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Guanosine 3′,5′-Monophosphate in Bone: Microscopic Visualization by an Immuno-Histochemical Technique

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Summary. Guanosine 3′,5′-monophosphate (cyclic GMP, cGMP) was localized in bone cells by the use of an immunoglobulin-enzyme bridge method. We observed that in cat alveolar bone most osteoblasts did not stain for cGMP, while adjacent periodontal cells displayed cytoplasmic as well as nuclear staining. Numerous osteocytes contained diffuse reaction products over most or all of the cellular area. The method used in this study may be helpful in identifying specific hard tissue cell types whose function(s) involve cGMP.

Key words: Bone cells — Cyclic GMP — Immunohistochemistry.

Introduction

Although the role of the cyclic nucleotides in bone metabolism has been studied [9, 11, 12, 14], little is known concerning the involvement of guanosine 3′,5′-monophosphate (cGMP) in regulating the response of bone cells to external stimuli [13]. Moreover, virtually nothing is known regarding the exact locations in bone of cells containing cGMP.

Recently, we have developed a method which enables microscopic localization in bone of cells containing adenosine 3′,5′-monophosphate (cAMP) [3]. We report here the successful utilization of a similar immuno-histochemical technique for the localization of cells containing cGMP in frozen, undecalcified sections of bone.

Materials and Methods

Preparation of Cat Jaw Sections: Young adult female cats 10–12 months old, were the three experimental animals. Following sacrifice the head was removed and immediately placed in liquid N_2. The frozen jaws were chiseled off and divided sagittally in two halves. One half of each jaw was randomly selected for the microscopic localization of cGMP and cAMP, while the other half provided bone samples for the measurement of the concentrations of the cyclic nucleotides at various sites. The selected jaw was then embedded in a 2% solution of methyl cellulose (Fisher Scientific Co., Fair Lawn, N.J.) in distilled water. Immediately after embedding, the medium containing the jaw was rapidly frozen in liquid N_2, then transferred to a freezer. Overnight storage in the freezer gradually elevated the tissue block temperature to −25°. Sagittal sections, 6 μ thick, were prepared with a Jung model K cryostat microtome and collected on an adhesive tape (No. 810, 3M/Co., St. Paul, Minn.).

During all of the following immuno-histochemical and staining procedures the sections remained attached to the adhesive tape. After final dehydration in alcohol and acetone, the sections were transferred to glass slides and immersed in Euparal (A.H. Thomas, Philadelphia, Pa.).

Preparation of anti-cGMP Antibodies: Sodium 2′0′-monosuccinyl guanosine 3′,5′-cyclic monophosphate (Sigma Chemical Co., St. Louis, Mo.) was coupled to either bovine or rabbit serum albumin (Miles Laboratories, Elkhart, Ind., Fraction V) as previously described [17]. Spectrophotometric analyses revealed a substitution ratio of 6 cGMP residues per albumin molecule in each case [17]. Antiserum to cGMP was produced in New Zealand white rabbits by repeated injections of cGMP-bovine serum albumin (1–4 mg) emulsified in complete Freund's adjuvant. Injections were given biweekly and blood collected 10 days after each injection. Pooled sera from rabbits were tested by radioimmunoassay [17] for reactivity against cGMP and cAMP. At least 50% of the rabbits responding to cGMP displayed cross-reactivity with cAMP. Pooled antiserum obtained from sequential bleedings of a single rabbit showing no cross-reactivity for cAMP (0% binding of cAMP by radioimmunoassay) were used for antibody isolation. Antibodies were isolated employing an immune-adsorbant column consisting of sepharose 2B coupled to rabbit serum albumin and the succinyl derivative of cGMP, as described previously for the isolation of cAMP antibodies [3]. The column was equilibrated in BBS (0.15 N NaCl–0.5 M borate buffer, pH 8.3) and the effluent was monitored at 280 nm. Hyperimmune serum (50 to 80 ml) was passed through the column, after which the column was extensively washed with BBS and the antibodies were eluted with 3.0 M KSCN. The fractions containing antibody were pooled and immediately dialyzed 4 times against 5000 fold excess amounts of BBS. Antibodies were concentrated by negative pressure ultrafiltration and quantitated spectrophotometrically using an extinction coefficient ($E^{1\%}_{280\,nm}$) of 15.0. In

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addition, the BBS eluate from the adsorbant column was reconstituted to the original serum volume. Both the BBS eluate and the concentrated antibody fractions were tested for reactivity to cGMP and cAMP by radioimmunoassay [17]. The BBS eluate showed a 40 fold reduction in binding activity for cGMP when compared with the starting serum. A second passage through a regenerated adsorbant column removed completely the cGMP reactivity from the subsequent BBS eluate. The purified antibodies showed enhanced binding activity with cGMP (due to the concentration effect) and no binding activity with cAMP. Immunoelectrophoresis revealed that the purified antibodies were IgG. Dilutions (concentrations ranging from 300–450 μg/ml) of purified antibodies (rabbit anti-cGMP, IgG class) were directly applied to tissue sections.

