ENZYMES OF THE ACCESSORY BORING ORGAN OF THE MURICID GASTROPOD UROSALPINX CINEREA FOLLYENSIS. I. AEROBIC AND RELATED OXIDATIVE SYSTEMS [1]

PHILIP PERSON, ANNE SMARSH, STEPHEN J. LIPSON AND MELBOURNE R. CARRIKER

Veterans Administration Hospital, Brooklyn, New York 11209; Veterans Administration Hospital, Boston, Massachusetts 02130; and Systematics-Ecology Program, Marine Biological Laboratory, Woods Hole, Massachusetts 02543

In continuation of studies of the demineralization mechanism of the accessory boring organ (ABO) of the shell-penetrating snail *Urosalpinx,* by Carriker, Scott and Martin (1963), Provenza, Nylen and Carriker (1966; and unpublished observations) demonstrated by means of electron microscopy that the secretory cells of the ABO contain dense concentrations of mitochrondria. Preliminary observations in the course of these studies suggested that numbers and size of mitochondria were significantly greater in secretory cells of ABO's from actively boring snails than of ABO's from non-boring or resting snails.

These observations are of interest because (1) mitochondria are the major sites of cell respiration, and (2) the secretory function of many exocrine glands is known to be dependent upon an active aerobic oxidative metabolism. As an example, in vertebrate salivary glands it has been well established that basal secretion, as well as any increases in *in-vivo* or *in-vitro* secretory activity, are obligatorily dependent upon oxygen utilization (for summarized references see Schneider and Person, 1960; Person *et al.,* 1961).

Because of the lack of available information in the literature concerning the intermediary metabolism of the ABO, a preliminary correlated histochemical and biochemical study of aerobic and related oxidative enzymes of the *Urosalpinx* ABO was undertaken. The objective of this was to determine whether the cytologic observations of dense populations of mitochondria in the secretory cells of the ABO indicate a significant aerobic metabolism in that organ. Observations and data reported in this paper show that the ABO does indeed possess a very active aerobic metabolism as judged by its cytochrome oxidase activity. It was also demonstrated in the laboratory that live snails will not penetrate the shell of oyster prey when their environment is depleted of oxygen.

Materials and Methods

Specimens of *Urosalpinx cinerea* (Say) were obtained locally on intertidal rock jetties in Woods Hole for preliminary studies; results reported in this study

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are based primarily on the large *Urosalpinx cinerea follyensis* Baker shipped alive *via* air mail from Wachapreague Bay, Virginia.

