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# RESORPTION OF NATURAL CALCIUM CARBONATE BY AVIAN OSTEOCLASTS *IN VITRO*

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## Abstract

Osteoclasts isolated from the endosteum of 2.5 to 3-week chick tibia were cultured on glass coverslips or natural $CaCO_3$ (*Tridacna*) wafers for 2 and 4 days. The cells were exposed to the pH-dependent dye, acridine orange, and fluorescence was measured by a light microscope photometer. Fluorescence intensity values were higher in cells adherent to *Tridacna* wafers than in those incubated on glass after 2 and 4 days of culture (three- and two-fold, respectively). Moreover, osteoclasts on *Tridacna* wafers were more flattened and were found to produce resorption pits. Acid production by osteoclasts cultured on *Tridacna* wafers was stimulated with $10^{-8}$ M parathyroid hormone and inhibited with $10^{-7}$ M acetazolamide or $10^{-7}$ M hydroxybenzoyl thiophene sulfonamide, as shown by changes in intensity of acridine orange fluorescence after 30, 60 and 120 minutes of treatment. These results indicate that osteoclasts cultured on natural $CaCO_3$ wafers mimic the behavior of osteoclasts cultured on other substrates. Further, the capacity to acidify was enhanced in cells cultured on $CaCO_3$ wafers. These results indicate that natural $CaCO_3$ *Tridacna* wafers provide a suitable substrate for osteoclasts in culture and demonstrate that carbonic anhydrase plays a role in carbonated substrate resorption.

**Key Words**: Osteoclasts, bone resorption, $CaCO_3$ wafers.

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## Introduction

A variety of calcified implant materials are currently used as bone graft substitutes. They are usually synthetic calcium phosphate materials such as hydroxyapatite and tricalcium phosphates. Krukowski and Kahn (1982) have reported the influence of substrate composition on osteoclast differentiation when particles of mineralized and demineralized human and rat bone, hydroxyapatite (HAP) and eggshell were grafted onto chorioallantoic membranes of chick embryos. These investigators concluded that the factor(s) responsible for osteoclast differentiation reside specifically within bone matrix and is intimately associated with the mineral phase. Their study supports the view that calcified substrates play an important role in osteoclast differentiation. Chambers *et al.* (1984) compared the resorptive activity of osteoclasts isolated from neonatal rabbit long bones which were cultured on untreated, demineralized or devitalized slices of human cortical bone. They concluded that osteoclastic contact with bone mineral is the stimulus that initiates bone resorptive behavior in osteoclasts. Jones *et al.* (1984) studied the behavior of rat, rabbit and chicken osteoclasts in addition to other cells isolated from bone marrow. These cells were incubated for 1 and 3 days on a variety of substrates, including natural and synthetic HAP, and natural calcium carbonate (calcite and aragonite). Their results demonstrated that osteoclastic resorption of mineralized substrates followed a scheme that was similar for both phosphate and carbonate compounds.

Natural coral skeleton, a calcium carbonate material, has been reported to be bioresorbable since it is progressively resorbed and gradually replaced by newly formed bone when grafted into bony sites (Guillemin *et al.*, 1987, 1989). Animals with natural coral implants who were treated with oral acetazolamide showed a partial inhibition of resorption of both coral and bone. Carbonic anhydrase, an enzyme present in osteoclasts (Simasaki and Yagi, 1960; Gay and Mueller, 1973), was assumed to play a key role in coral resorption. The fluorescent dye acridine orange becomes sequestered

within membrane-bound compartments when protonated. At neutral pH it appears green, but within an acidic compartment it is orange (Moriyama *et al.*, 1982). We have previously used the pH-dependent color change of acridine orange to study the dynamics of acidification in chick osteoclast cultures and have shown that it is a reliable indicator of acid production which is responsive to changes in carbonic anhydrase activity (Hunter *et al.*, 1988, 1991).

The purpose of the present *in vitro* investigation was to demonstrate the resorption of natural calcium carbonate by osteoclasts using an avian model, and to evaluate the role of carbonic anhydrase in the resorption of this substrate.