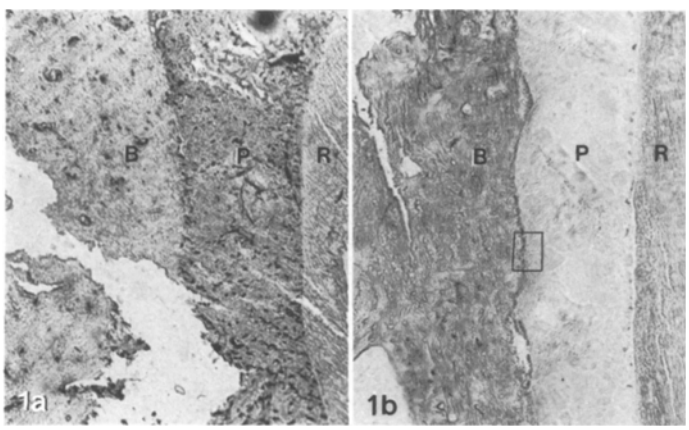
Microscopic Detection of Bone Cells Containing cGMP: Following our previous work on the histologic detection of cAMP in bone [3], we have used the immunoglobulin-peroxidase bridge method of Mason et al. [10] to detect cGMP in bone sections. In this method, the tissue sections are incubated with 3 immunoglobulins (IgG class) and horseradish peroxidase in the following sequence: (a) rabbit anti-cGMP antibodies; (b) goat anti-rabbit IgG (Cappel Laboratories, Dowingtown, Pa., lot no. 8576, antibody protein: 6.3 mg/ml, diluted 1:4); (c) rabbit anti-horseradish peroxidase (Cappel Laboratories, lot no. 7625, antibody protein: 2.3 mg/ml, diluted 1:4); and (d) horseradish peroxidase (Cappel Laboratories, lot no. 8264, 2 mg/ml, diluted to 1.25 mg/100 ml). The presence of peroxidase in the tissue sections was demonstrated by using 3,3′-diaminobenizidine tetrahydrochloride (DAB) (Sigma Chemical Co., St. Louis, Mo.) as described by Graham and Karnovsky [8], omitting, however, fixation with OsO_4. In the final step before dehydration and mounting, a light-blue green background staining was added by a 5 min incubation with a 0.1% fast green solution (pH 2.5).

Specificity Tests for cGMP Staining in Bone Sections: A number of control experiments were conducted to test the specificity of the staining for cGMP. Several sections were incubated with DAB only or with horseradish peroxidase and DAB, without prior incubations with any of the immunoglobulins. In other experiments, tissue sections were processed according to the bridge method, but the specific immunoglobulins used in each step were replaced by the corresponding normal, non-specific immunoglobulins. Finally, rabbit anti-cGMP antibodies were incubated at 4° for 4 h with the following compounds: cGMP (1×10^{-4} M, 1×10^{-5} M, and 1×10^{-6} M), cAMP (1×10^{-3} M, 1×10^{-4} M, and 1×10^{-5} M), 5′GMP, 5′AMP, GTP, ATP, adenosine (all at 5×10^{-3} M), or the equivalent amounts of DNA and RNA, and guanosine (1×10^{-3} M).

Results

Figure 1a presents a section of a jaw containing a root of a maxillary second premolar with its adjacent periodontal ligament (PDL) and alveolar bone. Many stained cells are visible in the PDL and bone (actual cellular stain is brown). Reaction products are also visible in the blood vessels of the alveolar bone. Figure 1b demonstrates a tissue section from the same site which was incubated with a preparation of non-specific, normal rabbit IgG, instead of the anti-cGMP antibodies. In this photomicrograph, as well as in a higher magnification of the bone–PDL junction (Fig. 1c), no cellular staining for cGMP is to be found.

