From: The Department of Surgery, School of Dentistry, University of Umeå, Umeå 2, Sweden.

THE EFFECT OF LEUKOCYTIC ENZYME ACTIVITY ON THE STRUCTURE OF THE GINGIVAL POCKET EPITHELIUM IN MAN

by

HOLGER THILANDER

INTRODUCTION

It is now fairly generally accepted that bacterial action is the precipitating factor in the occurrence of periodontal disease. A bacteriologically favourable environment is provided through the accumulation of hard and soft deposits (4, 5, 23). That tissue irritant substances are produced in these bacteria-rich deposits on the teeth and gingiva has been established by a number of workers (1, 3, 7, 11, 12, 13). In a series of studies it has likewise been shown that oral bacteria possess the ability to form enzymes that can act as catalysts in the hydrolysis of various structures in the underlying connective tissue (6, 14, 16, 17, 18). To penetrate to the periodontal connective tissue these tissue-destroying substances must, however, pass the barrier presented by the periodontal epithelium. It has been demonstrated (21) that such a penetration takes place and that it occurs both in the border zone between the pocket epithelium and the dental hard tissues and through the intercellular spaces of the pocket epithelium. This process may be promoted by a number of factors, one of which is the action of various bacterial enzymes on the structure of the epithelium. It has been shown (15) that hyaluronidase, applied

locally in the gingival pocket, causes a widening of the intercellular spaces of the epithelium.

The leukocytes contain a large variety of enzymes (19), including proteases, amylases, peroxidases and lipases. Leukocytes are common in the pocket epithelium and the gingival pocket in both clinically healthy and inflamed gingiva (9, 23). In the latter case the gingival pocket contains such large quantities of granulocytes that the pocket epithelium is separated from the plaque by large aggregations of these cells, many of which are in a state of necrosis (23). It is therefore of major interest from the aspect of the pathogenesis to understand the action of these enzymes on the structure of the pocket epithelium.

For that reason a study was performed to examine this action in man. The effect of hyaluronidase, the "spreading factor", both separately and in combination with the other enzymes mentioned was also explored.

METHODS

The study was performed on 12 healthy subjects (7 males and 5 females) with ages ranging from 21 to 25 years. In all 12 subjects, gingiva in the anterior segment of the lower jaw was clinically healthy.

Twenty millilitres of blood were drawn from each subject and spiral centrifuged to give a leukocyte fraction of 1.3 ml. Of this, 0.1 ml was used for leukocyte counts. The remainder was cooled with ice and submitted to supersonic treatment for 10 minutes in an MSE supersonic desintegrator, Model 60, to fragment the cells.

For each subject the following 4 tubes were prepared,

Tube 1. 0.5 ml of *own* leukocyte homogenate + 0.5 ml physiologic saline.

Tube 2. One millilitre of hyaluronidase solution (Hyalas, Leo).

Tube 3. 0.5 ml *own* leukocyte homogenate + 0.5 ml hyaluronidase solution.

Tube 4. One millilitre physiologic saline, to serve as a control.

The concentration of the leukocyte homogenates in tubes 1 and 3 varied for the different subjects and ranged from 3.8 to 34.2×10^6 leukocytes per ml. The hyaluronidase concentration was 200 I.U. per ml.

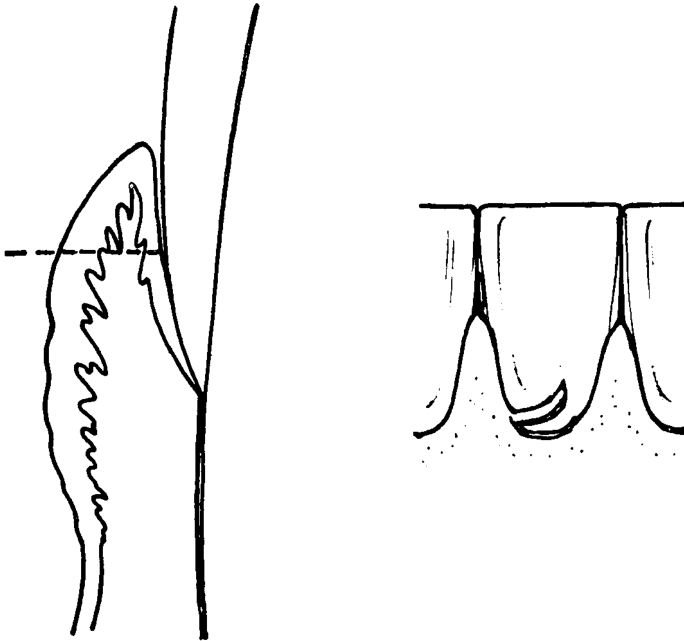


Fig. 1. Diagram showing the localization and size of the excised marginal part of the gingiva.

The experiments were performed on the four gingival front regions in the mandible (from the left to the right second incisor). The region to which the respective solutions should be applied for each patient was decided by random drawing.

The solutions were applied in the following manner: The area of the gingiva in question was dried by an air blast and the pocket was dried carefully with filter paper. A dry field was maintained throughout the experiment by means of a saliva ejector and cotton rolls. One drop of the solution was applied with a fine glass pipette on the facial aspect of the gingival pocket and on the border of the free gingiva. Care was taken not to traumatize the epithelium. This procedure was repeated every 5 minutes, the pocket being carefully dried between applications of solution. The experiment covered 60 minutes.

Immediately after this period, the marginal part of the gingiva was excised without anesthesia (Fig. 1). The tissue specimen was

divided in the middle (corresponding to the mesial and distal part *in situ*) for examination by light and electron microscopy. For the microscopic examination the upper part of the pocket epithelium was chosen since for obvious reasons only a narrow zone of gingival tissue could be excised.

The specimens for light microscopy were fixed for 24 hours in a solution consisting of one part of 40 per cent formalin and 3 parts of 95 per cent ethyl alcohol. After embedding in paraffin wax and sectioning by standard procedures, the sections were stained by the following methods: haemalum and eosin (Mayer), picrofuchsin (van Gieson), toluidin blue (0.1 per cent solution in 30 per cent ethyl alcohol), PAS (McManus), Astrablau (2) and mercuric chloride-bromphenol blue (8).

The specimens for electron microscopy were fixed for 1½—2 hours in one per cent solution of osmium tetroxide buffered with veronal (10) and, after dehydration, embedded in a mixture of metyl and butyl methacrylate (1: 9). Polymerization was carried out at 45° C. The sectioning was performed with a glass knife in an LKB ultrotome. An RCA EMU-2B electron microscope was used.

