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A relationship between alkaline phosphatase activity and the phagocytosis and degradation of collagen by the fibroblast

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INTRODUCTION

It is clear that both remodelling and turnover of connective tissue involves active synthesis and degradation of collagen (Lapière, 1967; Woessner, 1968) and that its cellular basis is most likely the fibroblast (Ten Cate, 1972; Deporter & Ten Cate, 1973; Ten Cate & Deporter, 1974). It is also clear that hydrolytic enzyme activity plays a significant role in collagen degradation. Thus, activity of the highly specific hydrolytic enzyme collagenase has been well documented in many tissues since the first description of the enzyme by Gross & Lapière (1962). This enzyme acts at a site three-quarters of the way along the collagen molecule from the *N*-terminal end at a pH between 7·0 and 9·0 (Fullmer & Lazarus, 1969). Unfortunately, activity of this enzyme cannot be demonstrated at the fine structural level. However, a relationship between collagenase and another hydrolytic enzyme, alkaline phosphatase, has been demonstrated (Robertson *et al.* 1972) and this fact was utilized by Deporter & Ten Cate (1973) to show, in part, the involvement of the fibroblast in collagen degradation. Finally, activity of another hydrolytic enzyme, acid phosphatase, has been used as a marker at the fine structural level (Parakkal, 1969; Deporter & Ten Cate, 1973) to demonstrate the relationship between lysosomal acid protease activity and intracellular collagen degradation after its initial cleavage by collagenase.

Currently it is believed (see Woessner, 1968, and Perez-Tamayo, 1970, for reviews) that collagen breakdown involves initial cleavage by collagenase at an alkaline pH and subsequent degradation by other less specific enzymes acting at an acid pH. If this is so, and also if there is a relationship between alkaline phosphatase activity and collagenase activity and if, in addition, fibroblasts can phagocytose collagen fibrils, then we may predict a sequence of events as outlined in Fig. 1. This figure shows (1) the phagocytosis of a collagen fibril by a fibroblast to form, at the structural level (2) a phagosome with a clear matrix, with the implication that collagenase is active at an alkaline pH in the severance of the selected fibril. Then one expects fusion of primary lysosomes, with their contained enzymes, to the phagosome to form a phagolysosome (3) characterized by an electron-dense matrix and in which ultimate collagen degradation occurs. The final stages of this series of events has already been shown to take place within the fibroblast (Deporter & Ten Cate, 1973).

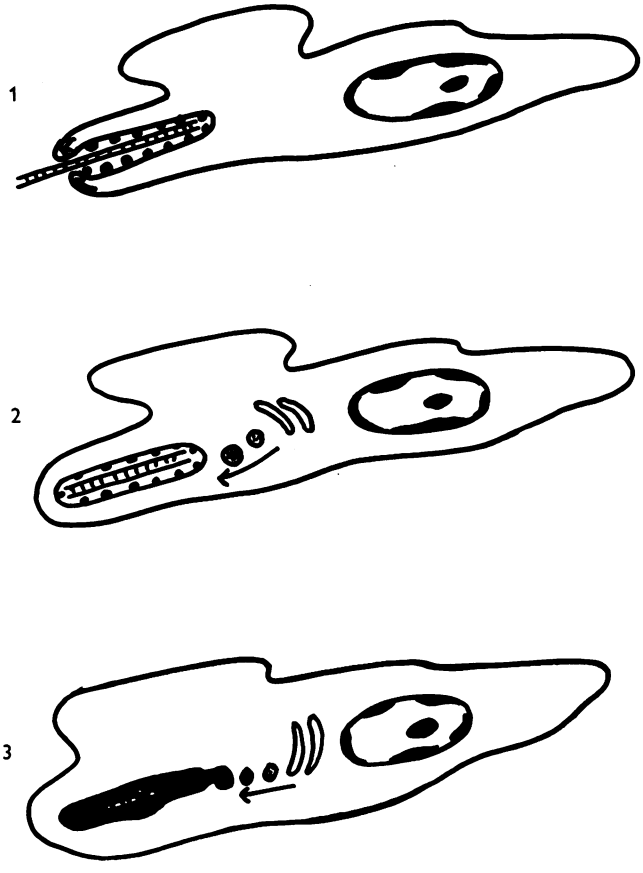


Fig. 1. Diagram illustrating the predicted events if the fibroblast phagocytoses extracellular collagen.

