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CELL RENEWAL IN THE GILL OF THE FRESHWATER MUSSEL, *MARGARITIFERA MARGARITIFERA*: AN AUTORADIOGRAPHIC STUDY USING HIGH SPECIFIC ACTIVITY TRITIATED THYMIDINE

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SUMMARY

High specific activity tritiated thymidine (50·3 Ci/mM and 56 Ci/mM) and autoradiographic techniques were used to study cell renewal in the gill epithelium of the freshwater mussel, *Margaritifera margaritifera*.

The cell renewal system in the gill epithelium of *M. margaritifera* appears to consist of a stem-type population in the gill furrow and gill furrow edges which supplies, through division, cells for a maturing, dividing transient transitional population along the proximal gill ridge sides which, in turn, supplies cells to a simple transient, differentiated, functional population on the distal gill ridge sides and tip. Loss of cells from the cell renewal system appears to be through cell death and/or extrusion from the gill ridge tip. No emigration or immigration of labelled cells out of, or into, the gill epithelium was observed. The minimum transit time from the dividing transient population to the functional population in the gill ridge tip may be no more than 24 h.

We were unable to detect any radiobiological effects or the presence of cytoplasmic labelling due to the use of high specific activities. However, such possibilities cannot be eliminated from consideration in further studies.

INTRODUCTION

Researchers have recently become interested in normal cell kinetics and cell renewal systems in molluscs of commercial importance and in determining the effects of ionizing radiations, various pathogens and environmental insults on normal cell kinetics and cell renewal systems in these molluscs. Autoradiography with tritiated thymidine (^{3}H-TdR) has been a valuable technique for such studies in plants and mammals (Cleaver, 1967; Feinendegen, 1967; Cameron & Thrasher, 1971). Unfortunately, there have been few successful attempts in using this technique to study cell renewal in molluscs and practically no studies have been reported on bivalve molluscs.

Mix (1971, 1972) and Mix & Sparks (1971*a*, *b*) have published a series of papers on the histopathological effects of gamma-irradiation on various tissues of the Pacific oyster, *Crassostrea gigas*, and on tissue repair and cell renewal systems in the digestive

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tubules, gonads, gut, and gills of this mollusc. However, unless cells can be labelled and traced, little can be definitely stated about cell renewal systems or the parameters of the cell cycle.

Cheney (1969), working with several bivalves, obtained virtually no cell labelling in *C. gigas* or the blue mussel, *Mytilus edulis*, although he subsequently utilized ^{3}H-TdR (3·0 Ci/mM and exposure times of 2–3 weeks) to study the morphology, morphogenesis, and reactive responses of Manila clam (*Tapes semidecussata*) blood cells. The reason for the labelling failure in *C. gigas* and *M. edulis* is not entirely clear since it is possible to observe mitoses in several tissues of these animals. Cheney (1969) and Mix (1972) have speculated that *C. gigas* may have a relatively long period of DNA synthesis and slow cell turnover times. Thus, since the time of availability of ^{3}H-TdR is assumed to be short, the resulting labelling in Cheney's studies may have been too low for detection with the exposure times and methods used.

Mix & Tomasovic (1973) have shown that in the freshwater mussel, *Margaritifera margaritifera*, high specific activity ^{3}H-TdR (50·3 Ci/mM and exposure times of 2–3 days) can be effectively used to obtain labelling in various cell types. However, radiobiological effects and cytoplasmic labelling may interfere with studies of cell renewal that utilize high specific activity ^{3}H-TdR.

The work reported here is a description of the cell renewal system in the gill epithelium of *M. margaritifera* using high specific activity tritiated thymidine and autoradiographic techniques. It also includes results of studies conducted to determine if radiobiological effects could be observed or if there was cytoplasmic labelling due to the use of high specific activity ^{3}H-TdR.

MATERIALS AND METHODS

Adult mussels of both sexes averaging 30 g in weight (wet weight without the shell) were collected in the Willamette River near Corvallis, Oregon, USA. The animals were acclimatized for 1 week in 27 × 20 × 10 cm plastic boxes containing aerated artificial stream water (Duodoroff, 1956). To maintain refrigerated conditions and still avoid excessive radioactive contamination, the boxes were placed in 183 × 56 × 15 cm fibreglass-lined wooden troughs containing circulating refrigerated water maintained at 12·5 ± 0·5 °C by a Westinghouse cooler.

