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TISSUE CULTURE IN INVITATION TO GROWTH AND DIFFERENTIATION

J. S. P. STRANGEWAYS

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BY THE SAME AUTHOR

THE TECHNIQUE OF TISSUE CULTURE "IN VITRO."

Tissue Culture in Relation to Growth and Differentiation

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Preface

THIS little work is based on the notes for a course of lectures delivered by the author to members of the University during the Michaelmas Term, 1923. It comprises a brief account of an extensive series of observations and experiments made by the writer upon animal tissues cultivated in vitro and in vivo, and also includes some suggestions as to the possible significance of the results described in relation to the general problems of growth and differentiation. The data and conclusions here recorded are published in the hope that they will be of interest to other investigators who perhaps may be stimulated to further labours in what has proved a most fruitful field of research.

References to the extensive literature on cell growth and differentiation have been intentionally omitted.

The author is much indebted to Miss H. B. Fell for her great assistance in the selection and writing up of the material for publication, and to the Medical Research Council for a grant towards the expenses in connection with the work described.

T. S. P. S.

CAMBRIDGE,
January, 1924.

Contents

Introduction

Until recent years cytology and histology were almost wholly confined to the study of dead material, and many of the results obtained were necessarily inconclusive. The evolution of a method whereby tissues could be grown and observed in vitro opened up a new field of investigation, and has enabled the cytologist to apply experimental methods to many of his most fundamental problems.

The earliest attempt to grow animal tissue in vitro appears to have been made by Leo Loeb. This worker noticed that epithelium would grow upon the clotted blood or lymph which exuded between the lips of an incision, and in 1902 he succeeded in growing epithelium on the surface of a blood clot contained in a glass vessel at the bottom of which had been placed a fragment of kidney. In 1906–07, Harrison obtained growth by placing a fragment of tissue in a drop of lymph upon a coverslip. The lymph clotted and formed a semi-solid medium in which the growth of the cells could be observed under the microscope. Burrows (1910) and Carrel (1911) improved upon this method by using clotted plasma as a medium, and finally succeeded in maintaining growth and cell division for an indefinite period by the addition to the plasma of extract of embryonic

tissues. Drew and others have recently modified this technique by growing tissues in a hanging drop of isotonic saline and tissue extract, and many observations have been made by the Lewis' on tissue grown in isotonic saline alone or with the addition of dextrose.

Whatever medium is used the technique for the cultivation of tissues by the coverglass method is essentially as follows. A fragment of tissue is planted in a hanging drop of the medium upon a coverglass, which is then inverted over a glass slide, into which a hollow has been ground, and sealed down with vaseline or melted paraffin wax. The whole procedure is carried out under strictly aseptic conditions, and the preparations thus made are incubated at the normal temperature of the animal from which the tissues were obtained. After a longer or shorter interval the first signs of growth appear, and a film of cells gradually forms round the original fragment. The growing tissue may be sub-cultivated repeatedly, and by this means it is possible to preserve the tissue in a state of life and growth for an indefinite period.

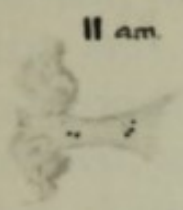
Chapter I

GROWTH IN VITRO

After a culture has been incubated for a few hours, a certain number of cells begin to wander out from the fragment by a form of amœboid movement, and if the culture is placed on a warm stage kept at body temperature the whole course of this outward migration can be watched under the microscope. A slender process of cytoplasm is seen to protrude from the edge of the implant; this process enlarges, the nucleus passes into it, and gradually the whole mass of cytoplasm detaches itself and passes out into the medium either freely or along the surface of the coverslip. Plate I. (Figs. 1–11) shows this process of detachment very clearly. The implant figured was taken from the choroid of an embryo chick, and was grown in plasma and embryonic tissue extract. As can be seen from the times given on the plate, the whole process occurred in two hours. In cultures which are growing favourably a number of cells can be seen migrating simultaneously, and a succession of others follows at short intervals until the whole fragment becomes surrounded by a more or less dense zone of cells. The cytoplasm of the outwandering cell appears

as a clear, homogeneous, jelly-like substance with no obvious cell wall, although the limits can be readily discerned both in cells lying free in the plasma and in those growing on the surface of the coverslip. The outline is constantly changing, and larger or smaller processes of cytoplasm are thrown out and withdrawn in amœboid movement; the cell is thus enabled slowly to change its position either in the medium or on the surface of the coverslip. The rate of movement varies in different cells, and also with the rate of growth of the culture itself. It is often so slow that it can be detected only by watching the cell for some minutes, whilst at other times it is relatively rapid, and can be easily observed. The amœboid movements take place over the whole surface of the cell, but are usually most active at the end furthest from the original mass of the growth. Fine rod-like filaments of cytoplasm, projecting from the cells and showing amœboid movement, may also be seen. The actual size of the cell appears to vary somewhat from hour to hour. Mitochondria are often visible as fine rod-like structures, which, on careful watching, may be seen moving in the cytoplasm. A number of minute refractile granules are also present; these constantly change their position, moving freely in the cytoplasm from one part to another; they sometimes pass around the nucleus, but do not penetrate the nuclear membrane. These granules vary in

Plate I.



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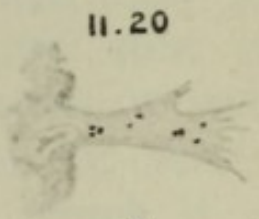

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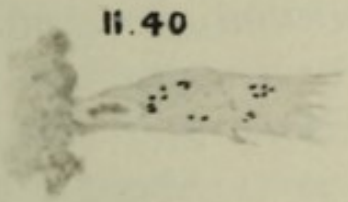

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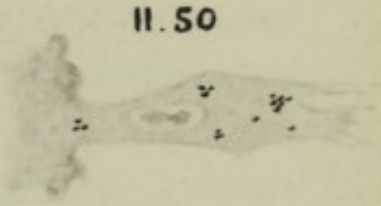

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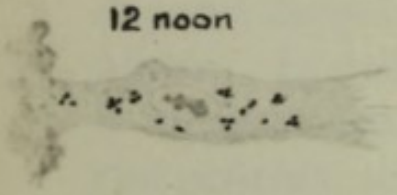

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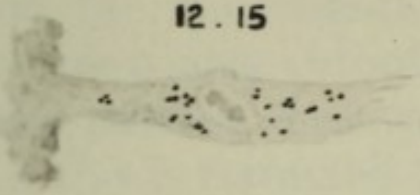

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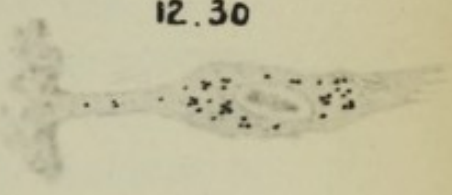

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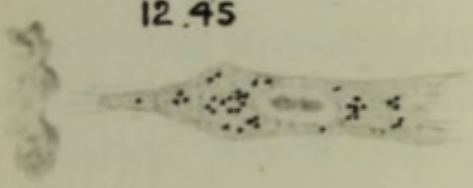

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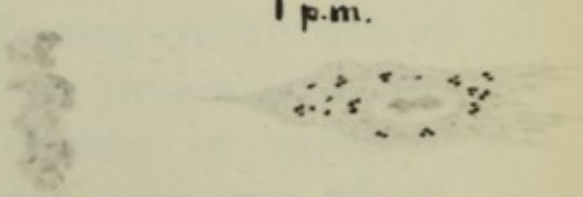

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number from time to time. In some cells clear droplets, which stain readily with the usual stains for fat, may be distinguished. These vary in size and number, but are often not seen in vigorously growing cells. The nucleus is well defined, usually oval in shape, and can be clearly seen lying in the cytoplasm from which it is sharply demarcated; the contour is generally oval, but is sometimes round. When first formed after mitosis the nucleus is relatively small, but it soon enlarges. The size is, however, not constant in a growing cell, and appears to vary somewhat with the size of the cell itself. The nucleus, although showing a tendency to assume a more or less central position, wanders freely about the cell, being sometimes in the centre, at other times at one or other pole, and its location appears to depend largely on the movements of the cytoplasm. In cells where a large cytoplasmic process is thrown out, the nucleus may move out into the process, and if this is retracted the nucleus is also withdrawn. The definite nuclear wall usually shown in fixed preparations is not seen in cells growing in vitro. The nucleus contains one or more irregular nucleoli, which are constantly altering in shape and size. During the growth of the cell a single nucleolus may divide into two parts, which for a time remain as separate bodies, and may then reunite—a process which may be repeated several times. In cells which are at rest, or show a lag in growth, or are growing

under abnormal conditions, it is possible to observe other changes—these, however, will be described later.

