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The Secretion of Enzymes into the Pericellular Environment [and Discussion]

J. T. Dingle and D. H. Leaback

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The secretion of enzymes into the pericellular environment

By J. T. Dingle
Tissue Physiology Department, Strangeways Research Laboratory, Wort's Causeway, Cambridge, CB1 4RN

Connective tissue cells are capable of both synthesizing and degrading the macromolecular components of the extracellular matrix. The degradation of proteoglycan and collagen has been shown to be associated with the extracellular release of proteolytic enzymes, some of which are of lysosomal origin. The identity in cartilage of two previously unrecognized proteases, capable of proteoglycan breakdown (CPGases), has recently been achieved by the use of a new assay for proteoglycan degradation. These enzymes have been shown to be synthesized and released in response to vitamin A. The third proteoglycan degrading enzyme of connective tissue cells, cathepsin D, has been located in the pericellular environment by trapping with specific antibody and the pattern of release studied in organ culture, experimental arthritis and in human rheumatoid tissues. The secretion of this enzyme and possibly also of the other CPGases is thought to be of importance in the local (pericellular) turnover of matrix macromolecules and, in association with collagenase, to be the cause of the excessive degradation in the pannus erosion of articular cartilage in rheumatoid arthritis.

Introduction

The maintenance of the structure and function of connective tissues requires the continual synthesis and destruction of both cellular and extracellular macromolecules. The rate of turnover of the various molecules can vary widely, from hours to perhaps hundreds of days, nevertheless this catabolic activity of cells in organized tissues is as characteristic of life as is cell division.

The degradation of extracellular macromolecules is of special interest to those concerned with diseases of connective tissues. It seems reasonable, as a working hypothesis, to view both the increased breakdown seen during physiological remodelling and that associated with pathological damage as a local exaggeration of a normal process. The process itself is thought to involve the combination of extracellular enzyme action and concurrent, or perhaps subsequent, endocytotic activity and intracellular digestion. The extracellular digestion phase is believed to be due to secretion from physiologically active cells, of specific degradative enzymes. The identification of these enzymes, their mode of secretion and site of action has been one of the principle goals of the Strangeways Laboratory for the past decade (for review, see Dingle 1973).

The connective tissue cell and its environment

In considering the possible action of enzymes upon the components of the extracellular environment it is important to know not only the nature of the substrates, but their physical state and ionic environment. In most tissues only the first of these parameters is known even approximately. It is clearly difficult in a short review to summarize the recent information on the chemical nature of the extracellular environment, but fortunately this has been considered

in the paper by Dr J. Scott in this Symposium. The only feature that should be stressed here is the ubiquitous nature of the two principle macromolecules that are present in all vertebrate connective tissues, namely collagen and proteoglycan. Both these molecules differ to some degree in various tissues and species; thus collagen shows variability in the amino acid sequence of the α chains, and proteoglycan may differ in the length of the polypeptide backbone and in the nature of the carbohydrate side chains. Both molecules, however, display one feature in common which is important in the understanding of connective tissue degradation: both are susceptible to proteolytic digestion. This type of degradation would appear to be of primary importance in the initial phases of extracellular catabolism.

Until recently the degradation of collagen has been considered to be due exclusively to the action of specific collagenases, similar in nature to the original tadpole tail collagenase by Gross & Lapiere (1962). Although it is probable that this type of enzyme is the most important extracellular hydrolase concerned in the degradation of collagen, the recent discovery in this laboratory by Burleigh, Barrett & Lazarus (1974) that cathepsin B1, but not cathepsin D, is capable of extensive degradation of collagen, may be of physiological and pathological importance. Cathepsin B1 as will be seen below, is present in most connective tissues and has a wide specificity. It is important to realize that the so called 'specific collagenases', though they undoubtedly display a high affinity for the collagen molecule, are nevertheless proteolytic enzymes and may be expected to attack other groups in a polypeptide chain, if only slowly. Conversely, other proteolytic enzymes may degrade collagen, more or less effective than 'collagenase'. Thus in this laboratory it has been demonstrated that a 'specific' synovial cell collagenase is effective in activating the α_2-macroglobulin trap mechanism. This effect is believed to be due to proteolytic cleavage of part of the macroglobulin molecule (Barrett & Starkey 1973). These results lend weight to the view that the naming of proteolytic enzymes from a substrate they have been shown to degrade is at best unsatisfactory and has sometimes been misleading.

