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Effect of Vitamin D Deficiency on in Vitro Labeling of Chick Intestinal Proteins: Analysis by Two-Dimensional Electrophoresis†

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ABSTRACT: Duodenal tissue from vitamin D_3 replete chicks was labeled in vitro for 2.5 h with ^3H-labeled amino acids and then combined with tissue from severely vitamin D deficient chicks incubated in an identical fashion with ^{14}C-labeled amino acids. Four double-labeled samples thus obtained were separated into pellet and cytosol fractions by centrifugation, the acidic proteins in each fraction separated by two-dimensional electrophoresis, and the separated proteins analyzed for changes in $^3H/^{14}C$ ratio by the McConkey method of double-label autoradiography. The $^3H/^{14}C$ ratios for proteins with high isotope incorporation were then determined by direct measurement of radioactivity. Of the 100 proteins resolved in the pellet fraction, two were found with $^3H/^{14}C$ ratios greater than base line. Protein 1 (M_r 27 000) was identified by comigration as membrane-associated, vitamin D induced calcium binding protein. Protein 23 (M_r 76 000 and unknown identity) was increased in $^3H/^{14}C$ ratio by 2.7-, 2.0-, 2.8-, and 1.4-fold over base line in samples I–IV, respectively; this protein stained very faintly with Coomassie blue. The $^3H/^{14}C$ ratio of β- and γ-actin was low in each sample, being 0.61, 0.54, 0.28, and 0.67 of the base-line ratio in samples I–IV, respectively. In the cytosolic fractions, the calcium binding protein was found to have a $^3H/^{14}C$ ratio approaching infinity in each sample. In addition, an unidentified cytosolic protein (M_r ~76 000) was found to be enriched 1.5-, 2.2-, 4.9-, and 1.7-fold over the base-line ratio. This protein also stained faintly with Coomassie blue. The observed deviations from the $^3H/^{14}C$ base-line ratios probably reflect differences in rates of protein synthesis brought about either directly by 1,25-dihydroxyvitamin D_3 or indirectly via changes in serum calcium or other serum factors.

The most pronounced physiological action of 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$][1] is to stimulate directly transcellular calcium transport across intestinal villi. Although several theories have been postulated concerning the mechanism of this stimulation (Zerwekh et al., 1976; Wilson & Lawson, 1977; Bickle et al., 1978; Lane & Lawson, 1978; Rasmussen et al., 1979; MacLaughlin et al., 1980), the precise cellular and molecular scheme of events leading to increased calcium transport in intestine is unknown.

One reasonable hypothesis (Zerwekh et al., 1976) is that since 1,25-$(OH)_2D_3$ is steroidlike in structure, possesses high-affinity intestinal receptors, and localizes in intestinal nuclei (Stumpf et al., 1979), its mechanism is similar to that of other steroid hormones, i.e., it acts via stimulation of transcription of specific genes (Gorski & Gannon, 1976). The vitamin D dependent calcium binding protein (CaBP) is one such gene product (Wasserman et al., 1978); however, the time

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[1] Abbreviations used: 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; CaBP, calcium binding protein; TEMED, *N,N,N',N'*-tetramethylethylenediamine; Tris, tris(hydroxymethyl)aminomethane; $NaDodSO_4$, sodium dodecyl sulfate; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; PPO, 2,5-diphenyloxazole.

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course of appearance of CaBP in chick intestine does not agree with the time course of appearance of calcium transport (Spencer et al., 1976). Two other proteins which appear to be induced by 1,25-$(OH)_2D_3$ and to correlate better with calcium transport have been identified in chick brush border by Wilson et al. (Wilson & Lawson, 1977, 1978).

In addition to these suggested primary effects on intestinal protein synthesis, it appears possible that 1,25-$(OH)_2D_3$ might indirectly influence intestinal protein synthesis as well as cell division in the villus crypts by modulating serum calcium levels (Perris, 1971; Spielvogel et al., 1972).

Recently methods have been described for (1) resolution of complex mixtures of proteins by two-dimensional electrophoresis (O'Farrell, 1975; Ivarie & O'Farrell, 1978), (2) increased sensitivity of detection of ^3H- and ^{14}C-labeled proteins in acrylamide slab gels by fluorography (Bonner & Laskey, 1974; Laskey & Mills, 1975), and (3) identification of proteins which vary between two similar complex mixtures by double-label autoradiography (McConkey, 1979). This report describes experiments which utilize these methods to analyze intestinal protein synthesis in severely vitamin D deficient and vitamin D treated chicks, the tissue having been labeled in vitro with ^{14}C- and ^3H-labeled amino acids. In this case, deviations in $^3H/^{14}C$ ratios of individual proteins from the base-line ratio might be directly due to vitamin D via 1,25-$(OH)_2D_3$; the changes might also be secondary to the vitamin due to differing levels of parathyroid hormone, serum calcium, or other serum factors. This work was undertaken as a first approach, to test the sensitivity of these methods for vitamin D induced CaBP and to test their general applicability to the complex problem of vitamin D induced transcellular calcium transport.