The cells composing the more or less compact zone of growth seen in a healthy culture differ markedly in size, shape and other cytological features from those of the original fragment, and lose entirely the histological arrangement characteristic of the organ from which they were derived. This is strikingly seen in cultures of the intestine, in which several diverse types of tissue are present—epithelium, connective tissue, plain muscle and sympathetic nerve fibres. The connective tissue and plain muscle cells assume a feather-like appearance and form a loose sheet of tissue, in which lie large areas of epithelium; the epithelial cells are usually round or oval in shape, and spread out on the under surface of the coverslip as a thin veil, whilst the sympathetic nerve fibres project far into the medium as long, fine processes. If articular cartilage is cultivated the disappearance of the characteristic structure of the implant is equally complete. The zone of growth consists of elongated feather-like cells, which, if grown under favourable conditions, contain a single nucleus; no intercellular substance is visible. If repeatedly sub-cultivated every 48 hours the cells of such implants maintain their altered structure indefinitely, and may be described as "dedifferentiated." It should be clearly understood, however, that the term "dedifferenti-

ation" is here used only to imply the latency, not the loss of those potentialities which enable cells to assume the specific structure and histological architecture of the organ of which they form part. As will be shown later, the dedifferentiated cells of a culture, by means of suitable technique, can be caused to redifferentiate into tissues similar to those from which they were derived. The rate at which the outward migration of cells from the implant proceeds depends upon several factors. Should the composition or concentration of the medium be unsuitable for a particular tissue, the outwandering is slight or absent. If dense intercellular substances are present in the implant, growth may be delayed for a considerable period, or even completely inhibited, since these structures impede protoplasmic movements, and at the same time prevent the rapid penetration of the medium to the cells, which therefore respond slowly to their altered environment.

After the cells have migrated into the medium, various changes take place in both cells and medium. In the case of the latter it is possible that the changes result from the liberation of certain cell products which alter the constitution of the medium. This alteration in the medium reacts upon the metabolism of the cells, and the physiological changes thus induced effect further chemical changes in the surrounding fluid, so that

a long series of interactions between the cells and medium ensue.

The future history of individual cells depends on environmental conditions. In favourable medium the cell which has wandered out from the implant, divides repeatedly for a certain period, after which division ceases, and the daughter cells pass into a vegetative condition. They may persist in this state, but do not again multiply unless the medium is changed. If the medium is unfavourable, mitosis may be completely absent, although the cells may wander out and live in a vegetative condition for some time. It thus appears that in cultures in vitro cells may show either amœboid migration, followed by reproduction or else amœboid migration followed by vegetation. Some workers maintain that there is a third possibility in the history of the outwandering cell, viz., fission, accompanied by amitotic division of the nucleus. Of this, however, the writer has been unable to find any definite evidence.

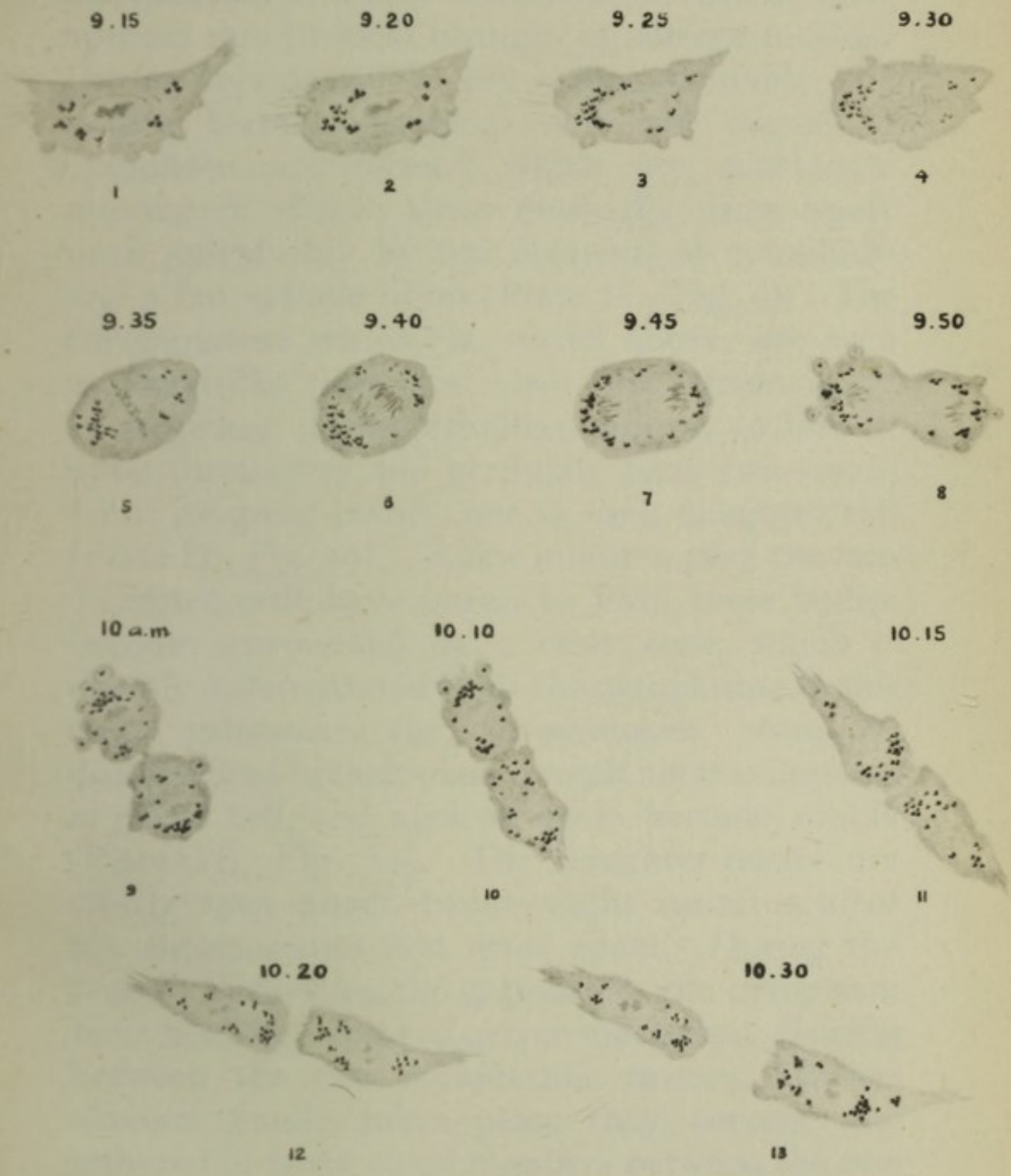
The increase in volume of a tissue may be brought about in several ways. It may be effected by (a) the multiplication of the cells, (b) the formation of intercellular substances consisting partly of the body fluids, and partly of solid or semi-solid substances which do not form an integral part of the cells, (c) hypertrophy of individual cells either by the accumulation in the cytoplasm of fluids and other materials

extraneous to the protoplasm or by actual increase in the protoplasm itself. In culture, as in the organism, increase in volume of tissue may result from any or all of these causes, but in normal cultures it is chiefly due to the first, i.e. the multiplication of the cells associated with mitotic division of the nuclei.

