JOURNAL OF BONE AND MINERAL RESEARCH
Volume 9, Number 3, 1994
Mary Ann Liebert, Inc., Publishers

Effects of Medium Acidification by Alteration of Carbon Dioxide or Bicarbonate Concentrations on the Resorptive Activity of Rat Osteoclasts

TIMOTHY R. ARNETT, ALAN BOYDE, SHEILA J. JONES, and M. LOUISE TAYLOR

ABSTRACT

Little is known about the extracellular conditions or factors that stimulate mature osteoclasts to resorb mineralized tissues. Isolated mammalian osteoclasts are strongly stimulated by protons in HEPES-buffered culture media in the absence of CO_2 and HCO_3^-, but it has been reported that cell-mediated Ca^{2+} release from bone organ cultures is increased only when media are acidified by reduction of HCO_3^- concentrations, and not by increasing P_{CO_2} (considered models of metabolic and respiratory acidosis, respectively). We investigated this question using disaggregated rat osteoclasts cultured on dentin slices for 24 h. The number of pits resorbed per osteoclast was stimulated in media acidified by manipulation of either HCO_3^- or CO_2 concentrations. In experiments in which incubator CO_2 was varied, resorption was almost abolished in the presence of 2.5% CO_2 at pH 7.61 but increased in a stepwise manner up to 1.3 pits per osteoclast when dentin slices were cultured with 10% CO_2 at pH 6.97. The depths and widths of pits, measured using a confocal laser reflection microscope, also tended to increase with increasing CO_2 and decreasing pH. However, in experiments where pH was lowered by reducing medium HCO_3^-, pit size decreased, partially offsetting the increased number of pits resorbed per osteoclast. These findings suggest that rat osteoclasts may be more sensitive to stimulation by CO_2 acidosis than by HCO_3^- acidosis, at least in the short term, and may possibly reflect local regulatory processes in bone.

INTRODUCTION

THE IMPORTANCE OF THE SKELETON in the maintenance of acid-base balance has long been recognized. The association of experimental acidosis with mineral loss and skeletal depletion was noted in the early part of this century.[1,2] Metabolic acidosis may be a contributory factor in renal osteodystrophy[3,4] and may play a role in the pathogenesis of osteoporosis.[5] Studies with rats made acidotic by ammonium chloride or hydrochloric acid feeding have suggested that the resulting bone loss and hypercalcemia may involve enhanced osteoclastic resorption.[6–8]

Dominguez and Raisz[9] examined the effects of changing H^+, HCO_3^-, and CO_2 concentrations on bone resorption in vitro by measuring ^{45}Ca release from cultured rat long bones. It was concluded that calcium release at low pH was accounted for by physicochemical dissolution of bone mineral and that cell-mediated resorption was unaltered between pH 6.9–7.5 but was inhibited outside this range. Experiments on the effects of pH in cultured neonatal mouse calvariae by Bushinsky and colleagues demonstrated differing results according to time in culture. In short-term (3 h) cultures, the net calcium efflux observed when pH was lowered by increasing P_{CO_2} (carbon dioxide pressure, a model of respiratory acidosis) or by decreasing the HCO_3^- concentration of the culture medium (a model of uremic metabolic acidosis) appeared to be due to physicochemical dissolution of bone calcium carbonate.[10,11] Evidence for proton stimulation of cell-mediated Ca^{2+} release was seen in long-term (96–99 h) cultures in response to lowered medium bicarbonate but not to increased P_{CO_2}.[12] Goldhaber and Rabadjija[13] reported large dose-dependent increases in calcium release from mouse calvariae cultured over 7 days in media acidified with hydrochloric acid; this effect was seen in live bones only and was blocked by calcitonin, implying osteoclast

Department of Anatomy and Developmental Biology, University College London, England.

involvement. Further work by this group led to the interesting conclusion that proton-stimulated resorption in bone organ cultures is dependent on prostaglandin E_2 and cyclic AMP production.[14]

The most direct evidence for modulation of osteoclastic function by protons comes from studies using disaggregated cell preparations. In media buffered with HEPES only (i.e., without HCO_3^- and CO_2), acidification of culture medium from pH 7.4 to 6.8 resulted in a 14-fold increase in the area resorbed by rat osteoclasts cultured for 24 h on cortical bone slices.[15]

In the present study, we have used an in vitro resorption assay to investigate possible differences in the response of rat osteoclasts to medium acidification by alteration of HCO_3^- or ambient CO_2 concentrations. Changes in resorption pit size were assessed using a video rate laser confocal microscope.

