COLLAGENOLYTIC ACTIVITY IN RAT BONE CELLS

Characteristics and Intracellular Location

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ABSTRACT

An assay system for estimating quantitatively collagenase-like activity present in bone cells has been developed as part of a more general investigation of mechanisms of bone resorption. Methods are described for preparing from bone a C^{14}-labeled collagen which is relatively pure and highly resistant to degradation by trypsin although readily broken down by bacterial collagenase. Collagenolytic activity in homogenates of bone cells harvested from rat metaphyseal bone was measured as the number of counts per minute released in ultrafiltrable form from the C^{14}-labeled collagen substrate after 40 minutes' incubation at 37°C and pH 7.3. Using these techniques, the presence of collagenase-like activity in whole bone cell homogenates was confirmed and the heat lability, partial cation dependence, pH optimum, and some other characteristics of the crude material were determined. Moreover, the major portion of the homogenate activity was found in a particulate fraction sedimenting in a centrifugal field between 700 and 15,000 *g*. The marked enhancement and solubilization of this activity by surface-active agents or freeze-thawing, together with the presence of considerable acid phosphatase activity in the same fraction and its sedimentation characteristics, suggested that it might be contained in lysosomes or similar bodies. The implications of these observations with respect to the physiology of collagen resorption in general and bone resorption in particular are discussed.

Though it is well known that skeletal tissue is constantly turning over, and the biosynthesis of new organic matrix by bone cells has been studied in some detail (2, 3, 5), little is known of the details of the resorptive mechanisms which form the other part of the turnover process. Such questions as whether mineral and matrix are removed simultaneously or sequentially, which of the bone cells are involved, and what factors control the over-all rate of breakdown still lack definitive answers. Although mechanisms potentially able to remove the mineral have been described, the presence in bone of enzyme systems capable of removing the components of the organic matrix have remained to be demonstrated (13).

Indeed, until recently, collagen—the major component of bone matrix as well as the most abundant protein in the body—appeared to resist the degradative action of all animal enzymes investigated unless it had been previously denatured. Specific collagenase, capable of degrading native collagen at neutral pH, had been found only in certain anaerobic bacteria. Then Gross and his coworkers, using tadpoles and tissue culture techniques, demonstrated the production of an enzyme which degraded salt-soluble guinea pig

skin collagen (10). This demonstration was followed a year later by a report from this laboratory of preliminary evidence suggesting the presence of an enzyme activity preformed in bone cells, capable of degrading bone collagen (17).

In the course of these earlier experiments (17) it became apparent that the method of assay employed was unnecessarily lengthy. Much more important, the method used to prepare the C^{14}-labeled collagen substrate from bone was open to criticism on the ground that the protein might have been denatured (by exposure to 0.1 N NaOH) in the course of preparation in such a way as to render it susceptible to degradation by non-specific proteases—a fact which necessitated a complete reexamination of the problem.

The present communication has been prepared, therefore, to describe (*a*) an improved method for the preparation of C^{14}-labeled bone collagen which results in a product without apparent denaturation, (*b*) a briefer assay, and (*c*) some experiments using these improved techniques which confirm the presence of collagenolytic activity in bone cells and suggest that it is confined in lysosome-like intracellular particles.

METHODS

Collagen labeled with C^{14} was prepared from rat bone as follows: Metaphyseal bone chips from 40 to 50-day old male rats of the Sprague-Dawley strain were incubated for 4 hours at 37°C in Krebs-Ringer bicarbonate buffered medium, pH 7.2, containing glucose 11.1 mM and proline-U-C^{14} 0.1 μc/ml. Carrier proline 0.5 mM was also present. In order to ensure that all the radioactive proline was passed out of the cells and into the collagen (3), the chips were washed and incubated in fresh Krebs-Ringer medium containing no radioactivity for a further 20 hours.

The bone fragments were then chilled to 3 to 4°C and kept at that temperature throughout all subsequent steps. After being washed thoroughly in medium and decalcified by shaking in three washes of 10 per cent EDTA for 24 hours, they were ground in a mortar for 5 minutes in 10 per cent EDTA solution in order to reduce the collagen to a fine suspension and to help get rid of adherent cellular material. Lipids were removed by shaking in a 1:1 mixture of ethanol and ether. Non-collagen proteins were extracted by shaking for 24 hours in 0.15 M NaCl. Finally, the collagen was washed with water and acetone and dried in air. The final product was divided into small portions which were kept frozen until used.

Bone cells for these experiments were also harvested from chilled minced metaphyseal bone from 40- to 50-day old male rats of the Sprague-Dawley strain. All operations were carried out at 3–5°C. Bone chips were ground in a mortar for 5 minutes with 1 to 3 times their volume of a medium containing NaCl 0.15 M, Ca^{++} 1.25×10^{-3} M, and P_i 1.25×10^{-3} M, adjusted to pH 7.4. The supernatant from this was decanted and allowed to stand for 30 minutes to allow fragments of calcified collagen to settle to the bottom. The supernatant solution from this step was then transferred with a pasteur pipette to a fresh centrifuge tube, and the cells suspended in it were sedimented by centrifugation at 1500 *g* for 10 minutes (4, 14, 17).

