ANCHORAGE - INDEPENDENT GROWTH OF

HUMAN TUMOUR CELLS

BY

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ABBREVIATIONS

F.C.S.	-	Foetal calf serum
C.F.E.	-	Colony forming efficiency
U.F.	-	Ultrafiltered
c.m.	-	Conditioned medium
I.M.S.	-	Industrial methylated spirits
EGF	-	Epidermal growth factor
TGF	-	Transforming growth factor
p.s.i.	-	Pounds per square inch
kPa	-	kilo Pascals
ATPase	-	Adenosine triphosphatase

ABSTRACT

The cell culture clonogenic assay appears to have considerable application in predicting individual cancer patient response to chemotherapy. There are several obstacles in the way of its successful application, however, including problems of extrapolating *in vitro* tests to the *in vivo* situation, and technical problems related to successful selective *in vitro* growth of primary human tumour cells. The work in this thesis addresses this latter area, using a human carcinoma line RPMI 2650 as a model system.

Different disaggregation methods were compared; after short-term incubation at 37°C, trypsin (0.25%) - EDTA (0.02%) produced the best viable single - cell suspension of RPMI 2650 cells. Following dissociation, RPMI 2650 cells and normal human fibroblasts were found to reaggregate spontaneously to form clusters. Clumps and clusters in cell suspensions contributed to final colony forming efficiencies; the significance of clumps and clusters in the clonogenic assay is discussed.

Use of low gelling temperature agaroses improved colony forming efficiencies under some conditions examined. Use of ultrapure water in the preparation of semi-solid media also improved colony formation.

Different incubation atmospheres were compared; RPMI 2650 colony formation was improved when the oxygen concentration was reduced from 20% to 3%. Colonies did not, however, grow under anaerobic conditions.

Medium without foetal calf serum did not support RPMI 2650 colony formation; growth was also poor in 1% and 2% f.c.s. Inclusion of HITES supplements did not reduce the requirement for serum. Other growth

factors tested include TCGF, EGF, insulin and hydrocortisone.

Use of feeder cells improved colony forming efficiencies considerably; best results were obtained with mitomycin C-treated feeder cells. Inhibition was observed where high densities of feeder cells were used with high test cell densities.

The contribution of cell cycle to final colony formation was examined but did not appear to be a contributory factor to the low cloning efficiencies observed.

Normal human fibroblasts did not grow in either agar, agarose or methycelluloses, thus confirming the selective nature of the assay used in this laboratory.

Colony formation in agarose was inhibited at a lower ouabain concentration than in monolayer; thioguanine inhibited colony formation in agarose and monolayer at the same toxic level. The significance of these findings is discussed and makes some contribution toward an understanding of the nature of anchorage-independent growth.

SECTION 1.

INTRODUCTION.

INTRODUCTION

1.1 Anchorage - Independent Growth

Normal cells, with a few exceptions (Peehl and Stanbridge, 1981), do not multiply in liquid or semi-solid suspension cultures. Cancer cells and many established cell lines will grow in suspension. MacPherson and Montagnier (1964) first described anchorage-independent growth as the ability of virus "transformed" cells to grow unattached to a solid surface. They used growth in semi-solid suspension cultures as an *in vitro* test of malignancy in polyoma transformed baby hamster kidney (BHK21) cells which could be grown as colonies in soft agar (unlike the untransformed parent BHK21 cells). Subsequently, it has been shown that colony formation in suspension is frequently enhanced following viral transformation (Shin *et al.*, 1975). The position regarding spontaneous tumours is less clear, however, in spite of the close correlation reported between biological potential for malignancy, as manifested by growth and tumour formation in an appropriate animal host, and anchorage - independent growth and colony formation in soft agar. Colburn *et al.*, (1978) studied transformation in BALB/C mouse epidermal cells following treatment with chemical carcinogens, and reported a strong correlation between anchorage - independent growth and tumour formation in syngeneic mice. Montesano *et al.*, (1977) compared the soft-agar growth of 22 tumours and non-tumourigenic rat liver epithelial cultures and found a good correlation with tumourigenicity. Freedman and Shin (1974) compared the tumourigenicity of permanent cell lines (which were originally derived from both normal and malignant tissues), in nude mice and anchorage - independent growth in methylcellulose and reported that anchorage - independent growth in a semi-solid medium was the *in vitro* property which consistently correlated with neoplastic growth in nude mice.

This correlation was further refined by Shin et al. (1975) who showed a strong relationship between anchorage independence and the tumourigenic properties of cells.

MacPherson (1973) suggests that the selectivity of agar suspension culture is due to the inhibitory effect of acidic and sulfated polysaccharides in most agars on normal cell multiplication. Neugut and Weinstein (1979) suggest that agar may allow only the most highly transformed cells to grow while agarose (lacking sulfated polysaccharides) is less selective. Normal cells also require attachment to a solid substrate in order to undergo cell division. Agar medium in which the polyanions have been complexed with DEAE-dextran is capable of supporting limited multiplication of some cell lines unable to grow in untreated agar. Conversely, Montagnier (1968) was able to show that untransformed BHK21 cells, which would grow in agarose but not in agar, could be prevented from growing in agarose by the addition of dextran sulfate.

Despite the frequently reported good correlation between malignant cellular behaviour and anchorage - independent soft-agar growth and the fact that anchorage - independent soft-agar growth is probably the currently best accepted in vitro criterion of malignancy, results from other studies dispute the general validity of growth in suspension as a property of only tumourigenic cells. Several investigators (Gallimore et al., 1977; Stanbridge and Wilkinson, 1978) have shown that certain transformed nontumourigenic cell lines exhibit anchorage - independent growth, whereas others have reported that a number of tumourigenic cell lines retain their anchorage dependence (Marshall, 1979). Hamburger and Salmon (1977) have shown that many human tumours contain a small percentage of cells (<1.0%) which are clonogenic in agar. Courtenay et al. (1977) attempted to culture 40 fresh human tumours from a variety of tissues in soft-agar. Colonies were noted in 22 of these soft-agar cultures with a plating efficiency

varying from 0.02 to 15%. Freshney and Hart (1982) and others (Laug et al., 1980) have shown that a number of normal cells will also clone in suspension with equivalent efficiency. In the cloning of malignant brain tumours by Thomas et al., (1982), suspension cloning was ultimately stopped as in their hands "normal brain cultures had similar cloning efficiencies to glioma cell cultures". Minor modifications of the culture medium may also support the cloning of non-malignant cells. In a report by Peehl and Stanbridge (1981) the addition of hydrocortisone and elevated serum levels to basal medium supported the cloning of normal fibroblasts. Asano et al., (1981), noted that while the use of PHA - conditioned medium significantly improved the colony formation of breast carcinoma in soft agarose, the colonies cultured were not of malignant cell origin, but T lymphocytes. These data stress the need to confirm the nature of cultured cells, particularly where added growth factors are used, and question the value of this technique for assaying for the presence of tumour cells in short-term cultures from human tumours.

Many of the properties associated with neoplastic transformation in vitro are the result of cell surface modifications (Hynes, 1974; Nicolson, 1976). For example,changes in the binding of plant lectins (Willingham and Pastan, 1975; Reddy et al., 1979) and in cell surface glycoproteins (Hynes, 1976; Llyod et al., 1979; Van Beek, 1979; Warren et al., 1978), may be correlated with the development of metastasis and invasion in vivo. Transformed fibroblasts lose fibronectin (LETS protein, large extracellular transformation sensitive protein) from the cell surface. Easty et al., (1960), suggest that the loss of fibronectin may contribute to a decrease in cell-cell and cell-substrate adhesion and subsequently to a decreased requirement for attachment and spreading for cells to proliferate (Dodson et al., 1983). This results in cells being able to grow detached from the substrate, either in stirred suspension culture or suspended in semi-solid media such as agar or methylcellulose

(MacPherson, 1973).

Anchorage - independent growth remains a valuable technique for assaying neoplastic transformation *in vitro* by tumour viruses and has also been used extensively by Styles (1977) to assay for chemical carcinogens. Zimmerman and Little (1983) transformed normal human fibroblasts to anchorage - independence by treatment with N - acetoxy - 2 - acetylaminofluorene. Other methods of transformation studied include X-ray induced transformation (Borek, 1980), propane sulfone induced transformation (Silinskas *et al.*, 1981) and ultraviolet light induced transformation (Sutherland *et al.*, 1980) of human cells to anchorage independent growth.

The disadvantages of carcinogenicity testing in this manner are that the cells most often used (e.g. mouse 3T3) have high rates of spontaneous transformation and are often tumourigenic before treatment, thus strict culture conditions are required to maintain anchorage - dependent growth; also, *in vivo* carcinogenesis is a multi-step process, and some of the steps - e.g. tumour promotion, may be easily reproduced *in vitro*.

1.2 Chemosensitivity Testing

It is possible to treat cells *in vitro* with drugs and chemicals which are routinely used in cancer chemotherapy and subsequently to determine cell sensitivity to treatment. The possibility has been considered (Hamburger and Salmon, 1977) that measurement of the chemosensitivity of cells derived from a patient's tumour might be used in predicting patient response to chemotherapy. Since different patients

frequently vary in their response to the same drug treatment, predictive chemosensitivity testing could be used in designing a chemotherapeutic regime on an individual patient basis.

However, one major problem associated with most chemosensitivity tests is that the end-points used to assess toxicity (for example, dye exclusion assays and morphologic assessment of drug-induced damage in monolayer) measure the effects of a drug on an entire population of cells which includes both normal and neoplastic cells. While results from assays which contain normal cells may be misleading, it has also become apparent that the population of neoplastic cells within a tumour is heterogenous in its growth characteristics. Cell kinetic studies of normal tissues have lead to the concept that for every renewing tissue in the body there is a subpopulation of cells that can reproduce themselves and also give rise to a differentiating line of mature and functional cells (Inoue et al., 1978; Woodruff, 1983; Heppner, 1984). These subpopulations of proliferating cells are frequently referred to as stem cells or "progenitor cells". It is possible that human tumours have a similar "stem" cell population which may have different growth characteristics compared to the total tumour cell population (Salmon, 1984; Salmon and Von Hoff, 1981). Proliferating cells from which a tumour may be propagated in immunosuppressed animals or in tissue culture may be referred to as clonogenic cells. If the "stem" cell theory is correct, then the best in vitro assay system for predicting responses to cytotoxic agents should selectively measure the effects of drugs on this cellular population.

A major development in culturing stem cells from human tumours came with the work of Hamburger and Salmon (1977). Using a modification of the two-layer soft agar system similar to that described by MacPherson (1973) with a bottom layer containing medium conditioned by spleen cells from BALB/C pristane-primed mice and a variety of other growth factors, colonies

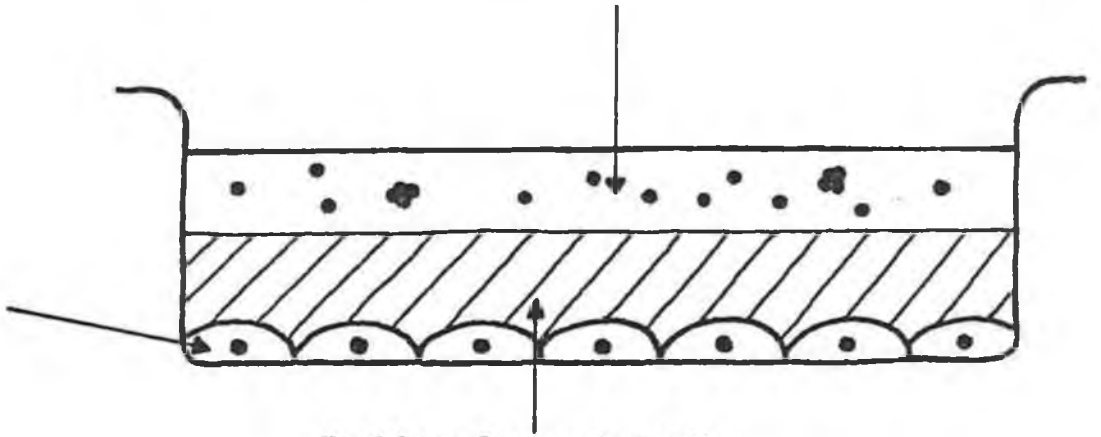
were grown from 75% of a large number of multiple myeloma bone marrow aspirate specimens. The in vitro clonogenic assay (Hamburger and Salmon 1977) is schematically illustrated in Figure 1.1. Following disaggregation procedures, a single cell suspension is prepared and mixed with culture medium and 0.3% agar (or agarose) at 40°c. One ml of the mixture is then plated, usually in triplicate, into 35mm plastic petri dishes over a base layer of 0.5% agarose (V/V) in culture medium that has hardened. Colonies (cell aggregates of 50 or more cells) are usually observed 7-21 days after plating and scored using an inverted phase microscope.

At the same time Courtenay and Mills (1978) also reported on the use of a soft agar assay for the culture of human tumours. Although similar in concept the technical aspects and design of the Courtenay assay were somewhat different from the Hamburger and Salmon assay (notably in the inclusion of August rat red blood cells and incubation in a low-oxygen atmosphere).

Since the initial reports of Hamburger and Salmon, many investigators have utilized the same culture system to assay for clonogenic cells in a variety of human tumours and in evaluating the in vitro chemosensitivity of tumour cells to a variety of cytotoxic compounds including agents in current use or under investigation (Alberts et al., 1980; Von Hoff et al., 1981; Meyskens et al., 1981; Rosenblum et al., 1982; Salmon et al., 1978; Hamburger, 1981).Salmon (1978), Salmon et al., (1978) and Salmon and Von Hoff (1981) describe in detail the methods used for in vitro chemosensitivity. Briefly, single cell suspensions obtained after dissociation of human specimens are incubated (usually for 1hr) with varying concentrations of drugs under study. After exposure, cells are washed free of drug and plated in the clonogenic assay. Following 2-3 weeks incubation, plates are counted and colony survival in control plates is compared with colony survival in drug treated plates. In most studies a mean colony count of 30 in

"Soft agar" growth medium containing

0.33%. AGAR(OSE) + Single-cell suspension.



"Feeder Layer"

of cells

(if used).

Hard base-layer of growth

medium containing 0.5% AGAR(OSE).

FIGURE 1.1.

CLONOGENIC ASSAY IN SEMI-SOLID MEDIUM

control plates was required for the assay to be considered evaluable. Tumours are classified as sensitive *in vitro* to specific drugs if tumour colony survival is 30% or less than is observed in control cultures at a specific concentration of drug. Colony formation in excess of 50% was considered to indicate resistance of tumour cells, while levels between 30-50% survival was considered to indicate "intermediate" sensitivity.

The predicting ability of this procedure has been tested in many laboratories (Buick *et al.*, 1980; Carney *et al.*, 1980; Von Hoff *et al.*, 1981; Salmon, 1982; Tveit *et al.*, 1982; Niell *et al.*, 1983; Fan *et al.*, 1984) since Salmon *et al.*, (1978) demonstrated a 95% patient correlation between drug resistance *in vitro* and a lack of tumour response *in vivo* and a 65% patient correlation between drug sensitivity *in vitro* and a positive clinical response. Currently, the selection of chemotherapeutic agents for individual patients is empirically made on the basis of how other patients with the same type of cancer have responded. This approach suffers from the large and unpredictable variation in response of different patients to the same drug treatment. Therefore, human tumour chemosensitivity in the soft-agar assay may indicate the proper choice of drugs and the effective concentrations so that unnecessary drug trials in the same patient can be avoided.

1.3 Limitations of the Clonogenic Assay

Since Hamburger and Salmon (1977) reported on the successful *in vitro* cloning of multiple myeloma specimens from bone marrow aspirates and the growth of a small number of other tumours,many investigators have evaluated this culture system for the growth of fresh tumour specimens (Kern *et al.*, 1982; Courtenay and Mills, 1978; Rosenblum *et al.*, 1978; Carney *et al.*, 1980; Carney *et al.*, 1981; Kitten *et al.*, 1982). Several different tumour types

have been cloned in semi-solid media; for example, lung tumours (Kitten et al., 1982), breast cancers (Sutherland et al., 1983), and ovarian tumours (Bertoncello et al., 1982), have been cloned with considerable success. Other types of tumour tissue cultured as colonies in semi-solid media include Wilm's tumour (Dow et al., 1982), malignant melanoma (Pavelic et al., 1980; Gupta et al., 1983; Joyce and Vincent, 1983; Meyskens et al., 1983), soft-tissue sarcoma (Kern et al., 1982; Pavelic et al., 1980), colon carcinoma (Kern et al., 1982), bladder cancer (Kirkels et al., 1982; Pavlik et al., 1983) and a variety of histologically unidentified tumours.

Carney and Winkler (personal communication) report that for a total of 7048 specimens evaluated, tumour cell colony growth has been observed in 14-95% of the tumours tested in different studies with colony forming efficiency (CFE) for most tumour types ranging from .001-0.1%, representing a colony number per 500,000 cells plated ranging from 5-500. If one assumes that a minimum of 30 colonies in each control plate is required to sucessfully evaluate the in vitro chemosensitivity of tumour cells to individual drugs, then the number of specimens suitable for in vitro drug testing falls to approximately 40% of all specimens (range 14-73%).

Although tumour cell colony formation has been observed for most tumour types in the clonogenic assay, a major problem is the poor colony forming efficiency for individual tumours (ranging from .001-0.1%). Even if one allows for the large fraction of admixed normal cells in a given biopsy, and recalculates the CFE based on the number of tumour cells plated, the CFE is still only 1-2% (Carney, 1981). Whether this low CFE is indeed a reflection of a small "stem-cell" compartment of individual tumours, or a result of the suboptimal conditions used both to disaggregate biopsies and to culture cells, remains uncertain. However, it is clear that many factors may influence the CFE of individual tumours.

Kitten et al., (1982) summarise the data which has been collected with regard to the clonogenic capacity of various pulmonary carcinomas.

Cell suspensions from solid tumour samples had a mean viability of 33% with a range from 0 to 98%, whereas fluid samples had a mean viability of 73% with a range of 0 to 100%. In all cases examined the colony forming efficiencies were less than 0.1%. Bertoncello et al., (1982) report upon the failure of some tumour cells to grow in soft agar. The reason for this poor cloning of solid tumours is unclear, but most likely reflects cellular damage that occurs during dissociation procedures but which may not be detected using dye exclusion techniques (e.g. trypan blue exclusion) to assay for cell viability.

A major problem that still exists with the clonogenic assay is the difficulty in obtaining a viable single cell suspension. While mechanical techniques may ultimately lead to a single cell suspension, in most cases aggregates of cells usually remain. Agrez et al., (1982) and Selby et al., (1983) have considered the effects of cell aggregates in the assay which can have major effects on the characteristics of the culture and on the measured response of the colonies to treatment with cytotoxic and anti-neoplastic drugs. To improve methods used to obtain single cells, many investigators have compared enzymatic techniques (using for example, combinations of DNase, pronase and / or collagenase) with mechanical techniques for the dissociation of tumour specimens. Pavelic et al., (1980) compared enzymatic and mechanical disaggregation techniques on a number of tumour types and the colony forming efficiencies which result in soft agar. Enzymatic disaggregation proved to be the most successful disaggregation technique for melanomas and sarcomas, giving a larger number of colonies in soft agar. However, in most reports, while the number of single cells obtained is greater with enzymatic techniques, no significant differences in CFE have been observed compared to that seen with mechanical dissociation (Kern et al., 1982; Pavelic et al., 1980).

Several reports suggest that factors which may improve the CFE of individual tumours are secreted by the admixed normal cells, in particular, macrophages (Salmon and Hamburger, 1978; Buick et al., 1980; Hamburger and White, 1981). In their initial studies of multiple myeloma, Hamburger and Salmon (1977) demonstrated that colony growth of these cells could be improved if conditioned medium from viable adherent macrophages was added to the culture medium. The effect was optimal in the presence of 2 - mercaptoethanol.

Since it has been reported that cell - free ascites may stimulate proliferation of neoplastic cells (Vaage and Agarwal, 1979), Uitendaal et al., (1983) tested the human tumour colony stimulating activity of cell free ascites on human ovarian tumour cells and found a marked increase in the plating efficiency. Similarly, other investigators (Hamburger and Salmon, 1981; Whelan and Hill, 1981) report upon the proliferative effect of lethally irradiated fibroblast feeder layers, concluding that feeder layers increase the cloning efficiencies of stem-cells in the assay. These studies suggest that normal/malignant cell interaction may be essential for optimum growth of tumour cells in vitro. The isolation and characterisation of such "growth factors" may lead to an overall improvement of tumour cell growth in the clonogenic assay.

While the above and other factors such as the nature of the semi-solid support (e.g. agar versus agarose versus methylcellulose (Pavlik et al., 1983)) may in a small way influence the CFE of individual specimens, it is most likely that improvement in the overall growth and CFE will emerge with improvement in the culture medium used for growth. While some factors such as epidermal growth factor may be mitogenic for different tumour types (Hamburger and Salmon, 1981; Pathak et al., 1982), Barnes et al., (1981) and others (Barnes and Sato, 1980; Carney et al., 1981; Calvo et al., 1983), have clearly demonstrated that specific growth factors

can significantly improve the growth of human tumours in vitro. In addition, requirements for individual tumours may vary from one histologic type to another (Barnes et al., 1981; Carney et al., 1981; Gazdar et al., 1983). Initially using established cell lines of a specific tumour type, and then testing the factors identified on fresh specimens of the same type, it has been demonstrated that the growth rate in culture of primary tumour specimens is substantially improved using specific growth factor/hormone supplemented media (Carney et al., 1981). Thus, while improvement may occur in the methods for obtaining and processing clinical tumour specimens, yielding cell suspensions of significantly higher viability, most improvements in the CFE of tumours will occur when growth conditions are optimised for each tumour type. Although it is likely that each histologic type will have different growth requirements for optimal growth, when these conditions are defined it will be a relatively simple matter (according to this hypothesis) to have each set of growth-factors "packaged" so that when a tumour of a specific histologic type is processed, then the specific and appropriate medium for optimal growth of that tumour will be used.

In addition to specific growth factors, several investigators have shown that, for a variety of tumour specimens, lowering of the oxygen tension from the usual of approximately 20% to as low as 3% will lead to significant improvement in the CFE (Gupta and Krishan, 1982; Kennedy et al., 1982; Gupta et al., 1983; Sridhar et al., 1983). Hill and colleagues (1980) have consistently observed that this improved the CFE of cells in the "Courtenay" assay (Courtenay and Mills, 1978) compared to the Hamburger/Salmon assay (1977). Similar results have been reported by Tveit et al., (1981). Thus, regulation of the oxygen tension may be an important consideration in the cloning of primary tumour specimens. However, modifications of growth conditions may not only alter the growth pattern of the plated cells, but may alter the pattern of cellular chemosensitivity observed in this assay.

Gupta et al., (1982) have shown that low oxygen tension will improve CFE of melanoma cells. However, a 4 - to 20 - fold enhanced sensitivity to a variety of chemotherapeutic agents including bleomycin, doxorubicin, cisplatin and 1,3 - bis (2 - chloroethyl) 1 - nitrosourea (BCNU) was seen in cells incubated under the low oxygen concentration. Other studies have also shown the importance of oxygen tension on in vitro cytotoxicity. Kennedy et al., (1983) using Sarcoma 180 and Em T6 cells in vitro, showed that doxorubicin, claunomycin and mitomycin C were selectively toxic to hypoxic cells. However, in their study BCNU exhibited equal cytotoxicity toward normally aerated and hypoxic cells while streptonigrin was selectively toxic toward normally aerated cells.

Similarly, the addition of hormones, particularly with "endocrine" sensitive tumours (e.g. breast and prostate cancer), may alter the sensitivity of these tumours to cytotoxic agents, although a study by Calvo et al., (1983) using breast carcinoma cell lines revealed that while the addition of growth factors and hormones improved the CFE of the cells, no alterations in the pattern of sensitivity to a variety of cytotoxic agents was observed. These studies show that when changes in growth conditions are made, one may have to alter in vitro definitions of sensitivity or resistance to cytotoxic agents.