RESULTS

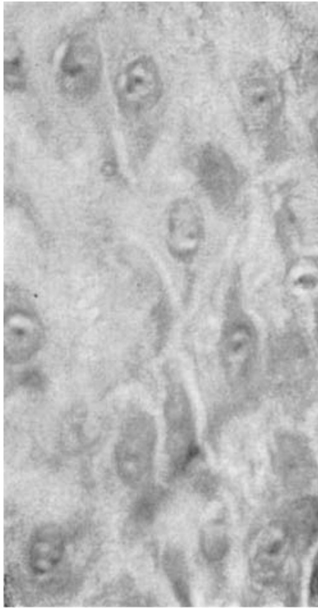
To simplify the description of the results the following notation will be used.

C-, L-, H- and LH-sections denote sections from the regions where the respective physiologic saline, leukocyte homogenate, hyaluronidase solution and leukocyte homogenate + hyaluronidase solution were applied.

Light microscope examination

Although the gingiva of the regions studied was clinically healthy certain cases displayed definite signs of an inflammatory

Fig. 2. The width and appearance of the intercellular spaces in the different sections. a) C-section, b) L-section, c) H-section, d) LH-section. The intercellular spaces in the L-, H- and LH-sections are wider than in the C-section. In this LH-section the boundary between intercellular spaces and cells is diffuse. Mayer's haemalum-eosin.



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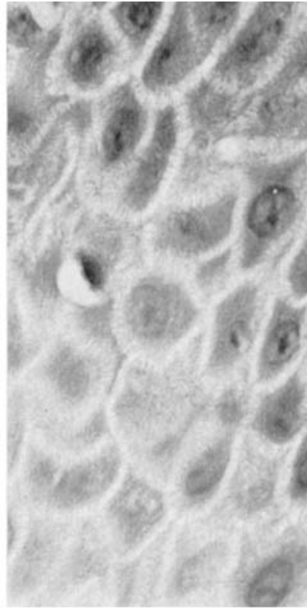

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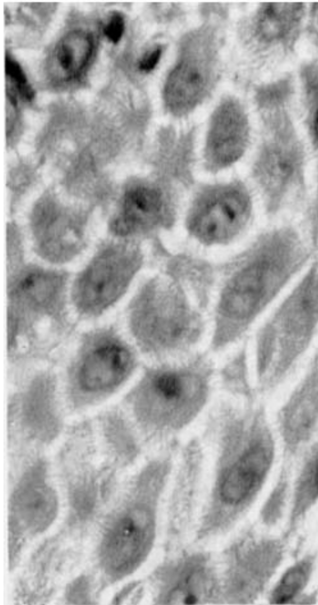

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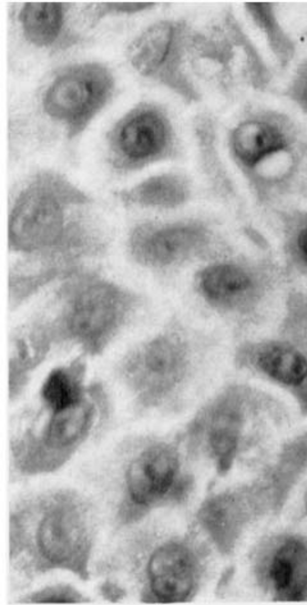

d.

Fig. 2.

reaction in the connective tissue, but these regions were uniformly distributed among the various treated tissues.

The intercellular spaces in the L-, H- and LH-sections were consistently wider than in the C-sections. The L- and H-sections displayed no difference in this respect, but compared with these two groups the intercellular spaces in the LH-sections were usually wider; in a few of these specimens, the boundary between intercellular spaces and cells was diffuse in small well-defined areas. The width and appearance of the intercellular spaces are illustrated in Fig. 2.

In all the PAS stained sections there was a weak positive reaction in the intercellular spaces. In the extreme marginal part of the pocket epithelium the cytoplasm exhibited an intense positive reaction, in several of the specimens in the surface layer and sometimes also in the stratum spinosum. This reaction was, however, considered to be associated with the presence of inflammation in the connective tissue rather than with variations in the experimental treatment of the tissue. No distinct difference between the C-, L-, H- and LH-sections stained with PAS was evident with respect to positive reactions.

Staining of the epithelium with Astrablau, to demonstrate acid mucopolysaccharides, was so weak that it was impossible to discern any differences between the sections as regards intensity or site of staining.

The mercuric chloride-bromphenol blue reaction for protein produced a more intense staining in the superficial than in the deeper layers of the epithelium. The peripheral cytoplasm of the cells was stained more intensely than their central regions. The only difference that could be discerned between the four groups was an often weaker staining intercellularly in the LH-sections, but the difference was not great.

Electron microscope examination

In the region used for the ultrastructural examination (Fig. 3), the stratum spinosum was chosen for comparison between the differently treated areas, for earlier electron microscope studies of the oral mucosa have shown that the epithelial cells in the outermost layer are often slightly separated from one another.

This has been ascribed to, for instance, an incipient physiologic desquamation and/or a continuous effect of the enzymes in the saliva (20).

In order to elucidate the structural differences observed within the stratum spinosum of the pocket epithelium on the enzyme-treated tissue, a short description of the morphology of the region will be given as seen in the C-sections (Figs. 4—6). Here the epi-

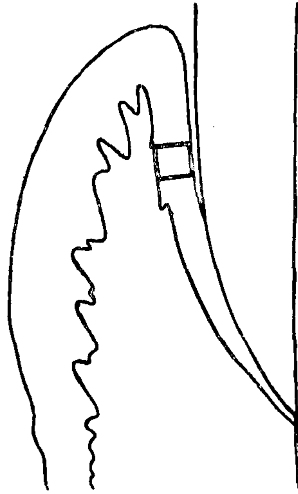

Fig. 3. The framed area illustrates the region used for the electron microscopic examination.

thelial cells were usually irregular in shape and numerous cytoplasmic processes in the form of fine microvilli gave the cell periphery an uneven appearance. These projections of adjacent cells often interlaced, with the result that the intercellular space was narrow and followed an often complex and tortuous course. Desmosomes were extremely numerous and appeared as electron-dense formations with a fairly regular distribution around the cells. The two opposing cell membranes in a desmosome were thickened and strongly osmiophilic, and in the space between them a fine granular, sometimes lamellar, intercellular substance was observed (Fig. 6). The cytoplasm contained numerous osmiophilic fibrils (tonofibrils), the finer elements — the tonofilaments — of which seemed to be intimately connected with the attach-