1. Phagocytosis of extracellular collagen. The arrows indicate the severance site of the collagen fibril. The black dots represent activity of collagenase.

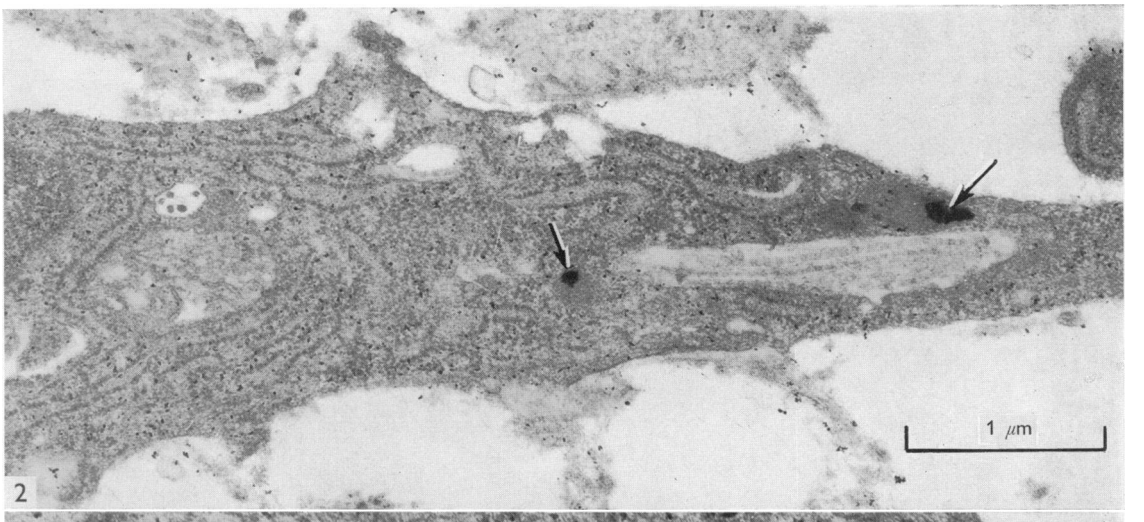
2. The banded collagen is now within the fibroblast in a phagosome surrounded by a clear matrix. Again the black dots indicate continuing activity of collagenase.

3. The banded collagen is now within a phagolysosome surrounded by an electron-dense matrix derived from fusion of primary lysosomes with the phagosome. Collagenase activity is replaced by lysosomal enzyme activity.

Fig. 2. Fibroblast with two clear profiles containing banded collagen. One is cut in cross section and the other is in longitudinal section. Adjacent to the longitudinal profile are two electron-dense lysosomal-like structures each containing precipitate of lead salt (arrows) indicative of acid phosphatase activity. × 26000.

Fig. 3. Fibroblast with an electron-dense profile containing residual banded collagen. The lead deposits, confined to the outline of the profile, are indicative of acid phosphatase activity. × 23000.

