16726

Technical Procedures for the Study of Organogenesis *in vitro* in Amblystoma.

CHARLES E. WILDE, JR. (Introduced by E. G. Butler.)

From the Department of Biology, Princeton University.

The purpose of this report is to present the technical procedures which have been developed in a study of the organogenesis and differentiation of small pieces of embryonic amphibian tissue. Explants from larval *Amblystoma punctatum* (*Ambystoma maculatum* (Shaw)) were used. The methods employed were modifications of existing tissue culture technics.

Among the many studies of explanted tissues *in vitro,* relatively few have dealt with potentialities and capabilities of explants to differentiate into formed structures, such as organs or organ systems. Fischer[1] first reported endodermal epithelial aggregates which differentiated into cellular structures of the alimentary tract. These structures, called *intestinal organisms,* have been reported occasionally since. Using the Ekman technic of explantation to tap water, Stöhr[2] reported several cases where heart-like structures developed from Bombinator explants. Some of these beat rhythmically within their epithelial sacs. Examination of published photographs of these experiments reveals little in the way of histological differentiation, or of the formation of anatomically proper lumina. All were very small. (Technical details are lacking in the report of Stöhr.) True morphogenesis has been demonstrated by Fell[3] in a series of remarkable papers dealing with the developmental mechanics of bones of the chick *in vitro.* Fell succeeded in effecting the development, in an essentially normal manner, of rudiments of femur, sternum and limb joints from explants of 4 to 9 day avian embryos. Holtfreter[4] has reported, in connection with his extensive studies on early amphibian morphogenesis, that certain pieces of head ectoderm and mesoderm, reared in isolation in salt solution (Holtfreter solution[5]) developed balancers, in some cases more than would normally have formed *in situ.* Discussion of the experimental results of our investigation will be reserved for future publication.

The technical details reported here readily fall into 3 topics and will be taken up in the order of their use in the experiments. The various technics to be discussed may well find application in many fields of investigation.

1. *Sterilization of tissue.* The cultivation of amphibian tissues, particularly those covered with epidermis, has always been hindered by the deleterious influence of the bacterial flora of the pond or tap water in which the animal or embryo is living. Larvae and adults are further contaminated by the passage into their immediate aqueous environment of their intestinal (bacterial) flora. In the past, this has limited the culture of amphibian surface and sub-surface tissues to short term studies or to the fortuitous occurrence of bacteria-free explants. Irradiation of amphibian tissue by ultraviolet light, with the aim of rendering tissues aseptic, always involves the danger of altering the tissue in some manner. The ideal method of rendering tissues sterile requires massive inhibition and death of contaminant bacteria, while the tissue to be studied is left in a normal or close to normal state.

The recent report of Detwiler, Copenhaver and Robinson[6] on the increased survival of

[1] Fischer, A., *J. Exp. Med.*, 1922, **36**, 393.

[2] Stöhr, P., Jr., *Arch. Entw. Mech.*, 1924, **102**, 426.

[3] Fell, H. B., *Proc. Roy. Soc. London*, Ser. B, 1934, **116**, 316; *Phil. Trans. Roy. Soc. London*, Ser. B, 1939, **229**, 407; *J. Roy. Microsc. Soc.*, 1940, **60**, 95.

[4] Holtfreter, J., *J. Exp. Zool.*, 1945, **98**, 161.

[5] Holtfreter, J., *J. Exp. Zool.*, 1943, **93**, 251.

amphibian eggs and embryos, following massive operations, when kept in a solution of 0.5 to 1.0% sodium sulfadiazine is of great value to the experimental microsurgeon. We have been able to confirm this finding. However, Detwiler and his associates were concerned with essentially whole animals, which presumably are endowed with a certain amount of tolerance or resistance to bacterial attack. Such a situation does not prevail in the case of excised pieces of organs and tissues of these animals. Not only is the orderly integrative effect of the "whole" animal lost, but also the ratio of wound surface to mass is increased. When such pieces as limb buds are transferred to nutrient medium following treatment in 0.5-1.0% sodium sulfadiazine, the onset of bacterial growth is delayed somewhat. It is, however, not completely inhibited, and at the end of 48 hours necrosis and cytolysis of the explant ensues, the result of a very visible growth of bacteria. When the contaminated culture fluid was plated out on nutrient agar, a large number of colonies appeared in 48 hours. These were found to consist of gram-negative, motile rods and, on further test (lactose fermentation, indole formation, etc.), proved to belong to the *Escherichia coli* group. Any culture of amphibian tissues contaminated by such bacteria is doomed to rapid cytolysis.