MATERIALS AND METHODS

Tissue culture media

Experiments were performed in minimum essential medium (MEM) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml of penicillin, 100 μg/ml of streptomycin and 0.25 μg/ml of amphotericin. In experiments in which initial bicarbonate concentrations were varied, MEM was reconstituted from powdered form with varying amounts of added $NaHCO_3$; media containing reduced levels of $NaHCO_3$ (i.e., below the standard concentration of 2.2 g/liter) were supplemented with osmotic equivalents of NaCl. All tissue culture reagents were purchased from GIBCO (Paisley, UK). Additional reagents were from Sigma (Poole, UK).

Cell isolation and culture

Cells were mechanically disaggregated from the long bones of 2- to 4-day-old Sprague-Dawley rat pups using procedures similar to those described previously.[15,16] Cells suspended in phosphate-buffered saline (PBS, pH 7.4) were allowed to sediment for 30 minutes onto 10 × 10 × 0.2 mm slices of dentin cut transversely using a low-speed saw (Buehler, Evanston, IL) from a hippopotamus canine tooth (kindly provided by Mr. D. Tomlin). Dentin slices were then rinsed with PBS to remove nonadherent cells before incubation for 24 h in test media (1 ml medium per slice). Experiments were repeated four or five times using cells derived from separate littermate pups. In experiments in which CO_2 levels were varied, slices were incubated in media preequilibrated for 90 minutes to the appropriate CO_2 concentration in 25 cm^2 flasks (four or five slices per flask). For insertion of dentin slices, flasks were briefly opened with the canted neck in a nearly vertical position to minimize loss of CO_2. Flasks were then sealed for the duration of the experiment. In experiments in which HCO_3^- concentrations were varied, slices were incubated in 24-multiwell plates. Experiments were terminated by fixing slices in 3% glutaraldehyde and 0.15 M sodium cacodylate for 5 minutes. Incubator CO_2 levels were calibrated using a Fyrite gas analyzer (Bacharach Instrument Co., Pittsburgh, PA); P_{CO_2} levels in media were measured using a blood gas analyzer (Radiometer, Copenhagen, Denmark). Medium HCO_3^- concentrations were calculated from the Henderson-Hasselbalch equation using a CO_2 solubility coefficient of 0.0301 and pH-corrected pK values between 6.099 and 6.120.[17]

Experimental analysis

Fixed dentin slices were stained for 15 minutes for tartrate-resistant acid phosphatase (TRAP) activity, and total numbers of TRAP-positive multinucleate osteoclasts were counted. Cells were stripped off wet slices by gentle rubbing, and total numbers of discrete resorption lacunae were counted following staining with toluidine blue (1% wt/vol in 1% wt/vol sodium borate), as previously described.[15,18,19]

The depths of the resorption pits were measuring using a Lasertec 1LM11 video rate confocal laser reflection microscope.[20–22] Dentin slices were searched systematically for resorption lacunae and a real-time image, obtained using a dry ×50 (numerical aperture = 0.95) lens, was displayed on a monitor. The deepest part of the pit was ascertained by manual focusing, and a sampling line, visible on the monitor, was placed across it. The dentin surface was reconstructed along this sampling line to give a vertical profile of the pit. Manual adjustment of pairs of horizontal and vertical cursor lines allowed the width of the pit at the sampling line and the maximum depth to be determined. The width measurement so obtained takes no account of the orientation of the pit along the sampling line and is thus not necessarily the maximum width of the pit. Measurements were made for the first 20 pits encountered on each slice, when this was possible. All measurements were performed on randomized, coded slices. Data were evaluated for statistical significance by one-way analysis of variance (toluidine blue-stained pit counts) or by Mann-Whitney U test (pit depths and widths).

RESULTS

Alteration of ambient CO_2 and, thus, culture medium pH was investigated in two sets of experiments. In the first series, the added $NaHCO_3$ concentration was 2.2 g/liter, and incubator CO_2 levels of 2.5, 5, 7.5, and 10% resulted in final medium pH values of 7.61, 7.32, 7.17, and 6.97; P_{CO_2} values were 18.4, 35.2, 50.4, and 82.0 mm Hg, respectively, with calculated HCO_3^- concentrations between 17.4 and 18.4 mM. Resorption was almost abolished in low CO_2-high pH conditions, but increased sharply with decreasing pH (Fig. 1A).

