Cultivation of Adult Teleost Tissues *in vitro*. (23250)

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Cultures of normal and abnormal tissue cells from warm-blooded vertebrates are routinely prepared in many laboratories, and there is abundant literature on the subject. In contrast, there is comparatively little information on culture or tissues from cold-blooded vertebrates. Osowski(1) observed cellular movement in fragments of tadpoles and trout which he maintained for as long as 24 hours in Ringer's solution. Sea water and fish bouillon were used as culture medium in other work with fish tissue, but the cultures were short-lived(2,3). Chlopin(4) cultured tissues from pike and crucian carp in rabbit plasma "diluted with homogeneous extract." Lewis and MacNeal(5) cultured pituitary glands from flounder, sculpin and angler fish. Their cultures were "prepared in autoplasma or homoplasma" to which chicken plasma was added. Recently, abnormal tissues of killifish (6) and goldfish(7) were investigated, and in these studies embryonic extracts were added to media composed of chicken, human cord or fish sera and salt solutions. Soret and Sanders(8) propagated the virus of eastern equine encephalomyelitis in fish embryos. There is no information on the culture of tissue cells from salmonids which require low environmental temperatures (10-15°C is the optimum range). Because the culture of host tissues would seem to offer promise as an effective tool in the study of those diseases of salmonids, suspected of being caused by a virus, the initial problem in this investigation was to develop simple and efficient methods of culturing tissues from normal fish. This communication describes the attempts that were made to cultivate salmonid and other fish tissues in certain commercial media.

Materials and methods. Tissues from hatchery-raised rainbow trout (*Salmo gairdneri*) were used for most of the work, but tissues of eastern brook trout (*Salvelinus fontinalis*), brown trout (*Salmo trutta*), and goldfish (*Carassius auratus*) were also cultured. Age of donors ranged from embryonic to adult, but cultivation of tissues from subadult and sexually-mature specimens was emphasized, because embryonic salmonid tissue is usually available only part of the year. Fish were killed just before use by severing the vertebral column immediately back of the head. Care was taken not to penetrate the body cavity. When gill tissue was needed, the arches were excised and washed in a stream of sterile salt solution from a syringe, which probably helped to remove surface bacteria. When sterile internal organ tissue was needed, the fish was immersed for 1 min. in a 1:1000 solution of benzalkonium chloride (Roccal). After draining, excess disinfectant was removed with cotton swabs saturated with 70% ethanol. Instruments were sterilized in boiling water followed by immersion in 70% ethanol and flaming immediately before use. Tissues or organs were removed aseptically, immediately submerged in cold Earle's solution, and maintained at about 5°C until minced with scalpels and placed in culture vessels. Mincing and all subsequent operations were performed in a hood. Throughout the manipulations the tissues and vessels were kept cool on or near trays of ice, partly because growth of trout tissue was not obtained at 26°C and above and partly because failure in early attempts to obtain growth was attributed to high temperatures within the hood during explantation. Because ozone and ultraviolet light also seemed to be deleterious, the hood was thoroughly flushed with fresh sterile air before use. Some tissues were cultivated directly on glass, but better results were obtained when tissues were embedded in a chicken plasma clot. Cultures were made in straight-necked 5 cm Carrel flasks or stationary 16 x 150 mm tubes, either with or without coverslips. Several 2-3 mm^2 x 1 mm explants were used in each culture vessel, with 1.5-2 ml of fluid media in flasks, 1-1.5 ml in tubes. When cultures were to be kept a week or longer, medium was changed in whole or in part every 4-7 days. Viability

could often be maintained with weekly renewals. Media used in this work always contained embryo extracts, generally at a level of about 5%. The media also contained from 10-20% serum, and the balance was medium 199 and salt solution with phenol red at 0.001%. Media for internal tissues contained 250-1000 units potassium penicillin G/ml. but penicillin alone did not inhibit contaminants in cultures of gill or fin tissue. Contamination in cultures of external tissues was controlled with 1000 units of penicillin plus 1000 mcg of dihydrostreptomycin sulphate/ml. Best results were usually obtained when the initial pH was adjusted with 5% CO_2 in air to 7.2-7.4, but growth was also obtained at pH 6.8-7.0. Temperature of incubation was 19°C. This investigation did not involve serial transplantation, but some cultures of trout tissue were kept growing for a month and longer, and one culture of goldfish tissue was kept alive for about 10 months.

Results. In general, trout tissues were readily cultivated, but comparable goldfish tissues grew more vigorously. It was necessary to keep trout tissues cool during removal, manipulation and incubation. Swim bladder from eastern brook trout grew well at 12° and 19°C, but more abundant growth was obtained at the latter temperature. No growth of trout tissue occurred at 26° and 37°C. (Maximum temperature tolerance for trout is generally below 26°C.)