The growing cells, as already stated, show every phase of division and reconstruction, and by keeping the culture on a warm stage, the complete process of mitosis can be observed under the microscope. In vigorously growing cells the changes preceding mitosis take place rapidly. The processes of cytoplasm retract, and the cell usually assumes an oval or rounded shape (Plate II, Figs. 1–4), the nucleoli become fainter, and hazy granules are seen in the nucleoplasm (Plate II., Fig. 3). The nucleus has now entered prophase. Under favourable conditions these changes occupy from two to ten minutes. Most of the granules in the nucleoplasm rapidly coalesce into a number of longer or shorter threads, which can be seen in active writhing movement, and from which the chromosomes are subsequently formed (Plate II., Fig. 4). After a short interval the outline of the nucleus vanishes suddenly, and the nucleoplasm and cytoplasm seem to become intermixed; the nucleoli usually disappear at the same time, although occasionally one of these bodies can be seen lying free in the mixture of nucleoplasm

and cytoplasm. The spindle can now be distinguished, and the chromosomes, which by this time are fully formed, gradually become arranged at the equator. The assumption of this configuration is by no means a simple falling into position of the individual chromosomes. They can be clearly seen arranged and re-arranged about the equator, and the writer has repeatedly observed them apparently pass into metaphase and draw apart into two separate groups as in anaphase, then come together again in true metaphase (Plate II., Fig. 5), and finally pass into true anaphase at right angles to the first plane of separation (Plate II., Fig. 6). The time intervening between early prophase and the appearance of the spindle varies somewhat, but is on the average about eight minutes. Anaphase ensues within five to ten minutes after the spindle is first seen; the chromosomes begin to draw apart towards the poles of the spindle, and are plainly visible as finger-like processes (Plate II., Fig. 7). The cell meanwhile assumes an oval or hour-glass shape. At this stage (early telophase) most interesting changes appear at the periphery of the cell. Small balloons of cytoplasm project from the surface, remain for a few seconds, and then collapse. The granules in the cytoplasm, which do not appear to take any active part in mitosis, can be seen flowing into these structures as they form and streaming out as they collapse (Plate II., Fig. 8). This ballooning movement

Plate II.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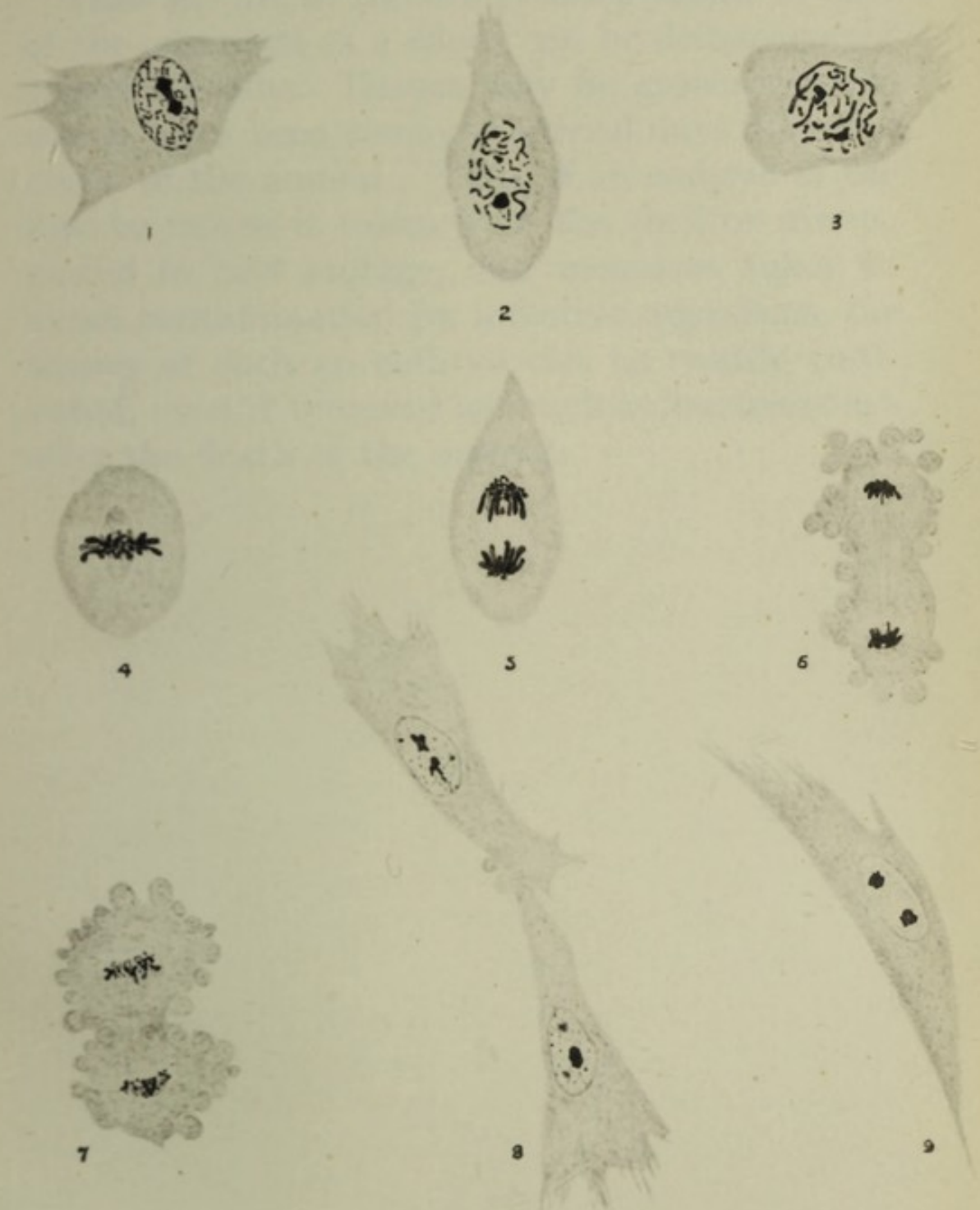
continues for about six minutes, new balloons being formed as the others collapse. This phenomenon is unlike amœboid movement, and appears due to local changes of surface tension. During this phase the cell begins to divide, the outline becomes irregular, and two masses of cytoplasm are formed which are constantly altering in shape; these gradually draw apart until joined only by fine filaments of cytoplasm and a few spindle fibres (Plate II., Fig. 9). The chromosomes which, as stated above, are seen as finger-like processes when first drawn apart at anaphase, during telophase appear to fold in upon themselves and gradually form two small, faint, irregular bodies, one in each daughter cell (Plate II., Fig. 10). A few minutes after the two daughter cells have begun to form these bodies become surrounded by a clear zone, which is sharply differentiated from the cytoplasm; in this clear substance the chromosomes seem to dissolve, and in their place a small nucleus appears in either cell, and nucleoli again become visible (Plate II., Fig. 11). The daughter-nuclei are clearly seen about twenty-eight minutes after the chromosomes first draw apart. During the process of division the granules in the cytoplasm have been in a state of active movement, flowing between the two cytoplasmic masses. Before division finally takes place they become distributed in fairly equal numbers between the two newly-formed cells (Plate II., Fig. 12). After

division is complete the irregular movement of the cytoplasm gradually diminishes, a reconstruction period, which usually occupies 1–2 hours, ensues, and the cells assume the characteristic shape and size of mature growing cells and contain nuclei of normal size (Plate III., Fig. 9). Cells which have been fixed and stained in different phases of mitosis are illustrated in Plate III., Figs. 1–9. Fig. 1—Early prophase; 2—Prophase; 3—Late prophase; 4—Melaphase; 5—Anaphase; 6—Early telophase; 7—Late telophase; 8—Daughter cells; 9—Fully formed cell.

The observations described in this chapter emphasise the important fact that the cell is essentially dynamic. Even in the vegetative stage it has been shown that no cell organ is wholly at rest, whilst mitosis marks a paroxysm of activity in which every structure is in almost violent movement.

The behaviour of the cells in "in vitro" cultures seems to indicate that each individual cell should rightly be regarded as an independent unicellular animal. Although somatic cells have existed in a state of close interdependence and functional association for untold generations of individuals, yet, as shown by tissue culture, they are capable of active amœboid movement, of reproduction by division, and of growth and reconstruction into normal daughter cells whilst existing in a free-living condition apart from the body. Moreover, they are able to maintain these

Plate III.



functions throughout innumerable cell-generations and for an indefinite period.

That the life of the cell is independent of that of the organism as a whole can be demonstrated experimentally. Tissues may be grown in vitro which have been removed several days after the death of the animal. Thus, if an embryo of the fowl or mouse is taken from the shell or uterus, placed in cold storage, and measures taken to avoid contamination by infective organisms, the tissues of such an embryo can be readily cultivated, even if removed as much as fourteen days after the death of the animal.

Chapter II

MITOSIS IN RELATION TO CELL PHYSIOLOGY

In the last chapter various changes which can be seen in the living cell during mitotic division were described. It now remains to discuss the functional significance of these changes. In the cell preparing for division, the nucleus not uncommonly increases in size, the two nucleoli, as stated previously, become fainter, and a number of fine granules appear scattered throughout the nucleoplasm. The appearance of these granules strongly indicates that they represent a separation of some of the colloids from the nucleoplasm. These granules can be seen gradually forming into a number of short threads which rapidly become more distinct, and thus is built up a number of fine but well defined filamentous structures which form the chromosomes. The outline of the nucleus suddenly vanishes and the cytoplasm and altered nucleoplasm appear to mix, and the chromosomes become arranged at the equator of the spindle; at the same time the nucleoli seem to go into solution and disappear. This apparent intimate commingling of the plasms to form a mixture which presumably differs in

physico-chemical constitution from either of the original plasms, may be of considerable importance in renewing the energy of the cell.

