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An Introduction to Animal Tissue Culture

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General Preface to the Series

It is no longer possible for one textbook to cover the whole field of Biology and to remain sufficiently up to date. At the same time teachers and students at school, college or university need to keep abreast of recent trends and know where the most significant developments are taking place.

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Reader's comments will be welcomed by the author or the Education Officer of the Institute.

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Preface

The cultivation of animal tissue *in vitro* was first shown to be a significant experimental procedure in 1907. Since then, tissue culture techniques have been refined, extended and applied by an increasing number of biologists, and tissue culture is now one of the most fruitful methods in biological and medical research. This short account of the basic knowledge and skills required for the successful cultivation of animal cells and tissues is designed to help the reader to appreciate the scope and limitations of some of the most commonly used culture techniques. Perhaps their greatest virtue is that they allow us to study the structure and behaviour of *living* cells, and I have included a brief discussion of some of the ancillary methods, such as phase contrast microscopy, which must be used to obtain information of scientific value from tissue cultures. The observation and interpretation of the activities of cultured cells is, to me, a source of unending fascination, and I have tried to convey a little of this in the final chapter, which illustrates some of the characteristic features *in vitro* of cells from each of the basic histological tissues.

Leeds, 1977

J. A. S.

Contents

1 Introduction

1.1 The birth and growth of tissue culture

At the beginning of this century, biologists who were interested in the development of the nervous system held conflicting views about the way in which the axons of nerve cells were initially formed in the embryo. The dispute had been going on for a long time; in 1886 the embryologist Wilhelm His had postulated that the primitive embryonic neuron or neuroblast produced its axon by the outgrowth of a process from its cytoplasm, which continued to elongate until its advancing tip made contact with a peripheral sense organ or muscle fibre. This view was supported in 1890 by Santiago Ramon y Cajal, who studied the development of neurons by applying a silver impregnation technique to sections of embryonic nervous tissue. The opponents of His and Cajal adhered to the 'cell-chain' theory, which proposed that the axon was produced by the fusion of a continuous chain of initially separate cells extending from the neuroblast to the peripheral structure it was destined to innervate.

An American zoologist, Ross Granville Harrison, was deeply interested in the development of the peripheral nervous system. Having already written a number of scientific papers on the results of his research into the histogenesis of peripheral nerves in the embryos of fishes and amphibians, he had come to the conclusion that His and Cajal were probably correct but, in his own words, 'that the ordinary methods of histology were inadequate to answer definitely the question of the origin of the nerve fiber'. He decided that the best way to solve the problem was to devise a method 'by which the end of a growing nerve could be brought under direct observation while alive, in order that a correct conception might be had regarding what takes place as the fiber extends during embryonic development from the nerve center out to the periphery'.

With this end in mind, Harrison first dissected out fragments of the primitive spinal cord from frog embryos and put them into a saline solution, but the tissue failed to survive and disintegrated. Later, he tried a semi-solid medium, gelatine, but this was also unsuccessful. Then, in the spring of 1907, he devised a method which did succeed. Here is his first description of it, published in the same year:

> 'The method employed was to isolate pieces of embryonic tissue known to give rise to nerve fibers, . . . and to observe their further development. The pieces were taken from frog embryos about 3 mm long, at which stage, i.e., shortly after the closure of the medullary folds, there is no visible differentiation of the nerve elements. After carefully

dissecting it out the piece of tissue is removed by a fine pipette to a cover slip upon which is a drop of lymph freshly drawn from one of the lymph sacs of an adult frog. The lymph clots very quickly, holding the tissue in a fixed position. The cover slip is then inverted over a hollow slide and the rim sealed with paraffine (Fig. 1–1). When reasonable aseptic precautions are taken, tissues will live under these conditions for a week and in some cases specimens have been kept alive for nearly four weeks. Such specimens may be readily observed from day to day under highly magnifying powers.'

