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Effects of 24R,25- and 1α,25-Dihydroxyvitamin D_3 on Mineralizing Growth Plate Chondrocytes

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Abstract Time- and dosage-dependent effects of $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ on primary cultures of pre- and post-confluent avian growth plate (GP) chondrocytes were examined. Cultures were grown in either a *serum-containing* culture medium designed to closely mimic normal GP extracellular fluid (DATP5) or a commercially available *serum-free* media (HL-1) frequently used for studying skeletal cells. Hoechst DNA, Lowry protein, proteoglycan (PG), lactate dehydrogenase (LDH), and alkaline phosphatase (ALP) activity and calcium and phosphate mineral deposition in the extracellular matrix were measured. In preconfluent cultures grown in DATP5, physiological levels of $24,25(OH)_2D_3$ (0.10–10 nM) increased DNA, protein, and LDH activity significantly more than did $1,25(OH)_2D_3$ (0.01–1.0 nM). However, in HL-1, the reverse was true. Determining ratios of LDH and PG to DNA, protein, and each other, revealed that $1,25(OH)_2D_3$ specifically increased PG, whereas $24,25(OH)_2D_3$ increased LDH. Post-confluent cells were generally less responsive, especially to $24,25(OH)_2D_3$. The positive anabolic effects of $24,25(OH)_2D_3$ required *serum-containing* GP-fluid-like culture medium. In contrast, effects of $1,25(OH)_2D_3$ were most apparent in *serum-free* medium, but were still significant in *serum-containing* media. Administered to preconfluent cells in DATP5, $1,25(OH)_2D_3$ caused rapid, powerful, dosage-dependent inhibition of Ca^{2+} and Pi deposition. The lowest level tested (0.01 nM) caused >70% inhibition during the initial stages of mineral deposition; higher levels of $1,25(OH)_2D_3$ caused progressively more profound and persistent reductions. In contrast, $24,25(OH)_2D_3$ increased mineral deposition 20–50%; it required >1 week, but the effects were specific, persistent, and largely dosage-independent. From a physiological perspective, these effects can be explained as follows: $1,25(OH)_2D_3$ levels rise in hypocalcemia; it stimulates gut absorption and releases Ca^{2+} from bone to correct this deficiency. We now show that $1,25(OH)_2D_3$ also conserves Ca^{2+} by inhibiting mineralization. The slow anabolic effects of $24,25(OH)_2D_3$are consistent with its production under eucalcemic conditions which enable bone formation. These findings, which implicate serum-binding proteins and accumulation of PG in modulating accessibility of the metabolites to GP chondrocytes, also help explain some discrepancies previously reported in the literature. J. Cell. Biochem. 98: 309–334, 2006. © 2006 Wiley-Liss, Inc.

Key words: 1,25-dihydroxyvitamin D_3; 24,25-dihydroxyvitamin D_3; growth plate chondrocytes; proteoglycans; lactate dehydrogenase; calcium and phosphate deposition; alkaline phosphatase

Vitamin D metabolites are known to be critical for normal calcium metabolism. Vitamin D_3 deficiency leads to a failure in the absorption of Ca^{2+} [Wasserman, 1964; Harris et al., 1965], which in growing animals leads to rickets, a failure of growth plate (GP) calcification. Under conditions of low blood Ca^{2+}, metabolism of $25(OH)D_3$ to $1,25(OH)_2D_3$ by kidney stimulates absorption of Ca^{2+} by the gut [Holick and DeLuca, 1974] and during prolonged Ca^{2+} deficiency causes release of Ca^{2+} from bone [Chambers, 1988]. Under conditions of eucalcemia, synthesis of $24,25(OH)_2D_3$ is increased and synthesis of $1,25(OH)_2D_3$ is reduced [Henry et al., 1976]. Thus, elevated levels of circulating $24,25(OH)_2D_3$ are a signal of Ca^{2+} sufficiency (eucalcemia), whereas increased $1,25(OH)_2D_3$ indicates Ca^{2+} deficiency (hypocalcemia) [DeLuca and Schnoes, 1976]. $1,25(OH)_2D_3$

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is considered to be "active," whereas 24,25 $(OH)_2D_3$ is commonly thought to be "inactive," but few studies have tested their direct effects on mineralization under defined conditions where Ca^{2+} and Pi, and the metabolite levels were controlled.

Both dihydroxyvitamin D_3 metabolites can be produced extra-renally. Schwartz et al. [1992] showed that rat costal chondrocytes and early growth-zone cells metabolize $[^3H]$-25$(OH)D_3$ to 1,25$(OH)_2D_3$ and 24,25$(OH)_2D_3$. They found that 1,25$(OH)_2D_3$ stimulates 24R-hydroxylase activity. Similarly, Seo et al. [1996] have shown that both dihydroxylated D_3 metabolites accumulate in GP cartilage when rats are injected with $[^3H]$-25$(OH)D_3$. These findings indicate that vitamin D metabolites may be important local regulators that contribute to the physiological development of the GP.

The purpose of this study was to determine the direct effects of the two principal dihydroxyvitamin D metabolites on the differentiation and mineralization of GP chondrocytes. That regulation of GP chondrocytes by these vitamin D_3 metabolites is not straight-forward is evident from inconsistencies reported on the activity of each. For example, 1,25$(OH)_2D_3$ has been shown in some studies to increase ALP activity [Manolagas et al., 1981; Majeska and Rodan, 1982; Schwartz et al., 1988a; Gerstenfeld et al., 1990; Farquharson et al., 1993] and in others to decrease it [Hale et al., 1986; Inao and Conrad, 1992]. Collagen synthesis also has been shown to be variably affected [Hinek and Poole, 1988; Gerstenfeld et al., 1990]. Although often regarded as inactive [DeLuca, 1988; Reichel et al., 1989; Holick, 1996], it has become apparent from several reports [Sebert et al., 1982; Nakamura et al., 1987, 1992; Schwartz et al., 1989] that 24,25-$(OH)_2D_3$ has distinct effects on cartilage, in particular on resting zone cells [Schwartz et al., 1995]. Factors in these apparent discrepancies appeared to be differences in culture media, source of the chondrocytes, timing of exposure, as well as levels of the metabolites studied. To clarify their physiological effects, we studied each of these variables using two different culture systems.

Based on the authors' previous analyses of electrolytes [Wuthier, 1969, 1971, 1977], amino acids [Ishikawa et al., 1985] and other constituents, a culture media was constructed that closely matches GP extracellular fluid. This culture medium, DATP5, fully supports proliferation, differentiation, and mineralization of primary cultures of GP chondrocytes isolated from rapidly growing broiler-strain chickens [Ishikawa et al., 1986; Wu et al., 1989, 1995; Ishikawa and Wuthier, 1992]. Of special importance are supplemental ascorbate [Wu et al., 1989], specific amino acids [Ishikawa et al., 1986], and inorganic phosphate [Ishikawa and Wuthier, 1992], although other factors present in serum are also known to be important. This *serum-containing* medium consistently supports calcification in the absence of organic phosphate supplements. As a control, we also used a standard commercially available *serum-free* culture medium (HL-1), which contains β-glycerophosphate and is also supplemented with ascorbate [Wu et al., 1989].

In the present studies, we investigated the effects of two major vitamin D_3 metabolites: 1,25$(OH)_2D_3$ and 24,25$(OH)_2D_3$ on chicken GP chondrocytes using these *serum-containing* and *serum-free* culture media. Since previous studies had indicated that the response of GP chondrocytes is stage-dependent [Schwartz et al., 1995], the metabolites were administered first to either pre- or post-confluent cells for various time periods. The metabolites were administered first to either pre- or post-confluent cells for various time periods, and studied at various levels ranging from deficient to physiological to supra-physiological. We report here the effects of 1,25$(OH)_2D_3$ and 24,25 $(OH)_2D_3$ on basic tissue parameters including DNA, general protein, proteoglycans (PG), lactate dehydrogenase (LDH), and alkaline phosphatase (ALP) activity, and mineralization.

MATERIALS AND METHODS

Materials

Vitamin D_3 metabolites, 1,25$(OH)_2D_3$ and 24,25$(OH)_2D_3$ were from Hoffman-La Roche, Inc.

Cell Culture

Epiphyseal growth plates were dissected from the tibiae of a large number of 6–8-week-old hybrid broiler-strain chickens; these were pooled and the chondrocytes isolated as previously described [Wu et al., 1995]. For these studies, the isolated cells were distributed into 320 identical primary cultures grown in 35-mm dishes in DMEM (2 ml per dish) with 10% fetal bovine serum for the first 3–4 days. Culture

media were changed and a supplement of fresh ascorbic acid (25–50 μg/ml) added every 3–4 days for the duration of the experiments. From day 7 onward for 160 of the cultures, the medium was changed to DATP5 mineralization medium [Wu et al., 1995]. To the other 160 dishes on day 7, the medium was changed to DMEM:HL-1 (1:1) and from day 10 onward, to HL-1 *serum-free* medium (Biowhittaker, Walkersville, MD). HL-1 chemically defined proprietary media contains transferrin, testosterone, sodium selenite, ethanolamine, saturated and unsaturated fatty acids, and stabilizing proteins (less than 30 μg/ml) and is supplemented with 10 mM β-glycerophosphate. DATP5 medium was prepared from DMEM basal medium by addition of eight amino acids [Ishikawa et al., 1985], insulin (5 μg/ml)-transferrin (5 μg/ml)-selenite (5 ng/ml), and 5% defined FBS; it had a total Pi level of 1.9 mM (0.9 mM from DMEM + 1 mM added Na_2HPO_4) and Ca^{2+} of 1.8 mM [Ishikawa and Wuthier, 1992]. All culture media contained 100 IU/ml of penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B.

Treatment Protocols

Administration of the vitamin D_3 metabolites was initiated either before the cells attained confluence (Day 7, pre-confluent tests), or after they had become confluent (Day 14, post-confluent tests), and continued as indicated. Stock solutions (2 mM) of the vitamin D_3 metabolites were prepared and further diluted in ethanol so that 2 μl of the test solution was added to 2 ml of culture medium.

Biochemical Assays

To analyze biochemical activities, cells were harvested from the 35-mm dishes as previously described [Wu et al., 1995] in 1 ml TMT buffer (50 mM Tris, pH 7.5, 0.5 mM $MgCl_2$, 0.05% Triton X-100), the samples being sonicated in a water-bath. Aliquots were taken from each sample for analysis of the following seven parameters: ALP and LDH activities, Hoechst DNA, Lowry protein, PG and Ca^{2+}, and Pi mineral content as previously described [Ishikawa et al., 1998]. In brief, ALP activity was assayed at 37°C in 750 mM AMP buffer, pH 10.3, containing 1 mM *p*-NPP and 0.25 mM $MgCl_2$ by monitoring the formation of *p*-nitrophenol for 510 min. ALP units were expressed as nanomoles of *p*-NPP hydrolyzed per minute, based on the extinction coefficient of *p*-nitrophenol of 18,450/M × cm [Tietz et al., 1983]. Cellular and matrix protein content was analyzed using the method of Lowry et al. [1951]. Bovine serum albumin was used as a standard. PG content of the cell matrix was analyzed using the dimethylmethylene blue reagent [Chandrasekhar et al., 1987] measuring the absorbency difference at 520 and 589 nm. The assay is based on the ability of the sulfated glycosaminoglycans to bind to the cationic dye, dimethylmethylene blue, in solution. DNA content of TMT sonicates was assayed fluorometrically in Millipore F plates after cell lysis by freezing in distilled water then mixing with Hoechst 33258 [Rago et al., 1990]. Hoechst 33258 is a bisbenzimide DNA intercalator that excites in the near UV (350 nm) and emits in the blue region (450 nm). Hoechst 33258 binds to the AT-rich regions of double stranded DNA and exhibits enhanced fluorescence under high ionic strength conditions. This method allows for determining DNA and is not affected by contaminating protein or RNA. For determination of LDH activity and mineral (calcium and phosphate) content, the TMT sonicates were centrifuged for 1 h to separate insoluble proteins and minerals. LDH activity in the TMT supernatant was assayed at 37°C in 0.2 M Tris buffer, pH 7.5, containing 0.2% Triton X-100, 0.15 mM NADH, and 1 mM sodium pyruvate. To extract mineral ions, 0.1 N HCl was first added to the culture dish, and then a precalculated aliquot was transferred to the sediment in the centrifuge tube equivalent to 1 ml/dish. After vortexing and standing at room temperature for 1 h, the tubes were centrifuged and the clear supernatants were separated for Pi and Ca^{2+} analyses. Pi concentration was determined spectrophotometrically at 820 nm using a modification of the ammonium molybdate method of Ames [1966]. Calcium was measured colorimetrically using a modification of the *O*-cresolpthalein complexone microdetermination method of Baginski et al. [1973]. Calcium reacts with *O*-cresolphthalein complexone in the presence of 8-hydroxyquinoline to form a purple chromophore. The intensity of the final color reaction is proportional to the amount of calcium in the sample.

Thus within each sample, the seven parameters were analyzed and subsequently compared. Overall statistical analysis of differences between the various treatment parameters was performed using ANOVA with post-hoc Tukey

HSD multiple comparisons. For specific comparisons, differences were analyzed using Student's *t*-test. Results are presented as the mean ± standard error of the mean.

RESULTS

DNA and Protein Content

Total DNA, measured by the Hoechst method, provides a measure of the extent of cell replication. Total protein, measured using the Lowry method, gives a broad measure of cell growth and general matrix synthesis, a picture of the general viability of the cultures. Effects on these parameters formed the basis for assessing the selectivity of the effects of the two vitamin D metabolites on more cartilage-specific entities.

