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Scope of Study: This report has been prepared to give helpful suggestions to inexperienced workers in the field of tissue culturing. It is almost impossible to explain every detail of such a subject as tissue culturing. However, a complete reference of material has been reviewed and should be helpful to those interested in tissue cultures. The suggestions are easily understood as well as being precise and practical for the average biologist.

Findings and Conclusions: A biologist can perform a tissue culture with inexpensive equipment and without being trained in the art of tissue culturing. With experience, the technique can be performed with accuracy and excellent results.

ADVISOR'S APPROVAL James H. Zant

TISSUE AND CELL CULTURE

By

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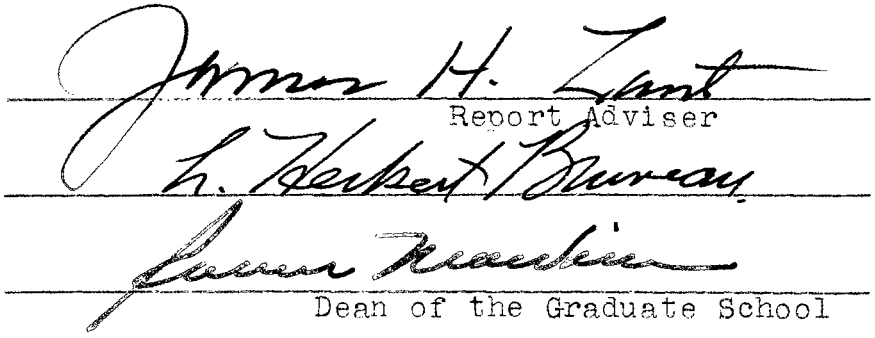

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PREFACE

Almost everyone in the biological sciences is familiar with the words tissue culture. Yet the average biologist has no knowledge of a tissue culture. This is unfortunate in the light of the fact that a great deal of our knowledge of cytology, pathological conditions, biochemistry, genetics, etc., is gained through the study of tissue cultures. Because of emphasis on such diseases as cancer and polio, tissue cultures have indeed become important. At the same time we are able to study the activities, the nutrient requirements and the life cycles of normal, healthy cells and tissues. Thus, the advantages of investigating biological and medical problems by the study of living cells *in vitro* is evident.

While only a brief outline of the entire field of tissue culture has been given, this paper emphasizes standard procedures and techniques. Although the presentation may seem somewhat incomplete, the paper is intended for those who are unacquainted with the more recent developments in tissue culture and for those who have had no experience with tissue cultures. Also, an effort has been made to give the reader a certain degree of orientation in literature reviews. The paper's main purpose is to show that with modest equipment and easily acquired skills, a tissue culture can be accomplished by most biologists.

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To Dr. Calvin Beames for his most helpful and kind criticisms and to the committee on selection of Institute participants for giving the author the opportunity to study at this university, appreciation is hereby gratefully extended.

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CHAPTER I

THE CELL AND ITS' ENVIRONMENT

Presumably the best environment for growing cells is one providing as nearly as possible the conditions they experience in vivo.

The environmental factors affecting tissues may be listed as follows:

1. Temperature
2. Osmotic Pressure
3. Hydrogen Ion Concentration
4. Other Inorganic Ions
5. Essential Metabolites
   - (a) Carbohydrates
   - (b) Dissolved gases
   - (c) Amino Acids
   - (d) Vitamins
   - (e) Proteins, Peptides
6. Antibiotics

Temperature

It is characteristic of living cells that they are rapidly destroyed by temperatures slightly in excess of those at which they operate best. In the case of most mammalian and avian tissues the temperature is between 37° and 38° C. If the temperature is raised as high as 45° C., the cells are killed within an hour. On the other hand, most cells will

survive cooling to a considerable extent. They may even be cooled to 4° C. and kept at this temperature for some time without apparent harm (other than delayed cell division). If cells are cooled below the freezing point they are destroyed due to the formation of ice crystals within the cytoplasm. But, if a protecting agent such as glycerol is added to the medium and the cells are frozen rapidly to a very low temperature, -20° C., they may be stored for months and will re-establish themselves after thawing.

Osmotic Pressure

The osmotic pressure of the medium is also critical. In the case of the mammalian cells the normal osmotic pressure at 38° C. is about 7.6 atmospheres (corresponding to a freezing point depression of about .63° C.). In general it is essential to try to keep within close limits of the normal. The osmotic pressure is due mainly to sodium chloride.

Hydrogen Ion Concentration

The pH of biological fluids has to be very near neutrality to permit survival. However, the average mammalian tissue will survive indefinitely in the range of 6.6 to 7.8.

Above 7.8, many cells will die within 24 hours and the same is true in many cases if cells are maintained in a medium more acid than ph 6.8 (26).

Monocytes from chick embryo spleen have been cultivated in pure strain in a fluid medium. Serum diluted with Tyrode

solution supplied all the necessary food material. At the end of two months cultivation, the monocytes were still in excellent condition, multiplying rapidly and giving every indication that they would continue to proliferate indefinitely under the same conditions. Cultivation in a dilute serum without a coagulum was made possible by immediately reducing the pH of the medium to 7.4 or 7.3 and not allowing it to fall below 7.0 or 6.8 during incubation. Monocytes embedded in a coagulum at a higher pH value survive only because their combined metabolism lowers the pH around them to this value or a lower one. The cells die in a fluid in which it is not possible for them to lower the pH to a sufficient extent. Even if the pH was 8.0, a large number of cells in a small chamber could reduce it to 7.2, especially if it were not well buffered.

In the first days of cultivation, the monocytes undoubtedly fed to some extent on the other cells present. However, cultivation was continued so long after the lymphocytes and polymorphonuclear leucocytes disappeared that it was evident that these other cells are not needed for food if fresh serum is supplied fairly often. So, evidently, diluted serum supplied all the essential nutritive substances. The cells remained in good condition when the pH was adjusted with either lactic acid, hydrochloric acid, or carbon dioxide. Adjustment with carbon dioxide was found to be more convenient and also more practical, since it does not destroy the buffer action of the medium (2).

(d) Iron - required for some of the respiratory pigments.

(e) Bicarbonates - essential for many fundamental biochemical processes in the cell and also is one of the main buffering substances in the medium.

(f) Phosphate - this ion is essential for energy metabolism (26).

Carbohydrates

A source of carbohydrates seems to be almost essential to cultured cells. The most commonly available source is glucose, although fructose and mannose may be substituted. In some cases, cells may be able to survive without glucose for some considerable time, especially those which possess proteolytic activity.

It has been observed that the metabolic patterns of a human epithelial carcinoma cell (strain HeLa) grown on a chemically defined medium supplemented with serum protein exhibited a high respiratory rate. Isotope tracer studies suggested that these cells metabolize glucose terminally via the tricarboxylic acid cycle. Recent work demonstrates that extracts of the HeLa cells contain all the enzymes involved in the Krebs citric acid cycle. The citric acid cycle is an important pathway for supplying the energy requirements of this mammalian carcinoma cell (1).

The amount of glucose used appears to have no direct relation to growth. This is due probably to the fact that higher amounts of glucose do not increase growth rate because it is already limited by factors such as permeability, rate of intracellular synthetic reactions, or availability of

essential foodstuffs. The enzyme systems for disposal of glucose appear to be stimulated to greater activity, possibly by a simple mass law effect. Although glucose consumption appears necessary for survival of cultures it may be used at a rate far in excess of that required for life and maximal growth. Lactic acid was found to be formed both in the presence and absence of glucose. Its formation increased with increased glucose utilization (10).

Gases

Both oxygen and carbon dioxide are probably essential for cell survival. Most systems require some control of the carbon dioxide tension in the gas phase and in the medium. This is achieved either by adding a special gas phase to the culture vessel or by sealing it tightly so that the carbon dioxide produced by metabolism is retained (26).

The respiration of mammalian tissue may be influenced by carbon dioxide tension. The optimal carbon dioxide tension is found to be around one per cent. One of the functions of CO_2 is to prime the Krebs cycle. The presence of CO_2 in the medium probably inhibits decarboxylation of oxaloacetic acid and hence stimulates respiration (8).

Amino Acids

It has now been established that the majority of cells have a specific requirement of 12 amino acids. These are--arginie, cystine, histidine, isoleucine, leucine, methionine,

phenylalanine, threonine, tryptophane, tyrosine, and valine. In some culture organs, fewer amino acids are required (26).

It was known that freshly-explanted chick embryonic heart tissues cultivated (in vitro) in a completely synthetic medium caused marked changes in the amino acid content of the culture fluid. Cystine, and the basic amino acids lysine, histidine, and arginine were almost completely removed from the medium. Less complete removal was observed with valine, phenylalanine, tryptophan, isoleucine, and leucine. Concurrently, increases in the concentration of glutamine, serine, glycine, threonine, alanine, and methionine was detected.

Tissues from 11-day-old-chick embryos were prepared. The kidneys and livers were dissected out separately under aseptic conditions. The tissue was chopped to a fine pulp with curved scissors and portions were transferred to the culture vessels by a capillary pipette. After a period of approximately 30 minutes, during which time the tissues became fixed directly to the glass without the use of plasma clots, the synthetic medium was added and the cultures were incubated at 37° C. Each culture contained 10 to 20 mg of tissue wet weight. All cultures were prepared in T-30 flasks and received 5.0 ml. of synthetic medium per flask. The medium used was synthetic M 150. The culture medium was renewed twice a week and the cultures were examined microscopically at the time of the fluid renewals to detect signs of degeneration. Only cultures of normal morphological appearance were used for the amino acid metabolism studies.

Changes in the amino acid composition of medium M 150 brought about by the tissue cultures were measured by paper chromatography. The used nutrient fluids from groups of cultures were pooled and 5.0 ml. samples used for analysis. These were concentrated to dryness (invacuo) over sulfuric acid and reconstituted in 0.2 ml. of deionized water. Five to twenty microliter quantities of this concentrated material were found sufficient for satisfactory analyses, with one dimensional descending paper chromatograms.

