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1,25-Dihydroxyvitamin D_3-Mediated Alterations in Microtubule Proteins Isolated From Chick Intestinal Epithelium: Analyses by Isoelectric Focusing

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Abstract Recent work has indicated that vectorial Ca^{2+} transport across the intestinal epithelium occurs in vesicles and may involve the participation of microtubules [Nemere et al., 1986]. Since 1,25 dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) stimulates this Ca^{2+} transport process, microtubule (MT) isotypes were studied as a potential regulatory point. The effect of $1,25(OH)_2D_3$ status on tubulin isotypes was analyzed by isoelectric focusing (IEF) gels of taxol stabilized MTs prepared from intestinal epithelium of vitamin D–deficient chicks dosed with vehicle (−D) or 1.3 nmoles of $1,25(OH)_2D_3$ (+D) 2.5, 5, 10, 15, or 43 h prior to sacrifice. Four bands, one of which was identified as α-tubulin on the basis of Western analysis, increased in Coomassie Blue staining intensity 5–15 h after $1,25(OH)_2D_3$, corresponding to the time course of augmented vesicular Ca^{2+} transport. Dose-response studies revealed similar changes in tubulin isotype profiles in IEF gels, again corresponding to doses known to elicit enhanced Ca^{2+} absorption (52–6,500 pmoles of hormone). The role of Ca^{2+} transport was also examined. Isoelectrically focused intestinal epithelial tubulin from −D chicks allowed to transport Ca^{2+} for 30 min revealed increased staining of bands relative to nonabsorbing −D controls. By comparison, Ca^{2+} transport in +D chicks resulted in fainter bands relative to nonabsorbing, +D controls. MTs prepared from fasted or fed chicks revealed similar changes upon IEF, but of much smaller magnitude. Enhanced phosphorylation did not account for the appearance of the more acidic bands, although $1,25(OH)_2D_3$ treatment resulted in decreased ^{32}P content of a presumptive non-tubulin component, relative to preparations from −D controls. Glucocorticoids, which are known to suppress $1,25(OH)_2D_3$-stimulated Ca^{2+} transport, led to severely diminished levels of total tubulin, as judged by SDS-PAGE, rather than altered tubulin isotypes. Thus, MTs of intestine are subject to regulation by hormonal status, as well as by the amount of Ca^{2+} available for transepithelial transport.

Key words: Ca transport, endocytic vesicles, lysosomes, α-tubulin, hormonal regulation, vectorial transport

The intestinal absorption of calcium is regulated by the hormonally active form of vitamin D_3, 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$). Our current hypothesis on the intracellular route by which Ca^{2+} is transported across intestine is one in which the cation is sequestered in vesicles. Uptake at the brush border is postulated to occur through endocytic vesicles, whose contents are subsequently transferred to lysosomes, and ultimately, exocytosis of the membrane delimited organelles at the basal lateral membrane completes the transport process [Nemere et al., 1986; Nemere and Norman, 1988, 1989]. Such vectorial transport is known to occur along intracellular tracks, particularly microtubules [Matteoni and Kreis, 1987; Nemere et al., 1984; Nassar et al., 1988]. Thus, an additional point of regulation of intestinal calcium transport by the seco-steroid hormone $1,25(OH)_2D_3$ could be the composition of such cytoskeletal elements, including tubulin isotype expression [Cleveland and Sullivan, 1985; Lewis et al., 1985; Miller et al., 1987]. Indeed, prolactin has been reported to regulate microtubule deployment in the anterior pituitary of rats [Ravindra and Grosvenor, 1986], and an earlier report from this laboratory [Nemere et al., 1987] documented regulation of α-tubulin mRNA expression by $1,25(OH)_2D_3$. The current work was undertaken to extend these latter observations by determining whether the seco-steroid hormone altered protein composition of chick intestinal epithelial microtubules, as judged by isoelectric focusing.

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MATERIALS AND METHODS

Animals and Surgical Procedures

White Leghorn cockerels (Lakeview Farms, Lakeview, CA) were obtained on the day of hatch and raised for 3.5 to 4.5 weeks on a rachitogenic diet [Norman and Wong, 1972]. Chicks (3–4 per group) were intramuscularly injected with $1,25(OH)_2D_3$ or vehicle at the times and doses described below prior to use. Dexamethasone (Sigma Chemical Co., St. Louis, MO) was injected intraperitoneally as described by Shultz et al. [1982].

On the day of experimentation, chicks were anesthetized with ether prior to surgical exposure of the duodenal loop. For studies on the effects of calcium absorption, the loops were ligated at the proximal and distal ends and instilled with 4 mg $CaCl_2$ in 0.4 ml of water, or an equivalent volume of water alone. The duodenal loop was replaced in the abdominal cavity, the incision closed, and transport allowed to proceed for 30 min prior to additional anesthesia and removal of the intestinal segment to ice-cold saline.

The mucosal scrapings within each group were pooled and homogenized on ice in 1–1.5 volumes of 100 mM PIPES (pH 6.6) using a Potter-Elvejhem homogenizer. The homogenates were subjected to a low speed centrifugation (20,000*g*, 20 min at 4°C) prior to preparation of microtubules by the taxol stabilization procedure of Vallee [1982]. The post-mitochondrial supernatant was further centrifuged at 180,000*g* for 90 min (4°C), and the final supernatant made 10 μM in taxol, 1 mM in GTP, and incubated at 37°C for 20 min for microtubule polymerization. Separation of polymerized microtubules from soluble components was accomplished by overlaying the incubation mixture on a 2 ml cushion of 15% sucrose in homogenization buffer, and centrifugation at 30,000*g*, 25 min at 37°C. The supernatant was decanted from the microtubule pellet, and while in the inverted position, the inside walls of the centrifuge tube were carefully swabbed with a Kimwipe to remove adherent soluble components. Earlier work [Field et al., 1984] has demonstrated that the inclusion of protease inhibitors is not required during the isolation procedure. The microtubule pellet was subsequently resuspended in double distilled water, and stored frozen until further use.

