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The basal levels of the nucleotide cAMP in gingiva and the relationship of these levels to gingival tissue health was determined in Rhesus monkeys. It was shown that tissue cAMP levels were negatively correlated to gingival inflammation resulting in lower tissue cAMP levels than seen in healthy gingiva. In addition, the effect of surgery on tissue cAMP levels was investigated. It was shown that the levels of cAMP in the tissues decreased as surgical flaps were reflected for 30, 60, or 120 minutes. It is proposed that the reduction in cAMP content of the gingival flaps may affect collagen synthesis and retard clinical healing of

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REGULATION OF GINGIVAL cAMP LEVELS: EFFECTS OF INFLAMMATION AND SURGERY

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ABSTRACT

→ The basal levels of the nucleotide cAMP in gingiva and the relationship of these levels to gingival tissue health was determined in Rhesus monkeys. It was shown that tissue cAMP levels were negatively correlated to gingival inflammation. Inflamed gingiva showed lower tissue cAMP levels than healthy gingiva. In addition, the effect of surgery on tissue cAMP levels was investigated. It was shown that the levels of cAMP in the tissues progressively decreased as flaps were reflected for 30, 60, or 120 minutes. It is proposed that the reduction in cAMP content of the gingival flaps may affect collagen synthesis and retard clinical healing of tissues after lengthy surgical procedures. ←

INTRODUCTION

Although the histopathology of inflammation in the gingiva (Weinmann, 1941; Goldman, 1957) and the reactions involved in the healing of the periodontum after full thickness gingival flaps have been extensively studied (Wilderman, et al., 1960; Kohler and Ramfjord, 1960), the exact nature of the biochemical signals involved in regulating the responses of the cell types making up the tissues and underlying bone have not been fully elucidated. It appears that one or more subcellular moderators of inflammation and regeneration must be present in order to initiate and regulate the inflammatory and repair process. In this study the role which the nucleotide cyclic AMP plays in regulating these processes and the basal metabolic health of the gingiva was examined.

Cyclic AMP is produced in the cell by the membrane associated

enzyme adenyl cyclase (Davoren and Sutherland, 1963; Pohl et al., 1969) by the conversion of ATP into cAMP which is then postulated to regulate intracellular metabolism (Robinson et al., 1968). Cyclic AMP is deactivated in the cell and hydrolyzed to 5' AMP by cyclic AMP phosphodiesterases in the cytoplasm.

The results of in vitro experiments utilizing tissue cultures of fibroblast have shown that intracellular concentrations of cAMP regulate many aspects of fibroblast metabolism. Cyclic AMP appears to regulate growth rate (Ryan and Heidrick, 1968; Otten et al., 1972; Johnson and Pastan, 1972), contact inhibition (Heidrick and Ryan, 1971), and morphology (Johnson and Pastan, 1972; Johnson et al., 1971; Hsie and Puck, 1971). Cyclic AMP apparently also regulates collagen synthesis by fibroblasts. Hsie et al. (1971), showed that 1 mM dibutryl cAMP produced a 6-10 fold increase in (³H) proline incorporation into collagen in CHO cells in tissue culture. Green et al. (1966), have shown that virally transformed fibroblasts have lower levels of cAMP and synthesize less collagen than normal cells. Peterkofsky and Prather (1974) confirmed this observation in transformed cells and demonstrated that dibutryl cAMP increased collagen synthesis in transformed cells. Goggins, et al. (1972) have reported similar results with respect to mucopolysaccharide synthesis as measured by (³⁵S) sulfate incorporation.

Grower et al. (1974) studied gingival wounds in Rhesus monkeys and found a coordinate increase in collagen and cAMP content as healing progressed. Decreased concentrations of cAMP were found to be associated with the proliferation of granulation tissue; only after cAMP concentra-

tions increased did differentiation and tissue maturity proceed. In addition, in biopsies of human gingiva, tissue from subjects with periodontitis was significantly lower in cAMP concentration than biopsies from healthy subjects (Schaeffer et al., 1974). Further, Bourne et al. (1974) have proposed that in vivo mediators of inflammation may regulate the character and intensity of inflammatory and immune responses by a general inhibitory action of cAMP on the inflammatory and immunologic functions of leukocytes.