The two solvent systems were a n-butanol-acetic acid-water mixture, and an ethanol-butanol-water mixture. Schleicher and Schuell No. 597 paper was found most suitable for the acidic solvent system, and Whatman No. 1 paper for the neutral solvent.

All chromatograms were routinely developed at room temperature for 16 to 18 hours and then dried at 110° C. for two to three minutes.

The isatin reagent of Acher, Fromageot, and Jutiez as modified by Saifer and Oreskes was also used as a general chromogenic agent, for amino acids. The most useful procedure was to dip the chromatograms in the isatin solution, allow excess reagent to run off, and heat in an oven at 100-110° C. for 10 to 15 minutes. When used under these conditions, the isatin reagent was found to be particularly valuable for detecting proline, glutamic acid, glutamine, aspartic acid, and lysine. Each of the amino acids present in M 150 was dissolved in the basic salt solution and run individually on paper

chromatograms, with the two solvent systems employed, in order to establish its relative position on the chromatograms under these experimental conditions.

From these experiments, the changes in amino acid content of the medium containing chick embryonic kidney tissue was as follows: A moderate uptake of cystine, tryptophan, and phenylalanine. A slight uptake of arginine, proline, tryosine, and isoleucine, and leucine. Increases in the concentrations of glutamine, serine, glycine, threonine, and alanine were noted. No changes in the concentrations of aspartic acid or hydroxproline could be detected. The values obtained for lysine, histidine, glutamic acid, methionine, and valine varied with different ages of cultures and consistent trends were not observed.

The amino acid changes during embryonic liver tissue culture were noted also. There was slight to moderate uptake of cystine, arginine, tryptophan, phenylalanine, isoleucine, and leucine. Slight to moderate increases in the concentrations in the medium of lysine, glutamine, aspartic acid, serine, glycine, threonine, alanine, and methionine were observed also.

The low level of activity of chick embryonic liver and kidney was somewhat unexpected, since these tissues are ordinarily considered to be intensely active metabolically. It is possible that synthetic medium M 150 may permit a prolonged "resting-state" type of survival, but not support active metabolism.

Arginine has been shown by these experiments also to be

an essential amino acid for all tissue cultures so far tested.

Chick embryonic heart tissue is characterized by a strong uptake of the basic amino acids and by slight release of glycine, asparatic acid, and glutamic acid. Chick embryonic kidney shows a pattern resembling that of chick embryonic liver except that a moderate uptake of proline occurs and no release of aspartic and glutamic acids can be detected (23).

Experiments pointed out that the growth-promoting substance in a culture consisted of a protein or was closely associated with a protein. It was also found that amino acids and other dailyzable nitrogenous compounds of the embryonic juice caused no increase in the mass of the tissues, although they had the property of stimulating cell migration and multiplication. Tissue cells obtain their nitrogen from proteoses and possibly from some of the other split products of the proteins. The smaller split products do not induce the rapid proliferation of the cells that the proteoses do. So far, no increase in the mass of a tissue in pure culture has been determined by a mixture of amino acids, or by the ultrafiltrate of embryonic juice. On the contrary, the larger fragments of the protein molecules can be used as food by tissue cells.

In these experiments it was found that fibroblasts do not feed upon the amino acids and other ultrafilterable constituents of the embryo juice, but upon the protein fraction. These experiments render it probable that for cell multiplication, the fact remains that these products have been obtained from impure proteins and, therefore, other substances may be

present which have some action on tissue growth (6).

Vitamins

Many vitamins, the majority are of the B group, are required for cell growth. Most of them are known to form essential parts of co-enzymes involved in metabolism and therefore a requirement for them is readily understood (26).

The synthetic culture medium "199" and adult rat mammary gland incubated for 5 days at 37° C. was used by Ellias to check the requirements of hormones for life. The pH of the medium was at about 8.4 and the hormones used were estrone, progesterone, cortisol, growth hormone, and mammotropic hormone. The crystalline steroid hormones were dissolved in 100-percent ethanol, and a quantity of this solution was added to medium "199" to give the desired hormone concentration in the final medium and a 0.5-percent ethanol concentration. The same amount of ethanol was added to all other cultures.

Mice of the C3H/He CRGL strain, at about the 14th day of pregnancy, provided prelactating mammary tissue with advanced lobulcalveolar development and marked secretary activity. Tissue samples of slightly less than 1 mm. in diameter were taken from a single area of one gland and distributed among the various culture media.

The initial results indicate that alveoli and terminal ducts are dependent on certain hormones for survival in vitro, whereas larger ducts survive in the absence of hormones. In

Ellias's experiments the hormone environment of the explanted tissue was almost wholly defined; the only questions concern the purity of the hormones used and the substances in the explants themselves. With these limitations in mind, it may be said that the hormonal combination of cortisol and mammotropin, acting directly on the explants, maintains and stimulates the secretory activity of prelactating and hyperplastic mammary lobules (14).

Supplementary Metabolites

In a great many instances we are not interested in the survival of the cell, but in the full development of the potentialities of that cell and this requires the addition of many other factors to the environment. For example, Vitamin A is essential for the differentiation of ciliated columnar epithelium since in its absence it changes to keratinized epithelium. It has been shown in other cases where supplementary metabolites are added there is better and faster growth (26).

Proteins and Peptides

It has not been established for certain that proteins are essential for growth of cells. However, no satisfactory medium has yet been produced in which cells will grow rapidly in the total absence of protein or a similar polypeptide (26).

Antibiotics

The growth of a nine day old chick embryo heart in Gey's solution was decreased with increasing concentration of terramycin. A concentration of terramycin in the nutrient medium, 3000 mcg/ml. and greater, completely inhibited the growth of embryonic cells.

Mouse heart grown in the antibiotic terramycin at a concentration of 100 mcg/ml. was perceptibly damaged and severe cytological changes of the mouse heart was produced by 160 mcg/ml. of aureomycin (20).

Nutritional Needs and Requirements

Media commonly used for culturing of fibroblasts and the human uterine carcinoma cell (strain HeLa) usually consisted of a "balanced" salt solution enriched with serum, embryonic tissue extracts, and ultrafiltrates of these materials in varying combinations. The omission of a single essential component results in the early death of the culture. It has now become possible to compare the growth requirements of normal and malignant human cells, to approach the problem of the specific metabolic requirements for the propogation of virus in such cells and to study the incorporation of various nutrilites into cellular protein and nucleic acid.

The amino acid requirement of both the mouse fibroblast and HeLa cells are remarkably similiar with respect to their amino acid requirement. For both, 13 amino acids proved to be essential (arginine, cysteine, lysine, methionine, phenylalanine,

threonine, tryptophan, tryosine, and valine). The optimal concentrations for the growth of the HeLa cell were from one to three times those required by the mouse fibroblast.

The optimal requirement for growth required 0.2 to 0.5 millimolar of glutamine for the L cells and one to two millimolar for the HeLa cell. An unexpected finding was the fact that glutamic acid at any concentration failed to permit the growth of the L-fibroblast. This was not due to the impermeability of the cell, for isotopically labeled glutamic acid could be shown to be actively incorporated into protein. With the HeLa cell, although glutamic acid did permit growth, about 10 to 20 times as much was required as of glutamine. With the HeLa cell glutamic acid may be active only by virtue of the fact that, at high concentrations, it can be transformed to glutamine in amounts adequate for growth. Proline, ornithine, alpa, ketoglutaric acid, and aoparagine, in any concentration tested, with or without NH_4^+ and ATP, failed to substitute for glutamine with either cell.

With both cell lines, a number of amino acids caused partial inhibition of growth in concentrations two to five times the maximally effective level. With both cell lines also, dipetides were just as effective as the component amino acids in promoting growth.

On the omission of a single amino acid from the medium, microscopic changes indicative of cell injury developed within two to three days, and the cells eventually died. These changes differed significantly with the individual amino acids,

perhaps reflecting their varying metabolic functions or differences in the amino acid composition and turnover rate of individual proteins.

In their early stages, the cytopathogenic effects of amino acid deficiencies were reversible. Cells that had been exposed to a medium deficient in a single amino acid and had largely degenerated in consequence could be revived on the restoration of the missing component.

To date, seven vitamins, (choline, folic acid, nictoinamide, pantothenate, pyridoxal, riboflavin, and thiamine), have proved essential for the growth of both the mouse fibroblast and the HeLa cell. On the omission of any one of these from the medium, degenerative changes developed after 5 to 15 days, and the culture eventually died. In their early stages, these specific vitamin deficiencies could be "cured" by the addition of the missing vitamin component to the medium. A few of the several vitamins which are interchangable are listed: Nicotinic acid and nicotinamide, pyridoxine, pyridoxamine, and pyridoxal; and cocarboxylase and thiamine. It must be emphasized that these seven vitamins are not necessarily the total requirement of these two cell lines.

It is possible that a number of other vitamins are essential, but probably more prolonged cultivation in an appropriately deficient medium would be necessary in order to produce the specific deficiency.

The ions demonstrably essential for the survival and growth of both cell lines were sodium, potassium, magnesium,

CHAPTER II

PLASMA, SERUM, AND OTHER BODY FLUIDS FOR CULTURING

It is assumed that plasma and body fluids contain and convey basic maintenance requirements to cells of the intact animal. This section will deal primarily with their practical merits and the methods of obtaining them.

The medium is by far the most important single factor in culturing cells and tissues (27).

Ever since the inception of the tissue culture method fifty six years ago, the elucidation of the nature of the nutrient medium has presented one of the major unsolved problems. Indeed, the ill-defined character of the nutrient is today one of the chief barriers to a really satisfactory and rational use of the method in the study of physiological questions. There is still today no nutrient described in the literature capable of supporting continued growth or even long continued survival of animal tissues which does not contain plasma, serum, bouillon, peptone, fibrin digest or tissue extract in some form.