The alveolar crest region of the tooth shown in Figure 1 is seen in Figure 2. Figures 2a and 2b are tissue sections which compare staining with toluidine blue (Fig. 2a) and staining for cGMP (Fig. 2b). It is apparent that while many cells are stained by toluidine



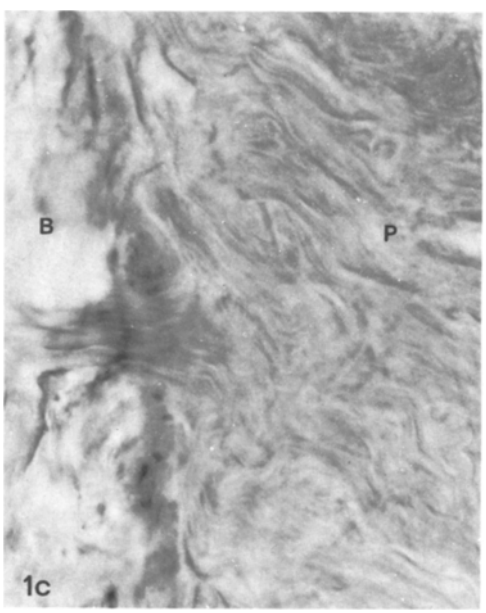


Fig. 1a–c. Alveolar bone (*B*), PDL (*P*) and root of a maxillary second premolar (*R*) of a 1-year-old cat. (a) Undecalcified, unfixed, 6 μ section, stained for cGMP by the immunoglobulin-enzyme bridge method (see text for details). Numerous reactive cells are visible in the bone as well as the PDL. (b) Section stained by the immunoglobulin-enzyme bridge method using normal rabbit IgG in place of the specific rabbit anti-cGMP antibodies. No cellular staining is seen in bone, PDL or root. ×80, 50. (c) Higher magnification of portion of (b) showing alveolar bone and adjacent PDL. ×1120

