sponsible for isoproterenol action. However, the evidence for the presence of sarcoplasmic vesicles in uterus is not great (*17*), and a recent report suggests that perhaps the microsomal calicum uptake system of smooth muscle is derived from the cell membrane (*18*). Definition of the cellular locus and chemical nature of the particulate kinase substrate will be of great importance.

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30 July 1973

Carbonic Anhydrase and Osteoclasts: Localization by Labeled Inhibitor Autoradiography

Abstract. *Autoradiography with tritiated acetazolamide indicates that osteoclasts of the hen and chick contain concentrations of carbonic anhydrase which are similar to those in pancreatic acinar cells. Grain counts of osteoblasts and osteocytes were not different from background. Thus, a sufficient quantity of carbonic anhydrase seems to be present in osteoclasts to be of physiological importance in bone resorption.*

Several studies (*1*, *2*) indicate that carbonic anhydrase (E.C. 4.2.1.1), an enzyme which is widespread in biological systems, may play a role in bone demineralization, possibly by decreasing *p*H in areas of bone resorption. A major deficiency in proving this hypothesis has been the lack of an unequivocal localization of carbonic anhydrase (CA) in osteoclasts or osteocytes, the cells which occur in areas of osteoclastic bone resorption and osteocytic osteolysis, respectively. Although several authors (*1*, *3*) have found significant amounts of CA in bone by chemical analysis, it is uncertain if the enzyme activity is associated with bone cells or the preerythrocytes of bone marrow, which are known to be rich in CA (*4*). Since bone mineral and marrow form an interlocking system, which cannot be separated, the best approach for determining if the enzyme is present in bone cells is cellular localization. The only localization of CA in bone cells (*5*) is based on a method which has been found to be invalid since staining is caused by CA-independent alkalinization of the medium (*6*).

We have developed an alternate procedure for localizing CA. The method, which has been described in detail elsewhere (*7*), involves labeling of CA in vivo with tritiated acetazolamide, a specific inhibitor (*8*, *9*). Tissues are then prepared for autoradiography.

A 1-year-old White Leghorn laying hen, two 10-day-old male commercial broiler chicks, both *Gallus domesticus*, and two 3-month-old laying quail, *Coturnix coturnix japonica*, were injected intravenously with sodium [acetyl-^{3}H]acetazolamide (160 mc/mmole, New England Nuclear, Boston, Massachusetts). The quail and chicks received 16.4 to 28.1 mc and the hen 2.3 mc per kilogram of body weight. This corresponded to an acetazolamide concentration of 22.8 to 39.1 mg per kilogram of body weight for quail and chicks. The injection received by the hen was supplemented with 10 mg of unlabeled sodium acetazolamide per kilogram of body weight so that the total drug concentration was 13.2 mg/kg. These dosages have been reported to cause maximum inhibition of CA in all organs of various species tested (*9*) and of egg shell formation in laying hens (*10*). Twenty-four hours later, tissues were removed from the hen and chicks. At this time the concentration of acetazolamide in plasma of the laying hen was about 100 times lower than that in CA-rich red blood cells (*7*), a finding which indicated that free and loosely bound acetazolamide was rapidly cleared from the plasma and most tissues. The quail were killed 3 hours after injection of the drug since experiments in our laboratory had shown that there is little inhibition of quail shell gland CA by acetazolamide after longer time intervals. Pieces of tissue about 2 mm^3 in volume were removed from birds which were under sodium pentobarbital anesthesia (25 to 100 mg per kilogram of body weight). The tissues were frozen immediately in isopentane at −160°C, freeze-dried, fixed with OsO_4 vapor, and embedded in Epon. Thus, the only liquid which came in contact with the tissues was a nonaqueous plastic resin.

Table 1. Average grain counts over osteoclasts, pancreas, and muscle. The values are corrected for background; S.E.M., standard error of the mean.