Experimental Procedures

Animals. White Leghorn cockerel chicks were obtained 1 day after hatching and maintained under incandescent lighting on a vitamin D deficient diet containing 1% calcium and 0.5% phosphorus (Omdahl et al., 1971). After 5.5 weeks, half of the chicks were dosed orally on alternate days for 1.5 weeks with 100 IU (2.5 μg) of vitamin D_3 in 50 μL of propylene glycol.

Reagents. Amopholines in the pH range 4–6 were purchased from Bio-Rad (Richmond, CA) along with acrylamide, bis(acrylamide), and *N,N,N′,N′*-tetramethylenediamine (TEMED). Amopholines in the pH 3.5–10 range were purchased from LKB (Stockholm, Sweden). All other chemicals were reagent grade.

The following proteins were purchased from Sigma Chemical Co. (St Louis, MO) and used as molecular weight standards: myosin (M_r 220000), β-galactosidase, (130000), phosphorylase A (94000), bovine serum albumin (68000), catalase (60000), ovalbumin (45000), α-actin (42000), trypsinogen (24000), and β-lactoglobulin (18400).

Vitamin D dependent CaBP in the denatured form was purified to homogeneity for use as a two-dimensional electrophoresis standard, as follows. Chicks were dosed orally with 100 units (2.5 μg) of vitamin D_3/day for 4 days. Intestinal cytosol was obtained by homogenization of duodenal scrapings in 5 mM Tris buffer, pH 7.4, followed by centrifugation at 105000*g* for 1 h. The cytosol was heated to 70–75 °C for 10 min (Bredderman & Wasserman, 1974), lyophilized, and dissolved in $NaDodSO_4$-containing buffer. Purified CaBP was obtained by preparative $NaDodSO_4$–polyacrylamide slab gel electrophoresis of the heat-treated cytosol.

In Vitro Incubations. Four vitamin D replete and four vitamin D deficient chicks were used to obtain the double-label samples. After a chick was killed by decapitation, the proximal half of the duodenal loop was removed, rinsed gently with saline, and opened onto a hardwood block where several 1.5 × 5 mm slices were cut. Slices from each chick were incubated separately at the surface of Trowel's T8 media as described by Kagnoff et al. (1972) under a humidified, O_2/CO_2 (95:5 v/v) atmosphere. Since chick intestine has essentially no submucosa (Hodges, 1974), removal of the mucosal layer from the underlying muscle as in the biopsy performed by Kagnoff et al. (1972) resulted in a loose suspension of villi which was difficult to manipulate; thus the intestinal slices were incubated intact, even though they tended to curl upon contact with the media.

Slices from vitamin D deficient chicks were incubated in media containing 50 μCi each of [^{14}C]leucine, [^{14}C]lysine, and [^{14}C]valine at a concentration of 75 μM, while slices from vitamin D replete chicks were incubated in media containing 500 μCi each of [^3H]leucine, [^3H]lysine, and [^3H]valine also at a concentration of 75 μM. The starting ratio of $^3H/^{14}C$ of amino acid was set at 10 so that the McConkey method (McConkey, 1979) of double-label autoradiography could be used to identify proteins enriched with ^3H. In this method, both ^3H-labeled and ^{14}C-labeled proteins are visualized by fluorography at −80 °C using Kodak XR5 film, but only ^{14}C-labeled proteins are detected by direct autoradiography at room temperature using Kodak No Screen film. Since the two films are exposed to the same gel, the resultant patterns are exactly superimposable and may be easily compared for differences. However, since for 0.75-mm-thick slab gels fluorography is about 10 times more sensitive for detection of the stronger β-emitter ^{14}C than for the weak β-emitter ^3H, the input ratio of $^3H/^{14}C$ on the gel must be adjusted to about 10 so that the limit of detectability by fluorography is the same for both isotopes. After 2.5 h of incubation, the tissues slices were passed through two rinses of 150 mM NaCl and blotted gently, and the mucosal layer was scraped off the muscle layer. Each set of scrapings from a vitamin D deficient animal was combined with similar scrapings from a vitamin D replete chick and the tissue homogenized in 7 mL of 0.25 M sucrose and 10 mM Hepes, pH 7.4, with three 10-s bursts of a polytron homogenizer (Brinkmann Instruments, Westbury, NY). The homogenates were centrifuged at 105000*g* for 1 h, the supernatants were saved, and the pellet was washed once by centrifugation. The pellets were resuspended by glass–Teflon homogenization in 2 mL of H_2O, analyzed for protein by the method of Bradford (1976) with bovine serum albumin as a standard, lyophilized, and redissolved in $NaDodSO_4$-containing lysis buffer at a concentration of 5 mg/mL as described by O'Farrell et al. (1977). The supernatants (cytosols) were stored at −80 °C for 2 weeks and then dialyzed for 24 h at 4 °C against four changes of 5 mM Tris-HCl, pH 7.4. The dialyzed cytosols were analyzed for protein, lyophilized, and redissolved in $NaDodSO_4$-containing lysis buffer as described for the pellet samples.

Two-Dimensional Electrophoresis. The samples were subjected to two-dimensional electrophoresis as described by O'Farrell (1975) except that pH 4–6 amopholines were substituted for pH 5–7 ampholines and the stacking gels were overlaid very carefully with water instead of Teflon strips. The 105-mm IEF gels, prefocused as described by O'Farrell, were focused at 400 V for 15 h and 800 V for 1 h; subsequent equilibration in "buffer O" was for 90 min. The second dimension was a uniform slab gel of 10% acrylamide of dimensions 95 × 140 mm.

