TISSUE CULTURE

E. N. Willmer

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THE GROWTH AND DIFFERENTIATION
OF NORMAL TISSUES IN
ARTIFICIAL MEDIA

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LONDON: METHUEN & CO. LTD.
NEW YORK: JOHN WILEY & SONS, INC.

First Published October 3rd, 1935
Second Edition, Revised and Reset, 1954
Third Edition, Revised, 1958

3.1
CATALOGUE NO. 4117/U (METHUEN)

PRINTED IN GREAT BRITAIN BY BUTLER & TANNER LTD
FROME & LONDON

PREFACE

TISSUE culture has in the past been the object of much misrepresentation and of many false conceptions. By some, optimists, it has been regarded as a magic key to the understanding of the life process; by others, pessimists, it has been slighted as an almost valueless craft which has contributed nothing to science. Needless to say, neither of these points of view is in the least justified. In this book an attempt is made to give as fair an account as possible of the part played by the method of tissue culture, for it must not be regarded as anything more than a method, in helping to elucidate some of the problems of normal growth and differentiation, and in furthering the knowledge of the processes involved in the normal development of the animal organism. No attempt has been made to give a full account of all the ways in which the method of tissue culture has been applied, which would indeed be a task far beyond the compass of this book, and no treatment will therefore be found of such subjects as the growth of malignant cells, of the relative sensitivity of these and normal cells to such external influences as radium and X-rays, nor of any of the applications of the method to problems of pathology.

Those who are engaged in researches in which the cultivation of tissues in artificial media outside the body plays a prominent part are conscious of a great change in outlook with regard to the conceptions of cellular structure and function which has been brought about by such a close study of the living cells as is possible by tissue-culture methods. The author has tried in this book to convey something of this more vital outlook to those interested in general problems of biological science, as well as to give to those working in the more restricted fields of tissue culture a summary account of the main advances which have been made in the knowledge of growth and differentiation processes, and for which the method has been responsible.

The author wishes to express his thanks to Dr. F. Jacoby, Dr. H. B. Fell and Dr. F. G. Spear for helpful criticism, and to Mr. and Mrs. H. G. Willmer for their assistance in the preparation of the manuscript.

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PHYSIOLOGICAL LABORATORY
CAMBRIDGE

April, 1935

PREFACE TO THE SECOND EDITION

DURING the last fifteen years the study of cellular behaviour has become a subject of the greatest importance and long strides have been made both in the techniques employed and in the results achieved. In physiology, the point of interest is shifting from the whole organ to the constituent cells, and the questions to be asked are not so much 'what does this organ do?' as 'how does it do it?' This necessarily involves a thorough knowledge and understanding of the properties of the component cells. The ultimate physicochemical organization of the cells in each group becomes the object of investigation, and in the solution of such problems the method of tissue culture which allows the study of isolated cells and groups of cells can naturally be expected to make significant contributions. Moreover a study of the behaviour of cells, as distinct from their form and arrangement, is possible by tissue-culture methods and this, combined with a study of the evolutionary development of the tissues concerned, gives a picture of cell physiology which is much more in perspective than those which could ever be obtained by the older methods.

The last two decades have seen the development of the phase-contrast microscope, the electron microscope and the reflecting microscope. They have witnessed the use of tracer elements in studying biological problems and also many other new methods of microanalysis and histo-chemistry. The time is therefore opportune to review once again the position of tissue culture in the study of growth and differentiation, and to consider how the subject has been affected by the lapse of time.

In the present edition, an attempt has been made to bring the earlier edition up to date without seriously altering the general approach, which is still essentially from the angle of general physiology, and to show where new methods have produced or are likely to produce significant advances.

Once again, I am greatly indebted to Dr. F. Jacoby for his ready help in reading the amended text and offering numerous suggestions. Dr. L. M. Rinaldini has also assisted in the same way and my thanks are equally extended to him.

