Calcif. Tiss. Res. 22, 303—313 (1977)

Isolation of Osteoclasts by Velocity Sedimentation at Unit Gravity

R. L. Nelson and G. E. Bauer

University of Minnesota

Received November 18, 1975, accepted May 27, 1976

A method is presented for separating osteoclasts from the heterogeneous population of bone and marrow cells. Cell suspensions were prepared from femora of young rabbits by mechanical dispersion. The starting cell suspension typically contained only $1.0\% \pm 0.5$ osteoclasts. Following an initial 45 min of unit gravity sedimentation in a lucite chamber osteoclasts were primarily distributed in fractions 2–5. A second 45-min sedimentation of these pooled fractions yielded cell suspensions containing greater than 30% osteoclasts (as much as a 50-fold increase over starting percentages). Linear scan analysis, however, revealed that osteoclasts accounted for $73.14\% \pm 0.58$ of the cell colume. Subsequent in vitro experiments demonstrated linear incorporation of ^{3}H-leucine into TCA precipitable protein for cells comprising the osteoclast fraction. Concomitant radioautographs revealed radioactive label in isolated osteoclasts.

Key words: Osteoclasts — Sedimentation — Isolation — Cells — Bone.

Introduction

Attempts at isolating viable osteoclasts from heterogeneous populations of bone and marrow cells have not met with much success. Crude cell suspensions containing osteoclasts have been noted following enzymatic digestion of bone [1] or bone cell fractionation procedures [27]. Using programmed gradient sedimentation, osteoclasts were concentrated over 50-fold from cell suspensions obtained from rat metaphysis and bone marrow [29], but neither the starting percentage of osteoclasts nor the final purity was reported. Walker [36], using microdissection, obtained osteoclasts from lyophilized imprints of metaphyseal bone. Several hundred osteoclasts were isolated per hour and plainometric analysis revealed that these cells represented 90% of the total cell mass. Although this technique enables histochemical assessment of the enzyme activity in normal and parathyroid hormone treated rats, it has limitations in cell yield and viability. Therefore, an effort was undertaken to explore an alternative isolation procedure.

A number of investigators have isolated cells using velocity sedimentation at unit gravity [13, 20, 24, 28, 30]. This technique exploits differences in sedimentation rates to separate cells on the basis of their size [24]. Because of the relatively large size of the osteoclast, we attempted cell separation using this method. The experimental procedure and results reported here have appeared elsewhere in abstract form [25, 26].

For reprints: G. Eric Bauer, Ph. D., University of Minnesota, Department of Anatomy, Jackson Hall, Minneapolis, Minnesota 55455, U.S.A.

Methods

Animals. Four littermates from New Zealand white rabbits which were 12–14 days old (average weight: 216 g) were used in all the experiments. The animals (delivered from the Oak Crest rabbitry, Minneapolis, Minn., on the morning of the experiment), were sacrificed by decapitation, and the femora removed by disarticulation. The femora were then cross sectioned at the epiphyses, cleaned of residual muscle attachments, and split longitudinally with a razor blade. The bone marrow was removed from the endosteal surface and the sectioned femora immediately placed in a dilute solution of neutral red (100 μg/ml) in phosphate buffered saline (PBS) for 30 min at 37°. This stained the osteoclasts on the endosteal surface, and their location and distribution could be assessed using a dissecting microscope. The staining solution was decanted, and the femora were rinsed and placed in cold PBS with 1.0 mM ethylenediamine tetraacetic acid (EDTA, Sigma, St. Louis, Mo.) and 0.1% bovine serum albumin (BSA, Sigma).

Preparation of Cell Suspension. Glassware and plastic instruments were pretreated with Siliclad (Clay-Adams, Parsippany, N. J.). Isolation and incubation media contained potassium penicillin G (Squibb, New York, N.Y.), 100 units/ml, and Streptomycin (Pfizer, New York, N.Y.), 50 μg/ml [12]. Individual femur sections were placed in a petri dish containing 10 ml of PBS with 1.0 mM EDTA and 0.1% BSA (PBSEB). With the aid of a dissecting microscope, the endosteal surface of each femur section was scraped with a curette (Clevedent #85, S. S. White Co., Minneapolis, Minn.) until stained osteoclsts could no longer be seen on the endosteal surface. The pooled scrapings were transferred to a test tube and the suspension permitted to settle 1 min to allow the bony debris and gross cellular clumps to settle to the bottom of the tube. The supernate was then carefully transferred to another test tube and spun at $160 \times g$ for 10 min at 4°. The pellet was resuspended in 1.0 ml of PBSEB and gently dispersed with a Pasteur pipette. Mechanical dispersion was continued until the medium became cloudy. This medium constituted the definitive cell suspension from which subsequent cell separation experiments were performed.