To achieve a lower pH operating range, a second series of experiments was conducted with an added $NaHCO_3$ concentration of 1.2 g/liter. In these experiments, ambient CO_2 levels of 2.5, 5, 7.5, and 10% yielded final pH values of 7.30, 7.20, 7.07, and 6.76, with P_{CO_2} values of 25.5, 32.0, 43.2, and 89.8 mm Hg, respectively, and HCO_3^- concentrations between 11.8 and 12.1 mM. A corresponding 7.7-fold increase in pits resorbed per slice (Fig. 1B) or 12.6-fold increase in pits resorbed per osteoclast was observed. Measurements by laser confocal microscopy for the experiments shown in Fig. 1A revealed increases in pit maximum depths, and widths at maximum depth, with increasing CO_2 and decreasing pH (see Fig. 3A).

In 5% ambient CO_2, added $NaHCO_3$ concentrations of 2.2, 1.7, 1.2, and 0.7 g/liter resulted in final culture medium pH

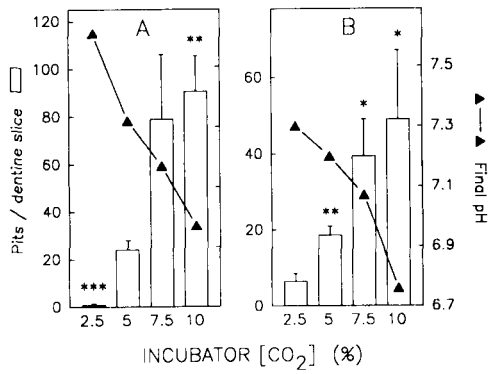

FIG. 1. Stimulatory effect of CO_2 acidosis on the numbers of pits resorbed by rat osteoclasts in dentin slices over 24 h. Culture medium contained 2.2 g/liter of added $NaHCO_3$ (A) or 1.2 g/liter of added $NaHCO_3$ (B). Numbers of TRAP-positive multinucleate cells per dentin slice were 69.2 ± 18.8, 81.7 ± 16.9, 81.5 ± 10.8, and 68.2 ± 19.2 (A) and 46.0 ± 13.7, 34.6 ± 12.1, 47.0 ± 14.7, and 33.2 ± 8.5 (B) for 2.5, 5.0, 7.5, and 10.0% CO_2, respectively. Resorption and cell number values represent mean ± standard error of the mean (SEM) for four experiments (A) or five experiments (B) analyzed by bright-field light microscopy. Significantly different from 5% CO_2 value (A) or 2.5% CO_2 value (B): $*p < 0.05$; $**P < 0.01$; $***P < 0.001$.

values of 7.48, 7.36, 7.24, and 7.03; calculated HCO_3^- concentrations were 21.2, 16.9, 12.0, and 7.4 mM, respectively, and P_{CO_2} levels were between 28.9 and 30.8 mm Hg. Over this range there was a corresponding 3.8-fold stimulation in pits resorbed per dentin slice (Fig. 2) and a 5.3-fold increase in the number of pits resorbed per osteoclast.

Maximum pit depths, however, decreased significantly with lowered $NaHCO_3$ and pH; pit widths also decreased, although not significantly (Fig. 3B). The significant decreases in pit size tended to offset partially the stimulation of the numbers of pits

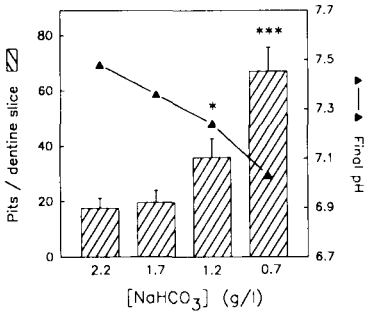

FIG. 2. Stimulatory effect of HCO_3^- acidosis on the total numbers of pits resorbed by rat osteoclasts cultured on dentin in a 5% CO_2 atmosphere for 24 h. Numbers of TRAP-positive multinucleate cells per dentin slice were 29.8 ± 5.9, 46.6 ± 2.9, 50.4 ± 6.9, and 34.8 ± 4.3 for 0.7, 1.2, 1.7, and 2.2 g/liter of $NaHCO_3$, respectively. Resorption and cell number values represent mean ± SEM for five experiments analyzed by bright-field light microscopy. Significantly different from 2.2 g/liter $NaHCO_3$ value: $*p < 0.05$; $***p < 0.001$.