Other factors which in a minor way can lead to improvement in colony growth include the elimination of some factors originally described in the Hamburger and Salmon assay which, while stimulatory for some cell types, are toxic to others (Hug et al., 1983); the use of different types of semi-solid materials (e.g. agar, agarose, methylcellulose) (Pavlik et al., 1983); the development of better ways to dissociate cells (Hamburger et al., 1981); and the use of density centrifugation techniques to eliminate unwanted or non-viable population of cells and which may select for the clonogenic

portion in the tumour cell population (Clynes et al., 1980; Mavligit et al., 1973; Hamburger et al., 1983).

1.4 Factors to be tested

Using fresh human tumour explants, Salmon et al., (1978) and Von Hoff et al., (1981) reported that in vitro inhibition of colony formation by chemotherapeutic agents correlated with clinical response of individual patients. Application of this technique has been limited (Bertoncello et al., 1982) by the low cloning efficiencies of many tumours tested. The poor success of the assay with respect to the cloning of solid tumours in semi-solid media (Kern et al., 1982) emphasises the need for optimization of the assay. In an attempt to optimize the assay, this project examined the effects of different factors on the anchorage-independent growth of RPMI 2650 cells. This cell line was chosen for experimentation for the following reasons:-

(A) RPMI 2650 cells are human tumour cells, established from a tumour which was diagnosed as an anaplastic squamous cell carcinoma (Moore and Sandberg, 1964). The cells are epithelial-like which is characteristic of primary tumours which are difficult to culture in vitro (carcinomas, or tumours of epithelial origin, constitute about 85% of human solid tumours).

(B) RPMI 2650 cells have a near normal karyotype and are available at a low passage level; also RPMI 2650 cells have low colony forming efficiencies; these properties are more characteristic of primary tumours than of cell lines which have been in culture for very many population doublings e.g. Hela cells.

Based on leads from the cell culture literature, and some novel ideas, we decided to investigate the influence of the following parameters on the growth of clones of RPMI 2650 in semi-solid media:

(a) Method of disaggregation (Section 3.2).
(Slocum et al., 1981; Pavelic et al., 1980).

(b) Influence on the assay of the spontaneous aggregation of cells (Section 3.3).
(Agrez et al., 1982; Wright et al., 1977).

(c) The gas phase (Section 3.6).
(Courtenay and Mills, 1978; Gupta and Krishan, 1982).

(d) Nature of the semi-solid support (Section 3.7).
(Dixon et al., 1981; Pavlik et al., 1983).

(e) Degree of purity of the water (Section 3.5).

(f) Serum levels (Section 3.8).
(Cowan et al., 1982; Peehl and Stanbridge, 1981).

(g) Use of growth factor - supplemented medium (Section 3.9).
(Carney et al., 1981).
Growth factors (Section 3.10).
(Todaro et al., 1981; Pathak et al., 1982; Hamburger et al.,1983).

(h) Use of feeder cell layers (Section 3.12).
(Hamburger et al., 1981; Whelan and Hill, 1981).

(i) Use of conditioned media (Section 3.13).
(Hamburger and Salmon, 1977).

(j) Stage of growth of cells (Section 3.14).

Some work was also undertaken relating to the nature of anchorage-independent growth (Peehl and Stanbridge, 1981) and towards applying the results obtained to chemosensitivity testing (Salmon, 1978; Salmon et al., 1978; Salmon and Von Hoff, 1981) (Sections 3.15 and 3.16).

1.5 Conclusion

There can be little doubt that clinical oncologists would like to have available to them a reliable system of predictive chemosensitivity testing. The facility of sending to the laboratory a small piece of tumour tissue from a readily accessible site and receiving back within a few days a piece of paper stating which of a panel of cytotoxic drugs will kill a high proportion of the malignant cells present in the sample at clinically achievable drug exposure levels would radically alter the basis of current chemotherapeutic practice. However, despite the large expansion of research directed towards this goal, for the bulk of common solid tumours, the availability of such a reliable and accurate predictive test remains a rather distant objective.

The problems associated with testing can be divided into those associated with particular methods and those which are basic to the whole concept of such testing. In the latter group one can mention in particular the relationship between cell response *in vitro* and tumour response *in vivo*, the sampling problem associated with tumour heterogeneity and the whole question of *in vitro* representation of *in vivo* pharmacology (Kern *et al.*, 1984; Von Hoff, 1983). However, it is to the former group of problems that this project was directed, in an attempt to optimise the clonogenic assay for a model epithelial cell system and gain some insight into the biology of RPMI 2650 cells and the nature of anchorage independent growth.

SECTION 2

(A) MATERIALS

AND

(B) METHODS

2. (A) MATERIALS

Materials were obtained from the companies listed below.

American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852:

RPMI 2650 cells.

Becton - Dickinson & Co. Ltd., Wembly, Middlesex, England:

'Plastipak' syringes and syringe needles, gauge 26G⅜ 10/45.

BDH Chemicals Ltd., Poole, England:

Industrial methylated spirits, acetone, formaldehyde, absolute alcohol, methanol, May and Grünwald stain, Leishmans' stain, sodium hydrogen carbonate (sodium bicarbonate), hydrochloric acid, potassium chloride, magnesium chloride, magnesium sulphate.

Boehringer Mannheim Gmbh - W. Germany:

Dispase (GradeII), DNase I (GradeII).

Bethesda Research Laboratories (UK) Ltd.,:

Mycoplasma Detection system (BRL Mycotect).

Calbiochem - Behring Corp. La Jolla CA 92037, U.S.A.:

Pronase (Activity : 45,000 PUK/g).

Chemical Products, R. Borghgraef s.p.r.l. Belgium:

RBS 25 Detergent.

Collaborative Research, 1365 Main St. Waltham, Mass 02154:

Epidermal growth factor (culture grade), insulin (non-cytotoxic), human T-cell polyclone (Activity: 1,150 half-maximal units of human T-cell growth factor activity per 10 ml.).

Corning, 490 San Antonio Rd. Palo Alto, CA 94306:

Corning isoenzyme electrophoresis kit.

Dow Chemical Company, Europe:

Methylcellulose (Methocel).

Difco Laboratories Ltd., Detroit, M: 4823, U.S.A.:

Bacto - Agar.

Edwards, U.S.A.:

Silicone high vacumn grease.

Fluka AG, Bucks, SG, Switzerland:

Methylcellulose (Viscosity, 4000cP).

F.M.C. Corporation, Marine, Colloids Div. Rockland, ME 04841, U.S.A.:

'Seaplaque' (low gelling temp. agarose), 'Seakem' agarose, 'Seaprep' (ultra low gelling temp. agarose).

Flow Laboratories, England:

Modular Incubator Chamber.

Gibco - Biocult Ltd., 3 Washington Rd, Sandyford Industrial Estate, Paisley, Scotland PA34EP:

Culture media, antibiotics, foetal calf-serum, trypsin, non-essential amino acids, trypan blue, balanced salt solutions, glass bottles.

Gibco Ltd., Grand Island N.Y. 14072, U.S.A.:

Amphotericin B (Fungizone).

George T. Gurr: Searle Scientific Services, High Wycombe, Bucks:

Giemsa stain.

Hopkin and Williams, Chadwell Heath, Essex, England:

Potassium phosphate.

I.C.I. Ireland Ltd., 59 Sth. Frederick St., Dublin 2.:

Sodium hypochlorite (Chloros).

Irish Industrial Gases, Bluebell estate, Tallaght:

Nitrogen gas, 5% CO_2 /air mix, 5% CO_2 /3% O_2 /N_2 mix, 10% CO_2 /N_2 mix.

Koch - Light Labs. Ltd., Colnbrook SL3 0B7 Bucks, England:

Sodium hydroxide.

Merck 6100 Darmstadt, Germany:

Nutrient agar (Standard II).

Millipore S.A. 78 Buc. France:

Sterile disposable filters.

Miles Laboratories Ltd., Stoke Poges, England:

Marine colloids (UK) agaroses; highest electroendosmotic agarose (HEEO), high electroendosmotic agarose (HE), medium electroendosmotic agarose (ME), Low electroendosmotic agarose (LE) and low gelling temp. agarose (LGT).

Novo Industri Als, Denmark: United Drug Distributors, Dublin Industrial Estate, Glasnevin, Dublin 11:

'Actrapid' insulin.

Nunc, Denmark:

Sterile tissue culture flasks, petri dishes.

Oxoid Ltd., Southwark Bridge Rd. London SE1 9HF:

Dulbecco's phosphate buffered saline tablets, resazurin anaerobic indicators.

Riedel - De Haen AG Seelze - Hannover:

Calcium chloride, sodium chloride, sodium phosphate.

Sigma Chemical Company P.O. Box 11508 St. Louis Mo. 63178 U.S.A.:

Type I (low electroendosmosis) agarose, type II (medium electroendosmosis) agarose, type III (high electroendosmosis) agarose, type IV (special high electroendosmosis) agarose, type VII (low gelling temp.) agarose.
Bovine serum albumin, HEPES, EDTA, collagenase type II, collagenase type IV, collagenase type V, transferrin (human), β-estradiol, sodium selenite (selenium), mitomycin C, ouabain octahydrate, citric acid, coomassie blue G-250, INT (2-(4- Iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride), Hoechst 33258 stain, thioguanine, DMSO (Dimethyl-sulfoxide) grade I.

Sterilin Ltd.,43-45 Broad St. Teddington Middlesex, England:

Plastic petri dishes, disposable sterile pipettes.

Upjohn Ltd., Fleming Way, Crawley, Sussex:

Hydrocortisone sodium succinate (Solu-Cortef).

All reagents and chemicals used were of analytical reagent grade where possible.

2. (B) METHODS

2.1. GLASSWARE.

Items of glassware used for dispensing and storage of media must be very carefully cleaned to avoid traces of toxic materials, contaminating the inner surfaces, becoming incorporated into the medium. Therefore, the requirements of tissue culture washing are higher than for general glassware.

The following procedure was observed for effective glassware washing:-

(1) Soiled glassware was not allowed to dry out but was soaked in a sterilizing solution of 0.1% sodium hypochlorite which removes any potential biohazard and prevents microbial contamination growing up in the water.

(2) 2% (V/V) RBS 25 was prepared in tap water at 50°C. RBS 25 is a surface active detergent which cleans effectively, rinses off easily and is non-toxic. Soiled glassware was soaked in this solution for 1-2 hrs.

(3) Glassbottles etc., were brushed by hand, rinsed three times in tap water, three times in reverse osmosis-purified water, and once in ultrapure deionized water, to prevent lime deposits forming, before drying.

(4) Finally, glassware was dried inverted in a drying oven before storing.

Glass bottles and aluminium screw-caps which were used for media preparation were supplied by GIBCO Ltd., Scotland. Reusable screw-caps

were lined with a synthetic non-toxic (silicone-type) seal, and both caps and liners were washed separately from glassware as the aluminium could possibly act as a glass contaminant.

2.2 STERILIZATION.

The major requirement that distinguishes tissue culture from other laboratory techniques is the need to maintain asepsis. This is accentuated by the much slower growth of cultured animal cells relative to most of the major potential contaminants. To eliminate micro-organisms and spores moist heat is very effective but does carry a risk of leaving a residue. For moist heat to be effective, steam penetration must be assured and for this the sterilization chamber must be evacuated prior to steam injection.

For large-scale sterilization of glassware, instruments and heat-stable solutions the following procedure was observed:-

(1) Ultrapure water was placed just above the level of the heating element in the autoclave (Portable Universal "Express" electric model, pressure steam sterilizer, Arnold & Sons Ltd., Basildon).

(2) Bottles for sterilization were loosely capped with screw-caps and labelled with 3M autoclave tape.

(3) The autoclave was brought to boiling and steam was allowed to escape before sealing.

(4) When the pressure had built upto 100kPa (15p.s.i.) the temperature was maintained at 120°C for 20 min.

(5) After cooling, screw-caps were tightened on the bottles.

Solutions which are heat-labile e.g. hydrocortisone, were sterilized by filtration through 0.22µm pre-sterilized disposable membrane filters. Filtration through a membrane of 0.22µm pore size excludes contaminants with the exception of certain viruses and mycoplasma. Several different filters from the Millipore Corporation were used in accordance with table 2.1

Table 2.2 demonstrates sterilization methods used and storage of routinely used reagents. Plastic culture ware which was used was pre-sterilized by the manufacturers by Y-irradiation, since plastics cannot be exposed to temperatures required by autoclaving or dry-heat sterilization. Exposure to 20,000 - 100,000 rad is the most suitable sterilization method for plastics.

TABLE 2.1

Specificity of Millipore Filter Units

Filter Unit	Description	Membrane Material	Uses
Millex-GS	Blue, Single-use filter unit. Non-pyrogenic, sterile. 0.22µm, membrane. Max. pressure 5 bars. Filter sixe:25mm diameter. Filter area: approx.3.9cm^2	M.F.membrane. Mixed esters of cellulose.	Sterilization/ ultracleaning of small volumes of aqueous fluids (tissue culture medium).
Millex-GV	Yellow (as above)	Hydrophilic Durapore membrane Polyvinylidene difluoride.	Sterilization of small volumes of dilute protein solutions(enzymes, hormones,interferons etc.)dispensed by syringe.
Sterifil-D	Blue disposable filter unit.0.22µm membrane. 150ml capacity. Vacumn pressure required.	M.F.-membrane Mixed esters of cellulose.	Sterilization of intermediate volumes of culture media, buffer and other aqueous based solutions.

TABLE 2.2

STERILIZATION AND STORAGE OF REAGENTS

* Autoclavable Instruments	* Autoclavable Liquids	Filter-Sterilizable liquids	Storage °C
Disposable Micro-Pipette Tips	Agars/Agaroses	Amino Acids	4
Glassware	Methylcelluloses !	Antibiotics	-20
Glass Bottles with screw-caps	EDTA	B.S.A.	4
Glass coverslips	HEPES	Collagenase	-20
Glass slides	1.5N HCl	Glutamine	-20
Forceps	1.5N NaOH	Serum	-20
Scalpels	$NaHCO_3$ (7.5%)	Transferrin	-20
Scissors	P.B.S.	Insulin	4
Magnetic stirring bars	Water	Estradiol	-20
Cloning rings	5N NaOH	Selenium	4
Silicone grease		Hydrocortisone	4
		Dispase	-20
		EGF	4
		DNase	-20
		Trypsin-EDTA	-20
		Human T-cell polyclone	4
		Mytomycin C (in dark)	4
		Ouabain	4

* Stored at room temperature.

! Stored at 4°C, autoclaved at 100kPa (15p.s.i.) for 40 min.

2.3 STERILE TECHNIQUE

All sterile manipulations of cells were performed in a vertical laminar flow cabinet (Intermed; MDH Ltd., Hampshire, England). The major advantage of working in laminar flow is that the working environment is protected from dust and contamination by a constant stable flow of filtered air passing over the work surface.

While working in laminar flow the following procedure was observed:-

(1) To remove any possible contaminants, the laminar flow was run for 15-20 mins. A microbunsen burner was lit and placed in the laminar flow at this stage to allow the vertical air-flow to equilibrate.

(2) The work surface of the laminar flow was swabbed down with 95% I.M.S. before and after use.

(3) All media bottles and instruments were swabbed with 95% I.M.S. before being introduced into the laminar flow cabinet.

(4) The necks of bottles were flamed and rotated in the bunsen flame prior to opening and closing.

(5) Disposable pipettes and the necks of disposable culture flasks were briefly flamed to avoid melting the plastic.

(6) All dirty glassware and used medium were autoclaved to remove possible biohazards.

(7) Glassware was then thoroughly cleaned as described previously.

2.4 STERILITY TESTING

Bacteria, yeasts, fungal spores and mycoplasma all appear as contaminants in tissue culture. They may be introduced via the operator, the atmosphere, work surfaces, solutions and many other sources. Contamination was minimized by observing the following procedures:-

(1) Cultures were checked on the microscope, using phase contrast, every time they were handled.

(2) Cells were cultured in antibiotic-free medium to reveal cryptic contaminations.

(3) Reagents were checked for sterility before use.

(4) Bottles of media were not shared for different cell lines.

(5) The standard of sterile technique was kept high at all times.

Commercially available media, sera and trypsin which were used had all been membrane filtered and screened for mycoplasma infection before sale. These preparations were further tested for bacterial contamination as constituents of complete medium as follows:-

(a) When the medium was complete it was tested for sterility by incubating at 37°C for 48 hrs. If the medium became cloudy the batch was discarded.

(b) Fresh medium was streaked on nutrient agar plates. The nutrient agar was composed of special peptone, sodium chloride and agar/agar and was prepared by suspending 25g in IL of distilled H_2O which was then allowed to soak for 15 mins. before heating in a boiling waterbath until completely dissolved. The nutrient agar

was then autoclaved (15 min. 100kPa) and poured into bacteriological grade petri dishes before setting. If colonies formed on the plates after streaking with medium, the batch was discarded.

When examined on a low-power microscope (X150), spaces between cells appear granular with bacterial contamination. Yeasts appear as separate round or ovoid particles which may bud off small particles. Fungi produce filamentous mycelia and may be visible with the naked eye. With toxic infection the cells may deteriorate and detach from the surface of the culture vessel.

Mycoplasmal infection, invisible under regular microscopy, presents one of the major threats of contamination. Superficial signs of chronic mycoplasmal infection include reduced rate of cell proliferation, reduced saturation density (Stanbridge and Doerson, 1978), and agglutination during growth in suspension. Mycoplasmas do not always reveal their presence with macroscopic alterations of the cells but can alter cell behaviour and metabolism in other ways.

Cultures were screened for mycoplasma infection using two techniques:-

(A) Fluorescent staining with Hoechst 33258 specific for DNA(Chen,1977).

REAGENTS:

1. Stain - Hoechst 33258, prepared as a 1mg/ml stock in B.S.S. without phenol red and frozen. For use it was diluted 1:20,000 (1µl in 20mls) in B.S.S. at pH 7.0. This chemical was treated as a potential carcinogen.
2. Fixative - 3 parts methanol, 1 part glacial acetic acid which

was prepared fresh each time and kept cold.

3. Buffer - McIlvaines Buffer pH 5.5 prepared from 0.2M Na_2HPO_4 and 0.1M citric acid. A 100ml volume was prepared by adding 56.85ml of 0.2M Na_2HPO_4 and 43.14ml of 0.1M citric acid together.

4. Hank's B.S.S. without phenol red -

NaCl	8g
KCl	0.4g
$CaCl_2.2H_2O$	0.14g
$MgCl_2.6H_2O$	0.10g
$MgSO_4.7H_2O$	0.10g
$Na_2HPO_4.2H_2O$	0.06g
KH_2PO_4	0.06g

All compounds were diluted separately and made upto 1L adding $CaCl_2$ last. The pH was then adjusted to pH 7.

PROTOCOL:

(1) Cells for testing were grown on coverslips at $1X10^5$ cells/ml for 2-3 days.

(2) Cells were fixed by adding an equal volume of acetic-methanol fixative to medium and coverslip in situ.

(3) Culture medium was then replaced with fresh fixative for 5 min.

(4) Fixative was replaced with fresh fixative for a further 5 min.

(5) The preparation was rapidly air-dried.

(6) 0.05µg/ml Hoechst 33258 in B.S.S. stained the cultures for 10 min.

(7) Two rinses in distilled water followed.

(8) The preparation was mounted in pH 5.5 McIlvaine's buffer, sealed and then examined by epifluorescence.

Since mycoplasmas contain DNA they can readily be detected by their characteristic particulate or filamentous pattern of fluorescence on the cell surface (Steiner and McGarrity, 1983) and if the contamination is heavy, in surrounding areas.

(B) BRL Mycotect, Mycoplasma detection system.

SAMPLE PREPARATION:

Cell lines to be screened for mycoplasma infection were grown in antibiotic - free media for at least 72 hr. prior to testing. The test culture should be very dense at the time of sampling. The test sample should consist of culture medium as well as a small number of cells (100-1000), because although some mycoplasma are shed into the culture medium, most are bound to the cell surface.

PROTOCOL:

(1) A 24-well tissue culture plate with mouse 3T6 indicator cells in DMEM+F.C.S. without antibiotics, at a concentration of $3X10^4$ cells per well in 1.5ml total volume was incubated at 37°C in a humidified 5-7% CO_2 atmosphere.

(2) 600µl of culture fluid containing cells for testing was collected.

(3) After indicator cells had attached (approximately 2 hr), test and control samples were added to the wells. Seeding media was added as a negative control. The pattern indicated in Figure 2.1 is recommended for sample addition.

FIGURE 2.1

RECOMMENDED SAMPLE ADDITION FOR BRL MYCOTECT

ROW \ COLUMN	1	2	3	4	5	6
1	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.
2	N.C.	1	2	3	4	5
3	N.C.	1	2	3	4	5
4	N.C.	1	2	3	4	5

Both row 1 and column 1 received 0.2ml of negative control media (N.C.). 0.2ml of a test sample was then added to rows 2-4 of one of the remaining columns. Five samples may be tested in a 24-well plate. The final volume per well was 1.7ml. The plate was then incubated at 37°C in a humidified 5-7% CO_2 atmosphere for 24 hr.

(4) After 24 hr. an aliquot of 1mM working solution of Mycotect was defrosted and kept on ice until ready to use.

(5) Each test sample and negative control received two concentrations of Mycotect; 15μM and 30μM. Rows 1 and 4 received no Mycotect. Row 2 received 25μl per well (15μM final concentration). Row 3 received 50μl per well (30μM final concentration). The plate was then incubated at 37°C, 5-7% CO_2 for 72hr until the cells in control wells

row 1 and column 1 - were confluent.

(6) The medium was removed from each well, taking care not to disturb the monolayer. 0.2ml of 0.2% crystal violet in 10% formalin was added to each well and incubated for 20min. at room temperature.

(7) The stain was removed and the wells were washed with distilled water, until the rinse water was clear. The plate was then air dried.

(8) The plate was inverted and the staining pattern in each well observed. Negative control wells should show a layer of confluent violet cells. If samples are positive for mycoplasma, the cell monolayers appear diminished by comparison with the controls. If the infection is extensive, the monolayer may be non-existent.

Mycoplasmas contain significant amounts of adenosine phosphorylase, an enzyme found in very small amounts in mammalian cells. Adenosine phosphorylase converts 6-methylpurine deoxyriboside (6-MPDR(MYCOTECT), a non-toxic analog of adenosine, into 6-methylpurine which is metabolished to 6-methylpurine riboside, both of which are toxic to mammalian cells. A mycoplasma-infected culture is thus detected by incubation with 6-MPDR and subsequent monitoring for toxicity.

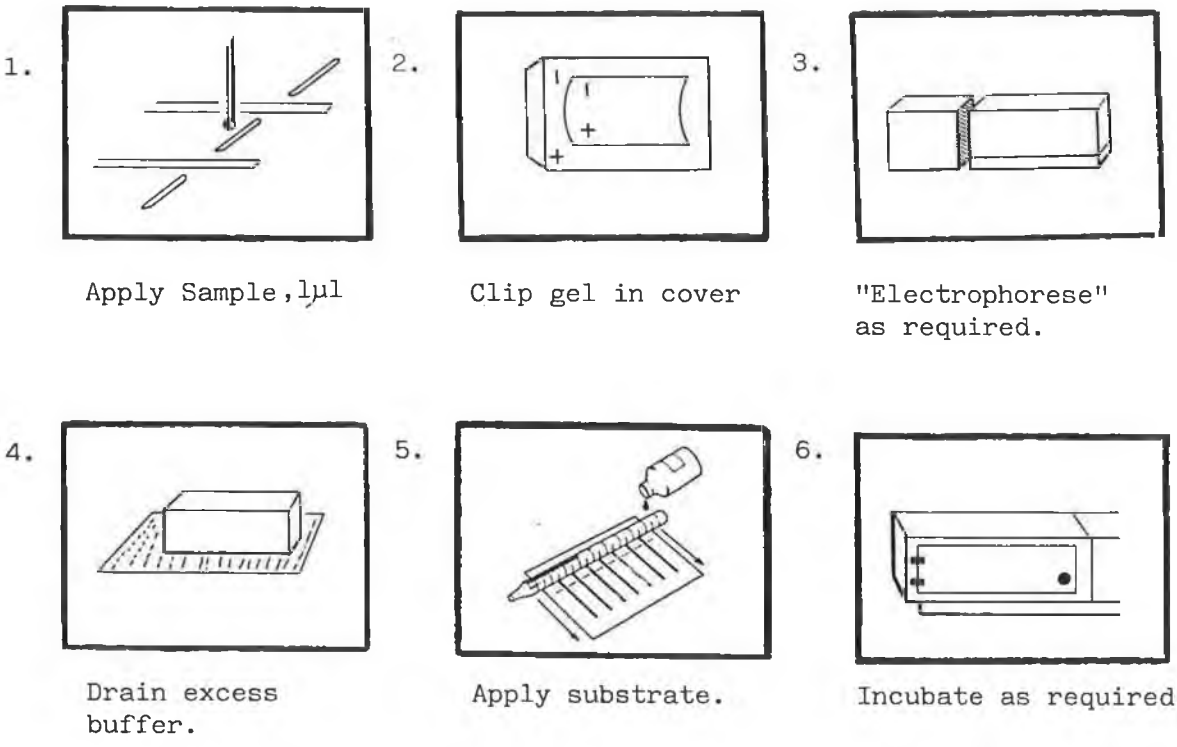
2.5 ISOENZYME ANALYSIS

In the course of cell culture, careful quality control is required to avoid inter-and intraspecies cell contamination. Halton et al., (1983) describe an electrophoretic technique that can be used routinely to monitor cell line integrity. The method involves the isoenzymatic separation of polymorphic enzymes which can be used for cell line species determinations and human cell line characterizations.