Sedimentation Procedure. The lucite sedimentation chamber (custom fabricated by Mr. J. Rowlands, National Cancer Institute, Bethesda, Md.) used in these studies was 15.5 cm high, 3.5 cm in diameter, and had a 60 ml capacity. A small baffle, located in the bottom section of the chamber, was used to disperse the incoming fluid. The chamber was filled with a 2–4% BSA gradient (in PBS with 1.0 mM EDTA) generated with a model 570 gradient former (Instrument Specialties Company, Lincoln, Neb.). The sedimentation chamber was emptied by displacement using 7.0% sucrose in 0.9% sodium chloride.

Sedimentation at unit gravity was conducted at room temperature. The procedure was initiated by allowing a small volume of gradient mixture to cover the baffle at the bottom of the chamber. The starting cell suspension (1.0 ml) was carefully layered on top of this solution with a Pasteur pipette, and the BSA gradient was then generated. After the appropriate sedimentation period (45 min), successive 5 ml fractions were collected by displacing the chamber contents with the sucrose-saline solution.

Procedures for unit gravity sedmentation were identical for those experiments which employed two separate sedimentation periods. However, some fractions collected by displacement were pooled to facilitate counting and identification of cell types.

Processing of Cells for Counting and Microscopy. Cells were concentrated by centrifugation at $250 \times g$ for 10 min in an International centrifuge (Model PR-2, #279 rotor). All pellets were resuspended in a small volume (0.3–1.0 ml) of PBSEB, and cell counts were made with a hemacytometer.

A cytocentrifuge (Shandon-Elliot Model SCA-0030 Cytospin, Camberley Surrey, England) was used to prepare the histologic slides. The cell suspensions (containing approximately 1.0×10^5 cells/ml) were spun at 1200 rpm for 5 min. Slides were air dried and immediately stained with Wright's stain.

Quantitation of Osteoclasts. The percentage of osteoclasts was determined by systematically scanning each slide. A template slide with five 1-mm markings was made to facilitate this procedure. The periphery of the cell suspension was located (at 100×) at each of these markings, the slide was moved laterally one visual field (via the mechanical stage), and the cells were counted at 400×. The slide was moved horizontally until 200 cells (1000 cells per slide) were examined. Osteoclasts were distinguished by their large size, multiple nuclei (with 1 or 2 nucleoli), and cytoplasm which was either homogeneous or foamy and vacuolated.

Volume Quantitation of Cell Fractions. A micrometer component quantitator [16] was used to calculate cell volume from slides of cell suspensions. This instrument consists of a microscope with a movable stage, a cross-hair reference point in the eyepiece, and a system of micrometers. The percentage of cell volume was calculated by dividing the sum of the intercepts for one cell by the total distance traversed across all cells [4, 5]. The direct relationship between two-dimensional linear analysis and volume is well documented [6]. It has also been reported that the accuracy and reproducibility of the linear scan method compares favorably with other quantitative methods [5].

The slides were scanned at 400× and volumes of three cell types—(1) osteoclasts, (2) blood and marrow cells, (3) bone cells—were recorded with the micrometers. Osteoclasts were identified by criteria previously mentioned. Cells which manifested obvious cytologic features of mature blood cells or developing precursor elements were placed in category 2. Osteoblasts, osteocytes, mesenchymal cells, fibroblasts, and unknown cells were included in the third category. Scanning was begun on the top portion of the slide just inside the periphery. A horizontal scan was made and intercepts recorded until the other periphery was encountered. The mechanical stage was then advanced in 1.0 mm vertical increments until the entire slide was examined. Typically, more than 45 mm of scan was recorded.

Protein Synthesis Studies. In vitro incorporation studies were conducted on cells collected after two 45-min sedimentation procedures. The cellular suspensions were spun at $250 \times g$ for 10 min at 4° and the pellets washed twice with Krebs Ringer bicarbonate buffer containing 70 mg% glucose, 2 mg% BSA, and a complete amino acid mixture (minus leucine). The pellets were resuspended in a small volume of this Krebs solution (500 µl) and added to test tubes containing 100 µCi of ^{3}H-leucine (specific activity 44.2 Ci/mM) which had been evaporated to dryness and kept at 4° prior to use. The test tubes were capped with rubber serum stoppers and incubated at 37° in a Dubnoff metabolic shaker. The tubes were gassed for 15 s every 30 min with a 95% O_2–5% CO_2 gas mixture. Duplicate aliquots (25 µl) of the incubation mixture then were transferred to 2.4 cm filter paper disks (Whatman No. 540) and processed by the method of Mans and Novelli [22]. The radioactivity of the disks was measured by liquid scintillation spectrometry in 10 ml of 3a 40 scintillation mixture (Research Products International Corp., Elk Grove Village, Ill.).

Radioautography. Radioautography was performed on cell suspensions following 2 h of incubation with ^{3}H-leucine. The cell suspensions were washed twice with KRB and resuspended in a small volume (200–300 µl) of KRB; slides (cleaned with acid alcohol) were prepared with a Shandon cytocentrifuge. Slides were fixed in methanol and coated with a thin layer of Kodak NTB_3 emulsion using a film drawing method [2]. Slides were developed 6 days later [2] and stained with Wright's-Giemsa or Mayer's hematoxylin and aqueous Eosin.