E. N. WILLMER

CAMBRIDGE, 1953

PREFACE TO THE THIRD EDITION

THE necessity to grow viruses in living cells, made urgent as a consequence of the increase in the incidence of poliomyelitis, has acted as a powerful stimulus to the development of new methods of tissue culture, and with these new methods the applications of tissue culture to physiological problems have been greatly widened. The present edition attempts to assess the position of the subject as it stands to-day.

The author is indebted to Miss M. E. Walker for her comments and suggestions for the revision of the text.

PHYSIOLOGICAL LABORATORY
CAMBRIDGE
1958

INTRODUCTION

HORTICULTURE dates back to the earliest days of human achievement, and two of its essential practices must be almost equally ancient, namely the striking of cuttings and the making of successful grafts. In the light of this it is perhaps a little surprising that the successful application of such methods to animals should have been so long delayed, although naturally the results might have been expected to be different. But the reasons for this delay are not far to seek. For the success of the cutting or graft certain requirements are made both as to the character of the scion or stock and as to the nature of the soil in which it is to be placed, and for experiments of the same kind on animals a far more rigorous set of conditions is necessary.

The evolution of the higher animals has largely been the evolution of a constant internal environment, so that although the animal as a whole may be subjected to a great variety of external conditions, yet its individual cells remain in much more constant surroundings. The cells so sheltered from the chances and changes of the outside world can then specialize in the performance of their functions, and develop physico-chemical processes and reactions which depend on a finely balanced set of conditions, and which would be completely impossible in cells not so carefully protected. One result of this specialization is that the cell groups tend to lose some of their independence for the good of the whole. A parallel may be drawn from human experience. In the small hamlet the inhabitants can mostly satisfy their needs by their own efforts and from the village shop, but in the more highly organized society the draper learns to rely on the milkman, the grocer on the butcher, and each upon the other, so that the community is dependent on the behaviour of all its members, on the railway, on the district council, and on the government. So in the body, the skin, the stomach, the liver, and the intestine could none of them function without the other, without the blood-stream, the peripheral nervous system or the brain. In recent years the problem of unemployment has made it only too obvious that when any given member of a highly organized human community is rooted up and transferred to an environment in which he has to fend for himself, he is seldom fitted for the conditions, and in the absence of help from others must needs perish. So it is that isolated groups of cells from the animal body, unless transferred to conditions extremely similar to those to which they are accustomed, very soon languish and die. The cells of plants are far less irreversibly specialized, and so are capable of life under much simpler conditions; but with animals it is different; and, as knowledge of their physiology advances, it becomes more and more evident that the success of the animal body depends on this proper balance of activity among its various parts, and the functioning of one organ becomes controlled by the conditions set up by another. The glycogen content of the liver, for example, is subject to the behaviour of the adrenal glands, the pituitary, and the pancreas; the respiratory movements are governed by the nature and amount of blood flowing in the carotid artery. It is not, however, necessary to enumerate further such examples of the balance of one system against another in the make-up of the higher animals. Among lower organisms the process has not proceeded so far. The internal environment cannot be maintained so constant, and therefore such delicate interrelationships are not possible. The cells cannot as a general rule become so specialized, and they must be able to carry out their functions in an environment in which greater or smaller changes are of constant occurrence. In short, the cells of lower organisms are less dependent on the constancy of an internal environment than are those of higher animals.

