Alterations of the γ-Carboxyglutamic Acid and Osteocalcin Concentrations in Vitamin D-deficient Chick Bone*

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The content of osteocalcin and protein bound γ-carboxyglutamic acid (Gla) was studied as a function of bone maturation and mineralization in normal and vitamin D-deficient, rachitic chickens. The Gla/Ca2+ ratio was elevated in rachitic bone, particularly in the most undermineralized regions. For example, there is a 10- to 20-fold elevation in Gla/Ca2+ in the newly synthesized, least mineralized rachitic bone fraction, which progressively decreases to a 1.5-fold elevation in the most highly mineralized areas of rachitic tissue. Osteocalcin, which is the principal Gla-containing protein of mature bone, was quantitated by radioimmunoassay using specific antiserum to the 5670-dalton chicken protein. Surprisingly, the osteocalcin concentration is decreased 50% in vitamin D-deficient bone. From this we infer that accumulated Gla-containing protein in vitamin D-deficient and poorly mineralized bone may possibly represent a precursor of osteocalcin.

Osteocalcin (1–3) is a vitamin K-dependent protein synthesized in bone containing 3 γ-carboxyglutamic acid residues/molecule (5200–5900 daltons). A small fraction of total body osteocalcin (10–14–10–10) is not bound to skeletal mineral but circulates in blood plasma (4). The amino acid sequences of osteocalcin from cow (2), human (5), chicken (6), and monkey (7) show that many structural features are identical. While a precise physiological role remains to be established, osteocalcin exhibits several interesting calcium binding properties in vitro, including the ability to inhibit hydroxylapatite formation from metastable calcium phosphate solutions (2) and a highly specific calcium-dependent binding to hydroxyapatite (8). In vivo this latter property is reflected in the constant Gla3/calcium molar ratio observed in both normal bone (9) and pathologically mineralized tissues (10–12).

Biosynthetic studies in chicken indicate that the M, 5670 osteocalcin might arise from a higher molecular weight Gla-containing protein. A 70,000 14C-labeled vitamin K-dependent protein is synthesized by chick bone in organ culture and bone microsomal preparations (13). Several Gla-containing proteins from 10,000 to >80,000 daltons have been shown to occur in embryonic bone by specific 3H-labeling of Gla residues (14). Finally, a M, 9000 intracellular protein has been identified in rat osteosarcoma cells with immunological identity to osteocalcin and has been tentatively identified as an intracellular precursor of osteocalcin (15).

The role of osteocalcin in the mineralization process appears controversial. For example, in studies of matrix-induced bone formation the amino acid Gla appears coincident with calcium deposition (16), while the appearance of osteocalcin is delayed (17). Osteocalcin has not yet been shown to be involved in the formation of the solid mineral phase; however, the appearance of Gla-containing proteins, including osteocalcin, coincident or just prior to mineralization of embryonic chick bone (18) and in fetal rat bone (19) suggest an important role for such proteins in bone formation and matrix maturation.

Several studies (20, 21, 23) have indicated that vitamin D affects osteocalcin. Plasma osteocalcin levels are increased in normal rats after 1,25-dihydroxyvitamin D3 injection (21), as in patients with vitamin D-dependent rickets and x-linked hypophosphatemia during vitamin D therapy. An indicator that osteocalcin synthesis may be regulated by vitamin D is the report that addition of 1,25-dihydroxyvitamin D3 to rat osteosarcoma cell cultures increases the in vitro level of osteocalcin (23). Our group has shown (20) that severely demineralized cortical bone from rachitic chickens contains significantly increased quantities of Gla-containing proteins when compared to bone from control chickens. From these preliminary observations we have inferred that Gla-containing proteins may be involved in preparing osteoid for mineralization. In this paper we explore in detail the relationship between the concentrations of osteocalcin and total Gla as a function of the degree of bone mineralization in control and vitamin D deficient, rachitic chicks.

