TISSUE CULTURE: A CRITICAL SUMMARY.*

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(*From the Department of Physiology.*)

CONTENTS

	PAGE		PAGE
1. The Conditions of Tissue Culture	131	6. Serological Considerations.	144
2. Outline of the Technique	133	7. Mechanical Factors in Tissue Culture	146
3. The Cultivation of Normal Tissues	134	8. Conclusions	148
4. Applications of Tissue Culture	138	9. References	149
5. Theoretical Considerations	140		

THE adjunct of the experimental method to histology, or to any other descriptive science, is always invaluable. In fact, the recent experimental trend of microscopical anatomy would seem to justify the assumption that the histological researches of the future will be based more on the physiology and dynamics of the cell than on descriptive morphology.

The *in vitro* cultivation of animal tissues is one of the latest experimental acquisitions of biology, and the aim of this article is to furnish a critical summary of the more important researches up to date, and the theoretical considerations to be derived therefrom with the minimum of personal bias.

1. The Conditions of Tissue Culture.

Certain conditions limiting the possibility of the *in vitro* cultivation of tissues have to be observed. They comprise:—

(1) **The Nature of the Culture Medium.**—For tissues to survive outside the organism it is necessary that they be surrounded by an "indifferent" (*i.e.* a more or less isotonic and non-toxic) medium, or better still, by one which is also nutritive. Thus, mammalian tissues will grow in hanging drop preparations in standard or modified Ringer-Locke solution, to which a small amount of glucose or bouillon is often added (Lewis and Lewis[46]; A. H. Drew[24]).

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The growth of tissues in purely inorganic solutions appears to be due to the absorption of nutriment by the living from the dead and disintegrating cells in the centre of the "implant." (I employ this word throughout this article to designate the piece of tissue under cultivation.) Clotted lymph (Harrison[34]) or clotted plasma (Carrel[2]; Burrows[1]; Champy[18]) are other media most frequently employed. The growth of tissues in these is due partly to the absorption of nutritive substances from the lymph or plasma, partly to the reason mentioned above. It was also found by Carrel[4] that the addition of "embryonic extract" (*i.e.* the substances derived from fœtal tissues by destruction of the cells by freezing) to the plasma markedly favoured growth.

(2) **Oxygen Supply.**—For the implant to obtain sufficient oxygen it is necessary that it be very small and that it should lie at, or immediately beneath, the free surface of the culture medium. The respiratory requirements of the tissue impose a limitation on the size of the fragments under cultivation, and, even when these do not exceed the size of a small pin's head, aseptic degeneration of the central portion of the tissues occurs sooner or later owing to oxygen want. The degree of aseptic necrosis varies considerably in different animal groups; thus, in Birds it is very great, in Mammals intermediate, and in Amphibia and Reptiles far less marked.

(3) **Temperature.**—The optimum temperature for the *in vitro* growth of tissues approximates, naturally enough, to body heat. Mammalian cells are therefore incubated at 37° or 38° C., while those of English frogs (*Rana temporaria*) were found by H. W. Drew[28] to cultivate best at room temperature, growth in fact being inhibited at 37° C. Lambert,[48] in the course of researches on the relation between the temperature and the growth-rate of the heart of chick embryos, has demonstrated that such tissues can be preserved, in a state of "suspended animation," in chick plasma or serum, or in isotonic saline media, at 6° C. for two weeks or more (but always under twenty days).

(4) **Asepsis.**—An adequate aseptic technique is a *sine qua non.* For, obviously, conditions such as the maintenance of body temperature, and the use of plasma or glucose-containing

media, are very favourable to infection by moulds or bacteria, while the anti-toxic and phagocytic powers of cell-conglomerations divorced from the other tissues of the body community are but feeble.

At the same time, the necessity for elaborate aseptic precautions has been over-emphasised. Speed in manipulating the tissues before placing them in the medium, and the minimum of exposure to the air-borne contaminations subsequent to this would appear to be the most effective measures against infection.

Furthermore, the necessity for tissues being sterile renders impossible the cultivation of many organs in the adult—*e.g.* alimentary canal—though the difficulty can be overcome by taking such tissues from fœtuses.

2. Outline of the Technique.

(In addition to the papers mentioned in the text, Erdmann's précis [29] on technique will be found most useful.)

