

A METHOD FOR THE PHYSIOLOGICAL STUDY OF TISSUES IN VITRO.

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PLATES 30 AND 31.

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The aim of the method described in the present paper is to maintain tissues in a condition of uninterrupted growth in a medium which does not deteriorate spontaneously. This simple idea has doubtless occurred to many experimenters, but great technical difficulties have so far prevented the development on this principle of a method of cultivating tissues. The problem consists of giving the cells the necessary food material and removing the catabolic substances from the medium without disturbing the tissues and without bacterial contamination. It has been solved by the construction of containers and instruments permitting the aseptic handling of the cultures, and by the use of a medium composed of two parts, solid and fluid, the solid medium being continually bathed by the fluid medium which is changed as often as necessary.

I.

Preparation and Handling of the Cultures.

1. *Containers and Instruments.*—The containers are flat, round flasks with narrow, oblique necks through which tissues and media may be introduced and removed. The neck is 3 cm. long and 1 cm. wide, and can be easily sterilized in a Bunsen flame. The width of the neck and its inclination are such that tissues and fluids are easily introduced with a pipette, and particles of dust cannot fall into the culture medium while the flask is opened. It is closed with absorbent cotton and a rubber cap. The flasks belong to five types (Fig. 1), and each type may be made in two sizes, 5 cm. or 8 cm. in diameter.

Type A bears a top opening 3 cm. in diameter, and is used for the cultivation of tissues which cannot be handled conveniently through the neck of the flask. The opening is closed by a disc of glass, fixed with shellac. Type B has two necks at the opposite sides of equal diameter and no top opening. It is convenient when the preparation of the culture requires the handling of the tissues with two instruments, which are introduced through the opposite necks. Type C has a long neck, a bottom opening closed by a thin mica plate, and also a top opening. It may easily be inverted under a microscope for photography or examination under high power of the living tissues. After the culture has been fixed in formaldehyde, the bottom of mica is excised with ordinary scissors or unsealed without disturbing the solid medium. Then the tissues and their medium, adherent to the thin mica plate, may be stained and studied under high magnification. Type D has only one neck and is commonly used for the cultivation of fibroblasts, epithelium, leucocytes, and virus, and for the study of cell secretions. Type E resembles Type D, and possesses a bottom opening covered with mica. Flasks A, B, and D are made of plain glass which allows the tracing of the cultures in a projectoscope and also low power microscopic examination. When cytological studies are required flasks of Types C and E are used.

The tissue fragments are handled and introduced into the flask with long platinum spatulas, straight or curved, and mounted on a glass rod (Fig. 2). Other kinds of spatulas are used for cutting the solid medium, when part of the culture has to be removed from the flask. The introduction of the fluids into the flask is made with short, graduated pipettes with rubber nipples (Fig. 3). The fluids are removed through a large hollow needle 4 cm. long, connected by rubber tubing with a vacuum apparatus (Fig. 3). The aspirating needle is also used when aeration of the culture is needed.

2. *Culture Medium*.—The culture medium is composed of two parts, solid and fluid. The solid medium consists of a coagulum of fibrin obtained from plasma or fibrinogen. When fibrinogen forms the basis of the clot, the fibrin is protected from digestion by a little serum or, when serum cannot be used, by a small amount of sodium linoleate or suspension of egg yolk. The volume of the solid medium in a flask 5 cm. in diameter should be 2 cc. If it is larger, the coagulum

becomes too thick and the tissues are isolated from the fluid medium by a layer of fibrin through which the diffusion of nutrient and catabolic substances is very slow. The volume of the fluid medium is generally 1 cc. The asepsis of the fluids used in the composition of the medium must be ascertained by the proper bacteriological tests, and the H ion concentration by the colorimetric method.