It is the general belief that embryonic tissues respond more satisfactorily to <u>in vitro</u> cultivation than do adult tissues. The most readily available, cheapest, and most easily handled vertebrate embryos are those of the chick. But, the decision as regards specific tissues is less simple. Most of

calcium, and chlorine.

Both the L-fibroblast and the HeLa cell grew well in a medium containing glucose as the only carbon source over and above the essential amino acids. Some carbohydrates such as galactose, mannose, and maltose were almost as active as glucose mole for mole.

A medium containing the 13 amino acids and the seven vitamins found to be essential for the growth of the L and HeLa cells, each at the optimum concentration, and appropriately supplemented with glucose did not permit growth unless a small amount of serum protein was added, conveniently supplied as dialyzed serum. For the L cell, the concentration of protein that permitted maximal growth was one part in 1500, and one part in 5000 sufficed for limited growth. Approximately three to four times these concentrations were required for the HeLa cells.

The function of the serum protein is not clear. It is obviously not supplying the amino acids and vitamins already shown to be essential. The possibility that the protein contributes trace elements or vitamins that are bound to the protein but slowly dissociate in the culture medium is under study.

In the following table the 27 factors which have been identified as essential for growth of the mouse fibroblast and human carcinoma cell are listed, together with the concentrations used (12).

TABLE I

BASAL MEDIA FOR CULTIVATION OF THE HELA CELL AND MOUSE FIBROBLAST

L-Amino Acids* (mM)			Vitamins (mM)		Miscellaneous	
Arginine	0.1		Biotin	10-3	Glucose 5 mM	
Cystine	0.05	(0.02)	Choline	10-3	Penicillin	0.005
Glutamine	2.0	(1.0)	Folic Acid	10-3	Streptomycin	0.005
Histidine	0.05	(0.02)	Nictotinamide	10-3	Phenol Red	0.000
Isoleucine	0.2		Pantothenic Acid	10-3		
Leucine	0.2	(0.1)	Pyridoxal	10-3	For studies of ce nutrition Dialyze horse serum, 1%	
Lysine	0.2	(0.1)	Thiamine	10-3	Dialyzed human serum, 5%	
Methionine	0.05		Riboflavin	10-4	For stock culture whole horse serum 5%.	
Phenylalanine	0.1	(0.05)			Whole human serum 10%.	
Threonine	0.2	(0.1)	Salts (mM)			
Tryptophan	0.02	(0.01)	NaCl	100		
Tyrosine	0.1		KCl	5		
Valine	0.2	(0.1)	$NaH_2PO_4.H_2O$	1		
			$NaHCO_3$	20		
			$CaCl_2$	1		
			$MgCl_2$	0.5		

*Conveniently stored in the refrigerator as a single stock solution containing 20 times the indicated concentration of each amino acid.
For mouse fibroblast.
Conveniently used as a single stock solution containing 100 or 1000 times the indicated concentration of each vitamin; kept frozen.
Conveniently stored in the refrigerator in two stock solutions, one containing Nacl, Kcl, NaH_2PO_4, $NaHCO_3$, and glucose at 10 times the indicated concentrations of each, and the second containing $CaCl_2$.
#Conveniently stored as a single stock solution containing 100 times the indicated concentrations of penicillin, streptomycin, and phenol red.

the literature deals with the so called " fibroblasts" which in the standard Carrel technique tend to over-run all other types of cells.

A more reliable criterion for judging physiological conditions is certainly needed. For example, heart muscle in vitro shows rhythmic contractions for long periods. Obviously, here would be a good tissue for testing to see if a medium contained the proper constituents, etc. to maintain life of the cells (30).

A report of six months for cultures of heart tissue from three day chick embryos in a plasma embryo juice medium has been reported (21). An equal period for 8-day chick embryo heart muscle in an embryo-juice Locke solution has also been reported (16).

A heart muscle which is beating strongly at a steady rhythm of 60-100 contractions per minute is certainly in good condition. If the rate is slow or fast then the condition in some respect is unsatisfactory. Another diagnostic criterion for conditions of survival can be judged by the establishment of new centers of cell accumulation.

If, in a flat sheet of cells either migrating or growing out on the substratum, there arise aggregations of cells which pile up into three dimensional masses, constituting new growth centers, we could say with considerable conviction that the culture is growing satisfactorily (30).

Although improved synthetic media will be produced in increasing numbers in the next few years, it seems certain that

natural media will form the cheapest and most convenient materials for the maintenance of cells for some time to come.

The natural media fall into three general categories: Coagula, such as plasma clots; biological fluids, such as serum, and tissue extracts, of which the commonest type is embryo extract.

Clotted plasma can be obtained commercially, but some people prefer to prepare their own. Any animal can provide plasma, but the fowl is generally used since fowl plasma gives a more solid clot and is easily obtained. The male bird is usually employed because the blood calcium is more constant and in certain procedures it is easier to operate on the male. A young bird is preferred and it is usually stated that it should be less than one year old and fed only water for one day before bleeding. Blood may be obtained from a wing vein, from the heart, or from a carotid artery. Clotting is prevented by the use of an anticoagulant such as heparin.

Bleeding from the Wing

When this procedure is once learned it is quite simple. It is advisable to treat the syringe, needle, and tubes for blood with heparin. It is also advisable to cool them in ice while the blood is being collected. A 20 ml. syringe may be used with an 18 or 19 gauge needle.

The feathers are plucked from the elbow of the wing while an assistant holds the head and feet. Clean the skin

with alcohol and then insert the needle into the large vein which crosses the elbow. This should be done with great care since it is very easy to go right through the vein and cause a large haematoma which will make subsequent exploration futile. Withdraw gently and slowly 30 ml. of blood, transfer to a prepared centrifuge tube and spin down as soon as possible.

Centrifuge at 3000 R. P. M. for 20 minutes and dispense one ml. aliquots of plasma to tubes. Insert rubber stoppers and store the plasma at four degrees C. if it is to be used within a few weeks. Place in the -20 degree C. freezer for longer periods of storage (27).

The solid medium of a plasma clot possesses certain disadvantages. In the first place it limits the rate of disposal of waste metabolites produced by the explants; the accumulation of such waste products may seriously handicap the growth and differentiation of the explant, especially when highly secretory tissue such as endocrine glands are cultivated. Moreover, certain tissues have the undesirable ability to liquify the plasma clot readily, producing a pool of fluid in which the explant becomes submerged and deprived of proper air supply.

It has been found that some brands of thin paper float indefinitely on the liquid medium and serve as a perfect support for small explants. A method has been developed which may combine the advantages of the classical watch-glass technique with those of using liquid medium. Because of the highly porous nature of the paper, nutrients and waste

products are easily diffused to and away from the tissue. During subcultivation the paper with the adherent explants can be readily lifted off the fluid, washed and put on a fresh medium, so that the disturbances and damage usually involved in freeing an explant from a clot and which often gravely interfere with its growth are eliminated. Embryonic pancreas and liver, which so far have never been successfully grown as organ cultures, differentiated almost normally and grew remarkably well. During subcultivation, the lens paper with its adherent explants is lifted bodily from the liquid, washed in Tyrode and laid on fresh culture medium (7).

Biological Fluids

The traditional and most commonly used fluid is serum. A percentage of sera always toxic but autologous sera may be just as toxic or non-toxic as heterologous sera. Serum is most commonly obtained from human adult or placenta cord blood, horse blood, or calf blood.

The preparation of serum consists of permitting whole blood or plasma to coagulate and thereafter removing the exuded serum. Serum thus collected has to be tested for sterility before it is used and it is also advisable to filter it.

There are several sources of serum. The placenta cord blood is allowed to drip from the umbilical cord and allowed to clot in a vessel. It is usually necessary to centrifuge it in order to remove the red blood cells. This serum is

considered particularly good for the growth of tissues which are very difficult to culture and is said never to be toxic.

Amniotic fluid from pregnant bovine, aqueous humor from the ox eye, and pleura fluid are all good sources of serum (27).

The Use of Embryo Extract

It has been known for many years that cells will grow without embryo extract. But embryo tissue extract has remarkable powers of promoting cell growth and multiplication. In 1912, Carrel found that subcultures would become smaller and smaller with each subsequent transfer, but with the addition of embryo tissue the strain continued to multiply for thirty-four years.

Cultures in serum and plasma put on more weight in four days than did similiar cultures in embryo extract and plasma. But, the latter grew to much greater areas and are known from other methods to show far more cell divisions. This increase in weight in serum was almost certainly not due in the main to increase in the number of cells, since serum has been shown not to increase the number of cell divisions. Embryo juice perhaps determines nuclear synthesis, while plasma and serum feed the cells and produce cytoplasmic growth and intercellular matrices. For prolonged growth both constituents are necessary (19).

There is little doubt that embryo juice hastens the glucose consumption and uptake of sugar by osteoblasts and to a less extent by heart fibroblasts and that much of this sugar

is converted into lactic acid. Tissues with high growth energy, like fresh heart explants from young chicks, reorganize quickly and spread their cells out into the medium without delay as soon as they are planted. The same occurs when tissues are planted into a medium containing embryo juice, but old colonies planted in plasma and Tyrode take longer for their cells to grow out. Embryo juice causes an initial increased glucose consumption which later falls below the level characteristic of a Tyrode-plasma medium. In general there is no absolute correlation between high growth rate and high sugar consumption or lactic acid production (31).

Previous work and observations have shown that tissues can use an amount of glucose greater than their own dry weight in 48 hours, and that the amount used decreases with the age of the tissue. Growth in cultures increases with increasing glucose concentration.

Previous workers have found extensive lactic acid production by chick fibroblasts. It has been noted that a decreased growth rate resulted in an increased ratio of lactic acid to size of cultures. About 60 percent of the glucose used in 48 hours has been accounted for as lactic acid, and glycolysis has been considered characteristic of the chemodynamics of the tissue cultures. It is to be expected that an unchanged medium loses efficiency by loss of essential constituents and by accumulation of end products. The increased life of cultures bolstered by additions of

glucose to an otherwise unchanged medium indicates that glucose loss may be a limiting factor in this regard. Daily changes of the whole medium increases the life span of cultures still further.