Phosphorylation Studies

Phosphorylation of microtubule proteins was undertaken by three approaches: (1) mucosal scrapings from vitamin D–deficient or $1,25(OH)_2D_3$-treated birds (3 chicks per group) were incubated for 30 min in carrier phosphate free Gey's balanced salt solution containing 2.5 mCi ^{32}P prior to the preparation of microtubules; (2) microtubules isolated from vitamin D–deficient or $1,25(OH)_2D_3$-treated chicks were incubated with 0.3 mCi of $\gamma^{32}P$-ATP; (3) the duodenal loops of anesthetized chicks were injected with 0.8 mCi ^{32}P for 30 min of in vivo phosphorylation prior to preparation of microtubules. Animals receiving the seco-steroid hormone were intramuscularly injected with 1.3 nmoles of $1,25(OH)_2D_3$ 15 h before experimentation. Isoelectric focusing gels (see below) were run in duplicate, stained, destained, dried, and placed on Kodak X-OMAT film for autoradiography at −80°C.

Isoelectric Focusing

For isoelectric focusing, an aliquot was removed and combined with an equivalent volume of sample buffer (9.16 M urea, 5% β-mercaptoethanol, 2% nonidet P-40, 30% glycerol) for solubilization [Field et al., 1984], and protein determined by the Bradford reagent (Biorad) against a buffer blank, and using bovine gamma globulin as standard (Biorad). To insure loading of equivalent amounts of protein in each lane, these protein determinations were made on the very same sample-buffer mixtures subsequently subjected to electrophoresis. Separation of tubulin isotypes was performed by a modification of the method of Field et al. [1984] using available vertical slab gel equipment. The IEF gels (165 × 120 × 0.75 mm) contained 4% acrylamide, 0.2% bisacrylamide, 9.16 M urea, 2% nonidet P-40, and ampholytes (1.73% pH 4–6, 0.27% pH 5–8; Sigma). Forty μls of overlay buffer (9.16 M urea, 2% pH 4–6 ampholytes, 2% nonidet P-40) were placed in each well for prefocusing at 250 V, 15 min, 300 V, 30 min, 400 V, 30 min. The anolyte was 0.05 M H_3PO_4 and the catholyte 0.05 M NaOH. Equivalent protein samples (40 μl, 100–200 μg per well) in sample buffer were then added to the wells and focused at 800 V for at least 19 h (15,000–20,000 V · h) at 16–18°C to prevent urea crystallization [Dunbar, 1987]. Prior to staining with Coomassie Brilliant Blue, the gels were fixed for at least 1 h

in 5% (w/v) trichloroacetic acid with agitation, and clarified (removal of urea) for at least 2 h in 5% acetic acid. Gels were destained in the presence of 0.5% $CuSO_4 \cdot 5H_2O$ to facilitate extraction of ampholytes.

Immunoblotting and Western Analyses

These were conducted on SDS-PAGE or on IEF gels of microtubules isolated by taxol stabilization. For SDS-PAGE (Laemelli, 1970) protein samples (50 μg/40 μl) were resolved on duplicate 8% polyacrylamide gels (0.75 mm thick). To optimize the electrophoretic transfer of proteins, dye-conjugated molecular weight standards (or naturally colored standards for IEF gels, both from Biorad) were included. The reference gels were fixed and stained as described above. The second gel was electroblotted immediately onto Immobilon P paper (Millipore, Bedford, MA) at 4°C, SDS-PAGE, or at 23°C (50 mA), for IEF gels, to prevent the crystallization of urea. Western analysis was performed using a method that relies on the presence of gelatin, BSA or Carnation® nonfat dry milk, and Tween-20 to prevent nonspecific protein interactions. The blotted paper was placed in buffer containing 0.2% gelatin, 0.05% Tween-20, and 1% Carnation [Tidball, 1987; blocking buffer] and washed for two 5 min periods with gentle agitation.

The primary antibody (either rabbit polyclonal anti-tubulin, or mouse monoclonal anti-alpha tubulin, ICN Biomedicals, Costa Mesa, CA) was diluted 1:500 in high salt Tris buffer (HST; 1 M NaCl, 0.5% Tween-20, 10 mM Tris-HCl, pH 7.4), and incubated with the filter for 18 h at 4°C with moderate agitation. Unbound antibody was removed by five sequential, 5 min washes with blocking buffer. Alkaline phosphatase conjugated secondary antibodies (Sigma) were incubated with the filters for 2 h at 23°C, followed by five additional washes, prior to reaction with substrate (BCIP and NBT, Bethesda Research Laboratories) according to the manufacturer's instructions.

RESULTS

Intestinal epithelial microtubule preparations (P_2) were electrophoretically compared to the original high speed supernatant (S_1) and post-microtubule supernatant (S_2) fractions. Similar analyses were also conducted on authentic chick brain tubulin. Figure 1A indicates that microtubule preparations from chick intestine contain two major bands (lanes 3, 6) of MW 52,000–55,000 corresponding to tubulin [Raybin and Flavin, 1975; Reddington et al., 1976] and ~40,000 which may represent actin [Putkey and Norman, 1983]. In contrast, brain microtubule preparations (lanes 9, 12) consist of a major band at MW 52,000–55,000 and a minor component at ~40,000. Differences in brain tubulin due to vitamin D status were not detectable upon SDS-PAGE.

Figure 1A further illustrates that microtubules prepared from vitamin D–deficient chick intestine after Ca^{2+} absorption (lane 3) were indistinguishable from those isolated from $1,25(OH)_2D_3$-treated chicks following Ca^{2+} absorption (lane 6). Likewise, SDS-PAGE failed to reveal differences in microtubules prepared from intestine of rachitic chicks dosed with vehicle (Fig. 1B, lane 3) or 1.3 nmols of $1,25(OH)_2D_3$ 10 h (lane 6) or 15 h (lane 9) prior to sacrifice. Moreover, the presence of Ca^{2+} transport (Fig. 1A, lane 3, −D chicks; lane 6, $+1,25(OH)_2D_3$ chicks) or the absence of absorption (Fig. 1B, lane 3, −D chicks; lane 9, $+1,25(OH)_2D_3$ chicks) had no effect on SDS-PAGE profiles.

Inspection of the supernatant fractions from which the microtubules were prepared revealed a corresponding band in brain homogenates (Fig. 1A, lanes 7, 10) that was depleted after the polymerization steps (Fig. 1A, lanes 8, 11). The amount of tubulin in supernatants of chick intestinal homogenates was miniscule by comparison, making analyses of recovery and enrichment difficult. However, since both co-precipitating bands were subjected to isoelectric focusing, a ratio of the proteins in the combined regions in the final pellet, relative to the corresponding initial supernatant bands, was estimated by laser densitometry.