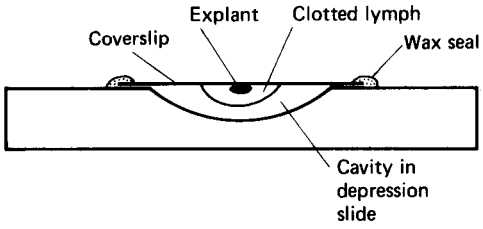

Fig. 1–1 Harrison's hanging drop culture method.

Not surprisingly, a problem Harrison had to overcome was the infection of his cultures by bacteria, which spoiled many of his first attempts. He persisted, and was eventually able to establish a reasonably aseptic procedure, although he found that 'the making ready of the apparatus consumes so much time, and the constant attention to the details of manipulation during operations is so fatiguing, that only a small number of preparations can be made in one day'.

Harrison's patience was rewarded; he succeeded in growing neurons outside the body in a medium (clotted lymph) where all possibility of contributions on the part of other living tissues was eliminated, and was able to see the outgrowth of axons from neuroblasts hour by hour, providing rigorous proof of the theory of His and Cajal (Fig. 1–2).

Harrison was not, in fact, the first person to keep cells alive outside the body. As early as 1885, Wilhelm Roux had explanted pieces of chick embryo into warm saline, where they survived for a few days; Arnold in 1885, and Jolly in 1903, had both transferred leucocytes (from frogs or salamanders) into saline or serum and had observed movement and division of the living cells. But Harrison is now generally regarded as the father of tissue culture because his experiments showed that, by adapting the hanging drop preparation on a depression slide—a technique already used by bacteriologists, it was not only possible to keep tissues alive *in vitro* for several weeks, but also that the procedure was a research method capable of making a fundamental contribution to biological knowledge.

Having succeeded, by ingenuity and patience, in developing a

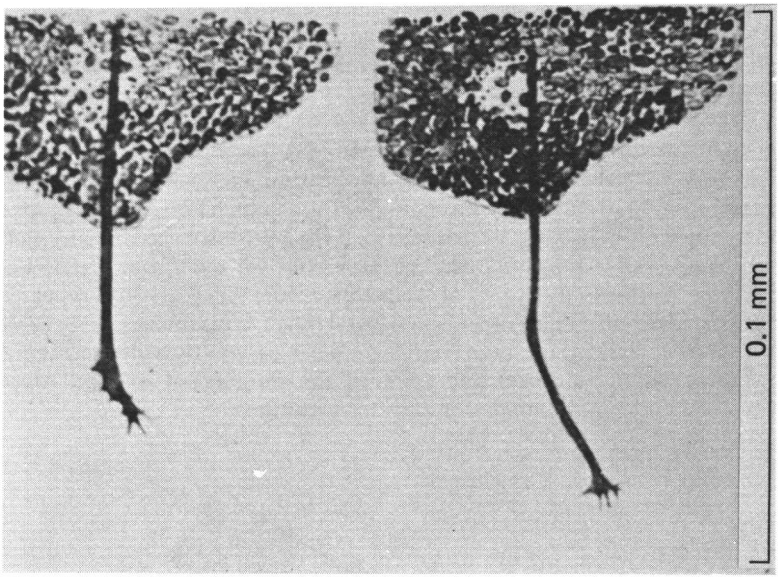

Fig. 1–2 One of Harrison's drawings showing the elongation, over a period of 25 minutes, of a nerve fibre *in vitro*. (Courtesy of the Wistar Institute.)

technique which enabled him to solve his immediate problem, Harrison himself played no further part in perfecting it, though indirectly through his published papers and lectures he made a valuable contribution by attracting the attention of other scientists to the potential importance of tissue culture. Indeed, the first major improvement in the method was introduced in 1910 by an American doctor, M. T. Burrows, who spent a few months of that year working in Harrison's laboratory in order to learn the technique. Burrows was interested in growing the tissues of warm-blooded animals. It was already clear that frog lymph left a good deal to be desired as a culture medium, partly because it did not produce a very firm clot and also because of the difficulty in obtaining it in sufficient quantities. Burrows' solution was to use chicken plasma to support and nourish explants of chick embryo tissues in hanging drop preparations. This proved to be much better than lymph, permitting good growth of nervous tissue, heart and skin.