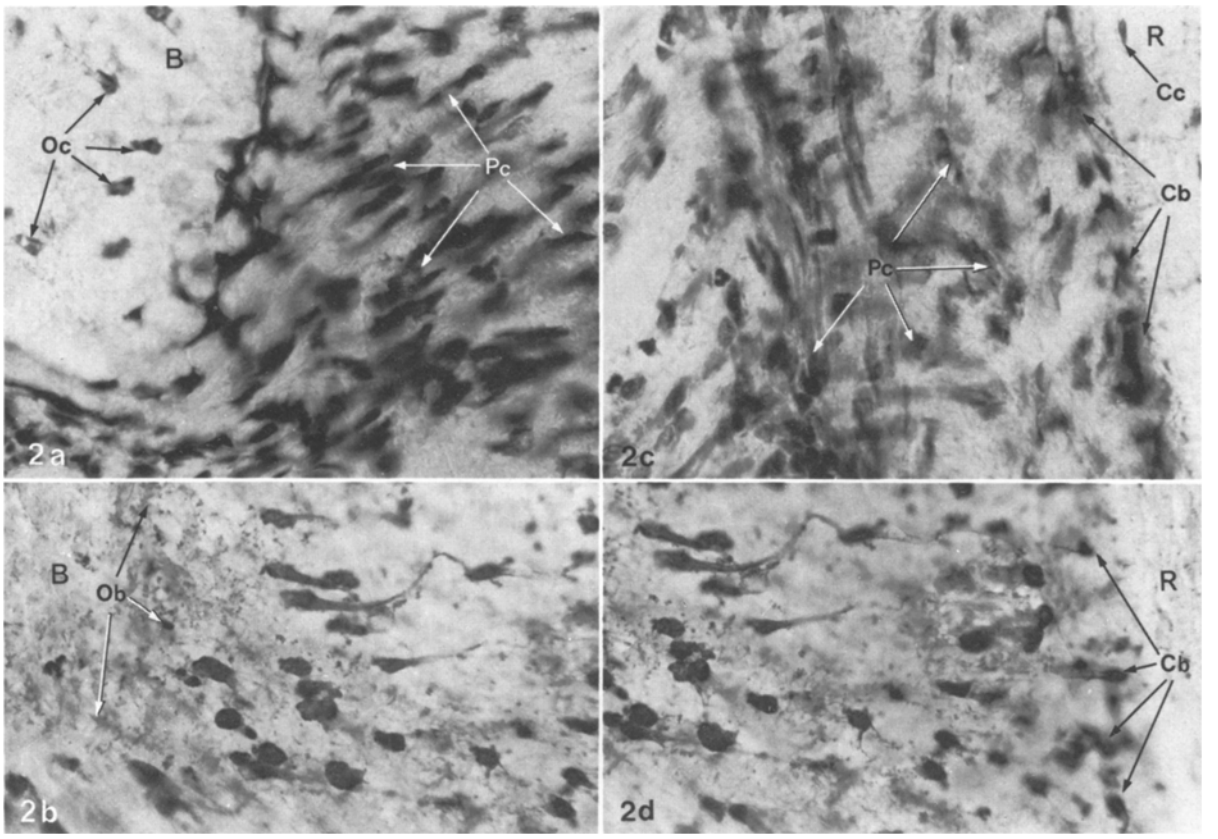


Fig. 2a–d. Crest of alveolar bone (*B*), PDL, and root (*R*) of maxillary second premolar of a 1-year-old cat. Cementoblasts (*Cb*), cementocytes (*Cc*), osteoblasts (*Ob*), oesteocytes (*Oc*), and PDL cells (*Pc*). Undecalcified, unfixed, 6 μ sections. (a) Toluidine blue stain showing bone and PDL cell population. (b) Immuno-histochemical staining for cGMP. Note that most oesteoblasts do not stain for cGMP. (c) Toluidine blue stain showing cementum and PDL cells. (d) Immuno-histochemical stain for cGMP. Note staining for cGMP in cementoblasts. ×462

blue, only a limited number of cells stain for cGMP. In particular, most of the osteoblasts fail to present cGMP staining. On the other side of the PDL, Figure 2c shows cementoblasts on the root surface which are stained by toluidine blue. The cementoblasts readily stain for cGMP (Fig. 2d).

In a higher magnification of the positively stained periodontal fibroblasts (Fig. 3) it is seen that in most cells the reaction product is uniformly distributed over the whole cellular area, including the long cytoplasmic processes and the nuclear region (Fig. 3a). The phase contrast micrograph (Fig. 3b) demonstrates that the staining found over the cellular body corresponds to the nuclear region as well as the cytoplasmic area. Some cells present well distinguished nuclear staining (Fig. 3c), which is much more intense than the cytoplasmic staining. The location of the nuclei in these cells is verified by the phase contrast micrograph in Figure 3d.

The alveolar bone osteocytes stain rather uniformly with toluidine blue (Fig. 4a), show no staining when incubated with non-specific rabbit IgG (Fig. 4b), and demonstrate a variable staining intensity when incubated with anti-cGMP antibodies (Fig. 4c). Nuclear staining is also visible in this type of cell, although less frequently than in the PDL cells.

In the cat, the apical zones of all the teeth are covered by a wide layer of cellular cementum. The cementocytes which populate these zones stain uniformly with toluidine blue (Fig. 5a), and some of these cells stain for cGMP (Fig. 5b). However, in comparison to osteocytes (Fig. 4c) the staining intensity of the cementocytes is weaker.

Specify Tests for cGMP Staining in Bone Sections: Figures 1b, 1c and 4b illustrate the results of one specificity test in which a sagittal maxillary jaw section was incubated with a non-specific IgG preparation instead of the anti-cGMP antibodies. This section does not contain stained cells. In the other control experiments when the remaining specific immunoglobulins of the bridge method were replaced by corresponding non-specific immunoglobulins, the cells failed to stain as well. When other serial tissue sections were incubated with DAB