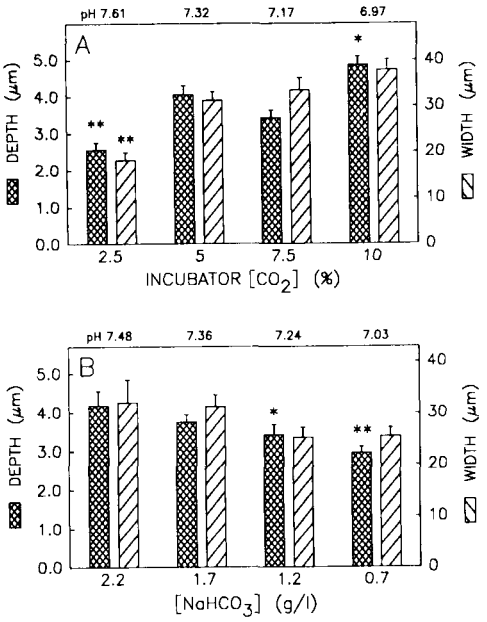

FIG. 3. (A) Effect of CO_2 acidosis on maximum pit depths and raster search widths at maximum depth. Measurements were made using a video rate confocal laser microscope. Values represent mean ± SEM for 19, 80, 80, and 85 pits measured for 2.5, 5, 7.5, and 10% CO_2 treatment groups, respectively. Significantly different from 5% CO_2 value: $*p < 0.05$; $**p < 0.01$. (B) Effect of HCO_3^- acidosis on maximum pit depths and raster search widths at maximum depth. Values represent mean ± SEM for 35, 81, 93, and 100 pits measured for 2.2, 1.7, 1.2, and 0.7 g/liter of $NaHCO_3$ treatment groups, respectively. Significantly different from 2.2 g/liter of $NaHCO_3$ value: $*p < 0.05$; $**p < 0.01$.

resorbed per osteoclast. Thus, these data are suggestive of different osteoclastic responses to HCO_3^- and CO_2 acidification.

Additional experiments confirmed that the number of pits resorbed by rat osteoclasts was increased at low pH in HCO_3^--free MEM and 10% fetal calf serum buffered with HEPES. However, mean pit depths and raster search widths were considerably lower in media buffered with HEPES only and were not significantly altered by pH. In one set of experiments using five separate bone cell preparations from littermate rat pups ($n = 5$), values for depth and width, respectively, were 1.11 ± 0.07 and 15.1 ± 0.90 µm at pH 6.84 and 1.20 ± 0.13 and 14.2 ± 0.96 µm at pH 7.47 (no significant difference). Values for pits/dentin slice and pits/osteoclast in these experiments were 14.2 ± 11.0 and 0.28 ± 0.18 at pH 6.84 and 6.2 ± 3.6 and 0.04 ± 0.11 at pH 7.47.

DISCUSSION

We demonstrated that the numbers of pits resorbed by rat osteoclasts in vitro are strongly stimulated under low-pH conditions resulting from increased P_{CO_2} or reduced culture medium HCO_3^- concentrations, whereas in alkaline conditions resulting

from reduced P_{CO_2}, resorption was almost abolished. Doubling ambient CO_2 from 5 to 10% resulted in a highly significant 3.5-fold stimulation of pits resorbed per osteoclast. The size of resorption pits, approximated using the formula $2/3\pi \times \text{depth} \times (\text{pit width}/2)^2$, also tended to increase with increasing CO_2 levels, such that the mean estimated amount of dentin substrate excavated by each osteoclast showed an overall increase of more than 1000-fold between pH 7.61 and 6.97, as a result of quadrupling ambient CO_2. However, HCO_3^--induced acidosis was associated with a 3.2-fold reduction in estimated resorption pit volume over the pH range 7.03–7.48, which partially offset the corresponding 5.3-fold increase in numbers of pits resorbed per osteoclast. Some caution is required in the interpretation of pit size data, since relatively few pits were formed in the high-pH treatment groups, particularly in the dentin slices incubated with 2.5% CO_2. Given this caveat, our results suggest that the overall rate of resorption by rat osteoclasts may be somewhat more sensitive to CO_2-related pH shifts than to HCO_3^--related changes, at least over a 24 h time period.

