Review

TISSUE CULTURE METHODS IN THE STUDY OF THE NERVOUS SYSTEM: A REVIEW

By

W. R. ASHBY, NORTHAMPTON

THIS paper is simply an account of what has been done in the field of the tissue culture of the nervous system. It is believed to be complete up to 1935. No attempt is made to deal with the whole field of tissue culture in general, but only the culture of the nervous system.

The first tissue culture ever made was by Harrison (1907). This, curiously, was a culture of nervous tissue. About 30 years ago, one of the outstanding problems in neural physiology was the question of the origin of the axiscylinder. Some held that the axon was an outgrowth of the nerve-cell, while others held that the axon was formed in situ by the surrounding sheath-cells. In attempting to answer this question, Harrison took small pieces of frog nerve and kept them aseptically in frog lymph. He observed that long filaments grew out from the nerve. These filaments were later shown to be true axiscylinders and the experiment thus settled the question of whether nerve-cells, without any other tissues, could grow axiscylinders.

Subsequent workers have since completely confirmed Harrison's observations (Harrison, 1910, a, b, 1912, 1914, 1928; Burrows, 1911 a; Speidel, 1933).

When embryo nerve-tissue is grown in clotted plasma, the tissue soon starts to throw out processes. These processes are very fine, $\frac{1}{2}$ to 1μ in diameter (Ingebrigsten, 1913, a), and they grow steadily outwards. Harrison found them to grow at the rate of $15-60\mu$ per hour (also Mossa, 1926 c, 1927). The fibre terminates in an expanded end, about $15 \times 25\mu$ in size, which is actively amoeboid. This amoeboid end continually throws out processes around it as though exploring the medium around. Along the length of the fibre are small varicosities and thickenings. Anastomoses were sometimes seen occurring between the fibres (Lewis, M. R., and Lewis, W. H., 1912; Ingebrigsten, 1916; Harrison, 1911). Sometimes the fibres branched.

At first it was assumed that these fibres were nerve-processes (axons or dendrons). Later Ingebrigsten (1916) suggested that some were from neuroglia cells. Burrows (1911, b) and Esaki (1929, a, b) stained the fibres

growing from embryonic brain and found that in all staining reactions they behaved identically with known (adult) nerve-fibres. That nerve-fibres do grow in this manner has recently been shown conclusively by Speidel (1933), who observed developing nerve-fibres in the living tadpole's tail and found them to agree in every respect with those observed in tissue culture.

It was soon found that various conditions were necessary for the successful culture of nerve-tissue. The first is rigid aseptis. The culture medium usually consists of plasma and embryo extract and is therefore an excellent medium for the growth of bacteria. Any contamination results in the decomposition of the culture in 24 hours.

Another feature which soon became apparent was the importance of the *mechanical* conditions in the culture. To allow the tissue to float free in liquid was found to lead to prompt cessation of growth and steady degeneration of the cells (Uhlenhuth, 1915 ; Loeb, 1921 ; Fischer, 1925). If cells are to grow in a fluid medium then either there must be very little fluid so that capillary attraction will hold the tissue firmly on to the coverslip, or a mass of cottonwool may be incorporated so that the tissue is held firmly in its meshes. This dependence of the cells on mechanical conditions is a factor which has not been widely appreciated, but the results of tissue culture have repeatedly shown it to be of fundamental importance. The usual method for supporting the tissues is to use plasma which is allowed to clot on the coverslip so as to hold the tissue firmly. When the cells or fibres grow they use the fine fibrin framework as a support.

The directing forces controlling the direction of growth of nerve-fibres have been the subject of much work. Here we shall refer only to work which has used the method of tissue culture. Ingvar (1920) reported that the outgrowth of fibres from nerve-tissue in culture was influenced by weak electric currents. This statement has been widely quoted and used extensively in theoretical discussions. Recently, however, Weiss (1929, 1934) has supplied much evidence that it is the orientation of the protein micelle which is the determining factor. He repeated Ingvar's work, using a variety of currents, and was entirely unable to find any specific electrical effect. He has certainly shown that mechanical conditions have a profound effect on the direction of growth of the nerve-fibre. By producing stresses in the clot in various ways he showed that these stresses have a marked effect on the direction of growth of nerve-processes. One ingenious method used by him was to make small wire frames of various shapes (triangle, rectangle, etc.). These were dipped in plasma to form a film across the frame and the nerve-tissue was placed in the centre so that when the film clotted the tissue was held in the film. Owing to the subsequent contraction of the fibrin, various stresses were set up in the film depending on the shape of the frame. In all cases the fibres developed mostly along the lines of stress. This property is not exclusive to nerve-fibres, however, since one remembers how, especially in the head of the femur, the bone spicules align themselves with the main lines of

stress. This is another example of the importance, in growth, of mechanical conditions.