The concentration of embryo extract present in the medium is a more important fact in determining the number of mitoses produced than is the time during which it acts and that it is not necessary for the division process. It has been made clear that cells do not divide just because they have reached a certain size. Cells divide only when stimulated to do so by special substances present in the medium, when these substances are absent, the cells become and remain larger.

The higher the concentration of embryo juice used in the culturing of chick fibroblasts, the greater is the number of cell divisions which occur. The addition of the embryo juice is followed within the first two hours by increased movement of the cells, which reaches its maximum in about ten hours, but it is not until this latter time that cell divisions begin again.

Embryo juice appears to increase many of the activities of the cells, it stimulates their movement; it causes them to divide; it accelerates the actual process of mitosis. The embryo juice initiates cell division after a latent period of 10-12 hours, and it is not necessary for the embryo juice to be present in any appreciable amount during the actual division process. The approximate minimum effective dose is 5% embryo juice in Tyrode solution acting for three

hours. Fifteen percent and forty percent juice produce marked effects when applied for only one hour.

The concentration of embryo juice is a more important factor in determining the number of mitoses produced than is the time for which it acts (17).

In the preparation of chick embryo tissue extract it is of utmost importance that the eggs show a higher percentage of fertility, that is to say, they should be of the grade sold expressly for breeding purposes. Under no conditions should they be procured by random selection in the market. Until ready for use the eggs are stored in a refrigerator but must not be allowed to freeze. They tend to become useless after refrigeration of about one week in a liquid state. They can be preserved for four or five months at -20 degree C. and for longer periods by lyophilization.

It is recommended that chick embryo extract be made from 10-11 day old embryos; the calcifying mechanisms are not very active yet (22).

Human placenta cord serum has at least one growth promoting factor in the euglobulin fraction of cord serum.

Besides the growth-promoting proteins of the globulin fraction, serum albumin is also needed in the tissue culture medium to prevent the appearance of a characteristic toxicity. It has been shown that: (a) Stearic, oleic, and linoleic acids are toxic to fibroblasts in tissue culture and that the unsaturated acids, oleic and linoleic, are more toxic than stearic acid. (b) This toxicity can be prevented by

serum albumin. (c) There are sufficient free fatty acids in the tissue culture medium to account for the toxicity observed in the absence of albumin and oleic acid. From this data, there is every reason to believe that albumin acts by binding the fatty acids, thus keeping the concentration of free fatty acids below the toxic level.

Therefore, toxicity is due to free fatty acids in the medium and serum albumin acts by binding the fatty acids (18).

Use of Chick Embryo Extract as a Medium

Crack the blunt end of the shell and remove the shell over the air space to expose the membrane. Remove the membrane with forceps. Slip a pair of forceps under the embryo's neck and deposit it very gently into a petri dish.

Rinse three times in a balanced salt solution to remove all blood and yolk. Drop several embryos into the barrel of a syringe and insert the plunger gently. Insert the tip of the syringe into a centrifuge tube and express the embryos. Add an equal volume of balanced salt solution to the pulp and stir with a sterile glass rod. Leave for 30 minutes at room temperature and then centrifuge for 20 minutes at 2,000 g.

Remove the supernatant by pipettes and distribute to small test tubes which have been sterilized. Store in the refrigerator if the extract is to be used the same day. For longer storage, up to 6 weeks, use the deep freeze. Before use, thaw slowly and recentrifuge for 10 minutes at 2,000 g.

Other tissues such as spleen, liver, bone marrow,

leucocytes, tumors, beef embryos are used but in some cases they have had inhibitory effects on growth.

All tissue extracts tend to deteriorate rapidly at room temperature and they will lose a great deal of their growth promoting potency within a week (27).

Hyaluronidase-treated chick-embryo extract was used in the maintenance of Earle's "strain L" cells for about 8 months. The cells required subculturing about every 10 days.

The procedure was as follows: Open a dozen fertile eggs, incubated 10 days. The embryos are collected in small petri dishes. They are then dropped into the barrel of a 20 ml. Luber syringe. The plunger is inserted and the tissue pulp forced into 15 ml. conical Pyrex centrifuge tubes, allowing about 5 ml. of pulp per tube. No complex grinders are needed. Then add an equal quantity of Earle's balanced salt-dextrose solution, stir thoroughly with a spatula or glass rod, and place in the refrigerator at $\not 5^{\circ}$ C. overnight. No freezing with CO_2 is required. The following day the extract is separated by centrifuging for 10 minutes at about 3000 r.p.m. The clear supernatant is decanted, and can either be used immediately in the preparation of nutrient, or can be stored in the freezing part of the refrigerator at -15° C.

The complete nutrient comprises one volume of extract, two volumes of horse serum, and seven volumes of balanced salt solution. About 10 mg. of hyaluronidase are added to each 100 ml. of nutrient, and the whole is filtered through a Selas candle of No. 03 porosity. A pressure filter was not

used. A water pump or vacuum pump is satisfactory, but the vacuum should be kept below 250 mm. of mercury by use of a bleeder valve. One hundred and fifty milliliters of nutrient will filter without clogging a 2-in-filter and with almost 100 per cent yield in about 10 minutes. The clear sterile fluid is drawn off into tubes and either used immediately or stored at $\not 5^{\circ}$ C. A dozen eggs will usually provide about 15 ml. of embryo extract (1:1) or 150 ml. of complete nutrient. The solution has a final composition of 10 per cent embryo extract, 20 per cent serum, and 70 per cent balanced salt solution. It is also to be noted that the nutrient was not "gased". Air was quite satisfactory.

This procedure eliminated the use of a wire screen in the syringe, preliminary freezing with CO_2 ice, grinding in a Potter-Eleuhjem grinder, ultracentrifugation, use of a pressure filter, and gassing of the culture flasks. The equipment needed is to be found in most laboratories. The procedure can be carried out by a student after a little instruction (29).

CHAPTER III

DEFINED MEDIA

An attempt will be made to define briefly some of the merits of defined or synthetic media. It is readily apparent that there will never be a universal medium for all cell types. For instance, ascorbic acid is an essential dietary constituent for man, but is not required by other lower forms of mammals.

The components essential for the immediate survival of cells and tissues have been precisely defined. It is essential to control osmotic pressure and pH to provide a source of energy for the briefest survival. These requirements are met by a combination of salts and glucose. A medium of this sort is referred to as a balanced salt solution.

For periods of longer time, mammalian cells require in addition to a balanced salt solution, all the essential amino-acids, oxygen, vitamins, and serum protein. In the complete absence of protein the cells do not survive long.

Many chemicals used in synthetic media are very sparingly soluble and it is essential to prepare them in such a way that they will come into solution. Therefore, the solids have to be made up in a special stock solution and mixed in with the other materials at a later stage in

preparation when the addition of acids will do no harm. Lipids are particularly difficult to dissolve and they have to be taken up in alcoholic solutions.

One of the most elementary difficulties concerns calcium phosphate which is highly insoluble, particularly in alkaline solutions. Therefore, in making up salt solutions it is essential that calcium and phosphate should not meet until the solution is quite dilute and that it should not be made alkaline until the last minute.

Heavy metals are particularly toxic to cells and thus care must be taken with materials which may have been purified by lead or mercury precipitation. On the other hand, minute traces of certain metals (zinc, colbalt) are essential.

Several of the compounds added to synthetic media are rather unstable and the only way to store them effectively for long periods of time is in the dry state, preferably in the cold. This is true of most labile compounds so that it is important to prepare fresh media or if they are stored it should be at very low temperature.

From the above considerations, it is apparent that if large amounts of media are to be made up for use and stored for any length of time difficulties are likely to be encountered. Only deep freezing facilities will permit the long term storage of large amounts of medium.

Balanced Salt Solutions

Balanced salt solutions (BSS) are used for rinsing tissue

cultures and for dilution of components of culture media or of compounds to be assayed in culture.

It is common practice to employ a single salt solution for cells of both mammalian and avian origin, ignoring certain natural differences in composition of body fluids.

The function of this salt solution is to maintain the pH and osmotic pressure in the medium and also to provide an adequate concentration of essential inorganic ions. All physiological salt solutions have been derived from the salt solution described by Ringer. The first one to be developed specifically for supporting the metabolism of mammalian cells was Tyrode solution. Most of them were devised for the object of preventing calcium deposition in the cultures or in order to obtain better buffering conditions in the medium. In practice none of these seem to have any demonstrable advantage over any other from the point of view of the maintenance of cell growth.

Salt solutions can be divided into two types, those intended to equilibrate with air and those which are designed to equilibrate with a gas phase containing a high carbon dioxide tension of the order of five per cent. The most useful type of the former is Hank's salt solution and the best example of the latter is Earle's balanced salt solution. Both of these have the great advantage that they are very easy to prepare by autoclaving of different parts. The best buffered salt solution is undoubtedly Earle's solution, but the fact that it requires a special gas mixture is sometimes

a disadvantage.

In making up salt solutions, it is advisable to use double glass distilled water. Ordinary tap water frequently contains a large number of ions, many of which are quite harmless, but some of which, such as lead, are toxic to the cells. It is a common practice to store glass distilled water in polyethene bottles to prevent the reaccumulation of metabolic salts from glass. Phenol red has been added to many salt solutions as a pH indicator since it has been found to be nontoxic in concentrations up to .005 per cent.

The main difficulty encountered in preparing a balanced salt solution is the formation of a precipitate of calcium and magnesium carbonate and phosphate. The avoidance of precipitation constitutes the only special art involved in preparing a salt solution (28).