Fig. 4. Fibroblast with two adjacent profiles both containing collagen. The lower, more electron-dense profile, contains reaction product indicative of acid phosphatase activity, whereas the upper clear profile (arrowed) exhibits no enzyme activity. × 88000.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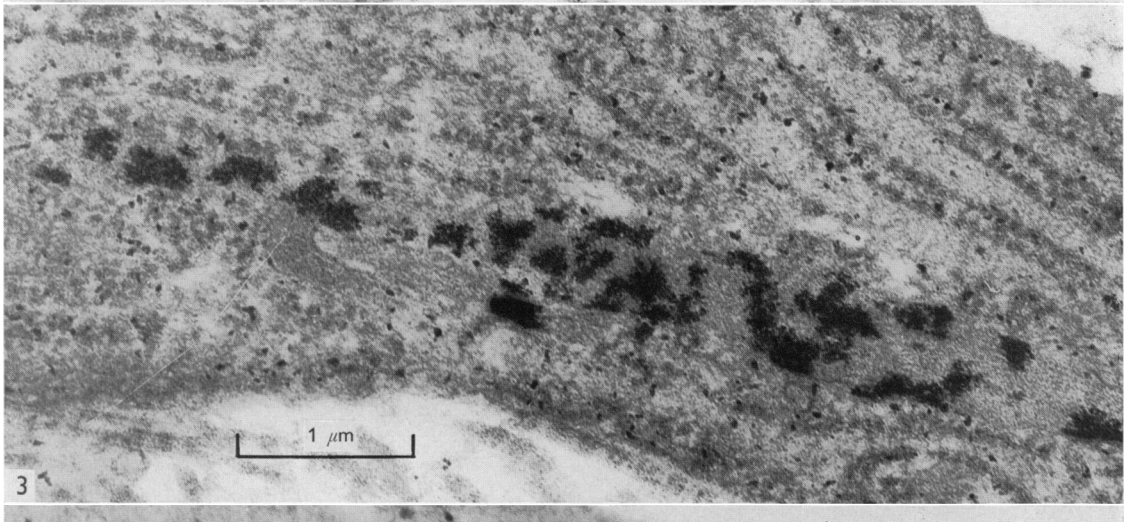
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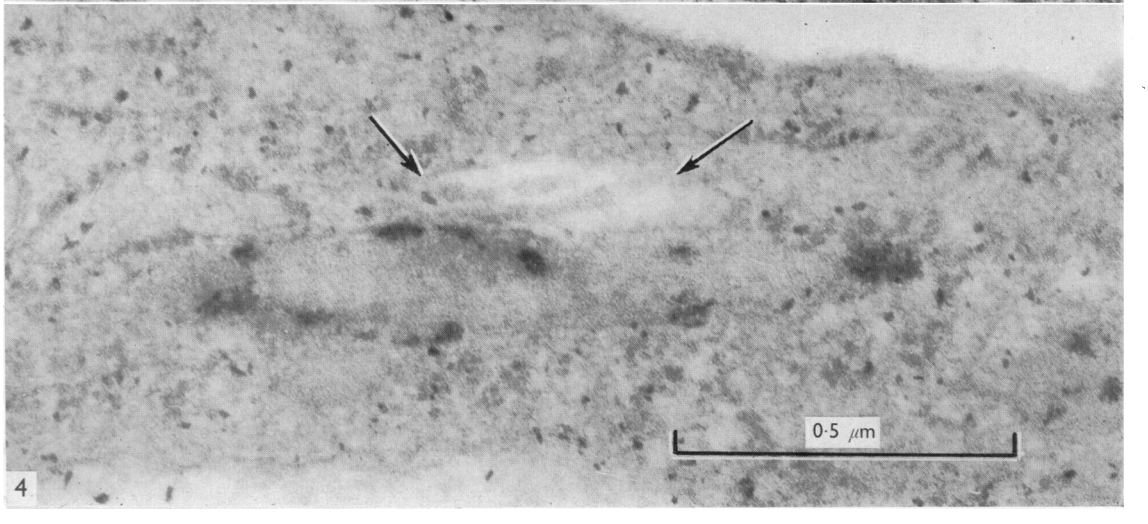
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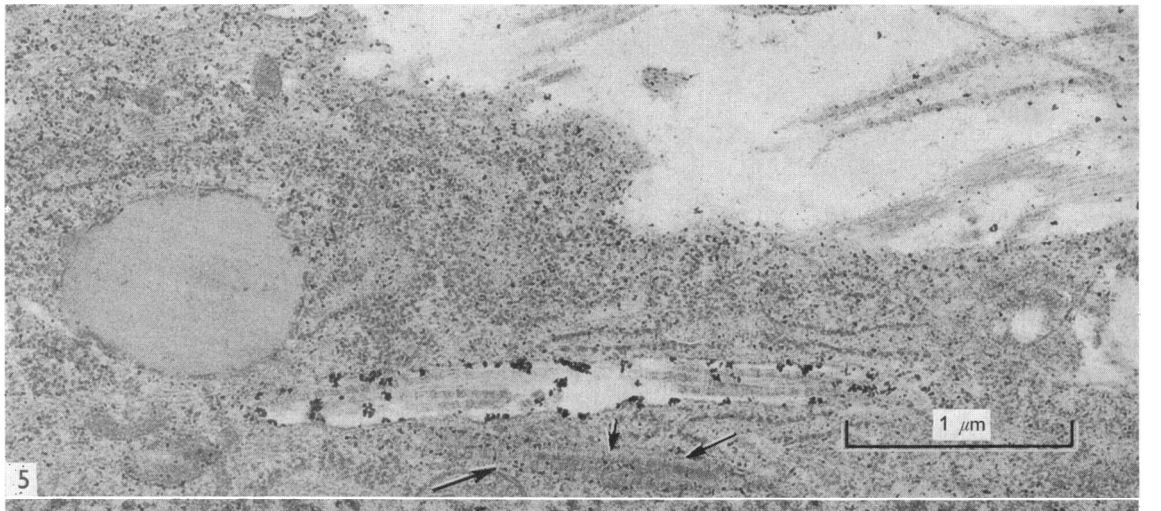