The cell button so obtained was suspended in approximately 10 times its volume of 0.25 M sucrose containing 1.0×10^{-3} M $MgCl_2$ and homogenized in a Teflon-glass homogenizer for about 3 minutes in the cold. Various cell fractions were then isolated by centrifugation. Nuclei, cell debris, and unbroken cells were removed by centrifugation at 700 *g* for 10 minutes. This has been called the "nuclear fraction." The supernatant from this was centrifuged at 15,000 *g* for 10 minutes, resuspended in fresh medium, and again centrifuged at 15,000 *g* for the same length of time. This sedimented a fraction containing mitochondria, rough endoplasmic reticulum, and smooth-walled vesicles which has been called the "large granule fraction." The supernatants from this procedure were finally centrifuged at 105,000 *g* for 60 minutes to remove remaining small particles which have been called the "small granule fraction." The particulate fractions were suspended in Krebs-Ringer bicarbonate medium at pH 7.3 for assay. The supernatant fraction was buffered and used directly.

Estimation of the amount of C^{14} label from the collagen substrate rendered ultrafiltrable by the presence of the preparation to be tested was again used as the assay system (17). However, the incubation time was reduced from 6 hours to 40 minutes, a time long enough for substrate breakdown by bacterial collagenase to be clearly demonstrable, yet too short either for the reaction rate of the system to become substrate dependent or for significant bacterial growth to occur even in contaminated systems. Total counts released in ultrafiltrable form in this system were directly proportional to the amount of enzyme present.

From the counts in the ultrafiltrate and the specific activity of the substrate, the actual amount of collagen rendered ultrafiltrable could be determined. Cell fractions for each experiment were standardized on the basis of their total nitrogen content measured by Nesslerization following micro Kjeldahl digestion.

RESULTS AND COMMENTS

Chromatography and chemical analysis of acid hydrolyzates (5) showed that collagen prepared from bone as described had an amino acid composition similar to that of mammalian collagen derived from other sources (12) and that all its radioactivity was contained in proline and hydroxyproline residues. Moreover, it was resistant to degradation by trypsin but was completely broken down by collagenase. The results of a time experiment illustrating this point are shown in Fig. 1. It was found that whereas trypsin released only a few counts and these only in the first minutes of incubation, collagenase continued to degrade the collagen in a linear fashion. Proline and hydroxyproline were found in equal amounts in the ultrafiltrate, and the process could be shown to continue until all the collagen was broken down.

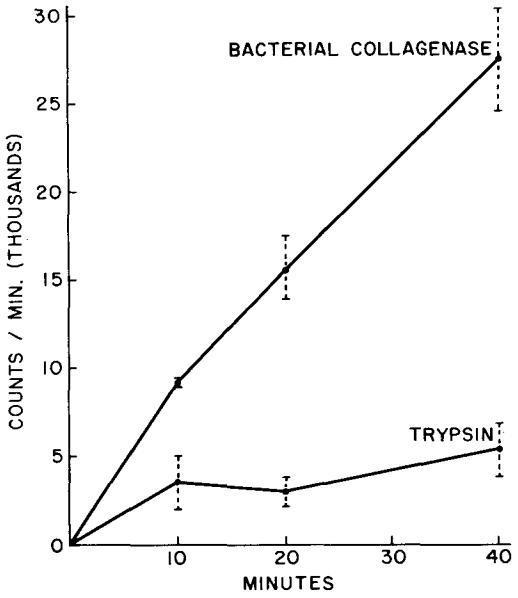

FIGURE 1 Degradation of collagen by bacterial collagenase and trypsin measured as counts per minute rendered ultrafiltrable after different periods of incubation at 37°C. Trypsin released a small amount of radioactivity in the first 10 minutes but had no significant effect thereafter; collagenase continued to act in a linear fashion during the full 40 minutes. The total amount of collagen present initially represented 110,000 to 150,000 counts per minute. 10 μg Sigma type I collagenase (*Clostridium histolyticum* enzyme) or 10 μg Sigma type I trypsin (from beef pancreas) were used in each flask. Each point represents the mean of 4 incubations (2 experiments), and the vertical bars indicate the limits of scatter of the results.

On the basis of these data three important conclusions regarding C^{14}-labeled bone collagen prepared as described could be drawn: The protein was relatively pure; the C^{14} label which passed the ultrafilter during the assay procedure could be considered to be derived virtually entirely from the degradation of this collagen; and, most important, the apparent resistance of bone collagen, so prepared, to degradation by trypsin indicated that the protein molecule was not significantly denatured in the course of isolation. Thus it seemed justifiable in subsequent experiments to ascribe breakdown of such labeled collagen, under conditions of physiologic pH, to specific collagenases rather than non-specific cathepsins.