The technique of tissue culture should aim at implanting very small pieces of tissue into the culture medium with the minimum of damage in the minimum of time. Generally speaking, the methods which make use of modified Ringer-Locke solutions are easier to manage than the plasma technique, since the difficulty of obtaining blood plasma without clotting is avoided.

With either method the tissues are cut into very fine fragments which are placed in a drop of the medium on a cover-slip. The latter, inverted over a slide bearing a concavity, constitutes a preparation of the hanging drop variety, which can be examined with quite high powers of the microscope. If it be desired to keep tissues growing over prolonged periods, subculturing is necessary, especially for the tissues of the higher vertebrates. This entails transplanting a portion or all of the original implant to fresh medium on another cover-slip. Cultures of the tissues of warm-blooded animals cease to grow, and then die, unless the toxic products of growth and degeneration be eliminated in some such manner.

Although artificial solutions only admit of the hanging drop technique, plasma, on account of its rapid clotting, renders

possible the cultivation of relatively large pieces of tissue embedded in large amounts of solid medium. Although the cover-slip technique is widely used for plasma cultures, Carrel[7] has obtained interesting results by means of plasma cultures on large glass plates kept in sealed damp boxes. A further refinement, introduced by Carrel,[8] consists in growing tissues in plasma on the under surface of the lid of a Gabritschewski box, the lower portion of which contains water.

Finally, Champy[18] has introduced a method of plasma cultures in small glass tubes.* The advantage of this technique is that a relatively large volume of plasma can be used, while toxic products can be eliminated by a daily washing with Ringer-Locke solution or saline. The need for subculturing is hence diminished or done away with.

The merits of artificially prepared solutions versus plasma are not yet quite clear. For while Drew[24] claims that an artificial medium is the best on account of its known and constant composition, plasma has the advantage of being the most natural medium for tissues already subjected to the very abnormal conditions of *in vitro* cultivation. It also admits of the cutting of serial sections of the original implant and the cells derived from it, a point, I think, of great importance.

On the other hand, experiments necessitating the adding of drugs to the medium are best made by the hanging drop technique. The artificial media and plasma methods are each best adapted for certain ends, and they would seem to be complementary rather than antagonistic.

3. The Cultivation of Normal Tissues.

Scientific discoveries are frequently foreshadowed before being actually achieved. Thus, the *in vitro* cultivation of tissues was attempted by several observers, and, notably, by Leo Loeb who, in 1897 and 1902,[49] noticed in the course of experiments on regeneration that transplanted epithelium would grow into the clotted blood or lymph filling the incision in the host's tissues. Placing pieces of kidney, skin, mouse carcinoma, etc., in blood clot and other media in glass vessels,

* See also, in this connection, Coca, *Espana Medica*, Jan. 1914.

Loeb noticed that the cells of these tissues grew over the surface of the nutrient medium.

It was not until 1910, however, that Ross Harrison[34] made the fundamental experiment on tissue culture. This observer isolated pieces of the spinal cord of frog and toad tadpoles in clotted lymph derived from the lymph sacs of adults. He made hanging drop preparations which he studied with a water immersion lens.

Harrison noted that nerve fibres (axones) grew out from the dissociated medullary tube and primary cranial ganglia, the free end of such axones being enlarged, pseudopodial and motile. The outgrowth of the nerve fibre from the nerve cells was observed and recorded; it varied from 15.6 μ to 56 μ per hour. The greatest growth observed, from start to finish, was 1.15 mm., and this took rather over two days. Amœboid movements of the nerve cells themselves were also observed. Apparently the nerve cells only *grew* but did not multiply.

To Harrison, then, is due the credit of having made a fundamental experiment, for he not only evolved the necessary aseptic technique, but succeeded, by direct observation, in brilliantly confirming the neurone theory.

