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Sensitive Assay for Measuring Tetracycline Levels in Gingival Crevice Fluid

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An increased interest in the clinical use of antibiotics as an adjunct to periodontal therapy has created a need to determine antibiotic concentrations in fluid obtained from the gingival crevice. For this purpose, an increase in sensitivity beyond that possible with current tetracycline assays is essential because sample volumes of gingival fluid typically obtained are less than 0.5 μl. This report describes the development of an agar-diffusion assay technique capable of measuring the concentration of tetracycline in samples of gingival crevice fluid in the range of 0.1 to 4.0 μg/ml. The assay will detect amounts of tetracycline in gingival crevice fluid samples as low as 50 pg. The high sensitivity of this assay was achieved by optimizing the medium depth, inoculum density, agar concentration, pH, period of prediffusion, and selection of basal medium. Use of this assay indicated that the concentration of tetracycline in gingival crevice fluid was greater than that found in blood and persisted at elevated levels for longer periods.

Destructive periodontal diseases appear to be caused by subgingival infection by specific microbial agent(s). Traditional therapy for these diseases has involved the elimination or suppression of subgingival microbial complexes by mechanical debridement such as repeated scaling and curettage or surgical procedures. With the recognition that the periodontal diseases may be caused by specific etiological agent(s), interest has grown in the use of antibacterial drugs for elimination of such agent(s) (8).

Tetracycline is an antibiotic which has been previously used as an adjunct to periodontal therapy. This has led to studies of its efficacy in human studies (7; R. J. Genco, S. Singh, G. Krygier, and M. Levine, IADR Abstr. 1978, 768, p. 266) and animal studies (R. Williams, M. Sandler, D. Nitzan, P. Aschuffenburg, and P. Goldhaber, IADR Abstr. 1978, 770, p. 267) as well as its effect on the microbial composition in the gingival crevice and periodontal pocket (S. Osterberg and B.L. Williams, IADR Abstr. 1978, 976, p. 378; H. Reynolds, P. Mashimo, J. Slots, N. Sedransk, and R. Genco, IADR Abstr. 1978, 769, p. 267). Doses and administration schedules utilized to date have been largely empirical, based on recommended doses for treatment of acute infection or acne. To optimize dosage and administration schedules of the antibiotic for periodontal disease therapy, it is useful to relate the drug concentration at the site of infection to the minimal inhibitory concentrations of antibiotics on the suspected etiological agents.

The major obstacle to measuring tetracycline in gingival fluid has been the limited quantity of fluid available for assay from a single gingival site. Sample volumes of gingival crevice fluid, which is derived in large part from plasma and local interstitial fluid, are generally less than 1 μl from each gingival site. Micromethods described in the literature for the determination of tetracycline concentrations in blood and other fluids require at least 10 μl of sample fluid, which is about two orders of magnitude more fluid than can be conveniently obtained from the gingival crevice. In this report, the conditions for an agar diffusion assay used for the determination of tetracycline in blood (10) have been optimized to provide the sensitivity necessary for measurement of tetracycline in gingival fluid samples from individual sites.

MATERIALS AND METHODS

Method of assay. The optimal assay procedure is summarized below. The parameters tested to develop this method are described in the results section.

Assay organism. A strain of *Clostridium perfringens* (SAL 19) was selected as the assay organism. A stock culture was maintained by weekly transfer in Todd-Hewitt (TH) broth (BBL Microbiology Systems, Cockeysville, Md.). Inocula was prepared by transferring 0.8 ml of the stock culture to 9.0 ml of TH broth and incubating in an atmosphere of 80% N_2, 10% CO_2, and 10% H_2 overnight at 37°C. A 0.4-ml amount of the overnight culture was then added to 9.0 ml of TH broth and incubated anaerobically at 37°C for ca. 3 h until the culture reached an optical density (OD) of 0.25 at 550 nm.

