The Changes of Bone γ-Carboxyglutamic Acid-Containing Protein in Bone and Serum of Developing Chick

Chiharu TSUTSUMI, Norimasa HOSOYA,¹ and Sachiko MORIUCHI²

¹Department of Nutrition, School of Health Sciences, Faculty of Medicine, University of Tokyo, Bunkyo-ku, Tokyo 113, Japan
²Department of Food and Nutrition, School of Home-economics, Japan Women’s University, Bunkyo-ku, Tokyo 112, Japan

(Received May 12, 1983)

Summary The changes of bone γ-carboxyglutamic acid-containing protein (BGP) levels in bone and serum were studied in relation to those in calcium metabolism using chick embryos and chicks aged from 13 days’ incubation to 8 weeks old. Chick BGP was determined by radioimmunoassay using antiserum to purified chick BGP. BGP levels in bone and serum increased significantly at hatching and then decreased until 3–5 days of age. Thereafter, BGP levels in bone and serum increased gradually until 8 weeks of age. These changes of BGP levels were well correlated with those of serum calcium and inorganic phosphorus concentrations, serum alkaline phosphatase activity and bone calcium content. The molecular size of increased serum BGP at hatching was not different from that of bone BGP. These results suggest that BGP plays a role in hatching and bone formation during chick development.

Key Words bone γ-carboxyglutamic acid-containing protein (BGP), chick development, hatching, calcium metabolism

Bone γ-carboxyglutamic acid-containing protein (BGP) is found in the extracellular matrix of bone (1, 2). BGP is purified from bovine (3), human (4), chick (1), swordfish (3), rabbit (5), and rat (6, 7) bone, and their molecular weights are estimated to be approximately 5,000–6,000. BGP contains 2–3 residues of vitamin K-dependent amino acid, γ-carboxyglutamic acid (Gla) and is one of the most abundant non-collagenous bone proteins in EDTA extracts of bone (8).

BGP content of bone has been estimated from Gla content, by amino acid analysis (9) or high-performance liquid chromatography (HPLC) (10), as a ratio of Gla/glutamic acid (Glu).

However, the recent development of radioimmunoassay for BGP has shown that a small fraction of total BGP circulates in blood (11).
Amino acid analysis of embryonic chick bone has shown that Gla appears in embryonic chick bone (mandible, calvalia, tibiotarsus, and femur), coincident with the first histologically observable deposition of bone minerals at 8 to 12 days after incubation and undergoes a rapid increase relative to other non-collagenous bone proteins (12). Moreover, it was shown by radioimmunoassay, for the measurement of BGP, that BGP levels in rat limb bone increased dramatically after birth to 14 days of age up to 5-fold (12, 13). Serum BGP levels of the young growing rats were extremely high and decreased dramatically until 22 weeks of age (14).

While the precise physiological role of BGP remains to be estimated, these observations suggest that BGP is of importance either in bone calcification or the regulation of calcium between bone and blood.

Therefore, the changes of BGP levels in bone and serum were studied in relation to those of calcium metabolism, such as the changes of serum calcium and phosphorus concentrations, bone calcium and phosphorus contents and serum alkaline phosphatase activity, during the aging of 13-day-old chick embryos to 8-week-old chicks. Results were discussed as regards the physiological role of BGP during development of the chick.

MATERIALS AND METHODS

1) Preparations of formic acid extracts from the chick femur. BGP was extracted from chick bone by the method of Poser et al. (4). Chick bone was obtained from White Leghorn chick embryo and chick right femur. Average chick weight was 7.5 g at incubation of 13 days and 700 g at 8 weeks of age. Embryos were removed from groups of five to eight eggs at intervals between incubation of 13 and 20 days. Developmental age was assumed from embryo morphology, weight and bone length and confirmed by hatching at 21 days. Bone samples were freed of connective tissues and muscles, homogenized with 10 volumes of redistilled water in Polytron (PT 10/35, KINEMATIKA), washed several times by redistilled water, and then lyophilized (bone dry powder). Bone dry powder (20 mg) was extracted with 20% formic acid (400 µl) for 3 h at 25°C (formic acid extracts).