Some of these gels were subjected to fluorography (Bonner & Laskey, 1974; Laskey & Mills, 1975) and exposed to

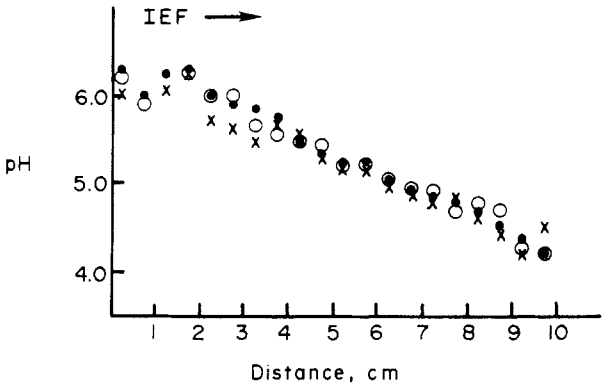

FIGURE 1: pH gradient of IEF tube gels. Tube gels (105 mm) were prepared with 1.6% amopholines with pH range 4–6 and 0.4% ampholines with pH range 3.5–10 and subjected to voltage gradients as described under Experimental Procedures. The gels were sliced into 5-mm sections, the sections were shaken in 2 mL of degassed distilled water for 3–4 h, and the pH was measured. The pH profiles of three gels (●, O, ×) are shown.

preflashed Kodak XR5 film at −80 °C or Kodak No Screen film at room temperature for periods ranging from 1 week to 12 weeks, according to the method of McConkey (1979).

Very few of the proteins on a single two-dimensional slab gel contained enough radioactivity for direct analysis of $^3H/^{14}C$ ratios. Thus, to obtain sufficient quantities of the ratio of $^3H/^{14}C$ in the more abundant and more highly labeled proteins, ten two-dimensional gels loaded with 100 μg each were prepared from each of the four double-label samples. Corresponding spots from the ten stained, dried gels were cut out, combined, combusted to $^{14}CO_2$ and ^3H_2O by means of a Packard Tri-Carb Model 306 sample oxidizer, and counted for radioactivity by using a Packard liquid scintillation counter. Similarly, corresponding spots from four dried slab gels for each of the four cytosol samples were pooled, oxidized, and counted. Counting efficiency was 25–35% for ^3H and 60% for ^{14}C. Oxidizer recoveries for both ^3H and ^{14}C were 95 ± 5%. All ratios are of disintegrations per minute (dpm).

Ampholines were removed during the staining of two-dimensional gels as described by O'Farrell (1975) except that the soak in 50% alcohol, 7% acetic acid, and 0.005% Coomassie blue was overnight with gentle shaking instead of 36 h.

Results

The serum calcium concentration for the vitamin D_3 treated chicks was 10.0 ± 0.8 mg% (n = 7) vs. 7.4 ± 1.2 mg % (n = 6) for the severely D deficient chicks (±SD; $p < 0.001$), confirming the vitamin D status of the two groups. Qualitatively the vitamin D deficient chicks were lethargic, with obvious muscle weakness and softened beaks; the vitamin D_3 replete animals had largely recovered from these symptoms after a week and a half of treatment.

Preliminary analyses of the four double-label pellet samples using one-dimensional electrophoresis ($NaDodSO_4$ slab gel electrophoresis) in combination with double-label autoradiography were not encouraging. Although the four samples could be conveniently run in adjacent wells on a single slab gel, no differences were detected between the patterns obtained by using Kodak XR-5 film and Kodak No Screen film.

The resolving power of two-dimensional electrophoresis is such that over a thousand proteins may be visualized on a single gel (O'Farrell, 1975; Ivarie & O'Farrell, 1978); however, such exceedingly complex patterns are very difficult to analyze thoroughly. In this work in order to simplify the analysis, only those proteins were studied whose synthesis was most likely to be affected by vitamin D, i.e., acidic proteins which might

Table I: Incorporation of ^3H and ^{14}C into Particulate Fractions from Chick Intestine[a]

sample	total mg of protein, pooled tissue	dpm of ^3H/mg	dpm of ^{14}C/mg	$^3H/^{14}C$
I	2.3	1.2×10^6	0.22×10^6	5.5
II	2.0	1.0×10^6	0.11×10^6	9.0
III	2.8	2.2×10^6	0.19×10^6	11.7
IV	2.5	2.7×10^6	0.13×10^6	20.8

[a] After incubation of intestinal tissue from vitamin D replete chicks with ^3H-labeled amino acids and tissue from vitamin D deficient chicks with ^{14}C-labeled amino acids as described under Experimental Procedures, the ^{14}C- and ^3H-labeled tissues were pooled, homogenized, and pelleted by high-speed centrifugation, and the protein content of the pellets was measured. The pellets were dissolved in $NaDodSO_4$-containing O'Farrell buffers (O'Farrell et al., 1977) and aliquots oxidized to determine ^{14}C and ^3H content per milligram of protein.