High specific activity tritiated thymidine was obtained from New England Nuclear (cat. no. NET-027Z; 40–60 Ci/mM) in sterile aqueous solution. Two lots were used in separate experiments; the first had a specific activity of 50·3 Ci/mM, the second 56 Ci/mM. Both lots were greater than 98 % pure and both were used within 1 month after the last radiochemical purity check.

Thirty-nine mussels were each injected with 30 μCi of ^{3}H-TdR. One to four mussels were serially sacrificed at 1, 2, 4, and 10 hours, and 1, 2, 3, 4, 7, 17, 21, 28, 35, and 42 days after injection; 6 control mussels received a sterile distilled water injection. Since it was desirable to distribute the isotope through the mussel's body quickly, those sacrificed within 4 h post-injection (PI) were opened by severing the adductor muscles and injected in the pericardial sinus. Mussels to be sacrificed later than 4 h PI were less likely to survive until sacrifice and were therefore injected in the blood sinuses of the visceral mass by inserting the needle through the shell gape.

Sacrificed mussels were fixed in cold (4 °C), neutral buffered formalin, cut into 0·5-cm thick tissue blocks, passed through a standard dehydration series, embedded in Paraplast, and sectioned at 6 μm. Both longitudinal and latitudinal cross-sections were taken from the body areas of interest. The slides were coated with Ilford K-5 gel emulsion in a darkroom, allowed to drain dry, and stored in light-tight slide boxes at 4 °C until test slides showed that sufficient

exposure time had elapsed. All the slides were then developed according to Gude (1968) and stained with Harris' haematoxylin and eosin or Gomori's trichrome.

In order to check for cytoplasmic labelling, some slides, known from previous studies to have heavy labelling, were treated with deoxyribonuclease (DNase) solution or Tris buffer (Luna, 1968) for 18–20 h at 37 °C prior to emulsion coating and then stained with methyl green-pyronin Y after development.

RESULTS

Autoradiographs of gill epithelium from mussels serially sacrificed from 1 to 48 h revealed: no labelling occurred prior to 4 h PI (it seems likely that labelling had occurred by 1 and 2 h PI, but that numbers of labelled nuclei remained low enough to escape detection in the sections taken at these times); by 4 h PI, labelled epithelial cell nuclei appeared in gill furrow and gill furrow edges (Fig. 1); by 10 h PI, labelled epithelial cell nuclei began to appear one third to one half the distance up the gill ridge sides; by 24 h PI, labelled epithelial cell nuclei were present from the gill furrow to the

Table 1. *Results of slide reading of autoradiographs of the gill epithelium of M. margaritifera*

Time PI	No. of slides read	No. of labelled nuclei per slides read/approx. no of grains per nucleus		
		Gill furrow	Gill ridge sides	Gill ridge tip
Hours: 1	3	None/NA*	None/NA	None/NA
2	3	None/NA	None/NA	None/NA
4	5	114/15–25	9/15–25	None/NA
10	3	26/60–70	35/50–60	None/NA
Days: 1	6	140/50–60	398/40–50	119/25–40
2	6	70/10–20	124/25–35	45/10–15
3	3	15/5–10	42/5–10	6/5–10
4	3	63/10–15	108/10–15	23/5–10
7	3	None/NA	None/NA	None/NA
17	3	4/5–10	3/5–10	None/NA
21	3	2/5–10	None/NA	None/NA
28	3	3/5–10	None/NA	None/NA
35	3	8/10–15	None/NA	None/NA
42	3	12/5–10	None/NA	None/NA

* NA-not applicable.

gill ridge tip (Fig. 2); by 48 h PI there was a decrease in the number of labelled cells and grains per nucleus from the gill furrow to the gill ridge tip. The proportions of cells in each area remained similar and there appeared to be a decrease in the number of grains over labelled nuclei from the gill furrow and gill ridge sides to the gill ridge tip. Numbers of labelled cells remained high from the gill furrow to the gill ridge tip through 4 days PI; by 7 days, there was a decline in observed labelled epithelial cell nuclei, particularly along the gill ridge sides and gill ridge tip; through 45 days PI, it was still possible to detect some labelled nuclei in the gill furrow (Table 1). No evidence

of emigration or immigration of labelled cell nuclei out of, or into, the gill epithelium was observed.

No clear radiobiological effects (e.g. growth delay, chromosomal aberrations, cell killing) were observed in any of the slides read.