2) Purification of chick BGP. BGP was purified from 6 to 8-week-old chick femur cortical bone by EDTA extraction, gel filtration on Sephadex G-100 and ion exchange chromatography on DEAE Sephadex A-25 described elsewhere (15). Purified chick BGP gave a single band on electrophoresis in 10% acrylamide gel at pH 7.4 in the presence of 1% sodium dodecylsulfate and its amino acid analysis showed 3 Gla residues/50 amino acid residues.

3) Preparation of antibody. Two rabbits were immunized by multiple site intradermal injection of purified BGP into their backs. An additional immunization was performed in the foot pad at 3 weeks after the first injection. Each injection was made using 0.1–0.5 mg of purified BGP emulsified in complete Freund’s adjuvant (Iatron Lab., Tokyo). Serum samples were withdrawn at regular intervals and tested for the titer of antibody to BGP by radioimmunoassay.
4) **Radioiodination of chick BGP.** Purified chick BGP was labeled with $^{125}$I by the method of Hunter and Greenwood (16). Iodination of chick BGP was performed at the Radioisotope Centre of the University of Tokyo. Labeled BGP was separated from unincorporated $^{125}$I by gel filtration on a Sephadex G-15 column (0.8 x 12 cm) equilibrated with assay solution (0.14 M NaCl, 10 mM phosphate, 10 mM EDTA, 0.1% gelatin, 0.1% Tween 20 at pH 7.4). The antiserum batch which bound 20% of the iodinated BGP at the highest dilution was used for all radioimmunoassays reported here.

5) **Radioimmunoassay of chick BGP.** Radioimmunoassay was performed by the method of Price and Nishimoto (11). Assay solution contained (in order of addition): a known amount of unlabeled BGP, diluted bone formic acid extracts or 10–20 µl of chick serum in 50 µl of assay solution, 50 µl of rabbit antiserum (a final 1:8,640 dilution) in 50 µl of assay solution and about 10,000 cpm of $^{125}$I-labeled chick BGP in 50 µl of assay solution. After incubation for 48 h at 4°C, the assay was terminated by precipitation of rabbit antibody on the addition of 10 µl of goat antiserum to rabbit γ-globulin in 20 µl of assay solution. After 24 h at 4°C, the reaction mixture was centrifuged to sediment $^{125}$I-labeled BGP bound to rabbit antibody and the supernatant was discarded. Background $^{125}$I-labeled BGP which nonspecifically adhered to the precipitate or to the assay tube was measured by incubation of $^{125}$I-labeled BGP with normal rabbit serum without specific antiserum followed by the usual second antibody precipitation. Total and antibody-bound $^{125}$I-labeled BGP were determined by use of a gamma counter (Aloca ARC-221). The fraction of $^{125}$I-labeled BGP bound to antiserum is defined as cpm in precipitate minus cpm in background divided by total cpm in assay (B/Bo).

6) **Gel filtration of the bone extracts and serum on Sephadex G-75.** Gel filtration was performed at 4°C using a column (1.5 x 30 cm) of Sephadex G-75 equilibrated with 50 mM Tris-HCl (pH 7.4). The bone EDTA extracts dissolved in an aliquot of 50 mM Tris-HCl (pH 7.4) and serum (hatching, 4-week-old chick) were applied to the column, and eluted with the same buffer (10 ml/h). After gel filtration, the BGP content of each fraction was assayed by radioimmunoassay.

7) **Chemical determinations.** Embryonic chick and chick left femur were dissected free of adhering connective tissues and muscles. The bone was dried at 110°C and heated at 600°C for 24 h for the determination of ash content. Calcium concentrations in bone ash and serum were determined by atomic absorption spectrophotometry using a Shimadzu AA-610S (17). Phosphorus concentrations in bone ash and serum were measured by the method of Chen et al. (18). Serum alkaline phosphatase activity was measured by the method of Lowry (19) using p-nitrophenylphosphate as a substrate. Total serum alkaline phosphatase activity, except that of intestinal origin, is inhibited by L-bromotetramisole (20). Thus, bone origin alkaline phosphatase activity was estimated from the difference of alkaline phosphatase activity between that in the absence and presence of 1 mM L-bromotetramisole, which represents total and intestinal origin alkaline phosphatase activity, respectively.