Synthetic medium 199 (SM 199) (Difco) and salt solutions were compared for their ability to maintain normal morphology in rainbow trout blood cells. Ringer's frog saline and Holtfreter's solution(9) proved to be unsatisfactory and appeared to be hypotonic. Earle's, Tyrode's, and Hanks' salt solutions and SM 199 gave satisfactory results. Earle's salt solution and SM 199 were judged to be best, and a mixture of the two was used in media for most subsequent fish tissue culture. Growth of trout tissue was obtained in media containing serum, embryo extract and either SM 199, Tyrode's or Earle's, but the results with Tyrode's were inferior. Growth was obtained with chick embryo extract (Difco, desiccated), but a saline extract of newly-hatched trout gave similar results in cultures of trout kidney and minced trout embryos. Beef embryo extract (Difco, desiccated) also supported growth of fish tissues. Either chicken serum or heparinized trout plasma was employed in much of the work, and serum from carp (*Cyprinus carpio*) supported trout kidney tissue. Many sera supported growth of trout swim bladder tissue, but results with horse serum were poor. Sheep and beef sera were slightly better. Chicken and rabbit sera gave fair results, and human serum was as good as homologous serum. Cellular quality and quantity were excellent in human cord serum; bovine amniotic fluid gave healthier cells, but multiplication was slower than in cord serum.

Tissues from kidney, spleen, peritoneum, gills, testes, ovary, fins, and swim bladder of trout were readily cultured. Heart and liver tissues of trout were less easily cultured. Tissue from a common idiopathic granuloma of eastern brook trout(10) yielded a few abnormal fibroblasts. Heart and kidney of goldfish were easily cultured; one heart culture was kept beating for 299 days in 20% chicken serum, 5% embryo extract, 30% SM 199, and 45% Earle's solution. It died when trout serum was substituted for chicken serum in the medium. Swim bladder explants usually exhibited smooth muscle movement for at least several days; one culture, for over 7 weeks. Ciliary movement was usually observed in explants of swim bladder, kidney and spleen. It was often maintained for several weeks, and for 58 days in one culture of trout swim bladder. Other workers(5) have made similar observations of cilia in fish tissues. Occasional individual cells and aggregations of cells which bore cilia separated from the explant and became motile. This phenomenon has been observed in frog epithelium by Duryee and Doherty(11). With the exception of fin epithelium, cells from tissues in a plasma clot usually adhered better and maintained a healthy appearance longer than those from the same tissue cultured directly on glass. Actively proliferating cultures usually produced only a slight drop in the pH of the medium.

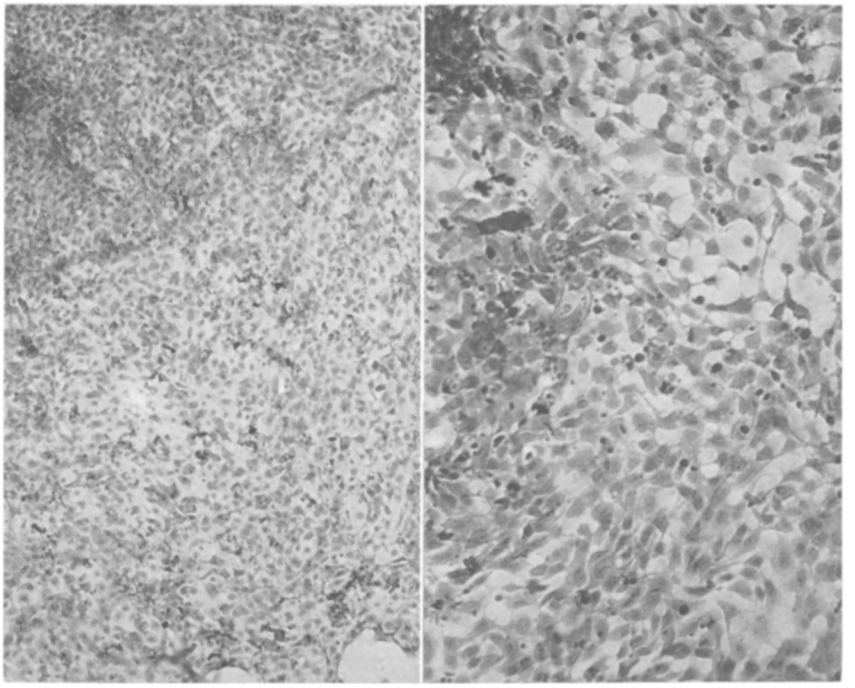

FIG. 1 (left). Cell sheet from 48 hr cultivation on glass of rainbow trout fin. H. & E. × 57.
FIG. 2 (right). Cells from rainbow trout fin in clot after 48 hr incubation. H. & E. × 140.