Next, in the course of a long series of researches beginning in 1910 and extending to the present day, Carrel and Burrows and their collaborators elaborated the plasma method of culture (already outlined on p. 66) whereby bird and mammalian tissues could be cultivated. Carrel obtained in his cultures not only cell-growth but also cell-multiplication of most of the tissues of the body. Owing to the greater growing power of embryonic as compared to adult tissues he employed especially the former. The discovery of the effects of embryonic extract on growth (already noted on p. 65), and the application of the technique of subculturing stand to his credit. Carrel's observations were made chiefly on living cover-slip cultures, or on such preparations fixed and stained in bulk. He distinguished in his cultures two kinds of cell: "Chaque organe et chaque tissu produisent deux différentes classes de cellules, les cellules de la charpente connective vasculaire et les cellules différenciées."[6] He apparently assumed that kidney produced kidney tubules *in vitro*, and thyroid fresh vesicles.[2] The

histological analysis of the growth-changes in his cultures was not attempted, nor, indeed, could it be with the technique he employed, for the only means of ascertaining the origin of the various cells growing out into the plasma and of studying their differential cytology is to make sections through both the implant and the newly-formed cells around it.

Carrel and his co-workers have also undertaken the making of pure cultures of tissues, notably mononuclear leucocytes [12] and embryonic connective tissue.[5] An original strain of the latter has been kept alive by Ebeling, by the aid of repeated subcultures, for ten years — a great technical achievement. No differentiation of the mesenchyme cells occurred at any time.

The large mononuclear leucocytes maintained their activity for nearly three months. Carrel and Ebeling [12] also noted that "differentiation of the large mononuclear leucocytes into cells assuming the appearance of fibroblasts took place under certain conditions." It is a pity that metamorphoses so important should not be more accurately described. A cell is either a fibroblast or it is not.

The absence—until quite recently—of the application of the sectional method is largely responsible for statements of this sort. The limitations of direct observation of living tissues *in vitro* are such that certain fundamental phenomena would appear to have been missed by Carrel. His earlier work especially abounds in technical innovation, but the interpretation of the histology of the growth-changes is often lacking.

Hadda,[33] while noting that the cells derived from the implant were not like those of the primary tissue, was unable to ascertain their nature or origin.

The very important study of the changes in the implant itself—changes which precede and often explain the subsequent cell-proliferation from it—was left untouched until taken up by Champy in 1913. This observer made sections through both the implant and the cells extending from it into the plasma. Further, by keeping the implants in tubes containing a relatively large volume of plasma he was able often to dispense with the need for subculturing, and this was advantageous in

that disturbance of the mutual relationship of the growing tissues was avoided.

Champy's interpretation of his results may be summarised as follows :—

The phenomena of tissue culture fall under two headings:— (*a*) *Multiplication of cells*, and (*b*) *their "dedifferentiation."* He emphasises the fact that while many tissues in the adult —*e.g.* non-striated muscle, kidney, thyroid, Müller's fibres in the retina—rarely (if ever) show evidence of mitosis, active cell-division appears in them under conditions of culture.[13, 14, 15, 16] This enhanced cell-division is accompanied by "dedifferentiation," as Champy terms it. The cells lose their morphological characteristics. For instance, non-striated muscle cells of the urinary bladder[18] under conditions of culture become swollen, lose their myofibrils, and, by the second or third mitosis, cease to show any of the characteristics of the previously highly differentiated muscle cells. Complete dedifferentiation to an indifferent condition has occurred, and such cells resemble the undifferentiated cells of the early embryo more than anything else.

Another important phenomenon of tissue culture, which Champy was the first to note, is the *phagocytosis* of tissue constituents by one another. Thus, in cultures of retina,[15] the neuroglial elements — the Müller's fibres — begin to divide mitotically on the third or fourth day, by which time the other retinal elements—rods, cones, and bipolar ganglion cells—have degenerated. Nor is this all, for, following the mitosis of the fibres of Müller, active phagocytosis by the latter of the other retinal elements occurs. Similar phagocytic phenomena have also been noted (Champy[17]) in cultures of testicle, where the Sertoli cells (particularly) first agglutinate and then ingest the more highly specialised spermatocytes. In fact, the cannibalism by relatively undifferentiated cells (*i.e.* Müller's fibres or Sertoli cells) of dedifferentiated—but previously specialised—elements is a common feature of certain tissue cultures.

Another frequent occurrence first observed by Champy in tissue cultures is the tendency for the epithelium to grow over the cut edges of the implant, thereby forming what this author has called an "épithélium de cicatrisation."

4. Applications of Tissue Culture.

The applications of tissue culture are many, and only a few will be mentioned here.

