Isolated Osteoclasts Resorb the Organic and Inorganic Components of Bone

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Abstract. Osteoclasts are the principal resorptive cells of bone, yet their capacity to degrade collagen, the major organic component of bone matrix, remains unexplored. Accordingly, we have studied the bone resorptive activity of highly enriched populations of isolated chicken osteoclasts, using as substrate devitalized rat bone which had been labeled in vivo with L-[5-^{3}H]proline or ^{45}Ca, and bone-like matrix produced and mineralized in vitro by osteoblast-like rat osteosarcoma cells.

When co-cultured with a radiolabeled substrate, osteoclast-mediated mineral mobilization reached a maximal rate within 2 h, whereas organic matrix degradation appeared more slowly, reaching maximal rate by 12–24 h. Thereafter, the rates of organic and inorganic matrix resorption were essentially linear and parallel for at least 6 d when excess substrate was available.

Osteoclast-mediated degradation of bone collagen was confirmed by amino acid analysis. 39% of the solubilized tritium was recovered as trans-4-hydroxyproline, 47% as proline. 10,000 osteoclasts solubilized 70% of the total radioactivity and 65% of the [^{3}H]-trans-4-hydroxyproline from 100 μg of 25–50-μm bone fragments within 5 d. Virtually all released tritium-labeled protein was of low molecular weight, 99% with $M_r \leq 10{,}000$, and 65% with $M_r \leq 1{,}000$. Moreover, when the 14% of resorbed [^{3}H]proline-labeled peptides with $M_r \geq 2{,}000$ were examined for the presence of TC^A and TC^B, the characteristic initial products of mammalian collagenase activity, none was detected by SDS PAGE. In addition, osteoclast-conditioned medium had no collagenolytic activity, and exogenous TC^A and TC^B fragments were not degraded by osteoclasts. On the other hand, osteoclast lysates have collagenolytic enzyme activity in acidic but not in neutral buffer, with maximum activity at pH 4.0. These data indicate that osteoclasts have the capacity to resorb the organic phase of bone by a process localized to the osteoclast and its attachment site. This process appears to be independent of secretion of neutral collagenase and probably reflects acid protease activity.

BONE resorption is a continuous, finely regulated process, fundamental to growth and remodeling of the skeleton and vital for maintenance of circulating [Ca^{++}] within narrow limits in land living vertebrates (20). This process, which involves solubilization of bone mineral and hydrolysis of dense bone collagen, is associated with the presence of osteoclasts, multinucleated cells derived from as yet uncharacterized hematopoietic precursors, thought to be members of the monocyte-macrophage family (1, 16).

Although circumstantial evidence strongly suggests that the osteoclast is pivotal to the resorptive process (14), the precise means by which the cell degrades bone, or if in fact it has the capacity to resorb both the inorganic and organic phases of bone, is unknown. This enigma probably reflects the complexity of in vivo and organ culture models which, until recently, were the bases of virtually all bone resorption experiments. Because these models contain numerous cell types, including osteoclasts, it has been difficult to attribute a particular role in bone resorption to a specific cell type. For example, enzymes are released into organ culture medium during the resorptive process (8), but the cells that produce these proteins have not been identified. More fundamentally, a functional role for these factors in bone resorption has yet to be confirmed.

Hence, the means by which osteoclasts degrade bone are unknown and, in fact, controversy exists regarding the collagenolytic capacity of these cells. Although it had been assumed that they degrade bone collagen, Heersche hypothesizes that osteoclasts mobilize only the inorganic phase of bone, whereas subsequent organic matrix degradation is a function of another cell type such as the fibroblast (12). Although his hypothesis is largely based on morphological observations, it has been strengthened by the fact that fibroblasts from other tissues can synthesize a neutral collagenase (22). Furthermore,

Heath et al. have recently shown that rat osteoblasts produce an apparently identical neutral collagen-degrading metalloprotease under the influence of parathyroid hormone (11).

Establishing the capacity of a bone cell to degrade specific matrix components therefore requires a system in which a given cell is present in sufficient purity to ensure that the appearance of matrix degradation represents the activity of that cell type. The recent development of techniques of Zambonin-Zallone et al. by which osteoclasts can be isolated and maintained in culture provides this opportunity (26). In the paper we describe the use of highly enriched populations of osteoclasts to assess the capacity of these cells to degrade the organic matrix of bone. We report that these cells do, in fact, have the capacity to resorb bone collagen, and appear to do so in a manner that reflects acidic protease activity and is independent of the vertebrate neutral collagenase.

Materials and Methods

All reagents were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise stated. Culture media were from Gibco (Grand Island, NY) and contained 100 U/ml of penicillin and streptomycin.

Isolation of Osteoclasts

Cultures of chicken osteoclasts were established using modifications of the methods of Osdoby et al. (18) and Zambonin-Zallone et al. (26). This method uses mature laying hens (*Gallus domesticus*) which were fed a calcium-deficient diet (Purina Test Diets, Richmond, IN) for 7 d before cell isolation. Hens were killed by CO_2 inhalation. The femurs and tibias were removed and placed into ice-cold calcium and magnesium-free phosphate-buffered saline, pH 7.2, containing 0.1% albumin (PBS); all subsequent steps using PBS were performed at 0–4°C. The bones were then split to expose the marrow and rinsed three times with PBS. The endosteum was then scraped with 18-gauge hypodermic needles into 50 ml PBS, and the resulting cell suspension was passed through loosely packed glass wool to remove large fragments, centrifuged for 10 min at 650 *g*, and resuspended in 5 ml PBS. This cell suspension was layered onto a seven-step (5 ml each) Percoll (Pharmacia AB, Uppsala, Sweden) gradient (1.01 to 1.07 g/ml) in a 50-ml centrifuge tube, centrifuged for 20 min at 650 *g*, and the fractions were collected by aspiration. The 1.05 g/dl fraction was washed twice with PBS and plated in tissue culture dishes (Corning Glass Works, Corning, NY) at up to 5,000 osteoclasts/cm^2, in Eagle's minimal essential medium, α-modification (α-MEM)[1], containing 10% heat-inactivated fetal calf serum. The plates were incubated at 37°C in 5% CO_2 and after 24 h were rinsed to remove nonattached cells (predominantly erythrocytes). In some experiments, osteoclast-rich fractions from the Percoll gradient were further enriched by differential attachment on devitalized bone. Cells were incubated with devitalized bone fragments for eighteen hours at 37°C. The bone fragments and attached osteoclasts were then separated from other cellular material by unit gravity sedimentation in PBS for 10 min at 25°C.

