Molluscan Cells: Dissociation and Reaggregation

Abstract. *Dissociation of the ovotestes of* Helix pomatia *requires the presence of carbohydrates as well as trypsin, but is independent of cation content. Dispersed cells obtained from the ovotestes reaggregate rapidly into histotypically organized tissue. The role of cellular adhesiveness is facilitated by centrifugation, but pseudopodial activity of the cells is suggested as another factor in reaggregation.*

Although organs and tissues of mollusks (*1*) have been cultured, there is virtually no information about the cultivation of cells of these animals. In attempting to culture snail cells in vitro, I sought a satisfactory method for obtaining dispersed cells. Current methods for dissociating vertebrate and invertebrate tissues, usually based on treatments that alter intercellular attachments (*2*), are unsuitable for snails.

Ovotestes were obtained aseptically from *Helix pomatia*, a terrestrial pulmonate snail. For each experiment, tissue (2 mm^3) from 25 adults was diced and placed in 125-ml stirring flasks; 20 ml of dissociation medium was added, and the entire mixture was stirred at 10 rev/min. Five times at 5-minute intervals the supernatant containing the dissociated cells was removed into centrifuge tubes, chilled to 4°C, and replaced with fresh dissociation medium. The cells so obtained were centrifuged at 1500 rev/min for 10 minutes at 4°C and washed twice in 10 ml of normal medium (*3*).

The cells were again centrifuged and suspended in 10 ml of a basal nutrient medium (*p*H 7.5) consisting of Chiarandini solution (*3*), 870 ml; fetal calf serum, 100 ml; egg albumen, 2.0 g; Eagle essential amino acids (*4*), 10 ml; and Eagle nonessential amino acids (*5*), 20 ml. A sample of the suspension was counted with the aid of a hemacytometer, and 0.1 percent of trypan blue was used for determination of cell viability.

In all instances 0.25 percent (weight to volume) of trypsin (*6*) was used in the dissociation medium; *p*H was adjusted to 7.8. The trypsin was originally suspended in Chiarandini physiological salt solution (*3*) consisting of 6.45 g of NaCl, 0.35 g of KCl, 0.94 g of $CaCl_2 \cdot 2H_2O$, 0.86 g of $MgSO_4 \cdot 7H_2O$, and 1.54 g of $NaHCO_3$ per liter. In the presence of this solution so few cells could be dissociated from the tissue that a cell count was impossible. When Ca was removed from the solution, some cells could be harvested (Table 1, experiment A) but most of the tissue remained intact. Trying to improve the physiological salt solution, I substituted sodium gluconate (6.45 g/liter) for NaCl; because the resultant solution was far more effective in disaggregating the tissue (Table 1, experiment B), the substitution was made for all subsequent studies. I designate this modification MPS (modified physiological salt solution).

For determination of the effect of calcium on the trypsin-MPS, this cation was deleted from the medium; calcium is of little significance in this system (Table 1, experiment B). Removal of Mg or K, or of both, produced similar results.

Because carbohydrates in the form of sodium gluconate facilitate disaggregation of the cells, the sugars glucose, galactose, and trehalose (1 g/liter) were used as supplements for determination of their effects on dissociation. Although cells could be obtained readily in each instance (Table 1, experiment C), galactose and trehalose were the only two sugars causing definite increase in cells obtained per milliliter. Subsequently galactose and trehalose together were added to the trypsin-MPS. Dissociation was best in the presence of trypsin-MPS plus galactose and trehalose (Fig. 1A).

To determine the effect of cations on cell dissociation when trypsin-MPS plus galactose and trehalose were used, Ca was eliminated from the solution—with no significant difference in dissociation (Table 1, experiment D); elimination of Mg or K, or of both, produced similar results.

The presence of galactose and trehalose enchances dissociation of gastropod tisues in solutions including NaCl instead of sodium gluconate (Table 1, experiment E). In the absence of Ca, somewhat more cells could be obtained, but dissociation of cells in the presence of NaCl is always less successful than with sodium gluconate.

