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TISSUE CULTURES IN BIOLOGICAL RESEARCH

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Preface

Thirty years ago—in 1931, to be exact—one of us was learning the technique of tissue culture from Amédée Borrel at the Institute of Hygiene of the University of Strasbourg, the one European source of knowledge at that time for anyone wishing to learn this technique, which was still considered a luxury.

Those were difficult times for tissue culture. Antibiotics did not exist and cultures easily became contaminated. Experiments based on cultures were often ruined by the inevitable unforeseen accidents that always occur when new techniques are introduced. But the cultures that were successful were brilliantly, and so provided a basis for observations that have been confirmed by the modern techniques now accessible to everyone.

Work with tissue cultures is now within the scope of any laboratory, and the use of this tool is becoming more and more important in the field of biology; tissue cultures are already indispensable to cytologists, geneticists, bacteriologists, virologists, parasitologists, entomologists, immunologists, pathologists, pharmacologists, radiobiologists, zoologists, physicians and veterinarians.

In the belief that further diffusion of tissue culture techniques would be encouraged, we have collected in this volume information that can be useful to all who wish to learn about tissue culture—theories, techniques and practical uses. An extensive and precise bibliography is included to facilitate the study of the sources.

It must be remembered that the field of tissue cultures is rapidly expanding and developing. What is true today may be superseded tomorrow. In this book we have tried to collect what is currently established in the theory, methodology, and practical applications of tissue cultures. If the careful reader finds deficiencies, omissions or mistakes in the book we beg his forgiveness; we also ask his cooperation

in communicating his observations, suggestions, and criticisms to us, and for this we offer our thanks in advance.

Rome, 1962

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PART 1

The cells in culture

CHAPTER I

General Considerations on Cells in Culture

To grow cells nowadays is as easy an endeavor as to grow any microorganism, as we will see in the following pages. It can be accomplished in any laboratory and for innumerable purposes. Workers who prepare themselves to use tissue culture must remember that, while these techniques facilitate and open up new possibilities in biological research, they also often make this research more complex and difficult.

A cell in culture is the most elementary manifestation of life of a superior animal. It is the most simple expression of life itself. A cell separated from the tissue to which it belonged and from the organ in which its functional activity was incorporated becomes free of all external influences, humoral, hormonal and neural. This cell in culture is a simple, vital element, that is an element that lives a life of its own without the influence of any organized structure.

On the basis of their biological characteristics, cells in culture have been used as a living substrate for the growth of parasitic microorganisms, such as viruses, that cannot multiply in an extracellular environment but are able to grow only by utilizing the metabolic machinery of the host cell. It is for this reason that tissue cultures have assumed such a high degree of importance nowadays in virological research. But the function of tissue culture is not limited to serving as a substrate for the growth of parasitic organisms. Cells in culture can and must form the basis for morphological, physiological, pathological and pharmacological studies. Innumerable examples can be furnished.

A chicken heart cell, separated from the animal, the organ and the tissue to which it belonged, continues to live in culture and to contract rhythmically. It continues to pulsate, and the pulsations are not regulated by the central nervous system, are not influenced by hormonal activity and not sustained by humoral products. These pulsations are

the primitive manifestation of a differentiated cardiac cell, independent of any other factor or external influence.

The physiologist will find in these cultures a source of research and of meditation, and not only the physiologist, but the pharmacologist as well. If the latter wishes to test the direct effect of a medicament on the cardiac cells, he will be able to do this only by testing this drug on the cells in culture. He will then be able to know the mechanism by which the drug acts on cardiac cells without the intervention of external elements of more specific regulation.

The bacteriologist who wishes to study the pathogenic action of a microorganism, such as Koch bacilli, at the most simple level can do so only on cells in culture. He will then be able to see how the toxin of this mycobacterium acts on the cells, how the cells are modified and change and finally die. He will be able to interpret the mechanism of the formation of the epithelioid cells and the giant cells. But he will never be able to study the formation of the tubercle that represents the collective reaction of several cells types in more complex tissues.

The immunologist, who knows that infinitesimal amounts of tetanic toxin can kill a man, will be surprised to observe that the tetanic toxin is inactive on cells in culture.

The pathology of a cell in culture does not always correspond to the pathology in tissues formed by these cells or the organs formed by these tissues. There surely exists a cellular pathology that precedes the tissue pathology but it is not identical to it, just as the pathology of the tissue cannot be confused with the pathology of the organ or of the whole living being, as in clinical medicine.

This is why we have stated that tissue culture on the one hand facilitates, extends and renovates biological research and on the other hand makes it more difficult and arduous.