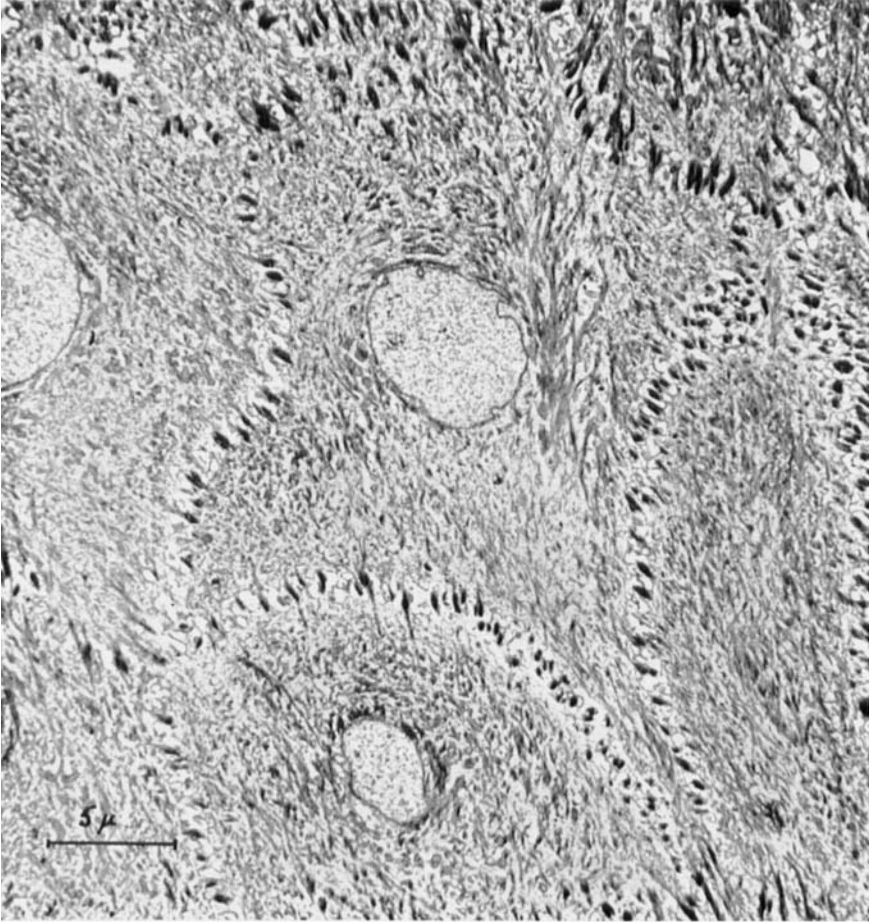

Fig. 4. Non-enzyme-treated tissue (C-section). Epithelial cells in the stratum spinosum in a low magnification. The cells are irregular in shape and numerous cytoplasmic processes give the cell periphery an uneven appearance. The intercellular space is narrow and follows a complex and tortuous course. The desmosomes are numerous with a fairly regular distribution around the cells. The cytoplasm contains numerous osmiophilic fibrils (tonofibrils).

ment plaques of the desmosomes. In the other parts of the intercellular space there were dispersed regions with a finely granulated substance of low electron density; no other signs of substance were observed.

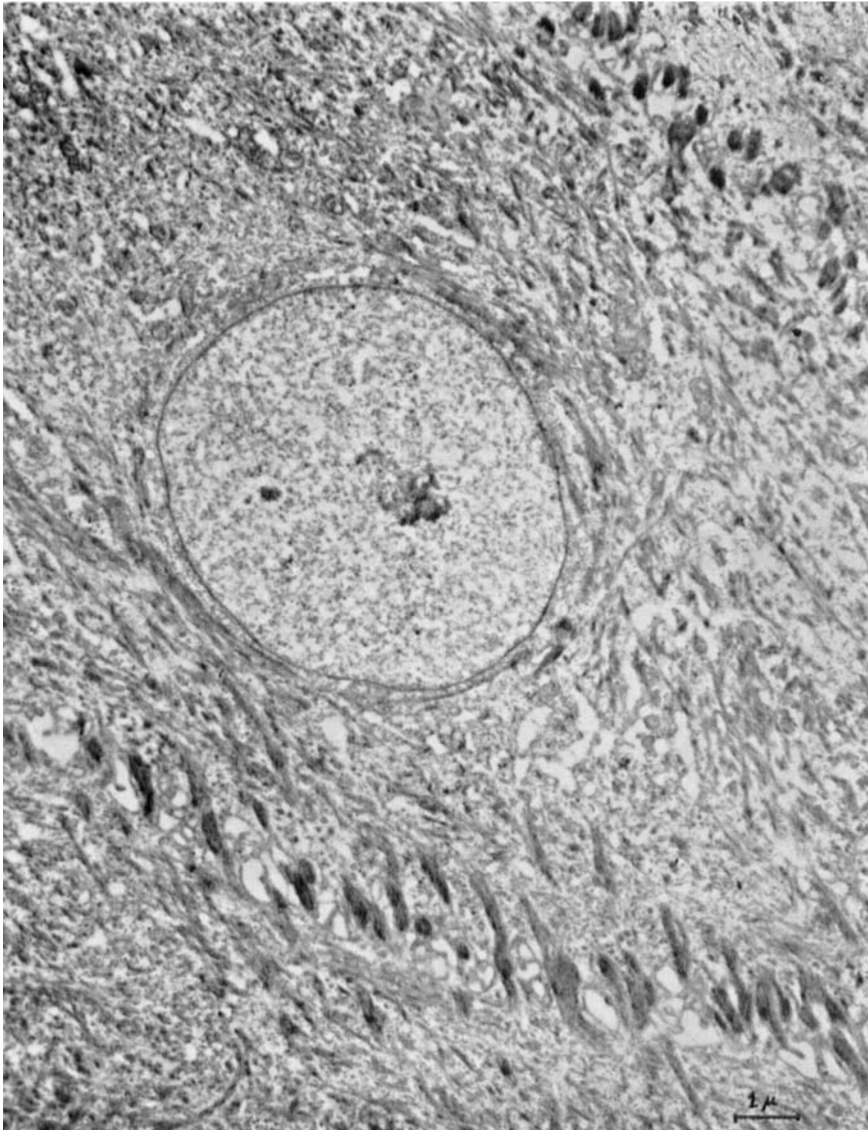

Fig. 5. Non-enzyme-treated tissue (C-section). Higher magnification than in Fig. 4.