The plasma is introduced first. Generally 0.5 cc. of plasma is injected into the flask, which is gently tilted in order that the whole bottom may be moistened. This prevents the loosening of the coagulum from the glass surface, which may otherwise occur after a few days. Then 1.5 cc. of Tyrode solution, containing 5 per cent tissue extract, is introduced and mixed with the plasma. When a fibrinogen suspension is used instead of plasma, it is introduced in the same manner. To 0.5 cc. of fibrinogen suspension is added 0.5 cc. of doubly concentrated Tyrode solution. Then 0.5 cc. of Tyrode containing a little sodium linoleate or serum, and 0.5 cc. of dilute embryonic tissue extract, are introduced. Before coagulation takes place, the fragments of tissues, carried on a platinum spatula, are placed in the medium. If there are many fragments, they are suspended in Tyrode solution and injected with a pipette. After coagulation has taken place, the fluid medium is poured on the surface of the clot, and the neck of the flask is flamed and tightly closed.

The fluid medium must be changed every 2nd, 3rd, 4th, or 5th day, according to the nature of the medium and the tissues. The flasks are brought into a room where the air has been sprayed and is practically free of dust. The rubber caps are removed and the neck is carefully flamed. Then the fluid is withdrawn by means of the aspirator (Fig. 3) or a pipette, and the new fluid introduced. The neck is again flamed and closed. The time required for changing a complex medium varies from 45 to 75 seconds. It is generally possible to handle about 60 flasks in 1 hour. If a fragment of the culture has to be transferred to another flask, the coagulum is detached with a spatula and removed through the neck of the flask. Then the tissues are dissected and a new culture is made.

3. Measurement of the Rate of Growth and Examination of the Cells.—The flasks are placed in a projectoscope and the outline of the tissue is traced every 1 or 2 days. As the thickness of the solid medium and

the density of the fibrin meshwork are about uniform, the increase in area of the tissue expresses the increase of its volume with some accuracy. When the area is plotted in ordinate and the time in abscissa, the curve expressing the growth of connective tissue or of epithelium is a parabola.

Microscopic examination of the tissue can be made with low power through the bottom of Flasks A, B, and D, and with high power through the mica sheets of Flasks C and E.

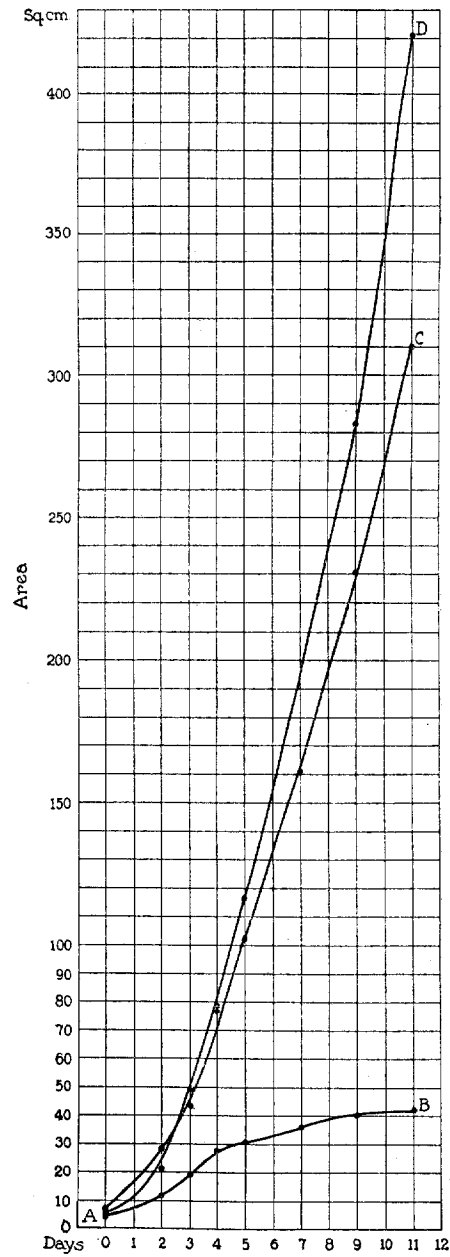
The tissues are fixed, the flask being still in the incubator, by warm Ringer solution containing 2 per cent formaldehyde. After fixation, the culture is washed and detached from the flask, then stained, or sectioned and stained. But the tissues are often damaged in the process of removal from the bottom of the flask. Therefore, when microscopic examination is needed, the tissues are grown on the bottom of Flasks C and E.