Assay medium. The assay medium was prepared by adding 0.5 g of agar (Ionagar, Colab Laboratories,

Glenwood, Ill.) to 100 ml of brain heart infusion (BHI) broth (BBL). The BHI-Ionagar medium was adjusted to pH 6.6, autoclaved, and cooled to 46°C, and 8.0 ml of defibrinated whole sheep blood (BBL) plus 2.0 ml of the standardized inoculum were added. Test plates were prepared by allowing 17.0 ml of medium to solidify in large petri dishes (150 by 15 mm; Van Lab, Boston, Mass.).

Preparation of standards. A stock solution of 1.0 mg/ml was prepared by dissolving tetracycline HCl (Sigma, St. Louis, Mo.) in distilled water. Lower concentrations were made by dilution with phosphate-buffered 3.5% bovine serum albumin (4). The diluent was prepared by dissolving 3.5 g of reagent-grade fraction V bovine serum albumin (Miles Laboratories, Elkhart, Ind.) in 100 ml of phosphate buffer (pH 8.0). The buffer was prepared by dissolving 16.73 g of K_2HPO_4 and 0.523 g of KH_2PO_4 in distilled water to make 1.0 liter. Fraction V bovine serum albumin was employed as a gingival fluid substitute instead of serum because of inhibitory substances frequently present in serum. Preliminary trials indicated that there was no statistically significant difference in zone diameters between standards prepared in human sera and the bovine albumin solution.

Standard solutions of tetracycline were prepared at concentrations of 0.125 μg to 4.0 μg per ml. Portions (0.5 μl) of the standard solutions were placed on filter paper strips (Periopaper, Harco Electronics, Winnipeg, Canada) using a 1.0-μl syringe (Hamilton, Reno, Nev.). The standards were stored at −20°C for a maximum of 2 weeks.

Sampling technique. Samples of gingival crevice fluid from subjects given tetracycline were obtained on filter strips by the intracrevicular sampling technique suggested by Golub (1). The volume of gingival crevice fluid on the strips was determined by measurement with a gingival fluid meter (Periotron, Harco Electronics, Winnipeg, Canada). The gingival fluid meter provides a nondestructive measure of fluid volume in the range of 0 to 0.5 μl to the nearest 0.005 μl through measurement of the dielectric constant of the wetted filter paper strip of standard dimensions (9). Volumetric calibration before each use was achieved by measurement of instrument response to measured volumes of serum applied to filter paper strips. Samples were stored until assayed at −20°C. Assays were performed within 2 weeks of sample collection.

Assay procedure. Sample strips and standards were placed on test plates and allowed to prediffuse anaerobically at room temperature for 30 min. Plates were then incubated in an anaerobic chamber at 35°C for ca. 4 h, and the diameters of the resultant zones of inhibition of hemolysis were measured with a vernier caliper to the nearest 0.1 mm. Because zones were occasionally elliptical, diameters were measured along the long and short axes and the average zone diameter was recorded.

Standard curves. Standard curves were fitted by linear regression using the method of Colquhoun (3). Because the volumes on each sample strip varied, the zone diameters were converted to absolute amounts of tetracycline on each strip, and the concentration in the gingival fluid was calculated.

RESULTS

Effect of medium depth on sensitivity. The effect of the medium depth on sensitivity of the assay was tested on plates (100 by 15 mm, Van Lab) prepared in duplicate which contained 6, 7, 8, 9, and 10 ml of medium. Filter paper strips containing known amounts of tetracycline were assayed on each plate. The standards were prepared by dipping filter paper strips into standard solutions of tetracycline. The volumes for each strip were measured by using the gingival fluid meter. Because the volume of fluid varied, each strip contained a different amount of tetracycline. The least amount of tetracycline that resulted in a measurable zone of inhibition for each medium depth is shown in Table 1. An inverse relationship between medium depth and sensitivity was observed. Plates containing 6.0 ml of medium detected the lowest amounts of tetracycline. However, 7-ml plates were almost as sensitive and were easier to pour at a uniform thickness than the 6.0-ml plates. Seven-milliliter plates were therefore selected for use in the following studies. Agar depth with this volume was ca. 0.5 mm.