The technique involves the preparation of enzyme extracts which are inoculated into pre-cut slots on agarose electrophoresis films, clipped into constant voltage cassette systems (Corning) in the appropriate electrophoresis buffers and finally stained with specific chromogenic substrates (Figure 2.2).

FIGURE 2.2

ISOENZYME ELECTROPHORESIS USING THE CORNING CASSETTE.



7. Dry gel (20 mins at 65°C)

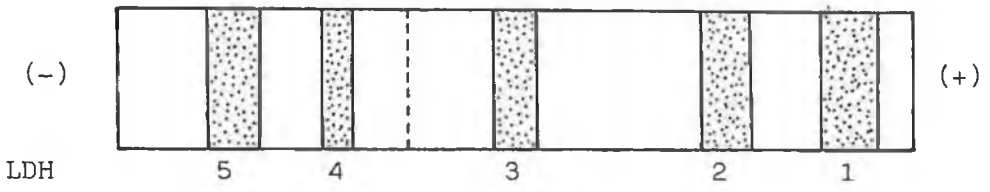
8. Interpret using appropriate scanning/viewing equipment.

The isoenzyme distribution patterns on stained gels were photographed for analysis.

The isoenzymes of Lactic Dehydrogenase (LDH) were examined using the Corning electrophoresis system. In mammalian tissue LDH exists as a tetramer of two types of units H (aerobic-found in myocardium, renal cortex and erythrocytes) and M (anaerobic-found in liver and sketetal muscle) which in combination produce five isoenzymes as shown in Figure 2.3.

FIGURE 2.3.

LACTIC DEHYDROGENASE



COMPOSITION	M_4	M_3H	M_2H_2	MH_3	H_4
NORMAL VALUES	5%	8%	27%	35%	25%

The nomenclature of isoenzymes is such that the fastest moving component towards the anode is designated number 1.

The following materials and methods were used for LDH isoenzyme electophoresis:-

(1) The gel:- Agarose Universal Electrophoresis Film, which is composed of 1% (W/V) agarose, 5% (W/V) sucrose, 0.035% (W/V) EDTA disodium salt in a 0.065M barbital buffer, pH 8.6.

(2) The buffer solutions:- Barbital buffer (Universal PHAB Buffer set) was supplied in the Corning

(2) The buffer solutions:- cont. Electrophoresis kit as a powder containing 17.7g sodium barbital, 2.6g barbital, 1.0g sodium chloride, and 0.7g EDTA disodium sucrose octaacetate, which reconstituted to 2 litres of working buffer solution (0.05M barbital, pH 8.6), without pH adjustment.

(3) Sample preparation:- Enzyme extraction was achieved as follows:-

(a) Confluent cell cultures were trypsinized for 15min. with trypsin-versene.

(b) The resulting cell suspension was pelleted at 1,000r.p.m. for 5 min.

(c) Cells were counted and diluted as required (a minimum of $3X10^6$ cells is required).

(d) Three washes in P.B.S. followed.

(e) 1-2ml aliquots of the resulting washed cell suspension were distributed into cryotubes.

(f) The cell suspension was centrifuged at 3,000r.p.m.(5 min).

(g) The pellet was resuspended in 300µl P.B.S. and freeze-thawed 3 times.

(h) Cell debris was spun down at 5,000r.p.m. (5min).

(i) Pellets were discarded. Supernatents were retained and stored at - 20°C until required for isoenzyme analysis.

(4) Electrophoresis:- 1.0µl of control and sample specimens were added to the sample wells of the agarose gel. The loaded agarose film was inserted into the cassette holder of the cell cover

(4) Electrophoresis:- cont.

(Figure 2.2), agarose side facing out, matching the anode(+) side of the agarose film with the anode (+) side of the cell cover. The cell cover was placed on the electrophoresis cassette cell base which was filled with 95ml of reconstituted pH adjusted Universal barbital buffer in each chamber of the cell base. The electrophoresis cassette cell was then connected with the 90V power supply and allowed to electrophorese for 35 min.

(5) Staining:-

Following the separation step the various isoenzymes are detected by their ability to catalyze the oxidation of lactate

$$\text{L-(+)-lactate} + \text{NAD} \underset{\text{pH9.1}}{\overset{\text{LD}}{\rightleftharpoons}} \text{pyruvate} + \text{NADH}$$

To make the reaction site apparent, the resulting NADH is used to reduce a tetrazolium salt to its corresponding formazan which is colored

$$\text{NADH} + \text{INT} \underset{\text{pH9.1}}{\overset{\text{pms}}{\rightleftharpoons}} \text{NAD} + \text{INF}$$

The regions within the electrophoretic medium which contain an enzyme are marked by a colored zone while those regions not containing the isoenzyme are colorless. The Corning system for colorimetric determination of lactate dehydrogenase isoenzymes consists of two reagents, the AMP lactate solution and the color reagent.

(5) Staining:- cont.

The AMP lactate solution contains L-(+)-lactate at 412mM concentration and sufficient 2-amino- 2-methylpropanol (AMP) (approx. 500mM) to adjust the pH to 9.1. One milliliter of this solution was used to dissolve one vial of the color reagent which contained 2mg nicotinamide adenine dinucleotide (NAD), 4mg iodonitrotetrazolium chloride (INT) and 0.05mg phenazine methosulfate (P.M.S.), 30min. prior to use. The substrate-dye mixture was then applied to the agarose film after electrophoresis (as shown in Figure 2.2.), and incubated at 37°C for 20min. to allow color development before evaluation of the isoenzyme distribution patterns.

2.6. CULTURE MEDIA.

Cultured cells were maintained in a sterile nutrient medium which is chemically defined before the addition of foetal calf serum. The chemically defined portion consists of a balanced salt solution, essential and non-essential amino acids, vitamins and glucose or another energy source.

Two types of culture media were used:-

(i) Eagle's Minimum Essential Medium (MEM) (Table 2.3) and

(ii) Dulbecco's modification of Minimal Eagle's Medium (DMEM) (Table 2.4.).

These media were obtained as 10X concentrates and diluted before use with sterile ultrapure water. Other concentrates were added to give final concentrations as follows:-

(a) 10% foetal calf serum.

(b) 20mM HEPES buffer (4-(2-hydroxyethyl)-piperazine ethane sulphonic acid).

(c) 2mM Glutamine.

(d) Pencillin G - 100 International units (I.U.)/ml.) Added in
Streptomycin - 100mg/ml.) combination.

(e) Sodium bicarbonate (7.5%).

PH 7.4-7.5 was achieved by the addition of 1.5N NaOH or HCl and was monitored by the presence of phenol red indicator in the medium. Exact pH was confirmed by aseptically removing a small portion of the medium and testing with a pH meter.

2.7. CELLS.

Two cell types were used:-

(i) RPMI 2650 cells and

(ii) Normal human fibroblasts.

The RPMI 2650 cell line was establised in July, 1962, by Moore and Sandberg (1964) from the pleural effusion of a patient with an extensive malignant tumour of the nasal septum. The tumour was diagnosed as an anaplastic squamous cell carcinoma. The cells are epithelial-like, very small and grow in dense clusters which fuse to form a thick layer of cells which are mucous-producing. RPMI 2650 cells were maintained in Eagle's Minimum Essential Medium supplemented with non-essestial amino acids (Gibco Cat. No. 043-1140) and 10% F.C.S.

Normal Human fibroblasts were obtained from newborn skin biopsy. The biopsy samples were prepared in accordance with Figure 2.4. Fibroblasts obtained from normal skin biopsy were spindle-shaped and were maintained in DMEM + 10% F.C.S.

TABLE 2.3.

COMPOSITION OF EAGLE'S MINIMUM ESSENTIAL MEDIUM

WITH EARLE'S SALTS

COMPONENT	mg/L	COMPONENT	mg/L
Inorganic salts		Amino acids	
$CaCl_2$ (anhyd)	200	L-Arginine:HCl	126
$NaHCO_3$	2200	L-Cystine	24
KCl	400	L-Glutamine	292
$MgSO_4:7H_2O$	200	L-Histidine HCl:H_2O	42
NaCl	6800	L-Isoleucine	52
		L-Leucine	52
		L-Lysine HCl	72.5
Vitamins		L-Methionine	15
D-Ca pantothenate	1	L-Phenylalanine	32.5
Choline chloride	1	L-Threonine	48
Folic acid	1	L-Tryptophan	10
i-Inositol	2	L-Tyrosine	36
Nicotinamide	1	L-Valine	46
Pyridoxal HCl	1	Other Components	
Riboflavin	0.1	Glucose	1000
Thiamine HCl	1	Phenol red	10

From Gibco Product Catalogue (Cat. No. 041-1095).

TABLE 2.4.

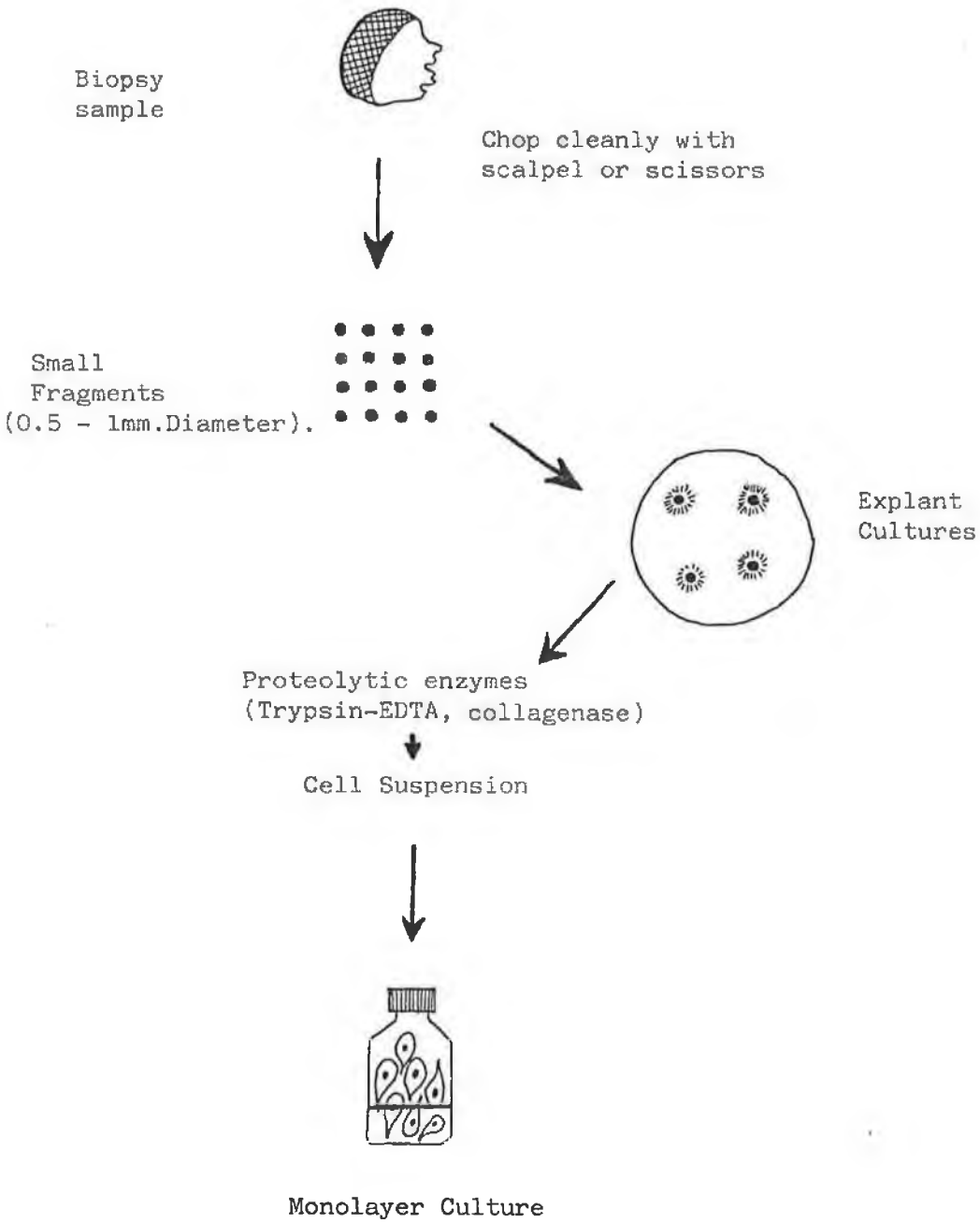
COMPOSITION OF DULBECCO'S MEM

COMPONENT	mg/L	COMPONENT	mg/L
Inorganic Salts		Amino Acids	
$CaCl_2$ (anhyd.)	200	L-Arginine:HCl	84
$Fe(NO_3)_3:9H_2O$	0.1	L-Cystine	48
KCl	400	L-Glutamine	580
		Glycine	30
$MgSO_4.7H_2O$	200	L-Histidine $HCl.H_2O$	42
NaCl	6400	L-Isoleucine	105
$NaHCO_3$	3700	L-Leucine	105
$NaH_2PO_4.H_2O$	125	L-Lysine HCl	146
		L-Methionine	30
Vitamins		L-Phenylalanine	66
D-Ca pantothenate	4	L-Serine	42
Choline Chloride	4	L-Threonine	95
Folic acid	4	L-Tryptophan	16
i-Inositol	7.2	L-Tyrosine	72
Nicotinamide	4	L-Valine	94
Pyridoxal HCl	4	Other Components	
Riboflavin	0.4	Glucose	1000
Thiamine HCl	4	Phenol red	15
		Sodium pyruvate	110

From Gibco Product Catalogue (Cat. No. 041-1885).

FIGURE 2.4.

TREATMENT OF BIOPSY SAMPLES



2.8 CELL PRESERVATION.

Storage in liquid nitrogen is currently the most satisfactory method of preserving cultured cells. The cell suspension, at high concentration ($2X10^6$ cells/ml), was preserved in the presence of dimethyl sulphoxide (DMSO) which was prepared as a 10% solution in medium complete with 10% F.C.S. The preservative solution (10% DMSO) was added gradually to the cell suspension in a 1:1 (V/V) ratio. 2ml of the preservative/cell suspension mixture was aseptically poured into a cryotube, labelled, and transferred to the vapour phase above liquid nitrogen (where the cooling rate is 2.5°C/min) for 3 hr. The frozen cells were then stored in liquid nitrogen until required.

When cells from frozen cultures were required a cryotube was removed from liquid nitrogen and thawed quickly at 37°C. The cryotube was then swabbed with alcohol, flamed briefly, and the contents were mixed with 5mls of serum supplemented medium. After centrifugation at 1,000 r.p.m. for 5min. the cell pellet was resuspended in medium + 10% F.C.S. and reseeded in a $25cm^2$ flask.

2.9. CELL DISAGGREGATION, COUNTING AND VIABILITY.

Monolayer cultures were disaggregated enzymatically using 0.25% trypsin, and 0.02% EDTA in P.B.S. The procedure is as follows:-

(1) Culture medium is removed and 1ml of trypsin-versene solution is added.

(2) After approximately 1 min. the excess solution is removed and 2ml of fresh trypsin-versene is added.

(3) The cells are incubated at 37°C for approximately 15min. until they have all detached from the flask surface.

(4) An equal volume of growth medium with 10% F.C.S. is then added and the cell suspension is centrifuged at 1,000 r.p.m. for 5 min. (F.C.S. contains a trypsin inhibitor and stops further trypsinization. Centrifugation allows the elimination of EDTA in the supernatent).

(5) Pelleted cells are resuspended in fresh medium, aspirated with a Pasteur pipette and passed through a fine gauge (26G⅜ 10/45) syringe needle to break down any cell aggregates which may be present.

Cell concentrations were determined by counting in a Weber haemocytometer (improved Neubauer type). The central position of the haemocytometer is 0.1mm below the level of the side portions and the large corner squares, which are divided into 16 equal squares each, have 0.1mm sides. Therefore, the volume of the squares is $1X10^{-4}cm^2$.

A coverslip was placed over the central portion of the haemocytometer and the cell suspension was introduced into the chamber so formed

using a Pasteur pipette. Cells were scored in each of the large corner squares (consisting of 16 small squares) and the cell concentration was determined as:-

$X \times 10^4$ = no. of cells/ml of suspension,

where X = average no. of cells/square

and 10^{-4} = volume of the chamber (cm^3)

Viability was estimated by trypan blue exclusion. 250µl of cell suspension was mixed with 50µl trypan blue and counts were made when the dye had been in contact with the cells for more than two but less than fifteen minutes. Since the cell suspension (250µl) was diluted by trypan blue (50µl), the final cell number was corrected by a factor of 1.2.

Thus,

$X \times 10^4 \times 1.2$ = the corrected no. of cells/ml of suspension.

2.10. AGARS, AGAROSES AND METHYLCELLULOSES.

Some cells, particularly virally transformed cells, will clone readily in suspension. To hold the colony together and prevent mixing, the cells are suspended in a semi-solid support. A range of agar, agaroses and methylcelluloses with different properties, described in Table 2.5., were used as semi-solid supports for cells.

1.4% agar and agaroses were prepared by dissolving 1.4g of agar (agaroses) in 80ml of boiling distilled water. The solution was stirred constantly to avoid charring until the agar (agaroses) was dissolved. After the volume was adjusted to 100ml by the addition of hot distilled water, the agar (agarose) solution was autoclaved

at 100kPa (15p.s.i.) for 20 min. at 120°C.

2.6% methylcelluloses were prepared by dispersing 2.6g of methylcelluloses in 30ml of hot (90°C) distilled water, followed by the addition of 70ml of cold (4°C) H_2O. The solution was stirred overnight at 4°C, then autoclaved at 100kPa (15p.s.i.), 120°C for 40 min. This solution was stored at 4°C for at least 24 hrs. before use.

TABLE 2.5.

PROPERTIES OF AGAR, AGAROSES AND METHYCELLULOSES.

Semi-solid support	EEO(-Mr)	Sulfate	Gelling temp.°C	Gel strength g/cm^2
Agar				
Bacto-agar(Difco)			36	
Agaroses(Sigma)				
Type I	Low .10-.15	< .35%	36	> 800
Type II	Medium.17-.20	< .35%	36	> 800
Type III	High .23-.26	< .35%	36	> 600
Type IV	Special High > .3	< .35%	36	> 450
Type VII	Low < .15	< .15%	< 30	> 150
(F.M.C. Marine Colloids)				
Seaplaque(LGT)	Low < .15	< .15%	25	
Seakem	Low .10-.15	< .35%	36	
Seaprep(ULGT)	Ultralow < .05	< .10%	15	
(Marine Colloids U.K.)				
HEEO	Highest > .3	< .30%	36	> 450
HE	High .23-.26	< .50%	36	> 500
ME	Medium .16-.20	< .35%	36	> 650
LE	Low .10-.15	< .35%	36	> 800
LGT	Ultralow < .15	< .15%	< 30	> 150
Methylcelluloses				
Methocel mc400cp				
Methocel mc 4000cp				
High substitution				
Low substitution				

From F.M.C. Corporation (marine colloids division),Miles Laboratories & Sigma.

2.11. PRE-TREATMENT OF TEST CELLS AND FEEDER LAYER PREPARATION.

Two days prior to use as test cells or feeder layers in the clonogenic assay, cells were pre-treated by trypsinization and passage into fresh medium (Section 2.9.). After pre-treatment two types of feeder layers were prepared as follows:-

(i) Live feeder layers were prepared at the required densities in 35mm petri dishes and incubated for a further 48 hr at 37°C in a 5% CO_2 humid atmosphere.

(ii) Mitomycin C-treated feeders were prepared by incubating sub-confluent cultures for 3 hr. in medium containing 4μg/ml of mitomycin C which was prepared from a stock mitomycin C solution of 200 μg/ml. After incubation the cells were trypsinized and reseeded at the required densities in fresh medium and incubated for 48 hr. at 37°C in a 5% CO_2 humid atmosphere.

After 48 hr incubation an agar (agarose) underlay, at 41°C, was poured over the feeder cell layer and pre-treated test cells were assayed in an upper agar (agarose) or methylcellulose layer as described in Section 2.12.

2.12. CLONOGENIC ASSAY; CLONING.

To clone cells in agar, agaroses, methylcelluloses and monolayer, the following procedures are used:-

(A) Cloning in Agar/Agaroses (Agar is taken as an example).

Agar becomes liquid at high temperatures but gels at 36.5°C. Cells are suspended in warm agar and when incubated after the agar

sets, will form discrete colonies which may be isolated and cultured as clones.

Protocol (Figure 2.5).

(1) Stock agar (1.4%, Section 2.10.) in a sterile bottle is melted in a boiling waterbath, air cooled briefly, and incubated in a waterbath at 44°C.

(2) Buffered 2X supplemented medium is equilibrated at 44°C for 30 min.

(3) 1.4% agar and 2X supplemented medium (Table 2.6.) are mixed (1:1.8 V/V) to give a final agar concentration of 0.5%. This mixture (0.5% agar/medium) forms the base layer.

(4) A cell suspension is prepared, counted and diluted to give 3X the final desired concentration (Section 2.9).

(5) Waterbath temperature is reduced to 41°C. If feeder layers are present the base layer temperature must be 41°C to avoid scalding the feeder cell layer. The base layer of agar (0.5%)/medium (3) is then poured (2ml/35mm petri-dish).