The normal growth of epithelial cells apparently is also controlled by the levels of cAMP found in them. Vorhees et al. (1973) observed that there were decreased levels of cAMP in the hyperplastic and abnormally differentiated epidermis of psoriasis as compared to normal epidermis.

The purpose of this study was, therefore, to study some of the parameters involved in modulating the basal levels of cAMP found in gingiva. The following parameters were studied:

1. The relationship between gingival cAMP levels and clinical status of the gingiva.
2. The effects of periodontal surgery on the cAMP levels and phosphodiesterase activity in gingiva.

These studies were conducted to determine if the change in cyclic nucleotide levels might be one of the mediators of healing and inflammation via its effects on cell proliferation and matrix turnover in the periodontium, thus regulating the metabolic state of the gingiva and the clinical responses seen after various surgical procedures.

MATERIALS AND METHODS

Rhesus monkeys weighing 5 to 8 kg were used in these studies and were fed a diet of monkey chow supplemented with apples, oranges, bananas, and peanuts. Prior to surgery, the monkeys were sedated with IM Sernlyn followed by the induction of general anesthesia using IV sodium pentobarbital.

The first part of this study investigated the correlation between clinical evaluation of the inflammatory status of the gingiva and its cAMP content. Inflammation of the facial gingiva of eight Rhesus monkeys, from the distal of the canine to the distal of the first molar on the right and left sides of both the maxilla and mandible, was evaluated using the Gingival Index of Loe (1967) as shown below:

G-0 = Normal gingiva

G-1 = Mild inflammation - slight change in color, slight edema.

No bleeding on probing.

G-2 = Moderate inflammation - redness, edema and glazing.

Bleeding on probing.

G-3 = Severe inflammation - marked redness and edema. Ulceration.

Tendency to spontaneous bleeding.

After scoring, intrasulcular incisions were made around the facial aspect of the teeth followed by a horizontal incision at the junction of the free and attached gingiva. The attached gingiva were removed by periosteal elevation. The excised tissue was immediately rinsed, placed in labeled plastic tissue containers, and frozen at -120°C in liquid nitrogen prior to determination of the cAMP content.

In the second part of this study the cAMP content of the attached gingiva of nine Rhesus monkeys was determined after periodontal surgery. At the start of the experiment (time 0) full thickness gingival flaps were made exposing 5-6 mm of mandibular facial alveolar bone extending from the right to the left lateral incisors. The attached gingiva in the flaps from the distal of the right lateral incisors to the mesial of the right central incisors (35-120 mg of gingiva) were removed at this time. The excised gingiva were immediately rinsed of excess blood and placed in labeled plastic tissue containers and frozen. The gingival flaps over the left central and lateral incisors were reflected away from the alveolar bone and 30, 60, and 120 minutes after the initial reflection of the flaps, the attached gingiva were excised from each of the three monkeys, as was done at time 0.

After collection of all the tissue samples the wet weights of the frozen samples were determined. The samples were re-frozen in liquid nitrogen and then pulverized in a steel mortar and pestle cooled to -40°C . (The pulverization was necessary in order to facilitate extraction of cAMP from the tissues.) After pulverization, the gingival samples were placed in Potter Elvehjem glass homogenizing tubes maintained in an ice bath at 4°C , and 2 ml of ice cold 6% TCA (Trichloroacetic Acid) were added. In addition, 0.1 ml of H_2O containing 0.022 μC of (^3H) cAMP (Adenosine 3', 5' cyclic phosphate ($8\text{-}^3\text{H}$), SA 20 c/mMole, Schwarz/Mann) was added to monitor the recovery of cAMP through the extraction procedures. The cyclic nucleotides in the tissue were extracted by homogenizing the tissue with a motor-driven teflon pestle and the homogenates

were transferred to conical glass centrifuge tubes and centrifuged for 15 minutes at 4000 RPM at 4°C to separate the precipitated proteins from the cyclic nucleotides present in the 6% TCA extract.