Effect of inoculum turbidity on sensitivity. The effect of changing inoculum density on the sensitivity of the assay was ascertained using media seeded with log-phase inocula of 0.10 to 0.75 OD at 550 nm. Each test plate contained 7.0 ml of medium at pH 7.4 and 1.0% agar. Filter paper strips with tetracycline were prepared as described above and were placed on each plate. ODs of 0.10 and 0.15 were achieved by diluting a log-phase culture of 0.25 OD with sterile TH broth. Media seeded with an inoculum standardized at 0.25 OD were most sensitive (Table 2). In media seeded with inocula of 0.5 OD and above, hemolysis was so rapid that the zones were either very small or else obliterated.

Effect of agar concentration on sensitivity and zone diameter. To test the effect of agar concentration on the detection limits of the assay, plates were made containing 0.5, 1.0, and 1.5% agar. The inoculum density was adjusted to 0.25 OD, the pH of the medium was adjusted to 7.4, and the medium volume was adjusted to 7.0 ml per plate (100 by 15 mm, Van Lab).

TABLE 1. *Detection limit with different volumes of media in a 100-mm plate*

Media vol (ml)	Lowest level of tetracycline detected (pg)
6	58
7	63
8	78
9	90
10	98

The greatest sensitivity was achieved with the lowest agar concentration. A 45-pg amount of tetracycline was detected on a medium containing 0.5% agar, whereas only 60 and 85 pg were detected at agar concentrations of 1.0 and 1.5%, respectively.

To determine the influence of agar concentration on the zone diameters, we compared standard curves by regression analysis (6) (Fig. 1). The slope of the regression line of the assay conducted at 0.5% agar was significantly greater ($P < 0.01$) than the slope of the regression lines of assays carried out in media with 1.0 or 1.5% agar. There was no statistically significant difference between slopes or intercepts of regression lines obtained using 1.0 or 1.5% agar.

TABLE 2. *Detection limit with different inoculum densities*

OD of inoculum	Lowest level of tetracycline detected (pg)
0.10	100
0.15	106
0.25	57
0.35	82
0.45	106
0.50	—[a]
0.60	——[b]
0.75	——[b]

[a] —, Zones were obliterated before the measurement was completed.
[b] ——, Complete hemolysis without zone formation.

Effect of prediffusion on sensitivity and zone diameter. Filter paper strips containing known amounts of tetracycline prepared in a diluent of distilled water were placed on the assay plates and allowed to prediffuse at room temperature for 0, 30, and 60 min before incubation at 37°C. Plates that had prediffused for either 0 or 30 min provided a detectable response to tetracycline levels as low as 50 pg. The lowest amount detected on plates allowed to prediffuse for 60 min was 60 pg. To determine whether zone diameters over the entire range of the assay were affected by this prediffusion step, we constructed and compared standard curves. Differences between slope and intercept of regression lines of standards were not statistically significantly different. However, it was observed that the zone edge was most clearly demarcated on the plates that were allowed to prediffuse for 30 min. On the other hand, plates that had prediffused for 60 min had an indistinct zone edge.