This relative independence renders it a comparatively easy matter to make grafts and to take cuttings of such lowly organisms as the flat-worms or planarians, in much the same way as is possible with plants. Their cells are as yet for the most part not so reliant on the other cells of the body, and are capable of much greater variation and adaptability in their behaviour. It is the same story as is told again and again in the history of various forms of parasitism. In becoming a parasite the free-living independent animal sacrifices more or less of its independence to gain a relatively constant set of conditions, under which it can devote itself more thoroughly to the reproduction of its species. In higher animals the individual cells are as it were parasitic on all the other cells of the body, and, by becoming thus subservient, they can function more efficiently in one particular direction. Just as a parasite becomes helpless when removed from its position on the host, so the isolated cells of higher animals are relatively incapable of independent existence when removed from their original situations. This 'parasitism' of the cells of the higher animals on one another, perhaps better described as 'symbiosis', is clearly responsible for the chief difficulty in making successful cultures of animal tissues away from their normal position. Lower animals and their cells avoid harmful environments by the method of escape, by surrounding themselves with impermeable membranes, &c. In higher animals the cells are by no means independent of their immediate environment but nevertheless can function properly because that environment has been specialized and made constant for them. To make cuttings of plants, all that is necessary is to remove part of the plant and place it in a fresh environment, and, given the necessary salts, water and light, certain of its cells will form new roots, new shoots or what-not, so that in time a whole new plant will be formed. The same operation may be performed with certain lower animals, but the more highly organized the animal the more difficult does the operation become; the cells have lost their independence, and in experiments on higher animals success is only possible when the cells are removed to an environment very closely similar to their normal one and with which they can quickly establish equilibrium. Moreover, except in animals very low in the evolutionary scale, a whole animal can never be regenerated from a part.

An intimate knowledge then of the make-up of the normal environment of the cells of an organism was a prerequisite for successful tissue culture. With the advance of physiological knowledge, enough is now known of the immediate surroundings of tissue cells within the body to allow such cells to be cultured and to grow for long periods of time in artificial surroundings outside the parent organism. There is still, however, a long road to be travelled before it will be possible to produce a completely synthetic environment containing only the necessary and sufficient ingredients for any given cell to function in tissue culture. In other words although it is possible to maintain cells alive, actively functioning and growing, outside the organism, it is now only beginning to be possible to decide exactly what are the particular necessities in the environment for each type of cell; tissue culture itself, however, must help to thread the labyrinth of such a problem.

Besides the difficulty of finding a suitable environment for the cells, a factor which delayed the beginnings of tissue culture was a lack of understanding of bacterial organisms and the consequent difficulty of establishing a suitable aseptic technique. The animal body as a whole has a variety of protective mechanisms against bacteria, &c., among which may be mentioned as an example the whole leucocyte system, by which the colourless and actively mobile cells of the blood and tissue fluids ingest and remove any foreign particles and bacteria, which may have penetrated below the skin or through the mucous membrane of the alimentary canal. These cells, although they may be found nearly all over the body, are essentially connected with the blood-stream, and are largely manufactured in certain definite regions such as the lymph nodes and the bone marrow. Tissue deprived of its blood-stream is, among other things, cut off from its main supply of leucocytes, and hence, in this direction at least, is relatively defenceless against bacteria. Media in which tissue cultures will grow or survive are media *par excellence* for the development of micro-organisms of various kinds, pathogenic and non-pathogenic, so that any slight infection of the medium of a tissue culture is almost certain, sooner or later, to prove fatal to the cells of the culture. Sometimes death may be due to the development of harmful metabolites and toxins, at other times it results from the exhaustion of the nutritional elements of the medium by the extremely rapid multiplication of the micro-organisms. It is thus obvious that a very rigid aseptic technique is a *sine qua non* for successful explantation experiments, and until the underlying principles of such a technique were firmly established, tissue culture was unlikely to be successful. The use of penicillin, the sulphonamide drugs and other antibiotics has been turned to advantage in recent years to combat infection in certain culture methods. It must, however, always be borne in mind in any physiological studies that these substances are not entirely without action on the cells of higher animals.

The first definite advances were made during the last century when it was established that balanced salt solutions, containing sodium, potassium and calcium ions in certain specified proportions were necessary for the regular pulsation of the isolated frog's heart; but it was then a long time before Harrison in 1907 [190] first successfully maintained frog's nerve tissue alive and active in a hanging drop of frog's lymph. This established the fact that, given suitable conditions, small groups of animal cells could function away from their position in the living animal, and it was with these experiments that tissue culture began.