Catabolism of the proteoglycan molecule can occur at either the polysaccharide side chains or the protein backbone of the molecule. Since however, the physical size of the molecule is probably the most important factor in retaining it in the extracellular matrix, the effect of enzyme action upon the sugar chains would not be manifested as a change in the integrity of the matrix until considerable cleavage had occurred. If however even very limited proteolytic cleavage of the polypeptide backbone occurs, the subsequent diminished size of the molecule would allow it to diffuse readily through and out of the connective tissue matrix. This type of cleavage is probably important also in the degradation of the type of 'hypermolecule' envisaged by Dr Muir in cartilage (see this volume). It seems possible but perhaps unlikely that the hyaluronic acid link would be susceptible to the action of hyaluronidase even if this enzyme were found to be present in cartilage, a view which is shared by Muir (personal communication) since it seems likely that the hyaluronic acid would be spatially protected from attack by the close packing of the adjacent proteoglycan sub-units. It is interesting however, to speculate on a possible proteolytic attack on the glycopeptide link region on the Muir hypermolecule. If such an attack occurred it would effectively diminish the structural resistance to diffusion which is displayed by the very large molecule. Nothing is known at present about enzymes displaying this degree of specificity for the link region.

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The identification of degradative enzymes in connective tissue metabolism

The question of what substrate specificity tissue proteinases may display in the degradation of connective tissue matrix has been paid scant attention apart from collagenase, where it may even have been overemphasized. In this laboratory Dr A. J. Barrett and I have for some time recognized that the conventional methods for the assay of the activity of tissue proteinases, i.e. the use of haemoglobin, casein, etc., as substrates, was open to serious theoretical objection. From time to time we and others have used intact cartilage as a substrate in an attempt to look for enzymes capable of degradation of the matrix of this tissue; such methods are very crude and at best only semi-quantitative. Viscometric assay of proteoglycan degradation has often been used, this again suffers from the disadvantage of being only semi-quantitative and is also unsuitable for assaying many samples at one time, as is necessary for the purification of an enzyme. To overcome these difficulties we have developed what is known as the 'bead' assay for proteoglycan degrading enzymes (PGases).

Essentially the method consists of polymerizing an emulsion of acrylamide and ^{35}S-labelled proteoglycan. The resulting droplets are washed and dried and it has been shown that by adjusting the concentration of acrylamide, a consistent pore size is obtained which allows the retention of the undegraded proteoglycan molecule but allows both the free access of enzyme and the exit of partially degraded proteoglycan. The method has been found to be reproducible for a variety of pure proteolytic enzymes and a standard assay system has been developed. The sensitivity of the assay is approximately 50–100 times that of the conventional protease assays with haemoglobin as substrate and the results are highly reproducible. Use of the assay has led to the recognition of a new tissue proteinase, tentatively named cathepsin F. The assay has also demonstrated the presence of a cathepsin B1-like enzyme in cartilage. Cathepsin F does not display appreciable activity on other protein substrates, unlike cathepsin B1 and cathepsin D, and this presumably is the reason it has not been previously identified.

Cathepsin F, like cathepsin D, has an acid pH optimum and a relatively low molecular mass. It is not inhibited by any of the conventional proteinase inhibitors including pepstatin, soya bean trypsin inhibitor, leupeptin, EDTA, etc. At the present time the only inhibition is that seen with α_2-macroglobulin, which displays its 'universal' inhibitory activity, and some degree of inhibition with an extract from egg white.

At the present time, cathepsins D, F and B1 are probably the best characterized tissue proteinases of cartilage, but since they are all acid proteinases they clearly present a problem with regard to environmental pH when their possible extracellular activity is considered; this will be discussed further below.

We have searched actively for neutral enzymes capable of proteoglycan degradation and though these are present in synovial tissue, skin, polymorphonuclear leucocytes, etc., we have not yet been able to demonstrate their presence in cartilage. Indeed cartilage resorption may reach a level of 75 % loss of extracellular matrix without any cellular neutral PGase activity being demonstrable with the present methods. It may be recalled that the 'bead' assay is some 50–100 times as sensitive as conventional proteinase assays and it would seem very unlikely that appreciable activity would have been missed by this procedure unless the postulated enzyme was either inhibited or being secreted in very small amounts indeed. Clearly the neutral enzymes are of considerable importance in the acute phase of tissue regression, where infiltrating

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cells may change the enzyme pattern of the cartilage. It is apparent that in tissues which apparently do not possess these enzymes resorption still occurs readily and this presents a challenge to the investigator which may not be resolved until a better understanding of the pericellular environment is achieved.