Vol. 29, No. 6, 1983
8) **Chemicals.** All chemicals used were of analytical grade (Wako Fine Chemical Co., Tokyo). $^{125}$I was purchased from New England Nuclear (U.S.A.). Anti-rabbit Ig-G goat serum was purchased from Miles-Yeda Ltd. (Israel). Sephadex G-15, Sephadex G-75, Sephadex G-100 and DEAE Sephadex A-25 were purchased from Pharmacia Fine Chemicals (Sweden).

![Fig. 1](image1.png)

**Fig. 1.** The effect of anti-serum dilution on radioimmunoassay for chick BGP. The effect of anti-serum at 2,160-fold (△), 8,640-fold (●), and 34,560-fold (■) dilution on radioimmunoassay for chick BGP. Relative fraction of $^{125}$I-labeled chick BGP bound to antibody ($B/B_0$) at increasing levels of BGP.

![Fig. 2](image2.png)

**Fig. 2.** Radioimmunoassay cross-reactivity. Relative fraction of $^{125}$I-labeled chick BGP bound to antibody ($B/B_0$) at increasing levels of purified BGP from rat (○), bovine (□), mouse (△), and chick (●).

*J. Nutr. Sci. Vitaminol.*
RESULTS

1) Radioimmunoassay for chick BGP

Rabbit antiserum to chick BGP was used for radioimmunoassay of BGP. As shown in Fig. 1, a standard curve was constructed by plotting the relative fraction of $^{125}$I-labeled BGP bound to antibody ($B/B_0$) against increasing amounts of unlabeled BGP (0–16 ng/tube). Using final 8,640-fold diluted antiserum, 0.5–16 ng of chick BGP per tube was determined. The sensitivity was 0.5 ng BGP/tube. Intra-assay and inter-assay variations were less than 5% and 7%, respectively.

Preparation of purified BGP from rat and bovine and EDTA extracts of mouse bone were tested for radioimmunoassay cross-reactivity by measuring the effect of

![Graph 1](image1)

![Graph 2](image2)

Fig. 3. Changes of BGP contents in femur cortical bone and serum of the developing chick. * The value at hatching is significantly higher than that at incubation for 18 days ($p < 0.02$), and also higher than that at 3 days of age ($p < 0.05$). ** The value at hatching is significantly higher than that at incubation for 18 days ($p < 0.01$), and also higher than that at 5 days of age ($p < 0.01$). The values are the means of 7 determinations ± SE.
different amounts of the respective BGP preparations on the binding of $^{125}$I-labeled chick BGP to antibody. As shown in Fig. 2, BGPs from rat, bovine and mouse bones did not displace labeled chick BGP from antibody. Thus, the chick BGP antibody obtained is specific for the chick BGP (Fig. 2).

2) The changes of BGP contents of bone and serum of developing chicks

Using chick embryos and chicks from 13 days' incubation to 8 weeks old, the changes of BGP contents of bone and serum were observed in relation to calcium metabolism, and, in particular, those around hatching, in which calcium metabolism is assumed to be very active, were examined in detail.

BGP content in bone increased from 13 days’ incubation (about 0.4 μg/mg dry bone) to hatching (about 1.22 μg/mg dry bone) and then decreased until 3 days of age (about 0.8 μg/mg dry bone). Thereafter, it began to increase with development. BGP content of serum also increased from 13 days’ incubation (about 0.1 μg/ml serum) to hatching (about 1.2 μg/ml serum) and then decreased until 5 days of age (about 0.5 μg/ml serum). The increase in serum was much higher than that in bone. Thereafter, the serum BGP content began to increase with development (Fig. 3).
Changes of calcium and phosphorus contents of serum of the developing chick.

* The value at hatching is significantly higher than that at incubation for 18 days (p<0.05), and also higher than that at 3 days of age (p<0.05). ** The value at hatching is significantly higher than that at incubation for 18 days (p<0.01), and also higher than that at 3 days of age (p<0.05). The values are the means of 10 determinations ± SE.

Calcium content of bone ash showed a slight increase at the time of hatching and then increased during development (Fig. 4). On the other hand, the phosphorus content of bone ash did not show a significant increase around hatching and, rather, increased gradually with development (Fig. 4).