Gill, swim bladder, kidney and fin tissues from trout regularly gave good cultures. Migration of macrophages was usually evident within hours after explantation and often became extensive. Migration was usually followed by a sheet-like growth of epithelium-like cells (Fig. 1). These sheets grew between the clot and the glass, and at times over the surface of the clot. Sheet-like growth of epithelium was often the first activity of fin, gill and swim bladder tissues. Cells comprising these sheets were polygonal to smoothly contoured in outline (Fig. 2). Their early appearance was hyaline, but with age and especially with media containing chicken serum they became granular and vacuolated. Sheets of older cells often tore or developed holes, which were rapidly covered by a sheet of new cells, and several such cycles were observed in a single culture. Hyaline spindle-shaped cells or cells with multiple processes were the last to appear. This cell type usually overwhelmed earlier appearing cells.

Summary. Normal tissues from trout—a coldwater teleost—have been successfully cultured for as long as 65 days at 19°C or lower in 20% serum, 30% SM 199, 45% Earle's solution and 5% embryo extract. Excellent results were obtained with human cord, human, homologous sera or bovine amniotic fluid when used at 20% level by volume. Manipulation and incubation temperatures below 22°C are thought to be essential.

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A Simple Method for Determination of Desoxypentose Nucleic Acid in Tissue Cultures. (23251)

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The numerous methods now employed for quantitative determination of desoxypentose nucleic acid (DNA) suffer from either a laborious purification procedure or the capriciousness of colorimetric assays for desoxypentose(1,2,5-8,10-14,16). The desirability of quantitation by means of ultraviolet absorption has been recognized(8,10,14), but these methods are either too laborious or their specificity has been questioned(7).

A new method is presented here which consists of a very simple, *but highly specific,* purification of DNA and quantitation by means of the ultraviolet absorption. The basis of the method is the fact that DNA, in concentrations as low as 5 μg per ml, can be quantitatively precipitated from .04 N sodium hydroxide solution by salmine. Interference by ribonucleic acid is completely eliminated by alkaline hydrolysis(12,15) before the DNA precipitation. The purines and pyrimidines are recovered from the salmine precipitate by extraction with an acid salt solution. DNA recovery is nearly 100%. The method as presented is designed for a lower limit of 2 μg of DNA. By manipulation with smaller volumes a lower limit of 0.2 to 0.5 μg should be feasible.

Experimental. Reagents are 0.4 N sodium hydroxide; 0.1 N sulfuric acid containing 10% sodium chloride (C.P. grade); 1% solution of salmine (General Biochemicals Inc.) *Procedure.* 1. Separate cells from culture medium and wash generously with physiological salt solution. For cultures firmly adhering to glass, centrifugation is not necessary and two 10 ml washings are more than adequate for cultures of 1 to 5 x 10^6 cells. 2. Take up the cells in a small volume of 0.4 N sodium hydroxide; heat in boiling water 1 hour, with a glass cap over the neck of the culture tube to minimize evaporation; cool and dilute the sodium hydroxide to 0.04 N. The volume of 0.4 N sodium hydroxide required in the cell extraction depends upon the number of cells in the culture. If too much alkali is used, dilution to 0.04 N will give a solution too dilute for quantitative precipitation of the DNA. If too little alkali is used RNA will not be completely hydrolyzed and a high value for DNA will be obtained. The best solution to this problem is to have an extra replicate culture for "range-finding." Extract the extra replicate with 5 ml of the acid salt solution in boiling water for 30 minutes, cool, centrifuge and read the absorbance at 268 and 330 mμ. Use the following guide for the alkali extraction:

Absorbance, 268 mμ–330 mμ	ml of 0.4 N NaOH	Dilution after heating
.050 to .100	.05	.5 ml
.100 .200	.1	1
.200 .400	.2	2
.400 .800	.4	4

Cultures of 2 to 3 x 10^6 strain L cells will require 0.4 ml of 0.4 N sodium hydroxide for the extraction. 3. To 0.4 ml of diluted cell extract, in a 0.4 ml graduated test tube, add 0.02 ml of salmine solution.* Mix thoroughly

* Volumes less than 1 ml were measured with micropipets made by Microchemical Specialties Co. The 0.4 ml test tubes were 8 mm O.D., 70 mm long, graduated by Paul O. Moeller, Universal Instrument Corp., Chicago.