identical with that of protein C of the supernatant fraction as revealed by both the densitometer tracing and gel photograph (Figure 6). However, the position of the calcium binding protein C′ may not be identical with the position of protein C, but may be displaced slightly toward the lower molecular weight region of the gel.

In a similar fashion protein A isolated by column methods (Drescher and DeLuca, 1971) is identical with protein A which appears altered in amount following vitamin D administration.

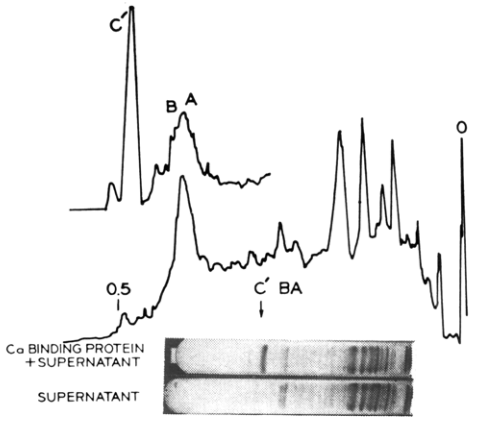

FIGURE 6: Migration of purified calcium binding protein (marked C′) and the proteins of intestinal supernatant fraction subjected to disc gel electrophoresis. A complete densitometric tracing of a supernatant fraction from vitamin D deficient rats as compared to a partial tracing above it of the coelectrophoresis of purified rat calcium binding protein with the same supernatant fraction as well as photos of the disc gels are included.

Discussion

Despite the now well established fact that calcium binding protein appears in intestine only after vitamin D administration (Wasserman, 1970b; Kallfelz *et al.*, 1967) and that the action of vitamin D on calcium transport in intestine requires protein synthesis (Zull *et al.*, 1965, 1966; Norman, 1965), our initial attempts to obtain evidence for *de novo* synthesis of calcium binding protein in response to vitamin D in the rat were not convincing. This puzzling situation began to be clarified when Tanaka and DeLuca (1971) found that the antinomycin D sensitive step in vitamin D action on the intestine is the conversion of 25-HCC[2] to 1,25-DHCC; the probable metabolically active form of vitamin D in the intestine (Holick *et al.*, 1971). The 1,25-DHCC has now been shown to act on intestinal calcium transport in the presence of actinomycin D suggesting that protein synthesis may not be involved in the initiation of calcium transport in response to the active form of vitamin D (Y. Tanaka *et al.*, 1971, unpublished results). The present report provides new evidence that the formation of calcium binding protein may not involve new protein synthesis but may result from the conversion of a preexisting protein (protein A) into the calcium binding protein (protein C).

[1] Some of the supernatant proteins, because of their large size, are not fractionated on these gels. For example, of the labeled proteins from the intestinal supernatant fractions, a total of 56% remain in the spacer gel region.

[2] Abbreviations used are: 25-HCC, 25-hydroxycholecalciferol; 1,25-DHCC, 1,25-dihydroxycholecalciferol.

The evidence for the above hypothesis is as follows. Both in our laboratory and elsewhere (Wasserman, 1970b; Kallfelz *et al.*, 1967) the only protein of the intestinal supernatant fraction to appear in response to vitamin D is the calcium binding protein. Furthermore, pure calcium binding protein isolated as described in the previous paper (Drescher and DeLuca, 1971) migrates in a manner almost identical with C protein on disc gel electrophoresis. Unfortunately the use of 9 M urea in the disc gel experiments precluded testing the calcium binding activity of the separated protein bands. In any case the evidence is strong that the C protein is the calcium binding protein which appears in response to vitamin D.

By means of visual observation and densitometric tracings of the electrophoretic disc gels, it is possible to demonstrate that as the calcium binding protein appears in response to vitamin D, the A protein decreases in intensity in an almost inverse manner. Finally the dual-label experiment demonstrates that vitamin D brings about a very definite diminution of ^{14}C in the A protein and an inversely proportional increase in ^{14}C in the calcium binding protein (C) of Figures 3 and 4. Although these results can be interpreted as vitamin D repressing the formation of A while stimulating the production of C, a more likely explanation is that protein A is a precursor of the calcium binding protein and that the metabolically active form of vitamin D in the intestine (1,25-DHCC) brings about its conversion into the calcium binding protein. Other experiments not reported here, in agreement with Corradino and Wasserman (1970), reveal that strontium prevents the appearance of calcium binding protein and inhibits the conversion of protein A into the calcium binding protein (protein C). Thus it appears that both vitamin D and calcium are involved in the conversion of protein A into calcium binding protein.

These results suggest that perhaps vitamin D and calcium ion in some way stimulate hydrolysis of a peptide fragment from precursor A to yield active calcium binding protein C. No direct evidence for such an enzyme has yet been found; however, the fact that the altered patterns occur in 9 M urea gels suggests the idea of covalent bond breakage, perhaps of the sort occurring in activation of digestive enzyme zymogens. It can further be suggested that the inactive precursor A would always be available, perhaps even rapidly synthesized. The idea of a precursor molecule for the chick calcium binding protein has been considered by MacGregor *et al.* (1970). These investigators at present, however, support the concept of *de novo* synthesis of chick calcium binding protein as opposed to a conversion from precursor. Clearly, additional, more direct experiments must be carried out to test these hypotheses.

The major basis for the suggestion that calcium binding protein of the rat is formed from a preexisting protein is on the basis of the dual-label experiment. Although an experiment of the type illustrated in Figure 3, part I, actually has an internal control, the double control experiment (Figure 3, part II) is a very stringent test for artifacts of the experimental procedure. The double control of Figure 3, part II, underscores the idea that vitamin D is responsible for the decrease in protein A and the increase in protein C. In addition, a duplicate set of gels of part I was electrophoretically destained for 24 hr to remove free amino acids after which they were analyzed as usual. The duplicates yielded results almost identical with those of the gels which had not been electrophoretically destained.

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