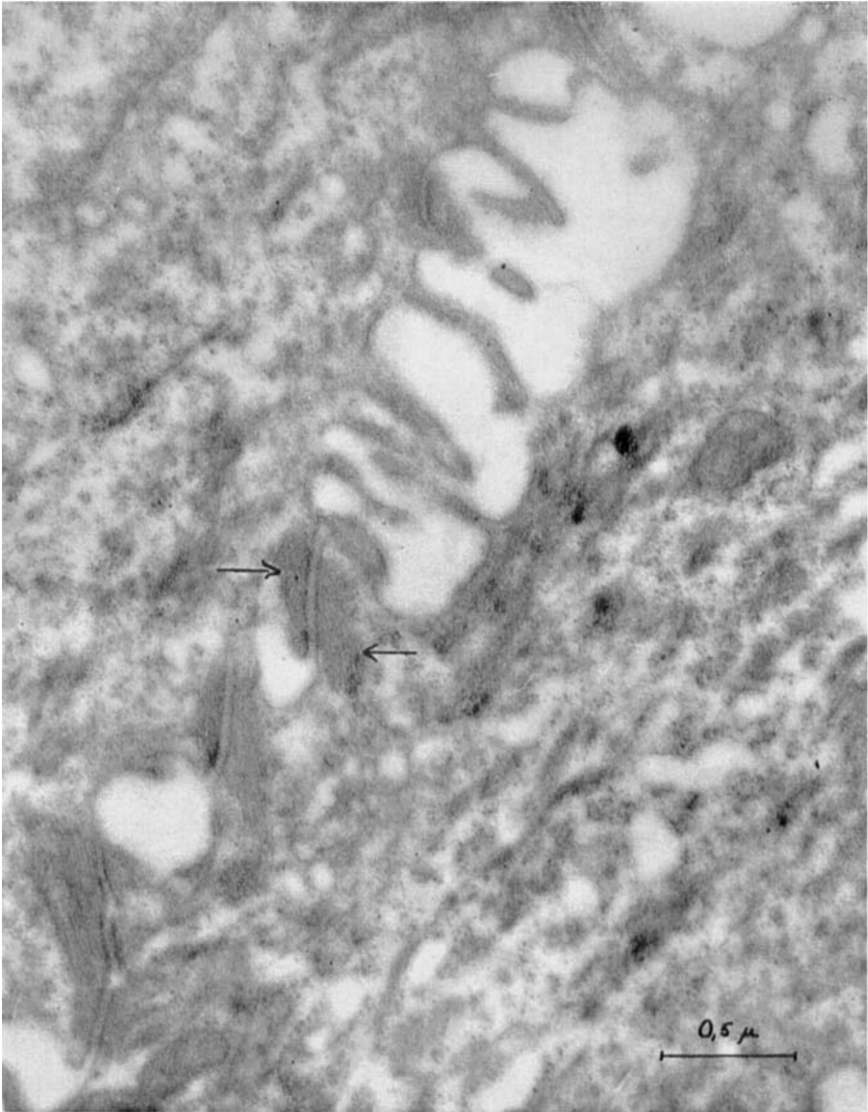

Fig. 6. Non-enzyme-treated tissue (C-section). A high magnification of an intercellular region. Cytoplasmic processes in the form of fine microvilli. The figure illustrates the structure of the desmosomes. The tonofilaments (arrow) seem to be intimately connected with the attachment plaques of the desmosomes.

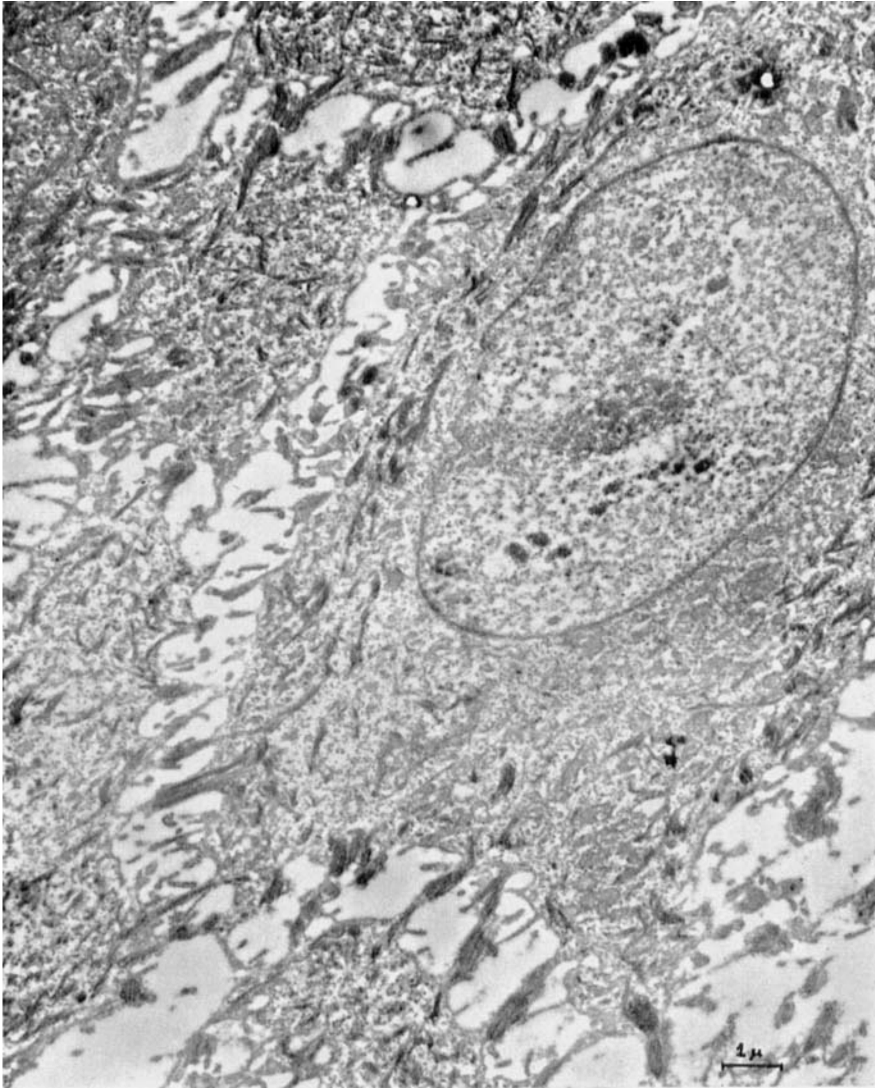

Fig. 7. Tissue treated with the intracellular enzymes of the leukocytes (L-section). Wide intercellular spaces. The desmosomes are connected with the respective cells by narrow cytoplasmic processes. In this figure there are fewer desmosomes than in Fig. 5.