Animal	Grains per 1000 $\mu m^2 \pm$ S.E.M. over		
	Labeled osteoclasts	Pancreas	Muscle
Hen	20.5 ± 2.4*	24.8 ± 2.7*	0.2 ± 0.7
Quail 1	16.5 ± 1.8*	52.3 ± 2.1*	3.5 ± 0.8
Quail 2	21.9 ± 2.6*	65.5 ± 5.0*	6.2 ± 1.1
Chick 1	7.7 ± 1.1	11.8 ± 0.9*	2.3 ± 0.7
Chick 2	15.4 ± 1.9*	15.5 ± 1.5*	2.6 ± 0.6

* Significantly different from background at $P < .05$ by Duncan's new multiple range test (*17*).

These precautions were taken to prevent loss of the water soluble enzyme-inhibitor complex. Sections 1 μm thick were cut with glass knives on a water surface, mounted on slides, and covered with Kodak nuclear track emulsion NTB-2. Grain counts were determined for ten randomly selected 1000-μm^2 areas of osteoclasts, pancreas, and skeletal muscle. When the osteoclast area was less than 1000 μm^2, areas were summated to make a total of 10,000 μm^2. Several other tissues containing CA were studied to validate the method, namely kidney, shell gland, proventriculus, and red blood cells (*7*). The exposure time for the sections from each animal was selected so that the grains in the most radioactive tissue (proventriculus) could be accurately counted (that is, each silver grain was distinct from all others). The exposure times selected were 143 days for the hen, 81 days for quail 1, 49 days for quail 2, 25 days for chick 1, and 41 days for chick 2.

The validity of the autoradiographic method is supported by the following results (*7*). (i) No significant amounts of label were found in tissues and cell organelles which have no CA activity, namely muscle, cartilage, adipose tissue, connective tissue, and nuclei. This suggests that tissue radioactivity was limited to CA sites and was not some breakdown product of acetazolamide, a conclusion which is supported by reports that acetazolamide is not metabolized in the rat, man, dog, rabbit (*9*), or cat (*11*). (ii) Grain patterns and concentrations over tissues from the three different classes of birds—hen, quail, and chick—were similar, except for the pancreas, which was more radioactive in the quail. (iii) The autoradiographic localizations are generally in agreement with concepts of physiological function and, in the proventriculus and shell gland, have been confirmed by a fluorescent antibody technique (*12*). (iv) There was no latent image fading or chemography during the exposure of the autoradiographs. (v) Grain counts in Epon adjacent to tissues and in connective tissue surrounding cell boundaries were similar to background (4.4 grains per 1000 μm^2), which indicates that loss or relocation of the isotope during processing was minimal. (vi) By using thin-layer chromatography it was shown that the tritium label chromatographed with acetazolamide and that the labeled drug was not contaminated by tritium-labeled impurities. In addition, decomposition of the tritiated acetazolamide was not detectable during storage.

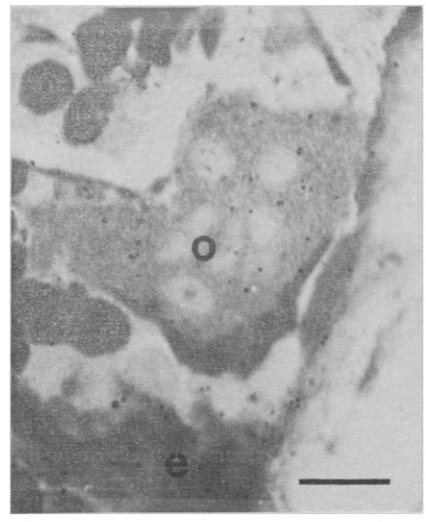

Fig. 1. Autoradiograph of medullary bone and bone marrow of the laying hen, showing label in an osteoclast (*o*) and in erythroblasts (*e*). Bone (right) is not labeled. The exposure time was 143 days; scale bar, 10 μm (× 1250).