Pre-confluent cultures. Visually the cells appeared to reach confluence by Day 14 in both *serum-containing* DATP5 and *serum-free* HL-1 media. This is a consistent feature of these cultures [Wu et al., 1989, 1995]. In both cultures, DNA and protein levels continued to increase throughout the culture period (Figs. 1 and 2) beyond the confluency stage. In control, *serum-free* HL-1 medium, DNA, and protein

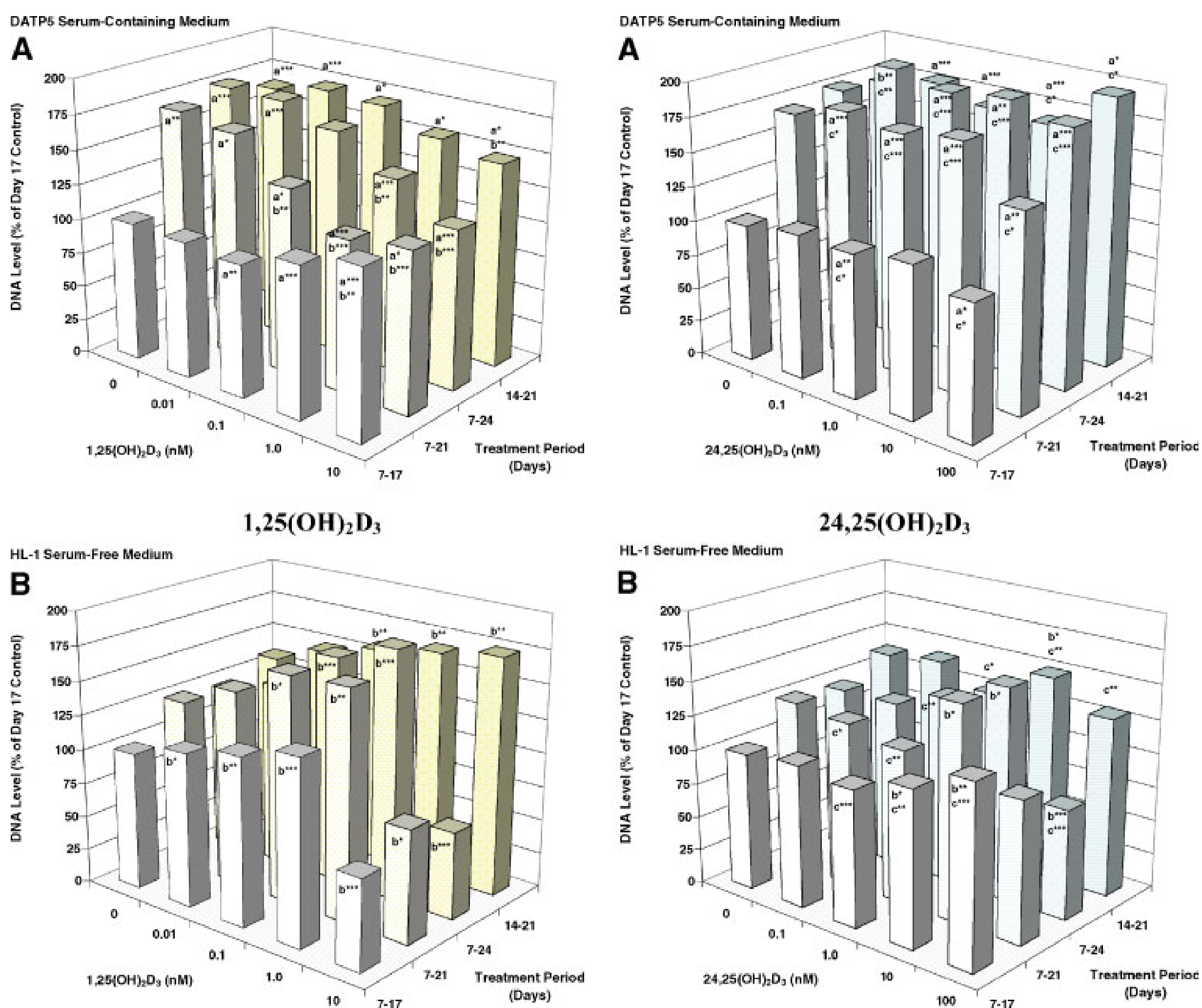

Fig. 1. Time- and dosage-dependent effects of $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ on DNA Levels of GP chondrocyte cultures compared in *serum-containing* DATP5 and *serum-free* HL-1 media. Times shown are for the end of the treatment period. Cultures were first treated with the metabolites on Day 7, which are considered to be on preconfluent cells. Values shown are mean of four samples each, expressed as the percentage of the Day 17 control. SEM are not shown on the graphs but included in the statistical analyses. Differences between indicated paired values are statistically significant as indicated. Equivalent levels of $1,25(OH)_2D_3$ versus $24,25(OH)_2D_3$ were compared: 0.01–0.10 nM, 0.1–1.0 nM, and 1.0–10 nM, respectively. $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$. ^{a} = (+) serum versus (−), ^{b} = Control versus metabolite-treated, ^{c} = $1,25(OH)_2D_3$ versus $24,25(OH)_2D_3$. Left Graphs—Effect of $1,25(OH)_2D_3$, Right Graphs—Effect of $24,25(OH)_2D_3$. **A:** DATP5 medium, (**B**) HL-1 medium. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

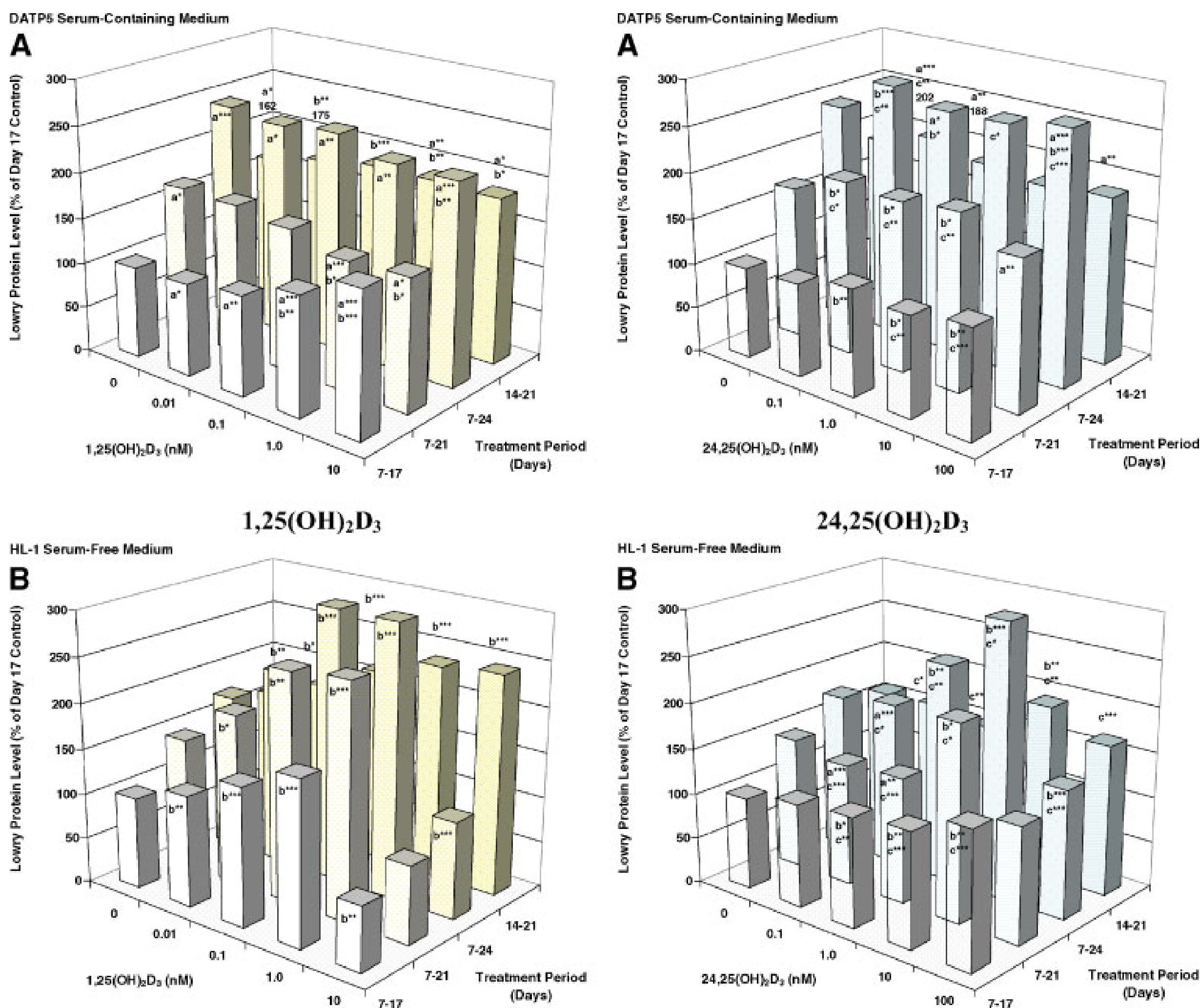

Fig. 2. Time- and dosage-dependent effects of $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ on Lowry protein levels of GP chondrocyte cultures compared in *serum-containing* DATP5 and *serum-free* HL-1 media. Times shown are for the end of the treatment period. Cultures were first treated with the metabolites on Day 7, which are considered to be on preconfluent cells. Values shown are mean of four samples each, expressed as the percentage of the Day 17 control. SEM are not shown on the graphs but included in the statistical analyses. Differences between indicated paired values are statistically significant as indicated. Equivalent levels of $1,25(OH)_2D_3$ versus $24,25(OH)_2D_3$ were compared: 0.01–0.10 nM, 0.1–1.0 nM, and 1.0–10 nM, respectively. $^*P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$. $^a = (+)$ serum versus (−), $^b =$ Control versus metabolite-treated, $^c = 1,25(OH)_2D_3$ versus $24,25(OH)_2D_3$. Left Graphs—Effect of $1,25(OH)_2D_3$, Right Graphs—Effect of $24,25(OH)_2D_3$. **A**: DATP5 medium, (**B**) HL-1 medium. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

levels rose less and leveled off at 65–75% of those seen in the more physiological *serum-containing* DATP5 medium.

In this physiological medium, $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ caused markedly different responses (Figs. 1 and 2). While $1,25(OH)_2D_3$ caused early small, dosage-dependent increases in DNA and protein, longer exposure caused dosage-dependent *decreases* in both. In contrast, $24,25(OH)_2D_3$ caused slower but persistent increases in DNA and Lowry protein levels that were significantly greater than those produced by $1,25(OH)_2D_3$.

In the β-glycerophosphate-containing *serum-free* HL-1 medium, $1,25(OH)_2D_3$ caused a greater response than $24,25(OH)_2D_3$. $1,25(OH)_2D_3$ caused dosage-dependent increases in DNA and protein levels throughout the culture period (Figs. 1 and 2). However, supra-physiological levels of $1,25\text{-}(OH)_2D_3$ (10 nM) led to marked reduction in DNA and protein levels. $24,25(OH)_2D_3$ caused lesser increases in DNA and protein levels, and the high dosage (100 nM) caused smaller reductions in DNA and protein.

These data reveal that preconfluent GP chondrocytes are more responsive to $24,25(OH)_2D_3$

than to $1,25(OH)_2D_3$ in in-vivo-matched DATP5 medium. It is also evident that serum proteins bind to and buffer the effects of both, affecting cell division and cell viability. In the absence of serum proteins, $1,25(OH)_2D_3$ had a greater effect, but at supra-physiological levels both metabolites lost their stimulatory effect and became inhibitory.

Post-confluent cultures. In both DATP5 and HL-1 cultures, post-confluent treatment with $24,25(OH)_2D_3$ had minimal effect on total DNA and Lowry protein levels. In contrast, $1,25(OH)_2D_3$ caused effects similar to those seen during pre-confluent treatment (Figs. 1 and 2). The major exception was that in *serum-free* cultures, the highest level of $1,25(OH)_2D_3$ (10 nM) was no longer inhibitory, but stimulatory to Lowry protein levels.

Proteoglycans

PGs are a specific component of all types of cartilage; in combination with type-2 collagen they are responsible for the viscoelastic properties of these tissues. They also provide an important diffusion barrier that buffers these cells from external influences.

Pre-confluent cultures. In control cultures grown in *serum-containing* DATP5 medium, PG levels progressively increased with time; in *serum-free* HL-1 medium, PG levels increased significantly less (Fig. 3).

In *serum-containing* DATP5 medium, early exposure (Day 7–17) to $1,25(OH)_2D_3$ led to a marked dosage-dependent increase in PG ($>$ fourfold over the control at 10 nM); at that time $24,25(OH)_2D_3$ caused minimal stimulation (Fig. 3A). However, longer exposure to $24,25(OH)_2D_3$ led to significant increases in PG, equal to or greater than those caused by $1,25(OH)_2D_3$.

In *serum-free* HL-1 medium, the effects of the vitamin D metabolites on PG levels were significantly greater (Fig. 3B). Maximal stimulation of $1,25(OH)_2D_3$ was seen at $\sim$1.0 nM and ranged from $\sim$ fourfold on Day 17 to nearly sevenfold by Day 24. Supra-physiological levels of $1,25(OH)_2D_3$ (10 nM), however, led to marked reduction in PG. $24,25(OH)_2D_3$ also stimulated PG formation in *serum-free* media. Its maximal effects, seen at 10 nM, were only about half of those with $1,25(OH)_2D_3$, but still highly significant. Supra-physiological levels of $24,25(OH)_2D_3$ (100 nM) were also inhibitory to PG formation in *serum-free* media. Thus, PG synthesis by GP chondrocytes is highly sensitive to the level of both $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$, especially in the absence of serum which contains proteins that buffer their availability.

Post-confluent cultures. The most striking difference between the effect of post-confluent and pre-confluent treatment with $1,25(OH)_2D_3$ was the finding that high levels were no longer inhibitory, but actually increased PG levels. This occurred in both *serum-containing* and *serum-free* cultures. In contrast, post-confluent exposure to $24,25(OH)_2D_3$ caused only small effects on PG. As will be discussed later, the lack of inhibition by high levels of $1,25(OH)_2D_3$ in the post-confluent state appears to result from the fact that the cells had already produced significant amounts of PG by Day 14 when the cells were first exposed to the metabolite.

Lactate Dehydrogenase Activity

LDH is an important enzyme involved in hypoxic metabolism of glucose. Upper GP tissue is largely avascular and generally hypoxic [Davis et al., 1989]. Under hypoxic conditions, LDH catalyzes the NADH-dependent reduction of pyruvate to lactate [Boiteux and Hess, 1981], regenerating NAD^+ needed for the continued metabolism of glucose via the glycolytic pathway.

Pre-confluent cultures. In control cultures in *serum-containing* DATP5 medium, LDH activity greatly increased, more than doubling from Day 17 to Day 24 (Fig. 4A). In contrast, over this same period, LDH activity increased much less in *serum-free* HL-1 medium. In *serum-containing* DATP5, exposure to physiological levels of both $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ led to progressive increases in LDH activity. Although occurring more slowly, the increase in LDH activity seen in $24,25(OH)_2D_3$-treated cells was significantly greater than that seen with $1,25(OH)_2D_3$. In contrast, in *serum-free* media, the response to $1,25(OH)_2D_3$ was generally greater than that seen with $24,25(OH)_2D_3$ (Fig. 4B). In addition, in *serum-free* HL-1 medium, cells treated with levels above the physiological range of either metabolite had significantly lower LDH activity compared to those treated with optimal levels.