**EXPERIMENTAL PROCEDURES**

**Animals**

Fifty or 100 1-day-old white Leghorn male chicks obtained from Spafas, Inc. (Norwalk, CT) were kept in wire-bottom brooders with constant heat source at 28 °C. The experimental chicks were maintained on a special diet (Reit Rachitogenic Diet, ICN Pharmaceuticals, Cleveland, OH) which contained no vitamin D3 and 0.9% calcium (low calcium, normal phosphorus). Control chicks were fed the same diet, but with vitamin D3 (400,000 units/g added) and a standard chick diet (No. 904603, ICN Pharmaceuticals). Weight gain was similar in both control groups, and bone formation as assessed by calcium and phosphorus were normal.


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‡ Osteocalcin has also been referred to as "bone Gla protein" or "BGP" in the literature (41).

¶ The abbreviation used is: Gla, γ-carboxyglutamic acid.
Deficiency—Animals maintained on the low calcium, vitamin D-deficient diet failed to grow as rapidly as control animals and developed the biochemical and histological changes as associated with vitamin D deficiency and rickets (Table I). The failure to mineralize bone normally was also evident from the histological appearance of widened osteoid seams.

Preparations of control and rachitic bone were analyzed before fractionation as shown in Table II. Chick membranous and endochondral bone exhibit biochemical changes characteristic of the rachitic state, including decreased calcium, increased protein-rich osteoid, and consistent with previous findings (34-37), hydroxylysine is elevated in the bone collagen of rachitic animals (Table II). When the total Gla content of calvaria and long bone diaphysis is measured by amino acid analysis (Table II), rachitic bone is not significantly different from the control. Only in the metaphyseal portion of rachitic long bone is Gla significantly decreased (p < 0.001). This is to be expected, since rachitic metaphysis is greatly enriched in cartilaginous tissue.

Changes in Osteocalcin by Radioimmunoassay—Measurements of osteocalcin in all rachitic bone samples show dramatic decrease from control bone. In rachitic chick diaphysis, where no significant changes in Gla content occur, a 58% decrease in radioimmunoassayable osteocalcin content is observed; metaphysis and calvaria show decreases in osteocalcin of 94 and 84%, respectively (Table II). Since osteocalcin concentration is expressed per gram of dry weight of bone, it is of interest to examine Gla content per gram of bone dry weight. Since rachitic bone has slightly more protein (and less mineral) expressed by per cent dry weight of the bone powder, Gla content of mid-diaphyseal cortical bone appears slightly decreased in rachitic bone (78 residues/10^6 amino acid residues) as a function of total protein because total protein is slightly elevated. Conversely, when nanomoles of Gla/mg of bone dry weight is calculated, the Gla content of rachitic, diaphyseal bone appears elevated (1.4 nmol of Gla/mg of bone versus 0.8 in control bone) as a function of total protein because total protein is slightly elevated. However, when nanomoles of Gla/mg of bone dry weight is calculated, the Gla content of rachitic, diaphyseal bone appears elevated (1.4 nmol of Gla/mg of bone versus 0.8 in control bone), since mineral is decreased in rachitic bone. Thus, normalized to similar parameters, Gla is slightly elevated in rachitic bone where osteocalcin is decreased 58%.

Bone Density Fractionation—To unmask significant changes that are occurring in recently synthesized and extremely rachitic bone of 6-week-old vitamin D-deficient chicks, density fractionation was performed. The histogram in Fig. 1 clearly depicts the defect in mineralization of diaphyseal bone. More than 65% of the rachitic bone consists of tissue with a density less than 2.0 g/cm^3 compared with 5% in normal bone. Variation in bone density is attributable to variable calcium phosphate mineral (hydroxylapatite) content, and this parameter has been monitored by calcium analysis. The calcium content of any given density fraction from normal bone is identical to the calcium content of the same fraction from rachitic bone. Fig. 2 illustrates that the concentration of Gla as a function of calcium content is significantly elevated in rachitic bone in all but the most heavily mineralized density fractions. At each density, control and rachitic bone are com-
Table II
Analysis of 6-week-old control and rachitic chick bone
Values are the means ± S.E.M. for four independent experiments with n = 12 birds/group.

