netics pattern of intercellular oxygen association and disassociations produced by the hypoxia of diving.

These results emphasize the importance of intracellular transients in defining respiratory gas exchange and suggest the value of the seal as a model for studying O_2 depletion on a broad evolutionary basis.

1964 #28

IN VITRO CULTIVATION OF CELLS DERIVED FROM MARINE ORGANISMS

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Quantitative cell culture methods have opened new approaches to the study of mammalian cells as isolated organisms or homogeneous populations. In general, these methods have contributed much to our knowledge about physiological, genetic, and biochemical regulatory mechanisms. In addition, in vitro cell cultures have provided ideal systems for the study of virus infection at the cellular and molecular level.

Several investigators have turned their attention to attempts to establish fish cells in continuous culture and to search for virus-like agents in fresh water or marine fishes. The promising results of these efforts have been summarized in a recent Symposium ("Viral Diseases of Poikilothermic Vertebrates," Ann. N. Y. Acad. Sci. 126, 1-680, 1965). Thus far, the greatest success has been obtained with cells from certain fresh water fishes and from a few marine teleosts. It has been the experience of all workers in this field that methods and media developed for cultivation of mammalian cells are, with minor modifications, also best suited to meet the requirements of fish cells. A number of fish viruses have been shown to grow and produce characteristic cytopathic effects in these cultures.

No reports have been published on the successful cultivation of cells derived from the more primitive cartilaginous fishes. Most of our work has been done with first year dogfish embryos (Squalus acanthias), but some success has also been obtained with skate fin tissue.

Embryos are collected aseptically from "candles," washed thoroughly in several changes of phosphate-buffered 0.22M NaCl solution (DF-PBS), and, after removal of the eyes, finely minced with scissros. The mince is thoroughly washed in $Ca^{\#}$- and $Mg^{\#}$-free PBS and then once or twice in a 0.025% trypsin solution in $Ca^{\#}$- and $Mg^{\#}$- free PBS (DF-trypsin). The thorough washing is essential to prevent the formation of mucous "ropes" which would otherwise entrap the cells during trypsinization.

The washed mince, suspended in 5 to 10 vol. of DF-trypsin, is placed in a fluted flask on a magnetic stirrer and agitated for repeated periods of 10 to 20 minutes each. The temperature is maintained at 18°C or less. The product of the first trypsinization period is discarded. Cell suspensions from subsequent periods are pooled, filtered through multiple layers of sterile gauze, and centrifuged. The cell sediment is resuspended in nutrient medium (DF medium), and the density adjusted to 0.25-1 x 10^7 cells/ml. Four ml portions of this suspension are seeded in 30 ml plastic tissue culture flasks (Falcon).

After much trial and error, the following medium has given the most promising results:

Basic medium L-15 (Leibovitz)*	100 ml
Human serum	10 ml
Fetal calf serum	10 ml
Egg ultrafiltrate	5 ml
0.2 M glutamine	2 ml
1.54 M NaCl	7 ml
Antibiotic mixture (40,000 units penicillin and 40,000 μg streptomycin/ml)	0.5 ml

"Conditioned D-F medium" is obtained as follows: Primary cultures are incubated with fresh medium for 4 to 7 days, then the medium is harvested and centrifuged for the removal of debris and floating cells. For complete elimination of cells, the medium is filtered through a Millipore membrane (pore size HA). To 100 ml of this "conditioned medium" is added 2 ml of 0.2M glutamine. It is then mixed in varying proportions (usually 1:1 with fresh medium, and the mixture is used as the growth medium of choice for primary cultures, for re-feeding, or for subcultures.

All cultures are incubated at temperatures ranging from 8 to 18°C. Use of L-15 medium obviates the need for continuous flushing with a CO_2-air mixture. Proper pH can be maintained in heavily seeded cultures by leaving the closures of the culture flasks loose.

Subcultures are prepared by removing cells from the plastic flasks with 0.25M trypsin and resuspending the centrifuged cells in medium.

Coverslip cultures are fixed with methanol and Giemsa-stained.

Cultures seeded with 1 to 4 x 10^7 cells give rise to confluent monolayers consisting mostly of fibroblastic strands forming extensive whorls. Occasional mitotic figures have been seen in such cultures 2 to 3 weeks old. However, the division rate of these cells must be very slow since no significant net increase in cell number has yet been found. This is probably due to the very heavy concentration at which cells have had to e seeded thus far. In this respect, dogfish embryo cells behave in a manner similar to primary chick embryo fibroblasts. As of this writing, viable cells have been maintained for over a year at 8 to 10° without more than occasional medium changes.

In preliminary experiments, monolayers of dogfish embryo cells in third subculture were inoculated with two human viruses at high input multiplicities (type 2 dengue virus, a group B arthropod-borne virus, and type 2 adenovirus). Periodic assays revealed no viral multiplication. Moreover, "infection" failed to induce cytopathic changes or to interfere with incidence of mitotic figures. "Infected" cultures could be subcultured with the same success as uninfected ones. These studies and experiments with known fish viruses will be pursued as soon as cultures can be secured in adequate numbers.

*Albert Leibovitz, Am. J. Hyg. 78, 173-80, 1963. Medium L-15 is an amino acid buffered medium for use in situations where an elevated CO_2 atmosphere is difficult to maintain or undesirable.