Evidence for proteinase secretion

The initial evidence for extracellular secretion of tissue proteinases was provided by the work of Dingle & Fell (reviewed by Dingle 1973) who used vitamin A (retinol) to stimulate the resorption of chick limb-bone rudiments in organ culture. The secreted enzyme was identified as lysosomal cathepsin D. Much subsequent work has demonstrated that this is a true secretion of enzyme, i.e. it is not due to the release of enzyme upon cell death, but active synthesis is a necessary prerequisite for secretion of lysosomal enzymes by cartilage cells.

A number of ways of stimulating protease secretion were subsequently discovered. Those which proved most useful as possible models for *in vivo* secretion and degradation, include retinol administered at about 2–3 times the normal serum level; antigen–antibody reacting in the presence of complement; and the endocytosis of indigestible but otherwise inert material. All these types of stimulation of the cartilage result in extracellular matrix degradation with retention of cellular viability. At the same time that matrix resorption began, the presence of secreted cathepsin D was observed in the extracellular medium, and rapid resorption and secretion was accompanied by increased cellular levels of the enzyme. Ultrastructural studies (Glauert, Fell & Dingle 1969) indicated that enzyme secretion was often accompanied by hypertrophy of the Golgi system. Measurements of enzymes such as acid phosphatase showed that to some extent other lysosomal enzymes were secreted at the same time as cathepsin D, but problems of stability and inhibition in the culture medium have made it impossible to resolve the question of whether or not all lysosomal enzymes are secreted at the same time. The development in this laboratory of immunoenzymic methods for other lysosomal (and non-lysosomal) enzymes should eventually answer this question (see below).

Measurements of lysosomal enzyme *in vivo* (as opposed to organ culture *in vitro*) have not as yet yielded much information about proteinase secretion. There is undoubted evidence for lysosomal proteinases in rheumatoid synovial fluid and in serum, but the origin of such enzymes is open to question. For example the recent demonstration that platelets contain cathepsin D, E and possibly B1 (Gordon, Ehrlich & Dingle, unpublished) and that these enzymes may be readily released, placed much more doubt upon the origin of the values obtained for serum levels of lysosomal proteinases. Similar difficulties apply to synovial fluid where a variety of cells in various stages of degeneration make it very difficult to decide whether synovial enzymes are secreted or are simply a product of cell death. The study of enzyme secretion by immuno-cytochemical means (see below) to some extent overcomes this difficulty.

The relation of enzyme secretion to matrix degradation

In organ culture systems of both embryonic and post-foetal skeletal tissues the secretion of cathepsin D does not occur for some 36 h after administration of retinol. The proteinase is released from the matrix almost simultaneously with the proteoglycan. This does not prove that cathepsin D is responsible for the observed digestion of the matrix, but merely that the

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two events occur at approximately the same time. A similar temporal relation between matrix breakdown and enzyme secretion is observed when other means of stimulating resorption are used. Inhibition of breakdown is observed with hydrocortisone treatment and is also correlated with inhibition of proteinase secretion. The relation of cathepsin D secretion to matrix degradation is not only temporal but also spatial. This has been demonstrated by the use of immunoenzymic methods.