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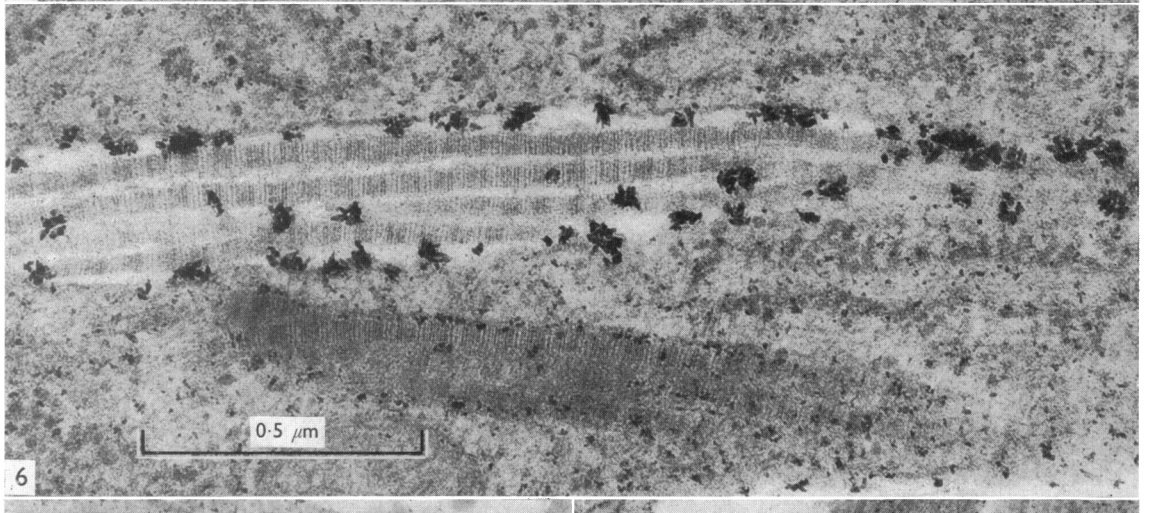

