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THE UNIVERSITY OF ALBERTA

IN VITRO STUDIES OF COLLAGEN CATABOLISM IN PROGRESSIVE PERIODONTAL DISEASE IN BEAGLE DOGS

BY

DONALD JAMES GATES

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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In Vitro Studies of Collagen Catabolism in Progressive Periodontal Disease in Beagle Dogs

submitted by DONALD JAMES GATES

in partial fulfilment of the requirements for the degree of

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in Experimental Surgery

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ABSTRACT

"*In Vitro* Studies of Collagen Catabolism in Progressive Periodontal Disease (PPD) in Beagle Dogs."

The destructive phase of chronic PPD is characterized by a decrease in collagen content of the gingival stroma and periodontal membrane. Bacterial plaque plays a major role in the initiation and progression of the disease. Beagle dogs routinely develop spontaneous gingivitis and subsequent periodontitis which closely resembles that found in humans. Explanted beagle dog gingival tissue has been used to assess proteinase physiology and collagenolysis in both healthy and diseased states, and has provided an *in vitro* model to study the effect of homologous dental plaque on these tissues.

Healthy and diseased tissue explants were cultured separately with or without dental plaque (100 µg plaque protein/ml) in Dulbecco's modified Eagle's medium with antibiotics for up to 10 days at 37°C. Media were changed daily and assayed for the following activities: active and latent collagenase, elastase, Cathepsin B-like thiol proteinase, plasminogen activator-like (PAL) proteinase, gelatinase, neutral proteinase, lysozyme and β-glucuronidase. Collagenolysis in the cultured explants was assessed by measuring the total and diffusable hydroxyproline released into the media. Cell viability was assessed by glucose utilization, release of lactate dehydrogenase (LDH) and histological examination of cultured explants at various times. The release of prostaglandin E_2 was also monitored in the explant media.

In general, diseased explants released higher levels of proteinase activities and collagen degradation products into the culture media than healthy explants. Collagen degradation was elevated in those cultures containing plaque and this was associated with increased levels of collagenase, neutral proteinase, plasminogen activator and

gelatinase. However, the response of healthy or diseased explants to plaque exposure varied with each enzyme; for instance, the increase in collagenase and PAL activities was significantly elevated in healthy tissues. Negligible elastase or lysozyme activities were found in either culture media, and cathepsin B-like thiol proteinase activities were similar for non-plaque-supplemented explant media. Prostaglandin E_2 was released early in culture and explant media levels were significantly higher for diseased tissues.

The healthy and diseased gingival tissues were also extracted directly and the biochemical and enzymatic components detailed above were assayed and compared. Higher levels of thiol proteinase and elastase were found for diseased tissues relative to healthy but no differences were found for LDH or β-glucuronidase activities. No collagenase, PAL or neutral proteinase activities were extractable from tissues either at the beginning or at the end of the culture period even when chaotropic, thermal or ionic dissociative methods were used. Histological observations of cell distributions and integrity of the stromal matrix supported biochemical data which showed slightly increased DNA and reduced amounts of hydroxyproline for diseased tissue.

We now have a better idea of the potential proteinase activity of the whole tissue, particularly the base levels and the extent of its response to soluble plaque products. This preliminary or background information now lends itself to further studies on specific cell:cell interactions involved in the degradative physiology of periodontitis and other inflammatory diseases in general.

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I am indebted to Dr. David Woolley for providing the opportunity to work on this project in Manchester, and for his expert guidance and continued perseverence without which this thesis would not have been possible.

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SYMBOLS AND ABBREVIATIONS

SYMBOL	DESIGNATION
○	Healthy tissues
●	Diseased tissues
□	Plaque-supplemented Healthy tissues
■	Plaque-supplemented Diseased tissues
▽▼	Trypsin-activated collagenase assays

Results have been grouped into related sets of activities wherever possible. The above symbols are used as indicated for figures 4-18.

ABBREVIATIONS

APMA	p-aminophenylmercuric acetate
BANA	N,αBenzoyl(D,L) Arginyl naphthylamide (Cathepsin-B-like proteinase substrate)
DAB	Diaminobenzoic acid
DABA	p-dimethylamino benzaldehyde
E'&EL	Active and Latent Collagenase, resp.
EDTA	Ethylenediamine(tetra-acetic acid)
FG/GBC	4 amino 3,1 dimethyl-azobenzene (fast garnet/GBC)
HPR	Hexazotized pararosaniline
HNF	Hexazotized New Fuchsin
HYPRO	Hydroxyproline
7AMC	7-amino-4-methyl coumarin
LEL-7AMC	MeO-Succ-L-Ala-L-Ala-L-Pro-L-Val-7AMC (Leukocyte elastase substrate)
MEL-7AMC	Acetyl-L-Ala-L-Ala-L-Pro-L-Ala-7AMC (Macrophage elastase substrate)
MA	Mersalyl; salyrganic acid
NA	Naphthylamide
αNAE	α,Naphthyl acetate esterase
NCA or CAE	Naphthol-(AS),D-Chloroacetate esterase
NAC	Naphthol AS, amino caproate esterase
PAL	Plasminogen activator-like activity
PAL-7AMC	N,benzoyl-L-Val-Gly-L-Arg-7AMC (PAL substrate)
PGE2	Prostaglandin E2
PMSF	Phenylmethylsulfonyl fluoride
PNP	p-nitrophenol
SBTI	Soy Bean Trypsin Inhibitor

INTRODUCTION

The development of gingivitis and its progression to periodontal disease has been extensively studied in several animal models, as well as in humans under experimentally-induced programs (Loe, etal, 1965; Lindhe, etal, 1973; Payne etal, 1975; Schroeder, 1977). Laboratory studies have included observations in mink (Lavine, etal, 1976); marmoset (Page etal, 1972); macaque (Pettigrew, 1980); dog (Lindhe, etal, 1973; Woolley, etal, 1978b; Soames etal, 1976) and bovine gingiva (Birkedal-Hansen, etal, 1976; Pettigrew etal, 1980). Many of the cellular and pathologic changes found in inflamed gingival tissues are common to other inflammatory diseases. The major effect of such pathophysiological changes in gingiva is the loss of connective tissue matrix and tooth support.

A highly organized array of collagen fibers in the periodontium provide anchorage of the gingiva to the alveolar bone and to the root cementum (for review, see Page & Narayanan, 1980). An attachment lamina produced by the junctional epithelial cells normally unites the gingival structures with the tooth surface (see Plate Ia at end of Results Section) at the cemento-enamel junction.

The microflora of the oral cavity and sulcular crevice is separated from the gingival stroma and its underlying supportive alveolar bone by a protective epithelium. A basement membrane connects the epithelial cells with connective tissue stroma. Enmeshed in the stroma, supported by highly

organized bundles of collagen, is a plexus of blood vessels which is found next to the junctional epithelium.

Vascularization of this region in the gingival stroma increases with advancing stages of the inflammatory disease, and is responsible for the extensive bleeding which accompanies probing to evaluate sulcular pocket depth (a major clinical diagnosis of the disease). Other features of the disease include apical advancement of the junctional epithelium, destruction of the periodontal ligament and bone resorption in the alveolar crest (for reviews of histopathology, see Schroeder, 1977; Payne, etal, 1975).

Many researchers have linked the pathologic alterations which occur in the stromal connective tissue with subsequent impairment of function (Page & Schroeder, 1981). During early stages of gingivitis the major site of collagen alteration occurs immediately subjacent to the junctional epithelium at the site of the inflammatory cell infiltrate (Page & Schroeder, 1976). This marginal tissue may be converted to granulation tissue with greatly increased numbers of blood vessels and dense populations of inflammatory cells in several animal models, but it is less common in human disease (Page & Narayanan, 1980). The cellular changes observed in the disease state accompany several well characterized matrix alterations which generally lead to a net reduction in collagen content (Narayanan, etal, 1980). The changes in collagen composition and content, destruction of the attachment between gingiva and tooth, and subsequent alveolar bone

resorption with loss of tooth support have been well documented in the literature (Page & Schroeder, 1981; Page & Narayanan, 1980).

The connective tissue stroma consists of highly organized collagen fibers and other constituents such as fibronectin, proteoglycans and glycoproteins (Kleinman, etal, 1981). Several changes in the collagen composition have been reported between normal and diseased gingiva (Narayanan ,etal, 1980). Type I collagen is the principal type found in both tissues, but alterations in its proportion to types III and V are evident in the diseased state (Narayanan & Page, 1976). A small amount of type III collagen (<10%) and trace amounts of type V (<0.5%) have been reported, together with a Type I trimer in the diseased tissues (Page & Narayanan, 1980) However, the most important feature is the overall reduction in collagen content of the stroma, including the insoluble, highly crosslinked portion. Increased amounts of poorly cross-linked collagen are found in diseased tissues which probably reflects increased synthesis and incomplete maturation of the collagen fibrils (Page & Narayanan, 1980;)

Non-collagen components of the gingiva include fibronectin, acidic glycoproteins and glycosaminoglycans. Fibronectin is an important factor for the interaction between gingival fibroblasts and collagen fibrils (Kleinman etal, 1981). Other glycoproteins comprise about 10-20 % of the ground substance of gingival connective tissues and probably

play a role in the regulation of collagen fibril organization. Proteoglycans, comprised of glycosaminoglycan subunits (chondroitin sulphate, dermatan sulphate, heparan sulphate) are also found in gingival tissues and are often linked with hyaluronic acid (Page & Narayanan, 1980). Proteoglycans are responsible for the degree of hydration of connective tissues. Recent evidence has suggested the early breakdown of proteoglycans in disease, as judged by measurement of diffusable GAG components in gingival washings from patients (G. Embery, personal communication).

The progression of the disease has been classified into three stages, with a fourth based on clinical rather than histological criteria (Page & Schroeder, 1976). The intial stage is manifested as an acute inflammation of the marginal gingiva, characterized by increased vascularization and permeability, enhanced PMN migration and accumulation in the sulcular crevice. Early inflammatory lesions (stage II) are characterized by cellular infiltrates which contain various lymphocytes, a few macrophages and plasma cells. The third stage, the established lesion, is characterized by the development of a sulcular pocket, with thinning of the sulcular epithelium and extension of the junctional epithelium apically along the root surface. Various ratios of T and B lymphocytes, plasma cells and macrophages, together with PMN's make up the inflammatory lesions throughout the later stages of gingivitis (Seymour, *etal*, 1979a). These lesions may disappear spontaneously, remain stable or transform into

the destructive lesion (periodontitis) with subsequent alveolar bone resorption. Other features include altered topography and permeability of blood vessels which may result in increased crevicular flow of gingival fluid.

Several workers have tried to elucidate the cellular mechanisms involved in initiation and progression of the disease (Seymour, etal, 1979a; Page etal 1980; Cowley, 1976). Although resident fibroblasts decrease in number in early gingivitis, their number recovers in periodontitis but there may be marked pathological changes in their appearance during late gingivitis, often increasing 2-3 times in size, and possessing swollen mitochondria and endoplasmic reticulum (Page & Narayanan, 1980). The influx and activation of PMN's and macrophages, together with immunological reactions initiated by mixed populations of lymphocytes, also contribute to the progression of the inflammatory lesion and subsequent loss of the connective tissue matrix. Tissues returned to health after removal of the stimulatory plaque appear normal histologically, but some differences in the collagen fibers ressembling those found in scar tissue have been noted (Page & Narayanan, 1980).

Microbial dental plaque and its metabolic products are the main etiologic factors of gingivitis and periodontitis (for review, see Krasse, 1977). Although there is rarely a direct microbial invasion of the periodontium (Ranney, 1978; Schroeder & Attstrom, 1980), a variety of toxic products have been characterized with regard to their ability to

induce tissue injury by direct or indirect action (Socransky, 1970; Slots, 1979; Ivanyi & Lehner, 1970; Baboolal, etal, 1970; Tsai, etal, 1979). Direct tissue injury usually results from cytotoxic effects of plaque components but pathologic alterations in various gingival cells may result from the direct action of proteolytic enzymes found in dental plaque (Alfano, etal, 1974; Holt, 1975; Tatevossian & Gould, 1976)

From research in microbial etiology, it is understood that the plaque microflora at subgingival sites of healthy tissues change from non motile gram-positive Streptococci and facultative Actinomycetes (Actinomycetes viscosus, A. naeslundii) to highly motile gram-negative anaerobes (Bacteroides gingivalis) in diseased crevicular pockets (for reviews, see Slots, 1979; Krasse, 1977). Other species have been found in very advanced diseased states, unaccompanied by clinically apparent inflammation (B. melaninogenicus (intermedius), Eikenella corrodens), but where inflammation is still evident, B. gingivalis, Fusobacterium nucleatum, and other Vibrios and pleomorphic Bacteroides are found in man. The presence of these and other species do not necessarily implicate them as causative agents, and as yet these still remain to be identified.

Dental plaque elements can stimulate a variety of host responses (see Figure 1 for this author's interpretation of reviews by Page & Schroeder, 1981; Seymour, 1979a; Wilde, etal, 1977; and Nisengard, 1977). The major effects are

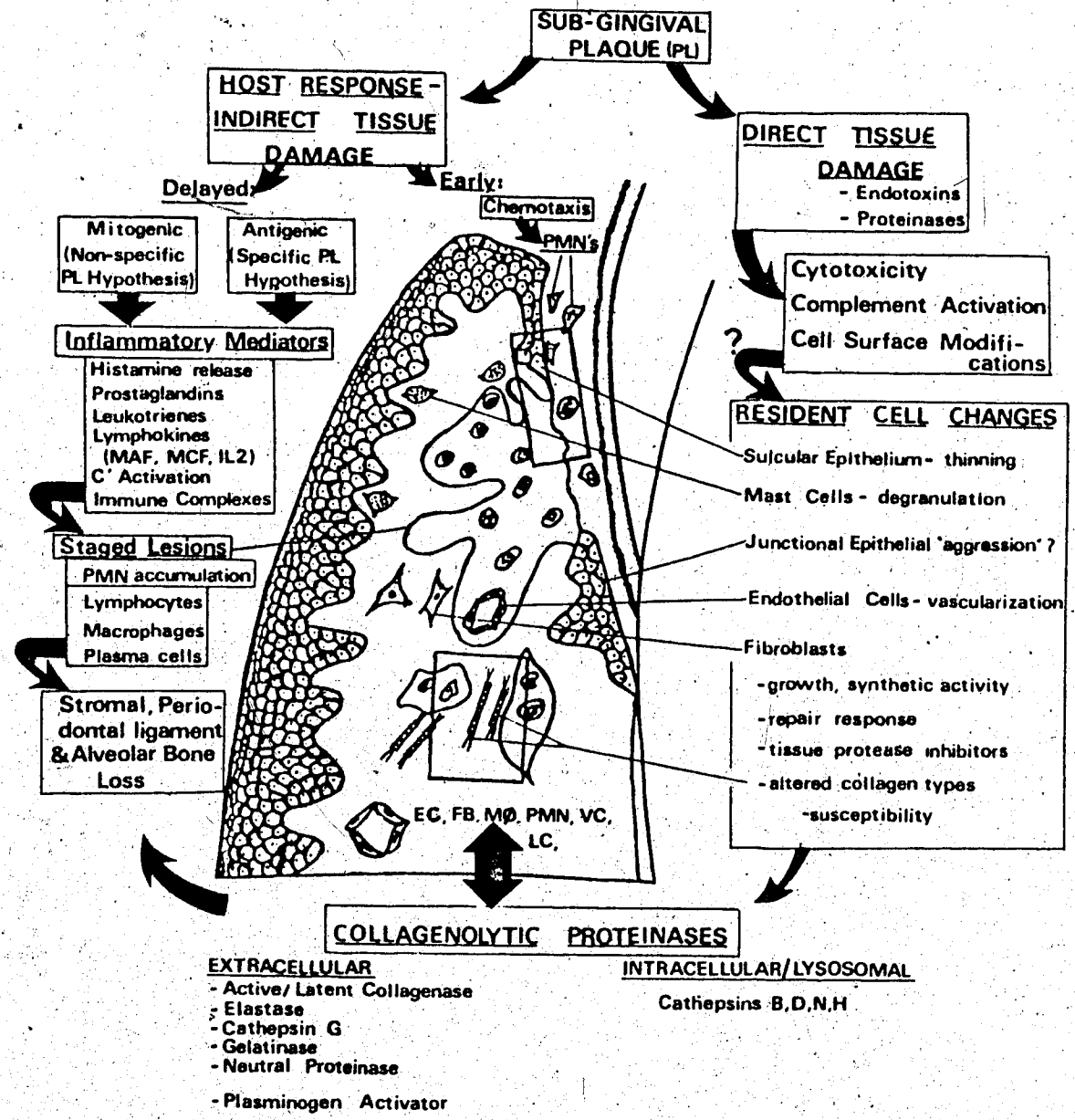


FIGURE 1. Some potential cellular mechanisms involved with connective tissue destruction in gingivitis and periodontitis. Cells that have been implicated in collagenolytic mechanisms include Epithelial cells (EC), Fibroblasts (FB), Macrophages (MØ), Polymorphonuclear leukocytes (PMN), Lymphocytes (LC) and Endothelial cells (VC).

initiated by (1) the secondary metabolites of plaque which activate the normal defence function, eg. PMN's; (2) activation of certain host cells with plaque antigens or non-specific mitogens which result in a continued immunologic response; and (3) a direct effect on specific cells such as macrophages which subsequently modify the response of other cells by the production of soluble mediators.

Some plaque elements may induce early inflammatory reactions by their chemotactic properties or by complement activation via the alternate pathway (Tsai, etal 1977;Johnson, etal, 1976; Holt, 1975). Complement activation may play an important role in mediation of periodontal disease because complement can be activated by bacterial plaque substances via the alternate pathway or by enzymes released by PMN's and macrophages (Allison, etal, 1976; Page & Schroeder 1981; Kahnberg, etal, 1976b). Recent experiments have shown that lymphocyte activation can attract and activate large numbers of PMN's and macrophages (Goodman & Sultzer, 1979).

Three mechanisms have been proposed for the participation of the immune system in gingival tissue destruction (Page & Schroeder, 1981). These may be summarized as: (1) Specific induction by antigens of T-cell mediated immune response and subsequent development in the tissue of a delayed hypersensitivity reaction, (2) activation of the complement cascade by immune complexes formed as a result of B lymphocyte activation; and, (3) stimulation of lymphokine

production by activated T or B cells. Current evidence favours the mitogenic components of plaque as the probable stimulatory mechanism for lymphocyte activation in the disease process (Page & Schroeder, 1981; Seymour, etal, 1979b).

The inflammatory reaction involves a variety of cell types such as PMN's, mononuclear phagocytes and macrophages, lymphocytes and mast cells (Nisengard, 1977). The distribution and frequency of each cell type is known to change throughout the course of the disease (Seymour, etal, 1979a&b; Schroeder, 1977; Payne etal, 1975).

PMN's are first-line protective agents against microbial invasion and act in the mediation of host tissue injury in inflammatory lesions (Attstrom, 1971; Baggliolini, etal, 1978). They are short lived (half lives of 24-36 hours) and although present in healthy gingiva and the sulcular crevice, they do not normally reside there (Schroeder, 1977). In disease they accumulate in the gingival stroma and crevicular space, probably through chemotactic attraction by N-formyl peptides (and other bacterial elements) or complement derived factors, and are probably responsible for local tissue destruction (Schiffmann, etal, 1978; Holt, 1975; Johnson etal, 1976). Diffusion of bacterial agents into the stroma may induce the release of proteolytic activity from PMN's- either by secretion or cell lysis (Cianciola, etal, 1977; Weissman, et al, 1979). Page & Schroeder,(1981), have suggested that such a reaction is probably more important in

early gingivitis.

Mast cells are normally resident in gingival stroma (Aeschlimann, etal, 1980; Schroeder, etal, 1973) and may provide an early defence mechanism to bacterial invasion (Schwartz & Dibblee, 1975 a&b). They contain electron dense granules which stain metachromatically with toluidine blue. These granules contain heparin, histamine and serotonin and other potent mediators of inflammation, as well as proteases of trypsin and chymotrypsin-like specificity (Schwartz & Austen, 1980).

Mononuclear phagocytes or macrophages are present in diseased gingival tissues and can remain resident in the stroma as histiocytes (Schroeder, etal, 1973; Spector, 1979). They can actively secrete collagenase, neutral proteinases and lysosomal acid hydrolases (Page, etal, 1973) and react directly to plaque bacteria (Page etal 1978b; Levine etal, 1973) or to lymphokines secreted by lymphocytes (Page & Schroeder, 1980). The macrophage not only produces proteinases but seems to have an important role in regulating the degradative activity of other cell types, for example the production of mononuclear cell factor (MCF) which stimulates fibroblast cells to produce prostaglandins and collagenase (Dayer etal, 1980; Meats etal, 1980; Mizel, etal, 1981).

Resident gingival fibroblasts also participate in matrix degradation, and intracellular collagen fibrils have been observed in various ultrastuctural studies (Ten Cate &

Deporter, 1975; Melcher & Chan, 1980;Soames & Davies, 1977).

The reduction of connective tissue elements observed in gingivitis is most likely caused directly by enzymatic degradation. Proteinases have received particular attention in the past decade for their possible roles in the destruction of tissue components during inflammation (Cimasoni & Kowashi, 1980; Poole & Mort, 1980).

There are four classes of proteinases, these being based on the chemical nature of the catalytic group or the cofactors required for the enzyme's activity (Barrett, 1980). The four classes are serine, thiol, metallo- and carboxyl proteinases. The term 'protease' infers both exo- and endo-peptidases; exo-peptidases are limited to those proteinases which hydrolyze small peptides near carboxyl or amino regions.

Serine proteinases (EC3.4.21) possess a serine residue in their active site and are sensitive to such inhibitors as diisopropyl phospho fluoridate (DiPF) and phenyl methyl sulfonyl fluoride (PMSF). Three important serine proteinases, neutrophil elastase, Cathepsin G and plasminogen activator are potentially important enzymes in connective tissue degradation associated with inflammation (Cimasoni & Kowashi, 1980; Baggiolini, etal, 1979).

Collagenase, thought to be the rate-limiting enzyme in many examples of collagenolysis, is a metallo-endopeptidase (EC 3.4.24). These enzymes require metal ions for activity, with Zn^{++} and Ca^{++} being essential for collagenase.

Gelatinase is another metalloproteinase which attacks single α-chain peptides resulting from either denaturated or collagenase-cleaved molecules. Chelating agents such as EDTA, EGTA and o-phenanthroline, are effective inhibitors. Macrophage elastase is also reported to be a metallo-enzyme (Werb, 1978).

Thiol proteinases(EC 3.4.22) such as Cathepsin B, require the presence of thiol groups and are inhibited by alkylating agents such as p-chloromercuric benzoate, various organomercurials, and other thiol blockers (eg, the halogenated ketones TLCK and TLCP).

Carboxyl endopeptidases (EC 3.4.23) function at low pH and are principally lysosomal in nature. One example, Cathepsin D, is known to degrade proteoglycans, but its major role is intracellular digestion (Poole & Mort, 1980) and its acid pH optimum probably limits its extracellular action.

Collagen is the major structural protein of all connective tissues and is resistant to attack by nearly all proteinases due to its triple-helical structure and organization into highly cross-linked fibrils (Gross, etal, 1980). Collagen molecules consist of three α-chain polypeptides arranged in a triple helix, and these aggregate into collagen fibrils where each molecule is stabilized by cross links located at the non-helical terminal extensions of each monomer (for review, see Jackson, 1980; Gross, etal, 1980).

Collagenase is the enzyme which selectively attacks molecules of types I, II and III collagens (Woolley,etal,

1978a) making a characteristic single cleavage across the triple helical monomer at a locus which produces 3/4 and 1/4 fragments of the molecule (Harper, 1980). At physiological temperature the two triple-helical reaction products thermally denature into gelatin polypeptides, which are then susceptible to other endopeptidases (Gross,etal, 1980; Harris & Vater, 1980).

Although collagenase is probably the rate-limiting step in many examples of collagen catabolism, and especially in processes such as remodelling, wound healing and normal collagen turnover, other enzymes may also play a role in pathological collagen resorption probably by solubilizing fibrillar collagen to facilitate the action of collagenase (Gross, etal, 1980). These other enzymes probably attack collagen molecules mainly at the telopeptide or cross-link regions, thereby solubilizing the triple helical portion of the collagen molecule (Barrett, 1978). Other physiological roles of some of these proteinases may be to activate latent enzyme extracellularly or to inactivate excess collagenase.

PMN elastase has been shown to attack gingival epithelial structures (Heineger & Cimasoni, 1980) and possesses significant collagenolytic activity preferentially degrading type III rather than type I collagen (Cimasoni & Kowashi, 1980). However, it has been shown to cleave type III collagen in a manner similar to that for collagenase (Gadek etal, 1980). PMN Cathepsin G, a serine endopeptidase with chymotrypsin-like activity, has a pH optimum of 7.5 and can

hydrolyze collagen (Starkey, 1977). Recent evidence suggests these enzymes can be found in gingival fluid (Cimasoni & Kowashi, 1980; Kowashi, etal, 1980).

Cathepsin B also possesses collagenolytic activity but its action is thought to be principally intra-lysosomal; despite retaining some activity at pH values approaching 7.0, it rapidly loses activity at neutral pH (Poole & Mort, 1980). Plasminogen activator may play an indirect role in matrix degradation through the generation of plasmin from plasminogen. Plasmin is a potential activator of latent collagenase (Werb etal, 1977) but no direct action of PA has been reported on latent collagenase.

In addition to the degradation of structural substrates, proteinases are also important in inflammation through their activation of the complement system, alteration of cell surface properties and stimulation of active inflammatory mediators (Kahnberg, etal, 1976b; Vaes, 1980a; Cimasoni & Kowashi, 1980).

The rapid and early turnover of collagen in inflamed gingiva justifies the attention that many investigators have paid to the presence of collagenase in gingival tissues. Several animal models of gingivitis and periodontitis using cell and tissue culture methods have been used to study the role of collagenase and these other proteinases in the diseased state. The beagle dog model has many similar features of progression of the disease in humans, and has been widely used (Lindhe etal, 1973;Tynelius-Bratthall & Attstrom

1976; Page & Schroeder, 1976; Clagett & Page, 1978; Soames etal, 1976). Such model systems have provided information on proteinase physiology either by direct assay of homogenates or by analysis of the proteinases elaborated in culture systems (Golub etal, 1978 & 1979; Woolley etal, 1978b; Birkedal-Hansen, 1980).

Collagenase has been identified in culture fluid of gingival tissues (Fullmer & Gibson, 1969; Birkedal Hansen, 1980; Woolley etal, 1978b; Geiger & Harper, 1980). Attempts to characterize the cellular origin of tissue collagenase (e.g. Fullmer etal, 1969; Birkedal-Hansen, 1976) have suggested several sources. Collagenase has been reported from both epithelial structures (Golub, etal, 1979; Birkedal-Hansen, 1980) and fibroblasts of the stroma (Birkedal-Hansen, etal, 1976; Frank, etal,1972) and immunolocalization studies have demonstrated the enzyme in diseased gingiva especially in association with inflammatory foci (Woolley & Davies, 1980). However, the relative contribution of collagenase from each cell type probably relates to the stage of the disease and evidence exists that collagenase can come from monocytes (Uitto & Raeste, 1978;Simpson, etal, 1980); neutrophils (Lazarus etal, 1968); fibroblasts (Birkedal-Hansen, etal, 1976; Rose & Robertson, 1977; Pettigrew, etal, 1980); macrophages (Werb, 1978); and epithelial cells (Golub etal, 1979; Birkedal-Hansen, 1980).

Tissue homogenates have been examined for proteolytic activities with rather limited success (Uitto & Saxen, 1978;

Uitto etal,1981; Golub etal, 1979). The presence of natural inhibitors and the binding of enzymes to their substrates provide the major problems in such studies. However, methods have been developed to demonstrate such masked or bound enzymes and these demonstrated that a significant amount of collagenase was tightly bound to collagen fibers in situ (Uitto & Saxen, 1978; Uitto etal, 1981; Golub, etal, 1979; Woessner, 1980).

Several workers have attempted to measure proteinase activity in order to obtain a clinical assessment or prognosis of gingival disease (Smith, etal, 1974; Golub, etal, 1976; Hidaka,etal, 1981; Ishikawa etal, 1972). For example, collagenase found in crevicular washings was reported to be associated with the progression or diminuition of the disease, as well as correlation of several clinical observations such as pocket depth and clinical index (Golub, etal, 1976 & 1979; Kowashi, etal, 1979). These and other clinical tests, such as the Phagocytic Collagenolytic Performance (PCP) index (Rose, etal, 1978), have obvious clinical relevance as the crevicular washings can provide a means to assess periodontal damage by non-surgical intervention. However, the measurement of **collagenase** activity in crevicular fluids, together with reports of elevated **elastase** (Kowashi, etal, 1980), **cathepsins G and D** (Cimasoni & Kowashi, 1980; Ishikawa, etal, 1972) and **fibrinolytic activities** (Hidaka, etal, 1981) and decreased **α_1-antitrypsin** levels (Paterson & Marsh, 1979) may serve only to reflect the

presence of PMN's and bacterial products in a variable quantity of gingival exudate (Cimasoni etal, 1980). Such measurements do not directly represent the degradative activity occurring in the stromal matrix.

AIMS:

The mechanisms of connective tissue destruction in gingival disease remain poorly understood, and most studies have been restricted to either one enzyme or the susceptibility of one particular substrate. As collagen is the major structural protein of gingival tissue, and its loss is a characteristic feature of chronic periodontal disease, this study attempts to elucidate which collagenolytic enzymes and other proteinases might be involved in gingival matrix degradation. Moreover, as dental plaque is an essential factor in the disease process it was important to examine its effects on proteinase production of gingival tissues. To this end, assay systems for seven different proteinases were established in the laboratory. These have been used to examine collagenolysis and proteinase physiology of cultured dog gingival explants for comparative studies between healthy and diseased tissues, both with and without exposure to dental plaque. Vigorous attempts have also been made to monitor tissue viability and friability in culture to ascertain the contributions of tissue necrosis in the appearance of measurable quantities of these enzymes in culture media.

MATERIALS AND METHODS

A. MATERIALS

Dulbecco's Modified Eagle's Medium (DMEM, 4.5mg/ml glucose, 25 mM HEPES), disposable plastic Nunclon culture flasks, bovine serum albumin and Fungizone (Amphotericin B) were purchased from Grand Island Biological Corp.,Uxbridge. Benzoyl-pencillin and streptomycin were manufactured by Glaxo Laboratories Ltd, Dagenham. Disposable plastic assay tubes(400 and 1400 l volume) and automatic pipette tips were from Eppendorf Int. Dialysis tubing (Visking Small, 8/32") was purchased from Scientific Instrument Centre, Liverpool. Euthanol (sodium pentobarbitol, thiopentanol sodium and acepromazine used for anaesthesia were purchased from May and Baker, Ltd., Dagenham.

Fluorogens: All coumarin-based fluorogenic substrates and standards were prepared by Bachem, Switzerland, and obtained through Cambridge Research Biochemicals,Ltd.(Harston, Cambs). These included N-acetyl-L-alanyl-L-alanyl-L-prolyl-L-alanyl-7-aminomethylcoumarin, methoxy-succinyl-L-alanyl-L-alanyl-L-prolyl-L-valyl-7-amino-methyl coumarin, N-benzoyl-L-valyl-glycyl-L-arginyl-7-aminomethyl coumarin and 7-aminomethyl coumarin standard.

Radiochemicals: (U-)^{14}C-Glycine, ^{3}H-Acetic anhydride and ^{3}H-Prostaglandin E_2 were obtained from Amersham International (Bucks.). ^{125}I- Fibrinogen coated plastic dishes were a gift from Dr. M. McGuire, Sheffield Medical School. Anti-PGE_2 antiserum was a gift from Dr. L. Levine, Brandeis

University. Readysolve II liquid scintillation cocktail fluor, minivials and low background glass vials were purchased from Beckman International.

Biochemicals: Sephadex G150 and Dextran T70 were obtained from Pharmacia Fine Chemicals. o-toluidine reagent, α,N,benzoyl D,L arginyl β-naphthylamide (BANA), β-naphthylamine (NA), hemoglobin, fibrinogen (Fraction I), activated charcoal, Mersalyl, phenyl methyl sulfonyl fluoride (PMSF), and DNA (Calf Thymus) were purchased from Sigma Chemical Company (Poole, Dorset). Trypsin/TPCK (L-1-tosyl amide-2-phenyl ethyl chloromethyl ketone), soy bean trypsin inhibitor and lysozyme test kits were purchased from Worthington Biochemical Corp; p-aminophenyl mercuric acetate and p-diaminobenzoic acid dihydrochloride (Gold label) from Aldrich Chemicals (Sillingham); and PNP-β,D glucuronide and p-nitrophenol from Calbiochem Ltd, (Herts). Lactate dehydrogenase-P/UV assay kits were purchased from Boehringer-Mannheim (Lewes) and bacterial collagenase (Cl. histolyticum) from Windsor Laboratories Ltd (London), previously known as International Enzymes, Ltd.

Histochemicals α,Napthyl acetate, naphthol-AS-D chloroacetate, naphthol-ε-amino caproic acetate, fast garnet GBC and p-rosaniline were obtained from Sigma Chemical Co.(Poole). Glutaraldehyde, p-formaldehyde, sodium cacodylate, Agar 100 embedding resin with DDSA (dodecenyl succinic anhydride), MNA (methyl nadic anhydride and BDMA (benzyldimethyl amine) in kit form were from Agar Aids Ltd (Starsted, Essex). Poly-sciences JB-4 embedding resin was purchased from Windsor Laboratories, Ltd (London). All other reagents were from British Drug Houses, (Liverpool), and were of AnalaR grade.

B. PROCEDURES

B.1. Animal protocols and tissue culture.

Beagle dogs were maintained at the Imperial Chemical Industries colony at Alderly Park, Ches., on regular chow and water ad lib. Four to six weeks prior to sacrifice, left sides of jaws of experimental animals were cleaned by ultrasonic scaling (Dentispray-Cavitron Model 700II) under anaesthesia (premedication with Acepromazine, 2 mg/ml, 0.25 ml/ 5 kg; thiopentone sodium, 25 mg/kg, intravenously) and subsequently restored to health by twice daily brushing with paste. Eight dogs provided comparative studies of both healthy and diseased gingival tissues and four dogs with chronic periodontitis were used without prior oral hygiene. Equal numbers of 2-4 year old male and female animals were used, all with well-established periodontitis at the beginning of each experiment.

Gingival tissues were removed following anaesthetic overdose (Euthanol, 100mg/kg intravenously) by reverse bevel incision between the molar and canine teeth of both upper and lower jaws. Tissues so removed were rinsed in chlorhexidine gluconate (0.2% w/v) and kept in DMEM containing double strength antibiotics (200U/ml Streptomycin,10 µg /ml benzylpenicillin and 10µg /ml Fungizone) for up to two hours until they could be prepared under sterile culturing facilities. Tissues were diced buccal-lingually into 0.5mm sections, washed in DMEM containing antibiotics and the explants were equally divided into seven portions. Five of

these were placed in culture flasks ($25cm^2$) with 2.0 ml DMEM (double antibiotics) and the remaining two tissue samples were kept for weight, extractable DNA, hydroxyproline and enzyme analysis.

Tissue explants were incubated at 37^oC in 5% CO_2 and a water-saturated atmosphere. Culture media were changed every 24 hours and aliquots were stored at -20^oC. Glucose, LDH and Cathepsin B-like thiol proteinase were assayed within two days of harvesting culture media, and assays for β-glucuronidase, plasminogen activator-like (PAL) proteinase, latent and active collagenase, gelatinase, neutral proteinase, elastase and fibrinolytic activities were all completed within 1-2 weeks. Prostaglandin E_2 and hydroxyproline levels in culture media were also determined.

The control tissue samples were accurately weighed and assayed for total DNA, LDH, β-glucuronidase, elastase, PAL- and Cathepsin B-like thiol proteinases, latent and active collagenase, neutral proteinase and total hydroxyproline content. Tissue explants after culture were stored at -20^oC and subjected to similar analysis. In two experiments, extra flasks were set up in parallel with those for biochemical studies, and explants from these were fixed for histological examination at various stages of the incubation period.

Tissue homogenates to be used for total enzyme content determinations were prepared by macerating samples in 3 ml of cold DMEM with a chilled ground glass tissue homogenizer

(3ml Virtis) and the resulting suspension centrifuged for 10 min at 3000 rpm, 4°C. Supernatants were assayed for activities described in Table I(a & b). Tissue explants examined at the end of the culture period for substrate-bound enzyme content were gently macerated and treated using various published dissociative methods (Woessner, 1980; Uitto, etal, 1981; Golub, 1979). Briefly, slurries of tissue explant material in 3 ml DMEM were incubated in 3M KCN or 2M NaCl (final concentration) for 1 hour at 4°C, centrifuged for 10 min at 3000rpm, 4°C to remove debris, and supernatants examined for active and latent collagenase, neutral proteinase, gelatinase, PAL and elastase activities. Another dissociative method employed was thermal agitation (60°C, 1 hr) of the tissue slurries in the presence of 100 mM Ca^{++}.

B.2. Plaque preparation

Homologous plaque samples were collected from animals at the time of scaling, stored in DMEM with antibiotics at 2-4°C for 2-3 h, and dispersed with ultrasound and irradiated with 2.5Mrads with a Linear Accelerator (courtesy of Paterson Laboratories, Holt Radium Institute, Christie Hospital, Manchester). Protein concentration was determined as outlined in Methods (section C.1.c), and aliquots of the plaque preparation were stored at -20°C to provide daily supplementation to selected explants of both healthy and diseased specimens. Plaque samples were assayed for the same enzyme activities as harvested culture media.

B.3. Radiochemical labelling

B.3.a. ^{14}C-labelled neutral salt-soluble collagen was extracted and purified using a slightly modified method to that previously described (Woolley, et al. 1975; Harris and Vater, 1980). Young Guinea pigs weighing approximately 300 grams were each injected intraperitoneally with 300µCi (11 MBq) of (U)-^{14}C-glycine (3.7 GBq/mmol)in 2 ml sterile saline and sacrificed 8-10 hours later. The skins were shaved, minced and soluble collagen was extracted with two neutral salt and two acetic acid extractions and purified as described by Gross (1958). All procedures were carried out at 0-4^{o}C.