It has been found that the addition of streptomycin in a concentration of 10 - 20 γ per milliliter to the sulfadiazine bath of Detwiler *et al.* will render small amphibians and their tissues completely aseptic in 24 hours in over 95% of attempts. The technic used in this laboratory is as follows: A 0.5% (for embryos) or 1.0% (for larvae) solution of sodium sulfadiazine is made up in tap water. To this is added streptomycin in a concentration of 10-20 γ/ml. This solution is placed in sterile covered stender dishes, in each of which one or two embryos or larvae are placed. The animals are kept in this solution for 24 hours at the end of which time they are sterile and ready for operation. They are anaesthetized, if need be, in sterile MS 222.* The operative removal of the organ anlagen is carried out aseptically under a plexiglass shield mounted just below the oculars of a dissecting binocular microscope. Surgery is carried out using sterile iridectomy scissors, microscalpels, and watchmakers forceps. Each explant is removed from the operating dish with a mouth pipette, and placed in a bath of sterile Holtfreter solution. This bath is employed to promote the wound healing of the epidermis of the tissue piece, and to permit the diffusion from the tissue of the sulfadiazine and streptomycin solution. Explants are kept in these baths individually for a minimum of two hours. Following this, the tissue is removed from the bath with an absolute minimum of salt solution (again by mouth pipette) and transferred to a drop of nutrient on a sterile cover slip. The preparation is then sealed to a depression slide with a sterile paraffin-vaseline mixture. Experiments thus set up are placed in a constant temperature chamber at 20°C for further study.

The sulfadiazine-streptomycin solution here employed has exhibited no toxic effects whatever. Detwiler, Copenhaver and Robinson[6] reported a retardation of growth of their animals when kept in 0.5-1.0% sodium sulfadiazine. Such an effect has not been noticed in our experiments. Both larvae and embryos continue to grow at normal rates, to undergo morphogenesis and to differentiate in a normal manner. Larvae and embryos have been kept for as long as one week in this solution with no visible ill effects. Young embryos (neurulae) may be removed from their capsules in this solution and develop normally.

The procedure outlined above does not inhibit the growth of fungi which may contaminate amphibia. However, if precautions are taken in the maintenance of the animals in the laboratory, fungus infection can be kept at a minimum (approx. 2%).

2. *Culture medium.* The medium used in

6 Detwiler, S. R., Copenhaver, W. M., and Robinson, C. O., *J. Exp. Zool.*, 1947, **106**, 109.

* MS 222 $C_{10}H_{15}NO_5S$. A meta-amino-benzoic acid-ethylester in the form of a methan-sulfonate. (Sandoz Chemical Works, Basel and New York).

this study consisted of a mixture of two fluids: 1) Citrated rabbit plasma obtained by sterile heart puncture, and 2) a solution of half strength Holtfreter solution, containing as additions 0.25% agar, 0.045% sodium nucleate and 0.77% glucose. Equal portions of these two fluids were mixed to form the final medium. This medium has sustained the growth and differentiation of amphibian limb buds for as long as 20 days. Routinely, cultures are kept for from 4 to 12 days. For these periods it does not need to be replenished. This medium is not by any means considered to be perfect for amphibian tissue, nor is it felt that all requirements for growth and differentiation are supplied. It is hoped that further experiments will result in a medium in which all of the constituents are known chemically. The small amount of yolk included in embryonic explants undoubtedly constitutes a part of the essential nutrient. Tissues do survive, however, beyond the point where yolk is visible microscopically either in the culture or in the cells.

The above medium was developed after experiments had been made with the synthetic medium of White.[7] It was found that White's medium alone would not support the growth of embryonic or larval amphibian tissue. Also, the inorganic salt solution of White, when used in place of Holtfreter solution in the above medium, would not support growth.

3. *Epidermal closure of the wound.* It has been found that the piece of tissue in culture must be covered by the epibolically active epidermis before organogenesis will take place. Holtfreter solution, as a bath for the explant prior to placing it in the hanging drop, serves well to enhance the wound healing capacities of the epidermis. In earlier stages this process may be observed under the low power microscope. When healing of the epidermis does not take place, the explant forms a culture characterized by migrating cells and loss of structural organization. The medium as used also favors epithelial permanence, *i.e.*, if the tissue is not in contact with the glass cover slip, but rather hangs down in the center of the drop. Epiboly of the epidermis is not hindered by the contact of the explant with the medium-air interface in the well of the slide. The agar contained in the medium tends to increase its viscosity to the consistency of a soft mush (Spratt[8]). The agar, plus the lack of fibrin strands to promote the outwandering of cells, is thought to assist in the maintenance and development of morphological organization of the explant within its epithelial sac.

By the use of the technics described above, it has been possible to study the differentiation *in vitro* of such complex organs and organ systems as limbs, hearts, gills, balancers, and eyes. Differentiation has been, in all cases where it occurred, essentially normal, although somewhat slower than in the intact controls.

Summary. 1. A technic is described for rendering surface and subsurface tissues of amphibia wholly sterile for use in tissue culture. Advantage is taken of the nontoxic selective antibiotic action of small concentrations of sodium sulfadiazine and streptomycin.

2. A medium which supports organogenesis, differentiation and growth of isolated organ and organ system primordia of amphibian embryos has been reported. The principal components of this medium are citrated plasma, salts, agar, glucose and sodium nucleate.

3. Morphogenetic activity in an explanted piece of tissue occurs only when the epidermis heals so as completely to cover the explant.

[7] White, P. R., *Growth*, 1946, **10**, 231.

[8] Spratt, N. T., Jr., *J. Exp. Zool.*, 1947, **106**, 345.