As shown in Table I, vitamin D status did not influence the enrichment of tubulin, nor the co-precipitating MW 40,000 protein. In brain, the enrichment of tubulin was smaller because of higher initial levels in the supernatant. However, polymerization and taxol stabilization reduced the amount of the MW 40,000 protein in the final pellet, leading to a combined enrichment factor equivalent to that observed in preparations from intestinal tissue.

Western analysis was also performed on microtubule proteins isolated by taxol stabilization, using a commercially available polyclonal anti-

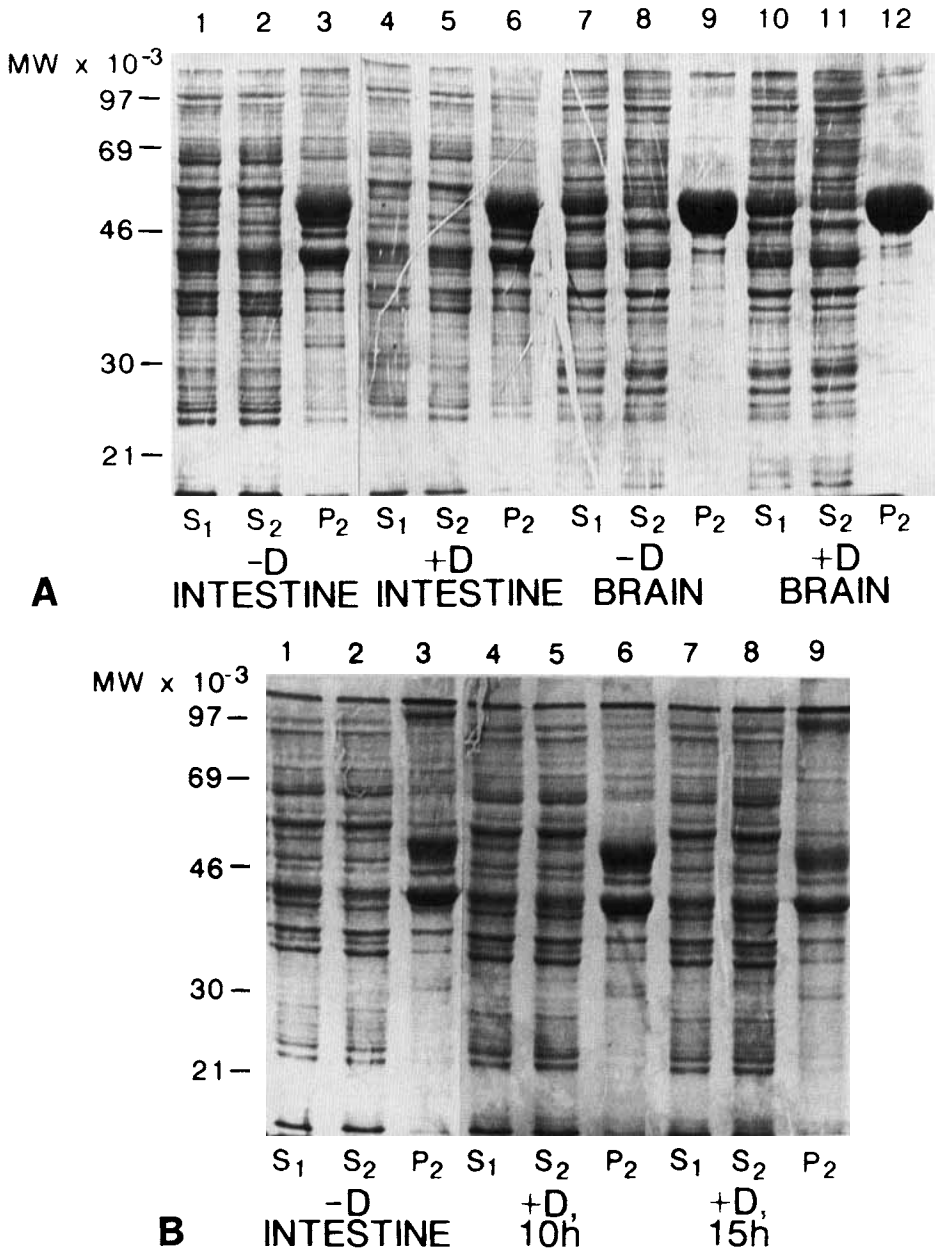

Fig. 1. Resolution of proteins in microtubules isolated from chick intestinal epithelium or brain by SDS-PAGE. **A:** High-speed supernatant fractions (S_1) were prepared from homogenates of intestinal mucosae of vitamin D–deficient (−D) or 1,25$(OH)_2D_3$-treated (+D) chicks after a 30 min Ca^{2+} absorption period. After incubation with GTP and taxol, further centrifugation yielded the post-microtubule supernatant (S_2) and microtubule pellet (P_2). Parallel fractions were prepared from brain tissue. **B:** Partial results of time course studies. Fractions were prepared from −D chicks dosed with vehicle or 1.3 nmoles of 1,25$(OH)_2D_3$ for 10 or 15 h prior to sacrifice. Each lane was loaded with 50 μg of protein.

tubulin antibody against chick brain tubulin. Reaction product from the alkaline phosphatase conjugated secondary antibody was apparent over the 2–3 predominant bands, and to a lesser extent, minor components (data not shown). Thus, traces of the 40,000 MW protein were present in the antigen preparation used to elicit the polyclonal antibody. No reaction was observed with molecular weight standards (unrelated proteins), or with primary antibody of different specificity.

