CELLS and TISSUES in CULTURE

METHODS BIOLOGY and PHYSIOLOGY

E. N. WILLMER

Cells and Tissues in Culture

METHODS, BIOLOGY AND PHYSIOLOGY

Edited by
E. N. WILLMER
Physiological Laboratory
University of Cambridge, England

VOLUME 1

1965
ACADEMIC PRESS
LONDON • NEW YORK

ACADEMIC PRESS INC. (LONDON) LTD
Berkeley Square House
Berkeley Square
London, W.1.

U.S. Edition published by
ACADEMIC PRESS INC.
111 Fifth Avenue
New York, New York 10003

Library of Congress Catalog Card Number: 64–14227

PRINTED IN GREAT BRITAIN BY
WILLMER BROTHERS AND HARAM LTD., BIRKENHEAD

Contributors

M. ABERCROMBIE, *Department of Zoology, University College London, England* (p. 177)

J. T. DINGLE, *Strangeways Research Laboratory, Cambridge, England* (p. 353)

HARRY EAGLE, *Department of Cell Biology, Albert Einstein College of Medicine, New York, U.S.A.* (p. 277)

HONOR B. FELL, *Strangeways Research Laboratory, Cambridge, England* (p. 659)

H. FIRKET, *Laboratoire d'Anatomie pathologique, Université de Liège, Belgium* (p. 203)

CLIFFORD GROBSTEIN, *Department of Biological Sciences, Stanford University, Stanford, California, U.S.A.* (p. 463)

T. C. HSU, *The University of Texas M.D. Anderson Hospital and Tumor Institute, Houston, Texas, U.S.A.* (p. 397)

ILSE LASNITZKI, *Strangeways Research Laboratory, Cambridge, England* (p. 591)

LEON LEVINTOW, *National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland, U.S.A.* (p. 277)

J. A. LUCY, *Strangeways Research Laboratory, Cambridge, England* (p. 297)

A. A. MOSCONA, *Department of Zoology, University of Chicago, Chicago, U.S.A.* (pp. 19 and 489)

JOHN PAUL, *Department of Biochemistry, The University, Glasgow, Scotland* (p. 239)

L. M. RINALDINI, *Instituto de Biologia Celular, Cordoba, Argentina* (p. 659)

J. SEED, *Department of Radiotherapeutics, University of Cambridge, England* (p. 317)

O. A. TROWELL, *M.R.C. Radiobiological Unit, Harwell, England* (p. 19)

CHARITY WAYMOUTH, *The Jackson Laboratory, Bar Harbor, Maine, U.S.A.* (p. 99)

M. WEBB, *Strangeways Research Laboratory, Cambridge, England* (p. 353)

E. N. WILLMER, *Physiological Laboratory, University of Cambridge, England* (pp. 1, 19 and 143)

ETIENNE WOLFF, *Laboratoire d'Embryologie Expèrimentale, Collège de France, Nogent-sur-Marne, France* (p. 532)

Foreword to Volume 1

Since 1907, Tissue Culture has been used and misused in a wide variety of ways and in a number of branches of biology. A few years ago, the idea was mooted that it was time to make an assessment of the achievements of Tissue Culture. This assessment should not only consider the technique in itself, but more particularly should evaluate the effects that Tissue Culture has had in the solution of problems in cellular and histological biology. What, indeed, are the contributions which Tissue Culture has made to biology, and what are they likely to be in the future? Has Tissue Culture altered our approach to cellular behaviour or opened up any new fields?

This book is an attempt to show what has been achieved so far and to discuss where, when and how the technique may be most profitably employed.

In 1960, a number of investigators in the various fields of Tissue Culture were approached and asked if they would evaluate the uses and applications of the technique in their particular areas of study. They were asked to assess what contributions the method had made, was making, or was likely to make to our understanding of normal or pathological cells and tissues, and to point out those features which, though they may be peculiar to life *in vitro*, are nevertheless of value to our understanding of cells and tissues in general.

The problem of selecting suitable authors for the task was, of course, both difficult and invidious. For the final choice, the editor accepts full responsibility, well-knowing that, despite his efforts, there will be many who would have chosen quite differently. Some investigators, whose contributions would have been most valuable, were unfortunately, for one reason or another, unable to accept the challenge; a few accepted the challenge and then found it impossible to carry on with the task; finally, in two cases, alas, illness and untimely death intervened.