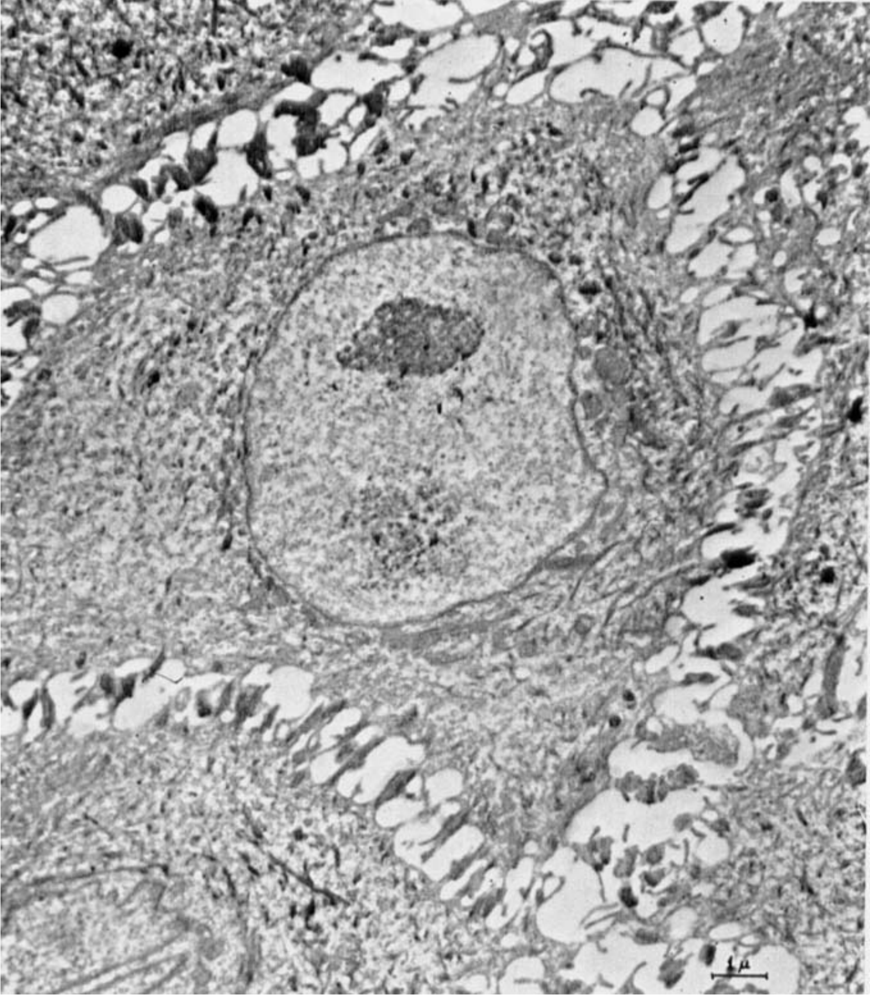

Fig. 8. Tissue treated with hyaluronidase. Wide intercellular spaces. Here the desmosomes are rather numerous.

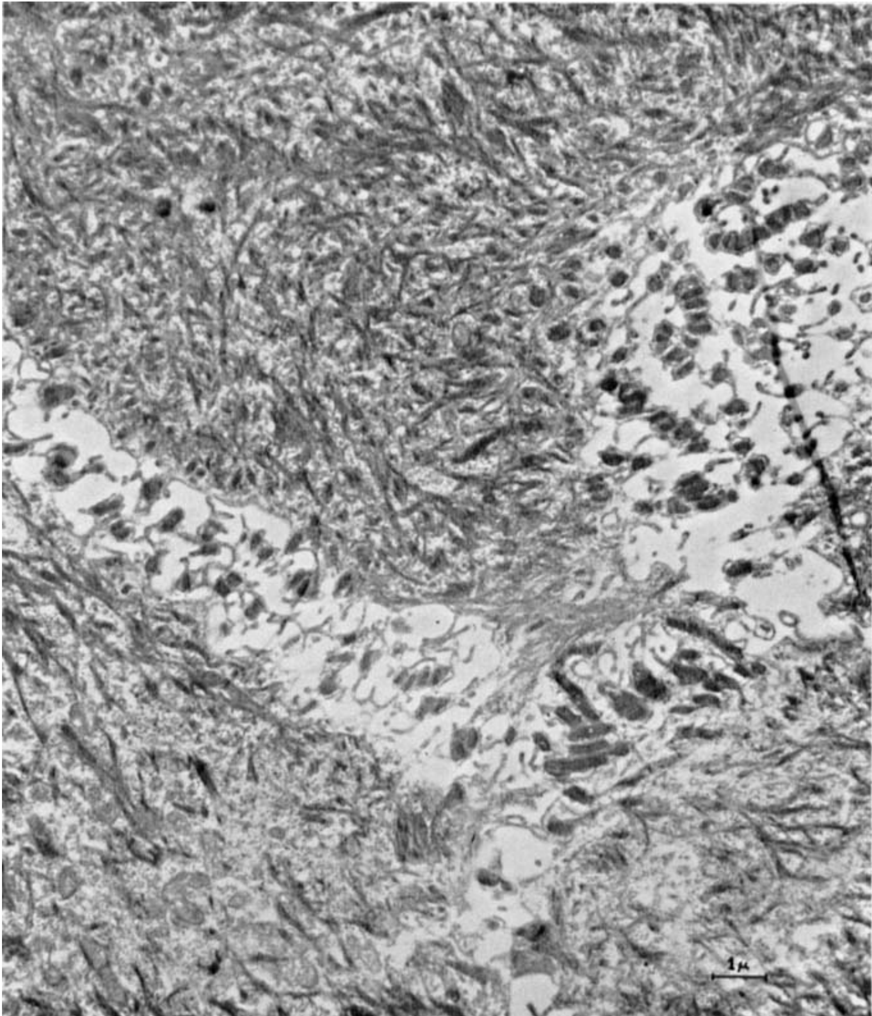

Fig. 9. Tissue treated with the intracellular enzymes of the leukocytes + hyaluronidase (LH-section). In these sections the intercellular spaces were widest. The numerous desmosomes lie like osmiophilic islands in the intercellular space, connected with the respective cells by narrow cytoplasmic processes.