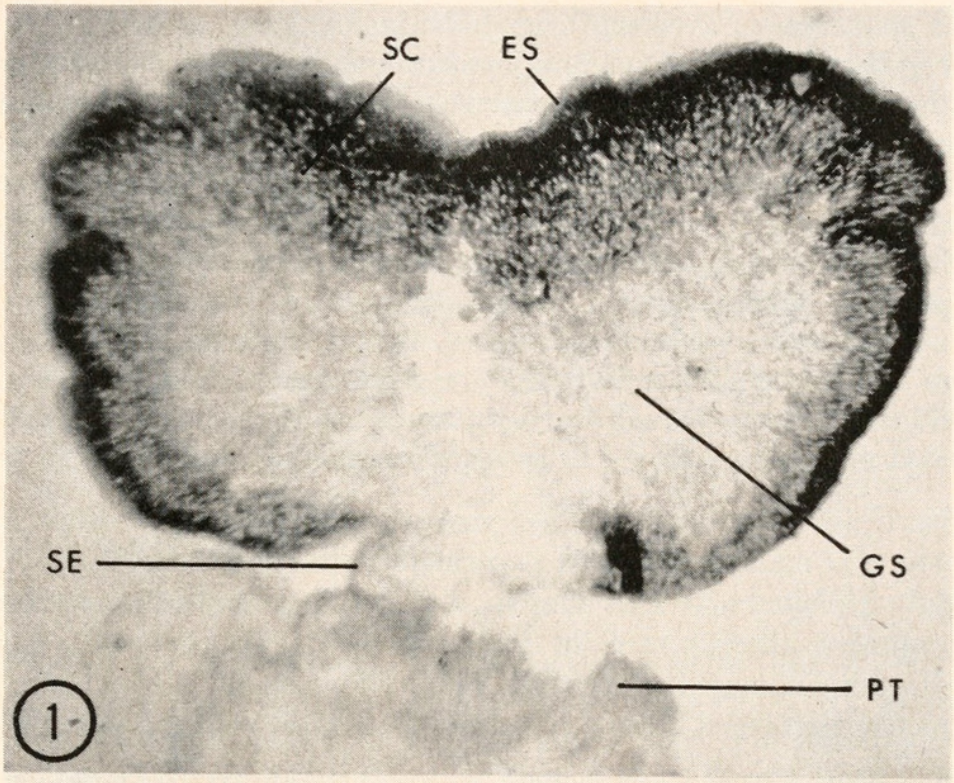
Snails were maintained in running sea water tables at the Marine Biological Laboratory, Woods Hole, where the research was conducted during the summer of 1966. For each enzyme determination, snails were removed from their shells by cracking, and the ABO's were excised cleanly under a binocular dissecting scope (see Carriker, Scott and Martin, 1963, for method). For histochemical studies, ABO's were severed at the region where the base of the ABO stalk is attached to the foot of the animal. Excised organs were immediately frozen with dry ice, and sections were cut at 8–10 μ using the cold-knife technique of Adamstone and Taylor (1948). The following histochemical procedures were performed: cytochrome oxidase (Burstone, 1960; with naphthol ASL-3G and added cytochrome *c*); succinate dehydrogenase (Nachlas *et al.*, 1957; with added menadione according to Wattenberg and Leong, 1960; and Morrison and Kronheim, 1962) and lactate dehydrogenase (Ogata and Mori, 1964). For biochemical assays, the distal secretory disk of the ABO was cut away from the stalk, leaving behind as much as possible of the non-secretory stalk tissues. Usually ABO's from 3 snails were pooled for each determination. Tissues were placed in the appropriate chilled (2°–4° C.) buffer for each assay and homogenized in a ground-glass tissue homogenizer. The following assays were performed: cytochrome oxidase (Wainio *et al.*, 1951); NADH-cytochrome *c*-reductase (Lenta and Riehl, 1960); succinate-cytochrome *c* dehydrogenase (Cooperstein *et al.*, 1950); lactate dehydrogenase (Kornberg, 1955) and glucose-6-PO_4 dehydrogenase (Noltman *et al.*, 1961). Protein determinations were by the method of Lowry *et al.* (1951).

A series of experiments were also performed in which snails were maintained in sea water, on, or in the presence of, live prey (*Crassostrea virginica*) under anaerobic conditions (N_2 atmosphere) for definite time intervals prior to biochemical and histochemical study of their ABO's. Details will be given in the appropriate section of the text.

Results

1. *Histochemical observations*

a. *Cytochrome oxidase.* The range of inhibition by cyanide was from 10^{-4} *M*, for complete, to 10^{-6} *M* for partial inhibition. Complete inhibition was given by 10^{-2} *M* azide, while inhibition by 10^{-4} *M* azide was only partial. Sections kept at 100° C. for 5 minutes were completely inactive. Omission of cytochrome *c* from the incubation mixture retarded color formation by a factor of 2 to 3. Figure 1 shows that cytochrome oxidase activity was confined almost entirely to the secretory cells (SC) of the ABO, and that neither the internal stalk tissues (GS), the stalk epithelium (SE), nor pedal tissue (PT) showed significant activity. In Figure 2 a higher magnification is shown. It may be seen in Figures 1 and 2 that most intense activity was present at the distal ends (*i.e.*, close to external surface) of the epithelial cells, and that a layer of secreted material external to the epithelial surface of the secretory cells (ES) also exhibited oxidative dye synthesis, although of lesser intensity. This secretory material presumably contains the substances which are active in chemically attacking the $CaCO_3$ crystals of shells which are



