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A Method for Tissue Culture of *Hydra* Cells.* (28335)

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Hydra is one of the simplest of the multicellular animals. It has also some characteristics of plants. *Hydra* provides, therefore, a unique material for cellular investigation. However, the presence of the cnidoblasts and nematocysts in *Hydra* has complicated the study of functional properties of constituent cells. A method to isolate the *Hydra* cell *in vitro* is highly desirable, and would be useful in studying effects of immediate environments, division, and differentiation. The culture of coelenterate tissue *in vitro* has not been successful until recently in the instance of anemone *Anthopleura elegantissima*(1). In the case of *Hydra,* Papenfuss and Bokenham (2) had studied the ectoderm and endoderm cultured independently in filtered pond water, and reported that the tissues always disintegrated. No information on tissue culture of fresh water *Hydra* cells in nutritive media has appeared in the literature. This communication describes a method for cultivation of *Hydra* cells *in vitro*.

Materials and methods. A preliminary study of the cell culture of *Hydra littoralis*§ revealed the presence of severe contamination by bacteria-like microorganisms, which could not be eliminated by carefully improving the aseptic techniques.‖ Our further studies showed that these contaminating bodies were, in fact, an intracellular parasite to the *Hydra,* the Microsporadia, previously tentatively identified as a species of *Plistophora*(3). One source of the infection was traced invariably to the artemia used for feeding the *Hydra*. Although Microsporadia-infected *Hydra* continued to bud and regenerated normally and showed no significant physiological or morphological changes, the spores of the Microsporadia rapidly germinated, multiplied and destroyed the host cells when the latter were cultured *in vitro*. Consequently it was imperative that the spores of the parasite be removed from the *Hydra* cells. Fumidil B was employed as this preparation has been demonstrated to be effective for this purpose(3). To prevent reinfection, subsequently, prior to the preparation of the *Hydra* cell suspension, the *Hydra* were cultivated without feeding for 2 days in the sterilized culture solution(4) containing 0.125 g neomycin, 0.125 g streptomycin, 0.200 g

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Fumidil B and 500 units penicillin, per liter, respectively.

The *Hydra* cell suspensions were prepared by means of trypsinization(5) with a magnetic stirrer(6) for 15 min and filtered through 4 layers of gauze. The filtrate was centrifuged at 700 r.p.m. for 10 min to remove the nematocysts, and the supernatant again centrifuged at 2200 r.p.m. for 15 min. The sediment was resuspended in *Hydra* culture solution and centrifuged at 2500 r.p.m. The *Hydra* cells thus obtained were resuspended in the culture solution containing all the above mentioned ingredients in a 10-fold concentration. The mixture was shaken occasionally for 1 hour and washed 3 times with cold standard culture solution without any added antibiotics, except Fumidil B at 200 mg/liter. The cells were examined under phase contrast microscope for completeness of removal of Microsporadia during the washing cycles. The cell suspension after washing was diluted to a concentration of approximately 64 $\times$ 10^3 cells/ml. One ml of the cell suspension was seeded per prescription bottle (125 ml), to which was added 5 ml of growth medium. In addition 0.1 ml of the above suspension was seeded per Leighton tube containing 1 ml of growth medium.

In the development of a growth medium for the culture of *Hydra* cells, Earle's saline solution was first used. It was found that the *Hydra* cells tended to shrink, largely due to too great an osmotic pressure of the medium. Consequently, the salt concentrations were reduced so as to provide as nearly as possible the conditions for *Hydra* growing *in vivo*. The *Hydra* cell growth medium as eventually developed consisted of 10 ml modified Eagle's medium (100 $\times$)(7), 100 ml horse serum, 500 units of penicillin and 200 mg Fumidil B, 100 ml of modified Earle's saline solution (10 $\times$) diluted to 800 ml with triple-distilled water, then the whole mixture was made up to 1 liter, including a 0.15 M photphate buffer to maintain the pH of the medium at 6.8. The composition of the modified Earle's saline solution (10 $\times$) is shown in Table I.

