CULTIVATION OF TISSUES IN VITRO AND ITS TECHNIQUE.*

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PLATES XXXVIII-XLVI.

I. DEFINITION.

A culture consists of a plasmatic medium inoculated with small fragments of living tissues. It is essentially characterized by an active growth of the cells from the original fragment into the plasmatic medium. Epithelial or connective tissue cells wander out in great number from the tissue into the coagulated plasma where they undergo direct or indirect divisions. They cover a wide area of the medium, and are often very densely packed. They grow during a period of time which varies from five or six days to more than twenty days, without any evidence of necrobiosis. The cells which have wandered into or have been born in the plasmatic medium can be transplanted into a fresh medium and produce a new and very luxuriant generation of cells. A culture transplanted into the body of an animal can take and grow rapidly. There is no growth of cells when serum is used instead of plasma as culture medium.

Those characteristics distinguish the culture of tissues from the phenomenon known as the survival of cells. The survival of cells outside of the body has been observed by many investigators; especially by Ljunggren (1), Jolly (2), Carrel (3), Volpino (4), and others. These authors placed pieces of tissues in serum or other fluids and observed the survival of the cells and even some mitotic divisions. But there was no active growth, while at the same time marked necrobiosis took place. Volpino (4) claims to have cultivated sarcoma in horse blood serum. In his experiments there

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was no cultivation of tissues according to our definition; there was only a survival of cells.

II. HISTORY.

The idea of cultivating tissues as previously defined is very far from being new. Many experimenters have already thought of the possibility of growing tissues outside of the body, and several have attempted to develop adequate methods for it. But these attempts were generally not recorded in the literature as they always met with failure. In 1897, Leo Loeb (5) stated that he had cultivated tissue cells outside of the body as well as in the body itself. Although thirteen years have elapsed since his announcement, he has not yet given the results and the technique of his method of cultivation of tissues outside of the body. In 1902, he published his researches on a second method; namely, the cultivation of tissue inside the body. In a series of ingenious experiments, he placed fragments of the skin of embryo guinea pigs in agar and in coagulated serum, and he inserted them into adult guinea pigs. He observed wandering and mitosis of the epithelial cells. In these experiments, the tissues and their media were grafted into a living organism and impregnated with its fluids. They cannot be considered as being strictly equivalent to a culture. Therefore, it is certain that the cultivation of tissues outside of the organism was accomplished for the first time by Harrison, in the Anatomical Department of Johns Hopkins University. In 1907, Harrison (6) demonstrated in a series of splendid experiments that embryonic tissue of the frog, transplanted into coagulable lymph, will develop normally. The central nervous system of a frog embryo, covered with fluid from the lymph sac of an adult frog, produces long nerve fibres. These experiments demonstrated that the nerve fibres are really an outgrowth from a central neurone. But they demonstrated also a very much more important fact, the possibility of growing tissues outside the body. At this time one of us (Carrel) was engaged in the study of the laws of redintegration of the tissues of mammals, and these researches required a method permitting the cultivation, with constant positive results, of mammalian tissues outside the body. Therefore he resolved to use
for this purpose the method of Harrison, after it had been adapted to the culture of mammalian tissues. Then, at Yale University, and under the guidance of Professor Harrison himself, Burrows (7) began to acquire and improve the technique of tissue cultivation by using plasma from the blood instead of lymph. Afterwards, he succeeded in adapting the method to the cultivation of tissues of the chick embryo. After having established this very important modification, he cultivated outside of the body the central nervous system, the heart and the mesenchymatous tissue of the chick embryo, or in other words, of a warm-blooded animal. Then in September, 1910, at the Rockefeller Institute, we succeeded in cultivating \textit{in vitro} adult tissues of mammals. The technique was rapidly improved as to details, and the results became practically uniformly positive. We used, at first, the culture method of Harrison, that is, of small pieces of tissue suspended in a hanging drop of plasma. Afterwards, we developed a method of culture on a plate, which permitted us to grow large quantities of tissues. In the intervening few months, it became, therefore, possible to observe many new facts. It was found at first, that almost all the adult and embryonic tissues of dog, cat, chicken, rat, and guinea pigs could be easily cultivated \textit{in vitro} (8). According to their nature, these tissues generate connective or epithelial cells, which grow into the plasmatic medium in continuous layers, or in radiating chains (plate XXXVIII, Fig. 1). The tissue fragments may surround themselves completely with dense new tissue, or, on the contrary the new cells may spread over the surface of the medium. We observed the direct division of the nuclei during the life of the cells, and many karyokinetic figures in the fixed and stained cultures (plate XXXIX, Fig. 2). Other experiments showed that the life \textit{in vitro} of the tissues, which varies from five days to about twenty days, can be prolonged by secondary and tertiary cultures (9), and that a second generation of thyroid, splenic, and sarcomatous cells can be obtained from cells which have developed outside the body (10). We succeeded quickly also in cultivating malignant tissues such as the Rous (11) chicken sarcoma (Fig. 1), the Ehrlich (plate XL, Fig. 4; plate XLI, Fig. 5; plate XLII, Fig. 6) and Jensen sarcoma of the rat, a primary carcinoma of the breast.
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(dog), and two human tumors, a sarcoma of the fibula (12), and a carcinoma of the breast. A culture in vitro of the Rous sarcoma transplanted into a chicken, caused the development of a sarcoma. Meanwhile the method has been applied successfully in the Laboratory of Professor MacCallum by Drs. Lambert and Hanes (13), who cultivated the Ehrlich sarcoma of the rat. In December, 1910, and in January, 1911, we applied the method of cultivation of tissues in vitro to several problems of the redintegration of normal tissues and of the biology of malignant tumor (14). Dr. Ruth found that fragments of skin with a small open wound in the center undergo in vitro a process resembling normal cicatrization. This new method of observing cicatrization of tissues outside of the body is very valuable for the study of the redintegration of normal tissues.