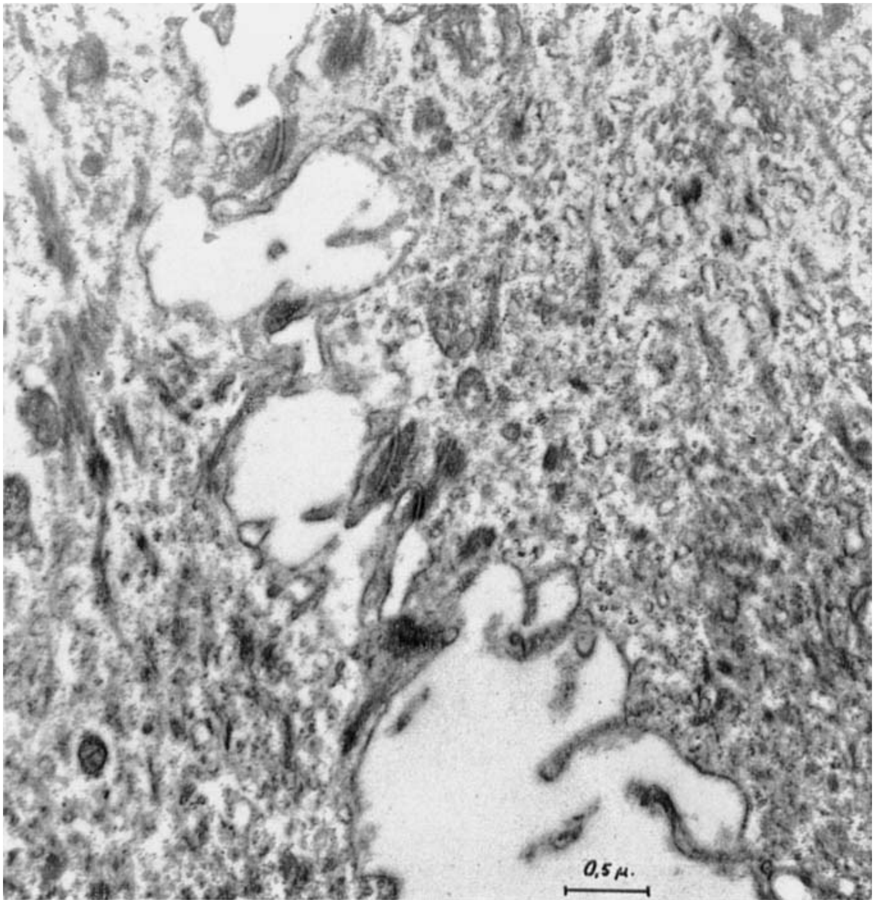

Fig. 10. A higher magnification from the same section as in Fig. 9. Here the finer structure of the desmosomes seems to be unaffected.

The pictures presented by L-, H- and LH-sections differed from this principally by virtue of the general widening of the intercellular spaces (Figs. 7—9). The desmosomes lay like osmiophilic islands in the wide spaces and were connected with the respective cells by narrow cytoplasmic processes. The fine structure of the desmosomes appeared to be largely unchanged (Fig. 10); in only a few of them there was apparently a separation of attachment plaques from one another. As in the C-sections, the intercellular

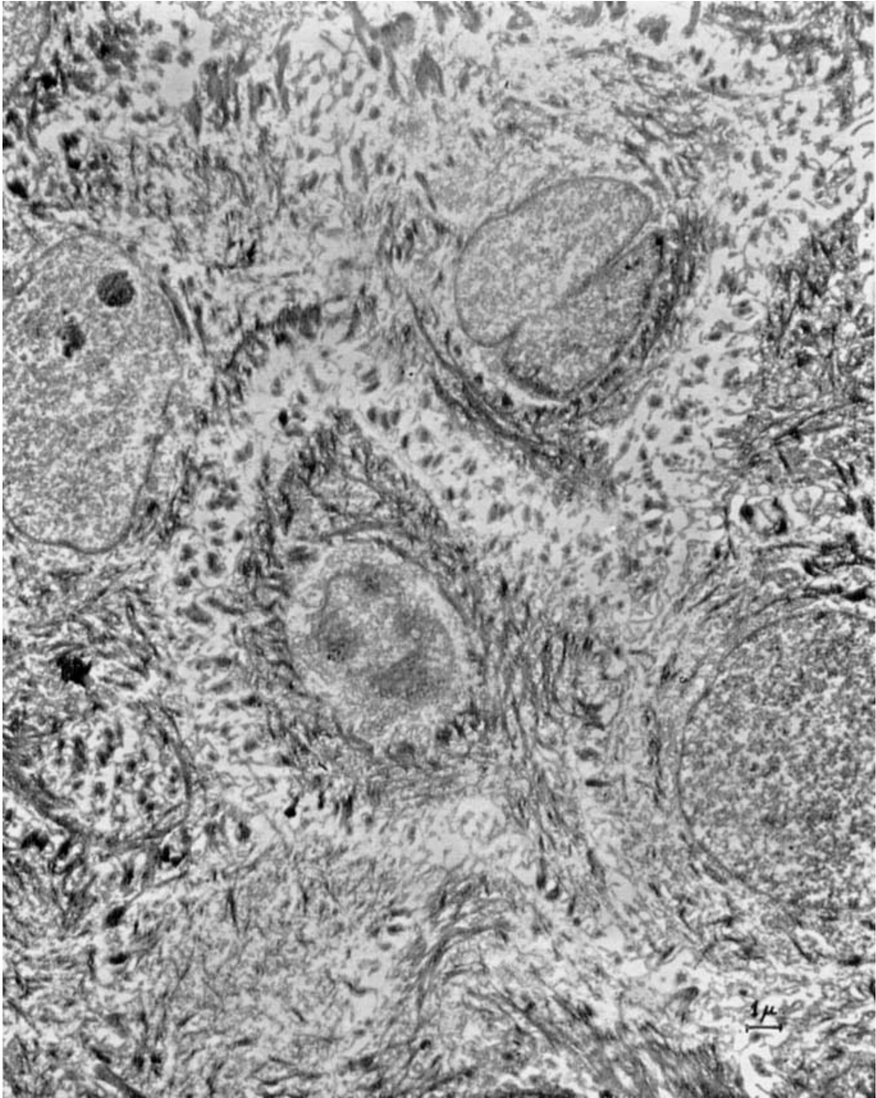

Fig. 11. Tissue treated with the intracellular enzymes of the leukocytes + hyaluronidase (LH-section). In this figure there are distinct cellular changes, the cell membranes and a nuclear membrane are diffuse.