Burrows then went on to collaborate with his colleague Alexis Carrel in an effort to extend tissue culture to mammalian tissues, and they soon succeeded in growing explants from adult dogs, cats, rats and guinea pigs, and also in growing malignant tissues. In addition, they showed that the life of cells *in vitro* could be prolonged by subculturing, that is by subdividing an established culture and transferring the living cells into

fresh medium. Carrel and Burrows also discovered that, by mixing a proportion of embryo extract (tissue 'juice' extracted from minced chicken embryos) with the plasma, even better survival and growth of their cultures was achieved.

It was Alexis Carrel who was largely responsible for the next phase of refinement of tissue culture procedures and, having been trained as a surgeon, he concentrated on the application of rigid asepsis to the manipulation of cultures. Indeed, the methods which he evolved began to give other biologists the impression that tissue culture was an extremely laborious and expensive business. However, he did show that it was feasible to keep a strain of cells alive for 34 years by repeated subculturing, an achievement which owed much to his invention (1923) of the 'Carrel flask' (Fig. 1–3), which made it easier to avoid accidental infection of the cultures and reduced the number of manipulations involved in the maintenance of long-term cultures.

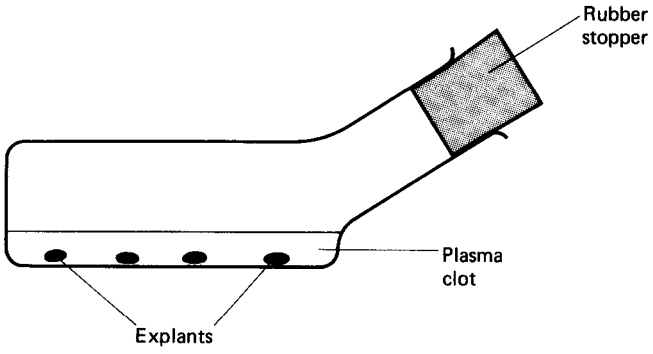

Fig. 1–3 The Carrel flask.

A different aspect of culture technique was investigated by two other Americans, W. H. and M. R. Lewis, who made the first attempt to replace natural media (plasma and embryo extract), whose composition could not be precisely defined, with synthetic media made up of known components. Their efforts, together with those of many others during the next 30 years, led eventually to the development of a number of synthetic media which can now be purchased in ready-made form. These have made it possible to reduce drastically the proportion of natural medium required and even, to a limited extent, to grow cells in a totally synthetic medium of precisely known composition.

The next major development in culture methods occurred not in America but in England, and made it possible to prepare cultures of a quite different kind in which the aim was to maintain small pieces of tissue, or entire embryonic organs, *in vitro* in such a way that their normal

histological structure was maintained and the component cells were discouraged from growing out of the explant in a disorganized fashion. This approach, generally known as Organ Culture, was perfected by Dame Honor Fell at Strangeways Research Laboratory in Cambridge. The method, described in a paper in 1929, involved placing organ rudiments from chick embryos on the surface of a clot formed by mixing chicken plasma and embryo extract in a small watch glass enclosed in a Petri dish containing moist cotton wool (Fig. 1–4). The explants drew their nourishment from the underlying plasma clot and their oxygen from the air to which they were freely exposed, and the tendency for the

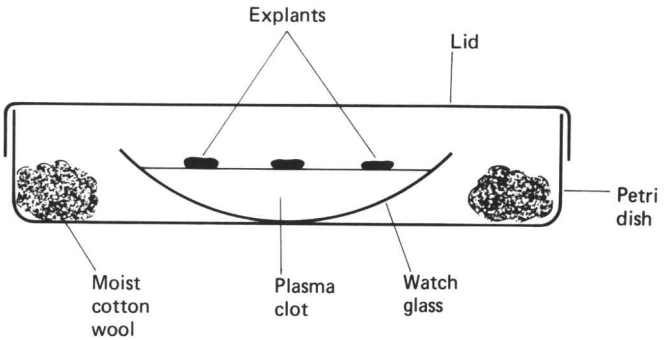

Fig. 1–4 The watch glass method for organ culture.

cells to migrate from the explants was minimized. By using this technique Fell and her collaborators were able to contribute a great deal to our knowledge about the development of bones and joints.

