Investigation of Substitutes for Foetal Calf Serum in Animal Cell Culture Media

by

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ABSTRACT

The purpose of this thesis was to develop a serum-free medium which would support the growth of a human tumour cell line, RPMI 2650. Serum is used almost universally as a medium supplement to support growth of cells in culture, but investigators in the field have long recognised the problems associated with the undefined, complex and variable composition of serum whereas serum-free media permit growth of cells in a precisely-defined environment.

In order to gain experience in the techniques used to grow cells in culture in the absence of serum, initial work was carried out on a canine kidney cell line (MDCK), for which a serum-free medium had already been developed. Growth of RPMI 2650 was initially examined in reduced serum conditions at clonal cell densities and the effect of various growth factors, hormones and other nutritional factors was examined. Significant growth stimulation was observed due to the addition of a number of factors (transferrin, insulin, EGF and selenium).

Growth occurred in dishes precoated with DEAE dextran in the absence of any serum supplement following addition of medium containing six components (transferrin, insulin, epidermal growth factor, selenium, ascorbic acid and a commercially-available amino acid mixture, MEM non-essential amino acids).

CONTENTS

1. INTRODUCTION

When attempts were first made to grow animal cells in culture, the media used were biological fluids. These included embryo extract, spinal or amniotic fluid (Barnes and Sato, 1980 a), lymph (Harrison, 1907) and plasma or serum (Brooks, 1975). It was thought that these materials resembled the natural environment of the cells. In time, a basal nutrient mixture of salts, sugars, amino acids and vitamins was found to support the growth of almost all cell types, but only when supplemented by a poorly defined biological fluid, generally serum. Sera from a number of sources are used - horse, bovine, human - but the most widely used serum is foetal calf serum (FCS).

1.1 Role of FCS

FCS is a complex mixture of proteins, hormones and nutrients (as well as salts and various metabolites). It contains some factors which are essential for the survival and growth of cells in culture, but our knowledge of the nature and mode of action of these factors is still incomplete, in spite of considerable research in this field.

Identification and purification of the various components in serum has proven very difficult for several reasons, for example :

(1) Serum contains a complex and diverse mixture of active components, including proteins:

(2) Many of the active components (e.g. hormones) are present in minute and variable amounts and may also, in some cases, be bound to serum proteins:

(3) There may be synergistic action between factors in the serum, which may be diminished or lost during fractionation procedures.

A considerable amount of information is, nevertheless, available on the active components of serum. Identified components can be divided into four groups :

- proteins and peptides (non-hormonal)
- hormones
- metabolites and nutrients
- trace elements

One important *in vitro* role for serum proteins is to act as carrier proteins for minerals, fatty acids and hormones. These proteins may stabilise and modulate the action of substances which they bind, or in some instances, they may detoxify the medium, as in the case of transferrin, which binds iron, thus rendering it less toxic but still available to the cells.

Serum contains a trypsin inhibitor; it has been suggested that the α_2-macroglobulin molecule is responsible for this action (Freshney, 1983). This, and possibly other protease inhibitors, play a very important role in protecting cells from proteases during passaging of cells *in vitro*. Serum also supplies factors which are involved in attachment and spreading of cells to and on the substrate. These include the proteins fibronectin and fetuin and possibly a number of other components which combine to act in a similar manner to stromal influences *in vivo*.

Certain polypeptides seem to play a critical role in promoting growth. One major contributor, platlet-derived growth factor (PDGF), is released from the platlets during clotting. This is thought to be the major growth promoter in serum for some cell types e.g. mouse Balb/c-3T3 cells (Antoniades, 1975). There is much speculation regarding some other polypeptide growth factors e.g. epidermal growth factor (EGF), fibroblast growth factor (FGF), multiplication stimulating activator (MSA) - which have been detected in the medium. It is not totally clear whether or not these are always present in minute quantities in the serum. It is clear in some instances that they are secreted by the cells into the medium during growth (La Rocca & Rheinwald, 1985) and act as autostimulators.

The hormonal composition of foetal calf serum is poorly defined. Insulin is known to be present in serum; growth hormone and the somatomedins are also thought to be present and may function synergistically to have a mitogenic effect (Freshney, 1983).

A number of nutrients such as glucose and amino acids are present in serum; these may be important when added to a simple basal medium, but cells growing in a complex basal medium have little need for these nutrients. However, serum vitamins may play a more critical role for some cell types e.g. biotin is absent from many basal media. Different cell types have different nutritional requirements and serum may supply a low, but critical level of a vitamin or some other nutrient. This specific nutritional requirement must be identified and supplied when growth is taking place in a serum-free environment.

Trace elements including iron, copper, zinc and selenium may be supplied to the cells by serum. These and possibly traces of many other minerals, may be bound to serum proteins.

1.2 Reasons for development of serum-free media

Almost all cell types require serum for growth in culture but there are numerous disadvantages associated with its use and these have led to increased interest in the development of serum-free media in the past fifteen years.

Workers in cell culture have long realised problems associated with the undefined, complex and variable composition of serum and their aim is to develop precisely defined environments for growth of cells. The concentration of various components varies considerably from batch to batch and this batch variation causes four main problems :

(1) the actual growth of cells - i.e. number of population doublings and time taken for each doubling to occur - can vary dramatically. This renders study of growth or any growth-related parameter very difficult.

(2) Specific cell types can be affected differently by batch variation. A batch of serum may preferentially support or inhibit growth of a specific cell type - thus, in a laboratory growing more than one cell type different batches of serum are required for optimum growth of different cell lines.

(3) Continuous long-term studies are disrupted when a change over to a new batch of serum occurs. Much time can be spent trying to achieve standard growth conditions with each new batch - and the nature and extent of growth will never match exactly that in the previous batch.

(4) If hormonal or nutritional studies on a serum component are being carried out, the serum contribution of this component can vary significantly from batch to batch.

Serum is also an expensive component, 100mls of FCS costing between £20 and £25 and a considerable portion of a cell culture laboratory's budget is spent on serum. The supply of FCS is also variable - in 1979 a world-wide crisis occurred which forced many laboratories to seek alternatives to FCS to maintain their stocks. There can also be variations in the manner in which the serum is processed prior to sale, thus increasing the variation associated with its use.

There have been reports of the presence of antibodies, bacterial toxins and growth inhibitors in serum (Harrington and Godman, 1981). Antibodies which were produced by the animal prior to collection of the serum remain in the serum after processing and may interfere with growth, possibly by binding to cell surface antigens. There may be some bacterial lipopoly-saccharides in some serum batches which may also affect growth. Some cell types grow better in the absence of serum, which suggests the presence of an inhibitor in the serum (Wille et al, 1984). It is also possible that physiological growth inhibitors may be present in varying amounts in different batches of serum. (Steck et al, 1979).

Cell culture concentrated initially on growth of fibroblasts and thus conditions were optimized for their growth. Serum stimulates fibroblast growth in culture but this is proving a major problem in primary culture work. Fibroblast overgrowth of other cell types is common and thus, a defined medium which would yield selective growth of different cell types would be a major step in the area of primary cultures.

Thus, the attractions of working with a defined medium are clear and these have led to numerous attempts at the development of such conditions.

1.3 Approaches to the development of a serum-free medium

When serum is omitted from culture medium substitutes must be found for all of the critical roles which serum plays, as described above. Three main approaches have been taken in attempting to minimise or replace the use of serum in cell culture media :

(1) Adaptation of cell lines to continually decreasing levels of serum, generally accompanied by the introduction of some known growth stimulators : Gradual adaptation to low serum levels may lead to selection of a sub-population of cells which will survive under these conditions. This is not desirable for many applications as the selected sub-population may vary significantly from the parent population in some important aspects. Thus, this method has proven generally unsatisfactory.

(2) Adjustment of basal medium components to provide cells with an optimal nutrient balance : All cells are cultured in a basal nutrient medium which consists of a balanced salt solution (which provides the correct ratio of various ions, an iso-osmotic environment for the cells and a buffering system), amino acids, vitamins and glucose or another carbon source. There are a number of commercially available basal media which are widely used in conjunction with serum for the growth of a variety of cell types. The laboratory of R.G. Ham, in particular, made considerable progress in optimising concentrations of the individual components of basal media. By sequentially optimising the precise concentration of each component in the basal medium, new media have been developed which support the growth of specific cell types in a totally defined medium. Peehl and Ham (1980) show that in two media developed in this manner from Ham's F12, human keratinocytes grow selectively in MCDB 151 medium and fibroblasts in MCDB 105.

This approach has led to the formulation of many useful chemically-defined media, some of which are now becoming available commercially. These optimal basal media often require supplementation with a much lower serum concentration than do standard media and replacement of serum often proves easier in such circumstances.

(3) Replacement of growth stimulatory activities of serum by a combination of hormones at the correct concentrations :
Many laboratories have followed the lead of Barnes and Sato (1980a) along this line of research. The first step involves choosing a basal medium which is rich in low molecular weight factors (e.g. vitamins, amino acids, trace metals), since serum supplies such nutrients. Ham's F12 (Ham, 1965) is one of the most widely used media, as its relatively complex nature seems suitable for growth of many cell types. It is often used in conjunction with other simpler media - e.g. a Dulbecco's Modified Eagles medium (DMEM) : Ham's F12 mixture forms the basis for a number of serum-free media (e.g. Taub, (1979) - canine kidney cells; Calvo et al.(1984) - human breast carcinoma cells; Morrison et al.(1981) - rat astrocytes). Dulbecco's medium contains high concentrations of many essential nutrients, whereas Ham's F12 contains a wider variety of nutrients at lower concentration.

A wide variety of hormones have been tested on a range of cell types. In general, workers have found that cells need factors other than hormones to grow in the absence of serum. Also, the combination of components for different cell and tissue types varies considerably. This is a fact, which, if exploited, could be of major significance in the area of primary culture work, where, as mentioned previously, fibroblast overgrowth proves a major obstacle to growth of epithelial cell cultures. A system allowing selective growth of epithelial cells over fibroblasts would be of considerable help. Thus, developing a serum-free medium for a specific cell type involves investigating the effects of all available hormones, binding proteins and attachment factors on the cells in vitro. Table 1.1 lists some of the factors which have been used in serum-free formulations to date.

TABLE 1.1

Hormones and Growth Factors	conc. used
Insulin	0.1 - 10µg/ml
Epidermal Growth Factor (EGF)	1 - 100ng/ml
Fibroblast Growth Factor (FGF)	1 - 100ng/ml
Platlet-derived growth factor (PDGF)	1 - 5ng/ml
Nerve growth factor (NGF)	1 - 10µg/ml
Hydrocortisone	1 - 100nM
Triiodothyronine	1 - 100pM
Progesterone	1 - 100nM
Glucagon	0.1 - 5µg/ml
Growth hormone	50 - 500ng/ml
Follicle stimulating hormone (FSH)	50 - 500ng/ml
Luteinizing hormone	0.5 - 2µg/ml
Thyrotropin releasing hormones	1 - 10ng/ml
Parathyroid hormone	1ng/ml
Somatomedin C	1ng/ml
Estradiol	1 - 10nM
Testosterone	1 - 10nM
Prostaglandin E_1	1 - 100ng/ml
Prostaglandin $F_{2\alpha}$	1 - 100ng/ml
Prolactin	5µg/ml
Glycyl-histidyl lysine	1 - 200ng/ml
Bombesin	1 - 200ng/ml
Binding proteins	
Transferrin	0.5 - 100µg/ml
Bovine serum albumin	0.1 - 10mg/ml
Attachment and Spreading Factors	
Collagen	precoating
Poly-D-lysine	precoating
Gelatin	precoating
Fibronectin	precoating/2-10µg/ml in medium
Laminin	precoating
Fetuin	1 - 5µg/ml in medium

TABLE 1.1 (cont'd)

Low-molecular weight nutrient factors/Trace elements

Selenium	10 - 100nM
Cadmium	0.1 - 1μM
Putrescine	100μM
Ascorbic Acid	10μg/ml
Trace Element mixtures	-
Ferrous sulphate	10^{-5}M

Table 1.1 : Serum-free media components
A list of some of the components used in serum-free media formulations.

Data compiled from Barnes and Sato (1980 a,b), McKeehan (1984), and Bottenstein et al. (1979).

As the work carried out in our laboratory has followed the latter approach, I will discuss the conventional pathway of investigation. In order to see some stimulation of growth when the various factors are added to the medium, the growth-promoting capacity of the serum supplement must be reduced in some way. However, total removal of serum initially is usually too dramatic a step as it may be difficult to see any growth at all with only a small number of randomly chosen factors - but a negative result at this stage does not necessarily mean that all the factors under study can be discarded as having no stimulatory ability. A vital factor may be missing, in the presence of which some or all of the original components may be growth stimulatory. In general, if a low level of serum is present, a growth stimulating component will be detected more readily by stimulating growth above the background level which the low serum concentration generates.

Thus, it is normal to first lower the serum concentration in the medium until growth, while still occurring, is significantly reduced. Then, under such suboptimal conditions, a wide range of factors can be tested individually and in combinations. As stimulatory factors are identified, the serum concentrations can be reduced and the optimal concentration of these factors worked out.

Table 1.1 lists many of the factors that have been used in serum-free media. Of these, insulin, transferrin, epidermal growth factor (EGF) and selenium are possibly the most commonly used components. Insulin, which is normally used at concentrations which are much higher than physiological levels, and transferrin are found in almost all serum-free media. They have been found to stimulate a wide range of cell lines e.g. SV40 Balb/c 3T3 (Rockwell 1984), human diploid fibroblasts (Kan and Yamane, 1982) and a human mammary tumour cell line MCF-7 (Barnes and Sato, 1979). Insulin and transferrin are also included in serum-free media used to establish some primary cultures e.g. human lung cancer cells (Carney et al., 1981) and canine kidney cells (Taub et al., 1979).

Epidermal growth factor (EGF) was originally isolated from male mouse submaxillary glands (Cohen, 1962). It is now commercially available and although relatively expensive, has been used in many serum-free media e.g. human keratinocytes (Tsao et al., 1982), rat hepatocytes (Enat et al., 1984) and Hela cells (Hutchings et al., 1978). EGF is a 53 amino acid polypeptide, with a molecular weight of approximately 6,000 Daltons. Its contribution

to growth in the absence of serum, varies between cell types; in some cases, EGF can replace another component e.g. in growing MDCK cells in culture, the PGF_1 requirement can be removed by adding EGF (Taub et al, 1984). In many serum-free media however, EGF seems to be required and cannot be replaced by other factors.

Selenium is a very interesting addition to serum-free media. It is added as a trace element in the form of sodium selenite or selenous acid and it has been shown to play a critical role in the growth of primary lung cultures (Carney et al., 1981), a lung cell line NCl-H69 (Carney et al., 1984), canine kidney cells (Taub et al., 1979) and MCF-7, a human mammary tumour cell line (Barnes & Sato, 1979). Addition of selenium is considered more as a modification to the basal medium than as an addition of a growth factor as such. It has been suggested that the requirement for selenium becomes greater as the level of FCS decreases (Ham, 1984b) which would support the idea that selenium is present as a trace element in FCS.

Other growth factors have been isolated and shown to stimulate growth of specific cell types. Platelet-derived growth factor (PDGF) is thought to be a major mitogen in serum for many cell lines, in particular those of mesenchymal origin. It has been used in serum-free media for Balb/c 3T3 cell line (Dicker et al., 1981) and has also been shown to be mitogenic for fibroblasts, smooth muscle cells and glial cells (Westermark et al., 1983). In the past two years much interest has been shown in this factor and it may come to play a much more prominent role in serum-free media in the near future.

Fibroblast growth factor (FGF) is used in many serum-free formulations to stimulate cell growth. A number of extracts from bovine neural tissue have proven to contain growth factors for cultured fibroblasts and vascular endothelial cells (Gospodarowicz, 1984). There are thought to be several factors present which have been given the name FGF; they are all single-chain proteins 14-18kDa in size (Goustin et al., 1986). Many fibroblastic lines have shown increased growth in the presence of FGF, e.g. Balb/c 3T3 (McClure, 1983), Swiss 3T3 cells (Shipley & Ham, 1983) and BHK kidney cells (Maciag et al., 1980) but other cell types also respond to FGF - e.g. a rat glioblastoma cell line, C6 (Bottenstein et al., 1979). Commercially available, FGF is generally isolated from bovine pituitary glands, as first described by Gospodarowicz (1975).

Glucocorticoids have been tested by various workers for growth-promoting ability. Hydrocortisone and its synthetic analogue, dexamethasone, are the two most commonly used. The former stimulates canine kidney cells (Taub et al., 1979), lung carcinoma cell lines (Carney et al, 1981) and human bronchial epithelial cells (Lechner et al, 1982), while dexamethasone is included in media for the growth of WI-38 fibroblasts (Allegra and Lippman, 1978) and a rat myoblast cell line, L6 (Florini et al, 1979).

Table 1.1 shows a wide range of other hormones that have been tested in serum-free media formulations. Some of these occur frequently in media for a range of cell types e.g. triiodothyronine, but some other hormones are less frequently used. An important factor to be noted is that specific cell types have very specific requirements and addition of a more unusual hormone, at what may appear to be a very low concentration, can prove to be the most crucial component in a serum-free medium. This hormone/growth factor may have no effect on a wide range of cell types but a given cell type may depend on its presence for survival in a serum-free environment. A typical example of this is the addition of a prostaglandin, PGE_1, to five other components, by Taub (1979) in developing a serum-free medium for MDCK cells. Growth is doubled on addition of PGE_1, at a concentration of 25ng/ml, yielding a level of growth comparable to that in 5% FCS.

A number of attachment factors are listed in Table 1.1. For many cell types attachment to the substratum is vital for growth and replication, but in the absence of serum fibronectin - thought to be the main serum component involved in attachment, many cells are unable to attach to the usual plastic tissue culture surfaces. Many, but not all, serum-free formulations involve precoating of dishes with one of a variety of factors. Collagen gels are often used to improve cell growth. Both native and denatured collagen have enhanced growth in different cell types - for example, in fibroblasts (Grinnell et al, 1978) and epithelial cells (Wicha et al, 1979). Many reports also show that interactions between collagen and other factors - fibronectin, laminin, chondronectin - enhance growth, where collagen on its own has no effect (Kleinman et al, 1981).

The natural factor that has so far been shown to have an effect with the widest range of cell types is fibronectin. This high-molecular weight plasma glycoprotein, which is commercially available, has been shown to enhance attachment in a wide variety of cells, e.g. fibroblasts, hepatocytes, epithelial cells (Grinnell, 1983). Precoating of dishes with collagen, fibronectin or some other factor prior to the introduction of cells and media, has proven a successful method of enhancing attachment in the absence of serum.

Interestingly, some adhesion factors work indirectly i.e. by incorporation into the growth medium and not by precoating. Examples of this is chondronectin with chondrocytes and epibolin with epidermal cells (Hewitt, 1980; Stenn, 1980) where precoating of the surfaces has no effect on cell growth but inclusion of the two factors in the media has significant stimulatory effects.

1.4 Miscellaneous considerations in using serum-free media

Apart from selection of the correct combination of components to replace the mitogenic action of serum, there are many other factors which have to be taken into account in the development of a serum-free medium. The whole growth system is much more sensitive in the absence of serum than in its presence and, thus, all aspects of culturing cells must be considered carefully.

In general, the shelf-life of powdered medium is longer than that of basal liquid media and for serum-free work it is advisable to use powdered medium, as any slight change in the medium composition due to instability of some factors may have much greater effect than in the presence of serum. This demands facilities for sterilising large volumes of medium, a facility that all laboratories may not have.

Many of the components used in serum-free media remain stable for a short period of time only. Stability at 4°C may be measured in hours for some components and, thus, medium has to be made up freshly from frozen

concentrated stock solutions immediately prior to use. Some of the components remain stable for a reasonable length of time only if they are in a lyophilised form, so even the stock solutions need to be replaced regularly. Some of the components used are very expensive, e.g. fibronectin, EGF, FGF and inclusion of these in serum-free media increases the cost of routine growth of cells in such media.

Another major problem is subculturing of cells. Serum contains a trypsin inhibitor which stops trypsin action once the cells have become detached and prevents damage of the cells. Soybean trypsin inhibitor is commonly used in serum-free work but some problems still exist, with cells suffering considerable damage. Low temperature trypsinization is a common method employed, while in some cases other proteases are used in an attempt to minimize cell damage.

1.5 Other biological supplements

Some work has been carried out on alternative biological supplements which would provide the same growth and attachment factors as serum. Bovine and human milk have been studied in the laboratories of Klagsbrun, Fassolitis and Baserga and some reports published on growth stimulatory activity. Klagsbrun reported that normal fibroblastic cell lines grew in the presence of bovine milk and fibronectin (Steimer and Klagsbrun, 1981), while Sereni and Baserga (1981) reported that a number of cell lines, including 3T3, grew in medium supplemented with milk and 0.5% FCS. Fassolitis (1981) discovered that when medium was supplemented with a non-fat dried milk supplement it supported the growth of a number of epithelial cell lines but not of two fibroblast lines.

Quite a number of workers have isolated growth factors from human milk (Zwiebel *et al*, 1986, Carpenter, 1980) and the mitogenic effect of these cells in culture has been recorded. Carpenter suggests that the growth factor involved is EGF, while Zweibel states that a transforming growth factor can be isolated from milk.

There have also been reports of growth stimulation due to bovine colostrum. Klagsbrun (1980) reports that this supports the growth and proliferation of epithelial cells but not of fibroblasts, but that milk taken from the same animal after eight days fails to influence either cell type.

1.6 Commercially available serum substitutes

A considerable amount of interest has been shown by the commercial sector in the developments in the area of serum-free media. Some companies have carried out their own research and a number of new products have come on the market in the past few years. The claims made by the manufacturers range from a cautious approach, suggesting that a product reduces the serum level required, to a bolder claim of totally removing any serum requirement.

Table 1.2 lists a number of commercial serum substitutes. A number of similar claims are made by the manufacturers, e.g.:

- guaranteed lot-to-lot uniformity - constant composition relative to FCS.
- eliminates pretesting time on a range of FCS batches (and the need to buy a large amount of serum at the one time to be assured of uniformity).
- frees valuable freezer space (most products are stored at $4^{o}C$ and in more concentrated forms).
- low protein levels makes purification of cell derived products easier.
- low protein levels decreases non-specific interference in monoclonal antibody screening.

Some information is available on the composition of the medium supplements. The exact components in "SerXtend" are listed (Table 1.2) but the exact concentrations of the polypeptide growth factors are not included. In most cases a minimum of information is given, e.g. UltraSerG.

The cell lines on which the serum substitutes have been tested quite extensive in some cases, e.g. Nu-serum, but some other supplements are optimized specifically for a smaller range of cell types. The Nutricyte products fall into the latter category; these are a series of biochemically defined supplements formulated to replace FCS in hybridoma or lymphocyte cultures. The original Nutricyte supplement is for general use in cultures of human, mouse or rat myelomas, hybridomas or lymphocytes where strict immunocompatibility is not an issue, Nutricyte H is for use in the in vitro immunization of human lymphocytes and for human monoclonal antibody production, while Nutricyte BHK is designed specifically for suspension cultures of BHK and Vero cells.

Much of the literature which accompanies the serum substitutes gives details on growth of cells for one passage only - many do not give evidence for continuous culture in the absence of serum. In other cases, claims are made that continuous culturing with a given medium supplement is possible - but only if a low level of FCS - 0.5 - 2% generally - is added. Some references are also made to changes in morphology, growth rate or production of cellular products, e.g. UltroSerG supports the growth of GH3 rat prolactin-producing cells but continuous growth requires the addition of "some hormones" (unspecified) and under these conditions prolactin production ceases.

Many of the manufacturers recommended a gradual adaptation of the cells away from serum-supplemented medium to medium supplemented with their products. The disadvantages of this procedure have already been discussed (See Sec.1.3), but almost all the literature recommends it as a standard technique which will yield better results with the serum substitutes.

Thus, while many serum substitutes are commercially available, their applications are somewhat more limited than their promotional literature suggests. At the present time some of the products are of considerable use in short-term work, but much more progress is necessary before the term serum-substitute can be accurately applied.

Name of Product	Data Available on Composition	Range of Cell Types Tested	Data Given On Subculturing	Adaptation Recommended	Protein Conc.	Comments
No FCS Required						
UltroSerG	Growth factors, Adhesion factors, Mineral "trace" elements, Hormones, Binding proteins, Vitamins, Trypsin inhibitor	Hela, Vero, Chondrocytes, GH_3 rat, Human fetal cells from amniotic fluid	Rabbit chondrocytes 3 subcultures	Recommended in some instances	1.5 mg/ml	Used in micro-carrier cultures also
Nu-Serum	EGF, ECGS, Insulin, Transferrin, T_3, Progesterone, Estradiol, Testosterone, HC, Selenium, Phosphoryl-ethanolamine, Glucose, Amino acids, Vitamins, Serum, Cell nutrients	Normal and neo-plastic human lines, Transformed and untransformed cell lines, Hybridomas, (e.g. Wl-38, FS-1230, MRC-5, Hela, BHK-21, Vero)	Data given for up to 8 passages - no FCS controls		50% of that in FCS	Much emphasis on uniformity of batches

TABLE 1.2 Details on a range of commercially-available serum substitutes. Data compiled from promotional literature.

Name of Product	Data Available on Composition	Range of Cell Types Tested	Data Given On Subculturing	Adaptation Recommended	Protein Conc.	Comments
Nutricyte	BSA, Insulin, Transferrin	Human, mouse or rat myleomas, Hybridomas, Lymphocytes, e.g. Sp 2/0-AG-14, Sp 2 derived hybrid	None	Very strongly recommended - vital	40 µg/ml	Suggests FCS additions are necessary in many cases and emphasise use of correct basal medium
Nutricyte H	HSA, Insulin, Transferrin	Immunization of human lympho-cytes, Production of monoclonals, Production of proteins secreted by human lymphoid cells.	None	Very strongly recommended -	40 µg/ml	Suggests FCS additions if cells are not growing well.
Nutricyte NS	BSA, Insulin, Transferrin	NS1, Ag8.653 and P3 x 63 derived hybridomas	None		75 µg/ml	

TABLE 1.2 (continued) Details on a range of commercially-available serum substitutes.

Name of Product	Data Available on Composition	Range of Cell Types Tested	Data Given On Subculturing	Adaptation Recommended	Protein Conc.	Comments
Nutricyte BHM	Albumin Insulin, Fn, Transferrin, Peptide and non-peptide hormones, Vitamins, Amino acids, Non-peptide substances	BHK cells and variants of this line, Vero	Suggests it can occur	Not necessary	20 μg/ml	
HB 101	None given	Sp2/0-Ag-14 P3x63-Ag8.653 Mouse myeloma & hybridoma cell lines 4 human myeloma lines	Suggests sub-culturing can be carried out	Recommended as an alternative		Seems very specific for cell lines mentioned. Some more grow with low FCS levels
HB 103	RPMI 1640 basal medium, HSA, Transferrin	Human mono-nuclear cells			700 μg/ml	
HB 104	HSA, Insulin	Human-human hybridomas				Specifically for production of human mono-clonals

TABLE 1.2 (continued) Details on a range of commercially-available serum substitutes

Name of Product	Data Available on Composition	Range of Cell Types Tested	Data Given On Subculturing	Adaptation Recommended	Protein Conc.	Comments
FCS/ Supplement						
SerXtend	MSA(800µ/ml), FGF(800µ/ml), EGF(800µ/ml), Insulin(3ng/ml), BSA(40ng/µl), Ethanolamine (240ng/ml), T_3 (3ng/ml), Se(120ng/ml), Trans (1ng/ml), HC(1.8 g/ml), Protein Stabilisers (2.8ng/ml)	MCR-5, MDCK, Vero 3T3				Use in 50:50 mixture with FCS
ITS	Insulin 5 µg/ml Transferrin 5 µg/ml Selenium 5 µg/ml	BHK-21 Vero Hela MDCK			10 µg/ml	Use in addition to FCS

TABLE 1.2 (continued) Details on a range of commercially-available serum substitutes

MSA - multiplication stimulating activator, T_3 - Triiodothyronine, Se - Selenium, HSA - Human serum albumin

1.7 Conclusion

There are still many limitations in the progress achieved in the development of serum-free media. A considerable amount of the work has been carried out only on cells at relatively high densities and in open petri dishes in 5% CO_2, rather than in closed flasks. In many cases continuous culture of cells has not yet been achieved in what are loosely called "serum-free media". The requirements for growth of some cell types over a single assay period, e.g. 7 days, may be insufficient to maintain growth over an extended period with many subculturings of cells.