The apparent purity and stability of the labeled collagen substrate having been established, the collagenolytic activity of homogenates of bone cells was reexamined in 11 experiments using the modified assay techniques described above. The homogenates used in these experiments were subjected to 5 minutes in a high speed Lourdes blender prior to assay, in view of previous experience (17) which indicated enhancement of activity following such treatment. The means of the results obtained are shown in Fig. 2. The data have been expressed as total counts rendered ultrafiltrable in 40 minutes of incubation. Twenty thousand to 40,000 CPM of collagen substrate in 0.5 ml of medium were used, with about 40 mg dry weight of homogenate where appropriate. It was immediately clear that the presence of cell homogenate increased 9-fold the mean release of ultrafiltrable radioactivity from collagen under these conditions. Although the variability from sample to sample was large, the difference between the two quantities was highly significant ($P<0.01$). Moreover, it was found in additional experiments that the apparent collagenolytic activity of such bone cell homogenates was completely abolished by heating to 70°C for 2 minutes and was inhibited 25 per cent by the addition of sufficient ethylenediamine tetraacetic acid (EDTA) to completely bind all the divalent cation in the incubation system.

Comparison with purified bacterial collagenase (Sigma type I) (Sigma Chemical Company, St. Louis, Missouri) revealed that 100 mg of dry homogenate contained activity approximately equivalent to that of 0.1 μg of dry enzyme. It should be noted, however, that this proved to be a minimal value for cell collagenolytic activity, for reasons which became apparent in subsequent experiments.

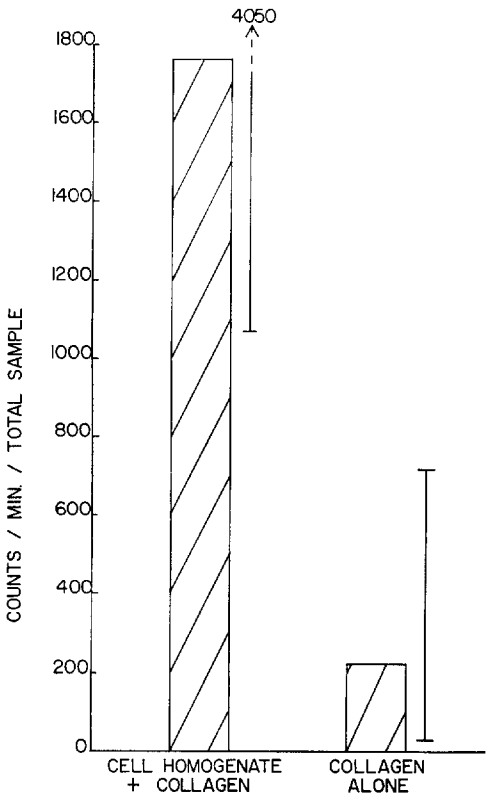

FIGURE 2 Radioactivity of ultrafiltrate derived from C^{14}-labeled collagen after 40 minutes' incubation with whole bone cell homogenate (about 40 mg dry weight in 0.5 ml Krebs-Ringer medium) and alone in 0.5 ml Krebs-Ringer medium. The total amount of collagen present initially in each incubation flask represented 20,000 to 40,000 counts per minute, and the results are expressed as counts per minute per total ultrafiltrate from each flask. The vertical bars indicate the range of results obtained from 11 experiments. The difference between the two columns is highly significant ($P < 0.01$).

The presence of collagenolytic activity in bone cell homogenates having been established it seemed desirable to reexamine the observation made in early experiments that this activity appeared to increase with time and violence of treatment in the blendor—an observation which first suggested that the enzyme might be associated with a subcellular particle. Bone cell homogenates were divided, therefore, into 4 fractions by differential centrifugation, and the collagenolytic activity of each was measured after 5 minutes in the blendor. The results of these experiments are shown in Fig. 3.

At this point it seemed important to settle upon some means of expressing experimental results which would permit quantitative comparisons of activities to be made. In order to compare the relative activities of the various subcellular fractions, calculation of the data on the basis of a milligram of the total nitrogen of each fraction—a quantity which could be measured more rapidly and accurately than the dry weight—seemed a simple and useful procedure. Moreover, estimation of collagenolytic activity in terms of equivalence to bacterial collagenase, though of general interest, appeared undesirable for two reasons. The available relatively crude bacterial enzyme varied considerably in activity from batch to batch, and the use of such a standard required additional controls in each experiment. It seemed much simpler to express activity directly in terms of milligrams of collagen substrate degraded per assay period, a value easily calculated from the total counts in the ultrafiltrate and the specific activity of the substrate used.

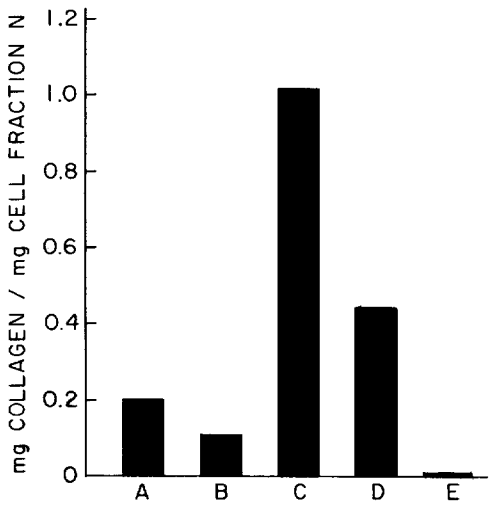

FIGURE 3 Relative collagenolytic activity of various fractions obtained from bone cell homogenates expressed as milligrams of labeled collagen degraded per milligram of nitrogen contained in the fraction. Columns represent the mean of 8 samples in 4 experiments. A, cell homogenate; B, nuclear fraction; C, large granule fraction; D, small granule fraction; E, supernatant. C and D are significantly greater than the others ($P < 0.01$), and C is significantly greater than D ($P < 0.01$). It should be noted that the *total* activity of any fraction of a given homogenate would be represented by the product of its relative activity and the total nitrogen contained in it.