Results

1. Sedimentation at Unit Gravity. Initial experiments assessed the distribution of osteoclasts following a single 45-min sedimentation period. The total number and percentage of osteoclasts present in fractions 1–10 are shown in Figure 1 and 2. Typically, no osteoclasts were present in fraction 1. Although the percent of osteoclasts was small, (1.4–3.4%), they were present in greatest number in fraction 2 because of the large total number of cells present here (Fig. 1). The highest percentage of osteoclasts (11.9–20.7) was seen in fraction 3 (Fig. 2). Fractions 4 and 5 had large percentages of osteoclasts but the number of all cells was decreased. The number and percent of osteoclasts continued to decrease in fractions 6–10. Moreover, significant cell clumping was more apparent in these later fractions. When considering the total number of osteoclasts recovered from the sedimentation chamber, more than 94% (94–98%) of these cells were present in fractions 2–5. Thus, the initial sedimentation period produced moderately enriched osteoclast fractions (fractions 2–5) while selectively reducing contaminating cells and cell aggregates. Longer sedimentation periods (2 h) did not improve the osteoclast purity.

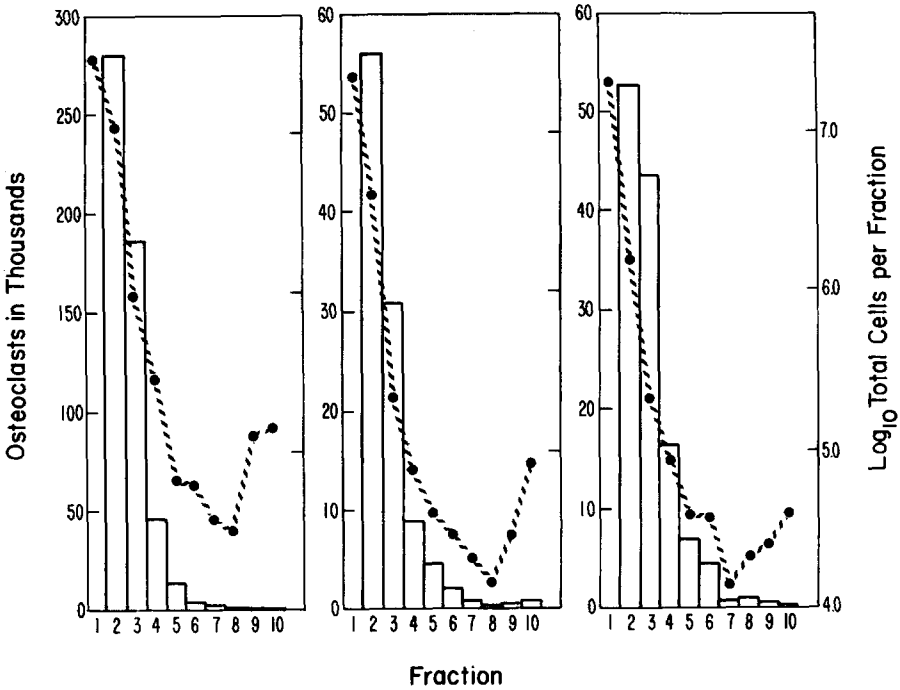

Fig. 1. Three separate experiments showing distribution of osteoclasts following initial 45-min sedimentation in a 2-4% BSA gradient in PBS with 1.0 mM EDTA. Points refer to total number of cells ($\log_{10}$) as determined from hemacytometer counts of the 10 fractions. Bars represent an estimate of number of osteoclasts per fraction extrapolated from microscopic quantitation (see Fig. 2)

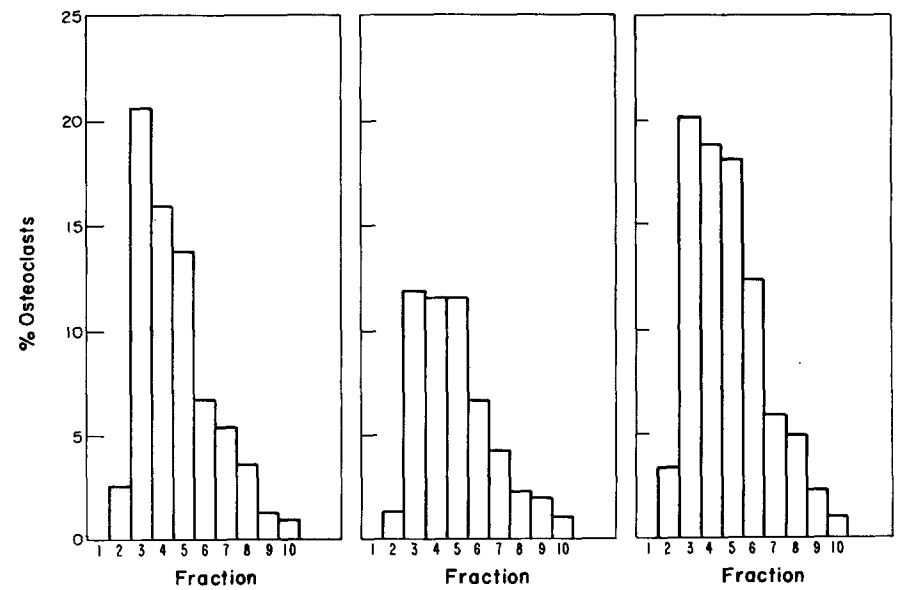

Fig. 2. Percentage of osteoclasts per fraction following initial 45-min sedimentation. Cell counts were determined from cytocentrifuge slide preparations