The samples were extracted three times with 5 ml of ethyl ether to remove the TCA present. The resulting aqueous solution was evaporated to dryness in a stream of air in a 60°C water bath. The residue was redissolved in 1 ml of pH 6.2 acetate buffer and the samples were assayed for cAMP content using the Schwarz/Mann cyclic AMP radioimmunoassay kit (^{125}I) (Schwarz/Mann, 1970) according to the procedures described by Steiner et al. (1969). The radioactivity in the (^{125}I) nucleotide antigen-antibody complexes obtained in the assays was counted in a Packard Auto-Gamma Spectrometer. The cAMP radioimmunoassay was sensitive enough to measure from 0.01 to 10 picomoles of cAMP per sample. The recovery of the cAMP was determined by adding an aliquot of the sample in pH 6.2 buffer to 10 ml of Aquasol (New England Nuclear) in a liquid scintillation vial and counting the (^3H) radioactivity in a Packard liquid scintillation counter. The efficiency of the extraction procedures for the cAMP content of gingiva was found to average $75 \pm 2\%$ (mean \pm SE of eight monkeys) based on the recovery of the (^3H) cAMP tracer added to the samples. In addition, the specificity of the radioimmunoassay for cAMP was determined by fractionating the extracted nucleotides on 1.0 ml columns of BioRad AG-1-X8, 200-400 mesh ion exchange resin equilibrated in 0.1 N formic acid. The columns were washed with 10 ml of 0.1 N formic acid and the cAMP was eluted with 10 ml of 2 N formic acid. The elute was lyophilized and the residue redissolved in 1 ml of acetate assay buffer

for analysis of the cAMP content. It was found that $79 \pm 3\%$ (mean \pm SE of eight observations) of the total cAMP content of the original gingival extract was eluted in the purified cAMP fraction.

The precipitated proteins remaining after TCA extraction of the gingiva were resuspended in 2 ml of 1.0 N NaOH, incubated at 60°C for two hours to solubilize them, and the protein content of the samples was determined by the micro method of Lowry *et al.* (1951).

The tissue preparation procedures utilized for determining the phosphodiesterase activity of the gingiva after surgery were similar to those utilized in the measurement of the cAMP content of the gingiva except that the gingival samples were homogenized in the following pH 7.0 buffer: 0.25 M sucrose, 0.001 M magnesium chloride, 0.05 M potassium chloride, 0.0033 M calcium chloride and 0.01 M Tris Base. After homogenization, the tissue suspension was centrifuged at 1200 RPM at 4°C to precipitate out unbroken cells and connective tissue. The supernatant was then assayed for its phosphodiesterase activity according to the procedure of Thompson and Appleman (1971). This is a two step assay in which (^3H) cAMP added to the homogenate [$0.25 \mu\text{C}$ (^3H) cAMP] is first converted to (^3H) 5' cAMP by the phosphodiesterases in the sample. 5' nucleotidase added to the sample converts this to (^3H) adenosine which is then assayed by liquid scintillation counting.

The phosphodiesterase activity in the homogenates was expressed as the picomoles of (^3H) cAMP hydrolyzed/10 min/mg of protein.

RESULTS

Clinical examination of the monkeys' oral tissues showed that the

maxillary gingiva was consistently more inflamed than the mandibular gingiva. Table I summarizes the results of systematic analysis of the inflammatory status of the monkeys using Loe's Gingival Index (1967). The average index of the maxillary gingiva was 1.4 ± 0.2 while that of the mandibular gingiva was 0.15 ± 0.07 . Table I also shows that the difference in the inflammatory state of the maxilla and the mandible was associated with a significant difference in the levels of cAMP in the maxilla and the mandible. The mandibular gingiva, which showed less inflammation, had levels of cAMP 26 - 33% greater than the maxillary gingiva, which were more inflamed. When regression analysis of the relationship between gingival index and cAMP content of the gingiva was done, as seen in Fig. 1, a linear relationship was observed. The correlation coefficient between gingival index and cAMP content of the gingiva was $r = -0.505$, which was significant at $p < 0.01$ ($df = 31$). Fig. 1 shows that the less inflamed tissue, indicated by lower gingival index numbers, exhibited higher cAMP levels than seen in inflamed tissue which had lower cAMP levels.