Serum calcium and phosphorus concentrations also increased significantly around hatching and did not show a significant change thereafter. The increase of serum phosphorus concentration at hatching was higher than that of calcium (Fig. 5).

As shown in the changes of bone and serum BGP contents, bone origin serum alkaline phosphatase activity increased from 13 days' incubation to hatching and decreased until 3 days of age. Thereafter it began to increase until 3 weeks of age and...
Fig. 6. Changes of alkaline phosphatase activity in serum of the developing chick.

-●-, total alkaline phosphatase activity; --- ○ ---, bone origin alkaline phosphatase activity. * The value at hatching is significantly higher than that at 18 days' incubation (p<0.01), and also higher than that at 3 days of age (p<0.05). The values are the means of 10 determinations ± SE.

Table 1. Correlation coefficient of BGP, calcium, phosphorus contents and alkaline phosphatase activity.

|                      | Bone BGP   | Serum BGP  |
|----------------------|------------|------------|
|                      | A | B | A | B | A | B |
| Bone BGP             | — | — | 0.72*** | 0.83** |
| Serum BGP            | 0.72**** | 0.83** | — | — |
| Serum calcium        | 0.88**** | 0.95**** | 0.79**** | 0.87*** |
| Serum phosphorus     | 0.69  | 0.75* | 0.76*** | 0.73* |
| Bone calcium         | 0.77*** | 0.93**** | 0.42  | 0.87*** |
| Bone phosphorus      | 0.78*** | 0.84*** | 0.42  | 0.49 |
| Alkaline phosphatase activity | 0.15 | 0.86*** | 0.35 | 0.84** |

(A) From 13 days' incubation to 8-week-old chick. (B) From 13 days' incubation to 5-day-old chick. The apparent positive correlations are p<0.05 (*), p<0.02 (**), p<0.01 (***), p<0.001 (****).

As shown in Table 1, there were significant correlations between bone BGP.

*J. Nutr. Sci. Vitaminol.*
BGP CHANGE IN DEVELOPING CHICK

Fig. 7. Sephadex G-75 column chromatography of the chick femur cortical bone EDTA extracts (A) and serum (hatching (B), 4 weeks of age (C)). Sephadex G-75 column (2.5 x 50 cm) equilibrated with 50 mM Tris-HCl, pH 7.4 at 4°C and eluted by the same buffer at 10 ml/h.

color content and serum BGP, calcium and phosphorus levels, bone calcium and phosphorus contents and bone origin serum alkaline phosphatase activity (only from 13 days' incubation to 5 days of age). Also, there were significant correlations between serum BGP content and serum calcium, phosphorus and bone calcium contents (from 13 days' incubation to 5 days of age) and bone origin serum alkaline phosphatase activity (from 13 days' incubation to 5 days of age).

3) Gel filtration of bone EDTA extracts and serum on Sephadex G-75

The elution profiles of BGP from serum (hatching, 4 weeks of age) on Sephadex G-75 gel filtration were not different from those of bone EDTA extracts, although protein profiles by absorbancy at 280 nm were not the same as each other (Fig. 7). These results indicate that BGP s in the bone EDTA extracts and in serum are protein of the same molecular weight, and that an increased serum BGP at hatching is also derived from bone proteins of the same molecular weight.

DISCUSSION

The changes of bone and serum BGP contents and calcium metabolism were
observed along with chick development in this study.

Hauschka et al. showed that Gla concentration in bone estimated by amino acid analysis increased in developing bones until well beyond hatching, which coincided with histologically detectable mineralization both temporally and topologically, but which levels off after about 3 to 6 weeks (9). However, in our study, which covered the developmental stages around hatching in detail and used radioimmunoassay for measuring BGP, we found that bone and serum BGP contents rose significantly around hatching and then increased again from 3–5 days after hatching. Moreover, bone ash calcium content, serum calcium and phosphorus concentrations and bone origin alkaline phosphatase activity changed in the same way as serum BGP content.

Radioimmunoassay did not distinguish uncarboxylated Gla protein, which cannot bind to bone mineral, from the native one (21). However, the increase of BGP in bone at hatching was, rather, observed in BGP content estimated from radioimmunoassay. Therefore, the difference between the changes of Gla and BGP contents in bone during hatching would not be ascribable to the delay in γ-carboxylation.