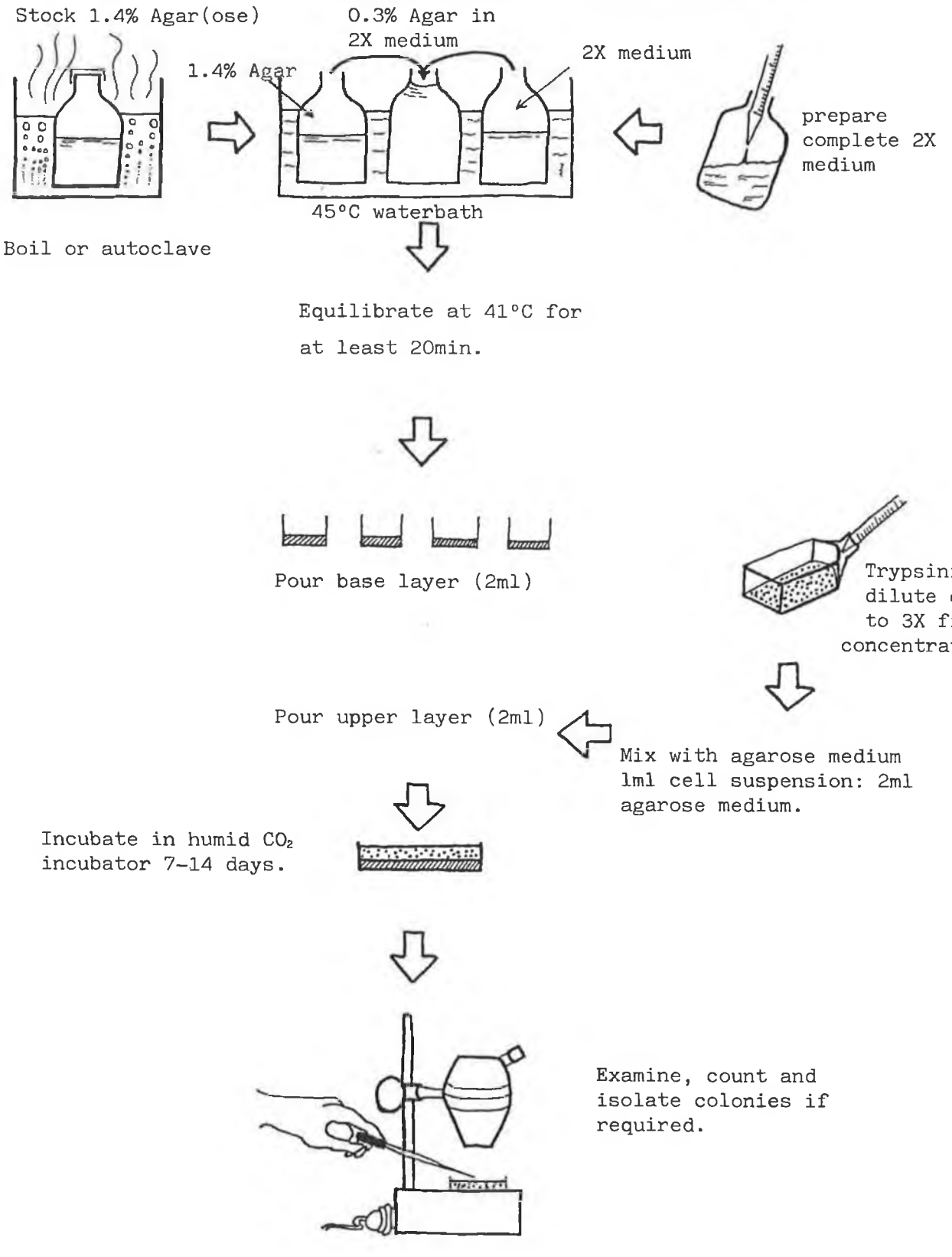
(6) The base layer is allowed to set at room temperature and is then equilibrated at 37°C in 5% CO_2.

(7) 0.5% Agar medium and 3X cell suspension are then mixed in the ratio of 2ml of agar medium to 1ml of 3X cell suspension giving the required cell concentration and a final agar concentration of 0.3%. 1ml of this mixture is then poured over the 2ml base layer.

(8) After all dishes have been poured, the agar gel is allowed to set at room temperature.

FIGURE 2.5

CLONING CELLS IN SUSPENSION IN AGAR(OSE)



(B) CLONING IN METHYCELLULOSE OVER AN AGAR BASE.

Cells suspended in medium containing methylcellulose are seeded into tissue culture grade dishes containing solid agar base layers.

Protocol

(1) Agar base layers are prepared as described previously (Section 2.12.(A)).

(2) A cell suspension is prepared, counted and diluted to give 2X the final required concentration (Section 2.9).

(3) Stock methylcellulose (2.6%) is prepared (Section 2.10.) and maintained at 4°C.

(4) The 2X cell suspension is diluted with an equal volume of (2.6%) methylcellulose and 1ml is poured over the (0.5%) agar underlay. Thus, final methylcellulose conc. is 1.3%.

(5) The dishes are incubated until colonies form. Since the colonies form at the interface between the agar and the methylcellulose, fresh medium may be added, 1ml per dish, after 1 wk. without disturbing the colonies.

Most cell types clone in suspension with a lower efficiency than in monolayer, some cells by two or three orders of magnitude. However, the isolation of colonies from semi-solid supports is much easier than from monolayer cultures (see Figure 2.5.).

The constituents of media used for cloning in suspension and monolayer are shown in Table 2.6.

TABLE 2.6.

CONSTITUENTS OF CLONING MEDIA

CLONING METHOD	MEDIUM USED	COMPONENTS	CONCENTRATION (ml/100ml)
Agar/Agarose Methylcellulose	2X Supplemented Medium	Buffered 2X medium	57.8
		* Foetal calf serum	22.2
		Pen-Strep	1.1
		Amphotericin B	1.1
		Glutamine	1.1
		Growth Medium or 10X additives	15.6
		Growth Medium + 10% F.C.S.	1.1

* This gives an actual working concentration of 14.3% Foetal calf serum.

2.13. COLONY STAINING AND SIZING.

Several types of staining were used, depending on the method used to culture colonies, as follows:-

(A) Monolayer colonies.

Colonies formed in monolayer cultures were stained using the May Grünwald - Giemsa method as follows:-

- Medium is removed from the cells.
- Colonies are fixed with absolute ethanol or methanol for 5 min.
- Cells are stained with May Grünwald (1:1 with distilled water), 5min.
- Giemsa stain is then added (1:17 with distilled water), 20min.
- Stain is removed and the monolayer is air-dried.

(B) Suspended colonies.

Colonies suspended in semi-solid media were stained using either a direct 'wet' staining method (Salmon and Liu,1979) or a metabolizable tetrazolium salt (Alley et al., 1982), as follows:-

Direct 'Wet' staining method.

- Colonies are fixed for 5min.with 10% formalin.
- 1-2ml of Coomassie brillant blue G-250 (0.4mg/ml) is pipetted onto the petri dishes with a Pasteur pipette.
- Petri dishes are incubated at 37°C for 1-2hr.
- Colonies, stained blue, are then examined under the microscope for colony counting.

Staining with a metabolizable tetrazolium salt.

By enzymatic reduction, viable cells transform 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT) which is water soluble into a formazan product which is water-insoluble and dark red. The dark red colour of colonies stained with INT facilitates detection under the microscope.

- Stock INT is prepared freshly, as required, at 1mg/ml distilled water.
- 1ml of INT solution is added to the surface of suspended cultures in agar.
- Petri dishes are reincubated at 37°C, 5%CO_2 and 100% relative humidity for 24hr.
- Colonies, stained red, are then examined under the microscope for colony counting and sizing.

Colonies formed in agarose were examined and scored using a Leitz Diavert microscope. Clones greater than or equal to 40μm were considered to be colonies as this size was estimated to represent 30 cells after 5 cell divisions from a single 'parent' cell and is in accordance with current colony sizing procedures (Endressen et al., 1985). Table 2.7. outlines the calculations on which the cut-off colony size was determined.

TABLE 2.7.

RPMI 2650 colony diameter (μm)	Volume (μm^3)	No.of cells (approx.)	No.of cell divisions
12.86	626.64	1	0
20	2357.14	4	2
25	4603.79	7	3
30	7955.36	12	4
40	18857.14	30	5
50	36830.4	58	

2.14. GROWTH IN DIFFERENT GAS ATMOSPHERES.

Cells in semi-solid media were cultured on a routine basis in a 5%CO_2/95% Air atmosphere at 37°C, 100% relative humidity, in CO_2 incubators. Experimental alteration of the gas atmosphere was achieved through the use of Flow Laboratories Modular Incubator Chamber (Figure 2.6.).

Before use, the chamber was swabbed down with 95% I.M.S. Petri-dishes containing sterile water were included with test petri-dishes to maintain humidity. Gas was then flushed through the chamber for 15min., at less than 2p.s.i. to avoid rupturing the chamber, before the inlet and outlet ports were closed. The chamber was then incubated at 37°C in a constant temperature room for 10-14 days with re-gassing at 5-7 days.

Four gas atmospheres were used in the incubator chamber:-

(1) Nitrogen gas (N_2)

(2) High oxygen concentration - 5%CO_2/Air (20% Oxygen Balance).

(3) Low oxygen concentration - 5%CO_2/3%O_2/N_2 Balance.

(4) 10%CO_2/N_2 Balance.

Gas flushing times were determined using an anaerobic indicator, resazurin, which is pink in the presence of oxygen and white in anaerobic conditions. The anaerobic indicator (Oxoid) comes in the form of a resazurin saturated pad, which must be moistened before use with distilled water. Nitrogen gas was flushed through the chamber, once the indicator was in place, until the indicator colour changed from pink to white. This took approximately 15min after which time the inlet and outlet ports of the chamber were closed.

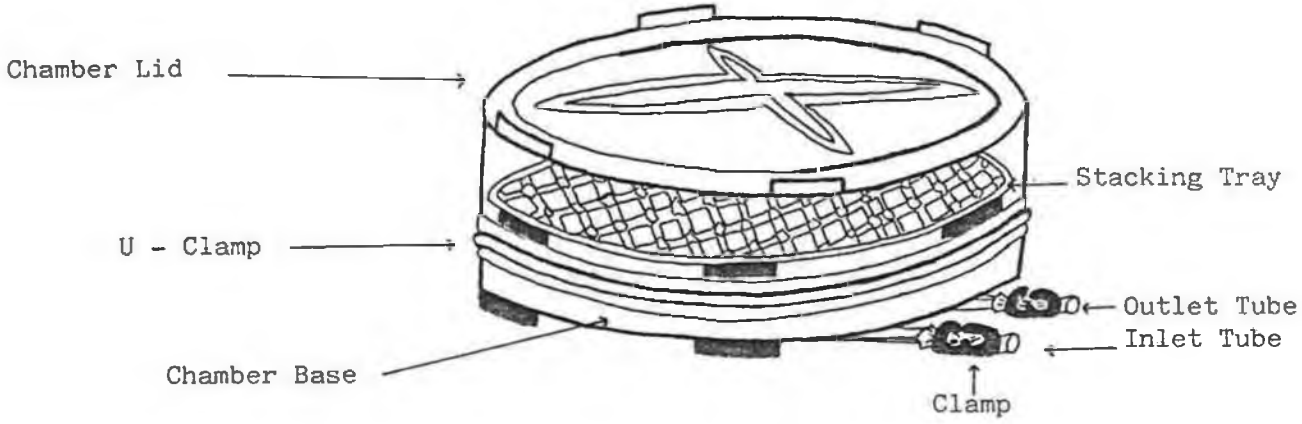


FIGURE 2.6.

FLOW MODULAR INCUBATOR CHAMBER

2.15. HORMONE AND GROWTH FACTOR SOLUTIONS

A number of hormone and growth factor solutions were used:

- HITES medium (Hamburger et al., 1979) which was prepared according to table 2.8. and included in both monolayer and semi-solid colony forming assays as 10X additives, in the proportions necessary to give the final desired concentrations.
- Insulin, stored as a stock solution of 40 i.u./ml prepared in P.B.S., was diluted to the final desired concentration with serum-free medium.
- Hydrocortisone, stored as a stock solution of 1mg/ml in ethanol and subsequently diluted as required with serum-free medium.
- EGF (Pathak et al., 1982), at a concentration of 100ng/plate, was included in both monolayer and semi-solid colony forming assays as a 10X additive which was prepared in medium from a stock solution of 1µg/ml, which was prepared in P.B.S.
- Human T-cell polyclone, from a stock solution of 50µg/ml, was diluted as required with serum-supplemented medium.

2.16. GROWTH INHIBITOR ASSAY

The presence of a cell produced growth inhibitor in the medium was assayed as follows:-

(i) Pre-treated cells at $2X10^6$ cells/25cm^2 flask and $2X10^5$ cells/25cm^2 flask were cultured for 4 days in 5ml of serum supplemented medium.

(ii) After 4 days the medium was removed and the cells were washed with P.B.S. before the addition of 5ml of fresh serum supplemented medium. Fresh medium was also

TABLE 2.8.

COMPOSITION OF HITES MEDIUM

Constituent	Concentration/ml	Diluent for stock solutions
Hydrocortisone	10^{-3}M	Ethanol
Insulin	5µg	P.B.S.
Transferrin	10^{-8}M	P.B.S.
Estradiol	5µg	Ethanol
Selenium	5ng	P.B.S.

added to cell-free control flasks.

(iii) At 24,48 and 96hr, 5ml samples of conditioned medium were collected from duplicate flasks and pooled. 10ml volumes of conditioned medium were then spun at 5,000r.p.m. for 20min. to remove cells.

(iv) Pelleted cells were discarded. The conditioned supernatents were transferred to sterile universals and stored at 4°C until required.

(v) Pre-treated cells at $1X10^4$cells/35mm petri dish were incubated at 37°C before the addition of test media.

(vi) Conditioned media were diluted as required and 2ml volumes tested by addition to pre-incubated cells at $1X10^4$cells/dish. Conditioned media were also added to cell-free control dishes to eliminate effects of any cells still remaining in the conditioned media. Control plates containing 2ml of fresh medium were also prepared to test cell growth in the assay.

(vii) After 10 days the colonies were stained with May-Grünwald-Giemsa stain, sized and scored.

2.17. ULTRAFILTRATION.

Ultrafiltration was carried out using an Amicon Ultrafiltration cell (model 8400) and a 5,000 molecular weight cut-off membrane (Diaflo YM5) conditioned and unconditioned medium was passed through the membrane under nitrogen gas pressure (50p.s.i.) Conditioned medium was ultrafiltered to 1/20 the initial volume and samples of both the filtrate and the retentate were filtersterilized before inclusion in the growth inhibitor assay (Section 2.16).

2.18. OUABAIN AND THIOGUANINE SENSITIVITY.

Cell sensitivity to ouabain and thioguanine was examined in both monolayer and suspension cultures for a range of ouabain and thioguanine concentrations. 10X concentrates of ouabain and thioguanine were included in both monolayer and suspension cultures in the proportions necessary to give the final desired concentrations. The final ouabain concentrations, included in the assays as 10X additives were prepared from a stock solution of 10^{-2}M (7.29mg/ml) ouabain. Stock thioguanine was prepared at a concentration of 100mM (16.72mg/ml) in 0.1N NaOH. Thioguanine and ouabain stock solutions were stored at 4°C until required.

2.19. STATISTICAL ANALYSIS: CLONO AND MINITAB

Clono (CLONO. BAS) is a BASIC programme which has several functions which may be described as follows:-

(i) It determines total colony score per plate from X no. of colonies scored per Y no. of quadrats per 35mm petri-dish.

(ii) From the total colony score per plate the colony forming efficiencies of cells inoculated into the bilayer clonogenic assay are determined according to the following formula-

C.F.E. (colony forming efficiency)

$$\text{C.F.E.} = \frac{\text{No.of colonies scored/plate X 100}}{\text{cell inoculum density.}}$$

(iii) CLONO tabulates total colony score results and C.F.E.'s for reference.

(iv) CLONO formats results for MINITAB (which is written in FORTRAN).

OPERATION OF CLONO

CLONO (CLONO.BAS) is a BASIC program (see Appendix). To use CLONO it is necessary to operate in BASIC mode. The computer which was used to perform statistical analysis using CLONO and MINITAB was the Vax II/785.

STEP 1: Recalling CLONO

- Call up BASIC mode

  $ BASIC

- Recall CLONO

  OLD (RETURN KEY)

  OLD FILE NAME: CLONO (RETURN)

  RUN CLONO (RETURN)

STEP 2: Processes of CLONO.

The following will appear on screen.

(Explanation of requests for CLONO).

System no: no. of different experimental parameters examined.

Block no: no. of horizontal blocks in Analysis.

Replicate no: no.of replicates per system.

No. of colonies scored: Colonies scored per X no. of quadrats.

Cell conc. U.L.: Upper layer initial cell inoculum density.

Cell conc. L.L.: Lower or feeder layer concentration.

No. of quadrats: no. of 4 sq.mm. grids scored/240 grids.

Press Y to continue? Y

(MINITAB)

The following must be carried out in DCL before MINITAB is used:-

$ Assign Woof.DAT FORØ2Ø (sets file as input data file for MINITAB).

$ Assign Val.DAT FORØ3Ø (sets file as output storage file)

$ MINITAB

-- Read FORØ2Ø into $C_1C_2.....C_N$ (Reads data from WOOF.DAT).

-- Outunit =Ø3Ø (All the following text is printed into Val.DAT).

Now follows the required tests, either;

-- Oneway Aov, OBS in C_1, Factor in C_2

OR

-- Twoway Aov, OBS in C_1, First Factor in C_2,Second in C_3

Further manipulations of data using MINITAB subcommands may be required at this stage.

-- STOP.

(Requests now follow)

- Type in the Date? (RETURN)

- For ONEWAYAOV type in 1, 2 for TWOWAYAOV ? (RETURN)

- Type in the no. of systems scored? (RETURN)

- Type in the no. of blocks? (RETURN)

- Type in the no. of replicate plates scored? (RETURN)

- Type in the no. of quadrats scored? (RERURN)

- Type in the no. of cells in the U.L.? (RETURN)

- Type in the no. of cells in the L.L.? (RETURN)

- Type in the no. of colonies scored? (RETURN)

- No. of colonies scored/plate =

- C.F.E.=

STEP 3: Files

The following files and their functions are created by CLONO.

(i) WOOF.DAT: Formatted results in columnar form with subscripts for MINITAB.

(ii) CPE.DAT: Tabulated results for reference purposes.

The following file contains output from MINITAB.

(iii) VAL.DAT: Results from ANOVA in tabular form.

STEP 4: Text

Clono contains explanations of input requests and additional information on the operation of MINITAB from data created by CLONO. Additional statistical tests may be performed on the data from WOOF.DAT. by re-formatting using manipulation commands and analysing further using the desired statistical tests.

SECTION 3.

RESULTS.

3.1. CHARACTERISATION OF RPMI 2650 CELLS

(A) Appearance of RPMI 2650 cells in vitro.

RPMI 2650 cells have been described previously (Section 2.7.) as anaplastic squamous carcinoma cells. The cells themselves are polygonal in shape with clear sharp boundaries between cells which gives a "pavement-like" appearance to monolayer cultures. This type of morphology is known as"epithelial-like"and is shown in Plate 3.1.

When RPMI 2650 cells are cultured as anchorage-independent clones, the morphology of the colony changes, as illustrated in Plate 3.2., since the cells are now growing in three-dimensions as opposed to two-dimensional growth characteristic of monolayer cultures. When these colonies are stained with INT (Section 2.13.) the distribution of viable cells within the colony may be seen (Plate 3.3.).

Human fibroblasts are elongated and spindle-shaped in appearance. In contrast with RPMI 2650 cells, their appearance in monolayer culture is irregular, as shown in Plate 3.4.

(B) Lactic Dehydrogenase Analysis.

Lactic dehydrogenase patterns for various cell lines were prepared (Section 2.5.). Serum samples were also run in the gel system and the pattern obtained was compared with the patterns obtained for the cell lines. The same pattern was obtained twice for the cell lines and serum sample examined. The isoenzyme patterns which were obtained

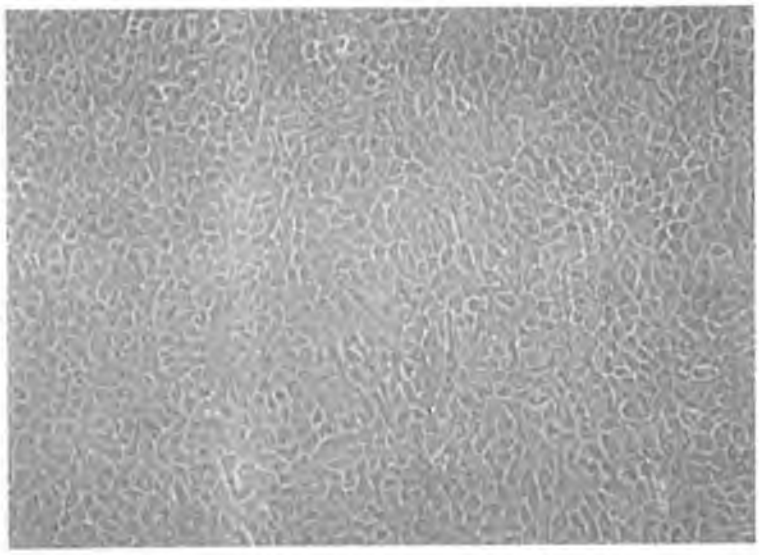


Plate 3.1

RPMI 2650 cells in monolayer; regular shaped polygonal cells give the monolayer a "pavement-like" appearance.

Plate 3.2

RMPI 2650 colonies suspended in agarose (shown using phase-contrast microscopy).

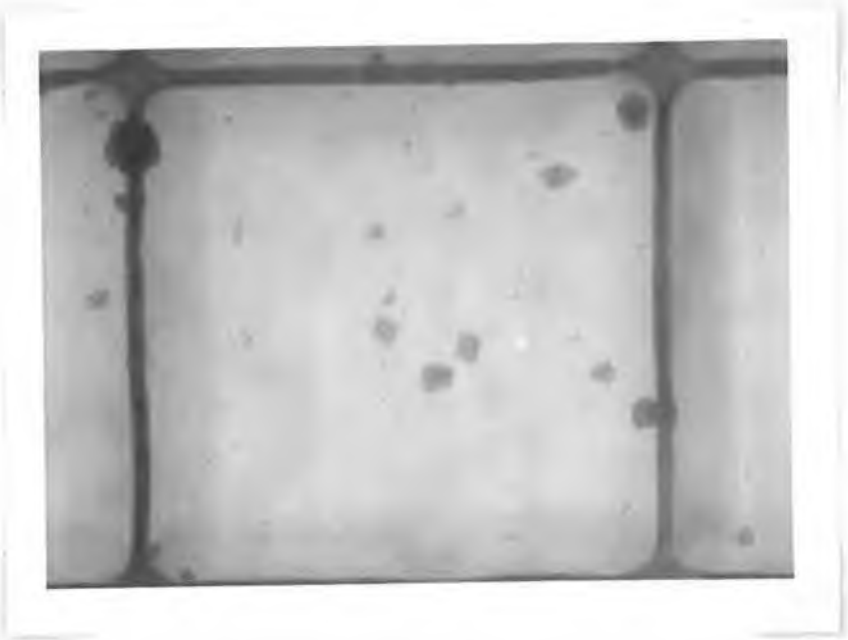


Plate 3.3

INT-stained colonies in agarose.

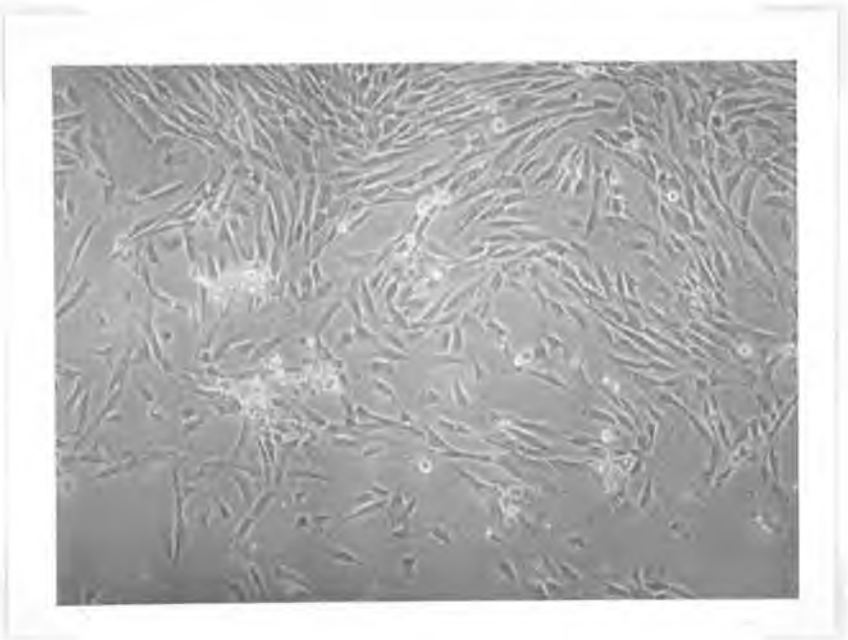


Plate 3.4

Normal human fibroblasts in monolayer culture showing the irregularity in shape and attachment typical of fibroblast growth.