In this brief introduction it has been possible to mention only a few of the many research workers who, during the last 70 years, have contributed to the improvement of tissue culture techniques and have brought them to bear on an increasing range of biological investigations. Some indication of the growth in the application of these methods can be gained from the fact that about 400 titles were listed in a bibliography on tissue culture published in 1927 (covering the two previous years), whereas the *Index of Tissue Culture* for 1974 contains over 30 000 titles of papers involving tissue culture which were included in the *Index Medicus* between January and December of that year. Examples of some of the most significant advances which have stemmed from the utilization of cultures of animal or human cells include the establishment, in 1956, of the true number of human chromosomes and the discovery shortly afterwards that Down's syndrome was due to the possession of an extra chromosome. In the field of virology the availability of cell cultures has simplified the cultivation of viruses and has made a vital contribution to

the development of immunization against poliomyelitis, and more recently our understanding of the mechanisms which produce cell movement has gained immensely from the application of electron microscopy to cells grown *in vitro*.

1.2 Definitions of tissue culture terms

An inevitable result of this expansion was a tendency for confusion to arise about the precise meaning of the various terms used in tissue culture, and this led to the establishment of a committee on nomenclature, which produced a report on 'Proposed Usage of Animal Tissue Culture Terms' published in 1967. Some of the most commonly used terms are defined as follows.

Animal tissue culture: is concerned with the study of cells, tissues and organs explanted from animals and maintained or grown *in vitro* for more than 24 hours. Dependent upon whether cells, tissues or organs are to be maintained or grown, two methodological approaches have been developed.

Cell culture: this term is used to denote the growing of cells *in vitro* including the culture of single cells. In cell cultures the cells are no longer organized into tissues.

Organ culture: this term denotes the maintenance or growth of tissues, organ primordia, or the whole or parts of an organ *in vitro* in a way that may allow differentiation and preservation of the architecture and/or function.

Other terms in common use include the following.

Cell line: arises from a primary culture at the time of the first subculture, and consists of numerous lineages of the cells originally present in the primary culture.

Cell strain: derived by the selection or cloning of cells having specific properties or markers, which must persist during subsequent cultivation.

Clone: a population of cells derived from a single cell by mitosis.

Explant: an excised fragment of a tissue or an organ used to initiate an *in vitro* culture.

Monolayer: a single layer of cells growing on a surface.

Primary culture: started from cells, tissues or organs directly taken from organisms.

Subculture: the transplantation of cells from one culture vessel to another.

Suspension culture: a type of culture in which cells multiply while suspended in medium.

2 Tissue Culture Media

The individual cells in a living metazoan animal exist in an environment which is very precisely controlled. The surface membrane of the cell *in vivo* is exposed to extracellular tissue fluid, from which it obtains the substances essential to its survival such as water, inorganic ions, amino acids, vitamins, glucose, oxygen and so on, and into which it discharges the products of its metabolism. Certain attributes of the tissue fluid are maintained within narrow limits; thus in mammals its pH is held at 7.4, its osmotic pressure is equivalent to that of a 0.9% solution of NaCl, and it is normally sterile. Furthermore, the temperature of the cell's environment is regulated, in birds and mammals, with considerable precision.