TABLE II

A BSS 10 STOCK SOLUTION

Components	For 1000 ml. 10x stock	Instructions	Composition of BSS 1x g/liter	mg%
1. $CaCl_2$	1.4g	1. Dissolve $CaCl_2$ in 200 ml water.	0.14	Ca 5.0
2. Glucose	10.0	2. Place other components in a beaker containing 100 ml. PR 0.2% and 800 ml. water; stir until dissolved.	1.00	100.0
NaCl*	80.0		8.00	
KCl	4.0		0.4	
$MgSO_4 \cdot 7H_2O$	2.0		0.2	Mg 2.2
KH_2PO_4**	0.6		0.06	P 2.5
$Na_2HPO_4 \cdot 2H_2O$**	0.6	3. Combine the two solutions. Add water to make total volume 1100 ml. to provide 10% water excess.	0.06	
PR 0.2%	100.0 ml.		0.02	

* Merck biological to avoid silver

**May be contaminated with aluminum.

If Na_2HPO_4 contains 12 H_2O, use 1.2g; select only clear crystals because opaque crystals have lost water of crystallization. If the solution is filtered, use only ash free papers.

Chloroform 3-4 ml/liter is used as a preservative. (Precautions below).

Autoclaving of BSS

The 10x stock is diluted in water and sterilized at 10 lbs. steam pressure, 115° C., for 10 minutes in convenient volumes in tubes or screwcap bottles (26).

It is convenient to prepare stock solutions of salt mixtures. A solution is made up with ten times the concentration of all components except sodium bicarbonate, which

is omitted. This is stored in a polyethylene bottle. If it is subsequently to be autoclaved it can be preserved with a little chloroform which will be driven off during sterilization. To constitute the BSS, distilled water is added to dilute the stock solution ten times (28).

CHAPTER IV

THE LABORATORY AND PREPARATION OF APPARATUS

The lab for tissue culturing should be located for sterile work as far as possible from the contaminated air of the outer laboratory. In a lab where several workers are to be, it is advisable to have two or more rooms.

Each culture room should be not less than 7 x 10 feet in size and should be supplied with forced filtered ventilation, overhead lighting and a sliding door. All other outlets should be sealed. The floors, walls, and ceilings should be washable.

The rooms should be kept clean with soap and water. Dry mops and brooms must not be used. Cleaning should be done at the end of the day so that there is ample time for dust to settle before the room is used again.

From time to time the culture rooms should be tested for their bacterial content by exposing petri dishes of nutrient agar for thirty minutes. If only four or five colonies appear in these dishes after 48 hours incubation, the rooms may be considered fit for use. If the count is higher than this, special attention should be given to the air filter and the floors.

Each culture room should be furnished with a long,

narrow operating table (18 x 16 inches) and one or two auxiliary tables and so arranged that two people may work facing each other. For general purposes, stools have been found to be more satisfactory than chairs.

A general preparation room should be provided in close proximity to the culture rooms. The preparation room should be equipped with facilities for washing, drying, and packing the glassware, instruments, and other materials. There should be enclosed cabinets and space for the storage of sterile materials and reserve supplies. Even though materials are wrapped in paper and cloth prior to sterilization, they must be well protected until used; otherwise, any dust that may accumulate on the outer coverings will be scattered in the culture room when the packages are opened.

A sterilizing room should be provided to house the hot air and steam sterilizers. Owing to the heat usually generated by sterilizers, this room is likely to be unfit for more general purposes.

In addition to the facilities already mentioned, there should be a chemical lab, a dark room and a general lab (24).

A laboratory is equipped with "sitting height" benches and chemist-type shelves above the working surfaces. Each of the desk spaces is furnished with gas, water, electrical outlets, and fluorescent lamps. One taller desk is used for demonstrations. Wall cabinets with sliding glass doors contain a stock of sterilized glassware and equipment.

Each student will keep in one drawer of his desk: straight

and curved forceps; small straight or curved scissors; scalpels (Bard-Parker handles No. 7, and blades Nos. 10 and 11); a platinum spatula; and rubber bulbs. A second drawer contains sterile supplies.

The instruments are sterilized by the Gey system, i.e., those in use are kept in a Coplin jar of 95 per cent ethanol. Each instrument is withdrawn from the jar and the ethanol ignited. This process is repeated once. Since each instrument could be sterilized as needed, only one of each type was required.

Glassware should be sterilized by dry air at 160° C. for two hours. Other equipment may be sterilized by autoclaving at 121° C. for 20 minutes. Time and labor are saved by omitting the wrapping of equipment in paper or plugging tubes with cotton. For convenience, most equipment is sterilized in cans (e.g., test tubes and Petri dishes), in glass tubes (e.g., pipettes), or in square screw cap jars (e.g., corks and rubber stoppers).

Contamination by microorganisms is avoided by several simple measures. To control dust, Filter-Aire window fans regulate and filter the air flowing into the room although these are not absolutely necessary; the work and supplies are organized to minimize traffic; the floor is wet mopped before each exercise; and the students are taught to polish all desk tops and work surfaces with a light grade of mineral oil before each operation. Simple, but rigid bacteriologic technics need to be enforced. The students are taught when

and how to flame equipment, to avoid talking or making unnecessary movements over exposed culture materials, and to keep all open tubes, jars or bottles in a slanted position. Surgical masks, caps, gowns or gloves are not used.

The test tubes employed for the course are 13 x 100 mm. and 16 x 150 mm. Pyrex. It would be desirable to replace the larger tubes with Pyrex screw cap test tubes 16 x 125 mm and 20 x 125 mm. Straight and curved tipped pipettes about 19 cm. in length can be made from 7 mm. outer diameter tubing so that the smaller opening is 1.5-2 mm. in diameter. Ordinary test tube racks are used for slanting test tubes. Wherever possible, rectangular or square cans or jars are used so that when opened, they can be placed on their sides without rolling (26).

A list of general equipment is listed below and is obtainable from nearly all biological supply houses.

General Equipment:

The following major items are essential or highly desirable:

One refrigerator with deep-freeze compartment.
One or more incubators.
One centrifuge.
One sensitive balance.
One sterilizing oven.
One autoclave or pressure cooker.
One pipette-washer.
One bottle-washer.
One all-glass still.
Buckets, pans, etc., for collecting and soaking used glassware.
Microscopes (for large-scale work an inverted microscope is particularly desirable).

Requirements for glassware and special apparatus are bound to vary very greatly. The following list of initial

requirements is reasonably representative for most purposes:

		Quantity	
Flasks (conical)	1 litre	1	
	500 ml.	4	
	250 ml.	6	
Beakers	50 ml. to 1 litre	1	each
Measuring cylinders	1 litre	1	
	500 ml.	1	
	250 ml.	2	
	50 ml.	4	
Volumetric flasks	1 litre	1	
	500 ml.	1	
	250 ml.	2	
Petri dishes	100 ml.	2	
	85 mm.	4	dozen
	60 mm.	4	dozen
Cans for sterilizing Petri dishes		2	
Universal containers		6	dozen
Medical flat bottles	16 oz.	6	dozen
	12 oz.	6	dozen
Test-tubes	6" x 5/8"	2	gross
	3" x ½"	1	gross
Stoppers for above (red rubber)		2	gross each size
Centrifuge tubes		6	dozen
Test-tube baskets		6	
Test-tube racks		6	
Pipettes, graduated	10 ml.	6	dozen
	5 ml.	6	dozen
Pipettes, bulb	1 ml.	6	dozen
	100 ml.	1	
	50 ml.	2	
	20 ml.	2	
Pipettes, Pasteur (or glass for making pipettes)		2	gross
Rubber teats	5 ml.		
Cans for sterilizing pipettes		10	
2 ml. syringes		2	
14 gauge needles		6	dozen
3" x 1" microscopic slides		1	dozen boxes
7/8" square No. 0 coverslips		6	oz.
Watchglasses (embryological)		1	dozen
Coplin dish		1	dozen
Cotton wool		2	lbs.
Gauze		10	yds.
Filter funnels (assorted sizes)		6	

Instruments:

Bard-Parker knife handles	4	
Knife blades No. 11	2	dozen
No. 23	1	dozen
Dissecting scissors	2	
Dissecting forceps	2	
Fine straight forceps	2	

Fine curved forceps	4
Fine scissors	2
Barber's scissors	1
Platinum wire in mounts	4
Coverslip forceps	4

Coverslip techniques:

Depression slides, large and small coverlips, racks for slides, double boiler, camel-hair brushes.

Roller-tube techniques:

Roller-drum, racks for roller-tubes (29).

Preparation of Apparatus

Contamination of media with bacteria is the greatest hazard in most tissue culture procedures, but almost as important is contamination with toxious chemical substances.

Glassware:

Although cells have been grown on many substrates, including cellophane, polystyrene and other plastics, the material most commonly used in glass, since this is so readily available. Some contain materials which are highly toxic to cells, such as lead and arsenic. Since these substances are slowly brought into solution by the slightly alkaline media used in tissue culture, toxicity to cells can sometimes be traced to this source. Both soda glasses and borosillicate glasses have been found to be highly satisfactory. Thus, it is best to find a brand of glass which has no toxic properties and to use it empirically. Two glasses which appear quite satisfactory are the borosilicate glass made by Pyrex and the soda glass used for most medical prescription bottles in this

country. It is worth mentioning here that new glassware drug is often found to be rather unsatisfactory for culturing cells. After it has been used at least once, it seems to give more satisfactory results, even when rigorous cleaning methods are used. The reason for this is obscure.

Stoppers for Culture Vessels:

Rubber stoppers contain substances which are toxic to tissue culture cells. This seems to be due to chemicals used in processing since stoppers made from virgin rubber and pure gum rubber are less toxic than ordinary black or red stoppers. In order to remove surface impurities, rubber stoppers (and all other rubber articles coming in contact with cells) should first be boiled in dilute alkali (5 per cent sodium carbonate) and exhaustively rinsed with water. Thereafter, they can be cleaned in the same way as glassware. It has been shown that even exhaustive cleaning does little to diminish the toxic properties of rubber and therefore care must always be taken to ensure that medium does not come in contact with rubber stoppers. This can be done either by using vessels designed to prevent it or by using stoppers made of some material other than rubber. The same consideration apply to rubber tubing for rubber stoppers.