After the dividing cell has passed through metaphase and anaphase the chromosomes in the daughter cells can be seen gradually forming a small clump, which eventually appears to dissolve completely. During this solution of the chromosomes the nucleus reappears—at first small, but rapidly increasing to its normal size, and the nucleoli again become visible. Thus the physico-chemical changes preceding cell division appear to have been reversed, with the result that the chromatin material apparently once more goes into solution. It would seem that the solvent of the chromosomes is the reconstituted nucleoplasm, which becomes separated out from the cytoplasm as the reconstruction of the nucleus commences.

The individual chromosomes may be regarded as built up of various specific, though closely allied substances, the constituents of which have affinity for others of the same group. When precipitation of the chromatin takes place this affinity causes the constituents of each group to come out of solution in intimate association, and in this manner the individual chromosomes are formed. Since the number and shape of the chromosomes appear to be relatively constant throughout the species, it would seem that each group of materials has a characteristic structure

which is probably subject normally to slight variations. It is important to note that the normal shape and structure of the chromosomes can be radically changed by subjecting the cell to abnormal influences, e.g. X-rays. Such changes are described in Chapter IV.

It seems that the chromosomes are inert structures, the formation of which begins very early in mitosis, and is, indeed, the first recognisable change that we can at present demonstrate. They remain inert during the complex turmoil which is taking place in the protoplasm, and thus the specific character of the various constituents of the chromosomes is preserved intact. It would appear that the chromatin exerts its manifold functions only when in solution. If correct, this interpretation of mitotic phenomena would have an important bearing upon certain aspects of modern genetical research. The results obtained by workers of the Mendelian school are sometimes explained on the assumption that the chromosomes persist throughout the life of the cell as structural entities or as particles distributed in a definite order, which, owing to the limitations of present cytological technique, can usually only be demonstrated as such during cell division. In the light of the present writer's observations upon the living cell this hypothesis seems unlikely.

We have now to discuss the nature of the factors which induce mitosis. In order to do so

it is first necessary to consider the various external influences which are known to produce cell division. It will be found that they may be classified under three heads as follows:—

(1) Union with another cell, or portion of another cell. This occurs only in the case of mature germ cells, and is known as fertilisation.

(2) Slight mechanical injury, e.g. if the unfertilised eggs of the frog are pricked with a needle segmentation occurs.

(3) Alteration in the environment. Innumerable examples of this are to be found in experimental embryology and pathology. Loeb, by placing the eggs of the sea-urchin for a short period in hypertonic saline, induced artificial parthenogenesis; alteration in the supply of nutritive material, trauma, bacterial infection, and many other pathological conditions, if not too severe, all tend to promote division of the tissue cells.

At first sight it is difficult to understand how the same response in the behaviour of the cell is effected by such widely different agencies as those indicated above. Although, however, these agencies are so numerous and so extraordinarily diverse, yet the effect that they are likely to have upon the cell would seem to be essentially the same in each case, viz., the disturbance of the physiological equilibrium of the cell, which, to

cope successfully with the new conditions of life, has to undergo a certain protoplasmic reorganisation. Fertilisation upsets this balance by the introduction of new protoplasm, mechanical injury by the need for readjustment to the changed medium.

As will be shown later there is experimental evidence to indicate that the cell is in its most impressionable condition when undergoing or about to undergo mitotic division, since it is most profoundly modified by the action of such external influences as X-rays during this period. It would seem, therefore, that if the physiological stability is disturbed to any considerable extent the cell automatically passes into its most plastic state in order that the protoplasm can be more effectually reorganised in adaptation to the new conditions. From this it would follow that tissue growth is only an incidental result, not the primary function of cell-division. Physiological adjustments necessitating a less radical reconstruction of the cell can of course be effected in the vegetative condition, as may be seen in many physiological and pathological processes, and, on the other hand, a too profound disturbance makes perfect readjustment impossible and abnormal mitosis, pathological changes, or dissolution ensue. If we are correct in regarding mitotic division as the immediate result of changes in environment, then the division of somatic cells would be directly comparable to artificial parthenogenesis.

If the foregoing argument is pursued a little further it will be found to offer a solution to the all important problem of the causes of the inhibition of growth. If mitosis is due to some disturbance of the physiological equilibrium usually resulting from an alteration in the environment of the cells, it is obvious that as soon as the cells become adjusted to the new conditions, the physiological equilibrium will be restored and growth will automatically cease. It is significant that it is found practically impossible to carry on successive sub-culture of tissues without periodically renewing the medium, that is altering the environment. Even when this is done, however, mitosis stops after the fluid has been changed a certain number of times, suggesting that the cells have adjusted themselves to, and are no longer affected by such slight environmental differences. This adjustment is probably aided by interaction with the surrounding cells. To maintain continuous mitosis the fragment of growing tissue must be displaced, cut in half and "planted" on a fresh coverslip. The probable explanation of this fact is that many of the cells are damaged in this process, and release substances which again alter the environment of the adjacent cells, and thus the physiological balance is once more disturbed.

As before stated, the cells composing tissue fragments grown "in vitro" wander out from the central mass into the surrounding medium,

where they proceed to grow and divide; they lose to a great extent the cytological structure characteristic of the tissue to which they belong. Correlated with this dedifferentiation there appears to be a gradual inhibition of all the normal functions except that of reproduction. It would seem probable that dedifferentiation and loss of functional activity are also the concomitants of cell division in the various tissues and organs of the body. The daughter cells resulting from division seem to possess a double potentiality—on the one hand for again passing into mitosis, and on the other for assuming their characteristic structure and function and co-operating with cells of a similar type in the building up of the appropriate tissues. The assumption that the physiological balance is unstable during mitosis affords a ready explanation of the fact that function and reproduction do not proceed synchronously in the same cell, since such metabolic instability would of necessity seriously interrupt those cell processes, the resultant of which is function.

Only when the daughter cells have completed mitosis and reconstruction and settled down in association with their differentiated neighbours could physiological equilibrium be maintained and functional activity become possible.

Chapter III

REDIFFERENTIATION

The worker engaged in the study of cells grown "in vitro" is naturally confronted with the question as to whether the highly atypical cells seen in proliferating cultures are normal, or whether they have become so modified by the altered environment as to lose all capacity for re-differentiation and function. The present chapter deals with certain experimental data, which indicate that the dedifferentiated cells in normal cultures retain most, if not all, of their original potencies.

As already stated, the daughter cells formed by division either divide again after a certain interval or settle down into a vegetative condition. The future history of the newly constructed cell is found to depend on certain experimental conditions. If mitosis occurs soon after the tissue has been placed in a fresh medium, continued observation of the daughter cells shows that almost invariably a second division takes place in twelve hours' time. On the other hand, should a cell pass into division in a culture which has not been "changed" for a considerable period, e.g. 48–72 hours, a second division of the daughter cells

rarely occurs. The number of dividing cells present diminishes with the age of the culture. Ultimately mitosis completely ceases and the cells, all of which may be considered to have completed reconstruction, tend to redifferentiate and to resume those functional activities hitherto in abeyance. This tendency is apparently due in part to the presence of substances which inhibit, and in part to the absence of substances which promote mitosis. Unless, however, the implant is transferred to fresh medium, the incipient redifferentiation gives place to obvious pathological changes, and finally death ensues. As stated in Chapter II., transplantation again brings on mitosis, and growth continues for another 48 hours or longer.

The writer has observed that if a tissue fragment is transplanted carefully during sub-culture it is possible to keep the cells in the centre of the implant healthy without inducing mitosis. In such cultures the central portion wholly or partially redifferentiates, and may be kept indefinitely in this condition. Simple tissues such as cartilage reassume the typical histological structure completely, and even the more complex tissues such as those of the choroid and glandular organs, show this phenomenon to a limited degree. This structural reorganisation can take place only if the various types of cells composing the original organ are present. Drew has published an interesting series of observations in

connection with this question. According to this author a pure culture of epidermal epithelial cells, from which all connective tissue has been eliminated, makes no attempt at redifferentiation, but spreads out as a structureless sheet of elongated or spindle-shaped cells. If a subculture of connective tissue is added, however, the epithelium begins to redifferentiate into its original form. The cells assume their characteristic rounded shape, and signs of commencing keratinisation are seen. Pure cultures of the epithelium of the kidney may be obtained in a similar way, and on the addition of connective tissue differentiation usually sets in within 48 hours, rudimentary tubules being formed. The same is true in the case of a mammary carcinoma of the mouse, a pure culture of which grows as a sheet of undifferentiated cells, whilst if connective tissue is added acini are developed, which closely resemble those of the normal mouse mamma.