Returning to Fig. 3, it can be seen that the activity was confined virtually entirely to the particulate fractions within the cells. The large granule fraction (sedimented between 700 and 15,000 *g*) had the highest relative activity, more than twice as large as that of the small granule fraction (15,000 to 105,000 *g*). Although the nuclear fraction (1 to 700 *g*) appeared to contain some activity, it was relatively very low, and the supernatant fraction (that remaining after 1 hour at 105,000 *g*) contained no detectable ferment at all. It must be noted, however, that a small amount of activity in this fraction (which represents the soluble cell proteins) has been found in other experiments. It probably was not detected in these experiments because of excessive dilution due to the accumulated volume of washings from previous steps in the fractionation procedure.

These data were of considerable interest from two points of view. They appeared to confirm the concept that the collagenolytic activity of bone cells was largely associated with intracellular particles. Moreover, the particulate fraction showing the greatest relative activity was sedimented in about the same centrifugal field as the lysosomes in liver described by de Duve and his collaborators (1), a fact which suggested that the collagenolytic activity might be contained in lysosomes or something very similar.

The effects of treatment of the large granule fraction with surface-active agents or freeze-thawing supported this view, as can be seen from the data in Fig. 4. In these experiments the large granule fraction was not treated in the blendor. Instead, it was assayed as isolated after treatment with saponin, Triton X-100, or thrice repeated freeze-thawing. It can be seen that the apparent activity of this fraction was increased approximately 3-fold by both saponin and Triton X-100, and that freezing and thawing had a similar but slightly smaller effect. Thus not only were the particles containing collagenolytic activity precipitated under the same conditions as lysosomes, but in addition the activity in this fraction was released or unmasked by the same agents which are known to release the lysosomal acid hydrolases.

Two further pieces of evidence were obtained which bore upon the question of the nature of these particles. These are shown in Figs. 5 and 6.

A fairly typical electron micrograph of the large granule fraction is shown in Fig. 5. The bulk of this fraction consisted of vesicles of the rough endoplasmic reticulum of various sizes, some of which

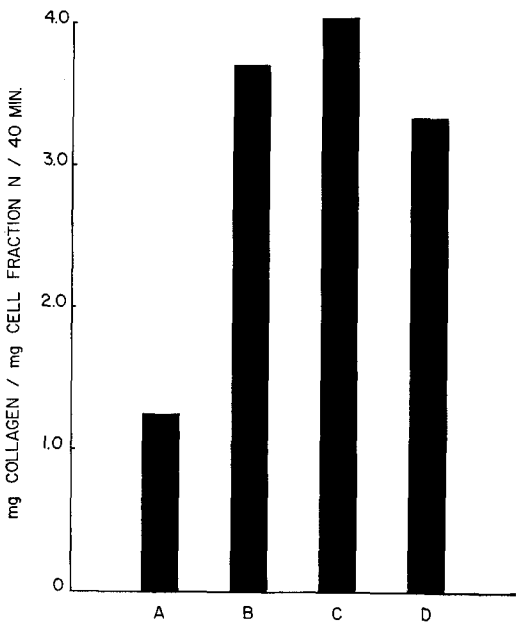

FIGURE 4 Amount of collagen degraded, per milligram of nitrogen, of large granule fraction after no treatment (A) and after treatment of the fraction with: saponin 1.5 per cent (B), Triton X-100 0.1 per cent (C), or freezing and thawing 3 times (D). Each column represents the mean of 3 experiments. Activity with both surface-active agents and freeze-thawing was significantly greater ($P < 0.01$) than in the untreated controls, but no significant difference among the three was found.

appeared to contain amorphous material whose nature is not yet known. A few mitochondria could also be identified in some fields. In addition, smooth vesicles of varying size and density were seen which did not have RNP particles adherent to their limiting membranes. Some of these appeared to contain myelin figures or fragments of membrane, or were simply filled with amorphous material (15). Though it is tempting to suggest that these bodies are the bone cell lysosomes recently identified by Vaes (16), proof of this idea is lacking at present. Moreover, evidence localizing the collagenolytic activity of the fraction to one or another of the structures visualized remains to be obtained.