II.

Characteristics of the Growth.

The fibroblasts were obtained from an 11 year old strain of connective tissue, or from the heart of chick embryos. They were imbedded in the coagulum covering the bottom of the flask and bathed in the fluid medium. When the fluid medium was composed only of Tyrode solution or serum, the fibroblasts used the food material stored in the tissue fragment itself. Then, the rate of growth became slower and ultimately the tissues died. The duration of the life of the culture in this non-nutrient medium expressed the residual activity of the tissue and its graphic representation is generally a long S-shaped curve (Text-fig. 1, Curve *AB*). When the fluid medium contained some nutrient substances, the tissues increased regularly in mass (Fig. 4). The diameter of the new colony might reach 1.8 or 2 cm. after 2 or 3 weeks, and the curve of the growth was a parabola (Text-fig. 1, Curves *AC* and *AD*).

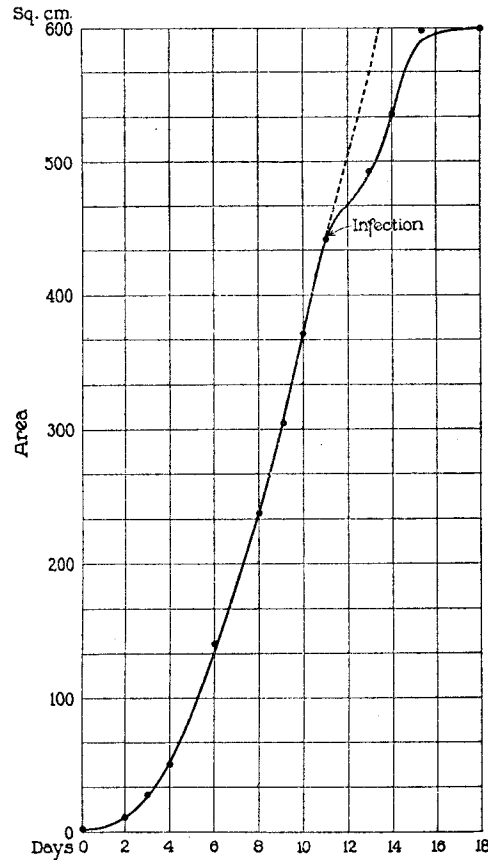
The rate of growth was modified by minute changes in the constitution of the medium. When bacterial contamination occurred, the rate of growth decreased and the tissues died (Text-fig. 2). Slight differences in the composition of the medium brought about marked differences in the rate of growth. During the first days of life of the



TEXT-FIG. 1. Curve *AB*, growth of fibroblasts in a medium containing no nitrogen. Curve *AC*, growth of fibroblasts in a medium containing 21 mg. of nitrogen per 100 cc. Curve *AD*, growth of fibroblasts in a medium containing 30 mg. of nitrogen per 100 cc.

cultures, these differences were not apparent, but after 2 or 3 weeks, they became evident (Text-fig. 1).

The changes in the rate of growth of a given tissue under the influence of another tissue could also be studied. For instance, when a colony of leucocytes, growing under the influence of serum, reached



TEXT-FIG. 2. Growth of a culture of fibroblasts which became infected on the 11th day and ultimately died.

the edge of a resting culture of fibroblasts, these cells began to proliferate again.

Epithelial cells grew as a membrane at the surface of the coagulum. The curve of growth was also a parabola. The rate of growth of epithelium was always less rapid than that of fibroblasts.

Leucocytes from the blood and wandering cells from the spleen were cultivated in Flasks D and E. From spleen fragments, about 1 sq. mm. of wandering cells migrated and in 30 or 35 days covered an area of about 2,000 sq. mm.; that is, the entire medium. Leucocytes from small blood clots grew closely packed together and later formed a number of small colonies scattered through the medium. These colonies originated by the grafting on the surface of the coagulum of groups of cells which floated into the fluid medium. They never aggregated in a tissue, and an accurate measurement of the area invaded by them was not possible.

III.

Accidental Causes of Death.