The cell *in vivo* is very much dependent on other tissues and organs; on the liver, kidneys and lungs for the control of the composition, pH and osmotic pressure of the tissue fluid, on the haemopoietic tissues for defence against infection, and on the nervous system for the regulation of temperature. When living cells are excised from an animal they are deprived at a stroke of all these physiological supporting and protecting mechanisms. If they are to be kept alive *in vitro* they must then be provided with an artificial environment which resembles their milieu *in vivo* as closely as possible, in much the same way that an astronaut must be enclosed within a protective capsule and equipped with life support mechanisms if he is to survive in outer space.

2.1 The essential features of a culture medium

The composition and properties of the culture medium are the most crucial factors in achieving the successful cultivation of cells *in vitro*, and the medium must fulfil the following essential requirements.

1. Nutrients

It must provide all the salts, amino acids, lipids, carbohydrates, vitamins, etc., needed by the living cell.

2. Buffering capacity

It must contain non-toxic buffers to maintain the pH at 7.0–7.3 in spite of the production of acidic substances by the cells.

3. Isotonicity

The concentration of substances dissolved in the medium must render it isotonic with extracellular fluid. If the medium is hypertonic the cells will lose water and shrink, while if it is hypotonic they will take up water and swell.

4. Sterility

The medium must be devoid of micro-organisms which, if present, will find the conditions in the culture ideal for their rapid multiplication, and they will soon outgrow and destroy the living cells.

Media may be classified into two distinct categories: natural media, which are derived from the body fluids or tissues of animals, and synthetic media, which are precisely defined mixtures of substances dissolved in pure water.

2.2 Natural media

2.2.1 Cockerel plasma

It was seen in the previous chapter that the first successful tissue cultures relied upon natural media, and of these cockerel plasma soon became the most popular. It serves two purposes, providing the nutrients for either avian or mammalian cells and also, by forming a clot, it produces a network of fibrin which gives physical support to the explant and to the cells which grow out from it. Plasma from a cock is preferable to that from a hen, which is subject to considerable fluctuations in calcium content during egg production. Although mammalian plasma can also be used, it suffers from the disadvantage that it produces a clot which is less firm and more prone to detach from the coverslip. It has the additional drawback of forming a coarser fibrin network, impairing the optical clarity of the culture.

Cockerel plasma can be collected fairly readily by withdrawing blood from the wing vein of a healthy young adult bird, using a sterile hypodermic needle and syringe and taking care to sterilize the skin with alcohol before the needle is inserted. The main problem is to prevent the clotting of the blood, but this can be achieved by coating the interior of the syringe with a dilute solution of the anticoagulant heparin, and by transferring the blood quickly from the syringe to sterile tubes containing a little heparin and cooled in ice. The blood is then centrifuged, and the supernatant clear liquid plasma is pipetted into sterile glass containers which are sealed and stored in a refrigerator. When cultures are being set up, a drop of the liquid plasma is usually mixed with a drop of embryo extract, which causes the plasma to clot firmly within a few minutes.

2.2.2 Serum

Although plasma continues to be used in certain types of tissue culture, it has largely been superseded as a natural medium by serum. The species from which serum is obtained is not particularly critical, but humans, calves or horses provide convenient sources and, generally speaking, serum from young donors sustains better growth of cultured cells. Foetal calf serum is often preferred, and has the additional advantage for some experiments that it contains no antibodies (gamma globulins). Human foetal serum can be obtained by collecting blood from the umbilical cord

and placenta after birth and is often used for maintaining delicate cultures of cells from the central nervous system. The preparation of serum is straightforward; blood is collected aseptically and allowed to clot, the clot is broken up and centrifuged, and the clear supernatant serum is transferred to sterile containers and stored in a freezer at – 20° C.

2.2.3 *Embryo extract*

Another natural medium which was widely used in the early days of tissue culture, but which has more restricted application today, is chick embryo extract. This provides a rich source of small molecular nutrients such as amino acids and nucleic acid derivatives and seems to stimulate the migration and mitotic division of cells *in vitro*. As already mentioned, it is often used in combination with cockerel plasma to produce a mixture having good supportive and nutritive properties. Chick embryos which have been incubated for ten days may be taken to prepare the extract. They are removed aseptically from the eggs, placed in a 20 ml syringe and disrupted by forcing them through the nozzle into a sterile tube. An equal volume of balanced salt solution is then added, the mixture is stirred, allowed to stand for an hour or two and then centrifuged. The supernatant liquid is collected and stored at – 20° C.