The minced tissue was initially extracted with 0.5M NaCl by stirring gently for 24 hours at 2^{o}C. The pH was adjusted to 7.6 with 1.0M Tris(hydroxymethyl) methylamine (TRIS) and the mixture filtered through cheesecloth. The insoluble residue was kept for subsequent extractions with 0.5M NaCl and 0.2N acetic acid. The crude, neutralized filtrate from each extraction was subject to the following procedure for collagen purification. Precipitation of collagen was achieved with 2.5N NaCl over 24 h and collected by centrifugation at 25000 x g for 1 hour. The pellet was dissolved in 0.4M phosphate buffer (pH 7.4) and dialyzed for 24 hours. After centrifugation at 12000 x g for 1 h, the supernatant was acidified with 30% TCA to pH 3.5 to remove non-collagenous protein which was removed by centrifugation. The supernatant was re-dialyzed against 0.4M phosphate buffer (pH 7.4) and then precipitated with ethanol (14% V/V, 1

hour). The precipitated collagen was centrifuged at 5000xg for 30 minutes and the pellet gently re-dissolved and dialyzed overnight against two changes of 0.2M acetic acid. The retentate was lyophilized and stored at -20°C prior to use. The specific radioactivity of these preparations was normally 40-50 MBq/mg. The purity of the collagen preparations was routinely analyzed by disc gel electrophoresis in 5% polyacrylamide gels containing SDS.

Collagen solutions were prepared as follows:lyophilized collagen was dissolved in 0.2 M acetic acid at a concentration of 4 mg/ml and dialyzed overnight at 2°C against the same buffer. Further dialysis against 0.4M phosphate buffer (pH 7.6) for 24 hours was followed by a final dialysis against two changes of 0.4M NaCl for 24 h. The collagen solution was centrifuged at 15000xg for 30 minutes at 0°C and stored at 2-4°C with 0.02% sodium azide as preservative. For collagenase assays, 25 µl of the collagen solution was pipetted into 400µl plastic tubes and reconstituted fibrilgels were formed by incubation in air at 37°C overnight. Each assay tube contained approximately 60-80µg of collagen (batch variation) with 40 Bq of radioactivity. Gels were only used on the day following the 37°C incubation and fresh collagen solutions were prepared every 2 weeks. Hydroxyproline content was measured for each collagen solution and concentration obtained by multiplying by 7.14. Final collagen concentrations so determined ranged from 2.5-3.5 mg/ml.

B.3.b. Preparation of ^{3}H-hemoglobin.

This neutral proteinase substrate was prepared by acetylation of hemoglobin with ^{3}H-acetic anhydride (Gisslow & McBride, 1975; Harris & Vater, 1980). 2.5mCi (90 MBq) of stock ^{3}H-acetic anhydride in toluene (18.5 GBq/mmol) was added to 5 ml of hemoglobin (50 mg/ml) in 0.1 M phosphate buffer, pH 7.1 containing 0.17M NaCl and 50mM sodium acetate. The reaction mixture was kept on ice for 1 hour and then dialyzed against 3 changes of cold phosphate-buffered saline over 24 hours to remove residual ^{3}H-acetic acid. Final specific radioactivity of the stock solution of hemoglobin was 0.1 MBq/mg. 100 µl of this labelled preparation was diluted with cold hemoglobin (1mg/ml in 20mM TRIS, pH 8.1, 0.17M NaCl, 10mM $CaCl_2$ to yield a working solution which contained 8 Bq/25µl per assay tube.

B.3.c. Preparation of ^{125}I-fibrinogen.

Labelled fibrinogen provided a second substrate for the assay of neutral proteinases and was prepared using a modified version of Greenwood and Hunter's method (1963).

To a reaction mixture containing 10µg fibrinogen in 20 µl of 0.5 M phosphate buffer (pH 7.4) and 0.5 mCi (18.5 MBq) of $Na^{125}I$ maintained at $0^{o}C$ was added 10 µl of Chloramine T (10 mg in 4.5 ml. of 0.05M phosphate buffer, pH 7.4). After 35 sec. the reaction was terminated by the addition of 20 µl of sodium-metabisulphite (same excess as chloramine-T) and 350 µl of 0.1 % potassium iodide in 0.05M phosphate buffer. The mixture was applied to a Sephadex G-

100 column equilibrated with 0.05 M barbitone (pH 8.6) containing 0.1% BSA and eluted with the same buffer. Eluant fractions (1.7 ml) were collected and the distribution of the free ^{125}I-peak and the ^{125}I-fibrinogen was determined using a bench top open well gamma counter. Specific activity of the recovered protein was about 10μCi/μg(0.4MBq/mg). A working stock was prepared from this labelled preparation by diluting 100μl with cold fibrinogen at 1mg/ml (20mM TRIS, pH 8.1, 0.17M NaCl) to yield a solution which gave 4KBq/mg.

^{125}I-fibrinogen-coated multiwell plates were a gift from Dr. M. McGuire, Dept of Medical Biochemistry and Human Metabolism, Sheffield Medical School, and were prepared according to the method of Unkeless, etal,(1973). Each well contained 20 μg fibrinogen which released 90 KBq/well upon trypsinization. Plates were allowed to dry for 5 days after preparation and stored for up to 3 months at -25^{o}C.

B.4. Histology

B.4.a. Fixation.

Representative samples of tissue specimens prepared for culture were placed in standard buffered fixative (1.5% glutaraldehyde in 0.1M sodium cacodylate/HCl, pH 7.4, 1mM $MgCl_2$). Specimens for enzyme histochemistry were fixed in 1.0% p-formaldehyde, 0.25% glutaraldehyde in 0.1M sodium cacodylate, pH 7.4, for 1 hour at 4^{o}, then stored in 0.15M sodium cacodylate, pH 7.4, for further processing.

Polysciences JB-4 methacrylate resin was used for rou-

tine histological or histochemical embedding of gingival tissues. Tissue blocks were embedded in JB-4 resin in Beem capsules at 4°C under N_2 atmosphere after acetone dehydration to ensure retention of enzyme activity. Sections (2-3 μm) were cut using glass knives, transferred onto glass slides and stained routinely with Toluidine blue for tissue orientation. Tissue sections to be processed for histochemistry were allowed to spread on a drop of distilled water on coverslips and dried at 30°C for 10-15 minutes.

B.4.b. Histochemistry.

Methods are available which identify specific cell types by their ability to hydrolyze different naphthyl esters (Yam etal,1971;Li etal,1972, M.Bromley, Dept. of Medicine, Withington Hospital, personal communication.) Macrophages and monocytes possess α-naphthyl acetate esterase (NAE) activity. Polymorphonuclear leukocytes and mast cells preferentially hydrolyze naphthol-AS-D-chloroacetate (NCA or CAE) and mast cells specifically hydrolyze the ester naphthol-AS-amino caproate (NAC). Free naphthols released by these esterases are coupled to insoluble diazonium salts for visualization of the reaction products.

The coupling reagents hexazotized para-rosaniline (HPR) and hexazotized New Fuchsin (HNF) were prepared by adding 4 drops of freshly prepared 4% $NaNO_2$ (w/v) to 4 drops of 4% para-rosaniline (w/v, 2M HCl) or 4 drops of 4% New Fuchsin (w/v, 2M HCl) and mixing for 30 seconds. The coupling reagents were filtered prior to use.

For α-naphthyl acetate esterase (NAE) activity, 0.5 ml α-naphthyl acetate (20 mg/ml in acetone) was added to 10 ml 0.1 M phosphate buffer, pH 6.3, and 50 µl of HPR, and tissue sections mounted on cover slips were incubated in this mixture for 1-2 hours at 37°C in Columbia jars. For α-naphthol AS-D chloroacetate (NCA or CAE) activity, sections were incubated in 0.5 ml of substrate (4 mg/ml in dimethyl formamide) in 10 ml phosphate buffer, pH 7.4 with 50 µl HNF under the same reaction conditions outlined for αNAE activity. Similarly, sections were incubated in 1.0 ml of naphthol AS amino caproate (NAC) (2 mg/ml in ethylene glycol monomethyl ether) with 10 ml of phosphate buffer (0.1 M, pH 7.4) and 50 µl HNF for 1-2 hr at 37°C for naphthol-AS-amino caproate esterase activity.

Following incubation, tissue sections on cover slips were rinsed in buffer, counterstained with Toluidine Blue or Gill's haematoxylin and permanently mounted on glass slides.

B.5. Instrumentation

Absorptiometric assays were analyzed on a Pye Unicam SP8000 Spectrophotometer equipped with a temperature controlled cell changer, 1805 Program Controller and linear chart recorder. Some colorimetric determinations were carried out on a Cecil 292 Digital spectrophotometer with a W/W Linear chart recorder. Fluorometric analyses were carried out on an Aminco-Bowman 4-8202 spectrophotofluorometer equipped with a 10-222 microphotometer and modified to read millivoltage output on a Fluke 8000 digital volt meter.

Fluorescence spectra were determined manually using a Brants 26000 A3 X-Y recorder and the dual-drive wavelength scanning system of the Aminco-Bowman fluorometer. All enzyme incubations were carried out in a Grant's #SB3 water bath.

Liquid scintillation measurements were provided by a Beckman LS250 with Automatic Quench Correction (AQC), equipped with ^{3}H-, ^{14}C- and combination Isosets. Channels ratio with external standard was used to establish quench correction curves for counting efficiencies. ^{125}I samples were counted using a Beckman Gamma 3000 spectrometer with automated ^{125}I program available. Eluant fractions from the gel filtration separation of $Na^{125}I$ and ^{125}I-fibrinogen were monitored with a bench-top open well gamma detector and MS310 count/rate meter (ERD Engineering and J&P Engineering, Slough).

Culturing facilities included a Howarth GB1H Grade II flow hood and Hot Pack CO_2 Digimatic incubator. All microassays were carried out using SMI-Digimatic 5-30 µl or SMI-Quik-Set (50/150 µl) automatic pipettes with siliconized glass disposable tips. Larger volumes were measured with Gilford Pipetteman P-200 or P-1000, with an Eppendorf multivolume dispenser (10µl-1.25ml) being used for routine dilutions in the fluorometric assays.

Histological sections were cut on either an LKB Ultratome 8800 III or an LKB Historange 2218 with glass knives prepared on an LKB 7801B Knifemaker. Photographic exposures were taken on either Kodak Ektachrome 160 or Panatomic X.

C. BIOCHEMICAL ASSAYS

These assays are summarized in Tables I(a) and I(b) at the end of this chapter.

C.1. Colorimetric Assays

C.1.a. Hydroxyproline was determined using a modified method of Woessner (1961), with internal standards, chloramine-T and p-dimethyl amino benzaldehyde (Woolley, etal, 1978b; Stegeman & Stalder, 1967). Typically, 300 µl samples were hydrolyzed overnight at 110°C with equal volumes of 12N HCl in sealed glass bijou bottles. Neutralized samples were aliquoted into tubes with 2.5 and 5.0 µg hydroxyproline internal standards in phosphate buffer, pH 6.0, with half-saturated NaCl and reacted with excess Chloramine-T and p-dimethyl amino-benzaldehyde for 12 minutes at 65°C. After cooling the samples, the absorbance was measured at [redacted] nm and concentrations were calculated from values obtained for the internal standards.

Levels of non-diffusible degradation products containing hydroxyproline were determined by dialyzing 1 ml samples of culture media against 20 volumes of DMEM overnight at 2°C, and measuring hydroxyproline in the retentate as outlined. Information regarding the "cut-off" size of the 8/32" Visking tubing is not available from the manufacturer.

C.1.b Glucose concentration in culture media were measured using the method of Hultman (1959). Ten µl of medium was added to 1.0 ml o-Toluidine reagent, the samples were incubated for 20 minutes in a boiling water bath, and

absorbance at 620 nm was measured. Concentrations were determined from a standard curve using 1 to 6 mg/ml glucose. The data was presented as :

µg glucose utilized/mg tissue (wet weight)/24 h.

C.1.c **Protein** was measured using Lowry's method (Lowry, etal, 1951) with bovine serum albumin as a standard.

C.1.d. **Lactate dehydrogenase** (EC.1.1.1.27) was measured kinetically with Boehringer Mannheim kit LDH-P/UV (Worthington Manual). The rate of oxidation of $NADH_2$ is followed as a decrease in E_{340} of a reaction mixture of 0.01M Na-pyruvate/0.002M $NADH_2$ in 0.03M phosphate buffer (pH 7.4) and activity was determined in units of moles $NADH_2$ oxidized/min at 25°C.

C.1.e. **β-Glucuronidase** (EC.3.2.1.31) was determined according to the method of Fishman (1963) by measuring the release of p-nitrophenol (PNP) from PNP-β,D-glucuronide (see also Kato etal, 1960). Fifty µl of medium was incubated with 150µl of acetate buffer (pH 4.0) and 50 µl of substrate (15 mg/ml in H_2O). Reaction mixtures were incubated for two hours at 37°C, diluted with 1 ml of 0.2M glycine/0.2% Dupanol (w/v), pH 11.7, and the released PNP was measured by absorbance at 420 nm to determine activities. One unit of activity was defined as 1 nmole PNP released/hr at 37°C.

C.1.f. **Lysozyme** (EC.3.2.1.17) was measured using the test kits available from Worthington Biochemical (Worthington Enzyme Manual). Micrococcus lysodeikticus cell walls at 0.1mg/ml in 0.06M phosphate buffer, pH 6.2, with 20mM NaCl

was mixed with 100 µl of culture medium, and the change in absorbance at 550 nm was followed for 5 minutes at 25°C. Rates of change in absorbance were compared with standard egg white lysozyme (EWL)at concentrations of 2 to 20 µg/ml. One unit of activity was equal to the change in absorbance/min brought about by 1 µg of standard EWL at 25°C.

C.2. Fluorometric assays

C.2.a. Cathepsin B-like thiol proteinase(EC.3.4.22.1) was measured using a modified method of Barrett (1972). Differences between the fluorescence spectra of the substrate BANA and the hydrolysis product naphthylamide allowed the latter to be monitored separately at 327nm excitation and 410nm emission without interference from unreacted substrate. This method proved more sensitive that the original colourometric procedure of Barrett.

For assay 100µl of medium was incubated with 10µl of the substrate Benzoyl,DL-arginyl naphthylamide, BANA, (32mg/ml in DMSO) and 100µl of 0.1M PO_4 buffer, pH 6.0 (10 mM cysteine and 1.5mM EDTA added), for 2 hours at 37°C. Reaction mixtures were diluted with 1.5 ml distilled water and fluorescence read at 410nm. Activities were determined from standard naphthylamine concentrations over the range of 1 to 100 nM, and 1 unit was defined as 1 nmole NA released per hour at 37°C.

C.2.b. Macrophage (EC.3.4.21.-) and leukocyte elastase (EC.3.4.21.11) activities were determined using a modified method of Zimmerman, etal (1977) in which the fluorogenic

compound aminomethyl coumarin is hydrolyzed from a synthetic peptide analogue of cleavage sites in elastin. The analogues used were either N-acetyl-L-alanyl-L-alanyl-L-prolyl-L-alanyl-7-aminomethyl coumarin (MEL-7AMC) for macrophage elastase, or O-methyl succinyl-L-alanyl- L-alanyl- L-prolyl-L-valyl-7-aminomethyl coumarin (LEL-7AMC) for leukocyte enzyme, both dissolved in dimethyl sulfoxide (DMSO) at 1 mg/ml. Reaction mixtures consisted of 100µl culture medium sample, 100 µl of buffer containing 20mM TRIS, 10mM $CaCl_2$ at pH 8.0, and 10 µl of substrate. After incubation at 37°C for 16 h, the assays were diluted to 1.5 ml with distilled H_2O and assessed fluorometrically. Excitation of the reaction mixtures at 383nm and fluorescence read at 460nm allowed for measurement of the free fluorogen as the labelled substrates do not interfere at these wavelengths (Baici, etal, 1981). Tissue homogenates were incubated for one hour due to high activities. K_m's of these reactions were in the order of 0.4-0.5 mM, and the reaction rates were linear over 24 h using commercial pancreatic elastase at these substrate concentrations. One unit of activity was defined as 1 nmole of 7AMC hydrolysed from either substrate/hr at 37°C.

C.2.c. **Plasminogen activator-like activity (PAL)** (EC.3.4.21.-) was measured using N-benzoyl-L-valyl-glycyl-L-arginyl-7-AMC (PAL-7AMC) as substrate (100µg/ml dissolved in DMSO), with fluorescence measured at 460nm (Zimmerman, etal, 1978). 100µl of culture medium was mixed with 100 µl of buffer containing 20 mM TRIS (pH 8.0) and 10mM $CaCl_2$ and

allowed to preincubate for 5 minutes at 37°C. Ten µl of substrate was then added to the mixture and after 1 h at 37°C the reaction was terminated with 10 µl of 20mM PMSF dissolved in DMSO. Each sample was diluted to 1.5ml and read at 460nm. Although the concentration of substrate was ten times lower than those used in the elastase assays, the reaction rate was not substrate limited and was linear for at least three hours. One unit of activity was defined as in the elastase assays, as 1 nmole of 7AMC hydrolysed from the substrate/hr at 37°C.

The Aminco/Bowman fluorometer was standardized by setting the microphotometer sensitivity range to 0.3 (coarse) and adjusting the relative fluorescence of a 1.0 nM solution of quinone sulfate in 0.1 M H_2SO_4 to a full-scale deflection (50 mvolts on a Fluke 8000 Digital Volt Meter, or 1 Relative Fluorescent Unit on the Microphotometer).

C.2.d. Tissue extraction and DNA determination.

Tissue DNA was extracted and measured according to the method of Setaro and Morley (1976). Approximately 20mg samples of tissue were homogenized with 3 ml of cold 10% trichloro-acetic acid and centrifuged for 5 min at 3000 rpm. Precipitated material was mixed with 2 ml potassium acetate in absolute alcohol (9.8 g/l) to remove fat. This slurry was allowed to stand at 20°C for 10 min, spun at 3000 rpm for 5 min, and the residual precipitate re-extracted with 2 ml absolute ethanol. Precipitates so extracted were digested in 3.0 ml 10N NaOH (60°C for 1 hour) and centrifuged for 15 min

at 3000 rpm. 100µl of the supernatant were reacted with 30mg of diaminobenzoic acid dihydrochloride (0.3g/ml in 0.1N HCl, 100µl per sample) at 60°C for 30 minutes. This mixture was diluted with 3.0 ml 1N HCL and fluorescence read at 420nm excitation and 520nm emission. Calf thymus DNA standards (0.1 to 10 µg/ml) were used with each determination.

C.3. Radiochemical Assays.

C.3.a. Collagenase activity (EC.3.4.24.7)was measured using the reconstituted ^{14}C-collagen fibril assay method described previously (Woolley, etal, 1975). Twenty-five µl aliquots of ^{14}C-collagen in 0.4M NaCl were allowed to form fibrils overnight at 37°C, and then incubated with 100 µl of culture medium sample and 100µl of buffer containing 20mM TRIS, pH 8.0, 0.17M NaCl and 10mm $CaCl_2$. Assays were usually incubated for 16 hours unless the collagen gels were visibly digested after 10 h, at which time the reactions were stopped by centrifugation at 15000 x g for 20 min at 20°C. 100µl of supernatant containing ^{14}C-peptides were counted in 3.0 ml ReadySolveII liquid scintillant in minivials for 10 min at 3% accuracy in a Beckman LS250 spectrometer.

Latent enzyme was activated by both proteolytic and organo-mercurial methods prior to assay. For proteolytic activation 100 µl of the latent collagenase sample was subjected to a 15 minute incubation with 10 µl of Trypsin/TPCK (100 µg/ml) which was terminated by the addition of 5-fold excess of Soy Bean Trypsin Inhibitor (5 µl of 1mg/ml

SBTI in H_2O). Alternatively chemical activation was employed by the addition of 10µl of 20mM APMA in DMSO to the collagenase assay. Trypsin (10µg/ml), SBTI, plaque and culture media controls were included in all experiments. One unit of collagenase activity is defined as 1 µg collagen digested/hr at 37°C.

C.3.b. **Gelatinase** activity (EC.3.4.24.-)was determined using thermally denatured ^{14}C-collagen (60°C for 1 hour) under similiar conditions as the fibril assay. After 16 h at 37°C, the reaction was terminated with 50 µl of 40% TCA-0.025% Tannic acid and kept at 0°C for 1 h prior to centrifugation at 15000 x g for 20 min at 20 °C to remove precipitated gelatin. 150 µl of supernatant containing degraded ^{14}C-gelatin-peptides were counted in 3 ml of Beckman Ready-Solve II scintillation cocktail fluor as described in the collagenase assay. One unit of gelatinase activity is defined as 1 µg gelatin digested/hr at 37°C.

C.3.c. **Neutral proteinase** activity (EC.3.4.24.-) was determined using ^{3}H-hemoglobin as substrate. Twenty-five µg of labelled substrate (0.1MBq/mg, 6 KBq in each assay tube) were incubated with 100 µl enzyme sample together with 100 µl of buffer (20mM TRIS, pH 7.5, 10mM $CaCl_2$) for 6h at 37°C. Reactions were terminated by precipitation with TCA/Tannin as described for the gelatinase assay. Similarly, radioactivity was determined as described for the gelatinase assay. One unit of neutral proteinase activity

is defined as 1 µg hemoglobin hydrolyzed/hr at 37 °C.

C.3.d. **Fibrinolytic** activity (EC.3.4.21.31) of samples was determined using the ^{125}I-fibrinogen plate assay (Unkeless, *et al*, 1973) in order to compare PAL activities obtained by fluorescence methods. ^{125}I-Fibrinogen-containing wells were pre-incubated with 200 µl fresh culture medium which was subsequently counted for background ^{125}I- wash-out of the plates. Acid-inactivated serum was included (10% V/V) in some of the reactions to assess plasminogen activator activity. Two-hundred µl of culture media samples were added to the wells and incubated for 16h at 37°C. The samples were removed with a fine tipped automatic pipette and transferred to plastic tubes for counting. Residual ^{125}I-counts on the plates were removed by trypsinization (10 µg trypsin/TPCK in 200 µl buffer, 20mM TRIS, pH 7.5, 10mM $CaCl_2$) for 3-4 h at 37°C, and total counts for each well determined. Fibrinolytic activity was expressed as % of total counts, but this method was found to prove unsatisfactory due to excessively high and extremely variable backgrounds.

C.3.e. **Prostaglandin E levels** in culture media were measured by radioimmunoassay in collaboration with Dr. D. Taylor. The anti-PGE_2 antisera was obtained as a gift from Dr. L. Levine (Brandeis University) and did not distinguish between PGE_2 and PGE_1. Although the antisera also cross-reacted with PGB_2, neglible amounts of this prostaglandin have been found in gingival tissues (D'Souza, *et al*, 1981). Radio-labelled tracer (5,6,8,11,12,14,15(n) ^{3}H-PGE_2 with a

specific activity of 180 Ci/mmol or 6.7 TBq/mmol and of radioactive concentration.= 0.2 mCi/ml) was obtained from Amersham International and diluted $1x10^3$-fold in RIA buffer to give $2x10^4$ cpm /100 µl. This diluted tracer solution was found to be stable for up to 3 days at 4°C. The RIA buffer contained 50mM phosphate(pH 7.4) 0.1 % (w/v) bovine serum albumin, 150mM NaCl and 0.05 % (w/v) Na azide. Standard PGE_2 at 10 ng/ml in RIA buffer was stored in 0.5 ml aliquots in the dark at -20°C until required, and then doubly diluted to yield 6 different concentrations between 0.15 and 5.0 ng/ml for a standard calibration curve of optimal reproducibility. Samples were usually diluted at least 5-fold with RIA buffer prior to assay.

One-hundred µl of PGE_2 standards or diluted culture medium samples were added together with 100 µl of tracer and 100 µl of diluted anti-PGE_2 antiserum to the assay tubes (12x75 mm polystyrene tubes) and mixed thoroughly. After 90 min at 4°C, 100 µl of charcoal suspension (1% (w/v) activated charcoal, 0.1% (w/v) dextran T70 in 0.05 M phosphate buffer, pH 7.4 with 0.05% Na azide) was added and mixed by vortex. After 10 min at 4°C, the tubes were centrifuged (2600 rpm for 10 min at 4°C, MSE-6L centifuge) and supernatants containing antibody-bound PGE_2 decanted into scintillation vials containing 3 ml Beckman ReadySolve HPII cocktail fluor. Care was taken not to disturb the charcoal pellet during decanting. Antibody-bound ^3H-PGE_2 in the samples was determined by counting for 5 minutes to 3%

accuracy in a Beckman LS250.

Calibration curves of percentage labelled PGE_2 bound versus concentration of unlabelled compound added to the reaction mixtures were obtained from a DP5500 mini-computer programmed for polygonal curve fitting. Values for samples were read from this non-linear calibration curve, and units expressed as percentages of that found in Day 1 media from diseased explants. Data was plotted as $\log_{10}$ (% ratio).

TABLE Ia

SUMMARY OF ASSAYS

ASSAY	TYPE	SUBSTRATE OR REAGENT	UNITS*
HYDROXYPROLINE	Spectrophotometric	Chloramine-T & p-dimethylamino-benzaldehyde.	µg/ml
GLUCOSE	Spectrophotometric	o-Toluidine	µg/ml
LACTATE DEHYDROGENASE	Spectrophotometric	Pyruvate (0.1mM) $NADH_2$ (2.0mM)	µmoles/hr/100µl
β-GLUCURONIDASE	Spectrophotometric	PNP-β,D-Glucuronide (15 mg/ml)	nmoles PNP/hr/100µl
LYSOZYME	Spectrophotometric	M. Lysodeikticus (0.1 mg/ml)	Std EWL equiv./100µl
CATHEPSIN B-LIKE THIOL PROTEINASE	Fluorometric	N,αbenzoyl-DL-arg-naphthylamide (32 mg/ml)	nmoles NA/hr/100µl
ELASTASE (Macrophage)	Fluorometric	N-Acetyl-L-Ala-L Ala-L-Pro-L-Ala-7-AMC (1.0 mg/ml)	nmoles AMC/hr/100µl
(Leukocyte)	Fluorometric	Me-Succ-L-Ala-L-Ala-L-Pro-L-Val-7-AMC (1.0 mg/ml)	nmoles AMC/hr/100µl

* SI or CGS Units where appropriate

TABLE Ib

SUMMARY OF ASSAYS

ASSAY	TYPE	SUBSTRATE OR REAGENT	UNITS*
PLASMINOGEN ACTIVATOR-LIKE	Fluorometric	N-Benzoyl-L-Val-Gly-L-Arg-7-AMC (0.1 mg/ml)	nmoles 7AMC/hr/100ul
DNA	Fluorometric	Diaminobenzoic acid (0.3 mg/ml)	µg/mg tissue
COLLAGENASE (Active and Latent)	Radiochemical	^{14}C-Collagen 40 KBq/mg (2.5-3.5 mg/ml)	µg/hr/100ul
GELATINASE	Radiochemical	^{14}C-Gelatin 40 KBq/mg (2.5-3.5 mg/ml)	µg/hr/100ul
NEUTRAL PROTEINASE	Radiochemical	^{3}H-Hemoglobin (0.1MBq/mg) (2 mg/ml)	µg/hr/100ul
FIBRINOLYSIS	Radiochemical	^{125}I-FIBRINOGEN (3.7 MBq/mg) (1 mg/ml)	%cts released/ 200µl/16hr
PROSTAGLANDIN E_2	Radioimmunoassay	^{3}H-PGE_2 3.7MBq/ml	ng/ml

*SI or CGS units where appropriate.

RESULTS

Previous experiments with this model suggested healthy gingival explants from beagle dogs retained the capacity to degrade endogenous collagen for periods of up to ten days while still retaining viability as judged by glucose uptake (Figure 2; Woolley, unpublished results). However, at that time it was difficult to compare the differences between healthy or diseased tissues, as data was not normalized to a wet weight, protein or DNA content basis. Nonetheless, evidence for the breakdown of collagen as measured by the appearance of soluble hydroxyproline in the culture media was apparently correlated with demonstrable collagenase activity. This activity was expressed only as cpm of ^{14}C-peptides solubilized from ^{14}C-glycine-labelled collagen by culture media samples, and not corrected to any tissue parameter or normalized to any standard enzyme activity unit. Improvements in the expression of this data were essential; this was achieved by correcting to a wet weight basis to compare healthy and diseased tissues in culture.

Tissue Viability:

Glucose utilization remained fairly stable throughout the culture period, decreasing slightly towards the end of the experiment (Figure 3). Lactate dehydrogenase (LDH) release was significantly higher on the first day of culture than any other time, and probably reflects tissue damage incurred during excision and dissection (Figure 3). From the

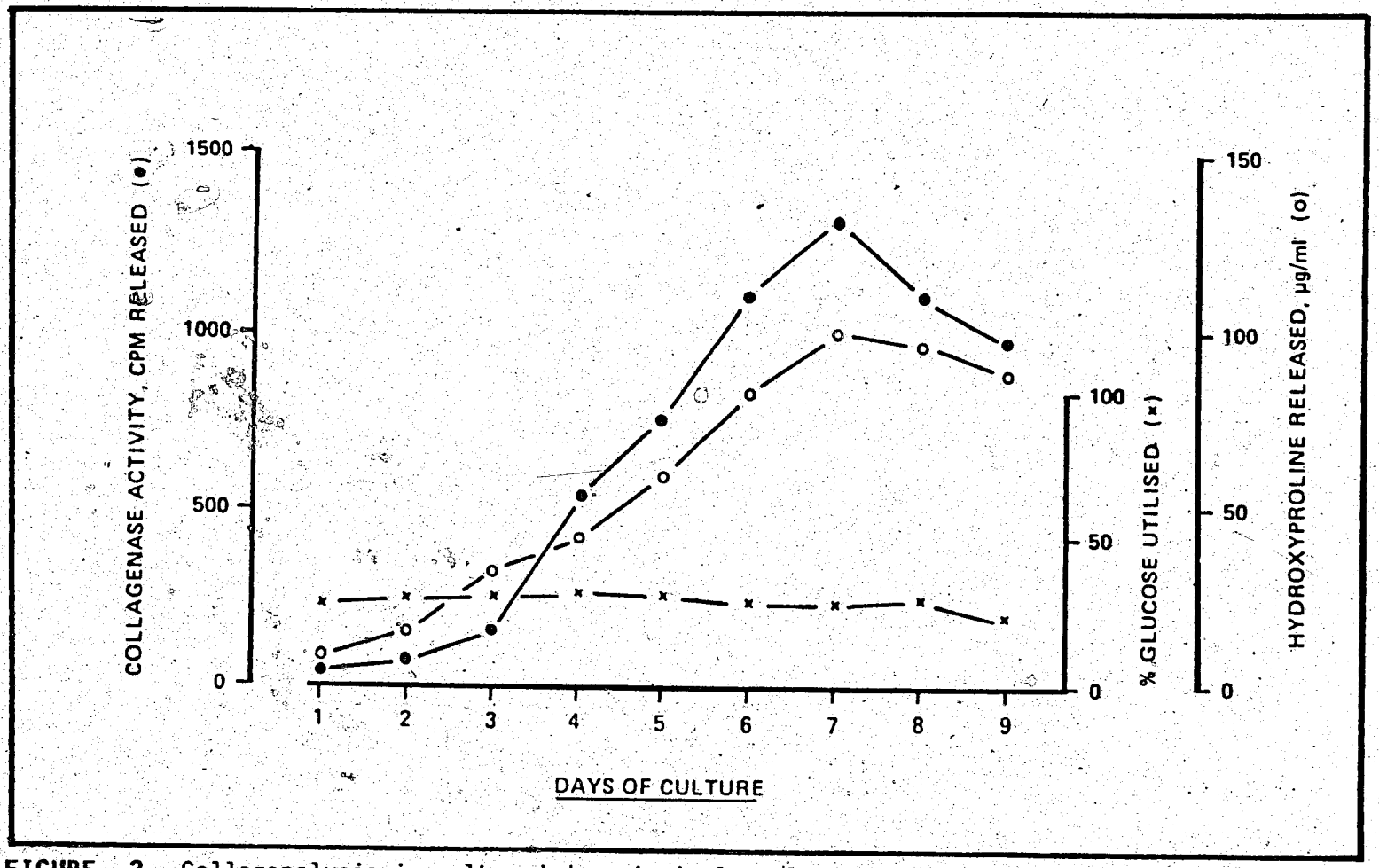


FIGURE 2. Collagenolysis in cultured dog gingival explants.
Release of collagenase activity and hydroxyproline from healthy dog gingival explants in culture. Medium harvested on successive days was assayed for collagenase activity (●), hydroxyproline content (O) and glucose utilization (X). Data uncorrected for any tissue parameters.

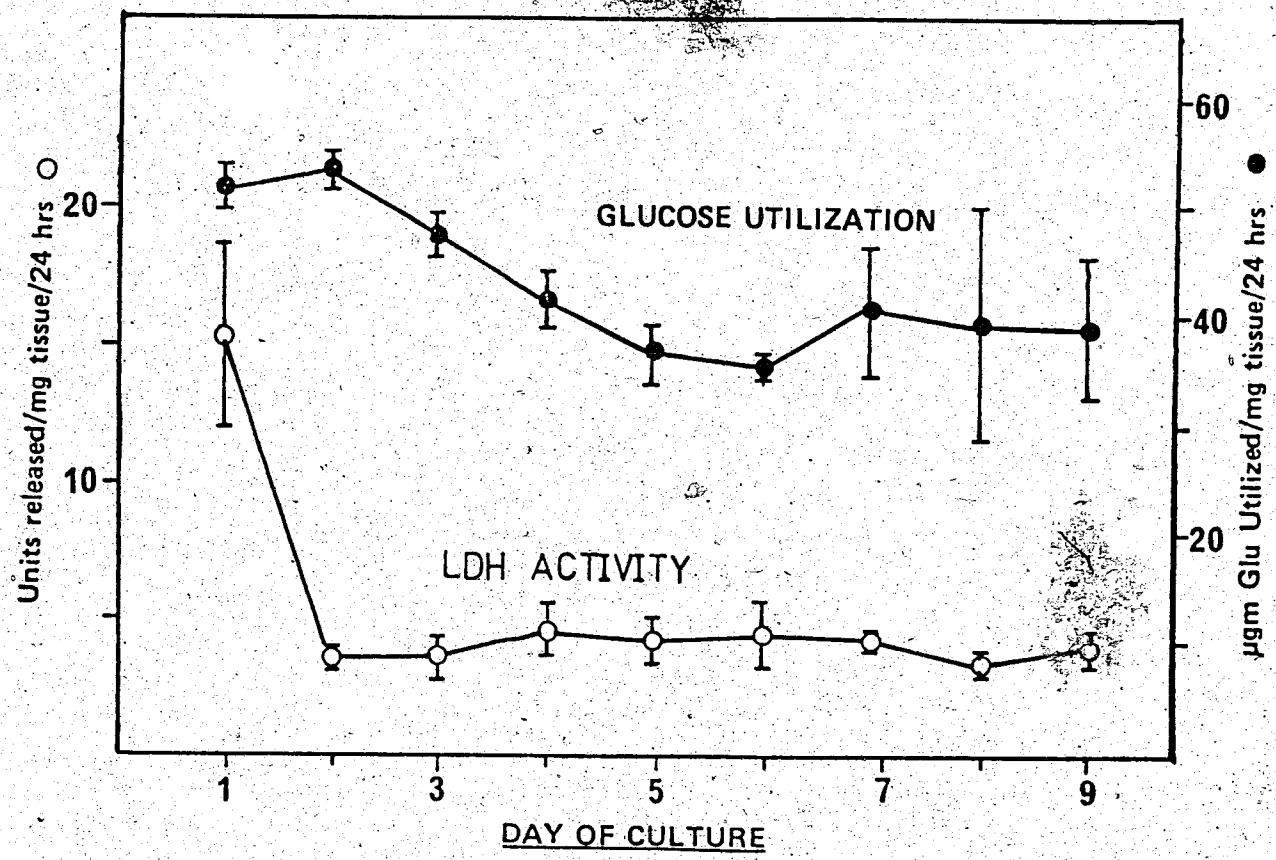


FIGURE 3. Assessment of tissue viability in vitro. Medium harvested on successive days was assayed for lactate dehydrogenase (○) and glucose (●). Glucose is expressed as μg utilized/mg/24 h which usually represented 20-30 percent of that available in the culture medium. Data points represent mean values ±1 SD for duplicate assays of 2 culture flasks of gingival tissue from each of 2 dogs.

second day of culture the LDH drops to a baseline value which approximates 0.2 % of total available LDH present in the tissue.

Histological observations of explanted tissues supported the biochemical assessment of viability (Plate II, end of Results) but numerous morphological and cytological changes were observed during culture. Some of these changes are apparent in the plates shown.

Experimental dogs: Most enzyme or other biochemical values to be presented are shown as averages of duplicate assays for at least 2 culture flasks from each of 2 to 4 animals. Several problems arose when averaging the data obtained for all of the animals, as each dog often showed different levels of enzyme activity, even though the general profiles were similar. Some enzyme activities from similarly treated dogs were usually in comparable ranges (eg. PAL) but others, such as collagenase and gelatinase often demonstrated extreme variability, even when corrected to a wet weight basis. In most cases, two dogs with similar treatments were run at the same time, and the experimental data was plotted and compared. The data presented usually represents that obtained from one or two dogs, but in all cases the pattern or profile of each parameter was confirmed by similar experiments with other dogs.

Collagenolysis from diseased explants.

Gingival explants released hydroxproline into the media after 1-2 days and retained this capacity for 5-7 days

(Figure 4a). Much of the hydroxyproline was found as diffusible products (Figure 4b) in the media suggesting further degradation by the presence of secondary aminopeptidase activity.

All gingival explants released hydroxproline into the medium but the profiles obtained for each animal often differed with respect to time and peak values. The profile shown in Figure 4a reflects this variance by the large standard deviations of the data obtained from 3 animals. In all media assayed for non-diffusible hydroxyproline there was a significant decrease in levels following dialysis suggesting degradation to small collagen peptides As no collagen is synthesized in culture owing to the lack of cofactors such as ascorbate and alpha keto-glutarate the media hydroxyproline is probably accounted for solely by degradative activity.

A typical profile for collagenase activity is shown in Figure 5a. Most of the enzyme is present in an active form with latent enzyme being found in the media only at later stages of culture. Both proteolytic and organomercurial activation methods were routinely used, with trypsin activation giving slightly higher values in most cases.