When the solid medium remains unaltered, and the proper nutrient fluid is added to it from time to time, the tissues invade the coagulum progressively. But several factors may interfere with their growth and bring about premature death. The more important of these factors are bacterial contamination, changes in the H ion concentration of the medium, and alteration of the coagulum.

The first is generally due to the contamination of the fluids composing the medium during their preparation; to non-sterile instruments, pipettes, or spatulas; to contact between the fluid medium and the stopper of the flask, and finally to contamination from the air. Plasma is never contaminated because, being received directly into tubes, it is not exposed to air and contaminating contacts in the course of its preparation. As Tyrode solution is sterilized by filtration through a Berkefeld filter, it is almost never a source of contamination. Tissue juice is often responsible for contamination. However, the following technique usually yields an aseptic preparation. The embryos are extirpated carefully from the egg or from the uterus, and immediately introduced into a Latapie apparatus. Then, the pulp is received in a graduated flask and the necessary amount of Tyrode solution is added. After centrifugation, the extract is placed in a number of small tubes in order that the contents of each tube may be used entirely, after the tube is opened. The condition of each tube must always be tested by cultivation of a sample of fluid in bouillon

and on agar. As some bacteria develop very slowly, these cultures have to be kept under observation for 5 or 6 days, before the fluids can be considered as sterile and used for the growth of tissues. Practically all the failures could be traced to a contamination of the tissue juice which had been overlooked in the examination of the bacterial cultures. The opening of the flasks, the aspiration of the fluid, and the introduction of new medium practically never give rise to contamination, because the neck of the flask and the spatulas are easily sterilized in a Bunsen burner flame, and the pipettes are easily kept aseptic. Contamination may also be traced to contact of the fluid medium with the cap when the flasks are handled without sufficient care. None occurs as long as the fluid medium does not penetrate the oblique neck during the transportation of the flasks. When the flasks are carried in a tray especially constructed, where they are placed vertically, with the neck up, there is no contact of the fluid with the stopper, and the number of bacterial contaminations is much lessened.

The infection by air appears to be very unusual. In the room where the flasks are opened and the fluid medium is replaced, the number of bacterial colonies found in half an hour on Petri dishes about 100 sq. cm. in area is only 3. As the section of the neck of the flasks is less than 1 sq. cm. and the time during which the flask is opened does not exceed 30 seconds, the chances of contamination by air are extremely small. Besides, if a few particles of dust should fall inside the neck, they would be destroyed by the flaming which always precedes the closing of the flask. When the air contained in the flasks is aspirated and replaced by the air from the room, contamination is also quite exceptional.

Another cause of death of the tissues is a change in the reaction of the medium. When the pH of the medium is higher than 8, the growth decreases and sometimes ceases entirely. The excess of alkali may come from the glass. Therefore, every new flask has to be tested from this point of view before being used. Generally the alkali comes from insufficient washing. A special apparatus has been made for rinsing the flasks after they have been cleansed with sodium hydroxide. Since its use, no losses due to excess of alkali have occurred. A more frequent source of accident is acid formation.

When the pH of the culture is lower than 7.2, the rate of growth is markedly modified and the tissues die. This is generally due to a spontaneous change of the tissue extract which occurs mainly when particles of tissues are suspended in the fluid. The H ion concentration of every fluid used in the cultures must be tested and adjusted to 7.6 or 7.8.

When the fluid medium is composed only of tissue juice, the fibrin may become partly digested after a few days. This accident is prevented by adding to the tissue juice a small amount of serum, which protects the fibrin against the proteolytic enzymes. Sodium linoleate or egg yolk may be substituted for serum. When the beginning of the digestion of the fibrin is observed, a few drops of plasma are placed at the surface of the coagulum and the tissues generally resume their normal growth.

IV.

DISCUSSION.