Other supposedly serum-free media include a low level of FCS (1-10% of the normal FCS level), which is necessary for attachment and growth of cells, while many serum-free formulations include foetal bovine serum protein (FBSP), i.e. a protein fraction isolated from foetal calf serum.

Thus, although much progress has been made in the area of serum-free media and some cell lines are continuously cultured in a totally serum-free environment, there is still a lot of work to be done.

The principal objectives of this work were:

(a) To investigate some of the limitations listed above.
(b) To repeat, if possible, the results referred to in section 1.5 on substitution of milk for serum.
(c) The principal objective was to develop a serum-free medium for a specific cell line, RPMI 2650 (Moorehead, 1965).

This epithelial cell line is quasi-diploid, has a low colony-forming efficiency (CFE) and is available at fairly low passage numbers and therefore may be more representative of primary human tumours than many of the lines (e.g. Hela), for which serum-free media are available. Development of a serum-free medium for RPMI 2650 may eventually contribute to the development of improved serum-free media for primary human tumour epithelial cells.

2. MATERIALS AND METHODS

2.1 Glassware

A good general principle to follow is that all glassware used in tissue culture should be reserved for that purpose alone. Traces of heavy metals or other toxic substances may adhere to the inside of glassware and can be difficult to detect, but will eventually lead to deterioration in cell quality. It is also recommended that separate washing of tissue culture glassware should occur.

The washing requirements for tissue culture are more stringent than for general glassware. Glassware was soaked for 1-2 hours in hot water in a special detergent RBS-25. The glassware was scrubbed and the detergent rinsed out. All glassware was then given three washes in reverse osmosis water and a final wash in water from a Millipore Milli-Q Ultrapure water system. The glassware was then dried in an oven.

The metal lids of bottles were treated in a similar manner, but were kept separate from the glassware during washing.

2.2 Water

High purity water is critical in cell culture work. The water used for preparation of all media and reagents passed through a Millipore Milli-Q Ultrapure water system. This system consisted of a reverse osmosis system which, with two prefilters removed ionic and non-ionic solutes and a further purification stage consisting of two ion-exchange filters, a carbon filter and a 0.22 μm cellulose acetate filter. The resulting water was of reagent grade and was regularly monitored by an on-line conductivity metre (10-18 megohms/cm was the acceptable resistivity).

2.3 Incubation

Cells were cultured in sealed flasks or in petri dishes which allowed pH equilibration to take place with the external environment. Incubators were maintained at 37°C. Where gaseous exchange with the external atmosphere occurred incubators were filled with 5% CO_2 (balance air) and a high humidity level was maintained by placing trays of sterile water on the base of the incubator. These incubators were cleaned regularly, as the high temperature and moist atmosphere caused increased risk of contamination.

2.4 Sterility

2.4.1. Contamination by microorganisms remains a major problem in tissue culture, in spite of the introduction of antibiotics. Bacteria, yeast and fungal spores may be introduced via the operator, the atmosphere, work surfaces, solutions and equipment. Many precautions were taken to minimize contamination.

All work with cells or reagents was carried out in a laminar flow cabinet with a vertical air-flow. All surfaces were swabbed with alcohol prior to use and all bottles, pipettes, flasks, etc., swabbed before being placed in the laminar flow. Operations were performed using the usual aseptic techniques.

Bottles were sterilised, with loosened lids, by autoclaving for 20 minutes at 15 p.s.i., using autoclave tape as an indicator. All plasticware used was purchased in a sterile condition. All waste was autoclaved prior to disposal to prevent contamination of either work or the worker. Water was autoclaved prior to use in making up media.

2.4.2. Sterile filtration

A number of different methods were used to sterilise solutions. The most successful method involved passing a solution through a Millipore Millex-GV 0.22 µm filter, using a syringe. These low-protein binding filters had a limited capacity - at times as little as 2 mls of a concentrated liquid could be forced through. Millex 0.22 µm filters were also used, but controlled experiments (Margaret Dooley, unpublished) showed that these bound more protein.

Sterifil-D units, another Millipore product, were used to sterilise larger volumes under water vacuum. Here, solutions were sucked from a non-sterile upper chamber into a sterile lower chamber via a 0.22 µm membrane. Sterivex filters were another Millipore product used, again with a 0.22 µm membrane. Here the actual filter was enclosed in a sterile plastic case and the end protected by a bell-shaped shield, which increased sterility. The Sterivex filters were used to sterilise large volumes of powdered medium; a peristaltic pump pumped the medium across the membrane, the area of which was four times that of the Millex-GV filters.

Some sterilization of milk was performed under N_2 pressure. A sterilization unit consisted of a clear plastic (polycarbonate) cylinder which could hold up to 600 ml . It had an inlet tubing adapter which could be connected up to a nitrogen cylinder and an outlet control valve at the other end, to which a swinnex adaptor was attached. A membrane (0.22 µm pore size) or a stack of membranes of decreasing pore size from 1.2 µm to 0.22 µm were placed in the swinnex and autoclaved prior to use. The swinnex was then attached to the polycarbonate cylinder under sterile conditions. The filtration unit was then connected to a nitrogen cylinder and filtration occurred under a pressure of 15 p.s.i. A Millex-GV filter (0.22 µm) was attached to the swinnex adaptor when it was discovered that this was necessary to ensure sterility. The integrity of the

membrane/membrane stack was tested by the bubble-point test. This test is based on detection of the minimum pressure required to force air through a wet membrane of a specific pore size. Bubble points, i.e. the minimum pressure have been calculated for all the filters used and details were available in commercial catalogues (Millipore).

Routine sterility checking of media and other solutions was carried out using the spread-plate technique on nutrient agar. This showed up bacterial and fungal contamination after incubation at 37° C for 24 hrs. Sometimes a very low-level contamination, often yeast or fungal in nature, did not appear visible to the eye until 5 mls of the solution had been incubated in a universal container at 37°C for 48-96 hours.

2.4.3. Mycoplasma detection

Mycoplasma infections cannot be detected by the naked eye, other than by signs of gradual deterioration in the culture. Mycoplasma can be slow-growing and may interfere with the host cell metabolism prior to any obvious deterioration in the appearance of the culture. Hence, routine periodic testing of all cell cultures for the presence of mycoplasma is very important.

The test used here involved fluorescent staining of DNA by Hoechst 33258 stain (Chen, 1977). Hoechst (2-[2-(4-hydroxyphenyl)-6 - benzimidazolyl] - 6 - (1 - methyl - 4 piperazyl) - benzimidazol - trihydrochloride) 33258 binds to DNA and, since mycoplasma contain DNA, they can be detected by observation of extra-nuclear fluorescence.

The method used, developed in this laboratory, was based on the assumption that if mycoplasmas were present in a culture they would be found, not only within the infected cells, but also in any medium which had been in contact with the cells. A normal rat kidney cell line (NRK) was used as the indicator cell line.

NRK cells were set up on sterile coverslips or multiwell slides, at a concentration of 1 x 10^4 - 1 x 10^5 cells/ml. These were incubated for 24-48 hours at 37°C and 5% CO_2. Medium was removed from cultures of all the cell lines to be tested and medium which had not come in contact with any cells was used as a control. A small volume (500 µl - 1000 µl) of the medium was added to the NRK cells and these were incubated for a further 2-3 days.

The coverslips/slides were removed from the dishes, excess medium poured off and the cells fixed in pre-cooled methanol for 8 minutes and acetone for 2 minutes at 20°C. A number of other fixation methods were tested unsuccessfully (Sec. 3.1). The slides were then rinsed for 5 minutes in PBS and 2 minutes in distilled water.

The Hoechst stain was diluted in a balanced salt solution to a concentration of 0.05 µg/ml. A few drops of this was added to the cells and incubated for 10 minutes in the dark. The cells were washed three times in distilled water and mounted in McIlvaines Buffer, pH 5.5. The cells were then examined for fluorescent staining of DNA, using a Nikon Optiphot Fluorescent microscope, with a wavelength of 405 nm. Observation under oil immersion was required to detect low level mycoplasma infection.

2.5 Preparation of Culture Media

2.5.1. Liquid Media

Liquid media were prepared from concentrated stock solutions (10x) of basal medium. 450mls of Milli-Q ultra-pure water were autoclaved and 50 mls of the 10x concentrate added aseptically. 1.7 g/l Sodium Bicarbonate, acting as the main buffer, 2 mM L-glutamine, an amino acid with a relatively short half-life in solution and 0.2 M 4 - (2 - hydroxy-ethyl) - 1 - piperazine -

ethane sulfonic acid (Hepes) were all added to the medium. Some other additions were made to specific media (see Table 2.1). The pH was adjusted to 7.4 - 7.6 with NaOH.

2.5.2. Powdered Media

Powdered medium made from a lyophilized basal medium is a better and more consistent quality medium, as the stability of some components is much greater when lyophilized than when stored in a liquid form. The lyophilized medium consists of a powdery mixture of all basal components - hence the title "powdered medium". Powdered Ham's F12 was made by dissolving one package of Gibco's powdered medium in 985 mls of Milli-Q water. 1.176 g/l sodium bicarbonate was added and the pH adjusted to 7.4 - 7.6.

The medium was passed through a Millipore Sterivex unit, (see section 2.4.2). The medium was collected in sterile glass bottles and stored for up to 4 weeks at $4^{o}C$.

TABLE 2.1 Media used to grow different cell types.

Medium	Cell types grown	Normal % FCS	Specific addition
Modified Eagles (MEM)	RPMI 2650	5%	MEM non-essential amino acids
	MDCK	5%	
	HEP-2		
Dulbecco's Modified Eagles Medium (DMEM)	3T3	5%	
	A431	5%	
McCoy's 5A	A549	10%	Hydrocortisone (0.4 µg/ml

2.6 Cell Lines

A number of cell lines were used in this work. The three main lines were RPMI 2650, 3T3 and MDCK. The RPMI 2650 cell line was established in 1962 from the pleural effusion of a patient with an extensive malignant tumour of the nasal septum. The 3T3 Swiss albino cell line is a mouse embryo fibroblast cell line, isolated initially from a mouse embryo, Todaro & Green (1963). The MDCK cell line was derived from a kidney of an apparently normal female Cocker Spaniel in 1958 and consists mainly of epithelial-like cells (Leighton et al., 1969).

Four other cell lines were also used to a lesser extent. A549 cell line was derived from a culture of a lung carcinomatous tissue of a Caucasian male patient, (Giard, 1973). HEP-2 cell line was produced from tumours that had formed in rats after injection with epidermoid carcinoma tissue from the larynx of a Caucasian male, (Moore, et al., 1955). It may have been contaminated with Hela cells. The SCC-9 line originated from a squamous cell carcinoma of the tongue (Rheinwald & Beckett 1981). The A431 cell line is another human cell line, having been derived from a human epidermoid carcinoma (Fabricant et al., 1977).

2.7 Maintenance of cells in culture

Cells were cultured routinely in 25 cm^2 flasks, growing attached to the flask surface. Cell lines were cultured in different basal media (see Table 2.1 - Sec. 2.5), supplemented with FCS, generally at a concentration of 5% v/v.

Cells were subcultured every 3-6 days, depending on the cell type. Subculturing was carried out by a trypsinization procedure. A 0.2% trypsin solution was made up in sterile PBS and EDTA added to give a final working concentration of 0.02% (known as Trypsin Versene, TV). This was incubated at 37^{o}C for 20 minutes. Medium was removed from the cells and the residual medium in the flask washed out with 0.5 mls of TV - this removes remaining FCS which, with its trypsin inhibitor, may interfere with enzyme

action. 2 mls of TV were then added to a $25cm^2$ flask and the flask incubated at $37^{o}C$ for 5-10 minutes until all the cells had detached. 3 mls of medium plus FCS was added to the flask and the contents then spun at 1,000 rpm for 5 minutes. The supernatant was discarded and the cells resuspended in 5 mls of medium.

Cell numbers were estimated using a haemocytometer. Stock flasks were then set up at the required concentration - this varied from $1x10^5$ cells/flask for HEP-2 cells to $5 \times 10^5/1 \times 10^6$ cells/flask for RPMI 2650.

A viability test was sometimes carried out on the cells using Trypan Blue (0.4%) dye. 250 µl of cell suspension was added to 50 µl of Trypan Blue, mixed well and allowed stand for 4-12 minutes. The cells were then counted on the haemocytometer - dead cells would have absorbed the blue dye - and hence percentage viability could be calculated.

2.8 Cell Freezing and Long-term Storage

2.8.1. Freezing Cells

Cells were trypsinized in mid to late exponential phase, before they had entered stationary phase, for successful freezing. A cell count of 2×10^6 cells/ml was required for freezing. A 10%(v/v) DMSO (dimethyl sulphoxide, a cryoprotective agent) solution was prepared in the growth medium of the cells. An equal volume of 10% DMSO was added dropwise to the high-density cell suspension. The addition of the DMSO was crucial, with very gradual addition and careful mixing of the cell suspension after the addition of each drop, to ensure correct freezing conditions were achieved.

The DMSO/cell suspension mixture was then dispensed into sterile cryotubes, 1.5 mls per cryotube. The cryotubes were placed in a neck plug which fitted into the top of a liquid-nitrogen-filled cryofreezer. The cryotubes were allowed stand in the liquid nitrogen vapour for $2\frac{1}{2}$-3 hours. After this, they were placed into the liquid nitrogen proper. Long-term storage in liquid nitrogen proved successful for all cell lines used.

2.8.2. Thawing Cells

It was important to check stocks 24-48 hours after freezing to ensure that the freezing was successful. A cryotube was removed from the cryofreezer and the contents thawed in a waterbath or incubator at 37°C as quickly as possible. The contents of the cryotube was then emptied into a universal containing 5 mls of medium and spun at 1,000 rpm for 5 minutes to remove the DMSO. The supernatant was discarded and the cells resuspended in 5 mls of medium and transferred to a 25 cm^2 flask. 24 h later this medium, containing any unattached cells was poured off and the flasks refed with 10 mls of medium. The flasks were examined at 48 h for cell attachment and growth.

2.9 Preparation of Growth Factors

Polypeptide growth factors, hormones, proteins and other components tested for growth promoting ability were carefully dissolved in a solute which allowed complete dissolution in a minimum length of time. Table 2.2 describes in detail the solvents used for different components.

Compounds were dissolved initially in water, PBS A or absolute alcohol and filter sterilised using 0.22 µm Millex-GV filter (see sec. 2.4.2.). Subsequent dilutions were made, if necessary, to bring the concentration of each component to 1,000 times the concentration required for use in an assay. Solutions were dispensed into 500 µl volumes and stored at - 20°C for up to 3 months.

Solvent used	Compound	Storage Conc.	Comments
Absolute Alcohol	Hydrocortisone	$1x10^{-5}$M	Diluted in PBS after initial dissolving. Higher conc. did not freeze at -20°C
	Estradiol	$1x10^{-4}$M	
	Progesterone	$1x10^{-4}$M	
	Retinoic Acid	$1x10^{-3}$M	Very acidic - came out of soln. in medium at high conc.
	PGE_1	2.5 µg/ml	
	$PGF_{2\alpha}$	20 µg/ml	
PBS A	Transferrin	5 mg/ml	
	Selenium(Sodium Selenite)	$1x10^{-4}$M	
	Bombesin	$1x10^{-4}$M	
	Glycyl-hystidyl-lysine	1 mg/ml	
	Ascorbic acid	1 mg/ml	Very short shelf life in soln. - not stored at -20°C.
	Bovine Serum Albumin	500 mg/ml	
	Glucagon	0.1 mg/ml	
	PDGF	1 unit/ml	
	EGF	10 µg/ml	
	T_3	$1x10^{-5}$M	100 µl/5 mls of NaOH required for dissolving
	Vitrogen (Collagen)	1 mg/ml	Commercial collagen in liquid gel at 3 mg/ml conc.
Dist.H_2O	Phosphoethalo-lamine	$1x10^{-2}$M	
	Poly-l-lysine	1 mg/ml	
	$Fe_2SO_4.7H_2O$	1×10^{-3}M	
	Dextran Sulphate	1 mg/ml)	All of these are attachment factors and after sterilization were stored at 4°C.
	DEAE dextran	1 mg/ml)	
	Methyl cellulose	1 mg/ml)	
	Gelatin	1 mg/ml)	
	L-lysine	1 mg/ml)	
	PVP	1 mg/ml)	
HCl	Insulin	5 mg/ml	Difficult to dissolve - leave stand at room temp. Dissolved at 37°C.
	Collagen	1 mg/ml	

TABLE 2.2 Preparation of growth and attachment factors. This table indicates the solvents used to make dilutions of the range of growth and attachment factors used.

2.10 Ultrafiltration

2.10.1 Tangential Flow Ultrafiltration of Milk

Milk from commercial dairies, was purchased in local retailers and ultrafiltered on the day of purchase. This process consisted of passing 2 l of milk through a Pellicon filtration unit across a 1 x 10^6 molecular weight membrane, which was contained in a stainless steel cassette. A peristaltic pump was used to pump milk into the cassette and, as it was a tangential flow system, a filtrate tube removed the filtrate and a retentate tube returned the retentate into the original container for recirculation. The unfiltered milk and the straw-coloured filtrate were kept on ice during the ultrafiltration procedure. 2 l of milk was reduced to 600-800 mls of retentate which became increasingly thick and creamy during the process. The filtrate was either filter sterilised immediately (see sec. 2.4.2.) or else ultrafiltered on an Amicon ultrafiltration unit (see sec. 2.10.2.).

2.10.2. Ultrafiltration on an Amicon ultrafiltration unit

Two ultrafiltration membranes were used, a 10,000 and a 5,000 molecular weight membrane. One of these was placed in an Amicon ultrafiltration unit and milk filtrate collected from the Pellicon ultrafiltration cassette (see sec. 2.10.1.) was poured into this unit. A magnetic stirrer was assembled to prevent clogging of the membranes. This filtration took place under N_2 pressure (60 p.s.i.) and the original volume reduced down to 10%. The filtrate was collected on ice and both it and the retentate were filter-sterilised immediately.

2.11 Freeze Drying

Preparations of growth factors in medium (see sec. 2.9) were freeze-dried before storing at - 20°C. The samples were dispensed into 2.5 ml volumes and frozen to - 20°C initially. Then they were immersed in liquid nitrogen for a short period of time (1-2 min.) before being transferred to the freeze dryer.

An Edwards Modulyo freeze dryer was used. Samples were placed in it and freeze drying took place overnight. The dessicated pellets were stored at - 20°C and reconstituted when required by addition of 2.5 ml of ultrapure water.

2.12 Preparation of Colostrum

Colostrum was collected from a cow within 4 hours of birth of a calf and was frozen at - 20°C immediately. Upon thawing, the colostrum was centrifuged in a RC-5 Sorvall centrifuge at 12,000g for 30 minutes. The fat floating on top of the centrifuge tube was discarded, as was cellular debris and other sediment that had pelleted at the bottom of the tube. The supernatant was prefiltered, using an AP25 prefilter (see sec. 2.4.2.) to remove any large pieces of debris, before passing it through the Pellicon ultrafiltration unit (see sec. 2.10.1). The clear, straw-coloured filtrate was sterilised by passing it through a 0.22 µm Millex-GV filter and stored at - 20°C until used.

2.13 Preparation of Dried Milk

A normal household brand of dried milk was used. A solution of 100 g/l was made up and dissolved using a magnetic stirrer. This was then passed through the Pellicon ultrafiltration unit and the filtrate sterilised using a Millex-GV filter. Baby food was made up and treated in an identical manner.

2.14 Isoenzyme Analysis

It is necessary in cell culture work to check routinely that no cross contamination between cell lines has taken place. An electrophoretic technique was used to characterize the different cell lines in use in the laboratory.

Cells were grown in 25 cm^2 flasks and trysinized (see sec. 2.7). A cell suspension of at least 3 x 10^6 cells was then centrifuged and washed twice in PBS. On the final centrifugation, the cells were resuspended in 500 µl of PBS and freeze thawed three times in liquid nitrogen at 37 ^oC. Centrifugation followed at 5,000 r.p.m. for 5 minutes and the supernatant was poured into a cryotube and stored at - 20 ^oC until used.

The isoenzyme detected was lactate dehydrogenase (LDH) which is present in serum and can be separated into five isoenzymes by the technique used. A sample of FCS was used as a control. The isoenzymes were separated by electrophoresis in a barbitol buffer (0.05M with 0.035% EDTA) at pH 8.6. Following separation, the isoenzymes were detected by supplying lactate as a substrate. LDH catalyses the oxidation of lactic acid to pyruvate and the NADH produced was used to reduce a tetrazolium salt to its corresponding formazan, which is coloured. Thus, the two reactions which occur are:

$$\text{L - Lactate + NAD} \underset{\longleftarrow}{\xrightarrow{\text{LDH}}} \text{Pyruvate + NADH}$$

and

$$\text{NADH + INT}^1 \underset{\longleftarrow}{\longrightarrow} \text{NAD + INF}^2$$

A commercial kit from Corning Electrophoresis was used. 1 µl of sample was applied to each well of the agarose gel and electrophoresis was carried out for 35 minutes. Incubation with the lactate substrate was carried out at 37 ^oC for 20 minutes in a moist environment. The agarose film was washed and dried at 37 ^oC for 20 minutes.

[1] INT - iodonitrotetrazolium chloride

[2] INF - iodonitroformazan

2.15 RPMI 2650 Monolayer Assay System

2.15.1. Setting up the assay

This assay was developed specifically to test the effect of various components on the growth of RPMI cells at clonal densities, in the presence of a low level of FCS. Much work was carried out to identify the most suitable background FCS level (see sec. 3.2). A background level was used in all experiments to investigate the effect of different factors.

The assay was carried out in 35 mm dishes placed on trays in 5% CO_2 incubators at 37°C. A DME: Ham's F12(1:1) mixture was the basal medium used on a routine basis. Dishes were set up in four replicates, with a final volume of 2 mls of medium per dish. The method used involved adding 1 ml of cell suspension in DME-FCS (unsupplemented DME) and 1 ml of Ham'S F12, which contained twice the required concentration of growth factors and/or FCS, to each dish.

Media for any four replicates was prepared in a universal. Growth factor stocks were diluted in medium to 100 times the final concentration required. Small additions were made to the Ham's F12 to achieve the required concentrations. This method reduced the quantity of solvent (e.g. absolute alcohol) which actually came in contact with the cells. FCS was also added to the Ham's F12 as required. The Ham's F12 medium was dispensed into the 35 mm dishes and allowed equilibrate in the 5% CO_2 incubator for 30 minutes.

During this period cells, which had been growing for 2-3 days at 1 x 10^6 cells/ml in 25 cm^2 flasks with 5% FCS were trypsinized (see sec. 2.7). When all the cells had detached an equal volume of sterile soybean trypsin inhibitor (1 mg/ml) was added and the cells centrifuged at 1,000 rpm for 5 minutes. The supernatant was discarded and the cells resuspended in DME-FCS and

and centrifuged again to remove any traces of FCS, trypsin or trypsin inhibitor. Cells were resuspended in DME-FCS and counted. A suspension of 1 x 10^4 cells/ml was made.

The dishes were removed after equilibration and 1 ml of cell suspension was added to each dish. The trays on which the 35 mm dishes were placed were covered with aluminium foil to reduce the risk of contamination and were placed in a 5% CO_2 incubator at 37° C.

2.15.2. Collection of Data

The growth period was generally 7 days. The trays were then removed from the incubator and medium discarded from the dishes. The cells were then rinsed with PBS and 1.5 mls of Leishmann's stain (0.3g/100 mls of methanol) added to each dish for 10 minutes. 1.5 mls of distilled water was then added for 10 minutes. This mixture was then poured off and the dishes rinsed twice in distilled water and inverted to dry. All cells, now growing in colonies, stained up a dark blue.

Counting of colonies was done using an AMS 40-10 Image Analyser. This consists of a camera lens, which observes the image of the darkly-stained colonies against a bright back-ground supplied by a light-box. This image is transferred to a television screen which is linked to a computer memory. The computer has the ability to count the number of colonies and to calculate the total colony area. Data from the assays was collected consistently using this image analyser.

2.16 Precoating of Dishes

Some of the experiments involved precoating of dishes with various substrates. The substrates were made up at the specified concentrations and were sterilised, generally by passing through a 0.22 μm Millex-GV filter (gelatin was an exception, it was autoclaved). 1.5 mls of the substrate was added

to each 35 mm dish. Precoating conditions varied - for 30 minutes, 4 hours or overnight, at 37°C, 4°C or room temperature.

The precoating materials were removed from the dishes and the dishes washed first with PBS and then with medium. Great care was required to avoid contamination. The assay was then set up in the normal manner in the precoated dishes. (See sec. 2.15.1).

3. RESULTS

3.1.1. Development of a cell fixation method for testing for mycoplasma contamination

The mycoplasma detection method described in Section 2.4.3. using the Hoechst stain required the development of a suitable cell fixation method. After fixation, the cells must remain intact and the membrane must be permeable to the Hoechst stain.

Numerous fixation methods were tested (Table 3.1). Dislodging cells by trypsinization and adding fixative to the cell pellet which forms after centrifugation proved very unsatisfactory. The cells, when fixed, were dropped onto a prewashed slide using a Pasteur pipette but this method resulted in total destruction of the cell membrane, regardless of the fixatives used, (Methods 1, 2 and 4, Table 3.1).

Attempts were then made to fix cells growing attached to a glass coverslip or a 35 mm dish. KCl: ethanol, methanol: acetic acid and formaldehyde: methanol fixatives all proved unsatisfactory (see Table 3.1, for details). Swelling of cells prior to fixation in ice-cold KCl or PBS A was found to have no effect on fixation.

The method which finally proved successful involved fixation in precooled methanol and acetone (Method 8). Cell morphology and number were of a consistently high standard to allow detection of mycoplasma, using an immunofluorescent technique (Sec. 2.4.3.).

Method Number	Fixation Method	Swelling of cells	Condition of cells during fixation	Treatment after Fixation	Results and Comments
1.	Ethanol Room Temp. 30 mins.	Ice-cold KCl (10 mins)	Cells trypsinized and centrifuged. Washed PBS x 3	Dropped onto slide. Stained with Giemsa	Cells totally broken open- no intact cytoplasms or nuclei. Low cell density.
2.	KCl: Ethanol Gradual fixation - initally 2:1 mixture, then 50:50 then 0:100 (KCl: Ethanol)	Ice-cold KCl (10 mins)	Cells trypsinized and centrifuged Washed in PBS x 3	Dropped onto slide. Stained with Giemsa	Some cells intact but majority broken open. Low cell number visible. Tried using a larger cell pellet - no effect.
3.	As in 2.	Ice-cold KCl (10 mins)	Cells grown in 35 mm petri dishes - washed in PBS x 3. Fixative added to cells in situ.	Air dried	Cells rounded up or split open - better than previous results but cytoplasm and nucleus not discernable. High cell density.
4.	Methanol:Acetic Acid (3:1) Added dropwise to pellet 2 x 10 mins	PBS A (10 mins)	Cells trypsinized and centrifuged Washed in PBS x 3.	Dropped onto slide & air dried	Fixative changed after 10 mins. by centrifugation. Very few cells formed pellet in methanol: acetic acid - hence very low cell number and very poor quality.
5.	As in 4.	PBS A (10 mins)	Cells grown in 35 mm petri dishes Washed in PBS x 3	Air dried	Quality and number of cells improved but cells split open during air drying.

TABLE 3.1 Fixation methods tested for suitability for use with NRK cells in detection of mycoplasmas

Method Number	Fixation Method	Swelling of cells	Condition of cells during fixation	Treatment after Fixation	Results and Comments
6.	Methods 2 and 4 both used	Varied	Cells grown in 35 mm petri dishes Washed in PBS x 3	Air dried	Varied swelling times - 0,5, 10 mins. in PBS and in KCl. No significant improvement in cell quality.
7.	Formaldehyde 10 min. Methanol 10 mins. at - 20°C	None	Cells grown in 35 mm petri dishes Washed in PBS x 3	Air dried	Very poor quality. All cells burst open.
8.	Methanol 8 mins. Acetone 2 mins. at - 20°C	None	Cells grown in 35 mm petri dishes Washed in PBS x 3	Air dried	Very good cell quality. Morphology well preserved, nucleus and cytoplasm intact.