Post-confluent cultures. Treatment of post-confluent GP chondrocytes with graded levels of $1,25(OH)_2D_3$ caused significant increases

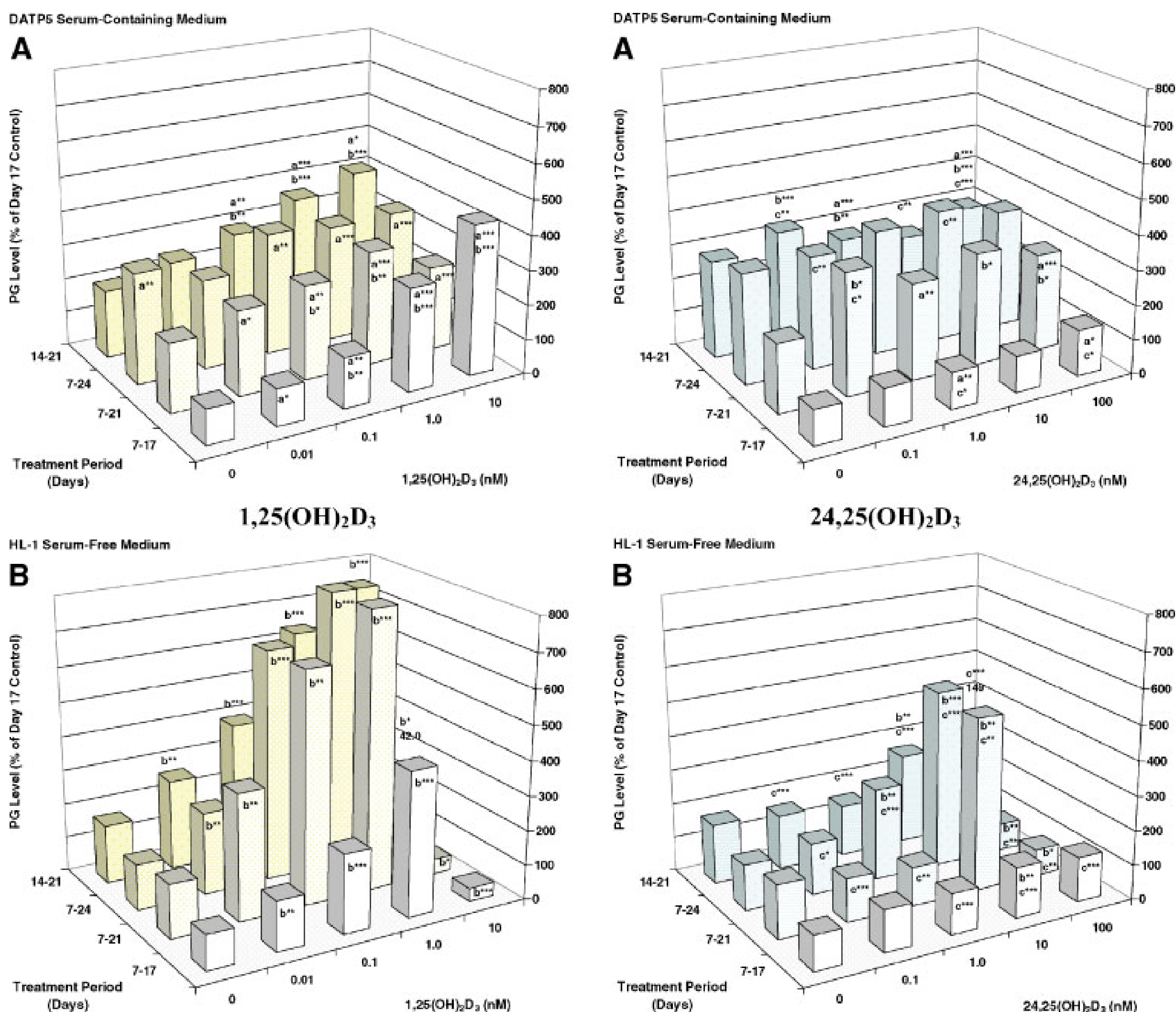

Fig. 3. Time- and dosage-dependent effects of $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ on PG levels of GP chondrocyte cultures compared in *serum-containing* DATP5 and *serum-free* HL-1 media. Times shown are for the end of the treatment period. Cultures were first treated with the metabolites on Day 7, which are considered to be on preconfluent cells. Values shown are mean of four samples each, expressed as the percentage of the Day 17 control. SEM are not shown on the graphs but included in the statistical analyses. Differences between indicated paired values are statistically significant as indicated. Equivalent levels of $1,25(OH)_2D_3$ versus $24,25(OH)_2D_3$ were compared: 0.01–0.10 nM, 0.1–1.0 nM, and 1.0–10 nM, respectively. $^*P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$. $^a = (+)$ serum versus (−), $^b =$ Control versus metabolite-treated, $^c = 1,25(OH)_2D_3$ versus $24,25(OH)_2D_3$. Left Graphs—Effect of $1,25(OH)_2D_3$, Right Graphs—Effect of $24,25(OH)_2D_3$. **A**: DATP5 medium, (**B**) HL-1 medium. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

in LDH activity in both *serum-containing* and *serum-free* media. But with $24,25(OH)_2D_3$, this delay in exposure abrogated the positive responses seen with preconfluent cells. Also, the inhibitory effects of high levels of $1,25(OH)_2D_3$ in *serum-free* pre-confluent cultures were not observed in post-confluent cultures.

Mineral Deposition

In this portion of the study, the direct effects of $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ on mineral deposition by primary GP chondrocyte cultures are documented. In addition, their effects on ALP, an enzyme that has long been associated with bone formation, will also be presented.

Mineralization is a key feature of GP cartilage, the site of longitudinal bone growth. The resulting calcified cartilage can be considered temporary scaffolding that provides mechanical strength to the growing bone until it is replaced by cancellous bone formed by osteoblasts.

Pre-confluent cultures. In control cultures grown in *serum-containing* DATP5 medium, Ca^{2+} and Pi levels progressively increased

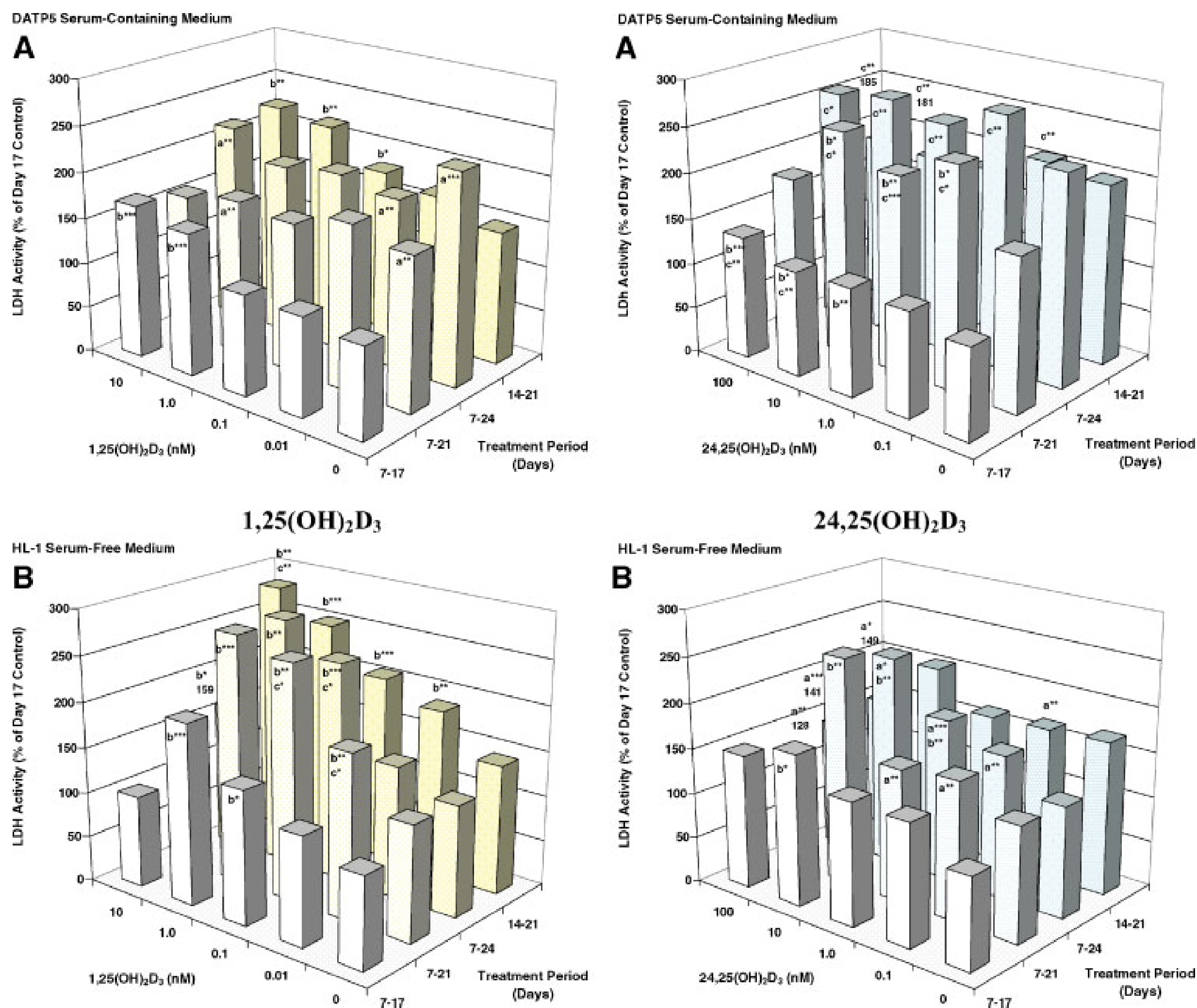

Fig. 4. Time- and dosage-dependent effects of $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ on LDH activity of GP chondrocyte cultures compared in *serum-containing* DATP5 and *serum-free* HL-1 media. Times shown are for the end of the treatment period. Cultures were first treated with the metabolites on Day 7, which are considered to be on preconfluent cells. Values shown are mean of four samples each, expressed as the percentage of the Day 17 control. SEM are not shown on the graphs but included in the statistical analyses. Differences between indicated paired values are statistically significant as indicated. Equivalent levels of $1,25(OH)_2D_3$ versus $24,25(OH)_2D_3$ were compared: 0.01–0.10 nM, 0.1–1.0 nM, and 1.0–10 nM, respectively. $^*P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$. $^a = (+)$ serum versus (–), $^b =$ Control versus metabolite-treated, $^c = 1,25(OH)_2D_3$ versus $24,25(OH)_2D_3$. Left Graphs—Effect of $1,25(OH)_2D_3$, Right Graphs—Effect of $24,25(OH)_2D_3$. **A**: DATP5 medium, (**B**) HL-1 medium. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

with time (Fig. 5). Physiological levels of $1,25(OH)_2D_3$ (0.01–1.0 nM) caused highly significant, dose-dependent inhibition of both Ca^{2+} and Pi deposition. In contrast, physiological levels (0.10–10 nM) of $24,25(OH)_2D_3$ caused significant (30–50%) increases in Ca^{2+} and Pi deposition. The dramatic differences between the effects of preconfluent exposure to $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ on mineralization can be seen in three-dimensional bar graphs of Ca^{2+} and Pi deposition (compare Fig. 5A,B).

On Day 7–17, even the lowest physiological dosage of $1,25(OH)_2D_3$ tested (0.01 nM) caused 70–75% inhibition of mineral deposition. Later, (Days 7–21 and 7–24) inhibition by this low level of $1,25(OH)_2D_3$ was reduced, but higher levels (0.1–1.0 nM) continued to be strongly inhibitory. The highest (supra-physiological) level tested (10 nM) caused >90% inhibition of both Ca^{2+} and Pi deposition throughout the culture period.

The stimulatory effects of $24,25(OH)_2D_3$ were not apparent until after it had been exposed to the cells for 14 days or longer (Days 7–21 and 7–24). Note that these stimulatory effects were largely dosage-independent—the lowest level of