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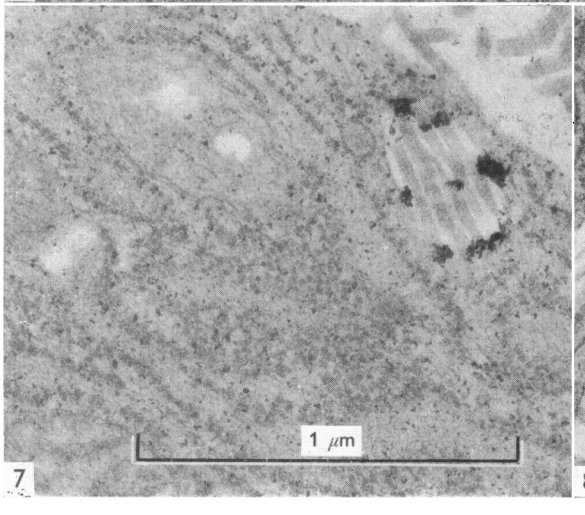

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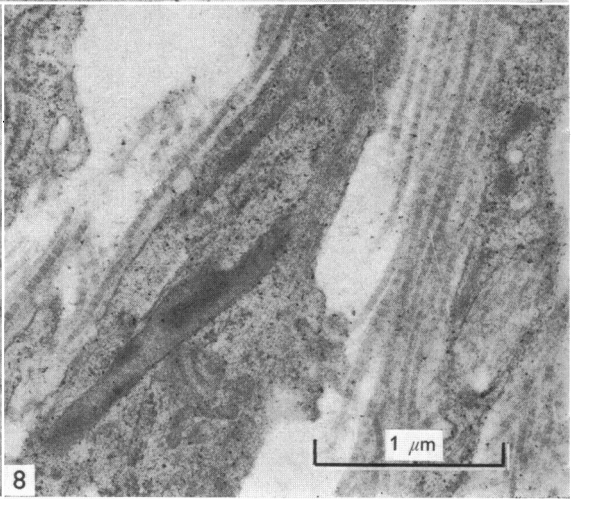

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The present paper shows that the initial stage also occurs, with the inference that the fibroblast is capable of phagocytosing extracellular collagen.

MATERIALS AND METHODS

The material for this study was the connective tissue overlying the non-erupted first molar of 12 to 14 day old mice. The animals were killed by decapitation and the lower jaws removed and fixed in 5% glutaraldehyde in 0·2 M cacodylate buffer for 4 hours at 4 °C. After fixation the specimens were demineralized in 10% EDTA in 5% glutaraldehyde for 72 hours, changing the solutions daily and stirring constantly. After demineralization the blocks were washed for a minimum of 24 hours in 0·05 M cacodylate sucrose solution, frozen by immersion in liquid nitrogen, and sectioned at 45 μm in a cryostat. The resulting sections were treated as follows. For the demonstration of acid phosphatase activity sections were incubated for 30 minutes using the Barka & Anderson (1962) modification of the Gomori medium. For the demonstration of alkaline phosphatase activity sections were incubated for 15 minutes, following the method outlined by Yoshiki & Kurahashi (1971) and Yoshiki, Umeda & Kurahashi (1972) which involves reactivation of enzyme activity by exposing sections to 10 mM $MgCl_2$ in 0·05 M trismaleate buffered sucrose for 24 hours before incubation. Control sections for both acid and alkaline phosphatase activity were incubated in the medium without substrate. After incubation all sections were washed in 0·05 M cacodylate sucrose, post-fixed in 4% osmium tetroxide, dehydrated and then embedded in Spurr. Ultrathin sections were cut and, after staining with 70% methanolic uranyl acetate, examined with a Philips 200 electron microscope.