During centrifugation to remove the trypsin solution, the cells clumped; each of the six centrifuge tubes per experiment contained a single clump or reaggregate. Contents of all six tubes were combined, washed, and recentrifuged, and the sizes of the reaggregates were compared. Tubes containing large reaggregates (1 to 1.5 cm) were always tubes in which dissociation had been good; reaggregates were smaller (0.4 to 0.5 cm) when dissociation had been poor, such as in the physiological solution with trypsin and NaCl but no sugar.

Histological sections of the reaggregates showed that all the cells normally found in the ovotestes of *H. pomatia* (that is, nurse cells, gametocytes, sperm, eggs, epithelial cells, and connective tissue) were present in the aggregates (Fig.1). Furthermore, despite the fact that centrifugation required only 10 minutes, the cells were in the same relation and architecture as were those seen in the normal ovotestes. The

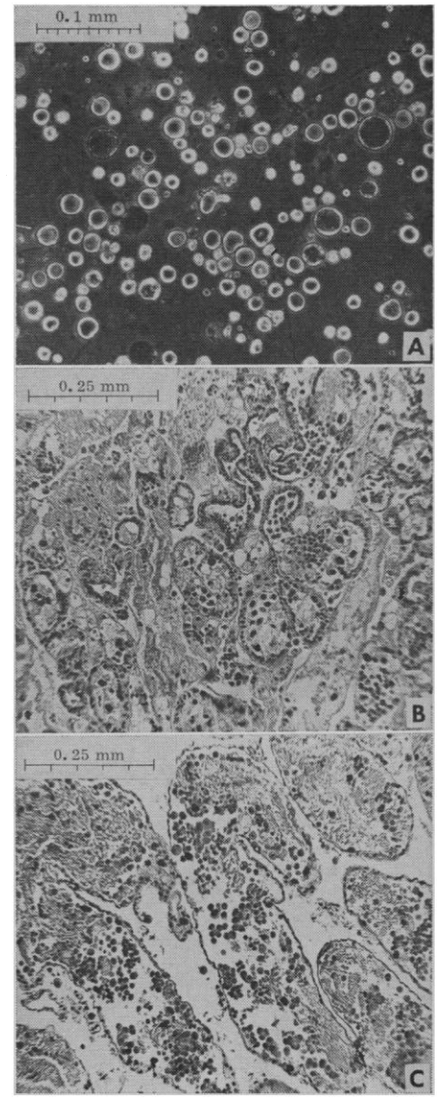

Fig. 1. (A) Ovotestes cells dissociated in the presence of MPS plus trypsin, galactose, and trehalose (note pseudopodia). Histological sections of reaggregated cells of the ovotestes of adult *Helix pomatia* after centrifugation for 10 minutes at 1500 rev/min (B) and of normal ovotestes (C).

Table 1. Effects of sugars and cations on the dissociation of *Helix pomatia* ovotestes. Numbers of experiments appear in parentheses. Chia, Chiarandini solution.

Dissociation medium	Cells ($\times 10^6$/ml)
Experiment A	
Chia + trypsin (4)	0
Chia + trypsin; no Ca (4)	0.25
Experiment B	
MPS + trypsin (4)	.95
MPS + trypsin; no Ca (4)	.94
Experiment C	
MPS + trypsin (11)	.90
MPS + trypsin + glucose (2)	.94
MPS + trypsin + trehalose (2)	1.04
MPS + trypsin + galactose (2)	1.20
MPS + trypsin + galactose + trehalose (11)	1.70
Experiment D	
MPS + trypsin + galactose + trehalose (4)	1.58
MPS + trypsin + galactose + trehalose; no Ca (4)	1.54
Experiment E	
MPS + trypsin + galactose + trehalose (5)	0.70
Chia + trypsin + galactose + trehalose (5)	1.64
Chia + trypsin + galactose + trehalose; no Ca (5)	.89

major differences between the normal ovotestes and the reaggregates were that in the latter (i) the hemocoel spaces were smaller, (ii) acinar lobes were fewer and smaller, and (iii) fewer cells were found per acinar lobe.