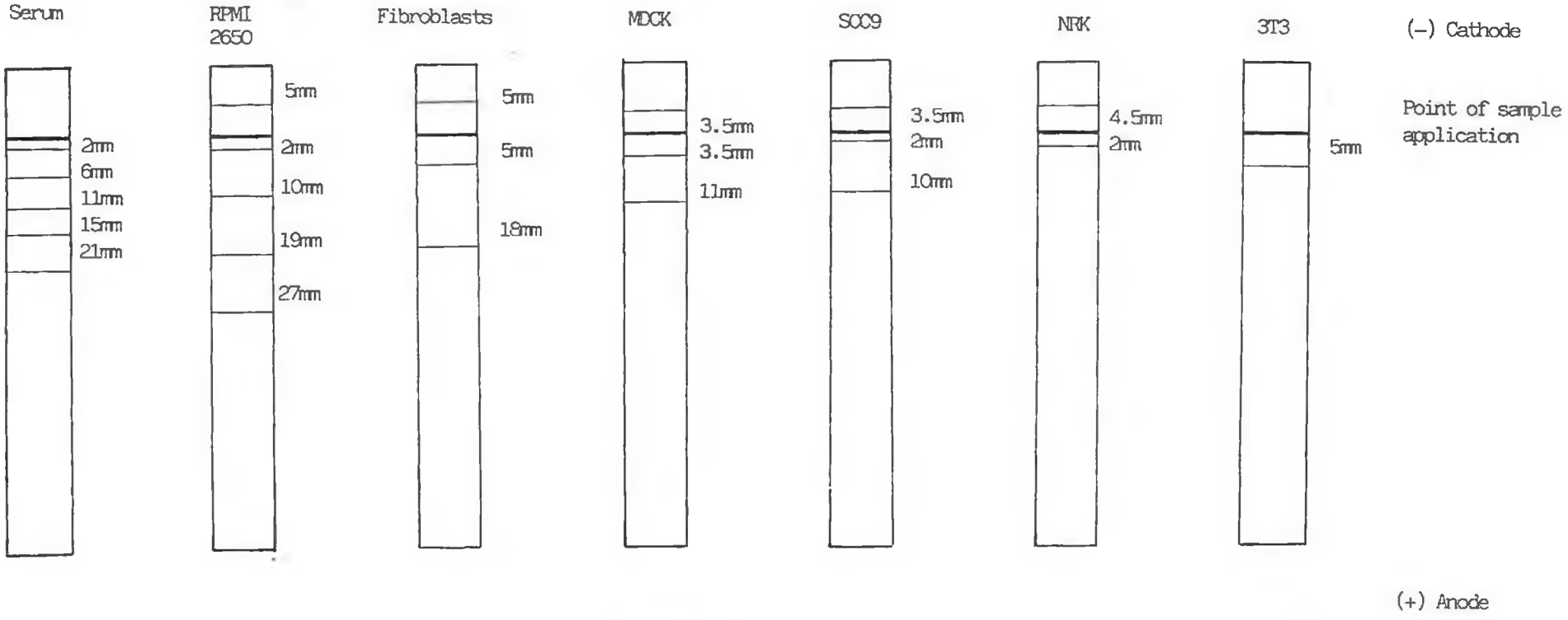


FIGURE 3.1.

LACTATE DEHYDROGENASE ISOENZYME PATTERNS.

TABLE 3. (A).

Cell line	Isoenzymes Present				
Serum	A_4	A_3B_1	A_2B_2	AB_3	B_4
RPMI 2650	A_4	A_3B_1	A_2B_2	AB_3	B_4
Fibroblasts	A_4	A_3B_1	A_2B_2	-	-
SCC9	A_4	A_3B_1	A_2B_2	-	-
MDCK	A_4	A_3B_1	A_2B_2	-	-
NRK	A_4	A_3B_1	-	-	-
3T3	-	A_3B_1	-	-	-

A_4 = LDH_5; A_3B_1 = LDH_4; A_2B_2 = LDH_3; A_1B_3 = LDH_2; B_4 = LDH_1

A = musle form of the sub-unit of lactate dehydrogenase.

B = heart form of the sub-unit of lactate dehydrogenase.

SCC9 - human tongue, squamous cell carcinoma.

MDCK - canine kidney cell.

NRK - normal rat kidney

3T3 - contact inhibited mouse embryo fibroblasts.

are shown in Figure 3.1. From the isoenzyme patterns which were obtained it was possible to name each band with reference to the position of the A_4 or B_4 band. Table 3. (A). shows the isoenzymes present in the cell lines examined. The A_3B_1 sub-unit appears to be by far the more common in the cell lines studied. The A_4 sub-unit is also very common and was found in all isoenzyme patterns examined except the isoenzyme patterns of 3T3 cells. RPMI 2650 was the only cell line to exhibit all five identified isozyme bands, which were also present in the serum sample examined. The B_4 band appeared only in RPMI 2650 cells and foetal calf serum, however, the positioning of the bands was different (Figure 3.1.).

Of the cell lines examined only two, RPMI 2650 and SCC9 cells were of human origin. Since the isoenzyme patterns obtained for all cell lines and the serum sample examined were different, it is clear that isoenzyme analysis is a useful tool in the characterisation of inter-and intraspecies differences which may be used to monitor cell line integrity.

3.2. DISAGGREGATION.

For soft-agar studies, the disaggregation method used must produce a high yield of freely suspended viable tumor cells and a suspension representative of the tumour cell population. Established cell lines are generally dissociated enzymatically. However, a universal enzymatic approach has not been identified since most enzymes are apparently specific for a particular type of tissue. In an effort to establish a method for the preparation of good single cell suspensions for clonogenic growth in monolayer and soft agar, several aspects of enzymatic disaggregation were examined.

(i) Enzyme preparation used.

A variety of enzyme preparations were tested on duplicate confluent monolayer cultures of RPMI 2650 and HT29 cells, which had been seeded with 5×10^5 cells per $25cm^2$ flask (Section 2.9.). Cells were disaggregated in 2mls of enzyme at 37°C until dissociated, stained with trypan blue and scored on a Neubauer haemocytometer as described previously (Section 2.9.).

Tables 3.1 (a) and 3.1 (b), which were arranged in order of increasing cell viability, show the effects of different enzyme preparations on monolayer dissagregation of RPMI 2650 and HT29 cells. The effects may be summarized as follows:-

(1) HT29 cells and RPMI 2650 cells reacted differently to the various enzyme preparations used. RPMI cells were easier to dissociate than HT29 monolayers and produced higher single-cell yields.

(2) From table 3.1 (a) it appears that there was an association between the method of dissociation,

TABLE 3.1 (a)

Effects of enzymes on cell suspensions prepared from monolayer cultures of RPMI 2650 cells.

Enzyme Concentration	Dissociation Time (min).	Viability %	Single Cell %	Doublets %	Triplets %	Clumps %
Dispase-DNase (1.2units/ml-0.02%)	Undissociated	-	-	-	-	-
Collagenase II -DNase I (0.8%-0.002%)	22	70	88	8	2	2
Trypsin-EDTA (0.25%-0.02%)	20	83	92	6	1	1
Collagenase II (1mg/ml)	70	85	94	2	2	2
Trypsin-DNaseI (0.25%-0.02%)	10	90	96	3	1	0
Trypsin (0.25%)	10	91	100	-	-	-
(1.25%)	8	91	98	0	1	1
Dispase (1.2units/ml)	60	96	99	1	0	0
Coll II-Pronase -DNase(0.04mg/ml-0.1mg/ml-0.04mg/ml)	13	98	97	3	0	0
Coll II-DNase I (0.02%+0.02%)	62	100	100	-	-	-

NOTE: Each percentage point is a mean value estimated from duplicate treatments.

TABLE 3.1 (b)

Effects of enzymes on cell suspensions prepared from monolayer cultures of HT29 cells.

Enzyme Concentration	Dissociation Time (min).	Viability %	Single Cell %	Doublets %	Triplets %	Clumps %
Dispase-DNase (1.2units /ml-0.02%)	Undissociated	-	-	-	-	-
CollagenaseII-Pronase-DNase (0.04mg/ml-0.1mg/ml-0.04mg/ml).	13	64	95	0	0	5
Collagenase II-DNase I (0.8%-0.002%)	32	81	89	0	0	11
Trypsin (0.25%)	10	89	79	7	2	12
Trypsin-EDTA (0.25%-0.02%)	20	91	93	4	2	1
Trypsin (1.25%)	5	97	84	8	0	8
Trypsin-DNase I (0.25%-0.02%)	10	98	78	19	1	2
Collagenase II (1mg/ml)	70	98	78	2	6	14
Dispase (1.2units/ml)	20	99	99	1	-	-
Collagenase II-(0.02%-0.02%)	70	100	92	-	-	8

NOTE: Each percentage point is a mean value estimated from duplicate treatments.

cell viability, and single-cell yield for RPMI cells. A similar association is not evident for HT29 cells from table 3.1 (b).

(3) All enzyme preparation except dispase (1.2 units/ml)-DNase I (0.02%) readily dissociated monolayers of both RPMI 2650 and HT29 cells.

(4) Reduction of the collagenase concentration of enzyme mixtures resulted in an increased incubation period before monolayers were dissociated.

(5) Different enzyme treatments had different incubation period requirements. Collagenase II (0.02%) - DNase I (0.02%) gave the best viable single-cell suspensions of RPMI 2650 and HT29 cells after 60min. incubation. By comparison, collagenase II (0.04mg/ml) - pronase (0.1mg/ml) - DNase (0.04mg/ml) produced the next best viable single-cell suspensions of RPMI cells after incubation at 37°C for 13 min. (Table 3.1 (a)).

(ii) Length of incubation with enzyme preparations.

The period of treatment of the cells was standarised by incubation at 37°C for 15min. Since trypsin - EDTA was the enzyme mixture routinely used, the effectiveness of trypsin - EDTA based enzyme preparations for the dissociation of RPMI monolayers was examined as described previously (Section 2.9.).

From table 3.2. it can be seen that all enzyme preparations readily dissociated the monolayers of RPMI 2650 cells. Enzyme mixtures composed of trypsin (0.25%) - EDTA (0.02%) and a third component gave the best viable suspensions and the poorest single-cell suspensions after 15min incubation.

TABLE 3.2.

Effects of enzymes on cell suspensions prepared after a 15 min. incubation period from monolayer RPMI 2650 cultures.

Enzyme Concentration	Viability %	Single-cell %	Doublets %	Clumps %
EDTA (0.02%)	70	77	18	5
Trypsin-EDTA (0.5%-0.04%)	89	93	5	2
Trypsin-EDTA (0.25%-0.04%)	90	90	9	1
Trypsin-EDTA (0.5%-0.02%)	92	86	12	2
Trypsin (0.25%)	94	71	19	10
Trypsin-EDTA (0.25%-0.02%)	95	85	12	3
Trypsin-EDTA-Collagenase V (0.25%-0.02%-0.1%)	99	58	23	19
Trypsin-EDTA-Collagenase II (0.25%-0.02%-0.1%)	99	57	23	20
Trypsin-EDTA-Dispase (0.25%-0.02%-0.1%)	100	60	22	18
Trypsin-EDTA-DNase (0.25%-0.02%-0.002%)	100	65	20	15
Trypsin-EDTA-Collagenase IV (0.25%-0.02%-0.1%)	100	59	22	19

NOTE: Each percentage point is a mean value estimated from duplicate treatments.

After 15 min. incubation, the best viable single-cell suspension was achieved by dissociation with trypsin (0.25%) - EDTA (0.02%). Unlike table 3.1 (a) there is no apparent association between viability and single-cell yield, however, the poorest single-cell suspensions exhibited the best viability, which would suggest that 15min. incubation was inadequate for complete dissociation by particular enzyme cocktails.

The ideal preparation would produce a good single-cell yield, viability between 90 and 100%, in the shortest incubation period. Collagenase (0.02%) - DNase (0.02%) produced the best viable single-cell suspensions of both HT29 and RPMI 2650 cells after 60min. incubation (Table 3.1(a) and 3.1 (b)). By comparison, trypsin (0.25%)-EDTA (0.02%) produced the best viable single-cell suspension of RPMI 2650 cells, after 15min. incubation (Table 3.2), when compared with other trypsin-EDTA based enzyme mixtures.

Overall, the effects of enzymes on single-cell yield appeared to be dependent on several factors, as follows:-

(a) Cell type,

(b) Enzyme preparation used,

(c) Concentration of enzyme preparation used,

(d) Length of incubation time.

The effects of these factors on viability and single-cell yield will be considered in the discussion.

3.3. REAGGREGATION.

So far, it has proved extremely difficult to prepare good single cell suspensions (Section 3.2.). Even when good single-cell suspensions are obtained, with no clumps or clusters noted in the cell suspensions, clumps and clusters appear in cultures examined soon after plating.

The following experiments were carried out to establish whether RPMI 2650 cells and normal human fibroblasts were reaggregating, to form clumps and clusters, after enzymatic disaggregation.

In the first experiment duplicate confluent cultures of normal human fibroblasts and RPMI 2650 cells were trypsinized (Section 2.9.). The resulting cell suspensions were treated as follows:-

(A) Cell suspensions were diluted with medium to $3X10^5$ cells/ml and then incubated at 37°C.

(B) Samples from the suspensions were taken without pipetting.

(C) Sampling was conducted over a period of 4hr.

The raw data obtained from duplicate cultures is shown in Table 3.3. as an indication of the variation which can occur between samples. Figures 3.2. and 3.3. show the results obtained from cell suspensions of RPMI 2650 cells and fibroblasts, respectively. Over the sampling period there was a decrease in single-cell yield, and an increase in doublet and clump yield in both RPMI and fibroblast cell suspensions. The greatest decrease in single-cell yield, in both types of cell suspension examined, was exhibited during the first hour of incubation. Initial fibroblast single-cell yield was poorer than RPMI single-cell yield, however, RPMI 2650 cells exhibited a greater rate of reaggregation than fibroblast suspensions over 4hr. The possible significance of

this difference in reaggregation rates will be discussed in Section 4.

The second experiment was prepared as follows:-

(i) Cell suspensions were prepared from duplicate monolayer cultures of RPMI 2650 cells which had been inoculated with 3 X 10^5 and 5 X 10^5 cells per $25cm^2$ flask.

(ii) Suspensions were diluted with medium to give 3 X 10^5 cells/ml.

(iii) Duplicate suspensions were then split; one suspension was passed through a narrow gauge syringe needle and the other suspension was untreated.

(iv) Before sampling, cell suspensions were pipetted 5-times.

(v) Sampling was carried out over a 60min period.

The raw data obtained from duplicate counts is shown in Table 3.4. The results, from Figure 3.4., show that syringed suspensions surpassed unsyringed suspensions with respect to single-cell yield and stability over an interval of 60 min. Cell suspensions prepared from confluent flasks (5 X 10^5 cells inoculum) of RPMI 2650 gave a lower single-cell yield and reaggregated quicker, when unsyringed, than suspensions prepared from subconfluent cultures (3 X 10^5 cells/flask), which would suggest that reaggregation is associated with cell density.

Overall the results from these experiments may be summarised as follows:-

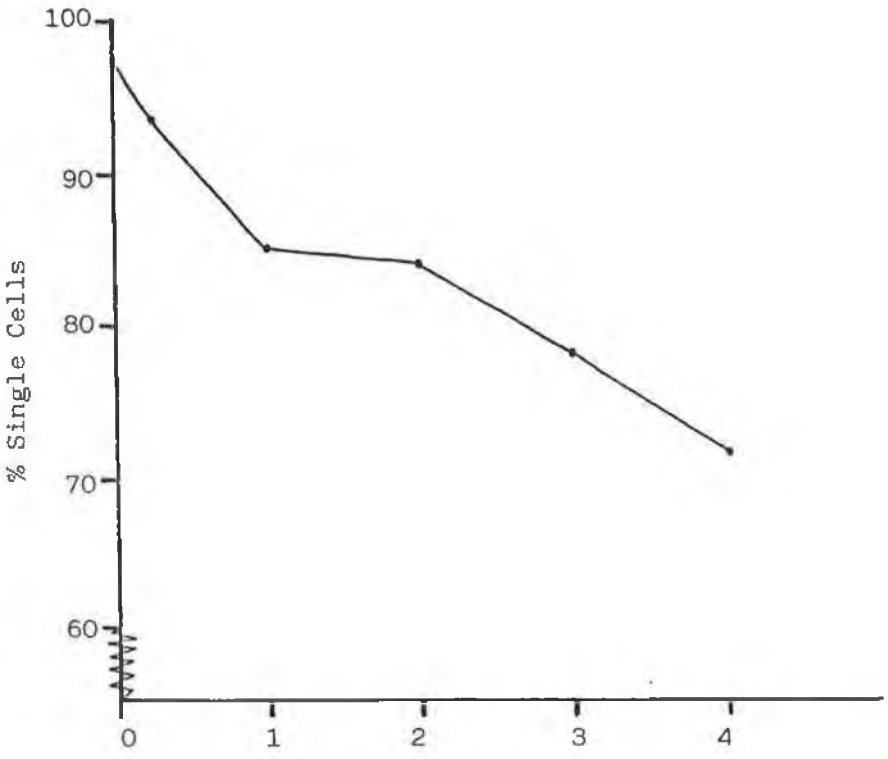
- RPMI 2650 cells and normal human fibroblasts did reaggregate in suspension.
- The extent to which reaggregation occurred depended on

TABLE 3.3

RAW DATA DUPLICATES FROM REAGGREGATION EXPT. 1.

Cell type:	RPMI 2650			FIBROBLASTS		
Time (hr)	% Single Cells	% Doublets	% Clumps	% Single Cells	% Doublets	% Clumps
0	98.33	1.67	0	92.71	4.29	3.0
	97.38	2.62	0	88.63	7.23	4.14
0.25	94.57	5.43	0	85.79	10.71	3.5
	92.56	7.44	0	80.21	15.79	4.0
1	84.57	7.97	7.46	81.37	13.33	5.3
	84.55	8.78	6.67	86.54	9.3	4.16
2	83.75	10.00	6.25	82.5	12.5	5.0
	84.81	10.64	4.55	82.95	11.5	5.35
2.5				80.46	13.64	5.9
				80.98	11.94	7.08
3	74.73	15.91	9.36	80.03	13.20	6.77
	81.08	10.0	8.92	81.72	10.64	7.64
4	68.89	14.44	16.67	79.81	13.20	6.99
	74.03	14.68	11.29	75.42	16.39	8.19

(A)



(B)

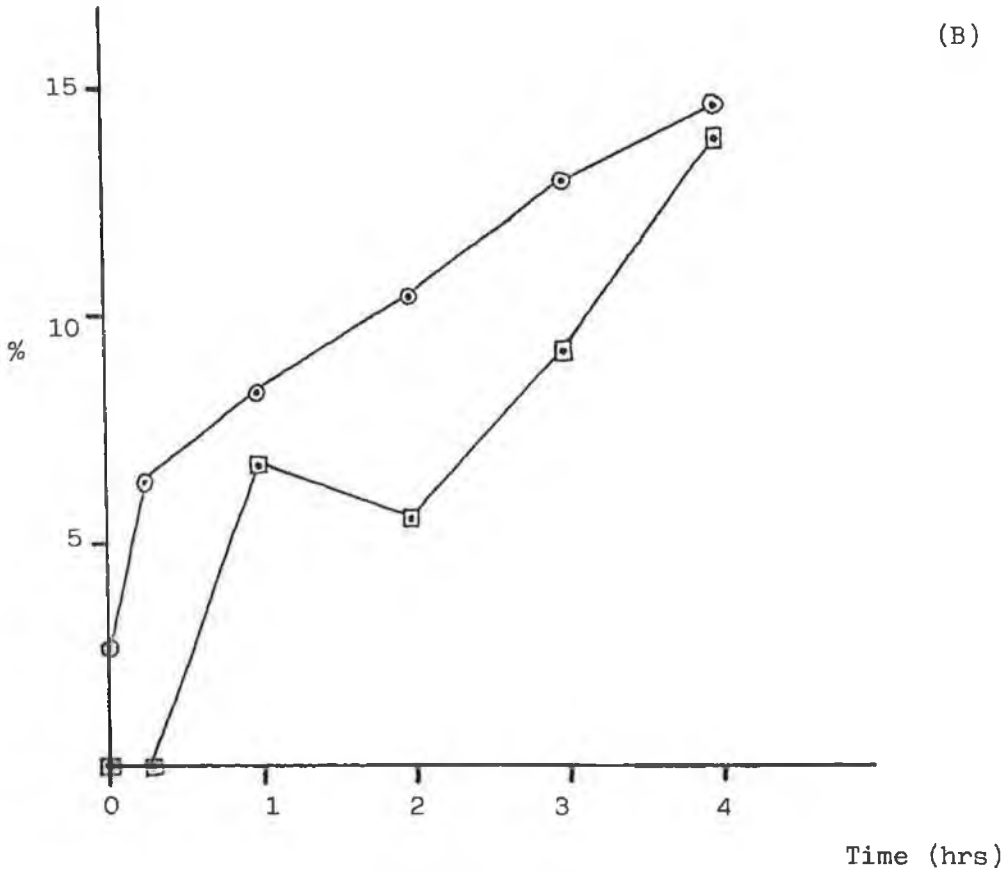


FIGURE 3.2.

(A) Effect of sampling time on the single-cell nature of RPMI 2650 cell suspensions.

(B) Effect of sampling time on cell doublets ⊙, and clumps ⊡, in RPMI 2650 cell suspensions.

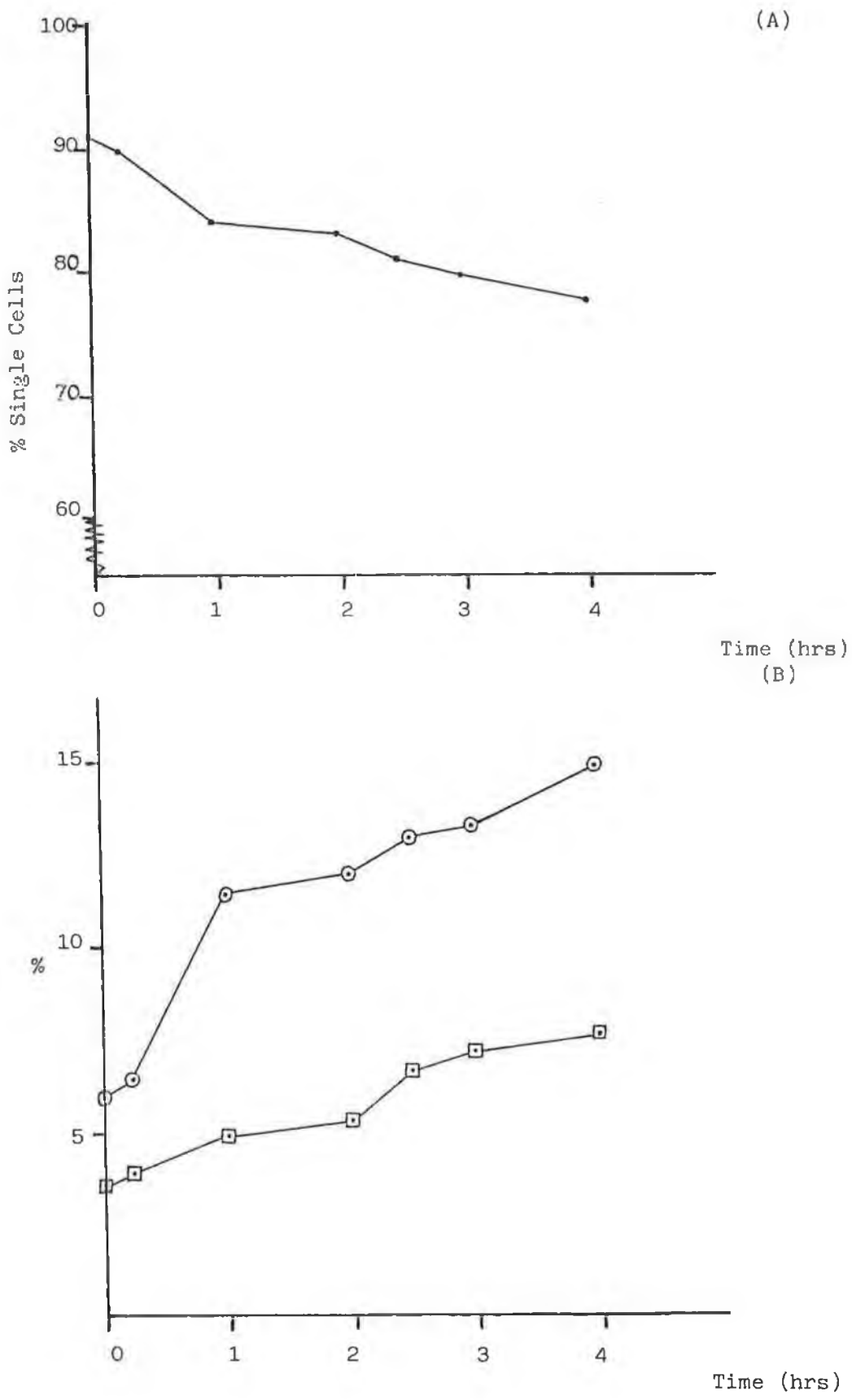


FIGURE 3.3

(A) Effect of sampling time on the single-cell nature of human fibroblast suspensions.