Active and total gelatinase is shown in Figure 5b. About 50% of the gelatinase is present as latent enzyme, but this ratio was variable between animals. Plasminogen activator-like activity is shown in Figure 6a. Most of the tissue culture profiles of PAL reached maximal values bet-

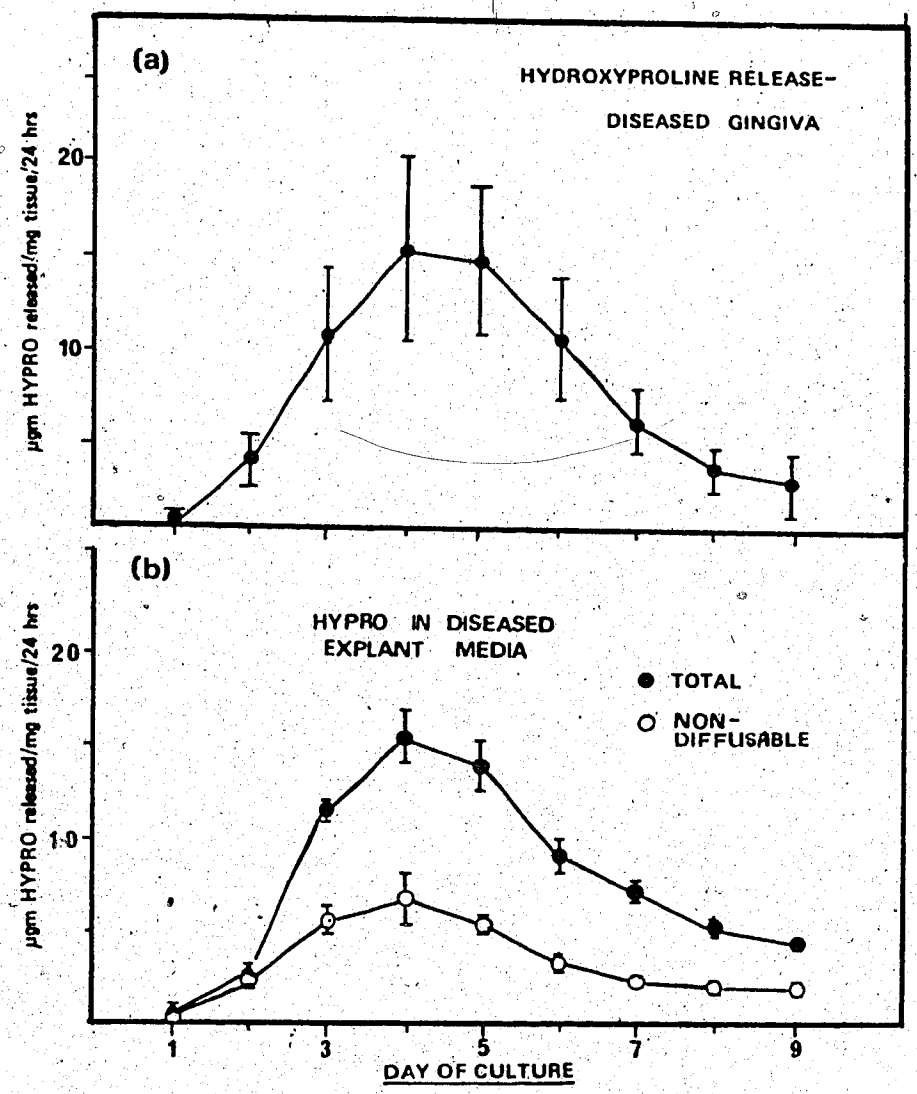


FIGURE 4. Release of hydroxyproline from gingival explants in vitro. (a). Total hydroxyproline released into culture medium for each day of culture. Data points represent mean values ±1 SD obtained from duplicate assays of 2 culture flasks of tissues from each of 3 dogs. (b). Total (●) and non-diffusable (○) hydroxyproline released into culture medium. Hydroxyproline in the culture medium was assayed before and after dialysis (16 h at 4°C). Data points represent means and range of values obtained from duplicate assays of two flasks from one experiment.

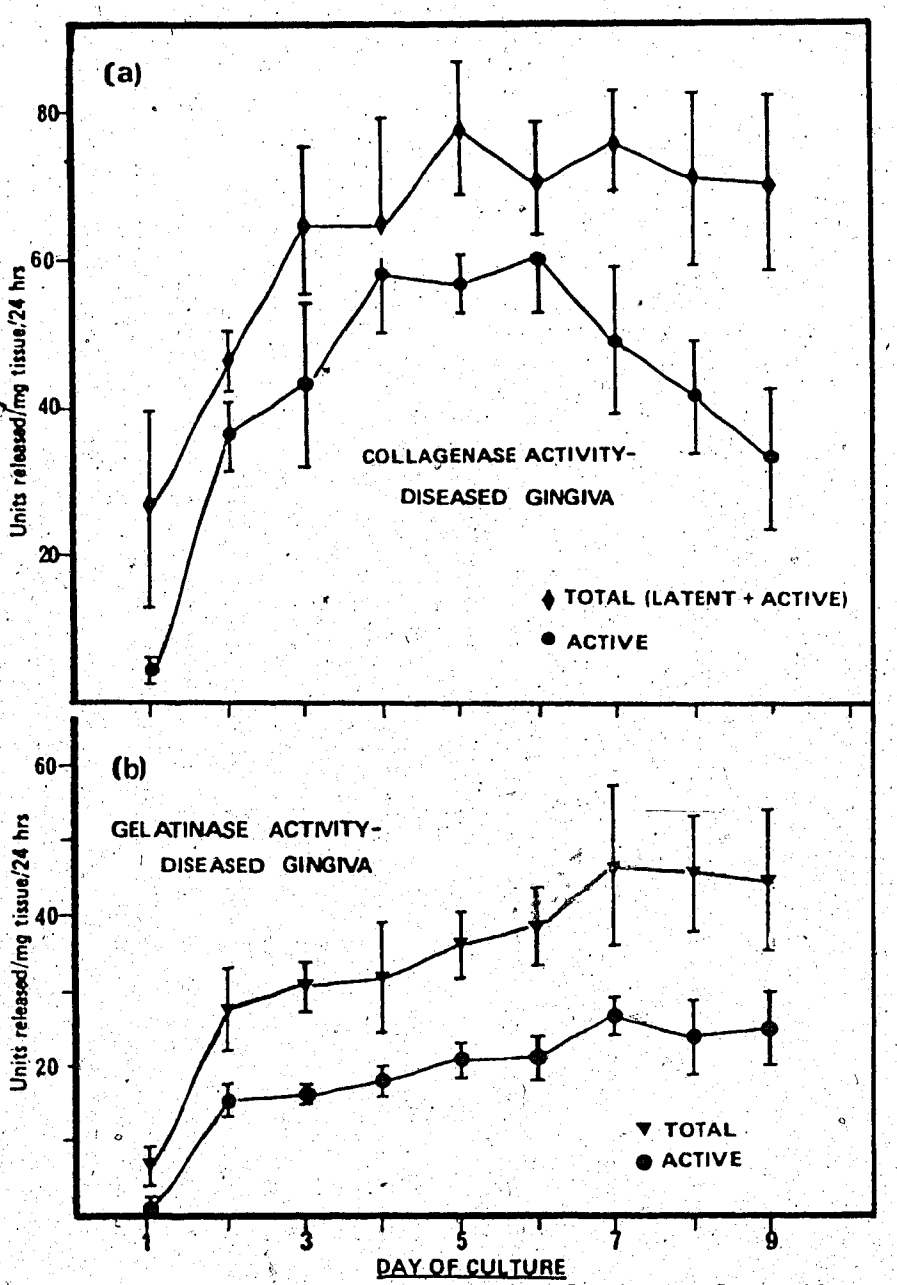


FIGURE 5. Release of collagenase and gelatinase from diseased gingival explants in vitro.
(a) Collagenase was assayed directly as active enzyme (●) or as total collagenase activity (♦) in culture medium. Both proteolytic and organomercurial activation was used. Data points represent mean values ±1 SD from duplicate assays of 2 tissue culture flasks from each of 2 animals.
(b) Active gelatinase activity assayed directly (●) or following proteolytic activation (▼) from diseased gingival cultures. Data points represent mean values ±1 SD of duplicate assays of 2 culture flasks from 1 animal.

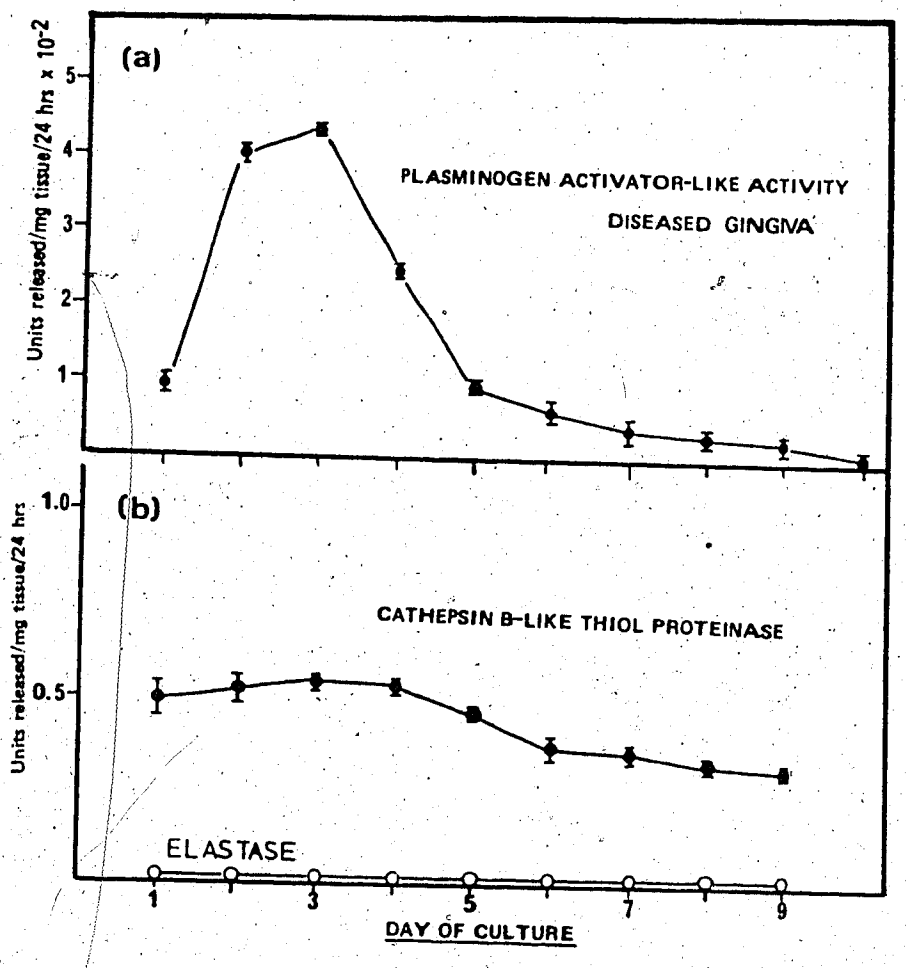


FIGURE 6. Release of plasminogen activator-like (PAL), cathepsin B-like thiol proteinase and elastase by diseased gingival explants in vitro.
(a).Plasminogen activator-like (PAL) activity (●) was released during the early stages of culture and this profile was similiar for all cultures assayed. Data points represent mean values ±1 SD of duplicate assays on 2 culture flasks of tissue from 1 dog.
(b) Assay of Cathepsin B-like thiol proteinase (●) and elastase (○) activities from the same cultures in (a). Data represents mean values ±1 SD. Maximal daily activity of Cathepsin B elaborated in culture was 0.5% of enzyme activity found in tissue homogenates. Both PMN and macrophage elastase activities were neglible.

ween days 2 and 4, and these peak activities were similar for all the cultures examined.

A representative profile of Cathepsin B-like thiol proteinase activity in diseased explant cultures is shown in Figure 6b. Comparatively small amounts of enzyme are released over the course of the culture period with a slight peak about the third day. One possible explanation for this is the poor stability of lysosomal Cathepsin B at neutral pH. Although the culture periods for many experiments were extended to 9 or 10 days no detectable elastase activity was ever present. This was surprising as tissue homogenates possessed significant levels of assayable leukocyte and macrophage elastase activity.

In general, the cultures of diseased gingival explants produced significant quantities of the enzymes collagenase, gelatinase and plasminogen activator, in contrast to elastase, Cathepsin B-like thiol proteinase, β-glucuronidase, lactate dehydrogenase and lysozyme.

B. Comparison of healthy and diseased tissues.

Comparative values for healthy and diseased tissue homogenate levels of the parameters presented in Figures 3-9 are shown in Table II. Tissue homogenates were prepared as described in Methods and the supernatants were subjected to enzyme analysis. In both diseased and healthy tissues an insoluble residue was left after the homogenization protocol, which remained even after TCA extraction of the tissue for DNA analysis. The hydroxyproline content of the insol-

TABLE II

COMPARATIVE STUDY ON HOMOGENATES AND EXTRACTS OF HEALTHY AND DISEASED GINGIVAL TISSUE AT TIME OF EXCISION.

PARAMETER	HEALTHY	DISEASED
LACTATE DEHYDROGENASE (U/mg)	2141±80	2304±42
β-GLUCURONIDASE (U/mg)	3118±11	3195±37
+HYDROXYPROLINE (µg/mg)	121.7±26	70.0±21
DNA (WET WT) (µg/mg)	2.31±0.04	2.53±0.03
(DRY WT) (µg/mg)	8.46±0.48	11.60±0.30
CATHEPSIN B (U/mg)	101.5±18	136.7±22
ELASTASE (U/mg)	48.5±6.0	70.5±8.1
PLASMINOGEN ACTIVATOR	0	0
GELATINASE	0	0
ACTIVE COLLAGENASE	0	0
LATENT COLLAGENASE	0	0
NEUTRAL PROTEINASE	0	0
LYSOZYME	0	0
PROSTAGLANDIN E	N.D.	N.D.

Tissue Activity in Units/mg wet weight (see Methods).
Values shown represent means ±1 SD (n=4 dogs for DNA, duplicates of 2 samples from each of 2 dogs for others).
N.D. Not Determined
+Hydroxyproline content determined on insoluble residue after solubilization in DMEM and TCA/DNA extraction profile.

uble residues indicated a large proportion of collagen, but sample variation in the quantity of this residue makes comparisons between relative collagen content of healthy and diseased tissues difficult. However, the insoluble nature of this residue may help to explain the decrease in hydroxyproline release into culture media towards the end of the culture period.

The higher DNA content of the diseased tissue almost certainly reflects the hyperplasia and granuloma of inflammed gingiva. The range of variability of the quantity of normal stromal components in diseased tissue precludes any quantitative correlations that might arise from this dat

Significant levels of Cathepsin B-like and elastase-like activities were found in tissue homogenates, but no activities were found for collagenase, neutral proteinase, gelatinase or plasminogen activator (Table II). This probably reflects either the presence of tissue- or serum derived inhibitors in the tissue or the fact that these enzymes were not present at the time of sampling. The measurement of cathepsin B-like and elastase activities in the homogenized extracts would suggest a large intracellular reserve of these enzymes in contrast to the 'cytosolic' proteinases. Homogenized extracts of tissue explants after 9 days in culture were treated with previously published dissociative methods (3M KCN, 2M NaCl, or 60^{o}C in the presence of 100 mM Ca^{++}; Uitto, Golub and Woessner, respectively) to determine whether proteinase activity was associated

or bound to the residual stroma. No significant activities were found for collagenase, neutral proteinase, gelatinase, or plasminogen activator, suggesting that tissue-bound enzymes contribute relatively little to the total enzyme produced in vitro.

A comparison of hydroxyproline released by diseased and healthy explants is shown in Figure 7a. Significantly more hydroxyproline is released by the diseased tissue, and collagenase levels (Figure 7b) show good correlation with this elevated hydroxyproline. One apparent discrepancy is evident on Day 2 levels, and could arise from different ratios of latent:active enzyme in healthy and diseased tissues. Similar observations of higher levels of neutral proteinase activities in diseased tissues are shown in Figure 8a. Although the profile is different to that for collagenase and active enzyme appears on the first day of culture, the same level of enzyme activity is maintained throughout the culture period. As this is a rather non-specific assay it is probably unable to distinguish between proteinases arising from dissection trauma and those elaborated either de novo or from storage release.

β-glucuronidase assayed in culture media was used as an indication for non-specific lysosomal enzyme release by the cultures (Figure 8b). Diseased tissues released higher amounts than healthy tissue explants, but the profile is similar to the LDH profile shown in Figure 3 for the healthy tissues. This possibly represents a more sensitive

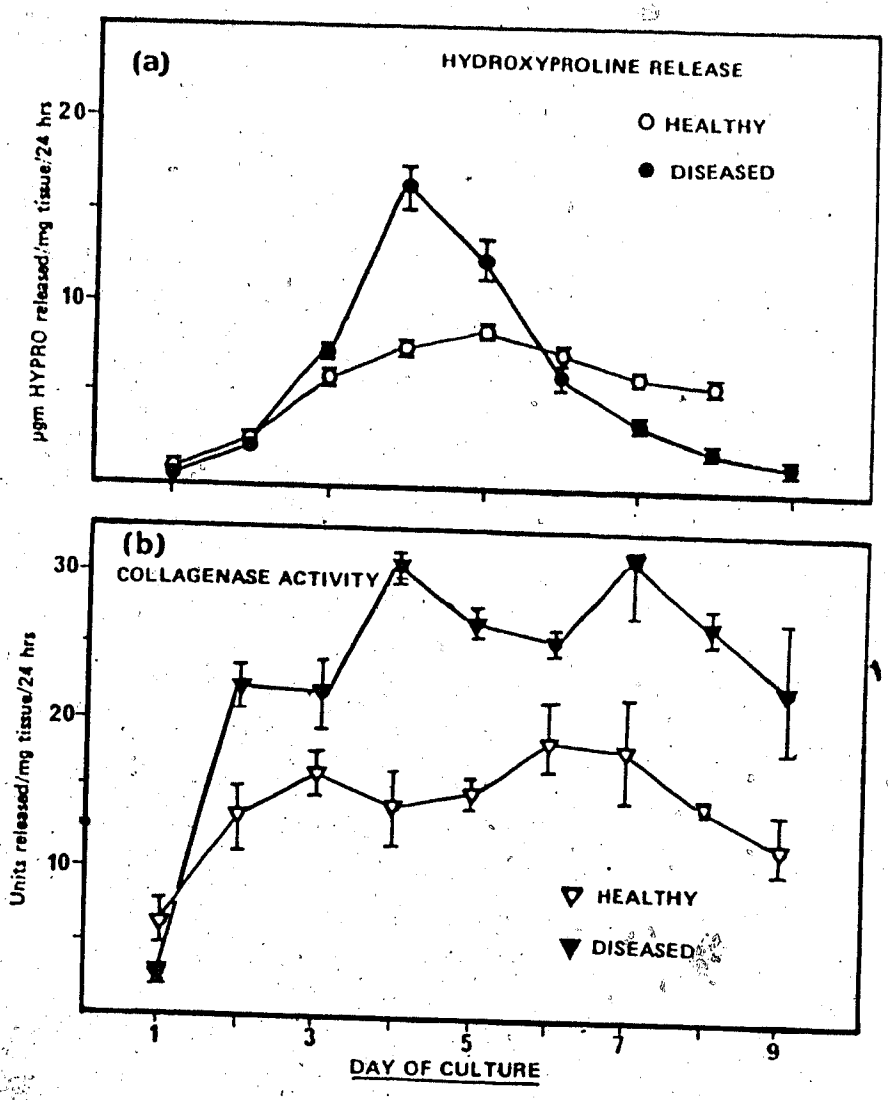


FIGURE 7. Collagenolysis in healthy and diseased gingival explant cultures.
(a)Hydroxyproline released into culture media by healthy (O) and diseased (●) explants. Data points represent means values (± range) for duplicate assays on 3 tissue culture flasks from one animal.
(b) Total collagenase activity (active + latent) elaborated by healthy (▽) and diseased (▼) tissues in same culture flasks used for hydroxyproline determination in (a). Data points represent mean values ±1 SD of duplicate assays for 3 culture flasks of tissue from one dog.

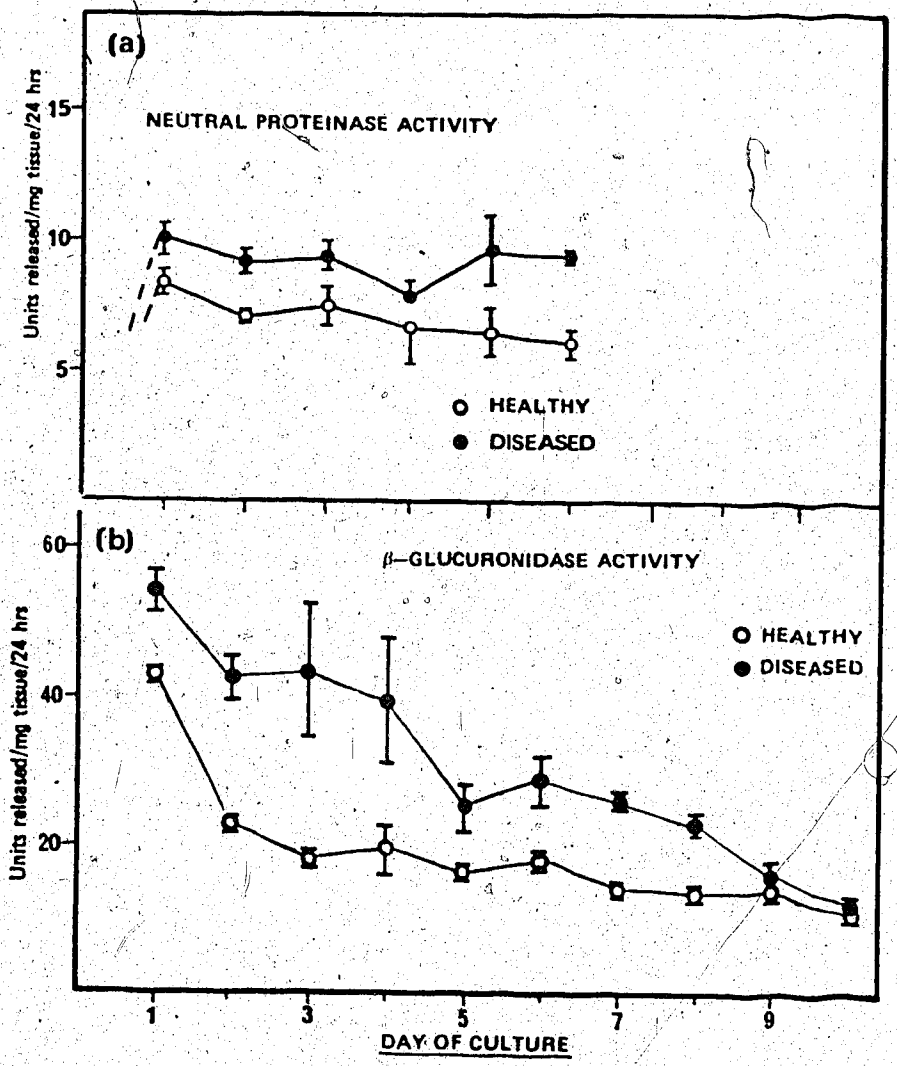


FIGURE 8. Release of neutral proteinase and β-glucuronidase from healthy and diseased gingival tissue cultures.
(a) Non-specific neutral proteinase activity from healthy (O) and diseased (●) cultures using ^{3}H-hemoglobin as substrate. Data represents mean values ±1 SD obtained from duplicate assays of 3 culture flasks from 1 animal.
(b) β-glucuronidase activity as a marker for non-specific lysosomal enzyme release. Maximal detectable β-glucuronidase activity in either healthy explant media (O) or diseased explant media (●) was equivalent to approximately 1.5% of the activity obtained from 1 mg of explant tissue homogenate. Data represents mean values ±1 SD of duplicate assays of two culture flasks from each of 4 dogs.

cell population in the diseased tissues, but when levels in culture media are compared with tissue content, no more than 2% of the enzyme available is released thoughout the entire culture period.

Comparative profiles of plasminogen activator-like activities released by healthy and diseased explant cultures are shown in Figure 9a. The profiles of this enzyme were similar in all animals, with peak values appearing about the same time between days 2-4. This was different from the other proteinases measured. The specific activities for healthy explants were usually about half that for diseased tissues but the profiles were similar in all cultures. Cathepsin B-like activities released into culture media are shown in Figure 9(b), and did not differ between healthy and diseased tissue cultures. However, these activities were insignificant when compared to the levels found in whole tissue homogenates.

Prostaglandin E levels released by explants (Figure 10) were elevated in diseased tissues. The method of expressing data as log percent ratio is convenient as the majority of PGE_2 is secreted in the first day in healthy tissues. Data is normalized to maximal amounts released by the diseased tissues (taken as $\log_{10} 100\ \% = 2$) on day 1 of culture, but a more accurate representation of the data is shown in Table III.

Cumulative amounts of all the biochemical and enzyme activity profiles measured are shown in Table III. Peak and

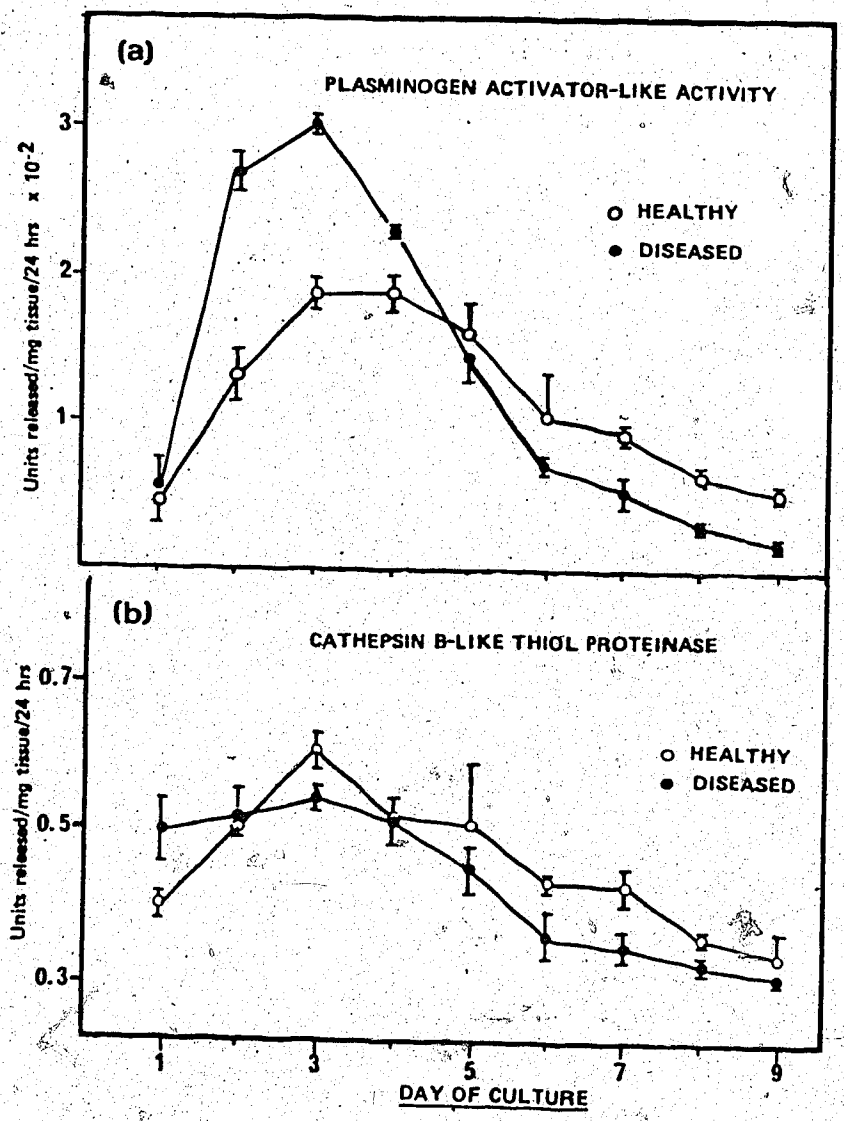


FIGURE 9. Release of plasminogen activator-like and Cathepsin B-like proteinase activities in vitro by healthy and diseased tissue explants.
(a) Plasminogen activator-like activity in healthy (O) and diseased(●) tissue explants. Data points represent mean values ±1 SD obtained from duplicate assays of 3 culture flasks of each type of tissue from each of two animals. Neglible PAL activity was found in tissue homogenates.
(b) Cathepsin B-like thiol proteinase levels; data represents mean values ±1 SD of cultures used in (a). Both healthy(O) and diseased(●) culture medium levels of Cathepsin B-like proteinase were equivalent to ~0.5% of the total activity found in tissue homogenates.

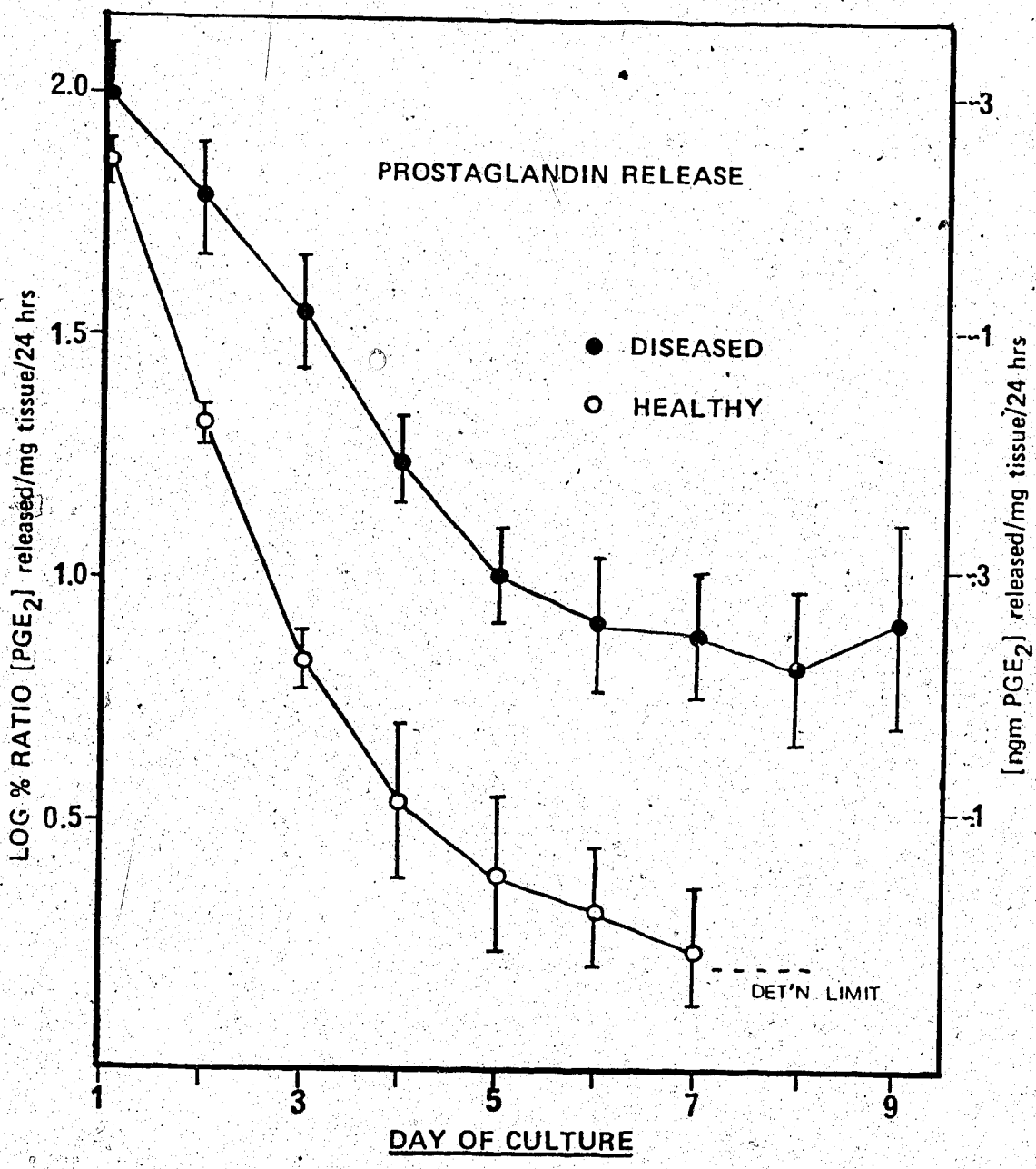


FIGURE 10. Release of Prostaglandin E from healthy and diseased gingivae in tissue culture. Concentrations of PGE_2 found in healthy(○) and diseased (●)culture media were normalized to Day 1 of diseased explant culture and converted to $\log_{10}$ (%released). Data points represent mean values ±1 SD of $\log_{10}$(PGE concentrations) determined in duplicate assays of three culture flasks from gingival tissues of one animal. Right ordinate indicates concentrations of PGE_2 equivalent to $\log_{10}$ (% ratio) values on left ordinate.

TABLE III: MEASUREMENT OF ENZYMES, HYDROXYPROLINE AND PROSTAGLANDIN E IN EXPLANT CULTURE MEDIA

(PEAK DAILY LEVELS AND TOTAL AMOUNTS RELEASED)

ASSAY	PEAK VALUE (Units/mg/day)		TOTAL RELEASED* (Units/mg/9 days)		TOTAL RELEASED AS % TISSUE CONTENT+
	HEALTHY	DISEASED	HEALTHY	DISEASED	
HYDROXYPROLINE (ug/mg/day)	8.5	17	45.5	54	7.6-11.4
PROSTAGLANDIN E (ng/mg/day)	1.9	3.5	3.5	8.12	‡Indeterminate
LACTATE DEHYDROGENASE	15	15	52	52	2.4
COLLAGENASE (ACTIVE)	15	30	129	208	Indeterminate
PLASMINOGEN ACTIVATOR-LIKE	180	300	1015	1175	Indeterminate
NEUTRAL PROTEINASE	8	10	39	54	Indeterminate
GELATINASE	23	29	170	210	Indeterminate
CATHEPSIN B	0.5	0.7	4.01	3.81	2.8-4.0
β-GLUCUR-ONIDASE	45	55	200	321	5.7-9.2

* Total amounts released in 9 days; see appropriate figures.
‡Indeterminate: no activity found in tissue homogenates for comparison.
\+ Total content of tissue homogenate/mg at time of excision.

accumulated values shown in this table are calculated from the profiles shown previously, and provide relative or comparative data rather than precise quantitation. In the last column of Table III are shown the relative amounts of enzymes released as a proportion of total enzyme content of tissue homogenates. LDH, Cathepsin B-like proteinase and β-glucuronidase activities elaborated in culture are low in comparison with the other proteinases. The term 'indeterminant' is used as no activities for these other proteinases were found in tissues, yet significant quantities were elaborated in culture. No activities were found for lysozyme or elastase in any of the culture media, and are omitted from Table III for clarity.

Quantitative comparisons between the diseased and healthy tissues presented in Table II (pg 51) are supported by histological observations of the tissues. Plate I (pg 77) shows gross comparative differences between healthy (a) and diseased (b) gingiva and demonstrates the increase in both cell numbers and specific types which has been well documented histomorphometrically. Plate III shows a higher microscopic magnification of healthy junction epithelium/stromal elements (a) and diseased inflammatory lesion/stromal interface (b). A later section in this chapter will detail subsequent identification of cell types found both in healthy and diseased tissues.

C. Effect of Dental Plaque on cell cultures.

As bacterial plaque plays an important [illegible] in the

pathogenesis of gingivitis and periodontitis *in vivo*, its effects on proteinase activities and other parameters in this model were examined to see if there was a direct effect on collagenolysis. Previous experiments done in this laboratory demonstrated an enhancement of collagenolysis as judged by increased hydroxyproline and collagenase released over a 5 day culture period of gingival tissue incubated with irradiated dental plaque (Figure 11,). The increase in collagenolysis was apparently proportional to the amount of dental plaque added to the media, with maximal enzyme and hydroxyproline release resulting from the plaque supplement being added at 100 µg/ml culture medium. The method of data presentation in these experiments shown in Figure 11 were again not comparable between cultures and animals as no standardization of calculations were employed or available. These experiments, however, provided the initial evidence that dental plaque somehow influenced the breakdown of gingival stroma, and established a working model to further investigate the phenomena observed in healthy and diseased tissues reported in Section B of these results.

Viability.

As certain components of bacterial plaque are cytotoxic even at low concentrations, culture viability was monitored in both healthy and diseased explants when exposed to plaque at 100 µg/ml, the concentration which provided maximal enzyme activity increase in Figure 11. Glucose utilization was increased slightly in cultures of both healthy and

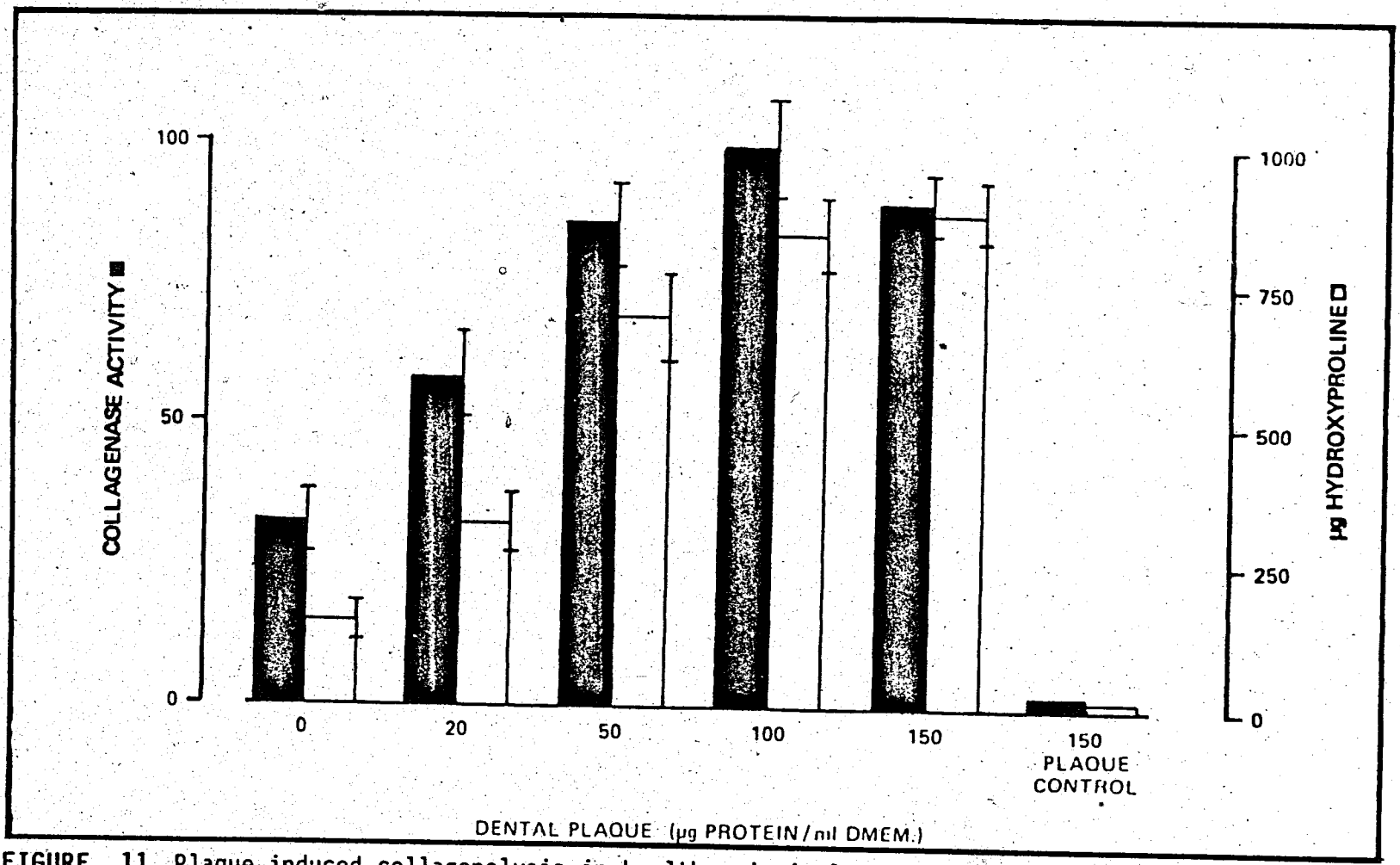


FIGURE 11. Plaque-induced collagenolysis in healthy gingival tissues. Cumulative values for collagenase activity (■, as % of maximal stimulation) and total released hydroxyproline(□) during 5 days of culture with increasing concentrations of irradiated dental plaque. Data points represent mean values ±1 SD of duplicate assays of culture flasks from two parallel experiments. Data not normalized to wet weights.

diseased explants exposed to plaque (Figure 12a). The plaque supplemented cultures showed increased utilization rates on day 5 but this is probably due to slight bacterial contamination rather than a direct effect on the metabolic rates of any of the cells in the cultured explants. No changes in pH of culture media (pH 7.8-7.9) were observed throughout the incubation.