space contained dispersed regions with a finely granulated substance, but otherwise no fine structure was observed. The most pronounced changes occurred in the LH-sections, where the intercellular spaces were consistently widest (Fig. 9). In a few specimens from this group there were distinct cellular changes. Certain structures such as the cell membrane, and sometimes also the nuclear membrane, were diffuse and the picture resembled that found in severe pathologic alterations (Fig. 11).

DISCUSSION

The increase in the width of the intercellular spaces observed in this investigation after local application of the different enzyme preparations was probably due to their effect on the intercellular substance and the mutual adhesion mechanism of the cells. It is well known that cells in tissue cultures lose their property of adhering to glass after treatment with trypsin and it has been shown that this adhesive property is restored when protein in the form of serum is added to the medium (24). This behavior has been ascribed to enzymatic digestion of a thin extracellular coating of the cell membranes consisting of polysaccharideprotein complexes (glycoproteins) (25).

Histochemical investigations by, for instance, *Wislocki, Fawcett & Dempsey* (1951) and *Thonard & Scherp* (1959), together with the positive reaction to PAS, metachromasia and the protein reaction which were observed to occur intercellularly in this investigation, suggest that the epithelium of the oral mucosa contains both polysaccharides and proteins intercellularly. An enzymatic breakdown of these substances (and the enzyme preparations used included proteases and hyaluronidase) may result in a modification of the primary and secondary forces (chemical bondings, "long range forces" etc.) which are thought to be responsible for the adhesion between cells. In the break-down of macromolecular substances such as proteins and polysaccharides, an increase in the uptake of water must occur to maintain constant tonicity. An increase in water uptake together with a weakening of the adhesion effect may well result in a widening of the intercellular spaces.

Except for the protein reaction the histochemical differences between the various treated specimens were not pronounced. This to some extent negative result of an attempt to verify histochemically the changes in the intercellular substance between the enzyme-treated and non-enzyme-treated specimens may be due to a low sensitivity of the methods for recording small changes in concentration in the substances.

No widening of the intercellular space was found in most of the desmosomes, since the distance between attachment plaques appeared to be for the most part unchanged. It would thus seem as if the most important cohesive forces between the cells are localized to these structures. The intercellular distance here is less than elsewhere, a factor that may have some bearing on, for instance, the magnitude of the primary and secondary forces of adhesion. Moreover, the intercellular substances here have a higher electron density and a different structure than the other intercellular substance. The relative difficulty in separating attachment plaques from one another has been thought to indicate the presence of a cohesive force far stronger than can be accounted for by a pure adhesion effect (26).

In occasional specimens treated with preparations containing both intracellular enzymes and hyaluronidase, cells were found that had undergone severe pathologic changes. It is possible that these cells were necrobiotic before the application of those preparations, but intense enzymatic digestion cannot be excluded.

The results of this investigation show that intracellular enzymes from leukocytes may be a factor of importance in the pathogenesis of periodontal disease. The widening of the intercellular spaces in the epithelium under the influence of these enzymes would promote the passage of irritant and destructive substances through the epithelium into the underlying connective tissue. If these enzymes are combined with hyaluronidase this cell-separating effect is increased. The property of hyaluronidase of causing a widening of the intercellular spaces in the epithelium confirms previous observations under the light microscope by *Schultz-Haudt, Dewar & Bibby* (1953).

SUMMARY

A light microscope and electron microscope study has been performed of the structural differences in the epithelium of the gingival pocket in man after local application *in vivo* of hyaluronidase, the intracellular enzymes of leukocytes, and a combination of these. The study was performed on 12 subjects with clinically healthy gingiva.

A widening of the intercellular space was observed which was most marked after application of intracellular enzymes + hyaluronidase.

This widening may be due to increased uptake of water in association with a reduction of the adhesive effect between the cells, following enzymatic breakdown of the macromolecular intercellular substance. Widening of the intercellular space did not affect a majority of the desmosomes.

The findings suggest that these enzymes may constitute a significant factor in the pathogenesis of periodontal disease. The widening of the intercellular spaces of the epithelium would be expected to promote the passage of irritant and destructive substances through the epithelium into the underlying connective tissue.

RESUMÉ

EFFET DE L'ACTIVITÉ ENZYMATIQUE LEUCOCYTAIRE SUR LA STRUCTURE DE L'ÉPITHÉLIUM DU SILLON GINGIVO-DENTAIRE CHEZ L'HOMME

Une étude au microscope optique et au microscope électronique a été faite sur les différences de structure dans l'épithélium du sillon gingivo-dentaire chez l'homme après application locale *in vivo* d'hyaluronidase, d'enzymes intra-cellulaires leucocytaires et d'une combinaison des deux. L'étude a été effectuée sur 12 sujets présentant une gencive cliniquement saine.

Un élargissement de l'espace intercellulaire a été observé, et ceci d'une manière plus accusée après application d'enzymes intra-cellulaires + hyaluronidase.

Cet élargissement peut être dû à une augmentation de l'absorption de l'eau associée à une réduction de l'effet d'adhésion entre les cellules, à la suite de la désintégration enzymatique de la sub-

stance intercellulaire macromoléculaire. L'élargissement de l'espace intercellulaire n'influait pas sur la majorité des desmosomes.

Les résultats de cette expérience incitent à penser que les enzymes peuvent constituer un facteur important dans la pathogénie des affections du parodonte. On pourrait supposer que l'élargissement des espaces intercellulaires de l'épithélium favorise le passage à travers l'épithélium de substances irritantes et destructrices dans le tissu conjonctif sous-jacent.

