potentials. Furthermore, no neuronal elements or end-plate organs were found in the cultured muscle. The spontaneous rhythmic discharges must arise at some point along the muscle fiber itself.

The above observations would suggest that the presence of a degenerated end-plate organ in denervated muscle is not important for the generation of fibrillation potentials. It is likely, as found by McIntyre and his associates, that a decrease in resting membrane potential occurs as a result of a change in muscle metabolism following denervation. The decrease in resting membrane potential leads to instability and oscillation of the membrane potential and, finally, to rhythmic spike discharges. It is probable that this sequence of events also occurs in the case of epileptic discharges from pathological nerve cells in the cerebral cortex (*15*).

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29 August 1960

Preparation of Monolayer Cell Cultures from Tissues of Some Lower Vertebrates

Abstract. Cold trypsin dispersion at *p*H 7.2 was used to obtain cultivable cells and cell groups from tissues of six species of fresh-water bony fishes, a frog, and a turtle. The cells readily attached to glass and were capable of at least limited, and in some cases extended, division in media consisting of commercially available components.

There are few fields of biological interest that have not found some use for the rapidly advancing techniques of cell and tissue culture. Paul's recent text on tissue culture (*1*) describes cultivation of tissues from cold-blooded animals, but the paucity of information reflects the scant attention that has been devoted to in vitro cultivation of tissues from the lower vertebrates. Madin *et al.* (*2*) reviewed the cultivation of tissues of domestic and laboratory animals but actually discussed only mammals. The recently compiled bibliography of tissue culture (*3*) contains more than 15,000 entries, but less than 0.002 percent deal with fish or fish tissue. Wolf and Dunbar (*4*) cited the principal works on fish tissue culture; all reports dealt solely with the technique of explantation.

The purpose of this report is to describe a method employed routinely in this laboratory during the past 9 months to obtain monolayer cultures of cells from several tissues of propagated trouts. Exploratory work with tissues from other easily obtained fishes, an amphibian, and an aquatic reptile has shown that the method is equally efficient for obtaining cultivable cells from these other animals.

Tissues were dispersed with a modification of the cold trypsinization described by Bodian (*5*). Since trout tissue explants prospered in vitro only when maintained at a *p*H between 7.2 and 7.4, the final *p*H of the digestion mixture was buffered at 7.2. Dulbecco and Vogt's phosphate-buffered saline (*6*) was modified to contain 1.065 g of Na_2HPO_4 and 0.249 g of KH_2PO_4 and constituted 87.5 percent of the digestion mixture. The balance consisted of a 2.5-percent trypsin solution (10 percent) (*7*) and, because it seemed to benefit the cells, human serum (2.5 percent). Potassium penicillin G and streptomycin sulfate were added at the rate of 200 unit/ml.

One to several volumes of saline-washed, scissor-minced tissue were used for each 10 volumes of the digestion mixture. All tissues were obtained as cleanly and aseptically as possible and, throughout preparation, were kept in vessels on or near ice. Most tissue was used soon after removal, but on occasion trout tissue was held at 4°C for 24 hours before digestion.