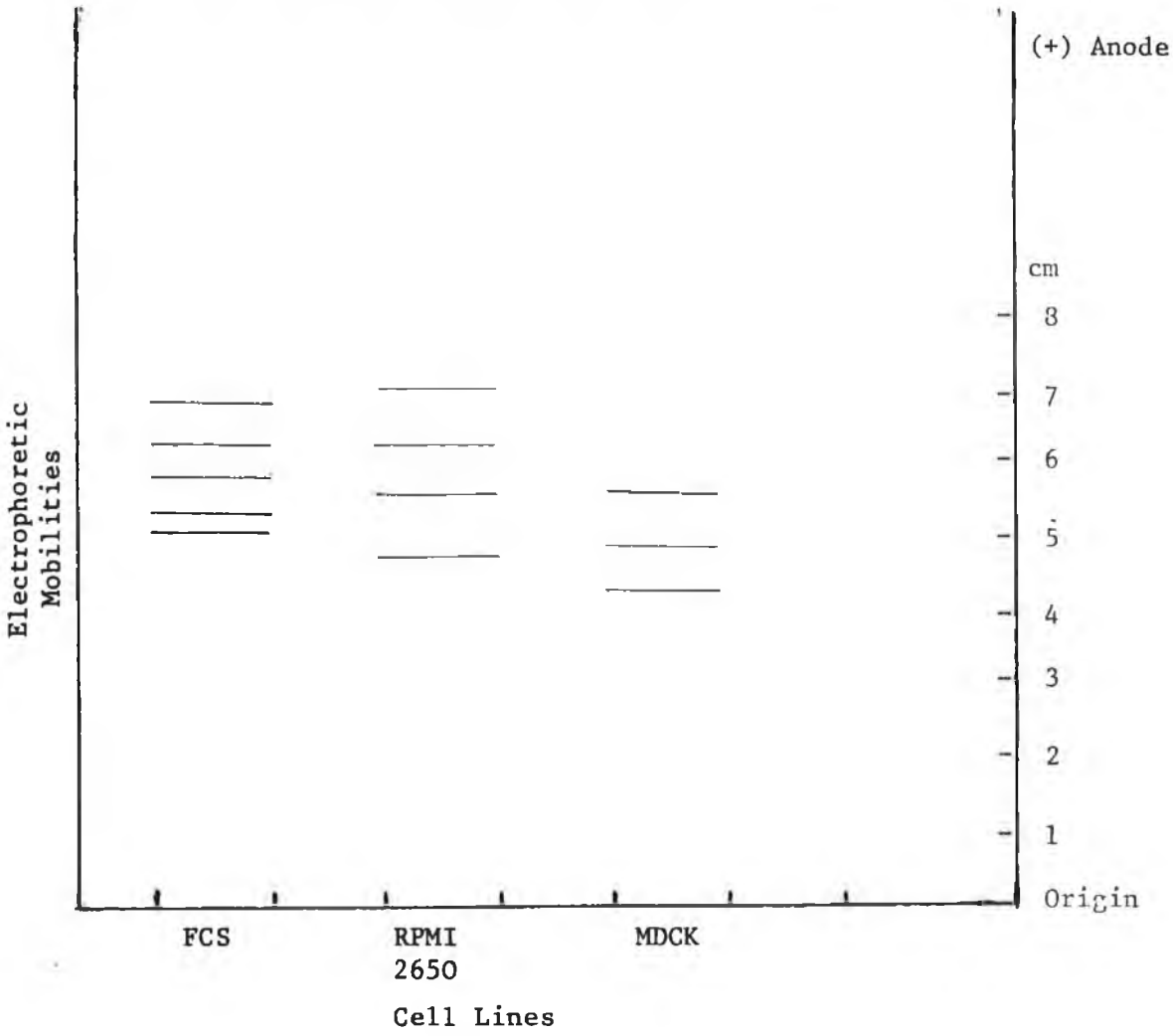
TABLE 3.1 (cont'd) Fixation methods tested for suitability for use with NRK cells in detection of mycoplasmas.

3.1.2. Isoenzyme analysis of RPMI 2650 and MDCK cells

Lactate dehydrogenase (LDH) enzymes were detected in RPMI 2650 and MDCK cell lines, by the electrophoretic technique described in Section 2.14. Foetal calf serum was used as a standard. The LDH of FCS can be separated into five isoenzymes, each one being a tetramer composed of a combination of H and M subunits.

Fig. 3.2 shows the electrophoretic mobilities of the LDH isoenzymes in FCS, RPMI 2650 and MDCK. Five isoenzymes are present in FCS, four in RPMI 2650 and three in MDCK. The differences in the number of isoenzymes and in their electrophoretic mobilities between RPMI 2650 and MDCK indicate clearly the presence of two distinct cell lines.

Fig. 3.2. Electrophoretic mobilities of the Lactate dehydrogenase isoenzymes in RPMI 2650 and MDCK.



3.2 Growth of MDCK cells in serum-free medium

The MDCK cell ine is grown routinely in MEM + 5% FCS. Taub et al (1979) reported the development of a serum-free medium which supports the growth of MDCK cells. The basal medium is a DME: Ham's F12 (1:1) mixture and this is supplemented with transferrin, hydrocortisone, insulin, triiodothyronine (T_3), selenium and prostaglandin E_1 (PGE_1);(called THITS (PGE_1)).

Experiments were set up to repeat the findings of this group in an attempt to establish the basic methods. MDCK cells were set up over a concentration range of $1x10^3$ to $5x10^4$ cells/35mm dish. The assays were set up in a similar manner to those described for RPMI 2650 (see Sec. 2.15.1). Growth occurred after 4-6 days in 5% FCS and in THITS (PGE_1). Some signs of attachment and a little growth were evident in unsupplemented medium. Results were collected either by trypsinization followed by a cell count (Table 3.2) or by staining with Leishmann's stain (see Sec. 2.15.2) and counting colony formation on an image analyser. (Table 3.3).

The growth achieved in THITS (PGE_1) medium varies from 37% to 63% of that achieved in 5% FCS. Cell morphology is similar in both media.

Many attempts were made to grow MDCK cells in 25 cm^2 flasks, but no growth at all was achieved in THITS (PGE_1). The THITS (PGE_1) medium turns very basic in sealed 25 cm^2 flasks during an assay period, but the use of control flasks fed with DME: F12 + 5% FCS supports normal MDCK growth, with the medium turning acidic as cell number increases.

Cells were set up over a range of concentrations; at $1x10^4$ - $5x10^4$ cells/ 25 cm^2 flask, growth in 5% FCS was normal but no growth occurred in THITS (PGE_1). Flasks were gassed with 5% CO_2 prior to sealing the caps, in an attempt to maintain a suitable pH for growth. Flasks were also placed in 5% CO_2 incubator with loosened lids to allow gaseous exchange which would maintain a suitable medium pH. Neither method helped growth in THITS (PGE_1), while growth in 5% FCS continued normally (Table 3.4). Controls set up in 35 mm dishes simultaneously (at $1x10^3$ cells/dish) always showed growth in THITS (PGE_1).

TABLE 3.2 Growth of MDCK in THITS (PGE_1)

Initial cell density:	$1.5x10^4$ Cells/ 35mm dish	$3x10^4$ Cells/ 35mm dish	$7.5x10^3$ Cells/ 35mm dish
Medium	Cell No.$x10^5$	Cell No.$x10^5$	Cell No.$x10^5$
5% FCS	14.0 ± 0.37	32.2 ± 0.5	13.0 ± 0.1
- FCS	0	0	0.08 ± 0.01
THITS (PGE_1)	6.3 ± 1.2	17.0 ± 1	5.56 ± 0.2
% Growth in THITS(PGE_1)*	45%	53%	43%

Note: T= Trans (5µg/ml), H= Hydrocortisone (50nM), I= Insulin (5µg/ml), second T= T_3(5pM), S= Selenium (50nM); PGE_1 (25ng/ml) used.

* Growth expressed as a percentage of growth in 5% FCS

Results expressed as average $\pm$ S.E.M. (n=3)

TABLE 3.3 Growth of MDCK at clonal concentrations in THITS (PGE_1)

Initial cell conc:	$1x10^3$ cells/35mm dish	$2x10^3$ cells/dish
	Colonies/dish	Colonies/dish
5% FCS	135 ± 25	400 ± 25
- FCS	0	0
THITS(PGE_1)	85 ± 5	170 ± 21
% Growth in THITS(PGE_1)	63%	43%

Note: Concentration as in Table 3.2. Colonies counted on image analyser.

*Growth expressed as a percentage of growth in 5% FCS

Results expressed as average $\pm$ S.E.M. (n=3)

TABLE 3.4 Summary of attempts to grow MDCK in THITS(PGE_1) in $25cm^2$ flasks

Treatments:	Medium	Results
Cells set up in sealed flasks at 37^oC	5% FCS	Normal Growth
	- FCS	No Growth
	THITS(PGE_1)	Some single cell attachment, no growth
5% CO_2 bubbled through medium prior to sealing flasks	5% FCS	Normal Growth
	- FCS	No Growth
	THITS(PGE_1)	No Growth
Loosened caps in 5% CO_2 incubator	5% FCS	Good Growth
	- FCS	No Growth
	THITS(PGE_1)	No Growth

Note: Cells set up over a range of concentrations from $1x10^4$ to $5x10^4$

3.3 Development of a serum-free medium for RPMI 2650

3.3.1. Choosing a suitable FCS batch

Batch to batch variation in FCS has always been highlighted as a major disadvantage in working with a serum-supplemented system. Hence it was necessary to screen a number of different batches of FCS to identify a batch which would consistently support growth of RPMI 2650 at a concentration of 1.0×10^4 cells/35 mm dish for continual use in an assay (see Sec. 2.15).

Five different batches of FCS were screened and growth in medium supplemented with 5% FCS examined. Fig. 3.2 indicates the range of variability that is found, both within a FCS batch and between batches. This diagram shows the average colony numbers per 35 mm dish. Batch number 30 Q is obviously consistently yielding higher colony numbers, while batch number 20 F appears to be very unsuitable for growth of RPMI 2650 cells. Very little growth occurs in many experiments using this batch of FCS.

Hence, 30 Q FCS is the best batch screened for supporting growth of RPMI 2650. But even with this batch there can still be large variations in the number of colonies that form from an initial innoculum of 1×10^4 cells/dish.

In some experiments two sets of replicates were independently set up at 1×10^4 cells/dish to examine the variance within a FCS batch. In Fig. 3.2., experiment number 8 shows that, using batch number 10 Q, the average number of colonies in identical sets of replicates can differ significantly. However, the variation within an experiment, using batch number 30 Q (Experiment 10, Fig. 3.2.), is reduced to a much more acceptable level.

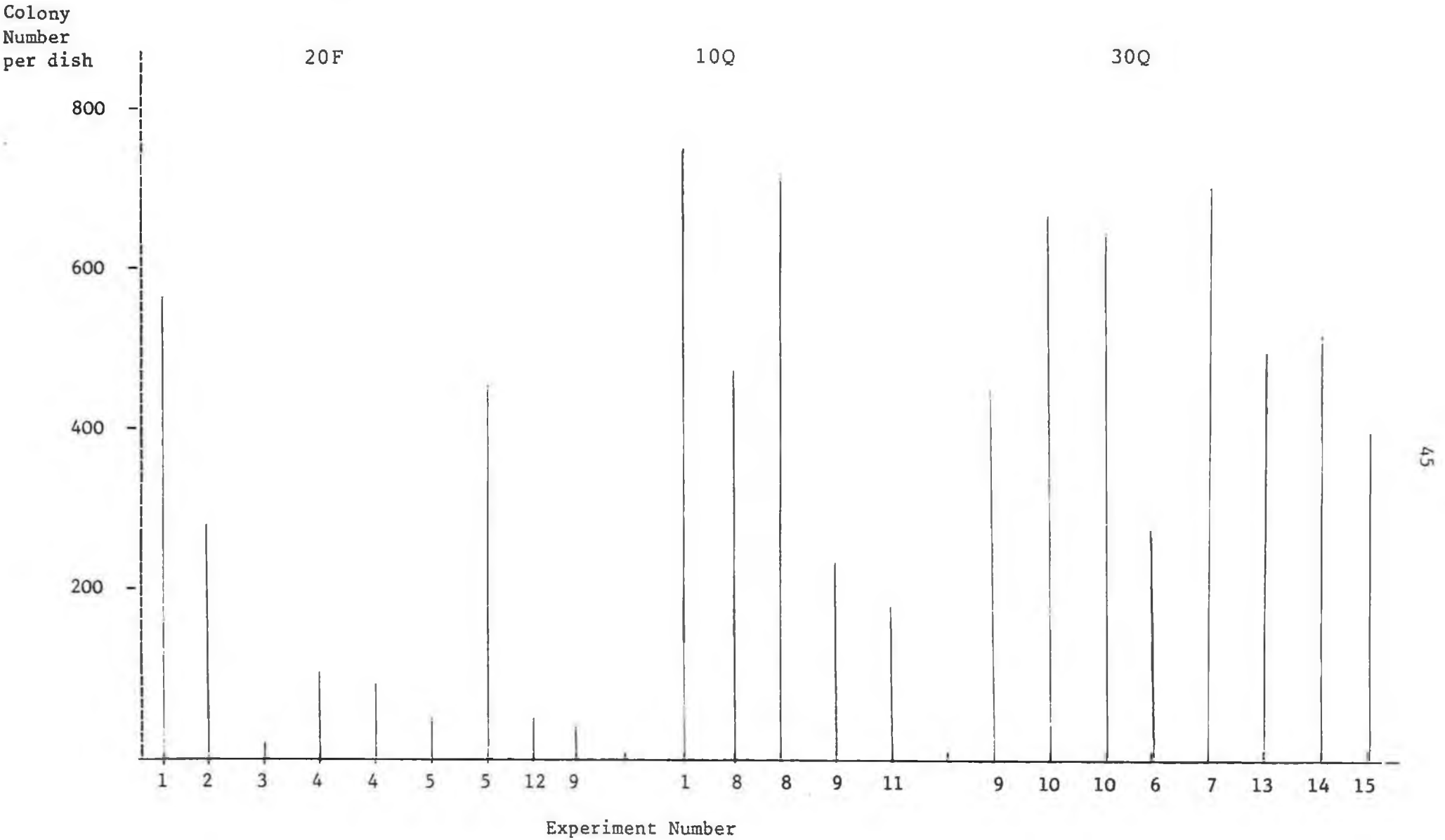


FIGURE 3.2: **Growth of RPMI 2650 in three different batches of FCS. Growth is in medium supplemented with 5% FCS.**

3.3.2. Detection of a minimum FCS level

To investigate the effects of various compounds on the growth of RPMI 2650 cells, a minimum level of FCS was required which would allow limited growth (see Sec. 1.3). Growth was examined initially using MEM as the basal medium and different batches of FCS. Growth was consistently observed in 3% FCS with batch number 30 Q and this seemed to be a suitable background FCS level to work with.

But developments in choosing a basal medium resulted in a lowering of the FCS level (see Sec. 3.3.3). Using DME:F12 (1:1) mixture, growth occurred in 1% FCS (Table 3.5). After 7 days growth, clonal growth was sufficient to allow staining with Leishmann's stain and the colonies could be counted using the image analyser (see Sec. 2.15.2).

When consistent growth was occurring at the 1% FCS level, using this batch of FCS, examinations were made into growth in 0.5% FCS and 0.25% FCS. At a concentration of 1×10^4 cells/dish growth occurs at both concentrations (Table 3.6). Either concentration is suitable for use as a minimum level of FCS which could be used to observe the response of RPMI 2650 to a variety of factors.

TABLE 3.5 Lowering FCS level for growth of RPMI 2650

	% Colony No.*	% Colony Area*	Colony No.	Total Colony Area (sq. mm)
5% FCS	100	100	525 ± 22	27.3 ± 1.1
3% FCS	65	61	343 ± 21	16.6 ± 0.7
2% FCS	60	55	315 ± 22	15.1 ± 1.5
1% FCS	24	16	128 ± 9	4.4 ± 0.2

Note: DME:F12 basal medium used

*Growth expressed as a percentage of growth of 5% FCS controls

TABLE 3.6 Growth of RPMI 2650 in low FCS concentrations

	% Colony No.*	% Colony Area*	Colony No.	Total Colony Area (sq. mm)
5% FCS	100	100	494 ± 28	47.8 ± 3.4
0.5% FCS	31	16	153 ± 20	7.8 ± 1.2
0.25% FCS	8	5	41 ± 7	2.2 ± 0.5

*Growth expressed as a percentage of growth of 5% FCS controls

In these and in all succeeding tables of this format (unless specified), results are expressed as average ± S.E.M. (n=4).

3.3.3. Choosing a basal medium

RPMI 2650 cells are grown routinely in Eagle's Minimal Essential Medium (MEM) + 5% FCS. Initial investigations into growth in reduced FCS concentrations were carried out using MEM. Having observed growth in 3% FCS, and having observed some stimulation due to ITS (see Sec. 3.3.4) at this FCS level investigations into different basal media were carried out to see if any other basal media would support growth to a greater extent in reduced FCS conditions.

Ham's F12 (Ham, 1965) is the basal medium most commonly used in serum-free media. Comparisons were made between growth in MEM and MEM:F12 mixtures. Table 3.7 shows that in the presence of Ham's F12, RPMI 2650 grow better than in MEM. At a 6:1 ratio with MEM, growth in 5% FCS is unchanged, but growth at 3% FCS shows a five-fold increase in colony number and a doubling in colony area. In a MEM:F12 (3:1) mixture, growth in both 5% FCS and 3% FCS is significantly better; the CFE and colony area are both increased significantly at the two FCS concentrations.

Thus Ham's F12 was seen to be contributing significantly to growth of RPMI 265(particularly in reduced FCS conditions. A MEM:F12 (1:1) mixture was then examined and growth in this mixture supplemented with 2% FCS was equal to the growth observed with MEM alone, supplemented with 3% FCS. Table 3.8 shows that colony number and colony area are equal in MEM:F12 + 2% FCS and MEM + 3% FCS.

Thus it seems that Ham's F12 is markedly influencing growth. Yet RPMI 2650 failed to grow in F12 + 0.5% FCS (Table 3.10). The recommended combination is DME:F12 (1:1) mixture - this is the most widely used in serum-free work. RPMI 2650 responded well to this mixture and growth was recorded consistently in 1% FCS (Table 3.9 and 3.5).

Investigations were then made into lowering the FCS concentration further (Sec. 3.3.2). Using 0.5% FCS a number of combinations of basal media were tested but, while some yielded equal growth to DME:F12 (1:1) yielded better

growth (Table 3.10). The basal media tested included one called Special Liquid Medium (SLM). This differs from the other basal media by having sodium pyruvate as the main energy source, where most basal media have glucose. SLM on its own in the presence of 5% FCS resulted in the same growth as DME:F12, yet a SLM:F12 mixture tested showed no great change in growth levels.

Thus DME:F12 was chosen as being the best basal medium for supporting growth of RPMI 2650. The mixture of these two media (not F12 on its own) seem to supply the optimum combination of nutrients for this cell line.

TABLE 3.7 Growth of RPMI 2650 in MEM:F12 v MEM

Medium	FCS Conc	% Colony No.*	% Colony Area*	Colony No.	Total Colony Area (sq. mm)
MEM[1]	5%	100	100	206 ± 48	11.25 ± 2.75
	3%	8	9	16 ± 6	1.0 ± 0.5
MEM:F12[1] (6:1)	5%	102	70	210 ± 20	7.9 ± 1.5
	3%	44	20	91 ± 9	2.2 ± 0.1
MEM:F12 (3:1)	5%	224	242	462 ± 2	27.2 ± 2.4
	3%	143	108	294 ± 60	12.2 ± 3.0

Note: Growth in MEM:F12 (3:1) is significantly greater ($p<0.01$) at 5% and 3% FCS than in MEM alone.

[1]MEM- Eagles Minimal Essential Medium; F12 - Ham's F12

*Growth expressed as a percentage of growth in 5% FCS

TABLE 3.8 Growth of RPMI 2650 in MEM:F12 v MEM

Medium	FCS Conc	% Colony No.*	% Colony Area*	Colony No.	Total Colony Area (sq. mm)
MEM	5%	100	100	1000 ± 8	151.5 ± 14.5
	3%	92	63	919 ± 16	95.5 ± 3.9
MEM:F12 (1:1)	5%	103	107	1034 ± 5	162.7 ± 4.4
	3%	97	86	965 ± 35	130.4 ± 17.0
	2%	92	64	915 ± 30	97.5 ± 5.3

* Growth expressed as a percentage of growth in 5% FCS and MEM

TABLE 3.9 Growth of RPMI 2650 in DME:F12 (1:1) basal medium

	% Colony No.*	% Colony Area*	Colony No.	Total Colony Area (sq. mm)
5% FCS	100	100	717 ± 6.5	67.7 ± 4.2
3% FCS	101	104	726 ± 6	70.7 ± 1.0
2% FCS	80	53	573 ± 40	36.1 ± 5.0
1% FCS	56	31	405 ± 10	21.2 ± 0.7

* Growth expressed as a percentage of growth in 5% FCS

TABLE 3.10 Growth of RPMI 2650 in a variety of basal media

Basal medium	% Colony No.*	% Colony Area*	Colony No.	Total Colony Area (sq. mm)
DME:F12 + 5% FCS	100	100	919 ± 32	204.2 ± 6.7
DME:F12 + 0.5% FCS	35	15	326 ± 12	29.7 ± 1.1
F12 + 5% FCS	26	7	239 ± 30	14.4 ± 2.1
F12 + 0.5% FCS	0	0	0	0
SLM + 5% FCS	97	97	888 ± 91	198.8 ± 15.4
SLM + 0.5% FCS	33	8	309 ± 50	17.1 ± 4.5
SLM:F12 + 5% FCS	91	78	839 ± 84	160.0 ± 36.8
SLM:F12 + 0.5% FCS	33	14	306 ± 60	30.3 ± 6.5
SLM:DME + 5% FCS	115	92	1053 ± 70	187.0 ± 14.9
SLM:DME + 0.5% FCS	21	4	193 ± 31	7.3 ± 2.1

* Growth expressed as a percentage of growth in 5% FCS

3.3.4 First observations of growth stimulation due to Insulin, Transferrin and Selenium

First investigations into the response of RPMI 2650 to insulin, transferrin and selenium were carried out using a FCS concentration of 3%; this was using MEM as a basal medium, prior to selection of the optimum basal medium. Assays were set up as described in section 2.15, at a concentration of $1x10^4$ cells/ 35mm dish. A mixture of insulin (5μg/ml), transferrin (5μg/ml) and selenium ($1x10^{-8}$M) (ITS) was found to stimulate growth, leading to a three-fold increase in colony number and a seven-fold increase in total colony area (Table 3.11). Two other compounds, hydrocortisone ($1x10^{-8}$M) and triiodothyronine (T_3), when added together to 3% FCS + ITS - supplemented medium, led to a decrease in cell growth, relative to that observed in the 3% + ITS supplemented dishes. In the absence of FCS, the ITS-mixture failed to support any growth at all.

TABLE 3.11 Growth stimulation of RPMI 2650 due to the addition of Insulin, transferrin and selenium (ITS)

	% Colony No.*	% Colony Area*	Colony No.	Total Colony Area (sq. mm)
5% FCS	100	100	688 ± 38	72.8 ± 5.4
3% FCS	29	13	200 ± 57	9.7 ± 3.8
3% FCS + ITS	98	87	677 ± 40	63.0 ± 8.9
FCS + $THIT_3S$	52	21	355 ± 67	15.1 ± 3.8
- FCS + ITS	0	0	0	0

Note: There is a significant difference in both colony number and area ($p<0.01$) between 3% FCS and 3% FCS + ITS; this was using MEM as a basal medium prior to selection of an optimum basal medium.

*Growth expressed as a percentage of growth in 5% FCS

3.3.5 Initial investigations into the action of transferrin, insulin, epidermal growth factor and selenium

Having established a background FCS level (see Sec. 3.3.2) and having observed some growth stimulation with higher FCS levels (see Sec. 3.3.4) due to the addition of ITS, we then attempted to identify compounds which consistently stimulated growth of RPMI 2650. The four compounds tested in detail initially were transferrin, insulin and selenium, (the most commonly used components in serum-free media) and epidermal growth factor (EGF).

Tables 3.12 and 3.13 show the contribution made by these factors. Table 3.12 shows that transferrin on its own contributes significantly to growth, increasing the colony forming efficiency almost eight-fold and the total colony area is six times greater than in the 0.5% FCS control. In combination with insulin, transferrin is even more effective. Data presented in Table 3.13 confirms the critical role played by transferrin. There is a significant increase in the colony number and colony area ($p < 0.05$) when transferrin alone is added to 0.5% FCS. In conjunction with selenium or with both EGF and selenium growth is significantly improved at this FCS background level also.

The increase in growth in the presence of insulin is less marked than that with transferrin, but when added at a concentration of 5 μg/ml to 5% FCS the colony number and colony area values are twice those with 0.5% FCS alone (Table 3.12). The exact extent to which insulin stimulated growth in RPMI 2650 merited considerable attention. Numerous sets of data initially showed insulin, in combination with transferrin, stimulating growth at 1 - 3% FCS concentrations (Tables 3.11 and 3.13). At lower FCS concentrations insulin is also shown to stimulate growth of RPMI 2650 (Tables 3.12 and 3.14).

Insulin is dissolved in 1M HCl (see Sec. 2.9) and can be difficult to dissolve. The first batch of insulin used for this work dissolved readily in a 1:5 mixture of 1M HCl and PBS A. The second batch used required the addition of some concentrated HCl to dissolve at the same concentration (5 mg/ml). Thus, even after diluting 1:1000 for addition to

the culture medium, it was considerably more acidic than the first batch used. The effect of increased acidity on cell growth is not clear.

The data presented in Table 3.15 shows that in three out of four instances insulin acted to stimulate growth of RPMI 2650 in the presence of transferrin, EGF and selenium. These sets of experiments were set up using the second batch of insulin. It appears that, in general, insulin stimulates growth of RPMI 2650, but its use should be approached with caution.

EGF (10 ng/ml) when added to transferrin and insulin, leads to a very significant increase in the colony number and colony area ($p<0.001$) with a FCS background of 0.5% (Table 3.12). This marked effect of EGF is confirmed by results presented in Table 3.13, especially at the lower FCS concentration. However, there is no detectable increase in cell growth when EGF is added on its own to FCS (Table 3.13). It appears to interact with other compounds (e.g. transferrin, selenium) in influencing growth of RPMI 2650 cells. Table 3.13 shows that, with a 0.5% FCS level, neither EGF nor selenium cause any increased growth when added on their own, yet when the two are added together there is a significant increase ($p<0.05$) over the 0.5% FCS control.

The exact contribution made by selenium is difficult to determine. Preliminary work at higher FCS background levels reveal no growth-stimulatory effects due to addition of selenium (Tables 3.13 and 3.16). At 2% and 3% FCS there is no increased growth on addition of selenium, while there is a very slight increase at a 1% FCS concentration (Table 3.13). But when the FCS level is lowered to 0.5% FCS and 0.25% FCS some effects of selenium become evident.

In most cases, there is a slight increase in the CFE and colony area when selenium is added in conjunction with another compound but no stimulation is evident when it is added on its own. Table 3.12 shows a significant increase in the colony number ($p<0.01$) when selenium is added to 0.5% FCS + TIE while the results in Table 3.13 also show an increase in the CFE ($p<0.05$). There is no significant increase in the colony area in either case. Thus, selenium appears to be influencing growth in some way which leads to an increase in colony number but not in colony area. The continual appearance of this effect by selenium on RPMI 2650 growth justifies including it as one of the components in the development of a serum-free medium formulation.

Thus, these four compounds - transferrin, insulin, EGF and selenium (referred to as TIES) are the basis of a serum-free medium formulation for growth of RPMI 2650. The results presented here show that in the presence of 0.5% FCS + TIES clonal growth of RPMI 2650 is maintained to some extent relative to growth in 5% FCS.