## Materials and Methods

### Materials

Bovine parathyroid hormone (PTH) was provided by the National Hormone and Pituitary Program of the National Institute of Arthritis, Diabetes and Digestive and Kidney Diseases (Bethesda, MD). It has a purity of 75% and a biopotency of 1300 USP units/mg. The carbonic anhydrase inhibitor, 5-[3-hydroxybenzoyl]-thiophene-2-sulfonamide (HTS), was provided by Merck, Sharp and Dohme (West Point, PA). Bovine pancreatic trypsin (3x crystallized) was obtained from Worthington Biochemicals (Freehold, NJ); human fibronectin from Biomedical Technologies Inc. (Stoughton, MA); calf serum, fetal bovine serum, salmon calcitonin (CT), hyaluronidase (type 1-S), collagenase (type 1-A), tosyl-lysine-chloromethylketone, acetazolamide, and minimal essential medium (MEM) (Eagle's modification) from Sigma (St. Louis, MO). MEM was modified by the addition of 26 mM L-glutamine, 2 mM $NaHCO_3$, 100 U/ml penicillin, 100 $\mu$g/ml streptomycin and additional $CaCl_2$ (~ 0.03 g/L), KCl (~ 0.05 g/L) and NaCl (~ 0.8 g/L) to adjust osmolality to 310 mOsm.

### Animals

Rapidly growing broiler chickens (Peterson x Arbor Acre) strain were obtained from the Metz Hatchery (Belleville, PA) and raised to 2.5-3 weeks of age on a normal chick starter diet by the Poultry Education and Research Center of the Pennsylvania State University.

### Calccium carbonate ($CaCO_3$) wafers

Natural calcium carbonate (NCC) wafers, derived from the bivalve *Tridacna*, were obtained from Inoteb (Paris, France). The wafers were disc-shaped, 15 mm in diameter by 1 mm thick and had a composition of 98-99% $CaCO_3$, 0.4-0.5% Na, 0.02-0.03% K, and 0.1-0.2% Sr. The remaining 0.25 to 1.5% included amino acids and trace elements, the composition of which has been described previously (Guillemin *et al.*, 1989).

### Cell isolation and culture

Osteoclasts were isolated from the endosteum of tibias from chickens which had been injected subcutaneously with salmon calcitonin (30 mU/100 g body weight) to facilitate release of the cells from bone surfaces (Ali *et al.*, 1984; Hunter *et al.*, 1988). The birds were sacrificed 30 minutes after calcitonin injection. Tibias were aseptically removed, cleaned with sterile gauze to remove attached tendon and muscle, and split longitudinally. The bone marrow was lifted out with forceps and the bones were then placed in MEM + 10% heat-inactivated calf serum (CS) and flushed by vigorous pipetting. The bones were then subjected to a sequential, mild, enzymatic digestion sequence consisting of 0.05% hyaluronidase in MEM + 10% CS for 10 minutes followed by rinsing 3 times in MEM; 0.03% trypsin in MEM for 20 minutes followed by rinsing in MEM + 10% CS; 0.1% collagenase in MEM + 10% CS + tosyl-lysine-chloromethyl ketone (an inhibitor of trypsin and papain, 0.0027%) for 75 minutes followed by a final rinse in MEM + 10% CS. All digestion steps were carried out in an incubator at 37°C, 5% $CO_2$, 95% air, saturated humidity. The endosteal surfaces were then gently scraped with a rubber policeman in MEM + 10% CS. The released cells were filtered through 250 and 105 $\mu$m polypropylene meshes (Small Parts Inc., Miami Lakes, FL) and centrifuged at 1800 rpm for 10 minutes. The pellet was resuspended in MEM + 5% heat-inactivated fetal bovine serum (FBS). The resulting cell suspension, in 8 ml aliquots, was then plated onto 10 cm Petri dishes which had been coated with human fibronectin as described by Engvall and Ruoslahti (1977). Following incubation for 20 minutes, non-adherent cells were discarded in order to remove blood cells. The cells that had attached loosely to the fibronectin plates were shaken into MEM + 5% FBS by gentle swirling and collected for plating onto NCC wafers or glass coverslips (12 mm diameter) in MEM + 5% FBS in 1.5 cm microtiter wells (1.0 ml medium/well). The purpose of the fibronectin adsorption was to remove fibroblasts, osteoblasts and other cells that have substantial numbers of fibronectin receptors and consequently adhere tightly to the Petri dishes. Osteoclasts were allowed to settle onto glass coverslips or NCC wafers for 24 hours. The cells were then rinsed in MEM and incubated for 2 and 4 days in MEM + 0.5% FBS. Approximately 4000-6000 osteoclasts were plated per well.