For the cytologist the method is very valuable in that the phenomena of mitosis, etc., can be studied *in vitro*. Strangeways,[53] working with cultures of choroidal and cartilage cells of eight to nine day chick embryos in adult chick plasma, has made the very interesting observation that the nuclear spireme, preceding the appearance of the chromosomes, shows active writhing movements. The existence of the mitochondria and, in one instance (Goldschmidt[52]), of the reduction divisions of the male germ cells has been confirmed by the intra-vitam observation of tissue cultures.

Again, Lewis,[48] from the *in vitro* study of degenerating mesenchyme cells of chick embryos noted that the centrospheres become greatly enlarged, and is of opinion that the cancer cell inclusions (Plimmer's bodies, etc.) are of a similar nature.

In pathology tissue culture has already yielded some promising results. Light has been shed on the controversy of the mode of formation of giant cells. These cells are formed in tissues around foreign bodies the phagocytosis of which is very difficult. Thus, spicules of bone, flint dust in the lungs, and tubercle bacilli are often enclosed within these giant cells.

Lambert[42] has cultivated chick spleens in plasma to which *Lycopodium* spores were added. He noted that the latter became surrounded by endothelial or pulp cells which then fused together, so as to form giant cells completely encircling the foreign bodies.

Of still greater interest, however, is the cultivation of tumour cells. That malignant tumours will grow *in vitro* has been shown by a number of observers. Leo Loeb[49] noted, but apparently never described, the growth of mouse carcinomata *in vitro*. Carrel and Burrows[6] have observed proliferation of a human sarcoma for six days, while Lambert and Haynes[41] report that differences in the type of growth of sarcomata and carcinomata are, to some extent, maintained *in vitro*. Growth in the former is by radiating strands, growth in the latter by a continuous sheet-like mass of cells. In other words, the

epithelial nature of the carcinomata tends to be maintained *in vitro*. These observers also state that the connective tissue can be distinguished from the epithelium in cultures of carcinomata. It would be interesting to know if this condition is persistent.

A. H. Drew[24] has made clear the necessity for frequent subculturing in the case of mouse sarcomata for these to survive *in vitro*. Such *in vitro* growths give rise to *in vivo* tumours when inoculated into mice. The necessity for frequent subculturing of mouse sarcomata is due, according to Drew, to special toxins elaborated by the tumour cells. Thus, he noted that while extracts of 37 S. inhibited growth of the tumour cells, they did not affect the growth of cultures of embryonic heart. An important fact, recently discovered by this author,[25, 26] is that while extracts of adult tissues prepared by destruction of the cells by freezing and subsequent filtration either do not—or only slightly—promote growth in tissue cultures, extract of adult tissues, provided these be first autolysed at 37° C., is a powerful growth stimulant if added to the culture medium. Probably the substances present in the autolysed extracts of adult tissues, which are of quite a different nature to those of embryonic extract, as shown by their far greater resistance to heat, are those responsible for the process of tissue repair within the organism. Furthermore, extracts of rapidly growing tumours, prepared by mechanical attrition in the cold, were found powerfully to stimulate the growth of normal adult tissues. Tumours of low malignancy yielded less active extracts. The outcome of these very interesting observations should be of great value in the unravelling of the factors of tumour growth. Presumably the weight of tissue—normal adult or tumour—and the volume of saline medium in which the extract was made were the same in both instances, as otherwise the results are not truly comparative.

Another suggestive observation in connection with the *in vitro* growth of tumours is that of Champy and Coca.[22] These authors cultivated a human adeno-carcinoma of the uterus in rabbit plasma. This tumour, reconstructed by the method of serial sections, was found to be a small pedunculated growth, the stalk of which was adenomatous, the free extremity

carcinomatous. The cultures were made from the adenomatous portion only. Yet the cells rapidly assumed the carcinomatous character of those of the free end of the tumour. Clearly, then, some restraining factor, present though ineffectual in the organism (since the cells furthest from the stalk were malignant), was absent in the cultures. As pointed out by Champy and Coca, the cultivation of benign—but only too often potentially malignant—tumours may well lead to more important results than the cultivation of malignant ones. The degree of dedifferentiation of the latter is too marked for much further change in this direction to take place. Of interest in this connection is the "malignant" appearance of sections of old cultures of highly dedifferentiated organs such as testis and thyroid (Champy [17, 16]). Such tissues multiply and dedifferentiate *in vitro* until the glandular elements form strands of cells of an embryonic character supported by a connective tissue stroma. Are the factors which produce dedifferentiation on the part of specialised tissues *in vitro* the same as those which produce the formation of malignant tumours *in vivo?* The evidence is not yet such as to warrant assertion or denial, but it is suggestive, and I will return to it later. Certainly further observations on the growth-changes of benign tumours *in vitro* would be of interest, and, possibly, of the greatest importance.