The first volume covers many of the more general fields of Tissue Culture, including such topics as the evaluation of the methods as such, the study of metabolic processes and growth, the action of hormones and vitamins and the use of the method in genetics. The second volume is devoted to studies of certain particular tissues or systems which have either been extensively investigated in themselves or which, for one reason or another, accentuate some achievement or contribution which

the method has made to biology. In the third volume, some of the uses of Tissue Culture in the study of invertebrate and plant tissues will be reviewed, together with some of the applications to pathology and virus research.

The editor wishes to express his most sincere thanks to the contributors, not only for their contributions but also for their forbearance and patience during the long period of gestation. He also wishes to thank all those authors and proprietors of journals who have kindly allowed their illustrations to be used to enrich the text. Dr. R. L. Tapp has provided invaluable assistance in the compilation of the index and his efforts will greatly increase the value of these volumes as a work of reference for years to come. Finally, the Academic Press and their printers are deserving of high praise for their splendid efficiency and patience.

PHYSIOLOGICAL LABORATORY
CAMBRIDGE

E. N. WILLMER

October 1964

Contents

CHAPTER 1

Introduction

E. N. WILLMER

Physiological Laboratory, University of Cambridge, England

The advantages, from an experimental point of view, of being able to observe cells and tissues in isolation and away from the controlling and modifying influences of other tissues in the body are clearly so great that it is at first sight surprising to find that Tissue Culture of animal cells and tissues was not attempted seriously until the very end of the nineteenth century, and really only launched successfully in 1907. In this respect there is a wide difference between animal tissues and the tissues of higher plants, for the latter are, on the whole, much more viable in isolation and the propagation of whole plants from isolated fragments, has of course, been carried out for centuries.

Tissue Culture, as it is practised today, can be profitably considered under three headings. There is first of all "tissue culture" proper, in which small fragments of tissue are explanted into a suitable medium and encouraged to grow in isolation, to form colonies, and perhaps to continue some of their normal functions. In such cultures, the original organization of the tissues may be lost, but the constituent cells emerge into the zone of outgrowth where their activities may be directly observed. Secondly there is "cell culture" in which the cells of a tissue, or even individual cells, are made to grow in much the same way as bacteria are grown: all the organization of the original tissues in such cultures is discarded as irrelevant and cell multiplication and growth in uniform populations are the dominant interests. The cells of different tissues tend to become "dedifferentiated" and relatively alike in appearance. Thirdly, in "organ culture", growth is only of minor interest, but embryological development and the maintenance of normal physiological functions are the chief aim and object. The outward growth and migration of "dedifferentiated" cells is positively suppressed and the maintenance of the normal organization of the tissue is of first importance.

The problems which arise in Tissue Culture* of animal material are in many ways different from those which arise when tissues from the higher plants are cultured. In general, they are more complex. Each cell from a higher animal is probably rather more dependent upon the presence of the whole of the rest of the animal than the individual cell of a higher plant normally is on the rest of the plant. As a rule, the whole animal must necessarily be present in order that the local environment of each of its constituent cells shall be properly stabilized. If animal cells or tissues are isolated, they have to be provided with a system by which their normal surroundings are very closely imitated. Thus the provision of these local environments of tissue cells in animals is a *sine qua non* for successful cultures. The viability of the cells of higher plants is probably much less dependent on an elaborate system controlling their environments, and the cells and tissues of higher plants survive in media that are relatively much more simple than those required by animal cells.

Animal cells and tissues also rely on defence mechanisms against infection by bacterial and other invading organisms, which are themselves dependent on the whole body remaining more or less intact, so that leucocytes, macrophages and antibodies can circulate freely from their places of origin to the sites of invasion. Plant cells and tissues are much more resistant in themselves and less dependent on a circulatory system for their defence.

The tissues from higher plants grow reasonably well in an organized way (cf. organ culture) so long as they are given the necessary salts, water and light. On the other hand, extensive outgrowth of normal plant cells in an unorganized way, like that which can now be fairly readily obtained in animal cell and tissue cultures, is, rather surprisingly, much more difficult to achieve, and has only been accomplished comparatively recently, and its applications are, at present, still somewhat limited.

In the light of these observations and in view of the applications of Tissue Culture to problems of human physiology and medicine it will be readily appreciated that most of this book must inevitably be devoted to Tissue Culture of animal material and the special problems of Plant Tissue Culture are confined to a relatively small section in Vol. 3.

L. Loeb, in 1897, was the first to maintain the cells of blood, connective tissues and some other tissues outside the body in a viable condition for any length of time and can thus be said to have taken the first steps in Tissue Culture. He used small tubes of serum or plasma. It was, however, Ross G. Harrison, in 1907, who, when working at

*Tissue Culture (with capital letters) will be used generally to include cell culture, tissue culture and organ culture.