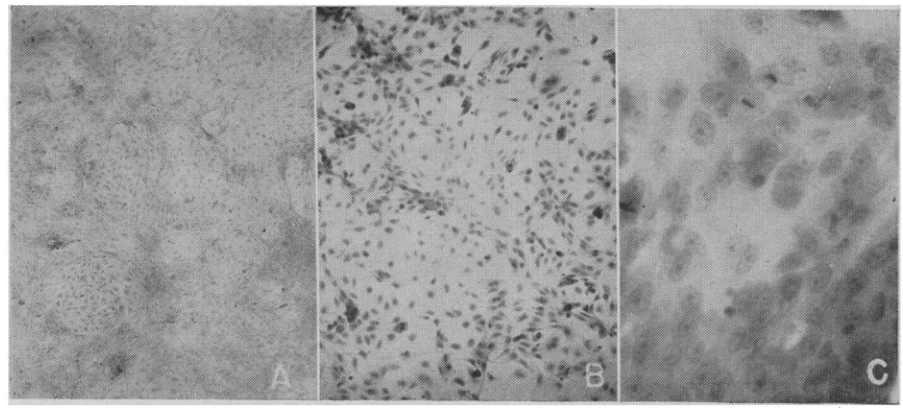

Fig. 1. *A*, Primary cultures of pooled gonad from immature rainbow trout (*Salmo gairdneri*) (× 24). *B*, Original cultivation of kidney from the bullfrog (*Rana catesbeiana*) (× 48). *C*, Original cultivation of ovary from the painted turtle (*Chrysemys picta*), showing cells in various stages of division (× 290). Hematoxylin and eosin stain.

Dispersion was effected at 4° to 6°C in fluted erlenmeyer flasks on magnetic stirrers which had separate rheostats and, therefore, did not heat the mixture. Cells obtained from the first hour's harvest generally gave poor results. In practice, the first hour's harvest was discarded and replaced with an equal volume of fresh digestion mixture. Depending upon the relative speed of dispersion, the process was continued for three or more hours. Best results were obtained when the time did not exceed 20 hours. Overnight treatment, which usually released from 10^5 to 10^6 or more cells per milliliter, became the routine procedure.

Cells were sedimented from the harvest fluid by cold centrifugation at 200*g* for 20 minutes. The resulting packs averaged 0.1 or more of the starting tissue volume. The cells were washed once in cold salt solution that contained 2 to 5 percent serum and were then suspended in the growth medium. Dilutions of 1:400 to 1:600 (1 to 3 × 10^5 cells per milliliter) were normally made, although considerably higher dilutions were possible. The initial *p*H was between 7.2 and 7.4, and static vessels were incubated routinely at either 12.5°C or, preferably, 19°C.

Mammalian-type media (*7*), used in all work, readily stimulated attachment and division of cells from fishes, a frog, and a turtle (Fig. 1). These results are in accord with the determinations of Phillips *et al.* (*8*), which first showed that, except for phosphorus, the inorganic constituents of trout blood are remarkably like those of human blood.

Our results substantiate Paul's prediction (*1*) that media intended for mammalian cells would probably suffice for in vitro cultivation of cold-blooded animal tissue.

Cells of all the animals used grew in a medium consisting of 10 percent human cord serum (2 to 5 percent serum was suboptimal; a 20-percent level was unnecessary); 5 percent whole egg ultrafiltrate (chick embryo extract was contraindicated); 30 percent medium 199, 0.5 percent lactalbumin hydrolyzate, 10 percent Hanks' balanced salt solution, 45 percent Earle's balanced salt solution (which contained one-half the prescribed $NaHCO_3$), and antibiotics (400 units each of penicillin and streptomycin and 50 units of nystatin per milliliter). Melnick's monkey-kidney medium A or B, with an additional 8 percent calf serum, was adequate for initial attachment and growth of rainbow trout cells but was not tried on cells of other animals.

Soft-organ tissue was used in all trials, and a variety of results was obtained. Attachment and division of cells from testes of mature trout was consistently poor. Except for a largemouth bass (*Micropterus salmoides*), whose ovaries were infested with cestodes, ovarian or pooled immature gonadal cells of the animals tested readily attached and divided; good results were obtained for painted turtle (*Chrysemys picta*), rainbow trout (*Salmo gairdneri*), eastern brook trout (*Salvelinus fontinalis*), brown trout (*Salmo trutta*), bluegill (*Lepomis macrochirus*), and goldfish (*Carassius auratus*). Mature reproductive tissues of the bullfrog (*Rana catesbeiana*) gave poor results, but the kidney and to a lesser degree the heart cells were readily cultivated. In work with the rainbow trout, excellent monolayers were prepared from larvae. There was fair attachment and some mitosis among cells of the swim bladder, spleen, and kidney of the adult rainbow trout, but liver, heart, intestine, and gills gave poor results.

Initial attachment of cells of all animals began almost immediately, even in the hemocytometer. Depending upon initial density, cell dispersions from reproductive tissue of fish gave uniform monolayers of epithelial-like and spindle cells in 1 to 6 days at 19°C (Fig. 1). Monolayers of frog and turtle cells formed more slowly.