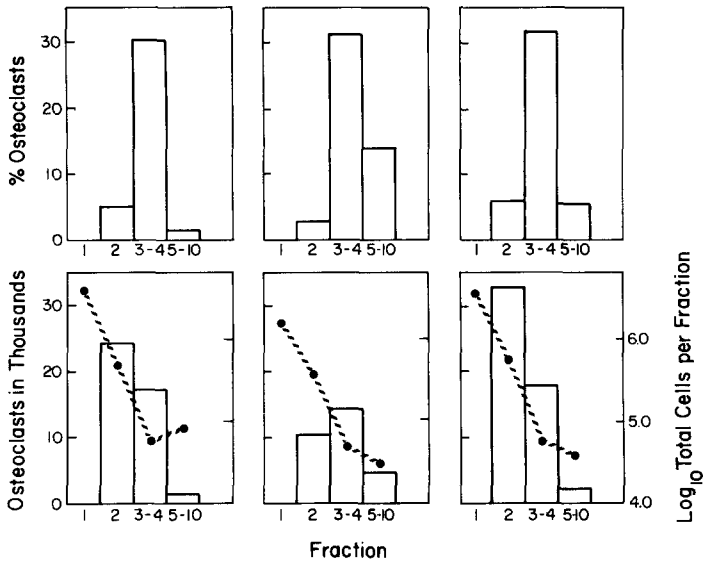

Fig. 3. Three separate experiments depicting distribution of osteoclasts per fraction following two separate 45-min sedimentation procedures. Data presented as in Figures 1 and 2. Highest percentage of osteoclasts (30.1–31.6) was consistently present in pooled fractions 3–4

A second series of experiments assessed the distribution of osteoclasts following two separate sedimentation procedures. Fractions 2–5 were pooled following an initial 45-min sedimentation and allowed to sediment an additional 45 min. The distribution of osteoclasts in the four fractions (1, 2, 3–4, 5–10) is shown in Figure 3. Again, no osteoclasts were present in fraction 1. Fraction 2 contained from 2.0 to 5.9% osteoclasts. The highest percentage of osteoclasts (30.1–31.6) was present in fractions 3–4 (Fig. 3). A cytocentrifuge slide preparation from pooled fractions 3–4 is shown in Figure 4. Since the starting percentage of osteoclasts was 0.6, 0.9, and 1.1, this represented 50.2-, 34.5-, and 28.7-fold increases, respectively. Fractions 5–10 contained a small number of cells including osteoclasts, but cell clumping was again evident.

2. Volume Quantitation of Enriched Osteoclast Fractions. Slides prepared from fractions 3–4 collected following two 45-min sedimentation procedures were subjected to linear scan analysis and the volume percentage of cell components was determined (Fig. 5). Osteoclasts represented 73.14 ± 0.58 volume percent of cellular components in fractions 3–4. Blood and marrow cells accounted for $11.34 \pm 0.12\%$ and bone cells $15.52 \pm 0.17\%$.

3. Protein Synthesis Experiments. Based on the results of the various isolation experiments, it was concluded that the purest fractions of osteoclasts were obtained following two 45-min sedimentation procedures. In an effort to evaluate potential metabolic capabilities for cells comprising these fractions, their ability to syntheisize protein in vitro was assessed. The results (Fig. 6) demonstrate linear incorporation of ^{3}H-leucine for fractions 1, 2, 3 (3–4), and 4 (5–10). Cycloheximide (final concentration 100 μg/ml) added after 2 h was inhibitory. In another experi-