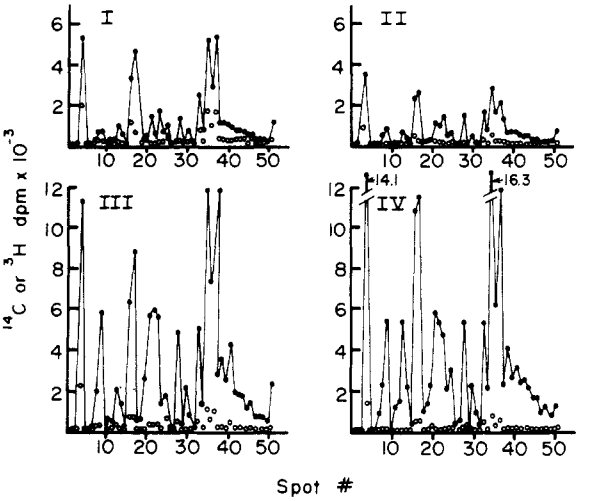

FIGURE 2: Ten two-dimensional electrophoresis gels (100 μg of protein/gel) were prepared from each of the four double-label pellet samples; the gels were stained with Coomassie blue as described under Experimental Procedures. A total of 51 corresponding protein spots for each sample were cut from each of the 40 gels, corresponding spots for each sample were combined, and the combined spots were analyzed for ^{14}C and ^3H content by combustion and scintillation counting. Roman numerals denote sample number; (●) ^3H content; (O) ^{14}C content per ten combined gel spots.

interact with calcium. Such proteins would be expected to be well resolved on the isoelectric focusing (IEF) pH gradient running from about pH 4 to 6.3 shown in Figure 1. About 100 proteins in the pellet fraction and about 200 proteins in the cytosolic fraction were resolved in this pH gradient.

As discussed under Experimental Procedures, the starting ratio of $^3H/^{14}C$ in the two incubation media was set at 10 so that the McConkey method of double-label autoradiography could be used for easy detection of vitamin D induced proteins on superimposable films.

However, other factors such as the total amount of tissue pooled from each intestine and the viability of the slices in vitro affected the final $^3H/^{14}C$ ratio of the double-label samples. Table I shows the absolute incorporation of ^3H and ^{14}C per milligram of protein and also the $^3H/^{14}C$ ratio for the four pellet samples. Both ^3H and ^{14}C incorporations varied about 2-fold so that the final $^3H/^{14}C$ ratio varied about 4-fold from 5.5 to 21.

Even though the $^3H/^{14}C$ ratios of the dissolved pellets varied 4-fold, Figure 2 shows that the general pattern of incorporation of label into cellular protein is similar from sample to sample. To obtain these data, ten two-dimensional slab gels were prepared from each of the four sample pellets, and 51 spots

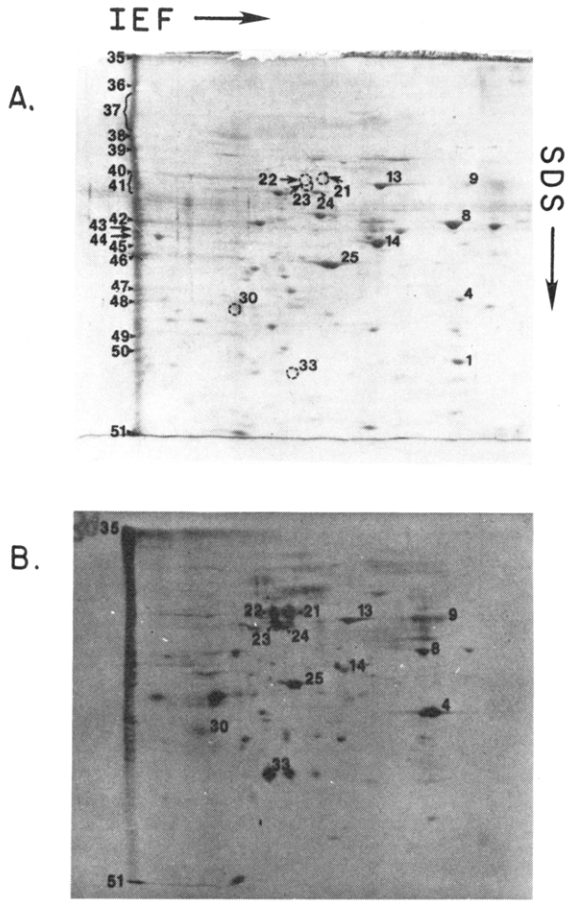

FIGURE 3: Comparison of acidic protein patterns from two pellet samples visualized by Coomassie blue staining (A) and fluorography using Kodak XR5 film (B). The numbered spots, with the exception of spot 1, are proteins whose ^3H content was 400 dpm or more per ten combined spots. Protein 1, which had a ^3H content of 70–210 dpm per ten combined spots, exactly comigrated with purified CaBP. Spot 4, the most highly labeled protein, contained a ^3H content of 3500–14 100 dpm per ten combined spots. The exposure time in (B) of the PPO-impregnated gel to preflashed XR5 was 4 weeks.