Effect of pH on sensitivity and zone diameter. To determine the effect of pH on sensitivity and zone diameter, we prepared test plates as previously described except that the pH was adjusted to 6.0, 6.6, and 7.4. Standards were prepared as described above except that the standard solutions of tetracycline were prepared with bovine serum albumin diluent. The test plate at pH 6.6 exhibited greater sensitivity than the plates at pH 6.0 and 7.4. Measurable zone formation occurred around strips of tetra-

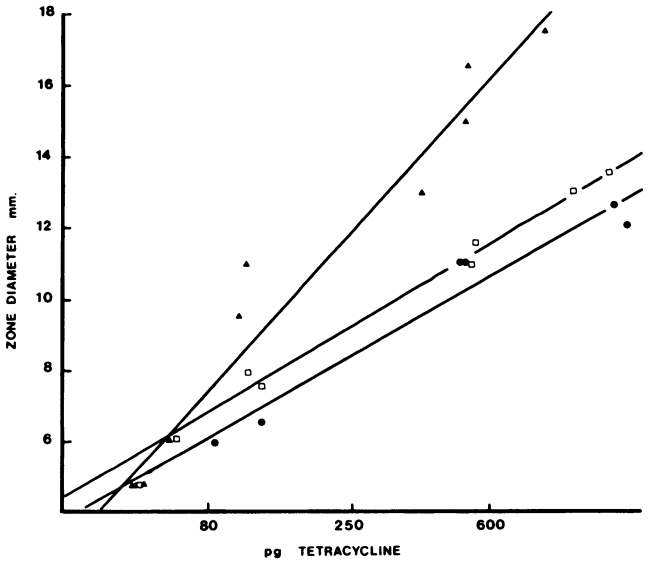

FIG. 1. *Effect of agar concentration on zone diameter. Zones of hemolysis inhibition with various amounts of tetracycline were determined at three agar concentrations (0.5% [▲], 1.0% [□] and 1.5% [●]) and compared by regression analysis. The slope of the response with 0.5% agar was significantly greater ($P < 0.01$) than the response with 1.0 or 1.5% agar.*

cycline containing 81, 48, and 90 pg on plates of pH 6.0, 6.6, and 7.4, respectively. Regression analysis revealed that the slope of the zone diameters obtained at pH 6.0 was significantly different from the slope of zone diameters obtained at higher pH's ($P < 0.01$) (Fig. 2). Differences in slope of the zone diameters were not statistically significant at pH 6.6 and 7.4. At the lower range of tetracycline concentrations, the zone diameters obtained at pH 6.6 were larger than at pH 6.0. Thus, although the significantly steeper slope ($P < 0.01$) at pH 6.0 permitted better discrimination at higher tetracycline concentrations, the larger zone diameters at lower concentrations on plates adjusted to pH 6.6 permitted greater sensitivity.

Sensitivity at pH 6.3, 6.6, and 6.9 was then similarly studied. The sensitivities of assays at pH 6.3, 6.6, and 6.9 were 48, 49, and 86 pg, respectively. At low concentrations of tetracycline, the test plate adjusted to pH 6.6 had the largest zone diameters. At higher concentrations, the largest zone diameters were achieved on the pH 6.3 plate. Regression analysis revealed no statistically significant difference in the slope and intercept of the regression lines of the zone diameters obtained at pH 6.3 and 6.9. A statistically significant difference was observed between the slope of the zone diameters in pH 6.6 plates and the plates at pH 6.3 ($P < 0.01$) and between the intercept of regression lines obtained at pH 6.6 and pH 6.9 ($P < 0.02$).

Effect of medium base on sensitivity and zone diameter. Test plates utilizing different bases were prepared and assayed as described above. The medium bases tested were BHI, Mueller-Hinton (MH) (BBL), Trypticase soy (TS) (BBL), TH, and heart infusion (HI) (Difco).

The assays run on BHI plates had greater sensitivity than assays run on the other bases tested. The sensitivity of assays on the BHI, TS, MH, HI, and TH plates were 47, 133, 145, 63, and 90 pg of tetracycline, respectively.

The standard curves are shown in Fig. 3. The BHI medium resulted in the largest zone diameters throughout the assay range. The slope of the regression line for the BHI assay was significantly greater ($P < 0.01$) than the slopes for the assays of each of the other media. There was a significant difference in slope between regression lines of zone diameters obtained on HI medium and TS ($P < 0.02$), TH ($P < 0.01$), and MH ($P < 0.01$) media. Differences in slope and intercept between zone diameters on MH and TS agars were not significant. Regression analysis indicated that the slopes of the assays conducted on TH, TS, and MH agar were not significantly different; however, the intercepts were significantly different at $P < 0.01$ (TH > TS = MH).