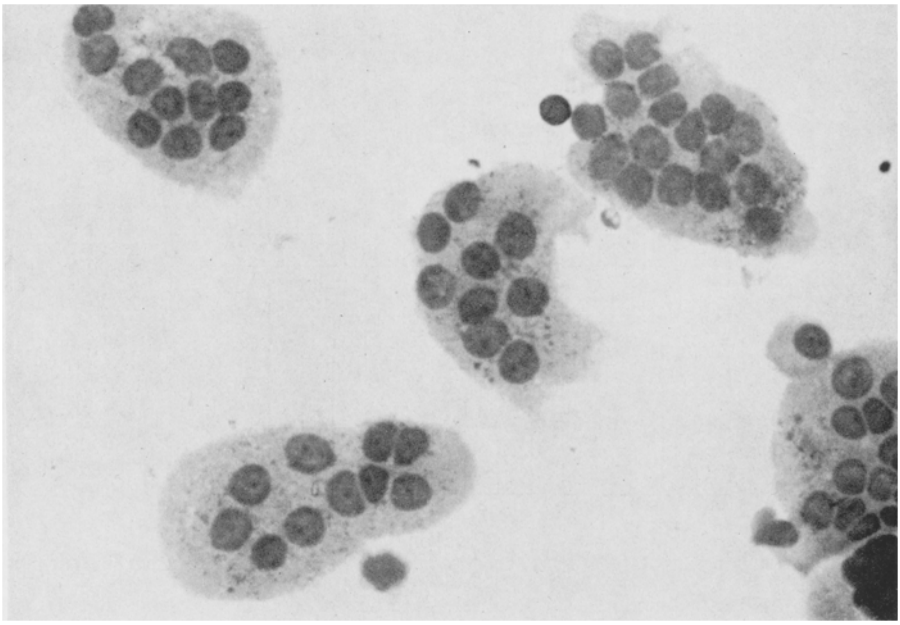

Fig. 4. Isolated osteoclasts from a cytocentrifuge slide preparation obtained from pooled fractions 3–4 collected after two 45-min sedimentation procedures (× 62.5)

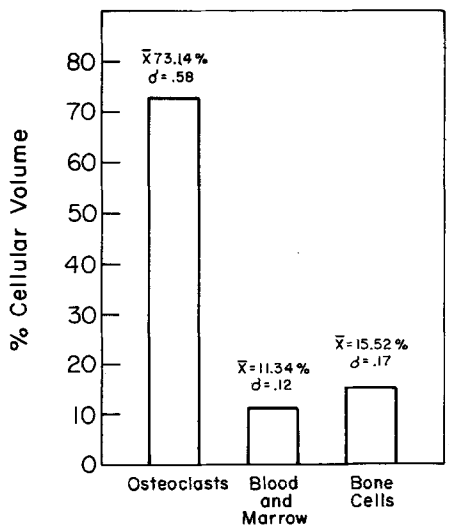

Fig. 5. Volume percent of cell components determined from linear scan analysis of cytocentrifuge slides prepared from pooled fractions 3–4 (see Fig. 3). In these three experiments, osteoclasts represented $73.14 \pm 0.58\%$ of total cell colume

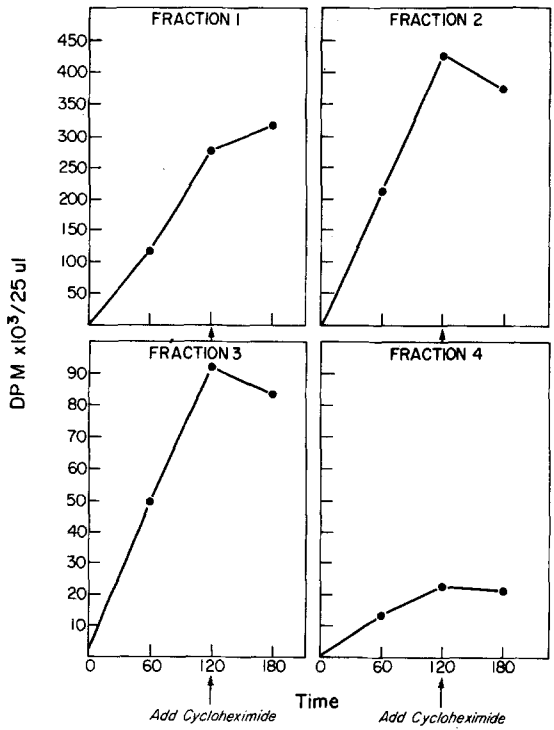

Fig. 6. In vitro incorporation of ^{3}H-leucine into cellular protein by cell fractions 1, 2, 3 (pooled 3–4), and 4 (pooled 5–10) collected after two separate 45-min sedimentation procedures. Cycloheximide (final concentration 100 μgm/ml) added after 2 h of incubation was inhibitory. Filter-paper disk method was used for protein extraction as detailed in materials and methods