Johns Hopkins, placed on a coverslip nerve tissue from the spinal cord of a tadpole in a medium of clotted lymph from the frog and inverted it over a hollow-ground microscope slide, sealed it with paraffin wax and demonstrated for the first time that nerve fibres grow out from nerve cells by a process not unlike the formation of pseudopodia by an amoeba. This was not just a random observation, but an experiment designed to provide data in the controversy which was raging at that time concerning the nature of nerve fibres, the relationship between nerve fibres and nerve cells and the problem of continuity or contiguity between nerve cell and nerve cell. The results of Harrison's experiments so dramatically answered some of these burning questions of the day that his method, the hanging-drop method, was soon followed up and applied to the solution of other problems. That was how Tissue Culture began.

Burrows (1910), Carrel (Carrel and Burrows, 1910) and Ebeling (1913), at the Rockefeller Institute, New York, were among the first to apply the method successfully to the tissues of warm-blooded animals. They used the hanging-drop method with fowl plasma or serum as a medium for the growth of tissues from the embryo chick. Fowl plasma was found to be specially suitable since it could be kept on ice without clotting until required for use, when it could be made to coagulate and thus enclose the tissue in a nutrient and protective medium. The embryo chick was ideal both because its tissues could be obtained free from bacteria and other infecting organisms and also because embryonic tissues grow more readily and actively than those of the adult organism, as these early experiments quickly showed.

Meanwhile W. H. and M. R. Lewis (1911) in Baltimore were also quick off the mark to make hanging-drop cultures of chick tissues in simple salt solutions (e.g. Ringer-Locke's solution) to which they added chick-bouillon. These were early pioneering days, and much had to be learned about the effects of various salts, pH, osmotic pressure, temperature and the utilization of glucose and other food substances. An account of much of this excellent early work, largely devoted to the study of the detailed cytology of the outgrowing cells and of how cells behave in culture, was published by the Lewises in "General Cytology", edited by Cowdry in 1924 and is still a scientific classic.

Tissue Culture became "headline news" just before the 1914–18 war when Carrel (1913) published his account of the artificial activation of growth and cell division by means of saline extracts from embryo tissues. In retrospect, it is interesting to speculate on the effects which this work had in orientating the history of cell biology. It immediately focused attention on the method of tissue culture as one which could be extremely suitable for the study of growth and, of course, this meant

for the study of cancer. Thus, by 1914, Tissue Culture was seen as a method which in the space of seven or eight years had not only solved many of the problems connected with the neurone theory of the nervous system, with the myogenic theory of the heart-beat (Burrows having seen the contraction of isolated cardiac muscle cells in 1912) but also answered many questions and raised others on cytological structure, cell movement, cell shape and cell division. Furthermore, Thomson (1914 a, b) had shown that embryonic organ rudiments could continue to enlarge and differentiate more or less normally *in vitro*, and it seemed likely that Tissue Culture would throw considerable light on the major human problem of cancer. The future was indeed rosy.

It was probably not by chance that Carrel, a highly skilled surgeon, well versed in the strict regimen of aseptic surgical technique, should be the one to initiate many of the basic methods of tissue culture. His training in surgery meant that he had the necessary experience and knowledge to overcome the technical problems of avoiding infection and of handling living tissues, but it also caused the method to be wrapped up from the beginning in a considerable cocoon of mumbo-jumbo, derived from the practices that were prevalent at that time in the operating theatres of the world. Thus Tissue Culture, though a delicate and exacting technique and one in which rigorous asepsis is absolutely essential, gained a spurious and unfortunate reputation for difficulty and almost for mysticism, while at the same time it was clearly of tremendous importance as a means of investigating, and perhaps eventually combating, the great scourge of cancer. Tissue Culture and Cancer Research were thus early linked together, both practically by the research workers themselves and also in the public mind. In consequence, Tissue-Culture laboratories were set up here, there, and everywhere throughout the world and an immense quantity of time and money was squandered on ill-directed research by adventurers who had climbed upon the band-waggon. It also meant that much of the effort in tissue culture was expended in trying to make cells grow fast and in the unorganized and rather abnormal way that they do in some malignant growths and in which they were found to do when suitably stimulated in tissue cultures. What was happening to the cells in the original tissue of the explant was at that time of minor interest as compared with the visible growth and division of the cells that moved out into the medium or onto the supporting surfaces provided. Though much of importance in our concepts of cellular behaviour has emerged from such studies of unorganized growth, the problem of malignancy is still with us and so also are many problems of cell differentiation, organization and function, for the solution of which Tissue Culture could have been used at a much earlier time and

which by now might have been solved had not the popular appeal directed research in the way that it did. More recent work has, of course, been along other lines, again partly determined by fear of a disease, this time by the fear of poliomyelitis, and it has amply shown that Tissue Culture had even greater possibilities in several other directions than that of cancer research.