2.2.4 *Collagen*

There is one further substance which deserves to be mentioned in this brief outline of natural culture media, and that is collagen, which has become increasingly important in recent years for the cultivation of certain types of tissues and cells. It differs from the natural media so far described in that it has little or no nutritive function, its main purpose being to provide physical support for cells *in vitro*, and in this role it has a number of virtues. It was introduced as an alternative to clotted plasma which tends to be digested by living cells. Collagen is not destroyed in this way, and its use simplifies the maintenance of certain types of long-term culture. In addition it seems to provide a more congenial surface on which cells readily adhere and migrate, but its most interesting property is its ability to promote and maintain the differentiation of highly specialized cells, like those of the nervous system, muscle and the liver, significantly better than the other materials used in tissue cultures.

Collagen is, unfortunately, more difficult to prepare than plasma. The original method was based on a solution of collagen produced by soaking the tendons from rats' tails in dilute acetic acid. The solution was then dialysed in distilled water to remove the acid. A drop of collagen solution was placed on a coverslip and induced to gel by exposure to ammonia vapour. Finally, the coated coverslip was washed in a liquid culture medium before the explants were placed on its surface. However, it has proved possible to simplify this procedure, and collagen is now widely used for the cultivation of cells of the types mentioned above.

2.3 Synthetic media

2.3.1 Balanced salt solutions

Turning now to the synthetic media, we must first consider the relatively simple mixtures generally referred to as 'balanced salt solutions', which are not only useful in themselves but are also important because they form the basis of the more complex complete synthetic media. In searching for a saline solution which would be tolerated by living cells, the early workers in tissue culture drew on the experience of physiologists who, by trial and error, had already devised solutions for the perfusion of excised organs. Ringer in 1883 used a solution of the chlorides of sodium, potassium and calcium which would keep an excised frog's heart beating for several hours. In 1895 Locke changed the concentration of these electrolytes to suit the mammalian heart, and in 1910 Tyrode produced a more complicated mixture which contained sodium bicarbonate as a buffer, and glucose in addition to the usual electrolytes.

The compositions of two of the most widely used balanced salt solutions, devised respectively by Earle and by Hanks, are shown in Table 1.

Table 1 Balanced salt solutions

Ingredients	*Earle* (mg/l)	*Hanks* (mg/l)
NaCl	6800	8000
KCl	400	400
$CaCl_2.2H_2O$	264	185
$MgSO_4.7H_2O$	200	200
$NaH_2PO_4.H_2O$	140	—
Na_2HPO_4	—	47·5
KH_2PO_4	—	60
$NaHCO_3$	1680	350
Glucose	1000	1000
Phenol red	17	17

Apart from furnishing the inorganic ions essential to the life of all cells, these balanced salt solutions provide an aqueous environment in which the correct osmotic pressure is established, chiefly by the sodium chloride; the pH is maintained at 7.2–7.4 by the buffering action of the sodium bicarbonate, and a convenient source of energy is available in the form of glucose. The indicator, phenol red, is included at a concentration which is not toxic to the cells but is sufficient to give visual warning of a significant change in the pH.