Previous work demonstrated that the numbers of pits resorbed in bone slices by rat osteoclasts is stimulated at low pH in media buffered with HEPES only.[15] However, the mean numbers of pits excavated per osteoclast over 24 h appear to be generally somewhat lower in non-HCO_3^-/CO_2-buffered media (Taylor and Arnett, unpublished results). Resorption pits were also a good deal smaller under such conditions. Maximum depths of resorption pits were relatively shallow (1.1–1.2 μm) and not significantly altered by pH, an observation consistent with earlier measurements by scanning electron microscope photogrammetry.[15] These data contrast with depth values ranging between 2.6 and 4.8 μm, depending on HCO_3^- concentration and P_{CO_2}, reported here. Pit widths were also somewhat reduced in the HEPES-buffered media and, again, not significantly altered by pH. These results are certainly in line with the trend toward reduced pit depth and width observed with decreasing P_{CO_2} and HCO_3^- concentration, although it is possible that a slight cytotoxic action of HEPES contributed to the effect. An important consideration here is that intracellular pH regulation may be perturbed in HCO_3^--free media, at least in renal mesangial cells[23]; under these conditions, Na^+/H^+ exchange appears to be the only operational pH regulatory mechanism.[24] Taken together, our data suggest that although extracellular protons appear to stimulate rat osteoclasts, mainly by increasing the absolute numbers of pits resorbed, CO_2 and HCO_3^- may primarily affect pit size. However, the present data do not allow us to distinguish the extent to which the effects observed reflect alterations in the proportion of activated to quiescent osteoclasts, changes in cell motility, or in the average time spent by each osteoclast per excavation, or simply in resorptive efficiency.

The effect of CO_2-induced pH changes on the resorption parameters measured may reflect to some extent the requirement of osteoclastic carbonic anhydrase for CO_2. This enzyme is thought to represent an important source of protons for resorption.[25] Our data also suggest that the presence of physiologic levels of CO_2 tends to facilitate resorption even at slightly alkaline pH (Fig. 2). The observed reduction in pit size with decreasing culture medium HCO_3^- concentration is less easy to rationalize. If HCO_3^- generated by carbonic anhydrase is exchanged for extracellular Cl^-, which then serves as a counterion for H^+ actively secreted at the ruffled border (see Ref. 26), then reduction of extracellular HCO_3^- may have been expected to facilitate cell-mediated resorption in a similar manner to elevation of ambient CO_2.

Several mechanisms could be involved in the stimulation of resorption by extracellular H^+ in addition to simple reduction of the gradient against which the osteoclast must pump protons to create and maintain the acid microcompartment necessary to effect resorption. Increases in extracellular proton concentration may be expected to depolarize osteoclasts by blockade of the K^+ inward rectifier, thus facilitating proton transport out of the cell.[27] In chicken osteoclasts, at least, falls in intracellular pH resulting from extracellular acidification may lead to a stimulation of active calcium efflux from the cell and enhanced expression of podosomes, the cell-matrix attachment structures located in the osteoclast clear zone.[28] The finding that H^+-stimulated bone resorption in neonatal mouse calvarial cultures is dependent on cyclic AMP and prostaglandin E_2 (PGE_2)[14] is intriguing but hard to interpret in the present context in view of the inhibitory effects of prostaglandins and cyclic AMP analogs on isolated osteoclasts.[16] The stimulatory effect of PGE_2 on resorption in bone organ culture is presumably indirect and is mediated through primary effects on other cell types, such as osteoblasts or fibroblasts. A further caveat to our own work relates to the impurity of the osteoclast cultures studied. Although the cell populations were distributed at quite low densities over the dentin slices, some osteoclasts undoubtedly contact or come into close proximity with osteoblasts or fibroblasts during the course of the 24 h assay. We therefore cannot exclude the possibility that the pH, CO_2, and HCO_3^- effects on osteoclasts may to some extent be indirect.