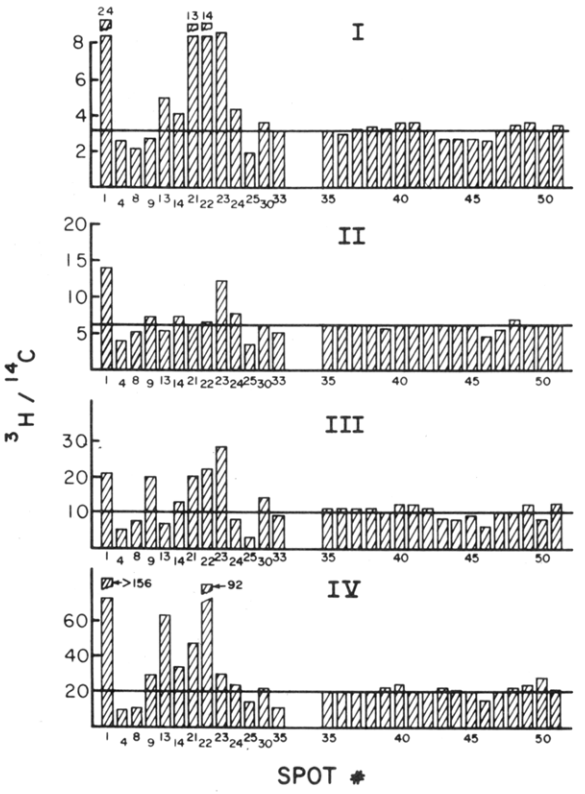

FIGURE 4: $^3H/^{14}C$ ratios of some acidic intestinal proteins separated by two-dimensional electrophoresis along with base-line ratios. The $^3H/^{14}C$ ratios of each of the proteins numbered in Figure 3 are shown for the four pellet samples; roman numerals indicate the sample number. The horizontal lines indicate the base-line ratio for each sample, i.e., the average ratio of proteins 35–51. Spot 1 comigrates with purified CaBP; spot 25 is presumptive β- and γ-actins on the basis of the position relative to α-actin (Garrels & Gibson, 1976). These actins are not completely resolved in this system.

or areas were selected from the Coomassie blue staining pattern. For each sample, corresponding spots from each of the ten stained gels were cut out, combined, and analyzed for radioactivity as described under Experimental Procedures. In general, the relative incorporation of both ^3H and ^{14}C into any protein spot relative to spot 4 (the most highly labeled spot) was about the same for each sample. Exceptions were spots 9 and 22 which showed considerable variation in ^{14}C and ^3H incorporations relative to spot 4; this variation is possibly due to glycoprotein charge heterogeneity.

Figure 3 shows two-dimensional gel patterns obtained by fluorography using Kodak XR5 and by staining with Coomassie blue. The numbered spots from 4 to 33 are acidic proteins with a minimum of 400 dpm of ^3H per ten combined spots. Once the radioactivity in a Coomassie blue spot was known, provided the level of radioactivity was sufficiently high, that protein spot could be matched with the corresponding fluorography spot by overlaying the film on the dried gel. The reverse was not true; many spots visualized by fluorography were not detectable by staining with Coomassie blue because of either low concentration or poor stainability.

Many proteins of varying molecular weight remained at the origin of the IEF tube gel; these more basic proteins with an isoelectric point (pI) of 6.3 or greater form the column of proteins on the left-hand side of the second dimension slab gel. Since the number of counts in these bands was relatively great and the staining pattern reproducible, the base-line $^3H/^{14}C$ ratio for each sample was determined from bands 35–51 within this column.

Figure 4 is a graphic representation of the $^3H/^{14}C$ ratio for spots shown in Figure 3 for each of the four samples. The $^3H/^{14}C$ ratios of protein bands 35–51 are fairly constant within a given sample. The average of the ratios of bands 35–51, i.e., 3.1 ± 0.34, 5.9 ± 0.53, 10 ± 1.7, and 20.9 ± 2.5 (±SD) for samples I–IV, respectively, is thus a reliable measure of the base-line ratio for that sample. With the exception of protein 1, the total ^{14}C and ^3H counts accumulated to obtain these ratios varied between 500 and about 14 000 and were usually above 1000. The corresponding counting error associated with the ratios was about 3%, with a maximum of 6%.

Protein 1 contained only 7–220 dpm of ^3H per 10 gels (100 μg of protein per gel or 1 mg total). Although the $^3H/^{14}C$ ratios measured for this protein were not entirely reliable due to the low number of counts per minute, the ratio in each of the samples was substantially higher than the base-line ratio in contrast to other spots with low radioactivity. Since protein 1 had an acidic pI and an approximate M_r of 26 000–27 000, it was suspected to be membrane-associated CaBP (Feher & Wasserman, 1978). This identity was confirmed by adding 0.5 and 1.5 μg of CaBP standard to 100-μg pellet samples and comparing the two-dimensional gel patterns to those of identical pellet samples without CaBP standard. Protein 1 exactly comigrated with purified CaBP prepared from chick cytosol. The physiological role of membrane-associated CaBP is uncertain; it is perhaps residual cytosolic CaBP trapped within membrane vesicles during homogenization. We calculate the