We still have to discover the mechanism of the primary action of microorganisms, toxins, hormones, humoral factors, drugs, and radiation on isolated cells suspended and actually growing in a medium. We still have to learn the general pathology of cells in culture, the primary basis for a better knowledge and understanding of the more complex phenomena occurring in tissues and organs within an organism.

This general pathology of the cells must be preceded by knowledge of the physiology of the cells in culture, a physiology that is pure and abstract: physiology of the cardiac cells, physiology of the hepatic

cells and of the muscular cells and so forth, *i.e.* physiology of the cells and not of the tissues.

For physiologists, pathologists, microbiologists, and pharmacologists, cells in culture represent the only possibility of conducting experiments with living cells of human origin.

But cells in culture are cells in revolt, cells that no longer obey the laws established for the organization of cells in tissues. Once free of the constrictions of collectivity, the cells no longer correspond to the organ from which they originated. Their specific physiological function ceases, the fixed number of chromosomes characteristic of any animal species changes, the atypical nuclear multiplications increase; thus at a certain point it is quite difficult to identify the type of cells with which one is working, since they can no longer be recognized on the basis of tissue of origin, and it is quite difficult to differentiate between normal and tumor cells.

We are only at the beginning of this new science of the cells, and only through the accumulation of observations we will be able to establish the foundation of the new science that will be the basis for any future biological research: *biocytology*. This is a science that studies the life of isolated cells liberated from the rules that govern tissues and organs in the life of a metazoan animal in all its complexity.

This new science of biocytology poses not only new technical and biological problems but also opens new possibilities for investigation of the eternal problem of life and death.

Cells in culture are living cells, with an active anabolic and catabolic metabolism; they are cells that multiply, giving origin to new cells, and this multiplication, theoretically infinite, goes on for generations and generations.

Living cells originate from living organisms, but they can also come from an organism that has died, such as an animal killed before removing the organ from which the cells were taken to be put in culture. This is the technique that is applied every day in growing poliomyelitis virus for the preparation of vaccine. The monkey is killed, the kidney is removed and fragmented and from these fragments come the cells on which the poliomyelitis virus is grown. These are live kidney cells derived from an organ that has been destroyed following removal from a dead animal. This can be done not only with cells from animals but also with cells from human beings. In our laboratories,

we are now able to maintain alive and multiplying cells of individuals who are no more. Their organized life is finished, their cellular life continues.

For the biologist, there is life in the cells that multiply in the test tube, cells that originated from an individual who is dead now.

What is life? What is death?

The life of a unicellular animal is the complex of the functions that regulate its preservation and multiplication. The death of a unicellular animal is the complete and irreversible cessation of these functions. From a unicellular animal that is dead, it is not possible to recover vital elements, *i.e.* particles able to maintain themselves and multiply; only biologically inert and chemically active products can be extracted.

The life of a multicellular animal is also characterized by a complex of functions for the maintenance and reproduction of the animal, but these functions are dependent upon a complex of organs harmoniously coordinated and subordinated to the whole organism, and for this purpose single organs exist. The death of a multicellular organism is the complete and irreversible cessation of the functions of maintenance and reproduction of the animal in his wholeness, *i.e.* cessation of the function of coordination and harmonious correlation between various constituent organs. But this systematic death is not the death of the single parts.

From a multicellular animal that is dead, vital elements can be isolated, tissues and cells still able to maintain themselves and multiply.

There exist, therefore, two types of death, or, better, two types of life of a multicellular animal: the life of the organism, *i.e.* the life of the organs related harmoniously among themselves and manifesting the characteristics of a particular biological species, and the cellular life that survives the life of the organism and is a purely vegetative and material manifestation.

In our laboratory, we work with cellular life that, although removed from the conscious, spiritual life, was part of this life and formed its indispensable substrate. Let us not forget this fact in our experiments when we work with human cells. These cells were part of a human being, once alive and now dead, and they survive beyond his death. "I am confident that there truly is such a thing as living again, and that the living spring from the dead." (Phaedo)

CHAPTER 2

History of Tissue Culture

Biologists of the 19th century had already realized that the death of the organism is not necessarily accompanied by the death of its single parts. Claude Bernard, in 1878, had formulated the theoretical principles for the creation of an artificial system in which organs could survive outside the influence of the whole organism.

The first successful transplant *in vitro* was done by Wilhelm Roux (1885), who kept alive a chick medullary plate in a physiological solution. He was also able to demonstrate in this system that the closure of the medullary canal was due to the direct activity of the cells and not to physical compression of the surrounding structures.

The era of tissue culture had then begun, and Arnold, in 1887, succeeded in studying the survival and migratory activity of frog leukocytes maintained in fragments of alder pits soaked in a physiological solution.

There is a 10-year lapse between these experiments and those of Ljungren, who, in 1898, kept fragments of human skin alive for several days and weeks in ascitic fluid.