The *age* of the animal providing the tissue is of some importance. With regard to the nervous system, Burrows (1911, a) found maximal activity in cultures from the neural tube when chick embryos were 48–72 hours old. Axiscylinders from brain-cells grew best when the chick embryos were 6–10 days old. Olivo (1927, b) found by culturing nerve-tissue from chick embryos of various ages that fibres grew only after several subcultures when taken from 4-day embryos and that they grew sooner as the egg was older. With 7–8-day embryos the fibres grew in the first culture. When the embryos were older than about 15–16 days no fibres would grow at all. He considered that the fibres obtained with young (4–5-day) embryos were new fibres, while those obtained from older (8–12-day) embryos were regenerated fibres growing from the ends of fibres cut while removing the piece of tissue.

With regard to the influence of *temperature*, embryo cells from the chick will generally tolerate low temperatures fairly well, living for many days at room temperature (Fischer, 1926). They may be kept for a day or two at 0° C. without permanent injury (Lambert, 1913; Drew, 1923). Adult tissues, however, seem to need more warmth. Pieces of adult rat brain when put in Locke's solution at 37° F. survive less than 24 hours (Lewis, W. H., and Lewis, M. R., 1924).

Pure cultures of nervous tissue have not yet been obtained. Several workers, especially Minea (1924), have attempted this, but without success. Over a dozen other tissues have been obtained in pure culture, and at the Rockefeller Institute there is a strain of chick fibroblasts which has been maintained in culture for over 24 years (it was started in 1912). But so far no one has obtained a pure culture of nerve-cells. This is chiefly due to the fact that it seems to be impossible to get single cells to divide or grow. In all cases it appears to be necessary that there should be a 'colony' to start with. Fischer (1925) has obtained in pure form the neuroepithelium of the chick retina, but it grew as a squamous epithelium instead of nerve-tissue. A further difficulty with nerve-tissue is, of course, that at an early age the spongioblasts differentiate from the neuroblasts, so that practically all nerve-tissue is 'contaminated' with either neuroglia or mesoblastic cells, or both.

Degeneration and regeneration of axons have been studied by tissue culture. First there was the pioneer work by Harrison mentioned above. Ingebrigsten (1913, a, b, 1916) found that when the nerve-processes were cut the central end retracted a little, but later started to grow again and sent out new processes. In the terminal, severed, part degeneration started after about 24 hours. By microdissection methods, Levi (1926) observed that if a fibre is cut the two ends may sometimes rejoin without subsequent degeneration.

It has been repeatedly found that growth in the *cellular* sense hardly

occurs at all in cultures of nerve-tissue. Levi (1926) has observed mitosis in nerve-cells in culture, but this is rare. A small amount of migration of nerve-cells occurs (Olivo, 1926, a, 1927, a) so that cultures may seem to increase slightly in size in culture, but it is the general rule that as soon as nerve-tissue is explanted, it stops growing. The morphology of the primitive cell and the question of whether differentiation occurs *in vitro* have been studied by Levi (1916, 1917, 1929, 1930, 1934), Olivo (1926 b, 1927 b), Grigorieff (1931, 1932), Lazarenko (1931), Bauer (1932), Mihalik (1932, d) and Serebriakow (1935). They observed various signs of partial differentiation but found that after repeated subculture the cells cease to throw out further processes and dedifferentiate to a circular neuroblast-like cell.

Spinal ganglia show a good growth of nerve-fibres and have been studied by Marinesco and Minea (1912, a, b, c, d, e, 1913, a, b), Minea (1930) and Mossa (1928). The results are essentially similar to those obtained with central nervous system.

Sympathetic nerve-fibres were seen to grow when embryonic liver, kidney, heart and intestine were cultured (Lewis, M. R., and Lewis, W. H., 1911, 1914; Lewis, W. H., and Lewis, M. R., 1924; Matsumoto, 1920; Campenhout, 1931; and Szantoch, 1935). Their growth was similar to that from the central nervous system.

Some work has been done from the pathological point of view by testing the reactions of cultured nerve-cells to various *noxious agents*. Mossa (1926, b) studied the effect of high temperatures; Lazarenko and Benenden (1932), the effect of ultraviolet light; Freifeld and Ginsburg (1930) and Verne and Sannié (1935), the effects of various chemicals. Martinovic (1930, 1932) studied the effect of cerebrospinal fluid on growth; he found that while the fluid appeared to be entirely harmless, yet it was not sufficient to support indefinite growth. Péterfi and Kapel (1928) studied, by microdissection, the consistencies of the ectoplasm, nuclear membrane, etc., and the effects of mechanical injury on the nerve-cell. Karssen and Sager (1934) and Péterfi and Williams (1934) studied the effects of electrical stimulation.

Some papers have appeared on purely technical questions. Thus Marinesco and Minea (1914) investigated the effect of the pH of the medium on the growth of nerve-fibres. Mossa (1926, a) studied the appearances of cultured nerve-cells under dark-ground illumination.

Questions relating to the origin of the various types of neuroglia and their morphological types have been studied by tissue culture (Verne, 1930; Marinesco and Minea, 1930; Wells and Carmichael, 1930; Costero, 1930, a, b; Mihalik, 1932, a, b, 1935).

Finally, Russell and Bland (1933) have applied the method to the study of the gliomata.