The distribution of acid phosphatase activity—the hallmark for identification of lysosomes—seen in the various centrifuge fractions of the bone cell homogenates is shown in Fig. 6. This was measured as the release of inorganic phosphate from β-glycerophosphate at pH 5.1. All fractions were treated

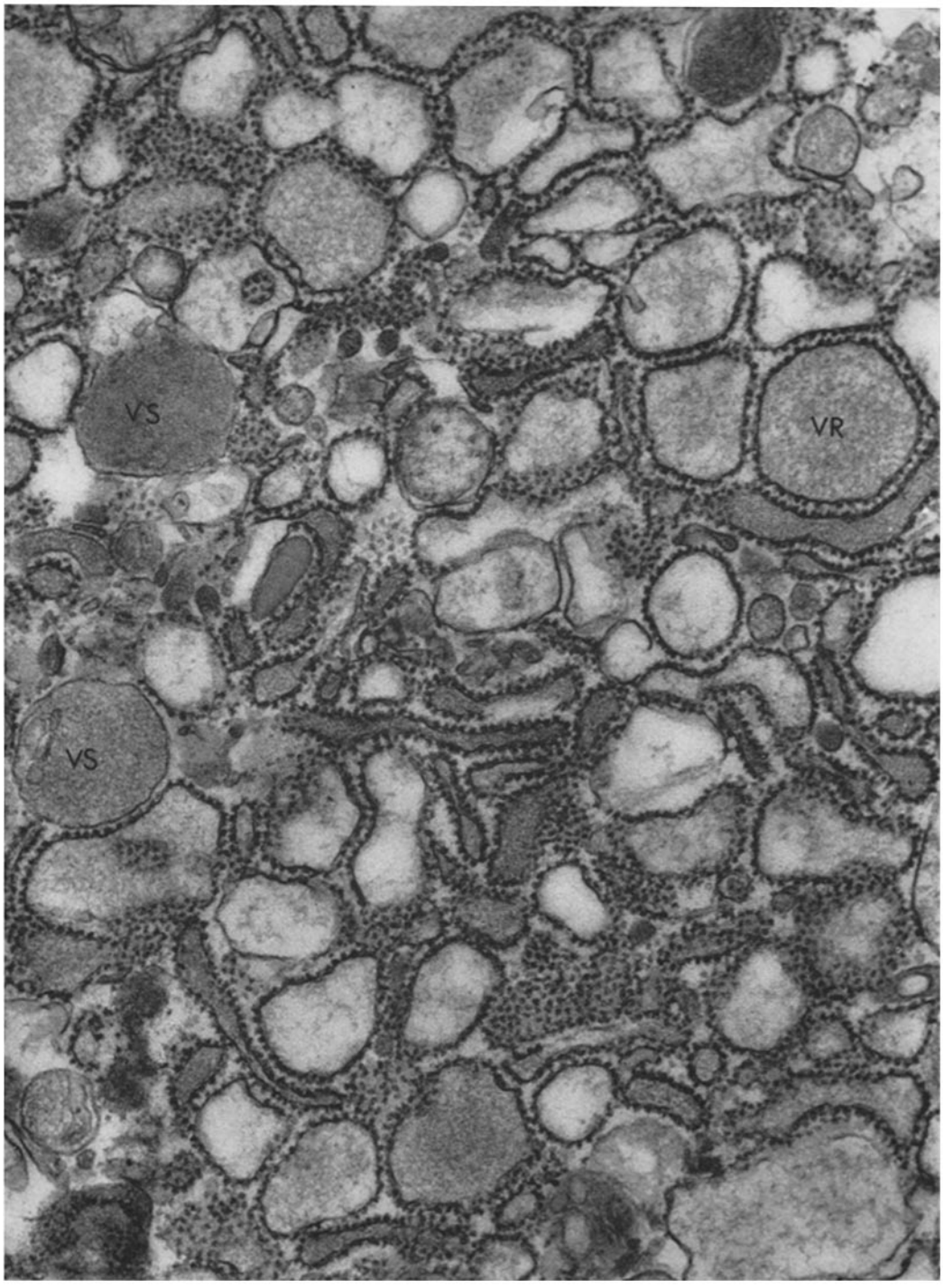

FIGURE 5 Electron micrograph of a section of a "large granule" pellet showing examples of the two types of vesicles which make up the bulk of this fraction: *VR*, vesicle of the rough endoplasmic reticulum; *VS*, smooth vesicle. A few mitochondria were also found in some fields. Pellet was fixed overnight in phosphate-buffered formalin, pH 7.4, stained with osmium tetroxide, and embedded in Vestopal. × 34,500.