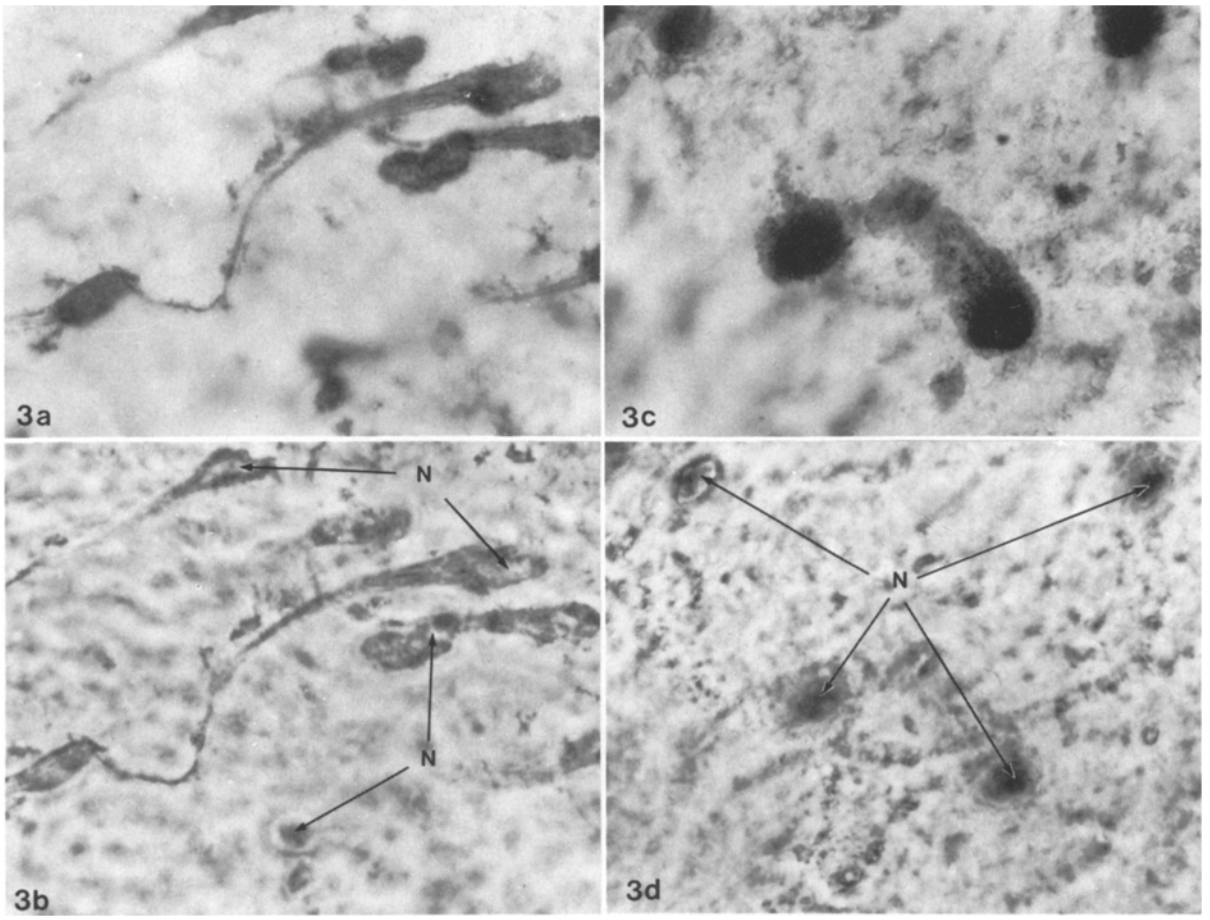


Fig. 3a–d. Higher magnification of PDL cells seen in Figure 2. Immuno-histochemical staining for cGMP. (a) DAB reaction products cover the cells including their nuclei and long cytoplasmic processes. (b) Phase-contrast micrograph of (a), indicating location of nuclei (*N*). (c) Periodontal cells, occlusal to the cells seen in (a). Diffuse granular cytoplasmic staining covers the cytoplasmic area and concentrated dark stain covers the nuclei. (d) Phase-contrast micrograph of (c), indicating location of nuclei (*N*). ×1120

alone or horseradish peroxidase and DAB, no tissue staining occurred. Only a few cells were found to be stained in the control experiments. These were mainly blood cells, which were stained due to their endogenous peroxidative activity.

Incubation of the anti-cGMP antibodies with solutions of cGMP in concentrations ranging from 1×10^{-4} M to 1×10^{-6} M competitively inhibited the cellular staining. Figures 6a and b show the staining of PDL cells and of osteocytes following incubation of the anti-cGMP antibodies with 1×10^{-6} M cGMP. Comparison of Figures 6a and b with Figures 3a and 4c respectively, demonstrates the reduction of cellular staining intensity resulting from prior incubation of the specific antigen (cGMP) with the antibodies. Incubation with cAMP, 5′GMP, 5′AMP, GTP, ATP, adenosine, guanosine, DNA and RNA did not prevent the specific cellular staining.