Staining of control (Tris buffer) and digested (DNase) slides with methyl green pyronin Y revealed that the DNase had removed DNA from the digested slides. One hundred and twenty-six labelled gill epithelial cell nuclei, with 10–25 grains over each nucleus, were observed in 3 Tris buffer-treated sections from 3 mussels sacrificed from 4 to 48 h PI. No label above background was observed in 3 DNase-treated sections (taken from positions in the sectioning ribbon adjacent to control sections) from the same mussels.

DISCUSSION

The gills of *M. margaritifera* consist of 4 demibranchs (folds) of tissue suspended from the visceral mass. Each demibranch appears V-shaped, with a descending lamella and an opposite ascending lamella. In a lateral view, each lamella of a demibranch appears as a sheet of parallel alternating ridges and furrows that extend vertically from the proximal to the distal demibranch. In a latitudinal cross-section the gill furrow epithelium varies with the location of the plane of the section. Most commonly, the epithelium consists of one to two layers of simple cuboidal cells. The epithelium on the gill ridge sides is transitional in appearance. The proximal portion may be simple cuboidal, or simple columnar epithelium. As the distal portion of the gill ridge side is approached, there is an apparent transition to a simple columnar ciliated epithelium. The gill ridge tip is a simple columnar ciliated epithelium with an extrusion region. Glandular cells (probably mucoid) are interspersed throughout the epithelium.

The gills are covered with morphologically specialized cells that are turned over (e.g. cell death, extrusion) as a result of normal function. The presence of labelled nuclei in the gill epithelium is associated with cell renewal activity. Cell renewal involves the continuous supply of new cells to replace those lost through cell death and extrusion (Thrasher, 1966).

The process of cell renewal in the gill epithelium of *M. margaritifera* appears to be in a steady state (i.e. the rate of cell production balances the rate of cell loss) (Thrasher, 1966). No emigration or immigration of labelled nuclei out of, or into, the epithelium was observed.

The epithelial surface can be divided into contiguous populations or compartments of cells that can be defined on the basis of morphology and location (Thrasher, 1966; Cleaver, 1967). Simple cuboidal cells located in the gill furrow and gill furrow edges appear to be a stem type population which, through proliferation, supplies undifferentiated replacement cells for other compartments. Simple cuboidal or simple columnar cells located on the proximal gill ridge sides appear to be a dividing transient, maturing population. Simple columnar ciliated epithelial cells located on the distal portions of the gill ridge sides and on the gill ridge tip appear to be a simple transient population

functional cells that eventually die and/or are extruded from the gill ridge tip. As cells migrate out of the gill furrow and up the gill ridge sides, they mature and undergo morphological and presumably physiological specialization to become functional gill epithelial cells. This transition makes sharp distinction between compartments difficult. The minimum transit time from the dividing transient population to the functional population in the gill ridge tip may be no more than 24 h.

Between 4 and 7 days PI, an obvious decline in observed labelled nuclei occurred. This decline was apparently due to repeated division of stem cell populations which reduced the number of grains per nuclei in some cells to an undetectable level. Migration and extrusion of cells may also have contributed to the decline of labelled nuclei.

It was possible to detect labelled nuclei at 42 days PI in the gill furrow areas while none were seen along the gill ridge sides or gill ridge tip. There are several possible explanations for this phenomenon.

Cleaver (1967) has shown that salvage of labelled breakdown products can become important in long-term *in vivo* experiments with ^{3}H-TdR. However, we found that this is not an important factor through 48 h PI and, if it occurred, it seems likely that such labelling would have continued to be seen throughout the renewal system. There are 2 other possible explanations.

Growth delay has been shown to be a radiobiological effect of X-irradiation (Cleaver, 1967; Mitchison, 1971). Some cells in the stem population may be labelled during synthesis of DNA and then undergo a radiation-induced growth delay that could result in their detection in autoradiographs after other label had been lost. However, this is not a clear example of a radiobiological effect. It has been shown that, in some cell renewal systems, some of the stem cells may enter a G_o phase (i.e. a population of cells that, after DNA synthesis, enter the cell cycle at a later time) (Cleaver, 1967). Such a reserve population may be present in gill furrow epithelium.

Incorporated labelled thymidine has been shown, under certain conditions, to cause a range of radiobiological effects including: growth delay, chromosomal aberrations, mutations and cell killing (Cleaver, 1967). Disintegration of a tritium molecule results in the emission of a beta-particle and transmutation of the emitting nucleus (^{3}H to ^{3}He). Transmutation of the emitting nucleus has a negligible effect since the vacated hydrogen atom sites in the pyrimidine ring are filled by hydrogen atoms present in living cells. Emission of beta-particles is responsible for any radiobiological effects (Cleaver, 1967).