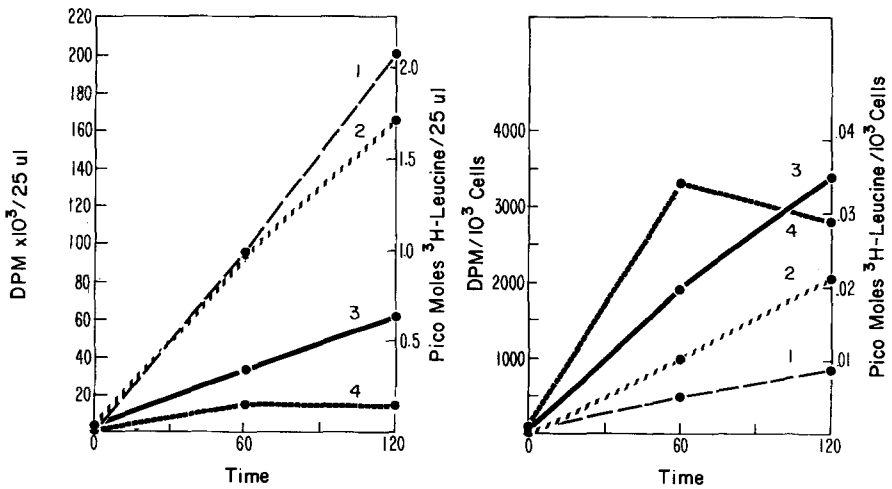

Fig. 7. In vitro incorporation of ^{3}H-leucine into cellular protein by cell fractions 1, 2, 3 (pooled 3–4), and 4 (pooled 5–10) separated by velocity sedimentation at unit gravity. Filter-paper disk method was used for protein extraction. Concentration of ^{3}H-leucine (in pico moles) was calculated from total radioactivity (DPM) incorporated. Cell number per fraction was estimated by hemacytometer

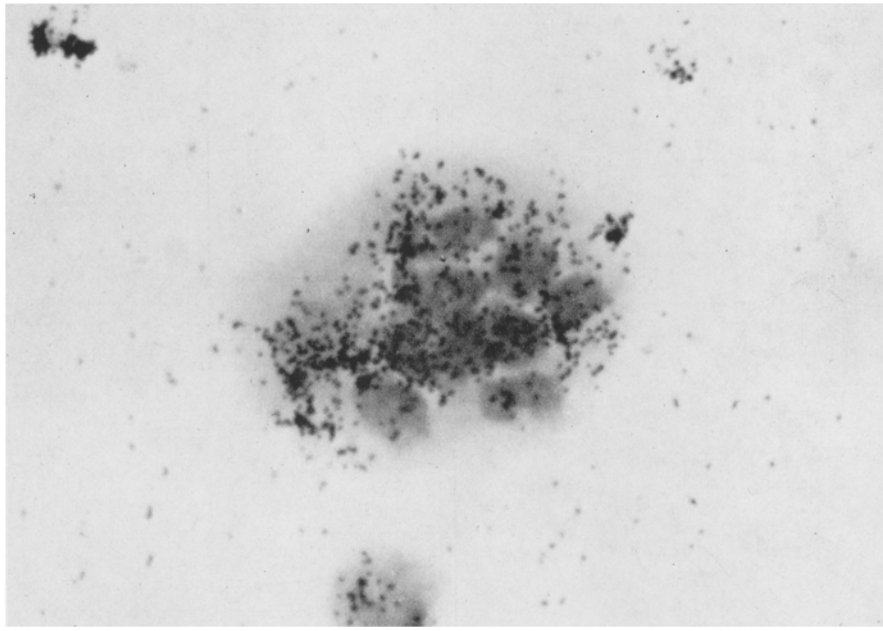

Fig. 8. Radioautograph of isolated osteoclast prepared from osteoclast fraction (fraction 3—Fig. 7) following 2 h in vitro incubation (× 52.5)

ment (Fig. 7), linear incorporation was again noted. In addition, fraction 3 (pooled fraction 3–4 containing the enriched osteoclast population) was found to be the most active with respect to picomoles of ^{3}H-leucine incorporated into cellular protein. Radioautographs prepared after the 2-h incubation demonstrated radioactive label in the osteoclasts present in this pooled fraction 3–4 (Fig. 8).

Discussion

That osteoclasts function in the resorption of bone, cartilage, and teeth, has been well documented [9], but how they resorb calcified tissue has not been entirely explained. The osteoclast has morphologic capabilities for both the ingestion and degradation of mineralized debris and macromolecules (endocytosis) [8, 10, 12, 14, 17, 18] and for the release of secretory substances which may solubilize bone (exocytosis) [18, 31]. Although the exact composition of the secretory substances is not known, acid and various hydrolytic enzymes may be involved in the process [3, 32–34, 36]. The presence of a mammalian collagenase has been suggested [37] but not demonstrated. Parathyroid hormone appears to stimulate the osteoclast's resorptive function [7, 11, 19, 33] whereas calcitonin has an opposite effect [7, 11, 15, 35].

To determine how these hormonal effects are mediated, a technique for separating osteoclasts from other bone-dwelling cells could be of value.

In the present study mechanical dispersion was used to remove osteoclasts from bone rather than enzymatic digestion because biochemical and morphologic

cell damage is less [23]. Following short-term incubation in neutral red, the stained osteoclasts could readily be removed with a curette. The average starting cell suspension contained approximately 4.4×10^7 cells with osteoclasts accounting for $1.0 \pm 0.5\%$ of these cells. Although mechanical dispersion, as used in these experiments, produced an adequate number of cells and osteoclasts, it was time-consuming (average 45 min), and a brief enzyme pretreatment [12] may prove applicable here.