The elucidation of the fundamental relations between the tissues and the humors of the organism would require an analysis of the action of the serum constituents on pure strains of cells, of the effect of the secretions of those cells on the composition of serum, and of the influence of the tissues upon one another. The main requisites of a method permitting such an investigation are to maintain pure cultures of cells in a condition of uninterrupted growth in a medium of practically unchanging composition. We already possess techniques by which strains of fibroblasts,¹ epithelial cells,² and leucocytes³ may be isolated and kept free from contamination by other cells. But these tissues must be maintained in a known condition; that is, in a medium which does not deteriorate spontaneously. As the activity of a tissue is a function of the concentration of certain substances in its medium,⁴ it is evident that the effect of a given factor on cell proliferation or morphology cannot be ascertained if at the same time the medium undergoes spontaneous and ill defined modifications.

¹ Ebeling, A. H., *J. Exp. Med.*, 1922, xxxv, 755.

² Fischer, A., *J. Exp. Med.*, 1922, xxxv, 367.

³ Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1922, xxxvi, 365.

⁴ Carrel, A., *J. Exp. Med.*, 1913, xviii, 287.

None of the techniques used so far fulfill these two main conditions. In the early method of so called cultivation of tissues, which is still employed by many experimenters, a fragment of fresh tissue, generally embryonic, is placed in a drop of plasma⁵ or saline solution,⁶ and for a few days migration and multiplication of cells may be observed. But the phenomenon is irregular and of short duration, and no increase in the mass of the tissues is observed. When the area of a pure culture of fibroblasts so cultivated is accurately measured, the curve of the growth resembles that of a unimolecular autocatalytic reaction. The second inflection of the S-shaped curve begins after 48 hours,⁷ and is due to a change of the medium which greatly restrains the rate of growth and brings about the death of the cells. During 48 hours only, the growth is uninfluenced by the progressive deterioration of the medium. Later, the action of a given factor on the growth of fibroblasts cannot be studied on account of the simultaneous effect of the modifications of the medium on the tissues.

In order to prevent the effect of the deterioration of the medium, a method was developed long ago⁸ in which the tissues were removed from the medium after 48 hours, washed in Ringer solution, and placed in a new medium. By the continual transfer of the tissues from medium to medium,¹ the cells were maintained in an identical condition, and the rate of growth of a strain of connective tissue, cultivated in this manner for more than 11 years, has not varied.¹ Today these fibroblasts multiply with the same velocity as 10 years ago. Many physiological problems have been investigated with the help of this technique. But it cannot be applied so effectively to more delicate structures such as epithelium and leucocytes. The amount of tissue is very small and the presence of secretions cannot be ascertained. Moreover, the section of the tissues and their transfer require a great deal of training and manual skill.⁹ As the growth is interrupted every 48 hours, the prolonged action of a given factor must

⁵ Carrel, A., and Burrows, M. T., *J. Exp. Med.*, 1911, xiii, 387.

⁶ Lewis, M. R., *Anat. Rec.*, 1915-16, x, 287. Lewis, M. R., and Lewis, W. H., *J. Am. Med. Assn.*, 1911, lvi, 1795.

⁷ Ebeling, A. H., unpublished experiments, 1919.

⁸ Carrel, A., *J. Exp. Med.*, 1912, xv, 516.

⁹ Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 231.

be observed during a number of passages. Thus, it involves much labor, and the process of growth cannot be expressed graphically in a satisfactory manner. For these reasons, an attempt was made to develop a better method.

It seems that the technique described in this article, although far from perfect, may be applied to the solution of many problems and fulfill most of the conditions required for a physiological study of tissues *in vitro*. However, the medium deteriorates in some measure because the substances contained in the fibrin do not diffuse entirely into the fluid which covers its surface. The coagulum, incompletely washed by the fluid medium, may be slowly modified by the substances which progressively accumulate in its meshwork. But the parabola expressing the increase of the tissues indicates that the growth proceeds at a constant rate for 2 or 3 weeks, instead of 2 days as in the hanging drop technique. A culture of fibroblasts or of epithelium can be kept in a D flask for about 3 weeks in a condition of active life. This space of time is longer than necessary for the study of most physiological phenomena. Leucocytes can remain in excellent condition for at least 35 days,¹⁰ and the coagulum is practically as transparent after this period of time as on the 1st day, although it may be completely invaded by the wandering cells. As fifteen or twenty fragments of tissues or of leucocytic film can be placed in a D flask containing 2 cc. of medium, the nature of cell secretions can be investigated in some measure.