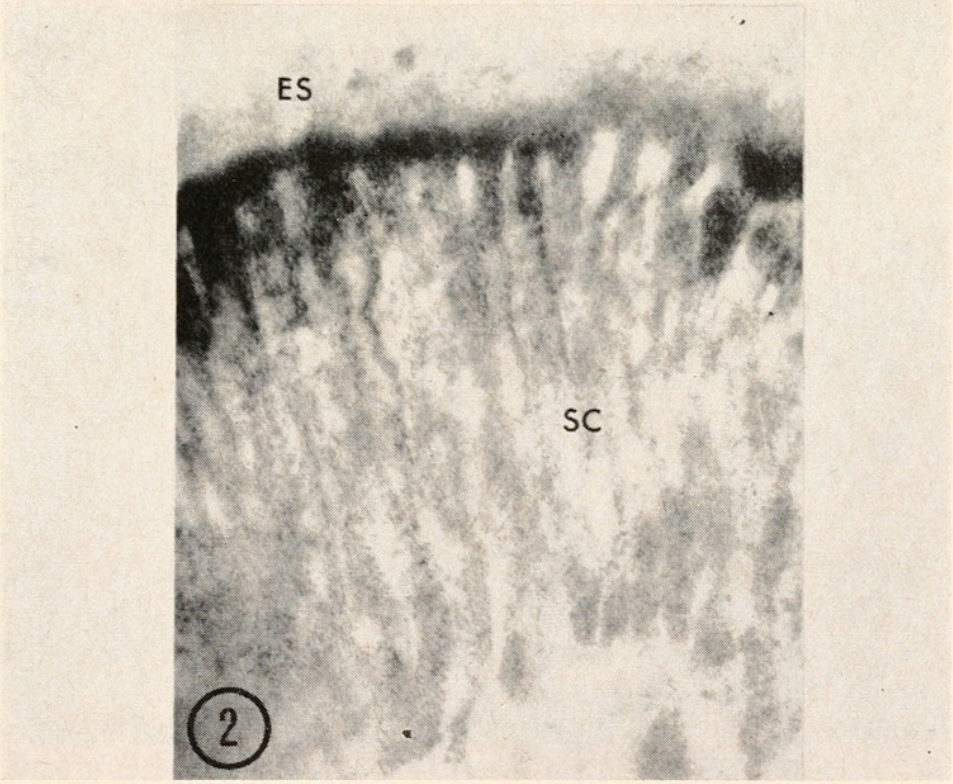


FIGURE 1. (Upper) Distribution of histochemical cytochrome oxidase in a sagittal freshly frozen section of *Urosalpinx* ABO. The secretory epithelial cells (SC) are intensely stained. Little or no activity is seen in the gland stroma (GS), in any of the stalk epithelium (SE) or in pedal tissue (PT). Note that externally secreted material (ES) at the outer surface of the secretory cells shows a slightly positive reaction. Magnification approximately 60 ×.

FIGURE 2. (Lower) Enlargement of secretory cell region from a comparable section to that seen in Figure 1. Magnification approximately 250 ×.

bored by the snails (see Carriker, Scott and Martin, 1963; and Carriker and Van Zandt, 1964). In view of the fact that cytochrome oxidase is almost always intracellular in localization, it is of considerable interest that extracellular oxidase activity should be associated with such secretions.

It should be mentioned that the rate of dye synthesis appeared to be more rapid than that customarily seen even with most actively aerobic mammalian tissues studied under similar assay conditions. Thus, in the epithelial cells of the ABO, dye formation was quite visible to the unaided eye in 5 minutes, and as seen in Figures 1 and 2 was markedly advanced by 15 minutes. Comparable color intensities in mammalian heart or kidney sections usually require 30 minutes or more of incubation time. It will be seen later that the biochemical data support this impression of greater activity in the ABO epithelial tissue.

b. *Succinate dehydrogenase.* When malonate, a classical inhibitor of succinic dehydrogenase, was made equimolar to succinate in the incubation mixture (*i.e.* 10^{-2} *M*), it strongly inhibited activity. Omission of succinate from the reaction mixture resulted in complete absence of color formation in the interval studied (*i.e.,* 3–10 minutes). Figure 3 shows that the distribution of succinate dehydrogenase activity almost completely paralleled that of cytochrome oxidase, with the possible exception