Dr. Jolly (15) of the Collège de France, in a recent communication to the Society of Biology of Paris, announced that we had not succeeded at all in cultivating tissues in vitro, and that we had observed only necrobiosis of the tissues and survival of a few cells.

III. TECHNIQUE.

The growth of tissue cells is obtained when small fragments of living tissue are placed at the proper temperature in fluid plasma, which will coagulate immediately. The cultures belong to two types: the small cultures in a hanging drop, similar to those of Harrison, and the large cultures in the surface of a plate, which can be compared to the plate cultures of bacteria. Theoretically, the technique is very simple, and it is very easy to obtain some growth in vitro of the tissues. But in order to obtain results which are uniformly positive, and which can be used for comparisons, the technique must be more elaborate in its details.

Tissues, especially the higher adult mammalian tissues, are easily killed by drying, chilling, and rough handling during the preparation of the culture. Bacterial infection is also detrimental to tissue growth. A rigid asepsis is necessary for the preparation of any tissue culture. The culture must be made in a warm humid operating room with the same care and rapidity as a delicate surgical operation. If the method is to give uniform results, not only must
the above precautions be closely followed but also the perfect teamwork of well-trained assistants is necessary.

In the following we shall describe the preparation of plasma, of the tissues, of the culture, and the methods of observing the growth of the cells.

1. Preparation of the Plasma.—The plasma is prepared from the blood of the animal whose tissues are to be cultivated or from another animal from the same species. Pure plasma or oxalated plasma can be used. Pure plasma gives far better results, and is to be preferred to oxalated plasma.

Pure plasma is prepared by a method similar to that used by Delezenne and by Gengou. The blood is taken from an artery or from a vein. When dogs, cats, chickens, guinea pigs, and rats are used, the carotid artery is ordinarily selected. For human beings, the blood is easily obtained from one of the superficial veins of the arm. The animal is etherized and the vessel is exposed and dissected from the surrounding tissue. The wall of the blood vessel is rubbed with dry gauze, and covered with olive oil, the circulation is then interrupted by a serre fine, the vessel wall is opened laterally, and a glass cannula, previously sterilized in olive oil is inserted into the lumen of the vessel. It is also possible to use on human beings a needle sterilized in olive oil and inserted through the skin into the vein.

The blood is collected in small tubes, carefully coated with paraffine, which have been previously cooled at 0° C. The tubes are immediately corked, placed in large tubes filled with ice, centrifuged for five minutes and deposited in a small ice-box at 0° C. The supernatant plasma is removed with pipettes coated with paraffine. It is generally used immediately, but it can be preserved for some time in a fluid condition if it is kept at a low temperature. Chicken plasma can be preserved for more than one week, human and dog plasma for a few days, while rat plasma always coagulates after a few hours.