Other Cell Cultures

Rat peritoneal macrophages were isolated and cultured as previously described (23). Human embryonic lung fibroblasts AG4393 were obtained from the NIA Aging Cell Repository (Camden, NJ), and rat osteosarcoma cells UMR-106-06 were kindly provided by Dr. T. J. Martin (University of Melbourne, Melbourne, Australia).

Purity of Cultures and Cell Number

Cells were counted under a phase-contrast microscope using a hemacytometer; osteoclasts were distinguished from contaminating cells (principally red blood cells) by size and morphology. The identity of cultured cells, attached to plastic or bone fragments, was confirmed by histochemical staining for acid phosphatase activity, ultrastructural morphology, and the binding of fluoresceinated osteoclast-specific monoclonal antibody (19).

Labeling of Substrates

^{45}C and [^{3}H]proline-labeled devitalized rat bone fragments, 23–45 μm, were prepared essentially as previously described (23). The proline-labeled bone was obtained from 60-g weanling rats injected intraperitoneally on alternate days with 1 mCi of L-5-[^{3}H]proline (Amersham Corp., Arlington Heights, IL) for 10 d and killed on day 12. Osteosarcoma cell-produced substrate was prepared according to a method developed by Dr. T. J. Martin. This approach entails plating 5×10^5 UMR-106-06 cells in 0.5 ml of α-MEM containing ascorbic acid 100 mg/liter, 10^{-10} M 1,25-dihydroxyvitamin D (provided by Dr. Milan Uskokovic, Hoffmann-LaRoche, Inc., Nutley, NJ), 1 μCi/ml L-[5-^{3}H]proline, and 10 mM β-glycerol phosphate, and 4% heat-inactivated fetal calf serum. After 10 d of culture in 5% CO_2 at 37°C, the preparation was dried, irradiated with ultraviolet light, washed with PBS for 3 d, rinsed three times, and dried again before use.

Bone Resorption Assays

100 μg of 23–43-μm fragments of ^{45}Ca or [^{3}H]proline-labeled bone was added to each culture of 10,000 osteoclasts/35-mm plate, in a total volume of 2 ml. Cultures were incubated at 37°C in 5% CO_2. 10% of medium was exchanged daily, and the radioactivity in each aliquot was measured. Parallel non-osteoclast-containing (bone alone) cultures were established to determine isotope release due to physical-chemical leakage, which was subtracted from total released counts to establish cell-mediated resorption. Total substrate radioactivity was determined by hydrolysis of labeled bone in 6 N HCl at 110°C overnight, neutralization, and counting of the solubilized radioactivity. For assaying collagenolytic activity in subcellular fractions, [^{3}H]proline-labeled bone collagen was prepared by decalcifying 23–45 μg ^{3}H-labeled bone in 120 mM EDTA at 37°C overnight; removing non-collagenous bone matrix proteins with trypsin, 200 μg/ml, 40 min at 37°C; neutralizing trypsin with excess soybean trypsin inhibitor; and washing the bone collagen to remove soluble material. Activity of the bone collagen substrate was 67 cpm/μg at the same quench as enzyme-assay fractions.

Radioactive Hydroxyproline/Proline Ratios

Samples were hydrolyzed at 110°C in 6 N HCl overnight, evaporated to dryness, and redissolved in 20 mM citrate buffer, pH 4.25. Amino acids were separated on a Beckman W-2 ion exchange column (6 × 310 mm), using a Beckman 119C amino acid analyzer (Beckman Instruments Inc., Palo Alto, CA) modified for fraction collection (17). Positions of elution of L-proline and trans-4-hydroxyproline were determined by chromatography of amino acid standards.

Fractionation of [^{3}H]Proline-labeled Material Solubilized from Radiolabeled Bone by Size

Initial fractionation of radioactive material solubilized from bone by osteoclasts was performed by ultrafiltration using Centricon centrifugal ultrafilters with exclusions of M_r 10,000 and 30,000 (Amicon Corp., Danvers, MA). Gel filtration of solubilized radioactivity was performed using a Biogel P-6 column, 1.5 × 29 cm, eluted with water at 19 ml/h, or a Sephadex G-75 column, 1.5 × 28 cm, eluted with water at 11.5 ml/h.

Analysis of Degradation Products of UMR-106 Matrix

200,000 cpm crude supernatant from osteoclasts cultured on UMR-106 matrix was dialyzed against deionized water using a Spectrapore 2,000 membrane (retention ~2,000 mol wt, Spectrum Medical Industries, Los Angeles, CA). The retentate, which contained 28,000 cpm of ^{3}H, was lyophilized and subjected to PAGE as described below.

Analysis of Collagen Production by Osteosarcoma Cells

Early confluent cultures of UMR-106-06 osteosarcoma cells were incubated for 18 h in serum-free α-MEM containing 100 μg/ml β-aminoproprionitrile (an inhibitor of lysyl cross-link formation), 100 μg/ml sodium ascorbate, and 10 μCi/ml L-[5-^{3}H]proline. The medium was harvested into protease inhibitors (0.2 mM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin, 10 mM ϵ-aminocaproic acid, 2.5 mM EDTA, and 2 mM N-ethylmaleimide), dialyzed against 0.1 M acetate, and lyophilized before analysis by SDS PAGE. Cell layers were extracted into acetic acid–containing inhibitors, dialyzed, and lyophilized as above.

SDS PAGE and Fluorescence Autoradiography

Proteins were examined by SDS PAGE on discontinuous 1.5-mm methylene-bis(acrylamide) slab gels containing 0.5 M freshly deionized urea. A 3% stacking

[1] *Abbreviation used in this paper:* α-MEM, Eagle's minimum essential medium, α-modification.

gel was used with a composite 6/10% separating gel. Vertebrate collagenase digestion products were resolved with a 15% separating gel. For fluorescence autoradiography, gel slabs were washed and fixed in 30% methanol-10% acetic acid, permeated with EN^{3}HANCE (New England Nuclear, Boston, MA), dried, and exposed at −70°C to sensitized Kodak XRP film (15). Protein bands were quantitated by densitometric scanning using a Corning model 760 densitometer.