Other investigators, reporting successful cell isolation using velocity sedimentation, have obtained purer cell suspensions [13, 28, 30]. However, the cells which were isolated always constituted a higher percentage (15–25%) of the initial suspension than was noted in our osteoclast preparations (0.5–1.5%). In addition, their initial cell suspensions contained cell concentrations below the streaming limit ($1.0–4.0 \times 10^6$ cells/ml), which facilitated cell separation. Attempts to dilute the osteoclast preparations were unsuccessful; cell streaming, therefore, was always apparent during sedimentation. Cell aggregation also hampered attempts to isolate osteoclasts. Cell clumping, though most notable after the first sedimentation period, was also present following the second procedure. Perhaps the use of deoxyribonuclease [12, 30] may help eliminate this problem. Despite the low initial percentage of osteoclasts, the high concentration of contaminating cells (above the streaming limit), and cell aggregation, highly enriched populations of osteoclasts were obtained.

The in vitro incubation experiments (Figs. 6 and 7) demonstrate that cells comprising the enriched osteoclast fractions (pooled fractions 3–4) are capable of protein synthesis. Although the cell type most active in protein synthesis in this heterogeneous cell population is not known, the radioautographs demonstrate the incorporation of labeled leucine into osteoclasts (Fig. 8).

The method employed to isolate osteoclasts compares favorably with other reported attempts to separate this cell type. Walker [36] reported that with his technique (lyophilized imprints), "several hundred" osteoclasts could be isolated per hour. With the present technique, several thousand ($1.5–1.8 \times 10^4$) were collected after approximately 4 h. Pretlow [29] reported a 50-fold increase in osteoclast purity following programmed gradient sedimentation but gave no information on the final purity of his preparation. In the present study, a 50.2-fold increase was noted in one experiment, with an enrichment to over 30% osteoclasts and accounting for greater than 72% of the cell volume. Therefore, quantitative biochemical, histochemical, metabolic, and ultrastructural studies of osteoclasts should be feasible. The effects of substances (such as parathyroid hormone or calcitonin) could be monitored in vitro or assessed in vivo after the cells are concentrated by velocity sedimentation.

References

1. Bard, D. R., Dickens, M. J., Smith, A. U., Zarek, J. M.: Isolation of living cells from mammalian bone. Nature (Lond.) **236**, 315–316 (1972)
2. Bevis, R. R.: An autographic and electron microscopic study of collagen synthesis during tooth movement in the supraalveolar fibers of the golden syrian hamster. Ph.D. thesis, University of Minnesota 1969
3. Cameron, D. A.: The ultrastructure of bone. In: The biochemistry and physiology of bone (Bourne, G. H., ed.), Vol. 1, pp. 191–236. New York: Academic Press 1972