Oxalated plasma was also used by Burrows (7) in his earlier work on the chick embryo. Sufficient blood was added to a 1 per cent. solution of sodium oxalate, making the solution 0.1 per cent. At the time of use the sodium oxalate was precipitated quantitatively
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from this plasma by the addition of calcium chloride, after which coagulation occurred. Although oxalated plasma does not give as good results as pure plasma, it can be used in cases of necessity.

2. Preparation of the Tissues.—The tissues used for cultures must be in normal condition. They are best if taken directly from the living animal or from an animal immediately after death. Positive results can still be obtained, however, when the tissues have been deprived of circulation for more than thirty minutes, but it is always better to put the tissues in the plasma as soon as the circulation is interrupted. With a cataract knife and a fine needle, a small fragment of tissue is dissected from the animal and placed on a glass plate. This piece of tissue is rapidly cut into small pieces about the size of a millet seed and transferred on the point of a needle to the surface of a cover glass. For the large cultures, the tissue is cut into small pieces with sharp scissors, or what is still better, into thin, broader pieces with a razor.

It must be remembered that Cristiani has demonstrated that a small piece of thyroid dies if exposed to the drying action of the air for more than ten seconds. Therefore, the section and the handling of the tissues must be very rapid, otherwise the tissue is killed. The dissection of the tissue may be made in a drop of serum, in order to prevent that accident.

3. Preparation of the Culture.—Two types of cultures have been prepared, the small hanging drop culture and the large plate culture. The small cultures are similar to those used by Harrison (6). One or two small pieces of tissue are transferred to a cover glass and quickly covered with a drop of plasma. It is best to spread the plasma in a thin layer over the cover glass. This is done with the needle before coagulation occurs. The cells grow, then, in a few planes (plate XLIII, Fig. 7) and in areas about the tissue. If the drop is thick the cells grow in many planes and it is difficult to measure the area of growth or to photograph and observe the growing cells. The cover glass is then inverted over a hollow slide to prevent the drop from touching the bottom, and sealed to the slide with paraffin to prevent drying. The finished slide is immediately placed in a small electric incubator which is used for transferring the cultures from the operating room to
the large incubator in the room where the study of the cultures are made. Coagulation of the plasma takes place either immediately upon the addition of the tissue or soon after the slides are placed in the warm oven.

To grow tissues on a large scale, the same general technique is used. A rigid asepsis here is most necessary as it is very easy to infect these large cultures. An entire chicken fetus of fifteen days, or small mammalian fetuses cut into small fragments may be used for these cultures. These fragments are spread in a thin layer over the surface of a large black glass plate and covered quickly with fluid plasma. As soon as coagulation of the plasma has taken place, the plates are placed in glass boxes with cotton sponges soaked in water, which preserve the proper humidity (plate XLVI, Fig. 11). The boxes are then carefully sealed with paraffine and kept in such a position that the fluid products of the culture may drain to the bottom.

4. Preservation and Observation of the Cultures.—During their growth, the cultures can be removed from the incubator for a few seconds without danger to their life. Certain tissues, like malignant tumor or spleen (Fig. 1 and plate XLIII, Fig. 7), grow and extend so widely that their condition can be observed without the use of the microscope. On a hollow slide, the new tissue of a culture of spleen appears as an opalescent area surrounding the primitive fragment. Even the beginning of growth can be diagnosed by the appearance on the sharp edges of the fragment of a very faint and narrow gray band. In the culture on plates the appearance of a whitish color around the fragments of the tissues shows that they are growing. But it is safer to make a few control cultures in hollow slides and to observe their growth with the microscope.

For the study of the cultures, we use a microscope placed in a warm stage, the temperature of which is kept constant. The slides can be kept under the microscope for a long time, if necessary, without any danger to the life of the tissue. Before the beginning of the growth, the fragment of tissue appears as an opaque, sharply outlined mass in the clear medium. In the surrounding clear medium, the growing cells are easily detected. They appear as fusi-
form or polygonal bodies isolated or united by filaments or densely packed together (Fig. 7 and plate XLIV, Fig. 8). Generally the cytoplasm is filled with refractile granules. The nucleus stands out as a clear and homogenous area (plate XXXIX, Fig. 3). It contains one or more darker nucleoli. When the cells grow in continuous layers, for instance in cultures of thyroid gland (plate XLV, Fig. 10), their individual outlines cannot be observed. They appear as a layer of small granulations, surrounding a great many clear spots. When the cells grow in continuous layers, for instance in cultures of thyroid gland (plate XLV, Fig. 10), their individual outlines cannot be observed. They appear as a layer of small granulations, surrounding a great many clear spots. When the culture is fixed and stained by hematoxylin, the outlines of the cells become distinct, and the clear spots appear to be the nuclei of the cells (Fig. 3). Often the movement of the living cells, their modification in shape, and the division of their nucleus can be readily and directly observed. Nuclear budding with formation of multinuclear cells have frequently been observed in the spleen (plate XLIV, Fig. 9).