The effects of beta-emission depend quantitatively on the number of ^{3}H-TdR incorporated into DNA, which is, in turn determined by the total concentration of thymidine (TdR) available, and the specific activity (Cleaver, 1967). Total concentration of available TdR was difficult to determine, but was similar to that available in many previous experiments in mammals (Thrasher, 1966; Cleaver, 1967; Gude, 1968). Specific activity was higher than the levels used in previous experiments and it may be suggested that this could increase radiobiological effects – a possibility that cannot be eliminated and was considered in all interpretations of data. It also should be noted that the radiation dose to the nucleus, as measured by the number of grains per unit

exposure time, was, in this study, greater than normally allowed in studies of mammalian cell renewal systems and could affect the parameters of cell kinetics. However the validity of extrapolating between mammalian and invertebrate studies is uncertain.

Labelled thymidine can undergo self-decomposition in storage to produce labelled by-products and degradation pathways of TdR can produce labelled breakdown products. Both of these processes can interfere with studies that rely on specific labelling of DNA. High specific activity increases the overall rate of self-decomposition. Rate of decomposition is closely proportional to the dose rate from emission of beta particles, which is dependent on the concentration of labelled molecules (specific activity). Non-sterile storage and subsequent infection by microorganisms can result in enzymic degradation, loss of labelled thymidine, and an accumulation of breakdown products (Cleaver, 1967). In order to minimize these 2 processes, solutions of labelled TdR were maintained under sterile, cold (4 °C) conditions and were used within 1 month of the last radiochemical purity check.

In the degradation pathway of thymidine, the labelled methyl group may be lost by demethylation of TdR or one of its breakdown products. These methyl groups can be incorporated into proteins and result in cytoplasmic labelling. However, the amount of label incorporated into proteins is low compared with that incorporated into DNA and is not usually detectable when short exposure times are used (as was the case in this study) (Cleaver, 1967).

That the above precautions and conclusions were successful and correct is supported by the results of the DNase digestion study. Since all label was removed from digested sections as compared to control slides, labelling by breakdown or self-decomposition products was not a factor in labelling through 48 h PI. All labelling observed was specific labelling of DNA.

There is a need for further studies comparing the effectiveness and utility of high and low specific activity tritiated thymidine on the same mollusc. It may be that a specific activity lower than that used in this study may reduce the possibility of radiation damage and still be high enough to overcome some of the difficulties faced in obtaining molluscan cell renewal data.

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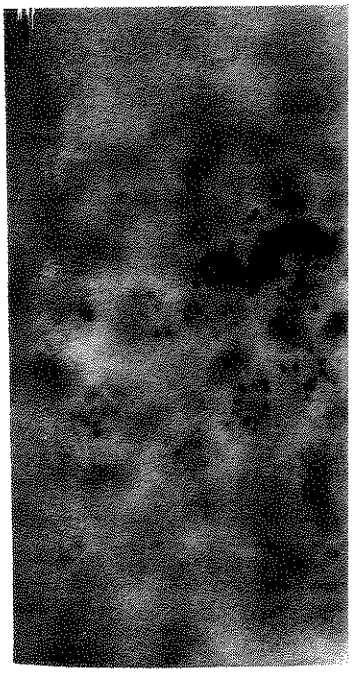


Fig. 1. Longitudinal cross-section containing numerous labelled epithelial cell nuclei in the gill furrow (*gf*) between 2 gill filaments in the posterior gill of *M. margaritifera* sacrificed 4 h after injection with ^{3}H-TdR. Haematoxylin and eosin. Exposure 3 days. × 1250.