Soon after the 1914–18 war, Tissue Culture was taken up in earnest in many countries. Carrel, Ebeling and the Lewises continued actively in America, Strangeways and his co-workers in England, Fischer in Denmark, von Möllendorff in Germany, Champy and Ephrussi in France, Chlopin in Russia and Levi in Italy were all early in the field, and provided many significant contributions to the subject.

The limitations of the original hanging-drop method, from the biochemical point of view, led to the development in 1923 of the Carrel flask (see p. 41), in which more tissue and more medium could be used and so facilitate chemical analyses. Growth stimulation and the problems connected with the provision of an adequate diet for pure strains of growing cells were, as already indicated, the main interests of Carrel, Ebeling, Fischer and subsequently of Parker. Others, like Strangeways, Chlopin, Levi and Champy were on the whole more interested in the then less popular problems of differentiation and cell behaviour although it should be noted that one of the earliest and most complete descriptions of the process of cell division itself was provided by Strangeways (1922), and it was he who probably inspired that versatile pathologist from St. Bartholomew's Hospital, R. G. Canti, to produce some of the most exciting and informative cinematographic films that have ever been taken of cells migrating and dividing in cultures. These were not the first films to be taken of cells in culture for Comandon, Levaditi and Mutermilch took some in France in 1913, but Canti's films (1928) were certainly a landmark in cell biology. The adaptation of the cine-camera for use at varying speeds with the microscope is an interesting example of how advances in one field of learning can quickly lead to advances in an entirely different field. The time-lapse camera of 1926 was, by modern standards, a primitive and cumbersome contraption, but it was the forerunner of one of the most powerful tools now in the hands of the cell biologist.

The Lewises (1914), in their elegant analyses of cell structure, cell behaviour and differentiation in cultures demonstrated, among other things, the presence of mitochondria in living cells in tissue culture by the process of vital-staining with Janus-green B, and they saw these by dark-field illumination in 1923. But it was the great technical advances in high-power dark-field microscopy which were made in the early twenties that allowed Canti (Strangeways and Canti, 1927) to show by

cinematography that mitochondria move about, change shape, and divide within the living, untreated and undamaged cell.

During the 1920's there were thus two main streams of research being pursued by means of Tissue Culture methods, one concerned primarily with problems of growth, cell nutrition and cell multiplication, the other more quietly investigating the differentiation of cells and the organized development of embryonic tissue.

While Rienhoff in 1922, and Drew, in 1923, showed that embryonic kidney could differentiate *in vitro* and that the epithelium grown *in vitro* could develop tubular structures more easily in the presence of connective tissue elements, thus confirming earlier observations by Champy (1914), it was Chlopin (1922), Ebeling and Fischer (1922) and Fischer (1922) who more or less simultaneously called attention to the differentiation of cells which went on within the central masses of mixed colonies of fibroblasts and epithelial cells. It may have been these latter experiments which caught the imagination of T. S. P. Strangeways whose extraordinary zeal and devotion to fundamental medical research, particularly at the cellular level, led to his initiating and founding a research hospital upon what nowadays would seem to have been a most inadequate shoe-string. Nevertheless, his efforts were rewarded, for this—at one time private—research hospital now enjoys a world-wide reputation as the Strangeways Research Laboratory and is a centre of cell biology for investigators from all over the world. Be that as it may, the self-differentiation of embryonic limbs, eye and ear rudiments was early followed by Strangeways and his team (1926), which included Honor B. Fell, and a major advance was made by the use of the so-called watch-glass technique for "organ culture" (Fell and Robison, 1929). Indeed this technique, and a similar one developed by Maximow (1925), were really the beginnings of "organ culture", as opposed to "tissue culture" and "cell culture" though, as mentioned above, Thomson had pioneered in this direction some ten years earlier. The watch-glass technique was destined to develop as the dominant method for investigating the problems of embryogenesis and organogenesis. It has been extensively used by such investigators as Waddington (1932), Spratt (1947), Wolff (1952) and many others, and is now the basis for one of the major fields of study (Chapter 15). It, or some modification of it, is also the method of choice for numerous physiological problems, such as the actions of vitamins and hormones on cells and tissues, and many related problems in endocrinology (Chapters 16, 17).

Meanwhile, in the 1920's, the facility with which cells in tissue culture could be directly observed in the living state invited the application of the microdissection apparatus, then recently developed to assist the already capable hands of Robert Chambers (1921, 1924, 1931), and