(B) Effect of sampling time on cell doublets ⊙, and clumps ⊡, in normal human fibroblast suspensions.

TABLE 3.4

RAW DATA DUPLICATES FROM REAGGREGATION EXPT. 2.

Culture Concentration	RPMI $3X10^5$ cells/$25cm^2$ flask					
Suspension Type:	Syringed			Unsyringed		
Time (min).	% Single cells	% Doublets	% Clumps	% Single cells	% Doublets	% Clumps
0	100	0	0	94.5	5.5	0
	100	0	0	93	7.0	0
20	100	0	0	90	10.0	0
	100	0	0	85	15.0	0
40	100	0	0	83.56	7.98	8.46
	100	0	0	83.12	8.68	8.20
60	100	0	0	82.06	8.69	9.25
	100	0	0	81.58	9.49	8.93
Culture Concentration	RPMI $5X10^5$ cells/$25cm^2$ flask					
Suspension Type:	Syringed			Unsyringed		
Time (min).	% Single cells	% Doublets	% Clumps	% Single cells	% Doublets	% Clumps
0	95.85	4.15	0	97.7	2.3	0
	95.75	4.25	0	96.3	3.7	0
20	95.7	4.3	0	94.1	5.9	0
	96.1	3.9	0	93.1	6.9	0
40	95.64	4.36	0	90.0	6.0	4.0
	95.16	4.84	0	85.0	8.22	6.78
60	93.7	6.3	0	89.48	7.63	2.89
	94.3	5.7	0	87.00	8.5	4.5

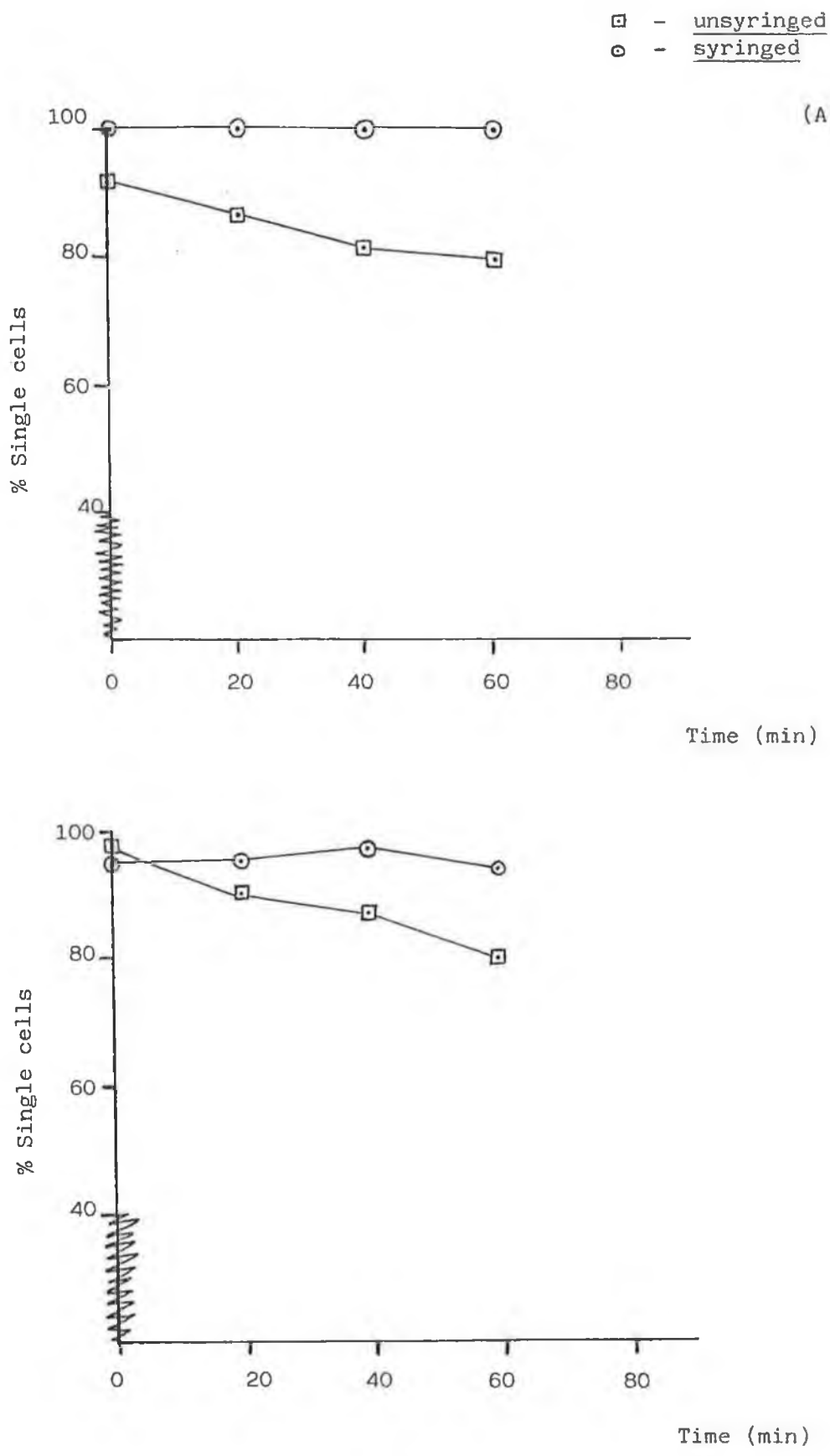


FIGURE 3.4.

Effects of syringing on the single-cell nature of RPMI 2650 cell suspensions at $3X10^5$ cells/ml (A), and $5X10^5$ cells/ml (B)

(a) the degree of initial pipetting

(b) the density of the cell suspension used and

(c) the degree of confluency to which the monolayer had grown before disaggregation.

- Reaggregation was reduced by lowering the working cell density and by passing the cell suspension through a narrow-gauge syringe needle.

3.4. CELL CLUMPS IN THE CLONOGENIC ASSAY.

Often, microscopic examination of agar cultures just after plating reveals many clusters or clumps of cells; these are often large enough and numerous enough to count immediately so that the "background" can be subtracted from the final colony count. However, this correction addresses only one of the problems created by the presence of clumps and clusters, since they can have major effects on the characteristics of the cultures and on the measured response of the "colonies" to treatment with anti-neoplastic drugs.

In this experiment, the contribution of single cells, doublets and clumps of RPMI 2650 cells, to the final colony score was examined by comparing the growth in agarose of good single-cell and poor "clumpy" suspensions.

Good single-cell suspensions were prepared by passing the cell suspension through a narrow-gauge syringe needle (Section 2.9); untreated cell suspensions remained as poor "clumpy", suspensions. Cells were cultured in agarose at approximately 1×10^4 cells/35mm plate as described in 2.12. The progress of 100 single-cells, 50 doublets and 25 clumps over 12 days was monitored using a microscope

(Leitz diavert) which had reference scales and an eye-piece micrometer to facilitate the location and sizing of cells and colonies. The features of this experiment are summarised in Table 3.5.

The growth curves obtained for the single-cells, doublets and clumps observed are shown in Figure 3.5. The results can be summarised as follows:-

(1) Single cells doublets and clumps grew in agarose to contribute to the final colony score.

(2) Passing the initial cell suspension through a narrow-gauge syringe needle had the greatest effect on clumps, which were reduced in size and growth rate by comparison with clumps from untreated cell suspensions.

(3) Clump growth from untreated cell suspensions ceased after 6 days incubation. By comparison, clumps from syringed suspensions increased in size throughout the 12 day period examined. The clump size distribution, for syringed and unsyringed cell suspensions,over 12 days is shown in Figure 3.6.

Not all single cells, doublets and clumps monitored grew to form colonies in agarose. The percentages of single cells, doublets and clumps monitored which increased in size, are shown in Table 3.6. "Clumpy", unsyringed suspensions grew approximately twice as much as syringed single-cell suspensions, which suggests that syringing may damage cells in suspensions and reduce their capacity for growth in agarose. In both good single-cell and "clumpy" cell suspensions, single cell and clump contribution was equivalent, however, cell doublet contribution to colony formation was approximately half that of single cell and clump contribution to final colony formation.

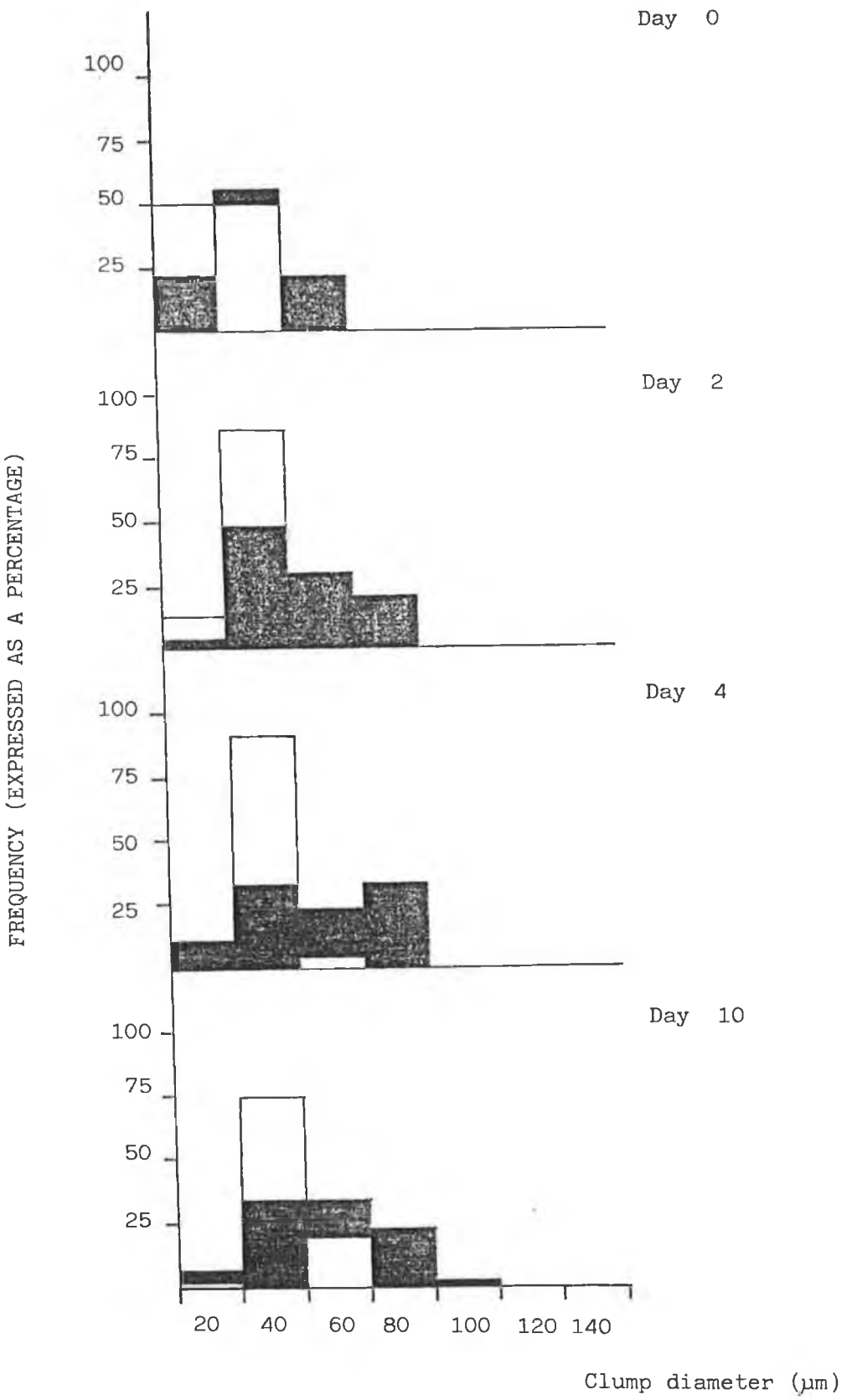


FIGURE 3.6

RPMI 2650 clump size distribution patterns from unsyringed (■) and syringed (□) cell suspensions over 10 days.

TABLE 3.5.

EXPERIMENTAL FEATURES OF "CLUMPOGENIC" ASSAY.

Serum Batch	10F4049A
No.of Replicates	4
Incubation Period	12 days
Cell Passage Level	44
Cell Concentration	1 X 10^4 cells/35mm dish.

TABLE 3.6.

PERCENTAGE GROWTH OF OBSERVED SINGLE CELLS, DOUBLETS, AND CLUMPS.

Cell Grouping	No.Observed	Syringed Suspension	"Clumpy" Suspension
Single-cells	100	38%	88%
Doublets	50	25%	57%
Clumps	25	42%	88%

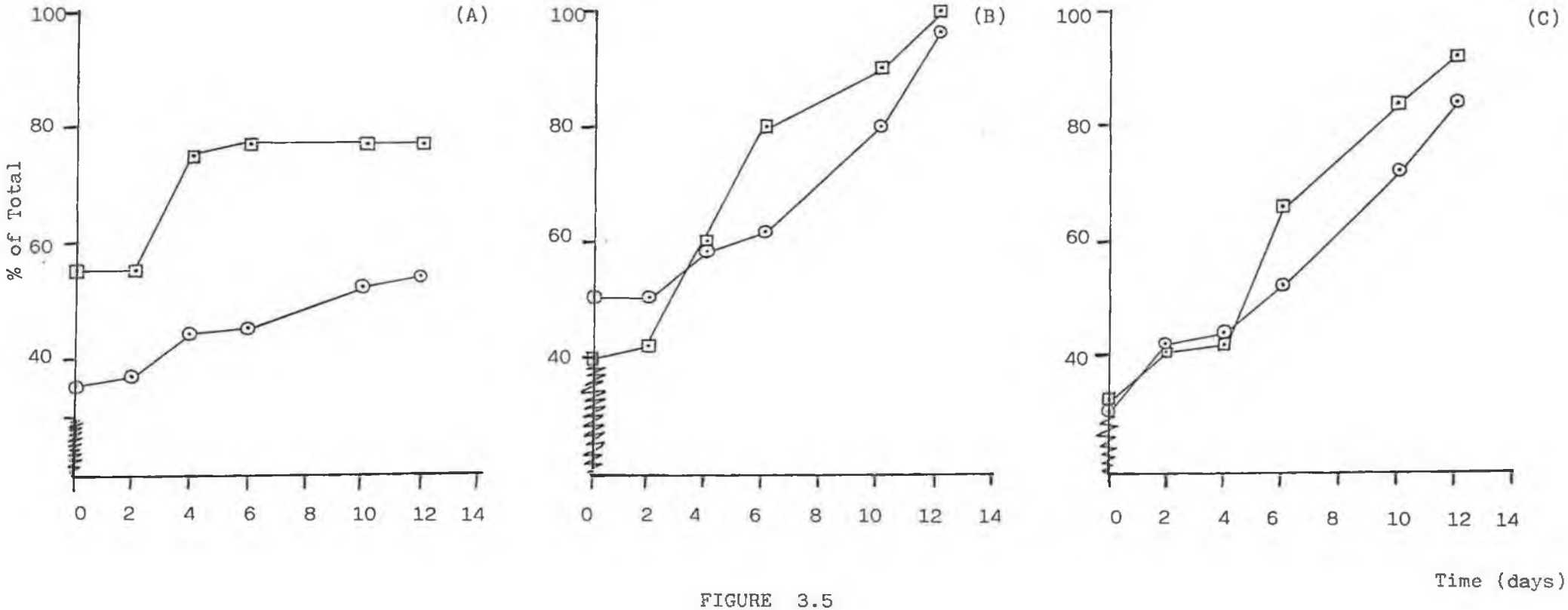


FIGURE 3.5

Increasing diameter in agarose (with time) of (A) cell clumps; (B) cell doublets and (C) single cells derived from syringed ⊙ and unsyringed ⊡ suspensions of RPMI 2650 cells.

The initial compositions of syringed and untreated cell suspensions and the final colony forming efficiencies obtained are shown in Table 3.7. From Table 3.7. it may be seen that:-

(A) The initial doublet, triplet and clump concentrations influenced the final colony forming efficiencies.

(B) Passing cell suspensions through a syringe needle improved the single-cell yield of the suspension.

Overall, the following conclusions may be made:-

- Single cells, doublets and clumps grow in agarose.
- Doublets appear to contribute less than single cells and clumps to final colony formation.
- Syringing improves the single-cell yield of cell suspensions but reduces the final colony formation. This would suggest that syringing,
  - (a) causes cell damage by shearing and / or,
  - (b) removes cell clumps and clusters which may produce factors required for growth.

These results emphasise the desirability for a protocol which corrects for the presence of clumps and clusters. As mentioned previously, clumps and clusters counted immediately after plating as "background", may be subtracted from the final colony count. However, a method of cell disaggregation which produces a single-cell suspension without damaging the cells (Section 3.2.) would overcome the problems of "background" clumps and clusters and those encountered when evaluating the effects of anti-neoplastic drugs on "clumpy" agarose cultures.

TABLE 3.7.

Composition of cell suspensions and final C.F.E.'s obtained.

Features	Syringed Suspension	Unsyringed Suspension
Viability	99%	97%
Initial single-cell conc.	86%	54%
Initial doublet conc.	8%	21%
Initial triplet conc.	3%	18%
Initial clump conc.	3%	5%
Total doublet/triplet/clumps.	14%	44%
Final colony forming efficiencies	11%	44%

3.5. WATER PURITY EFFECTS.

Only water of the highest purity should be used in the preparation of the defined portion of media (Section 2.6.). It is necessary, however, to realise that even the purest components have traces of impurities which may be important. Heavy metals are particularly toxic to cells and may occur in minute quantities in tap, deionized and distilled water. On the other hand, very small traces of certain substances may be essential to the cells for survival (e.g. zinc, cobalt, manganese).

To examine the effects of different purity grades of water on colony formation in the clonogenic assay, batches of agarose (Sigma type II), Eagle's minimum essential medium and buffered 2X medium (Section 2.12.) were prepared using the following:-

(1) Tap water,

(2) Water purified by reverse osmosis (Millipore, Fisons) and

(3) Water purified by reverse osmosis, high-efficiency deionization and carbon filtration.

The effects of different purity grades of water on RPMI colony formation, in monolayer and agarose were examined. Pre-treated cells, prepared as monolayers, were included at several concentrations in the clonogenic assay (Section 2.11.). Table 3.8. summarises the cell concentrations in both monolayer and agarose assays and other features which were considered in this experiment.

The colony-forming efficiencies obtained are shown in Table 3.9. Before scoring, monolayer colonies were fixed in methanol and stained with May - Grünwald giemsa, as described previously (Section 2.13.).

TABLE 3.8.

Experimental features of Water Purity Experiment.

Features	Agarose	Monolayer
F.C.S. Batch	10G1033S	10Q2048S
No.of replicates	6	3
Initial single-cell %	89.62	93
Initial doublet %	7.58	5
Initial clump %	2.8	2
Viability %	96	96
Feeder passage no.	75	-
Test cell-passage no.	76	-
Monolayer passage no.	-	66
Feeder cell conc.	$0, 3X10^4, 1X10^5$ cells/dish	-
Test cell conc.	$1X10^5$ cells/35 mm dish	-
Monolayer conc.	-	$3X10^3, 1X10^4$ cells/35mm dish
Incubation period.	14 days	10 days

TABLE 3.9.

Effects of different purity grades of water on colony forming efficiencies of RPMI 2650 cells in monolayer and agarose assays.

C.F.E.'s obtained for agarose assay. (n = 6)

Test cell concentration. (cells/35mm dish) = 1×10^5

Water Grade	Tap Water	R.O.	R.O./deionized/ filtered.
Feeder cell conc. (cells/plate)			
0	3.30 ± 0.28	6.18 ± 0.84	8.85 ± 2.25
3×10^4	4.20 ± 0.72	6.73 ± 0.61	14.00 ± 1.71
1×10^5	2.61 ± 0.23	4.53 ± 0.52	13.062 ± 1.57

C.F.E.'s obtained for monolayer assay. (n = 3)

Water Grade	Tap Water	R.O.	R.O./deionized/ filtered.
Test cell conc. (cells/plate)			
3×10^3	54.93 ± 6.11	65.60 ± 14.22	58.67 ± 2.44
1×10^4	33.44 ± 3.6	35.20 ± 0.37	38.80 ± 4.20

These results were analysed using ANOVA (Section 2.19) which found that differences in the agarose assay due to water type were significant at the 1% level. However, the differences in monolayer due to water type were not significant at either the 5% or 1% levels.

Figure 3.7. shows the size-distribution of colonies formed in agarose, in the different purity water grades examined, from an initial cell density of 1 X 10^5 cells/35mm dish without a feeder cell layer.

From Table 3.9. it may be seen that:-

- The best colony forming efficiencies in agarose were obtained when ultrapure water was used. By comparison, tap water and reverse osmosis water inhibited colony formation.
- Tap water and water purified by reverse osmosis, included in the monolayer assay, did not stimulate or inhibit colony formation of RPMI 2650 cells when compared with colony formation in medium prepared with ultrapure water.
- Where feeder cells were present in the agarose assay, both stimulatory and inhibitory effects were evident (Section 3.11.).
- Low inoculum density cultures in monolayer (3 X 10^3 cells/35mm dish) had greater (approx. 1.5 - fold) C.F.E.'s than high inoculum density cultures.
- Colony formation in monolayer was higher than colony formation in agarose. Possible explanations for the difference between colony forming efficiencies of monolayer and agarose cultures will be discussed in Section 4.

Colony size (Figure 3.7.) also appeared to be affected by the purity of water included in the agarose assay. Where tap and reverse osmosis water were included in the assay, a wide range of colony sizes were found. By comparison, inclusion of ultrapure water in the assay resulted in a narrower range of colony sizes, which could be an important consideration in chemosensitivity testing where colony uniformity with respect to size is desirable.

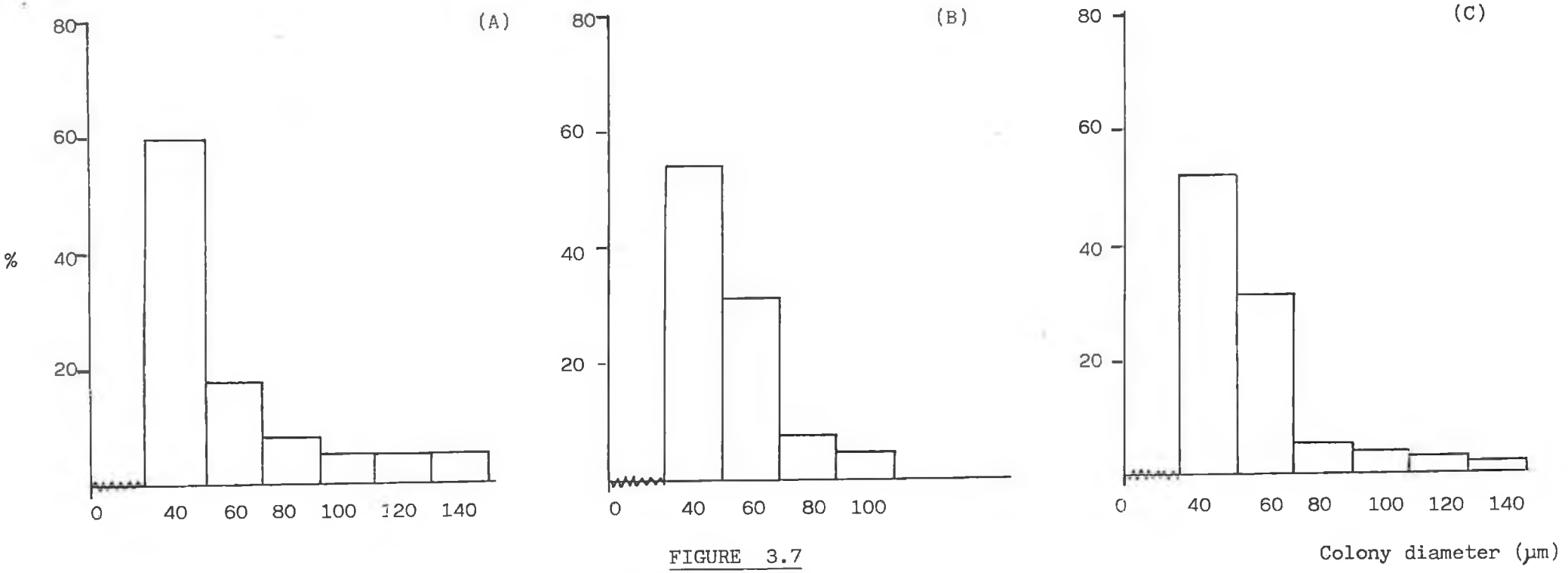


FIGURE 3.7

Effects of (A) - Reverse osmosis water,
(B) - Ultrapure water, and
(C) - Tap water on colony size in agarose.