<table>
<thead>
<tr>
<th></th>
<th>Ca*</th>
<th>Protein*</th>
<th>100 Hyl/Hyp</th>
<th>Gla*</th>
<th>Osteocalcin*</th>
<th>mg/g bone dry wt</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>%</td>
<td></td>
<td>%</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>Calvaria</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>Control</td>
<td>20 ± 0.6</td>
<td>23 ± 1.4</td>
<td>3.9 ± 0.2</td>
<td>55 ± 3</td>
<td>1.16 ± 0.2</td>
<td></td>
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<tr>
<td>Rachitic</td>
<td>14 ± 0.4</td>
<td>36 ± 2.1</td>
<td>7.4 ± 0.2</td>
<td>49 ± 4</td>
<td>0.184 ± 0.09</td>
<td></td>
</tr>
<tr>
<td>Long bone</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diaphysis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>24 ± 0.3</td>
<td>20 ± 2.0</td>
<td>5.6 ± 0.3</td>
<td>82 ± 3</td>
<td>1.8 ± 0.3</td>
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<tr>
<td>Rachitic</td>
<td>23 ± 0.4</td>
<td>25 ± 1.3</td>
<td>10.1 ± 0.2</td>
<td>78 ± 5</td>
<td>0.75 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>Metaphysis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>23 ± 0.5</td>
<td>22 ± 1.9</td>
<td>7.4 ± 0.3</td>
<td>76 ± 4</td>
<td>1.3 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>Rachitic</td>
<td>14 ± 1.2</td>
<td>30 ± 2.4</td>
<td>13 ± 0.7</td>
<td>56 ± 4</td>
<td>0.08 ± 0.04</td>
<td></td>
</tr>
</tbody>
</table>

*Expressed relative to dry bone weight.
*Gla residues/10^5 amino acid residues.
*Determined by radioimmunooassay.

Elevated Gla and Decreased Osteocalcin in Rachitic Bone

Reproducible in terms of total mineral and protein content. Paradoxically, this dramatic increase in Gla content of rachitic bone density fractions is obscured by the analysis of whole unfractonated bone. To explain the Gla increase a calculation of total weighted Gla content has been performed. As illustrated in Table III, when the Gla concentration of each density is multiplied by the weight per cent of that fraction and the computed Gla of each fraction totaled, the total Gla content computed from all the fractions of rachitic bone (112 residues/10^5 amino acids) is equivalent to the total Gla content of the fractions from normal bone (112 residues/10^5 amino acids).

Thus, there is a shift in the distribution of the Gla protein, accumulating in the low density, undermineralized portion of rachitic bone, rather than a net increase in Gla protein. Osteocalcin content was measured in each density fraction to determine if its concentration paralleled the changes in Gla content (Fig. 3). As shown in Fig. 3, osteocalcin content was decreased in every fraction of rachitic bone, while Gla content of these same fractions was elevated. A 30-50% decrease was found in the higher density fractions (1.8-2.1 g/cm³). In the lowest density fractions, the decrease in osteocalcin is even more pronounced when compared to the 10- to 15-fold elevated Gla content.

Extractability of Gla versus osteocalcin—Table IV shows

![Distribution of bone of various densities in 6-week-old normal (A) and rachitic (B) chicks. The increased proportion of incompletely mineralized bone tissue in chicks raised on a vitamin D-deficient and low calcium diet is very striking. Bars indicate the range of values in three independent preparations.](image_url)

![The change in Gla content in the density fractions of control (○) and rachitic (●) bone plotted as a function of the calcium content of the tissue fraction. Also shown is calcium content (○) of each fraction which is identical in rachitic and control fractions.](image_url)
results of analysis for both Gla and osteocalcin in 0.5 M EDTA (plus protease inhibitors) extracts of bone. Total extracted Gla/unit of solubilized protein is equivalent in rachitic and control bone (49 residues of Gla/1000 glutamic acid), but the amount of osteocalcin/mg of total bone which can be detected by radioimmunoassay is decreased by 40%. Additionally, more Gla remains in the residue of rachitic bone (17% of total) after EDTA extraction than control (8%). Partial solubilization of Gla remaining in EDTA-insoluble residue is achieved with 4 M guanidine hydrochloride (Table III), but this extract from rachitic bone also shows decreased osteocalcin by radioimmunoassay. These results were reproduced in several experiments with separate groups of chicks.