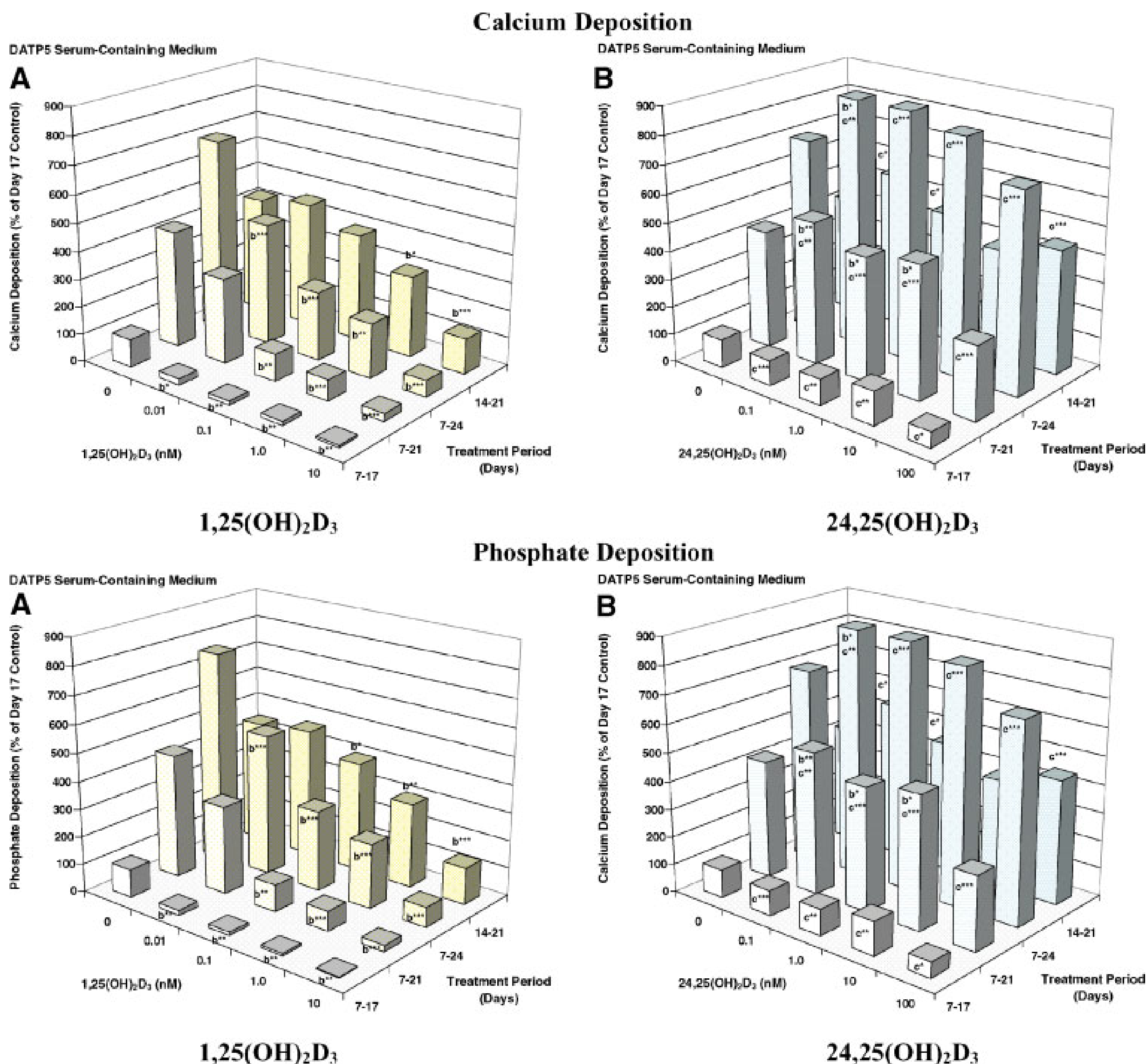

Fig. 5. Time- and dosage-dependent effects of $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ on calcium and phosphate deposition by GP chondrocytes cultured in *serum-containing* DATP5 medium. Times shown are for the end of the treatment period. Cultures were first treated with the metabolites on Day 7, which are considered to be on preconfluent cells. Values shown are mean of four samples each, expressed as the percentage of the Day 17 control. SEM are not shown on the graphs but included in the statistical analyses. Differences between indicated paired values are statistically significant as indicated. Equivalent levels of $1,25(OH)_2D_3$ versus $24,25(OH)_2D_3$ were compared: 0.01–0.10 nM, 0.1–1.0 nM, and 1.0–10 nM, respectively. $^*P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$. ^{a} = (+) serum versus (−), ^{b} = Control versus metabolite-treated, ^{c} = $1,25(OH)_2D_3$ versus $24,25(OH)_2D_3$. Left Graphs—Effect of $1,25(OH)_2D_3$, Right Graphs—Effect of $24,25(OH)_2D_3$. **A**: DATP5 medium, (**B**) HL-1 medium. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

$24,25(OH)_2D_3$ tested (0.10 nM) was equal or more stimulatory than the higher dosages. The supra-physiological dosage of $24,25(OH)_2D_3$ (100 nM), however, caused some inhibition of both Ca^{2+} and Pi deposition.

Post-confluent cultures. Exposure of post-confluent cells to $1,25-(OH)_2D_3$ caused a less inhibitory response; in fact the lowest dosage tested (0.01 nM) appeared to have a slight stimulatory effect (Fig. 5). Higher dosages caused progressively inhibition, but only about one-third of that seen with preconfluent cells. Exposure of post-confluent cells to low levels of $24,25(OH)_2D_3$ (0.10 nM) modestly stimulated Ca^{2+} and Pi deposition (~20–30%). Thus in post-confluent cells, the effects of $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ on Ca^{2+} and Pi deposition were qualitatively similar to those seen in preconfluent cells, but were generally smaller.

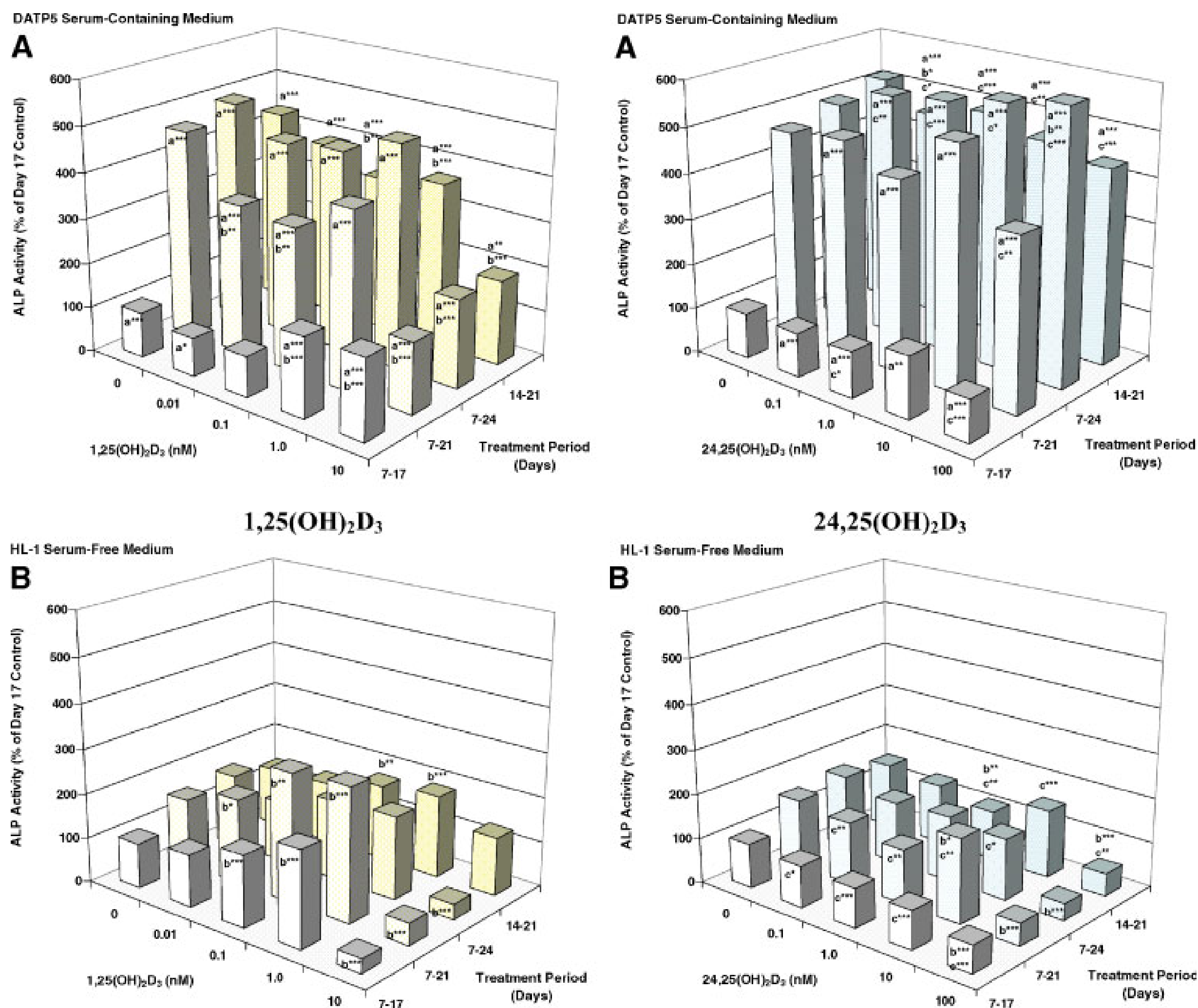

Fig. 6. Time- and dosage-dependent effects of $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ on ALP activity of GP chondrocyte cultures compared in *serum-containing* DATP5 and *serum-free* HL-1 media. Times shown are for the end of the treatment period. Cultures were first treated with the metabolites on Day 7, which are considered to be on preconfluent cells. Values shown are mean of four samples each, expressed as the percentage of the Day 17 control. SEM are not shown on the graphs but included in the statistical analyses. Differences between indicated paired values are statistically significant as indicated. Equivalent levels of $1,25(OH)_2D_3$ versus $24,25(OH)_2D_3$ were compared: 0.01–0.10 nM, 0.1–1.0 nM, and 1.0–10 nM, respectively. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$. ^{a} = (+) serum versus (−), ^{b} = Control versus metabolite-treated, ^{c} = $1,25(OH)_2D_3$ versus $24,25(OH)_2D_3$. **A**: Effect of $1,25(OH)_2D_3$, (**B**) Effect of $24,25(OH)_2D_3$. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Alkaline Phosphatase Activity

Pre-confluent cultures. In control *serum-containing* DATP5 medium, ALP activity increased rapidly from Day 17 to Day 24 (Fig. 6). In control *serum-free* HL-1 medium, ALP activity was initially ~60% of that in *serum-containing* DATP5 medium, but increased only slightly from Day 17 to Day 24, reaching a maximum of only about 20% of that in *serum-containing* DATP5 medium.

In *serum-containing* DATP5 medium, early exposure (Day 7–17) to $1,25(OH)_2D_3$ caused dosage-dependent increases in ALP activity up to a maximum of ~180% at 1–10 nM (Fig. 6). But longer exposure to physiological levels (0.01–1.0 nM) of $1,25(OH)_2D_3$ led to 10–30% reduction in ALP activity, while supra-physiological levels (10 nM) lead to ~60% reduction. Physiological levels of $24,25(OH)_2D_3$ (0.1–10 nM) caused a 10–25% increase in ALP activity, primarily after 17-day exposure. Initially, supra-physiological levels (100 nM) slightly reduced it. On Days 21–24, ALP activity in $1,25(OH)_2D_3$-treated cells was only about 70% of that in cells treated with equi-

valent levels of $24{,}25(OH)_2D_3$. This was also true of post-confluent treated cells. Thus, $24{,}25(OH)_2D_3$ exerted a slower but more persistent stimulation of ALP activity than did $1{,}25(OH)_2D_3$.

In *serum-free* HL-1 medium, 10–14-day exposure to physiological levels (0.1–1.0 nM) of $1{,}25(OH)_2D_3$ caused near doubling of ALP activity, but even so levels were less than half of those seen in *serum-containing* DATP5 medium. This stimulatory effect was lost upon treatment beyond Day 7–21. Also in *serum-free* HL-1 medium, supra-physiological (10 nM) $1{,}25(OH)_2D_3$ caused a marked (~70%) decrease in ALP at all treatment times (Fig. 6). Physiological levels (0.1–10 nM) of $24{,}25(OH)_2D_3$ caused generally minor effects on ALP activity; however supra-physiological levels (100 nM) caused significant reduction similar to that seen with $1{,}25(OH)_2D_3$.

Post-confluent cultures. In *serum-free* HL-1 medium, $1{,}25(OH)_2D_3$ and $24{,}25(OH)_2D_3$ caused more variable effects on ALP activity. $1{,}25(OH)_2D_3$ produced 20–30% increase at 0.1–1.0 nM; $24{,}25(OH)_2D_3$ caused inconsistent minor effects except for ~65% reduction at 100 nM.

Relationships Between Components

Consistent effects were produced by both vitamin D metabolites on most of the tested factors; however, it was not immediately obvious whether these were primary or secondary to effects on cell division and/or general protein expression. Initially, scatter plots were constructed with regression analyses of trend lines; these showed close relationships between many of these parameters. To facilitate interpretation of the data, ratios of PG and LDH to either DNA or Lowry protein, or to each other, were calculated. The effects of physiological levels of $1{,}25(OH)_2D_3$ (0.01–1.0 nM) and $24{,}25(OH)_2D_3$ (0.10–10 nM), in the presence or absence of serum, were averaged and compared statistically (Table I).

Lowry protein/DNA. The calculated ratios reveal that $1{,}25(OH)_2D_3$ increased protein significantly more than DNA in both *serum-free* and *serum-containing* media. $24{,}25(OH)_2D_3$ exerted a less specific effect on protein levels (Table I).

PG/DNA and PG/Lowry protein. Control cells grown in *serum-containing* DATP5 medium had significantly higher PG levels relative

TABLE I. Ratios of Chondrocyte Components

Ratio	Control (0 nM)	$[1{,}25(OH)_2D_3]$ (0.01–1.0 nM)	$[24{,}25(OH)_2D_3]$ (0.10–10.0 nM)
Protein/DNA			
(+) Serum	(32) 33.24 ± 0.92	(48) 36.71 ± 0.84^{b,‡}	(48) 34.94 ± 0.68
(−) Serum	(32) 33.19 ± 0.90	(48) 41.45 ± 1.24^{a,‡,b,†}	(48) 37.00 ± 1.14^{b,*,c,‡}
PG/DNA			
(+) Serum	(32) 1.84 ± 0.10	(48) 2.65 ± 0.12^{b,†}	(48) 2.14 ± 0.08^{b,*,c,†}
(−) Serum	(32) 1.46 ± 0.04^{a,†}	(48) 3.90 ± 0.24^{a,†,b,†}	(48) 2.06 ± 0.15^{b,‡,c,†}
PG/Protein (100×)			
(+) Serum	(32) 5.48 ± 0.19	(48) 7.19 ± 0.27^{b,†}	(48) 6.13 ± 0.08^{b,‡,c,*}
(−) Serum	(32) 4.45 ± 0.13^{a,†}	(48) 9.24 ± 0.45^{a,†,b,†}	(48) 5.43 ± 0.28^{b,‡,c,†}
LDH/DNA			
(+) Serum	(32) 9.10 ± 0.28	(48) 10.20 ± 0.23^{b,‡}	(48) 10.30 ± 0.20^{b,†}
(−) Serum	(32) 7.78 ± 0.18^{a,†}	(48) 10.14 ± 0.24^{b,†}	(48) 10.08 ± 0.23^{b,†}
LDH/Protein (10×)			
(+) Serum	(32) 2.75 ± 0.06	(48) 2.81 ± 0.06	(48) 2.97 ± 0.06^{b,‡,c,*}
(−) Serum	(32) 2.38 ± 0.07^{a,†}	(48) 2.51 ± 0.06^{a,†}	(48) 2.82 ± 0.09^{b,‡,c,‡}
LDH/PG			
(+) Serum	(32) 5.14 ± 0.15	(48) 4.07 ± 0.13^{b,†}	(48) 5.06 ± 0.17^{c,†}
(−) Serum	(32) 5.43 ± 0.15	(48) 2.97 ± 0.14^{a,†,b,†}	(48) 5.69 ± 0.29^{c,†}

Values shown are the average ± SEM of the indicated control and metabolite-treated cultures. They comprise all of the treatment periods, including both those initiated in the pre- and post-confluent states. The numbers of individual samples are shown in parenthesis. (+) Serum indicates cultures grown in DATP5 medium; (−) Serum indicates cultures grown in HL-1 medium. Equivalent levels of $1{,}25(OH)_2D_3$ versus $24{,}25(OH)_2D_3$ were compared: 0.01–0.10 nM, 0.1–1.0 nM, and 1.0–10 nM, respectively.
Differences between the indicated paired values are statistically significant as indicated:
*$P \leq 0.05$.
‡$P \leq 0.001$.
†$P \leq 0.01$.
[a](+) Serum versus (−) Serum.
[b]Control versus metabolite-treated.
[c]$1{,}25(OH)_2D_3$ versus $24{,}25(OH)_2D_3$.