TABLE 3.12 Effects of transferrin, insulin, EGF and Selenium on RPMI 2650 cells

	% Colony No.*	% Colony Area*	Colony No.	Total Colony Area (sq. mm)
5% FCS	100	100	534 ± 53	154 ± 204
0.5% FCS	0.7	0.2	4 ± 0.5	0.3 ± 0.06
0.5% FCS + I (5μg/ml)	1.6	0.4	9 ± 0.5	0.6 ± 0.3
0.5% FCS + T (5μg/ml)	5.5	1.2	29 ± 7	1.9 ± 0.8
0.5% FCS + TI	9.7	2.2	52 ± 8	3.4 ± 0.9
0.5% FCS + TI E (10ng/ml)	30.5	13.2	163 ± 17	20.4 ± 0.7
0.5% FCS + TIE S (10nM)	33.3	14.2	178 ± 30	21.9 ± 4.0

Concentrations tested: Transferrin (T) - 5μg/ml, Insulin (I) - 5μg/ml, EGF (E) - 10ng/ml, Selenium (S) - 10nM

*Growth expressed as a percentage of growth in 5% FCS

TABLE 3.13 Response of RPMI 2650 to transferrin, insulin, EGF and selenium with varying FCS backgrounds

	% Colony No.*	% Colony Area*	Colony No.	Total Colony Area (sq. mm)
5% FCS	100	100	718 ± 6.5	67.7 ± 5.0
2% FCS	80	53	573 ± 41	36.1 ± 5.0
2% FCS + IT	109	97	783 ± 30	65.9 ± 8.3
2% FCS + ITS	106	87	762 ± 31	59.0 ± 8.0
2% FCS + TIES	121	134	867 ± 42	90.6 ± 5.0
1% FCS	56	31	405 ± 11	21.2 ± 1.2
1% FCS + IT	74	45	529 ± 27	30.4 ± 3.8
1% FCS + ITS	77	53	554 ± 33	35.5 ± 0.9
1% FCS + TIES	79	62	569 ± 35	42.0 ± 1.2
5% FCS	100	100	695 ± 13	46.3 ± 2.9
0.5% FCS	31	20	216 ± 15	9.2 ± 0.7
0.5% FCS + T	44	32	303 ± 13	14.8 ± 2.8
0.5% FCS + EGF	22	24	156 ± 20	11.3 ± 2.1
0.5% FCS + S	35	22	241 ± 25	10.3 ± 0.5
0.5% FCS + TS	49	35	343 ± 32	16.0 ± 2.8
0.5% FCS + ES	52	40	366 ± 14	18.5 ± 2.0
0.5% FCS + TES	63	49	441 ± 47	22.8 ± 1.8

* Growth expressed as a percentage of growth in 5% FCS

TABLE 3.14 Growth of RPMI 2650 in the presence of insulin

	% Colony No.*	% Colony Area*	Colony No.	Total Colony Area (sq. mm)
5% FCS	100	100	1153 ± 24	192.7 ± 10.9
0.5% FCS	61	22	705 ± 76	43.0 ± 6.9
0.5% FCS + I	80	41	926 ± 49	79.0 ± 9.9
0.5% FCS + T	96	64	1104 ± 62	124.0 ± 9.0

Insulin (5μg/ml) and Transferrin (5μg/ml) both show significant increases ($p<0.05$) over 0.5% FCS control

* Growth expressed as a percentage of growth in 5% FCS

TABLE 3.15 Growth of RPMI 2650 in TIES v TES

	% Colony No.*	% Colony Area*	Colony No.	Total Colony Area (sq. mm)
5% FCS	100	100	953 ± 48	180.0 ± 11.0
0.5% FCS	40	16	385 ± 30	28.3 ± 2.1
0.5% FCS + TES	54	28	516 ± 54	50.1 ± 4.4
0.5% FCS + TIES	75	34	718 ± 47	60.3 ± 9.1
5% FCS	100	100	871 ± 67	135.8 ± 13.0
0.25% FCS	3	0.5	25 ± 12	0.75 ± 0.4
0.25% FCS + TES	29	9	248 ± 37	11.6 ± 1.7
0.25% FCS + TIES	45	17	395 ± 34	21.75 ± 2.2
5% FCS	100	100	1060 ± 125	180.8 ± 62.3
0.25% FCS	19	4	197 ± 24	7.7 ± 1.7
0.25% FCS + TES	33	10	346 ± 4	18.3 ± 0.6
0.25% FCS + TIES	54	25	575 ± 48	44.5 ± 4.2
5% FCS	100	100	410 ± 29	35.5 ± 2.6
0.5% FCS	11	9	45 ± 9	3.3 ± 1.8
0.5% FCS + TES	65	45	268 ± 17	15.9 ± 2.6
0.5% FCS + TIES	60	47	246 ± 23	16.7 ± 3.2

* Growth expressed as a percentage of growth in 5% FCS

TABLE 3.16 Response of RPMI 2650 to selenium in the presence of 3% and 2% FCS

	% Colony No.*	% Colony Area*	Colony No.	Total Colony Area (sq. mm)
5% FCS	100	100	525 ± 22	27.3 ± 0.8
3% FCS	65	61	344 ± 21	16.6 ± 0.7
3% FCS + IT	105	96	551 ± 20	26.2 ± 1.0
3% FCS + ITS	106	122	556 ± 18	33.3 ± 1.5
2% FCS	60	55	314 ± 30	15.1 ± 1.5
2% FCS + IT	86	89	453 ± 20	24.4 ± 1.4
2% FCS + ITS	83	90	436 ± 31	24.7 ± 0.3

*Growth expressed as a percentage of growth in 5% FCS

3.3.6 Varying concentrations of the main components

Experiments were set up to investigate the optimum range of the four components found to consistently produce the greatest stimulation to growth of RPMI 2650 in a low FCS background level, i.e. Transferrin, Insulin, EGF and Selenium.

Table 3.17 shows the effect of varying the concentration of insulin. A concentration range of 5 μg/ml - 10 μg/ml resulted in no obvious change in either the CFE or colony area, while insulin at a lower concentration range - 1 - 2.5 μg/ml was shown to be less effective. Hence, a concentration of 5 μg/ml was chosen for routine use as the lowest concentration of insulin which gave maximal effect.

Table 3.17 also shows the effect of varying EGF concentrations from 1ng/ml to 100ng/ml. Between 1ng/ml and 50ng/ml there is no significant difference in either the colony area or the colony number. At a higher concentration of 100ng/ml, EGF is less effective; there is a significant decrease in both the CFE and the colony area ($p<0.02$).

Table 3.18 shows growth of RPMI 2650 under varying transferrin and selenium concentrations. Transferrin was tested over a range from 1 μg/ml to 10 μg/ml but growth was almost the same under all conditions. A value in the middle of the range, 5 μg/ml, was chosen for routine use.

Selenium is present in most serum-free formulations as a trace element. Hence, a range from 5×10^{-9}M to 5×10^{-8}M was examined. Greatest growth was observed in the middle region of the range (1×10^{-8}M - 2×10^{-8}M); therefore a concentration of 1×10^{-8}M was used on a routine basis.

TABLE 3.17 Varying concentrations of Insulin and EGF

	% Colony No.*	% Colony Area*	Colony No.	Total Colony Area (sq. mm)
5% FCS	100	100	208 ± 18	10.2 ± 2.1
0.5% FCS	6	3	13 ± 1	0.3 ± 0.02
0.5% FCS + TES +:				
Ins (1 µg/ml)	25	23	64 ± 6	2.4 ± 0.4
Ins (2.5 µg/ml)	30	29	63 ± 5	3.0 ± 0.4
Ins (5 µg/ml)	62	55	128 ± 16	5.6 ± 0.9
Ins (7.5 µg/ml)	55	41	115 ± 9	4.2 ± 0.7
Ins (10 µg/ml)	64	56	133 ± 14	5.7 ± 1.0
5% FCS	100	100	489 ± 26	43.2 ± 2.7
0.5% FCS	25	12	124 ± 21	5.5 ± 3.5
0.5% FCS + TIS +:				
EGF (1ng/ml)	69	47	337 ± 27	20.1 ± 2.4
EGF (10ng/ml)	82	68	399 ± 5	29.4 ± 0.4
EGF (20ng/ml)	66	61	324 ± 37	26.4 ± 3.8
EGF (50ng/ml)	79	66	388 ± 15	28.6 ± 4.0
EGF (100ng/ml)	55	31	271 ± 26	13.2 ± 2.7

Concentrations of Trans (5 µg/ml), Selenium (10nM) and either insulin (5 µg/ml) or EGF (10ng/ml) were kept constant to observe the effect of changing insulin or EGF concentrations.

* Growth expressed as a percentage of growth in 5% FCS

TABLE 3.18 Varying concentrations of Transferrin and Selenium

	% Colony No.*	% Colony Area*	Colony No.	Total Colony Area (sq. mm)
5% FCS	100	100	501 ± 52	31.8 ± 6.9
0.5% FCS	16	10	77 ± 25	3.2 ± 1.8
0.5% FCS + TIE +:				
S ($5x10^{-9}$M)	76	61	382 ± 52	19.4 ± 3.8
S ($1x10^{-8}$M)	72	59	363 ± 48	18.9 ± 5.3
S ($2x10^{-8}$M)	80	67	405 ± 42	21.3 ± 4.4
S ($5x10^{-8}$M)	58	49	289 ± 84	15.5 ± 4.2
0.5% FCS + IES+:				
T (1 µg/ml)	71	50	354 ± 42	16.0 ± 3.0
T (2.5 µg/ml)	58	44	289 ± 44	14.0 ± 2.6
T (5 µg/ml)	72	59	363 ± 48	18.9 ± 5.3
T (10 µg/ml)	77	62	385 ± 30	19.7 ± 2.0

Concentrations of insulin (5 µg/ml), EGF (10 ng/ml) and either transferrin (5 µg/ml) or selenium (10nM) were kept constant to observe the effect of varying transferrin and selenium concentrations.

* Growth expressed at a percentage of growth in 5% FCS

3.3.7 Examination of the effects of a range of compounds on growth of RPMI 2650 cells

Experiments were set up to investigate the activity of a large number of compounds, in the presence of a background level of FCS, on the growth of RPMI 2650. The background level of FCS varied from 0.25% FCS to 0.5% FCS. Compounds were tested over a range of concentrations and usually on their own and in conjunction with transferrin (5 μg/ml), insulin (5 μg/ml), EGF (10ng/ml) and selenium (10nM)(TIES). In some cases, experiments were carried out without insulin (TES), as investigations were being carried out simultaneously into the exact role of insulin (see Sec. 3.3.5). Growth was measured in terms of both the colony number and the colony area. (The colony number is a reflection of the colony forming efficiency (CFE) and at times is referred to as such). The colony area is given in square millimetres (mm^2).

3.3.7.1 Hydrocortisone

A number of experiments were set up to test the effect of hydrocortisone (HC). In early developmental work with higher background FCS levels, HC seemed to be stimulating growth when added with some other factors (Table 3.19). But this stimulation, while observed on occasions with lower FCS background levels, was not evident consistently and hence it was concluded that HC did not play a critical part in supporting RPMI growth in the absence of FCS (Table 3.20).

TABLE 3.19 Hydrocortisone (H) as a medium component. Effect on RPMI 2650 with 3% FCS

	% Colony No.*	% Colony Area *	Colony No.	Total Colony Area (sq. mm)
5% FCS	100	100	410 ± 22	89.4 ± 2.1
3% FCS	33	20	135 ± 15	17.9 ± 1.8
3% FCS + TIES	64	45++	262 ± 4	40.4 ± 2.5
3% FCS + THIES	70	54++	287 ± 10	48.3 ± 2.7

++ Differences in CFE and area are significant at 5% level ($p<0.05$)

* Growth expressed as a percentage of growth in 5% FCS

TABLE 3.20 Effect of Hydrocortisone (HC) on growth of RPMI 2650 (0.5% FCS level)

	% Colony No.*	% Colony Area *	Colony No.	Total Colony Area (sq. mm)
5% FCS	100	100	501 ± 52	31.8 ± 6.9
0.5% FCS	16	10	77 ± 25	3.2 ± 1.8
0.5% FCS + TIES	72	59	363 ± 48	18.9 ± 3.8
0.5% FCS + TIES:				
HC (5×10^{-8}M)	45	27	227 ± 74	8.7 ± 4.0
HC (2.5×10^{-8}M)	71	59	355 ± 27	18.7 ± 3.8
HC (1×10^{-8}M)	73	58	365 ± 44	18.6 ± 3.6
HC (5×10^{-9}M)	58	39	293 ± 57	12.4 ± 3.3
HC (1×10^{-9}M)	56	44	283 ± 64	14.0 ± 2.0

* Growth expressed as a percentage of growth in 5% FCS

3.3.7.2 Triiodothyronine and the Prostaglandins

Triiodothyronine (T_3) and PGE_1 are present in the serum-free medium which supports the growth of MDCK (see Sec. 3.2). Hence, experiments were carried out to examine the effects of these compounds on growth of RPMI 2650. Another prostaglandin, $PGF_{2\alpha}$, was also tested. All three were tested individually in the presence of 0.5% FCS or 0.25% FCS + TIES. T_3 and PGE_1 were tested in combination with each other and with hydrocortisone (HC).

Table 3.21 shows that at two different concentrations, T_3 has no effect on the growth of RPMI 2650, in the presence of 0.5% FCS + TIES, while Table 3.22 shows that T_3 (2×10^{-12}M) and HC(5×10^{-8}M) when combined in the presence of 0.5% FCS + TIES make no major impact on growth of RPMI 2650. However, when T_3 and PGE_1 are combined there is a significant decrease in the CFE ($p<0.001$) and the combination of HC, T_3 and PGE_1 inhibits growth to almost one third that obtained in the 0.5% + TIES control.

PGE_1 (25 ng/ml) when added to 0.5% FCS + TIES, seems to cause some reduction in growth (Tables 3.21 and 3.22), but when PGE and either T_3 or HC are added to 0.5% + TIES more significant growth inhibition occurs. Both the CFE and colony areas are affected and when all three are supplied to the medium together, the growth inhibition is greatest. This pattern was detected on other occasions, with PGE_1 inhibition being more pronounced at times (results not given).

$PGF_{2\alpha}$ was added at a range of concentrations to 0.5% + TIES. It has no effect on either the CFE or the colony area (Table 3.23).

TABLE 3.21 Effect of Triiodothyronine (T_3) on growth of RPMI 2650

	% Colony No.*	% Colony Area*	Colony No.	Total Colony Area (sq. mm)
5% FCS	100	100	647 ± 60	70.7 ± 13.3
0.5% FCS	33	20	213 ± 4	14.0 ± 0.7
0.5% FCS + TIES	58	40	374 ± 50	28.6 ± 2.9
0.5% FCS + TIES T_3 ($1x10^{-8}$M)	56	37	363 ± 21	26.4 ± 3.8
0.5% FCS + TIES T_3 ($2x10^{-12}$M)	55	37	358 ± 66	26.0 ± 5.7
0.5% FCS + TIES PGE_1	43	35	281 ± 41	24.7 ± 3.5
0.5% FCS + TIES HC T_3 PGE_1	32	18	205 ± 19	12.9 ± 1.7

PGE (25 ng/ml) and HC ($5x10^{-8}$M) were used in this experiment.

*Growth expressed as a percentage of growth in 5% FCS

TABLE 3.22 Response of RPMI 2650 to additions of PGE_1, hydrocortisone and T_3

	% Colony No.*	% Colony Area*	Colony No.	Total Colony Area (sq. mm)
5% FCS	100	100	534 ± 53	154.0 ± 20.4
0.5% FCS	0.7	0.2	4 ± 0.5	0.3 ± 0.06
0.5% FCS + TIES	33	14	178 ± 31	21.9 ± 2.4
0.5% FCS + TIES T_3	33	17	179 ± 21	26.5 ± 3.7
0.5% FCS + TIES (PGE_1)	29	14	155 ± 16	22.1 ± 4.25
0.5% FCS + TIES HC	35	16	190 ± 7.4	24.9 ± 2.1
0.5% FCS + TIES HC T_3	31	14	165 ± 12	21.6 ± 5.1
0.5% FCS + TIES HC (PGE_1)	16	7	85 ± 13	10.0 ± 4.0
0.5% FCS + TIES T_3(PGE_1)	22	12	116 ± 3	17.9 ± 2.3
0.5% FCS + TIES HC T_3 (PGE_1)	13	6	72 ± 14	9.0 ± 1.4

Concentrations used: HC(50nM), T_3(2pM), PGE_1(25ng/ml)

* Growth expressed as a percentage of growth in 5% FCS

TABLE 3.23 Growth of RPMI 2650 in the presence of $PGF_{2\alpha}$

	% Colony No.*	% Colony Area*	Colony No.	Total Colony Area (sq. mm)
5% FCS	100	100	799 ± 10	172.0 ± 4.6
0.5% FCS	81	27	653 ± 10	45.7 ± 1.7
0.5% FCS + TIES	89	34	712 ± 54	58.4 ± 7.9
0.5% FCS + TIES +:				
$PGF_{2\alpha}$ 2ng/ml	88	32	705 ± 30	55.0 ± 2.5
$PGF_{2\alpha}$ 20ng/ml	82	29	653 ± 31	50.1 ± 2.0
$PGF_{2\alpha}$ 100ng/ml	88	29	705 ± 49	50.3 ± 5.7

*Growth expressed as a percentage of growth in 5% FCS

3.3.7.3 Ascorbic Acid

Addition of freshly-dissolved ascorbic acid to medium supplemented with 0.25% + TES increases growth of RPMI 2650. Table 3.24 shows the effect of adding ascorbic acid over a concentration range from 1 µg/ml to 100 µg/ml. At concentrations of 1 µg/ml and 10 µg/ml of ascorbic acid both the CFE and the colony area are significantly increased ($p<0.05$). Over twice the number of colonies form and over three times the colony area is recorded, compared to the 0.25% FCS + TES controls. At the higher concentration of 100 µg/ml there is no significant increase in growth.

3.3.7.4 Bombesin

Bombesin was tested over a range from $1x10^{-6}$M to $1x10^{-8}$M, both with 0.5% FCS only and with 0.5% FCS + TIES or TES. This compound seems to influence growth of RPMI 2650 to some extent, but consistent growth stimulation was not recorded with bombesin. Table 3.25 shows the results of five experiments carried out to examine the effect of bombesin on growth. In medium supplemented with 0.5% FCS only, bombesin stimulated RPMI 2650 growth significantly ($p<0.05$) at 10^{-7}M and 10^{-8}M, in experiment (1) but in experiment (3) stimulation is seen at 10^{-6}M.

In medium supplemented with TIES or TES, with both 0.25% FCS and 0.5% FCS backgrounds, stimulation by bombesin is recorded, but under similar conditions, experiments (4) and (5) show no significant increase in RPMI 2650 growth due to bombesin.

These results are difficult to interpret. There is too much evidence for stimulation to suggest that its appearance is due to experimental error, yet it does not appear consistently. It seems that there is some inherent variation in the role of bombesin as a medium supplement which cannot be detected within this assay system.

TABLE 3.24 Growth of RPMI 2650 in the presence of Ascorbic acid

	% Colony No.*	% Colony Area*	Colony No.	Total Colony Area (sq. mm)
5% FCS	100	100	731 ± 129	296.0 ± 11.3
0.25% FCS	17	2	123 ± 70	7.1 ± 3.4
0.25% FCS + TES	26	6	193 ± 10	16.6 ± 1.2
0.25% FCS + TES +:				
Ascorbic Acid 1 μg/ml	62	13	456 ± 29	38.0 ± 4.6
Ascorbic Acid 10μg/ml	69	21	505 ± 104	63.4 ± 18.9
Ascorbic Acid 100 μg/ml	32	5	236 ± 57	15.5 ± 6.8

This experiment was carried out using TES (no insulin)

*Growth expressed as a percentage of growth in 5% FCS

TABLE 3.25 Growth of RPMI 2650 in the presence of bombesin

	% Colony No.*	% Colony Area*	Colony No.	Total Colony Area (sq. mm)
(1)				
5% FCS	100	100	953 ± 48	180.0 ± 11.0
0.5% FCS	40	16	385 ± 30	28.3 ± 2.1
0.5% FCS + B(10^{-6}M)	47	15	448 ± 53	27.3 ± 2.9
0.5% FCS + B(10^{-7}M)	52 +	16	498 ± 54	29.5 ± 7.3
0.5% FCS + B(10^{-8}M)	58 +	19 +	549 ± 37	33.8 ± 4.1
0.5% FCS + TES	54	28	516 ± 54	50.1 ± 4.4
0.5% FCS + TES B(10^{-6}M)	62	30	587 ± 28	54.8 ± 4.8
0.5% FCS + TES B(10^{-7}M)	86 +	49 +	818 ± 30	88.3 ± 11.0
0.5% FCS + TES B(10^{-8}M)	95 +	54 +	901 ± 2	97.3 ± 8.6
(2)				
5% FCS	100	100	883 ± 33	237.5 ± 16.7
0.25% FCS	14	9	123 ± 12	20.2 ± 1.4
0.25% FCS + TIES	33	14	287 ± 76	33.2 ± 4.9
0.25% FCS + TIES +:				
B(10^{-6}M)	55 +	21 +	489 ± 47	49.1 ± 6.2
B(10^{-7}M)	43 +	17	377 ± 25	41.1 ± 3.2
B(10^{-8}M)	47 +	19 +	415 ± 54	45.3 ± 6.4
(3)				
5% FCS	100	100	705 ± 67	155.4 ± 18.4
0.5% FCS	22	13	156 ± 76	19.5 ± 8.0
0.5% FCS + B(10^{-6}M)	43 +	20 +	305 ± 65	30.8 ± 5.9
0.5% FCS + B(10^{-7}M)	23	12	163 ± 65	19.2 ± 11.4
0.5% FCS + B(10^{-8}M)	30	14	208 ± 60	21.4 ± 4.0
0.5% + TIES	77	48	544 ± 38	75.0 ± 15.1
0.5% + TIES +				
B(10^{-6}M)	90 +	64 +	635 ± 41	99.6 ± 19.2
B(10^{-7}M)	86 +	56 +	605 ± 21	86.8 ± 17.0
B(10^{-8}M)	93 +	61 +	657 ± 70	94.5 ± 22.5

+ This indicates a significant increase ($p<0.05$) in either colony number or colony area.

* Growth expressed as a percentage of growth in 5% FCS

p.t.o.

TABLE 3.25 (cont'd) Growth of RPMI 2650 in the presence of bombesin

	% Colony No.*	% Colony Area*	Colony No.	Total Colony Area (sq. mm)
(4)				
5% FCS	100	100	871 ± 67	135.8 ± 13.0
0.25% FCS	3	0.5	25 ± 12	0.75 ± 0.4
0.25% FCS + TIES	45	16	395 ± 34	21.75 ± 2.2
0.25% FCS + TIES +:				
B(10^{-6}M)	34	9	297 ± 32	12.9 ± 2.0
B(10^{-7}M)	32	9	285 ± 7	12.6 ± 1.3
B(10^{-8}M)	33	10	288 ± 32	13.1 ± 2.4
(5)				
5% FCS	100	100	911 ± 37	101.0 ± 8.6
0.5% FCS	21	7	191 ± 25	7.4 ± 0.8
0.5% FCS + TES	53	29	479 ± 23	28.8 ± 3.3
0.5% FCS + TES B(10^{-6}M)	37	17	333 ± 31	17.2 ± 2.1
0.25% FCS	1	0.3	9 ± 2	0.3 ± 0.15
0.25% FCS + TES	20	6	185 ± 32	6.3 ± 0.5
0.25% FCS + TES B(10^{-6}M)	23	8	211 ± 16	8.3 ± 0.6

Three experiments included insulin, two excluded insulin.

* Growth expressed as a percentage of growth in 5% FCS

3.3.7.5 Glycyl-hystidyl-lysine (GHL)

Glycyl-hystidyl-lysine is a polymer that has been used in some serum-free formulations. Experiments were set up to examine its effect on growth of RPMI 2650. GHL was tested over a range of concentrations (10ng/ml - 200ng/ml) in the presence of 0.5% FCS + TIES. Under these conditions, no stimulation due to GHL was recorded (Table 3.26).

3.3.7.6 MEM non-essential amino acids and sodium pyruvate

MEM non-essential amino acids is a commercially available mixture of amino acids which are added to the basal MEM medium, at a 1:100 dilution, in which RPMI 2650 cells are grown routinely (for composition, see Table 3.27). Experiments were set up to investigate the effect of adding this amino acid mixture to the DME:F12 basal medium, supplemented with 0.5% + TES. The non-essential amino acids were diluted 1:100 in the medium.

Table 3.28 shows that the amino acid mixture enhances growth of RPMI 2650. There is a significant increase in both colony area and colony number ($p < 0.05$) with the greatest increase being in colony number. Thus it would appear that there are some amino acids in this mixture which either are not present, or are not at a sufficiently high concentration in the DME:F12 basal medium, supplemented with 0.5% FCS + TES.

Sodium pyruvate is an alternative carbon source found in some basal media. Experiments were set up to see if its inclusion in medium, in conjunction with the normal carbon source glucose, would have any effect on cell growth. Sodium pyruvate was added at a concentration of 1.1mM. These showed that it had no significant effect on growth of RPMI 2650 in the presence of 0.5% FCS + TES, (data not presented).

TABLE 3.26 Growth of RPMI 2650 in medium supplemented with glycyl-hystidyl-lysine (GHL)

	% Colony No.*	% Colony Area*	Colony No.	Total Colony Area (sq.mm)
5% FCS	100	100	806 ± 9	154.8 ± 7.5
0.5% FCS	34	17	275 ± 52	26.6 ± 4.3
0.5% FCS + TIES	53	25	429 ± 28	38.1 ± 6.2
0.5% FCS + TIES +				
GHL(10ng/ml)	47	20	379 ± 28	31.6 ± 4.3
GHL(50ng/ml)	49	23	394 ± 28	35.4 ± 2.1
GHL(100ng/ml)	40	18	325 ± 29	28.5 ± 4.7
GHL(150ng/ml)	50	25	406 ± 22	38.7 ± 5.4
GHL(200ng/ml)	53	22	427 ± 37	34.6 ± 8.7

* Growth expressed as a percentage of growth in 5% FCS

TABLE 3.27 Composition of MEM non-essential amino acids

Ingredient	mg/l
L-alanine	8.90
L-asparagine .H_2O	15.00
L-aspartic acid	13.30
Glycine	7.50
L-glutamic acid	14.70
L-proline	11.50
L-serine	10.50

TABLE 3.28 Growth of RPMI 2650 in medium supplemented with MEM non-essential amino acids (NEAA)

	% Colony No.*	% Colony Area*	Colony No.	Total Colony Area (sq.mm)
5% FCS	100	100	953 ± 48	180.0 ± 11.2
0.5% FCS	40	16	385 ± 30	28.3 ± 2.1
0.5% FCS + TES	54	28	516 ± 54	50.7 ± 4.4
0.5% FCS + TES NEAA	88	42	840 ± 77	75.8 ± 10.2
0.5% FCS + TES	72	31	689 ± 57	55.3 ± 4.5

* Growth expressed as a percentage of growth in 5% FCS

TABLE 3.29 Growth of RPMI 2650 in medium supplemented with PDGF and Retinoic acid (RA)

	% Colony No.*	% Colony Area*	Colony No.	Total Colony Area (sq.mm)
5% FCS	100	100	920 ± 49	165.7 ± 16.7
0.5% FCS	48	22	442 ± 120	36.9 ± 17.6
- FCS	0	0	0	0
0.5% + TIES	74	44	683 ± 30	73.7 ± 3.4
- FCS + TIES	0	0	0	0
0.5% + TIES(PDGF)	72	45	661 ± 96	74.5 ± 10.7
0.5% + TIES +				
RA 10^{-9}M	74	40	680 ± 48	65.9 ± 10.5
RA 10^{-8}M	68	35	622 ± 78	57.7 ± 10.0
RA 10^{-7}M	35 x	16 x	323 ± 37	27.0 ± 4.8
RA 10^{-6}M	46 x	22 x	419 ± 37	37.0 ± 6.7
RA 10^{-5}M	0 x	0 x	0	0
RA 10^{-4}M	0 x	0 x	0	0

x Decrease in growth significant at $p<0.01$

* Growth expressed as a percentage of growth in 5% FCS

3.3.7.7. Other factors tested

Platelet derived growth factor (PDGF)

This polypeptide growth factor was available only in a very small quantity. Thus it was possible to test it at one concentration only. The PDGF came from Bioprocessing Ltd. and a concentration of 1 unit/ml was recommended. No details were supplied on the protein concentration present at 1 unit/ml. At this concentration, this preparation of PDGF showed no effect on growth of RPMI 2650 (Table 3.29). The growth in PDGF-supplemented medium is identical to that in the 0.5% FCS + TIES control.

Retinoic Acid

Retinoic acid (vitamin A) was tested over a wide concentration range. At lower concentrations (10^{-8} - 10^{-9}M) retinoic acid has no significant effect on RPMI 2650 growth (Table 3.29). At concentrations of 10^{-7}M and 10^{-6}M growth inhibition has begun. There is a significant reduction in cell number and colony area and when the concentration is further increased to 10^{-5} - 10^{-4}M, no viable colonies remained after 7 days.

Progesterone

Experiments were set up to test the effect of progesterone over a range of concentrations. With a background FCS level of 0.25%, progesterone on its own has no stimulatory effect and at higher concentrations (10^{-7} - 10^{-8}M) growth is inhibited. In medium supplemented with 0.25% FCS + TIES, progesterone, over a concentration range of 10^{-7}M to 10^{-9}M has no effect at all on either colony number or colony area (Table 3.30).