4. Carpenteur, A.-M.: Scanning methods: volume quantitation of tissue, cells, and subcellular components. J. Histochem. Cytochem. **14**, 834–841 (1966)
5. Carpenteur, A.-M., Lazarow, A.: Component quantitation of tissue sections. II. Study of the factors which influence the accuracy of the method. J. Histochem. Cytochem. **10**, 329–340 (1962)
6. Chaynes, F.: Determination of relative volume by sectional analysis. Lab. Invest. **14**, 987–995 (1965)
7. Doty, S. B., Schofield, B. H., Robinson, R. A.: The electron microscopic identification of acid phosphatase and adenosinetriphosphatase in bone cells following parathyroid extract or thyrocalcitonin administration. In: Parathyroid hormone and thyrocalcitonin (Talmage, R. V., Belanger, L. E., eds.), pp. 169–181. Amsterdam: Exerpta Medica Foundation 1968
8. Gothlin, G., Ericsson, J. L. E.: Observations on the mode of uptake of thorium doixide particles by osteoclasts in fracture callus. Calcif. Tiss. Res. **10**, 216–222 (1972)
9. Hancox, N. M.: The osteoclast. In: The biochemistry and physiology of bone (Bourne, G. H., ed.), Vol. 1, pp. 213–250. New York: Academic Press 1972
10. Hancox, N. M., Boothroyd, B.: Motion picture and electron microscope studies on the embryonic avian osteoclast. J. Cell Biol. **11**, 651–661 (1961)
11. Holtrop, M. E., Raisz, L. G., Simmons, H. A.: The effects of parathyroid hormone, colchicine, and calcitonin on the ultrastructure and the activity of osteoclasts in organ culture. J. Cell Biol. **60**, 346–355 (1974)
12. Hopkins, C. R., Farquahar, M. G.: Hormone secretion by cells dissociated from rat anterior pituitaries. J. Cell Biol. **59**, 276–303 (1973)
13. Hymer, W. C., Evans, W. H., Kraicer, J., Mastro, A., Davis, J., Griswold, E.: Enrichment of cell types from the rat adenohypophysis by sedimentation at unit gravity. Endocrinology **92**, 275–287 (1973)
14. Kallio, P. M., Garant, P. R., Minkin, C.: Evidence of coated membranes in the ruffled border of the osteoclast. J. Ultrastruct. Res. **37**, 169–177 (1971)
15. Kallio, D. M., Garant, P. R., Minkin, C.: Evidence for an ultrastructural effect of calcitonin on osteoclasts in tissue culture. In: Calcium, parathyroid hormone, and the calcitonins (Talmage, R. V., Munson, P. L., eds.), pp. 383–385. Amsterdam: Exerpta Medica Foundation 1972
16. Lazarow, A., Carpenteur, A.-M.: Component quantitation of tissue sections. I. Characterization of the instruments. J. Histochem. Cytochem. **10**, 324–328 (1962)
17. Lucht, U.: Absorption of peroxidase by osteoclasts as studied by electron microscope histochemistry. Histochemie **29**, 274–286 (1972a)
18. Lucht, U.: Cytoplasmic vacuoles and bodies of the osteoclast. Z. Zellforsch. **135**, 229–244 (1972b)
19. Lucht, U., Maunsbach, A. B.: Effects of parathyroid hormone on osteoclasts in vivo. Z. Zellforsch. **141**, 529–541 (1973)
20. Mage, M. G., Evans, W. H., Peterson, E. A.: Enrichment of antibody plaqueforming cells of spleen by sedimentation at unit gravity. Proc. Soc. exp. Biol. (N.Y.) **127**, 478–481 (1968)
21. Malkani, K., Luxembourger, M.-M., Rebel, A.: Cytoplasmic modifications at the contact zone of osteoclasts and calcified tissue in the diaphyseal growing plate of foetal guinea pig tibia. Calcif. Tiss. Res. **11**, 258–264 (1973)
22. Mans, R. J., Novelli, P. G.: Measurement of the incorporation of radioactive amino acids in protein by a filter-paper disk method. Arch. Biochem. Biophys. **94**, 48–53 (1961)
23. Mateyko, G. M., Kopac, M. J.: Cytophysical studies on living normal and neoplastic cells. Ann. N.Y. Acad. Sci. **105**, 183–286 (1963)
24. Miller, R. G., Phillips, R. A.: Separation of cells by velocity sedimentation. J. Cell Physiol. **73**, 191–202 (1969)
25. Nelson, R. L., Bauer, G. E.: Isolation of osteoclasts by sedimentation at unit gravity. J. dent. Res. **54**, 106 (1975a)
26. Nelson, R. L., Bauer, G. E.: Isolation of highly enriched fractions of rabbit osteoclasts by velocity sedimentation at unit gravity. Mineralized Tiss. Res. Comm. **1**, 246 (1975b)
27. Park, H. Z., Talmage, R. V.: Comparison of the effects of calcium and endogenous parathyroid hormone on RNA synthesis in rat bone. In: Parathyroid hormone and

thyrocalcitonin—calcitonin (Talmage, R. V., Belanger, L. F., eds.), pp. 203–215. Amsterdam: Exerpta Medica Foundation 1968
28. Peterson, E. A., Evans, W. H.: Separation of bone marrow cells by sedimentation at unit gravity. Nature (Lond.) **214**, 824–825 (1967)
29. Pretlow, T. G. II., Boone, C. W., Riley, W. B.: Cell separations using programmed gradient sedimentation. Fed. Proc. **28**, 684 (1969)
30. Romrell, L. J., Coppe, M. R., Ito, S.: The isolation of viable, highly enriched fractions of mouse parietal cells by velocity sedimentation. Anat. Rec. **175**, 427 (1973)
31. Scott, B. L.: The occurrence of specific cytoplasmic granules in the osteoclast. J. Ultrastruct. Res. **19**, 417–431 (1967)
32. Vaes, G.: The role of lysosomes and of their enzymes in the development of bone resorption induced by parathyroid hormone. In: Parathyroid hormone and thyrocalcitonin-calcitonin (Talmage, R. V., Belanger, L. F., eds.), pp. 318–328. Amsterdam: Exerpta Medica Foundation 1968
33. Vaes, G.: Lysosomes and the cellular physiology of bone resorption. In: Lysosomes in biology and pathology (Dingle, J. T., Fell, H. B., eds.), Vol. 1, pp. 217–253. Amsterdam: North-Holland Publishing Co. 1969
34. Vaes, G.: Enzymatic and other biochemical events accompanying bone resorption in tissue culture. Calcif. Tiss. Res., Suppl. **4**, 57–60 (1970)
35. Vaes, G.: Inhibitory actions of calcitonin on resorbing bone explants in culture and on their release of lysosomal hydrolases. J. dent. Res. **51**, 362–366 (1972)
36. Walker, D. G.: Enzymatic and electron microscopic analysis of isolated osteoclasts. Calcif. Tiss. Res. **9**, 296–309 (1972)
37. Woods, J. F., Nichols, G.: Collagenolytic activity in rat bone cells. J. Cell Biol. **26**, 747–757 (1965)