Proteolytic Cleavage of Extracted Proteins

For pepsin digestion, 100,000 cpm lyophilized protein was suspended in 900 μl of 0.5 M acetic acid at 4°C, and pepsin (3352 U/mg, Worthington Biochemical Corp., Freehold, NJ) was added to a concentration of 100 μg/ml. After incubation for 20 h with stirring at 4°C, the reaction was terminated by addition of a twofold molar excess of pepstatin. The sample was lyophilized and examined by SDS PAGE. For bacterial collagenase digestion, the lyophilized protein was suspended in 25–50 μl of 25 mM Tris-HCl, 10 mM $CaCl_2$, pH 7.2. Approximately 60 U purified bacterial collagenase (Form III, Advance Biofactures, Lynbook, NY) was added in 25 μl buffer, and the sample was incubated for 2 h at 37°C. The reaction was terminated by the addition of an equal volume of 2× SDS PAGE sample buffer and heated for 2 min at 100°C before electrophoresis.

Processing of Reconstituted Guinea Pig Collagen by Osteoclasts

Substrate Preparation. Salt extracted [^{14}C]glycine-labeled guinea pig collagen was prepared as previously described (10). To form fibrillar collagen gels, labeled guinea pig collagen, 1 to 2 mg/ml, pH 7.4, was incubated at 37°C in a humidified atmosphere overnight.

Assay of Osteoclast-conditioned Medium. 250 μl supernatant from 3-d osteoclast cultures, 5,000 osteoclasts/ml, was analyzed for collagenolytic activity, at pH 7.4, by incubation overnight at 37°C on 50 μl collagen gels. After centrifugation, soluble ^{14}C counts were compared with substrate controls incubated with fresh medium. Activated purified rat collagenase was used as a positive control (21). Tests were run with and without activation of osteoclast supernatant using 50 μg/ml trypsin for 10 min at 37°C. Trypsin was neutralized with excess soybean trypsin inhibitor.

Assay of Osteoclast Cultures. 2,000 osteoclasts were plated on 200 μl collagen gels in 2-cm^2 tissue culture wells. After 3 d of incubation, supernatant ^{14}C activity was compared with that of cell-free controls.

Processing of Vertebrate Collagenase–derived Collagen Fragments by Osteoclasts

To prepare labeled collagen fragments, salt-extracted [^{14}C]glycine-labeled guinea pig collagen, prepared as described above, was digested overnight at 25°C with rat collagenase (21). The reaction was terminated with EDTA, and the solution was dialyzed against cell culture medium. The resulting collagen fragments had a specific activity of 100,000 cpm/mg protein, and 92% of label migrated as authentic TC^A and TC^B on SDS PAGE (see Results).

Degradation of TC^A/TC^B by Osteoclasts

10,000 cpm (0.1 mg) TC^A/TC^B fragments were incubated at 37°C in 5% CO_2 with the following: confluent fibroblasts (250,000/2 cm^2 well), rat peritoneal macrophage (100,000/2 cm^2 well) or osteoclasts (10,000/2 cm^2 well), with 1.5 ml α-MEM/well. The capacity of these cells to degrade the radiolabeled collagen fragments to smaller fragments was assessed after 1, 2, and 3 d of culture by counting total and nondialyzable (M_r < 10,000) label in the medium. In addition, another preparation of 50,000 cpm of [^{14}C]glycine TC^A/TC^B fragments was incubated for 72 h with 20,000 osteoclasts (2,500 cells/cm^2) on unlabeled bone in 6 ml α-MEM. At the end of incubation, proteins were precipitated out of solution with 35% saturation $(NH_4)_2SO_4$, and this fraction, containing 85% of radioactivity, was desalted, lyophilized, and run on SDS PAGE. Osteoclasts on labeled bone, without added TC^A/TC^B (resorption control), showed degradation of 200 μg of bone collagen to low molecular weight fragments during the course of incubation with TC^A/TC^B.

Collagenolytic Activity of Osteoclast Lysates

Fresh osteoclast-rich populations were lysed by freeze-thaw in 20 mM citrate, pH 4.5, and clarified by centrifugation. Aliquots representing 1.5 × 10^5 cells were assayed for collagenolytic activity using 60 μg [^{3}H]proline-labeled 23–45-μm bone collagen. Citrate buffered assays were adjusted to final pH (3.0–5.0) using 1 N HCl or 1 M KOH. Lysate aliquots were incubated with the bone collagen for 18 h at 37°C; particulate matter was then removed by centrifugation, and soluble radioactivity was determined. No-lysate blanks, incubated identically to lysate assays, were subtracted from the total to arrive at net collagen solubilized by lysates. No lysate blanks indicated that <1.5% of total tritium label was solubilized in 18 h at 37°C in pH 4 citrate buffer, <0.2% in 18 h at 37°C in PBS (pH 7.4).

Statistical Methods

Data are expressed as mean ± standard deviation. All comparisons of groups used the Student's *t* test.

Results

3 d after isolation, 98% of the total nuclei in the osteoclast-enriched bone cell cultures were confined to large, multinucleated acid phosphatase-rich cells which contained an average of 4.8 nuclei (Fig. 1). When examined by transmission electron microscopy, these cells were seen to have the abundant mitochondria and the extensive membrane ruffling characteristic of osteoclasts (Fig. 2). Binding of osteoclast-specific antibody confirmed that the cells were osteoclasts (Fig. 3).

Isolated osteoclasts, incubated with ^{45}Ca or L-[5-^{3}H]proline-labeled bone, degraded both its inorganic and organic phases, although the lag time before the onset of resorption of each matrix differed (Fig. 4). When radiolabeled bone particles were added to the osteoclast-bearing fraction of the Percoll gradient, net (cell-mediated) mobilization of ^{45}Ca reached its maximal rate in 2–4 h, whereas attaining the maximum rate of ^{3}H release required 12–24 h. The maximum rate of organic matrix resorption, however, paralleled that of mineral mobilization, and in the presence of excess substrate, degradation of both phases continued for at least 6 d (Fig. 5). Under these conditions, the mean resorption rate of osteoclasts was 0.15 ng bone/d per nucleus. By comparison, exudative peritoneal macrophages resorbed <0.01 ng/cell per d, and bone matrix degradation by lung fibroblasts was negligible.