RESULTS

Examination of the connective tissue over erupting teeth revealed that the fibroblasts contained electron-dense collagen-containing profiles corresponding to previous descriptions of connective tissue remodelling (Ten Cate, 1972) and connective tissue turnover (Ten Cate & Deporter, 1974). In addition, clear vesicles containing collagen profiles were seen (Fig. 2). Sections stained for acid phosphatase activity demonstrated the enzyme in the electron-dense profiles (Fig. 3) as previously reported (Deporter & Ten Cate, 1973). Activity of this enzyme was absent from the clear

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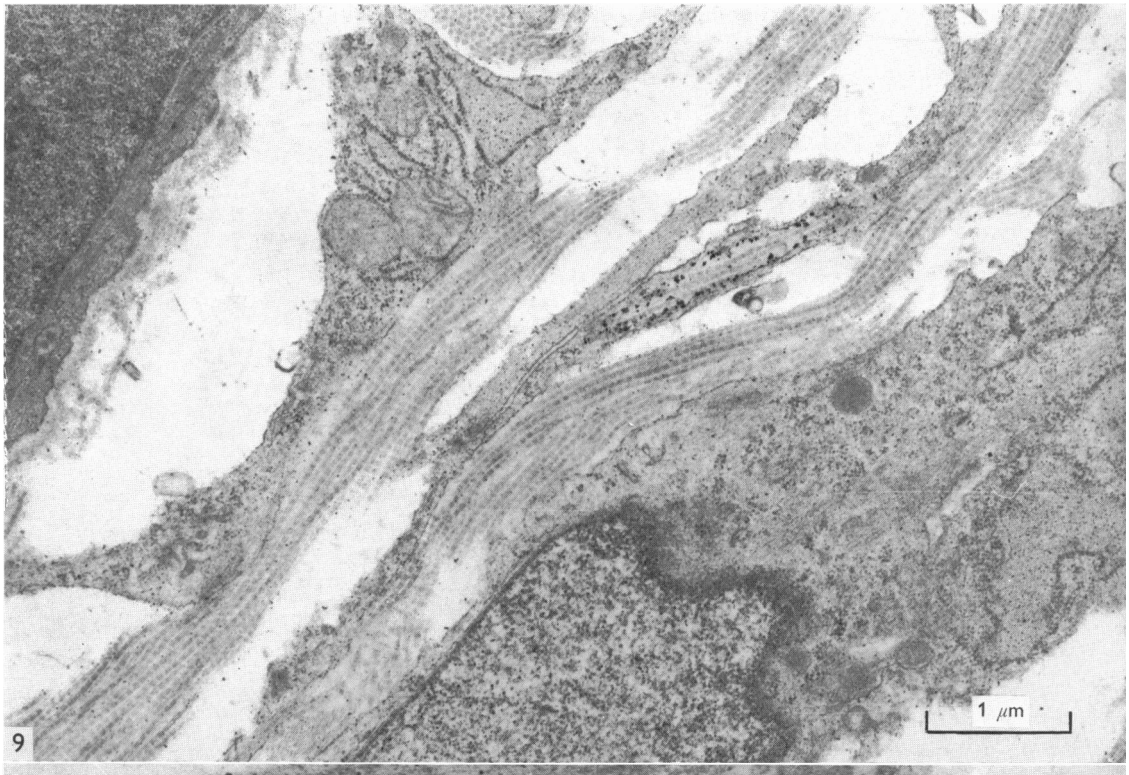
Fig. 5. Fibroblast with two adjacent profiles both containing collagen. The upper, clear, profile contains many deposits of reaction product indicative of alkaline phosphatase activity whereas the lower electron-dense profile (arrowed) contains little reaction product. Note also the absence of precipitate at the cell surface. × 29500.

Fig. 6. Another example of two collagen-containing profiles within the same fibroblast. The reaction product, indicating alkaline phosphatase activity, occurs principally in the clear profile. × 73000.

Fig. 7. A clear profile cut obliquely. The reaction product indicating alkaline phosphatase activity is confined to the profile. × 50500.

Fig. 8. An electron-dense profile in which residual banding of collagen fibrils is just discernible. This section has been stained for alkaline phosphatase activity and reaction product is absent from the profile. × 25000.