Discussion

Two methods have been used in a number of laboratories to localize tissue cyclic nucleotides, i.e., immunofluorescence [1, 6, 18, 19] and the immunoglobulin-enzyme bridge method [3]. The advantage of the immunoglobulin-peroxidase bridge method over the fluorescence technique is that the peroxidase molecule, being an enzyme, is capable of converting a large number of DAB molecules to the typical brown reaction product, while the immunofluorescence method provides only one marker molecule per molecule of antigen. Although Spruill and Steiner [15], using an immunofluorescent technique have successfully demonstrated the localization of cAMP and cGMP in developing rat testes, we have previously found [4] that in bone the immunoglobulin enzyme bridge method is preferable for the detection of cAMP be-

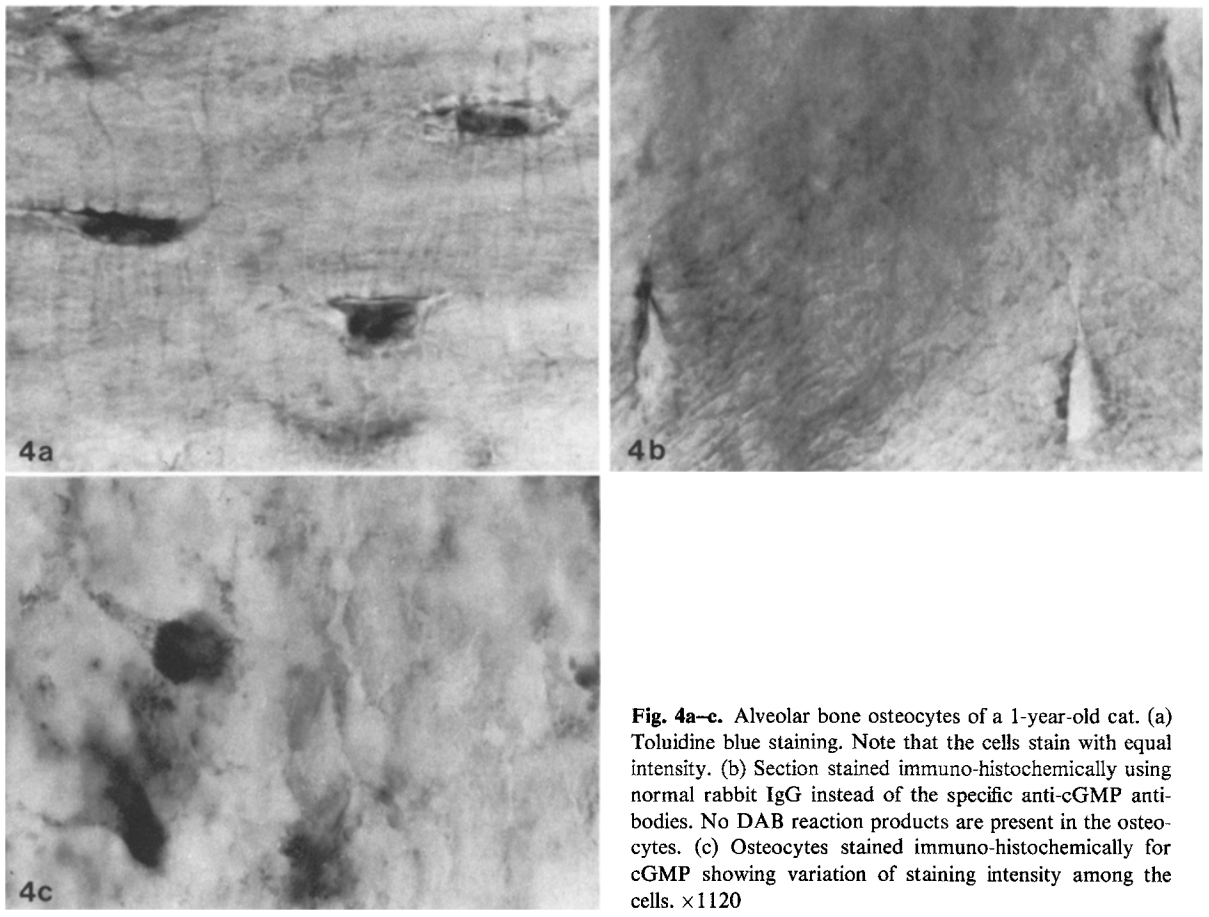


Fig. 4a–c. Alveolar bone osteocytes of a 1-year-old cat. (a) Toluidine blue staining. Note that the cells stain with equal intensity. (b) Section stained immuno-histochemically using normal rabbit IgG instead of the specific anti-cGMP antibodies. No DAB reaction products are present in the osteocytes. (c) Osteocytes stained immuno-histochemically for cGMP showing variation of staining intensity among the cells. ×1120

cause the paucity of cells in calcified tissues requires a technique which provides amplification of the antigen-antibody reaction site. In the present study we used the bridge method for the detection of cGMP in bone cells since the concentration of this nucleotide is known to be even lower than that of cAMP.