These experiments illustrate the capacity of osteoclasts to degrade the organic matrix of bone which, although largely collagenous, also contains noncollagenous proteins. Hence, total isotope release from [^{3}H]proline-labeled substrate offers no information regarding specificity of the target protein. This issue was addressed by amino acid analysis of the osteoclast-mobilized, ^{3}H-bearing peptides. As illustrated in Fig. 6, 39% of ^{3}H resorbed from bone appeared in the form of trans-4-hydroxyproline, an amino acid virtually unique to collagen, and 47% in proline. This result indicates that most of the solubilized substrate represents degraded collagen.

Although these data document the collagenolytic capacity of osteoclasts, they provide no insight into the mechanism by which the cells degrade collagen. One possibility is neutral collagenase, an enzyme that cleaves collagen into well-characterized, discrete fragments of 75,000 and 25,000 mol wt (TC^A and TC^B). We first addressed this issue by determining the size of the collagen fragments that resulted from osteoclast processing. Interestingly, the collagen-degradation products produced by osteoclasts were of low molecular weight. When subjected to ultrafiltration, 99% of cell-mobilized tritium passed membranes with nominal exclusion of M_r < 10,000. Furthermore, when the hydroxyproline-containing resorption medium was chromatographed on a Biogel P-6 column, 29% of the tritium-bearing material was excluded (M_r > 4,000), whereas 65% eluted with the included volume M_r < 1,500 (Fig. 7). 33% of the tritium in the excluded volume was in

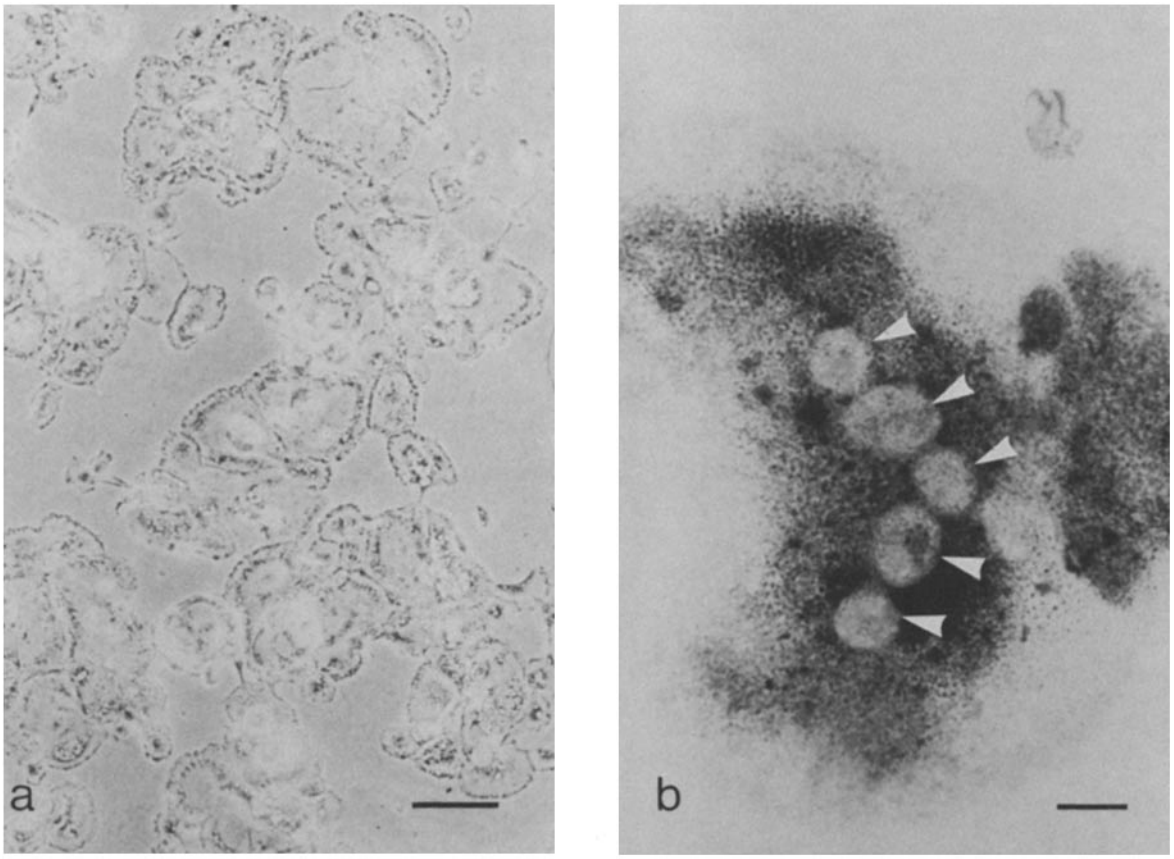

Figure 1. Osteoclasts attached to a plastic culture dish. (*A*) Phase-contrast photograph. Bar, 100 μm. (*B*) Histochemical stain for acid phosphatase; dark granular material indicates acid phosphatase activity. Five nuclei are seen by negative contrast (arrowheads). Transmitted light photograph. Bar, 10 μm.

the form of trans-4-hydroxyproline, and 41% of the isotope eluting with the included volume was in trans-4-hydroxyproline. When the void volume fraction from the Biogel P-6 column was chromatographed on Sephadex G-75, essentially all of the radioactivity eluted with the included volume (Fig. 7).

Since these experiments used devitalized bone particles as a resorption substrate, we were concerned that the bone milling process might alter the susceptibility of the matrix to degradation. We therefore examined the capacity of osteoclasts to degrade an alternative bone-like substrate synthesized by osteoblast-like tumor cells, which had not been subjected to fragmentation. The nature of the proteins synthesized by the osteoblast-like osteosarcoma cells was determined by SDS PAGE of their radiolabeled acid-soluble products. As shown in Fig. 8, these cells synthesized predominantly type I collagen, which is also the principal protein in authentic bone matrix. SDS PAGE of secreted protein showed disulfide-bonded, bacterial collagenase-sensitive components consistent with type I procollagen. After pepsin digestion, chains were identified that migrated as α1 (I) and α2 (I). There was, however, an excess of α-1 chains consistent with the secretion of type I trimers. In long-term cultures containing heat-inactivated serum but not the anti-cross-linking agent β-aminoproprionitrile, substantial amounts of fibrillar, insoluble collagen appeared (not shown).