5. Theoretical Considerations.

That the growth of tissues is normally controlled within the organism is a matter of common knowledge. That this growth-restraining factor is often absent in tissue cultures appears to be certain. Of interest in this connection is Champy's statement [16] that dedifferentiated and multiplying tissue cultures, if grafted beneath the skin or into the peritoneum of animals of the same species, cease to multiply and tend to redifferentiate. A. H. Drew,[26] however, claims that his cultures, when inoculated into animals, rapidly disappeared. Further observations are needed on this point.

Strangeways (unpublished data) informs us that he has regularly obtained redifferentiation from subcultures of articular cartilage, intestine, skin and choroid of embryonic chick and

mouse tissues, and of articular cartilage from the adult fowl, by subcutaneous inoculation into animals of the same species.

The cells of the inoculated subcultures multiply rapidly for a few days and form a distinct tumour, then multiplication gradually ceases and at the same time redifferentiation occurs; in the case of articular cartilage true bone is formed.

Where, then, are the excitatory and inhibitory factors of growth to be found, and what is their nature? There is little doubt that adjacent tissues exercise such influences on one another. As an epithelium grows the underlying connective tissue keeps pace with it. Even under pathological conditions the same phenomenon may occur: in a fibro-adenoma of the breast the adjacent connective tissue grows concurrently with the mammary epithelium. Experimental embryology has already furnished some striking instances of the interactions of developing tissues. In the course of the development of the vertebrate eye, the lens is derived from the epidermis, the optic vesicle from the primary fore brain. If the optic vesicle be excised, no lens is formed, while, on the other hand, if pieces of optic vesicle are grafted beneath the epidermis, the latter attempts to form a lens. Clearly the formation of the lens is dependent on the tissue of the optic vesicle. Nor is this all, for a lens grafted beneath the skin of the salamander dedifferentiates *unless* a piece of retina be grafted with it, in which case the overlying epidermis loses its pigment, as normally occurs in the formation of the cornea.

As to the nature of the substances inducing and upholding the degree of differentiation of tissues, nothing is known. That such substances are formed by adjacent tissues appears undoubted, but their biochemical constitution, like that of the antigen or amboceptor of the blood is, as yet, purely a matter of conjecture.

Now, many of the phenomena of tissue culture would seem to fall into line with this conception of the mutual dependence of tissues. Note, for example, the behaviour (already described) of the nervous and neuroglial elements of the retina, of epithelium and its underlying connective tissue.

As a result of these observations, Champy has suggested that the reason for overgrowth of one type of tissue is to be

sought for in some reciprocally inhibitive action of tissues on one another. Thus, active proliferation of Müller's fibres only begins after the degeneration of the rods and cones, and of the bipolar ganglion cells. Similarly, according to Champy,[19] epithelium and connective tissue, when present in the same implant, proliferate but slightly. Once the connective tissue has died, the epithelium rapidly proliferates and undergoes dedifferentiation. A similar condition is obtained by the growing of epithelium without or with only the minimum of connective tissue. Thus, connective tissue would appear to limit the growth of the epithelium and to uphold its degree of differentiation.

A. H. Drew[25, 26] has carried the above investigations yet further by adding connective tissue to dedifferentiated cultures of kidney, thereby inducing tubule-formation. Similarly, the addition of connective tissue to skin causes keratinisation to appear. And, finally, carcinomata also react to connective tissue, in that those of the tumour cells which come into contact with the connective tissue conglomerate to form acini. The latter, according to Drew, "strongly resemble ordinary mouse mamma" (in culture or *in vivo?*), though the figures illustrating this point are not very demonstrative.