Thus, the inclusion of ultrapure water in the agarose assay appears to increase colony formation and produce more uniformly sized colonies than tap or reverse osmosis water.

3.6 THE GAS PHASE.

This experiment examined the effects of nitrogen gas, low oxygen tension (3% O_2), high oxygen tension (20% O_2) and a 10% carbon dioxide/nitrogen gas mixture on the colony formation of RPMI 2650 cells as anchorage-dependent monolayers and anchorage-independent colonies in agarose.

Pre-treated cells were prepared in 35mm plates, as monolayer and semi-solid suspended cultures, at a concentration of 1×10^4 cells/plate (Section 2.11.) Cultures were incubated in a humidified incubator chamber (Section 2.14.) which had been flushed through with the test gas mixture. Control cultures were incubated in a LEEC CO_2 incubator in a 5% CO_2/air balance atmosphere. Colonies formed in agarose were scored directly, those formed in monolayer were scored after staining with May-Grünwald Giemsa (Section 2.13.).

Table 3.10. summarises the features of this experiment. The colony-forming efficiencies of RPMI 2650 cells, obtained after incubation in the gas mixtures examined, are shown in Table 3.11. After 12 days incubation at 37°C the following observations were made:-

- Agarose and monolayer assays grown in 5% CO_2/Air and 5% CO_2/ 3% O_2/N_2 atmosphere were orange/yellowish in colour indicating that pH was suitable for cell growth.
- Assays cultured in an atmosphere of 10% CO_2/N_2 were yellowish in colour indicating that pH was low.

TABLE 3.10

FEATURES OF GAS PHASE EXPERIMENT

Features	Experiment No. 1	Experiment No. 2	Experiment No. 3
F.C.S. Batch no.	k223901A(309)	10F4049A	10F4049A
No.of replicates	3	3	3
% Single cells	93	95	92
% Doublets	5.5	5	5.8
% Clumps	1.5	0	2.2
% Viability	92	94.5	95
Cell passage no.	P37	P45	P32
Test cell conc.	$1X10^4$ cells/plate	$1X10^4$ cells/ plate	$1X10^4$ cells/ plate
Incubation period	12 days	12 days	12 days

TABLE 3.11

C.F.E.'s of RPMI 2650 cells in altered gas phases.

Experiment 1. (n=3)

Gas Phase	High Oxygen ($5\%CO_2$ /Air balance)	Low Oxygen ($5\%CO_2$ /$3\%O_2$ /N_2)	Nitrogen
Monolayer	18.28 ± 1.43	33.44 ± 1.86	No growth
Agarose	9.72 ± 0.26	16.76 ± 0.24	27.76 ± 2.28

Experiment 2. (n=3)

Gas Phase	High Oxygen ($5\%CO_2$ /Air balance)	Low Oxygen ($5\%CO_2$ /$3\%O_2$ /N_2)	Nitrogen
Monolayer	18.11 ± 0.86	29.14 ± 2.32	No growth
Agarose	9.8 ± 0.07	17.5 ± 0.70	26.54 ± 2.29

Experiment 3. (n=3)

Gas Phase	$5\%CO_2$ /Air in LEEC Incubator	$5\%CO_2$ /Air in incubator chamber	$10\%CO_2$ /N_2
Monolayer	14.28 ± 0.34	16.31 ± 2.38	7.68 ± 1.02
Agarose	12.3 ± 0.3	14.04 ± 2.29	5.97 ± 2.57

- Monolayer and agarose assays incubated in N_2 gas were pink in colour indicating that pH was high.

From Table 3.11 it may be seen that:-

(a) Incubation in a nitrogen gas atmosphere gave the best colony forming efficiencies in agarose, however, no cells attached to form colonies in monolayer.

(b) Overall, the best colony-forming efficiencies for both monolayer and agarose clonogenic assays, were obtained under hypoxic conditions, in a gas atmosphere of 5% CO_2 /3%O_2 /N_2 balance. By comparison, C.F.E.'s were lower in control cultures incubated in a gas atmosphere of 5% CO_2 /Air balance.

(c) The poorest colony forming efficiencies were obtained from cultures incubated in a 10% CO_2 /N_2 gas atmosphere. However, by comparison with colony formation in a nitrogen gas atmosphere, which inhibited monolayer colony growth, incubation in 10% CO_2 /N_2 permitted colony formation in both agarose and monolayer.

(d) C.F.E.'s in the control atmosphere of 5% CO_2 /Air, in the Modular Incubation Chamber (Section 2.14.) and the LEEC incubator were compared. 95% confidence intervals indicated that the differences in colony forming efficiencies observed (Table 3.11. Experiment 3) were insignificant.

Differences in colony formation, in both agarose and monolayer assays, due to the types of gas atmosphere used, were found from ANOVA, (Section 2.19.) to be significant at the 1% level.

Figure 3.8. shows the effect of different gas atmospheres on colony size in agarose. The widest range of colony sizes occurred in the control gas atmosphere of 5% CO_2 /Air. By comparison, the range of colony sizes was reduced dramatically in atmospheres of N_2 and 10% CO_2 /N_2 . The range of colony sizes was reduced in 5% CO_2 /3%O_2 /N_2 , however, the frequency of colony sizes decreased in a linear fashion as the colony size increased, and correlated very closely with the size distribution curve obtained in 5% CO_2 /Air. The size-distribution curves of colonies incubated in N_2 ,10% CO_2 /N_2 and 5% CO_2 /Air atmosphere were non-linear with colonies of 40μm diameter occuring most frequently.

Overall, the following conclusions may be made with respect to the gas atmospheres examined:-

- Anaerobic conditions were unsuitable for RPMI 2650 colony formation.
- 5% CO_2 /3% O_2 /N_2 produced the highest C.F.E.'s.
- Colony formation in the control atmosphere of 5% CO_2 /Air produced C.F.E.'S which were comparable with C.F.E.'s produced in 5% CO_2 /3% O_2 /N_2 .
- Colony size was adversely affected by N_2 and 10% CO_2 /N_2 atmospheres.

For the purposes of chemosensitivity testing the best gas mixtures to use for RPMI 2650 colony formation appear to be 5% CO_2 /3%O_2 /N_2 and 5%CO_2 /Air, since these atmospheres produce good colony forming efficiencies and colonies large enough to analyse.

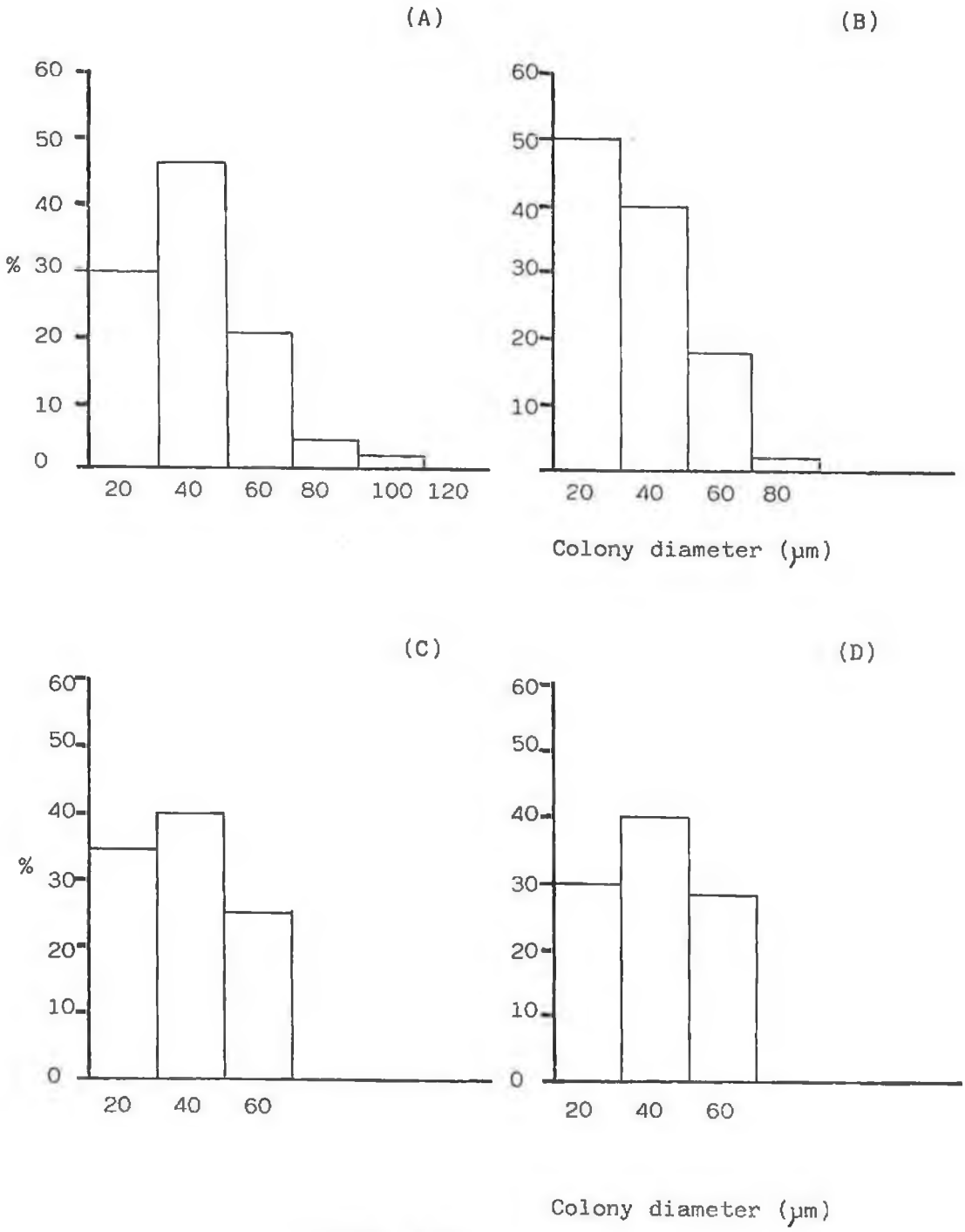


FIGURE 3.8.

Effects of different gas atmospheres on colony size in the clonogenic assay.

(A) : 5% CO_2 /Air (control)

(B) : 5% CO_2 /3% O_2 /N_2

(C) : N_2

(D) : 10% CO_2 /N_2

3.7. THE SEMI-SOLID SUPPORT.

The *in vitro* environment of the clonogenic assay consists essentially of liquid nutrient media and agents that render the media semi-solid so that anchorage independent growth might be achieved. In this experiment, the effects of different gelling agents on the growth and colony formation of RPMI 2650 cells were examined in an attempt to identify a gelling agent which enhanced colony forming efficiencies.

Pre-treated cells were prepared (Section 2.11.) as monolayer feeders when required. Cells were tested in 0.3% and 0.15% agaroses and 1.3% methycelluloses under the following conditions:-

(i) AGAROSE.

(A) With feeder cells, without adjustment for gelling properties (Section 2.10.).

Table 3.12. summarises the features considered in this experiment. The results obtained are shown in Table 3.13., which is arranged in order of decreasing colony forming efficiency in 0.3% agaroses without feeders. Differences in colony forming efficiencies due to the type of agarose used were shown by ANOVA to be significant at the 1% level. 95% confidence intervals indicated significantly different groups of agaroses under the conditions examined, which are shown in order of increasing C.F.E.'s in Table 3.14. ANOVA also showed no significant difference in colony formation between 0.3% and 0.15% agarose or in 0.3% agarose in the presence or absence of feeder cells. However, the presence of feeders in 0.15% agarose was shown to significantly

TABLE 3.12.

FEATURES OF AGAROSE EXPERIMENT (A)

Serum Batch No:	10A11301D
No. of Replicates:	6
Assay incubation period	12 days
Initial singlet conc:	90%
Initial doublet conc:	6.5%
Initial clump conc:	3.5%
Viability	96%
Feeder cell concs:	$3X10^5$ cells/35mm plate
Suspended cell concs:	$1X10^5$ cells/35mm plate
Agarose Incubation Temp.	44°C - 41°C

TABLE 3.13.

EFFECTS OF AGAROSE TYPE, AGAROSE CONCENTRATION AND FEEDERS ON THE C.F.E.'s OF RPMI 2650 CELLS.

Agarose Conc.	0.3% Without Feeders	0.3% With Feeders	0.15% Without Feeders	0.15% With Feeders
Agarose Type	C.F.E.	C.F.E.	C.F.E.	C.F.E.
* Seakem (F.M.C.)	$3.23^{+}0.47$	$3.01^{+}0.38$	-	-
Sigma type I	$2.04^{+}0.28$	$0.81^{+}0.15$	$3.75^{+}0.82$	$1.57^{+}0.314$
* Seaplaque (F.M.C.)	$1.96^{+}0.39$	$1.71^{+}0.35$	-	-
Sigma type VII	$1.57^{+}0.45$	$1.46^{+}0.23$	$2.52^{+}0.65$	$0.87^{+}0.14$
Sigma type III	$1.3^{+}0.19$	$1.18^{+}0.33$	$3.97^{+}0.93$	$1.57^{+}0.11$
Sigma type II	$1.29^{+}0.43$	$2.61^{+}0.49$	$1.29^{+}0.31$	$2.53^{+}0.65$
HEEO(marine)	$0.87^{+}0.27$	$0.87^{+}0.26$	$1.11^{+}0.34$	$0.96^{+}0.29$
Sigma type IV	$0.72^{+}0.13$	$0.74^{+}0.10$	$2.42^{+}0.26$	$1.07^{+}0.11$
ME (marine)	$0.42^{+}0.11$	$0.47^{+}0.10$	$0.53^{+}0.11$	$0.49^{+}0.061$
LE (marine)	$0.18^{+}0.05$	$0.23^{+}0.04$	$1.26^{+}0.27$	$1.198^{+}0.29$
* Seaprep (F.M.C.)	-	-	-	-

(n=6)

* Seaprep did not gel under any conditions examined. Seakem and Seaplaque did not get at the lower concentration of 0.15%.

TABLE 3.14.

AGAROSE GROUPS FOUND FROM 95% CONFIDENCE INTERVALS

Group No.	0.3% Without Feeders	0.3% With Feeders	0.15% Without Feeders	0.15 With Feeders
1	Seakem agarose	Seakem Sigma Type II	Sigma Type I Sigma Type III	Sigma Type II
2	Remaining agaroses	Remaining agaroses	Sigma Type VII Sigma Type IV	Remaining agaroses except
3			Remaining agarose	Seakem Seaplaque Seaprep

(P=0.01) decrease C.F.E.'s when compared with C.F.E's in 0.15% agarose without feeders.

(B) Without feeder cells, without adjustment for gelling properties.

The features considered in this experiment are shown in Table 3.15. and the results obtained are shown in Table 3.16, which was also arranged in order of decreasing colony forming efficiency in 0.3% agaroses. Differences in colony forming efficiencies due to agarose type were shown to be significant (P=0.01). However, no significant difference was found between colony formation in 0.3% and 0.15% agaroses. 95% confidence intervals identified significantly different groups of agaroses at 0.3% and 0.15% which are shown in Table 3.17.

(C) Without feeder cells, with temperature adjustment for gelling properties.

In this experiment, the method used for the preparation of clonogenic semi-solid layers (Section 2.12.) was altered to allow for the properties of the low gelling temperature agaroses which were examined. Agar and agaroses used in the clonogenic assay are generally prepared at 44°C and cooled before use to 41°C (Section 2.12.). Use of low gelling temperature agarose prepared at 41°C and cooled before use to 37°C may reduce the risk of "scalding" both feeder cell layers and cells tested in the assay. The features of this experiment are shown in Table 3.18., and the results obtained are shown in Table 3.19. These results were analysed using ANOVA (Section 2.19.) which demonstrated that differences due to the type

TABLE 3.15

FEATURES OF AGAROSE EXPERIMENT (B)

Serum Batch No:	20Q8833A
No. of replicates:	4
Assay incubation period:	14 days
Suspended cell conc:	$1X10^4$ cells/35mm plate
Initial single-cell conc:	89.17%
Initial doublet conc:	7.53%
Initial clump conc:	3.3%
Viability:	95%
Cell passage no:	46
Agarose incubation temp:	44° - 41°C

TABLE 3.16

EFFECTS OF AGAROSE TYPE AND CONCENTRATION ON C.F.E.'s OF RPMI 2650 CELLS.

Agarose conc:	0.3%	0.15%
Agarose type	C.F.E.	C.F.E.
Sigma type III	39.36±4.20	30.84±2.13
Sigma type VII	24.30±7.63	20.34±3.73
Sigma type II	22.98±5.98	32.52±5.91
HEEO (marine)	21.82±2.59	15.34±3.52
Sigma type I	17.10±4.07	40.4±4.72
LE (marine)	15.19±5.12	28.87±1.01
Seakem (F.M.C.)	14.34±6.89	12.96±1.00
Sigma type IV	12.78±2.82	1.44±0.59
Seaplaque (F.M.C.)	12.12±1.47	7.50±2.72
ME (marine)	12.10±3.04	15.90±2.39
LGT (marine)	9.54±2.88	16.02±2.62

(n=4)

TABLE 3.17.

AGAROSE GROUPS FOUND FROM 95% CONFIDENCE INTERVALS.

Group No.	0.3% Agaroses	0.15% Agaroses
1	Sigma type III	Sigma type I
2	Remaining agaroses	Sigma type II
		Sigma type III
		LE (marine)
3		Remaining agarose

of agarose used were significant at the 1% level. In 0.15% agarose, 95% confidence intervals (C.I.) identified four significantly distinct groups of agaroses, which are, in order of decreasing C.F.E.'s, as follows:-

(1) Seaplaque agarose.

(2) Sigma type VII and Marine Colloids LGT agarose (Section 2.10).

(3) All the remaining agaroses except,

(4) Seaprep agarose.

ANOVA also showed no significant difference between colony formation in 0.3% and 0.15% agaroses.

Colony size (Figure 3.9.) appeared to be affected by the agarose concentration and the type of agarose used in the assay. 0.15% agarose appeared to increase the range of colony sizes obtained for a number of agaroses which had very narrow ranges in 0.3% agaroses. The importance of colony size in relation to chemosensitivity testing will be examined in the discussion.

(ii) METHYLCELLULOSES.

The colony forming efficiencies of RPMI 2650 cells in two different methylcelluloses (Section 2.10) were compared with the colony forming efficiencies obtained in Sigma type II agarose. Cells in suspension were prepared as described previously (Section 2.9.). The features of this experiment and the results obtained are shown in Section 3.15.

The observed effects of semi-solid supports on RPMI 2650 colony formation may be summarised as follows:-

- Seaprep agarose (F.M.C. Corporation) did not get at 0.3% or 0.15%

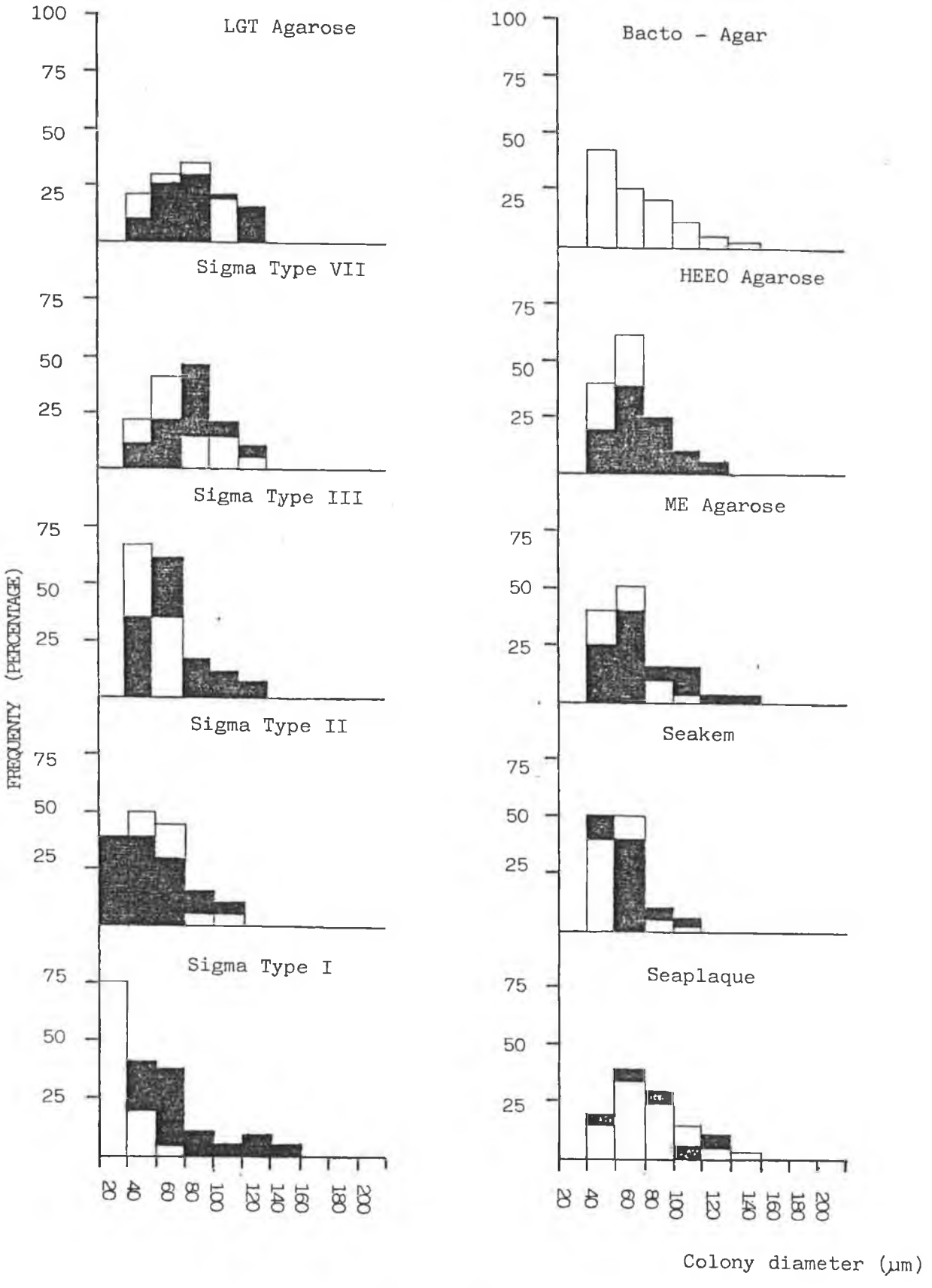


Figure 3.9.

Colony size distributions of RPMI 2650 cells in 0.15% (■) and 0.3% (□) agaroses.

TABLE 3.18.

FEATURES OF AGAROSE EXPERIMENT (C) WITH TEMPERATURE ADJUSTMENT.

Serum Batch No:	10F4049A
No. of Replicates:	4
Assay Incubation period:	14 days
Suspended cell conc:	$1X10^4$ cells/35mm dish
Initial single-cell conc:	98%
Initial Doublet conc:	2%
Initial clump conc:	0%
Viability	99%
Cell passage no:	48
Agarose incubation temp.	In accordance with gel properties (Section 2.10).