ZUSAMMENFASSUNG

DIE AUSWIRKUNG DER LEUKOZYTENAKTIVITÄT AUF DAS EPITHEL DER ZAHNFLEISCHTASCHEN BEIM MENSCHEN

Es wurde eine lichtmikroskopische und elektronenmikroskopische Untersuchung der Strukturunterschiede im Epithel der Zahnfleischtaschen beim Menschen nach einer Lokalapplikation in vivo von Hyaluronidase, von intrazellularen Enzymen aus Leukozyten und einer Kombination von diesen ausgeführt. Die Untersuchungen wurden auf 12 Personen mit klinisch gesundem Zahnfleisch vorgenommen.

Man beobachtete eine Expansion des Interzellular-Gewebes, die nach einer Applikation von intrazellularen Enzymen + Hyaluronidase am ausgesprochensten war.

Diese Expansion kann auf eine Aufnahme von Wasser in Verbindung mit einer Reduktion des Adhäsionsvermögens der Zellen infolge eines enzymatischen Abbaus der makromolekularen Substanz zurückzuführen sein. Die Expansion des Interzellular-Gewebes hatte keine Einwirkung auf eine Mehrheit der Desmosomen.

Die Befunde lassen darauf schliessen, dass diese Enzyme in der Pathogenese der parodontalen Krankheiten eine wichtige Rolle spielen können. Man muss annehmen, dass die Expansion des Interzellular-Gewebes im Epithel die Passage der Reiz- und destruktiven Stoffe durch das Epithel in das darunterliegende Bindegewebe fördert.

REFERENCES

1. *Beckwith, T. D., A. Williams & E. J. Rose*, 1929: The role of bacteria in pyorrhea. Med. J. Rec. *129:* 333.
2. *Bloom, G. & J. Kelly*, 1960: The copper phthalocyanin dye Astrablau and its staining properties, especially the staining of mast cells. Histochemie *1:* 48.
3. *Graham, J. W.*, 1937: The toxicity of sterile filtrate from parodontal pockets. Proc. roy. Soc. Med. *30:* 1165.
4. *James, W. W. & A. Counsell*, 1928: The primary lesion in so-called pyorrhoea alveolaris. Brit. dent. J. *49:* 1129.
5. *Krasse, B. & N. Brill*, 1960: Effect of consistency of diet on bacteria in gingival pockets in dogs. Odont. Revy *11:* 152.
6. *Lucas, R. B. & J. C. Thonard*, 1955: Action of oral bacteria on collagen. J. dent. Res. *34:* 118.
7. *Macdonald, J. B., R. M. Sutton & M. L. Knoll*, 1954: The production of fusospirocheatal infections in guinea pigs with recombined pure cultures. J. infect. Dis. *95:* 275.
8. *Mazia, D., P. P. Brewer & M. Albert*, 1953: The cytochemical staining and measurement of protein with mercuric bromphenol blue. Biol. Bull. *104:* 57.
9. *Orban, B. & E. Mueller*, 1929: The gingival crevice. J. Amer. dent. Ass. *49:* 177.
10. *Rhodin, J.*, 1954: *Correlation of ultrastructural organization and function in normal and experimentally changed proximal convoluted tubule cells of the mouse kidney.* Thesis. Stockholm.
11. *Rosebury, T., A. R. Clark, S. Engel & F. Thergis*, 1950: Fusospirochetal infection. I. Pathogenicity for guinea pigs of individual and combined cultures of spirochetes and other anaerobic bacteria derived from the human mouth. J. infect. Dis. *87:* 217.
12. *Rosebury, T., A. R. Clark, F. Thergis & S. Engel*, 1950: Studies of fusospirochetal infection. II. Analysis and attempted quantitative recombination of the flora of fusospirochetal infection after repeated guinea pig passage. J. infect. Dis. *87:* 226.
13. *Rosebury, T., A. R. Clark, J. B. Macdonald & D. C. O'Connell*, 1950: Studies of fusospirochetal infection. III. Further studies of guinea pig passage strain of fusospirochetal infection including the infectivety of sterile exudate filtrates, of mixed cultures through ten transfers and of recombined pure cultures. J. infect. Dis. *87:* 234.
14. *Schultz-Haudt, S. D.*, 1957: *Observations on the acid mucopolysaccharides of human gingiva.* Thesis. Oslo University Press, Oslo.
15. *Schultz-Haudt, S. D., M. Dewar & B. G. Bibby*, 1953: Effects of hyaluronidase on human gingival epithelium. Science, *117:* 653.

16. *Schultz-Haudt, S. D. & H. W. Scherp*, 1955: Production of hyaluronidase and beta glucuronidase by viridans streptococci isolated from gingival crevices. J. dent. Res. *34:* 924.

17. —»— 1955: Symbiotic production of phenol sulfatase by human gingival bacteria. J. Bact. *69:* 655.

18. —»— 1955: Lysis of collagen by human gingival bacteria. Proc. Soc. exp. Biol. (N.Y.), *89:* 697.

19. *Seelich, F.*, 1962: The metabolism of normal and abnormal leukocytes. From: *The Physiology and Pathology of Leukocytes.* Ed. *H. Braunsteiner & D. Zucker-Franklin.* Grune & Stratton, New York p. 152.

20. *Sognnaes, R. F. & J. T. Albright*, 1958: Electron microscopy of the epithelial lining of the human oral mucosa. Oral Surg. *11:* 662.

21. *Thilander, H.*, 1961: *Periodontal disease in the white rat. Experimental studies with special reference to some aethiologic and pathogenetic features.* Thesis. Trans. Royal Schools Dent. Sthlm Umeå, No. 6.

22. *Thonard, J. C. & H. W. Scherp*, 1959: Characterization of human gingival epithelial intercellular cementing substance. J. dent. Res. *38:* 711.

23. *Waerhaug, J.*, 1952: *The gingival pocket. Anatomy, pathology, deepening and elimination.* Thesis. Odont. T., Suppl. I.

24. *Weiss, L.*, 1959: Studies on cellular adhesion in tissue culture. I. The effect of serum. Exp. Cell Res. *17:* 499.

25. —»— 1960: The adhesion of cells. Internat. Rev. Cytol. *9:* 187.

26. *Weiss, P.*, 1958: Cell contact. Int. Rev. Cytol. *7:* 391.

27. *Wislocki, G. B., D. W. Fawcett & E. W. Dempsey*, 1951: Staining of stratified epithelium of mucous membranes and skin of man and monkey by the periodic acid-Schiff method. Anat. Rec. *110:* 359.