Our results indicate that CO_2-related (respiratory) acidosis in the presence of normal levels of HCO_3^- maximizes cell-mediated resorption not only by increasing the number of pits resorbed by disaggregated rat osteoclasts over 24 h but also by increasing the depth and width of these excavations. In contrast, low-pH conditions resulting from reduction in HCO_3^- concentration (metabolic acidosis) increased the numbers of pits resorbed per osteoclast but were associated with a progressive reduction in the size of excavations. These findings are clearly at variance with the data of Bushinsky,[12] who reported that Ca^{2+} efflux from cultured neonatal mouse calvariae is stimulated at low pH only when medium HCO_3^- is also reduced, but not as a consequence of increased P_{CO_2}. However, the Ca^{2+} effluxes measured by Bushinsky had both physicochemical and cell-mediated components. The earlier studies of Dominguez and Raisz[9] failed to demonstrate any effect of H^+, CO_2, or HCO_3^- on the cell-mediated release of ^{45}Ca from cultured, prelabeled fetal rat long bones. These discrepancies may well be accounted for by the dissimilarities of the various in vitro assay systems and their time courses.

From a methodologic standpoint, our results highlight the potential sensitivity of the disaggregated rat osteoclast resorption assay to pH-related artifacts[15,26,29] and show that although simple pit counting may be the best and most efficient single index for assessing resorption episodes in vitro, such data by themselves may to some extent be misleading. Confocal microscopy now offers a realistic method by which accurate three-dimensional measurement of resorption pits can be made,[20–22] although useful if less accurate depth measurements can also be obtained using simple reflected light microscopy.[30]

Osteoclasts are relatively sparsely distributed in vivo, often functioning in groups, but in close proximity to a variety of metabolically active cells, including osteoblasts, fibroblasts, and endothelial and immune cells. A central concern in bone cell biology has been the elucidation of the mechanisms by which these cell types influence osteoclastic activity during the processes of formation and remodeling. It is possible that small local shifts in extracellular pH, HCO_3^- concentration, or P_{CO_2}, perhaps related to growth factor, cytokine, or hormone action on target cells in bone, could influence the resorptive activity of adjacent osteoclasts in vivo. Furthermore, local reduction in extracellular pH owing to the activity of osteoclasts themselves may also be expected to result in self-stimulation, particularly in osteoclast "clusters." Testing these ideas in living bone microenvironments is clearly no easy task, but confocal microscopy in conjunction with pH-sensitive dyes may offer one potential approach.

ACKNOWLEDGMENTS

The authors gratefully acknowledge support from Research into Ageing (UK), the Wellcome Trust and the Research Corporation Trust (UK). We thank Mr. S. Komiya of Lasertec Corporation (UK) for the kind loan of the 1LM11 microscope system. Presented in preliminary form at the Workshop on Osteobiology, Brindisi, Italy, June 2–5, 1991.