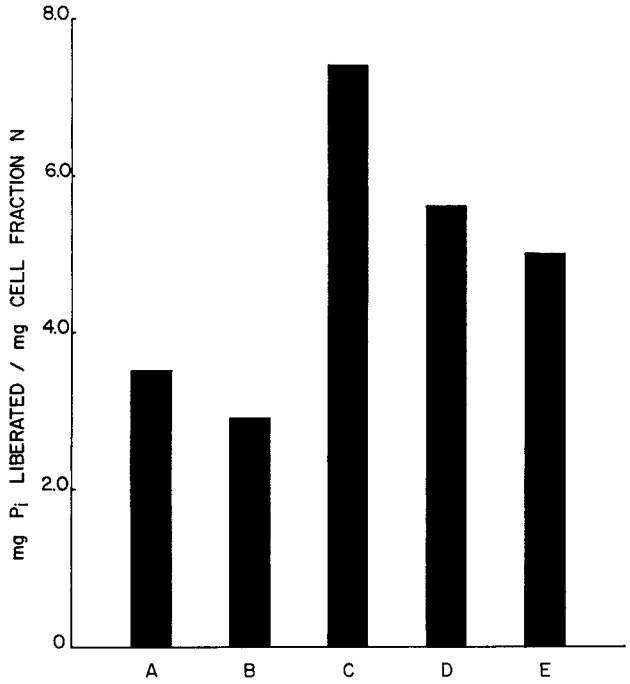

FIGURE 6 Relative acid phosphatase activity of various fractions of bone cell homogenates measured in terms of milligrams of inorganic phosphate released from β-glycerophosphate at pH 5.1 after 30 minutes' incubation at 37°C. A, whole homogenate; B, nuclei; C, large granule fraction; D, small granule fraction; E, supernatant. Each column represents the mean of 2 incubations. All fractions were treated with saponin 1.5 per cent.

with saponin in these experiments. It can be seen that although the relative acid phosphatase activity was highest in the large granule fraction, considerable activity was also found in the small granule and supernatant fractions.

Although the peak relative activities of both the collagenolytic and the acid phosphatase systems were found in the large granule fraction, which also appeared to contain bodies with some characteristics of lysosomes, it must be emphasized that these data cannot be interpreted as indicating the presence of both ferments in the same particle. Indeed, the differences in distribution of activity of the two ferments between large and small granule fractions suggest either that two types of "lysosomes" are present in these cells, only some of which contain both enzymes, or that the two enzymes are contained in quite separate particles.

Implicit in the lysosome concept is the idea that the lysosome hydrolases are released in soluble form when the membranes which contain them are ruptured or destroyed by surface-active agents. In order to see whether or not the increase in collagenolytic activity of the large granule fraction induced by saponin and other substances was due to the release of soluble enzyme, the large granule fraction was treated with saponin at 4°C for 5 minutes. A particulate and a supernatant fraction were then separated by centrifugation at 105,000 *g* for 60 minutes, and each was assayed for collagenolytic activity. The results of these experiments are shown in Fig. 7. Considerable activity could be found not only in the supernatant but also in the precipitate. Thus, although it was clear that saponin released some collagenolytic activity in a soluble form, another portion of activity seemed to remain bound to the particles. The precise meaning of these data is unclear. Whether there are two types of collagenase—one soluble, the other particle bound—in the large granule fraction or whether the saponin treatment was too short or carried out at too low a temperature to lyse all the membranes of the fraction are questions which remain to be answered.

In addition to the experiments with whole bone cell homogenates in which thermolability and partial cation dependence were examined, experiments to determine some further characteristics of this collagenolytic system were carried out using the relatively purer activity found in the large granule fraction. Saponin was present in the assay system in these experiments in order to make sure that total enzyme activity was estimated and to eliminate any effects due to changes in membrane stability alone.

The influence of temperature of incubation on

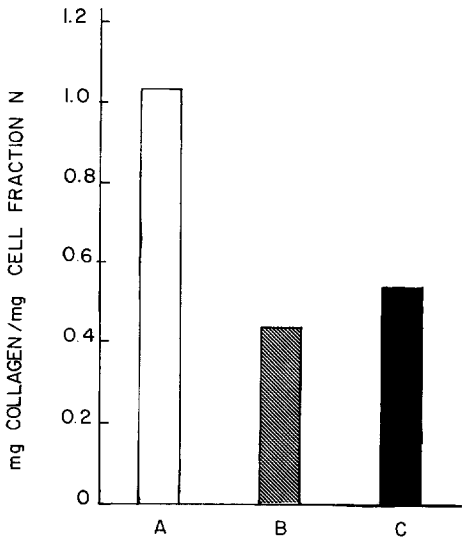

FIGURE 7 Relative collagenolytic activity of a whole large granule fraction (A) treated for 5 minutes at 4°C with 1.5 per cent saponin, and of the pellet (B) and supernatant (C) from centrifugation of the same fraction for 60 minutes at 105,000 *g*. Considerable activity was found in both supernatant and pellet.

activity is shown in Fig. 8. At 37°C the activity was increased 4.5 times over what was seen at 22° or 1°C. Indeed, at the lower temperature the additional radioactivity above that of controls rendered ultrafiltrable by the presence of the enzyme preparation was barely measurable.

Collagenases from bacterial and animal sources (9, 10, 12) characteristically have pH optima between 6 and 8. In order to determine whether this was also true for the activity in bone cells, the system was tested over a pH range of 5 to 8, using phosphate buffers. The pH of the flask contents was measured before and after incubation and was found to vary no more than 0.3 when the large granule fraction of the cells was used.[1] The results of these experiments are shown in Fig. 9. The differences in the amount of ultrafiltrable collagen between the controls and experimental incubations have been plotted, and the graph therefore illustrates collagenolytic activity independent of the physical conditions of the assay. Plotting the results in this way was important, for when the pH of the assay system was below 5 or above 8 the collagen substrate appeared to undergo partial spontaneous breakdown. This resulted in the release of variable but significantly increased amounts of radioactivity in the control flasks. The pH optimum of the collagenolytic activity over the range in which it could be tested can be seen to have been about 6, with considerable but less activity at pH 7 and 5. Below pH 5 and above pH 8 the net activity appeared to be virtually zero.