When the two solutions in Table 1 are compared, the most noticeable

difference is the greater quantity of sodium bicarbonate in Earle's saline. This is due to the fact that Hanks' saline is designed to be used in a sealed culture system containing air, while Earle's saline is intended for use in culture systems exposed to a mixture of 5% CO_2 in air. These differences stem from the fact that sodium bicarbonate is a relatively inefficient buffer; it tends to dissociate, releasing CO_2 into the atmosphere and hydroxyl ions into the medium, which consequently becomes too alkaline. Thus the buffering capacity of the culture medium is better maintained if the proportion of CO_2 in the gas phase inside the culture vessel is fairly high. The situation is further complicated by the fact that some types of cell produce considerably more metabolic CO_2 than others. Cells of the first type will tend to grow better if cultured in Hanks' saline exposed to air in a sealed container, where the CO_2 which they release into the enclosed air will help to slow down the dissociation of the sodium bicarbonate. Cells of the second type will generally do better in Earle's saline exposed to a gas mixture containing 5% CO_2, which compensates for their low production of CO_2.

These difficulties in the use of sodium bicarbonate have led to the introduction of different types of buffer, of which the most useful is probably a compound with the unfortunate name of *N*–2–Hydroxyethylpiperazine–*N*′–2–ethanesulphonic acid, more often referred to simply as HEPES. Briefly, the advantages of this compound are that it does not require an atmosphere enriched in CO_2; its pKa (i.e. the midpoint of its buffering range) is 7.3 at 37° C, hence it is a more efficient buffer than sodium bicarbonate at the temperature and near neutral pH required for tissue cultures. Its molecular weight is 238.3, and at a concentration of 20 mmol/l it is non-toxic to cells and produces a physiological osmotic pressure.

There is a variety of salt solution which is used specifically in the treatment of tissues with the enzyme trypsin or with the chelating agent EDTA in order to provide a suspension of separated, individual cells. Cells are more easily detached from each other in the absence of calcium and magnesium ions, and the actions of trypsin and EDTA are enhanced in solutions lacking these ions, such as the calcium- and magnesium-free version of Dulbecco's phosphate buffered saline.

2.3.2 *Complete synthetic media*

Although cells will remain alive for several hours in a balanced salt solution, such simple mixtures can do no more than permit them to survive for a limited time. Many more ingredients must be included in a synthetic or defined medium if it is to be capable of supporting not merely the survival but the proliferation of cells for significantly longer periods. Much time and effort have been expended by a considerable number of scientists in trying to achieve the ultimate goal of producing a completely defined medium in which cells will grow indefinitely. This ideal has not

yet been realized, except in the case of a few highly selected and rather 'artificial' cell types, but a wide range of synthetic media are now available which, with the addition of only a small proportion of natural medium (usually serum), will supply all the requirements for the long-term growth of cells *in vitro*.

An example of a complete synthetic medium, one of several in general use at present, is Eagle's Minimal Essential Medium, and the specification for this is set out in Table 2. In common with other media of this kind, Eagle's M.E.M. is based on a balanced salt solution, usually either Earle's or Hanks', which is omitted from this table.

Table 2 Eagle's minimal essential medium. (Balanced salt solution omitted.)

Ingredients	*mg/l*
L-arginine HCl	126.40
L-cystine	24.02
L-glutamine	292.30
L-histidine HCl.H_2O	41.90
L-isoleucine	52.50
L-leucine	52.50
L-lysine HCl	73.06
L-methionine	14.90
L-phenylalanine	33.02
L-threonine	47.64
L-tryptophan	10.20
L-tyrosine	36.22
L-valine	46.90
D-Ca pantothenate	1.00
Choline chloride	1.00
Folic acid	1.00
i-inositol	2.00
Nicotinamide	1.00
Pyridoxal.HCl	1.00
Riboflavin	0.10
Thiamin.HCl	1.00

The composition of this medium, published by Eagle in 1959, was determined by studying the nutritional requirements of pure populations of established mammalian cell lines. Eagle found that, if any single component was omitted, the cells degenerated and died, but the complete medium, together with the electrolytes and glucose supplied by a balanced salt solution, was sufficient for the indefinite propagation of cells *in vitro* in the presence of only 5–10% of serum. It will be seen that the medium consists of 13 amino acids and eight vitamins. The amino acids are the L–isomers (laevo-rotatory), since D–amino acids are not incorporated into mammalian proteins.