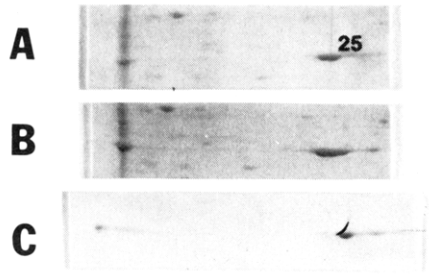

FIGURE 5: Identification of protein spot 25 as β- and γ-actins. (A) Spot 25 as it appears after two-dimensional electrophoresis of 50 μg of pellet sample III. (B) Spot 25 as it appears after two-dimensional electrophoresis of a mixture of about 1.5 μg of α-actin and 50 μg of pellet sample III. (C) The pattern obtained from two-dimensional electrophoresis of about 1.5 μg of α-actin. For each gel, the spots were stained with Coomassie blue as described under Experimental Procedures. Since β- and γ-actins have been shown to be a major component of chick intestinal brush border (Wilson & Lawson, 1978; Mooseker & Tilney, 1975) sedimenting in the pellet fraction and since Garrels & Gibson (1976) having shown that α-, β-, and γ-actins migrate during two-dimensional electrophoresis as a tight cluster of pI ~5.4, with α-actin the most acidic, then spot 25 consisting of two incompletely resolved protein components must be composed of β- and γ-actins.

membrane-associated CaBP fraction to be approximately 6–7% of the total ^{3}H-labeled CaBP in the initial homogenates.

Protein spot 23 contained from 1400 to 5600 dpm of ^{3}H and from 120–200 dpm of ^{14}C per mg of protein (ten combined spots). This unidentified protein of M_r ~71 000 was found to be increased over the base-line ratio in each of the four samples, 2.7-, 2.0-, 2.8-, and 1.4-fold for samples I–IV, respectively.

Proteins 21 and 22 of M_r ~76 000 and pI similar to spot 23 appear to be charge variants of a single glycoprotein species (Baumann & Doyle, 1979). These proteins were enriched in ^{3}H in three out of four samples, from about 2 to 5-fold. One possibility for protein (21 + 22) is that it is a denatured subunit of intestinal alkaline phosphatase; alkaline phosphatase is known to be a heterogeneous glycoprotein of M_r 157–160 000 existing as a dimer of identical or similar subunits in the rat (Maliki & Butterworth, 1976). Although proteins 21, 22, and 23 contained a relatively large amount of radioactivity, they stained poorly with Coomassie blue, suggesting either low abundance or poor stainability.

Wilson & Lawson (1978) working with chick intestinal brush borders have recently reported that 1,25-$(OH)_2D_3$ increases the incorporation of [^{3}H]leucine into β- and γ-actins (or actinlike proteins) by 60% within 3 h after dosing. This increased incorporation is presumably in the actin core filaments of the intestinal brush border microvilli (Mooseker & Tilney, 1975). Garrels & Gibson (1976) have shown that α-, β-, and γ-actins migrate in close proximity on a two-dimensional electrophoresis slab gel; the three forms have slightly different isoelectric points near pH 5.4, with α-actin being the most acidic, followed by β- and γ-actins. In order to determine if actin synthesis was stimulated in vitamin D_3 replete animals in this study, we identified β- and γ-actins on the two-dimensional gels by using α-actin purchased from Sigma Chemical Co. as a standard.

Figure 5 shows that spot 25, which is composed of two incompletely resolved proteins of M_r ~43 000, migrates in a position consistent with β- and γ-actins. The β and γ forms are present in approximately equal amounts as assessed by staining intensity, as was observed by Wilson & Lawson (1978). However, the ^{3}H/^{14}C ratio of these two proteins combined is not increased but rather is significantly decreased when compared to the base-line ratio in each of the four