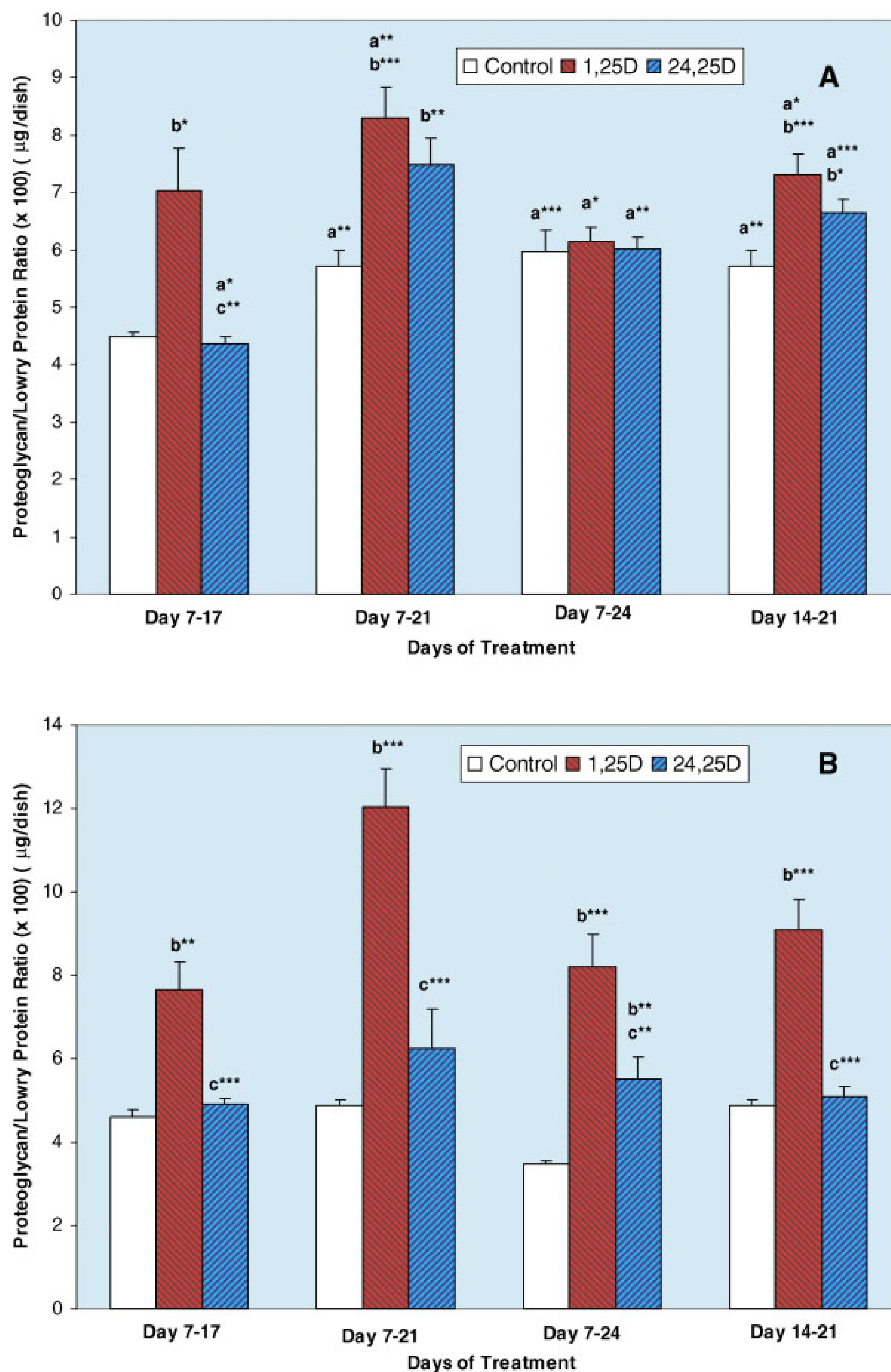

Fig. 7. Comparison of the effects of physiological levels of $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ on the ratios of PG to Lowry protein in primary cultures of GP chondrocytes. Cultures were grown in 35-mm diameter dishes in *serum-containing* DATP5 medium (**A**) or *serum-free* HL-1 medium (**B**). Shown are the average ± SEM of the ratios of PG/Lowry protein (100×) expressed as µg/dish. Averaged for each time period were: controls, 4–8 replicates; $1,25(OH)_2D_3$-treated cultures, 12 replicates (4 each for 0.01, 0.10, and 1.0 nM); $24,25(OH)_2D_3$-treated cultures, 12 replicates (4 each for 0.10, 1.0, and 10 nM). Differences between the indicated paired values are statistically significant as indicated: $^*P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$. [a] = (+) Serum versus (−) Serum, [b] = Control versus metabolite-treated, [c] = $1,25(OH)_2D_3$ versus $24,25(OH)_2D_3$. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

to DNA or protein than did those grown in *serum-free* HL-1 medium (Table I). $1,25(OH)_2D_3$ significantly increased PG relative to both DNA and protein, especially in *serum-free* media. $24,25(OH)_2D_3$ also significantly increased PG relative to DNA or protein, but its effects were less obvious. Time-dependent effects of both metabolites on the PG/Lowry protein ratios, shown in Figure 7, revealed a specific stimulation of PG by $1,25(OH)_2D_3$, which is particularly apparent in *serum-free* HL-1 medium.

LDH/DNA and LDH/Lowry Protein

Control cultures grown in *serum-containing* DATP5 medium had significantly higher LDH activity relative to DNA and protein than did those grown in *serum-free* HL-1 medium (Table I). Although $1,25(OH)_2D_3$ increased both Lowry protein and LDH activity, the LDH/Lowry protein ratios in both *serum-containing* or *serum-free* media were the same as in the control. This indicates that the stimulatory effect of $1,25(OH)_2D_3$ on LDH was dependent on its effect on general protein levels, rather than on LDH expression. More surprising was the finding that $24,25(OH)_2D_3$ caused increases in the LDH/DNA ratios equal to those of $1,25(OH)_2D_3$, and when LDH/Lowry protein ratios were compared, $24,25(OH)_2D_3$ caused a significantly greater effect than $1,25(OH)_2D_3$ (Table I). The time-dependent effects of both metabolites on the LDH/Lowry protein ratios, shown in Figure 8, reveal that $24,25(OH)_2D_3$ caused a specific stimulation of LDH activity. This was especially evident in *serum-free* media.

LDH/PG

Comparison of the LDH/PG ratios was used to clarify the selective effects of $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$. Treatment with $1,25(OH)_2D_3$ caused a highly significant *decrease* in the LDH/PG ratios, especially in *serum-free* cultures (Table I). These time-dependent effects are more obvious in Figure 9 where it is evident that $24,25(OH)_2D_3$ selectively stimulated LDH activity relative to PG in both culture media under all experimental conditions studied. These differences between $24,25(OH)_2D_3$ and $1,25(OH)_2D_3$-treated cultures are highly significant statistically (Table I). They can be largely attributed to two factors: (1) $1,25(OH)_2D_3$ strongly stimulated PG in these GP chondrocyte cultures, but had minimal effect on LDH expression. (2) $24,25(OH)_2D_3$ was less stimulatory to PG, but significantly increased LDH activity relative to DNA or Lowry protein. Thus, $24,25(OH)_2D_3$ selectively enhanced LDH activity in both *serum-containing* and *serum-free* cultures.

Mineral Deposition

To more accurately interpret the time-dependent effects of the vitamin D metabolites, plots were made of their effect on the relationship between mineral deposition and various parameters including Hoechst DNA, Lowry protein, ALP, and LDH activity. Shown first is their effect on Ca^{2+} versus Pi deposition (Fig. 10). As expected, there was a close linear relationship between these mineral ions. The progressive stimulation of Ca^{2+} deposition by $24,25(OH)_2D_3$ and the strong inhibition by $1,25(OH)_2D_3$ become readily apparent.

Figure 11 illustrates effects on Ca^{2+} deposition relative to those on Hoechst DNA and Lowry protein. The time-dependent, progressive stimulatory effect of $24,25(OH)_2D_3$, and the rapid inhibitory effect of $1,25(OH)_2D_3$ on Ca^{2+} deposition follow a linear continuum. By calculating the ratios of Ca^{2+} and Pi deposition to Hoechst DNA, or Lowry protein, it became evident that these metabolic effects were specific and statistically significant (Fig. 12). $1,25(OH)_2D_3$ caused rapid and profound reductions in both the Ca^{2+}/Lowry and Pi/Lowry protein ratios, revealing its specific inhibitory effect on mineral deposition; $24,25(OH)_2D_3$ caused progressive increases in both Ca^{2+} and Pi/Lowry ratios, revealing a specific stimulation of mineral deposition, over and above its anabolic effect on protein synthesis. Also evident in Figures 11 and 12 is the fact that if exposure was delayed until after the cells attained confluence, the effects of both $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ were diminished.

To shed further light on the relationship between mineral deposition and enzyme activity, the effects of $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ on Ca^{2+} deposition relative to their effects on ALP and LDH were also plotted (Fig. 13). Several features become evident from these plots. (1) The inhibitory effects of $1,25(OH)_2D_3$ on Ca^{2+} deposition were not closely related to its effects on ALP or LDH activity. Early on (Day 7–17) $1,25(OH)_2D_3$ stimulated both ALP and LDH activity, but strongly inhibited Ca^{2+} deposition. Later (Day 7–21

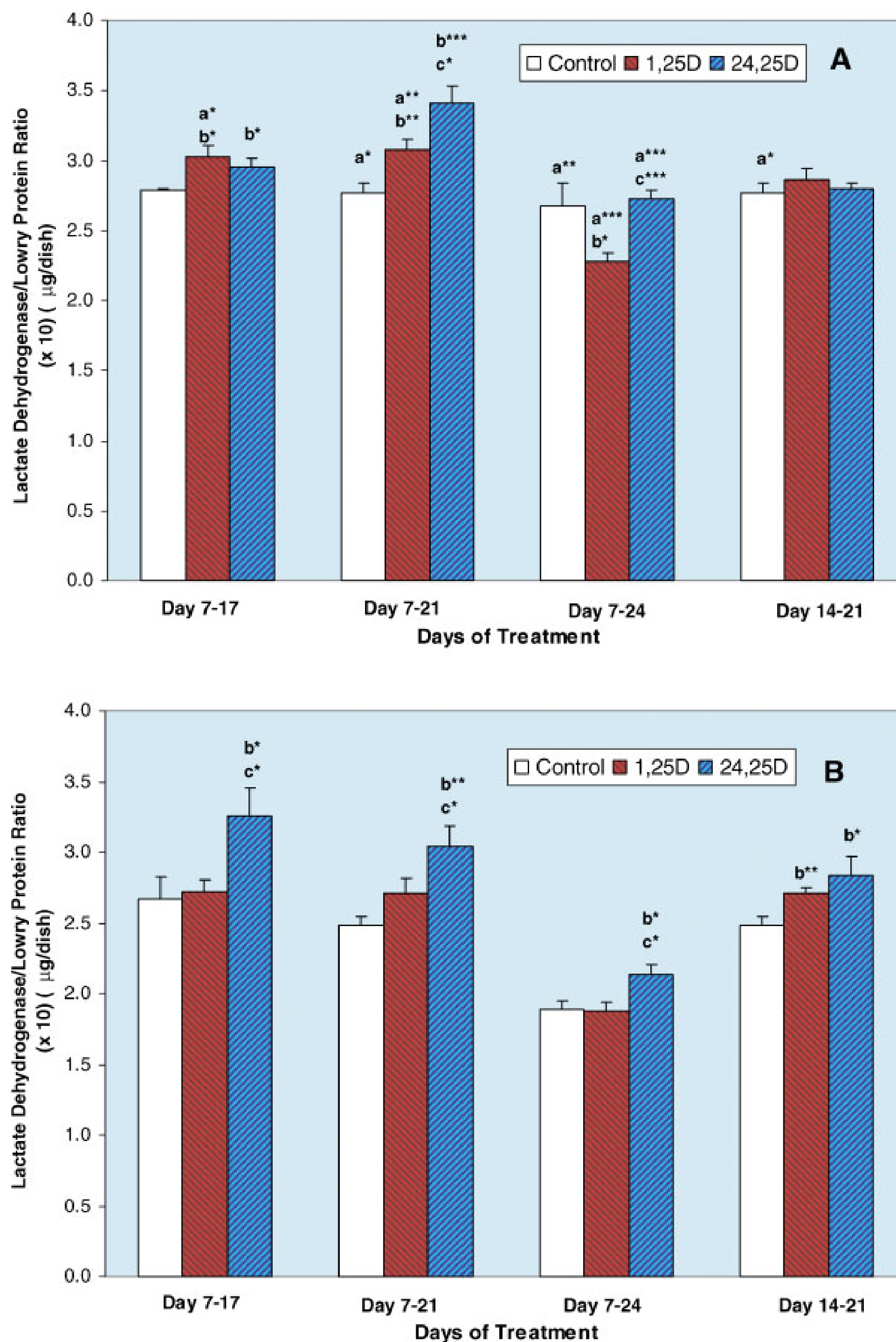

Fig. 8. Comparison of the effects of physiological levels of $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ on the ratios of LDH activity to Lowry protein in primary cultures of GP chondrocytes. Cultures were grown as indicated in Figure 1, *serum-containing* DATP5 medium (**A**) and *serum-free* HL-1 medium (**B**). Shown are the average ± SEM of the ratios of LDH/Lowry protein (100×) expressed as units of activity/µg protein. Averaged for each time period were: controls, 4–8 replicates; $1,25(OH)_2D_3$-treated cultures, 12 replicates (4 each for 0.01, 0.10, and 1.0 nM); $24,25(OH)_2D_3$-treated cultures, 12 replicates (4 each for 0.10, 1.0, and 10 nM). Differences between the indicated paired values are statistically significant as indicated: $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$. ^{a} = (+) Serum versus (−) Serum, ^{b} = Control versus metabolite-treated, ^{c} = $1,25(OH)_2D_3$ versus $24,25(OH)_2D_3$. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