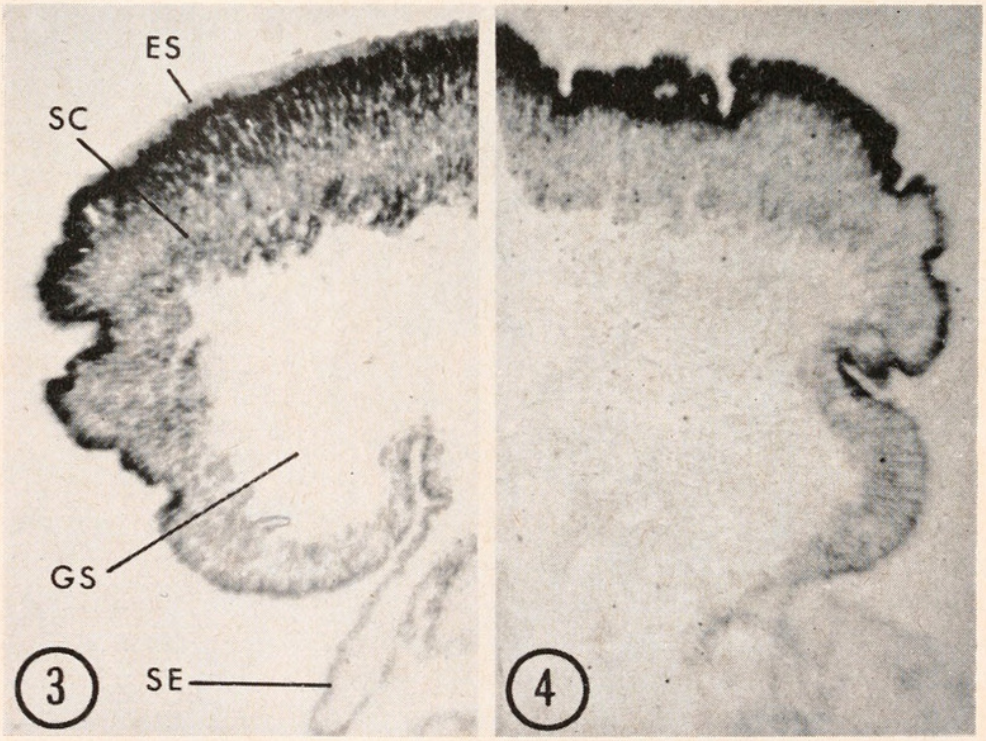


FIGURE 3. (Left) Distribution of histochemical succinate dehydrogenase in a saggital freshly frozen section of *Urosalpinx* ABO. Activity appears in locations similar to that seen for cytochrome oxidase in Figures 1 and 2; only secretory epithelial cells (SC) show activity, while gland stroma (GS) and stalk epithelium (SE) are negative. Externally secreted material (ES) at the outer surface of the secretory cells shows a positive reaction. Magnification approximately 60 ×.

FIGURE 4. (Right) Distribution of histochemical lactate dehydrogenase in a sagittal freshly frozen section of *Urosalpinx* ABO. Activity is found only in epithelial secretory cells, and primarily at the distal edge. Magnification approximately 60 ×.

that in some cells in the former system dye formation was occasionally visible in nuclei (not shown in illustrations). As was the case with cytochrome oxidase, activity was widespread throughout secretory cells, and was not seen in stalk components. Again, such activity was especially concentrated at the proximal and distal ends of cells, with the greatest concentration at the latter. Very intense dye formation occurred in 3–5 minutes.

c. *Lactate dehydrogenase.* Figure 4 shows that positive reactions for this enzyme also occurred, with widespread distribution throughout secretory cells and little if any activity in stalk components. As was the case with cytochrome oxidase and succinate dehydrogenase activities, color formation was greatest at the epithelial surface end of the cells; but unlike the former two enzymes, activity at the proximal (stalk) end of the cells was much reduced.

2. *Biochemical assays*

a. Cytochrome oxidase

1. *Preliminary observations.* Homogenates kept at 100° C. for 5 minutes were completely inactive. Oxidase activity was inhibited by 10^{-4} *M* cyanide and 10^{-4} *M* azide to the extent of 76% and 60%, respectively. On standing at 1°–2° C., homogenates progressively lost activity, so that after 3½ hours, only 40% of initial activity remained. In beginning assays, snails were taken at random from the waters surrounding Woods Hole, and also from different sea tables in different laboratories (in which variable populations of other invertebrates from a variety of phyla were also maintained). On 18 different days, 18 separate oxidase assays were performed using 3 pooled ABO's per assay. The range of specific activities in this series of determinations was from 0.190 to 2.10 μM cytochrome *c* oxidized/mg. protein/minute (in 15 of the 18 assays); in 3 of the 18 assays absolutely no cytochrome oxidase activity could be measured. It was found that ABO's from the snails shipped from Wachapreague Bay (*U. c. follyensis*) were more consistently active, so that in the experiments which follow the gastropods of the Wachapreague Bay subspecies were employed. They were all maintained under closely similar conditions with reference to availability of live food, running sea water and temperature.

2. *Influence of physiologic status of snails on oxidase activity.* A series of experiments were performed to determine the influence of the snail's physiologic status upon oxidase activity. Snails in the sea-water table were classified according to whether they were "resting" (*i.e.*, no contact with oysters), "drilling" (*i.e.*, mounted on live oyster with evidence of incompletely drilled hole in shell) or "feeding" (*i.e.*, mounted on oyster, with perforation of shell and feeding on oyster). Table I shows that the oxidase activities of the three groups were quite similar, with no detectable differences outside the range of standard deviations.