In the second part of this study the levels of cAMP and cyclic nucleotide phosphodiesterase present in gingival tissues during surgery were studied.

Comparison of the levels of cAMP in the gingiva removed at time 0 with the levels seen in the tissues taken at 30, 60, and 120 minutes after surgery, utilizing the one-tailed students' "t" test for paired samples (Snedecor and Cochran, 1967), revealed that the cAMP content of the gingival flaps showed significant changes during surgery. Fig. 2 graphically depicts the changes observed in the cAMP content of the

flaps. (The levels of cAMP are expressed both as picomoles per milligram of wet weight of tissue and as picomoles per milligram of protein contained in the samples) Fig. 2 shows that as the length of surgery was increased, the levels of cAMP in the gingival flaps decreased. Based on the cAMP content per mg of gingival tissue, at 30 minutes there was a 46% decrease in cAMP content from time 0 ($p < .05$), at 60 minutes a 56% decrease ($p < 0.025$) and at 120 minutes a 75% decrease ($p < 0.025$).

In order to determine the possible mechanisms responsible for the decrease in cAMP content of gingival tissue after surgery, the phosphodiesterase activity of the gingiva was determined. Table II shows the phosphodiesterase activity of gingival homogenates prepared from tissue reflected for 120 minutes. Table II shows that surgery caused a 20% decline in the phosphodiesterase activity of the maxillary gingiva compared to the control samples taken at time 0, and a 25% decline in the phosphodiesterase activity of the mandibular gingiva compared to the control samples.

DISCUSSION

This study has shown that inflammation in monkey gingival tissues is associated with a decrease in the cAMP content of the tissues from that seen in noninflamed tissues. In addition, it has been shown that the trauma of gingival surgery results in a progressive reduction in cAMP levels dependent on the duration of the surgery.

The early phases of chronic inflammatory gingival and periodontal disease are characterized by the accumulation of inflammatory and lymphoid cells in the tissues, alteration in the quality and quantity of collagen in the tissues, and the downgrowth of epithelium to form

pockets (Page & Schroeder, 1973). Microbial plaque appears to play a key etiologic role in the initiation and progression of these changes (Socransky, 1970), but the exact mechanisms responsible for producing the tissue changes have not yet been elucidated. In this paper we propose that the decreased levels of cAMP which occurred with increased inflammation as shown in this study (Table I, Fig. 1) may be one of the cellular mediators of the tissue changes seen in gingival inflammation and periodontal disease.

Schroeder, et al. (1973) demonstrated that infiltrated connective tissue showed a collagen content 70% less than noninflamed connective tissue. This reduction in tissue collagen levels could have been due to either decreased production of collagen or increased destruction of the collagen since Page (1972) has shown that gingival collagen has a high turnover rate. In vitro studies by Hsie, et al. (1971) showed that exogenous cAMP was able to stimulate collagen production by fibroblast in tissue culture; thus the lowered levels of cAMP seen during inflammation (Table I, Fig. 1) and after surgery (Fig. 2) may contribute to a lowered rate of collagen synthesis.

Narayanan & Page (1976) showed that fibroblasts derived from diseased gingiva synthesize collagen of a different composition than that synthesized by fibroblasts derived from normal gingiva. The lowered levels of cAMP which occur in gingival inflammation (Table I, Fig. 1) may have led to cell transformation or cell selection which may have changed the population of fibroblasts in the tissues sampled. The possibility that cAMP is involved in these changes is supported by Green, et al., (1966) who showed that virally transformed fibro-

blasts synthesize less collagen than normal cells and by Peterkofsky & Prather (1974) who showed that exogenous cAMP could increase collagen production in transformed cells.

Increased destruction of tissue collagen may also be one of the mediators of the changes seen in gingival inflammation. Page & Schroeder (1973) have proposed that the increased destruction of collagen may be caused by endogenous tissue collagenase. Ten Cate & Deporter (1974) have proposed that the fibroblast itself may function in such a degradative role. Gustafson et al. (1977) have recently shown in cats that the levels of cAMP in the gingiva around erupting teeth, where collagen breakdown is occurring, is lower than in normal tissue. This suggests that lowered levels of cAMP could play a role in modulation of the synthetic or degradative role of fibroblasts.