### Stimulation or inhibition of acidification

After 2 and 4 days of culture, osteoclasts on NCC wafers were treated with $10^{-8}$ M bovine PTH (1-84), $10^{-7}$ M acetazolamide or $10^{-7}$ M HTS in MEM for 30, 60 and 120 minutes at 37°C prior to acridine orange staining.

### Acridine orange staining and fluorescence quantitation

Acridine orange staining and fluorescence quantitation were performed as described previously (Hunter *et al*, 1991). After 2 and 4 days of culture, osteoclasts on NCC wafers or glass coverslips were neutralized with 0.02 M $NH_4Cl$ in MEM for 15 minutes, rinsed in MEM (3 times, 30 seconds each), and allowed to recover in the presence or absence of stimulators or inhibitors of acidification.

After treating with PTH or carbonic anhydrase inhibitors, the cells were incubated for 10 minutes at 37°C in MEM which contained 10 mM acridine orange plus stimulatory or inhibitory agents where applicable. The wafers were then rinsed for 1 minute in MEM and mounted in a live-cell observation chamber which contained MEM plus the stimulatory or inhibitory agent. The NCC wafers were quickly scanned under fluorescence optics, osteoclasts were identified on the basis of morphology and staining characteristics and fluorescence measurements were made. Nuclei number was not routinely assessed because their presence is often masked by the intense orange staining. The fluorescence intensity of individual osteoclasts was determined using a Leitz microscope MPV compact photometer with an H2 filter cube plus a GG455 cutoff filter (excitation range 455-490 nm, emission 510 nm, suppression at 515 nm). A neutral density filter (N16, 6.25% transmittance) was used to reduce light intensity and fading. Twenty cells per time point were measured. The fluorescence values correspond to the overall fluorescence emitted within the entire area of the photometer window. The area of the window was set to fall within single osteoclasts and was kept constant for all experiments. Controls included using neutralized, acridine orange-stained cells on both an NCC wafer and a glass coverslip per experiment to provide a reference level of fluorescence after neutralization. A second set of NCC wafers with attached cells were neutralized, stained with acridine orange and incubated without any inhibition or stimulation to provide data on untreated cells. The experiments were performed in triplicate (20 osteoclasts per NCC wafer; three wafers per time point per treatment). Statistical significance was determined by analysis of variance and the Tukey-Kramer multiple comparisons test.

### Toluidine blue staining

In order to demonstrate resorption pits, NCC specimens with adherent osteoclasts were sonicated for 30 seconds in 0.25 M $NH_4OH$, treated for 30 seconds in acetone, air-dried, and then immersed for 5 minutes in 1% toluidine blue in 1% sodium borate. The NCC specimens were briefly rinsed in distilled water twice and air-dried.

### Scanning electron microscopy (SEM)

At the end of the culture period, NCC wafers with adherent osteoclasts were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer. Half of the samples were brushed with a fine paint-brush and sonicated in 0.25 M $NH_4OH$ for 10 minutes prior to fixation to remove adherent cells. The wafers were washed with 0.1 M cacodylate buffer, dehydrated in a graded ethanol series and critical point dried. Samples were sputter coated with gold and examined in an AMRAY 1000A SEM operated at an accelerating voltage of 5 kV at a tilt angle of 35°.

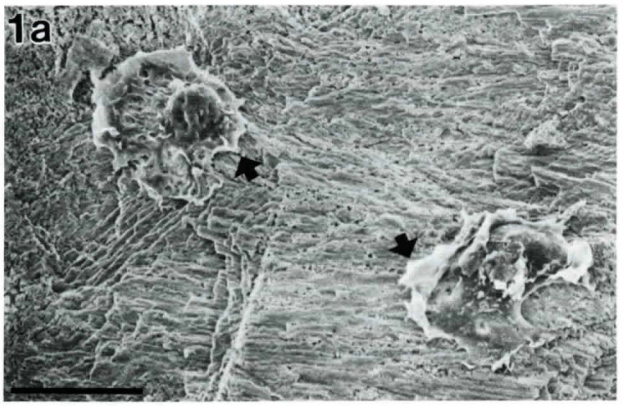
## Results

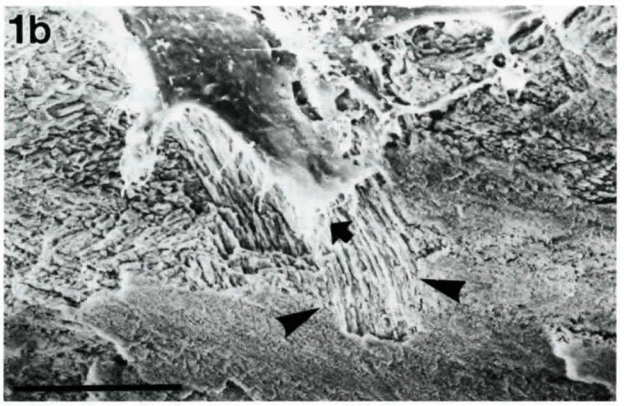
### Morphology of osteoclasts on NCC wafers