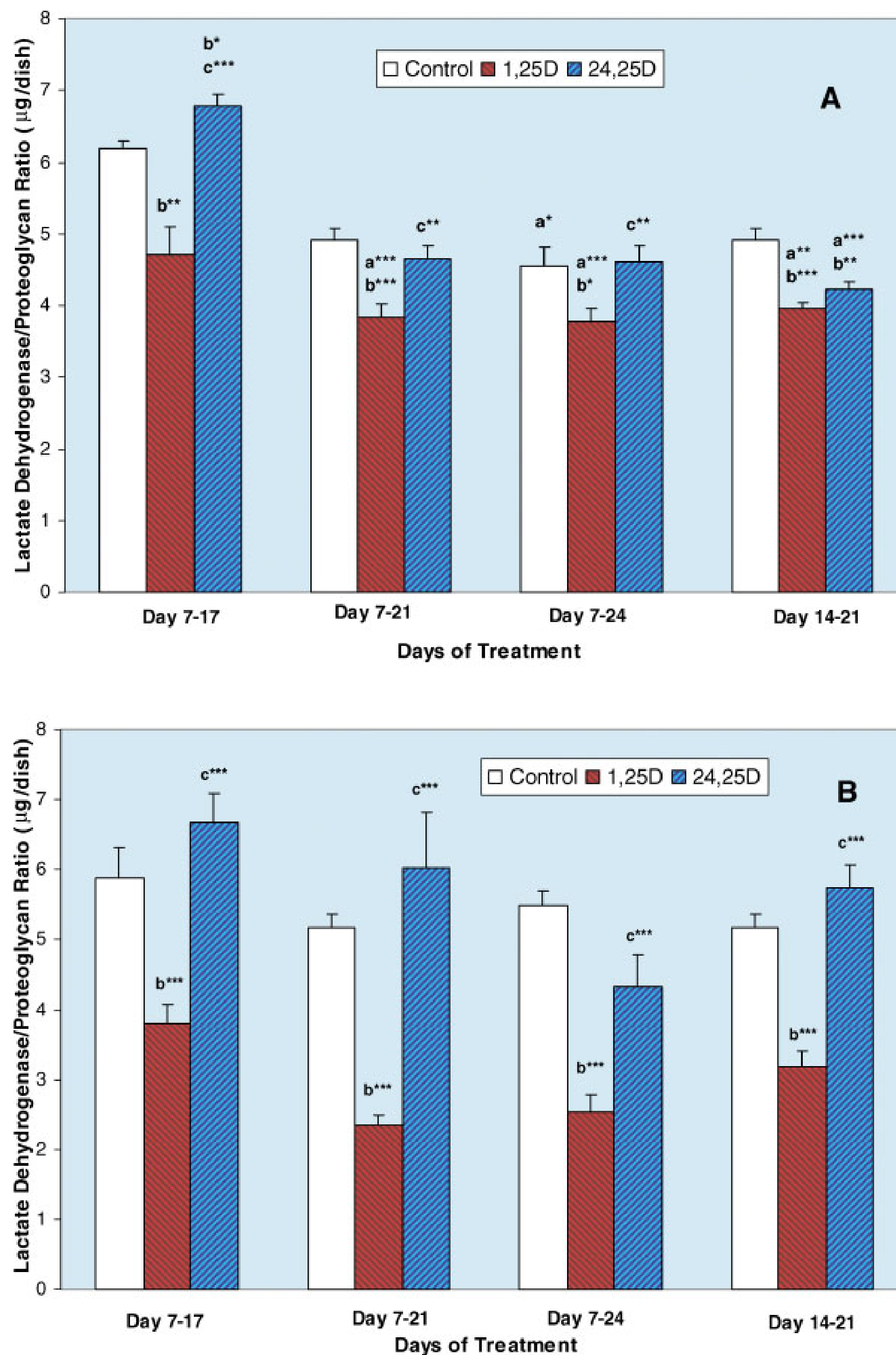

Fig. 9. Comparison of the effects of physiological levels of $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ on the ratios of LDH activity to PG in primary cultures of GP chondrocytes. Cultures were grown as indicated in Figure 1, *serum-containing* DATP5 medium (**A**) and *serum-free* HL-1 medium (**B**). Shown are the average ± SEM of the ratios of LDH/PG expressed as units of activity/µg PG. Averaged for each time period were: controls, 4–8 replicates; $1,25(OH)_2D_3$-treated cultures, 12 replicates (4 each for 0.01, 0.10, and 1.0 nM); $24,25(OH)_2D_3$-treated cultures, 12 replicates (4 each for 0.10, 1.0, and 10 nM). Differences between the indicated paired values are statistically significant as indicated: $^*P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$. [a] = (+) Serum versus (−) Serum, [b] = Control versus metabolite-treated, [c] = $1,25(OH)_2D_3$ versus $24,25(OH)_2D_3$. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

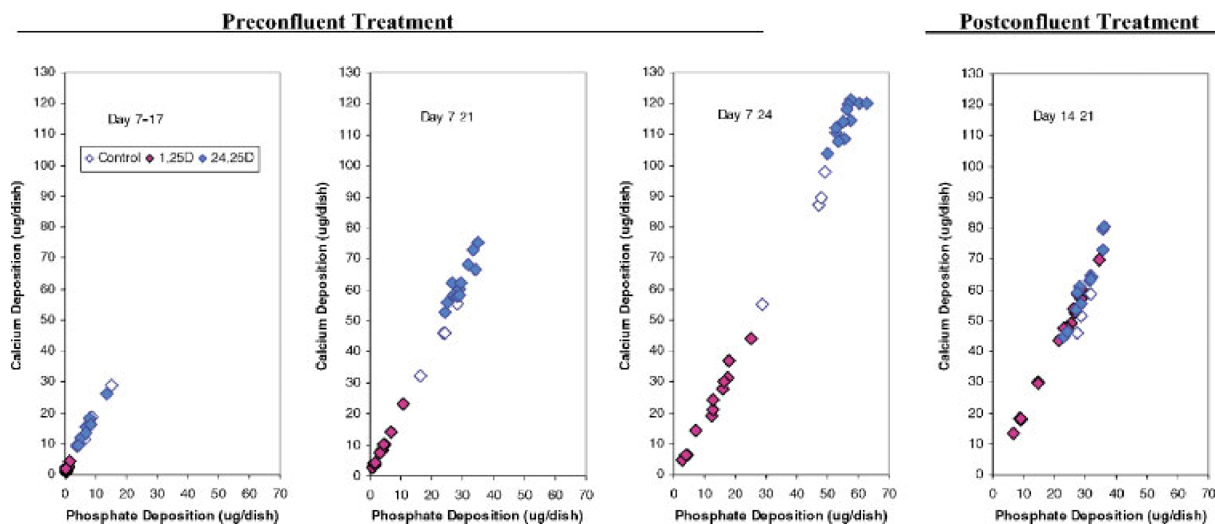

Fig. 10. Time-dependent effects of $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ on calcium relative to phosphate deposition by GP chondrocytes cultured in *serum-containing* DATP5 medium. Cultures first treated with the metabolites on Day 7 are indicated as being on preconfluent cells; those treated from Day 14 are indicated as being on postconfluent cells. Shown are paired values of analyses of individual cultures from the untreated control (4) or the full range of metabolite-treated (16) cells: 4 cultures each for 0.01, 0.10, 1.0, and 10 nM $1,25(OH)_2D_3$; 4 each for 0.10, 1.0, 10, and 100 nM $24,25(OH)_2D_3$. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

and 7–24), although $1,25(OH)_2D_3$ became inhibitory to both enzymes, the degree of inhibition was smaller than its powerful effect on Ca^{2+}. (2) The stimulatory effects of $24,25(OH)_2D_3$ on Ca^{2+} deposition were visibly greater than its stimulus to either ALP or LDH activity, and (3) became progressively more obvious when the cells had been treated for 14–17 days. (4) If exposure to the metabolites was delayed until after confluence, the differential effects of the two metabolites became blurred.

Finally, to clarify whether these metabolites had a specific effect on ALP activity per se, their comparative effects on ALP versus Lowry protein in both *serum-containing* DATP5 and *serum-free* HL-1 media were plotted. Figure 14 reveals that in *serum-containing* DATP5 medium, while $1,25(OH)_2D_3$ had an early (Day 7–17) stimulatory effect on ALP relative to Lowry protein, upon longer exposure this was reversed. In contrast, while $24,25(OH)_2D_3$ initially had minimal effect, with time its specific stimulation of ALP relative to Lowry protein became more and more apparent. This was true in both pre- and post-confluent treated cells. Further, in *serum-free* HL-1 medium, whereas $1,25(OH)_2D_3$ was generally stimulatory to ALP, $24,25(OH)_2D_3$ failed to stimulate enzyme activity. But most conspicuous was the markedly lower levels of ALP in *serum-free* HL-1 medium.

DISCUSSION

The current study focused on the effects of $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ administered in-vitro to primary cultures of chicken GP chondrocytes. These metabolites were provided at levels ranging from deficient, to physiological, to supra-physiological and were given at different stages of culture development (e.g., preconfluent and postconfluent) in the presence or absence of serum, and for varying periods of time ranging from 7 to 17 days. The findings of this comprehensive study help clarify divergent findings reported in the literature, revealing that $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ exert significantly different dose- and time-dependent effects on the proliferation and differentiation of GP chondrocytes.

Of our various findings, in this aspect of the study, four have particular physiological interest: (1) the selective, remarkable stimulatory effect of $1,25(OH)_2D_3$ on PG levels, (2) the small general stimulatory effect of $24,25(OH)_2D_3$ on basic cellular parameters (DNA and Lowry protein levels) in *serum-containing* media, (3) the selective stimulatory effect of $24,25(OH)_2D_3$ on LDH activity, and (4) the close correlation between LDH activity and the PG levels under a wide variety of culture conditions.

Previous studies have reported diverse effects of $1,25(OH)_2D_3$ on PG synthesis ranging from

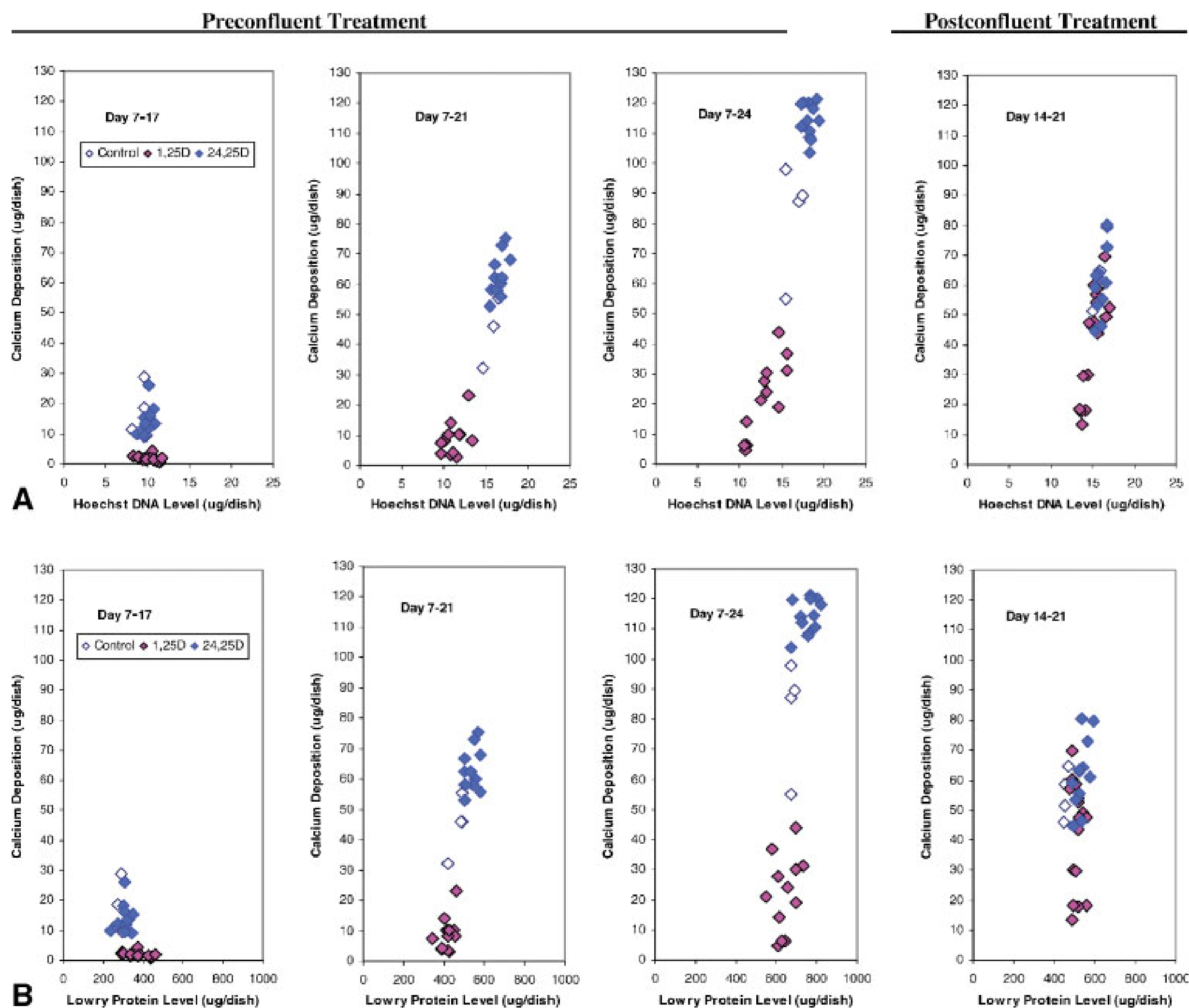

Fig. 11. Time-dependent effects of $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ on calcium deposition relative to Hoechst DNA and Lowry protein levels in GP chondrocytes cultured in *serum-containing* DATP5 medium. Cultures first treated on Day 7 are considered to be preconfluent; those treated from Day 14 are considered to be postconfluent. Shown are paired values of analyses of individual cultures from the untreated control (4) or the full range of metabolite-treated (16) cells: 4 cultures each for 0.01, 0.10, 1.0, and 10 nM $1,25(OH)_2D_3$; 4 each for 0.10, 1.0, 10, and 100 nM $24,25(OH)_2D_3$. **A**: Hoechst DNA, (**B**) Lowry protein. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

inhibitory [Takigawa et al., 1988; Horton et al., 1991; Bagi and Miller, 1992] to no effect [Hinek and Poole, 1988] to mild stimulation [Kato et al., 1990]. These varying responses can now be explained by differences in the cells used, the levels of $1,25(OH)_2D_3$ tested, as well as the timing (developmental stage) when exposure occurred. Our studies reveal that GP cells are exquisitely sensitive to $1,25(OH)_2D_3$, their responses depending on the level, timing, and medium in which exposure occurred. Preconfluent GP cultures showed large, dosage-dependent increases in PG levels in both *serum-free* and *serum-containing* media. In early cultures in *serum-containing* media, supra-physiological levels of $1,25(OH)_2D_3$ (10 nM) were stimulatory, whereas in *serum-free* media they were highly inhibitory. In contrast, in postconfluent cultures $1,25(OH)_2D_3$ caused dosage-dependent increases in PG in both *serum-free and serum-containing* media. The inhibitory effect of high levels of $1,25(OH)_2D_3$ in preconfluent cells cultured can be explained by cellular toxicity: in *serum-free* media 10 nM $1,25(OH)_2D_3$ strongly inhibited DNA, Lowry protein, and most other parameters tested. The paradoxical stimulation of PG in *serum-containing* media (and in postconfluent cultures) can be explained by the tight binding of $1,25(OH)_2D_3$ by both albumin and D-binding protein [Bikle et al.,