In order to determine whether or not non-oxidase protein from the ABO and associated stalk tissues might be masking changes in specific activity of the homogenates, experiments were performed to minimize such contamination. To obtain sufficient tissue material, ABO's from 10 resting and 10 drilling *Urosalpinx* were pooled in two groups, and each group was separately homogenized. For each homogenate system, intact cells and debris were first sedimented in a centrifuge

at 600 *g* for 20 minutes. The supernatant fluid was recovered and spun at 30,000 *g* for 30 minutes. The 30,000 *g* supernatant fluid was now completely devoid of oxidase activity and was discarded. The pellet was resuspended in buffer and again spun at 30,000 *g* for 30 minutes. The washed pellet was then resuspended and used for assay. This time the specific activities were: resting, 0.639; drilling, 1.040, showing that there were indeed differences between specific activities of resting and drilling glands which had been masked by indifferent protein in the homogenates.

3. *Influence of lack of oxygen upon boring behavior of snails and oxidase activity of their ABO's.* To determine the effect of oxygen deprivation upon boring behavior, two types of experiments were performed. In the first, all snails were maintained in running sea water for several days in a resting state prior to the experiment, *i.e.*, neither in contact with, nor near oysters. Approximately one dozen snails were then placed in each of two desiccators containing fresh live oysters in sea water. To set up an anaerobic group, one desiccator was alternately evacuated and flushed with N_2 atmosphere to remain at atmospheric pressure. The aerobic group was maintained in air at atmospheric pressure. In the second experiment, all snails were maintained in a drilling state prior to the experiment. They were then placed in their respective desiccators while mounted on the live

TABLE I

Cytochrome oxidase activity of homogenates of the accessory boring organ of Urosalpinx with reference to physiologic status of animals. (Specific activity = μ moles cytochrome c oxidized/mg. protein/minute)

Status of snails	No. of experiments	Snails per experiment	Mean specific activity	Std. deviation
Resting	8	3	0.528	0.119
Drilling	8	3	0.732	0.165
Feeding	4	3	0.531	0.092

oysters, and the anaerobic group was established as described above. In both experiments, following placement in the desiccators, snails were maintained aerobically or anaerobically for a period of 24 hours, following which they were removed from the oysters and their ABO's were excised for analysis. In each experiment, snails in the anaerobic desiccators were never securely attached to the oysters and were easily removed, whereas snails mounted on oyster shells under aerobic conditions were always securely attached and were removed only with considerably greater effort. It should also be noted that in the second experiment, many of the initially drilling animals, when placed under anaerobic conditions, disengaged from their underlying oyster shells and fell off. Results of oxidase determinations are given in Table II, from which it can be seen that the physiological (nutritional) status of snails prior to experiment appears to exert a significant influence upon oxidase activity of ABO's. Thus, when animals were initially resting prior to experiment (Table II, experiment 1), ABO homogenates from all aerobically maintained animals were twice as active as ABO homogenates from anaerobically maintained animals. However, when animals had been drilling prior

TABLE II

Cytochrome oxidase activities of homogenates of the accessory boring organ of Urosalpinx following maintenance under anaerobic and aerobic conditions. (Specific activity = μ Moles cytochrome c oxidized/mg. protein/minute)

Status	No. of experiments	Snails per experiment	Mean specific activity	Std. deviation
Experiment 1. Snails initially resting				
Anaerobic	2	3	0.592	0.058
Aerobic	2	3	1.368	0.012
Experiment 2. Snails initially drilling				
Anaerobic	2	3	0.640	0.036
Aerobic	2	3	0.435	0.090

to the experiment, there was no significant difference in oxidase activity of ABO's from aerobic or anaerobic groups (Table II, experiment 2).

b. Other oxidative enzymes

On several occasions attempts to detect succinate-cytochrome *c* dehydrogenase, lactate dehydrogenase and glucose-6-PO_4 dehydrogenase activities in ABO homogenates by the assay procedures listed under MATERIALS AND METHODS were consistently negative. Results with NADH-cytochrome *c* reductase were extremely variable, and will therefore not be described in this paper.