Page & Schroeder (1973) also suggested that collagen breakdown could be due to digestion by lysosomal hydrolases and collagenase from neutrophilic granulocytes or digestion by hydrolytic enzymes released by macrophages since gingival inflammation is associated with a dense infiltrate of inflammatory and lymphoid cells. Changes in the levels of cAMP in tissues could affect the function of these cells by a number of mechanisms. The decreased tissue levels of cAMP seen in inflammation (Fig. 1 and Table I) might act to allow an increased influx of inflammatory cells into the tissues since Hill et al. (1975) showed that increased intracellular concentrations of cAMP inhibited the leukotactic response of neutrophils to bacterial

chemotactic factor. The decreased levels of cAMP in the tissues might also stimulate the selective secretion of lysosomal enzymes by the polymorphonuclear leukocytes, a response which can be inhibited by agents which increase intracellular levels of cAMP (Kaliner & Austen, 1974). In addition, Ichikawa et al. (1972) showed that exogenous cAMP could suppress carrageenin induced edema and granuloma formation in rats.

Voorhees et al. (1972) have suggested that decreased tissue levels of cAMP may play a pathophysiological role in the rapid epidermal cell cycle kinetics of psoriasis, a disease which is marked by accelerated proliferation and glycogen accumulation of epidermis. It is possible that the lowered levels of cAMP seen in gingival inflammation (Table I, Fig. 1, and Schaeffer et al. 1974) trigger the overgrowth of epithelium in the periodontal pocket.

This study showed that gingival surgery (Fig. 2) resulted in a decrease in tissue cAMP levels similar to that seen due to gingival inflammation (Table I, Fig. 1). In addition, Fig. 2 shows that the levels of cAMP showed a more profound decrease as the length of surgery was increased. Since the initial repair of gingival flaps occurs through collagen fiber production by pre-existing fibroblasts (Staffileno 1974), a reduction in tissue cAMP levels might delay this process. Support for this concept comes from the work of Hsie et al. (1971) and Peterkofsky & Prather (1974) who showed that cAMP increased collagen production in fibroblasts and that of Goggins et al. (1972) who showed that mucopolysaccharide synthesis was also increased by cAMP.

Later healing of flaps is associated with cellular proliferation and tissue remodeling (Staffileno, 1974; Levin et al. 1977). Grower et al. (1974) reported this later process appeared to be associated with an increase in cAMP content of the gingival tissues.

Schilling (1976) has claimed that any complication in the early hours or days of wound repair increases the statistical probability of future complications. Support for this concept and the hypothesis that the decrease seen in tissue cAMP might be one of these complications was recently presented by Levin et al. (1977) who reported that full thickness flaps reflected for 90 minutes in dogs showed lower collagen levels during healing than flaps reflected for only 15 minutes.

The levels of cAMP determined in this in vivo study were from tissues composed of a mixed population of cells. Therefore, the question of which cells were the source of the tissue cAMP and which cell types showed the most changes has to be considered. Voorhees et al. (1973) found the levels of cAMP in human epidermis to be 12.3 ± 1.7 picomoles of cAMP/mg protein, while that of whole blood was 300 times lower, i.e., 0.042 ± 0.006 picomoles cAMP/mg protein (Voorhees et al. 1972). Sheppard (1972) found that the content of cAMP in fibroblasts (3T3) in culture was 24.4 ± 3.4 picomoles cAMP/mg protein. The levels of cAMP determined in the monkey gingiva as seen in Table I are similar to that seen in skin and fibroblasts but 400 to 500 fold higher than seen in blood; thus the inference is strongly supported that the tissue levels measured were epithelial and

collagenous origin and not due merely to changes in blood volume of the tissues.