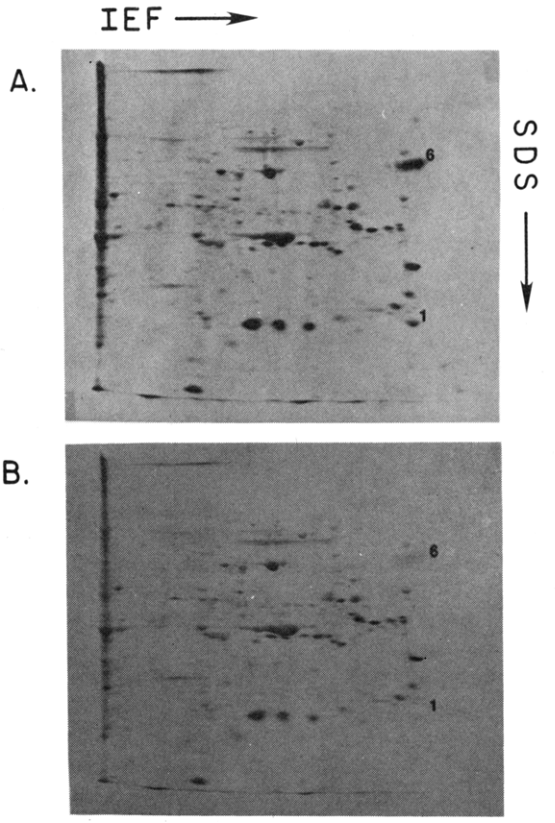

FIGURE 6: Double-label autoradiography of acidic proteins from the cytosol fraction. For this figure, the acidic proteins of 100 μg of sample III cytosol were separated by two-dimensional electrophoresis, and the resultant slab gel was impregnated with PPO for fluorography and double-label autoradiography (Laskey & Mills, 1975; McConkey, 1979). In (A), Kodak XR-5 film was exposed to the PPO-impregnated gel for 1 week at −80 °C for visualization of ^{14}C- and ^{3}H-containing proteins. In (B), Kodak No Screen film was exposed to the same gel at room temperature for 3 weeks for visualization of ^{14}C-containing proteins selectively. Of the approximately 200 proteins scanned by this method, only proteins 1 (CaBP) and 6 (unknown identity, M_r ~76 000) appeared to be present in (A) and absent in (B), suggesting a ^{3}H/^{14}C ratio much greater than that of the background.

samples, being 0.61, 0.54, 0.28, and 0.67 of base-line ratio in samples I–IV, respectively.

The usefulness of the McConkey approach in analysis of complex two-dimensional gel patterns is best seen in Figure 6 which shows film obtained by double-label autoradiography. For Figure 6, 100 μg of cytosolic protein from sample III (base-line ratio 10.0) was subjected to two-dimensional electrophoresis followed by fluorography as described under Experimental Procedures. The pattern obtained when presensitized Kodak XR5 was exposed to the PPO-impregnated gel for 7 days is shown in Figure 6A; this film detects both ^{14}C and ^{3}H. Figure 6B shows the similar pattern obtained when Kodak No Screen film was exposed to the same gel for 3 weeks at room temperature; under these conditions, No Screen film detects only ^{14}C-containing proteins. Thus, by difference, those spots appearing only in Figure 6A are highly enriched in ^{3}H. Of the approximately 200 proteins detectable under these conditions of exposure, only two were of significantly different intensity on the two films, proteins 1 and 6. Spot 1 was easily visualized by Coomassie blue staining while spot 6 stained extremely faintly.

For quantitation of the radioactivity and ^{3}H/^{14}C ratio for these spots, four two-dimensional slab gels (100 μg of protein/gel) were prepared from each of the four samples, and the corresponding spots were cut out, combusted, and counted for radioactivity as before. Table II shows the absolute in-

Table II: Incorporation of Radioactive Amino Acids into Cytosolic Protein 1 (CaBP) and Protein 6[a]

sample	dpm/four gels ^3H	dpm/four gels ^{14}C	$^3H/^{14}C$	fold increase over base-line ratio
I, 1	122	0	>122	>39
II, 1	626	0	>626	>106
III, 1	1560	4	390	39
IV, 1	1120	5	224	11
I, 6	780	167	4.7	1.5
II, 6	681	53	12.8	2.2
III, 6	6270	128	49	4.9
IV, 6	4990	139	36	1.7

[a] Four two-dimensional gels (100 μg of protein/gel) were prepared from each of the four double-label cytosol samples and stained with Coomassie blue as described under Experimental Procedures. Proteins 1 and 6 were cut from each of the four dried gels; the gel spots were combined and combusted to determine the ^{14}C and ^3H content.

corporation of ^{14}C and ^3H in the proteins along with the ratio and fold enrichment. Protein 1, (M_r ~27 000, acidic p*I*, with essentially no ^{14}C incorporation is certainly CaBP. In these samples, the presence of labeled CaBP, a cytosolic protein known to be induced by 1,25-$(OH)_2D_3$, confirms that the intestinal tissue is still hormonally responsive during the in vitro incubations. Protein 6, M_r ~76 000, is enriched between 1.5- and 5-fold in ^3H; the total ^3H incorporation into this protein is considerably higher than that for CaBP, suggesting a higher rate of synthesis.

Discussion

Wilson & Lawson (1978) have reported that one of the earliest effects of 1,25-$(OH)_2D_3$ is to increase either the rate of synthesis of brush border β- and γ-actins by 60% or the rate of incorporation of newly synthesized actin into microvilli by that amount. Our results suggest that the synthetic rate of β- and γ-actins in vitamin D replete chicks as compared to severely vitamin D deficient chicks is decreased by about 50%, not increased. However, our results are not necessarily in disagreement with those of Wilson & Lawson since these workers were analyzing brush border actins compared to our analysis of total cell actins, and they were looking at effects occurring within 3 h after 1,25-$(OH)_2D_3$ treatment compared to our study of steady-state conditions, D-replete vs. D-deficient states.

The reason for the decrease in ratio, observed for several unidentified proteins as well as β- and γ-actins is not clear. Possibly it stems from the increase in intestinal villus height, which is as much as 30% after prolonged vitamin D treatment (Spielvogel et al., 1972). A gradual decline in protein synthetic activity from intestinal crypt cells to villus tip cells is known to occur (Altmann, 1976). If vitamin D increased the number of more highly differentiated cells in the upper part of the villus, that is, increased the number of cells with decreased synthetic activity, this might be manifested in our experiments as a decrease in $^3H/^{14}C$ ratio for some proteins.