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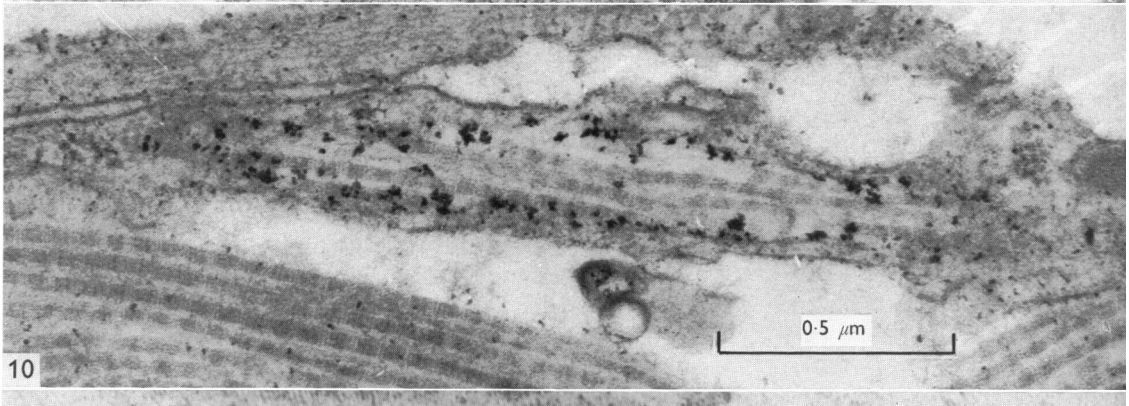

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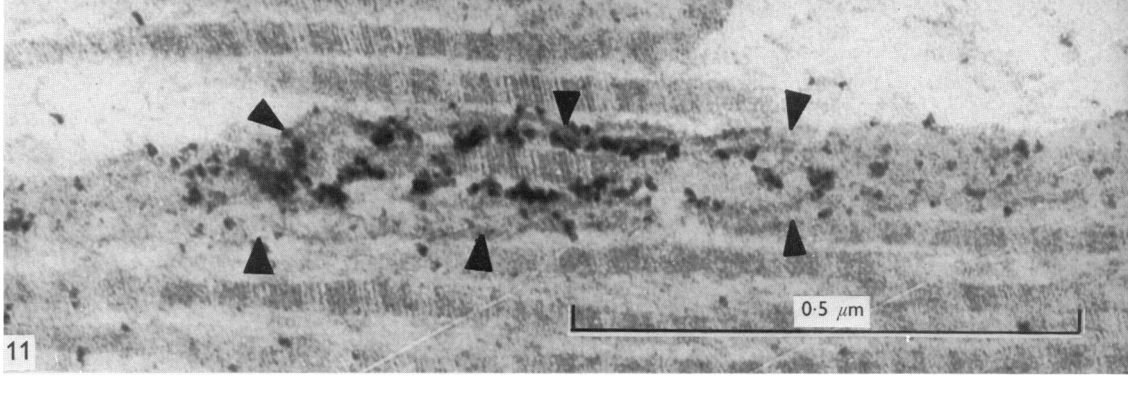

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collagen-containing profiles although present in the same cell in lysosomes (Fig. 2) and in adjacent collagen-containing electron-dense profiles (Fig. 4). In contrast, sections stained for alkaline phosphatase activity showed a reverse pattern of distribution. Thus enzyme activity was evident within the clear, collagen-containing vesicles (Figs. 5, 6, 7) and showed a declining gradient of activity down to complete absence in the electron-dense collagen-containing profiles (Figs. 5, 6, 8).

In addition, collagen fibrils were noted surrounded by tenuous cell processes of the fibroblast. Activity of the enzyme alkaline phosphatase was seen in some instances on, or at least close to, the plasma membrane of fibroblasts where the cell membrane was in association with a collagen fibril (Figs. 9, 10, 11). Control sections were negative.