Control plaque solution at 100 µg/ml in DMEM was incubated at 37°C daily to monitor potential proteinases and other enzymes. No activity was detected for most of the enzymes, except for traces of lysozyme and LDH.

Lactate dehydrogenase release was enhanced in plaque treated diseased explants, but no difference was observed for the healthy tissues. When compared with the total amount of LDH available in the explants (Figure 12b and Table II), only a small proportion (less than 2 %) of this enzyme was released into the media, essentially all on day 1 of the culture period. This probably represents the recovery of the tissue explants following excision.

Histological examination of tissues exposed to plaque support the biochemical evidence for lack of plaque-induced cytotoxicity (Plate IV). Obvious alterations in tissue morphology are evident by day 9 of the culture period.

Comparisons between hydroxyproline release in control and plaque-challenged cultures are shown in Figure 13 (a&b). Plaque added to both healthy and diseased explants caused an enhanced hydroxyproline release in both cases, but no change

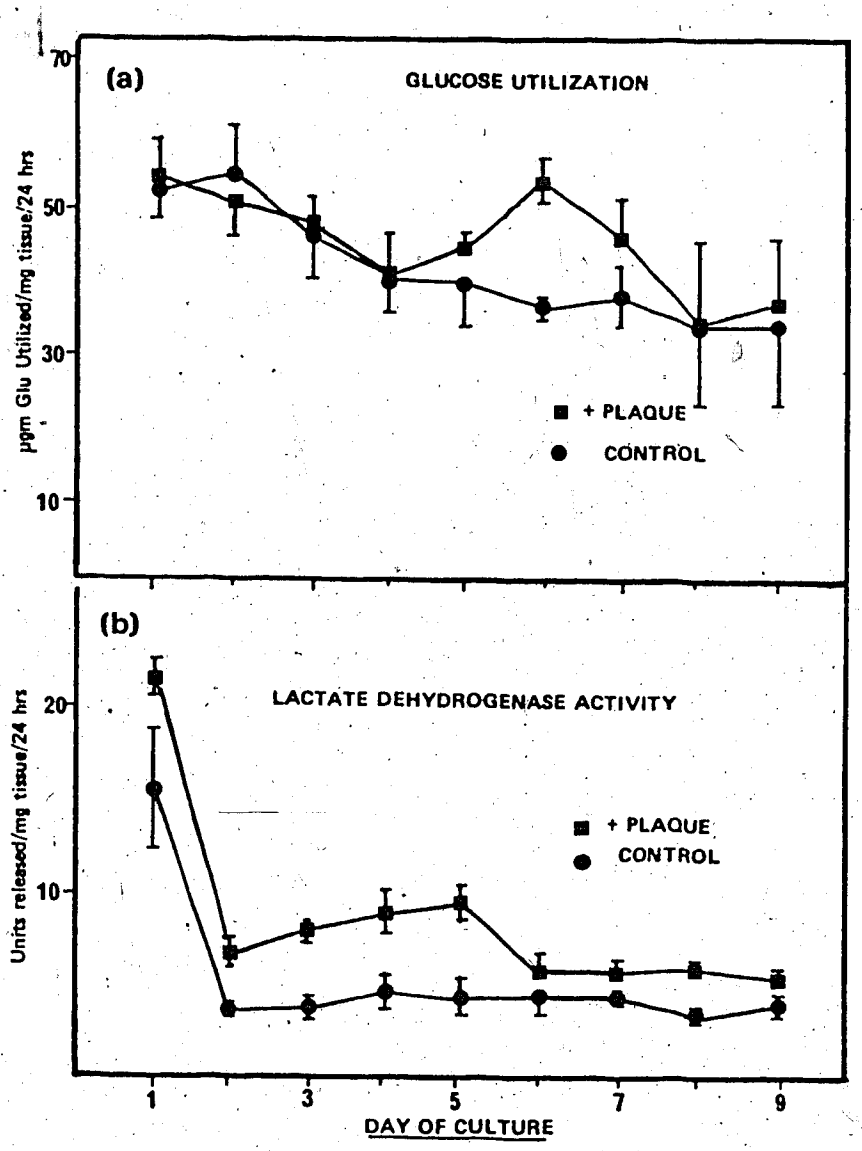


FIGURE 12. Assessment of cell viability in presence of plaque.
(a) Glucose utilization by diseased explants (●) and those with plaque added to the cultures at 100 µg/ml (■). Data represents mean values ±1 SD obtained from duplicate assays of 3 culture flasks from each of two dogs.
(b) Lactate dehydrogenase activity in media from diseased explant cultures. Values ±1 SD were obtained from duplicate assays of 3 flasks without plaque (●) and 2 flasks supplemented with plaque (■) at 100 µg/ml. Less than 2% of total available LDH activity in tissue homogenate was found in explant media on day 1 of all cultures.

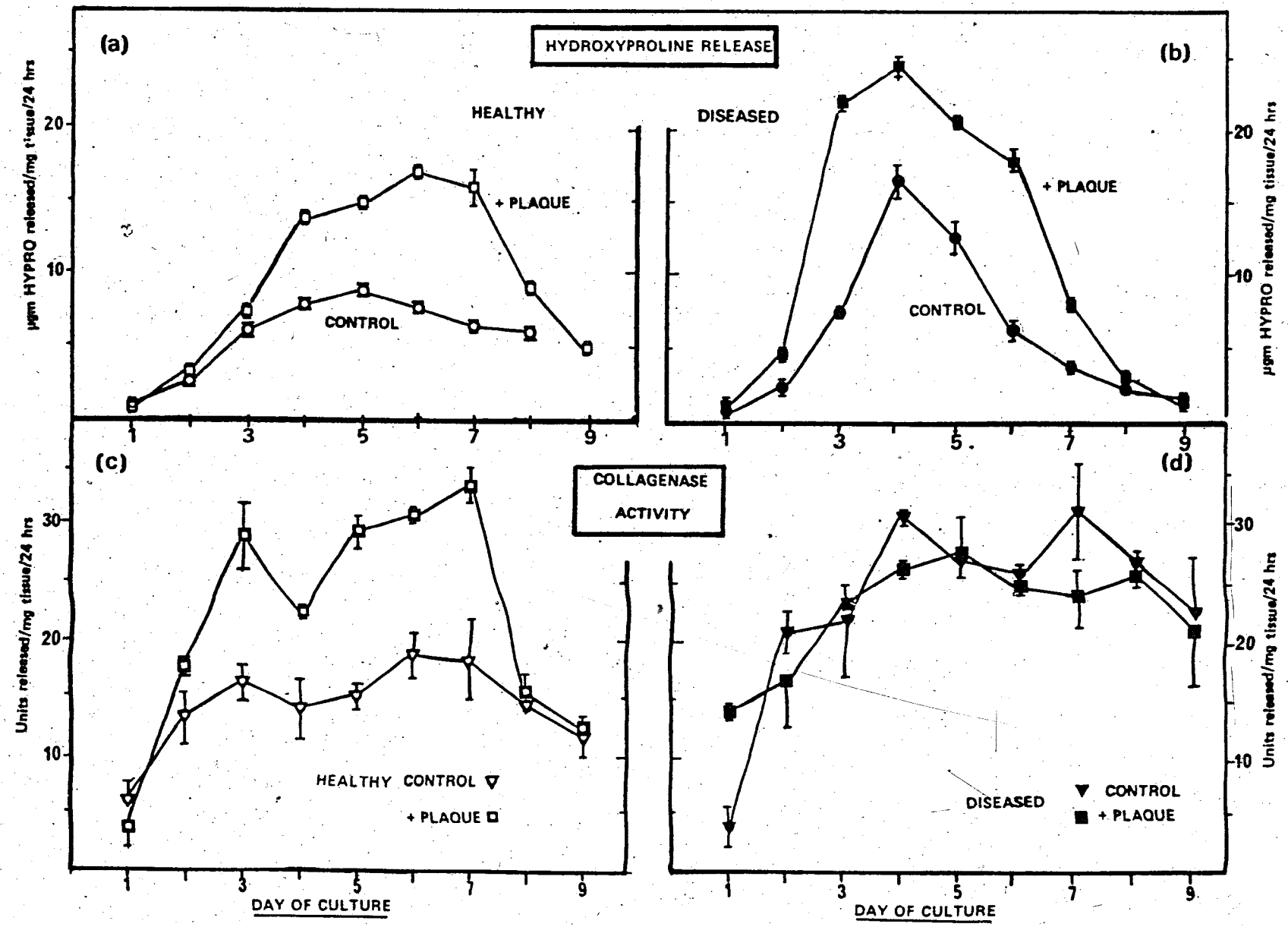

(a)
HYDROXYPROLINE RELEASE
(b)
HEALTHY
DISEASED
+ PLAQUE
CONTROL
µgm HYPRO released/mg tissue/24 hrs
(c)
COLLAGENASE ACTIVITY
(d)
HEALTHY CONTROL
+ PLAQUE
DISEASED
CONTROL
Units released/mg tissue/24 hrs
DAY OF CULTURE

FIGURE 13. Effect of dental plaque on hydroxyproline release and collagenase activity in cultures of healthy and diseased gingiva tissues.

All plaque-supplemented explants were exposed to 100 µgm of irradiated plaque protein/ml of culture medium which contained no detectable hydroxyproline or collagenase activity.

(a) Hydroxyproline released into culture media by healthy control (○) or plaque-supplemented (□) explant tissues. Data points represent mean values obtained from duplicate assays (± range) of two culture flasks for each experiment.

(b) Hydroxyproline released into culture media by diseased control (●) or plaque-supplemented (■) diseased tissues. Data points represent mean values obtained from duplicate assays (± range) of two culture flasks for each experiment.

(c)Total collagenase activity (active and latent) secreted into the media by healthy control cultures (▽) or plaque-supplemented healthy cultures (□); Data points represent mean values ±1 SD obtained from duplicate assays of two culture flasks of each tissue.

(d)Total collagenase activity secreted into the media by diseased control explant cultures (▼) and plaque-supplemented cultures (■). Data points represent mean values ±1 SD obtained from duplicate assays of two culture flasks of each tissue.

in the time course of this release was observed. The healthy explant media profiles are paralleled by increases in collagenase. However, the enhanced collagenolysis in diseased tissues is not accompanied by increase in total collagenase and this might reflect a different susceptibility of the collagen in diseased tissues to hydrolysis by other proteinases. No change in the ratios of active enzyme present in the media to total enzyme (as measured by proteolytic activation) were observed, apart from the slight increase in latent enzyme towards the end of the culture period.

Other proteinase measurements such as gelatinase, neutral proteinase and plasminogen activator were elevated in both plaque-treated healthy and diseased cultures, although the increase was different for each of the various enzymes. Plasminogen activator from healthy tissues was enhanced to a greater degree by plaque than the response exhibited by diseased explants (Figure 14). The PAL activity released into the media by healthy tissues reached the same levels as the diseased tissues, suggesting that healthy tissue may be primed for rapid elaboration of this enzyme in response to plaque challenge.

Gelatinase activities are shown in Figure 15 for healthy and diseased explant media. Increased levels are observed after the first day in culture in the presence of plaque in both types of explants. Unlike the collagenase profile, the levels remained maximal throughout the culture period

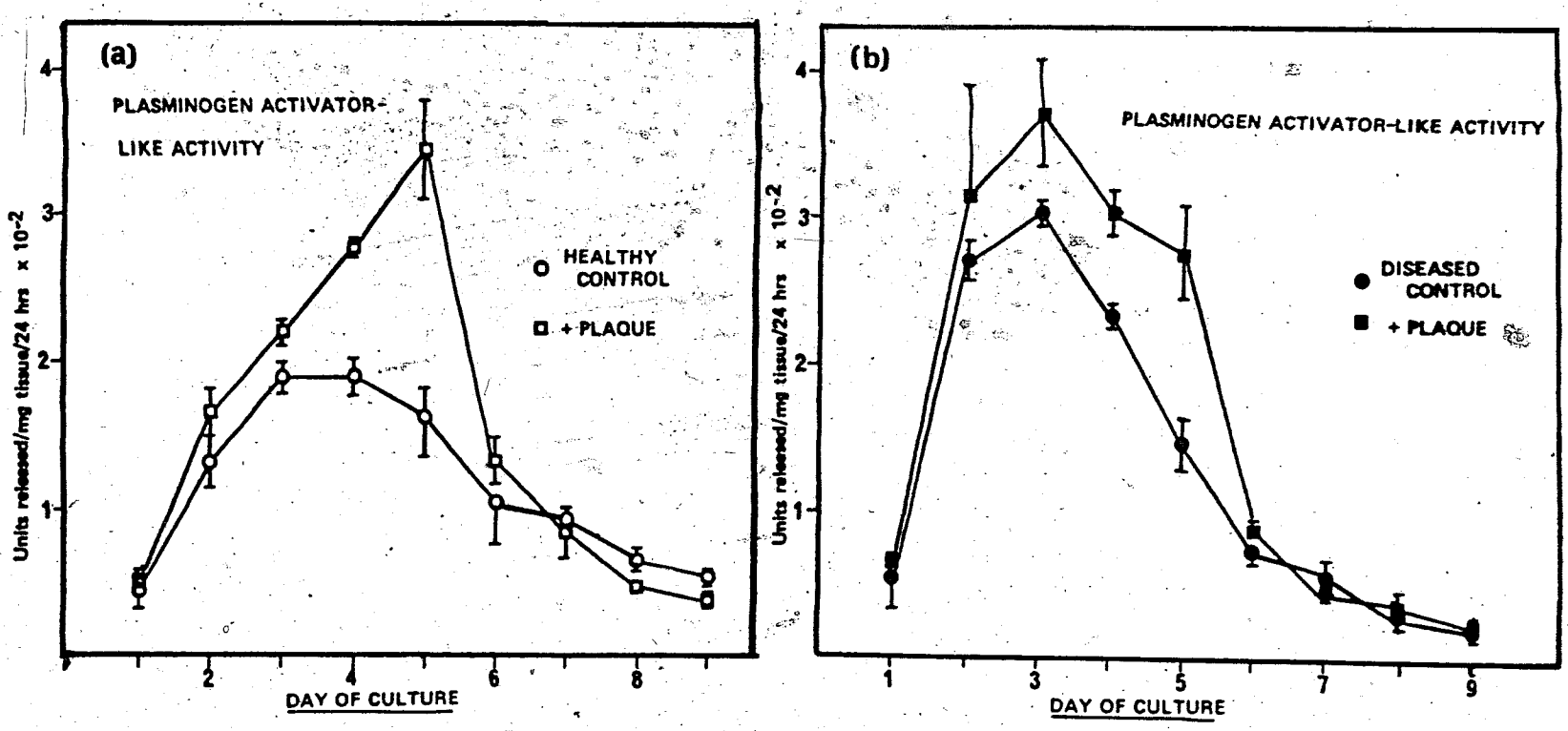


FIGURE 14. Effect of dental plaque on plasminogen activator-like (PAL) activity released from healthy and diseased gingival explants.
(a) PAL activity released into media of healthy control(○) and plaque supplemented (□) explants; (b) PAL activity released into media of diseased control (●) and plaque supplemented diseased (■) explants. Values in (a) and (b) represent mean ±1 SD of duplicate assays of 5 culture flasks of each of the healthy and diseased explants. Two flasks were plaque-supplemented in each experiment.

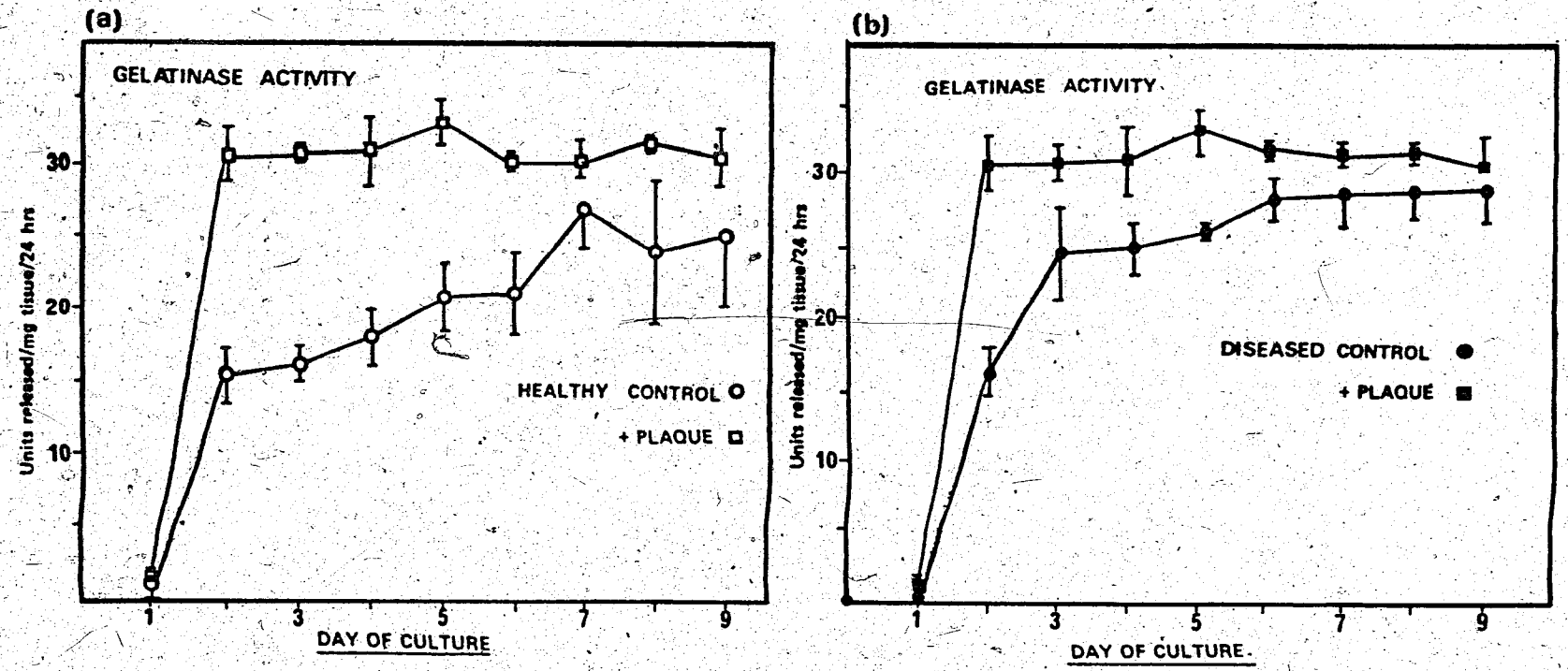


FIGURE 15. Effect of dental plaque on gingival gelatinase activity released into culture media of healthy and diseased explants.
(a) Gelatinase levels in media from healthy control (O) and plaque supplemented (□) explants.
(b) Gelatinase levels in media of diseased control (●) and plaque supplemented explants (■). Data points in (a) and (b) represent mean values ±1 SD of duplicate assays of 5 culture flasks of healthy and diseased explants. Two flasks were plaque-supplemented in each experiment.

for both tissue types and did not decrease towards the end of the incubations. Usually the ratios of active to total enzyme stayed the same or were slightly higher (i.e., more active enzyme in the cultures) in the plaque supplemented culture media.

Neutral proteinase profiles (Figure 16) of all cultures had significant activity on the first day of culture, and both healthy and diseased explants released more enzyme in response to plaque exposure. These levels remained high throughout the culture period.

Cathepsin B-like thiol proteinase levels increased in response to plaque only in the diseased tissues, possibly suggesting that different cells are responding to the plaque for the elaboration of this enzyme (Figure 17). Healthy tissues exhibited no change in levels for this proteinase. Only a small amount of the enzyme activity measured in homogenates was released into culture media, but this is not surprising as this is a lysosomal proteinase. High activities found in tissues most likely are due to the non specific nature of the substrate used, but could also arise from the instability of the enzyme at neutral pH. β-glucuronidase levels were similar for healthy and diseased tissue culture media to those shown in Figure 8. It therefore meant that plaque exposure did not enhance non-specific lysosomal release from either healthy or diseased tissues.

Prostaglandin secretion in both healthy and diseased explants was elevated with plaque exposure, although dis-

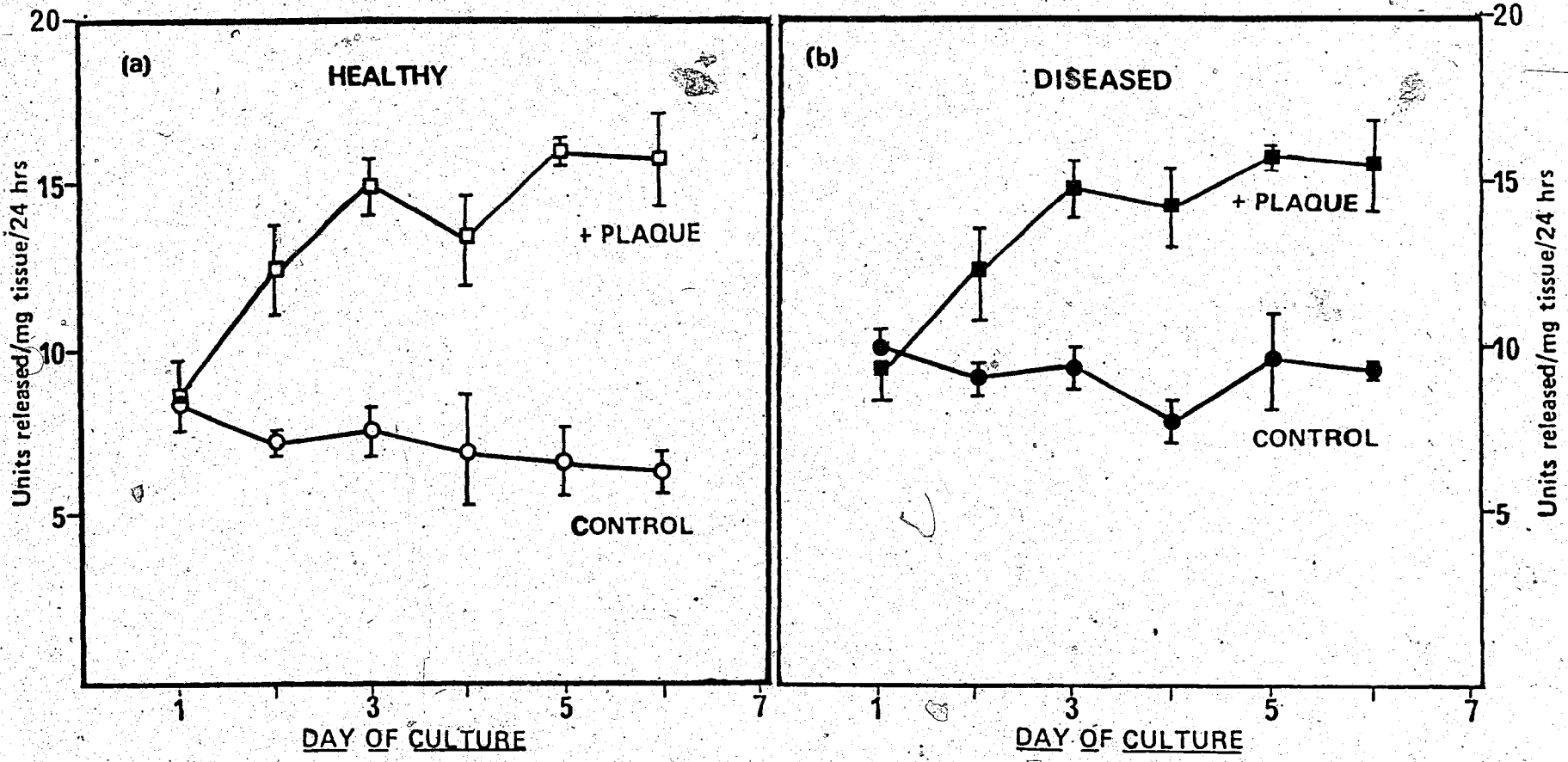


FIGURE 16. Effect of dental plaque on neutral proteinase activity release into culture media of healthy and diseased gingival explants.
(a) Neutral proteinase activity of healthy control (○) and plaque supplemented healthy explants (□). (b) Neutral proteinase released by diseased explants (●) and plaque supplemented diseased (■) explants. Data points from (a) and (b) represent mean values ±1 SD of duplicate assays of five culture flasks from each tissue type. Two cultures in each experiment were supplemented with plaque protein.

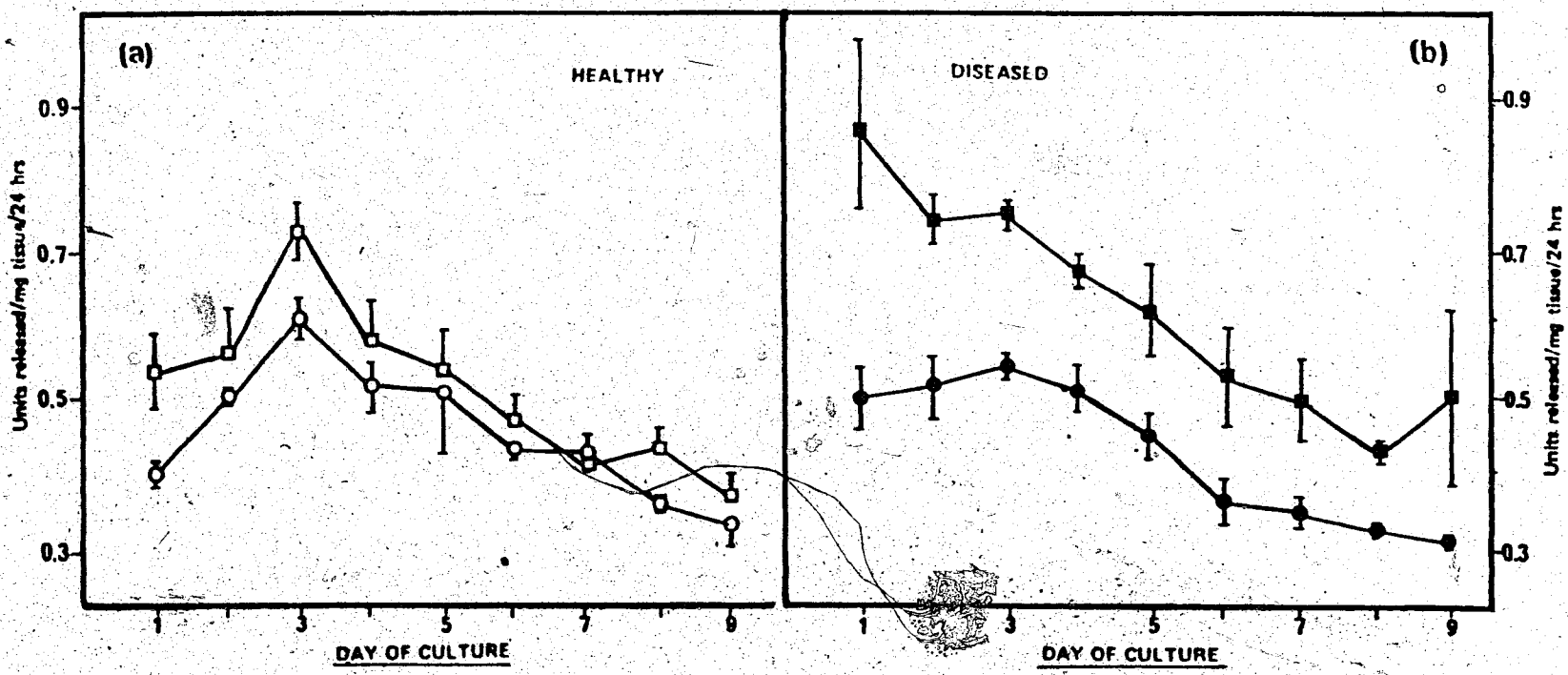


FIGURE 17. Effect of plaque on the release of Cathepsin B-like thiol proteinase activity from healthy and diseased gingival explants. Maximal levels of released enzyme activities were less than 0.3% of the total available from tissue homogenates.
(a)Release of Cathepsin B-like thiol proteinase activity into culture media by healthy control(○) or plaque supplemented healthy explants(□).
(b)Release of Cathepsin B-like thiol proteinase activity into culture media by diseased control (●) and plaque-supplemented diseased explants (■). Data points in (a) and (b) represent mean values ±1SD of duplicate assays on five culture flasks from each tissue type. Two culture flasks in each experiment were supplemented with plaque protein.

eased explant media had a much greater response than healthy (Figure 18 a&b). This may not be apparent from the differences in profiles, but the use of a logarithmic scale factor for concentrations masks the differences, notably the 3 fold increase in diseased tissue secretion of PGE_2 over the duration of the experiment. Levels of PGE_2 might have been higher if serum had been present in the media, as it is known that serum lipids provide a source of prostaglandin precursors.

Summary. A schematic representation of profiles presented in Figures 3 through 18 is shown in Figure 19. Relative activities or levels in culture media of all the parameters measured in this study are shown on the left, with the effect of plaque on peak levels found in the media indicated in the chart to the right. This data is meant to be qualitative and shows the major effects of the disease process and exposure to plaque on the proteinase physiology of the explants. The three groups of profiles shown include the major collagenolytic parameters in the top group, secondary proteinases in the middle, and related parameters in the lower panel.

There are numerous differences in the profiles indicative of various mechanisms that may be influencing the release or elaboration of each of these enzymes or markers in different ways. Observations from this *in vitro* model might be extended to the understanding of events *in vivo* by comparison of the notable differences in the healthy and

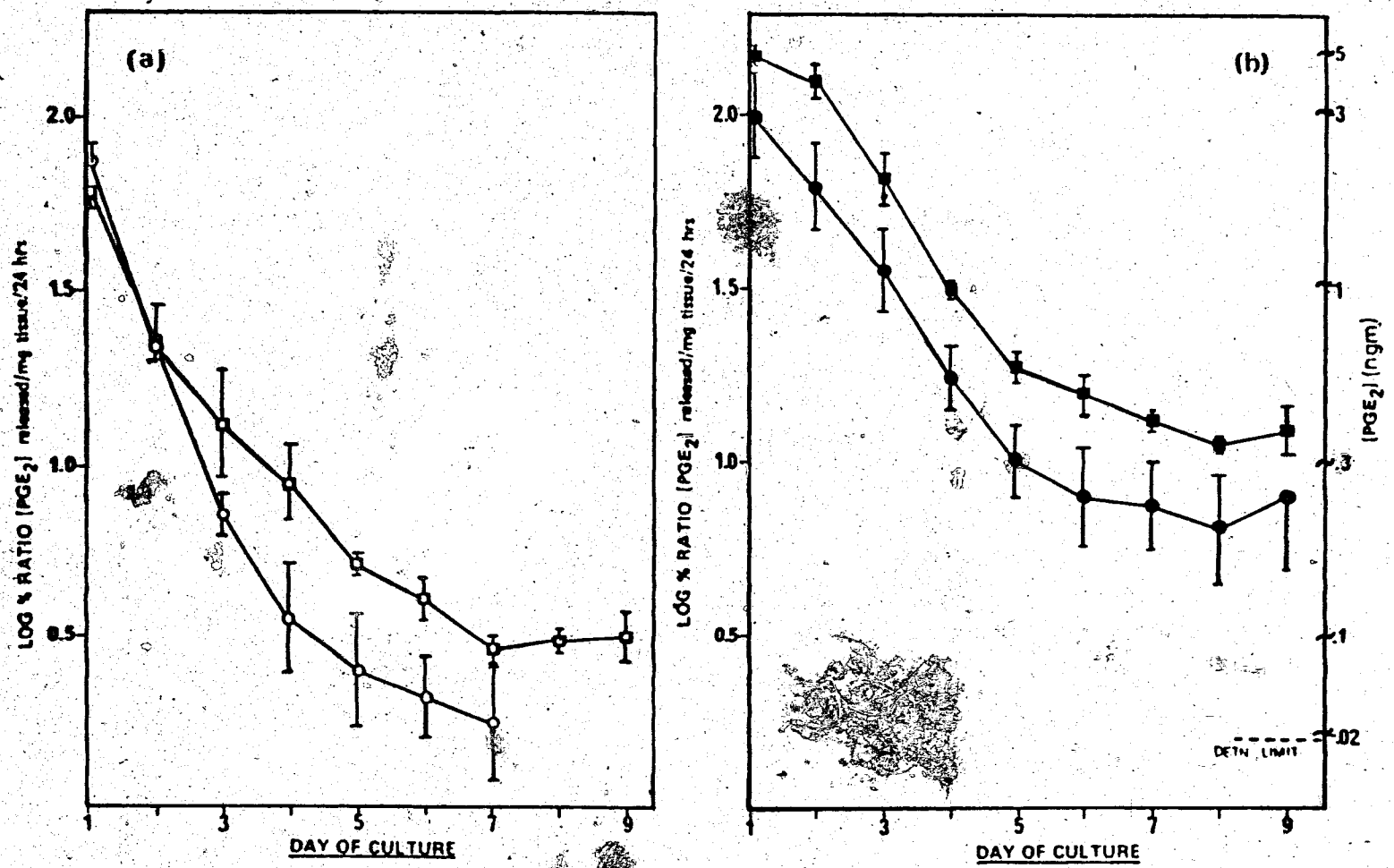
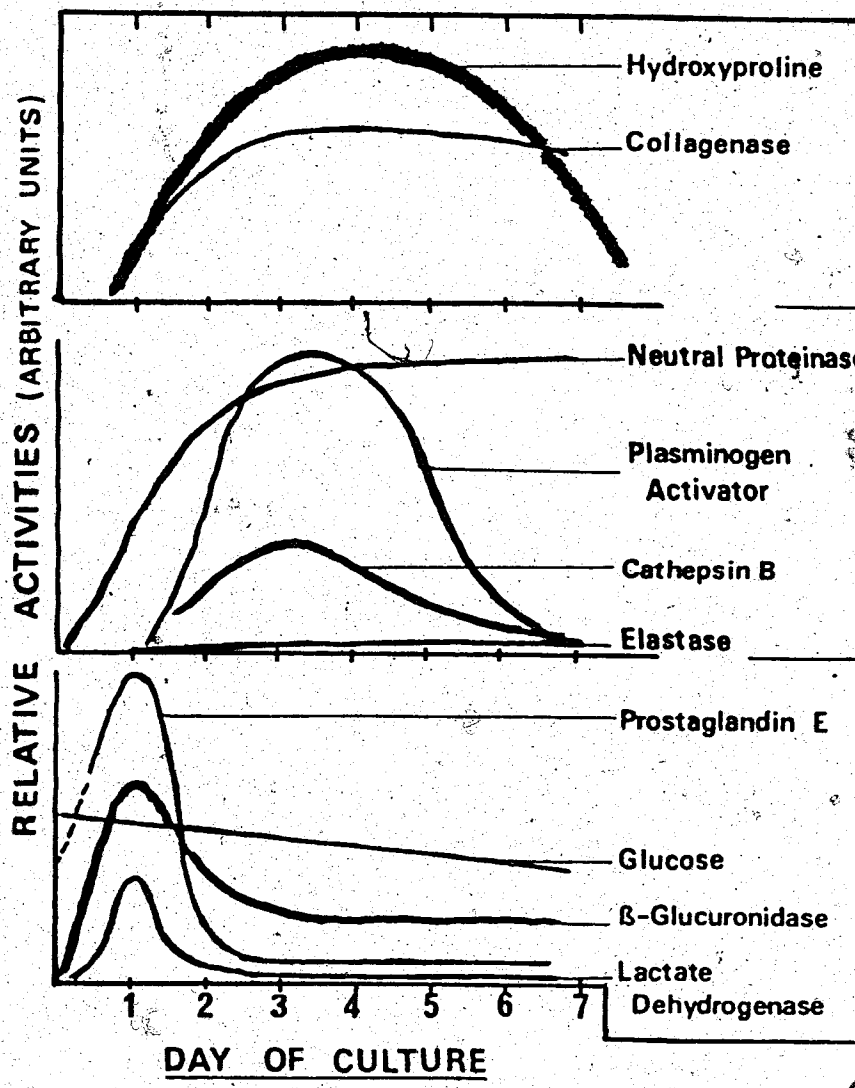


FIGURE 18. Effect of plaque on Prostaglandin E release into culture media from healthy and diseased gingival explants.
(a) Prostaglandin E levels in culture media from healthy gingival tissue (○) and from similiar tissue exposed to plaque (100 µg/ml) (□).
(b) Prostaglandin E levels in culture media from diseased gingival tissue (●) and from similiar tissue exposed to plaque (100 µg/ml)(■). Prostaglandin levels are presentedas $\log_{10}$ (% ratio) normalized to Day 1 of diseased culture explant media. Equivalent concentrations of prostaglandins are indicated on the right. Mean values ±1 SD were obtained from duplicate assays on 5 culture flasks of healthy and diseased tissues, 2 flasks from each being plaque-supplemented.



	HEALTHY		DISEASED	
	Control	+Plaque	Control	+Plaque
Hydroxyproline	(1)	2	2	3
Collagenase	(1)	2	1.5	2
Neutral Proteinase	(1)	2	1+	2
Plasminogen Activator	(1)	2	2	2
Cathepsin B	(1)	1+	1	2
Elastase	(1)	1	1	1
Prostaglandin E	(1)	1	2	3
Glucose	(1)	1	1	1
ß-Glucuronidase	(1)	1+	1+	1.5
Lactate Dehydrogenase	(1)	1+	1	1+

Relative Peak Values in Media

FIGURE 19. Schematic representation of changes in profiles of biochemical parameters measured in gingival explant media. Relative peak levels found in healthy, diseased or plaque supplemented cultures of either are indicated on the chart to the right.

diseased tissues. Some of the profiles are clearly a result of the insult to the tissue though excision and dissection and others due principally to cells not surviving under the culture conditions. Some of the enzyme profiles may be influenced by the absence of serum or the presence of tissue inhibitors, whilst others may show strong interactions with substrates and remain bound to the tissue matrix.

Some of the cells which may be responsible for producing the various enzymes and collagenolysis *in vitro* are shown in Plate V. These cells are labelled on the plate and comprise PMN's, fibroblasts, epithelial cells and inflammatory cells. Also shown are specific staining methods for mast cells (metachromasia for Toluidine blue) in Plate V(c) and napthol chloro-acetate esterase activity specific for PMN's in Plate V(d).