The question of the dedifferentiation of tissues *in vitro* has recently been taken up at the Rockefeller Institute, and elsewhere. Fischer[30] has cultivated a strain of embryonic chick epithelium, derived from the iris, for three months. This author states that no dedifferentiation occurred. Lewis and Lewis[45] have described various membrane formations derived from four to ten day chick embryos cultivated in saline solutions of various sorts. They also are of opinion that such membranes "retain their differentiation and do not return to their embryonic type." Again, Ebeling and Fischer[28] have cultivated implants of fibroblasts and epithelium side by side. These dual cultures were subcultured every forty-eight hours for seven generations, at the end of which period Ebeling and Fischer noted that both epithelium and fibroblasts remained individual, and that dedifferentiation and transitional forms were lacking. And this, of course, is what one would expect in view of the observations of Champy and Drew already

mentioned. On the other hand, Fischer[81] has cultivated over a period of three months cartilage cells from the eye of the chick embryo. The hyaline substance disappeared while the cells became spindle-shaped. Similarly, Carrel and Ebeling[13] have described (though without adequate detail) the "differentiation" of pure cultures of mononuclear cells into cells "assuming the appearance of fibroblasts."

Much of the confusion regarding dedifferentiation appears to be due to a misunderstanding on the part of these authors of the meaning of the term "*differentiation.*" By this word is meant the process of specialisation, functional and structural, of cells and tissues. Inversely, "*dedifferentiation*" *implies the return of previously specialised elements to a simpler and more embryonic type.* The criticism consequently seems justified that statements as to the absence of dedifferentiation in embryonic tissues are not to the point, *since such tissues, being embryonic and undifferentiated, cannot dedifferentiate.* It is also noteworthy that cartilage, taken from the eyeball of (presumably) late chick embryos *did* dedifferentiate, in that the intercellular substance disappeared while the cells lost their characteristic shape, although Fischer apparently does not consider such a change as being dedifferentiation.

And, finally, I must stress once more the need for accurate histological investigation of these changes. Intra-vitam observations on plasma cultures, supported by gross methods, such as staining in bulk of the implant, are not sufficiently accurate methods of controlling the fine cell changes involved in dedifferentiation.

It would be interesting to know whether dedifferentiation occurs in invertebrates. According to the observations of Goldschmidt[82] on the *in vitro* behaviour of the testes of the pupa of a butterfly (*Samia cecropia*), the germ cells underwent normal differentiation through the stages of spermatogenesis. Only when the cells reached the stage of transformation into spermatozoa did abnormalities appear. Here, then, the behaviour of the testis is totally different from that of mammals and birds, where dedifferentiation rapidly occurs. It looked as if the conditions responsible for spermatogenesis

in the vertebrates were inseparable from the organism, whereas in the invertebrate tissue studied by Goldschmidt the factors of development were largely in the germ cells themselves. Also of interest in Goldschmidt's experiments is the fact that differentiation of the germ cells only occurred if the investment of follicle cells was intact.

Wilson [55] and Huxley [86] have noted that the tissues of certain sponges, after being passed through fine gauze, dedifferentiate. The cells then agglutinate to form "restitution masses" which may subsequently undergo transformation into normal regenerates.

Pure cultures of collar cells may be obtained by appropriate methods of isolation. Dedifferentiation has been noted in higher forms than sponges—viz., in the Ascidian Clavellina—by Schultz [51a] and Driesch [20a] after transverse bisection of the animal. Sometimes, in such experiments, the component containing the pharynx underwent complete dedifferentiation into a sphere of cells which subsequently redifferentiated into a perfectly normal individual.

Certainly the widespread extension of tissue culture to the lower organisms should yield valuable results.

6. Serological Considerations.

Although the study of the growth-changes within the implant is, in itself, of great interest, equally important is the relation between the composition of the medium and the type of growth.

Plasma cultures fall into three groups: (i) those in which both tissues and plasma are furnished by one and the same animal; (ii) cultures in which plasma and tissues are taken from different animals of the same species; (iii) cultures in which plasma and tissues are provided by individuals belonging to different species. Such cultures are respectively termed autospecific, homospecific, and heterospecific (also autogenic, homogenic, etc.).

Carrel and Burrows,[8] in 1911, made the important observation that chick embryo tissues would grow nearly—but not quite—as well in the plasma of man, dog, and rabbit as in chicken plasma. Much research has been centred on the factors facilitating or retarding growth, and the problem has

been studied in a model way along quantitative lines by Carrel and his collaborators.