DISCUSSION

Although our specimens were demineralized in EDTA, frozen in liquid nitrogen, thawed and incubated at pH 9·2, the preservation of fine structural detail was reasonable. Based on the study of control material we are confident that the lead deposits in our sections are indicative of enzyme activity. Also, diffusion of reaction product must be considered as a possibility in any histochemical reaction. However, the precise localization of enzyme activity to specific structural features of the cell, and to specific segments of the plasma membrane, suggests that little, if any, diffusion has occurred.

The finding of fibroblast alkaline phosphatase activity on the cell membrane and in the clear phagosome, but acid phosphatase activity in the phagolysosome, equates with the predictions proposed in Fig. 1, and supports the occurrence of phagocytosis of extracellular collagen by fibroblasts. The importance of establishing unequivocally the occurrence of phagocytosis of extracellular collagen was stressed in our previous publication (Deporter & Ten Cate, 1973). It was pointed out that there was still a slight possibility that our findings could be interpreted as the production of excess procollagen by the fibroblast which is then aggregated intracellularly and subsequently degraded. In the light of the present evidence this seems very unlikely.

On the basis of our results we also suggest that activity of the enzyme alkaline phosphatase has some function in phagocytosis and degradation of collagen. We base this statement on the precise localization of the enzyme to parts of the cell which, on a morphological basis, can be equated with phagocytosis. In particular the occurrence of enzyme activity in that part of the plasma membrane of the fibro-

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Fig. 9. Electron micrograph illustrating several fibroblasts and the associated extracellular compartment. The section has been stained for alkaline phosphatase activity. The reaction product is localized specifically to one part of the field where a fibroblast cell process has a close relationship to three collagen fibres. × 19000.

Fig. 10. Higher magnification of the site of enzyme activity illustrated in Fig. 9. Enzyme activity is confined to the surface of the cell membrane in contact with the collagen fibrils. × 60500.

Fig. 11. Another example of the intimate relationship between alkaline phosphatase activity, fibroblast cell process and a collagen fibril. In this picture a peripheral fibril of a collagen bundle has become surrounded by a tenuous extension of the fibroblast. Enzyme activity is confined to the cell membrane in association with the collagen fibril. The arrowheads outline the fibroblast cell process. × 112000.

blast in contact with the peripheral fibril of a collagen bundle is regarded as significant.

We can only speculate as to the role of alkaline phosphatase in collagen degradation. Although there is a relationship between activity of this enzyme and collagenase activity in the polymorphonuclear leucocyte, and a predicted relationship in our model system, these two enzymes have totally different functions. Thus collagenase hydrolyses a peptide bond whereas alkaline phosphatase hydrolyses a phosphate ester. As collagenase activity disrupts a peptide bond and as the collagen molecule after such rupture contains no phosphate groups, it is most likely that alkaline phosphatase exerts its effect *before* collagenase. It may, therefore, be involved in the removal of some phosphate group associated with intact collagen or, as alkaline phosphatase activity is associated with calcium transport (Russell *et al.* 1972), with the provision of calcium ions necessary for collagenase activity (Fullmer & Lazarus, 1969).

The demonstration of alkaline phosphatase activity in relation to collagen degradation rather than to collagen synthesis in the connective tissue studied in this report and in the developing periodontal ligament (Deporter & Ten Cate, 1973), does not seem compatible with the long-standing suggestion (Bradfield, 1951) that alkaline phosphatase is related to collagen fibre synthesis unless of course it is involved in both synthesis and degradation. In any case, rapid synthesis of collagen and collagen degradation often go on side by side (Lapière, 1967).

SUMMARY

The activities of both alkaline and acid phosphatase have been demonstrated in fibroblasts apparently engaged in degrading collagen. Alkaline phosphatase activity is present in the cell membrane and the phagosome, but acid phosphatase activity is shown by the phagolysosome. This pattern of enzyme activity is in keeping with the idea of phagocytosis of collagen by fibroblasts and of a role for alkaline phosphatase in collagen breakdown.

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