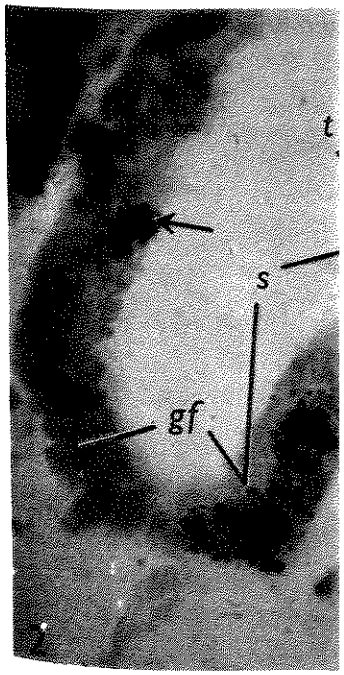
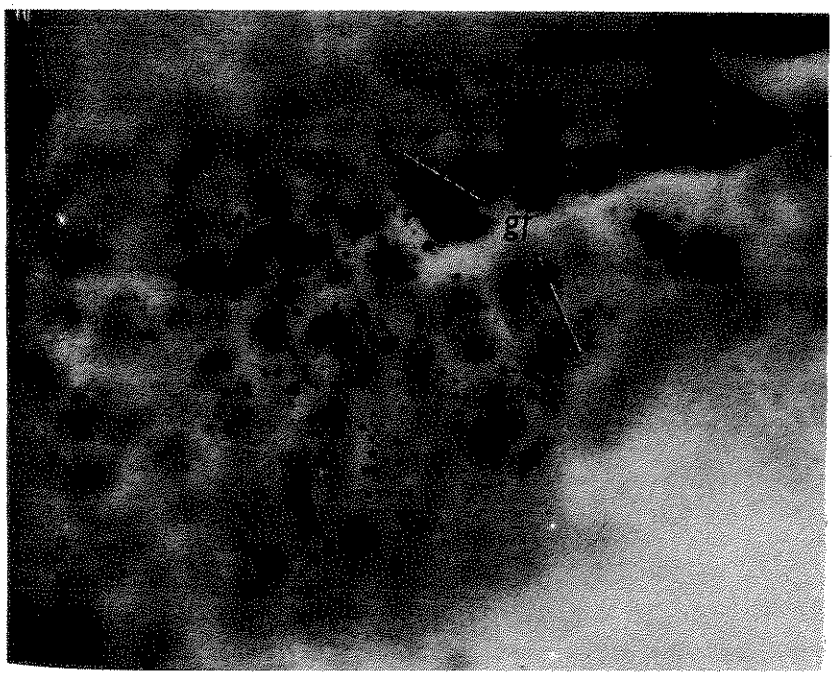
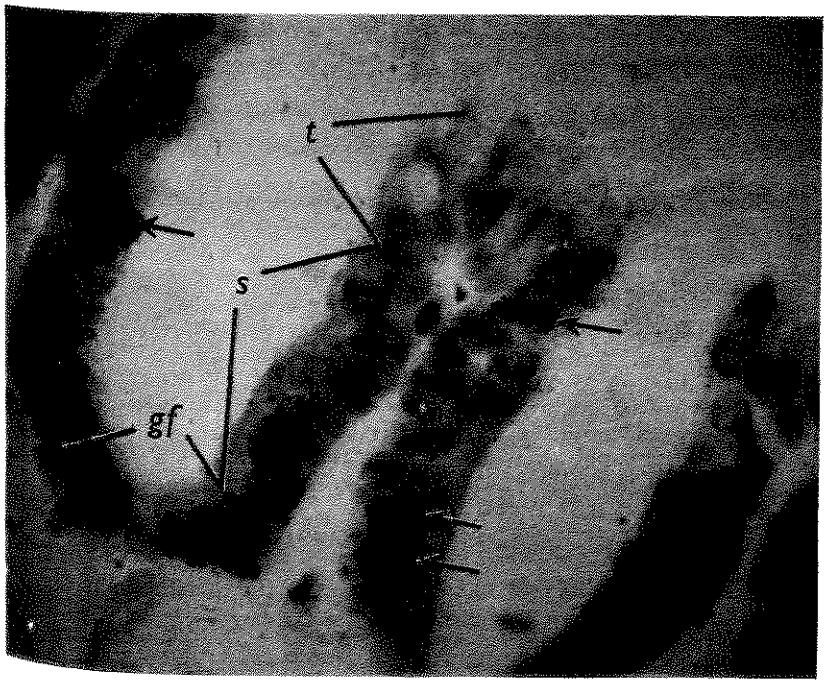


Fig. 2. Latitudinal cross-section containing labelled epithelial cell nuclei (arrows) from gill furrow to gill ridge tip in the posterior gill of *M. margaritifera* sacrificed 24 h after injection with ^{3}H-TdR. Haemotoxylin and eosin. Exposure 3 days. *gf*, gill furrow; *s*, gill ridge side; *t*, gill ridge tip. × 500.





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