Phosphoethanolamine

Phosphoethanolamine (PEtn) was added to the culture medium at concentrations of 10^{-5}M - 10^{-7}M. In the presence of 0.5% + TIES, PEtn addition at all three concentrations tested, leads to a reduction in growth (Table 3.31). Both the CFE and the total colony area are reduced in the presence of PEtn.

Ferrous sulphate

Ferrous sulphate is an iron source which cells can sometimes utilise in place of the more conventional iron supply routes. Initial tests at a concentration of 10^{-5}M suggested that $FeSO_4$ was inhibitory to cell growth (see Table 3.31). Growth was markedly reduced when $FeSO_4$ was added to medium with a 0.5% FCS background.

$FeSO_4$ was tested over a range of concentrations, 10^{-5}M to 10^{-9}M, in the presence of transferrin. When medium was supplemented with 0.5% FCS + TIES, addition of ferrous sulphate at a concentration of 10^{-5}M inhibits growth severely while lower concentrations have no effect (Table 3.31).

TABLE 3.30 Growth of RPMI 2650 in the presence of Progesterone (P)

	% Colony No.*	% Colony Area*	Colony No.	Total Colony Area (sq.mm)
5% FCS	100	100	731 ± 129	229.5 ± 94.5
0.25% FCS	17	3	122 ± 70	7.1 ± 3.4
0.25% + TIES	26	7	193 ± 10	16.6 ± 1.2
0.25% +				
P (10^{-9}M)	21	5	157 ± 32	12.0 ± 3.4
P (10^{-8}M)	6	1	42 ± 13	2.8 ± 0.5
P (10^{-7}M)	5	1	38 ± 4	1.4 ± 0.2
0.25% + TIES +				
P (10^{-9}M)	27	7	199 ± 10	15.5 ± 2.1
P (10^{-8}M)	29	6	211 ± 17	13.9 ± 1.7
P (10^{-7}M)	26	7	192 ± 75	17.25 ± 5.6

* Growth expressed as a percentage of growth in 5% FCS

TABLE 3.31 Effect of Phosphoethanolamine and Ferrous Sulphate ($FeSO_4$) on growth of RPMI 2650 cells

	% Colony No.*	% Colony Area*	Colony No.	Total Colony Area (sq.mm)
5% FCS	100	100	410 ± 29	35.5 ± 2.6
0.5% FCS	11	9	45 ± 9	3.3 ± 1.8
0.5% FCS + TIES	60	47	246 ± 23	16.7 ± 3.2
0.5% FCS + TIES +				
PEtn 10^{-5}M	32	21	130 ± 13	7.4 ± 1.3
PEtn 10^{-6}M	36	22	147 ± 18	8.1 ± 0.1
PEtn 10^{-7}M	30	17	122 ± 17	5.9 ± 0.6
5% FCS	100	100	508 ± 36	86.2 ± 10.9
0.5% FCS	62	32	313 ± 25	27.9 ± 3.2
0.5% + $FeSO_4$ (10^{-5}M)	3	1	14 ± 4	0.9 ± 0.6
0.5% + Trans (5 µg/ml)	67	39	342 ± 42	33.5 ± 6.8
0.5% + TIES	79	61	402 ± 10	52.4 ± 4.7
5% FCS	100	100	799 ± 10	172.0 ± 4.6
0.5% FCS	81	27	653 ± 10	45.7 ± 1.7
0.5% FCS + TIES	89	37	712 ± 54	63.2 ± 7.9
0.5% FCS + TIES +				
$FeSO_4$ (10^{-5}M)	2	0.5	17 ± 3	0.6 ± 0.2
$FeSO_4$ (10^{-7}M)	78	29	624 ± 37	50.1 ± 1
$FeSO_4$ (10^{-9}M)	85	28	681 ± 61	47.3 ± 8

*Growth expressed as a percentage of growth in 5% FCS.

3.3.7.8 Inclusion of attachment factors as media components

Some compounds can stimulate growth of different cell types either through precoating of dishes or by adding the compounds into the medium. Thus, three of the compounds that were used for precoating (see Sec. 3.3.8.) - bovine serum albumin (BSA), collagen and fibronectin (Fn) were also added to the cells as medium supplements.

When Fn is added to medium containing 0.25% FCS + TIES at a concentration of 10 μg/ml, there is a significant increase in the colony number ($p<0.01$) but the change in colony area is not significant. An identical pattern is observed, at 0.5% FCS background, the CFE value increasing (p 0.02) but with no significant increase in colony area.

Collagen at a concentration of 10 μg/ml influences growth positively, with the CFE value almost doubling and an increase is also recorded in the colony area. The results at a concentration of 1 μg/ml are most likely due to experimental error (Table 3.32).

Bovine serum albumin was added to 0.25% FCS + TES and its influence tested on growth of RPMI 2650. Over a concentration range from 0.5 mg/ml to 5 mg/ml there was no significant difference in growth, assuming that the low readings at the intermediate concentrations of 1 mg/ml are due to experimental error. Thus, it appears that the inclusion of BSA in the medium has no influence on growth of RPMI 2650.

TABLE 3.32 Addition of collagen, fibronectin and BSA to medium

	% Colony No.*	% Colony Area*	Colony No.	Total Colony Area (sq.mm)
5% FCS	100	100	635 ± 65	73.8 ± 14.0
0.5% FCS	14	6	91 ± 19	4.3 ± 1.5
0.5% FCS + TIES	28	11	177 ± 38	8.1 ± 2.2
0.5% FCS + TIES +:				
Collagen 10µg/ml	49	18	312 ± 34	13.0 ± 2.6
Collagen 1µg/ml	2	0.5	12 ± 4	0.3 ± 0.1
5% FCS	100	100	736 ± 13	184.2 ± 7.7
0.5% FCS	33	7	242 ± 4	12.3 ± 0.8
0.5% FCS + TIES	72	23	529 ± 52	42.9 ± 5.0
0.5% FCS + TIES (Fn)	90	29	662 ± 48	54.3 ± 10.4
0.25% FCS	35	12	261 ± 39	22.3 ± 5.2
0.25% FCS + TIES	54	17	398 ± 16	32.2 ± 3.2
0.25% FCS + TIES (Fn)	68	20	502 ± 45	37.2 ± 6.6
5% FCS	100	100	731 ± 129	296.0 ± 11.3
0.25% FCS	17	2	122 ± 70	7.1 ± 3.4
0.25% + TES	26	6	193 ± 10	16.6 ± 1.2
0.25% + TES +:				
BSA 500mg/ml	21	5	154 ± 33	13.75 ± 2.8
BSA 1mg/ml	5	1	39 ± 14	2.2 ± 0.3
BSA 5mg/ml	29	7	213 ± 55	20.3 ± 9.2

* Growth is expressed as a percentage of growth in 5% FCS

3.3.8 Testing attachment factors

Various factors were examined to see their effect on cellular attachment. Experiments were carried out as described in Section 2.16.

3.3.8.1 Collagen

Collagen Type III (acid soluble) was tested initially at two concentrations - 1 mg/ml and 100 μg/ml. Table 3.33 shows the growth recorded in collagen-coated dishes. At a concentration of 100μg/ml, collagen precoating occurred overnight (16 hrs) at 37°C or at room temperature. At the higher concentration, (1 mg/ml) growth was reduced relative to the 0.25% FCS + TIES control. The collagen was dissolved at 1 mg/ml in 1M HCl and diluted to 100 μg/ml in PBS A. It is possible that the precoating at 1 mg/ml concentration created an acidic surface which was unsuitable for growth.

Further tests were carried out at 100 μg/ml concentration. The length of time for precoating was varied and the temperature at which the dishes were left during precoating was also varied. Table 3.34 shows that precoating under any conditions enhances cell attachment and clonal growth. Precoating at room temperature for 16 hrs. or at 37°C for 4 hrs. increases both the CFE and the colony area to the greatest extent. There is almost a four-fold increase in the area covered by colonies and the number of colonies which form is more than doubled. Precoating at 37°C for longer - 16 hours - or shorter - 30 minutes - periods also leads to considerably more growth while precoating at 4°C or at room temperature also enhances colony formation and growth to varying degrees.

3.3.8.2 Vitrogen

A commercially-available solution of collagen, Vitrogen, was also used to precoat dishes. A range of concentrations was used, 0.1 mg/ml - 3 mg/ml at 37°C, room temperature and 4°C. Table 3.35 shows that at the highest concentration, 3 mg/ml, at 37°C for 16 hrs., there is a significant increase in both colony number and area ($p<0.05$) and an increase in growth is also evident at a concentration of 100 μg/ml at room temperature. Precoating at other temperatures at varying concentrations had little effect on cell growth.

TABLE 3.33 Growth of RPMI 2650 in dishes which had been precoated with collagen

	% Colony No.*	% Colony Area*	Colony No.	Total Colony Area (sq.mm)
Controls - no precoating				
5% FCS	100	100	548 ± 27	170.1 ± 28.1
0.25% FCS	10	2	56 ± 23	4.0 ± 2.1
0.25% FCS + TIES	26	8	144 ± 29	14.0 ± 5.3
Collagen 100 µg/ml				
16 hrs. 37°C	58	29	319 ± 25	49.8 ± 5.0
16 hrs. Room Temp.	46	26	250 ± 10	44.0 ± 12.8
16 hrs. 4°C	11	4	59 ± 2.6	7.0 ± 1.4
Collagen 1 mg/ml				
16 hrs. 37°C	13	2	73 ± 30	3.6 ± 1.6
16 hrs. Room Temp.	15	3	80 ± 7	4.8 ± 0.2
16 hrs. 4°C	10	3	55 ± 19	4.8 ± o.2

Details of precoating given in key. Controls were not precoated.
Cells are growing in 0.25% FCS + TIES in dishes precoated with collagen.
* Growth expressed as a percentage of growth in 5% FCS

TABLE 3.34 Growth of RPMI 2650 in collagen-coated dishes

	% Colony No.*	% Colony Area*	Colony No.	Total Colony Area (sq.mm)
Controls - no precoating				
5% FCS	100	100	635 ± 65	73.8 ± 14.0
0.5% FCS	14	6	92 ± 20	4.3 ± 1.5
0.5% FCS + TIES	28	11	177 ± 38	8.1 ± 2.2
- FCS - TIES	0	0	0	0
- FCS + TIES	0	0	0	0
Collagen 100 µg/ml				
16 hrs. 37°C	58	27	369 ± 95	19.7 ± 7.3
16 hrs. Room temp.	67	42	426 ± 39	31.0 ± 9.0
16 hrs. 4°C	38	15	242 ± 80	11.2 ± 5.2
4 hrs. 37°C	67	41	425 ± 56	30.6 ± 14.8
4 hrs. Room temp.	48	30	307 ± 13	22.2 ± 1.2
4 hrs. 4°C	38	20	239 ± 34	15.0 ± 5.2
30 mins. 37°C	73	34	464 ± 45	25.1 ± 1.3
30 mins. Room temp.	31	25	196 ± 49	18.4 ± 1.4
30 mins. 4°C	54	38	346 ± 57	28.2 ± 2.4

Cells are growing in 0.25% FCS + TIES in dishes precoated with collagen.

* Growth expressed as a percentage of growth in 5% FCS

TABLE 3.35 Growth of RPMI 2650 in Vitrogen-treated dishes

	% Colony No.*	% Colony Area*	Colony No.	Total Colony Area (sq.mm)
Controls - no precoating				
5% FCS	100	100	548 ± 27	170.0 ± 28.1
0.25% FCS	10	2	56 ± 23	4.0 ± 2.1
0.25% FCS + TIES	26	8	144 ± 29	14.0 ± 5.3
Vitrogen - 37 °C				
3 mg/ml	37	18	203 ± 20	30.0 ± 3.6
1 mg/ml	29	9	159 ± 66	15.5 ± 8.5
100 µg/ml	14	4	75 ± 26	6.7 ± 0.7
Vitrogen - Room Temp.				
3 mg/ml	16	6	89 ± 8.5	9.6 ± 2.6
1 mg/ml	17	5	94 ± 6	8.4 ± 0.7
100 µg/ml	36	16	195 ± 58	27.1 ± 4.4
Vitrogen 4°C				
3 mg/ml	14	7	78 ± 14	11.6 ± 2.2
1 mg/ml	21	7	113 ± 29	12.2 ± 1.9
100 µg/ml	12	8	66 ± 6	13.6 ± 1.1

Cells are grow ing in 0.25% FCS + TIES in dishes precoated with Vitrogen.

* Growth expressed as a percentage of growth in 5% FCS

3.3.8.3 DEAE Dextran

DEAE dextran is a basic polymer, of approximately 500,000 molecular weight. Initial precoating at 1 mg/ml and 100 μg/ml showed some stimulation at the latter concentration, but total inhibition of growth at the higher concentration (Table 3.36). Further work was carried out at 100 μg/ml, using an FCS background level of 0.5% FCS. Results from precoating at room temperature or 37°C (Table 3.36) show high levels of growth in both instances - the colony area was greater than that in 5% FCS while the colony number was also significantly increased.

Further investigations with DEAE dextran precoating were carried out, with the level of FCS being reduced to 0.25% FCS and also removed completely. Table 3.37 shows that at an FCS level of 0.25% FCS, precoating at room temperature resulted in over a three-fold increase in CFE and a ten-fold increase in colony area. Precoating at 37°C, while less dramatic also led to significant increases in cell growth, as measured by both of these parameters.

Growth in TIES - supplemented medium in the absence of FCS was also achieved when dishes were precoated with DEAE dextran, for 4 or 16 hours. Although growth levels were low compared to 5% FCS, no growth at all is recorded in uncoated dishes in the absence of FCS.

TABLE 3.36 Growth in dishes precoated with DEAE dextran for 16 hours

	% Colony No.*	% Colony Area*	Colony No.	Total Colony Area (sq. mm)
Controls - no precoating				
5% FCS	100	100	755 ± 74	119.8 ± 12.1
0.5% FCS	23	9	176 ± 18	11.0 ± 2.7
0.5% + TIES	53	25	398 ± 74	30.0 ± 9.7
Precoating (100μg/ml)				
37°C	100	184	760 ± 19+	220.0 ± 21.6+
Room temperature	78	103	588 ± 54+	124.0 ± 17.5+
Precoating (1mg/ml)				
37°C	0	0	0	0
Room temperature	0	0	0	0

+ Growth stimulation significant at $p<0.05$. Cells growing in 0.5% FCS + TIES.

* Growth expressed as a percentage of growth in 5% FCS.

TABLE 3.37 Growth of RPMI 2650 in dishes precoated with DEAE dextran

	% Colony No.*	% Colony Area*	Colony No.	Total Colony Area (sq. mm)
Controls - No precoating				
5% FCS	100	100	1325 ± 114	273.5 ± 28.9
0.25% FCS	7	1	94 ± 23	2.8 ± 0.8
0.25% FCS + TIES	22	6	289 ± 109	16.1 ± 7.0
- FCS + TIES	0	0	0	0
Cells in 0.25% FCS + TIES:				
37°C, 16 hours	44	23	580 ± 105	63.2 ± 20.6
37°C, 4 hours	33	15	443 ± 50	41.9 ± 5.0
Room Temp. 16 hours	67	64	891 ± 82	173.9 ± 40.5
Room Temp. 4 hours	69	72	914 ± 93	196.9 ± 35.5
Cells in - FCS + TIES:				
37°C, 16 hours	6	2	83 ± 10	4.9 ± 1.0
37°C, 4 hours	23	4	305 ± 48	16.1 ± 3.0
Room Temp. 16 hours	11	2.2	146 ± 29	10.7 ± 2.4
Room Temp. 4 hours	12	1.4	161 ± 18	11.7 ± 1.4

Precoating conditions varied - details in table. After precoating, cells were set up either in medium + 0.25% FCS + TIES or in medium unsupplemented with TIES only (no FCS).

* Growth expressed as a percentage of growth in 5% FCS.

3.3.8.4 Dextran Sulphate, Gelatin and other polymers

Dishes were precoated with dextran sulphate at concentrations of 1 mg/ml and 100 μg/ml. At the former concentration, almost total inhibition of growth occurs after precoating at room temperature and 37°C. At the lower concentration, some attachment occurs but growth is considerably less than in controls (Table 3.38). These results are from cells set up in 0.25% FCS + TIES. Dishes precoated with 100 μg/ml dextran sulphate and in which cells were set up in - FCS + TIES showed absolutely no growth (data not presented).

Gelatin is another factor used widely for precoating dishes. At a concentration of 1 mg/ml, there was no significant inhibition or stimulation of growth when precoating was carried out at 4°C, room temperature or 37°C for 4 or 16 hours (Table 3.39). These results are confirmed by those presented in Table 3.40, where gelatin precoating is for 16 hours at room temperature and 37°C only.

Table 3.40 shows the effect of precoating dishes with a variety of basic polymers at room temperature and 37°C for 16 hours. Methyl cellulose precoating makes no major impact on cell growth. Polyvinylpyrolidone (PVP) and bovine serum albumin (BSA) have an inhibitory effect on cell growth; precoating at 37°C results in no growth at all with either compound, while precoating at room temperature supports a low level of growth, but both colony number and colony area are significantly smaller than the controls.

Poly-D-lysine is one of the most effective precoating polymers in widespread use. In the absence of poly-D-lysine, precoating with poly-L-lysine and L-lysine were carried out. Both result in marked reduction in growth (Tables 3.40 and 3.41). At two different concentrations, (20μg/ml and 200μg/ml) and both at room temperature and 37°C, poly-L-lysine precoating leads to a significant decrease in both colony number and colony area. L-lysine similarily inhibits growth at room temperature and 37°C.

3.3.8.5 Fibronectin precoating

Fibronectin precoating has proved to be very successful in promoting growth of some cell types in the absence of serum. In the present work Fibronectin (Fn) was tested at a concentration of 10 µg/ml. Precoating with Fn alone and with a Fn:Collagen (100µg/ml) (1:1) mixture were both examined for their contribution to growth of RPMI 2650 in the presence of both 0.25% FCS + TIES and 0.5% FCS + TIES. Table 3.42 shows that Fn on its own has no significant effect but a combination of Fn and Collagen does enhance growth at both FCS levels. It is not clear what contribution Fn makes here, as Collagen, at twice the concentration present here, has been shown to increase both the CFE and colony area considerably (see Sec. 3.3.8.1.). Fn was also tested as a medium supplement (see Sec.3.3.7.8.)..

3.3.8.6 FCS precoating

Precoating of dishes with FCS is a common treatment employed to allow cells to grow in a serum-free environment while still supplying some of the attachment factors supplied by the FCS. It is thought that some of these attachment factors bind to the dishes and are not removed through the PBS washes. Thus, binding sites are available to the cells when they are added to the dishes in serum-free media.

Dishes were precoated with undiluted FCS for 4 hours at 37°C and room temperature. After washing the precoated dishes, cells were added in medium supplemented with 0.25% FCS + TIES. Precoated controls were fed with unsupplemented medium (-FCS-TIES) to investigate the growth stimulatory effects of any residual FCS in the dishes.

Table 3.43 shows that precoating with FCS greatly enhances growth, both in terms of CFE and colony area, at 37°C and room temperature. Dishes were also precoated with FCS and subsequently fed with unsupplemented medium (-FCS-TIES). This was to investigate the effect of any residual FCS which remained in the dishes after the normal washing procedure had taken place. In these dishes, relatively high levels of growth were recorded. This suggests that residual FCS contributes significantly to the growth recorded in dishes supplemented with 0.25% FCS + TIES. Thus the level of FCS in these dishes is

undefined and if precoating with FCS is to be used to allow growth in serum-free conditions, even a very strict washing regime may not be sufficient to remove all residual FCS.

TABLE 3.38 Precoating with Dextran Sulphate

	% Colony No.*	% Colony Area*	Colony No.	Total Colony Area (Sq. mm)
Controls - no precoating				
5% FCS	100	100	905 ± 34	303.0 ± 24.9
0.25% FCS	17	2	154 ± 18	5.3 ± 0.2
0.25% FCS + TIES	41	6	371 ± 18	18.2 ± 0.3
- FCS + TIES	0	0	0	0
- FCS - TIES	0	0	0	0
Precoating, 1mg/ml				
Room Temp	4	2.5	36 ± 11	7.76 ± 2.4
37°C	0	0	0	0
Controls - no precoating				
5% FCS	100	100	1325 ± 114	273.5 ± 28.9
0.25% FCS	7	1	94 ± 23	2.8 ± 0.8
0.25% FCS + TIES	22	6	289 ± 109	16.1 ± 7.0
Precoating 100 μg/ml				
37°C, 16 hours	4	0.6	56 ± 15	1.7 ± 0.7
Room Temp. 16 hours	19	3	256 ± 88	9.0 ± 1.5
37°C, 4 hours	0	0	0	0
Room Temp. 4 hours	9	2.5	123 ± 46	6.8 ± 3.2

Cells are growing in 0.25% FCS + TIES.

*Growth expressed as a percentage of growth in 5% FCS

TABLE 3.39 Growth of RPMI 2650 in dishes precoated with gelatin.

	% Colony No.*	% Colony Area *	Colony No.	Total Colony Area (sq. mm)
Controls - no precoating				
5% FCS	100	100	922 ± 50	288.0 ± 24.7
0.5% FCS	82	54	754 ± 12	156.0 ± 7.9
0.5% FCS + TIES	92	84	850 ± 33	242.0 ± 22.6
Precoating conditions				
16 hours Room Temp.	83	93	766 ± 56	268.0 ± 26.0
16 hours 37°C	97	63	893 ± 109	181.0 ± 24.0
16 hours 4°C	81	36	745 ± 39	102.3 ± 12.2
4 hours Room Temp.	77	106	710 ± 39	304.4 ± 31.7
4 hours 37°C	97	60	898 ± 21	172.0 ± 11.0
4 hours 4°C	98	49	899 ± 76	140.4 ± 23.1

Cells are growing in 0.5% FCS + TIES in dishes precoated with gelatin.

*Growth expressed as a percentage of growth in 5% FCS.

TABLE 3.40 Precoating with a range of polymers

	% Colony No.*	% Colony Area *	Colony No.	Total Colony Area (sq. mm)
Controls - no precoating				
5% FCS	100	100	755 ± 74	119.8 ± 12.1
0.5% FCS	23	9	176 ± 18	11.0 ± 2.7
0.5% FCS + TIES	53	25	398 ± 74	30.0 ± 9.71
Precoating at Room Temp.				
BSA 1mg/ml	14	5	109 ± 6	6.1 ± 0.6
Methyl Cellulose 1mg/ml	47	25	357 ± 41	30.8 ± 2.1
Gelatin 1mg/ml	17	90	134 ± 34	11.5 ± 0.5
PVP 1mg/ml	10	4	72 ± 8.6	4.7 ± 1.1
L-Lysine 1mg/ml	38	16	285 ± 86	18.3 ± 1.9
Precoating at 37°C				
BSA 1mg/ml	0	0	0	0
Methyl Cellulose 1 mg/ml	36	11	276 ± 1	13.3 ± 0.4
Gelatin 1mg/ml	44	20	330 ± 77	24.0 ± 5.9
PVP 1mg/ml	0	0	0	0
L-Lysine 1mg/ml	0	0	0	0

PVP = Polyvinylpyrrolidone

Cells are growing in 0.5% FCS + TIES in all precoated dishes

* Growth expressed as a percentage of growth in 5% FCS

TABLE 3.41 Growth of RPMI in poly-L-lysine-coated dishes

	% Colony No.*	% Colony Area*	Colony No.	Total Colony Area (sq. mm)
Controls - no precoating				
5% FCS	100	100	799 ± 10	172.0 ± 4.6
0.5% FCS	81	27	653 ± 10	45.7 ± 1.7
0.5% FCS + TIES	89	37	712 ± 54	63.2 ± 7.9
Precoating				
16 hours Room Temp.				
20 µg/ml	11	3	88 ± 21	4.3 ± 0.9
100 µg/ml	39	7	310 ± 48	11.65 ± 4
16 hours 37°C				
20 µg/ml	27	7	218 ± 29	12.0 ± 2.2
100 µg/ml	28	8	226 ± 40	13.6 ± 3.3

Cells growing in 0.5% FCS + TIES in dishes precoated with poly-L-lysine

*Growth expressed as a percentage of growth in 5% FCS

TABLE 3.42 Precoating with Fibronectin

	% Colony No*	% Colony Area*	Colony No.	Total Colony Area (sq. mm)
5% FCS	100	100	736 ± 13	184.3 ± 7.7
0.5% FCS	33	7	242 ± 3.5	12.3 ± 0.8
0.25% FCS	35	12	261 ± 39	22.3 ± 5.2
0.5% + TIES	72	23	529 ± 52	42.9 ± 5.0
0.25% + TIES	54	17	398 ± 16	32.2 ± 3.2
Fn -> 0.25% + TIES	58	15	426 ± 57	28.2 ± 6.5
Fn -> 0.5% + TIES	69	27	509 ± 52	48.9 ± 5.8
Fn: Coll ->0.25% + TIES	65	23	476 ± 28	41.65 ± 1.7
Fn: Coll ->0.5% + TIES	82	28	607 ± 16	50.7 ± 4.3

*Growth expressed as a percentage of growth in 5% FCS.

TABLE 3.43 Growth of RPMI 2650 in dishes precoated with FCS

	% Colony No.*	% Colony Area*	Colony No.	Total Colony Area (sq. mm)
5% FCS	100	100	905 ± 34	303 ± 24.9
0.25% FCS	1	2	153 ± 22	5.1 ± 0.32
0.25% + TIES	41	6	373 ± 20	19.5 ± 2.4
- FCS + TIES	0	0	0	0
- FCS - TIES	0	0	0	0
Precoating with FCS, 37°C				
0.25% FCS + TIES	96	42	868 ± 90	127.7 ± 11.3
- FCS - TIES	39	10	353 ± 25	29.9 ± 4.5
Precoating with FCS, Room Temp.				
0.25% FCS + TIES	104	45	942 ± 89	135.0 ± 31.9
- FCS - TIES	47	9	425 ± 42	27.9 ± 3.3

*Growth expressed as a percentage of growth in 5% FCS

3.3.9. Testing stimulation by TIES over a range of cell concentrations

Cells are normally set up at a concentration of $1x10^4$ cells/35mm dish and clonal growth is measured after 7 days. Investigations into growth at lower cell concentrations show that stimulation due to TIES is visible at any concentration at which cells form colonies. These assays were counted after 12 days. Over a range of concentrations from $1x10^4$ cells/dish to $5x10^2$ cells/dish, greater growth is recorded in medium supplemented with 0.5% FCS + TIES than with 0.5% FCS alone (Table 3.44).

At $1x10^3$ cells/dish and $5x10^2$ cells/dish almost equal levels of growth are observed in 0.5% FCS + TIES, where colony number is approx. 60% of the 5% FCS control and colony area is slightly over one-third that in the 5% FCS control. At higher cell concentrations, the relative growth in 0.5% FCS + TIES is greater than at the lower concentrations, but this is due to the fact that, after 12 days the colonies have grown so big that a monolayer has almost formed. Thus, growth at $1x10^4$ cells/dish is being limited by the dish size, in 5% FCS and 0.5% FCS + TIES. At $1x10^2$ cells/dish no colonies formed at all.

TABLE 3.44 Varying concentration of cells

	% Colony No.*	% Colony Area*	Colony No.	Total Colony Area (sq. mm)
$1x10^4$ cells/dish				
5% FCS	100	100	983 ± 59	234.0 ± 53
0.5% FCS	93	28	919 ± 10	65.0 ± 2.7
0.5% FCS + TIES	104	97	1627 ± 38.7	227.2 ± 32
$5x10^3$ cells/dish				
5% FCS	100	100	755 ± 31	277.4 ± 7.3
0.5% FCS	36	9	275 ± 38	24.7 ± 2.5
0.5% FCS + TIES	90	64	678 ± 14	178.5 ± 1.5
$1x10^3$ cells/dish				
5% FCS	100	100	324 ± 17	99.6 ± 8.5
0.5% FCS	9	2	30 ± 6	2.0 ± 0.5
0.5% FCS + TIES	62	35	200 ± 10	35.3 ± 5.5
$5x10^2$ cells/dish				
5% FCS	100	100	182 ± 18	46.0 ± 8.5
0.5% FCS	8	2.5	15 ± 4	1.2 ± .1
0.5% FCS + TIES	60	39	109 ± 15	17.8 ± 2.1
$1x10^2$ cells/dish				
5% FCS)				
0.5% FCS) No countable colonies				
0.5% FCS + TIES)				

This assay was left for 12 days (instead of 7) to allow colonies grow large enough to be counted.