REFERENCES

1. Goto K 1918 Mineral metabolism in experimental acidosis. J Biol Chem **36:**355–376.
2. Jaffe HL, Bodansky A, Chandler JP 1932 Ammonium chloride decalcification as modified by calcium intake: The relation between generalized osteoporosis and ostitis fibrosa. J Exp Med **56:**823–834.
3. Avioli LV 1978 Renal osteodystrophy. In: Avioli LV, Krane SM (eds.) Metabolic Bone Disease, Vol. 2. Academic Press, New York, pp. 149–215.
4. Cunningham J, Fraher LJ, Clemens TL, Revell PA, Papapoulos SE 1983 Chronic acidosis with metabolic bone disease. Am J Med **73:**199–204.
5. Barzel US 1976 Osteoporosis II: An overview. In: Barzel US (ed.) Osteoporosis II. Grune and Stratton, New York, pp. 1–6.
6. Barzel US, Jowsey J 1969 The effects of chronic acid and alkali administration of bone turnover in adult rats. Clin Sci **36:**517–524.
7. Kraut JA, Mishler DR, Kurokawa K, Warech E 1984 Effect of colchicine and calcitonin on the calcemic response to metabolic acidosis. Kidney Int **25:**608–612.
8. Chan Y-L, Savdie E, Mason RS, Posen S 1985 The effect of metabolic acidosis on vitamin D metabolites and bone histology in uremic rats. Calcif Tissue Int **37:**158–164.
9. Dominguez JH, Raisz LG 1979 Effects on changing hydrogen ion, carbonic acid and bicarbonate concentrations on bone resorption in vitro. Calcif Tissue Int **29:**7–13.
10. Bushinsky DA, Krieger NS, Geisser DI, Grossman EB, Coe FL 1983 Effects of pH on bone calcium and proton fluxes in vitro. Am J Physiol **245:**F204–209.
11. Bushinsky DA, Lechleider RJ 1987 Mechanism of proton-induced bone calcium release: Calcium carbonate dissolution. Am J Physiol **253:**F998–F1005.
12. Bushinsky DA 1989 Net calcium efflux from live bone during chronic metabolic but not respiratory acidosis. Am J Physiol **256:**F836–842.
13. Goldhaber P, Rabadjija L 1987 H^+ stimulation of cell-mediated bone resorption in tissue culture. Am J Physiol **253:**E90–E98.
14. Rabadjija L, Brown EM, Swartz SL, Chen CJ, Goldhaber P 1990 H^+-stimulated release of prostaglandin E_2 and cyclic adenosine 3′,5′-monophosphoric acid and their relationship to bone resorption in neonatal mouse calvaria cultures. Bone Miner **11:**295–304.
15. Arnett TR, Dempster DW 1986 Effect of pH on bone resorption by rat osteoclasts in vitro. Endocrinology **119:**119–124.
16. Arnett TR, Dempster DW 1987 A comparative study of disaggregated chick and rat osteoclasts in vitro: Effect of calcitonin and prostaglandins. Endocrinology **120:**602–608.
17. Severinghaus JW 1965 Blood gas concentrations. In: Fenn WO, Rahn H (eds.) Handbook of Physiology, Sec. 3, Vol. 2, Respiration. American Physiology Society, Washington, D.C., pp. 1475–1487.
18. Boyde A, Ali NN, Jones SJ 1984 Resorption of dentine by isolated osteoclasts in vitro. Br Dent J **156:**216–220.
19. Murrills RJ, Dempster DW 1990 The effects of stimulators of intracellular cyclic AMP on rat and chick osteoclasts in vitro: Validation of a simplified light microscope assay of bone resorption. Bone **11:**333–344.
20. Boyde A, Dillon CE, Jones SJ 1990 Measurement of osteoclastic resorption pits with a tandem scanning microscope. J Microsc **158:**261–265.
21. Boyde A, Jones SJ 1991 Pitfalls in pit measurement (Editorial). Calcif Tissue Int **49:**65–70.
22. Boyde A, Jones SJ 1992 Really close up! Surveying surfaces at sub-micrometre resolution: The measurement of osteoclastic resorption lacunae. Photogrammetric Rec **14:**59–84.
23. Ganz MB, Boyarsky G, Sterzel RB, Boron WF 1989 Arginine vasopressin enhances pH_i regulation in the presence of HCO_3^- by stimulating three acid base transport systems. Nature **337:**648–651.
24. Thomas RC 1989 Bicarbonate and pH_i response. Nature **337:**601.
25. Minkin C, Jennings JM 1972 Carbonic anhydrase and bone remodeling: Sulfonamide inhibition of bone resorption in organ culture. Science **176:**1031–1033.
26. Arnett TR, Dempster DW 1990 Protons and osteoclasts. J Bone Miner Res **5:**1099–1103.
27. Sims SM, Dixon SJ 1989 Inwardly rectifying K^+ currents in osteoclasts. Am J Physiol **256:**C1277–C1282.
28. Teti A, Blair HC, Schlesinger P, Grano M, Zambonin-Zallone A, Kahn AJ, Teitelbaum SL, Hruska KA 1989 Extracellular protons acidify osteoclasts, reduce cytosolic calcium and promote expression of cell-matrix attachment structures. J Clin Invest **84:**773–780.
29. Chambers TJ 1988 The regulation of osteoclastic development and function. In: Evered D, Harnett S (eds.) Cell and Molecular Biology of Vertebrate Hard Tissues, Ciba Foundation Symposium 136. John Wiley and Sons, Chichester, pp. 92–100.
30. Walsh CA, Beresford JN, Birch MA, Boothroyd B, Gallagher JA 1991 Application of reflected light microscopy to identify and quantitate resorption by isolated osteoclasts. J Bone Miner Res **6:**661–671.

Address reprint requests to:
Timothy R. Arnett
Department of Anatomy and Developmental Biology
University College London
Gower Street
London WC1E 6BT, UK

Received in original form November 30, 1992; in revised form May 24, 1993; accepted July 28, 1993.