That the morphological changes induced by the altered environment have in no way affected the potentialities of the dedifferentiated cells of actively growing cultures is further shown by certain observations of the present writer. If completely dedifferentiated implants are returned to an environment more nearly akin to that from which they were originally taken, the cells will tend to resume to some extent at least their original shape, arrangement and functions. If

chick material is employed, the easiest, though possibly not the best method, is to graft the culture subcutaneously into the connective tissue of a young chick of the same parentage as the embryo from which the implant was obtained. Cartilage, and such relatively simple tissues, return readily to their original structure, but in the case of cultures of complex organs such as the gut, the process of redifferentiation is not so easily demonstrated. For the complete and typical redifferentiation of such a structure as the intestine, it would be necessary for the secreting epithelium, lymphoid cells, smooth muscle, connective tissue, and endothelium to be present in the growing implant in the proportions in which they occur in the normal embryonic organ. Since some types of tissues grow more vigorously "in vitro" than others, this condition is in practice by no means fulfilled. An orderly arrangement of the various tissues in the culture would also be important, whereas actually the different types are intermixed in a confused medley of cells. Finally, the culture medium must have been consistently favourable to normal growth, otherwise abnormalities will be produced in the sub-cultures, which will hinder and even arrest growth and redifferentiation in vivo. In successful grafts, a tumour about the size of a small shot appears within 24–48 hours after operation. During the next few days this tumour gradually increases in size, and may

become as large, or even larger, than a small pea. Growth usually ceases at the end of a fortnight or three weeks, and the tumour may then remain stationary for a few weeks or longer, during which time it becomes distinctly firmer in consistency. It may remain in this condition for many months, or even permanently, but more often shrinks and vanishes within a month. If such grafts are fixed and sectioned at various stages, evidence of great reproductive activity in all the cells is seen in tumours of a few days growth, but this is less marked in those removed 10–14 days after inoculation in which the first traces of redifferentiation are found. In one experiment a culture of adult articular cartilage which had been subcultivated 23 times, and in which no trace of the original implant remained, was grafted subcutaneously under the wing of a young chick. On the 14th day the graft, which had increased to the size of a large shot, was removed for histological examination, and was found to consist entirely of hyaline cartilage in which calcification had commenced.

In another experiment a culture of the skin of a mouse embryo was inoculated into an adult mouse under the skin of the abdomen. After 31 days' growth the graft, which had attained the size of a large shot, was removed and sectioned. It was found that the epithelium had assumed the characteristic squamous structure of the epidermis, and showed keratinisation.

A slightly different series of experiments was also carried out, in which a portion of the tumour was removed and regrafted a second time. In one such experiment a culture of the embryonic choroid and sclerotic of the fowl, which had been sub-cultivated seven times, was implanted subcutaneously on the underside of the wing of a young chick, where it gave rise to a small tumour about the size of a large shot. At the end of 16 days this was removed, and a portion was reimplanted under the wing of a second chick. After 36 days' growth this fragment again formed a small tumour about as large as a small hempseed. This was removed for histological examination, and was found to contain nodules of cartilage and pigment cells. In another case a culture of the embryonic intestine was implanted into the connective tissue beneath the skin of a chick, and after 7 days' growth reached the dimensions of a small pea. It was then removed, and a portion was reimplanted in a second chick. Here it again attained the size of a pea, and on the 5th day after the second operation it was dissected out and sectioned. Histological preparations showed well developed tubules lined by columnar epithelium and others, surrounded by masses of undifferentiated cells, were in course of formation.

In all experiments dealing with the redifferentiation of cultures in vivo, it is important first to ascertain that no trace of the original implant is

included in the graft. If this precaution is neglected the resulting tumours will represent not redifferentiation, but merely growth of an unaltered fragment of the original embryonic organ. It is significant, however, that the tumours found in such uncritical experiments differ but slightly in subsequent history and ultimate histological structure from those obtained by the inoculation of completely dedifferentiated cells.

The various observations and experiments briefly outlined in the preceding pages show conclusively that the abnormality of the growing cells of normal "in vitro" cultures is only superficial, since under appropriate environmental conditions the characteristic morphology and function of the original tissue may at any time be resumed. These observations also supply important data concerning the interrelationship of tissues of different types in organ-formation.

Chapter IV

EXPERIMENTAL MODIFICATION OF THE CELL

In the previous chapter it was pointed out that in order to obtain tissue cultures which will continue to grow and multiply normally, it is of the utmost importance that the medium in which they are cultivated should be carefully adjusted to their requirements. Information of considerable interest can, however, be obtained from the observation of cells grown in medium of a composition not wholly favourable to normal growth, since many interesting abnormalities in mitosis and cell structure appear in such cultures. Precisely similar modifications of growth and division can be experimentally produced by several methods, e.g. by the addition to the medium of various substances such as potassium iodide, bacterial toxins, and extracts of different tissues (endocrine glands, etc.), and by infection. The cells may also be affected directly without necessarily altering the medium, by growth at an unfavourable temperature, or by subjection to the influence of radium and X-rays. The writer has found the most exact and reliable modifying agent to be the X-rays. The results obtained by

subjecting organs and tissues to the rays, either before or after culture "in vitro," can be graduated by varying the duration and intensity of the dose, and experience enables the worker to foretell what effects will be produced under any given set of experimental conditions.

The conditions under which "in vitro" cultures of tissues can be subjected to the influence of the X-rays are very varied. The dose may be administered either before or after growth has commenced; tissues which have been exposed may be either fixed and examined at once, or may be returned to the incubator and later sub-cultivated. The beam of X-rays can be graduated in both intensity and quality by varying the length of the spark-gap of the coil, the milliamperage passing through the tube, the distance of the tissue from the anticathode and by the use of suitable filters or of anticathodes built of different metals. The intensity of the beam may be measured roughly by a pastille, or more accurately by an ionto-quantimeter such as Friedrich's[1] (a really satisfactory instrument for measuring a unit of X-rays under all conditions has, however, yet to be devised). It is thus possible to record the approximate dose given

[1] By the use of this instrument the "dose" or "exposure" received by an object placed in an X-ray beam is measured by absolute units. The unit employed, designated by "I e," is that amount of energy of beam which will produce in 1 c.c. of air under normal conditions an amount of ionisation that is equivalent to a change of one electrostatic unit of quantity of electricitv in the electrometer.