We found that osteoclasts completely degraded this matrix as manifested by total mobilization of the matrix-incorporated [^{3}H]proline (Table I). Furthermore, amino acid analysis of osteoclast-mobilized peptides showed 31% of solubilized ^{3}H label in the form of trans-4-hydroxyproline, indicating that collagen degradation occurred during osteoclast degradation of osteosarcoma matrix.

Because the specific activity of osteosarcoma matrix synthesized in the presence of 1 μCi/ml of [^{3}H]proline was ~100 times that of bone particles, it was practical to use SDS PAGE to study the osteoclast resorption products using this substrate, even though the vast majority of matrix fragments had $M_r <$ 10,000. Starting with 200,000 cpm tritium resorbed from UMR-106 matrix by osteoclasts, a nondialyzable fraction (nominal exclusion of membrane, M_r 2,000) was obtained with 28,000 cpm of ^{3}H activity, which was run on SDS PAGE and examined by fluorography. The retained material, containing 14% of the resorbed radioactivity, formed no discrete bands. All radioactivity was diffusely distributed in peptides of relatively low molecular weight similar to those mobilized from authentic bone (Fig. 9).

These experiments indicated that the ultimate products of osteoclast organic matrix degradation were of relatively low M_r. However, they did not distinguish collagenolysis as a result of enzyme degradation at the site of osteoclast attachment from that due to collagenolytic enzymes released, by these cells, into the tissue culture medium. There was, however, no measureable collagenolytic activity in osteoclast-conditioned medium at pH 7.4, using reconstituted guinea pig collagen as substrate, with or without trypsin activation.

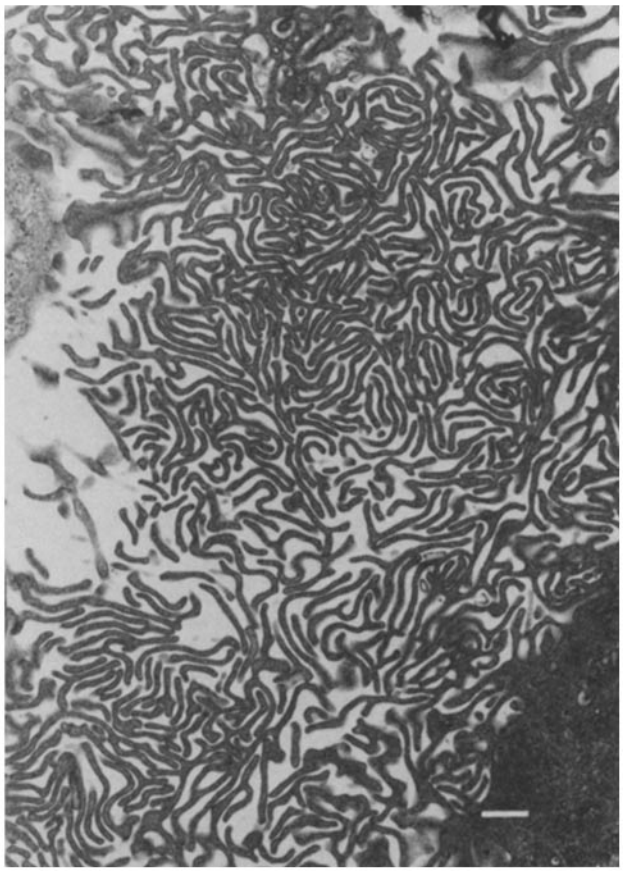

Figure 2. Electron micrograph of a portion of an isolated osteoclast in cell culture, showing the characteristic ruffled border. Bar, 0.2 μm.

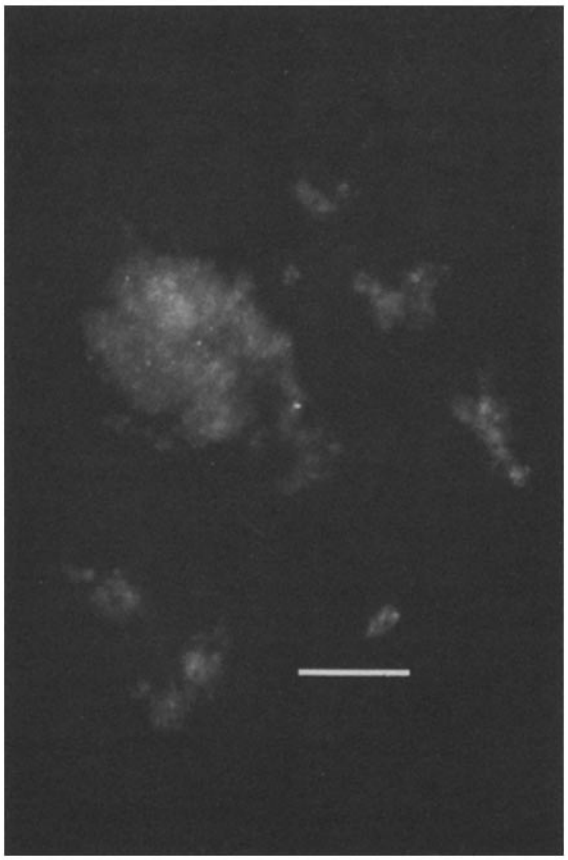

Figure 3. Osteoclasts, adherent to 25–50-μm bone fragments, 24 h after isolation, reacted with mouse anti-chicken osteoclast antibody and visualized with fluoresceinated anti-mouse antibody. Bar, 100 μm. Macrophages, stained by the same method, and controls without mouse anti-chicken osteoclast antibody showed no staining (not illustrated).

Furthermore, there was no measureable collagenolysis when osteoclasts were plated directly onto reconstituted guinea pig collagen gels, to which they do not attach. Hence, osteoclast collagenolysis cannot be attributed to release of proteolytic enzymes into the general tissue culture medium. On the other hand, the process of collagen degradation by these cells is probably a compartmentalized event occurring at the osteoclast-bone attachment site.