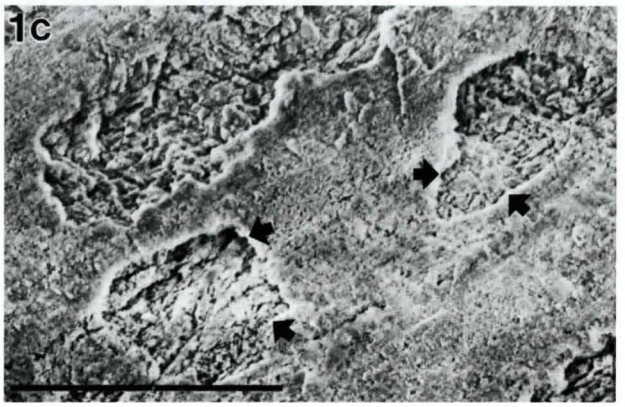
Osteoclasts cultured on NCC wafers had a fried-egg shape with a raised central area surrounded by a broad cytoplasmic apron when viewed after 2 days of culture (Fig. 1a). After 4 days of culture, areas of erosion were evident beneath cells in which cell margin retraction had occurred (Fig. 1b) and on wafers from which the cells had been removed (Fig. 1c). Although examination of control wafers which had not had cells plated onto them revealed a rough, irregular surface (Fig. 1d), the same degree and type of erosion observed in the cultured wafers was not seen. Cells were commonly found nestled in the depressions and valleys of the NCC wafer surface. Toluidine blue-staining of NCC wafers revealed light blue resorption pits evident after 4 days of culture (Fig. 2). Any remaining adherent cells were dark blue. Areas of erosion were found following 2 days of culture, but their increasing extensiveness with increasing culture length made identification easier and more reliable after 4 days of culture.

Morphological observation of fluorescent staining indicated that larger, more spread-out osteoclasts were routinely seen on the NCC wafers than on glass coverslips although this was not quantitatively assessed (Figs. 3a and 3b). It was possible to discern many small orange granules and intracytoplasmic canaliculi in cells cultured on NCC wafers whereas cells plated on glass tended to be less spread out and to have larger more centralized orange granules. Differences in sizes of osteoclasts cultured on NCC wafers and glass coverslips appeared to be the same after both 2 and 4 days of culture.

Osteoclasts cultured on NCC wafers were approximately two times more fluorescent than osteoclasts cultured on glass at both 2 and 4 days (Fig. 4). The levels of fluorescence for cells cultured on glass at both days 2 and 4 were similar. However, in four day cultures of cells on NCC wafers, fluorescence was slightly reduced compared with 2 day cultures on the same substrate.



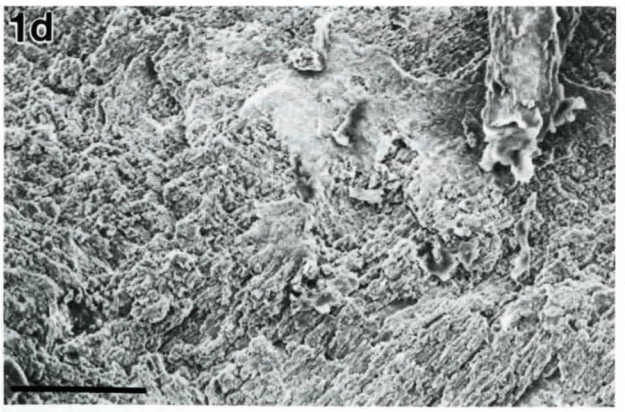




**Figure 1 (at left).** Scanning electron micrographs of NCC wafers. **(a)** 2-day culture showing two osteoclasts (arrows); **(b)** 4-day culture, arrow indicates retraction fibrils from the cell, the arrowheads indicate areas of resorption; **(c)** NCC wafer from which cells were removed after 4-day culture, arrows indicate areas of resorption; and **(d)** control NCC wafer. Bar = 10 $\mu$m.