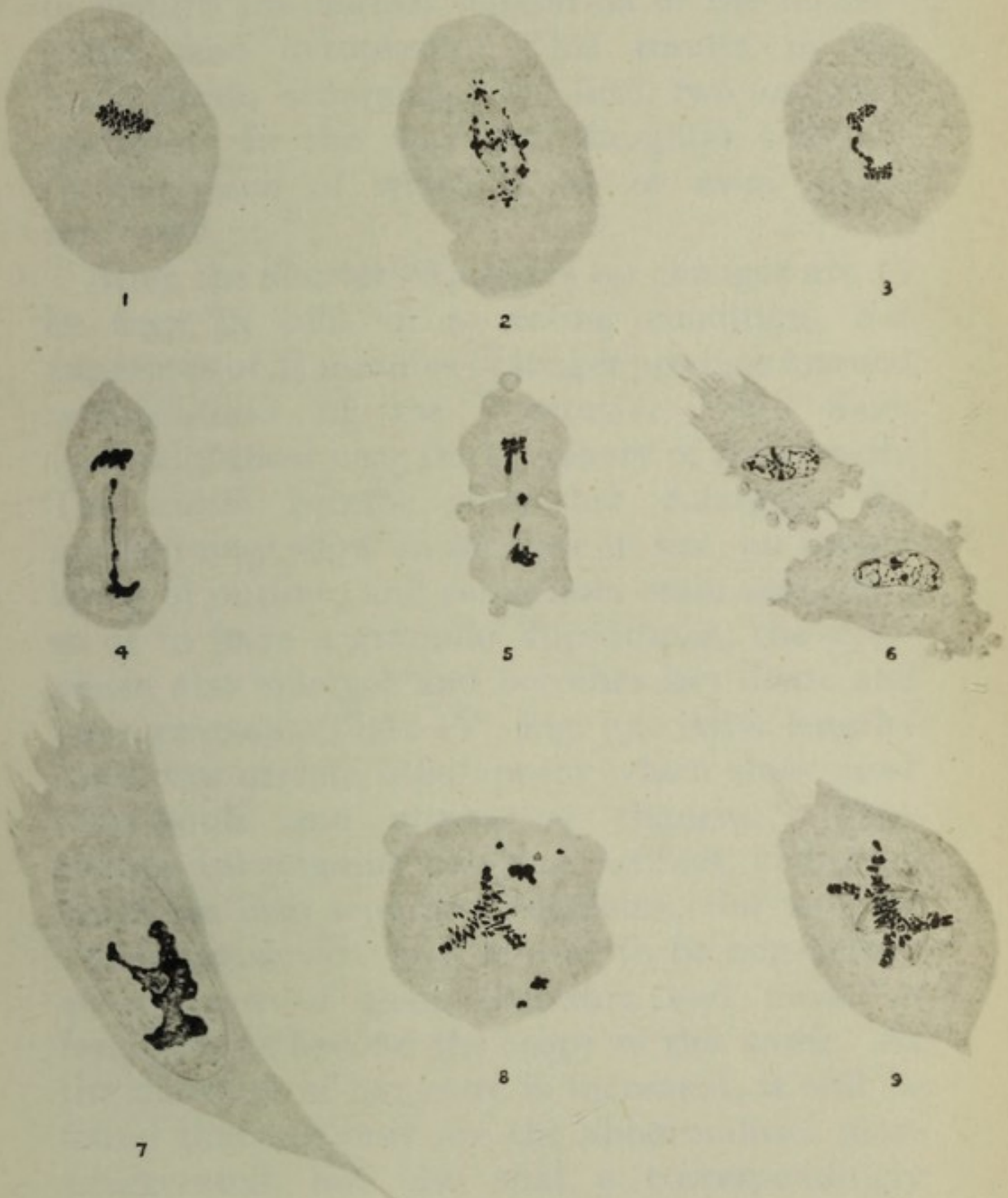
so that it may be repeated at will. The duration of the exposure may also be varied. The tissues may be exposed at the optimum temperature, which is easily kept constant by the use of a suitable thermostat. The apparatus should not be built of metal, but of wood, to avoid as far as possible the production of secondary rays.

It will thus be seen that the technique permits of almost endless modifications. For the production of the first series of changes described below, the writer employs the following methods. The cultures are exposed at a temperature of 37°C. to a beam of X-rays; a 12 inch coil is used with a spark gap of 8 cm., and a gas tube with a current of 1 milliamp. The cultures are placed at a distance of 24 cm. from the anticathode; the "dose" is regulated by varying the length of the exposure. Under these conditions the incident beam is of such an intensity that "I e" (Friedrich) is given per minute. Four cultures are exposed simultaneously, and in this way changes noted in one can be controlled by comparison with the other three. The cultures are returned to the incubator for 80 minutes, before fixation and staining. Any tissues may be used, but the present writer usually employs the embryonic choroid and adult articular cartilage of the fowl. After an exposure of 5 minutes, the only noticeable difference is the diminution in the number of prophase figures. After 10 minutes' exposure the number of cells showing the later phases of

mitosis decreases, and after exposures of half-an-hour or longer, mitotic figures are rare. If subjected to the rays for not longer than 5 minutes, most of the cells already showing prophase complete division normally. After 10 minutes' exposure definite changes appear in the chromosomes from metaphase onwards, and become more pronounced as the period of exposure is increased. Certain chromosomes show a varicosity, which, after prolonged exposure often becomes so exaggerated that the chromosomes break down into numerous granules and larger fragments (Plate IV., Figs. 1–2). At anaphase these altered chromosomes no longer split into apparently equal halves, but divide unequally so that one daughter cell may receive more chromatin than the other (Plate IV., Fig. 3). Another mitotic abnormality which is commonly seen in cultures which have been exposed for 15 minutes or longer, is a lag in division. Metaphase may persist for an hour or longer, and in anaphase the chromosomes pass towards the poles so slowly that telophase is well advanced before the poles of the spindle are reached. Anaphase and telophase thus merge into a single stage (Plate IV., Fig. 4). Associated with this lag in division is a striking lack of synchronism in the separation of daughter chromosomes, and granules and even large fragments of chromatin material may be found lying on the spindle threads after the division of the cell is almost

complete (Plate IV., Fig. 5). When the reconstruction of the daughter nuclei takes place these pieces of chromatin either remain in the cytoplasm or are thrown out of the cell. The reconstruction period may be protracted, but ultimately apparently healthy daughter cells are formed, one of which may be definitely larger than the other (Plate IV., Fig. 6). Cells are not uncommonly found showing abnormalities in telophase which result in the formation of bi-nucleated cells. The complete process can be watched under the microscope, and it is found that these cells may be produced, at least in some instances, as the result of abnormal mitosis, and not as is generally thought by a form of amitosis. Prophase, metaphase and anaphase are found to be as usual, and the only noticeable difference from the normal dividing cell is seen in the increased violence of the "ballooning" and amœboid movements during anaphase. In telophase, however, mitosis shows three types of abnormality, any one of which may cause the formation of a bi-nucleate cell. In the simplest type, the daughter cells, when almost separated, reunite to form a single cell containing two nuclei; in the second type, which is somewhat similar to the first, the cytoplasm divides unequally into two portions, one of which contains both daughter nuclei; the non-nucleated fragment disintegrates and dissolves some hours after it becomes detached, whilst the larger mass

Plate IV.



enlarges to form a perfect bi-nucleate cell. In the third type the "ballooning" and cytoplasmic movements are so pronounced as apparently to disturb the normal separation of the nucleoplasm and cytoplasm. This results in the nucleoplasm separating out into two or more portions. In this way two daughter cells are formed, each of which is bi- or even multi-nucleated.

After the shorter exposures no changes are to be seen in cells in a resting condition, but exposures of 45 minutes or longer produce marked modifications of the vegetative cells, more especially those near the periphery of the growth. The nuclei become somewhat enlarged; the nucleoli may show an increase in size, an irregularity of outline, and sometimes stain unequally so as to have a granular appearance; the cytoplasm also enlarges and becomes less dense and more granular (Plate IV., Fig. 7). After lengthy exposures certain cells appear which show most remarkable and interesting changes. They assume varying and complex outlines, and often break up into separate fragments, but as such "breaking down" cells appear to be non-viable, an account of their structure and mode of formation is beyond the scope of this work. As the duration of exposure is increased, it will be found that not only are the abnormalities more exaggerated, but also that a correspondingly greater number of cells are affected.

Experiments may be carried out in which the "dose" is increased. For such experiments the writer employs a standard Coolidge tube, through which a current of 2·5 milliamps is passed with an alternative spark gap of 15 cm., and the cultures are placed at a distance of 15 cm. from the anticathode. Under these conditions a relatively brief exposure produces the same results as those obtained by a long exposure to a beam of lower intensity. It is more convenient to increase the intensity of the beam than to prolong the exposure. More critical results will also be obtained in this way, since during a long exposure various normal processes are being carried out by the cells alongside those resulting from the experimental modification of the cell.

In order to ascertain whether there is a latent period before the changes described above can be demonstrated, four cultures may be simultaneously exposed, after which two may be returned to the incubator for a certain period, whilst the other two are fixed and stained immediately. Comparison of the changes visible indicates that there is a latent period of from 15 to 20 minutes before the modifications are recognisable.

In all cultures in which the dose given is sufficient to effect changes, it is found that individual cells show marked differences in their reaction to the influence of X-rays—indeed, no two cells can be said to respond in a precisely

similar way. This difference in reaction is found to be due in part to inherent physiological differences in the cells, and in part to the fact that the cells are at different stages of growth and mitosis at the time of exposure.

It has already been stated that cultures which have been exposed to X-rays may be returned to the incubator and sub-cultivated. If the culture is changed within 24 hours after exposure, it is found that the cells grow readily. The number of sub-cultures which can be obtained after exposure appears to depend to some extent upon the dose. After 300 e units sub-cultures may be repeated apparently indefinitely; after 500 e units only about a dozen times; after a dose of 700 e units only a few sub-cultures can be made.