Figure 1 shows an autoradiograph of a typical osteoclast. Of the 105 osteoclasts examined, 83 contained label in excess of background levels. In all of these cells the label was evenly distributed throughout the cytoplasm. Because of the small size and scattered location of osteoblasts and osteocytes, no grain counts were obtained for these cells. However, examination of several hundred osteoblasts and osteocytes showed that only approximately 1 percent was associated with any grains in the overlaying emulsion. This can be attributed to background and indicates that these cells were essentially unlabeled (Fig. 2). Newly formed bone adjacent to osteoblasts frequently contained label (Fig. 2), whereas the surfaces of older bone, whether free or covered by osteoclasts (Fig. 1) or inactive flattened cells, were usually not labeled.

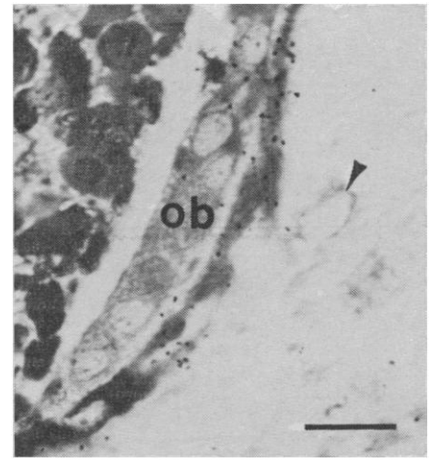

Fig. 2. Autoradiograph of medullary bone and bone marrow of the laying hen. Osteoblasts (*ob*) and an osteocyte (arrow) are not labeled. Bone (right) is not labeled, except for the surface below the osteoblast layer. The exposure time was 143 days; scale bar, 10 μm (× 1250).

Table 1 shows the average grain counts over osteoclasts, pancreas, and muscle. Except for chick 1, the average grain counts over osteoclasts were significantly greater than background, which averaged 4.4 grains per 1000 μm^2, and the counts over muscle, which contains no CA (*13*). In the hen and the two chicks the grain counts over osteoclasts were not significantly different from those over the pancreas, but quail pancreas contained significantly more CA than chicken pancreas or osteoclasts.

The qualitative findings reported here show that CA is localized in osteoclasts but not in osteoblasts or osteocytes. Since the grain counts for chicken osteoclasts and pancreas were similar, it appears that a sufficient quantity of CA is present in osteoclasts to be of physiological importance in bone metabolism. It is unlikely that the presence of [^{3}H]acetazolamide in osteoclasts is due to the ingestion of labeled bone particles by phagocytic activity. The phagocytosis is due to the activity of the ruffled border of osteoclasts and phagocytic vacuoles occur primarily in the region of cytoplasm below this border (*14*). Thus, if the labeling of osteoclasts were caused by phagocytosis, grains should have been concentrated, at least in some cells, along and below the ruffled border. However, as mentioned earlier, the label was always evenly spread throughout the cytoplasm of osteoclasts (Fig. 1). Furthermore, if the presence of label was due to phagocytosis, the grain counts in osteoclasts should have been proportional to the amount of [^{3}H]-acetazolamide injected and to the time during which these cells were in contact with labeled bone, since it has been found that osteoclasts progressively concentrate plutonium previously deposited on bone surfaces (*15*). However, as shown in Table 1, the average grain counts over osteoclasts were similar for all birds, with the exception of chick 1, although the dose of [^{3}H]-acetazolamide varied from 2.3 to 28.1

mc/kg and the interval between drug administration and killing was 24 hours for chickens and 3 hours for quail. The finding of grains along bone surfaces beneath osteoblasts but not along inactive and resorbing surfaces also argues against phagocytosis of the label, and provides an alternate explanation for the presence of grains in newly formed bone. That is, [^{3}H]acetazolamide may have been initially adsorbed on bone surfaces but, as shown by the quail data, was cleared from inactive and resorbing surfaces within 3 hours or less, while in areas of bone formation it was covered by layers of new bone before the loosely bound drug could be cleared completely.

The results of this study demonstrate that CA occurs in osteoclasts in physiologically significant quantities. This is in contrast to some earlier results with mammals (*16*) which indicated that chronic CA inhibition had no effect on bone growth or on blood calcium and phosphate. However, our results support more recent evidence (*1*, *2*) suggesting that CA may be important in bone resorption.