**Figure 2 (above).** Resorption pits (arrows) on a typical NCC wafer stained with toluidine blue after removal of osteoclasts cultured for 4 days. Bar = 20 $\mu$m.

## Influence of treatments

Osteoclasts from three different isolations incubated on NCC wafers were examined after 2 and 4 days of culture following neutralization and recovery in the presence of PTH or carbonic anhydrase inhibitors. Ammonium chloride treatment followed by acridine orange staining resulted in the total disappearance of the yellow and orange fluorescence. Following neutralization, osteoclasts will fully regain their normal acid content in a 1 hour recovery period at 37°C (Hunter *et al.*, 1991).

The ability of osteoclasts cultured on NCC wafers to form acid after ammonium chloride neutralization and in the presence of agents which either stimulate or inhibit acid production is shown in Figsures 5a and 5b. All cells in both 2-day and 4-day cultures show a dramatic recovery of acid production during the first 30 minutes after neutralization, indicating that the cells have not been irreversibly affected by neutralization and that membrane integrity has been maintained. The untreated values remain the same (2-day) or increase gradually (4-day) over the next 90 minutes.

Treatment with $10^{-8}$ M PTH produced a significant increase in acidity by 120 minutes in 2-day cells. The effect of PTH is more pronounced in 4-day cultures where a significant difference from the untreated, neutralized cells was observed at all time points. At two days of culture the cells recovered their ability to acidify

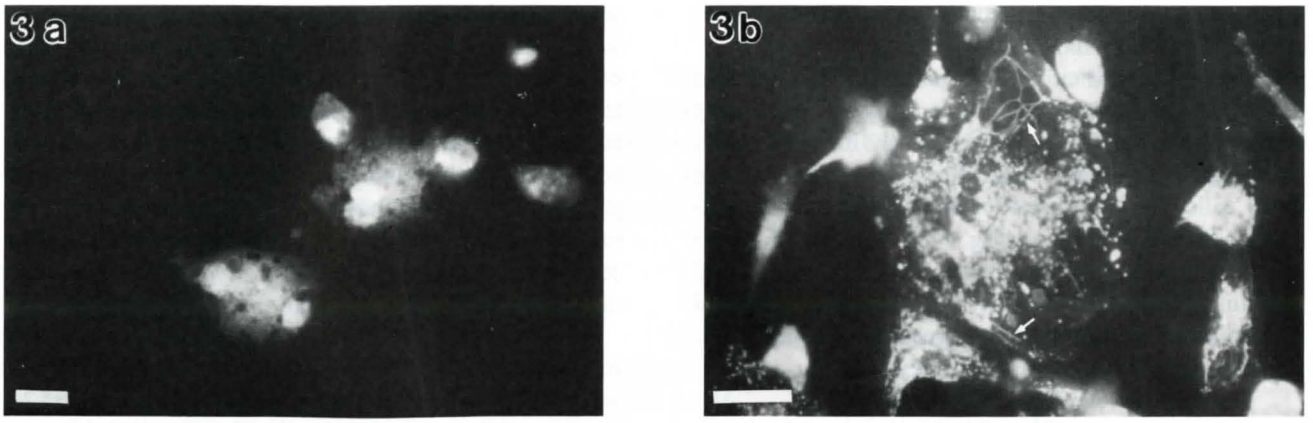



**Figure 3.** Typical acridine orange-stained 4-day osteoclasts on (a) glass and (b) an NCC wafer. Cells on NCC wafers were usually flatter and contained more orange granules and intracytoplasmic canaliculi (arrows). Bar = 20 $\mu$m.

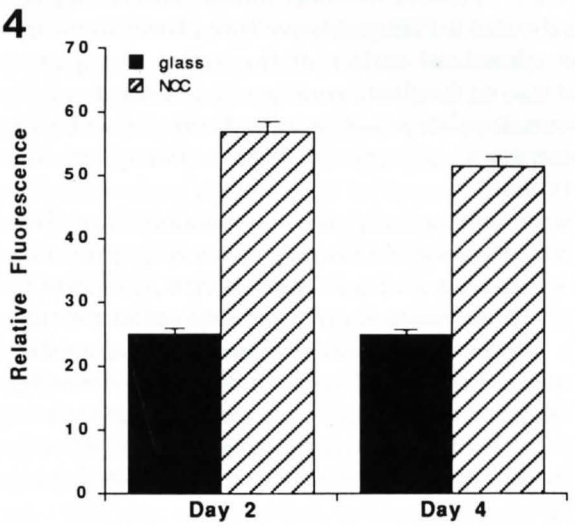



**Figure 4.** Mean relative fluorescence of osteoclasts cultured on NCC wafers or on glass coverslips for 2 and 4 days. For each of the four groups, n = 120 osteoclasts. Error bars are standard error of the mean. Differences in fluorescence intensity between glass and NCC wafers were statistically significant on days 2 and 4, $p < 0.001$.