*Growth expressed as a percentage of 5% FCS

3.3.10 Growth of RPMI 2650 in reduced serum in flasks

Many attempts were made to grow RPMI 2650 in 25 cm^2 tissue culture flasks in medium with a low FCS level, supplemented with TIES. The surface area (25 cm^2) is approximately three times that in the 35mm dishes. Clonal growth occurred consistently at $1x10^4$ cells/dish in the 35 mm dishes, hence cells were set up initially at $3x10^4$ cells/25 cm^2 flask. But growth in 5% FCS was very poor at this concentration.

Therefore, growth at higher concentrations was examined. Cells were set up at $5x10^5$ cells/flask in medium supplemented with 0.5% FCS + TIES. No growth at all was recorded on two occasions at this concentration in either the 0.5% FCS control or the medium supplemented with 0.5% FCS + TIES. When the cell concentration was increased to $1x10^6$ cells/flask, growth in 0.5% FCS alone was very extensive - a complete monolayer formed and on trypsinization, relatively little change in cell number was visible in 0.5% FCS + TIES (see Table 3.45), part (3)).

With 0.25% FCS concentration and cells seeded at $1x10^6$ cells/flask, no growth occurred in 0.25% FCS or 0.25% FCS + TIES. The 5% FCS control grew normally. On any occasion when no growth occurred in reduced serum conditions, the medium appeared very basic. Hence, it was hypothesized that there might be a pH/buffering problem in the 25 cm^2 flasks. Ham's F12 does not contain any Hepes buffer, so the buffering capacity of the DME:F12 mixture is less than that of MEM, the basal medium in which RPMI 2650 cells are usually grown.

Cells were then set up at $1x10^6$ cells/ml, with 0.25% FCS background, in 25 cm^2 flasks and the flasks were partially sealed and placed in a 5% CO_2 atmosphere. This allowed gaseous exchange with the external (5% CO_2) atmosphere. After 6 days colonies were stained and counted (Table 3.45, part (3)). A significant difference ($p<0.05$) in growth was observed between the 0.25% FCS controls and 0.25% FCS + TIES. There was a doubling in colony number but a four-fold increase in total colony area when TIES was added to the medium. The medium changed to a slightly acidic colour, which is to be expected where growth is occurring.

This experiment was repeated, at a concentration of $5x10^5$ cells/flask, with a 0.25% FCS level. Some flasks were sealed totally while replicates were partially sealed. Growth occurred in the 5% FCS controls in sealed and unsealed flasks in a 5% CO_2 incubator. No growth occurred in any flasks in medium supplemented with 0.25% FCS. In partially-sealed flasks supplemented with 0.25% FCS + TIES many colonies formed (Table 3.45, part (6)), but no growth at all occurred in sealed flasks supplemented with 0.25% FCS + TIES.

Thus, the need for interaction with the external CO_2 - rich atmosphere for growth of RPMI 2650 cells in flasks with 0.25% FCS + TIES was confirmed. It appears that the buffering capacity of the DME:F12 medium is insufficient to support growth of the cells in reduced serum concentrations, even at high density, in 25 cm^2 flasks which are totally sealed. Further work is required at low cell densities to investigate growth patterns in flasks.

TABLE 3.45 Growth of RPMI 2650 cells in reduced serum in $25cm^2$ flasks

(1)	Initial cell concentration:	$3x10^4$ cells/flask - sealed flasks	
	5% FCS	Very few colonies after 7 days - none greater than 8 cells	
	0.5% FCS	No growth	
	0.5% FCS + TIES	No growth	
(2)	Initial cell concentration:	$5x10^5$ cells/flask - sealed flasks	
	5% FCS	$2.4x10^6$ cells/flask	
	0.5% FCS	No growth	
	0.5% FCS + TIES	No growth	
(3)	Initial cell concentration:	$1x10^6$ cells/flask - sealed flasks	
	5% FCS	$3.4x10^6$ cells/flask	
	0.5% FCS	$1.5x10^6$ cells/flask	
	0.5 FCS + TIES	$1.65x10^6$ cells/flask	
(4)	Initial cell concentration:	$1x10^6$ cells/flask - sealed flasks	
	5% FCS	$3.0x10^6$ cells/flask	
	0.25% FCS	No growth	
	0.25% FCS + TIES	No growth	
(5)	Initial cell concentration:	$1x10^6$ cells/flask - partially-sealed flasks	
		Colony No.	Total Colony Area
	5% FCS	1749 ± 394	408.9 ± 107
	0.25% FCS	889 ± 258	46.2 ± 11.3
	0.25% FCS + TIES	1895 ± 105	156.5 ± 29.5
(6)	Initial cell concentration:	$5x10^5$ cells/flask	
	Sealed flasks	Colony No.	Total Colony Area
	5% FCS	1045 ± 94	267.0 ± 52.1
	0.25% FCS	0	0
	0.25% FCS + TIES	0	0
	Partially-sealed flasks		
	5% FCS	1175 ± 54	212.8 ± 8.7
	0.25% FCS	0	0
	0.25% FCS + TIES	238 ± 20	24.8 ± 1.8

Cells were trypsinized or colonies stained after 6-7 days

3.3.11 Optimization of media components for growth of RPMI 2650 in reduced serum conditions

Two final experiments were set up to find the optimum conditions for growth of RPMI 2650 cells in reduced serum concentration. Apart from transferrin, insulin, EGF and selenium, two other media supplements consistently stimulated growth of RPMI 2650 - i.e. MEM non-essential amino acids (NEAA) and ascorbic acid (see Tables 3.24 and 3.28, Sec. 3.3.7.). These components were tested as individual medium supplements in the presence of 0.25% FCS and in conjunction with TIES and each other. DEAE dextran had proven to be a very suitable precoating material, hence it was included in the final optimization experiment and the action of NEAA and ascorbic acid examined in conjunction with DEAE dextran precoating.

Tables 3.46 and 3.47 show the growth recorded under these conditions. NEAA and ascorbic acid (AA) both stimulated growth when added to 0.25% FCS or to 0.25% FCS + TIES. Ascorbic acid worked equally well at both concentrations tested (1 μg/ml and 10 μg/ml), with no significant difference in growth at the two concentrations. A significant increase ($p<0.05$) was observed in both the colony number and total colony area when it was added to 0.25% FCS and to 0.25% FCS + TIES. NEAA stimulated to a greater extent when added to 0.25% FCS alone, and showed almost equal stimulatory ability to ascorbic acid when added to 0.25% FCS + TIES.

When both NEAA and ascorbic acid (10 μg/ml) were added simultaneously to 0.25% + TIES, a significant increase in both colony number and colony area were recorded, but the stimulation is not additive. When both are added to 0.5% FCS + TIES, an increase in cell growth is also recorded.

Precoating dishes with DEAE dextran again yielded very good growth of RPMI 2650 (see also Section 3.3.8.). Growth in the absence of any FCS background was very significant, in medium supplemented with TIES NEAA AA. The colony number is 74% of the 5% FCS control, while the total colony area is 66%. The growth observed here is considerably greater than that observed previously in the absence of FCS with DEAE dextran precoating (Table 3.37, Section 3.3.8.). In this instance NEAA and ascorbic acid are additional medium supplements

(only TIES were present in previous work). Thus, it seems that NEAA and ascorbic acid play a very important role in the absence of FCS.

In the presence of 0.25% FCS and 0.5% FCS, DEAE dextran precoating enhances growth significantly. In medium supplemented with 0.25% FCS + TIES, a doubling in colony number occurs but a very large increase in total colony area occurs. The total colony area recorded is a twenty-fold increase over the uncoated control. It is also significantly greater than the total colony area in 5% FCS, but microscopic observations show considerable piling up of cells in colonies in 5% FCS compared to the cells in colonies in DEAE-dextran precoated dishes where cells within a colony are spread out to a greater extent over the surface.

Somewhat conflicting results were recorded in medium supplemented with NEAA and ascorbic acid. In 0.5% FCS, the addition of these two components to TIES causes an increase in colony number and total colony area and DEAE-dextran precoated dishes show a very significant increase ($p<0.01$) in total colony area over the uncoated control.

In uncoated dishes, growth in 0.25% FCS + TIES NEAA AA was less than that in 0.25% FCS + TIES (Table 3.47). This must be due to experimental error as there is sufficient other data (growth with a 0.5% FCS background in this experiment, growth with both 0.5% FCS and 0.25% FCS backgrounds in Table 3.46 and results presented in Table 3.28) which shows NEAA and ascorbic acid stimulating RPMI 2650 growth.

The results obtained in precoated dishes in the presence of NEAA and ascorbic acid also exhibit an unusual characteristic. Under both precoating conditions, the addition of NEAA and ascorbic acid to 0.25% FCS + TIES causes an increase in the colony number, but a decrease in the colony area, compared to precoated dishes with medium supplemented with 0.25% FCS + TIES. This decrease in colony area could be due to a decrease in the number of cells per colony or a decrease in cell spreading.

When DEAE dextran was added as a medium component, at a concentration of 1 μg/ml, a significant increase in both colony number and total colony area was recorded. Growth recorded was comparable to that in DEAE dextran precoated dishes.

TABLE 3.46 Optimization of RPMI 2650 medium with reduced FCS concentration

	% Colony No.*	% Colony Area*	Colony No.	Total Colony Area (sq. mm)
5% FCS	100	100	685 ± 72	136.1 ± 12.2
0.5% FCS	21	12	141 ± 43	16.6 ± 6.5
0.5% FCS + TIES	89	35	611 ± 36	47.1 ± 4.4
0.5% FCS + TIES NEAA AA	93	38	640 ± 10	51.3 ± 4.1
0.25% FCS	18	3	125 ± 64	4.5 ± 2.4
0.25% FCS + NEAA	45	11	309 ± 4	15.5 ± 0.1
0.25% FCS + AA (1 μg/ml)	28	5	192 ± 3	7.1 ± 0.3
0.25% FCS + AA (10 μg/ml)	31	7	210 ± 5	9.2 ± 0.4
0.25% FCS + TES	39	10	270 ± 41	13.7 ± 3.7
0.25% FCS + TIES	59	19	406 ± 5	26.5 ± 0.8
0.25% FCS + TIES NEAA	72	26	496 ± 23	36.0 ± 3.0
0.25% FCS + TIES AA (1μg/ml)	71	27	488 ± 62	37.0 ± 3.0
0.25% FCS + TIES AA (10μg/ml)	72	28	493 ± 25	38.7 ± 4.3
0.25% FCS + TIES NEAA AA (10μg/ml)	68	26	469 ± 21	35.9 ± 3.6

*Growth expressed as a percentage of growth in 5% FCS.

TABLE 3.47 Optimization of growth conditions for growth of RPMI 2650 in reduced serum concentrations - DEAE dextran precoating

Uncoated Controls	% Colony No.*	% Colony Area*	Colony No.	Total Colony Area (sq. mm)
5% FCS	100	100	639 ± 59	83.6 ± 13.5
0.5% FCS	44	23	279 ± 43	19.4 ± 3.6
0.5% FCS + TIES	87	53	553 ± 70	44.3 ± 10.4
0.5% FCS + TIES NEAA AA	92	57	588 ± 28	47.6 ± 4.0
0.25% FCS	11	4	68 ± 20	3.6 ± 1.2
0.25% FCS + TIES	41	17	262 ± 29	14.6 ± 2.3
0.25% FCS + TIES NEAA AA	18	6	116 ± 20	4.6 ± 1.6
0.25% FCS + TIES NEAA AA + DEAE dextran (1 µg/ml)	91	151	580 ± 4	126.5 ± 4.9
- FCS	0	0	0	0
- FCS + TIES	0	0	0	0
DEAE DEXTRAN PRECOATING				
37°C - 4 hrs				
- FCS + TIES NEAA AA	74	66	474 ± 36	55.3 ± 3.5
0.25% FCS + TIES	90	311	577 ± 66	260.0 ± 21.6
0.25% FCS + TIES NEAA AA	100	139	639 ± 90	116.4 ± 29.9
0.5% FCS + TIES NEAA AA	94	270	598 ± 31	226.0 ± 4.0
Room Temp. - 4 hrs				
- FCS + TIES NEAA AA	66	43	421 ± 30	35.8 ± 10.4
0.25% FCS + TIES	95	241	605 ± 48	201.7 ± 19
0.25% FCS + TIES NEAA AA	102	190	653 ± 27	158.6 ± 29.0
0.5% FCS + TIES NEAA AA	90	270	575 ± 38	226.2 ± 18.6

Precoating with DEAE dextran (100 µg/ml) - details given in key.

Ascorbic acid (AA) concentration - 10 µg/ml. DEAE dextran (1 µg/ml) added as a medium component (no precoating) in one instance.

*Growth expressed as a percentage of growth in 5% FCS.

3.3.12 Growth of cell lines in TIES-supplemented medium

Experiments were set up to detect the effect of TIES, with a low FCS concentration on a number of different cell lines.

A431 cells

A431, a human epidermoid carcinoma cell line, was grown at two clonal concentrations in DME:F12 + TIES with both 0.25% FCS and 0.5% FCS. Growth occurred in the reduced FCS concentrations but there was no growth stimulation due to the presence of TIES. At the higher cell concentration, No differences were evident (Table 3.48).

A549 Cells

A549 cells, a human lung carcinoma cell line, was set up at two concentrations, $1x10^4$ cells/dish and 5×10^4 cells/dish. This cell line forms very diffuse colonies and using the image analyser, while the measurement of total colony area was very accurate, that of colony number was not an accurate reflection of growth. But interpretation of the values for total colony area gave a very clear picture (see Table 3.49). Some growth occurs in reduced FCS concentrations, but there is a total inhibition of growth in the presence of TIES. This was found at both concentrations. The results for $5x10^4$ cells/dish are not presented as a complete monolayer had formed, even in 0.25% FCS and hence, counting on the image analyser yielded no real results. But, in spite of a very high level of growth in 0.25% FCS, no growth at all was recorded in the presence of TIES.

HEP-2 Cells

HEP-2 cells, a human epidermoid cell line, was set up at a clonal concentration of $5x10^3$ cells/dish. Growth occurred in DME:F12 + 0.25% FCS, but when TIES was added to this, growth inhibition occurred. Some colonies did form but there was a significant decrease in both colony number and colony area. (Table 3.50).

TABLE 3.48 Growth of A431 cells in TIES

	% Colony No.*	% Colony Area*	Colony No.	Total Colony Area (sq. mm)
$2x10^3$ cells/dish				
5% FCS	100	100	428 ± 6.2	110.6 ± 17.3
0.25% FCS	48	24	204 ± 30	26.6 ± 5.4
0.25% FCS + TIES	57	19	245 ± 37	21.1 ± 6.9
0.5% FCS	64	38	275 ± 23	41.7 ± 0.7
0.5% FCS + TIES	81	34	345 ± 37	38.1 ± 6.9
$5x10^3$ cells/dish				
5% FCS	100	100	615 ± 32	276.8 ± 41.0
0.25% FCS	93	34	570 ± 83	94.2 ± 16.5
0.25% FCS + TIES	103	32	635 ± 10	87.3 ± 10.0
0.5% FCS	98	65	602 ± 13	179.3 ± 18.4
0.5% FCS + TIES	102	39	630 ± 39	108.9 ± 7.9

*Growth expressed as a percentage of growth in 5% FCS

TABLE 3.49 Growth of A549 cells in TIES

	% Colony No.*	% Colony Area*	Colony No.	Total Colony Area (sq. mm)
$1x10^4$ cells/dish				
5% FCS	-	100	-	140.0 ± 10.0
0.25% FCS	-	7	-	10.5 ± 1.2
0.25% FCS + TIES	-	0	-	0 ± 0
0.5% FCS	-	65	-	90.4 ± 9.4
0.5% FCS + TIES	-	0	-	0 ± 0

*Growth expressed as a percentage of growth in 5% FCS

TABLE 3.50 Growth of HEP-2 cells in TIES

	% Colony No.*	% Colony Area*	Colony No.	Total Colony Area (sq. mm)
5×10^3 cells/dish				
5% FCS	100	100	63 ± 0.5	5.52 ± 0.3
0.25% FCS	43	29	27 ± 7	1.6 ± 0.5
0.25% FCS + TIES	14	6	9 ± 4	0.32 ± 0.2
5% FCS	100	100	88 ± 7	19.7 ± 2.0
0.25% FCS	52	25	46 ± 5	5.0 ± 1.4
0.25% FCS + TIES	32	17	28 ± 4	3.4 ± 1.2

*Growth expressed as a percentage of growth in 5% FCS

3.3.13 Growth stimulatory activity of milk

There have been some reports in the literature on the growth-promoting effects of bovine milk on cells in culture (see Section 1.5). Investigations were carried out into growth of a number of cell lines with milk-supplemented medium.

3.3.13.1. 3T3

The majority of the analysis of the growth stimulating properties of milk was carried out using mouse 3T3 cells as an indicator cell line. Bovine milk was prepared by ultrafiltration (see Section 2.10) and sterilised as described (see Sec. 2.4). It was tested as a medium supplement (10% v/v concentration) in the presence of a low FCS background level and on its own.

Initial work was carried out at monolayer concentrations of 3T3 cells. 3T3 cells grow normally in $25cm^2$ flasks, in DME + 5% FCS, at concentrations of $5x10^5$ cells/flask to $1x10^6$ cells/flask. Numerous attempts were made to grow 3T3 cells in this concentration range in 0.25% FCS + 10% milk and in 0.5% FCS + 10% milk.

Table 3.51 gives a sample of the results obtained by trypsinizing flasks after 5-9 days. At a 0.5% FCS background level, a significant increase in growth due to milk was observed on occasions, but not consistently. At 0.25% FCS, stimulation due to milk was recorded on a more regular basis, but the extent of stimulation varied greatly. In experiment numbers 1, 4 and 6 there is approximately a four-fold, a ten-fold and a 19-fold increase in the presence of 10% milk + 0.25% FCS over the 0.25% FCS control. Growth in medium supplemented with milk only (no FCS) also varies considerably.

These results do suggest that some stimulatory factors are present in milk, but the exact extent of their action is not clear. Further work was carried out at clonal concentrations of 3T3. These assays were set up in 60mm dishes in 5% CO_2, at cell concentrations of 50, 100 and 250 cells/dish. Investigations were made into choosing a suitable system which would

consistently yield growth. The initial results looked promising (Table 3.52), with stimulation showing consistently due to the addition of milk at a FCS background level of 1% and 2%. Many different batches of milk were tested but there was considerable variation within batches and some variation between batches.

These results led to attempts to identify the stimulating factor. Ultrafiltration on an Amicon ultrafiltration cell (see Sec. 2.10) yielded filtrates and retentates which were tested for growth stimulatory ability. But no consistent results were obtained. In some cases, non ultra filtered milk (from a batch which had been ultrafiltered and had previously shown growth stimulatory ability), failed to stimulate growth and in other cases, there were discrepancies between results obtained from the different ultrafiltration fractions. Table 3.53 gives the results from a typical experiment. No growth occurs at 250 cells/dish in the 1% FCS + 10% Milk control but growth occurs in retentate containing components between 5,000 and 30,000 molecular weight, and in the filtrate after filtration through a 5,000 membrane.

Other treatments were carried out on milk to try to identify what, if any, growth stimulating factors were present. Dialysis, heat treatment, trypsin treatment, pH treatments and variation of storage conditions (details not given) all failed to yield a consistent answer.

Other milk products were tested on 3T3 cells. Baby food, dried powdered milk and colostrum were prepared as described (see Sections 3.12 and 3.13) and added into assays similar to those described above, at a 10% concentration. Baby food and dried milk failed to show any growth stimulatory ability, and while colostrum showed some slight stimulation, it could not be repeatedly identified.

TABLE 3.51 Growth of 3T3 cells in medium supplemented with milk

Exp. No.	Medium	Cells/Flask x 10^{6*}
1	5% FCS	4.67
	0.2% FCS	0.78
	0.2% FCS + 10% Milk	2.85
	- FCS + 10% Milk	1.65
2	5% FCS	3.21
	0.5% FCS	1.40
	0.5% FCS + 10% Milk	3.87
3	5% FCS	1.93
	0.5% FCS	1.30
	0.5% FCS + 10% Milk	1.40
4	5% FCS	1.93
	0.5% FCS	1.30
	0.5% FCS + 10% Milk	0.90
5	5% FCS	4.50
	0.2% FCS	.25
	0.2% FCS + 10% Milk	2.43
	- FCS + 10% Milk	0.90
6	5% FCS	2.90
	0.2% FCS	0.15
	0.2% FCS + 10% Milk	2.78
	- FCS + 10% Milk	2.64
7	5% FCS	1.87
	0.2% FCS	0.65
	0.2% FCS + 10% Milk	0.70

*Cells were set up at $5x10^5$ cells/flask and trypsinized after 5-9 days. These results are the average of duplicate flasks.

TABLE 3.5 2 Growth of 3T3 at clonal concentrations in milk-supplemented medium

	Exp. 1		Exp. 2		Exp 3	
	Col. No.	Col. Area mm^2	Col. No.	Total Col. Area mm^2	Col. No.	Total Col. Area mm^2
50 cells/dish						
5% FCS	26±2	15.0±1.4	63±5	58.0±7.1		
- FCS	0	0	0	0		
0.5% FCS	0	0	0	0		
0.5% FCS + 10% Milk	0	0	8±2*	30.0±1.0*		
1% FCS	8±0.5	1.8±0.2	10±1	2.5±0.1		
1% FCS + 10% Milk	31±2*	13.5±1.4*	70±3*	12.0±1.2*		
2% FCS	21±3	18.0±1.8	54±2	35.0±1.1		
2% FCS + 10% Milk	52±7*	33.0±4.0*	83±4*	54.0±2.7*		
100 cells/dish						
5% FCS	41±3	78.4±1.4			39±4.0	85.0±4.0
- FCS	0	0			0	0
0.5% FCS	5±1	1.2±0.1			0	0
0.5% FCS + 10% Milk	5±1	0.8±0.2			0	0
1% FCS	7±2	1.9±0.4			0.5±0.1	0.25±0.1
1% FCS + 10% Milk	8±1	1.7±1.3			6±1*	2.30±1.4*
2% FCS	24±4	13.3±1.9			0.5±0.5	1.0±0.5
2% FCS + 10% Milk	39±5	20.4±2.0			39±2*	50.5±4.2*
250 cells/dish						
5% FCS	44±1	62.0±14.4	92±4	221.0±4.2		
-FCS	0	0	0	0		
0.5% FCS	0	0	5±1	1.5±.5		
0.5% FCS + 10% Milk	0	0	5±1	1.4±.5		
1% FCS	1±.5	1.0±0.5	32±0.5	23.0±6		
1% FCS + 10% Milk	61±4*	45.5±0.5*	66±4*	38.5±0.5*		
2% FCS	41±12	50.1±3.8	68±10	120.0±10.0		
2% FCS + 10% Milk	91±10*	100±1.1*	91±7*	131.4± 8.4*		

* Indicates stimulation due to addition of milk

TABLE 3.53 Effect of ultrafiltrated milk on growth of 3T3

	Colonies/dish	Total colony area (mm^2)
250 cells/dish		
5% FCS	41 ± 3	204.0 ± 13.7
1% FCS	4 ± 1	30.0 ± 2.5
- FCS	0	0
1% FCS + 10% Milk	0	0
1% FCS + R_1	3 ± 1	15 ± 1.9
1% FCS + R_2	1 ± 1	1.5 ± 2.5
1% FCS + R_3	20 ± 3	21.4 ± 4.0
1% FCS + F_1	0	0
1% FCS + F_2	50 ± 2	56.4 ± 5.9
1% FCS + F_3	6 ± 2	1.3 ± 0.5
- FCS + 10% Milk	0	0

Key: Milk was ultrafiltered on both 30,000 and 5,000 membranes.

R_1 Retentate from 30,000

R_2 " " 5,000

R_3 " " 5,000 after ultrafiltering filtrate from 30,000 (F_1)

F_1 Filtrate from 30,000

F_2 " " 5,000

F_3 " " 5,000 after ultrafiltering filtrate from 30,000 (F_1)

3.3.13.2 MDCK

Some experiments were set up to investigate growth of MDCK cells in milk-supplemented medium. Assays were done both at monolayer-forming concentrations and at clonal concentrations in 35mm dishes. No stimulation due to the addition of milk was evident at higher concentrations (Table 3.54) but, as in 3T3, some stimulation was evident at clonal concentrations - but it was not consistently observed. Table 3.54 shows an increase in the colony number, at two cell concentrations, with a 0.2% FCS background. But not all assays showed growth stimulation due to milk.

3.3.13.3 RPMI 2650

Growth of RPMI 2650 was examined in milk-supplemented medium. Table 3.55 shows three sets of typical results recorded. In no instance, with 0.5% FCS or 0.25% FCS backgrounds, is growth stimulation recorded due to the addition of 10% milk. Thus on its own, milk does not appear to stimulate RPMI 2650 cells.

However, the effect of milk in conjunction with TIES and a low FCS background was observed. With a concentration of 0.5% FCS, no increase in colony number or colony area is recorded. But with a 0.25% FCS background, growth recorded in 0.25% FCS + TIES + 10% milk is greater than that in 0.25% FCS + TIES. This stimulation was recorded on a number of occasions which lends support to the idea that bovine milk does contain some growth stimulatory ability.

TABLE 3.54 Growth of MDCK in milk-supplemented medium

Initial cell conc:	7.5×10^3 cells/35mm dish
	Cells/dish x 10^4
5% FCS	11.0 ± 0.2
- FCS	0.1 ± 0.05
0.25% FCS	6.0 ± 0.5
0.25% FCS + 10% Milk	6.1 ± 0.4
10% Milk	0 0

These dishes were trypsinized and cell counts done on a haemocytometer.