Two other characteristics of the ferment have been investigated in a preliminary way. The tight binding of the bacterial and tadpole collagenases to collagen (6, 9, 10, 12) appears to be a characteristic of the bone cell enzyme also. Labeled collagen was shaken at 4°C for 5 minutes with saponized preparations of the large granule fraction, washed repeatedly with large volumes of fresh cold medium, and incubated for 40 minutes at 37°C in small volumes of medium. In each of two experiments significantly more counts were released from

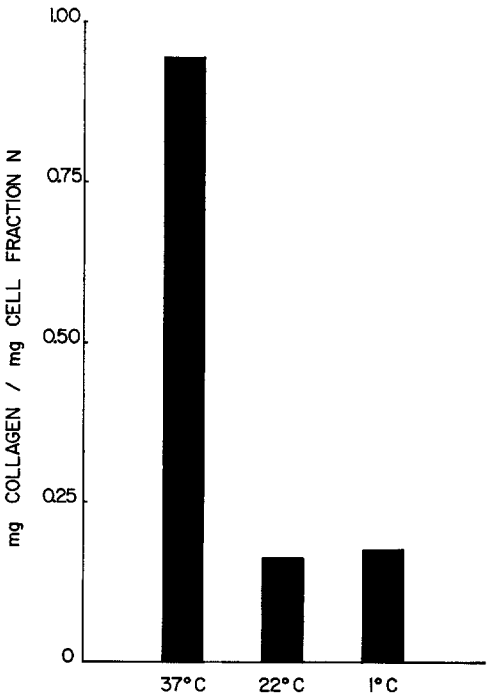

FIGURE 8 The effect of temperature of incubation upon the collagenolytic activity of the large granule fraction of the cells was determined by incubating flasks at 37°, 22°, and 1°C. Each column represents the mean of 4 incubations, except the 1°C column, which represents the mean of 3. The amount of collagen degraded at 37°C was significantly greater than at either 22° or 1°C ($P < 0.01$).

[1] Much more difficulty in maintaining a stable pH was encountered when whole cell homogenate was used for the assay, the final pH in such experiments usually being between 6 and 7.

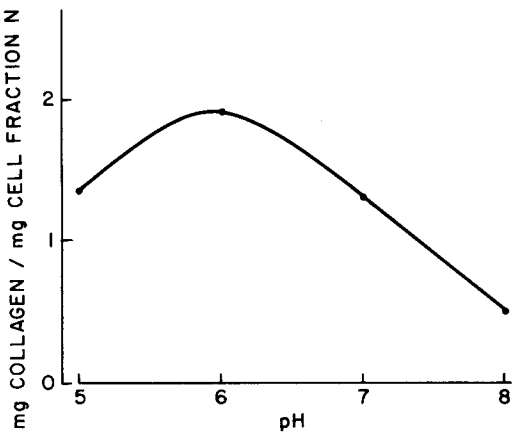

FIGURE 9 Activity of cell fractions following treatment with saponin, when incubated with collagen over a pH range in phosphate-buffered media. Each point represents the mean of 4 incubations. Significantly more activity was found at pH 6 than at pH 5 or 7 ($P < 0.05$).

such treated collagen than from untreated collagen controls.

Finally, the effects of bacterial collagenase inhibitors, such as $HgCl_2$ 2×10^{-4} M, $CuCl_2$ 10^{-3} M, and cysteine 10^{-2} M, on the collagenolytic activity in the large granules were examined in duplicate experiments using a single enzyme preparation. No inhibition was demonstrated, and indeed cysteine seemed, if anything, to enhance activity. Further experiments using different concentrations of these agents will be needed, however, before their lack of effect can be taken as evidence of differences between the bacterial and bone enzymes.

DISCUSSION

Collagen, although apparently a relatively stable fibrous protein highly resistant to non-specific proteolytic digestion in its native state, is nevertheless denatured quite rapidly by heat or by exposure to solutions at pH's above 8 and below 5. Once denatured, it becomes susceptible to degradation by most non-specific cathepsins. Thus stringent requirements must be met with respect both to the preparation of the collagen substrate and to the conditions of assay if the degradation of this substrate is to be taken as evidence of the presence of collagenolytic activity at neutral pH—the only type of collagenolytic activity which, by the same token, can be considered truly specific. As Mandl (12) has pointed out, neglect of these essential requirements has been responsible for many reports claiming to have demonstrated collagenase in various mammalian tissues which subsequently could not be substantiated. It was with these ideas in mind that the methods reported here were developed. As has been noted, it proved possible to isolate from bone a collagen substrate which was reasonably stable to at least one non-specific protease, trypsin, and which also contained sufficient C^{14} for adequate assay sensitivity, by careful control of pH and maintenance of low temperature during the procedure. The importance of controlling pH was well illustrated by the apparently spontaneous release of counts from this collagen in incubations at pH's above 8 or below 5. Similarly, pH and temperature were closely controlled during the assay, and the time of incubation was kept sufficiently short to eliminate contamination with growing bacteria or limited substrate availability as significant causes of erroneous results.