The preparation in this laboratory of antisera specific to cathepsin D has allowed immunocytochemical localization studies to be made (Poole, Hembry & Dingle 1974). As is to be expected, the conventional immunofluorescent staining of sections of cartilage or synovial tissue show that cathepsin D is located in lysosomal organelles in the cell cytoplasm. This technique does not give any information on the localization of secreted enzymes and a new method has been developed to investigate this problem. Briefly, the living tissue is incubated, either *in vivo* or *in vitro*, with specific anti-cathepsin D antiserum which traps the secreted enzymes in the approximate area in which they are released. It must be emphasized that the tissue is alive during this procedure and that the antiserum is not taken up to any extent by the cells, but remains as an immunoprecipitate in the extracellular environment. The formation of these immunoprecipitates takes place in a similar manner to that in an immuno-diffusion plate. At the end of the experiment, which may last between 1 and 24 h, the tissue is removed from the animal or from the organ culture, washed, fixed, sectioned and stained with a second antibody which is labelled with a fluorescent dye. The second antibody is usually either purified IgG or a digestion product of IgG, since this smaller fragment diffuses more readily into the tissue. The resulting immunoprecipitate localizes the released enzyme in a clear manner. This work has shown that not all cells in a tissue secrete enzyme at the same time, and the enzyme is often closely opposed to discrete areas of the cell membrane. It has been found that in a variety of situations, both physiological and pathological, greatly increased enzyme release is closely correlated with the areas in which the matrix is being actively eroded. This has been demonstrated in such diverse situations as retinol-stimulated cartilage in organ culture, *in vivo* in experimentally arthritic joints in the rabbit and in rheumatoid synovial pannus excised at synovectomy from the human knee joint. These last studies, which have been carried out in collaboration with a group at the Royal National Hospital for Rheumatic Diseases in Bath and with Dr Poole and Miss Hembry of this laboratory, have demonstrated a relation between the clinical state of the joint and the degree of enzyme secretion. This work has shown that synovial cells in the region of eroding pannus are the agents principally responsible for extracellular secretion of cathepsin D. Similar techniques are currently being applied in this laboratory to the localization of hyaluronidase, glucuronidase, collagenase and elastase.

The synovial cell as a source of extracellular enzyme

Werb & Reynolds (1974) of this laboratory have shown that monolayers of rabbit synovial cells, under certain conditions, secrete large amounts of active collagenase. Sufficient enzyme has been obtained in such culture medium to allow its purification and the subsequent raising of a specific antiserum against rabbit collagenase. The stimulation of collagenase secretion is occasioned by the feeding of indigestible material to the cells. Latex particles are particularly effective, but almost any non-digestible material will probably suffice. The material is endocytosed and accumulated in the lysosomal system in the normal manner, but a most curious

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feature of the cells' response is the high level of secreted collagenase which is not accompanied by a similar increase in lysosomal enzyme secretion, even though it is the lysosomal system which becomes grossly overloaded by the feeding of the latex particles. In this system there is also evidence of the secretion of a metal-dependent neutral proteinase which co-chromatographs with the collagenase and which readily degrades proteoglycans. This enzyme however, like the collagenase, is not thought to be of lysosomal origin. It is hard to understand the mechanism by which the synthesis and secretion of non-lysosomal enzymes occur by interference with lysosomal digestion. This is particularly curious because much work in this and other laboratories has demonstrated that when cartilage chondrocytes endocytose non-digestible material, they actively secreted lysosomal enzymes, an observation quite unlike that obtained with the synovial cell cultures.

Consideration of these findings led to the prediction (Dingle 1975) that interference with lysosomal function observed in lysosomal storage diseases should lead to the synthesis and secretion of collagenase. Experiments in collaboration with Dr W. Sly and Z. Werb have recently confirmed this prediction. Monolayers of human fibroblasts from several types of mucopolysaccharidases have been found to release very high activities of collagenase. The mechanism of the relation between lysosomal storage and the production of an important non-lysosomal enzyme requires urgent study.

Since synovial cells of the rheumatoid joint readily phagocytose insoluble materials, and in particular immune complexes, it is interesting to speculate further that the perhaps unique properties of the synovial cells in such a situation may result in the secretion of two enzymes, i.e. the neutral metal proteinase and collagenase which could initiate changes characteristic of pannus erosion.

Another important effect of synovial tissue in the catabolism of cartilage was observed by Dingle, Horsfield, Fell & Barratt (1974) who demonstrated that the presence of soft connective tissue obtained from the joint capsule increased the resorption of cartilage matrix in the presence of retinol. Biochemical analysis demonstrated that cartilage alone was capable of proteoglycan resorption but the breakdown of collagen only occurred when invading marrow on synovial connective tissue was present. These experiments also demonstrated that proteoglycan was lost from the tissue several days before collagen was released. This work emphasized the importance of the soft connective tissue in the process of matrix resorption and also indicated that the secretion of degradative enzymes (i.e. collagenase and proteoglycanase) may occur not only from different tissues but possibly at different times in the pathological process. It should perhaps be stressed again that all the above experiments were performed on living tissue and are not due to an autolytic release of enzymes, but to a secretory process almost certainly linked with the synthesis of fresh enzyme.