It is unfortunate that the quantitative method is so difficult in application to microscopical problems. At the Rockefeller Institute for Medical Research the study of the influence of different sera and plasmata has been pursued along truly quantitative lines in its relation to the growth-rate of *in vitro* tissues.

From the observations made up to the present it seems certain (Carrel and Ebeling[9]) that homospecific serum is increasingly inhibitive to chick embryo fibroblasts as the age of the donor increases. In other words, the older the animal which furnished the plasma, the less the growth on the part of the particular strain of embryonic fibroblasts employed in these experiments. Further, these authors have shown,[10] in the case of heterospecific sera, that there also exists a relation between the growth-rate, the concentration of the heterospecific serum, and the age of the animal furnishing the medium. The effect of heat on the growth of fibroblasts, as shown by Carrel and Ebeling,[11] is curious. Heterospecific serum heated to 56° or 70° C. is a better medium than the same medium unheated (as already shown by Ingebrigtsen[87]).

The importance of pure strains of cells from the view-point of standardisation is illustrated by the above experiments, which were performed with the same strain of chick fibroblasts, now under cultivation for over nine years.

The effect of alien plasma on the histological changes within the implant, and in the growing zone around it, has been studied by Champy[14] who, in 1914, showed that mammalian kidney may be grown, within the widest limits, in heterospecific plasma, without any important difference in the growth-changes. Only when the species furnishing the tissue and plasma belonged to widely separated zoological groups were degenerative changes noted—*e.g.* pigeon's kidney and cat plasma. It is interesting that, in regard to the testicle, Champy[17] found that degenerative phenomena were more marked in heterospecific plasma, although cultures of this organ, in plasma of females of the same species (rabbit) were only slightly, if at all, modified.

It would therefore seem that the effect of heterospecific

plasma or serum upon *in vitro* tissues is relatively slight—within wide limits—in contrast with the sensitivity of the organism as a whole towards such media.

Another important factor upon *in vitro* growth would appear to be the hydrogen-ion concentration. Mendeleef[50, 51] has shown that the P_H of maternal guinea-pig serum is 7.4, while that of the fœtal blood serum and also of the amniotic and allantoic fluids is 5.8. By putting up cultures of embryonic tissues in the maternal serum treated by the Bordet method of "Gélosage," the P_H of maternal serum can be reduced, and tissue cultures proliferate more abundantly in serum so treated than in normal serum. The increased acidity of the medium favours the growth of embryonic tissues. Presumably some such change whereby the P_H is decreased—and the maternal serum consequently rendered acid—must occur, as suggested by Mendeleef, in the placenta.

7. Mechanical Factors in Tissue Culture.

The amount, and the type, of newly-formed tissue around the implant is also influenced by various mechanical factors, chief of which are (i) the consistency of the medium and (ii) the cover-slip.

A careful study has been made by Uhlenhuth[54] of the morphological characters of the epidermis *in vitro* in relation to the consistency of the medium. Uhlenhuth studied the epidermis of the leopard frog (*Rana pipiens*) in the following media :—

(i.) Hard medium = frog plasma + frog muscle extract + chicken plasma + chick embryo extract.

(ii.) Semi-hard medium = frog plasma + frog muscle extract + chicken plasma.

(iii.) Soft to fluid medium = frog plasma + frog muscle extract.

In a hard medium the cells of the growing zone were polyhedral and formed a compact membrane; with the semi-hard medium the cells at the edge of the membrane were fusiform and migrated individually into the plasma; in a fluid

medium cell-migration was so marked that the membrane contained holes, while the cells themselves were round.

Uhlenhuth's conclusion that the morphological changes in the cells were solely due to physical reasons is hardly rational, since he obviously modified his medium chemically as well as physically by the abstraction of the chick embryo extract from the semi-fluid, and both chicken plasma and chick embryo extract from the fluid variety. Nevertheless, his description of the growth-changes in response to variations in the consistency of the medium may be correct for the following reason. Erdmann[20] mentions the fact that all the changes described by Uhlenhuth may be seen in the course of the progressive liquefaction of the plasma in one and the same cover-slip culture (though here also chemical changes certainly accompany the liquefaction).