Taxol-stabilized microtubules prepared from chick brain and intestinal epithelium were also compared by isoelectric focusing (Fig. 2). Al-

TABLE I. Enrichment of Proteins in Isolated Microtubules Relative to Initial Supernatants as Determined by Laser Densitometry of SDS-Polyacrylamide Gels

	P_2 Protein/S_1 Protein[a]			
Group	MW 52,000–55,000	MW 40,000	Combined	n
Intestine: −D	8.09 ± 2.71	2.24 ± 0.45	3.53 ± 0.18	3
+D, 10 h[b]	8.33 ± 0.20	2.86 ± 0.13	4.06 ± 0.21	2
+D, 15 h[b]	8.66 ± 2.04	2.49 ± 0.97	3.52 ± 1.09	2
Brain: −D	3.68	0.45	2.93	1
+D[b]	4.12	0.40	3.31	1

[a]Ratio of the areas under the curves determined by laser densitometry of Coomassie Blue–stained gels of microtubular protein (P_2) over the corresponding value in the initial supernatant fraction (S_1). Values represent mean ± SD.
[b]1.3 nmoles of 1,25(OH)$_2$D$_3$.

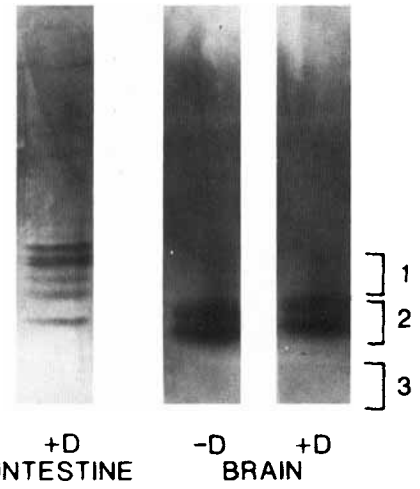

Fig. 2. Comparison of microtubule proteins isolated from +D chick intestine and −D, and +D chick brain by isoelectric focusing. Chicks were dosed with vehicle or 1.3 nmoles of 1,25(OH)$_2$D$_3$ 15 h prior to sacrifice. Solubilized microtubules (100 μg/lane) were resolved at 800 V for 18 h.

though resolution of the various brain tubulin isotypes was not possible under the conditions employed, comparison with an intestinal microtubule preparation indicated that the bands in region 2 were tubulin, whereas those in regions 1 and 3 were probably co-precipitating non-tubulin proteins. However, as indicated below, the consistency of the bands in region 1 provided a suitable internal control.

Time Course Studies

Intestinal epithelial microtubules were prepared as a function of time after 1.3 nmoles of 1,25(OH)$_2$D$_3$. SDS-PAGE analysis of the major bands in the microtubule pellet (P_2) relative to the initial supernatant (S_1) yielded enrichment factors of 3.64, 2.82, 3.39, 3.85, 2.43, and 4.35 and 0, 2.5, 5, 10, 15, and 43 h after seco-steroid hormone.

Further analysis of the effect of vitamin D status on tubulin was undertaken with the technique of isoelectric focusing. Figure 3 presents representative results of time course experiments. Vitamin D–deficient chicks were dosed with vehicle or 1.3 nmols of 1,25(OH)$_2$D$_3$ 2.5, 5, or 10 h prior to sacrifice (Fig. 3A), and 15 or 43 h prior to use (Fig. 3B). Isoelectric focusing of taxol-stabilized microtubules prepared from intestinal epithelium of such chicks yielded banding patterns with several regions of interest. Region 1 (Fig. 3) usually appeared as a triplet, and was believed to be a non-tubulin protein on the basis of comparison with brain tubulin (Fig. 2). Consistent vitamin D–mediated differences were not observed in this region. The more acidic protein bands evident in region 2 (Fig. 3A,B) were tentatively identified as tubulin on the basis of migration, and revealed increasing staining intensity as a function of time after seco-steroid, as did the bands in region 3. The more acidic protein bands were faint or absent in microtubules prepared from intestinal mucosa of vitamin D–deficient chicks, and chicks injected with 1,25(OH)$_2$D$_3$ 2.5 h prior to sacrifice. Augmented levels of these proteins were evident at 5, 10, and 15 h after seco-steroid, corresponding to times of enhanced intestinal calcium absorption, as well as at 43 h when calcium absorption has declined towards control levels [Nemere and Norman, 1988]. Similar qualitative results were observed in three independent experiments.

Densitometric analysis of the gels depicted in Figure 3 were undertaken, and values for each region normalized to the corresponding value for the lane containing proteins from −D chicks

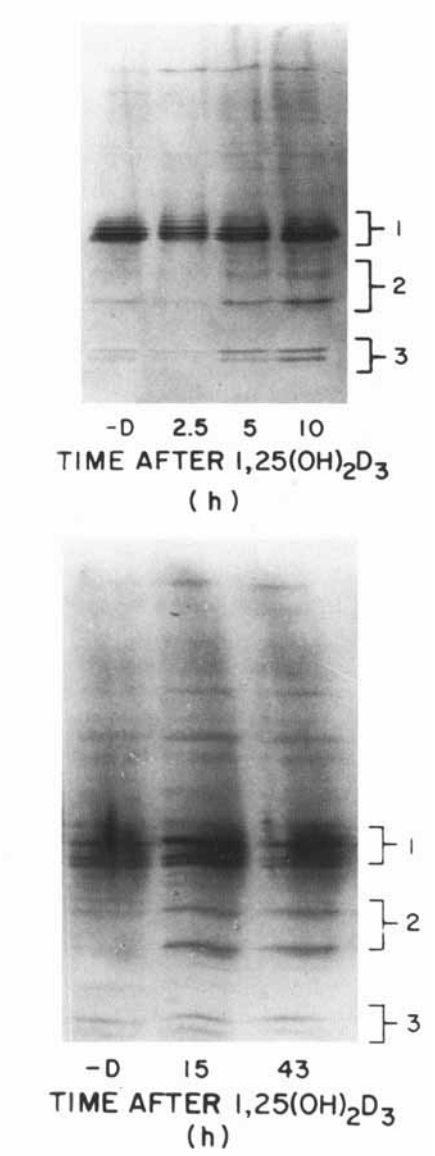

Fig. 3. Time course of $1,25(OH)_2D_3$ effect on microtubule proteins isolated from chick intestinal epithelium and separated by isoelectric focusing. Microtubules were prepared by taxol stabilization from chicks dosed with **A:** vehicle (−D) or 1.3 nmoles of $1,25(OH)_2D_3$ 2.5, 5, 10 h before sacrifice. Samples were focused at 400 V for 18 h. **B:** In a separate series of experiments, the time course was extended to include 15 and 43 h of exposure to $1,25(OH)_2D_3$ in vivo. Samples were focused for 18 h at 800 V.