**DISCUSSION**

Bone is heterogeneous in terms of the age and maturity of both its mineral and organic matrix constituents. In the case of rapidly growing animals, bone is in a physiologically dynamic state with constant remodeling and turnover of the tissue. This is evidenced by the heterogeneous density fractions obtained from an anatomically homogeneous and intact region of bone, the mid-diaphysis (Fig. 1A). Since analyses on whole bone samples represent a weighted average of microscopic portions of tissue of different physiological maturity, metabolic changes occurring in a small portion of the total bone mass may not be detected. This is illustrated with results obtained for the concentration of Gla in the composite protein matrix of normal or rachitic bone (Table III). These data show that while there is little difference in the overall concentration of Gla in the composite protein matrix of rachitic bone as compared to normal bone, analyses of the individual low density fractions reveal that, in fact, during the early stages of mineralization rachitic bone contains a significantly higher concentration of Gla-containing protein than does normal bone. In rachitic bone the rate and the amount of mineral deposited in the tissue are diminished and much more of the bone consists of tissue which is undermineralized (Fig. 1). Thus, in the analysis of unfractionated rachitic bone the net Gla content is comparable to or slightly lower than normal bone, but the concentration of Gla in the fractions of maturing bone is distributed differently (Figs. 2 and 3 and Table III). These data point out very clearly the limitations of using whole bone analyses to detect certain changes in the composition of bone matrix constituents in circumstances where the changes occur for limited time periods and involve alterations in bone metabolism, matrix and mineral deposition, and resorption.

These studies provide evidence that a clear distinction must be made between osteocalcin, the M, = 5670–5900 protein as measured by radioimmunoassay, and other forms of vitamin K-dependent Gla-containing proteins in bone which contribute to the total Gla content. From these measurements an apparent paradox has emerged: nearly normal levels of Gla are present in rachitic bone, but the osteocalcin content is severely diminished. Further, density fractionation of rachitic bone powder reveals a significantly elevated content of Gla in the undermineralized rachitic osteoid compared to normal bone of the same density, but this is unaccompanied by an increase in osteocalcin. Other situations exist where bone Gla and osteocalcin levels are not coupled (14, 16, 17, 19, 38, 39). For example, immature embryonic bone of chick (14, 38) and

![Graph](http://www.jbc.org/)

**Fig. 3. Gla and osteocalcin concentration in fractionated cortical bone from 6-week-old control and rachitic chicks.** A, total Gla content as a function of total protein. B, osteocalcin concentration measured by radioimmunoassay of the 0.5 M EDTA extracts of the same fractions. Bars indicate the range of triplicate determinations.

**Table IV**

<table>
<thead>
<tr>
<th>Solubilization of γ-carboxylglutamic acid and osteocalcin</th>
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<td>Values are means ± S.E.M. for triplicate determinations of one experiment.</td>
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</table>