Precision and accuracy of assay. The precision of the assay was determined by measuring zone diameters produced by replicate standards on filter paper strips. The standard strips were prepared by placing 0.5 μl of tetracycline solution standards on filter paper strips with a 1.0-μl Hamilton syringe. Six strips of each of the six

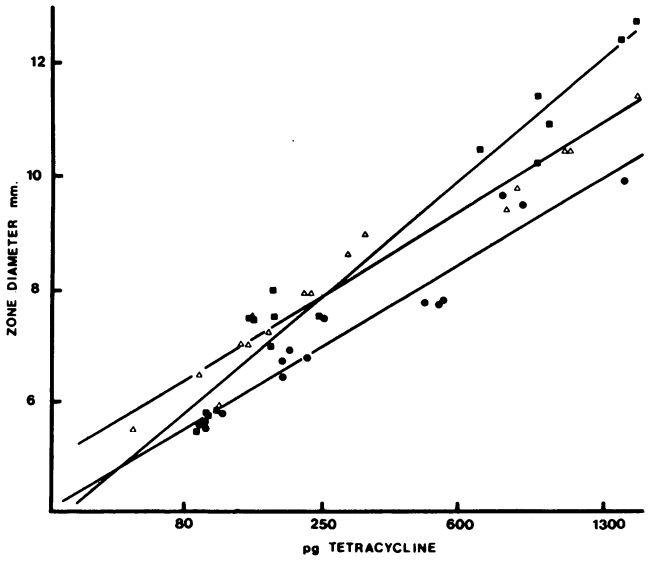

FIG. 2. *Effect of pH on zone diameter. The slope of the tetracycline zone formation response at pH 6.0 (■) was significantly greater than the response at pH 6.6 (△) or 7.4 (●). However, the zone diameters with low concentrations of tetracycline were larger at pH 6.6 than at pH 6.0.*

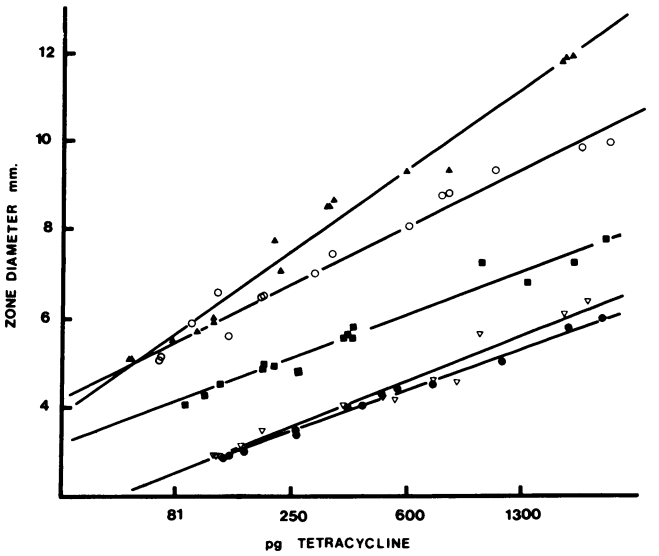

FIG. 3. *Effect of basal medium on zone diameter. The slope of tetracycline zone formation response on BHI agar (▲) was significantly greater by regression analysis (P < 0.01) than assays conducted on HI (○), TH (■), TS (▽), and MH (●) agars, respectively.*

concentrations of tetracycline were assayed on the same plate. Figure 4 shows the results of six replications at each concentration of tetracycline and the prediction interval (6) for a new value. The confidence interval, which is narrower than the prediction interval, ranged from ±4% at the highest concentration of tetracycline to ±10% at the lowest concentration.