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Mycoplasma-Like Structures in the Aquatic Fungus Aphanomyces astaci

Abstract. *The hyphae of a nonzoosporulating strain of* Aphanomyces astaci *contain numerous structures which most closely resemble published micrographs of certain mycoplasmas. Two normal strains of the same species do not contain these mycoplasma-like bodies.*

Numerous plant and animal diseases appear to be caused by mycoplasmas (*1*, *2*) but, although fungi frequently show various disease symptoms, only viruses have so far been reported as disease-causing agents of fungi (*3*). Unestam and Svensson (*4*) reported a strain (G_1) of the crayfish plague fungus, *Aphanomyces astaci*, which had lost the ability to produce zoospores in culture. If this defect was genetically controlled such a strain could yield information on the zoosporulation process when compared to normal strains. Alternatively, the defect could be due to an infective agent. In order to differentiate between these possibilities an ultrastructural investigation was initiated.

Isolates G_1, J_1, and Si (*4*) of *Aphanomyces astaci* Schikora (a member of the Oomycetes) were grown aseptically in liquid PG 1 medium (*5*) from infected PG 1 agar cubes at 25°C for 4 days. On this medium they only grow vegetatively. Zoosporulation was induced by transfer from PG 1 to "dilute salts" (*6*). Colonies at the appropriate growth stages were fixed by adding to the liquid growth medium an equal volume of 5 percent glutaraldehyde in M/15 Sorenson's phosphate buffer. After 5 minutes in this mixture at about 24°C the colonies were transferred to similarly buffered 5 percent glutaraldehyde for 65 minutes. After three 20-minute rinses in the buffer mentioned above

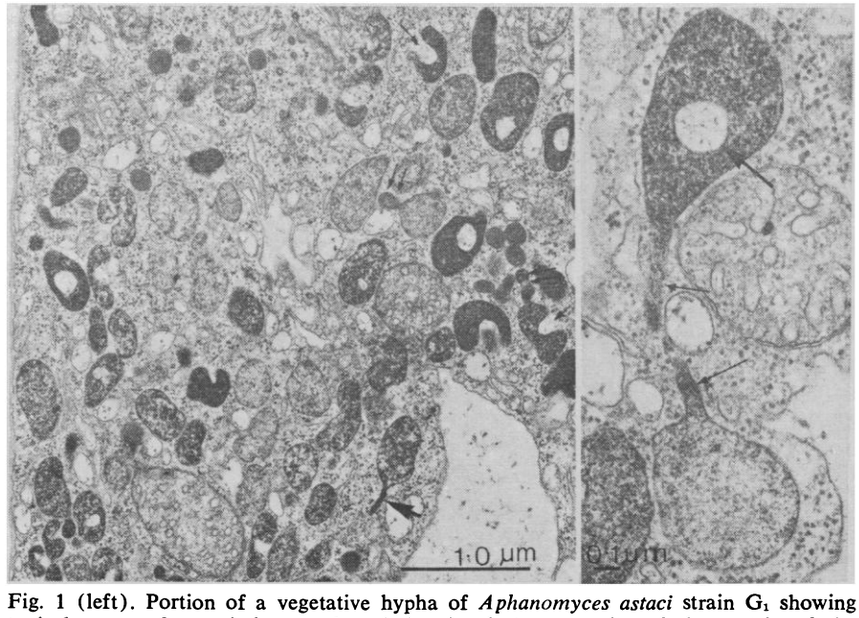

Fig. 1 (left). Portion of a vegetative hypha of *Aphanomyces astaci* strain G_1 showing typical range of morphology and variation in electron opacity of the matrix of the mycoplasma-like structures. Note frequent invaginations (small arrows), fingerlike projections (large arrow), and morphological suggestions of budding (double arrow). Fig. 2 (right). Detail of two mycoplasma-like bodies from a hypha of strain G_1 showing typical fingerlike projections (small arrows) and an enclosed cavity in the more electron-opaque body (large arrow). Serial sections verified that this cavity was enclosed and not an invagination comparable to those seen in Fig. 1.