TABLE I. Composition of Modified Earle's Saline Solution (10$\times$).

Material	Conc (mg/liter)
NaCl	58
$MgSO_4 \cdot 7H_2O$	1000
$NaHCO_3$	1680
KCl	75
$CaCl_2$	1110
Dextrose	10000
Phenol red	100
Streptomycin	125

Following the above procedures, cultured cells could be obtained in small numbers, yet most of them could not adhere to the walls of the container. Since a previous finding has shown that *Hydra* bud extract reduces the latent period for the bud to grow and initiate its own new bud(8), such extract was prepared and added to the growth solution with entirely satisfactory results. The preparation of the extract consisted in homogenizing the *Hydra* buds in their culture solution(4), and filtering through gauze and microfilter. The concentration of the extract was 50 buds/ml and 1 ml of which was added to 5 ml of growth solution.

All *Hydra* cell cultures were then incubated at 22°C in Leighton tubes, and bottles, cotton-plugged. Harvested after 24 hours these cultures showed a continuous sheet of integrated cells with excellent growth. These cultures have been serially transferred for more than 10 times either by trypsinization or mechanical manipulation and maintained in the growth medium, *Hydra* bud extract omitted, for more than 5 days.

Results. After a few transfers, *Hydra* cells that grew on the coverslip in the Leighton tube for 12 hours apparently appeared only as interstitial cells of *Hydra in vivo*(9), although the initial cell suspensions were heterogeneous, consisting of interstitial cells, gastrodermal cells, epitheliomuscular cells and very young cnidoblasts. The cytoplasm of the cultured cells did not show double membranes. Their nuclei were evenly granular with one or more prominent nucleoli. In some of the cells, the mitotic process, seldom so clearly demonstrated *in vivo*, could be observed distinctly (Fig. 1, a-d). One may note the large increase in size of the

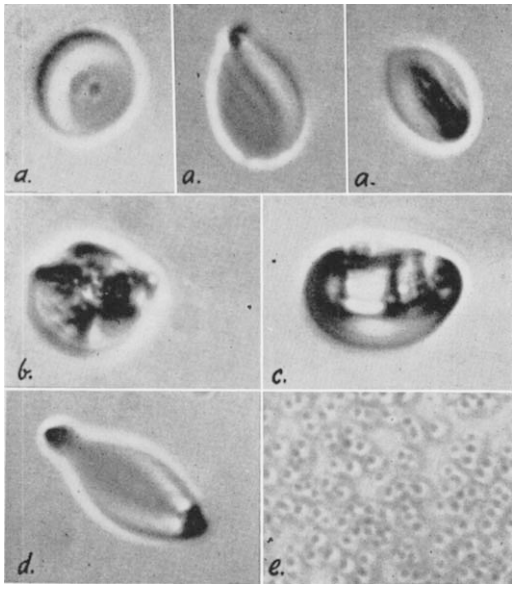

FIG. 1.¶ Cultivated *Hydra* cells after 72 hr incubation, × 1,000. (*a*)-(*d*), freshly mounted in glycerol, the main stages during mitosis: (*a*) prophase, (*b*) metaphase, (*c*) anaphase and (*d*) telophase. (*e*) Wright's stain smear, interphase.

cells during mitosis compared with those at the interphase. As the time of incubation was increased from 12 to 72 hours, the cells appeared to be syncytial as the cell boundaries became less prominent. However, for cells growing in the bottles with large population after the same 72 hour interval, such cell boundaries remained well defined when observation was made by smearing technique (Fig. 1, e). In fresh preparations, some of these cells exhibit amoeboid mobility.

Discussion. In developing the present method for tissue culture of *Hydra* cells, the early recognition of contaminating Microsporadia spores permits prompt application of necessary corrective measures. Although it is quite feasible to use modern aseptic techniques without the use of antibiotics, this has not been possible in the present case. The methods devised for culturing vertebrate tissues also needed modifications. Conditions of temperature, osmotic pressure, hydrogen ion concentration, nutrient requirement and other factors had been specially considered to promote the growth of *Hydra* cells.