The phosphodiesterase activity of the gingival flaps was measured after 120 minutes of surgery to see if the decrease seen in tissue cAMP levels might have been due to increased breakdown of cAMP due to phosphodiesterase activation. The decrease seen in phosphodiesterase activity noted (Table II) suggests that surgery apparently caused a general decrease in enzyme activity of the tissues. The reduction of cAMP levels may be due to a decrease in adenyl cyclase activity, although this cannot be stated unequivocally since adenyl cyclase was not measured in the study. An alternative possibility for the decrease observed in both cAMP levels and phosphodiesterase activity is that since fibroblasts have several forms of phosphodiesterases with different K_m 's (d'Armiento, et al., 1972), the cellular conditions associated with surgery may have favored the intracellular activation of one form over the other. The phosphodiesterase activated may not have been detected in the in vitro assay used even though in vivo it may have acted to reduce tissue cAMP levels.

The results of this study and the work of others discussed in this paper have caused us to propose that cAMP may be one of the sub-cellular mediators of inflammation and wound repair in the periodontium. It appears that decreased levels of cAMP are associated with increased inflammation. Both the local etiologic effects of gingival inflammation, and prolonged surgery appear able to reduce tissue cAMP levels. It is proposed that, the decreases seen in the tissue cAMP levels may result in decreased collagen content of the

tissues. While this hypothesis appears to be an attractive one, it is at the present time based partly on circumstantial evidence. Further work concerning the changes in both cAMP and collagen level of flaps during healing has yet to be completed. In addition, the effects of endogenous and exogenous modulation of tissue cAMP levels on gingival inflammation and wound healing also require further study before this hypothesis can be accepted as valid.

* * * *

In conducting research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals" as promulgated by the Committee on the Revision of the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Research Council.

Commercial materials and equipment are identified in this report to specify the investigative procedure. Such identification does not imply recommendation or endorsement, or that the materials and equipment are necessarily the best available for the purpose. Furthermore, the opinions expressed herein are those of the authors and are not to be construed as those of the Army Medical Department.

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TABLE I. GINGIVAL INDEX AND cAMP CONTENT OF THE MAXILLARY AND MANDIBULAR GINGIVA OF RHESUS MONKEYS

| | <u>Picomoles cAMP</u> mg gingiva (a) | <u>Picomoles cAMP</u> mg protein | Gingival Index (b) |
|----------|---|-------------------------------------|-----------------------|
| Maxilla | 1.95±0.16 | 18.82±1.70 | 1.4±0.2 |
| Mandible | 2.59±0.23 | 23.75±2.89 | 0.15±0.07 |
| | (c) p<.02 | p<.02 | p<.001 |

a. Mean ± SE of 17 observations. Gingival biopsies were taken from 8 different monkeys. Four of the monkeys had duplicate samples taken from the same location after a six month period.

b. Loe, 1967.

c. Level of significance using the Wilcoxon matched-pairs signed-ranks test (2-tailed) Siegel, 1956).

TABLE II
EFFECTS OF SURGERY ON PHOSPHODIESTERASE LEVELS
IN MONKEY GINGIVA

| | Picomoles [³ H] cAMP hydrolyzed/10min/mg protein (a) | |
|----------|---|--------------|
| | Control (b) | 120 min flap |
| Maxilla | 18.7±2.3 | 13.8±1.8 |
| Mandible | 16.1±1.9 | 12.8±1.0 |

(a) Three tenths of a milliliter of assay buffer containing $1.7 \times 10^{-7} M$ cAMP (0.25 μC [³H] cAMP) was incubated with 0.1ml of sample containing 100-150 μg of protein for 10 min at 30°. The reaction was stopped by boiling for 2.5 min. The contents of the tube were then incubated with 0.1ml of snake venom (1.6 units of 5' nucleotidase) for 10 min at 30°. The reaction was stopped by adding 1.0ml of a slurry of Bio-Rad resin AG1-X2, 200-400 mesh. The contents of the tube were spun at 1200 RPM and 0.5ml of the supernant was added to 10ml of Aquasol and counted in a liquid scintillation counter.

(b) Mean \pm SE of observations from four monkeys.

Figure 1. Relationship of cAMP content of monkey gingiva to gingival index.