PLATE I a

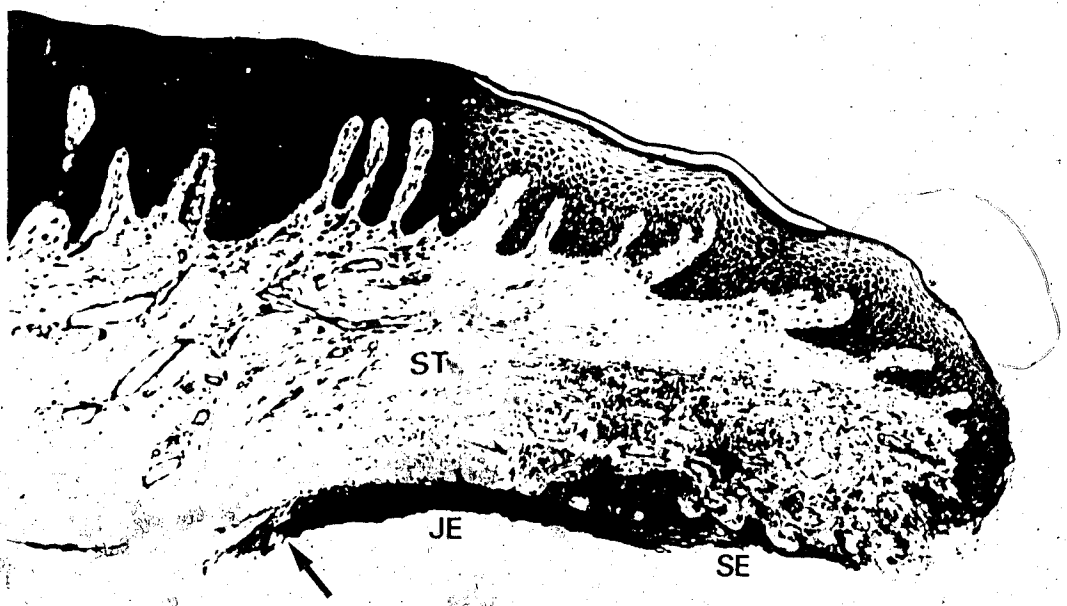


PLATE I b

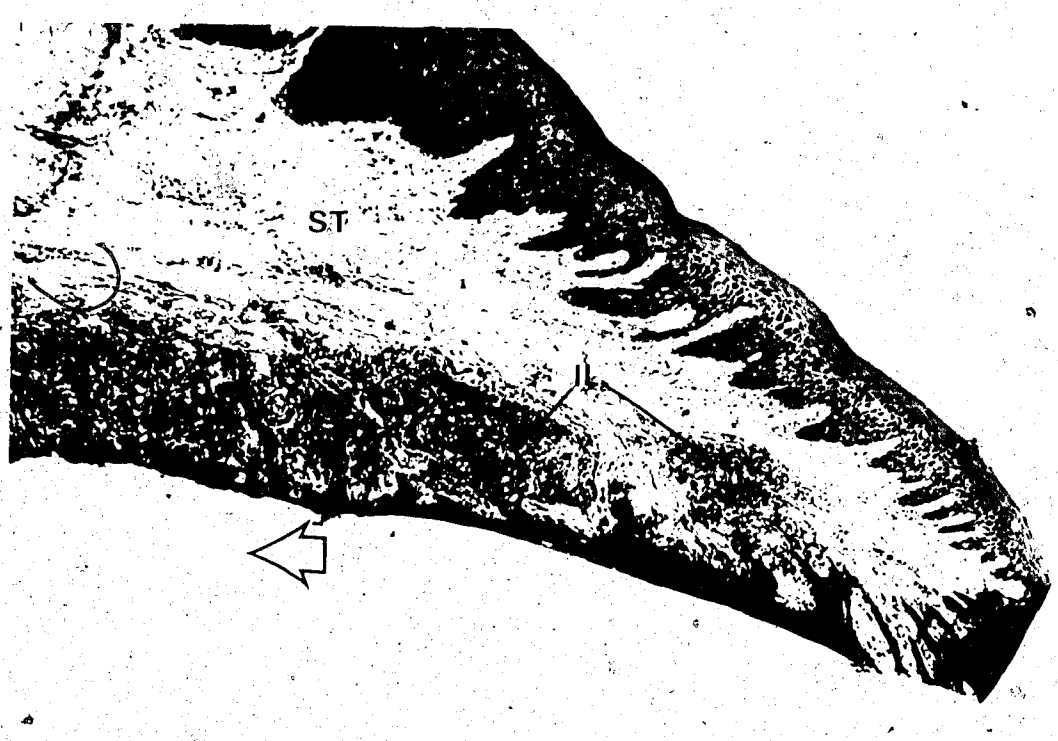


Plate Ia. Normal gingival tissue, showing Junctional (JE), sulcular (SE), and oral epithelium (OE), stromal matrix (ST). Arrow indicates point of attachment to cementum.
Toluidine blue staining, mag X 80

Plate Ib. Diseased beagle dog gingival tissue, showing inflammatory lesion (IL), reduced stroma (ST) and oral epithelium (OE). Note apical extension of JE for sulcular depth (open arrow).
Toluidine blue staining, mag X 80

Plate IIa. Healthy gingival tissues fixed at time of excision, showing normal appearance of oral epithelial (EC) and stromal cells (ST). Stain toluidine blue. magx 1050

Plate IIb. Section of healthy explant tissue after 3 days in culture to show the appearance of cells in epithelial (EP) and stromal (ST) regions. Toluidine blue staining; mag x 1050

Plate IIc. Section of diseased explant tissue after 9 days in culture illustrating viable cells of the stromal (ST) and epithelial (EP) regions. Toluidine blue staining; mag x 1050

PLATE II

PLATE III a

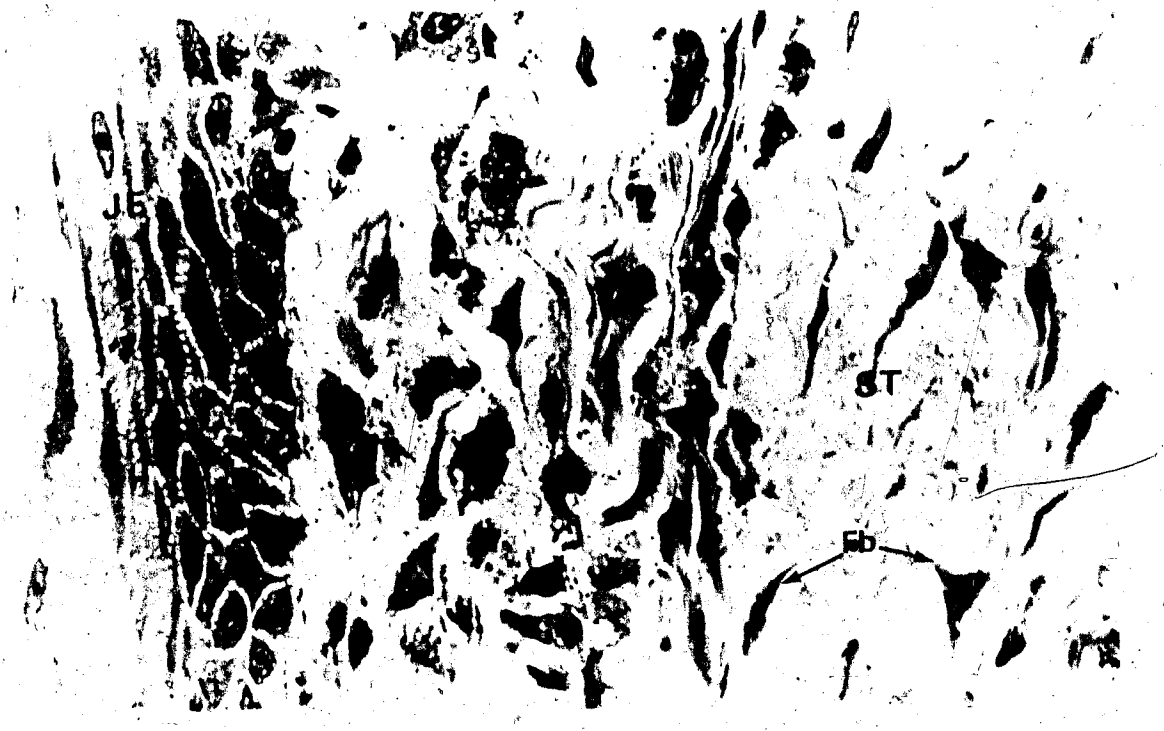


PLATE III b

Plate IIIa. Section of healthy tissue after excision showing junctionalepithelium (JE)and stromal (ST) interface. Note overgrowth of oral epithelium (OE) and characteristic appearance of fibroblasts (Fb). Toluidine blue stain. mag x 1050

Plate IIIb. Diseased tissue showing border of inflammatory lesions (IL) with stroma (ST). Note increased numbers and mixed populations of inflammatory cells. Toluidine blue stain. mag x 1050

Plate IVa. Healthy tissue explant exposed to plaque for 3 days in culture. Oral epithelial cells (OE) and gingival fibroblasts (FB) show normal appearance. Note presence of mast cell (MC) containing granules. (V, blood vessel)
Toluidine blue stain, mag x 1050.

Plate IVb. Healthy tissue exposed to plaque for 9 days in culture, showing appearance of oral epithelial (OE) and stromal cells (ST). Toluidine blue stain. mag x 1050.

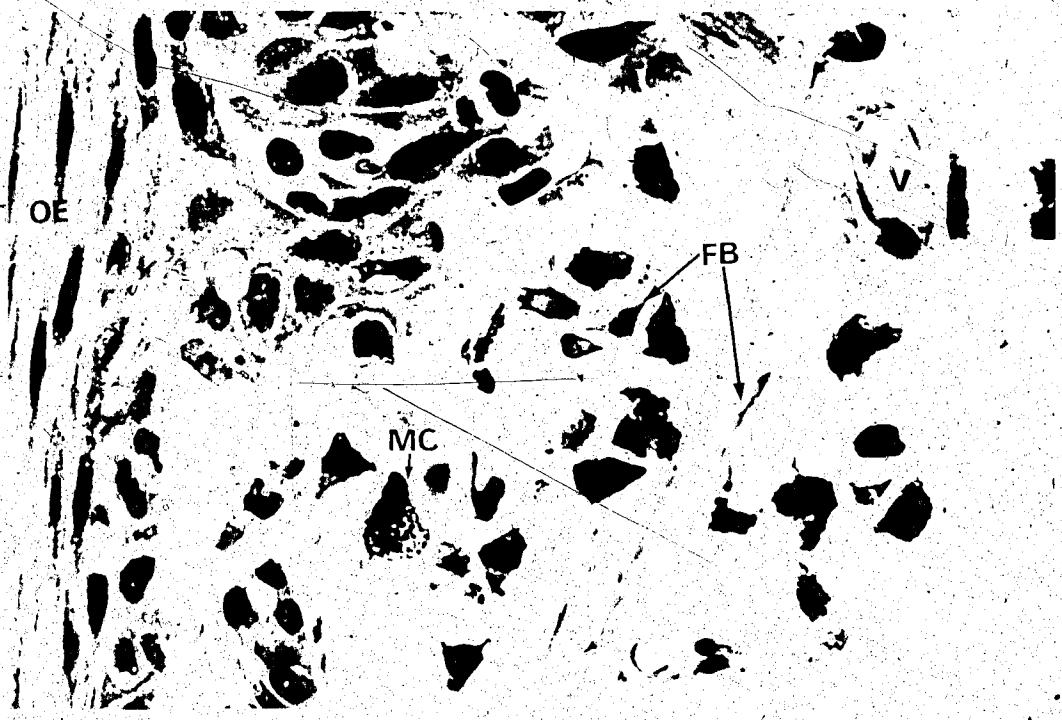


PLATE IV a

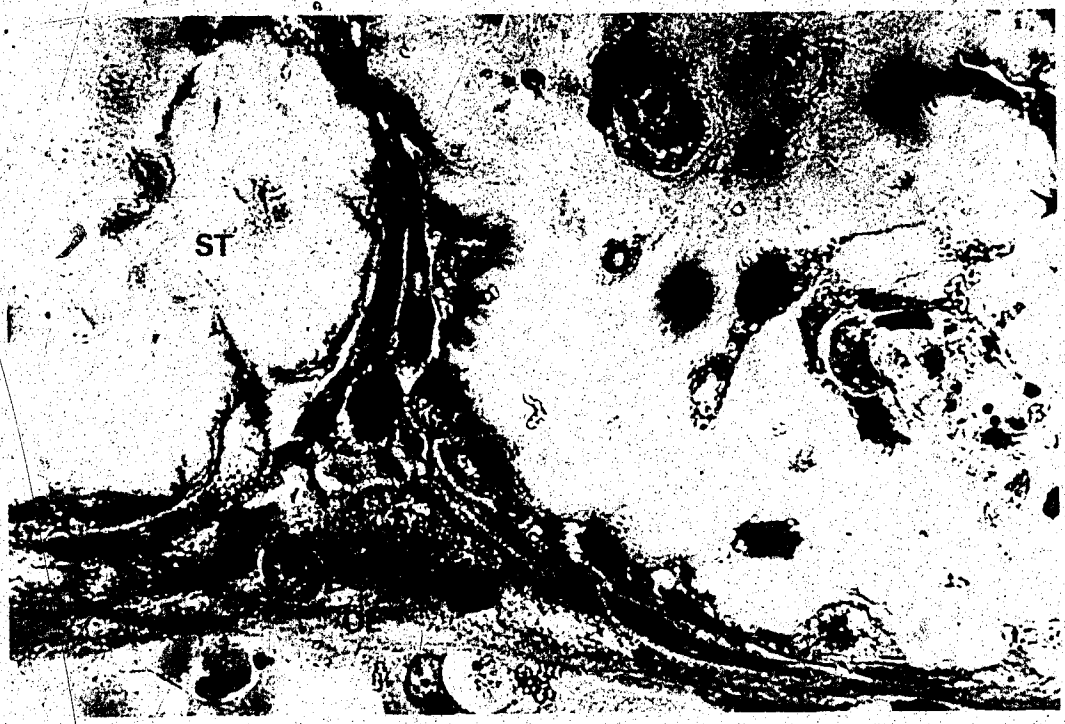


PLATE IV b

PLATE V

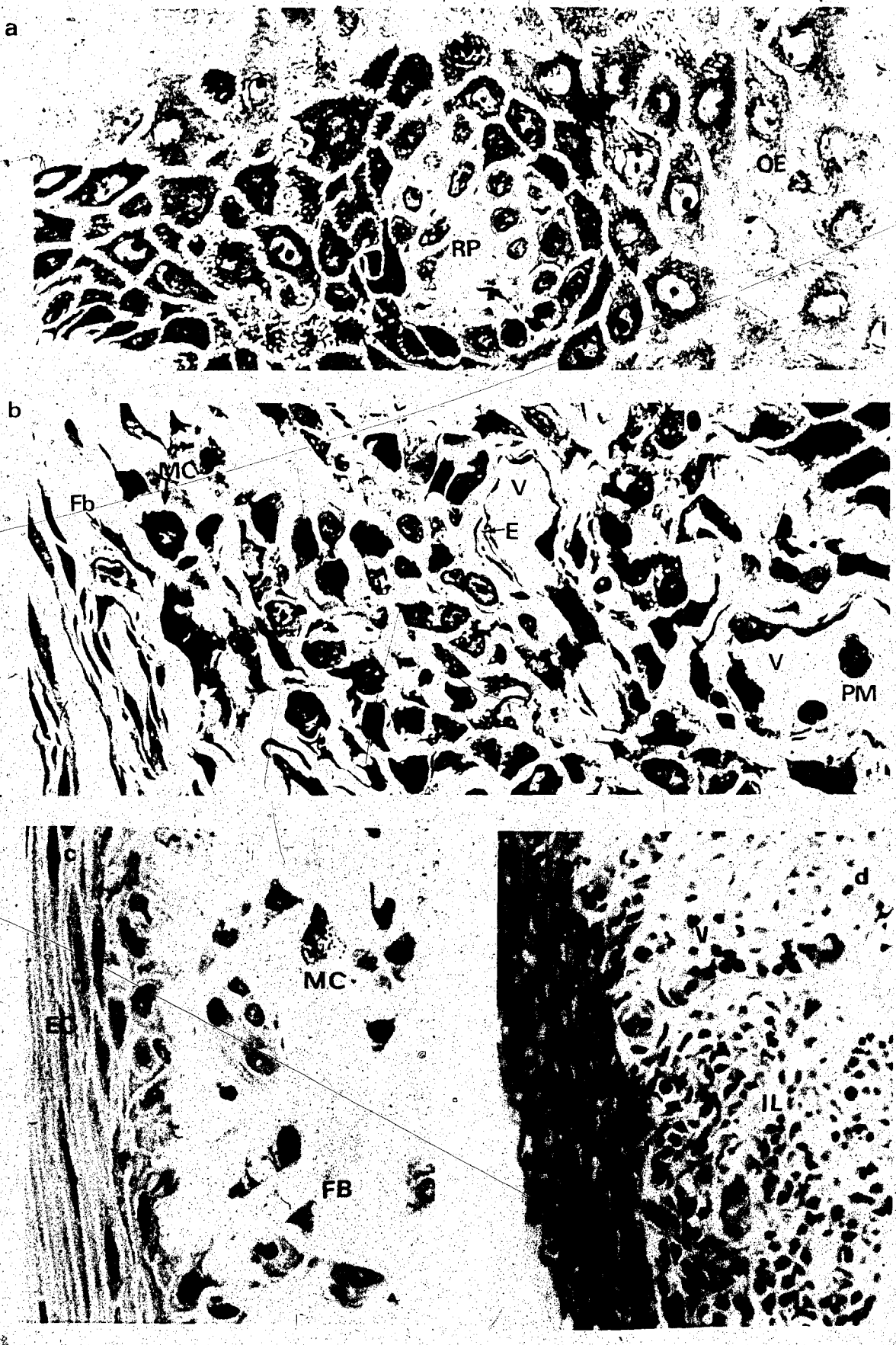


Plate Va. Section through diseased gingival explant after 3 days in culture, showing variable appearance of oral epithelium. Note difference between laminal epithelial cells (OE) and Rete peg regions (RP). Toluidine blue stain. mag x 1050

Plate Vb. Section of diseased gingival tissue showing inflammatory lesion with variable cell types. Note increased vascularity (V), endothelial cells (E), Mast cell (MC), polymorphonuclear leukocytes (PMN) and fibroblasts (Fb). Toluidine blue stain. mag x 1050

Plate Vc. Specific staining of a mast cell from healthy gingival tissue after 3 days in culture. Note metachromatic granules of mast cell (MC) stained with toluidine blue. EC, epithelial cell; FB, fibroblast
mag x 1050

Plate Vd. Section of healthy gingival tissue at time of excision, stained for naphthol-chloroacetate esterase activity. Note red stain of PMN, both in blood vessels and oral epithelium.
mag x 425

DISCUSSION

Over the last decade many investigators have attempted to study the role of proteinases in inflammatory periodontal disease (for review, see Cimasoni & Kowashi, 1980). In most cases each study has only concentrated on one or at most two enzymes (eg. Birkedal-Hansen, 1980; Geiger & Harper, 1980; Pettigrew *etal*, 1980). Such studies are somewhat limited as it is now recognized that matrix degradation probably represents a concerted action by several proteolytic enzymes (Gross, *etal*, 1980; Vaes, 1980; Barrett, 1978). The approach which forms the basis of this thesis has been an attempt to obtain an overall 'profile' of seven proteinases from cultured normal or diseased gingival tissues in order to assess the relative importance of each in matrix degradation. In addition, because diseased gingival tissue is known to contain different cell populations (eg. inflammatory cells in hyperplastic granulations), an attempt has been made to relate the various proteinase activities to altered cellular composition.

The experimental data contained in this thesis are best discussed under 3 sections, *viz*. in relation to the work of others, a critical appraisal of current methodology, and the interpretation of the results in relation to the pathophysiology of the disease.

Experimental Data:

One feature common to all the gingival explants was the release of hydroxyproline into the media during the culture period. Hydroxyproline measurement as an index of collagen degradation has been used by many researchers for the study of connective tissue breakdown *in vitro* (eg. Geiger & Harper, 1980; Golub *etal*, 1979; Woolley *etal*, 1978). The hydroxyproline profiles observed in cultures of normal or diseased tissues allow an assessment of the collagenolytic potential of each tissue. Significantly higher levels of solubilized hydroxyproline were found in diseased explant cultures, suggesting higher collagenolytic activities.

The release of hydroxyproline in culture may have several explanations, among them the possibility of newly synthesized collagen being secreted by the cultured explants. Although collagen synthesis has been shown to be elevated in diseased gingival tissues (Page & Narayanan, 1980) this can be disregarded under the culture conditions employed as the essential cofactors keto-glutarate, ascorbic acid and Fe^{++} required for collagen synthesis are absent from the culture medium (Udenfriend, 1966). As different collagen types and their respective ratios have been shown to be associated with different stages of disease (Page & Schroeder, 1981, Narayanan *etal*, 1980), the amounts of hydroxyproline released during culture may reflect the different susceptiblies of the various collagen types to enzymatic degradation. As diseased tissue specimens contain variable granulomatous content and newly synthesized collagens resulting

from repair activity, it is possible these are more vulnerable to degradation and could account for the elevated hydroxyproline values, rather than increased enzyme activity per se.

Although exhaustive characterization of the degradation products from collagenolysis was not carried out in this study, much of the solubilized hydroxyproline released into the culture medium was shown to be in diffusible form. This was not surprising in view of the relatively high levels of proteinase activity found in the media. The presence of enzymes such as gelatinase, cathepsin G and neutral proteinase would be expected to complete the degradation of the primary collagenolytic products brought about by collagenase. The increased hydroxyproline values found in media of diseased or plaque-supplemented gingival tissue cultures appears to be correlated with increased production of certain degradative enzymes such as collagenase, neutral proteinase and plasminogen activator.

Collagenase was consistently produced by cultured gingival tissues and in most cases this enzyme closely paralleled the appearance of hydroxyproline in culture media, suggesting a close relationship with collagen degradation. The increase in collagenase activity observed in cultures of diseased tissue explants is in good agreement with elevated levels of enzyme reported in crevicular fluids from diseased patients (Golub, _etal_, 1979; Kowashi, _etal_, 1979), extracts of diseased gingival tissues (Uitto & Saxen, 1978; Uitto, _etal_, 1981), and other _in vitro_ studies using explanted

gingival tissues (Birkedal-Hansen, 1980; Woolley, etal, 1978b; Geiger & Harper, 1980). Such increased collagenase levels may be explained by the contribution by inflammatory cells (Cowley, 1972; Simpson etal, 1980), by the stimulation of resident cells of the gingiva (Birkedal-Hansen, 1980; Robertson, etal, 1973; Taylor, 1971), or most likely, by a combination of both sources.

The stimulation of collagenase production from normal gingiva upon exposure to dental plaque suggests an inherent capacity of resident gingival cells to respond to plaque elements. As yet the stimulatory components have not been identified but other workers have suggested an important role for such factors as Lipid A from lipopolysaccharide (LPS), lipotechoic acids, N-formyl methionyl peptides or bacterial proteases to elicit responses in healthy gingival tissues (Wicker & Knox, 1975, Taichman etal, 1966, Rosenstreich, etal, 1977).

The neutral proteinase activities reported were obtained using ^{3}H-hemoglobin as a substrate and probably reflect a certain degree of non-specificity. The various enzymes which contribute to this activity probably include the serine and metallo-proteinases; the thiol and carboxyl proteinases would be excluded on account of their cysteine-activation or low pH requirements, respectively. Other workers have reported a similar appearance of neutral proteinases in gingival cultures from various species such as porcine (Pettigrew, etal, 1978), bovine (Birkedal-Hansen, 1978) and human (Geiger & Harper, 1980; Kowashi, etal, 1979)

gingival tissues.

Neutral proteinase activities were always found in significant quantities even after one day in culture. This was a consistent feature with levels being higher in diseased explants. Neutral proteinase usually showed a different profile to that monitored for collagenase, but the response to bacterial plaque challenge was similar for both enzymes. Neutral proteinases are probably important in the breakdown of such matrix components as proteoglycan, fibronectin and laminin of the gingival stroma (Vaes, 1980a; Kleinman, etal, 1981). Such degradative activity, by removing ground substance of the matrix, could well facilitate exposure of collagen bundles or fibrils to the lytic action of other, more specific enzymes.

Plasminogen activator levels elaborated from gingival tissue explants showed a markedly different profile from the other proteinases measured. Peak levels were observed midway through the culture periods, and fell to background levels after 5 or 6 days in culture. Higher PA levels were found in diseased explant rather than in healthy explant cultures. This may be correlated to the increased vascularization expected in diseased tissues, as endothelial cells are thought to be the principal source of PA in these tissues (Christman, etal, 1977) Hidaka, etal, (1980) reported no difference in PA activity for diseased and healthy human tissues using gingival biopsy tissue explants on fibrinogen plates, whereas different fibrinolytic activities were found for the crevicular washings taken from the same patients. It

was therefore suggested that other mechanisms of enzyme regulation existed in vivo which could not be reproduced by in vitro conditions (Hidaka, etal, 1980). Such observations are probably explained by the absence of humoural factors and serum inhibitors which would be lost after a few days in culture, and further complicated by the synthesis of specific inhibitors by gingival tissues at later stages in culture (Simpson & Mailman, 1981; Pettigrew, etal, 1980).

The response of healthy gingival explants to secrete elevated PA levels after plaque stimulation resembles that for collagenase. However, diseased tissue explants challenged with plaque did not increase PA secretion, and the enzyme levels attained by the healthy, plaque-challenged cultures approximated those in diseased explant media. Such results suggest that endothelial cells were not necessarily the primary source of enzyme in this system. Macrophages have been shown to increase their synthesis of PA when stimulated (Unkeless, etal, 1974). Fibroblasts obtained from gingival explants have been shown to produce PA using the less sensitive fibrinogen-plate assay, but this activity was found to be membrane- or cell-associated (D'Souza etal, 1981). PA activities measured in this study were restricted to culture media; little activity was found in tissue homogenates and no experiments were carried out on cell-bound enzyme.

Elastase activity was not detectable in any of the gingival explant culture media, even after prolonged periods of culture.This was surprising since polymorphonuclear leuk-

ocytes are known to produce relatively large quantities of elastase (Barrett, 1978; Baggiolini etal, 1978) and these cells are present in high numbers in diseased tissue (Page etal, 1972; Payne etal, 1975). Cimasoni, Barrett and others have shown that human PMN elastase can degrade collagen, but very low levels of this enzyme activity were found in crevicular washings of diseased patients (Cimasoni & Kowashi, 1980; Barrett, 1978; Kowashi etal, 1980). These results tend to discount the potential role postulated for elastase as an alternative collagenolytic activity, but there may be other explanations for absence of elastase in this system. Although elastase is known to attack type III collagen (Gadek etal, 1980), it does not seem likely that this enzyme could account for the changed collagen type I/III ratios in diseased gingival tissues (Page & Narayanan, 1980).

In this context, other collagen type-specific enzymes, notably the 'collagenases' for type IV and V collagen, may be important in breakdown of the stromal and epithelial matrix (Liotta, etal, 1981; Mainardi, etal, 1980a&b). Their contribution to the hydroxyproline levels measured in this in vitro system may well be limited due to the proportionately small content of these collagen types in the explanted tissues. Assays for type IV or V 'collagenases' were not attempted in this study. Such assays still require certain improvements (Garbisa, etal, 1980), but they will be useful for later studies on specific gingival cell cultures.

An assessment of lysosomal enzyme release into culture media was obtained by measuring β-glucuronidase, lysozyme

and Cathepsin B activities. The marked absence or low levels of these enzymes found primarily during the early stages of culture (probably reflecting the trauma of explantation) suggested that relatively little lysosomal enzyme activity contributed to extracellular matrix degradation. The main function of lysosomal enzymes is generally thought to be intracellular degradation, but an extracellular role resulting from cell lysis or conditions of stress cannot be discounted (Cimasoni & Kowashi, 1980). Important considerations regarding their contribution to extracellular degradation include both the stability of lysosomal enzymes and effective pH of the extracellular environment.

Cathepsin B activity in the media, arising from culture conditions or plaque challenge, may lose stability when exposed to lengthly incubations at neutral pH (Poole & Mort, 1980). The slight increase in Cathepsin B-like proteinase levels in media of diseased tissues explants exposed to plaque may be explained by the exposure of all tissue cells to a stressful insult, or by the response of a minor cell population to the plaque challenge.

Prostaglandin E levels have been found to be elevated in other inflamed tissues besides gingiva (Elattar, 1979; Loning, etal, 1980). The levels secreted from diseased gingival explants were much higher than those from healthy tissues, and plaque challenge resulted in even greater production. Prostaglandins have been shown to have an effect on the production of proteolytic enzymes, especially in inflammation, and recent evidence suggests that PGE_2 is

important in the regulation of collagenase production by macrophages (McCarthy etal, 1980) and synovial cells (Dayer etal, 1980). The high levels of PGE found in this study may be an important factor in the regulation and stimulation of enzyme production and associated tissue destruction. The role of PGE and other prostaglandins in the modulation of specific proteinase synthesis and release is poorly understood at present, and it is possible that specific cell types may react differently to each of these inflammatory mediators (Page etal, 1978a).

Attempts to extract proteinase activity directly from freshly excised gingival tissue met with limited success as activities were detected only for Cathepsin B and elastase. Extractable levels of the known lysosomal marker enzymes β-glucuronidase and lysozyme were not in agreement as no lysozyme activity could be found in the homogenates. Similarly, when explant tissues (from 9 day cultures) were extracted using dissociative procedures, no detectable levels were found for collagenase, neutral proteinase or PAL. Such findings suggest that little of the enzyme produced during culture remained confined to the tissue. Although collagenase activity has been extracted directly from diseased tissues by others, and levels were correlated with the disease process, no latent or active collagenase was isolated from extracted tissue in this study (Golub, etal, 1979; Uitto & Saxen, 1978, Uitto, etal, 1981).

Gingival tissues and cells have been shown to secrete inhibitors of collagenase and neutral proteinase in culture

(Pettigrew, etal, 1978;Simpson & Mailman, 1981). Both serum and tissue inhibitors are present at early stages of culture, but the serum components would tend to be lost within a few days. Their presence probably explains the lag phase observed for such enzyme activities as collagenase and plasminogen activator, for only when the available inhibitors are saturated with enzyme would proteinase activity manifest itself in the culture medium. The lack of a lag phase for the appearance of neutral proteinase most likely results from the non-specificity of the assay, and certain activities may not be inhibited early in the culture period. Another potential masking phenomenon involves the binding of enzyme to respective substrates such as collagen or elastin in the explanted tissue, but the dissociative extractions described above would suggest this is a minimal effect.

There are numerous excellent histomorphometric studies which describe the onset of gingivitis and its progression to peridontal disease (Loe etal, 1965; Lindhe, etal, 1973; Schroeder etal, 1973; Schroeder, 1977; Schroeder & Attstrom, 1980; Soames, etal, 1976). The histology used in this investigation was intended to assess the viability of cultured tissues and to identify specific cell types which might facilitate the interpretation of the enzyme activities produced in culture. The identification of specific cell types in the inflammatory lesion, especially during its development, helped to provide a better understanding of the distribution and involvement of cell types such as macrophages, monocytes, PMN's and mast cells. Such observations also

served to confirm the state of health or disease for each of the animals used in the study.

Appraisal of Methodology

Although the techniques used in this study have much to offer, there are certain limitations which have to be recognized. In vitro systems without serum supplement are known to restrict growth and caution has to be exercised when attempting to project the findings to in vivo conditions (Barnes & Sato, 1980). The development of enzyme assays and the interpretation of the enzyme data must take into account problems of substrate specificity and the 'proportionality' of relative sensitivities of different assay systems.

A major concern in this study was an appreciation of the dramatic physiological alterations imposed on tissues by explantation and potential necrosis. The viability of explants was monitored throughout the culture period, and other workers have observed that similar tissues so cultured retained viability over similar time periods (Birkedal-Hansen, etal, 1976; Woolley, etal, 1978b). Moreover, it has been shown that such explants retain outgrowth when serum-free cultures were eventually supplemented with serum after two weeks (Birkedal-Hansen, 1980).

The initial shock of excision and dissection on the explanted tissue probably results in immediate responses by tissue cells. The elevated LDH and β-glucuronidase levels observed on the first day of all cultures, and possibly some of the early neutral proteinase activity, probably repre-

sents cell damage and injury due to explantation. It is likely that all tissues placed in culture recognise the new environment as foreign, and without the various tissue and serum factors normally essential for homeostasis, explants may respond in a manner similar to wound healing.

The absence of serum in the culture system is an important consideration. The main reason for not adding serum is to eliminate the natural proteinase inhibitors such as alpha$_2$-macroglobulin (α_2M) and alpha$_1$-antiproteinase (α_1AT) which would mask potential enzyme activities elaborated in culture (Murphy & Sellers, 1980).

The absence of serum factors in culture are known to affect numerous cell functions (Barnes & Sato, 1980). Some of these include the lack of cell division and the prevention of lysozyme production and prostaglandin synthesis. The absence of serum can cause the alteration of the ratio of type I/III collagen synthesis (Page & Narayanan, 1980) Moreover, the long-term absence of serum often accounts for the fragile nature of certain gingival cells such as epithelial, endothelial and some inflammatory cells. Each cell type requires special growth conditions for successful culture, but the culture period used in this study is relatively short term and conditions must allow for measurement of proteolytic activity.

Some of the cells in the excised tissues usually possess a short life-span, and would normally not be expected to survive in vitro for any longer. The best example would be the polymorphonuclear leukocytes, which possess short

half lives of approximately 24-36 hours, and certain lymphocytes which often appear fragile in culture. Macrophages are also known to lose such characteristic markers as Fc receptors and lysozyme production after four days of culture (Dayer, etal, 1980). Mast cells seemed stable under these conditions, as there was evidence for similar distributions of undegranulated cells before and after culture. As reported in the literature, this distribution of mast cells was markedly different for both diseased and healthy explants (Aeschlimann, etal, 1980; Schroeder, 1977). Histological examination of tissue samples taken after 9 days in culture showed some signs of tissue necrosis; caution should therefore be exercised regarding enhanced synthetic rates of the enzymes as a possible explanation for increased activities observed in culture media. However, biochemical evidence for cell viability did not suggest a marked degree of necrosis.

The inter-animal variation observed for some of the experimental measurements are characteristic features throughout biological systems. Normally this variation is dealt with by a large sample size but the nature of these studies precluded such an approach. Experimental data was therefore compared between healthy and diseased tissues from the same animal. The amounts of collagenase activities released in culture varied between animals, but plasminogen activator when corrected for wet weight and culture media dilution, were similar for healthy tissues from all animals. Increases in PAL for diseased or plaque supplemented cultures therefore are interpreted as significantly elevated.

Although tissue sampling presented some problems, as did the preparation of explants with uniform area/volume ratios, these considerations did not seriously affect the reproducibility of triplicate cultures. The distribution of inflammatory lesions was not uniform in all diseased tissues and others have used gingival tissues apically located from clinically diseased sites as normal control tissue (Page *etal*, 1980). Gingival tissue about 3-5 mm from the coronal border were excised for these studies and the tissues remained intact for the duration of the culture period suggesting that a high proportion of gingival stromal elements remained throughout the culture period. As most of the diseased tissue explants contained a high proportion of what might be regarded as uninflamed, healthy gingiva (the apical regions) it is probable that the differences found between the diseased and healthy explants are underestimated.

DNA determinations and total collagen content in relation to wet weight of tissue support the observation of increased cell numbers and decreased collagen content in chronically diseased tissues (Page & Schroeder, 1981). However, the increased DNA content of diseased tissue was usually no more than 10 percent higher than that for normal tissues, in relation to wet weight, and the approximate doubling of various measurements cannot be explained solely by increased cell numbers.

The method of using wet weight basis of tissue to express enzyme activities was found to be more convenient than presenting enzyme data in relation to DNA or total

protein (see also, Page, etal, 1980). The latter methods would have to be determined on explants at the end of the culture period. Much of the original DNA and tissue protein may have been solubilized into culture medium by this time.

There are several factors which may modify apparent enzyme activities released into the culture media, but the most important are probably enzyme stability, the presence of inhibitors and the problems of latency (Harper, 1980; Woolley, etal, 1978b, Murphy & Sellers, 1980; Pettigrew etal, 1978) The stability of several enzymes under culture conditions have been examined and Cathepsin B was found to be irreversibly inactivated at pH 7.8. Other reports of the lack of secretion of this enzyme in vitro may also be explained by its poor stability (Poole & Morte, 1980) and those studies reporting Cathepsin B activity may be complicated by the presence of a Cathepsin B-like thiol proteinase recently reported by Poole and Mort (1980).

Proteinase inhibitors are present in gingival tissues and may also be secreted in culture (for reviews, Birkedal-Hansen, 1980; Cimasoni & Kowashi, 1980). Whereas the serum inhibitors are likely to be washed out and saturated by proteinases during early stages of culture the tissue inhibitors may be produced throughout culture (Birkedal-Hansen, 1974; Pettigrew etal, 1980). The specificity of such tissue inhibitors has yet to be established although it is known that collagenase is susceptible (Pettigrew, etal, 1980; Simpson & Mailman, 1981). Such inhibitory potential complicated the question of latency, especially when inter-

preting collagenase activity, and for this reason activation treatments were commonly used in this study (Harris & Vater, 1980; Murphy & Sellers, 1980).

Another potential masking phenomenon for proteinases involves the binding to natural substrates such as collagen or elastin of the explanted or extracted tissue, thereby preventing access to the culture medium. However, in this study negligible activities were released after dissociative treatments of residual cultured explants.

The need for standardization of enzyme and biochemical measurements is essential, as illustrated by the problems of inter-animal variation, high and low sensitivities of specific assays, and the need to determine the optimum conditions for specific enzyme assays especially with regard to linear ranges and enzyme:substrate concentrations. The need to determine the range of linear response to both enzyme concentration and in relation to time of incubation is essential for all proteinase assays (Pardue, 1977). Other considerations are exemplified by problems recognized for the collagen fibril assay; these include batch variation of specific radioactivity, concentration and chemical purity of the substrate, and its behavior with other enzymes. Such considerations have recently been reviewed for collagenase assay methods by Harris and Vater, (1980). Although the soluble collagen assay (viscometric) method provides more accurate specific activities for collagenase preparations, the radioactive fibril assay is more convenient for the assay of large numbers of samples for comparative studies.

Provided that such variables in the collagenase assay as substrate preparation, fibril formation, incubation temperatures, hydration and aging of the collagen gels are recognized, the fibril assay provides a useful method for the measurement of collagenase.