In general, we presume that both the increases and decreases in $^3H/^{14}C$ ratios of specific proteins described under Results are due either directly to vitamin D via the active metabolite 1,25-$(OH)_2D_3$ or indirectly to the vitamin via increased serum calcium. Other serum constituents which fluctuate with 1,25-$(OH)_2D_3$ levels such as parathyroid hormone might also be a factor in intestinal protein synthesis. Since the in vitro incorporation time was for 2.5 h, it is possible that alterations in degradation rate are a factor in some of the ratio changes.

The obvious advantage of two-dimensional electrophoresis in combination with double-label autoradiography is that large numbers of proteins may be surveyed for changes in rate of synthesis. Problems with this approach encountered in these experiments designed to visualize effects of vitamin D on intestine are also apparent. The absolute incorporation of ^3H- and ^{14}C-labeled amino acids into cellular proteins in vitro was relatively low and somewhat variable from sample to sample; thus, autoradiography for detection of ^{14}C-labeled proteins took a minimum of 3 weeks. For direct measurement of radioactivity, multiple gels had to be prepared for each sample to obtain sufficent radioactivity by combination of factions. Even so, only proteins with relatively high incorporations could be directly analyzed.

These problems are probably not insurmountable. The use of more complex mixtures of ^3H- and ^{14}C-labeled amino acids in combination with [^{35}S]- and [^3H]methionine, which may be obtained in very high specific activity, should substantially increase isotope incorporation. Preliminary experiments with rat intestine where the villus layer may be removed from underlying muscle before incubation with labeled amino acids suggest that this biopsy procedure both increases the extent of isotope incorporation and reduces variability. Increased incorporation is especially important in that shorter incubation times (e.g., ≤30 min) should be used to reduce intracellular degradation as a factor affecting $^3H/^{14}C$ ratios.

For the work described here, analyses of differences in the XR5 and No Screen film pattern were performed by eye. Although virtually all the 200 resolved cytosolic proteins and 100 resolved pellet proteins could be compared without difficulty, only extremely pronounced differences were reliably detectable, e.g., 5-fold or greater. However, we feel that the exact superimposability of the two films in double-label autoradiography should facilitate the analysis of gels by computerization. For example, Bossinger et al. (1979) describe a computerized system where two-dimensional film density distributions are recorded by means of a high-speed rotating drum scanner, and the data are digitized, converted to radioactive intensity per resolved spot by means of a standard curve, and visualized on a numbered contour map. But even with this elegant system, these authors describe difficulty in comparing one complex pattern with another with respect to minor protein components, due to a small unpredictable variations from gel to gel. Uncontrollable factors such as slight stretching of the IEF tube gel during transfer to the slab gel agarose account for such variations. Since in the McConkey method all the common protein spots from two different samples are identical in shape and position, computerized analysis of the two films should permit quantitative comparisons of the ratios of all resolved proteins, major and minor, with concomitant increases in sensitivity and useful information.

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Calorimetric Study of the Rabbit Hepatic Galactoside Binding Protein: Effects of Calcium and Ligands†

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ABSTRACT: Differential scanning calorimetry has been used to examine the thermal denaturation of rabbit hepatic galactoside binding protein. In the absence of Ca^{2+} or ligands, the inactive binding protein shows a single transition with a T_m of 46 ± 0.5 °C and an enthalpy of denaturation of 0.891 cal g^{-1}. In the presence of 20 mM $CaCl_2$, the active binding protein has a single transition with a T_m of 61 °C and an enthalpy of denaturation of 2.67 cal g^{-1}, indicating that Ca^{2+} markedly stabilizes the protein toward thermal denaturation. The T_m values of the binding protein–Ca^{2+} complexes with asialoorosomucoid or lactose are 64 and 63 °C, respectively. The enthalpy of denaturation in the presence of 20 mM lactose is 3.39 cal g^{-1}, indicating that an additional stabilization (~27%) toward denaturation is provided by binding of specific ligands. Furthermore, the differences in the shape of the denaturation profiles in the presence and absence of ligands suggest that ligand binding influences the denaturation process. Calcium binding, however, stabilizes the galactoside binding protein to thermal denaturation to a greater extent than does ligand binding. Thermal denaturation transitions attributable to the A or the B subunits of the binding protein are not observed, suggesting that the two subunits may be structurally similar.

The rabbit galactoside binding protein, discovered by Ashwell and co-workers (Morell et al., 1968, 1971; Hudgin et al., 1974; Kawasaki & Ashwell, 1976), contains two types of polypeptide chains, designated the A (apparent M_r 48 000) and B chains (apparent M_r 40 000), in a molar ratio of one A and two B chains, as determined by sodium dodecyl sulfate gel electrophoresis. The detergent-solubilized rabbit protein appears to have the subunit structure A_2B_4, in the absence of calcium, with a molecular weight of 234 000, as determined by sedimentation equilibrium (Andersen et al., 1981). The binding protein from rat liver has a similar molecular weight (Andersen et al., 1981), although it contains identical subunits (M_r 47 000; Tanabe et al., 1979). The binding proteins from all species, however, require Ca^{2+} for binding activity. Molecular weight studies indicate that the binding protein with a subunit structure A_2B_4 aggregates on binding of Ca^{2+} to give the active binding protein $(A_2B_4)_{2-3}$. Moreover, Ca^{2+} binding is accompanied by quenching of the intrinsic fluorescence of the protein.

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