Initial cell conc:	7.5×10^2	1.0×10^3
	Colonies/dish	Colonies/dish
5% FCS	115 ± 5	130 ± 10
- FCS	0	0
0.2% FCS	1 ± 0.5	10 ± 5
0.2% FCS + 10% Milk	29 ± 2	45 ± 9
- FCS + 10% Milk	3 ± 1	0 ± 0

These dishes were stained with Leishmann's and counted on the image analyser

TABLE 3.55 Growth of RPMI 2650 in milk-supplemented medium

	% Colony No.*	% Colony Area*	Colony No.	Total Colony Area (Sq. mm)
(1)				
5% FCS	100	100	1079 ± 33	332.0 ± 16.0
0.5% FCS	91	44	978 ± 40	146.9 ± 18.5
0.5% FCS + 10% Milk	88	41	950 ± 30	136.9 ± 25.5
0.5% FCS + TIES	98	50	1061 ± 10	164.6 ± 28.5
0.5% FCS + TIES + 10% Milk	99	56	1064 ± 43	187.2 ± 12
0.25% FCS	53	16	571 ± 53	53.8 ± 18.2
0.25% FCS + 10% Milk	44	14	471 ± 84	47.6 ± 15.4
0.25% FCS + TIES	71	21	766 ± 54	71.3 ± 7.2
0.25% FCS + TIES + 10% Milk	88	47	950 ± 34	154.9 ± 14.1
(2)				
5% FCS	100	100	613 ± 42	47.4 ± 5.5
0.5% FCS	8	5	50 ± 10	2.23 ± 0.65
0.5% FCS + TIES	48	32	296 ± 41	15.1 ± 2.4
0.5% FCS + 10% Milk	9	4	55 ± 22	2.0 ± 0.5
0.5% FCS + TIES + 10% Milk	44	35	268 ± 40	16.7 ± 3.2
0.25% FCS	2	0.6	14 ± 0.5	0.28 ± 0.02
0.25% FCS + TIES	17	10	104 ± 70	4.6 ± 3.3
0.25% FCS + 10% Milk	0	0	0	0
0.25% FCS + 10% Milk + TIES	31	20	193 ± 44	9.5 ± 0.4
(3)				
5% FCS	100	100	1015 ± 64	108.9 ± 19.4
0.5% FCS	33	15	336 ± 61	16.0 ± 5.3
0.5% + TIES	46	21	469 ± 37	23.3 ± 2.3
0.5% + TIES + 10% Milk	40	19	410 ± 38	21.0 ± 3.0

*Growth expressed as a percentage of growth in 5% FCS

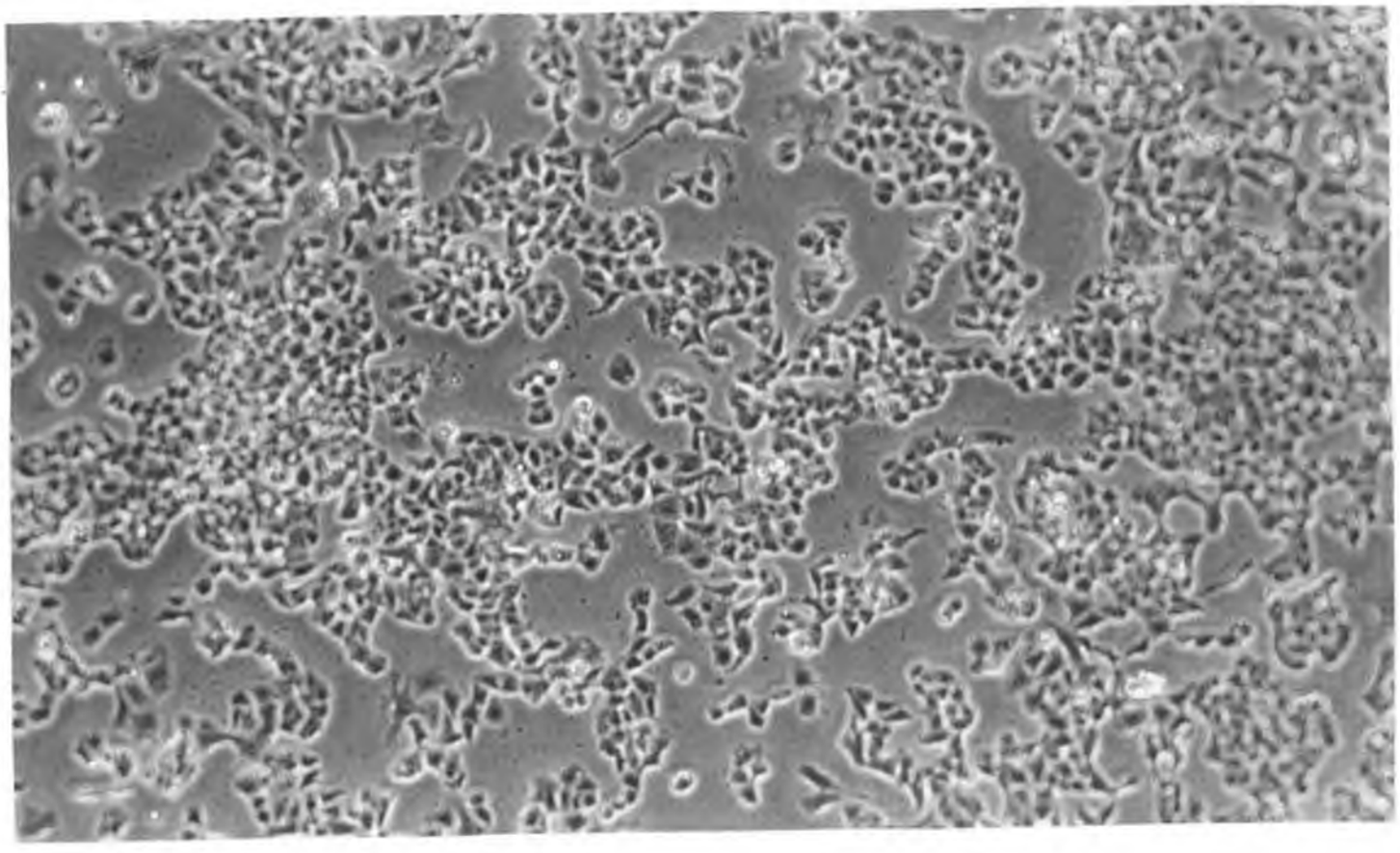


Fig. 3.3. RPMI 2650 cells growing in medium supplemented with 5% FCS. Subconfluent monolayer. Notice variety in shape and size of colonies.

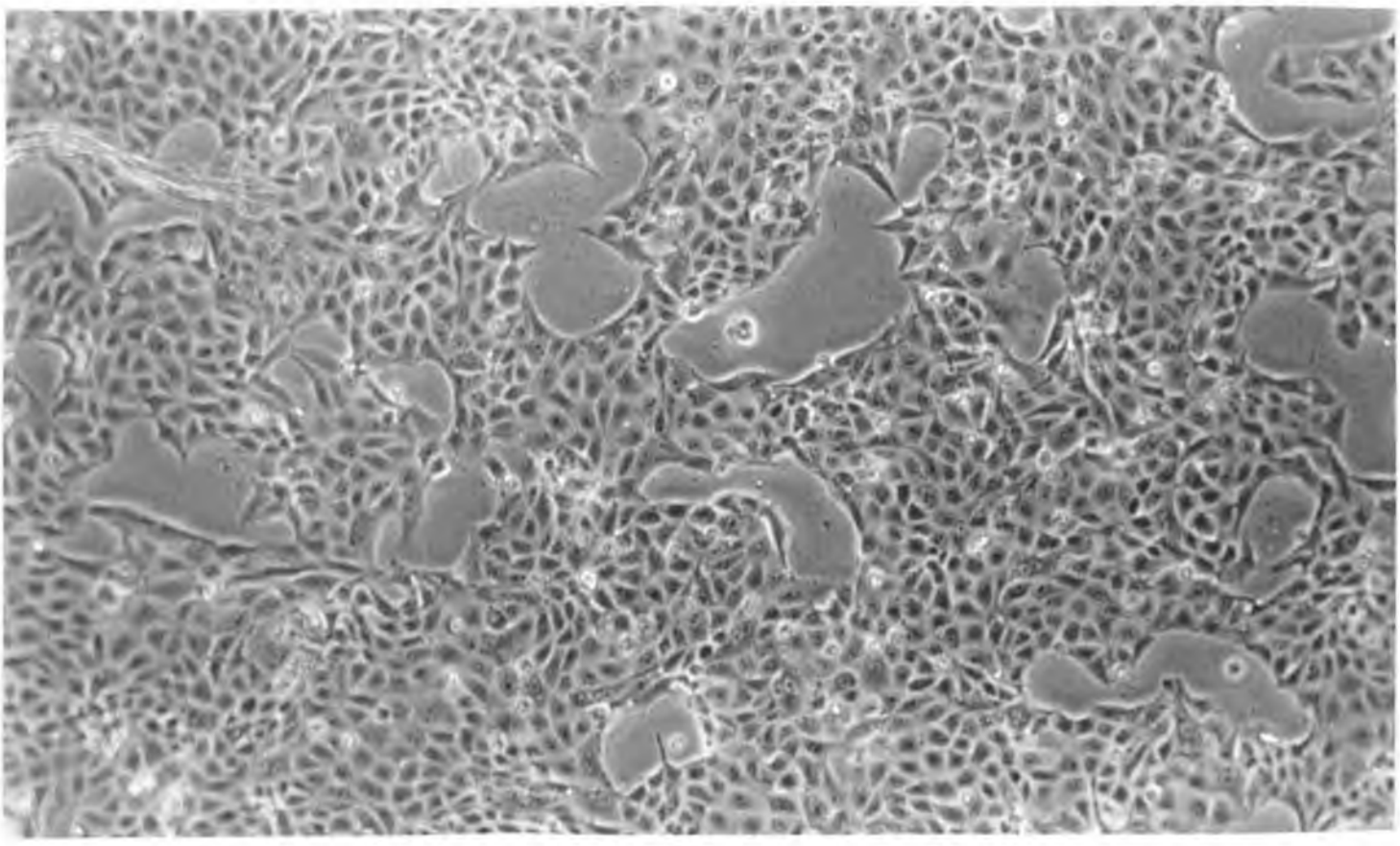


Fig. 3.4. Monolayer growth of MDCK (canine kidney) cells in medium supplemented with 5% FCS. Notice epithelial-like appearance of cells.

4. DISCUSSION

4.1

Growth of virtually all types of cells in culture requires the presence of serum. Despite numerous attempts in many laboratories, efforts to find a universal replacement for serum in the culture medium have met with limited success. The complex, but undefined, nature of serum appears to supply the cells with a wide range of nutritional and growth requirements, all of which must be supplied in the absence of serum. In addition, serum has the ability to supply the varying requirements for a wide range of cell types. The advantages of working with a serum-free system have already been discussed (see Sec. 1.2); suffice it to say that the attractions of working in a precisely-defined and reproducible environment have provided the stimulus to many laboratories to develop serum-free media.

4.2 Foetal Calf Serum Background

RPMI 2650 is an epithelial cell line, isolated originally from a human nasal tumour (Moorhead, 1965) and one that is in continuous use in this laboratory. Normally grown in Eagles Minimal Essential Medium (MEM), it is dependent on the addition of 5% foetal calf serum for continual growth and subculturing.

Steps were taken to develop a serum-free medium in which RPMI 2650 would proliferate. The variable nature of FCS was clearly observed throughout this work. Initially, the variability between FCS batches was very evident (see Fig. 3.1), but even after choosing a batch which supported good growth of RPMI 2650, major variations were observed within this batch also. It was necessary to identify a low level of FCS which would allow limited growth of the cells (see Sec. 3.3.2.). It was established that FCS concentrations of 0.5% or 0.25% were both suitable levels to observe growth stimulation by hormones or other media supplements. The results presented throughout section 3.3. show the great range of growth recorded at these levels of FCS. Growth in 0.5% FCS varied from 3% to 81% of that recorded in 5% FCS.

This emphasises the variable nature of FCS. It strengthens the argument for development of serum-free media, where known concentrations of purified components are added to the medium. The varying levels of growth in the 0.5% or 0.25% FCS controls made it difficult on some occasions to interpret results confidently. In some cases, e.g. Table 3.39, where gelatin pre-coating was under observation, growth in the background FCS level was so high that it was more difficult to observe stimulation.

4.3 Identification of growth requirements for RPMI 2650 cells

Much emphasis has been placed by some workers on optimization of the basal medium in the development of a serum-free medium (Ham, 1984). Workers who follow this line of approach (see Sec. 1.3) generally develop a chemically-defined serum-free medium, e.g. Peehl and Ham (1980) developed a medium MCDB 151, which selectively supports the growth of human keratinocytes in the absence of FCS, by qualitative and quantitative adjustments to Ham's F12 basal medium.

While not strictly following this line of approach, some investigations were carried out to choose a suitable basal medium for growth of RPMI 2650. Having compared a number of commercially available basal media and one medium prepared in this laboratory, Special Liquid Medium (SLM)(Margaret Dooley, unpublished), a 1:1 mixture of DME:F12 was chosen (see Sec. 3.3.2.).

This basal medium is the most widely used in serum-free media. DME is one of the simpler media, having high nutrient concentrations but fewer constituents than F12, but it seems to make a vital contribution when used in conjunction with the more complex F12. F12, originally designed for clonal growth of cells, contains a larger number of different amino acids and vitamins although the nutrient concentrations are, on the whole, low. Thus, the richness of F12 and the higher concentrations of basal nutrients in DME have been used in a 1:1 mixture by many workers, e.g. Taub *et al.* (1979) grew MDCK epithelial cells, Barnes and Sato (1979) report the growth of human mammary tumour epithelial cells in basal medium and HeLa cells are also grown in a DME:F12 basal medium (Hutchings and Sato, 1978). This medium has also been used with a wide

variety of cell types other than epithelial, e.g. with cells of mesenchymal origin, NIH/3T3 cells (Chiang et al, 1984) and human and rat glial-derived cells (Michler-Stuke and Bottenstein, 1984). DME:F12 has also been used to establish primary cultures, e.g. canine renal epithelial cells, (Taub et al, 1984).

Having chosen the optimum basal medium available, various hormones, growth factors and nutrients were added to the medium and changes in the growth rate of RPMI 2650 recorded.

Two parameters were used to record cell growth, colony number and total colony area. An image-analyser was used to count dishes which had been stained with Leishmann's stain (see Sec. 2.15).The colony number, which is directly related to the colony forming efficiency (CFE), gives an indication of the number of cells that have attached and undergone cell divisions to form colonies. The total colony area gives information on the extent of cell division, i.e. the area of the individual colonies varies considerably, both within a dish and between dishes which have had different treatments.

It is necessary to look at both of these parameters when assessing growth. Different factors influence cell attachment and cell growth. Hence, when looking at changes in growth levels, it is important to look separately at changes in the number of cells attaching and at the number of cell divisions occurring. Counting colonies with an image analyser also has some limitations, e.g. if two colonies are joined together at one point this is interpreted as being a single colony. Hence, in situations where growth is extensive and many colonies are touching each other, the number of colonies present is underestimated. But by looking also at the second parameter, total colony area, misinterpretation of data is less likely.

The total colony area in a given dish is given in square millimetres. A conversion factor is fed into the computer memory of the image analyser and it converts picture points recorded to the required units. The area of individual colonies varies enormously, even within one dish. The average colony area can readily be obtained from the data presented throughout Section 3, but to estimate the number of cells per colony, which indicates how many cell divisions take place, is much more difficult.

TABLE 4.1 Relationship between growth parameter measurements

Dish	Colony No.	Total Colony Area (mm^2)	Av. Colony Area (mm^2)	Av. No. of Cells/Col
A	753	70.2	0.093	208
B	543	52.8	0.09	238
C	827	66.0	0.08	150
D	771	53.97	0.07	94

The colony number and total colony area were recorded from randomly chosen dishes using the image analyser, and the average colony area calculated from these values. The number of cells per colony are counted microscopically and the size distributions recorded.

TABLE 4.2 Size distribution of colonies*

Dish	Average No. of Cells/Colony	Number of cells per colony					
		16	32	64	128	256	512
A	208	0	1	4	2	2	1
B	238	0	0	3	3	2	2
C	150	0	1	1	5	3	0
D	94	0	2	4	4	0	0

* Size of colonies expressed in terms of number of cell divisions which have occurred. Microscopic counts carried out on a random selection of dishes from typical assays.

It is clear from Tables 4.1 and 4.2 that by calculating the individual colony areas, the numbers of cell divisions can be fairly closely approximated. A colony area of 0.09 mm^2 (approx.) gives colonies that have undergone seven to eight cell divisions, in the examples given in this table. However, it is difficult to make broad generalizations about the number of cells per colony in all dishes by simply looking at colony area. This is due to the fact that when growth at high levels is occurring, cells tend to pile up on top of each other - this piling of cells is especially evident in dishes fed with 5% FCS. Therefore, the two parameters used to measure growth, while giving a considerable amount of information on changes in growth levels, do not give a totally accurate measure of cell growth. Accurate haemocytometer counting of such low cell numbers, however, would not be feasible and the method employed here allows rapid assessment of growth stimulation.

Using this system to analyse growth, some factors were clearly identified as making a significant contribution to growth in reduced serum conditions.

Transferrin was the first component thus identified. It quite clearly plays a vital role in supporting growth of RPMI 2650 in low serum conditions. It caused significant stimulation when added on its own or in combination with other components to 0.25% or 0.5% FCS (see Table 3.12, Sec. 3.3.5.). Transferrin is used in serum-free media of almost every cell type - canine kidney epithelial cells (Taub et al., 1979), human breast carcinoma cell lines (Calvo et al, 1984), lung cancer cell lines and primary cultures (Carney et al., 1981) and NIH/3T3 cells (Chiang, 1984).

The relative importance of transferrin varies in different serum-free media, depending on the cell type. Omission of transferrin from a serum-free medium (with four other factors) used to grow MCF-7 cells (mammary tumour cells) reduces growth of these cells by 20% (Barnes and Sato, 1979), whereas omission of transferrin in the serum-free growth of MDCK cells results in a dramatic reduction to only 10% of the original growth level (Taub et al., 1979).

Transferrin is generally used at 5 µg/ml, which was the concentration chosen for use here, but in some cases other levels are used. 25 µg/ml is the

optimum concentration used for growth of MCF-7 cells, yet growth survives at 80% of the original level when transferrin is removed. Transferrin is used at a concentration of 10 μg/ml for growth of NCI-H23, a small cell lung carcinoma cell line, where it is essential for cell replication - cell number is markedly reduced (by 95%) when transferrin is removed (Brower et al., 1986). Thus there is no correlation between the concentration of transferrin and its contribution to growth relative to other components in a serum-free medium.

The results presented here suggest that transferrin is the most important factor in this medium. Cell growth occurs in its absence but it is significantly reduced (see Table 3.13, Sec. 3.3.5.). Over a concentration range of 1-10 μg/ml, very little change in growth stimulation was observed (see Table 3.18, Sec. 3.3.6.). Thus it is possible that it is present in excess and possibly a lower concentration might be sufficient. Barnes and Sato (1979) report that there is approximately 250 μg/ml of transferrin present in medium supplemented with 10% FCS. Thus the levels used in serum-free media are considerably less than this.

In some cases where transferrin was used routinely at 1-10 μg/ml increasing its concentration led to increased growth stimulation. Simms et al., (1980) developed a serum-free medium for growth of NCI-H69 cells using RPMI 1640 basal medium. Hence, transferrin at 5 μg/ml was used routinely in conjunction with four other factors and deletion of it from the medium resulted in immediate and dramatic differences in growth. When the concentration of transferrin was increased to 100 μg/ml there was a significant increase in cell growth. This high concentration was used for further work on this cell line, but when a different basal medium, F12 was used transferrin at 100μg/ml was inhibitory compared to transferrin at 5 μg/ml. Medium F12 contains iron, whereas RPMI 1640 medium does not. Thus it would appear that if iron is available in excess to the cells growth inhibition occurs. Thus, where these results show ferrous sulphate (10^{-5}M) to be toxic to the cells, it may be due to the presence of excess iron (see Table 3.31, Section 3.3.7.).

Transferrin functions in iron transport in vivo and it is thought to act similarily in vitro. In vivo, iron is transported to the bone marrow and other sites bound to plasma transferrin. The plasma transferrin iron pool

is in equilibrium with the iron in storage forms in the gastrointestinal tract and the reticuloendothelial cells. Both bovine and human transferrin are used in serum-free formulations. Transferrin binds to a specific cell surface receptor, which functions to transport the iron to intracellular compartments for synthesis of heme, which in turn is required for production of mature cytochromes, myoglobin and haemoglobin.

Iron is present at a concentration of 0.834 mg/l in Ham's F12, in the form of $FeSO_4.H_2O$ (ferrous sulphate), but it is not present in DME. There is a strong possibility that iron is removed from the medium as an insoluble ferric hydroxide during sterile filtration (Ham, 1984); thus, although a media formulation may state that ferrous sulphate is a component, it may no longer remain in the medium after filtration. It has been suggested to add a solution of freshly prepared sterile ferrous sulphate solution to the medium after filtration. Tsao et al, (1982) added ferrous sulphate (10^{-5}M) solution to the final medium after it had been sterilised and they found that much of the transferrin requirement could be replaced by this. (In growing normal epidermal keratinocytes in a chemically-defined basal medium, MCDB 152, these workers report the need to add 10 μg/ml of transferrin, in conjunction with six other components).

Thus, it seems that transferrin functions primarily to supply iron in a usable form to the cells in culture. Transferrin preparations are not 100% purified and possibly contain a range of unknown contaminants. It is possible that it owes some of its mitogenic ability to the presence of these contaminants, particularly when operating in a totally serum-free, chemically-defined medium.

Insulin is the second component which has widespread use in serum-free media. The requirement for insulin by RPMI 2650 cells is not absolute, yet insulin does contribute significantly to growth. These results show that the contribution of insulin to growth can be detected in the absence of any other factors (see Tables 3.12 and 3.14, Section 3.3.5.), with a 0.5% FCS background level. When insulin is in combination with transferrin, the growth stimulation which occurs is greater than the sum of the individual contributions (see Table 3.12, Section 3.3.5.). Therefore, insulin and

transferrin appear to interact in a synergistic manner to increase growth of RPMI 2650.

Growth of RPMI 2650 will occur in the absence of insulin, in reduced serum conditions. Growth in medium supplemented with 0.5% FCS + TES is compared to growth in 0.5% FCS + TIES (see Table 3.15, Sec. 3.3.5.) and it is clear that although growth does occur in 0.5% FCS + TES, there is a significant increase due to the addition of insulin. This pattern is found in a number of other serum-free media formulations, e.g. Simms et al, (1980), shows that, while insulin does lead to a significant increase in growth of a lung carcinoma cell line, the removal of insulin totally from the medium results in submaximal, but significant growth.

Insulin is found in almost all serum-free media and stimulates a wide range of cell types, e.g. a prostatic epithelium cell line (Chaproniere et al, 1985), a colon carcinoma cell line, HT29, (Zirvi et al, 1986), canine kidney (Taub et al, 1979) and monkey kidney cells (Taub, 1984) and Swiss 3T3 (Shipley and Ham, 1981). As with transferrin, the extent to which cells are dependent on insulin varies. As already mentioned, both the RPMI 2650 and a carcinoma cell line (Simms et al, 1980) still exhibit growth in the absence of insulin, but with some other cell lines insulin plays a much more critical role, e.g. with the MCF-7 mammary tumour cells deletion of insulin from the serum-free medium reduces growth by 66% (Barnes & Sato, 1979).

Insulin, (a polypeptide of molecular weight circa 5,000), is synthesized (in the form of a high molecular-weight precursor) by the β cells in the islets of Langerhans in the pancreas. The half life of insulin in the blood is only 3-4 minutes, which may be of significance to its inclusion in serum-free media. Insulin is known to act on a variety of tissues (liver, fat, muscle). Insulin preparations are standardized in units by measuring their effect on the blood glucose of rabbits. The international standard unit contains 24 units per mg recrystallized insulin. The insulin content of serum is reported to be 25 μU/ml, which is approximately equivalent to 1 ng/ml. Insulin is used at concentrations of 1-10 μg/ml in most serum-free media. Therefore, if commercially-available insulin is as purified as standard insulin, concentrations used are far above physiological levels.

Insulin plays an important role in metabolism, causing increased carbohydrate metabolism, glycogen storage, fatty acid synthesis, amino acid uptake and protein synthesis. Insulin receptors are found on the cell surface; these are glycoproteins of 300,000 molecular weight, to which insulin binds. It is thought that this high affinity binding is vital for insulin action *in vivo*; binding to the plasma membrane of target cells leads to increased uptake of amino acids, lipids and ions.

The relationship between the effect of insulin on metabolism and its positive effects on growth of cells in culture are at present poorly understood. The fact that growth-promoting effects of insulin are visible often at supraphysiological levels suggest that insulin may be binding to receptors other than its own (Coppack and Straus, 1984).

There is a group of insulin-like growth factors called the somatomedins. Human serum contains two classes of somatomedins; they are of M.W. approx. 7,500kDa and are distinguishable only by their isoelectric points. There is considerable evidence to show that the receptors that bind insulin and at least one of the somatomedins, insulin-like growth factor I, also called Somatomedin C, are immunologically, structurally and functionally related (Perdue, 1984). Some work carried out on adult human fibroblasts (Conover *et al*, 1985) demonstrate that insulin and IGF-1(SM-C) at physiological concentrations are both mitogenic for these cells. These workers suggest that when both compounds are present at physiological concentration (nanomolar concentrations) they each act through their own receptors. When insulin levels are increased to supraphysiological levels (10-100 µg/ml) increased stimulation is recorded. It is hypothesized that the increased mitogenic action is due to interaction of insulin with IGF-I receptor (Rosenfeld & Dollar, 1982).

Insulin can also modulate various cell functions. Rat pancreatic acinar cells are maintained in a serum-free medium supplemented with albumin, EGF, dexamethasone and hepes. But the addition of insulin at 1 μg/ml concentration significantly increases intracellular and secreted amylase activity after 3 days (Brannon et al, 1985). Thus, insulin may be playing a role, when present in medium, that is not detected by looking at changing growth levels.

The results presented here show that selenium plays a certain role in growth of RPMI 2650 at reduced serum concentrations. The contribution of selenium was not evident at higher FCS concentrations (see Table 3.16, Sec. 3.3.5.), but at reduced (0.5%) FCS levels selenium consistently increased growth to a relatively small extent. Ham (1984) in a review article on growth of human diploid fibroblasts (HDF), reports that selenium is of major importance for serum-free growth of HDF but that this requirement is masked in most serum-containing systems but becomes very important when the concentration of serum is lowered. Thus, our observation of stimulation in reduced serum with an epithelial cell line is similar to Ham's report for fibroblasts.

Early developmental work on optimized basal media led to the development of F10 (Ham, 1963) and F12 (Ham 1965). Chinese hamster ovary and lung cell lines grew in the complete absence of protein supplementation in F12 medium but later (Hamilton and Ham, 1977) it was discovered that cellular multiplication of these cells in F12 medium without supplementation was dependent on the presence in the medium of selenium as a trace element.

Selenium is known to be an antioxidant. This may be important when the cells are exposed to an oxygen-rich environment of 95% air and 5% CO_2. When adrenocortical cells grow in a serum-free defined medium, α-tocopherol (Vitamin E) and $NaSeO_3$ have a stimulatory effect on cell proliferation (and also affect energy metabolism and differentiated function)- cells did not survive in the absence of these antioxidants (Gill et al, 1984). Selenium is known to act as a cofactor for glutathione peroxidase which converts lipid hyperoxides to alcohols (Tappel, 1980).

The importance of selenium in media formulation varies. In growth of primary cultures of bronchogenic epidermoid carcinoma cells in serum-free conditions selenium is added (with insulin, T_3, retinoic acid and glucagon) at a concentration of 2.5×10^{-8}M (Miyazaki et al, 1984). Of the four factors selenium was the most important when cells were cultivated at low density. In the absence of selenium growth was very limited, regardless of the addition of the other factors. The precise nutritional and growth requirements of individual cell lines is emphasized in work carried out by Michler-Stuke & Bottenstein (1984). Here, growth of three different glial derived cell-lines in a serum-free medium is recorded. Selenium (sodium selenite) is one of the components. Deletion studies show that in the absence of selenium growth of one cell line, U-251 (human glioma), is reduced to 80% of that in selenium-containing medium, growth of the C6 (rat glioma) cell line is reduced by more than 50%, while growth of the third line, RN-22, a rat schwannoma cell line is actually increased in the absence of selenium.

Selenium is not normally included in media originally formulated for use with serum and is not in DME or F12. It is postulated that traces of selenium may be present in FCS (Freshney, 1983), but the only real evidence for this is the fact that a selenium requirement has been demonstrated in quite a number of cell lines. McKeehan et al. (1976) were one of the earliest to demonstrate requirement for selenium when they showed that growth of normal fibroblast was stimulated by selenium. α-tocopherol (Vit. E) and selenium have been shown to be synergistic in vivo so it is possible that addition of Vit. E (not normally a serum-free media component, although there are some reports (Bettger et al, 1984), working with human fibroblasts) may enhance the effect of selenium in culture.

Our results show selenium having a consistent effect on cell growth (Tables 3.12 and 3.13, Sec. 3.3.5.). Selenium appears to increase the CFE, but not the total colony area, when added on its own or when added in conjunction with other components with 0.5% FCS background level. This suggests that selenium is acting as an attachment factor, increasing the number of cells which attach and form colonies but not affecting the number of cell divisions which take place. This is an interesting hypothesis, as the exact role of selenium, when present as a trace element in many serum-free media, is not known.

In growth of RPMI 2650 epidermal growth factor plays an important role. Although EGF on its own does not cause any significant increase in cell growth, when it is added in conjunction with transferrin or selenium significant growth stimulation is evident (see Table 3.13, Sec. 3.35). Again, like insulin, growth occurs in the absence of EGF, but its contribution to growth of RPMI is very significant when added at a concentration of 10ng/ml.

EGF, a polypeptide growth factor (molecular weight 6,000 Da) was first described as a peptide which would stimulate precocious eyelid opening and tooth eruption in newborn mice. EGF has been used to support serum-free growth of a variety of cell types - HeLa cells (Hutchings & Sato, 1978), BHK-kidney cells (Maciag et al. 1980), W1-38 lung fibroblasts (McKeehan et al. 1977).

The cellular receptor for EGF is the best understood growth factor receptor and is present on a large number of cell types. First purified from A431 cells, the receptor is a membrane protein (170-kDa) that has an extracellular binding region to which EGF binds. The receptor is known to have an associated tyrosine kinase activity and it is thought that the inner portion of the receptor is involved in this function.

EGF belongs to a diverse family of polypeptide growth factors which share common amino acid sequences; these include TGF-α and the insulin-like growth factors (IGF-1 and IGF-2) and vaccinia virus protein (VVP). No clear-cut mechanism of action has been identified for these factors but it seems certain that, while sharing some characteristics, each has its own individual mechanism of action. EGF is known to require a relatively long exposure time (18 hrs) to induce mitogenesis in cells (James, 1984). EGF is thought to bind to the surface receptor region and it seems that internalization of the bound ligand occurs, which may then trigger off the biological response.