A potential source of error open to misinterpretation is the activation of culture medium samples by proteolysis. The use of trypsin for activation may result in variable levels of activation, depending on trypsin activity and protein concentration of the sample. The soy bean trypsin inhibitor used to terminate the activation must also be active in the appropriate molar ratio. Such treatment should always be performed at the same time under identical conditions for accurate comparison of samples. Optimal conditions for trypsin/SBTI activation used in this study were determined by titration of latent enzyme in culture media for trypsin concentration and time of preincubation.

One major area of concern is the lack of substrate specificity for certain enzyme activities. The BANA substrate has been used for a variety of enzymatic assays with variable pH and co-factor conditions, and is commonly used for trypsin-like, Cathepsin B and thiol proteinase activities (Heresby & Smith, 1980; Recklies, etal, 1980). The assay developed for these studies was essentially described by Barrett (1972) for Cathepsin B activity, but with improved sensitivity by adapting the assay to fluorometry. Although metallo-proteinase activity was excluded by use of phosphate buffer and EDTA, it is possible that some thiol

proteinase activity resembling Cathepsin B might also be measured (Poole & Mort, 1980).

Similar problems of substrate specificity occurs for the other fluorometric assays (eg. PAL and elastase activities). Limited information is available at present regarding the coumarin substrates but the literature suggests such substrates are highly specific and very sensitive (Zimmerman, etal, 1977 & 1978; Baici, etal, 1981). However, the high activities found in tissue homogenates for PMN elastase reflects some non-specificity as normal healthy tissues contained a paucity of PMN's.

Pathophysiology

It is only in recent years that gingivitis and periodontitis have been considered as separate bacterial infections due largely to the different bacterial populations which characterize the various disease stages (Page and Schroeder, 1976). The host reaction to plaque varies throughout the course of the disease and it is likely that different plaque elements play specific roles in gingival pathophysiology (Socransky, 1970; Krasse, 1977).

Soluble preparations from homologous whole plaque were used in these studies, but no attempt was made to characterize or identify the stimulatory components. It is possible that normal healthy gingival cells may not have the capacity to spontaneously recognize or respond to plaque elements from diseased tissue due to the lack of immune or inflammatory cells. However, the results show that normal healthy

explants did respond to plaque elements by increasing proteinase production and collagenolysis, but it is uncertain whether the specific plaque elements responsible were identical to those which stimulated diseased gingival explants. The latter, with all the various inflammatory cells, are better 'equipped' to respond to plaque antigens or endotoxins, elements against which the tissue is probably already sensitized. Such factors have to be taken into account when attempting to relate the experimental findings to the pathophysiology of gingivitis and periodontal disease. It is probable that the enzymes produced by normal and diseased explants do not derive entirely from a single 'collagenolytic' cell type, but result from the overall response of all the tissue cells to different stimuli, be they primarily (ie., plaque derived) or secondarily (ie, cell mediator) induced. However, the fibroblastic or stromal cells are known to be the major contributors of proteinase activity in culture (Birkedal-Hansen, 1980; Stricklin, et al, 1977).

Degradation of gingival connective tissues has been explained by the pathologic alteration of fibroblasts in early disease and also by the influx and activation of macrophages and PMN's with concomitant release of collagenase and other hydrolytic enzymes (Page & Narayanan,1980). Fibroblastic growth and synthetic capacity may be modified by the extent of vascularization, lymphokines, prostaglandins and other biologically active agents which result from the disease process. Such factors are probably invol-

ved in the alterations of stromal fibroblast populations during the various stages of the disease.

The polymorphonuclear leukocyte has long been recognized as the first-line defence against bacterial invasion through the sulculus (Attstrom, 1971). Page and Schroeder (1981) have recently suggested that early gingivitis arises from PMN accumulation and activation within the gingival stroma rather than in the gingival sulculus. Activation of PMN's would result in the release of hydrolytic enzymes and the rapid and early destructive phase of the disease. Later stages have been associated with increased numbers of macrophages and altered matrix composition generated by fibroblast selection (Page & Schroeder, 1981). The elimination of fibroblasts by inflammatory cells such as lymphocytes (K-cells) would also result in an inability to maintain the normal tissue matrix (Lehner, 1977; Seymour, etal, 1979b).

In the experimental studies the PMN's of diseased explants may provide a source of the enzymes released early into the media by plaque-challenged tissues. As their viability is limited it is unlikely they would contribute proteinase activity later in culture. Neutral proteinase activity was found to be elevated even after one day in culture and it is likely that the PMN's were largely responsible for this by discharging pre-formed or packaged proteinases (Baggiolini, etal, 1978 & 1979).

Macrophages increase in numbers with progressive inflammatory stages and react to plaque challenge by selective release of lysosomal enzymes (Page & Schroeder, 1981). They

are also capable of producing a variety of degradative enzymes which may also be released into the culture media as a result of plaque challenge (Page etal, 1973; Spector, 1979). Perhaps the major function of macrophages in inflammation is the production of mediators such as mononuclear cell factor (MCF) and prostaglandins (PG) which regulate proteinase production by other cells such as stromal fibroblasts (Dayer, etal, 1980; Loning, etal, 1980).

Mast cells are present in healthy and diseased gingival tissues, but plaque did not appear to induce degranulation in culture as judged histologically. It is therefore uncertain whether mast cell proteinases contribute to matrix degradation either in vivo or in vitro, but it is possible that plaque could induce the release of enzymes or mediators such as heparin or histamine without degranulation (Swartz & Dibblee, 1975 a&b).

Fibroblasts are known to produce collagenase and other degradative enzymes in culture, and this response may be increased when exposed to plaque elements. Stromal fibroblasts apparently decrease in early gingivitis and this may be explained either by the toxicity of plaque factors or by their elimination by lymphocytic K-cell activity, especially as fibroblasts may bind specific bacterial antigens to their surface which may lead to attack by resident immune cells (Engel etal, 1978; Page & Schroeder, 1981).

A considerable body of evidence now supports the idea of phenotypic heterogeneity in fibroblasts resident in the gingiva (Page & Narayanan, 1980). Clonal selection studies,

mediated by numerous factors, have provided some explanations for the various matrix alterations which occur in chronic inflammatory diseases (Page, etal, 1978a; Ivanyi, etal, 1972; Wilde, etal 1977) and these might account for some of the increased collagenolytic observations in diseased tissues.

The increased proportions of lymphocytes and plasma cells in inflammatory disease suggested an active role for these immunocytes in the degradative phase. Such cells probably function by releasing factors which can modulate enzymatic potential in PMN's, macrophages and fibroblasts. It appears that lymphocytes can indirectly modulate matrix composition or destruction by their ability to produce either stimulatory or inhibitory mediators (Page & Schroeder, 1981). It is therefore possible that in culture lymphoid cells would interact with plaque elements, with subsequent production of mediators which serve to regulate the proteinase potential of other cells (Goodman & Sultzer, 1979).

A variety of factors have recently been identified as playing a role in the mediation of proteinase production. Mononuclear cell factor (MCF) produced from monocyte macrophages, interleukin 1, lymphokines, connective tissue activating protein (cTAP), osteoclast activating factor (OAF) and catabolin (for reviews, see Dayer etal, 1980; Mizel etal, 1981; Castor, etal, 1971;Jalkanen, 1981; Dingle, 1981) to name but a few. As yet the relative importance of each of these, and their relationship to each other, has still to be established.

Prostaglandin E_2 can influence growth characteristics of gingival fibroblasts or synthetic capacity of macrophages for collagenase production (Page & Schroeder, 1981; McCarthy etal, 1981). It can also selectively inhibit growth and synthetic activity of 30-40% of gingival fibroblasts in cultured explants (Page & Narayanan, 1980). PGE may also have importance in determining the phenotypic expression of subset populations of fibroblasts, which might alter matrix composition (Page & Narayanan, 1980). Similarily, complement component C_1 can also mediate fibroblast growth in certain sub-populations (Korotzer, etal, 1980) and would provide a mechanism for alteration of matrix at sites of inflammation. Some of these factors would be excluded from the experimental studies reported here, especially the complement system and other serum elements which are lost after 1-2 days of culture However, the presence of lymphocytes, mononuclear cells, mast cells and others may well respond to plaque exposure by producing factors which modulate proteinase production of the tissue explant. A potential role could be postulated for mast cells in inflammation as degranulation has been shown to be regulated by PGE from eosinophils (Hubscher, 1975).

The prostaglandin levels produced in culture are difficult to interpret but the differences observed in diseased and healthy explant culture media are in agreement with those obtained by extraction techniques (Elattar, 1978). Plaque induced a dramatic increase in the amount of the prostaglandin E secreted; this may be explained by the

activation of macrophages, for McCarthy, etal, (1981) have shown that these cells secrete large quantities of PGE on exposure to endotoxin. Moreover, they concluded that the PGE produced subsequently stimulated proteinase release.

The response of the host tissue to bacterial plaque challenge is clearly a sequence of complex interactions, and the hierarchy of the regulatory mechanisms have yet to be determined. An understanding of proteinease regulation in response to plaque would seem to be essential for effective pharmacology to be successful. Promising avenues of further study would include the use of specific cell cultures in order to determine the response of each celltype to plaque elements. Subsequent co-culture experiments, or studies on the effects of specific cell products on different cell cultures, would help to elucidate the cell:cell interactions involved in proteinase physiology.

Specific plaque elements have been isolated and their direct effect on specific cell types have been reported (Johnson, etal, 1976; Kahnberg, etal, 1976a; Krasse, 1977). Characterization of the plaque elements or functional difernces (eg., heat lability) is more likely to provide answers on the action of plaque rather than providing the identification of specific antigens. However, as some plaque elements probably act synergistically, producing greater tissue damage than single plaque components, it would seem that soluble whole plaque products would be more beneficial in initial specific cell culture experiments. Products of specific cell cultures such as macrophages,

lymphocytes or mast cells may also be tested in plaque-free fibroblast or epithelial cell cultures; such studies would attempt to determine the cellular and biochemical events which result in increased proteinase production upon plaque exposure.

Summary:

The establishment of the in vitro model and seven enzymatic assays to examine the various proteinases involved in collagenolysis and matrix degradation has provided interesting information on the proteolytic response of healthy and diseased tissues to plaque. We now have a better idea of the potential proteinase activity of the whole tissue, particularly the base levels and the extent of its response to soluble plaque products. The experimental findings showed that diseased gingival tissue released a significantly greater amount of proteinase activity and collagen degradation products than normal gingival explants. Both normal and diseased tissues responded to plaque exposure by a doubling in collagenolytic activity, with collagenase, neutral proteinase and plasminogen activator making the major contribution to proteolytic activity.

This preliminary or background information now lends itself to further studies on specific cell types in order to determine some of the potential cell:cell interactions involved in the degradative physiology of periodontal disease and inflammation in general.

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APPENDIX

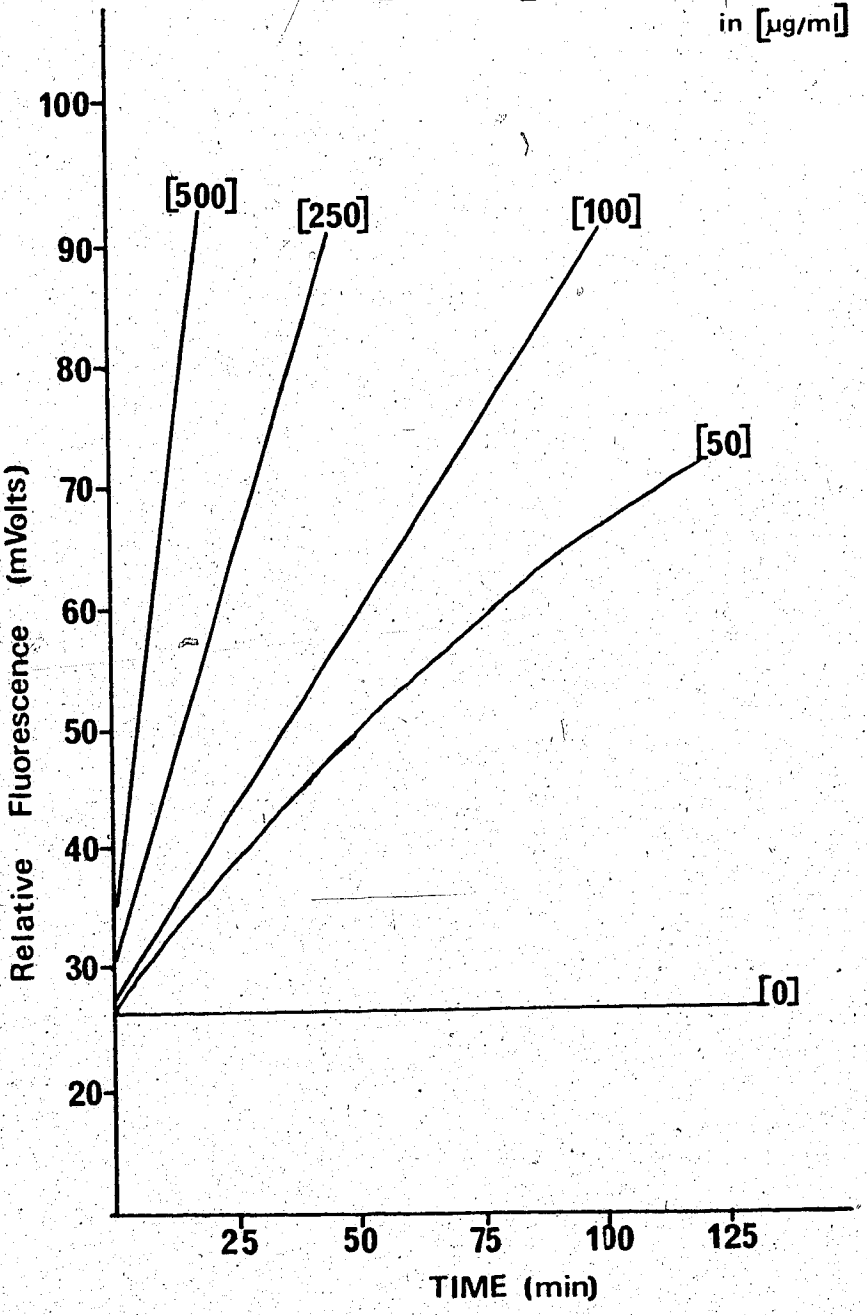
STANDARDIZATION DATA FOR METHODOLOGY

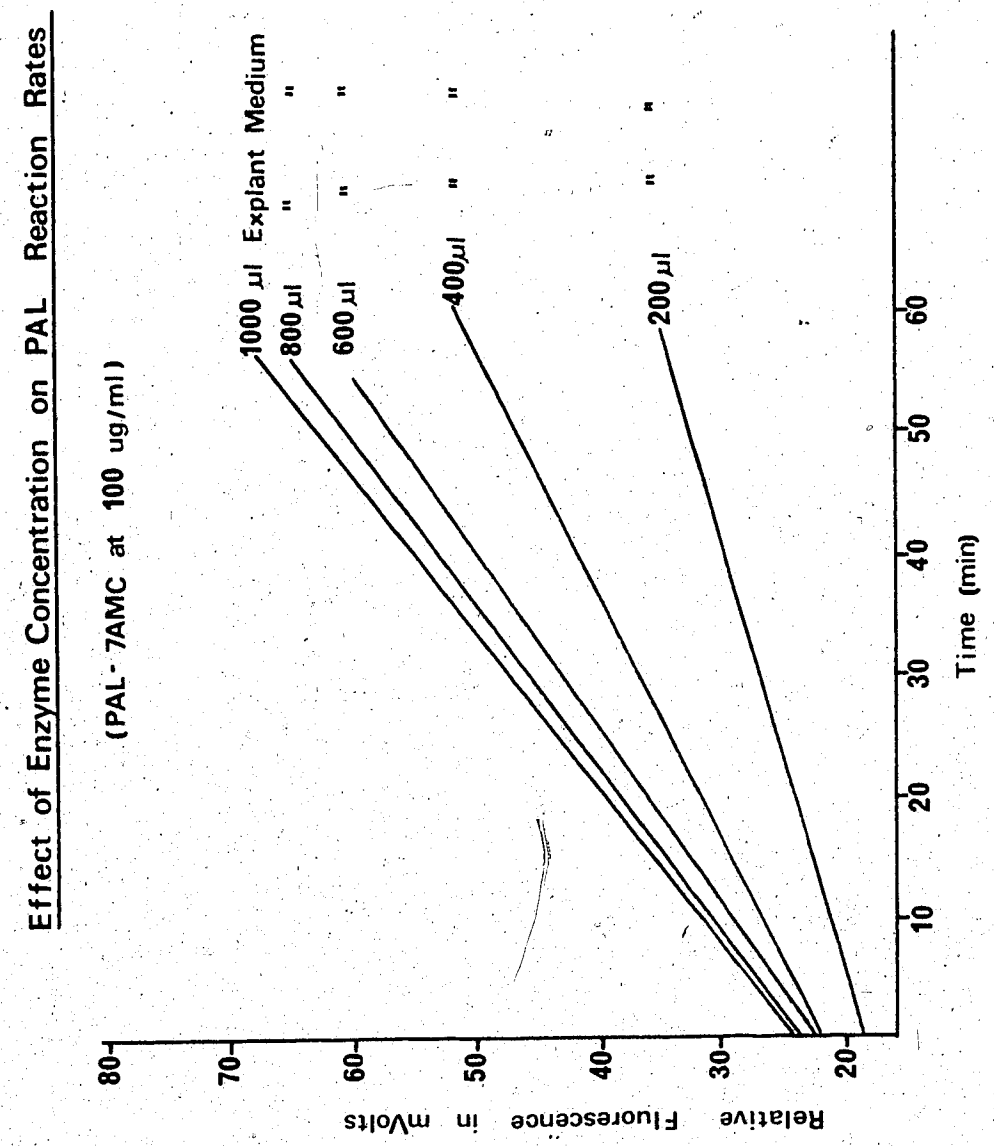
A. FLUOROMETRIC ENZYME ASSAYS: REACTION CONDITIONS (pp. 34-35, 99).

1. PAL ACTIVITY: SUBSTRATE CONCENTRATION EFFECT.
2. PAL ACTIVITY: ENZYME CONCENTRATION EFFECT.
3. ELASTASE ACTIVITY: ENZYME CONCENTRATION EFFECT.
4. CATHEPSIN B: STABILITY AT pH 7.8 (pp. 91, 98-101).
5. CATHEPSIN B: CUMULATIVE ACTION AT pH 6.0 (pp. 91, 100).
6. INTER-ANIMAL VARIATION IN PAL ACTIVITIES (pp. 88, 96, 99).

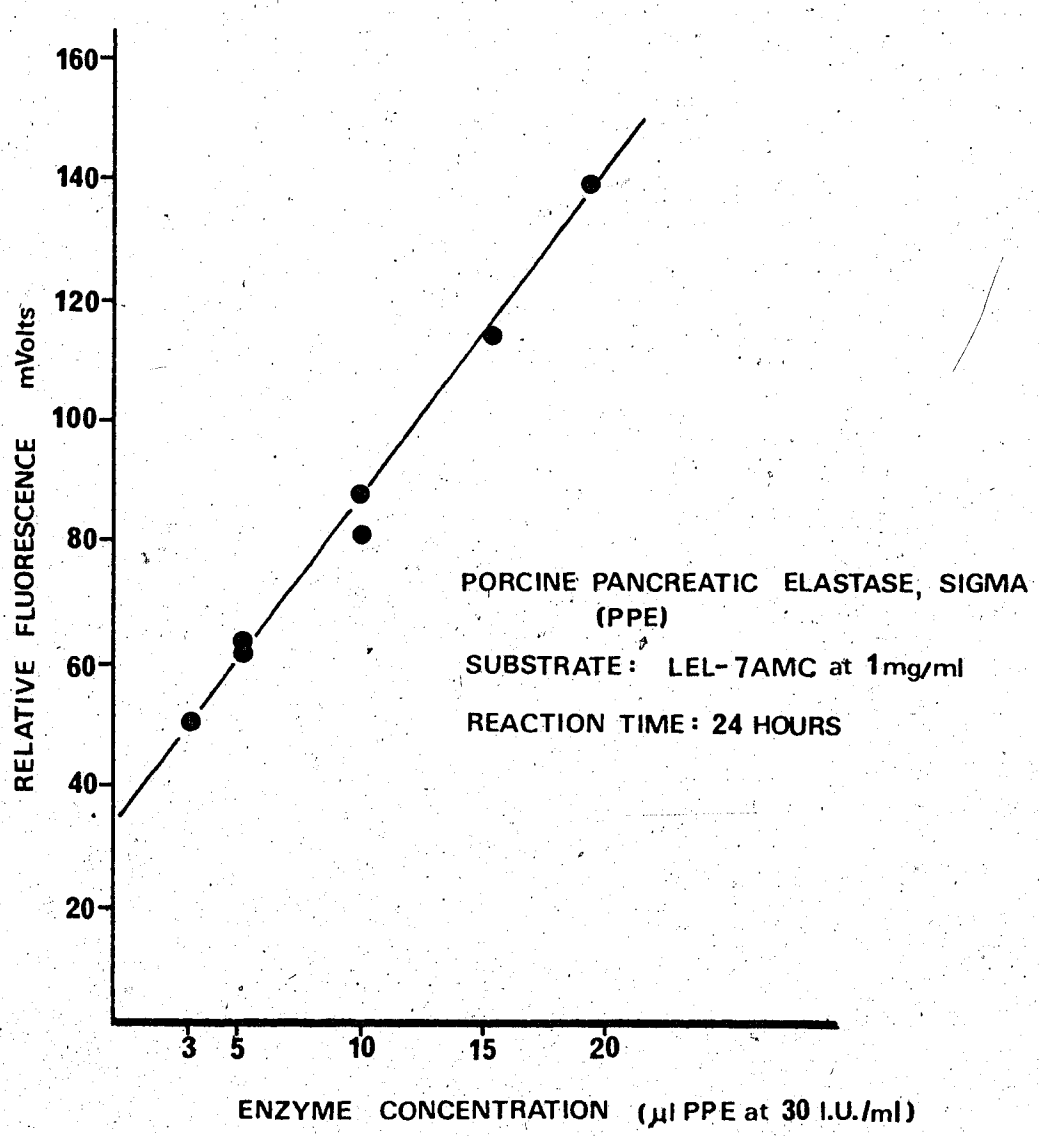
Effect of Substrate Concentration on PAL Activity in 1ml of Explant Medium

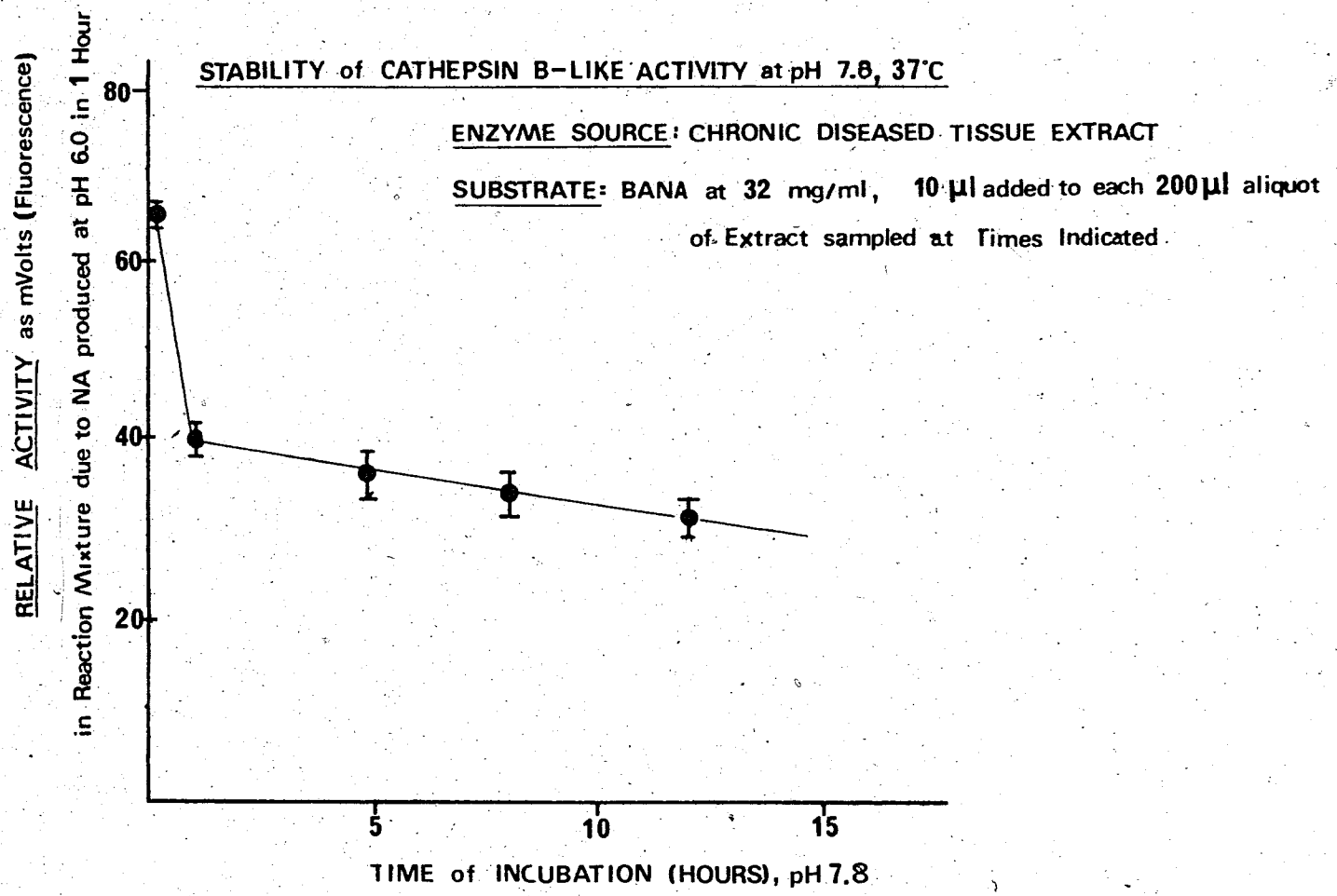
10 µl [PAL-7AMC] at Concentrations Shown in [µg/ml]



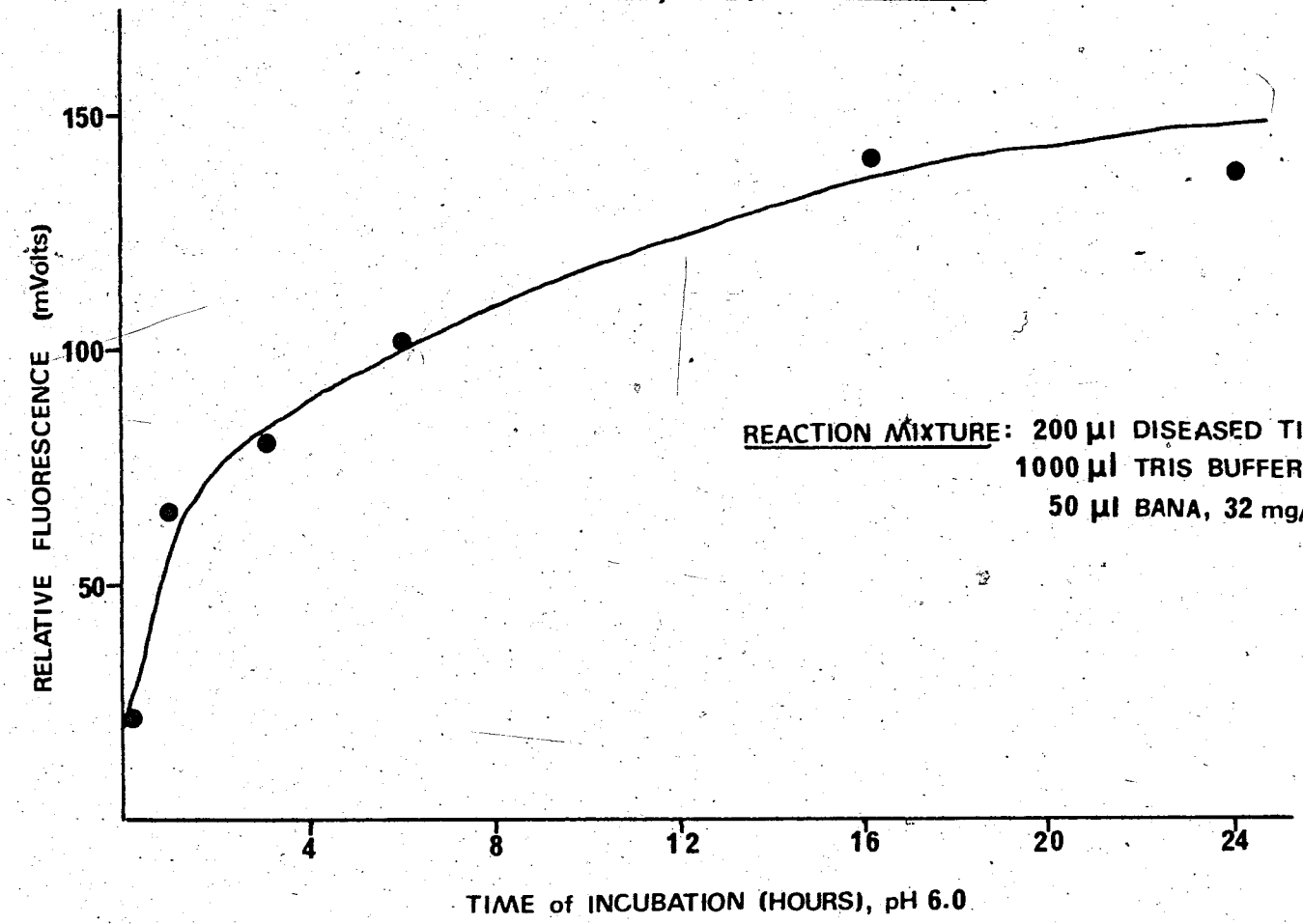

Effect of Enzyme Concentration on PAL Reaction Rates
(PAL- 7AMC at 100 ug/ml)
1000 µl Explant Medium
800 µl " "
600 µl " "
400µl " "
200 µl " "
Relative Fluorescence in mVolts
Time (min)
80
70
60
50
40
30
20
10
20
30
40
50
60

EFFECT OF ENZYME CONCENTRATION on ELASTASE REACTION PRODUCT FORMATION

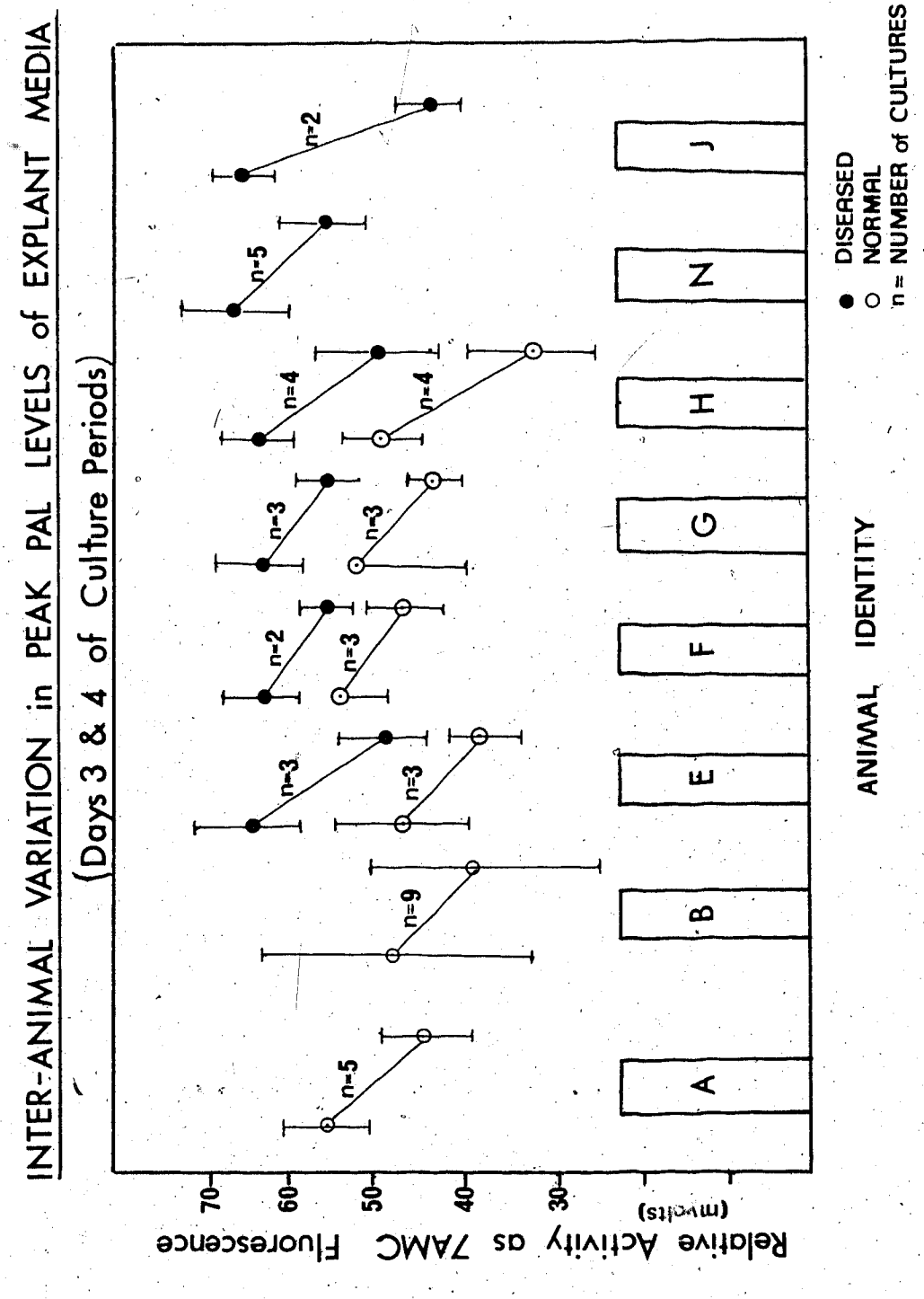



STABILITY of CATHEPSIN B-LIKE ACTIVITY at pH 7.8, 37°C
ENZYME SOURCE: CHRONIC DISEASED TISSUE EXTRACT
SUBSTRATE: BANA at 32 mg/ml, 10 µl added to each 200 µl aliquot of Extract sampled at Times Indicated
RELATIVE ACTIVITY as mVolts (Fluorescence) in Reaction Mixture due to NA produced at pH 6.0 in 1 Hour
80
60
40
20
5
10
15
TIME of INCUBATION (HOURS), pH 7.8

Cathepsin B-Like Activity at pH 6.0, 37°C



REACTION MIXTURE: 200 µl DISEASED TISSUE EXTRACT
1000 µl TRIS BUFFER, pH 6.0, 100mM CYSTEINE
50 µl BANA, 32 mg/ml


INTER-ANIMAL VARIATION in PEAK PAL LEVELS of EXPLANT MEDIA
(Days 3 & 4 of Culture Periods)
Relative Activity as 7AMC Fluorescence
(micrits)
70
60
50
40
30
A
B
E
F
G
H
N
J
ANIMAL IDENTITY
n=5
n=9
n=3
n=3
n=2
n=3
n=3
n=4
n=4
n=5
n=2
● DISEASED
○ NORMAL
n = NUMBER of CULTURES

B. RADIOCHEMICAL ENZYME ASSAYS: REACTION CONDITIONS (pp. 36-37, 99).

1. COLLAGEN FIBRIL ASSAY: GINGIVAL EXPLANT COLLAGENASE CONC.

2. COLLAGEN FIBRIL ASSAY: INCUBATION TIME

3. TCA/TANNIC ACID PRECIPITATION CURVES:
   FINAL CONCENTRATION IN REACTION MIXTURES.

4. GELATINASE SUBSTRATE PRECIPITATION CURVE

5. NEUTRAL PROTEINASE SUBSTRATE SENSITIVITY:
   B. subtilis PROTEASE CONCENTRATION (pg. 87).

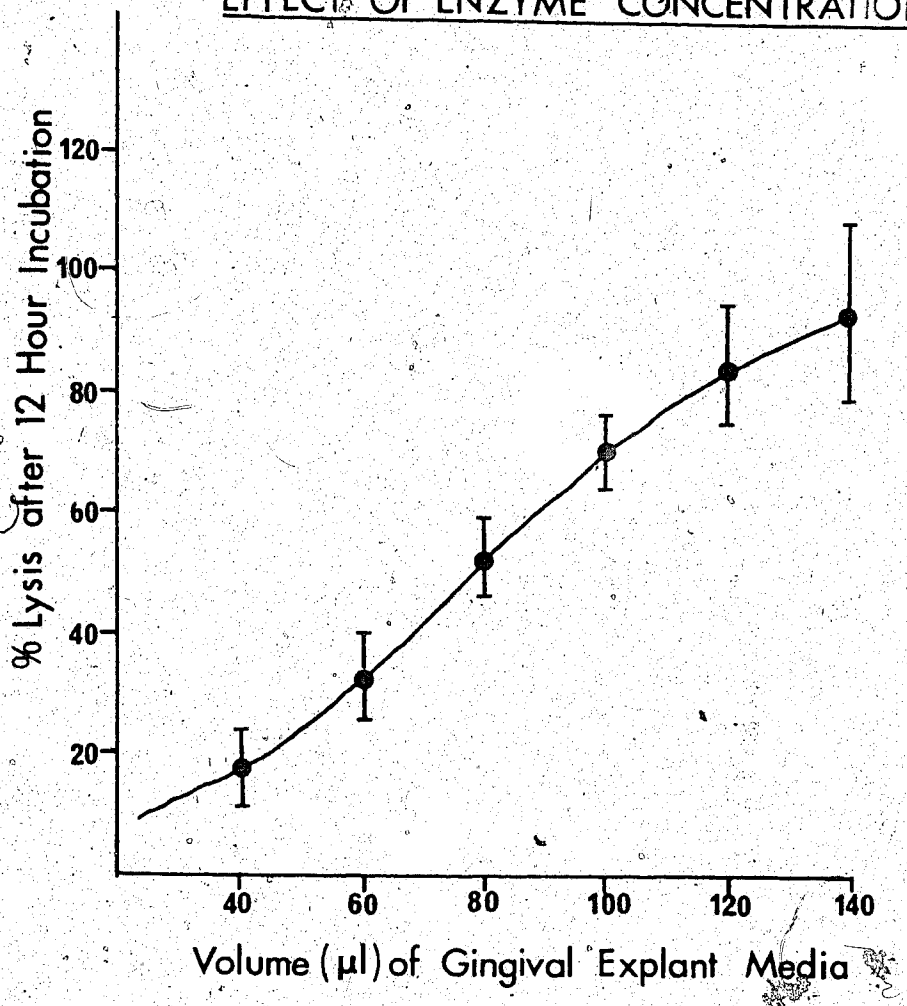
6. NEUTRAL PROTEINASE: GINGIVAL EXPLANT MEDIA CONCENTRATION

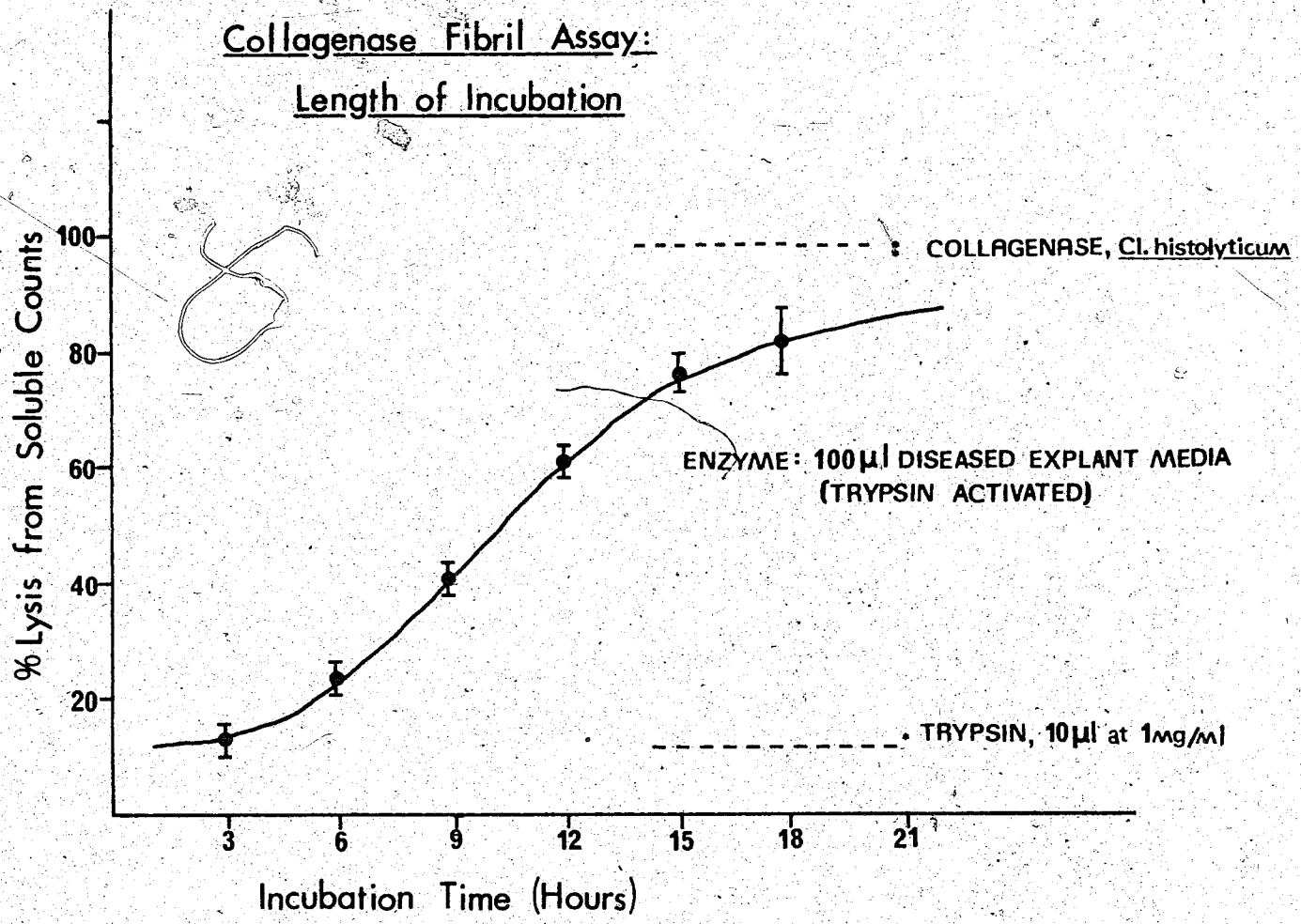
7. NEUTRAL PROTEINASE: TIME OF INCUBATION