DISCUSSION

The histochemical and biochemical data and observations presented above indicate the existence of a very active aerobic metabolism in the secretory cells of the *Urosalpinx* ABO which is consonant with the extended and active use of the gland in boring through the shell of bivalve prey (Carriker, Scott and Martin, 1963; Carriker and Martin, 1965). The bipolar concentration of oxidase activity (at the proximal and distal ends of the cells) suggests that transport and secretory processes at these respective sites may be supported by the aerobic systems. The similar histochemical distribution of succinate dehydrogenase and cytochrome oxidase activities is in keeping with the well known localization of both these enzymes in mitochondria. Our inability to detect succinate dehydrogenase by the biochemical assay method may have resulted from (a) the presence of endogenous inhibitors (for the biochemical assay system) in the homogenates, or (b) a requirement for a different assay system with a more suitable "poise" such as the phenazine methosulfate procedure of Singer and Kearney (1957). Similar considerations may apply in connection with the positive histochemical results for lactate dehydrogenase, and the negative results with the biochemical assay for this enzyme. These problems will be treated in a separate study in the future.

The range of specific activities of cytochrome oxidase in the biochemical assay is unusually high (0.190–2.10) for crude tissue homogenates. In two other

experiments using ABO's which were frozen in dry ice immediately following excision and stored at −20° C. for 3 weeks prior to assay, homogenate specific activities were as high as 3.82. Such high cytochrome oxidase activities in whole homogenates from any animal tissues have never been reported previously to our knowledge. For comparison we have calculated specific activities for heart muscle homogenates from data in the literature obtained with similar assay methods (Dobson and Kasahara, 1964; Lang *et al.*, 1963). These authors' data convert to specific activities of 0.043–0.062 and 0.0025–0.0027, respectively, for rabbit and mouse heart muscle whole homogenates. In our own laboratory (P.P.) we obtain specific activities of 0.256–0.574 for isolated beef heart mitochondria, and values in the region of 2.30 for purer deoxycholate-solubilized and fractionated beef heart oxidase preparations (Person, Schneider and Scapa, 1961).

The lessened ability of drills to remain firmly attached to oyster shells and their inability to bore under anaerobic conditions also emphasize the aerobic nature of these phenomena and correlated well with conditions in the natural coastal habitats of the snails, where under usual conditions the water around them is highly oxygenated (Carriker, 1955).

With respect to the question asked at the initiation of this work, namely, do the electron microscopic cytologic observations of increased mitochondrial populations in secretory cells of drilling snails indicate an enhanced aerobic metabolism on the part of the gland, the present data and observations answer in the affirmative. In addition, the importance of aerobic processes in predation by the snails is also emphasized by the experiments with the live intact gastropods, in which mounting of prey and drilling behavior virtually ceased under anaerobic conditions. The exact relationship of aerobic processes within the gland to demineralization events is not yet known. It should be kept in mind, however, that shell penetration by snails involves predominant use of the ABO as compared with the radula. In this connection Carriker and Martin (1965) and Carriker and Van Zandt (1964) have shown that a typical cycle of the boring behavior of *Urosalpinx* involves apposition of the gland to shell for a period ranging from 30 to 90 minutes, during which weakening of the shell by demineralization occurs, followed by withdrawal of the gland, and rasping of the weakened shell by the radula for an interval of a few seconds to 5 minutes. Successful penetration of shelled prey involves alternate use of gland and radula in this fashion for periods ranging from a few hours for thin-shelled prey, to as long as 7 days of continuous activity for large thick-shelled prey.

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Summary

1. Histochemical observations of the aerobic metabolism of the accessory boring organ (ABO) of *Urosalpinx* showed that cytochrome oxidase, succinate dehydro-

genase and lactate dehydrogenase activities are localized in the secretory cells of the distal secretory disk.

2. Correlated biochemical assays of cytochrome oxidase activity of whole homogenates of the ABO prepared from active snails gave specific activities which ranged between 0.190 to 2.10.

3. There were no differences in cytochrome oxidase specific activities using ABO whole homogenates from resting or drilling snails. However, the specific activities of mitochondrial-rich particulate fractions isolated from whole homogenates by high speed centrifugation showed that oxidase activities of drilling specimens were 1.040 as opposed to 0.639 for samples from resting specimens.

4. Snails did not bore shells of the oyster *Crassostrea virginica* when maintained in a N_2 atmosphere, and ceased boring begun prior to transfer to anaerobic conditions.

5. These data and observations were discussed in relation to physiological and ecological factors involved in predation and demineralization activities by *Urosalpinx*.

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