EGF has widespread applications in serum-free media and in many cases interesting interactions with other compounds have been recorded. Lechner et al. (1982) show that in a chemically-defined medium, MCDB 151, growth of

normal epithelial cells is stimulated due to the addition of EGF (5ng/ml). Insulin stimulates these cells also, yet the addition of both EGF and insulin does not yield an additive effect. EGF displays synergistic action with hydrocortisone.

EGF is included in a serum-free medium for growth of MCF-7 cell line, but its omission from the medium does not have a very significant effect, if cells are counted after 7 days. After a longer incubation period EGF deletion had a noticable effect, (Barnes & Sato, 1979).

EGF stimulates the growth of certain cells in primary culture. Rat hepatocyte cultures responded greatly to the addition of EGF and the mitogenic response was greatly increased due to the addition of insulin and glucagon (Bucher, 1984). Although EGF is thought to be present in serum, LaRocca and Rheinwald (1985) suggest that, if EGF is present in serum at all, it is present at exceptionally low and non-mitogenic levels (0.1ng/ml). EGF may modify Ca^{++} and Mg^{++} requirements for some cell types (McKeehan, 1984).

Two other additions to the medium caused significant increase in growth of RPMI 2650. MEM non-essential amino acids, when added at the concentration used in MEM basal medium, led to a significant increase in cell number (see Table 3.28, Sec. 3.3.7.). MEM non-essential amino acids (see Table 3.27 Sec. 3.3.7.) are added to MEM basal medium in which normal growth of RPMI 2650 occurs in 5% serum. Although not a regular serum-free media supplement, the decision to add NEAA to the medium was taken on the grounds that (a) of the amino acids in the mixture, five (L-alanine, L-asparagine, L-aspartic acid, L-glutamic acid and L-proline) are not present in DME and although present in Ham's F12, the final concentration is only half the normal concentration and L-asparagine is not present in Ham's F12 at all. Hence, as RPMI 2650 are normally supplied with sufficient quantities of these amino acids when growing in serum-supplemented medium, it seemed logical to supply the cells with at least the same amino acid composition in the more stringent, serum-free environment. (b) No optimization of the basal medium had been carried out - yet, as optimization generally involved qualitative and quantitative alterations in the amino acid composition, the addition of this amino acid mixture could be considered a "bulk" optimization procedure.

The results obtained justified the addition of this mixture of amino acids and could justifiably be included in a serum-free media formulation for RPMI 2650.

Ascorbic acid was another supplement which yielded a positive response by RPMI 2650. Ascorbic acid is very unstable in solution and is not a component of either DME or Ham's F12 medium. It has been known to stimulate some cells in serum-free conditions, e.g. Gill et al, (1984) report that the addition of ascorbic acid (100 μg/ml) greatly enhances the growth of adrenocorticoid cells. Weinstein et al.(1982) added ascorbate to a DME:F12 mixture to enhance human diploid fibroblast growth (in the presence of 6 other factors).

Ascorbic acid (Vitamin C) is the least stable of the vitamins, but is stable to freezing, which is of great relevance for its potential use as a serum-free medium supplement. Ascorbic acid is a reducing agent and functions in vivo in some oxidation-reduction reactions. It is present in large amounts in the adrenal cortex, (which is interesting, in view of its action in culture on adrenocorticoid cells mentioned previously) but its function there is not known. It is known to play a role in collagen biosynthesis. It is not known how it functions in vitro, but it may stimulate cell attachment, due to its stimulation of collagen synthesis.

4.4 Attachment

Many cell types in culture are classed as anchorage-dependent and grow only when attached to a suitable surface. Cell attachment to various substrates that are in common use (generally negatively-charged polystyrene) is often inadequate and this problem is intensified at low cell density and in reduced serum/serum-free conditions.

The results presented here (sec. 3.3.8.), show that optimization of cellular attachment is a critical step that should be investigated in the development of a serum-free medium. Lack of any growth of RPMI 2650 in the absence of serum (in medium supplemented with TIES only) suggests that the low FCS level may be involved primarily in supplying attachment factors.

Significant growth stimulation is recorded when dishes are precoated with collagen, Vitrogen or DEAE dextran. This, even in the presence of reduced FCS, suggests that neither this level of FCS nor the four growth factors present (TIES) are supplying sufficient attachment factors to achieve maximum cell attachment.

Precoating dishes with collagen stimulated RPMI 2650 growth, over a wide range of precoating conditions. Precoating at 37°C (for 30 mins. to overnight) gave the best results. The precoating procedure used (see Sec. 2.16) involved a fairly stringent washing regime, yet cell binding factors must obviously bind to the dish surface in such a way that washing does not remove them. A collagen gel was not visible on the surface of the dishes, even after the longest precoating period (16 hours).

Collagen has been shown to enhance growth of a variety of cell types, when used as a precoating material, e.g. T-84, a human colon carcinoma cell line (Barnes and Sato, 1980 b) and chicken fibroblasts (Gey et al, 1974) showed increased growth on collagen-treated surfaces. Vitrogen, a commercial bovine dermis collagen product, which also proved successful as a precoating material with RPMI 2650, has been used by other workers instead of collagen with considerable success, e.g. Calvo et al.(1984) with human breast cell lines.

Collagen is a protein with a very characteristic structure. There are at least five isotypes of collagen molecules which are quite similar yet each has an identifying characteristic. Type III Collagen was used in our work; this acid-soluble collagen is most prominent in blood vessels, skin and connective tissue of internal organs. It (and types I and II) is known to be synthesised in precursor form, called procollagen, which is modified after synthesis. This modification of procollagen requires ascorbic acid and the ferrous iron.

Most cells in culture synthesise collagen (Kleinman et al., 1981) the amount varying with the cell type. The addition of ascorbic acid to the medium is known to enhance collagen synthesis. This may explain why ascorbic acid, when added to the medium, led to significant proliferation of RPMI 2650 cells in culture (Sec. 3.3.7.). Cell-produced collagen can also stimulate attachment.

It is thought that a collagen-mediated increase in cell growth is likely to be due to an interaction between surface glycoproteins on the cells and the collagen substratum. Fibronectin is generally accepted as being the major protein functioning in this manner; it is present on most cell surfaces and also in serum. Thus it is likely that the collagen-mediated increase in cell growth is due to the improved binding of cellular fibronectin, although fibronectin levels are thought to be greatly reduced on the surfaces of malignant cells. The action of ascorbic acid may cause increased collagen secretion which stimulates cell proliferation also.

Precoating dishes with fibronectin has also led to increased growth of various cell types, e.g. Lechner et al. (1982) showed increased CFE and growth rates in bronchial epithelial cells when dishes were precoated with Fibronectin (Fn). Fn precoating did not enhance growth of RPMI 2650 when added as a precoating substance.

When Fn and collagen were used in a 1:1 precoating mixture growth stimulation occurred but it was not significantly greater than growth stimulation recorded in other experiments with collagen precoating alone. Fn and collagen have been reported to interact to produce greater growth stimulation when both are used as precoating agents than when either are used alone (Grinnell, 1983) but our results show that this does not occur with RPMI 2650 cells.

DEAE (diethyl-amino-ethyl) dextran proved the most successful attachment factor tested. The DEAE dextran, in a chloride form with a nitrogen content of 3.2%, when used as a precoating substance led to very significant growth stimulation. Growth was increased so much that the possibility of eliminating the serum background was suggested. Growth in dishes precoated with DEAE dextran in medium supplemented with TIES in the absence of FCS, yielded some growth. This was the first recorded growth of RPMI 2650 in the absence of serum and it was confirmed in a subsequent experiment by more extensive growth in the presence of TIES + non-essential amino acids and ascorbic acid (see Table 3.28, Sec. 3.3.7.6.).

DEAE dextran is a basic polymer, which, along with many other basic polymers, has been tested for its ability to modify the negatively-charged polystyrene surfaces to enhance cellular attachment. There have been some reports of enhanced cell growth with DEAE dextran-precoating (McKeehan, 1984, working with WI-38 fibroblasts), but it has not, as yet, been tested in a large number of systems. Some microcarriers are composed of DEAE dextran.

Various other basic polymers have been tested for an effect on cell growth. Precoating with poly-D-lysine is one of the most commonly used methods, and one of the most successful. Swiss 3T3 cells showed increased colony number and colony size in dishes precoated with poly-D-lysine, in the presence of low levels of foetal bovine serum-protein, Shipley and Ham, (1983). McKeehan (1984) shows a doubling in cell number of WI-38 fibroblast cells with poly-D-lysine precoating but precoating with L-Lysine had no effect on cell growth.

As no poly-D-lysine was available, we tried L-lysine and poly-L-lysine but no growth stimulation of RPMI 2650 occurred (see Tables 3.40 and 3.41, Sec. 3.3.8.). McKeehan (1984) states that, while either the D- or L- isomers of polylysine are effective, monomeric lysine or dilysine shows no stimulatory effect. McKeehan suggest that a minimum polymer length of 10 amino acids is necessary in the case of polylysine to stimulate cell growth. Our results show L-lysine inhibiting growth, while poly-L-lysine has no effect on this epithelial cell line.

Various other polymers were tested with RPMI 2650 (see Sec. 3.3.8.4.), but none showed any great growth stimulatory activity. Gelatin (denatured collagen) is a substance that has stimulated growth in numerous systems - e.g. Richler and Yaffe, (1970) used it to culture myoblasts and Haas et al., (1984) grew fibroblasts on gelatin-coated dishes. Gelatin has, however, been used more by workers studying cellular attachment (e.g. Haas et al., 1984), than for continuous culture of a cell line. Gelatin provides a simple, uniform and well-defined model collagenous substratum and it binds more fibronectin than does undenatured collagen, thus explaining its use in cellular attachment studies.

The manner in which any basic polymer-coated surface enhances cell growth is not known. In some cases the effect of this treatment is more apparent at low cell densities and low serum concentrations (Michler-Stuke and Bottenstein, 1984). This has led to suggestions that the coated substrate is substituting for serum-proteins or cell-derived factors but no studies have been reported which identify exactly what is happening. Cell spreading has been recorded as being much more extensive on coated substrates (McKeehan and Ham, 1976 with fibroblasts) and this may serve to increase the cell surface area to affect transport and receptor sites for nutrients and hormones in the culture medium. It is not clear why, for example, RPMI 2650 should grow better in dishes precoated with DEAE dextran, or why growth occurs in these dishes in the absence of serum, while a low level of serum is vital for growth in dishes which have a sulphonated polystyrene surface only.

In some cases attachment factors are included as medium components in solution. The addition of collagen to medium supplemented with TIES and a reduced FCS level stimulated growth of RPMI 2650 significantly. When fibronectin was added to the medium growth stimulation occurred (where precoating had no effect) and when DEAE dextran was added to the medium extensive growth stimulation was recorded.

4.5 Other developmental work

Cells are known to produce autostimulatory growth factors and these factors can act in conjunction with any growth factors which are present as medium supplements to stimulate increased cell growth. There are also some instances where the nutritional requirements of cells at high densities differ from those of cells at lower densities, e.g. transferrin at 100 µg/ml increases growth of lung cells at high density but not at low density (Simms et al., 1980). Therefore, growth was observed over a range of cell concentrations from $1x10^2$ cells/dish to $1x10^4$ cells/dish. The lowest concentration of cells at which growth occurred in medium supplemented with 5% FCS was $5x10^2$ cells/dish and this may indicate an involvement of autostimulatory growth factors. At this concentration, as well as at $1x10^4$ cells/dish, growth in 0.5% FCS + TIES was significantly greater than in 0.5% FCS alone.

Many problems arose when attempting to culture cells in flasks in medium with a low serum concentration and supplemented with TIES (see Sec. 3.3.10.). It would appear that growing cells in an open dish, allowing gaseous exchange to occur with the 5% CO_2 atmosphere, is a superior manner of culturing cells in low serum conditions or in the absence of serum. Most workers who report long-term subculturing of cells in serum-free media carry out subculturing in open petri dishes (30 mm or 60 mm), e.g. Taub et al.,(1979), This method appeared very inaccurate for use with RPMI 2650, as cell counts were low from small dishes - almost too low to detect using a haemocytometer. Therefore, considerable time was spent trying to grow RPMI 2650 in 25 cm^2 flasks. The fact that growth was eventually achieved only when partially-open flasks were used in a 5% CO_2 atmosphere suggests that there was insufficient buffer present in the medium or alternatively relates to O_2/CO_2 exchange. This suggests that not only the nutritional but also the gaseous requirements of cells should be taken into account when subjecting the cells to suboptimum conditions.

Many compounds, apart from transferrin, insulin, EGF and selenium were examined for ability to stimulate RPMI 2650 growth. None of them consistently stimulated growth although some, e.g. bombesin and hydrocortisone did stimulate under particular conditions. It is common in developing a serum-free medium to examine a large number of compounds and to eliminate those that are definitely inhibitory or having no effect. To a large extent, if a compound appears to be contributing anything at all to growth of the cell line in question, workers retain this compound in their serum-free medium formulation. There is always the possibility that a slight stimulatory action will be potentiated by the random addition at a later date of another compound. This mode of action can be seen in many of the deletion studies (some examples given in Sec. 4.3.), where omission of a component from a serum-free formulation makes little difference. Brower et al.(1986), for example, describe a serum-free medium, ACL-3, which contains TIES, hydrocortisone, T_3 and sodium pyruvate, in which a lung carcinoma cell line grows in the absence of serum. But deletion studies show that, in the absence of either T_3 or sodium pyruvate, growth continues at 90% of the original value. Yet, in spite of the small contribution these factors are making, they are included in the serum-free formulation.

4.6 Optimal conditions for growth of RPMI 2650 in reduced serum conditions and in the absence of serum

The results of a final experiment carried out to detect the best combination of factors for growth of RPMI 2650 show that DEAE dextran precoating of dishes greatly enhances growth of RPMI 2650, supporting growth in the absence of FCS when the medium is supplemented with TIES, MEM, non-essential amino acids and ascorbic acid. The CFE in the precoated dishes was an average of 70% of that in the 5% FCS control, while the total colony area was over 50% of that in 5% FCS. These results represent a major breakthrough in the development of a serum-free medium for RPMI 2650.

Once growth has been achieved in the total absence of FCS, increasing the extent of this growth is a relatively easy matter. An adequate set of attachment, nutritional and growth requirements of this cell line have now been identified and optimization of these factors, with the addition of possibly a few more compounds, should yield consistently high levels of growth, comparable to that in 5% FCS. It is possible that some of the compounds rejected previously as having no consistent growth stimulatory ability for RPMI 2650 (particularly bombesin and hydrocortisone) may now, in the absence of FCS, perform some function that was previously masked by the low level of FCS present.

The inclusion of DEAE dextran as a medium component, in the absence of precoating, in medium supplemented with 0.25% FCS + TIES, non-essential amino acids and ascorbic acid, yielded growth comparable to that in DEAE dextran precoated dishes (see Table 3.47, Sec. 3.3.11.). The inclusion of an attachment factor as a medium component is obviously more advantageous for use in a routine system. Hence, investigation into growth in the absence of FCS with DEAE dextran as a medium component would certainly be of interest.

The growth obtained in the presence of 0.25% FCS and 0.5% FCS backgrounds, in DEAE dextran coated dishes was excellent (see Table 3.47, Sec. 3.3.11.). The total colony area in these dishes not only exceeded that in the non-coated controls but also that in the 5% FCS controls. Microscopic

observation of the colony morphologies showed a distinct difference in the number of cells per colony between the coated and uncoated dishes. Colonies in 5% FCS are more densely packed than in the precoated dishes and thus the difference in total colony area is not an accurate reflection of the difference in cell number. Further analysis involving cell counts would be needed to obtain more information. However, microscopic observations suggest that there is a real increase in cell number between precoated and non-precoated dishes fed with TIES - supplemented medium.

Thus it appears that growth equivalent to that in 5% FCS can be achieved in dishes precoated with DEAE dextran (100 μg/ml) in DME:F12 medium supplemented with 0.25% FCS and transferrin (5 μg/ml), insulin (5 μg/ml), EGF (10 ng/ml), selenium ($1x10^{-8}$M), ascorbic acid (10 μg/ml) and MEM non-essential amino acids (1:100 dilution).

A rough estimate of the cost of 100 mls of medium (and DEAE dextran coating of 50 dishes) shows no marked reduction in costs compared to using medium supplemented with 5% FCS. At current prices, transferrin (£23.62/100 mg), insulin (£22.50/100 mg), EGF (£94.60/100 μg) selenium (£10.54/1 mg), ascorbic acid (£10.54/100 mg), MEM non-essential amino acids (£5.21/100 mls) and DEAE dextran (£7.81/10g), supplemented with 0.25% FCS, costs approximately £2, (including V.A.T.), for 100 mls of this medium. 100 mls of medium supplemented with 5% FCS and MEM non-essential amino acids costs approximately £1.60. But the composition of this medium is much more precisely defined and hence is a better system to work with.

Development of a serum-free medium is not complete until continuous growth of the cells is examined in this environment. The nutritional requirements for short-term survival of cells may be inadequate for continual cultivation of cells in this environment. It is always advisable to maintain stocks in serum-supplemented medium while long-term growth in a newly-developed serum-free medium is being tested, as the nutritional and growth requirements may change with subculturing. Simms et al. (1980) showed that, when growing NCI-M69 lung carcinoma cells in serum-free conditions, omission of insulin does not reduce growth greatly, when cells have been freshly subcultured from serum-containing medium. But after the cells have been maintained for six months in this serum-free medium, deletion of insulin from the medium led to a cessation of growth.

The growth achieved here in reduced serum conditions with RPMI 2650 cells has been tested only over a single growth period (7 days). No subculturing has been carried out. This is an area that would have to be thoroughly investigated before claiming to have identified reduced serum growth conditions for this cell line. Apart from changing growth and nutritional requirements, there is also the problem of trypsinization. Serum contains a trypsin inhibitor which protects the cells from further trypsin action once serum-containing medium is added to the cells. In the absence of serum, the cells must be protected from destructive trypsin action. Soya-bean trypsin inhibitor may afford sufficient protection - this was used routinely in setting up cell assays. This would have to be checked over a number of trypsinizations, to define the optimum conditions for continuous growth of RPMI 2650 in the absence of serum.

Investigations are currently being carried out in this laboratory into the production of autocrine growth factors by RPMI 2650. Medium which has been conditioned by the cells is collected and examined for the presence of growth factors. This work may benefit from the development of a serum-free medium, as a more accurate knowledge of the cellular environment would be available in which to test the activity of these factors.

4.7 Growth of other cell lines in TIES-supplemented medium

The results presented in Sec. 3.3.12. emphasize the problem of development of a universal serum-free substitute. The complex nature of serum provides the growth requirements for a very extensive range of cell types and it is proving very difficult to mimic this action with serum-free formulations. Many workers report a serum-free formulation which will stimulate a number of cell types of similar nature, e.g. Calvo *et al.*(1984) developed a serum-free medium for one human mammary cell line and found that this medium also supported the continuous growth of four other human breast cancer cell lines. But little success has been reported in development of serum-free formulation, with widespread applications.

Growth of A431 cells was unchanged in the presence of TIES. A431 cells, in spite of having an increased number of EGF receptors, is actually inhibited by EGF at nanogram concentrations (Barnes, 1984). Thus although transferrin

and insulin are both stimulatory to A431 cells (Barnes, 1984), the TIES combination yields no significant growth stimulation. One possibility is that the EGF is negating any positive stimulation of transferrin and insulin but time did not permit testing of this hypothesis.

The growth inhibition recorded in the HEP-2 cell line is somewhat surprising. It is generally accepted that this cell line is contaminated with HeLa cells. Hutchings and Sato (1978) developed a serum-free medium for HeLa cells which consisted of Ham's F12 basal medium, supplemented with transferrin, insulin, EGF, hydrocortisone and a trace element mixture which included selenium. The growth requirements of the HEP-2 cells must obviously differ somewhat from those of HeLa cells, in spite of supposed contamination.

Total inhibition of growth in A549 cells occurred in the presence of TIES. Carney (1984) reports the growth of A549 in medium supplemented with TIES, hydrocortisone and albumin and involving serum precoating: this is the LA medium developed by Barnes (1981) . Carney also reports growth of A549 in TIES and hydrocortisone (HITES) - supplemented medium but states that it is the only non-small cell lung cancer cell line that grows in HITES. No details are given on the importance of hydrocortisone in this latter medium. The total inhibition of growth in 0.5% FCS + TIES or 0.25% FCS + TIES must be due either to the presence of the low level of FCS or the absence of hydrocortisone.

Thus, none of these lines tested showed a similar response to the TIES-supplemented medium. While, the developmental work carried out with the RPMI 2650 cells identified the growth requirements of this cell line, these clearly are not the growth requirements of A431, A549 or HEP-2 cell lines.

4.8 Milk as a medium supplement

Milk or milk products have been used as a medium supplement by a small number of workers. Sereni and Baserga (1981) report growth of several cell lines, including mouse 3T3, in both dairy farm milk and pasteurised skim milk. They added a low level of serum, 0.5% calf serum, to help cellular attachment. Fassolitis *et al.* (1981) used 5% powdered milk to support the growth of a number of epithelial cell lines in the absence of serum. They reported that

cell growth was slower than in serum-supplemented medium but the same saturation densities were obtained. They also claimed that two fibroblast cell lines failed to grow in milk-supplemented medium, which opened up the possibility of a selective medium supplement.

Steimer and Klagsbrun (1981) reported the growth of certain fibroblastic cells in medium supplemented with milk in dishes precoated with fibronectin. Previously, Klagsbrun (1980) stated that MDCK cells grew in medium supplemented with colostrum over a period of five months with continuous subculturing, but failed to grow in milk obtained one week after the birth of the calf.

We attempted to test milk-supplemented medium on some of the cell lines in our laboratory. The first problem we encountered was sterilization of the milk. Milk from commercial sources was used and attempts to repeat the work of Sereni and Baserga failed completely. They claimed that, after centrifuging milk to remove fat and cellulose debris and diluting it to 10% in DME, sterility was achieved by passing it through a 0.44 μm and a 0.22 μm Millipore filter. In our experience, milk failed to pass through a 0.22 μm filter even after having been passed through a stack of membranes of larger pore size. This is probably due to the presence of micelles. Milk filtered through 0.45μm only was usually contaminated.

We then used a Pellicon ultrafiltration unit, with a $1x10^6$ molecular weight membrane (see Section 2.10) which retained the micelles without clogging the filter, due to the tangential flow in the apparatus. Filtrate from here was passed through a 0.22 μm filter under N_2 pressure but this did not consistently produce sterile milk. A membrane stack, while increasing the volume of milk filtrate that could be sterilised, did not increase the sterility of the milk. The membrane stack repeatedly failed an integrity test.

The addition of a 0.22 μm Millipore-GV filter (presealed) to the end of the membrane stack resulted in sterile milk being consistently obtained. Having overcome the technological problem of achieving a sterile milk solution, we then tested it on a number of cell lines (see Section 3.3.13.).

As reported in Section 3.3.13, great inconsistencies were recorded in the growth-stimulatory ability of milk-supplemented medium. Out of a total of 41 attempts to grow mouse 3T3 cells in milk-supplemented medium in the presence of a low FCS level, 21 positive results were obtained and 20 negative results. It was obvious that milk has some growth-stimulating property, but the manner in which it operates remains a mystery. Similar results were obtained with MDCK cells and RPMI 2650 cells. With the latter, no stimulation was ever recorded when milk was added on its own to medium with 0.5% FCS. But when milk was added to medium with 0.5% FCS + TIES growth recorded on a few occasions was greater than in 0.5% FCS + TIES; again this stimulatory action of milk was not consistently observed.

Growth in colostrum, dried milk or baby food showed no stimulatory action. The results we obtained using milk as a medium supplement need to be compared with those published in the literature. We found that the sterilization methods used by Sereni and Baserga were not sufficient to produce a sterile milk solution, thus raising some questions relating to their results. Steimer and Klagsbrun (1981) reported that milk failed to support growth of fibroblastic cells, and Klagsbrun (1980) showed growth in milk-supplemented medium only in the presence of fibronectin. Therefore, our lack of convincing positive growth-stimulation due to the addition of milk is not in total conflict with other literature reports.

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ABBREVIATIONS USED THROUGHOUT THESIS

BSA	-	Bovine serum albumin
CFE	-	Colony forming efficiency
DEAE	-	Diethylaminoethyl
DMEM/DME	-	Dulbecco's Modified Eagles Medium
EDTA	-	Ethylene diamine tetra acetic acid
EGF	-	Epidermal growth factor
FCS	-	Foetal calf serum
Fn	-	Fibronectin
Hepes	-	4-(2-hydroxyethyl-1-piperazine ethane sulphonic acid)
I	-	Insulin
ITS	-	Insulin, transferrin, selenium
MEM	-	Minimal Essential Medium
MDCK	-	Mandin Darby Canine Kidney Cells
PBS	-	Phosphate buffered saline
PDGF	-	Platelet-derived growth factor
PGE_1		Prostaglandin E_1
RPMI 2650	-	Roswell Park Memorial Institute - cell line
S	-	Selenium
T	-	Transferrin
TV	-	Trypsin versene

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SOURCES OF CHEMICALS AND EQUIPMENT

Amicon Corporation, Scientific Systems Divisions, Danvers, MA, U.S.A.
Amicon ultrafiltration unit; ultrafiltration membranes.

Bioprocessing Limited, Consett No. 1 Industrial Estate, Consett, Co. Durham.
PDGF.

BDH, Chemicals Ltd., Poole, England.
Methyl cellulose, Schiff's reagent, DMSO.

Boehringer Corporation Ltd., Bilton House, 54-58 Oxbridge Rd., Ealing, London W5 2TZ.
Oxford Micropipettes.

Chance Propper Ltd., Smethwick, Warley, England.
Microscope slides and coverslips.

Chemical Products, R. Barghraef, Belgium.
RBS-25.

Corning Ltd., Halstead, Essex, CO9 2DX, England.
Electrophoresis kit.

Edwards High Vacuum, Manor Royal, Crawley, West Sussex, England.
Freeze dryer.

Flow Laboratories Ltd., Second Ave, Industrial Estate, Irvine, Ayrshire, Scotland.
Cell lines.

Gibco-Europe Ltd., Trident House, Renfrew Road, Paisley, PA3 4EP, Scotland.
Culture media, foetal calf serum, L-glutamine, trypsin, ascorbic acid, powdered medium; Nunc plasticware, EGF.

Grant Inst. (Cambridge Ltd., Barrington, Cambridge, CB2 5QZ, England.
Waterbath.

Heraus Christ GmbH, D3360 Osterode am Harz, Germany.
Varifuge centrifuge.

Irish Industrial Gases Ltd., Bluebell Industrial Estate, Dublin. 12.
CO_2 gas, nitrogen gas.

Koch-Light Laboratories, Coinbrook, Bucks, England.
NaOH, HCl.

LEEC Ltd., 7 Private Road, Colwick Industrial Estate, Nottingham, NG4 2AJ, England.
37°C incubators, 5% CO_2/37°C incubators; drying oven.

Millipore Corporation, Ashby Road, Bedford, MA 01730, U.S.A.
Pellicon ultrafiltration unit, Millex-GV and Millex 0.22um filters; sterilising unit, swinnex adaptors, membrane and depth filters; Milli-Q Ultrapure water system and replacement cartridges.

Nippon Kogaku, K.K., Fugi Bld., 2-3, 3 chome, Marunouchi, Chiyoda-ku, Tokyo 100, Japan.
Microscopes.

Oxoid, Ltd., Southwark Bridge Road, London, SE1 9HF, England.
Dulbecco's A phosphate buffered saline tablets.

Pharmacia Ltd., Paramount House, 75 Uxbridge Road, London, England.
DEAE dextran, dextran sulphate.

Reidel de Haen, D-3016 Seelze Hanover, Germany.
Absolute alcohol, acetone, methanol.

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Sodium bicarbonate, Hepes buffer, EDTA, dimethylsulphoxide (DMSO), trypan blue (0.4%), Hoechst stain, insulin (bovine pancreas), human transferrin, (Catalogue numbers T1147 and T5391), sodium selenite, estradiol, PGE_1, $PGE_{2\alpha}$, hydrocortisone, progesterone, retinoic acid, bombesin, glycyl-hystidyl-lysine, glucagon, triiodothyronine, phosphoethanolamine, collage Type III. (Acid soluble from calf skin).

Sterilin Ltd., 43-45 Broad Street, Teddington, Middlesex, England.
25 cm^2 flasks, 35mm dishes, 9cm petri dishes, 10ml pipettes, 30ml universals, autoclave bags, cryotubes.

Stuart Ltd., U.K.
Magnetic stirrer.

Union Carbide, U.K. Ltd., Aycliffe Ind. Estate, Nr. Darlington, Co. Durham.
Liquid nitrogan freezer unit.