Subcultures of fish cells have been prepared also by mechanical dispersion, by a 10-minute cold digestion with 0.25-percent trypsin, but preferably by 10-minute cold dispersion with disodium versenate (20 mg/100 ml) followed by immediate "neutralization" with the medium in which the cells had been grown.

Subcultures of fish cells have been carried on the media described, on the growth medium of Puck *et al.* (*9*), and on media consisting of 10 percent human cord serum and either 90 percent Eagle's basal medium, Eagle's minimal essential medium, or NCTC-109.

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18 August 1960

Hormology in Nutrition

Abstract. Discrepancies in the growth curves for the sodium requirement of the cricket indicate that hormology may play an important role in the response to essential nutrients as well as to toxic compounds. Differentiation between stimulation of growth by a nonnutritive action and support of growth by an essential nutrient is difficult.

Discrepancies in data obtained during the determination of the quantitative requirement for sodium in crickets (*1*) suggested the possibility that this and other unexplained phenomena in nutrition may be due in part to hormology. Hormology has been defined as the study of stimulation and excitation (*2*). The general term includes hormoligosis, the stimulation resulting from any harmful agent present in minute quantities. Hormones are presently being studied as typical systems of hormology. Hormetics (*3*) are toxic compounds which excite in low concentrations. Many reactions of pharmacology show typical dose-response curves predicted by hormoligosis (*4*).

Multiphasic response curves were obtained by Richet (*5*) with concentrations of heavy metals which were 10 to 1000 times less than those which exhibit the harmful oligodynamic action reported by Nägeli (*6*). Schultz (*7*) and Branham (*8*) have shown that most of the classical bactericidal compounds stimulate bacteria when present in very dilute concentrations. Antibiotics sometimes exhibit this phenomenon. The myriad reactions reported for antibiotics in nutrition (*9*) cannot all be attributed to the intestinal microorganisms—for example, the decreased mineral requirement, or the changes seen in germfree plants (*10*) and animals (*11*). Other unexplained phenomena in nutrition include (i) the observation that certain derivatives of vitamin B_{12} are more active than the vitamin itself (*12*) and (ii) the increased growth in pigs that is attributed to copper sulfate (*13*). To this list may be added the reactions obtained by adding graded levels of sodium chloride to the diet of crickets. The dose-response curves obtained are extremely atypical for an essential nutrient, but they strikingly resemble the responses obtained in hormoligosis.

Day-old crickets, *Acheta domesticus* (*L*), were placed in glass or plastic specimen jars covered with cheesecloth. Each jar contained a crumpled 7-cm filter paper on the floor for cover; a cotton-stoppered 25-ml erlenmeyer flask with distilled water for drinking; and dry, powdered diet—grass (*14*) with graded amounts of sodium chloride—placed in one corner. The crickets were the offspring of several females and were all fed from the same mix. Sodium chloride solution was mixed into the grass with washed neoprene gloves. More grass was added to give the desired concentration of Na^+ in the food. The jars were placed at random in one tray of an egg incubator at 37°C and relative humidity of 65 percent. The crickets were weighed individually at 30 days, and the average weights were either plotted in milligrams or converted to relative weights (the weights of crickets fed unsupplemented grass was taken as unity).

The response to graded low levels of sodium chloride resulted in multiple stimulation peaks—a growth pattern which resembled that of hormoligosis rather than the smooth response curve expected for an essential nutrient. These data (Fig. 1) are quite similar to those obtained by Richet with different concentrations of salts of heavy metals in lactose fermentation. The two lower curves are based on data obtained in one laboratory; the two upper curves were derived in another laboratory. As the same batch of diet, the same hatch of crickets, and the same time periods were used in the two experiments, the differences are not easily explained. The results at 60 and 100 days were similar to those presented in Fig. 1. The data indicate an instability of the effect of sodium upon the growth of grass-fed crickets.

The experiment was repeated with fresh materials. Average results for 18 crickets (three separate cages of six