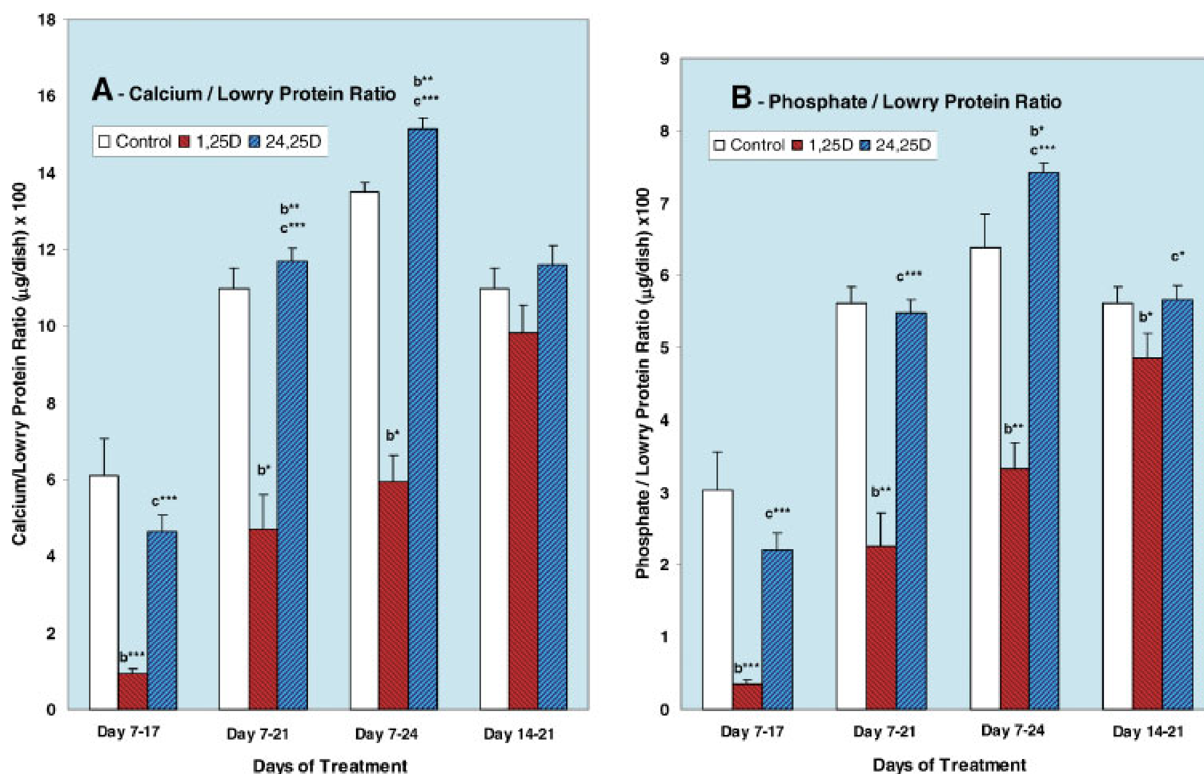

Fig. 12. Time-dependent effects of $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ on the ratios of calcium and phosphate deposition to Lowry protein in GP chondrocytes cultured in *serum-containing* DATP5 medium. **A**: Calcium/Lowry protein ratios, (**B**) Phosphate/Lowry protein ratios. Cultures first treated on Day 7 are considered to be preconfluent; those treated from Day 14 are considered to be postconfluent. Values shown are the mean ± SEM of 4–8 samples of the control and 12 samples of the metabolite-treated cultures. $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ are compared at physiologically equivalent levels: 0.01–0.10 nM, 0.1–1.0 nM, and 1.0–10 nM, respectively. Differences between the indicated paired values are statistically significant as indicated: $^*P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$. ^{b} = Control versus metabolite-treated, ^{c} = $1,25(OH)_2D_3$ versus $24,25(OH)_2D_3$. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

1985] that would complex most of the metabolite present and thus reduce its activity and/or accessibility to the cells. More will be made of this later. We also found that $24,25(OH)_2D_3$ increased PG levels significantly, especially in *serum-containing* media specifically designed to promote GP development.

Why should elevated levels of $1,25(OH)_2D_3$ stimulate extracellular matrix PG synthesis? Serum levels of $1,25(OH)_2D_3$ are inversely correlated with Ca^{2+} levels [Holick, 1996; Hamada et al., 1998]. Our findings reveal that levels of $1,25(OH)_2D_3$ causing increased DNA, Lowry protein, and PG encompass the range found by Bikle et al. [1985] in hypocalcemic serum. Thus, in a hypocalcemic state, GP expansion would continue in anticipation of restoration of Ca^{2+} that would enable mineralization to resume. The specific increase in PG would increase water content and turgidity (mechanical strength since water is noncompressible) to the tissue in the absence of mineral deposition. This would explain expansion of the GP in the absence of calcification during development of rickets.

Another interesting finding of this study was that GP chondrocytes treated before attaining confluence with physiological levels of $24,25(OH)_2D_3$ in DATP5 media for 14–17 days had higher levels of DNA, Lowry protein, PG, and LDH activity than did those treated with $1,25(OH)_2D_3$. The stimulatory effects of $24,25(OH)_2D_3$ were seen over its physiological range (0.10–10 nM), were most obvious after 14-day treatment, and occurred primarily in *serum-containing* DATP5 medium. Stimulation of LDH activity by $24,25(OH)_2D_3$ was confirmed when ratios of LDH/DNA were compared with the control, and LDH/Lowry protein, or LDH/PG ratios were compared with those of $1,25(OH)_2D_3$. These levels of $24,25(OH)_2D_3$ are characteristic of a normocalcemic state, and appear to signal conditions permissive for normal GP development.

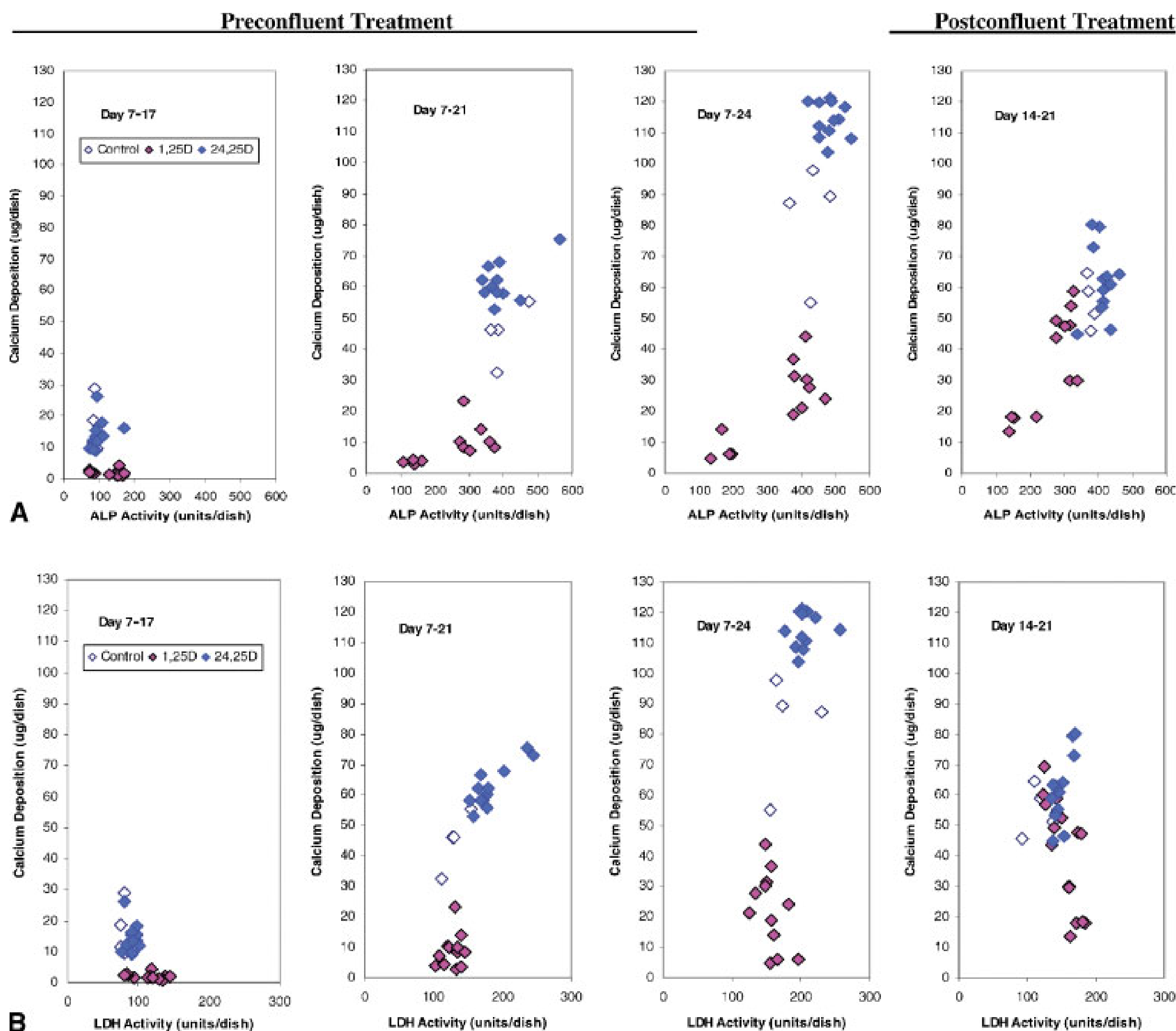

Fig. 13. Time-dependent effects of $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ on the relationship between calcium deposition and (**A**) ALP or (**B**) LDH activity in preconfluent GP chondrocytes cultured in *serum-containing* DATP5 medium. Cultures first treated on Day 7 are considered to be preconfluent; those treated from Day 14 are considered to be postconfluent. Shown are paired values of analyses of individual cultures from the untreated control (4) or the full range of metabolite-treated (16) cells: 4 cultures each for 0.01, 0.10, 1.0, and 10 nM $1,25(OH)_2D_3$; 4 each for 0.10, 1.0, 10, and 100 nM $24,25(OH)_2D_3$. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

The stimulatory effects of $24,25(OH)_2D_3$ were not large and were for the most part, lost if the timing of exposure was delayed until the postconfluent period. This is in accord with previous findings by Schwartz et al. [1992, 1995] that indicated chondrocytes are most sensitive to $24,25(OH)_2D_3$ prior to development of the GP phenotype. Thus, exposure of GP chondrocytes to $24,25(OH)_2D_3$ at a time when they are becoming established prior to confluence appears to be required for optimal sensitivity to the metabolite.

Of what significance would the small stimulation of LDH by $24,25(OH)_2D_3$ have in GP physiology? First, LDH activity is a prominent feature in cartilaginous tissues [Patzschke, 1967]; it plays a critical role in glycolysis [Boiteux and Hess, 1981]. Glycolysis provides the primary energy source for GP differentiation and mineral deposition [Hoffman and Wertheimer, 1928; Futami et al., 1979]. Glycogen, stored in large amounts in GP cells [Lewinson, 1989; Townsend and Gibson, 1970], provides the glucose metabolized by glycolysis. Second, LDH expression is strongly upregulated under hypoxic conditions [Firth et al., 1995; Gleadle et al., 1995]. We find that LDH activity closely correlates with PG levels in

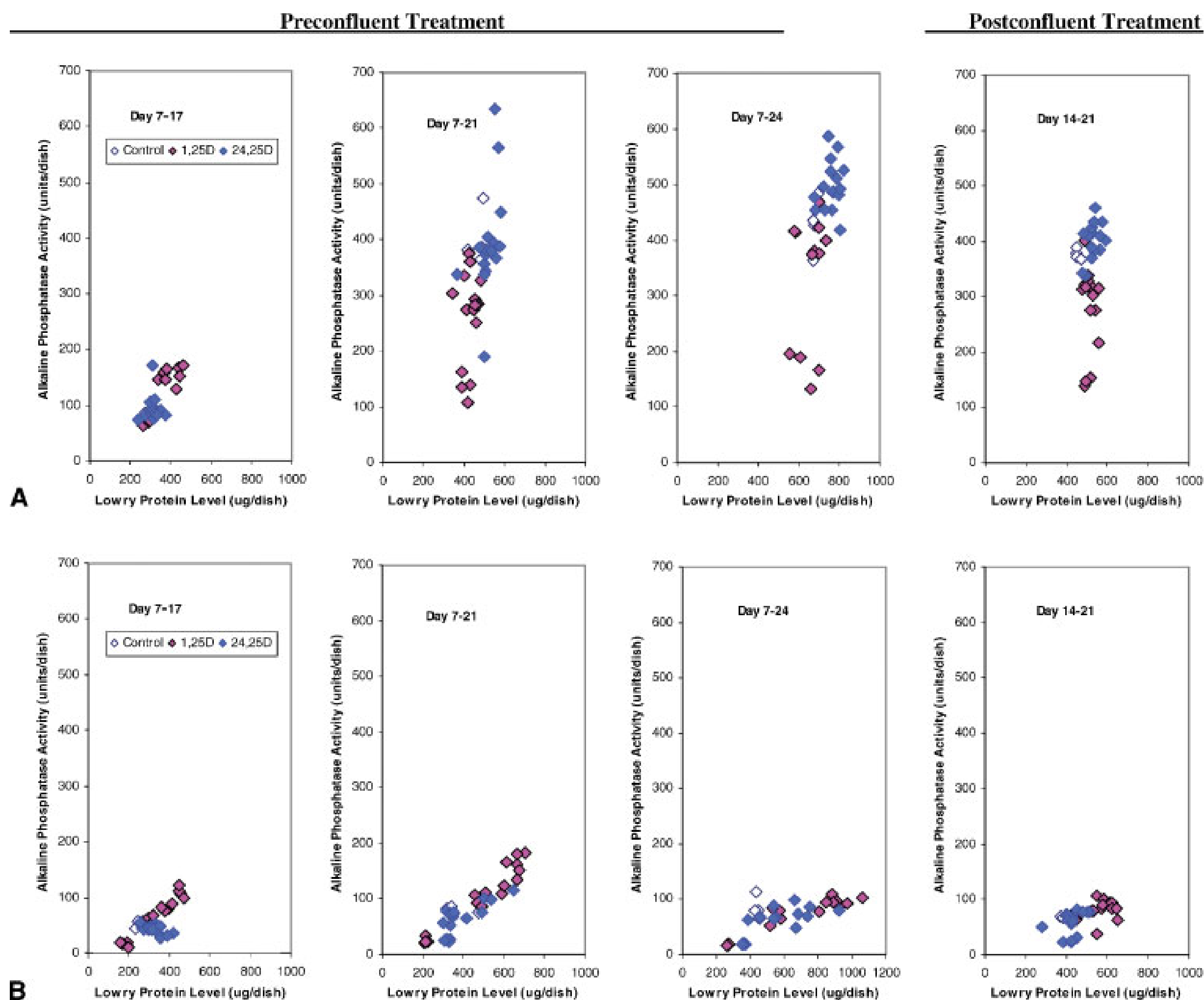

Fig. 14. Time-dependent effects of 1,25$(OH)_2D_3$ and 24,25 $(OH)_2D_3$ on the relationship between ALP activity and Lowry protein in (**A**) *Serum-containing* DATP5, and (**B**) *Serum-free* HL-1 medium. Cultures first treated on Day 7 are considered to be preconfluent; those treated from Day 14 are considered to be postconfluent. Shown are paired values of analyses of individual cultures from the untreated control (4) or the full range of metabolite-treated (16) cells: 4 cultures each for 0.01, 0.10, 1.0, and 10 nM 1,25$(OH)_2D_3$; 4 each for 0.10, 1.0, 10, and 100 nM 24,25$(OH)_2D_3$. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