It is now possible to maintain cells alive outside the organism for an apparently indefinite time, to examine them in the living condition under the highest powers of the microscope, to perform experiments upon them and, in general, to study their behaviour under a variety of conditions. This range of conditions is not, however, as great as might be desired owing to the very definite limitations which are imposed by the techniques so far developed. It is not surprising, however, now that the fundamental requirements for tissue culture have been established, that the method should find applications in several biological fields, and indeed there is hardly a branch of biological science which has not taken advantage of the possibilities afforded by the technique. Physiology and anatomy have perhaps reaped the greatest harvest, and particularly where they meet on the common ground of embryology; but, in more recent years, the method has become of inestimable value in the study of the growth and behaviour of viruses. In the ensuing pages the plough of tissue culture is followed in its rather uncertain and erratic course across the field of physiology. Two particular aspects will be reviewed: one, unorganized growth, concerned with the survival and proliferation of cells, and the other, organized growth, dealing with the differentiation of growing tissues and the development of their functional capabilities.

It is both fortunate and unfortunate that the domestic fowl is a prolific bird, and that its embryo develops in a shell, isolated from the contaminations of the outside world. It is fortunate because in the chick embryo are present active living cells in various stages of development and practically free from possible infections, cells therefore which are in a state highly suitable for many kinds of tissue culture experiment. It is unfortunate in that, as a direct consequence of this, by far the greatest amount of work has been done on avian cells in an embryonic condition and there is a tendency, sometimes justifiable sometimes not, to generalize from results obtained on such tissue and to infer similar behaviour for adult or even mammalian tissue.

In the development of the chick, as indeed of all other animals, there are three main stages. Its cells first increase in number, then they become specialized for the performance of their particular functions, in other words they differentiate, and finally they function. What therefore may be expected of embryonic cells and tissues when removed from their natural habitat to cultural conditions outside the body? At first the main interest of tissue culture centred round the fact that such cells from chicks and embryonic animals in general would remain alive and multiply in artificial conditions; then the centre of attraction in the picture shifted to the problems of differentiation and function, and now once again it is essentially problems of cell metabolism and growth which are receiving attention. With improved technique and greater understanding of cellular behaviour it is possible to some extent to control the activities of cells and tissues when removed into artificial conditions, and to determine whether on the one hand they shall grow and divide, or whether on the other they shall remain relatively quiescent, differentiate and even in some cases start to function. These two main types of behaviour have been classified as unorganized and organized growth, and it is on those lines that tissue culture methods have had most to contribute to physiological knowledge; but much has also been learned from direct observation and experiment on tissues which are simply in a state of survival in artificial media, and in this connexion it is perhaps unfortunate that embryonic cells have played such a prominent part.

Adult tissues can be cultured, but the results are not so spectacular; the cells do not grow as readily and the tissue tends to remain relatively quiescent. There is, however, undoubtedly much to be gained by the study of the behaviour of isolated adult tissues, though up to the present time comparatively little has been achieved in this direction.[104]

There is also another field to which tissue culture has much of importance to contribute, namely the study of malignant cells, and both carcinoma and sarcoma tissues of various types have been grown in vitro. The requirements of such tissues are rather special, being often quite different from those of normal cells, and indeed the presence of the latter either dead or alive is sometimes necessary for the successful culture of malignant cells. The growth rates are quite different; the tissues show many peculiarities of metabolism, and though it is probably impossible to point to any one particular histological distinction between normal and malignant cells which is always valid, yet they form a class of tissues by themselves, and offer their own peculiar problems. It is important, however, to remember that when the metabolism of malignant cells is under investigation, it should always be compared with that of normal cells belonging to the same tissue, because recent work has tended to emphasize the metabolic differences between the cells of different normal tissues. For these reasons it is impossible to discuss the behaviour of malignant cells in the pages that follow, and attention is therefore restricted simply to problems set by normal tissues, first in connexion with their growth, and secondly in connexion with their differentiation, organization and acquisition of their respective functions.

CONTENTS