In 1902, Loeb published the results of experiments he had begun 5 years previously. He described how he was able to maintain, in the subcutaneous tissue of a rabbit, small sections of guinea-pig skin embedded in blocks of agar or coagulated plasma.

In 1903, Jolly cultivated leukocytes of the salamander for over a month in a hanging drop and was able to follow their division for several generations.

Beebe and Ewing, in 1906, grew explants of a dog lymphosarcoma in serum of immunized and non-immunized animals.

The first successful tissue culture—in the modern sense—was realized by Ross Harrison in 1907. He removed aseptically small fragments from the wall of the frog neural crest and cultivated them in coagulated

frog lymph. In this system active growth continued for several weeks, and he observed growth of nerve fibers from the central body toward the periphery of the explant. Harrison not only described the technique used and the results obtained, but suggested that such a technique could be of great help in the solution of several important biological problems.

Burrows, one of Harrison's students, developed a new technique using coagulated plasma for the growth of chicken embryo cells (1910). This medium was suggested to him by the observation that isolated fragments of skin transplanted on a wound were able to grow and reach the edges of the wound, provided that fibrin was present. With this technique Burrows was the first to observe and describe mitosis *in vitro* and to point out that cells were able to grow as far as the limit of the coagulated plasma.

In collaboration with Carrel in the following years (Carrel and Burrows, 1911) he discovered that embryo extract has a strong growth-promoting action for certain cells. In this way, the technique of tissue culture with embryo extracts on coagulated plasma was introduced in several laboratories. The technique is very simple and is still widely used in the following manner: explants are fixed in plasma on a cover slip. This is mounted upside down on a Koch slide.

At about the same time M. and W. Lewis (1911) introduced the first liquid medium with some controlled components, such as different salts and peptones. They also used sea water, serum and aqueous embryo extracts in different concentrations. In these media the cells grew as a thin layer, excellent for microscopic observation, but only for a short period.

It was A. Carrel who led the advances in tissue culture technique. He immediately realized (1912) the great possibilities that were offered by applying to these techniques the principles formulated by C. Bernard.

Carrel's first contribution was the introduction of surgical methodology in the handling of tissues, particularly aseptic and antiseptic techniques. Later, by himself (1913, 1923, 1929, 1931) and in collaboration with Baker (Carrel and Baker, 1926; Baker and Carrel, 1928), he studied the composition of media by identifying the different substances favoring cell growth. Finally, he perfected the instrumentation for tissue culture, inventing the flask that bears his name and is still in use.

The great importance of Carrel's work resides in the fact that it

demonstrated for the first time that any type of tissue can be maintained and grown *in vitro* for an indefinite period of time through successive subcultures. For almost 30 years he was the apostle of this technique and expounded its importance for the study of biological problems. The only drawback, if any, in his writing was the constant emphasis on very meticulous surgical procedures to ensure asepsis. These procedures were too expensive to be easily followed and required instruments which were not currently available. Thus, for several decades tissue culture was considered a difficult and expensive methodology, the privilege of a few select laboratories. But Carrel's methods were, and still are, sound, and for a number of years they have been the only ones available.

In later periods, several other authors worked along the same lines as Carrel, introducing technical improvements and using new culture media. Burrows and Neymann (1917) studied the effect of egg yolk hydrolysates. Baker, in 1929, introduced casein hydrolysates. Willmer and Kendal, in 1932, demonstrated the necessity of introducing numerous amino acids in the growth medium. Vogelaar and Ehrlichman, in 1933, successfully introduced the first synthetic medium composed of peptone, hemin, cystine, insulin, thyroxin and glucose. This medium was later improved by Baker (1936) and Ebeling (1939).

The search for better synthetic media was pursued with considerable success by Simms (1936), Fischer (1939, 1941, 1946, 1948, a, b, 1949) and collaborators (Fischer and Astrup, 1942; Astrup, Fischer *et al.*, 1945; Fischer, Astrup *et al.*, 1948) and by White (1946). Of particular importance has been medium 199, introduced by Morgan *et al.* (1950). All synthetic media commonly used nowadays originate from these studies.

While considerable effort was spent in the study of metabolic requirements of cells growing *in vitro*, new techniques were introduced to facilitate the growth of large masses of cells. Thus, Gey, in 1933, used roller tubes for growth of cells. This method was further improved by Lewis (1935) and by G. Gey and M. Gey (1936) and has been widely and very successfully utilized ever since.