within the same gel. At 2.5, 5, 10, 15, and 43 h after 1.3 nmols of $1,25(OH)_2D_3$ the +D/−D ratios for region 1 were 0.43, 0.92, 1.28, 1.01, 1.04, respectively; for region 2 the ratios were 0.59, 2.02, 3.20, 1.41, 2.21, respectively; for region 3 the ratios were 0, 2.46, 2.64, 1.43, 1.67, respectively. The unusually low +D/−D ratio for region 1 2.5 h after $1,25(OH)_2D_3$ suggested that less of the 40,000 MW protein was present and this was confirmed by SDS-PAGE (data not shown). However, since equivalent amounts of protein, as judged by the Bradford assay, were loaded in each lane, the bulk of the material may have had pIs outside of the gradient range. The observation that region 1 ratios were mainly close to 1.0 suggested that $1,25(OH)_2D_3$-mediated increases in proteins of region 2 and 3 were not due to unequal loading of material. The reproducibility of the +D/−D ratios (mean ± SD, $n = 2$) for microtubules prepared 10 h after $1,25(OH)_2D_3$ was 1.18 ± 0.10 (region 1), 3.07 ± 0.13 (region 2), and 2.74 ± 0.01 (region 3). For microtubules prepared 15 h after seco-steroid, the ratios were 1.10 ± 0.9 (region 1), 2.02 ± 0.61 (region 2) and 1.65 ± 0.22 (region 3).

Dose-Response Studies

Taxol-stabilized microtubules from chick intestinal epithelium were also evaluated as a function of increasing doses of $1,25(OH)_2D_3$. Enrichment factors (P_2/S_1) determined from SDS-PAGE were 3.17, 3.25, 3.31, 2.65, 4.27, and 3.61, respectively, for microtubules prepared from chicks dosed with vehicle or 10, 52, 260, 1,300, or 6,500 pmoles of $1,25(OH)_2D_3$ 10 h prior to sacrifice. Following isoelectric focusing the bands in region 1 (Fig. 4) showed no effect of vitamin D status: +D/−D ratios obtained by densitometry were 1.03, 1.16, 1.21, 1.08, and 1.61 for 10, 52, 260, 1,300, and 6,500 pmoles, respectively. The bands in regions 2 and 3 re-

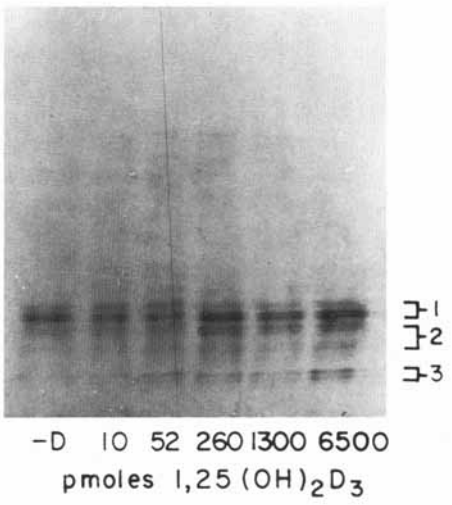

Fig. 4. Effect of increasing doses of $1,25(OH)_2D_3$ on microtubule proteins isolated from chick intestinal epithelium and separated by isoelectric focusing. Chicks were dosed with vehicle or 10, 52, 260, 1,300, or 6,500 pmoles of $1,25(OH)_2D_3$ 10 h prior to sacrifice. Samples were focused for 18 h at 800 V.

vealed a faint presence in microtubules prepared from vitamin D–deficient chicks and birds dosed with 52 pmoles of seco-steroid hormone. An increased intensity of staining was evident between 260 and 6,500 pmoles of 1,25(OH)$_2$D$_3$, corresponding to doses known to produce optimal stimulation of net intestinal calcium transport. The +D/−D ratios for region 2 were 2.05, 2.76, 3.18, 2.94, and 6.12, respectively, for microtubules prepared after 10, 52, 260, 1,300, and 6,500 pmoles of 1,25(OH)$_2$D$_3$. In region 3, the same doses resulted in +D/−D ratios of 1.33, 2.50, 3.17, 2.83, and 5.00.

Effect of Calcium Transport

Since one of the major effects of vitamin D–repletion in rachitic animals is a marked increase in intestinal calcium absorption, experiments were undertaken to determine whether tubulins were altered by conditions known to promote net transport of the cation. Briefly, chicks were dosed with vehicle or 1.3 nmoles of 1,25(OH)$_2$D$_3$ 15 h prior to experimentation, and fasted during this time interval. Under ether anesthesia, the exposed duodenal loop was ligated, and intraluminally injected with water (no transport) or $CaCl_2$ in water. After 30 min of transport, microtubules were prepared as described above, but with buffer containing 1 mM EGTA. Enrichment factors for microtubular proteins relative to starting supernatants (P_2/S_1) were 3.77, 3.96, 4.61, and 5.38 for preparations from vitamin –deficient chicks without absorption (−DØ), vitamin D–deficient chicks after absorption (−D+Ca^{2+}), and 1,25(OH)$_2$D$_3$-treated chicks without absorption (+DØ), and 1,25(OH)$_2$D$_3$-treated chicks after Ca^{2+} absorption (+D, +Ca^{2+}), respectively. When IEF gels were run as shown in Figure 5, calcium transport in vitamin D–deficient chicks yielded an enhanced staining of bands in regions 2 and 3, relative to non-transporting rachitic controls, and comparable to the intensity of equivalent protein bands in non-transporting, 1,25(OH)$_2$D$_3$-treated chick intestinal microtubules. By comparison, calcium transport in birds dosed with the seco-steroid hormone resulted in fainter bands in regions 2 and 3 (Fig. 5). For region 1, the +D/−D ratios in the IEF gel were 1.35, 1.19, and 1.25 for −D +Ca^{2+}, +DØ, and +D +Ca^{2+}, respectively. The same experimental conditions yielded region 2 ratios of 3.27, 3.00, and 2.64, and region 3 ratios of 1.00, 1.87 and 1.31.