The results of our present study demonstrate that the microscopic localization of cGMP-containing cells is possible in unfixed, undecalcified, frozen bone sections, kept undistorted by an adhesive tape, as in the procedure for the localization of cAMP. However, since the same enzyme label (horseradish peroxidase) is used for both cyclic nucleotides, it is impossible at the present time to stain differentially for cAMP and cGMP in the same tissue section. For this purpose separate labels should be utilized.

The major problem in the application of the immunoglobulin-enzyme bridge method to bone sections is the diffusion of reaction products out of the cells or over cellular areas which may not normally contain cGMP. This problem is apparently not confined to cyclic nucleotides, but is characteristic for many enzyme-labeled antibodies, especially when DAB is utilized as the label. Yasuda [20] in a recent critique of this technique noted that in most cases the sections appear more widely colored in the light microscope than with the electron microscope, probably due to diffusion of the antibodies or that of the end product of the DAB reaction. In the case of cGMP, diffusion may also result from its high water solubility. To cope with this problem we have drastically reduced the incubation and rinsing time in all the reaction steps. Mason et al. [10] used 30 min incubation periods for each antibody and enzyme, as well as 30 min rinses between reactions in phosphate-buffered saline. However, we found that reducing the incubation time to 7.5 min each, and the rinses to 5 min, substantially decreased the stain diffusion without adversely affecting the intensity of the cellular staining.

A second problem inherent in our method was the maintenance of tissue integrity. To prevent tissue disintegration during processing we attempted to fix the sections prior to the application of the anti-cGMP antibodies. A 4% paraformaldehyde-picric acid solution [16] was used, causing marked improvement in the integrity of the tissues. However, the effect of fixation

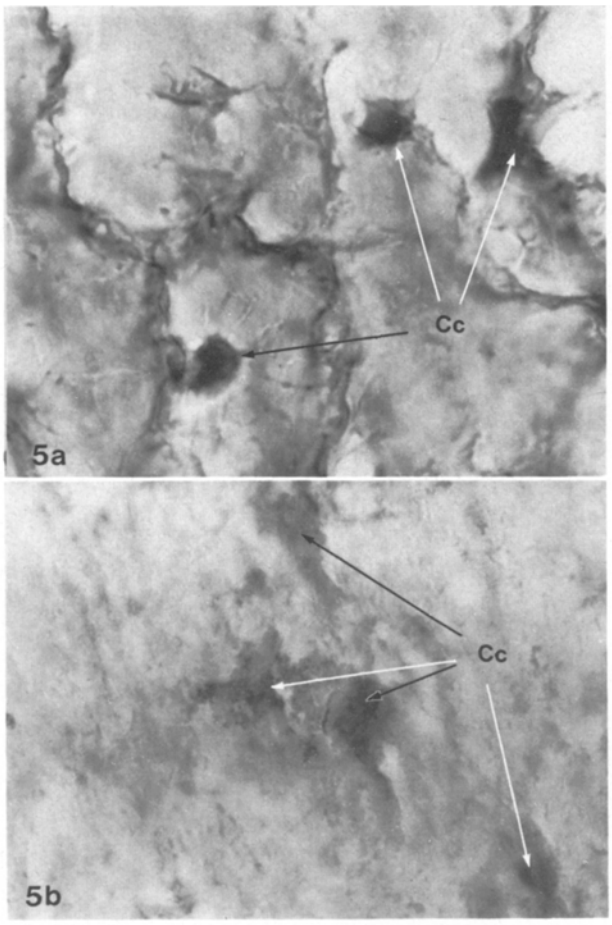


Fig. 5a and b. Root cementum of a 1-year-old cat. (a) Section stained with toluidine blue, presenting cementocytes (*Cc*). (b) Immuno-histochemical staining for cGMP. Cementocytes are stained more weakly than the alveolar bone osteocytes seen in Figure 4c. ×1120