Sub-culture, cultures which have received heavy doses—300 e to 700 e units Friedrich's—present modifications even more striking than those previously described. The most noteworthy changes are: hypertrophy of the vegetative cells, marked fragmentation of the chromosomes in division, the occurrence of tripolar and multipolar mitosis (Plate IV., Figs. 8–9), and the presence of binucleate and multinucleated cells in some of which as many as twenty nuclei are present. Cells undergoing multipolar mitosis may contain six or more spindles, and consist of a large rounded mass of protoplasm. In such cells a number of separate nuclei suddenly make their appearance, much amœboid movement is seen, and a large

multinucleate cell is formed. After an interval this may divide into two daughter cells, each containing several nuclei.

The most significant fact in connection with the experiments described in this chapter is that no technique has yet been devised whereby the abnormal cells of exposed cultures can be induced to return to the normal condition seen in the controls. The cells appear to be permanently modified, and, if the changes are not too violent in character, their viability and reproductive capacity is in no way affected. From this it seems legitimate to conclude that new types of cells can be experimentally produced which, throughout an indefinite number of generations, may be said to "breed true."

Chapter V

DIFFERENTIATION AND CELLULAR VARIATION

Cleavage of the fertilised or parthenogenetic ovum is initiated by its division into two equal halves, the physiological values of which are in some species at least apparently equivalent, since either cell if detached from the other may continue to divide and thus give rise to a pair of perfect embryos or identical twins. Normally, the separation does not occur, and segmentation proceeds to the 4–, 8–, 16–, cell stages, and so on. There is experimental evidence to show that in a few species each of the blastomeres in the 4–, and even 8–, cell stages are, if disassociated, capable of forming a perfect although smaller individual. As the number of cells composing the embryo multiplies, however, the potentialities become progressively narrowed except in the case of those set aside for reproduction, i.e. they gradually become specialised or differentiated. Various series of cells are formed, the members of which, whilst retaining their capacity of division, can adopt only that structure and arrangement characteristic of the tissue of which they are destined to form part, for example, epithelial

cells become differentiated and give rise to epithelium only.

The mechanism whereby differentiation is brought about constitutes one of the most complex and difficult, and at the same time perhaps the most fundamental study in biology. The experimental work described in the preceding chapters seems to shed a little more light on this important and perplexing problem, and to indicate certain of the conditions requisite for differentiation.

The limitations and modifications of the potentialities of cells must result from alterations in cell structure. The experimental data recorded in Chapter IV. afford a few suggestions as to when and how such structural changes may be brought about. It would seem from these results that the modifications are produced mainly during mitosis, since external influences such as abnormality of medium or X-rays cause the profoundest effects upon those cells which are about to undergo division. This would be anticipated on theoretical grounds since, as already pointed out, the complete protoplasmic reorganisation which apparently takes place in mitosis must be associated with considerable physiological plasticity. The nature of the forces primarily responsible for the production of structural variation during normal embryonic differentiation remains a mystery, but it is probable that the body fluids, through the agency

of which is brought about the complex physiological interaction of the cells composing the various organs of the body, play an important part in the process. This is supported by the fact that modifications in cell structures can be experimentally produced by growing tissues in abnormal media. Further evidence to this effect is afforded by the fact that in "in vitro" cultures, which have completely lost the characteristic histological structure of the original implant on inoculation into a living animal give rise to tumours composed of more or less typical differentiated tissue.

As previously pointed out, one of the most important conditions for the differentiation of individual cells is reproductive quiescence. Mitosis involves a complete reconstitution of the protoplasm, and it is therefore not surprising that during this period the cell loses its characteristic cytological features and becomes incapable of functional activity.

Drew's experiments have shown that although a group of cells may possess the necessary potentialities, may be grown in favourable body fluids or other media, and may be in a state of reproductive quiescence, they are, nevertheless, incapable of differentiation into their specific tissue if this is of a complex type. A fourth factor is required, viz., interaction with cells of the other tissues composing the organ. The laws controlling this interdependence of different types

of cells are obscure, and appear to be partly physiological and partly mechanical.

An interesting and important observation in connection with the experimental modifications of the cell structure, to which reference has already been made, is the dissimilarity in the response of cells which have been affected by the modifying agent at the same period in their life history. For example, if in an irradiated culture 10 cells showing metaphase are examined, it will be found that the chromosomes are affected somewhat differently in each case—in some cells fragmentation is inconspicuous, in others marked, and so on. From this it seems justifiable to conclude that each cell in the body possesses slight individual characteristics wherein it differs from neighbouring cells of the same type. If the foregoing suggestions are correct it follows that any two daughter cells resulting from division—either germinal or somatic—will differ somewhat in structure and physiology both from each other and from the parent cell. In other words the division of animal cells results in the reproduction of individuals of the same type, but not of absolutely identical potentialities. It seems probable that these differences arise during mitosis. Cytological and genetical evidence indicates that the complex molecules of which the cell is composed are segregated into various groups having some degree of independence. It is almost inconceivable that such molecular

aggregates should be exactly equally halved at cell division, or that they should be reconstituted in a precisely similar way in the two daughter cells during reconstruction. A qualitative and quantitative inequality of division would mean that a pair of daughter cells would differ slighty from one another and from the parent cell. In the case of the somatic cells, as far as we know at present, the individual differences resulting fortuitously from mitosis are of little significance. In the case of the germ cells, on the other hand, the results must be of far-reaching importance, as each of the two cells produced by the division of a gonocyte would possess certain potentialities in which it differed from the other. Ordinarily the individual differences between cells must be very slight, and in the case of the germ cells are probably manifested in the small individual variations which occur within a species of pure breed, or, if rather more pronounced, are represented by the more striking variations usually known as mutations.

It has been demonstrated experimentally that cells may be modified by external agencies such as alteration in the medium or by exposure to X-rays and that after the influence of the agent is removed, the cells will continue to show these modifications in all subsequent generations. This observation appears to be of fundamental importance, and gives strong reasons for believing that alterations in the somatic cells and body

fluids, by producing an altered environment may cause slight or even profound changes in the germ cells. Such modified germ cells would give rise to individuals differing from the parent. It should be clearly understood, however, that although it seems not improbable that somatic modifications of the parent may, in this way, result in a variation in the offspring, we have no reason whatever for believing that the offspring will exhibit the same type of variation as that produced in the somatic tissues of the parent. That it is possible to upset the potentialities of the mature germ cells by the application of external influences, and thus produce marked abnormality in development has been demonstrated experimentally by O. Hertwig, who found that if the normal ovum of the frog is fertilised by spermatazoa which have been exposed for a certain period to the action of radium, segmentation occurs, but the resulting embryo is markedly atypical, the abnormality being apparently proportional to the duration of the exposure. If, however, the spermatazoa are exposed for a relatively long interval, segmentation occurs more or less normally, but in this case the male gamete appears to act merely as a mechanical stimulus to parthenogenetic development.

The question naturally arises — are these experimental modifications of the cell of a pathological nature, and should they be described

as such? The writer has given much thought to this question, and feels that the use of this term is often extremely misleading, and should therefore be avoided where possible. Although the label "pathological" is applied to many of the changes observed in tissue cells and fluids, yet the boundary-line between physiology and pathology is an imaginary one. Many processes at present described as pathological are in reality normal reactions of the cell, which for some reason have become modified, and which, far from being injurious, may be necessary for the continued well-being both of the individual cells involved, and of the organism of which they form part.

Chapter VI

TISSUE CULTURE IN RELATION TO INFLAMMATION AND REPAIR

The experimental study of growth and differentiation of animal tissues by the method of tissue culture "in vitro" is found to shed further light on many of the processes involved in inflammation and repair in the living organism.

Under normal conditions the cells composing the various tissues of the body remain in a more or less vegetative state throughout adult life in some animals for 20, 40, 80 or even 100 years. At any moment, however, cells are able to pass out of this quiescent existence in response to such abnormal influences as trauma, the products of bacterial infection, etc., and their reactions under such circumstances are included under the terms inflammation and repair. Many of the histological changes seen in an inflamed area may be reproduced experimentally in tissues cultivated "in vitro," and some of the more interesting and important examples of this will be given here.

(1) As already described in Chapter I., a tissue fragment may be implanted in a medium whose composition is such that although not favourable to mitosis, it promotes amœboid movement of

the peripheral cells. Cytoplasmic processes are protruded from the cut edges of the implant, and some of the cells wander out into the medium. The appearance of the edges of the implant in such cases closely resembles that of the lips of a small incised wound (e.g. a cut with a razor whilst shaving), in which union takes place within a few hours, leaving no trace of the injury.