The results obtained from absorption studies prompted an investigation into whether changes

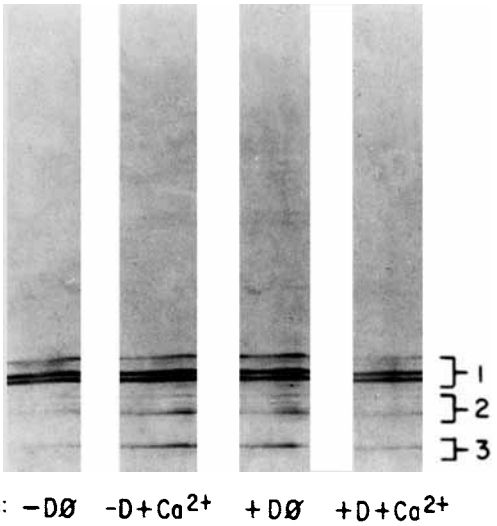

Fig. 5. Effect of vitamin D status and calcium transport on microtubule proteins isolated from chick intestinal epithelium and separated by isoelectric focusing. Vitamin D–deficient chicks (3–4 per group) were dosed with vehicle (−D) or 1.3 nmoles of 1,25(OH)$_2$D$_3$ (+D) 15 h prior to transport studies. Under ether anesthesia, the duodenal loop was surgically exposed and the lumen injected with 0.4 ml water (θ transport) or an equivalent volume containing $CaCl_2$ (+Ca^{2+}). Transport was allowed to proceed for 30 min prior to removal of the duodenal loop and preparation of taxol-stabilized microtubules in the presence of 1 mM EGTA. Samples were focused at 800 V for 18 h.

in band appearance in isoelectric focusing gels presumed to be due to vitamin D status were actually the result of enhanced food intake (calcium transport). Microtubules were prepared from fasting or fed vitamin D–deficient chicks, as well as from fasting or fed 1,25(OH)$_2$D$_3$-treated birds. Resolution of the proteins on isoelectric focusing gels indicated that changes mediated by vitamin D were predominant, with only faintly discernible alterations due to food intake (data not shown).

Immunoblot Analyses

In an attempt to identify the vitamin D–regulated proteins discerned upon isoelectric focusing, gels were electroblotted and probed with anti-tubulin antibodies. The left lane in Figure 6 shows a Coomassie Blue–stained sample on the replicate gel, the center lane depicts the results of analyses with polyclonal anti-tubulin, and the right lane the staining observed with monoclonal anti-α-tubulin. The polyclonal antibody was found to recognize bands within regions 1 and 2, but not 3, whereas the anti-α-tubulin antibody reacted with a single band in region 2.

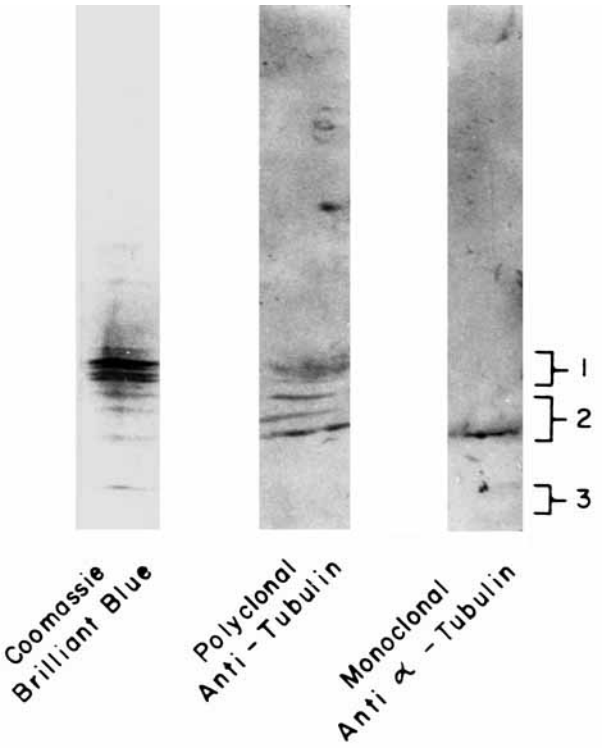

Fig. 6. Western analysis of microtubule proteins isolated from +D chicks and separated by isoelectric focusing. **Left lane**: Coomassie Blue–stained bands. **Middle lane**: Immunoblot probed with polyclonal anti-tubulin. **Right lane**: Immunoblot probed with monoclonal antiα-tubulin.

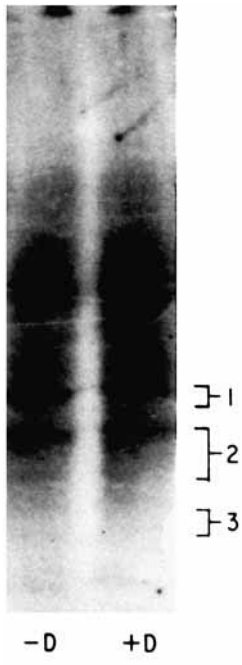

Fig. 7. Incorporation of ^{32}P into microtubule proteins of chick intestinal epithelium in vivo. Vitamin D–deficient chicks (3 per group) were dosed with vehicle (−D) or 1.3 nmoles of $1,25(OH)_2D_3$ 15 h prior to ether anesthesia and surgical exposure of the duodenal loop. Each loop was injected with 0.8 mCi of ^{32}P in water and labelling allowed to proceed for 30 min *in vivo* prior to preparation of microtubules and resolution on isoelectric focusing gels for autoradiography.

Phosphorylation Studies

One of the better known post-translational modifications of tubulin is phosphorylation [Reddington et al., 1976; Eipper, 1972; Diaz-Nido et al., 1990]. In brain slices, both α- and β-tubulin are phosphorylated [Reddington et al., 1976], whereas only β-tubulin is phosphorylated in vivo [Diaz-Nido et al., 1990]. Also, many phosphorylation events are under hormonal regulation. Thus, an assessment of vitamin D status and phosphorylation was undertaken in scraped mucosae, isolated microtubules, and in vivo. Figure 7 illustrates the autoradiogram of microtubules isolated after phosphorylation in vivo and resolution on isoelectric focusing gels. Bands in region 1 were phosphorylated under all conditions tested and revealed a $1,25(OH)_2D_3$-induced alteration in phosphate content (see below). The upper band of region 2 was also phosphorylated in vivo and in isolated microtubules, but not in microtubules isolated from scraped mucosae incubated with ^{32}P in vitro.