We next considered the possibility that large collagen fragments, such as those produced by neutral collagenases, were further processed in the medium by collagenase or other gelatinolytic enzymes potentially secreted by osteoclasts; since these fragments are not cross-linked or polymerized, they might provide a less resistant substrate than reconstituted native collagen. To examine this issue, we incubated ^{14}C-TC^{A}/TC^{B} fragments, representing the largest products of purified neutral collagenase activity, with osteoclasts, macrophages, or fibroblasts. 3 d later, >90% of the applied radioactivity was recovered from each culture after dialysis (M_r > 10,000), and there was no measurable difference (<2% of total counts) between recovery of nondialyzable material after incubation with osteoclasts, fibroblasts, or macrophages. The bulk of this activity appeared in discrete bands of TC^{A} and TC^{B}, on SDS PAGE after precipitation from the cell culture medium with ammonium sulfate (Fig. 10). The initial TC^{A}/TC^{B} preparation contained 68% of protein migrating as TC^{A} fragments (M_r 75,000), 24% migrating as TC^{B} fragments (M_r 25,000), and 8% at other molecular weights. After exposure to osteoclasts, 32,000 cpm was recovered from 50,000 cpm of TC^{A}/TC^{B} after precipitation, dialysis, and lyophilization

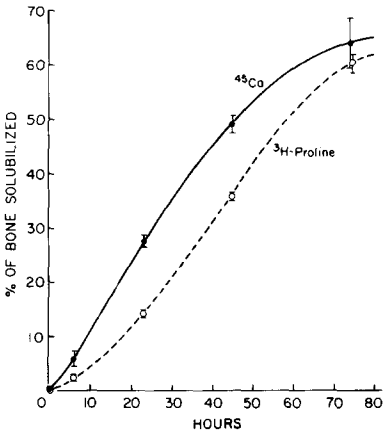

Figure 4. Resorption of 100 μg ^{45}Ca or L-[5-^{3}H]-proline labeled bone by 10,000 osteoclasts.

(64% recovery). 73% of recovered tritium migrated as TC^{A}, 11% as TC^{B}, and 16% at other molecular weights; there were no other discrete bands. The relative increase in high molecular weight recovered material may represent greater efficiency of recovery of these fragments. The slight decrease in the amount of TC^{A}/TC^{B} as compared with total labeled protein in recovered material may reflect some degree of proteolytic degradation or relative loss during preparation of

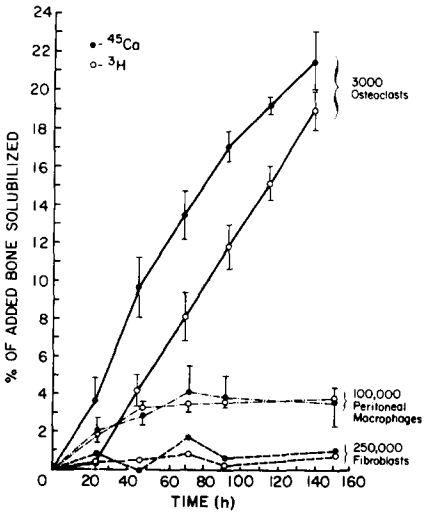

Figure 5. Comparison of bone resorption by 3,000 multinucleated osteoclasts, 100,000 freshly isolated peritoneal macrophages, or 250,000 fibroblasts. All cell types except the osteoclasts are mononuclear. 100 μg ^{45}Ca or L-[5-^{3}H]proline-labeled bone was added at time zero.

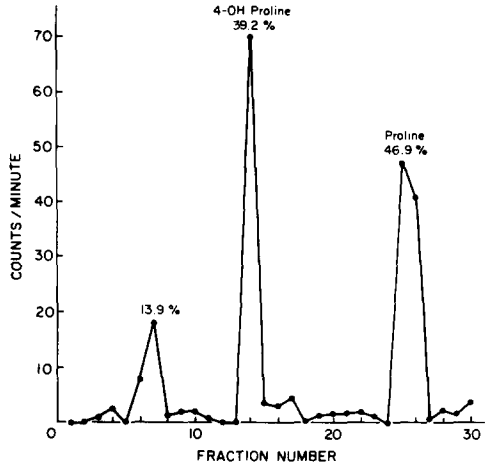

Figure 6. Fractionation of ^{3}H activity from osteoclast supernatant into 4-hydroxyproline (specific for collagenolysis) and proline. Counts of fractions were from an amino acid analyzer. Positions of proline and 4-hydroxyproline were determined using amino acid standards.

sample for SDS PAGE. Nevertheless, this pattern is quite distinct from that obtained after the degradation of bone collagen by osteoclasts (99% M_r < 10,000, Fig. 7) and indicates that osteoclast collagenolysis is not mediated by release, into the supernatant compartment, of neutral collagenase.

These experiments indicated that osteoclasts degrade collagen to low molecular weight fragments despite the lack of significant gelatinolytic activity in cell-culture supernatants. Thus, an enzyme (or enzymes) localized to the osteoclast or to its bone-attachment site probably mediates this collagen degradation. To test this hypothesis, we measured the collagenolytic activity of osteoclast lysates on 23–45-μm fragments of decalcified, trypsinized bone collagen. No evidence of collagen degradation was seen when lysates were prepared and assayed in PBS at pH 7.4 (not shown). However, when lysates were prepared and assayed in citrate buffers in the lysosomal pH range, significant collagenolytic activity was seen, with maximal collagen degradation at pH 4.0 (Fig. 11).

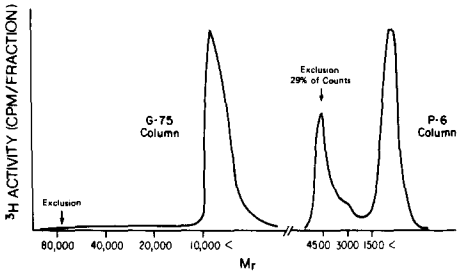

Figure 7. Size distribution of [^{3}H]proline-labeled fragments from bone resorption by isolated osteoclasts. G-75 and Biogel P-6 columns were used. Ordinate: ^{3}H activity (relative counts per minute per 2-ml fraction). Abscissa: M_r, determined with protein standards. All activity was below the useful molecule size range of G-75 (M_r < 10,000), and 65% was below the useful range of P-6 (M_r < 1,500).