Camera lucida drawings of the cells can be made when the tissues develop slowly like cartilage or peritoneum. But even in these cases, the motion of the cells and the changes in their shape require that the sketches be made rapidly. The growth of sarcoma or of spleen is often so rapid that it renders impossible an accurate camera lucida drawing.

The best method of recording the morphology of the living cultures is to photograph them. But this is often very difficult, because the new tissue is dense or the cells are faintly seen; and chiefly because the cells do not grow on the same plane. Generally in a very actively growing culture no cell can be seen distinctly. They are disposed in chains closely packed and radiating from the original fragment as a center (Fig. 1). Even when the outlines of the cells can be distinguished easily under the microscope, a sharp photograph of them may be impossible if they are surrounded by cells which have grown on slightly different planes. Mitotic figures have never been detected in a living culture, and they have become visible only after staining the fixed specimen (Fig. 2).

For exact cytologic study, the cultures are fixed and stained. The cover glass, to which the culture is adherent, is separated from the hollow slides and immersed in corrosive sublimate, acetic acid, or formalin, or the various preparations of potassium bichromate.
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solutions. Afterwards, they are stained in hematoxylin. When the culture medium is spread on the cover glass in a very thin layer, and when the culture is not too old, the cells appear very distinctly and all their structural details are easily observed (Figs. 5, 6, 8, 9, 10). In many places, beautiful mitotic figures are present (Fig. 2). When the plasmatic medium is thick, and when the cells have grown in many different planes, serial sections of the hardened culture are required.

The histological characteristics of the large cultures on glass plates can be studied only by serial sections. The purpose of a culture on a plate is not the observation of the morphology of the cells, but the study of the dynamic changes undergone by the cells during their life outside of the organism, and the nature of their secretions.

When the technique is applied carefully in all its details, the results of the cultures are practically uniformly positive. If some of the details are neglected, the tissues do not grow or their growth is altered. Great accuracy in the technique is required when the method of cultivation of tissues in vitro is employed for the study of such important problems as the redintegration and growth of normal tissues and the growth of malignant tumors.

**BIBLIOGRAPHY.**

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EXPLANATION OF PLATES.

PLATE XXXVIII.
Fig. 1. Living culture of the Rous chicken sarcoma, twenty-four hours old. The central opaque mass represents the original fragment of tissue. The new cells are radiating in great numbers from the tissue. The irregular outer dark areas are reflections from water of condensation on the bottom of the culture.

PLATE XXXIX.
Fig. 2. Culture of Wolfian body of a chick embryo. Mitosis of the new-grown cells. Stain hematoxylin.
Fig. 3. Isolated living connective tissue cells. The cytoplasm of these cells is filled with refractile fat granules. Nucleus is the clear oval area. In some of the nuclei faintly staining nucleoli can be made out.

PLATE XL.
Fig. 4. Culture of the Ehrlich rat sarcoma. The central and completely opaque mass is the original tumor fragment. The new cells are arranged irregularly throughout the surrounding medium. Stain hematoxylin.

PLATE XLI.
Fig. 5. Small area of the new-grown cells of the living culture of the Ehrlich rat sarcoma shown in Fig. 4.

PLATE XLII.
Fig. 6. Photograph of the culture of the same cells as seen in Fig. 5. Stain hematoxylin.

PLATE XLIII.
Fig. 7. Culture of spleen.

PLATE XLIV.
Fig. 8. Same culture as Fig. 7. An area of the new-grown connective tissue cells. Stain hematoxylin.
Fig. 9. Isolated cells from a culture (Fig. 7) of adult spleen (chicken). The cells are chiefly multinuclei and filled tightly with large fat granules. Stain hematoxylin.

PLATE XLV.
Fig. 10. Culture of thyroid (adult dog). A layer of epithelial cells spreading out from the border alveoli of the thyroid. Isolated connective tissue cells are seen in the clear medium beyond. Stain hematoxylin.

PLATE XLVI.
Fig. 11. Large plate culture in its sealed moist chamber.
Fig. 1.
FIG. 6.