In six experiments any undissociated fragments of tissue were removed by expression of the cells through a 60-mesh stainless steel grid in the base of a 30-ml hypodermic syringe before centrifugation. Sections of the resultant reaggregates yielded results similar to those obtained without use of the grid. Thus the cells are able to form organized tissue during centrifugation.

The reaggregates could be dispersed with a pipette by vigorous aspiration of the solution in which they were bathed. The cells in such suspensions had pseudopodia (Fig. 1A). If the suspension was centrifuged briefly (about 0.5 minute) and a sample was removed and examined without a cover slip, almost all cells showed this activity. There were two kinds of pseudopodia: a broad, slow-moving lobopodium and a thin, flexible, active filopodium. Furthermore, the cells tended to orient on any substrate, such as cotton fibers or sperm tails, with which they came in contact.

The role of the pseudopodia formed by the dissociated molluscan cells is not clear. Trinkaus (*7*) states that both vertebrate fibroblasts and epithelial cells in culture exhibit cytoplasmic extensions at free surfaces. These extensions apparently act as the locomotive organ of the cell and seem to be the means of movement of many cells engaged in morphogenetic cell movements (*7*). The descriptive behavior of these extensions is very similar to that of the pseudopodia of the snail cells; thus it is possible that these pseudopodia function in the rapid reaggregation of the ovotestes cells.

Centrifugation and cellular adhesiveness appear to be the most important factors in rapid reaggregation of the ovotestes cells. The extensive reorganization of the cells in such a short time suggests that adult *H. pomatia* cells retain the ability to (i) recognize a morphogenetic tissue pattern and (ii) maintain cellular adhesiveness after dissociation. Although the reaggregation of dissociated cells has been extensively studied, most research has been concerned with embryonic cells, for only one other group of animals, the sponges, have been reported to have cells showing aggregation behavior in the adult. Sponges can be dissociated both mechanically (*8*) and chemically by removal of divalent cations from the solution (*9*). Moscona (*10*) suggests that, in both sponges and vertebrates, extracellular materials function in the selective attachment and organization of cells. In the case of the sponges, this idea appears to be supported by the fact that cell-free extracts having cell-aggregation activity were obtained from suspensions of sponge cells washed in cold sea water lacking divalent cation (*11*). Such extracts consisted largely of glycoproteins whose activity depended on calcium. Chemical analysis revealed that sponge-cell aggregation depended primarily on the presence of disulfide groups and on protein integrity, although it was stated that these experiments could not entirely rule out a possible role of carbohydrates (*12*).

Most workers (*13*) on reaggregation agree that the regrouping of dissociated cells into coherent multicellular bodies is probably due to the mutual adhesiveness of the cells, and conditions affecting reaggregation have been interpreted as affecting cell adhesion. A study of *Helix* cells similar to that of sponge cells (*11*, *12*) might clarify the reaggregation of dissociated molluscan cells.

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LSD in Mice: Abnormalities in Meiotic Chromosomes

Abstract. *Meiotic chromosomes of six mice injected with high doses of lysergic acid diethylamide (LSD-25) and of six controls were studied. Several breaks, gaps, and unidentifiable fragments were found in the treated but, with a few exceptions, not in the control animals. Secondary constrictions were more numerous in the treated than in the untreated mice. Possible consequences are discussed.*

Lysergic acid diethylamide (LSD-25) has been shown to cause abnormalities in the chromosomes of human leukocytes (*1*). The drug has other teratogenic properties in production of congenital abnormalities and increased rates of abortion in rats and mice (*2*). The possible effect on human embryos has been discussed (*3*). Our preliminary results suggest that LSD can also cause changes of the meiotic chromosomes in germ cells.

Meiotic chromosomes were studied from six healthy male mice injected with LSD-25 (group A); the mice were aged between 6 and 8 weeks. Identical mice were used as controls (group B). The animals were coded, and the investigator did not know the code. But the mice were matched in pairs; one member of each pair received LSD and one did not—in some in-