On the other hand, the chorioallantoic membrane has the role of calcium carrier in egg shell, and vitamin K-dependent calcium-binding protein is found in the chorioallantoic membrane (22). Its molecular weight is reported to be approximately 100,000 (22). However, Sephadex G-75 gel filtration showed that the increased serum BGP at hatching was protein of the same molecular weight as bone BGP. Thus, the increased serum level of BGP at hatching is unlikely to originate from chorioallantoic membrane. These results suggest that the transient rise of BGP at hatching is ascribable to the increase of native BGP and that the reason why Hauschka and Reid (9) missed this phenomenon is that they observed only bone, because the change of BGP content in bone is rather smaller than that in serum. Moreover, they determined Gla weekly after hatching.

It is not clear whether the changes of BGP contents in bone and serum were ascribable to the result of marked calcium metabolism during hatching, or vice versa. However, why do BGP contents of bone and serum show an elevation at hatching? The recent finding that 1,25-(OH)2-D3 regulates the synthesis of BGP by rat osteosarcoma cells has raised the possibility that BGP may mediate some action of vitamin D on bone (23). 1,25-(OH)2-D3 administration elevates serum BGP levels in the rat (24). Moreover, Seino et al. (25) demonstrated that serum 1,25-(OH)2-D3 concentration is maximal at hatching and decreases thereafter. The possible explanations of these effects are that 1,25-(OH)2-D3 increases the synthesis of BGP and that this increased BGP synthesis results in the elevated serum BGP levels at hatching. Moreover, the elevation of serum BGP and 1,25-(OH)2-D3 concentration at the same time would be required for hatching.

On the other hand, there was a significant correlation between BGP content of bone and calcium and phosphorus contents of bone. This suggests that BGP has a biological role concerned with bone formation.

J. Nutr. Sci. Vitaminol.
REFERENCES

1) Hauschka, P. V., Lian, J. B., and Gallop, P. M. (1975): Direct identification of the calcium binding amino acid \(\gamma\)-carboxyglutamate. *Proc. Natl. Acad. Sci. U.S.A.*, 72, 3925–3929.

2) Price, P. A., Otsuka, A. S., Poser, J. W., Kristaponis, J., and Raman, N. (1976): Characterization of a \(\gamma\)-carboxyglutamic acid-containing protein from bone, *Proc. Natl. Acad. Sci. U.S.A.*, 73, 1447–1451.

3) Price, P. A., Otsuka, A. S., and Poser, J. W. (1978): Comparison of \(\gamma\)-carboxyglutamic acid containing protein from bovine and swordfish bone, in Calcium Binding Proteins and Calcium Function, ed. by Wasserman et al., Elsevier/North Holland Inc., Amsterdam, pp. 333–337.

4) Poser, J. W., Esch, F. S., Ling, N. C., and Price, P. A. (1980): Isolation and sequence of the vitamin K dependent protein from human bone. *J. Biol. Chem.*, 255, 8685–8691.

5) Price, P. A., Epstein, D. J., Lothringger J. W., Nishimoto, K. S., Poser, J. W., and Williamson, M. K. (1979): Structure and function of the vitamin K-dependent protein of bone, in Vitamin K Metabolism and Vitamin K-dependent Proteins, ed. by Suttie, J. W., University of Park Press, Baltimore, pp. 219–226.

6) Otawara, Y., Hosoya, N., Moriuchi, S., Kasai, H., and Okuyama, T. (1980): Purification and characterization of calcium-binding protein containing \(\gamma\)-carboxyglutamic acid from rat bone. *J. Nutr. Sci. Vitaminol.*, 26, 209–219.

7) Otawara, Y., Hosoya, N., Moriuchi, S., Kasai, H., and Okuyama, T. (1981): The NH\(_2\)-terminal amino acid sequence of a \(\gamma\)-carboxyglutamatic acid-containing protein from rat femur cortical bone. *Biomed. Res.*, 2, 442–446.

8) Hauschka, P. V., and Gallop, P. M. (1978): Purification and calcium binding properties of osteocalcin. The \(\gamma\)-carboxyglutamate containing protein of bone, in Calcium Binding Proteins and Calcium Function, ed. by Wasserman et al., Elsevier/North Holland Inc., Amsterdam, pp. 338–347.