All glassware should be placed in water immediately after use. It is also good practice to place new untrained assistants in the washing and sterilizing division of the laboratory and to have them remain there until they have mastered that phase of the work. It is almost needless to say that a tissue

culture laboratory comprised of individuals who are specialists is an inefficient lab. Each member of the staff should be able to take over any function during an emergency (29).

Used Glassware:

Used glassware is rinsed under the tap to dislodge all solid matter, then boiled for ten minutes in water and soap jelly. After it has been rinsed in warm tap water, it is boiled again with soap as before. It is not thoroughly rinsed with warm running water, placed for a time in distilled water, then in 95 per cent alcohol and set to drain in a drying oven. New, unused glassware is treated in the same manner except that it is first placed in dilute HCl (about 1 per cent) for several hours to remove any free alkali.

Glass coverslips, Carrel flasks and other glassware too fragile to be boiled, are rinsed with water, immersed in concentrated sulphuric acid containing small amounts (0.5 per cent) of sodium nitrate ($NaNO_3$) and sodium chlorate ($NaCLO_3$), and heated to 90° C., after which the cleansing action is allowed to continue overnight at room temperature. The following morning all of the articles except the flasks are again rinsed with many changes of tap water, then with distilled water and, finally, with 95 per cent alcohol. The flasks are treated with the alcohol before the final rinsing with distilled water. The coverslips are dried with a towel; the other glassware is allowed to drain in the drying oven.

Used Serological Pipettes:

Used serological pipettes are rinsed under the tap and placed in acid cleaning fluid overnight. They are then washed

for a day in an automatic pipette washer, left overnight in distilled water, and allowed to drain in the drying oven. Be sure there is an abundant supply of pipettes since a large quantity of them will be used at one time.

Instruments:

Instruments are washed with soap and warm water, thoroughly rinsed with running water, placed in 95 per cent alcohol, and dried in an oven. Instruments are cleaned immediately after use, to prevent any organic material from drying on their surfaces. If it is necessary to scour them, it should be done with the finest nonabrasive material. Used knives are sharpened with a good razor strap. From time to time, it will be necessary to send them to be an instrument maker for reconditioning. It would be wise to clean all material immediately after use.

New Unused Rubber Stoppers:

The stoppers are boiled in dilute sodium hydroxide (about N/2), rinsed in tap water, then boiled in dilute hydrochloric acid (about N/2), and finally rinsed very thoroughly in tap water. This treatment removes sulfur "bloom" and other surface impurities.

New Rubber Tubing:

New rubber tubing that might come in contact with culture media is boiled in dilute sodium hydroxide (about N/2), rinsed in tap water, then boiled in dilute hydrochloric acid (about N/2). During the process of boiling, the hot fluids are circulated through the tubing with a syringe. This is done

several times. After washing the outside of the tubing with hot tap water, the tubing is attached to a faucet and flushed with tap water for thirty minutes. A funnel is then attached to one end of the tubing and not less than 1 liter of distilled water is passed through it.

Used Rubber Tubing:

Used rubber tubing is washed thoroughly in warm tap water, then attached to a faucet and flushed with tap water for thirty minutes. Finally, a funnel is attached to one end of the tubing and not less than 1 liter of distilled water is run through it.

At the National Cancer Institute, large steam kettles filled with concentrated sulfuric acid are used for cleaning all the glassware. Very few labs use this technique, however (24).

Sterilization of Materials

Contamination of materials can be prevented in two ways:

(1) Sterilization - This term applies to the removal of microorganisms already present.

(2) Aseptic technique - This term implies the prevention of contamination of materials already sterile.

In tissue culture work the sources of contamination are as follows:

(1) The apparatus
(2) The culture medium
(3) The tissue itself
(4) The atmosphere
(5) The operator

Sterilization can be achieved by:

(1) Dry heat, moist heat, and radiation.
(2) Chemical destruction of micro-organisms by antiseptics and antibiotics.
(3) Physical removal of micro-organisms by filtration, centrifugation, and washing.

Most apparatus is sterilized by dry or moist heat. In general, apparatus which is not damaged by high temperatures is sterilized by dry heat since this is the most convenient method. Where excessive heat can damage the material, moist heat is used. It is more efficient than dry heat because of steam coagulating the proteins of microbes. Autoclaving is usually used for solutions, rubber ware, etc. Simple boiling is used for instruments.

Solutions can be sterilized by ultra-violet radiation. This requires special apparatus and has not been widely used in tissue culture.

For laboratory benches and tables alcohol is commonest and the tables can be swabbed down with it to reduce the number of bacteria present. Oil forms a slightly adhesive layer on benches and prevents dust from being disturbed. It is a good practice to have laboratory floors treated with oil every few days for this reason.

As a rule it is not necessary to sterilize air since contamination from this source can be prevented by ordinary aseptic techniques.

To sterilize with dry heat, a simple domestic oven is used. Consequently, a rather high temperature for a rather

long time is recommended and 90 minutes at 160° C. is usually employed.

A domestic pressure cooker is sometimes more convenient for sterilizing small amounts. The recommended pressure is 15 lbs. for 20 minutes at a temperature of 121° C. Seventy per cent alcohol is a good antiseptic.

By far the most useful antibiotic is penicillin. Penicillin is the most commonly used and at a concentration of 20-50 units per ml. It is completely harmless to all cell types and is inhibitory to the vast majority of bacteria.

Hand Sterilization:

One should carefully wash his hands with soap, water, and a brush.

Contamination from Tissue:

Contamination from tissue may be avoided simply due to the fact that most internal tissues of animals are already sterile.

If no mistakes in technique have been made in preparation of the tissue, media, and apparatus, then subsequent contamination can only arise from the air in the room in which the operations are carried out or from the operator.

A "hood" is commonly used to work in and prevent contamination from the air, hair, and dust. This hood is simply a glass or plastic structure with two openings for the hands to fit in. Inside should be made germ free by use of 70% alcohol, ultraviolet light, etc., before operations. It is recommended that every laboratory have at

least two "hoods". The hood should be large enough so that transferring, transplanting, etc. can be done under it. The hood should be at least 30" in height, width, and length.

CHAPTER V

SOURCES AND CHARACTERISTICS OF TISSUES

Tissues are usually obtained from the adult and embryo. Embryonic tissue is sterile, they tend to grow easily, migrate rapidly, and undergo mitosis very soon after explantation. Fibroblasts tend to grow out from them more rapidly than other types of cells. Adult material has to be specially treated to ensure its sterility before culturing and is harder to culture because of the strict nutrient requirement. There is a latent period of several days before any outgrowth becomes apparent and there is also less tendency for fibroblasts to grow. Tumor tissues from adult animals behave in many ways like normal adult tissue, but on the whole they tend to grow more rapidly in the first days (30).

Most tissues when cultured show a movement of cells from the explant into the surrounding medium after a varying latent period. This period varies with the age of the donor organism, the medium used, and the type of tissue. Thus, the migration of cells in a culture of blood leucocytes begins in less than an hour while no cells may appear for four days in cultures of connective tissue of an adult animal. The migrating cells of the embryo appear in bundles or sheets in cultures of connective tissues, glandular, and

muscular organs. In cultures of hematopoietic organs and of blood, the cells migrate at first as individual leucocytes and macrophages; only later do sheets of cells appear in such cultures. Nervous tissue is characterized primarily by the movement of numberous nerve cell processes and of only a few cells into the medium (3).

A fibroblast is a flattened cell with tapering processes which may be in contact with those of adjacent cells. The cytoplasm is smooth and usually contains a few granular mitochondria. The nucleus is oval and quite large.

With the exception of nervous tissue, practically all types of tissue when cultured give rise to a large number of cells which suggest by their appearance the fibroblast of the normal loose connective tissue. Remember then almost all tissues give rise not to fibroblasts, but to fibroblast-like cells. It should also be pointed out that blood leucocytes can develop into fibroblast-like cells in vitro. So this would be one disadvantage of using blood as a medium. Fibroblast-like cells also arise in cultures of embryonic bone but does not develop.

When most of the epithelium-containing organs of embryos, and to a lesser extent of adult animals, are cultured, there is an outgrowth of epithelium and connective tissue, and usually some endothelium. After a few days the outgrowth of connective tissue predominates greatly over the epithelium unless certain experimental means are employed to permit the epithelium to grow. Although in the mammary gland of the

mouse the epithelium grows faster than the connective tissue in culture.

Epithelium in culture usually grows in thin membranes or solid cords; only rarely does it grow out of the explant as a tubular structure. But, in all of these instances the epithelium preserves one of the characteristics of this type of tissue, namely, adjoining cells are in immediate contact.

Fibroblasts of both embryonic and adult connective tissue may grow in sheets in which adjacent cells are in as close contact as those in any epithelium. Accordingly, there seems to be no difference between epithelium and connective tissue. Both of them may grow as "epithelial" membranes and they may both grow as isolated or loosely connected cells. When these cells are grown in glass flasks, they may be viewed under the microscope.

Pure cultures of epithelium may grow for months in sheets on free surfaces, while it may grow in cords or as isolated cells when buried deep in the fibrin of the culture medium. Pure cultures of epithelium grow in tubes and cords when connective tissue is added to them. Isolated epithelial cells have been observed to turn into typical phagocytes which cannot be distinguished morphologically from the free macrophages of the reticular tissue in vitro.

Muscle Tissue:

Adult cardiac muscle does not grow and dies after a short time in vitro common. However, in explants of 24 to 30 hour

old undifferentiated heart primordium of the embryonic chick, the myoblasts develop cross striations and contract rhythmically. They persist for several weeks and then redifferentiate, lose their characteristic structure and appear as fibroblast-like cells. In cultures of older embryonic heart (3 days) the histologically differentiated myocardial cells migrate into the medium where they soon lose their cross striations, although they continue to beat rhythmically. In plasma alone, such cultures might contract for over 60 days.