CONCLUSIONS

From this survey it will be seen that tissue culture methods are far from exhausted as a method of approach to various problems in regard to the

nervous system. It may be of interest to mention a few of the obvious further possibilities. There is, for instance, the possibility of growing neurotropic viruses in cultures of the nervous system. In this connexion it may be noted that chick embryo is an excellent culture medium for the vaccinia virus. This possibility appears to be entirely unexplored. Then there is the possibility of studying in the living nerve-cell the action of the 'classical' nerve poisons—syphilis, tetanus, diphtheria, lead, alcohol, ergot, etc. In neuroembryology, the problem of why nerve-fibres grow to the muscles is still largely unsolved: tissue culture methods, by studying the directing effects of various conditions and substances, should offer plenty of material here. Further, the tissue culture of adult cells is practically an untouched subject. To be able to study living adult nerve-cells would obviously open up tremendous fields of research. But it may be concluded generally that tissue culture methods offer many openings for research. Not the least advantage of these methods is that they study the living, and not the dead, cell.

REFERENCES

- BAUER, K., *Z. mikrosk.-anat. Forsch.*, 1932, **28**, 47.
 BURROWS, M. T., a, *J. exp. zool.*, 1911, **10**, 63.
Idem, b, *J. exp. zool.*, 1911, **9**.
 CAMPENHOUT, E. VON, *Arch. de biol.*, 1931, **42**, 480.
 COSTERO, J., a, *Arch. Staatsinst. exper. Ther. Frankf.*, 1930, **23**, 27.
Idem, b, *Mem. R. Soc. esp. Hist. nat.*, 1930, **14**, 125.
 DREW, A. H., *Brit. J. Ex. Path.*, 1923, **4**, 46.
 ESAKI, S., a, *Z. Mikrosk.*, 1929, **46**, 369.
Idem, b, *C. R. Assoc. anat.*, 1929, **24**, 223.
 FEDEREW, B. G., *Biol. Zbl.*, 1933, **53**, 41.
 FISCHER, A., *Tissue Culture*, London, 1925.
Idem, *Arch. f. exp. Zellforsch.*, 1926, **2**, 303.
 FREIFELD, H., and GINSBURG, A., *Arch. f. exp. Zellforsch.*, 1930, **10**, 128.
 GRIGORIEFF, L. M., *Arch. f. exp. Zellforsch.*, 1931, **11**, 483.
Idem, *ibid.*, 1932, **13**, 195.
 HARRISON, R. G., *Proc. Soc. exp. biol. med.*, 1907, **4**, 140.
Idem, a, *J. exp. zool.*, 1910, **9**, 799.
Idem, b, *Arch. f. Entwicklungsmech. d. Org.*, 1910, **30**, 15.
Idem, *J. exp. zool.*, 1911, **9**, 787.
Idem, *Anat. Rec.*, 1912, **6**, 181.
Idem, *J. exp. zool.*, 1914, **17**, 521.
Idem, *Arch. f. exp. Zellforsch.*, 1928, **6**, 4.
 INGEBRIGSTEN, R., a, *J. exp. med.*, 1913, **17**, 182.
Idem, b, *ibid.*, 1913, **18**, 412.
Idem, *ibid.*, 1916, **23**, 251.
 INGVAR, S., *Proc. Soc. exp. biol. med.*, 1920, **17**, 198.
 KARSSSEN, A., and SAGER, B., *Arch. f. exp. Zellforsch.*, 1934, **16**, 255.
 LAMBERT, R. A., *J. exp. med.*, 1913, **18**, 406.
 LAZARENKO, T., *Arch. f. exp. Zellforsch.*, 1931, **11**, 555.
 LAZARENKO, T., and BENENSON, M., *ibid.*, 1932, **13**, 412.
 LEVI, G., *R. Acc. Lincei*, 1916, **25**, serie 9, 1 sem.
Idem, *Mem. Acad. Lincei*, 1917, **12**, 6.

- Idem*, *Arch. f. exp. Zellforsch.*, 1926, **2**, 244.
Idem, *R. Acc. Lincei*, 1929, **9**, 942.
Idem, *Monit. zool. ital.*, 1930, **40**, 302.
Idem, *Psychiat. en neurol. bl.*, 1934, **38**, 761.
LEWIS, M. R., and LEWIS, W. H., *Anat. Record*, 1911, **5**, 277.
Idem, *ibid.*, 1912, **6**, 7.
LEWIS, W. H., and LEWIS, M. R., *General Cytology*, edited by E. V. Cowdray, Chicago, 1924.
LOEB, L., *Science*, 1921, **53**, 261.
MARINESCO, G., and MINEA, J., a, *Rev. neurol.*, 1912, **22**, 469.
Idem, b, *Bull. Acad. de méd.*, **68**, 78.
Idem, c, *C. R. Soc. de biol.*, 1912, **73**, 668.
Idem, d, *ibid.*, 1912, **73**, 346.
Idem, e, *Bull. Acad. de méd.*, 1912, **68**, 37.
Idem, a, *ibid.*, 1913, **69**, 91.