<table>
<thead>
<tr>
<th></th>
<th>Whole midshaft</th>
<th>EDTA extract</th>
<th>EDTA residue</th>
<th>Guanidine extract</th>
<th>Guanidine residue</th>
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</thead>
<tbody>
<tr>
<td><strong>Gla</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>8.05 ± 0.05</td>
<td>48.8 ± 1.0</td>
<td>1.33 ± 0.1</td>
<td>1.6 ± 0.5</td>
<td>0.78 ± 0.09</td>
</tr>
<tr>
<td></td>
<td>(100)</td>
<td>(88)</td>
<td>(8)</td>
<td>(3)</td>
<td>(7)</td>
</tr>
<tr>
<td>Rachitic</td>
<td>8.11 ± 0.06</td>
<td>49.8 ± 1.5</td>
<td>3.09 ± 0.2</td>
<td>12.3 ± 0.3</td>
<td>1.6 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>(100)</td>
<td>(70)</td>
<td>(17)</td>
<td>(25)</td>
<td>(5)</td>
</tr>
<tr>
<td><strong>Osteocalcin</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.38 ± 0.15</td>
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<td>0.1244 ± 0.002</td>
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<tr>
<td>Rachitic</td>
<td>0.84 ± 0.11</td>
<td></td>
<td></td>
<td></td>
<td>0.0554 ± 0.003</td>
</tr>
</tbody>
</table>

| *a* Per cent recovery based on total nanomoles of Gla on original bone sample. |
| *b* Determined by radioimmunoassay. |
rat (19, 39) exhibits a greater ratio of total Gla/osteocalcin than does adult bone. During matrix-induced bone formation osteocalcin content lags behind the appearance of Gla in the interstices of the implanted matrix (16, 17).

There are several possible explanations for the disparity between total Gla and osteocalcin in rachitic bone. The decreased level of radioimmunoassayable osteocalcin could be accounted for by 1) lower molecular weight degraded osteocalcin fragments, or 2) higher molecular weight Gla-containing proteins, both of which could be weakly immunoreactive or nonimmunoreactive to the osteocalcin antisera. Regarding the first possibility, Gla-containing peptide fragments of osteocalcin are known to have an affinity for bone mineral but are also readily solubilized by EDTA (2, 38). In contradistinction, rachitic bone is deficient in mineral and potential Gla-peptide adsorption sites, yet the lowest density (osteoid) fractions of this tissue have the most dramatically elevated Gla levels (Fig. 1). Furthermore, the Gla-containing proteins in this tissue are only poorly solubilized by EDTA (Table IV). Therefore, the second possibility mentioned above appears to be the more likely one.

Thus, in rachitic bone there could be either 1) abnormal synthesis of a Gla-containing protein completely unrelated to osteocalcin (for which there is as yet no evidence), or 2) accumulation of another form of osteocalcin which is not immunoreactive. Perhaps this form may be a putative pro-osteocalcin (14) which is dependent on either vitamin D or a normal bone mineral content for conversion to osteocalcin peptide. By analogy, in the well studied case of prothrombin activation, the Gla-rich fragment 1 portion of this protein is released by proteolytic cleavage (40). In support of the latter interpretation, it is known that proteins are synthesized in bone which contain Gla and are of higher molecular weight than osteocalcin. For example, in chick bone cultures and bone microsomal preparations a 70,000-dalton Gla-containing protein can be demonstrated which is labeled by NaH\(^{14}\)CO\(_3\), in a vitamin K-dependent reaction (13). In extracts of embryonic chick bone, specific \(^{14}\)H-labeling of Gla residues has allowed identification of several higher molecular weight species of Gla-containing proteins (10,000 and 30,000–80,000) which are immunologically related to osteocalcin but only weakly cross-reactive with osteocalcin antiserum (14). In rat osteosarcoma cells an intracellular 9000-dalton protein is found to cross-react with antiserum to the 5900-dalton osteocalcin and has been tentatively identified as a precursor of osteocalcin (15). In view of positive evidence of osteocalcin precursors, it is possible that vitamin D regulates the biosynthetic rate of osteocalcin either directly (23) or indirectly by affecting the molecular processing of a putative pro-osteocalcin. Whether or not the changes in Gla and osteocalcin observed in this vitamin D-deficient, rachitic animal model are due directly to vitamin D or to hypocalcemia and/or other changes associated with the vitamin D- and Ca\(^{2+}\)-deficient state remains to be established. Studies are in progress to delineate the sequence of events in osteocalcin biosynthesis and its dependence on vitamin D metabolites, calcium, and other physiological mediators.

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