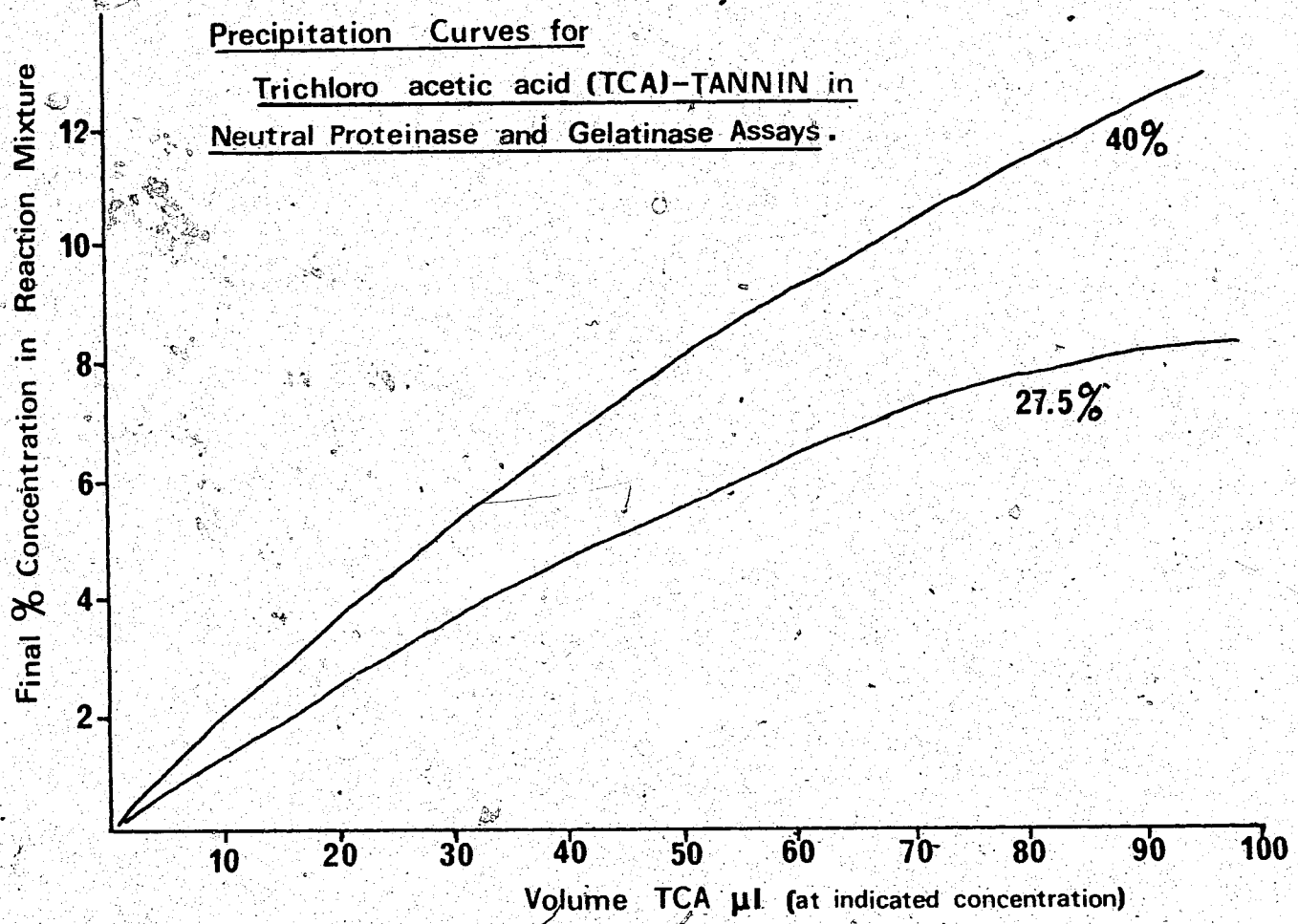
8. NEUTRAL PROTEINASE: TCA/TANNIC ACID PRECIPITATION OF
   ^{3}H-HEMOGLOBIN SUBSTRATE

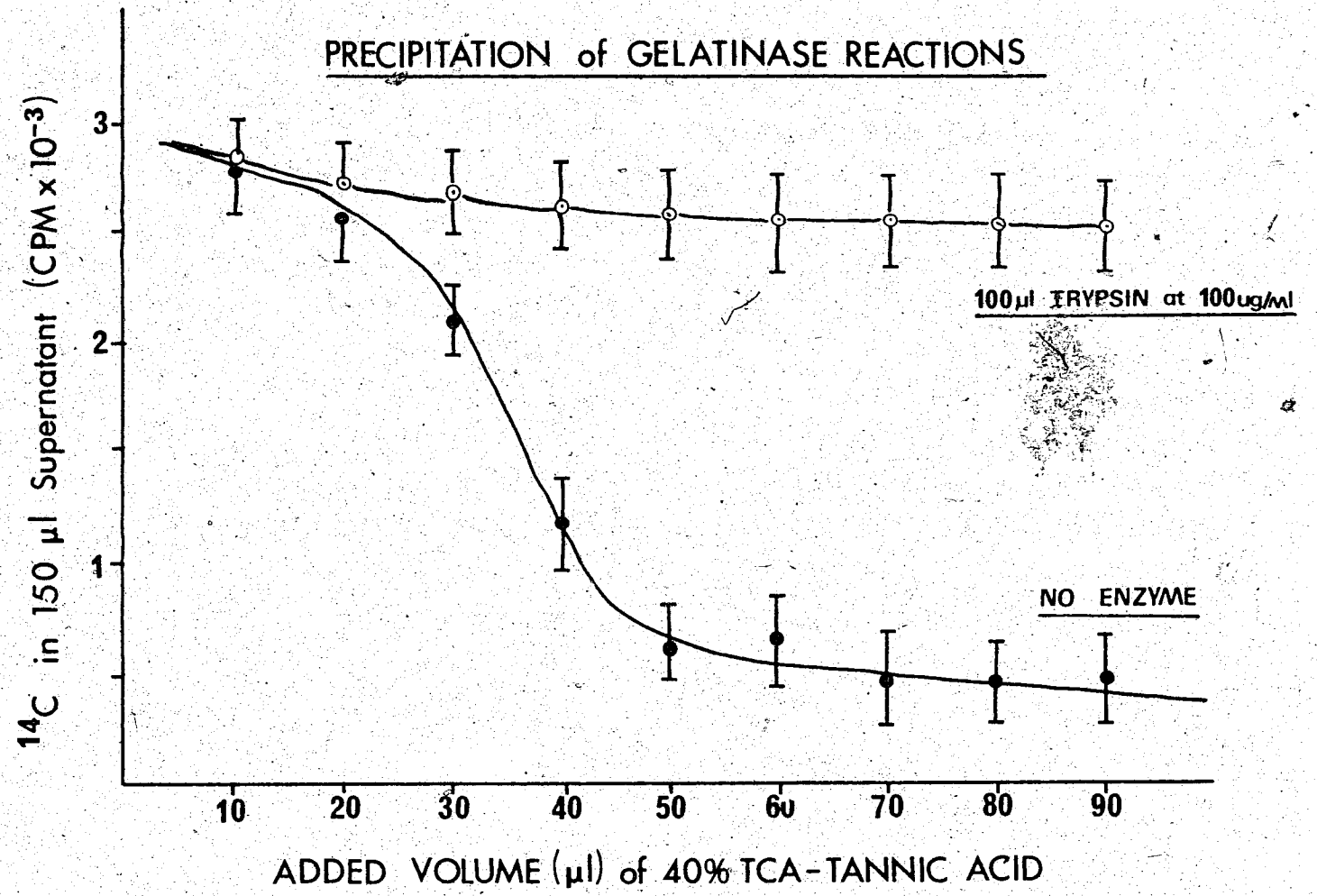
COLLAGENASE FIBRIL ASSAY -

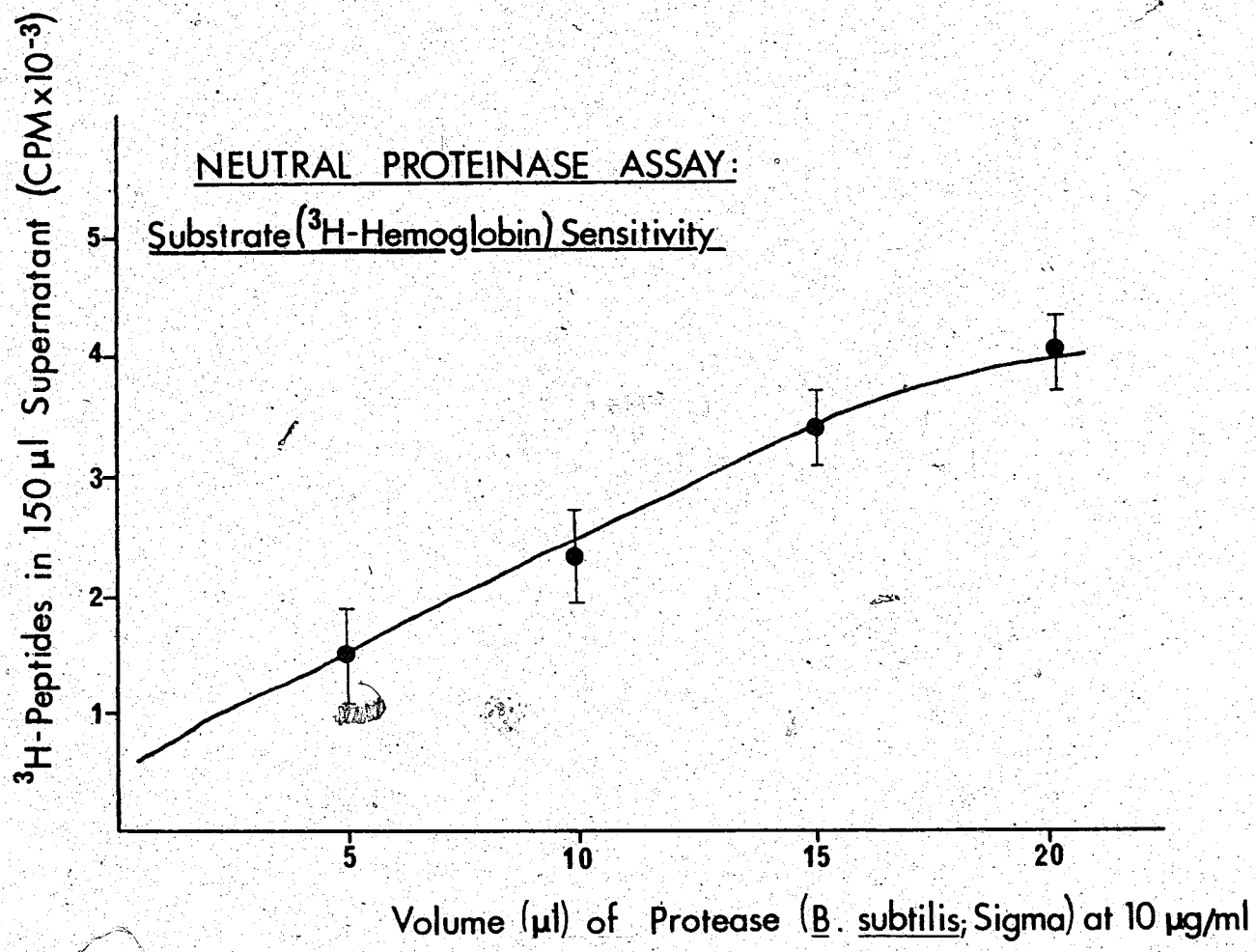
EFFECT OF ENZYME CONCENTRATION

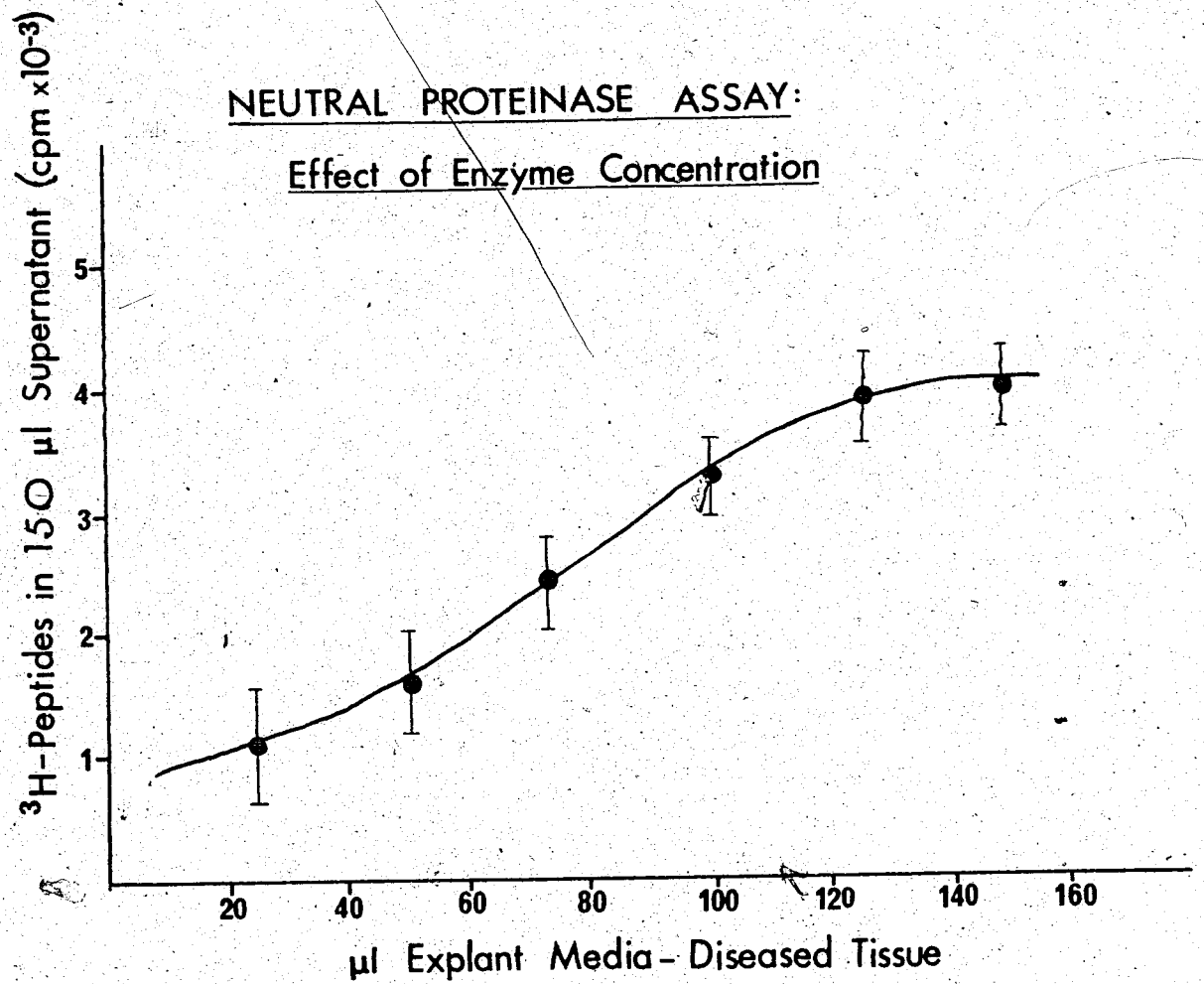


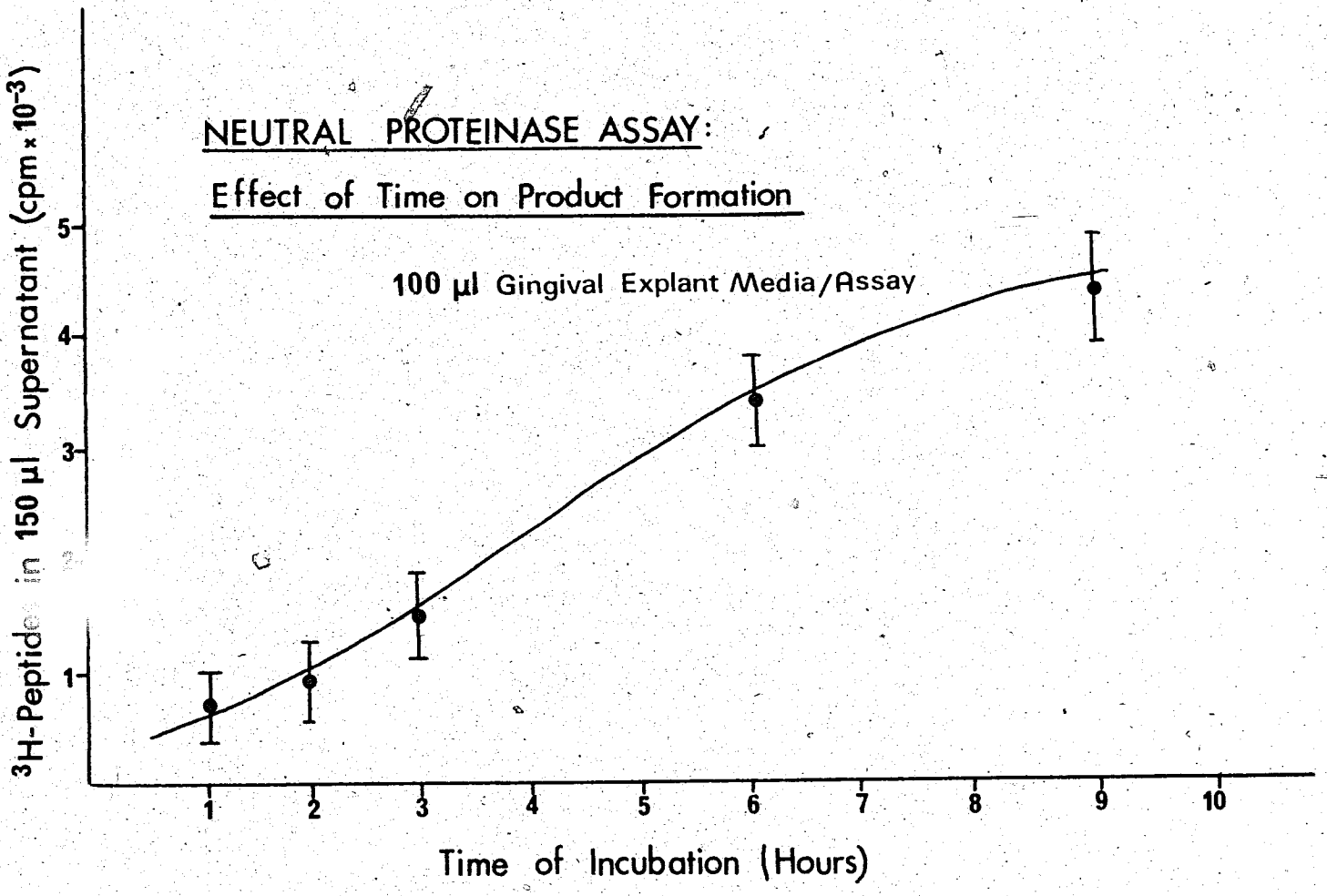

Collagenase Fibril Assay:
Length of Incubation
% Lysis from Soluble Counts
100
80
60
40
20
3
6
9
12
15
18
21
Incubation Time (Hours)
COLLAGENASE, Cl. histolyticum
ENZYME: 100µl DISEASED EXPLANT MEDIA
(TRYPSIN ACTIVATED)
TRYPSIN, 10µl at 1mg/ml

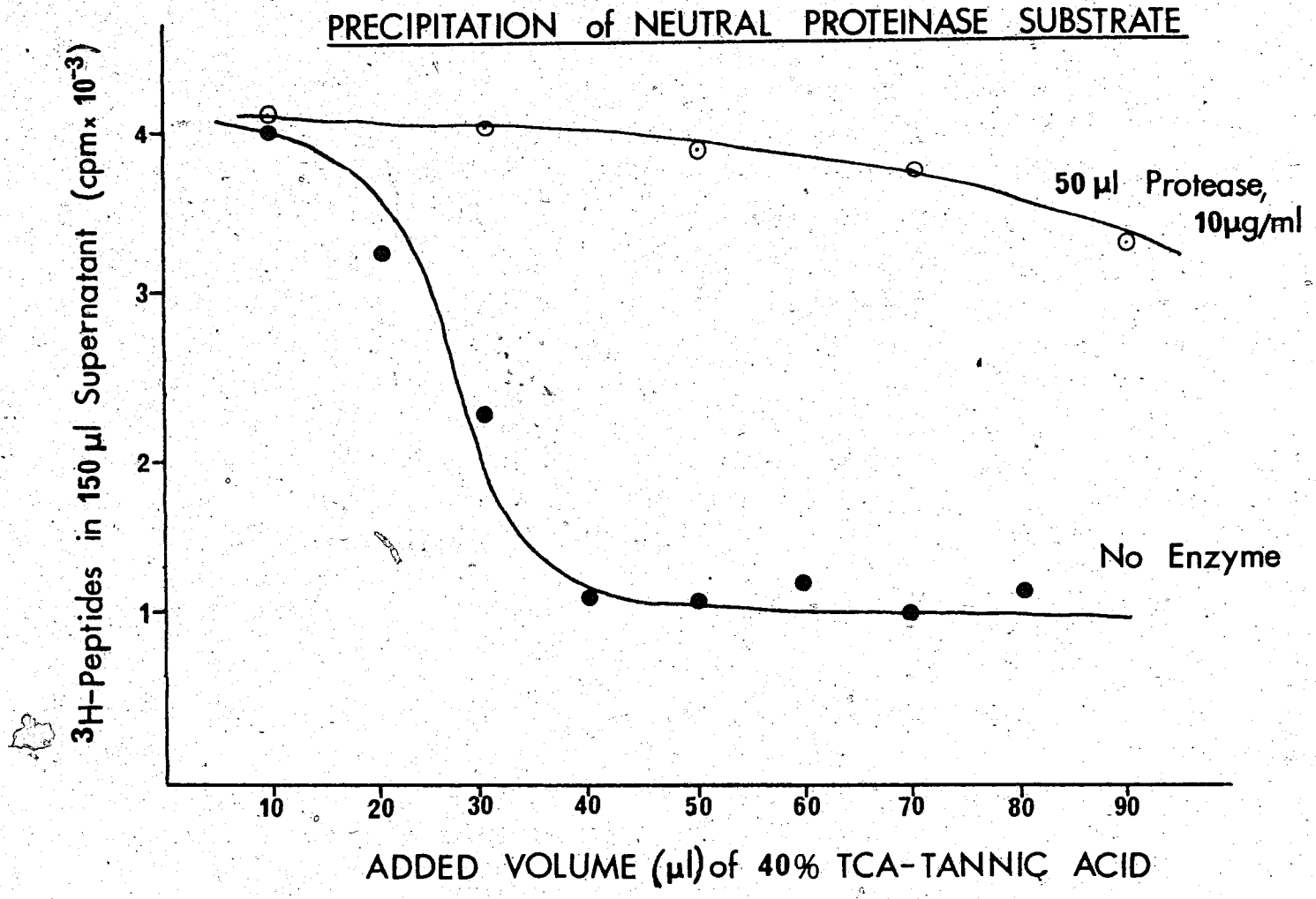

Precipitation Curves for
Trichloro acetic acid (TCA)–TANNIN in
Neutral Proteinase and Gelatinase Assays.
Final % Concentration in Reaction Mixture
12
10
8
6
4
2
40%
27.5%
10
20
30
40
50
60
70
80
90
100
Volume TCA µl (at indicated concentration)


PRECIPITATION of GELATINASE REACTIONS
^{14}C in 150 µl Supernatant (CPM x 10^{-3})
3
2
1
100µl TRYPSIN at 100ug/ml
NO ENZYME
10
20
30
40
50
60
70
80
90
ADDED VOLUME (µl) of 40% TCA-TANNIC ACID


NEUTRAL PROTEINASE ASSAY:
Substrate (^{3}H-Hemoglobin) Sensitivity
^{3}H-Peptides in 150 µl Supernatant (CPM x 10^{-3})
1
2
3
4
5
5
10
15
20
Volume (µl) of Protease (B. subtilis; Sigma) at 10 µg/ml

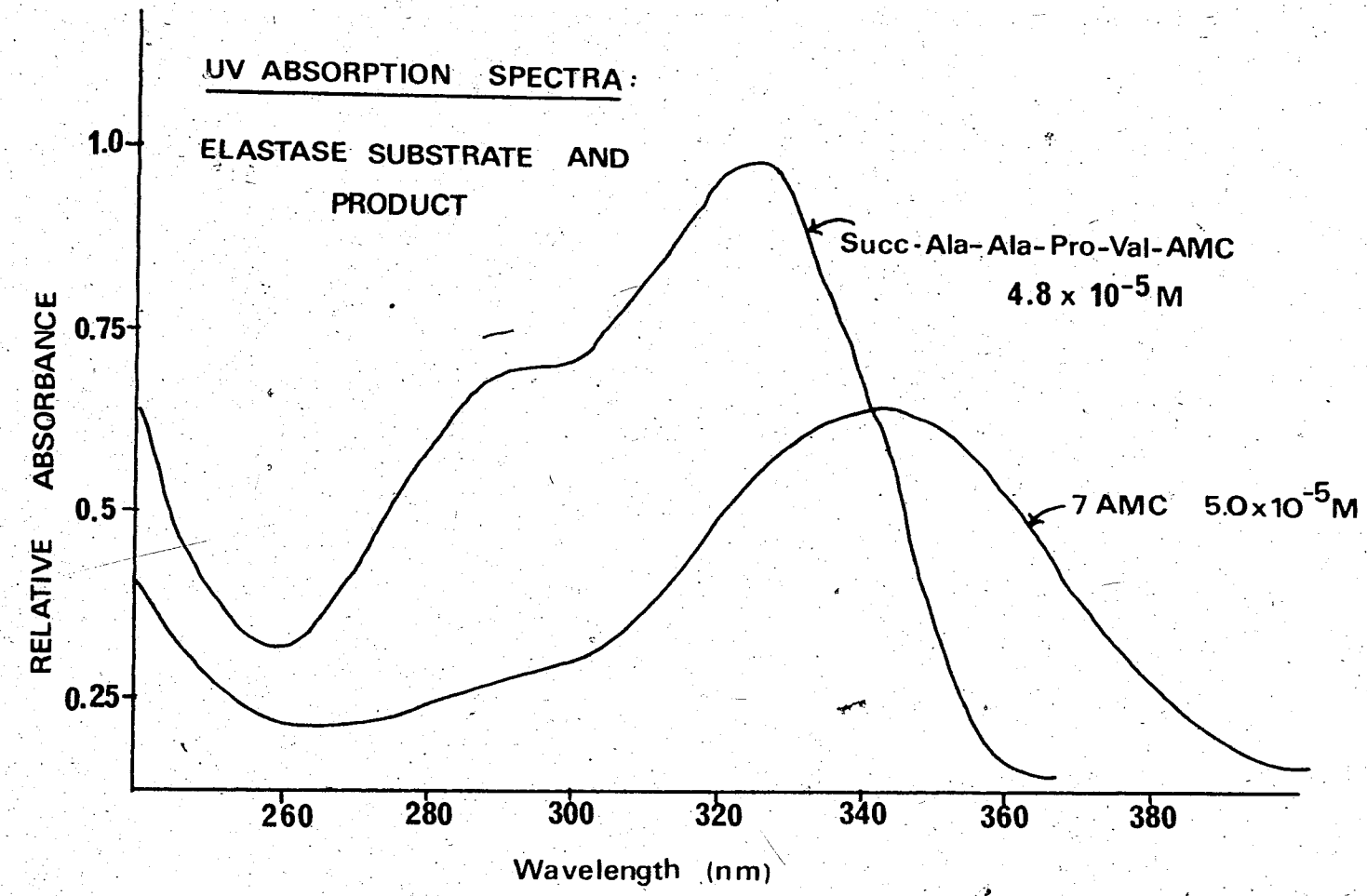

NEUTRAL PROTEINASE ASSAY:
Effect of Enzyme Concentration
^{3}H-Peptides in 150 µl Supernatant (cpm x10^{-3})
1
2
3
4
5
20
40
60
80
100
120
140
160
µl Explant Media - Diseased Tissue

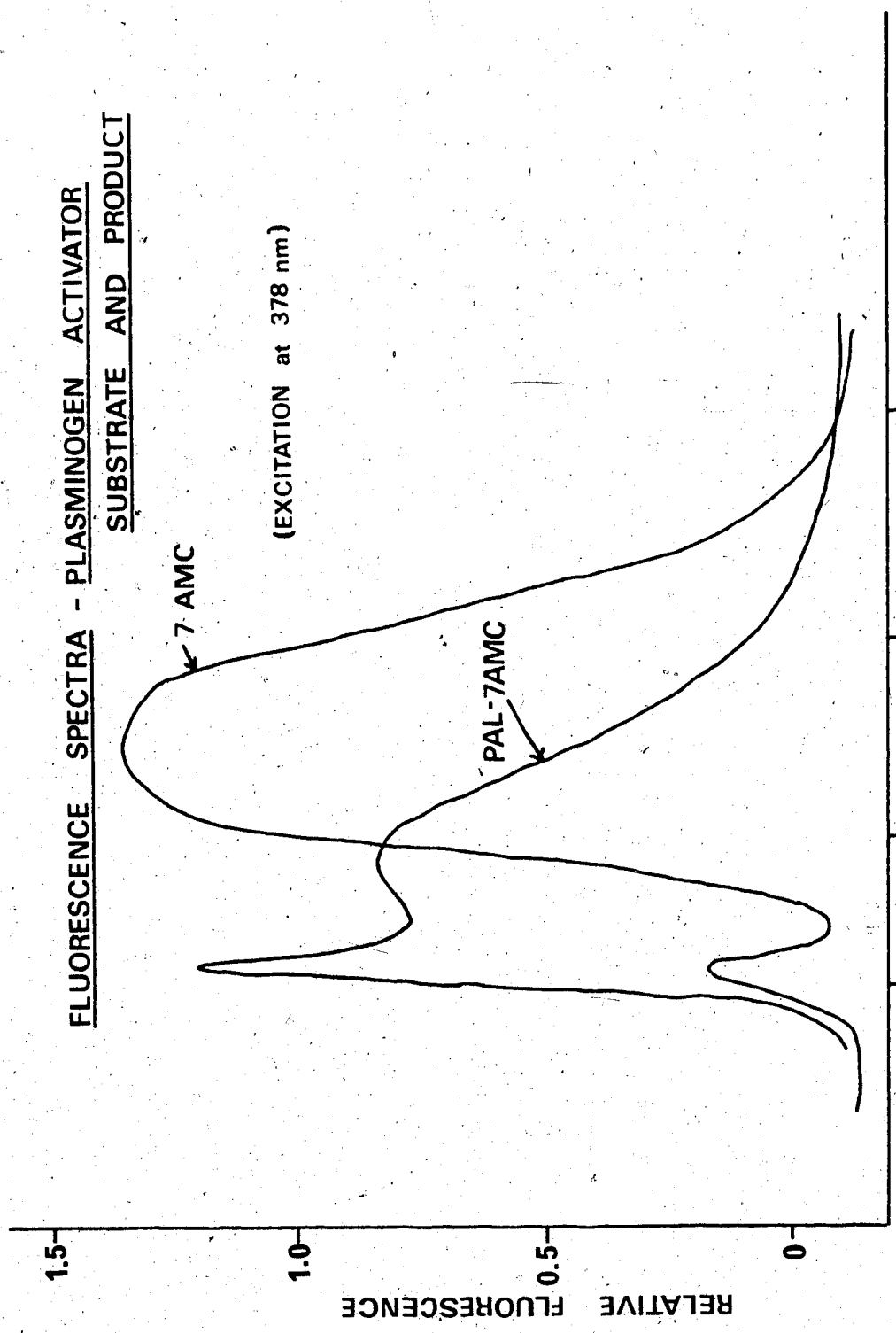

NEUTRAL PROTEINASE ASSAY:
Effect of Time on Product Formation
100 µl Gingival Explant Media/Assay
^{3}H-Peptide in 150 µl Supernatant (cpm × 10^{-3})
1
3
4
5
1
2
3
4
5
6
7
8
9
10
Time of Incubation (Hours)

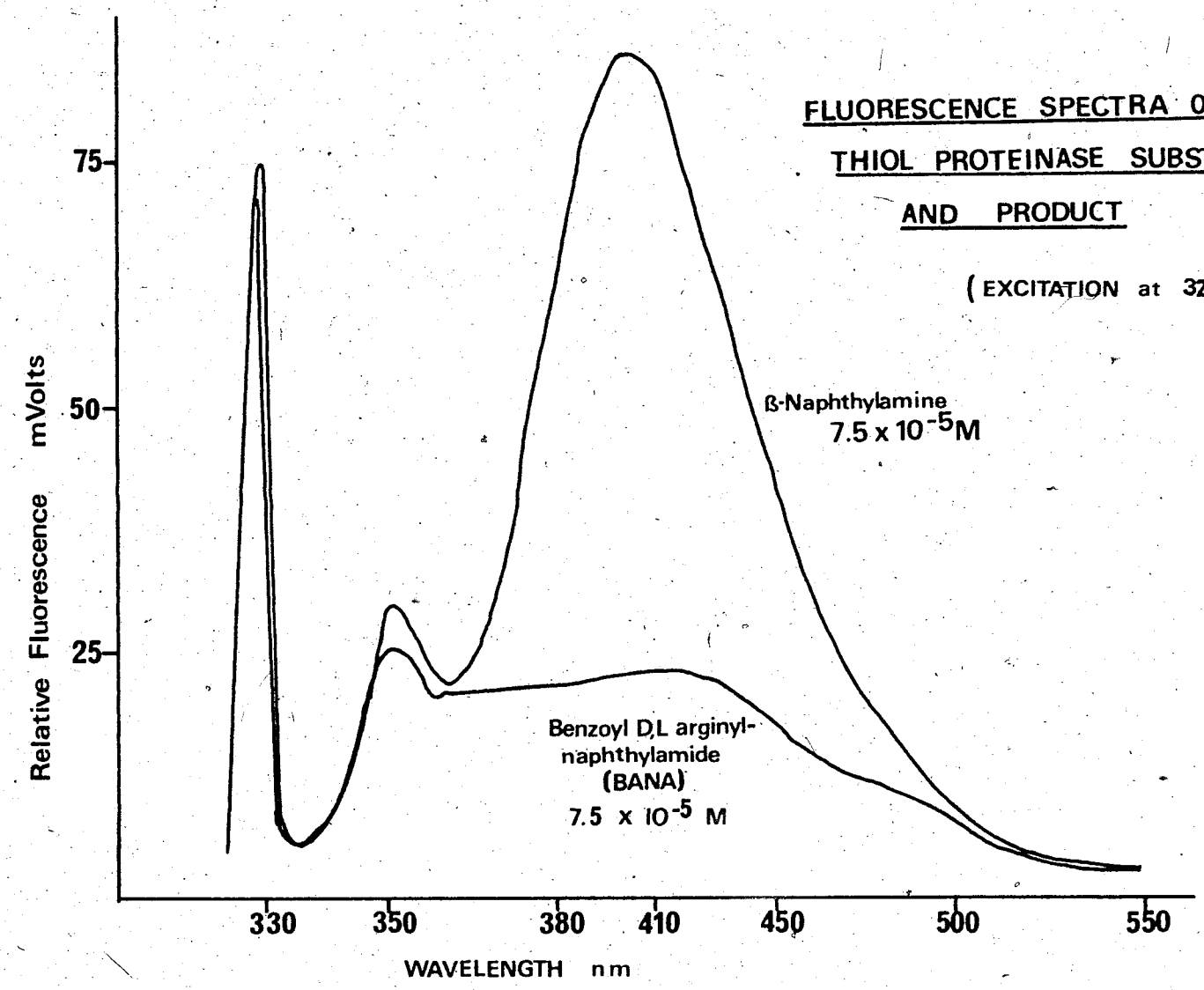

PRECIPITATION of NEUTRAL PROTEINASE SUBSTRATE
3H-Peptides in 150 µl Supernatant (cpm× 10-3)
4
3
2
1
50 µl Protease, 10µg/ml
No Enzyme
10
20
30
40
50
60
70
80
90
ADDED VOLUME (µl) of 40% TCA-TANNIC ACID

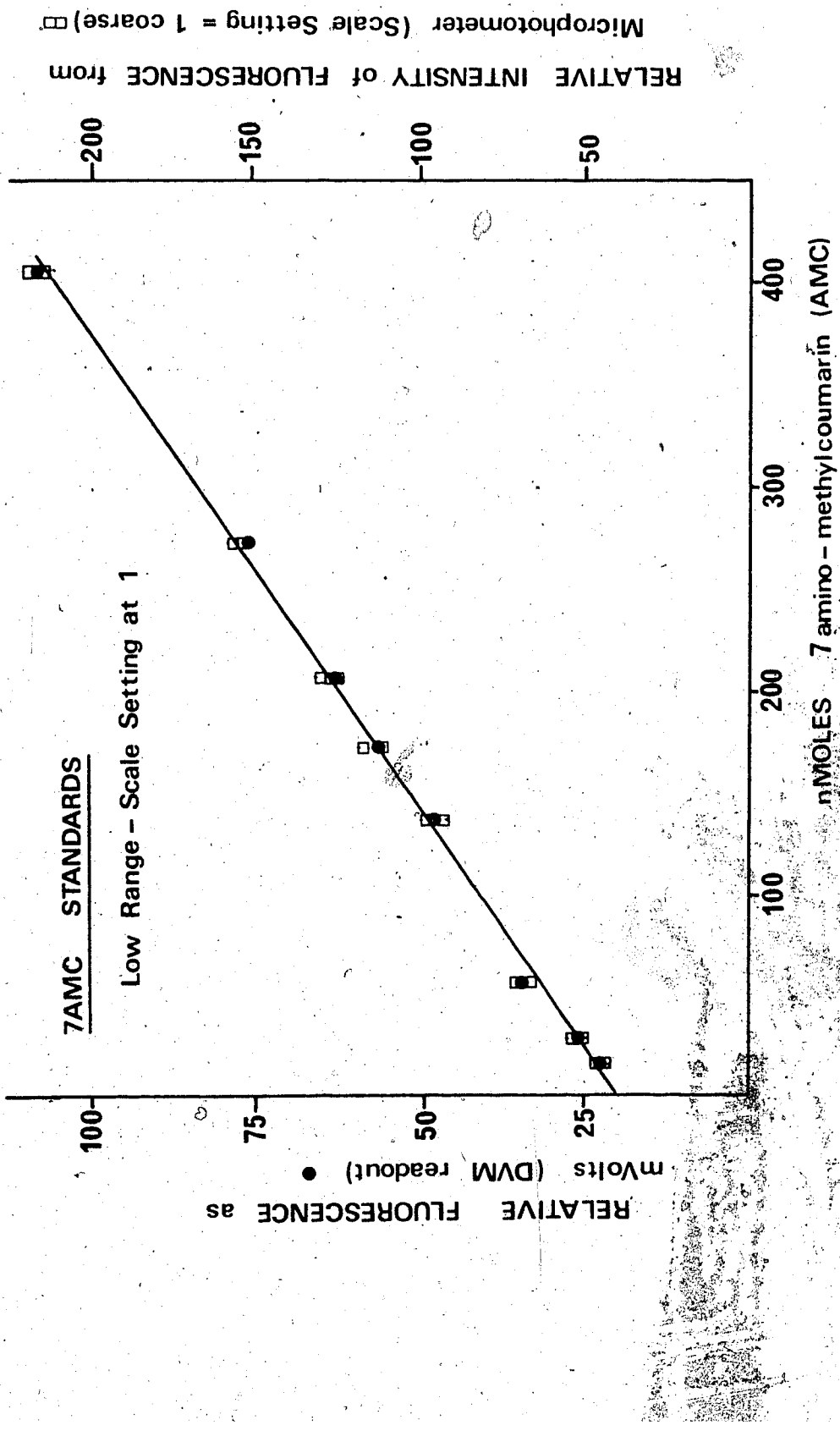
C. FLUORESCENCE SPECTRA AND STANDARD CURVES FOR FLUOROGENIC SUBSTRATES AND PRODUCTS (pp. 33-36).