9) Hauschka, P. V., and Reid, M. L. (1978): Timed appearance of a calcium-binding protein containing \(\gamma\)-carboxyglutamic acid in developing chick bone. *Develop. Biol.*, 65, 426–434.

10) Kuwada, M., and Katayama, K. (1980): A high-performance liquid chromatographic method for the simultaneous determination of \(\gamma\)-carboxyglutamic acid in proteins, bone and urine. *Anal. Biochem.*, 117, 259–265.

11) Price, P. A., and Nishimoto, S. K. (1980): Radioimmunoassay for the vitamin K-dependent protein of bone and its discovery in plasma. *Proc. Natl. Acad. Sci. U.S.A.*, 77, 2234–2238.

12) Price, P. A., Lothringger, J. W., and Nishimoto, S. K. (1980): Absence of vitamin K-dependent bone protein in fetal rat mineral. *J. Biol. Chem.*, 255, 2938–2942.

13) Price, P. A., Lothringger, J. W., Baukol, S. A., and Reddi, A. H. (1981): Developmental appearance of the vitamin K-dependent protein of bone during calcification. Analysis of mineralizing tissues in human, calf and rat. *J. Biol. Chem.*, 256, 3781–3784.

14) Otawara, Y., Hosoya, N., and Moriuchi, S. (1983): Effect of aging and castration on the changes in the levels of bone \(\gamma\)-carboxyglutamic acid-containing protein in bone and serum of female rat. *J. Nutr. Sci. Vitaminol.*, 29, 249–260.

15) Sakaguchi, C., Otawara, Y., Hosoya, N., Moriuchi, S., Kasai, H., and Okuyama, T. (1982): The effect of vitamin D on the properties of vitamin K dependent calcium-binding protein in chick femur cortical bone. *Vitamins* (in Japanese), 56, 13–18.
16) Hunter, W. M., and Greenwood, F. C. (1962): Preparation of iodine-131 labeled human growth hormone of high specific activity. *Nature*, 194, 495–496.

17) Willis, J. B. (1961): Determination of calcium and magnesium in urine by atomic absorption spectroscopy. *Anal. Chem.*, 33, 556–559.

18) Chen, P. S., Toribara, T. Y., and Warner, H. (1956): Microdetermination of phosphorus. *Anal. Chem.*, 28, 1756–1758.

19) Lowry, O. H. (1957): Micromethod for the assay of enzyme. II Specific procedures, alkaline phosphatase, in Method in Enzymology IV, ed. by Colowick, S. P., and Kaplan, N. O., Academic Press, New York, pp. 371–372.

20) Borgers, M., and Thone, F. (1975): The inhibition of alkaline phosphatase by L-p-bromoteramisole. *Histochemistry.*, 44, 277–280.

21) Price, P. A., Williamson, M. K., and Lothringer, J. W. (1981): Origin of the vitamin K-dependent bone protein found in plasma and its clearance by kidney and bone. *J. Biol. Chem.*, 256, 12760–12766.

22) Tuan, R. S., and Scott, W. A. (1978): Calcium-binding protein of chorioallantoic membrane. Identification and developmental expression. *Proc. Natl. Acad. Sci. U.S.A.*, 74, 1946–1949.

23) Price, P. A., and Baukol, S. A. (1980): 1,25-Dihydroxyvitamin D₃ increases synthesis of the vitamin K-dependent bone protein by osteosarcoma cells. *J. Biol. Chem.*, 255, 11660–11663.

24) Price, P. A., and Baukol, S. A. (1981): 1,25-Dihydroxyvitamin D₃ increases serum levels of the vitamin K-dependent bone protein. *Biochem. Biophys. Res. Commun.*, 99, 928–935.

25) Seino, Y., Yamaoka, K., Ishida, M., Yabuuchi, H., Ichikawa, M., Ishige, H., Yoshino, H., and Avioli, L. V. (1982): Biochemical characterization of 1,25-(OH)₂-D₃ receptors in chick embryonal duodenal cytosol. *Calcif. Tissue. Int.*, 34, 265–269.