the extracellular matrix. Elevated levels of PG would create a diffusion barrier impeding diffusion of O_2 to the cells; the imposed hypoxia would induce expression of LDH. Thus, even that small increase in LDH activity caused by 24,25$(OH)_2D_3$ should help these rapidly growing cells to better tolerate the hypoxic state that results from the dense PG-rich matrix that surrounds these cells [Evans et al., 1981; Rajpurohit et al., 1996].

Regarding the long controversy over 24,25 $(OH)_2D_3$, data presented in this report show that it can significantly stimulate GP chondrocytes. In fact, 24,25$(OH)_2D_3$ increased DNA, Lowry protein, and LDH activity more than 1,25$(OH)_2D_3$, but only under special conditions, which may largely explain previous failures to discern these stimulatory effects. First, 24,25$(OH)_2D_3$ exerts its stimulatory effects only under culture conditions that very closely match those occurring in vivo. These conditions are not easily met. Second, the anabolic responses to 24,25$(OH)_2D_3$ are not large and require considerable exposure time (generally >10 days). Few studies have provided this length of exposure to 24,25$(OH)_2D_3$. Third, if exposure to 24,25$(OH)_2D_3$ is delayed until after the cells attained confluence, again there is little or no effect. This last finding is in agreement with Schwartz et al. [1992, 1995] who showed that only the early cells are sensitive, and that 24,25$(OH)_2D_3$ promotes

advancement to the GP phenotype. Thus, timing, level of administration, length of exposure, and critically, the nature of the culture medium, all strongly influence the observed response.

The notable differences in cellular responses to $1{,}25(OH)_2D_3$ in *serum-containing* and *serum-free* medium merit further discussion. In human serum, vitamin D metabolites are bound with high affinity by α-globulin and with high capacity by albumin [Haddad, 1979; Bikle et al., 1985, 1986]; over 99% of the total $1{,}25(OH)_2D_3$ present in serum is bound [Bikle et al., 1985]. Thus, in *serum-containing* media, most of the $1{,}25(OH)_2D_3$ would be sequestered, markedly lowering its ambient concentration. This helps explain the muted response of preconfluent cells to $1{,}25(OH)_2D_3$ in *serum-containing* medium. But during cell culture, synthesis of PG also shields the cells from protein-bound $1{,}25(OH)_2D_3$. GP chondrocytes produce a PG-rich matrix that forms a diffusion barrier to protein-bound metabolites [Kuettner and Kimura, 1985; Comper and Williams, 1987; Torzilli et al., 1997; Christakos et al., 2003]. This would explain why postconfluent cells were not inhibited by high levels of $1{,}25(OH)_2D_3$. Chondrocytes synthesize and export PG into their extracellular matrix, shielding themselves from external influences. A striking example of this is the finding that normal GP chondrocytes in vivo manifest clear signs of essential fatty acid deficiency, even in the face of normal serum fatty acid levels [Adkisson et al., 1991]. This finding, observed in a wide variety of species, can be most readily explained by the fact that fatty acids are carried in blood by serum albumin, which cannot diffuse through the PG barrier.

Another striking finding from this aspect of the study was the remarkable inhibitory effect that $1{,}25(OH)_2D_3$ had on mineral deposition. Its effects were rapid, dosage-dependent, and profound. Equally interesting was the finding that $24{,}25(OH)_2D_3$ had a specific stimulatory effect on mineral formation that exceeded its general anabolic effect on DNA and Lowry protein levels. The degree of stimulation was not large and required considerable exposure time, but it was consistent, specific, and persistent. Thus, these two major vitamin D metabolites exert diametrically opposite effects on mineralization. What makes these findings especially interesting is the fact that they make good physiological sense.

Careful studies by Bushinsky et al. [1985a,b] have documented that the level of $1{,}25(OH)_2D_3$ formation is inversely proportional to the degree of hypocalcemia; other studies indicate that hypocalcemia may induce $1{,}25(OH)_2D_3$ formation indirectly via parathyroid hormone [DeLuca, 1988; Holick, 1995]. In any event, $1{,}25(OH)_2D_3$ has long been known to stimulate intestinal Ca^{2+} absorption [Holick, 1996]. Under hypocalcemic stress, $1{,}25(OH)_2D_3$ also causes release of skeletal Ca^{2+} by indirect activation of osteoclasts [Merke et al., 1986]. Both of these actions serve to replenish Ca^{2+} levels in the blood. What our data now also show is that $1{,}25(OH)_2D_3$ can conserve Ca^{2+} by directly inhibiting mineral deposition during bone development (endochondral calcification). The extent of inhibition depends of the level of $1{,}25(OH)_2D_3$; this enables the body to rapidly suppress mineral deposition in proportion to the degree of Ca^{2+} deficiency. There is no information available on whether $1{,}25(OH)_2D_3$ directly affects osteoblastic mineralization under physiological conditions [DeLuca, 1988].

In contrast, while $24{,}25(OH)_2D_3$ has been considered to be inert by many [DeLuca, 1988; Reichel et al., 1989; Holick, 1996], there are persistent reports that it does have biological activity [Sebert et al., 1982; Nakamura et al., 1987, 1992; Schwartz et al., 1989]. Our findings strongly support this latter concept, and document that $24{,}25(OH)_2D_3$ can play a significant stimulatory role in GP mineralization. However, its functionality depends heavily on the circumstances in which it is presented. If given in *serum-free* HL-1 medium, or after the GP cells have attained confluence, $24{,}25(OH)_2D_3$ was largely without effect. However, when presented to preconfluent cells in a *serum-containing* medium that supports mineralization, $24{,}25(OH)_2D_3$ not only had a general anabolic effect on cell proliferation and protein synthesis, but over and above this enhanced mineral deposition. The effects were not large or immediate; they took 14–17 days exposure before full stimulation of mineral deposition was evident. This critical time-dependency for exposure to $24{,}25(OH)_2D_3$ is consistent with previous studies by Schwartz et al. [1995] which indicate that only in the earliest stages of differentiation are GP chondrocytes sensitive to the metabolite.

From a physiological perspective, the rapid response to $1{,}25(OH)_2D_3$ and the relatively slow

measured response to $24,25(OH)_2D_3$ are reasonable. A decrease in blood Ca^{2+} is an emergency that requires immediate remediation. Thus, it makes sense to rapidly shut down new bone formation when this condition occurs. On the other hand, bone formation requires a steady, adequate supply of both Ca^{2+} and Pi. While their simple presence enables mineralization to occur, the persistent presence of $24,25(OH)_2D_3$ clearly stimulates bone development. Our data show is that this metabolite is not inert, but exerts a significant stimulatory effect that would enhance bone formation. Thus, in-vivo the presence of an adequate supply of Ca^{2+} would signal production of $24,25(OH)_2D_3$ from $25\text{-}OH\text{-}D_3$, which in turn would directly stimulate bone formation. This interpretation is consistent with the slow persistent anabolic effects of $24,25(OH)_2D_3$ on in vivo bone formation reported by Nakamura et al. [1992].

Our study also shows that the inhibitory effect of $1,25(OH)_2D_3$ on mineralization is direct. The effects of vitamin D_3 metabolites on mineralization were carried out in primary cultures of chicken GP chondrocytes in which adequate levels of Ca^{2+} and Pi were continuously present. Despite the fact that mineralization progressed readily in the controls, $1,25(OH)_2D_3$ powerfully inhibited it in a dosage-dependent manner. During the incipient stages of mineral formation, even the lowest level of $1,25(OH)_2D_3$ tested (0.01 nM) markedly inhibited Ca^{2+} and Pi deposition. Higher levels of $1,25(OH)_2D_3$ caused progressively greater and more persistent reductions. These findings are consistent with a primary function of $1,25(OH)_2D_3$ in maintaining normal Ca^{2+} levels in the blood. Our studies now show that $1,25(OH)_2D_3$ conserves Ca^{2+} by potently blocking mineralization of the growth plate.

Also of interest was the effect of the two vitamin D metabolites on ALP activity, in as much as recent gene knockout studies in mice unequivocally establish the importance of this enzyme in mammalian skeletal mineralization [Narisawa et al., 1997; Fedde et al., 1999]. From these studies, insight into the role of inorganic pyrophosphate (PPi)-generating enzymes have also become evident, pointing to the importance of a balanced rate of production of PPi and the activity of ALP in local destruction of this inhibitor of mineralization [Hessle et al., 2002]. Nevertheless, very recent studies reveal that other factors also must be involved in *long bone* mineralization, because double knock-out of the genes for both ALP and nucleotide pyrophosphatase phosphodiesterase 1 (NPP1), a key PPi-generating enzyme in skeletal tissues, failed to correct the osteomalacia of the long bones of these mice [Anderson et al., 2005]. This reveals that ALP must have a role beyond its action as a phosphatase for PPi. This is in accord with our current findings, which failed to find a close correlation between the effects of the two vitamin D metabolites on ALP *activity* and mineralization.

Also of direct pertinence here is the finding in ALP-deficient mice that mineral forms normally within matrix vesicles (MVs), but fails to emerge and trigger general tissue mineralization [Anderson et al., 1997]. This reveals that mineral formation within MV is not dependent of ALP, and suggests that ALP must somehow be required for the egress of mineral from MV. Indications of such a role were uncovered nearly a decade ago when it was discovered that there was a progressive die-off of ALP activity during MV mineralization, which was closely correlated with the extent of mineral formation [Genge et al., 1988]. Thus, because of the intimate association between MV and ALP, mineral formation within MV followed by penetration through the vesicle membrane may result in ALP denaturation or occlusion and subsequent loss of activity.

The question then arises how $1,25(OH)_2D_3$ brings about such profound inhibition of mineral deposition. Since inhibition occurs relatively rapidly, a logical target would be MVs, which trigger mineral formation by GP cells. Extensive studies have been made on the effects of $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ on MVs derived from passaged cultures of GP chondrocytes [Schwartz et al., 1988b; Dean et al., 1996; Greising et al., 1997; Maeda et al., 2001]. These studies recently focused on the rapid activation of phospholipase A_2 (PLA_2) by membrane receptor-mediated action of $1,25(OH)_2D_3$ [Boyan et al., 2002]. Thus, $1,25(OH)_2D_3$ may inhibit mineralization by activating PLA_2, which could destabilize MVs before they were able to form mineral within their lumen. But the timing is critical. PLA_2 has been shown to stimulate mineral formation in a unilamellar liposomal model of MV calcification—if the vesicles are preincubated to allow mineral to form within the lumen [Blandford et al., 2003]. Note in the current study that

inhibition by $1,25(OH)_2D_3$ was most profound early in the mineralization process.

An equally important question is how $24,25(OH)_2D_3$ stimulates mineralization. The fact that this was a relatively slow process requiring many days of exposure to the metabolite suggests an indirect mechanism. Also, $24,25(OH)_2D_3$ was effective only in *serum-containing* DATP5 medium, raising the possibility of a serum-derived cofactor. However, DATP5 also contains other components designed to mimic the extracellular fluid of avian GP cartilage, including the slightly elevated levels of Pi (~2 mM). Recent studies reveal that Pi is an important regulator of numerous genes involved in osteoblast differentiation [Beck, 2003; Beck and Knecht, 2003; Beck et al., 2003; Conrads et al., 2005]. It is possible that $24,25(OH)_2D_3$ in combination with the slightly elevated Pi levels exerted its general anabolic effect via this mechanism.

Finally, it is important to note that the control values for mineral deposition by GP cultures in *serum-containing* DATP5 medium in the current study were comparable to those observed previously with chicken GP chondrocytes cultured in the same medium [Wu et al., 1997a,b] However, in the current study, Ca^{2+} and Pi deposition in *serum-free* HL-1 medium was minimal (data not shown). This lack of mineralization had not been observed previously in HL-1 medium [Wu et al., 1997a,b]. One possibility is that these cells did not express adequate levels of ALP activity, perhaps because they were less mature and did not remain in contact with serum for an adequate period of time. Factors in serum have been shown to be essential for the normal expression of ALP activity in GP chondrocytes [Ishikawa et al., 1987; Ishikawa et al., 1991]. In any case, the levels of ALP in cells cultured in the *serum-free* HL-1 medium were only a fraction (~20%) of those seen in *serum-containing* DATP5 medium. In our earlier studies, the levels of ALP in HL-1 medium also tended to be low, and yet mineralization occurred. However, again, in those studies the extent of mineralization was not closely correlated with ALP activity in either DATP5 or HL-1 medium.

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