Nervous Tissue:

After several subcultures, nerve cells begin to die and the residual, undifferentiated neural epithelium begins to grow until finally a pure culture of epithelium may be obtained. It is also possible that nerve cells may gradually turn into fibroblast-like cells. In addition to nerve cell processes, neuroglial cells and microphages migrate from explants of embryonic nerve tissue. The question of the nature of the microglia or cells which support nervous tissue is still unsettled. Mitoses are probably completely absent in nerve cells in the body and are exceedingly rare in nerve cells in culture, having been described by only a few observers. Nervous tissues are harder generally speaking to culture than other types.

In summary, the cells of embryonic nervous tissue have marked powers of self-differentiation in culture. In nerve cultures, the differentiated cells die after a few weeks while the undifferentiated cells persist as a pure culture of

epithelium (3).

Strains of thyroid cells can be isolated and maintained in pure condition, probably for an unlimited period of time. Their cultural requirements are similiar to those of fibroblasts. Embryonic tissue juice must be present in the culture medium if they are to multiply indefinitely. According to the condition of the medium, the cells assume the appearance of pavement epithelium or of fibroblasts, but they do not dedifferentiate. When the cells are growing in thin membranes at the surface of the clot, generally no evidence of the secretion of the colloid material can be found, but when they grow within the coagulum, they again assume a glandular appearance and form acini filled with colloid material. This change occurs spontaneously whenever the cells grow inside the clot, the presence of fibroblasts being unnecessary. Not only do the cells fail to dedifferentiate, but they keep the property of secreting growth promoting substances for fibroblasts. The growth-promoting substances are probably identical with those contained in thyroid extracts, the action of which an connective tissue was observed long ago, in vivo as well as in vitro.

A pure strain of thyroid epithelium was isolated and maintained in active condition by Ebeling for seven months. At the end of the experiment, the rate of cell multiplication was as great as at its beginning (13).

The most readily embryonic tissue is that of the chick and mouse. The classical source is the chick (36).

CHAPTER VI

PRIMARY EXPLANATION TECHNIQUES

Primary explantation denotes the cultivation of pieces of tissue fresh from the organism. Most of the techniques are similiar in principle, the main difference being in the vessels used for growing the tissue. The main group of methods for growing tissue are depression slides, Carrel flasks, and roller tubes of which there are many variations.

Due to the length of this report, I will illustrate only the chick embryo as a source of tissue and explain three culture methods.

A chick embryo of 10-12 days is a good supply of tissue. Crack the shell and gently lift the embryo out with a pair of curved forceps and place it on a petri dish. Lay the embryo on its back and severe one leg and expose the thigh muscles by peeling the skin off. A section of muscle is then removed, transferred to a glass plate and cut into a number of small fragments with a cataract knife. Each fragment should not be more than one cubic mm. in size. The liver, heart, and intestines may be removed by splitting open the abdomen and should be treated as the thigh muscle was. The head should be cut off also. Commencing near the tail of the embryo apply two pair of wide forceps. Work these forward, one after the other so as to squeeze out the

spinal cord, rather like toothpaste from a tube. The spinal cord is sliced in small pieces as is the skin.

Slide Cultures:

This is the oldest method of tissue culture, but is less frequently used now. It has a number of advantages such as it is simple and relatively inexpensive. Also the cells spread out for favorable microscopic examinations and they grow directly on the coverslip so that they are easily fixed, strained, and made into permanent preparations. The disadvantages are the small supply of oxygen and nutrients that are very rapidly exhausted, the medium quickly becomes acid and this necessitates that rapidly grown tissues must be transferred frequently. In addition, it is difficult to maintain sterility for long periods of time. But the beginner should master this technique before doing more complicated ones.

Technique:

The medium consists of chicken plasma and the chick embryo extract diluted with Tyrode's solution or equivalent. The plasma should be kept chilled until just before use.

The explants are placed on 22 mm. square coverslips in one drop of plasma and then afterwards a drop of embryo extract is added. Next the medium and explants are thoroughly mixed by rotating the tip of the pipette in a small circle. The liquid medium should be placed and stirred with a small pipette. The tissue should lie in the center of the medium.

Meanwhile several pairs of round depression slides are

prepared with vaseline. Only a trace of vaseline is used since an excess is likely to run into the culture upon incubation. This is applied with a sterile glass rod. The slide is then oriented, depression slide down, over the coverslip. By this time the medium will have coagulated and it will be possible to turn the preparations over. Finally, they are all sealed with melted paraffin and set away for incubation. The paraffin is applied with a brush around the entire margain of the coverslip. If the preparations are not completely sealed, the cultures will evaporate.

The cultures are then incubated and can be examined periodically for growth under the scope.

The hanging drop method is the best method for minute high power observation of the behavior of individual cells. But, the nutrient needs to be changed every 48 hours or so and the small size of the nutrient drop precludes accurate control of the chemical or physiocochemical changes which the nutrient undergoes.

With practice and sterile techniques, the hanging drop method can be quickly done. Penicillin should be added to prevent any contamination present from spreading. If the beginning student can master the hanging drop technique it will give him confidence and encouragement. The main thing is to avoid contamination. If the plasma, explants, etc. are all prepared under a hood, contamination will be greatly reduced. Make sure the paraffin seals the coverslip down real tightly. The hanging drop is also suited for viewing under the microscope.

To wash the cultures, the coverslip bearing the tissue is removed by a razor blade and thumbnail. Then flip it over and transfer it to a Columbia staining dish (provided with vertical grooves for the accomodation of four coverslips) containing Tyrode's solution. After the culture has been washed, the coverslip is reattached to a new slide which has had new media added. Again, this should be done under the hood. By washing in Tyrode's waste products are removed (36).

Cultivation for Prolonged Periods

Tissue extracts are prepared from adult chicken heart muscle and from adult chicken brain. The tissue is minced finely with scissors and suspended in four times its volume of Tyrode solution. The resulting suspension is allowed to stand for 24 hours in the refrigerator, and after centrifugation the supernatant fluid is decanted. Heart extract is used in the original concentration. Brain extract is diluted before use with an equal quantity of Tyrode solution.

Cultures are made from heart muscle of adult fowls and fragments of uniform size are implanted in Carrel flasks. The medium consists of .5 cc. adult fowl plasma diluted with 1 cc. Tyrode solution. Two drops of adult heart extract are added in order to coagulate the plasma. Every fourth day the cultures are washed in Tyrode solution and fresh tissue extract is added. At intervals the coagulum is reinforced by the addition of fresh plasma diluted with Tyrode solution. The cultures are then transferred every 16-20 days (10).

Embryonic extracts stimulate cell proliferation as well as enable prolonged growth of fibroblasts in vitro. Extracts of adult tissues are not capable of maintaining indefinitely the life of the fibroblasts in vitro, as fresh embryonic juice does. However, fibroblast colonies will grow quite well in medium composed of adult plasma to which either adult heart or brain extract is added. These colonies may be transferred without difficulty from flask to flask.

It is evident that adult cells may be cultivated outside the organism permanently, completely free of embryonic extract and composed entirely of adult plasma and adult tissue extract (4).

The longevity of a tissue culture is not dependent on culture size. The problem of longevity may be defined in terms of preventing deficiencies in nutrient or accessory substances. Insofar as nutrition is concerned, prolonged survival depends on supplying the greatest tolerated food reserve and also upon excluding stimulating or readily utilized substances such as fresh embryo juice, human placenta serum etc. Among the substances studied, the best longevity occured in approximately 15-20% of chicken serum and of pasteurized embryo juice with glucose levels at 400 mg. per cent. The presence of low concentrations of sodium citrate was found to repress both the rate of growth and of acidification, and thus, to be advantageous. Whether its effect was to modify the rate of glycolysis or on the concentration of certain cations is not known.

It is interesting that chick tissue cultures maintained at approximately 30° C. not only produce more cells than at 37° C., but are capable of completely invading and utilizing the plasma of the medium, and thereafter, of surviving on the glass walls of the vessels, whereas at higher temperatures they die long before this is accomplished.

Reduced temperature tends to minimize certain unfavorable conditions such as rapid enzyme decay, diversified metabolism, etc. At the higher temperatures diffusion becomes a limiting factor, since even in liquid media there is less dissolved oxygen to supply a greater demand while in solid media such as agar or plasma, the problem is not only considerably more critical with respect to oxygen, but includes also the rapid local depletion of nutrients and the higher local concentrations of acid, which in turn hastens enzyme decay. The more acid the medium, the more the enzymes are inactivated. When cells are growing leisurely, whether at reduced temperature or in vivo, they benefit not only from the more favorable relations between diffusion and demand, but are also less confined to the sources of energy, since there is time and opportunity to hydrolyze complex substrates and to utilize metabolites which may serve as secondary sources of energy. It is not possible to know whether greatest importance should be attached to the factor of slow growth or to the temperature ranges of effective enzyme synthesis.

It has been shown that chick tissue cultures survive for 3 or 4 months at 30° C. when supplied with only 0.6 ml.

of medium. Their survival was not prevented by an accumulation of toxic metabolites other than hydrogen ions, but was dependent on the supply of utilizable nutrients. During slow growth at reduced temperatures the substrate was utilized much more effectively and completely than it was during rapid growth. It would be profitable to culture tissues at both room and lowered temperature and determine which temperature gives the best results (15).

Carrel Flask Technique:

A good Carrel flask has excellent optical properties and this is an advantage if it is desired to follow growth of the cells microscopically. The flask should also have a wide neck. The main advantages of this flask is that the tissue can be maintained in the same flask for months or even years and they do not need to be changed so often. Large number of cultures can be prepared and grown at the same time.