To test the accuracy of the procedure, we prepared 47 strips with known concentrations of tetracycline. Standards were run in triplicate, and the zone diameters of standards and unknowns were determined. The method of Colquhoun (3) was used to aid in determining the best fit of the regression line and predict the values of the unknowns. The absolute differences between the predicted and actual concentrations on the strips were determined and expressed as a percentage of the known concentrations. The percent error was 11.6 ± 10.3 (mean ± standard deviation).

Use of assay in monitoring crevicular fluid tetracycline concentration. A representative clinical use of this assay is presented in Fig. 5. A single dose of 500 mg of tetracycline hydrochloride was orally administered to a volunteer. Crevicular fluid was sampled from four sites at 1-h intervals for 22 h. The data presented include the sites showing the highest and lowest levels of tetracycline of the four sites sampled. Tetracycline levels in crevicular fluid peaked at 5 to 6 h, reaching levels of 5.6 to 18.6 μg per ml, and declined slowly to ca. 2.0 μg/ml by 16 h. In contrast, the concentration of tetracycline in blood obtained by finger puncture peaked at 3 h, reaching a peak level of 2.69 μg/ml, and declined to 0.2 μg/ml at 16 h. The sharp peak response at 5 h did not appear to represent experimental error since similar responses were observed in most subjects tested (Gordon et al., in preparation).

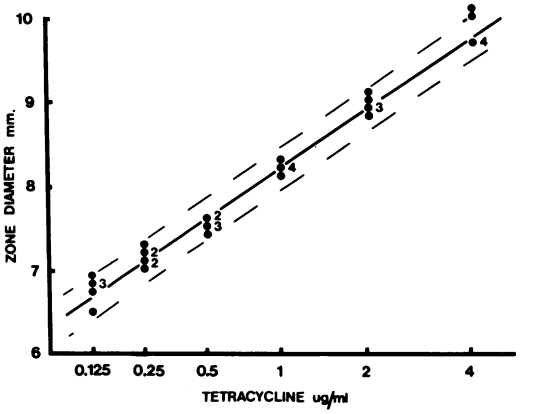

FIG. 4. *Precision of the tetracycline assay. The response of six replicates at each of six concentrations over the useful assay range was determined. The regression line (solid) and 95% prediction interval (dashed line) are illustrated. The numbers in the figure represent the number of coincident points.*

DISCUSSION

Gingival crevice fluid seeps slowly from the tissues into the gingival crevice and out into the saliva. The flow rate of gingival fluid is low in

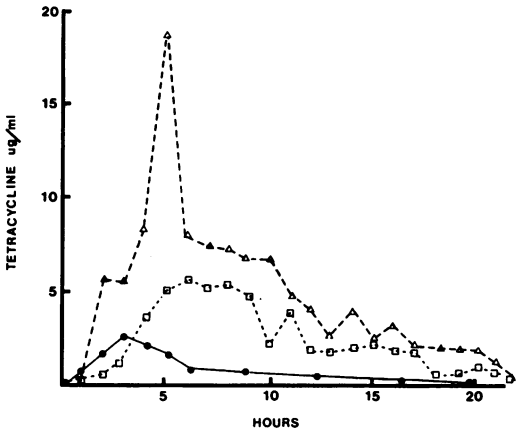

FIG. 5. *Tetracycline concentrations in gingival crevice and blood resulting from oral administration of 500 mg of tetracycline. Blood (●) was obtained by finger puncture. The gingival fluid values illustrated are from the sites showing the maximum (△) and minimum (□) concentrations of tetracycline in that individual.*

healthy sulci, but increases with increased severity of inflammation (2). Because this fluid bathes the subgingival microbial masses, measurement of antibacterial concentration in it would be a reflection of the level of systemically administered antimicrobial agent presented to the subgingival microorganisms.