It would seem that the synovial cell is of considerable interest to those involved in the study of resorption mechanisms in articular tissues. Nevertheless the ability of the cells of isolated cartilage to degrade at least one component of the matrix and the apparent absence of collagenase or neutral proteinase in this tissue makes it valuable material in which to study the mechanisms of matrix degradation.

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The site of matrix degradation

The work briefly reviewed above indicates that cartilage contains at least 3 acid proteinases and no appreciable collagenase or neutral proteoglycanase. On the other hand synovial tissue and other soft connective tissues contain at least one neutral metal dependent proteoglycanase as well as collagenase, in addition to the full complement of acid cathepsins. The question which must be asked at this point is how and where do these enzymes act, either when resorption takes place in isolated cartilage or when synovial or other tissue interacts with the cartilage?

Since isolated cartilage appears to contain only acid proteinases the problem of intracellular pH is clearly very important. In any attempt to understand the possible action of the cathepsins (cathepsin D has an optimum of pH 5 on proteoglycan, cathepsin B1 of pH 5.5 and cathepsin F of 4.5 on this substrate) one is tempted to look for regions, perhaps very local, in which the pH could be between 5 and 6. The pH of intercellular vacuoles (secondary lysosomes) has been shown to change from approximately 7 to as low a value as 4.5 as the vacuole moves into the cell (Bainton 1973). This change in pH would allow the sequential action of the full complement of proteases within the lysosomal environment. The possibility that a similar local pericellular pH may be maintained at least temporarily, may be considered. Electron micrographs of connective tissue cells show local regions which might be described as indentation or partial infoldings which may be analogous to those observed in the osteoclasts. In this cell it has been suggested that such areas may provide a specific micro-environment in which acidic conditions may be maintained and which would allow resorption of mineral (Vaes 1969). Cartilage has a considerable anaerobic metabolism and releases much lactic acid. If the proton secretion occurred in the same local area as the enzyme secretion, in an analogous manner to the acidification of the lysosomal digestive vacuole, conditions might be suitable for acid catheptic activity. This is of course speculation and the evidence given by direct measurement of the intracellular pH (as opposed to that of the pericellular areas now being considered), does not provide direct support for this concept; as yet, however, it has not proved possible to make measurements for the area close to the cell surface where the concentration of hydrogen ions or organic acids might be presumed to be greatest. A definitive study of this problem is long overdue.

There is one other theoretical site of cartilage digestion which up to now has not been seriously considered by those studying matrix turnover. Investigations, particularly with the high voltage electron microscope (Glauert, personal communication), have shown that cells in cartilage may project long processes into the intercellular territory. Limited movements of the cell surface in relation to the extracellular matrix may thus be possible. If the surface of connective tissue cells contains bound enzymes as part of the cell membrane, these enzymes could be brought into contact with extracellular molecules, without being actively secreted into solution. Studies with enzymes bound to insoluble particles have shown that they are still capable of actively digesting large molecular substrates. If this type of digestion were operating at the cell/extracellular boundary mediated by proteinases attached to the cell membrane, as has been postulated in tumour cells, then the pericellular digestion of proteoglycan might be a quite different phenomenon from that previously envisaged, and it must be recognized that as yet we may not have discovered the nature of the enzyme or enzymes responsible. If this type of digestion takes place then the observed secretion of other lysosomal enzymes would be

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a secondary phenomenon, perhaps related to differences in interaction between the cell membrane and the altered (partially digested) pericellular matrix.