Movement of cells in hanging drop preparations of fluid media—defibrinated serum or modified Ringer-Locke solutions—only occurs if the cells are in contact with the cover-slip or some other solid object, such as a piece of spider's web (Ross Harrison[35]).

The observations on the *in vitro* extension of axones (Ross Harrison[34]; Ingebrigtsen[33, 39]; Lewis and Lewis[46]) suggest that stereotropism *may* be one of the directing influences in the connecting together of the axone and the sensory or motor structures during ontogeny. But the nature of the forces guiding the nerve fibre, after section, through the scar tissue of a wound are, as pointed out by Sherrington,[52] even more mysterious. The *in vitro* study of the axone and of the factors influencing its growth is hence of the greatest interest.

Again, according to Lambert,[43] the thinner the drop of fluid, the thinner the cells, while those cells actually in contact with the cover-slip are the thinnest of all.

Obviously, then, stereotropism—the influence of contact—modifies both the amount and type of growth of tissue cultures. Of interest in connection with the mechanical factors of tissue culture are the observations of Lake[40] and others that the pulsation of the embryonic cardiac muscle fibres, under conditions of culture, is dependent upon the tension exerted by

the contracting fibrin threads in the plasma — evidence in support of the myogenic theory of the heart beat. This author is further of opinion that the fibroblastic cells of embryonic heart studied by the American observers are not of a connective tissue but a muscular nature. Here he would appear to be wrong, since Ebeling and Fischer have conclusively shown that the cells of a ten year old strain of fibroblasts, derived from the chick's heart (*i.e.* according to Lake, cardiac muscle cells), stained in section as typical collagen connective tissue with Van Gieson's stain! Yet another instance of the necessity for controlling histological observations by adequate histological methods.

A final comment: no little misunderstanding must arise in the minds of those who read occasional articles on Tissue Culture from the lack of bibliographical references to the work already done. Some of the American, and particularly the English, papers are the worst offenders in this respect. As can be seen from the literature quoted in the present summary—and only the more important papers have been mentioned—much work has been done since Ross Harrison's fundamental memoir in 1910. The days are gone when a scientific worker could launch out into research and be sure that what he observed had never been observed before. Consequently the authors who omit adequate reference to the work of their colleagues do an injustice both to science and to previous workers.

8. Conclusions.

Briefly to summarise the main lines of thought derived from the above paragraphs:—

The tissues of the higher vertebrates may be cultivated *in vitro*; apparently, in some instances, indefinitely. The life of chick embryo fibroblasts has been prolonged for ten years, a period at least as long as the life of the hen itself! The growth-rate remained practically the same throughout (Ebeling).

Undifferentiated—that is to say embryonic or malignant—tissues proliferate *in vitro* with slight or no histological change.

More highly specialised tissues often show extensive cell-division—even those tissues which do not normally reproduce

themselves in the adult organism—and ultimately dedifferentiate to an indifferent (or embryonic) type (Champy).

The presence of living connective tissue in a mixed culture would seem to be the chief factor in restraining cell-multiplication and upholding differentiation. The addition of connective tissue to dedifferentiated cultures causes redifferentiation (Drew).

The only benign tumour (adenoma) cultivated up to date *in vitro* rapidly dedifferentiated and became malignant in character (Champy).

The phagocytosis of dedifferentiated, but previously specialised, cells by less specialised elements is a common feature in certain tissue cultures (Champy).

The technique of tissue culture provides a means of differentiating between the exogenous and the endogenous factors acting upon tissues.

The phenomena of nerve-regeneration, as pointed out by Sherrington, may well be enlightened by those of tissue culture, for in both cases is specialisation lost, but cell-multiplication provoked.

The effects of drugs and of tissue extracts can be observed in relation to growth and other phenomena. A. H. Drew has made the important contribution that extracts of adult autolysed tissues, as well as extracts of malignant tumours, activate the growth of normal adult tissues *in vitro*.

And, lastly, the localisation of the factors restraining, inhibiting, or stimulating the growth of tissues can be approached experimentally through the technique of tissue culture. Research along these lines is fundamental, since all such endeavour is in direct contact with the problems of causal embryology and, doubtless, with the cancer problem (Champy ; Drew).

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