The major problem in such an assay is the amount of fluid available. One to 2 μl of fluid can be collected by inserting glass capillaries into the area for periods of 15 to 30 min. However, this technique is laborious, subject to salivary contamination, and may not truly reflect gingival fluid, but rather a fluid resulting from injury to the pocket wall by inserting the device into the intracrevicular area. The method of collecting gingival fluid with standardized filter paper strips obviates many of these problems. Volumes obtained by this method using 3-s sampling periods are typically between 0.01 and 0.5 μl.

Due to the limited volume of fluid obtainable from the gingival crevice, an assay capable of measuring the levels of tetracycline in crevicular fluid must have a greater sensitivity than assays utilized for the measurement of tetracycline in blood. The greater sensitivity of the present assay was achieved by optimizing several factors known to affect the sensitivity of agar diffusion assays. The factors investigated were medium depth, agar concentration, inoculum density, pH, prediffusion, and choice of basal medium.

Jay and Sherris (5), using high concentrations of tetracycline, observed zones which were 5.0- to 8.0-mm greater in diameter on MH media than on TS agar. In our assay system, MH and TS yielded similar zone diameters and sensitivity. Both MH and TS plates had the least sensitivity and smallest zone diameters of the bases tested. This apparent failure of MH agar in this assay may be due to the more rapid growth of *C. perfringens* on this medium. The increased rate of growth resulted in earlier zone formation compared to other media. This allowed less time for the antibiotic to diffuse from the paper strip, resulting in much smaller zones and decreased sensitivity. It is possible that if other factors, especially inoculum density, were changed to increase the time of the assay, MH media might yield acceptable zone diameters and sensitivity.

The present assay has a sensitivity of 50 pg of tetracycline per strip which allows detection of tetracycline at levels as low as 0.1 μg of tetracycline per ml in 0.5 μl of gingival fluid. Use of this assay on samples taken after systemic administration of a single dose of 250 or 500 mg of tetracycline revealed peak gingival fluid levels which were higher than peak blood levels and persisted for a longer period of time. The reasons for this elevated concentration of tetracycline in gingival fluid are not clear.

ACKNOWLEDGMENTS

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LITERATURE CITED

1. **Chilton, N. W., and M. F. Miller.** 1977. Diagnostic methods, p. 93–118. *In* International Conference on Research in the Biology of Periodontal Disease. University of Illinois, Chicago.
2. **Cimasoni, G.** 1974. The crevicular fluid. Monogr. Oral Sci., vol. 3. S. Karger, Basel.
3. **Colquhoun, D.** 1971. Lectures on biostatistics. Clarendon Press, Oxford.
4. **Grove, C., and W. A. Randall.** 1955. Assay methods of antibiotics. A laboratory manual. Medical Encyclopedia Inc., New York.
5. **Jay, V. C., and J. C. Sherris.** 1971. Differences in tetracycline zone sizes on routine and reference media. Report for antibiotic sensitivity testing. Report of International Collaborative Study. Acta Pathol. Microbiol. Scand. Sect. B Suppl. **217**:30.
6. **Kleinbaum, D. G., and L. L. Kupper.** 1978. Applied regression analysis and other multivariable methods. Duxbury Press, California.
7. **Listgarten, M. A., J. Lindhe, and L. Hellden.** 1978. Effect of tetracycline and/or scaling on human periodontal disease. Clinical microbiological and histological observation. J. Clin. Periodontol. **5**:246–271.
8. **Socransky, S. S.** 1977. Microbiology of periodontal disease—present status and future considerations. J. Periodontol. **48**:497–504.
9. **Suppipat, N., and N. Suppipat.** 1977. Evaluation of an electronic device for gingival fluid determination. J. Periodontol. **48**:388–394.
10. **Walker, C. B., D. Nitzan, and T. D. Wilkins.** 1977. Chemotherapy of an experimental *Bacteroides fragilis* infection in mice. Antimicrob. Agents Chemother. **11**: 435–440.