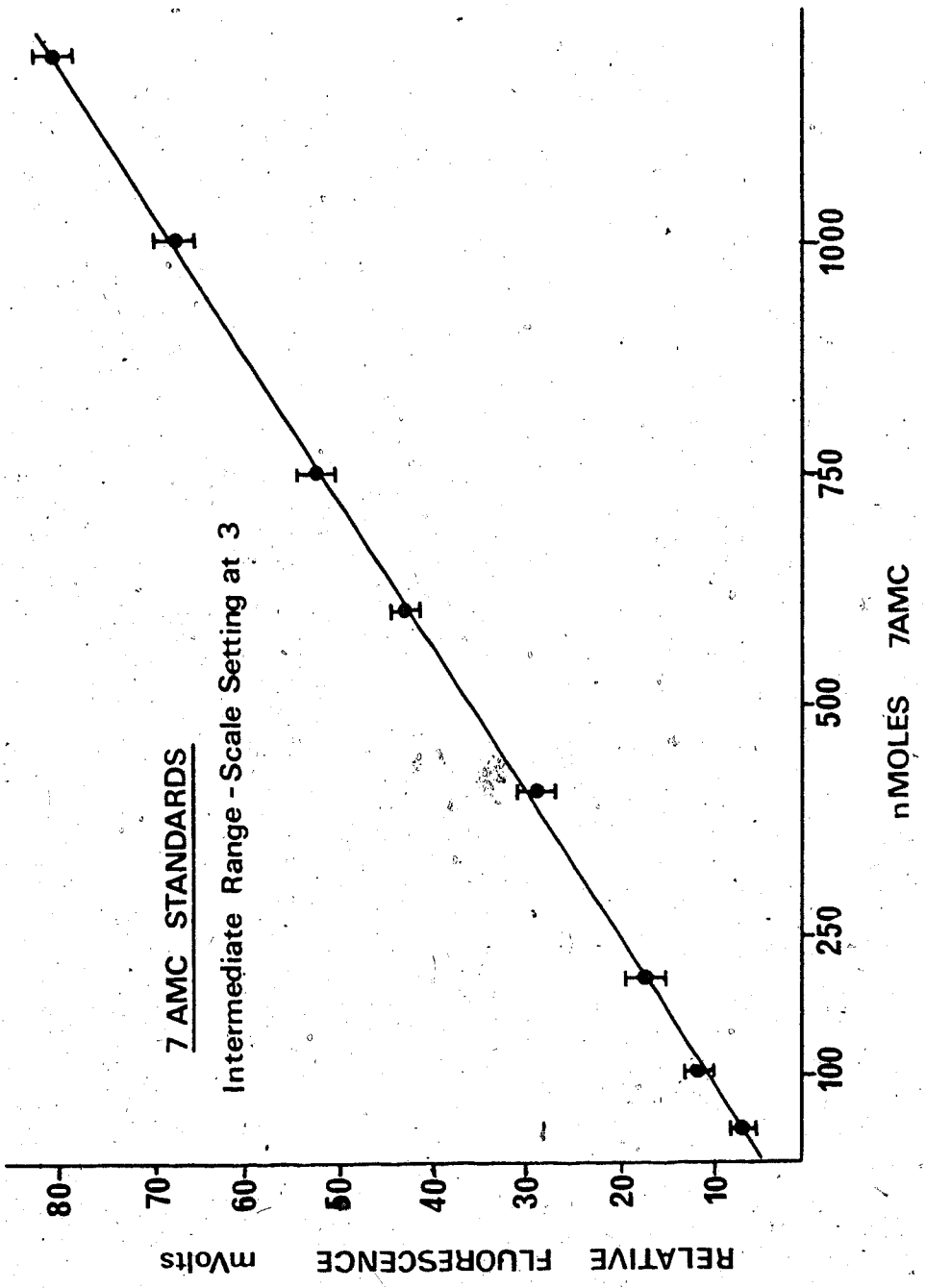
1. UV SPECTRA (ABSORPTION) FOR ELASTASE SUBSTRATE AND 7-AMC.
2. FLUORESCENCE SPECTRA FOR PAL SUBSTRATE AND 7-AMC.
3. FLUORESCENCE SPECTRA FOR CATHEPSIN B-LIKE THIOL PROTEINASE SUBSTRATE (BANA) AND PRODUCT (ß-NAPHTHYLAMINE).
4. 7-AMC STANDARDS: LOW CONCENTRATION RANGE.
5. 7-AMC STANDARDS: MEDIUM CONCENTRATION RANGE.
6. 7-AMC STANDARDS: HIGH CONCENTRATION RANGE.
7. BANA AND NA FLUOROMETRIC STANDARD CURVE.
8. DNA STANDARD CURVE.

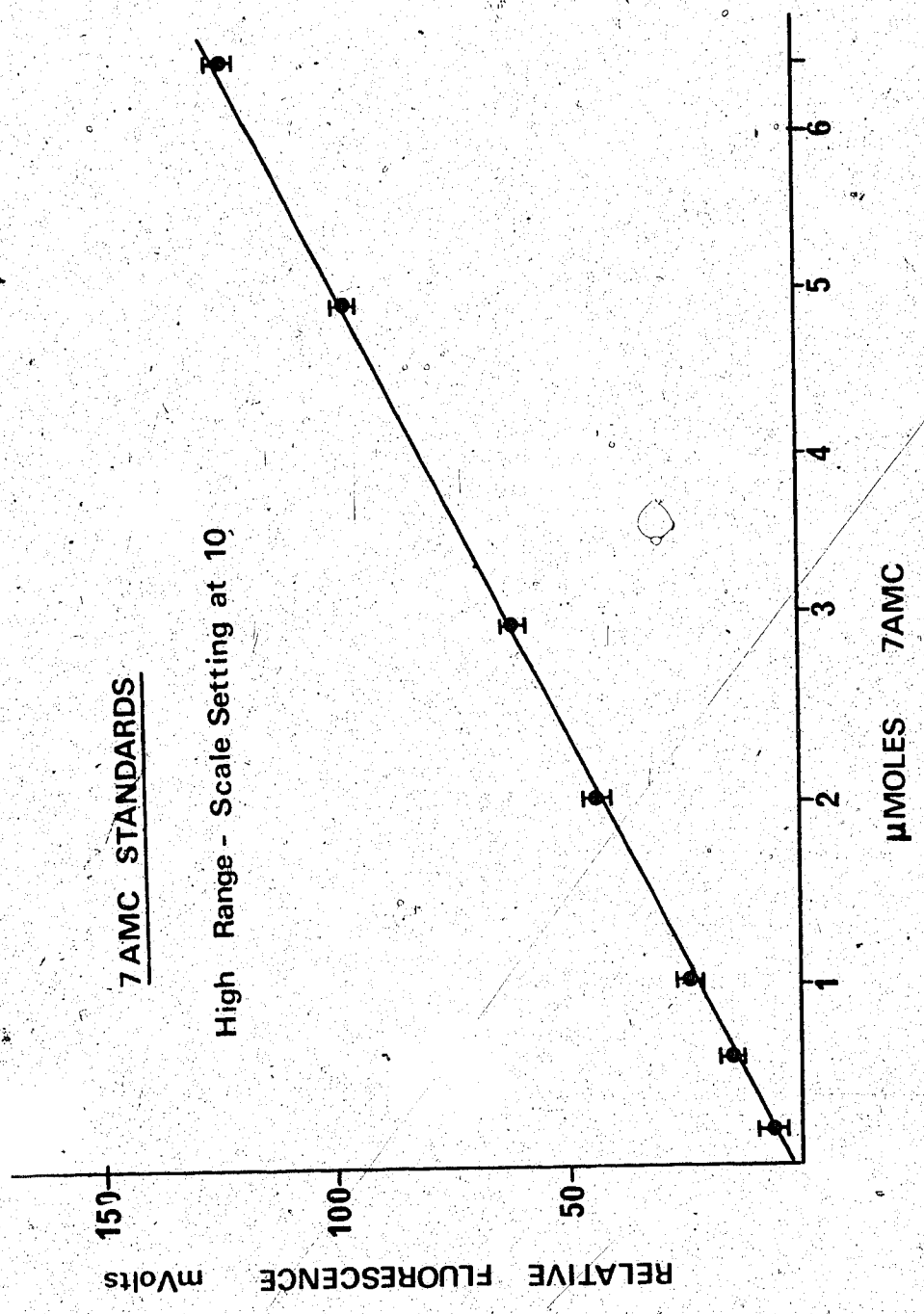

UV ABSORPTION SPECTRA:
ELASTASE SUBSTRATE AND PRODUCT
Succ-Ala-Ala-Pro-Val-AMC
4.8 x 10-5 M
7 AMC 5.0x10-5M
RELATIVE ABSORBANCE
1.0
0.75
0.5
0.25
260
280
300
320
340
360
380
Wavelength (nm)

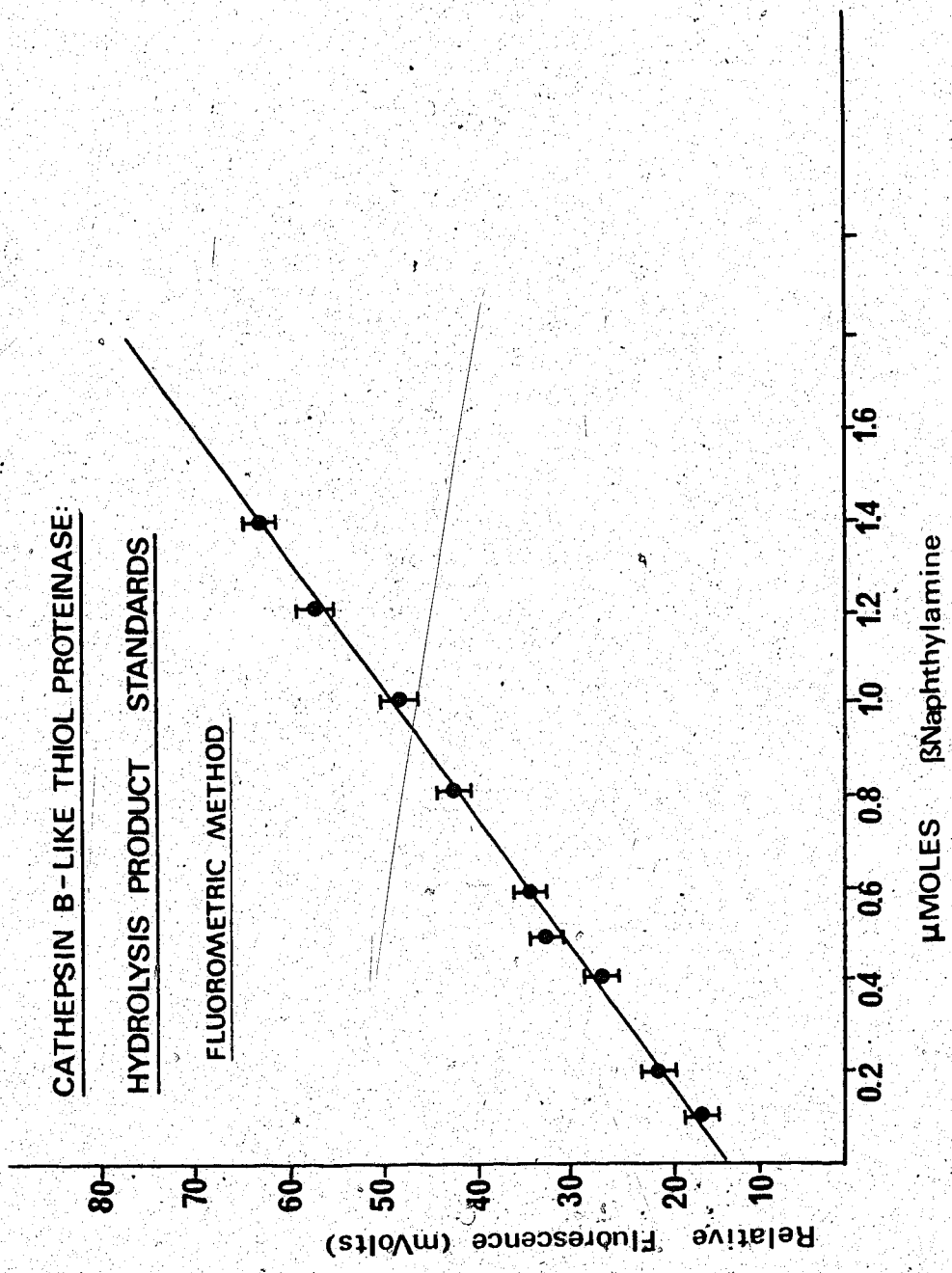

FLUORESCENCE SPECTRA - PLASMINOGEN ACTIVATOR SUBSTRATE AND PRODUCT
(EXCITATION at 378 nm)
7-AMC
PAL-7AMC
RELATIVE FLUORESCENCE
1.5
1.0
0.5
0
370
400
460
550
WAVELENGTH (nm)

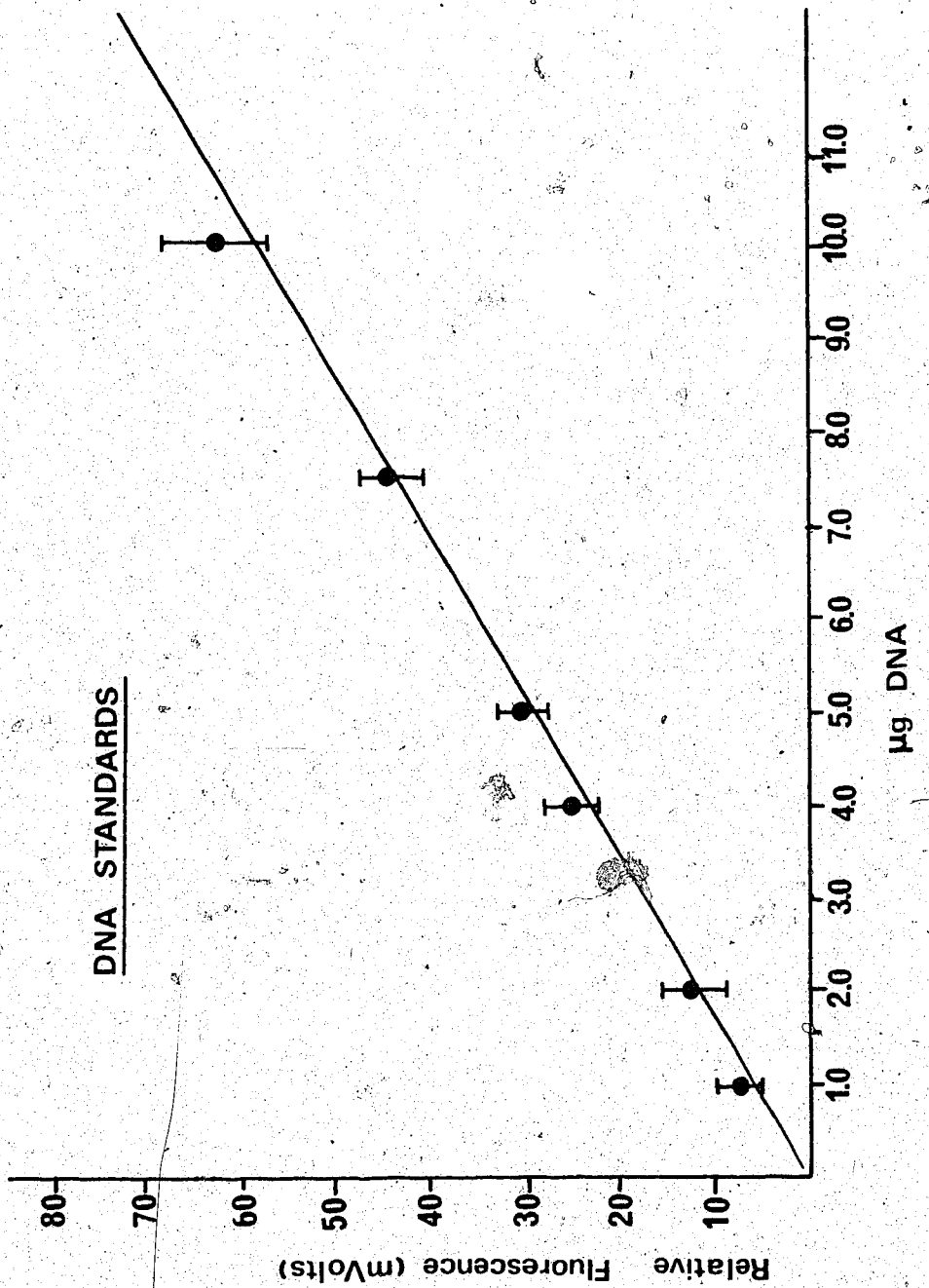

FLUORESCENCE SPECTRA OF
THIOL PROTEINASE SUBSTRATE
AND PRODUCT
(EXCITATION at 327 nm)
ß-Naphthylamine
7.5 x 10-5M
Benzoyl D,L arginyl-
naphthylamide
(BANA)
7.5 x 10-5 M
Relative Fluorescence mVolts
75
50
25
330
350
380
410
450
500
550
WAVELENGTH nm


RELATIVE INTENSITY of FLUORESCENCE from
Microphotometer (Scale Setting = 1 coarse)
200
150
100
50
RELATIVE FLUORESCENCE as
mVolts (DVM readout) ●
100
75
50
25
7AMC STANDARDS
Low Range – Scale Setting at 1
100
200
300
400
nMOLES 7 amino – methylcoumarin (AMC)


7 AMC STANDARDS
Intermediate Range - Scale Setting at 3
RELATIVE FLUORESCENCE mVolts
nMOLES 7AMC
10
20
30
40
50
60
70
80
100
250
500
750
1000

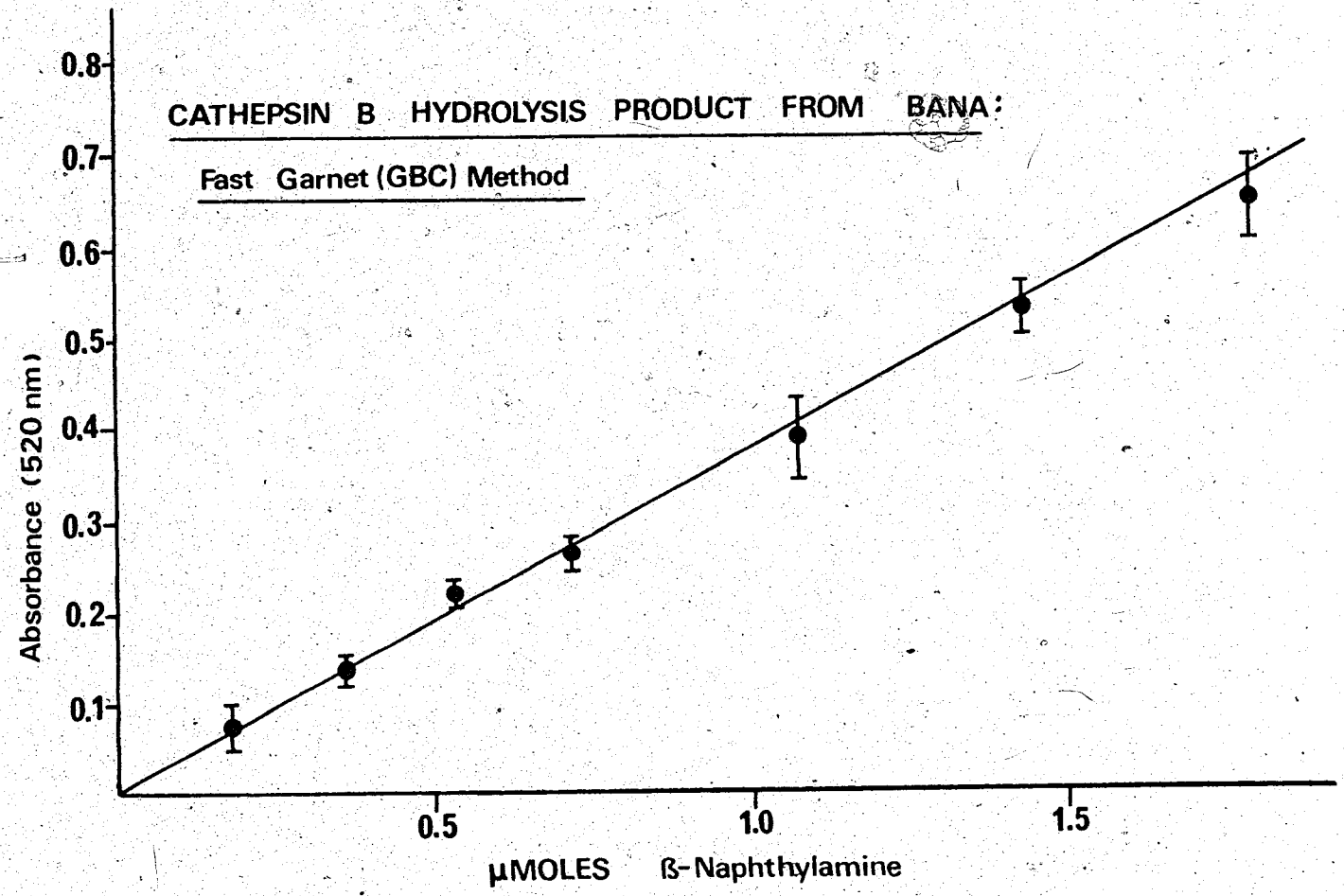

7AMC STANDARDS
High Range - Scale Setting at 10
RELATIVE FLUORESCENCE mVolts
150
100
50
1
2
3
4
5
6
µMOLES 7AMC

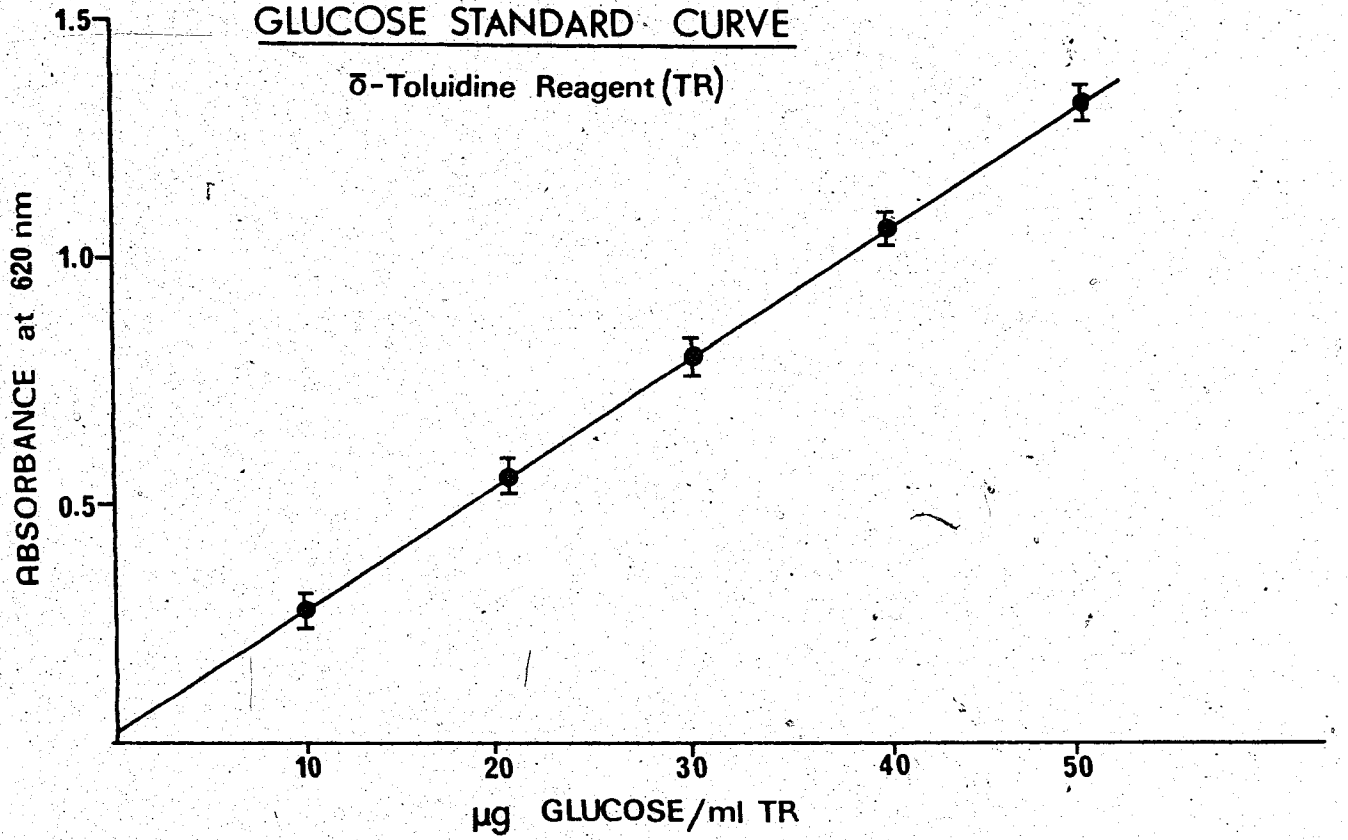

CATHEPSIN B-LIKE THIOL PROTEINASE:
HYDROLYSIS PRODUCT STANDARDS
FLUOROMETRIC METHOD
Relative Fluorescence (mVolts)
10
20
30
40
50
60
70
80
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
µMOLES βNaphthylamine

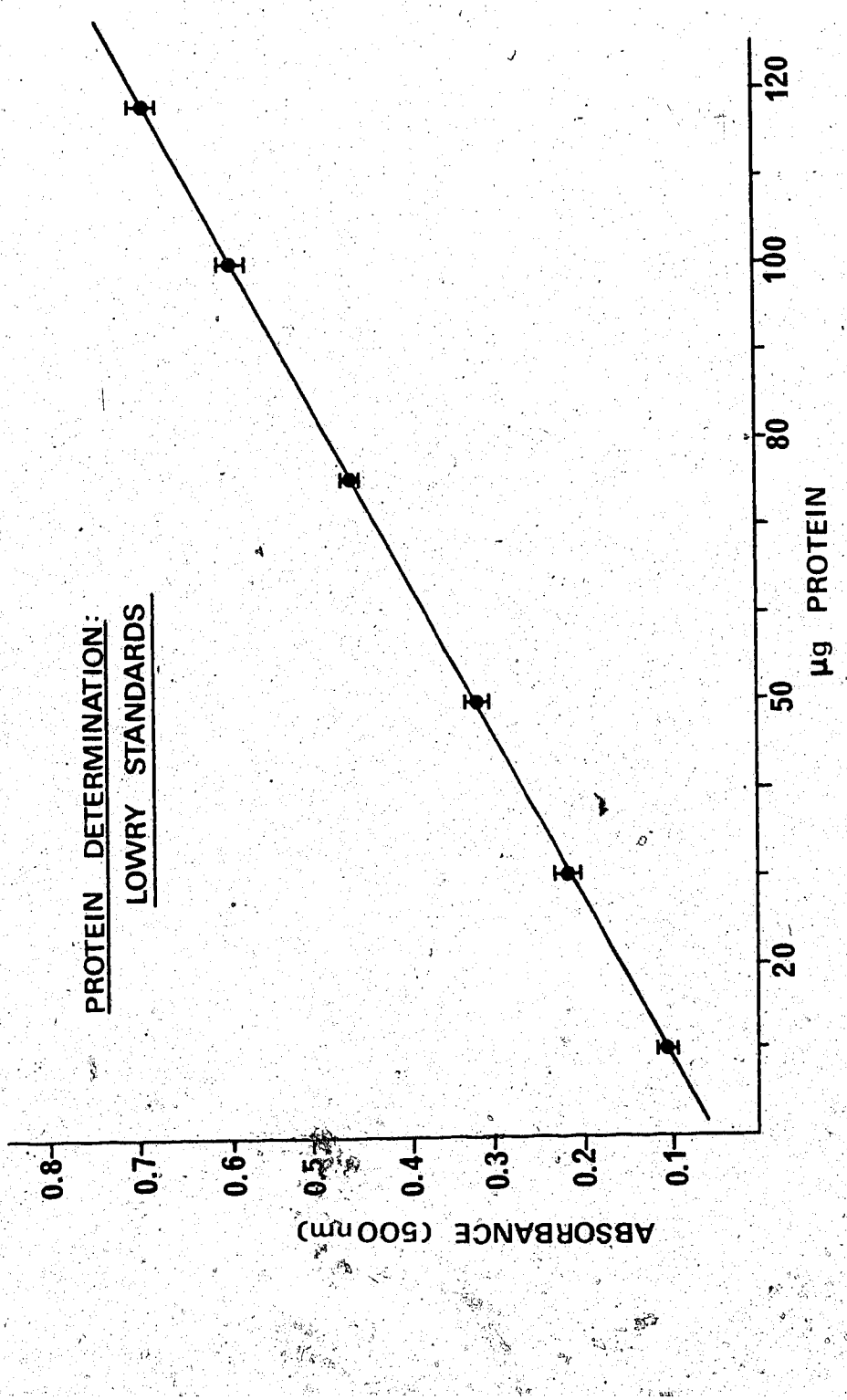

DNA STANDARDS
Relative Fluorescence (mVolts)
10
20
30
40
50
60
70
80
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
10.0
11.0
µg DNA

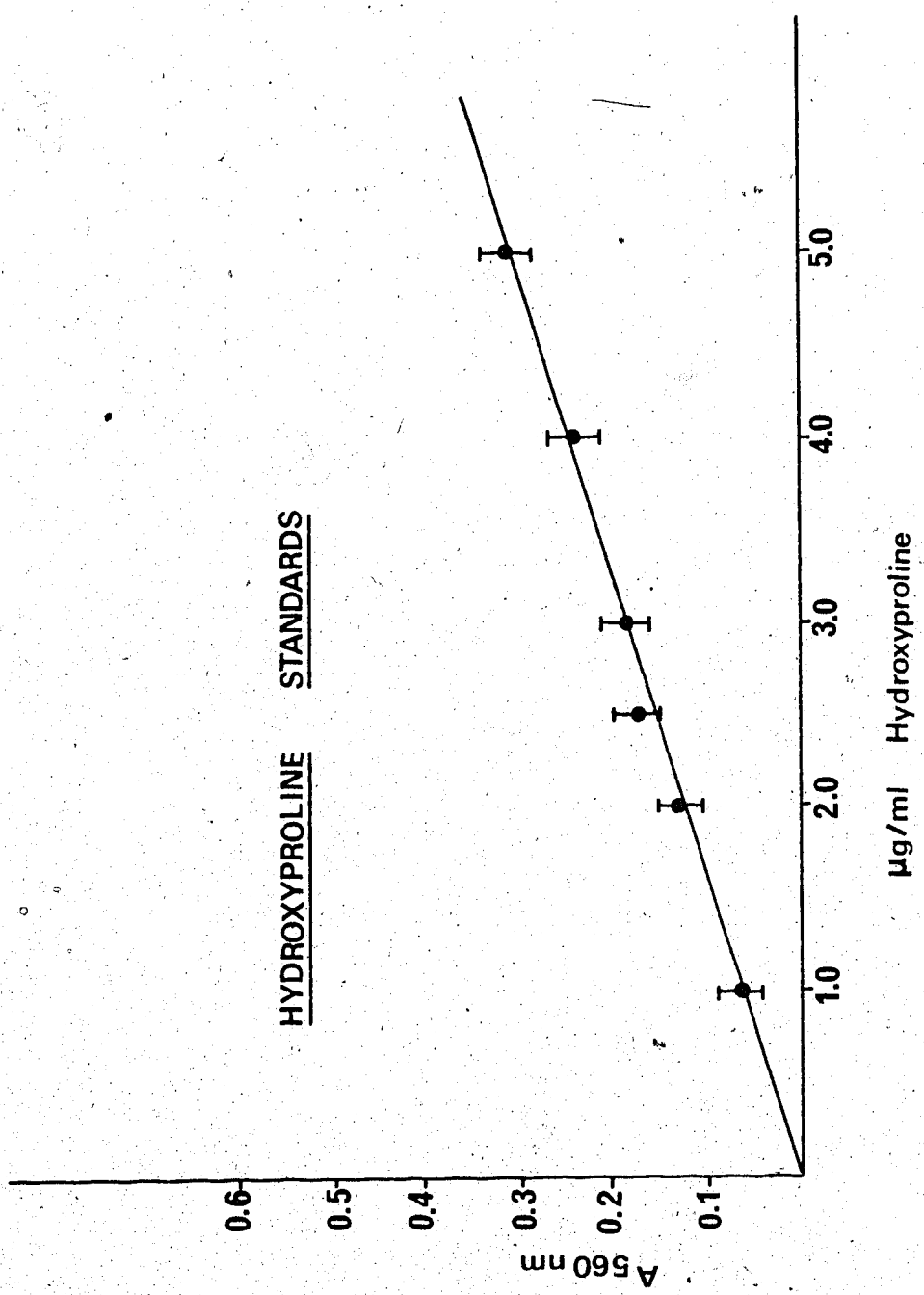
D. STANDARD CURVES FOR COLORIMETRIC ASSAYS (pp. 31-33, 35-36, 100).

1. ß-NAPHTHYLAMINE STANDARD (FAST GARNET-GBC METHOD).
2. GLUCOSE (o-TOLUIDINE METHOD).
3. PROTEIN CONCENTRATION (LOWRY METHOD).
4. HYDROXYPROLINE CONCENTRATION (CHLORAMINE T/ pDAB METHOD).


CATHEPSIN B HYDROLYSIS PRODUCT FROM BANA:
Fast Garnet (GBC) Method
Absorbance (520 nm)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.5
1.0
1.5
µMOLES ß-Naphthylamine


GLUCOSE STANDARD CURVE
ō-Toluidine Reagent (TR)
ABSORBANCE at 620 nm
1.5
1.0
0.5
10
20
30
40
50
µg GLUCOSE/ml TR


PROTEIN DETERMINATION:
LOWRY STANDARDS
ABSORBANCE (500nm)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
20
50
80
100
120
µg PROTEIN


HYDROXYPROLINE STANDARDS
A 560 nm
0.6
0.5
0.4
0.3
0.2
0.1
1.0
2.0
3.0
4.0
5.0
µg/ml Hydroxyproline