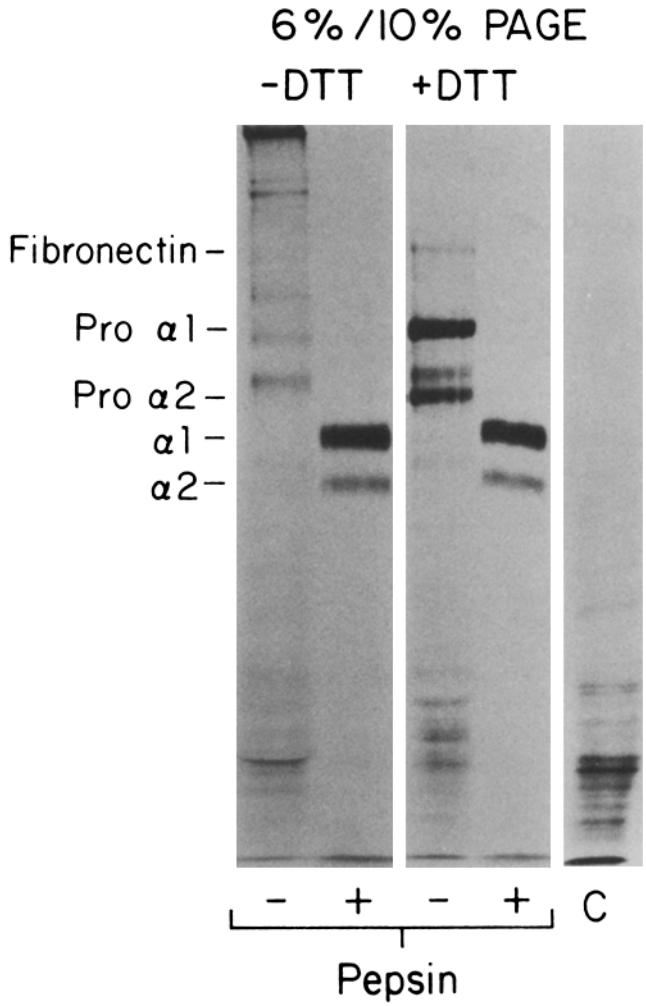

Figure 8. Fluorogram of [^{3}H]proline-labeled proteins secreted by UMR 106-06 cells. Proteins were resolved by SDS PAGE with or without prior reduction of disulfide bonds with dithiothreitol (*DTT*). Parallel aliquots were digested with pepsin or purified bacterial collagenase (lane *C*). Positions of reduced fibronectin, procollagen (*Pro α1*, *Pro α2*), and collagen ($\alpha 1$, $\alpha 2$) chains are indicated to the left of the figure.

Table I. Degradation of Osteosarcoma Matrix by Osteoclasts

Time	^{3}H in supernatant	Percentage of total ^{3}H counts in supernatant
h	*cpm less blank*	
0	0 ± 100	0
50	2,500 ± 300	57
120	3,600 ± 400	99

4,000 osteoclasts were plated on 1-cm^2 layers of ^{3}H-labeled osteosarcoma matrix, consisting predominantly of type I collagen (see text).

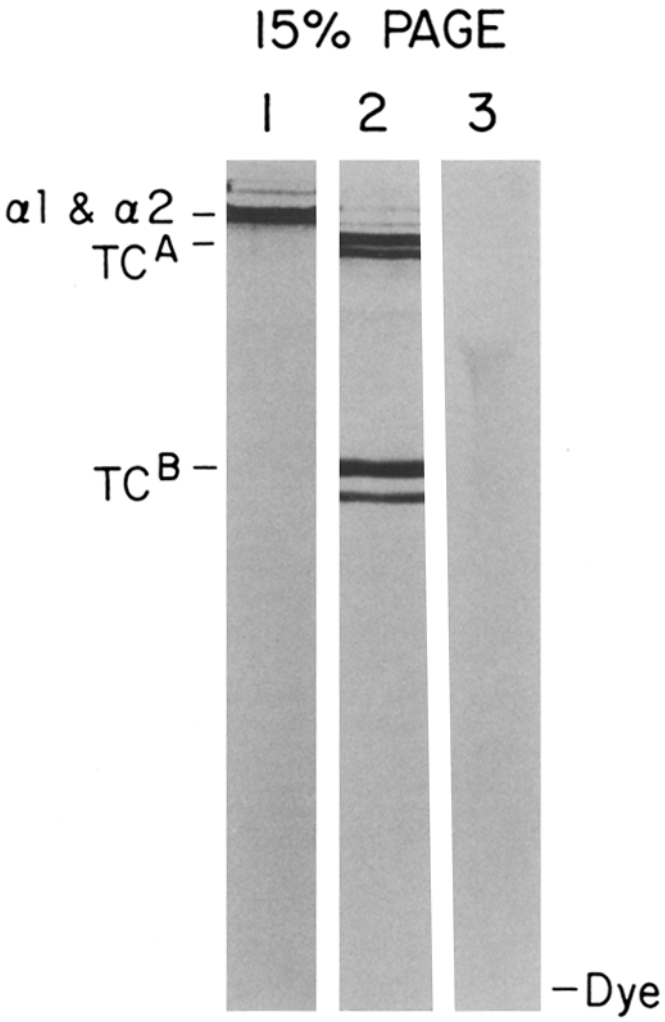

Figure 9. Fluorogram showing comparison of protein fragments resulting from osteoclast degradation of [^{3}H]proline-labeled UMR 106-06 matrix to intact [^{14}C]proline-labeled collagen and products of neutral collagenase digestion of collagen. Proteins were resolved by SDS PAGE 5%/15% slab gels. Lane *1*, type I collagen; lane *2*, neutral collagenase partial digestion products; lane *3*, nondialyzable fraction ($M_r \geq 2{,}000$) solubilized from UMR matrix by osteoclasts. Positions of collagen chains ($\alpha 1$ and $\alpha 2$) and collagenase fragments (TC^A and TC^B) are indicated to the left of the figure.

Discussion

Our studies demonstrate that osteoclasts have the capacity to resorb both the inorganic and organic matrices of bone and do so in a temporal sequence consistent with electron microscopic observations that the mineral phase is mobilized before collagen degradation (3). The use of intact osteosarcoma cell-

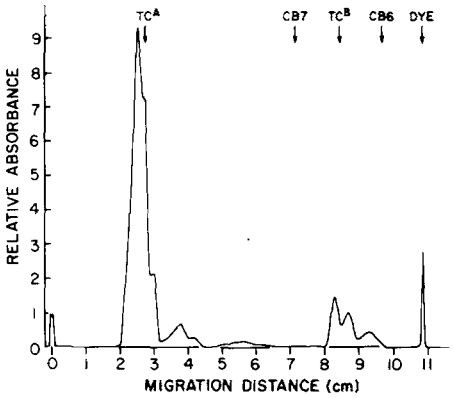

Figure 10. Recovery and TC^A and TC^B fragments after a 72-h incubation with resorbing osteoclast cultures. 50,000 cpm of ^{14}C-labeled TC^A/TC^B was incubated with 30,000 osteoclasts. The medium was collected at 72 h. Proteins were reprecipitated with 35% saturation $(NH_4)_2SO_4$, desalted, lyophilized, and examined by PAGE. During this period the cultures resorbed 200 μg bone collagen. Arrows indicate the migration distances of TC^A and TC^B, and of CNBr fragments (CB7 and CB6), from other lanes on the same gel.