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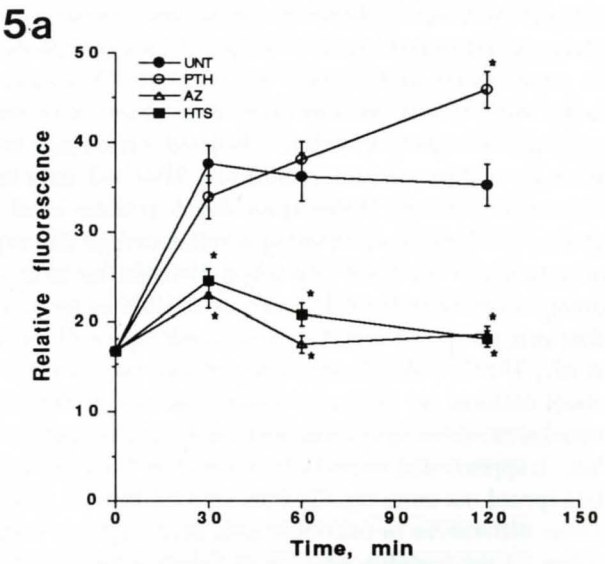
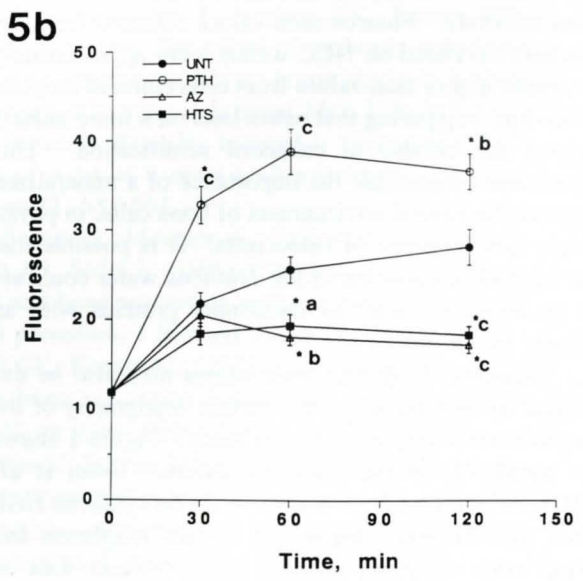
**Figure 5 (at right).** Typical time course of recovery of acidification following ammonium chloride neutralization of untreated cells and cells treated with $10^{-8}$ M PTH, $10^{-7}$ M acetazolamide, or $10^{-7}$ M HTS. Statistical comparisons were made between neutralized, treated and neutralized, untreated cells. Time is minutes after neutralization ended. (a) Cells were cultured for two days. * denotes statistical significance, $p < 0.001$. (b) Cells were cultured for 4 days. * denotes statistical significance, a: $p < 0.05$; b: $p < 0.01$; c: $p < 0.001$. Error bars are standard error of the mean.



and to respond to PTH gradually over the period from 60 minutes to 120 minutes.

Treatment with either carbonic anhydrase inhibitor produced significant reductions in acidity. The acidity levels in 2-day cells were significantly lower than untreated cells at all time points (Fig. 5a) and the levels in 4-day cells were reduced after 60 and 120 min of treatment with either inhibitor (Fig. 5b). Acetazolamide almost completely prevented re-acidification over the 120 min time frame in 2-day cells (Fig. 5a). Both HTS and acetazolamide were effective in blocking reacidification, more so at two days than at four days.