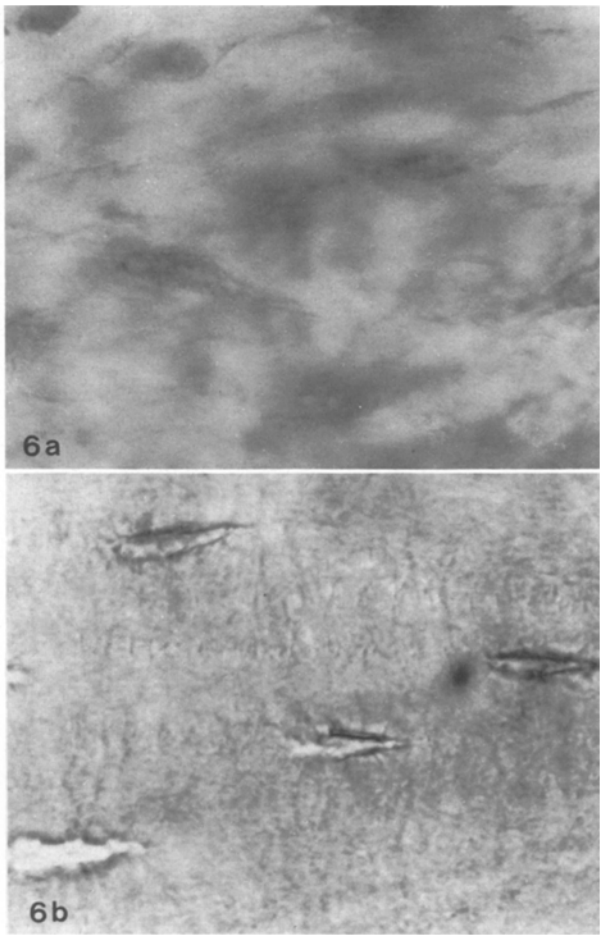


Fig. 6a and b. Alveolar bone and PDL of a 1-year-old cat. Undecalcified, unfixed, 6 μ section, stained immuno-histochemically for cGMP, after prior incubation of the anti-cGMP antibodies with a solution of 1×10^{-6} M cGMP (4 h at 4°). Competitive inhibition of staining is evident in (a) PDL cells and (b) osteocytes. Compare these photomicrographs to Figures 3a and 4c. ×1120

on the cellular staining for cGMP was a total loss of staining, thus forcing elimination of the tissue fixation altogether. The problem was solved to a large extent by the drastic reduction in the incubation and rinse time. Thus the bone sections which were treated by the short incubation schedule presented a high quality of tissue integrity.

The wide range of staining intensity for cGMP seen in cells, particularly in the osteocytic population, suggests that concentrations of this cyclic nucleotide may differ from cell to cell. It is also possible that diffusion may affect the intensity of the staining observed in various cells. However, the finding that most of the stained PDL cells present a rather uniform staining intensity seems to preclude the influence of diffusion. Unfortunately, it is impossible at the present time to estimate the exact concentration of cGMP in single positively stained bone cells.

The finding of a distinct nuclear staining for cGMP in some bone cells is in agreement with previous reports on the localization of cyclic nucleotides in a variety of soft tissues [6, 18]. Steiner et al. [18] studied the localization of cyclic nucleotides in rat tissues and found that in the adrenal gland nuclear staining was seen in cells of the zona fasciculata when stained for cGMP. In the testis, cGMP was found predominantly on the cell membrane, but not in the cytoplasm. In meiotic spermatocytes, cGMP was found lining chromosomes. In the present experiment, the nuclear staining seen in some bone cells may suggest involvement of cGMP in cellular proliferative activities. Diamantstein and Ulmer [5] found that cGMP increased thymidine incorporation into the DNA of mouse spleen cells. A similar effect in murine lympho-

cytes was observed by Gillette et al. [7]. However, our observation that most bone and PDL cells presented diffuse cytoplasmic staining for cGMP suggests that this cyclic nucleotide may play a role in a number of cellular processes, not necessarily related to proliferation. The diffuse nature of this cytoplasmic staining prohibits identification of specific sites of the cyclic nucleotide. Electron microscopic investigations may help to shed light on this question.

The finding that most of the osteoblasts appeared unstained for cGMP is of interest. The absence of cGMP from this cell type points to a basic metabolic difference between these cells and the adjacent bone and PDL cells. Moreover, we have previously reported [4] that osteoblasts do stain for cAMP both in untreated as well as in mechanically stressed cat jaws. This apparent dichotomy between the cAMP and cGMP profile of osteoblasts warrants further investigation.

The experiments reported here demonstrate the usefulness of the modified immunoglobulin-enzyme bridge method for localizing cGMP in mineralized tissues. The numerous tests which were performed indicate that this method is highly specific, reliable and informative. Together with our previously developed technique for the localization of cAMP, it provides a research tool which may be used to study the role of the cyclic nucleotides in mineralized tissues under a variety of physiologic, experimental and therapeutic conditions.

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