The growth, being uninterrupted, may be represented graphically, and the part played by the constituents of the medium in the growth analyzed without difficulty. The characters of the curve vary according to the nature of the medium. In a nutrient medium the growth is expressed by a parabola and in a preservative medium by an S-shaped curve. This curve is the measure of the residual activity of a tissue. It is known that the activity of a tissue at a given instant is function of at least three independent variables, the inherent cell activity at the preceding instant, and the concentrations of growth-activating and growth-inhibiting substances in the medium. The inherent activity of a tissue may be defined as that which would be displayed during an instant in a medium deprived of all nutrient substances. The energy used by a tissue in that condition would be supplied by the

¹⁰ Carrel, A., unpublished experiments, February, 1923.

food material stored in the cells or in the interstitial lymph, and not by the medium. This inherent activity cannot be measured directly. But it is certainly proportional to the residual activity; that is, to the increase in area of the tissue and to the duration of its life in a preservative non-nutrient medium. The residual activity being known, it is easy to measure the part which is played in the process of growth by the nutrient substances of the medium. The area *ABD* in Text-fig. 1 represents the increase of the tissue due to the action of the medium; that is, the amount of protoplasm built up by the cells from the nutrient substances contained in the medium.

There is no doubt that this method permits the measurement, conveniently and without great labor, of the effect of many factors on cell proliferation, and will therefore help in the study of physiological and pathological problems.

V.

CONCLUSIONS.

1. A method has been developed which allows the continuous growth of pure strains of fibroblasts, epithelium, and leucocytes in a medium which undergoes but slight spontaneous deterioration.
2. The principle of the method is to leave the tissues undisturbed while the medium is changed. This was realized by special containers allowing the change of the medium without bacterial contamination and by the simultaneous use of a solid and a fluid medium.
3. The curve of growth of pure cultures of fibroblasts and epithelial cells in a nutrient medium is a parabola; in a non-nutrient medium, it is S-shaped and expresses the residual activity of the tissues. Leucocytes invade the culture medium progressively, as do bacteria, but never aggregate in a tissue.
4. The method is used for the study of the morphological and dynamic changes occurring in tissues under the influence of chemical and physical factors.

EXPLANATION OF PLATES.

PLATE 30.

FIG. 1. Flasks A, B, C, D, and E.

FIG. 2. Introduction of tissues into a D-5 flask with a spatula.

PLATE 31.

FIG. 3. Aspiration of the fluid.

FIG. 4. Culture of fibroblasts in a D flask.



D A B C E

FIG. 1.



FIG. 2.

(Carrel: Physiological studies of tissues *in vitro*.)



FIG. 3.

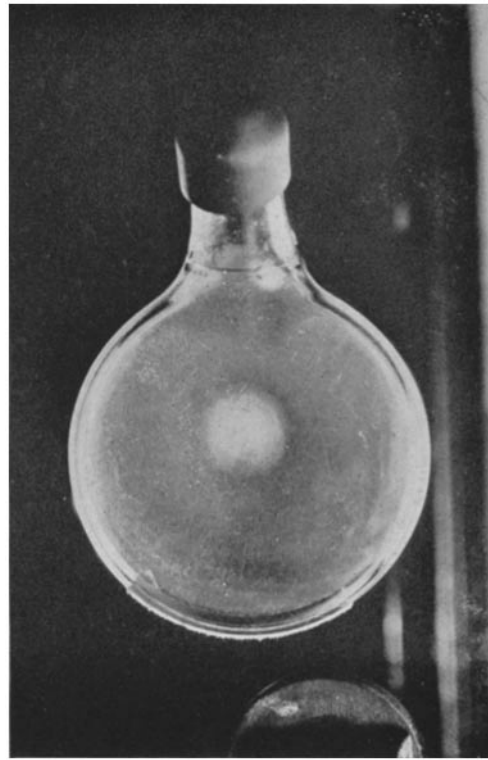


FIG. 4.

(Carrel: Physiological studies of tissues *in vitro*.)