The introduction of tissue trypsinization by Moscona in 1952 is another landmark in the history of tissue culture technique. This author digested a fragment of chicken embryo in a 3% solution of trypsin and observed that the cellular clumps obtained were still able to grow *in vitro*. Actually, Moscona's observation was preceded by that of

Rous and Jones, who, in 1916, digested with trypsin a plasma coagulum in which a tissue explant had been grown. The cells obtained after trypsin digestion could still be grown in a new coagulum. The observations of Rous and Jones did not find a practical application until Vogelaar and Ehrlichman, in 1933 and 1939, utilized trypsin to detach cells from the walls of roller tubes.

This has been the evolution of the techniques for culture of cells *in vitro*, but it cannot be fully understood without taking into consideration the use of tissue culture in the field of general biological research.

Cancer was one of the first problems for which tissue culture has been extensively used. Volpino, in 1910, cultivated *in vitro* cells from mouse adenocarcinoma and found that these cells reproduced tumor when reinoculated in the mouse. Carrel and Burrows (1911a) and Lambert and Hanes (1911) grew *in vitro* chicken Rous sarcomata which also were transplantable into the homologous species; nevertheless, this initial research did not shed much light on the cancer problem. Actually, the use of tissue culture somewhat complicated the problem when Fischer, in 1926, and Bisceglie, in 1928, observed malignant transformation of cells *in vitro*. This phenomenon was later confirmed by several other authors. Des Ligneris, in 1935, produced 'cancerization' *in vitro* of normal cells after treatment with dibenzenanthracene. Experimentation with carcinogenesis *in vitro* is directly connected with the study of tumor viruses. The literature on this subject is quite abundant, from the initial work of Carrel (1924, 1926) to the more recent work of Stewart and collaborators (1957, 1958) with polyoma.

The problem of antibody production was investigated early in the development of tissue culture. Carrel and Ingebrigtsen (1912), successfully studied the formation of antibodies (hemolysin) *in vitro*. He was followed by Lüdke, who, in 1912, demonstrated the production *in vitro* of agglutinin for *Salmonella typhosa* using explants obtained from animals vaccinated with this bacterium. Similar results were obtained by Przygode (1913), Reiter (1913) and, more recently, by Meyer and Loewenthal (1927), Parker (1937) and Salle and McOmie (1937).

Another problem that was investigated quite early in the development of tissue culture was the action of bacterial toxin on the cells. Levaditi und Mutermilch, in 1913, and Okabe and Teruuchi, in 1930, studied neutralization of toxin with homologous antitoxin in cells

in vitro. These problems have been reinvestigated with more modern techniques by Penso and Vicari (1957). It is now possible to use tissue culture for very precise dosimetry of toxin and antitoxin.

The first attempts to cultivate viruses in cells *in vitro* were made by Steinhardt, Israeli *et al.* In 1913, they demonstrated that the vaccinia virus could survive for over a month in fragments of rabbit cornea maintained in coagulated plasma. These experiments with vaccinia were continued by other workers (Parker and Nye, 1925; Maitland and Maitland, 1928; Li and Rivers, 1930). Ever since, others have worked with numerous viruses: Carrel (1924) with Rous sarcoma virus; Findlay (1928) with avian vaccinia; Andrewes (1929) with virus III; Parker and Nye (1925a), Rivers, Haagen *et al.* (1929) and Andrewes (1930) with herpes; Maitland and Maitland with foot-and-mouth disease virus (1931); Hallauer (1931) with fowl plague; Haagen and Theiler (1932) with yellow fever; Bland and Canti (1935) with psittacosis; Sabin and Olitzky (1936) with poliomyelitis; Parker and Hollender (1945) with rabies; Koprowski and Lennette (1946) with West Nile encephalitis. After the work of Enders, Weller *et al.* in 1949 on the growth of poliomyelitis virus, the use of tissue culture for biological studies has become universal.

The idea of producing vaccine by growing virus in tissue culture was first conceived by Carrel and Rivers, who, in 1927, produced a vaccinia vaccine with this technique. Rivers and Ward, in 1935, first reported results of vaccination done with vaccinia grown *in vitro*. Since then viral vaccines produced *in vitro* have been in common use, particularly the poliomyelitis vaccine developed by Salk in 1953.

Throughout the years tissue cultures have been utilized in connection with many types of biological problems. Tissue cultures were used by Levaditi and Mutermilch (1913b) for the study of snake poison; by Lewis (1920) for bacteriological studies; by Champy (1922) for hormone research; by Hogue (1928) for protozoology; by Bucher (1940) for toxicology; by Fell (1940) for embryology; by Pomerat, Drager *et al.* (1946) for pharmacology; by Brues and Stroud (1951) for radiobiology; by Penso *et al.* (1961) for the standardization of antiviral chemotherapy.

The literature on the use of tissue culture is increasing every year, demonstrating that this technique has become indispensable for any biological research at the present and in the future.