¶ The assistance of Mr. Orris Rivers in the preparation of photographs is acknowledged.

Our findings that apparently only interstitial cells were obtained from the cell cultures are of interest. In an attempt to obtain regeneration from the ectoderm and endoderm of *Hydra* when cultured independently(2), the negative experimental results were attributed to the limited formative powers of the unspecialized interstitial cells. This seems to have support from our observation that although these interstitial cells grew well in the culture media, no further cell differentiation was observed. However, whether these interstitial cells were derived entirely from the initial interstitial cells or also from the transformations of differentiated cells would require further studies. The phenomenon of dedifferentiation of the heterogeneous cells into homogeneous undifferentiated cells was also often observed in other cultured tissues discussed by Paul and Fischer(10,11). The difference in morphological features between *Hydra* cells growing in the bottle and in the Leighton tubes as observed in the present study could involve the different growth environments resulting from the different population density which is often a major factor influencing cell morphology(12).

Summary. A method for tissue culture of *Hydra* cells is described. The removal of parasitic microsporadia from the *Hydra* cells and addition of *Hydra* bud extract to the culture medium are essential for a successful growth *in vitro*. Under the proposed procedures, the cultivated cells apparently have the features only of interstitial cells. Mitotic division in some of the cultured cells has been demonstrated.

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Localization of Glycogen in the Opacity Characterizing Decidualization in the Cleared Hamster Uterus.* (28336)

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Uteri of pregnant hamsters, and of pseudopregnant hamsters in which decidualization has been induced, reveal areas of opacity when viewed by oblique light after clearing by the benzyl-benzoate process. These opacities have been shown to be associated with decidua(1-3), and the presence of glycogen in them has been suggested(2).

During gestation, the first of these areas which appear opaque after clearing develop coincidentally with decidua and mark implantation or decidualization regions prior to visible external swelling. They increase in mass, distending the uterus as localized swellings, late on the sixth day of gestation. They reach a peak in terms of mass per conceptual swelling on the seventh day of development, and begin to regress from the antimesometrial area on the eighth day, *i.e.*, at 7 days and 12 hours.

This paper reports results of chemical analyses of these tissues in comparison to the remainder of the uterus.

Materials and methods. At the relative peak of development of the areas in question (sixth and seventh days), it is possible to make a transverse slit in the uterus at the swollen gestational sites and, with fine forceps, peal from the endometrium the denser mass of the embryo and its surrounding decidua. Examination of material separated by the above technique and then cleared, showed that the peeled out region comprised almost all the opaque material (Fig. 1 and 2). Such "peeled out regions" subsequently are referred to as "opacities," whether or not they have been subjected to the clearing process and thus actually appear opaque. Samples of the remaining portions of the uterus, from which all mesometrium was removed, were analyzed for comparison.

One series of determinations was carried out on material which had been cleared prior to the separation of the opaque regions. This cleared material was pooled from the tracts of 2 animals killed at 5 days and 23 hours and 2 animals killed at 6 days and 12 hours developmental age. Subsequent determinations were made on material from animals freshly killed by exsanguination. Opacities and uteri from these animals were frozen with dry ice immediately after dissection but the tissues were not cleared except for one swelling from each animal which was used as a control (*i.e.*, cleared but not analyzed). Two animals, both at 6 days and 12 hours, were used for study; one showed 12 and the other 13 conceptual swellings.

Since deciduomata also appear opaque, they were produced by air injection (0.05 ml) into the uteri of 2 hamsters made pseudopregnant by mating with vasectomized males. This air injection was done at 3 days 20-21 hours post ovulation and the animals were autopsied at 6 days 21 hours post ovulation. One deciduomata from each hamster was retained as a control (*i.e.*, cleared but

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