This speculation, and it must be emphasized that it is pure hypothesis, has been presented because the action of the present known and characterized enzymes of cartilage is still in doubt. This is due mainly to the results of experiments on the effects of inhibitors of cathepsin D or B1, on the resorption of living tissue. At present no effect of such inhibitors has been observed reproducibly. It has proved possible to inhibit completely or almost completely the autolytic breakdown of cartilage by the use either of antiserum specific against cathepsin D or of pepstatin, a very potent, low molecular mass inhibitor of cathepsin D (Dingle, Barrett, Poole & Stovin 1972). In this system therefore it is reasonable to believe that cathepsin D is the enzyme responsible for the breakdown of matrix. This type of experiment however is a very poor model for living tissue; not only are all the enzymes released at once in the autolytic system, but there is no synthesis of new enzyme or maintenance of pH or ionic conditions. In living tissue in which resorption has been stimulated, e.g. by vitamin A, many experiments have been made with a wide range of inhibitors for the known acid proteinase, serine proteinases, thiol proteinases and the metal proteinases, but without apparent effect on resorption. Various reasons may be advanced for the ineffectiveness of these inhibitors, thus α_2-macroglobulin which is an almost universal inhibitor of proteinases is clearly too large to penetrate the cartilage matrix, the same is probably true for the anti-cathepsin D IgG as it is presently being used, though future experiments with inhibitory fragments of the molecule might prove informative. It might be thought that the low molecular mass inhibitors such as pepstatin would readily enter the cartilage matrix, but recent experiments in collaboration with Barrett and Knight of this laboratory, using labelled pepstatin, have demonstrated that this molecule does not readily penetrate cartilage matrix, and thus lack of inhibition by this agent does not necessarily preclude the action of cathepsin D. It is possible that the other inhibitors that have been tried may also have difficulty in penetration or interaction with enzymes in the presence of the high concentration of substrate, i.e. proteoglycan, which is present in the matrix. Nevertheless these experiments do cast doubt on the function of the known extracellular soluble enzymes in the resorptive mechanism and one is continually reminded that unrecognized enzyme or enzymes may be present. The possibility also exists that extracellular digestion as we understand it may not occur to any great extent and that the diffusion of apparently degraded molecules from the matrix may in fact be due to the breakage of the link between proteoglycan and hyaluronic acid perhaps by some physicochemical means. There is absolutely no evidence for this suggestion, but the nature of the released proteoglycan does not entirely preclude the possibility. It might be suggested also that the increased catabolism of matrix in cartilage during the process of degradation, might be due exclusively to endocytotic activity of the cells and subsequent breakdown within the lysosomal systems. This has been postulated as the second phase of digestion (Dingle, Fell & Glauert 1969); in the present state of our knowledge, however, it seems unlikely to be the exclusive mechanism of action, since such an intracellular digestion would hardly explain the observed release of small proteoglycan molecules into the culture medium from cartilage undergoing resorption.

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Conclusions

It is clear from the above that we are not yet in a position to formulate the precise enzymic mechanism concerned in the degradation of cartilage matrix. We have been able to recognize three enzymes capable of such degradation which are present in cartilage and two other enzymes, notably collagenase and a neutral proteinase which are present when mixed tissue, i.e. pannus, is participating in the resorption. The extent to which any of these enzymes are acting either singly or synergistically is still uncertain. The search for other enzymes which may be capable of cleaving the proteoglycan backbone or the link region is continuing, and the methods that have been developed for the present study should allow their recognition if the appropriate extraction conditions can be elaborated. Up till now, the heterogeneity of the proteoglycan in cartilage has made analytical experiments on the nature of the fragments released somewhat unproductive. If a means for studying the precise changes in the proteoglycan molecule can be discovered, this might give a valuable clue to the nature of the enzyme or enzymes responsible.

Although in this paper attention has been directed deliberately to the uncertainties that still exist about the mechanism of cartilage degradation, this should not detract from the substantial advances that have been made in this field during the past few years. The identification of new enzymes capable of degrading proteoglycan and studies on the secretion of these enzymes are being actively pursued in this and other laboratories and the techniques now available should enable us to construct a more complete picture of the changes that occur during the resorption of cartilage in the near future.

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Discussion

D. H. Leaback (*Institute of Orthopaedics, Stanmore, Middlesex*). You seem concerned that your cartilage proteinases have acid pH optima but no detectable activities at neutral pH so that it is difficult to account for the action of such enzymes extracellularly. Is this a matter of the lack of sensitivity of your assay technique? Could there be residual activity at neutrality which you are not detecting and yet which could be significant physiologically? I ask because I work with glycosidases from cartilage which have acid pH optima and yet, with sufficiently sensitive assay techniques, I can still study such activities above pH 8.

J. T. Dingle. I do not think this is a question of sensitiveness, since the bead assay is some 50 to 100 times as sensitive as the conventional assays. One cannot of course discount the possibility of endogenous inhibitors.

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