(2) If an implant is grown in a medium which will induce normal mitosis, the migrating cells divide repeatedly, and, if subcultured, continue to do so indefinitely. A similar migration and proliferation of the cells takes place during the healing of clean wounds, in which an actual loss of tissue has occurred, and during the healing of infective inflammatory processes.

(3) If a tissue fragment is cultivated in a medium which is unfavourable to normal growth, or if before implantation the tissue is exposed to X-rays, many of the newly-formed cells show modifications in size and structure, and examples of abnormal mitosis are often not uncommon. Similar cytological abnormalities are found in the fixed cells lying near the surfaces of infected wounds and in tissues in which the inflammatory changes are the result of infective organisms.

(4) The experimental production of multi-nucleated cells in cultures by the use of slightly unfavourable medium or by response to the influence of X-rays or radium is comparable to the formation of giant cells commonly seen in

certain specific lesions such as tubercle and in certain types of chronic inflammation.

(5) If a fragment of spleen is implanted into a plasma medium, the leucocytes migrate into the plasma, and under favourable conditions form a relatively large zone of cells around the implant. A similar migration of leucocytes from the capillaries into an inflamed area is a constant feature of acute inflammatory processes, and to this is largely due the formation of abscesses and the presence of pus on an inflamed surface.

(6) If a fragment of spleen is implanted near to a growing fragment, e.g. of cartilage, within a few hours the leucocytes migrate from the former implant, wander across the intervening space and infiltrate the newly-formed cells proliferating from the latter in precisely the same way as leucocytes infiltrate the tissue of an inflamed area in what is usually described as "round cell" infiltration.

(7) If a 24 hours culture of connective tissue such as cartilage is exposed to a moderately heavy dose of X-rays or radium, or grown in a modified medium, in the course of a few hours certain cells show definite abnormality, and eventually appear as small round cells lying among the normal elements of the culture. This transformation of connective tissue cells into round cells is of interest in connection with the round cell infiltration of inflamed tissues. The above experiment shows that connective tissue

elements, under abnormal conditions, are able to give rise to round cells, and it seems probable, therefore, that a certain number of the round cells in an inflammatory area are derived from modified fixed cells.

(8) If a fragment of spleen is implanted in a medium containing micro-organisms or particles of carmine, the leucocytes which migrate from the implant are seen to act as phagocytes and ingest a few of the foreign particles in much the same way as the leucocytes phagocytose micro-organisms in an acute inflammatory area.

(9) That some of the fixed tissue cells can also act as phagocytes can be demonstrated by implanting a fragment of cartilage or a pure culture of endothelium in a medium in which foreign particles are present. Under suitable conditions many of the newly-formed cells may be seen to ingest foreign particles—a phenomenon which is particularly marked if the culture has been modified by exposure to X-rays, in which case not only do newly-formed cells ingest micro-organisms or carmine granules, but also erythrocytes or fragments of disintegrated cells. Multinucleated cells may also ingest micro-organisms or carmine particles. The fixed and giant cells of inflammatory areas may act as phagocytes in the same way.

(10) If an "in vitro" culture of 24 hours' growth is exposed to a heavy dose of X-rays or radium many of the cells undergo a more or less

rapid disintegration, and in the course of a few hours many appear to dissolve completely in the surrounding medium. Cells showing the same type of change are common among both the fixed and wandering cells of acutely inflamed areas.

(11) In small tissue fragments growing in a plasma medium the intercellular material in the course of a few hours is seen to become granular and gradually dissolve, leaving no trace, whilst the tissue cells separate from one another, and may proceed to divide. The intercellular structures of acutely inflamed areas undergo precisely similar changes.

(12) If two fragments of connective tissue are implanted near each other in plasma medium the cells growing from the adjacent edges of the implant will interlock, and thus unite the two fragments. Such union of separated tissues is seen in the healing of incised wounds.

The observations recorded above serve to indicate the close resemblance between many of the experimental results to be obtained by means of tissue culture "in vitro" and the various phenomena of inflammation and repair, and it would seem, therefore, that the factors at work in the two conditions are essentially the same. In order to compare the course of inflammation and repair with growth "in vitro," it is first necessary to give a brief outline of the former process.

In the early stages of inflammation, especially when the reaction is acute, many of the cells in

the centre of the area disintegrate and dissolve. There is usually a zone between an acutely inflamed area and the normal tissue in which the reaction is less pronounced, although the cells exhibit modifications of size, shape and arrangement, and the fixed cells show some degree of proliferation. As inflammation subsides owing to the removal or dilution of the causal agent by the body fluids or because of an acquired immunity of the cells of the affected region, cellular disintegration ceases, the cells of the intermediate zone become more normal, repair of the damaged tissue commences. Eventually a new tissue is formed from the remaining fixed cells, partly by their rearrangement and partly by proliferation, which is nourished by capillaries arising as endothelial buds from adjacent capillaries. The number of wandering cells present gradually diminishes owing in part to disintegration and in part to their migration into the lymphatics by which they are removed from the area. Eventually the fixed cells settle down into a vegetative condition. The structure of the newly-formed tissue depends partly upon the extent of the damage inflicted and partly upon the type of tissue affected. When the damage is slight repair may be so complete that no trace of injury remains, but in the higher vertebrates at least this is rarely the case, and usually some evidence of injury persists throughout life.

The process of inflammation and repair may

be divided into two stages. The first stage consists in the dedifferentiation and proliferation of the cells in the extravasated lymph or blood of the injured area, and may be regarded as comparable to culture "in vitro"; the second stage is the attempt on the part of the newly-formed tissue to redifferentiate into the same type of histological structure as that which originally occupied the site of injury. The second stage, which is usually correlated with the re-establishment of circulation, might be said to correspond to the redifferentiation "in vivo" of dedifferentiated "in vitro" culture. The phenomena characterising stage one will first be discussed.

From the close similarity in the behaviour of cells "in vitro" and in inflammatory areas, it would seem that the cytological changes observed are in both cases the result of environic disturbance. As shown above the characteristic features of inflammation—amœboid movement, proliferation, cellular disintegration, leucocytic infiltration, etc.—may all be induced experimentally (*a*) by removing tissue fragments from their normal environment, the body, and placing them in an abnormal environment, the culture medium, or (*b*) by altering the composition of the medium. They represent the response of the cells to new conditions of life, and are associated with a complex series of intercellular reactions in the affected tissue. The nature and extent of the

changes induced in tissue cells in the body by a pathogenic agent depends upon the nature of the agent, the duration and intensity of its action, the type of tissue affected and upon whether the tissue is so modified as to possess immunity—hereditary or acquired—to the agent involved. These qualifications also apply to the experimental modification of tissues grown "in vitro." When the reaction of the cell to abnormal conditions either in the body or "in vitro" is too violent, the normal physico-chemical balance cannot be regained, and, as previously described, death and dissolution ensue. If, however, the effect upon the metabolism is relatively slight, the resulting modifications of structure and behaviour are less profound and are of a transient character. The cell passes from a vegetative state into a more active condition, and exhibits amœboid movements, which may culminate in actual migration. A slightly greater environic alteration may induce mitosis, and the resulting cells may again divide, undergo further modifications, or return to a vegetative existence and resume their normal functions.

It has been said above that the second stage of inflammation and repair—that of redifferentiation of proliferated cells—corresponds to some extent with the behaviour of dedifferentiated implants which have been grafted into the living organism. While such redifferentiation of "in vitro" cultures is readily induced in the case of

cultures of simple connective tissues such as cartilage, grafts of mixed cultures of connective and epithelial tissues are found to contain a disproportionate quantity of connective tissue when compared with the normal organ, and in extreme cases may consist of connective tissue only. This is due to the fact that connective tissue grows more readily both "in vitro" and "in vivo" than does epithelium, and in consequence the latter tends to become obliterated, and, as described in Chapter III., redifferentiation is therefore incomplete and atypical. In the higher vertebrates this tendency for connective tissue to proliferate at a greater rate than epithelium is a noteworthy feature of inflammation and repair. In the case of serious injury the connective tissue completely overwhelms the epithelium, repair is effected by the connective tissue elements alone, and a scar is formed. Where the damage is less great the epithelial elements may to some extent compete with the connective tissue, and in such cases atypical epithelium is formed in association with the connective tissue. Occasionally the resulting histological structure may present a fairly normal appearance. It will thus be seen that the course of events during the final stages of tissue repair in the body is exactly parallel to the history of "in vitro" cultures when grafted into the living animal.

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