## Discussion

### Effect of the substrate

The support-related parameters that may explain the differences observed between osteoclasts incubated on glass coverslips and *Tridacna* wafers include differences in composition and surface topography. Typically, larger more spread-out osteoclasts were observed on the calcium carbonate substrate. Isolated osteoclasts on glass coverslips are still rounded-up after 1-2 days in culture but become flattened by day 5 (Hunter *et al.*, 1989). Previous work showing acridine orange staining of osteoclasts on glass coverslips revealed an intense orange-staining in the cell center with little or no acridine orange spread into the cytoplasmic apron (Hunter *et al.*, 1988). The present study shows that in osteoclasts cultured on *Tridacna* wafers, the orange stained vesicles are more spread-out and individually recognizable. It appears that osteoclasts cultured on *Tridacna* wafers spread out more rapidly than cells cultured on glass. These differences in osteoclast area may originate from either (i) the inability of cells to firmly adhere to the glass substrate or (ii) the inability of cells to resorb the glass substrate. Fluorescence values obtained from osteoclasts incubated on NCC wafers were approximately two times higher than values from cells cultured on glass coverslips, suggesting that osteoclasts on a more natural support are capable of enhanced acidification. This experiment emphasizes the importance of a mineralized support, the natural environment of bone cells, in physiologic investigations of osteoclasts. It is possible that the mineral composition of the *Tridacna* wafer could act as an attractive pole for membrane proteins with an affinity for calcium.

Variations in fluorescence values may also be explained by differences in the surface topography of the two substrates employed in this study. Figure 1 shows the roughness of the *Tridacna* wafers. Gomi *et al.* (1993) cultured rat bone marrow cells on synthetic HAP discs with different degrees of surface roughness and found more resorbing cells on rough surfaces than on smooth surfaces. They concluded that the resorptive susceptibility of the substrates could be critically influenced by surface topographical effects rather than substratum chemistry alone. It is possible that the roughened, microporous surface of *Tridacna* shell could also contribute to the differences observed in the present studies. It has been shown that isolated osteoclasts cultured on a synthetic calcite crystal substrate will produce resorptive cavities on the smooth crystal faces (Jones *et al.*, 1986)

Another explanation for the differences could reside in the culture method employed. Cells were seeded onto wafers or glass coverslips and incubated for the first 24 hours in medium supplemented with 5% FBS. After 24 hours, cells adherent to substrates were transferred on their supports into medium supplemented with 0.5% FBS only. Proteins from the former medium supplemented with 5% FBS could have been adsorbed onto the rough mineralized surface of the wafers to a greater extent than on the glass coverslips. In this manner, cells on mineral wafers would have been exposed to higher concentrations of growth factors throughout the experiment.

Since acridine orange is a non-ratiometric dye, fluorescence values can be affected by quenching, particularly as a consequence of specimen thickness, photobleaching and dye distribution, making precise quantitation difficult. However, comparisons of treated specimens with untreated samples would have validity for demonstrating inhibitory and stimulatory effects, as shown in the next section. The use of acridine orange to detect intracellular acidification has been reviewed recently by Zelenin (1993).

It has been demonstrated that medium acidification by manipulation of either $HCO_3^-$ or $CO_2$ resulted in an increase in the number of pits resorbed by disaggregated rat osteoclasts (Arnett *et al.*, 1994). In our system, the small volume of cells in combination with the large buffering capacity of the medium and the stability of the $Pco_2$ level within the incubator would be expected to produce minimal changes in the resorptive behavior of the cells.

### Influence of treatments

In this investigation we examined the action of agents known to influence carbonic anhydrase activity in osteoclasts. Acetazolamide and HTS are well established inhibitors of carbonic anhydrase. PTH appears to activate carbonic anhydrase indirectly through a cell signaling mechanism (Dietsch, 1987; May *et al.*, 1993; Silverton *et al.*, 1987). The results of the present study demonstrate that acidification was stimulated by PTH and inhibited by acetazolamide or HTS treatment when osteoclasts were incubated on natural calcium carbonated

*Tridacna* substrate. Previous studies have shown an increase in osteoclastic carbonic anhydrase activity when PTH was added to bone organ culture (Anderson *et al.*, 1985) or chicken osteoclasts in culture (Silverton *et al.*, 1987). Total or partial inhibition of the enzyme has been shown with acetazolamide in organ and cell cultures (Hall and Kenny, 1985; Hunter *et al.*, 1988; Kenny, 1985) as well as with HTS in organ (Raisz *et al.*, 1988) and cell (Hunter *et al.*, 1991) cultures. Our data indicate similar action by these agents on isolated osteoclasts cultured on a calcium carbonate substrates. It is interesting that fluorescence levels in the cells cultured for 4 days on NCC wafers were less than the fluorescence values found in cells cultured for 2 days. This observation may be the consequence of the 4-day old cells being able to secrete acid and thereby reduce the level of trapped acridine orange dye.