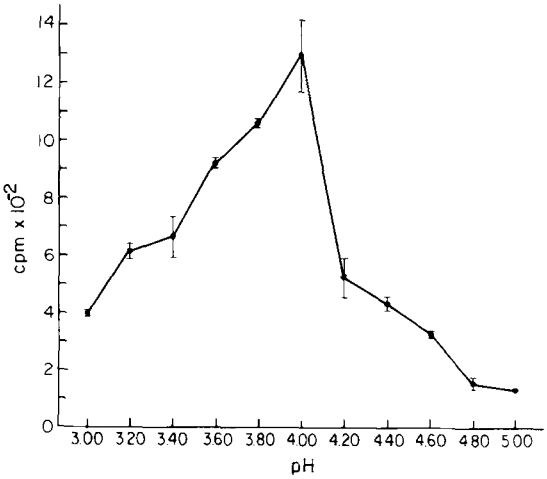

Figure 11. Collagenolytic activity of osteoclast lysates as a function of pH in the lysosomal range. Aliquots of fresh osteoclast lysates (1.5 × 10^5 cells) were incubated with 60 μg bone collagen at 37°C for 18 h, and the solubilized fraction of the collagen was determined by measuring ^{3}H in supernatants after insoluble bone collagen was removed by centrifugation. pH-matched substrate controls, incubated with buffer only, have been subtracted. The peak at pH 4.0 represents hydrolysis of 20 μg collagen.

produced matrix as an alternative resorptive substrate indicates that milling of the rat bone fails to affect qualitatively its susceptibility to osteoclast degradation.

Since 5–10% of bone proteins are noncollagenous (13), the mobilization of isotope from [^{3}H]proline-labeled matrix is insufficient to establish collagenolysis. On the other hand, the appearance of ^{3}H-trans-4-hydroxyproline in the culture medium (Fig. 6) is specific for collagen degradation. Since the ratio of proline to hydroxyproline in type I collagen is ~1.2 (5), ~90% of osteoclast-solubilized ^{3}H from bone represents collagenolysis.

Vertebrate collagenase, a neutral metalloprotease, is believed to play a central role in type I collagen turnover in soft tissues. Previous reports, especially those of Vaes (24), suggest, however, that collagenase activity is not enhanced in bone

organ culture stimulated to undergo resorption. Alternatively, other enzymes, including acid hydrolases, may also degrade collagen (4), and an acidic environment may alter the structure of collagen such that acidic proteases, not classically associated with collagen degradation, may become the primary catalysts for this process during bone resorption. Moreover, that bone degradation can be blocked in the rat calvarial explant model by thiol protease inhibitors (7) also supports collagenase-independent process responsible for at least the major component of the resorptive process. The collagenase issue is, however, by no means resolved, since Heath et al. have observed that osteoblasts synthesize this enzyme in response to the bone resorptive agent, parathyroid hormone (11). These collective findings raise the possibility that different cells may degrade bone collagen by various enzymatic means.

Our data suggest that osteoclastic activity is independent of secretion of collagenase. Using various sizing techniques, we have shown that the osteoclast-produced, bone-derived ^{3}H-bearing peptides are virtually all <10,000 M_r and hence, much smaller than the TC^A and TC^B fragments characteristic of limited collagenase digestion. Collagenolytic activity is not detectable in osteoclast-conditioned media, and failure of these cells to process exogenous TC^A and TC^B also speaks against masking of a classical collagenase by gelatinase activity. Moreover, attempts were also made to demonstrate collagenase release by osteoclasts using a rabbit anti-rat collagenase antibody. No immunoreactive collagenase was seen, either by enzyme-linked immunosorbent assay in osteoclast supernatants or by peroxidase-anti-peroxidase staining of fresh osteoclasts, although this finding may reflect insufficient homology of the chicken enzyme to the rat collagenase against which the antibody was raised. Probably our most compelling argument that osteoclastic collagenolysis reflects the activity of an enzyme (or enzymes) other than neutral collagenase is the pH optimum of osteoclastic collagenolytic activity.

We found that lysates of osteoclasts contain abundant collagenolytic activity at acidic, but not at neutral, pH. In keeping with this finding, an acidic microenvironment probably exists at the osteoclast-bone resorptive interface. This cell attachment site is anatomically similar to its macrophage-related counterpart which has been shown by Wright and Silverstein to be a sealed compartment capable of excluding proteins (25). Recently, Fallon has employed direct microprobe technology to explore the site of osteoclast attachment and has documented the existence of a pH of ~5.0 (9). Baron et al. have achieved similar results by an indirect chemical method (2). These observations, taken with the low molecular weight of bone collagen degradation fragments produced by osteoclasts and the lack of soluble collagenolytic activity in medium conditioned by resorbing osteoclasts, suggest that the major collagenolytic enzymes are compartmentalized at the bone resorption interface and are acid hydrolases. One such enzyme, cathepsin B, has been shown to cleave collagen into small, polydisperse fragments at low pH (6). It is important to realize that although lysosomal enzymes are detectable in media of resorbing bone explants (see, for example, reference 7), the mere presence of these enzymes does not establish their functional role in bone collagen degradation and, in fact, soluble collagen fragments are not degraded in the culture supernatants (Fig. 10).

In conclusion, our data support a model in which the osteoclast degrades both the organic and inorganic components of bone in an acidic microenvironment localized at the osteoclast-bone interface. The characterization of the hydrogen pump for acidification of the osteoclast attachment site, and of its bone-degrading acid hydrolases are therefore the ultimate goals of detailing osteoclastic bone resorption.

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Note Added in Proof: Fresh osteoclast lysates solubilized 12–16 μg collagen/18 h per mg lysate protein at pH 4.2, 37°C, while HLF 3114 fibroblasts or UMR 106-01 osteosarcoma cells solubilized <1 μg collagen/18 h per mg lysate protein under the same conditions.

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