The autoradiograms and Coomassie Blue–stained gels were subjected to laser densitometry, and the results for bands of region 1 analyzed (Table II). In each of the three test situations, $1,25(OH)_2D_3$ caused a decrease in the level of ^{32}P incorporated per mg protein in bands of region 1, achieving values of 54–70% of controls.

Effect of Dexamethasone

Since glucocorticoids are known to suppress vitamin D–stimulated intestinal calcium transport and inhibit the synthesis of calbindin-D_{28K}, the effect of a 48 h treatment with dexamethasone on microtubule proteins was studied. In three such experiments, glucocorticoid treatment was observed to result in reduced microtubule yields and an absence of the usual bands on isoelectric focusing gels. This prompted an analysis of the sample composition by SDS-PAGE (Fig. 8). Although each lane was loaded with the equivalent of 50 μg of Lowry- or Bradford-reactive material, samples prepared from intestinal epithelium of chicks treated with dexamethasone revealed a profound decrease in proteins with molecular weights greater than 15,000 regardless of vitamin D status. Microtu-

TABLE II. Incorporation of ^{32}P Into Microtubules of Chick Intestinal Epithelium*

Labelling conditions	D status[a]	^{32}P	Protein	^{32}P/protein	Control (%)
Scraped mucosae	−	0.76	2.94	0.26	
	+	0.45	3.24	0.14	54
Isolated microtubules	−	4.19	6.64	1.59	
	+	6.69	7.54	1.12	70
In vivo	−	3.14	3.50	1.12	
	+	2.68	2.11	0.78	70

*Tissue was labelled with ^{32}P and isolated microtubules with γ^{32}P-ATP for 30 min, the final microtubule pellet prepared, and dissolved in sample buffer for isoelectric focusing (200 μg per lane). The stained gels and autoradiograms were scanned with a laser densitometer for quantification. Values represent areas under peaks, or the ratio of such areas to yield an estimate of specific activity.

[a]Vitamin D–deficient chicks were dosed with vehicle or 1.3 nmoles of 1,25(OH)$_2$D$_3$ 15 h prior to experimentation. Each group consisted of 3–4 chicks.

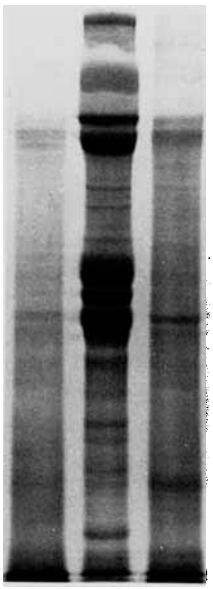

Fig. 8. Effect of dexamethasone in vivo on microtubules isolated from vitamin D–deficient (−D) and 1,25(OH)$_2$D$_3$-dosed (+D) chick intestinal epithelium. Proteins were resolved on 8% polyacrylamide gels under denaturing conditions.

bular proteins from vitamin D–deficient chicks gave the usual banding patterns (data not shown).

DISCUSSION

Earlier work [Nemere et al., 1987] has documented a 1,25(OH)$_2$D$_3$-induced change in α-tubulin mRNA expression. The current study extends these observations by monitoring secosteroid–mediated alterations in the tubulin proteins by gel electrophoresis.

Isolation of intestinal epithelial microtubules by taxol stabilization yields a pellet enriched in proteins not only of MW 52,000–55,000, but also proteins of MW ~40,000. Vitamin D status apparently had no effect on enrichment, as judged by SDS-PAGE, although this is difficult to quantitate due to the exceedingly low levels of the 52,000–55,000 MW proteins in the starting supernatant fractions. Thus, it cannot be conclusively determined whether differences observed upon isoelectric focusing are attributable to vitamin D–mediated differences in isotype levels, or whether all isoforms are available, but secosteroid–altered changes in the intracellular environment result in differential polymerization and recovery.

In contrast to the absence of vitamin D–mediated effects evident by SDS-PAGE, band differences were discernible by IEF. A number of these proteins are non-tubulin in nature, as judged by comparing the bands with isoelectrically focused authentic brain tubulin. However, the levels of the most alkaline (region 1) bands served as valuable internal controls. Since the +D/−D ratios of these bands were always close to 1.0, the changes in regions 2 and 3 were not due to differential loading of proteins. A notable exception was found during the time course studies in that all visualized proteins in samples prepared 2.5 h after 1,25(OH)$_2$D$_3$ were reduced. Although the reason for this is unknown, the phenomenon was observed in 3 out of 3 experiments. Treatment of rachitic chicks with 1,25(OH)$_2$D$_3$ was found to cause an increase staining intensity of bands in regions 2 and 3 in isoelectric focusing gels of microtubules isolated at 5–43 h after hormone. These findings are noteworthy within the context of net intestinal calcium transport and vesicular calcium content. At 2.5 h after 1,25(OH)$_2$D$_3$ administration to the chick, net transport as judged by serum

$^{45}Ca^{2+}$ is not augmented, nor is the level of the radionuclide in lysosomes enhanced relative to controls [Nemere and Norman, 1988]. A gradual increase in both parameters is detectable at 5 h, with maximal transport and lysosomal content of $^{45}Ca^{2+}$ at 10–15 h, and a decline to control levels 43 h after hormone [Nemere and Norman, 1988]. By comparison, radionuclide specific activity in putative endocytic vesicles was enhanced relative to controls at 2.5 h, and maximally elevated by 5 h [Nemere and Norman, 1988]. Despite the early effect on endocytic vesicles, it was postulated that transfer of contents to lysosomes and hence stimulation of net transport did not occur due to lack of appropriate microtubule "tracks." The current work indicates that 5 h after $1,25(OH)_2D_3$ the composition of intestinal epithelial microtubules is indeed altered.

Another discontinuity was noted in dose-response studies where lysosomal $^{45}Ca^{2+}$ specific activity was fully stimulated at the lowest dose of $1,25(OH)_2D_3$ tested, yet net transport was only fully stimulated at doses greater that 52 pmoles of seco-steroid hormone [Nemere and Norman, 1989]. In the present study, microtubule protein profiles are gradually altered over the same range of doses.