Bone collagen was selected as the substrate in these experiments for several reasons. Methods for labeling it with C^{14} *in vitro* had already been developed and evidence was in hand indicating that residual label in the cells of the bone samples could be removed by "chase" techniques. Moreover, the known insolubility of any significant fraction of bone collagen in salt solutions permitted extensive washing and extraction procedures to be carried out under suitable conditions without fear of losing the newly synthesized and therefore most highly labeled fraction of the substrate. Finally, since the collagenolytic activity being investigated was the one presumed to be in bone cells, it seemed desirable to examine its capacity to degrade the substrate which it would be expected to attack under natural conditions. The ability of collagen in bone to calcify as well as to resist solubilization has long suggested differences between it and collagen found in other connective tissues, a view which has now been elegantly confirmed by the work of Glimcher and co-workers (7, 8). In addition, it was clear that the collagenase of bone cells would of necessity have to attack solid collagen fibers if it were concerned in bone resorption. Therefore, the use of a solid substrate in the assay systems rather than collagen in solution seemed to provide a more precise model of the natural events under study. It should be noted that the use of a solid substrate in an enzyme assay introduces an additional set of problems in the standardization of the assay, since particle size and therefore the surface

area available for enzyme binding may be very important in determining apparent reaction rate. This proved to be the case in these experiments and considerable care had to be taken in preparing batches of labeled collagen to ensure that the particles were sufficiently small and relatively uniform. However, with suitable precautions results of reasonable reproducibility could be easily obtained.

A unique feature of these experiments is that they demonstrate the presence preformed in bone cells of a system capable of degrading collagen to ultrafiltrable fragments. In contrast, all previous experiments demonstrating animal collagenase (9–11) have depended on *de novo* synthesis or release of the enzyme from fragments of viable tissue maintained under tissue culture conditions. Indeed, Gross has reported the repeated failure of attempts in his laboratory to extract an active collagenase from tissues in which considerable activity could nevertheless be demonstrated in culture (9). Although the reasons for the differences between Gross's experience and the present experiments cannot be stated with certainty, they may well lie in the differences between the methods used to extract the activity from the tissue and in the avidity with which collagenase binds to its substrate. In the experiments reported here, the collagenase-like activity measured was found encased in particles derived from homogenates of cells which were intact at the time they were separated from the calcified collagenous bony matrix. Thus any contact between this "collagenase" and the preformed collagen of the tissue to which it might bind was effectively prevented. In Gross's experiments, on the other hand, attempts to extract active cellular collagenase were made in the presence of liberal amounts of tissue collagen—a situation in which the small amounts of preformed cellular collagenase might be expected to be effectively sequestered.

A number of features of the collagenolytic activity described in these experiments suggest that it is indeed due to an enzyme which bears at least a close resemblance to the skin enzyme described by Gross (9, 10) and to bacterial collagenase. These features include its marked temperature dependence, thermolability, need for divalent cations for maximum activity, and ability to bind tightly to its collagen substrate—all characteristics of the purer preparations of these enzymes. Other features suggest that the bone cell enzyme may be somewhat different. These include its slightly lower pH optimum and its apparent resistance to inhibition by Hg, Cu, and cysteine in concentrations which inhibit the *Clostridium* enzymes.

Though the final answers to these questions will have to await purification of the bone enzyme, its pH optimum of 6 is of particular interest in relation to bone metabolism. Bone cells are known to produce large quantities of lactic and other acids (13), a production which is stimulated by factors such as parathyroid hormone which increase bone resorption. It is likely, therefore, that the pH of the environment at the resorbing surface in the bone is considerably lower than that of the rest of the extracellular fluid and may well be in the vicinity of 6.5 or even lower.

Perhaps the most important observation reported here is the demonstration that this enzyme activity is seemingly confined in a particulate fraction within the bone cell from which it can be released by physical means or surface-active agents. The resemblance between the fraction containing maximal activity and lysosomes in terms of behavior in a gravitational field not only seems to fit with Vaes' observations but suggests that the collagenase of bone cells is contained in the very same bodies which contain the acid hydrolases which he has described (16). On the other hand, the slightly different distribution of acid phosphatase and collagenolytic activity among centrifuge fractions may indicate that more than one type of lysosome-like body is present. A number of possibilities with respect to the biosynthesis and release of collagenase and other hydrolases in bone cells and their relationships to the bone resorptive process are suggested by these observations—possibilities which will require further experiments to evaluate.

Though it is obviously too early to define precisely the manner in which the collagenase activity is related to bone resorption, it now seems clear that the bone cells contain, preformed, an enzyme capable of degrading collagen in its native state at physiological pH. Thus the mechanism vital to the turnover of the most abundant protein in the body and the connective tissues of which it forms the structure has been shown to exist. On the basis of these observations it seems safe to predict that this enzyme will be found, at least in trace quantities, in all tissues in which collagen turnover exists, once assays of sufficient sensitivity to detect it have been perfected.

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