Technique:

A convenient number of flasks to handle at one time is six. Place one drop of plasma on the floor of each flask. Using a platinum spatula spread this plasma out in a circle extending just short of the edge. With the spatula transfer the number of explants to a respective plasma pool. Clotting should occur.

Extra medium can be added now. One-two ml. of dilute serum is added instead of plasma. The flasks should then be gassed with 5% CO_2 in air. While going in and out of the

flask, do it under the hood and make sure all instruments are sterilized. This technique is just as simple as the hanging drop.

Renewal of Medium:

(1) Draw off the old fluid with a pipette.

(2) 1-2 ml. of fluid is added to replace this.

(3) The flask is again gased.

Common Errors in Preparation of Tissue Cultures:

1. Crushing and tearing. The tissue extracted should be made with a single straight clean cut edge. Use of odd shaped explants leads to colonies of irregular form.

2. Failure to rinse properly results in a plasma clot which is cloudy with erythrocytes and cellular debris. This interferes with examination. Wash at least two or three times in Tyrode's or a balanced salt solution.

3. Bubbles in the plasma may occur due to unskillful use of the pipette when adding transplants. If bubbles do form they may be eliminated before the plasma clots by touching it with a hot platinum spatula.

4. Premature coagulation of the plasma. After the embryo extract has been added it is necessary to mix the ingredients and position the explants quite rapidly. If coagulation begins, strands of fibrin may bind the tissue to the platinum spatula. The explants will then pull away when this instrument is removed from the culture. This part of the procedure requires practice. Try to place the explants in the middle so all the cells will have an equal

distribution in the plasma. The longer the medium and glassware is exposed the greater the chance of contamination.(26).

Test Tube Cultures:

Test tubes are very cheap and convenient vessels for the cultivation of cells and tissues. They can be set up in either roller drums or in stationary racks. But, the optical conditions are poor and also there is a risk of contamination due to leakage of air between the stoppers and tubes. This may occur on opening tubes which have been removed from the incubator when there is an inrush of air. If old test tubes are used, soak them overnight in acid, wash in soap and water, then put in autoclave for 2-3 hours.

Plasma Clot Techniques:

1. With a Pasteur pipette, place a drop of plasma near the bottom of each tube.

2. Then put the explants in the plasma and let clotting occur.

3. After they are fixed, add medium, .5-1 ml. per tube.

4. Stopper, label, place in rack or on a roller drum which should be in an incubator. Here again, screw type test tubes would help prevent contamination. Try to do all the procedures as quickly as possible to prevent contamination.

The roller drum is simply a motor driven drum with holes fitted for test tubes. As it slowly turns the medium is applied to the explant.

A roller drum can be built quite easily. A circular piece of board or any material with holes drilled in it to accomodate the test tubes, is connected to an electric motor.

The motor is set to rotate the board about 10 or 15 revolutions a minute. This procedure allows the medium to bathe the cells and keep them moist at all times. The board can be made to hold as many test tubes as desired.

By use of the roller drum more cultures can be prepared and cultured at once. The beginner should do the hanging drop and Carrel flask techniques first for experience. At the same time this will prevent expensive loss of material due to only a few cultures being done.

Feeding Test Cultures:

Simply remove the supernatant fluid and replace it with fresh media. This will of course be done with sterile pipettes.

Transfer of cultures from test tubes:

Cultures can be removed from the test tubes by cutting around them with a bent Pasteur pipette. Then gently push on the margins of the dissected plasma the small disc containing the colony. This should free the explant from the glass. The tissue is then freed of pheripheral plasma by a razor blade.

Flying Coverslips in test tubes:

Flying coverslips are small narrow coverslips (11 mm. wide, 40-22 mm. long). These can be inserted into ordinary 6" x 5/8" test tubes. Cultures are prepared on them in the standard manner for coverslip cultures, but instead of mounting them on depression slides, they are slid into test tubes. A fluid medium is added to the tubes (1 to 2 ml. each) which

may then be put in the roller drum.

In those techniques described, the cells are allowed to migrate. This produces very flat cells spread over a large area, but often the surface area for growth is limited as with test tubes, flasks, etc. This migration can be seen with the naked eye and will look like a dull, gray mass (30).

CHAPTER VII

CELL STRAINS

When a colony of cells has been subcultured many times in vitro, and continues to divide rapidly so that there is no reason to doubt that it can be carried indefinitely, it is called a strain.

After an initial phase of rapid growth, cells slow down and stop growing. In many cases they die out, but in some, after a static period, there is a sudden outgrowth of new cells. It is generally agreed that cells should survive twelve to twenty generations without any evidence of alteration of the growth pattern before they can be considered a strain.

Some cells which came from the original culture may be different in metabolism and growth patterns, shape, etc. At the present time it is not known whether these cells arise by selection, adaptation, mutation or some other undefined process. Almost all strains always grow as colonies, layers, or suspensions.

The cells may be maintained in any flat bottomed dish, such as a Petri dish, which is properly sterilized and sealed for use. Use a dish which has good optical properties for microscopic examination also. On the cells may be cultured as previously described. Care should be taken in identifying

a strain. Only after thorough knowledge and several different characteristics have positively identified should the cells be called a strain. This report is not concerned with identification of cell strains so I will not dwell very much on this subject.

Feeding is very simple, consisting merely of removing all or part of the used medium and replacing it with fresh medium. In transferring the cells a suspension is required.

There are two methods for bringing cells to a suspension in order to remove them. One is by physical means--scraping from the glass, shaking, etc. Another way is by chemical means--usually by proteolytic enzymes.

Some cell stains are very loosely adherent to the glass surface and may be suspended by agitating the flask gently. Others are dislodged by scraping with a rubber or plastic rod. When the dislodged flakes are sucked up and down once or twice in a pipette a suspension or single cell is formed.

The Trypsin Techniques:

A trypsin solution consisting of 5 per cent trypsin in BBS, is diluted down one-tenth in BBS, and then incubated. This is to raise the temperature up to 37° C. The medium is removed from the cells and replaced with the trypsin solution. The vessel is rocked gently from time to time for 10 minutes and then as soon as the cells begin to leave the vessel wall the suspension is transferred to a sterile centrifuge tube and spun down at about 1000 R.P.M. for a few minutes. The supernatant is removed and replaced with medium. The cells

are then sucked up once or twice to form a suspension. This method is the one most commonly used to make a suspension and is not difficult to do.

The pH is important during typsinisation. Below pH 7, trypsin is inactive. Above pH 8, the cells are damaged. With delicate cells, excessive pipetting is to be avoided.

For a great many purposes the best way to grow cells is in suspension. It provides more uniform growth and permits more accurate sampling and therefore provides the ideal system for biochemical and kinetic studies. Also, for the production of large numbers of cells it is most likely to be the best.

Most of the cells grown in suspension now are gently vibrated or kept in suspension by means of some type of shaker. For instance a cytostat consists of cells growing in a 1 litre flask. A stirrer driven by a motor keeps them in suspension. A mechanical timing arrangement, linked to a pump, adds a volume of medium and removes an equal volume of cell suspension at determined intervals. By regulating the system so that the cells are removed at the same rate as they are produced, logarithmic growth can be maintained indefinitely.

Feeding of Cell Strains:

Normally cells grow to a maximum population in a vessel of given size and will increase no further, although if the medium is renewed regularly they will survive in a healthy state. Thus, the rate of growth of the cultures can be controlled by varying the size of the inoculum. Rapidly growing

cells will increase about a hundred-fold in a week and it is common practice with some strains to inoculate fresh vessels one-twentieth to one-fiftieth of the final population.

A convenient routine for the maintenance of cells is to handle them twice a week, feeding them on one occasion and transferring them on the other.

CHAPTER VIII

PRESERVATION AND STORAGE OF LIVING CELLS

A major problem is the maintenance of the large number of cultures necessary to carry several strains simultaneously. A further difficulty is the tendency for cell-types to change after they have been kept in a state of proliferation for some years.

Cells can be maintained at slightly reduced temperatures, refrigerator temperatures or very low temperatures (-70° C.). Room temperature and refrigerator storage are sometimes used for short term maintenance. For a long term storage, very low temperatures are necessary. In the deep frozen state metabolism is suspended completely. At 20° C. very infrequent renewal of the medium is required.

Sheets of cells and single cells do not survive well for more than a few days in temperature of 2-6° C. But, tissue fragements will survive for several weeks in this temperature. The main practical use of this method is the preservation of tissue before explantation, usually only a few days.

If a tissue is deep frozen ice crystals tend to form and lacerate the cells. If some substance such as glycerol or ethylene glycol is added to the medium and allowed to freeze slowly, it will work nicely. Others contend that the rate

of freezing is unimportant while others have shown convincingly that slow freezing gives better results. All authorities agree that it is best to store cells at the lowest possible temperature and -70° C. is usually employed. They usually die if stored for any length of time at a temperature higher than 20° C. Cells have survived from two-five years in this condition.

Cells can be transported over long distances via mail now. If they are sent by air the only difficulty encountered will be the variations of temperature. So it is best to send the cells in insulated package (foam plastics, powdered cork, and cotton) (31).

CHAPTER IX

MORPHOLOGICAL STUDIES

Almost all cells in a tissue culture tend to lose their identity; however, some, such as neurones and the HeLa cells do retain their characteristics.

After a tissue culture has been established, the cells may be stained and fixed for histological studies. In order to fix and stain the cells, Grays "Basic Handbook of Microtechnique" or Guyers "Animal Microscopy" are two sources of information.

CHAPTER X

CONCLUSION

The study of a tissue culture is a science in itself. Only special, intensive training, and research will yield a full understanding of the cells' functions, activities, and requirements for life. But, the potential usefulness of the culture is unlimited both to biological and medical science.

The technics and knowledge which required years to develop are now being used by people who are not professionals in this field and with further research many of the age-old problems of disease and health will be discovered.

This paper is meant to demonstrate for a non-experienced worker how to do a tissue culture. It is by no means complete, but it does explain a few of the basic procedures.

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