Immunofluorescence microscopy [Nemere, Leathers, Thompson, Luben, and Norman, manuscript, in press] has revealed that tubulin staining intensity not only increases with vitamin D repletion, but is also affected by the physiological process of calcium absorption. Briefly, it has been found that 30 min of calcium uptake in intestinal tissue prepared from vitamin D–deficient chicks resulted in greater tubulin immunofluorescence than in sections prepared in the absence of calcium transport. In $1,25(OH)_2D_3$-treated chicks, however, calcium transport was found to diminish tubulin staining relative to sections prepared in the absence of absorption. Isoelectric focusing gels of microtubules prepared under equivalent conditions essentially agree with these findings.

The identity of the microtubule protein bands has been more difficult to assess. Although a number of such bands reacted with polyclonal anti-tubulin upon Western analysis, only one of the $1,25(OH)_2D_3$-sensitive protein components was found to react with monoclonal anti-α-tubulin. There are a number of explanations for this observation, two of which involve the limitations of the technology. Since electroblotting depends on size rather than isoelectric point, many of the finer bands could have been over-transferred. Currently available protein staining techniques are not sensitive enough to detect the fine bands formed during isoelectric focusing after transfer to membranes. In addition, the monoclonal antibody may not have recognized various isoforms. Alternatively, the unreactive bands are not tubulin. This is presumed to be true of region 1 and 3 bands, as discussed above. The more alkaline bands that are tentatively identified as non-tubulin on the basis of migration were also found to be substrates for phosphorylation in vitro and in vivo. Although this modification did not appear to produce the more acidic bands, a portion of the phosphorylation events are under hormonal control, possibly either through decreased kinase activity or increased phosphatase activity.

Finally, glucocorticoids, which inhibit intestinal calcium transport, are thought to exert their action through inhibition of CaBP synthesis. From the present work, however, it is also clear that the glucocorticoid dexamethasone inhibits tubulin synthesis. It remains to be determined whether the two phenomena are linked by regulatory mechanisms, or whether they represent a more generalized inhibition or indeed a pathophysiological response to the high glucocorticoid levels employed.

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REFERENCES

Cleveland DW, Sullivan KF: Molecular biology and genetics of tubulin. Ann Rev Biochem 54:331–365, 1985.

Diaz-Nido J, Serrano L, Lopez-Otin C, Vandekerckhove J, Avila J: Phosphorylation of a neuronal-specific β-tubulin isotype. J Biol Chem 265:13949–13954, 1990.

Dunbar BS: Two-Dimensional Electrophoresis and Immunological Techniques. Plenum Press, New York, 1987.

Eipper BA: Rat brain microtubule protein: Purification and determination of covalently bound phosphate and carbohydrate. Proc Natl Acad Sci USA 69:2283–2287, 1972.

Field DJ, Collins RA, Lee JC: Heterogeneity of vertebrate brain tubulins. Proc Natl Acad Sci USA 81:4041–4045, 1984.

Laemelli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685, 1970.

Lewis SA, Lee MG-S, Cowan NJ: Five mouse tubulin isotypes and their regulated expression during development. J Cell Biol 101:852–861, 1985.

Matteoni R, Kreis TE: Translocation and clustering of endosomes and lysosomes depends on microtubules. J Cell Biol 105:1253–1265, 1987.

Miller FD, Naus CC, Durand M, Bloom FE, Milner RJ: Isotypes of α-tubulin are differentially regulated during neuronal maturation. J Cell Biol 105:3065–3073, 1987.

Nassar CF, Khairallah EW, Nasser MG, Habbal ZM: Cytoskeletal control of calcium absorption across the rat small intestine. Comp Biochem Physiol 89A:197–201, 1988.

Nemere I, Leathers VL, Norman AW: 1,25-Dihydroxyvitamin D$_3$-mediated calcium transport across the intestine: Biochemical identification of lysosomes containing calcium and the calcium binding protein (calbindin-D$_{28K}$) J Biol Chem 261,:16106–16114, 1986.

Nemere I, Norman AW: 1,25-Dihydroxyvitamin D$_3$-mediated vesicular transport of calcium in intestine: Time course studies. Endocrinology 122:2962–2969, 1988.

Nemere I, Norman AW: 1,25-Dihydroxyvitamin D$_3$-mediated vesicular transport of calcium in intestine: Dose-response studies. Molec Cell Endocrinol 67:47–53, 1989.

Nemere I, Theofan G, Norman AW: 1,25-Dihydroxyvitamin D$_3$ regulates tubulin expression in chick intestine. Biochem Biophys Res Commun 148:1270–1276, 1987.

Nemere I, Yoshimoto Y, Norman AW: Calcium transport in perfused duodena from normal chicks: Enhancement within 14 minutes of exposure to 1,25-dihydroxyvitamin D$_3$. Endocrinology 115:1476–1483, 1984.

Norman AW, Wong RG: The biological activity of the vitamin D metabolite 1,25-dihydroxycholecalciferol in chickens and rats. J Nutr 102:1709–1718, 1972.

Putkey JA, Norman AW: Studies on the mode of action of calciferol: XLV. Vitamin D: Its effect on the protein composition and core material structure of the chick intestinal brush border membrane. J Biol Chem 258:8971–8978, 1983.

Ravindra R, Grosvenor: Microtubules are mobilized in the lactating rat anterior pituitary gland during suckling. Endocrinology 118:1194–1199, 1986.

Raybin D, Flavin M: An enzyme tyrosulating α-tubulin and its role in microtubule assembly. Biochem Biophys Res Commun 65:1088–1095, 1975.

Reddington M, Tan LP, Lagnado JR: The phosphorylation of brain microtubular proteins *in situ* and *in vitro*. J Neurochem 27:1229–1236, 1976.

Shultz TD, Bollman S, Kumar R: Decreased intestinal calcium absorption *in vivo* and normal brush border membrane vesicle. Proc Natl Acad Sci USA 79:3542–3546, 1982.

Tidball JR: α-Actinin is absent from the terminal segments of myofibrils and from subsarcolemmal densities in frog skeletal muscle. Exp Cell Res 170:469–482, 1987.

Vallee RB: A taxol-dependent procedure for the isolation of microtubules and microtubule-associated proteins (MAPS) J Cell Biol 92:435–442, 1982.