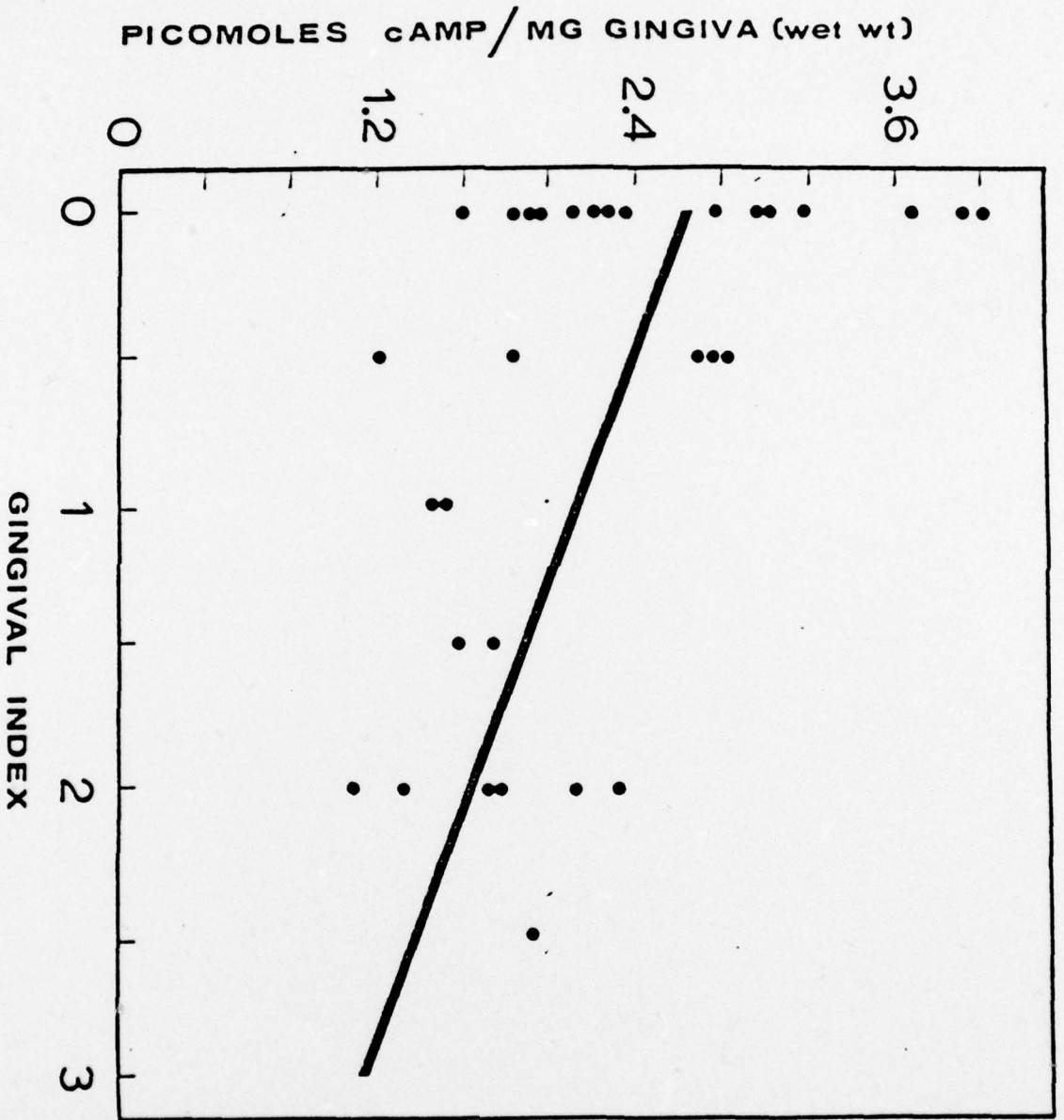


Figure 2. Changes in Gingival cAMP Levels After Surgery.

The cAMP content of the gingiva is represented as the percent change from the control level which is represented by the 0 line. The horizontal lines show the

cAMP content based on the protein content of the gingival flaps while the diagonal lines show the cAMP content based on the wet weight of the tissues. Each observation is the mean of paired analysis done on three monkeys.