Whole animal studies which investigated the role of carbonic anhydrase on resorption of natural coral skeleton fragments grafted into bone have also been carried out (Guillemin *et al.*, 1987). Histological analysis demonstrated that the coral structure was initially invaded by bone marrow elements accompanied by blood vessels, followed by osteoclastic resorption and finally by osteoblastic bone formation. Acetazolamide treatment of some of the animals implanted with natural coral led to a reduction in coral resorption and a lack of bone repair. However this experiment could not directly demonstrate that the decrease in resorption was related to inhibition of osteoclastic activity. The present study shows that acetazolamide directly affects the activity of osteoclasts in contact with a material similar in composition to coral skeletons. One may conclude that, *in vivo*, acetazolamide has a direct effect on osteoclastic resorbing activity which results in the observed reduction of coral resorption. However, other possible routes of action cannot be excluded, such as the renal effect of acetazolamide on calcium and phosphate metabolism, as suggested by Gram *et al.*, (1990). The present study is in concert with the whole animal experiments.

We conclude from this investigation that natural calcium carbonate from *Tridacna* shell is a biomaterial that can undergo dissolution through the mechanism of osteoclastic resorption. Osteoclasts exhibit similar sensitivity to stimulatory or inhibitory substances when cultured on natural calcium carbonate as when cultured on natural or synthetic calcium phosphate. Further experiments are necessary to correlate increases or decreases in carbonic anhydrase activity with changes in substrate resorption. It also remains to be investigated whether the resorbing cell area is related to the resorbability of the substrate. Finally, it would be of interest to investigate the role played by the carbonated portion of the bone apatite crystal in the osteoclastic resorption process.

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## Discussion with Reviewers

**S.F. Silverton:** Except for Figure 3b, all the avian osteoclasts shown on this substrate appear to be less than 10 $\mu$m in diameter. This is relatively small for the average avian osteoclast which has 4-6 nuclei. Did you see any indication that osteoclast size was affected by this particular substrate?

**Authors:** We have generally found our isolated osteoclasts to be on the small side of the osteoclast size range. We interpret this to be a reflection of our isolation technique which seems to cause damage and loss of larger cells. The cells shown in Figure 1a are approximately 12 $\mu$m in diameter. These are 2-day cells that are probably not fully spread. The 4-day cells shown in Figures 3a and 3b have diameters between 50-70 $\mu$m. In **Discussion**, the differences in cell size found with different substrates are discussed.

**S.F. Silverton:** Toluidine blue has been used to indicate osteoclast resorption pits on several calcified substrates. Its usefulness is presumed to be due to binding to exposed proteins after osteoclasts dissolve the calcium and partially degrade the matrix. In the case of this substrate, do the authors feel the toluidine blue is binding to proteins from the calcium carbonate substrate, or from proteins secreted by the osteoclast during resorption?

**Authors:** The NCC wafers have essentially no protein and a very low amino acid content (see **Methods**, $CaCO_3$ wafers). Toluidine blue is thought to be binding to proteins secreted from the osteoclast. Localization of tartrate-resistant acid phosphatase (TRAP) at the electron microscope level has revealed its presence in the resorption lacunae beneath the ruffled border area of osteoclasts (Doty and Shofield, 1972).

**S.F. Silverton:** The authors comment that no pits were found after two days of culture. What was the authors' rationale for this observation?

**Authors:** In our previous work (Hunter *et al.*, 1988, 1989, 1991), we have not used cultured osteoclasts prior to day 4 of the culture period. The fluorescent staining and imaging works best with flattened, well-spread cells that have been in culture several days. We have observed what we believe to be eroded areas on *Tridacna* wafers after 2 days of culture but the cumulative nature of their assessment makes identification more reliable after 4 days of culture.

**S.C. Miller:** If the osteoclasts are not neutralized first, can they be further stimulated or are the "maximally" stimulated when cultured on the wafers?

**Authors:** Our previous work demonstrated stimulation of osteoclast acid production following PTH treatment in osteoclasts cultured on glass coverslips which was not neutralized prior to acridine orange staining (Hunter *et al.*, 1988). A similar but longer lasting PTH response was observed in a rat calvarial system (Anderson *et al.*, 1985, 1986).

**M. Kneissel:** Do you plan to investigate the size and shape of the resorption pits in the differently treated groups? Would the pits created in these $CaCO_3$ wafers be suitable for this type of analysis?

**Authors:** Although the sizes and shapes of the resorption pits on the *Tridacna* wafers could be analyzed, we have not begun those studies at the present time.

Please see next page for **Additional References**.

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