TABLE 3.19

EFFECTS OF AGAROSE TYPE AND CONCENTRATION ON THE C.F.E.'s OF RPMI 2650 CELLS.

Agarose conc.	0.3%	0.15%
Agarose type	C.F.E.	C.F.E.
* LGT (marine)	41.76±15.50	31.38±8.68
* Sigma type VII	40.70±2.57	32.40±7.51
* Seaplaque	39.24±8.08	48.87±1.29
Seakem	33.73±1.31	20.21±2.23
Sigma type II	29.68±0.55	20.82±2.48
Agar	25.80±1.47	24.58±2.65
Sigma type III	19.56±1.07	17.04±2.76
HEEO	18.36±0.82	10.33±0.16
HE	16.92±2.33	7.32±1.25
ME	16.32±2.74	16.91±1.32
Sigma type IV	14.24±0.44	13.20±1.70
Sigma type I	13.83±1.04	18.58±0.91
LE	10.72±0.45	17.59±1.40
* Seaprep	-	-

(n=4)

* Low gelling temperature agaroses prepared at 37°C and allowed to set for 5min at 4°C.

N.B. Note large difference in C.F.E.'s between Table 3.12 and 3.19. Different serum batches used may provide at least a Partial explanation.

under any conditions examined and did not support colony growth.

- Optimum colony forming efficiencies were obtained using low gelling temperature agaroses when the temperature was adjusted to account for individual gelling properties.
- Decreasing the concentration of the semi-solid support to 0.15% did not promote a significant increase in colony formation.
- Where high cell concentrations were used (1 X 10^5 cells/35mm plate) the colony forming efficiencies observed were lower than those obtained from cultures inoculated with 1 X 10^4 cells/35mm plate. Where feeder cells were included no significant increase in colony formation was observed in 0.3% agaroses, however, feeders in 0.15% agaroses significantly decreased colony formation.
- The colony forming efficiencies obtained in agar and Sigma type II agarose, which was routinely used, were comparable. However, the colony size distributions were different.
- Fluka methylcellulose encouraged colony formation to a greater extent than Dow methylcellulose (Section 3.14. Table 3.48) and Sigma type II agarose.

3.8. SERUM EFFECTS.

The factors controlling the growth of human tumour cells in soft-agar are poorly understood. However, it has been demonstrated (Peehl and Stanbridge, 1981) that serum provides factors which promote anchorage-independent growth.

The following experiments were undertaken to determine whether the ability of RPMI 2650 cells to form colonies in soft agarose was dependent on the presence of serum.

In the first experiment, duplicate confluent and sub-confluent, pre-treated, (Section 2.11.) cultures of RPMI 2650 cells were trypsinized (Section 2.9.). The resulting cell suspensions were then diluted, with medium containing either 2% or 10% foetal calf serum, to 1 X 10^5 cells/25cm^2 flask. Monolayer growth, in terms of cell number per flask obtained after trypsinization, was monitored over a period of 11 days. Cell viability over this interval was also monitored using the trypan blue dye-exclusion test (Section 2.9.). The monolayer growth and viability curves obtained under the following conditions are shown in Figures 3.10. and 3.11., respectively:-

A Confluent monolayer, subcultured at 48hr; set up @ 1 X 10^5 cells/25cm^2 flask in MEM (Section 2.7.) + 10% F.C.S.

B Monolayer subcultured at 0hr @ 2 X 10^5 cells/25cm^2 flask in MEM + 10% F.C.S., and at 48hr @ 1 X 10^5 cells/25cm^2 flask in MEM + 2% serum.

C Confluent monolayer, subcultured at 48hr; set up @ 1 X 10^5 cells/25cm^2 flask in MEM + 2% F.C.S.

D Monolayer subcultured at 0hr @ 2 X 10^5 cells/25cm^2 flask

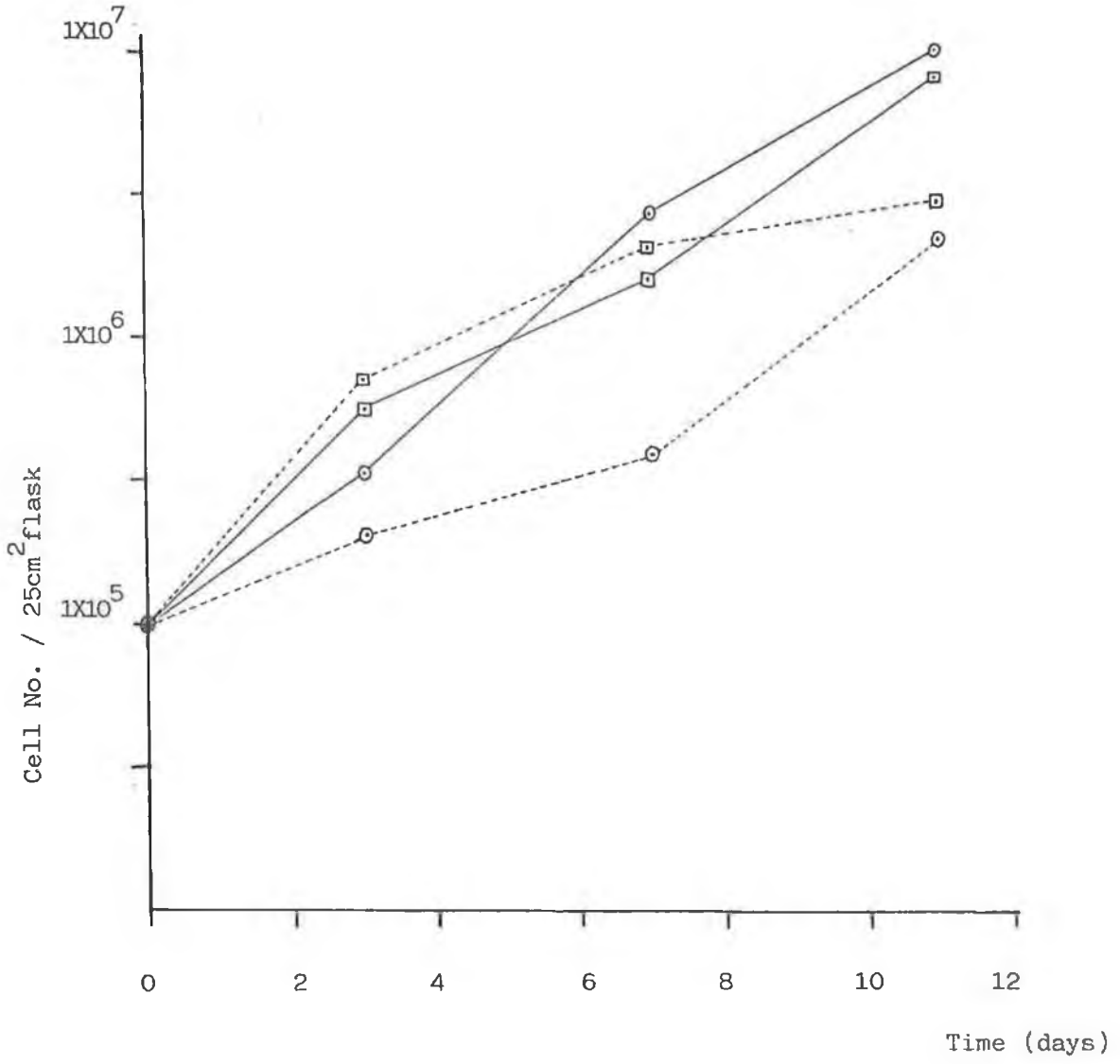


FIGURE 3.10

Effects of 10% F.C.S., — and 2% F.C.S., ----- , on monolayer growth of RPMI 2650 cells taken from pre-treated, subconfluent, ⊙ , and untreated, confluent, ⊡ , monolayer cultures.

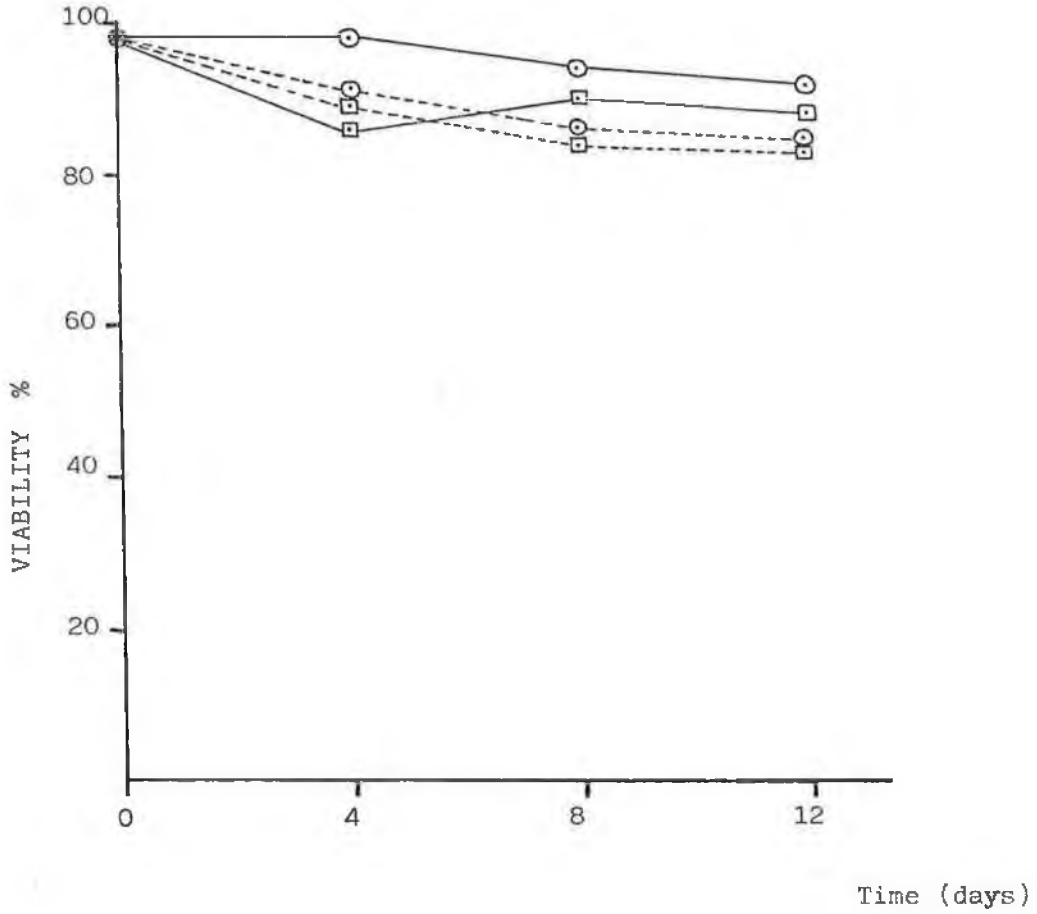


FIGURE 3.11

Effect of serum concentration (2%F.C.S.= ⊙ ; 10%F.C.S.= ⊡) on viability of RPMI cell cultures grown from confluent -----, and subconfluent ———, monolayers.

in MEM + 10% F.C.S., and @ 48hr @ 1 X 10^5 cells/flask in MEM + 10% F.C.S.

From Figures 3.10 and 3.11.the following observations may be made:-

(1) RPMI 2650 cells routinely cultured in medium containing 10% F.C.S. grew in medium containing 2% F.C.S., however, growth in 10% F.C.S. was better overall than growth in 2% F.C.S.

(2) Cell viability decreased slightly over the interval monitored, however, there were no major differences between the viability of cells

(a) cultured in 2% F.C.S. and 10% F.C.S.,

or

(b) taken from pre-treated subconfluent and untreated confluent cultures.

(3) In MEM containing 10% F.C.S. cells from pre-treated subconfluent and confluent RPMI 2650 cultures behaved in the same way. From an initial cell density of 1 X 10^5 cells/25cm^2 flask, growth progressed in an exponential fashion to 1 X 10^7 cells/flask after 11 days. It appears that pre-treatment of cells (Section 2.11.) did not affect growth in 10% serum.

(4) Cells from pre-treated subconfluent and confluent RPMI 2650 cultures behaved differently in MEM containing 2% F.C.S. Cells from pre-treated subconfluent cultures appeared to be in a lag phase of growth by comparison with cells from untreated confluent cultures, which appeared to grow exponentially before entering a stationary growth phase.

The second experiment examined the effects of different serum concentrations on colony formation of RPMI 2650 in agarose over a period of 14 days. Pre-treated RPMI 2650 cells for cloning in agarose (Section 2.12.) were prepared as described previously (Section 2.11.). The quantities of growth medium (MEM) and serum used in buffered 2X medium (Table 2.6.) were adjusted to give the required serum concentrations. Table 3.20 summarises the features of this experiment.

The results obtained are shown in Figure 3.12. Colony formation on day 4 was low at all concentrations of serum. ANOVA found that no significant differences were present between treatments at the 5% or 1% levels on day 4. However, ANOVA found that differences between serum treatments were significant at the 1% level on day 7 and day 14.

The greatest colony forming efficiencies were obtained in 20% serum, the poorest in medium supplemented with 1% or 2% F.C.S. MEM + 1% F.C.S. supported little colony formation over 14 days, however, in media supplemented with 2% or higher serum concentrations an increase in colony formation with time was evident. Between day 4 and day 7 there was a 10-fold (approx.) increase in colony formation, however, this large increase in colony formation was not evident between day 7 and day 14.

The third experiment also examined the effects of different serum levels on the colony forming efficiency of RPMI 2650 cells in agarose. The features of this experiment are summarised in Table 3.21.

Figure 3.13. shows the growth response of RPMI 2650 cells in agarose at the different serum concentrations examined. The best growth, with respect to colony forming efficiency, was obtained

TABLE 3.20

FEATURES OF SERUM RANGE EXPERIMENT (2).

Serum Batch No:	20Q8833A
No. of Replicates:	3
Cell passage level:	31
Initial singlet conc:	93.74%
Initial doublet conc:	5.1%
Initial clump conc:	1.16%
Viability	98%
Test cell conc:	$1X10^5$cells/35mm plate
Serum conc:	1%,2%,5%,10%,15%,20%
Assay incubation period:	14 days

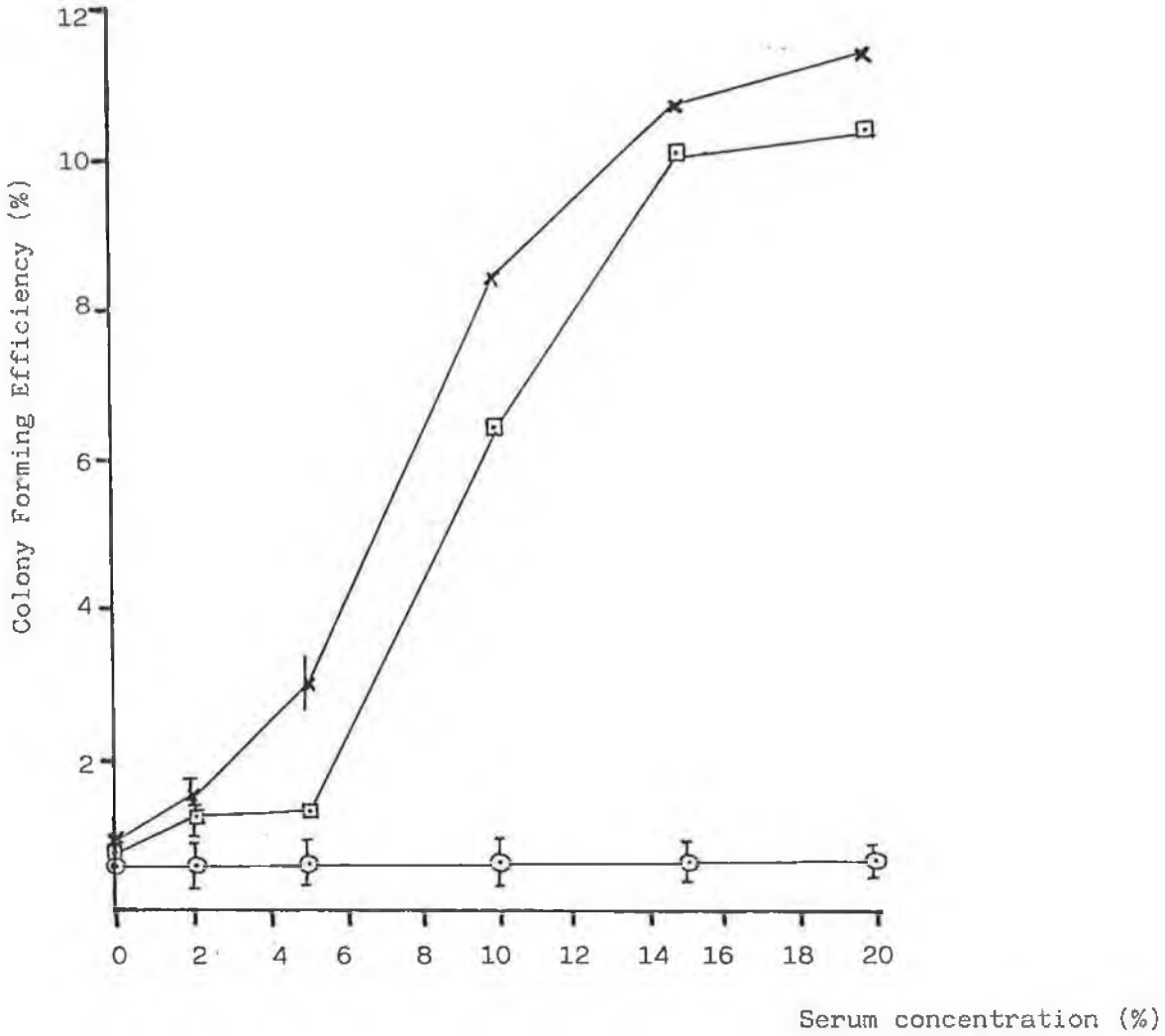


FIGURE 3.12.

RPMI 2650 colony forming efficiency in different serum concentrations on days 4 (⊙); 7 (⊡); and 14 (X).

TABLE 3.21.

FEATURES OF SERUM CONCENTRATION EXPERIMENT (3).

Serum Batch:	20Q8833A
No. of Replicates:	3
Cell passage level:	33
Initial singlet conc:	87.17%
Initial doublet conc:	6.25%
Initial clump conc:	6.58%
Viability	97%
Test cell conc:	$1X10^4$cells/35mm plate
Serum concs:	1%,2%,5%,7%,10%,15%,20%
Assay incubation period:	14 days

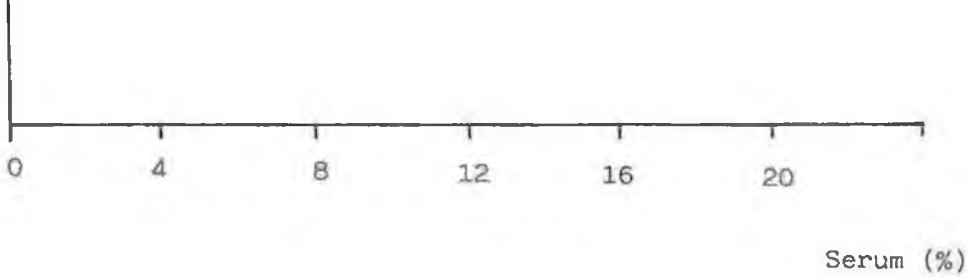
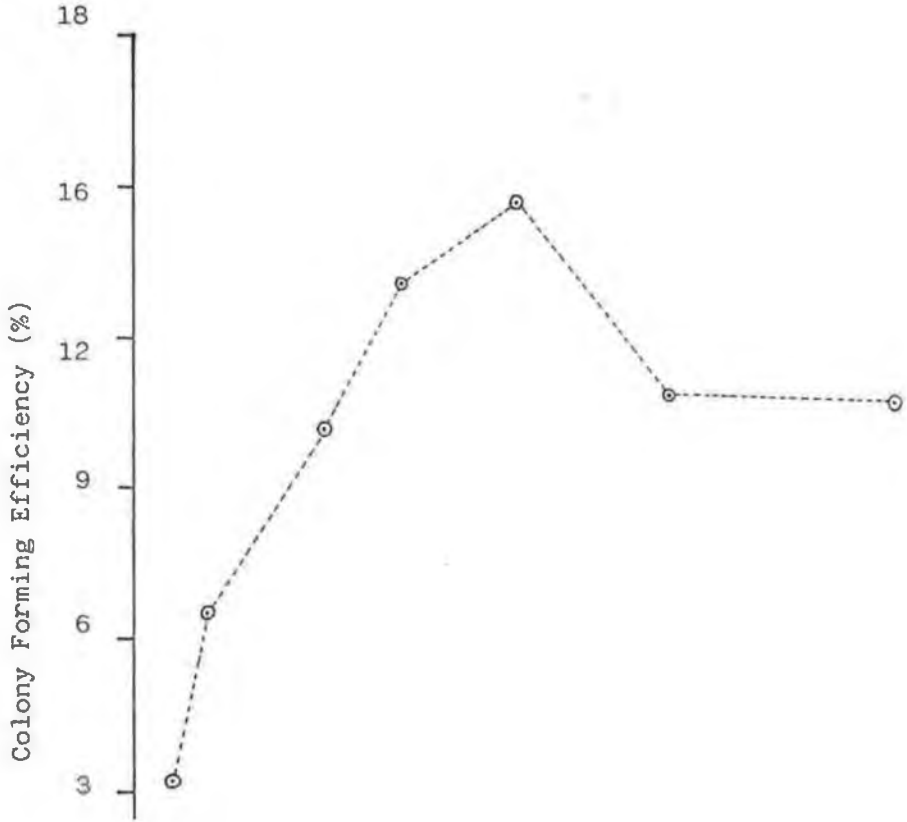


FIGURE 3.13

Effects of serum concentration on the C.F.E. (Colony forming efficiency) of RPMI 2650 cells suspended in agarose.


Colony Forming Efficiency (%)
18
16
12
9
6
3

when 10% F.C.S. was incorporated in the medium. At higher serum concentrations there was a decrease in colony forming efficiency which will be considered in the discusssion in addition to the generally higher colony forming efficiencies obtained in this experiment by comparison with the second experiment.

3.9. GROWTH FACTOR SUPPLEMENTED MEDIUM.

Selective *in vitro* growth of tumor cells using defined, hormone supplemented, media has already been examined by Carney (1981) for small cell lung cancer cells. The hormone supplemented medium examined by Carney is specific for SCCL cells alone and does not allow the growth of non-malignant cells. Simms *et al.*, (1980), reported similar results for a range of growth factors including HITES (Hydrocortisone, insulin, transferrin, estradiol, selenium), epidermal growth factor (EGF), parathyroid hormone and luteinizing hormone-releasing factor.

In this experiment, the effects of HITES components on the growth of RPMI 2650 cells in monolayer and agarose, under a variety of conditions were examined as follows:-

(A) The effect of HITES medium on the monolayer growth of RPMI 2650 cells.

RPMI 2650 cells (Passage level 30), pre-treated as described previously (Section 2.11.), were prepared at 1 X 10^5 cells/ 25cm^2 flask, in medium supplemented with HITES (Table 3.22.). Cells were cultured in the absence of serum, at 1%, 2% and 10% serum with or without HITES supplementation. Growth was monitored over 12 days visually and by harvesting and counting the cells (Section 2.9.). The results obtained are shown in Table 3.23. and Figure 3.14.

TABLE 3.22.

CONCENTRATION OF HITES COMPONENTS.

Component	Concentration / ml
Hydrocortisone	10^{-8}M
Insulin	5µg
Transferrin	10^{-8}M
Estradiol	5µg
Selenium	5ng

TABLE 3.23.

DESCRIPTION OF CELL GROWTH

Day	Supplements	Cell Description
6	0	Few attached cells,most cells in suspension. Attached cells shrivelled in appearance.
	0+HITES	Cells were poorly attached and shrivelled.
	1%F.C.S.	Attachment,with some growth. Cells appeared to be attached as normal; cells formed very small colonies.
	10%F.C.S.+HITES	Many cells attached and growing as colonies which were substantial in places.
	2%F.C.S.	Good colony growth.
	2%F.C.S.+HITES	Good colony growth.
	10% F.C.S.	Good colony growth,cells very healthy in appearance.
11	0	Cells shrivelled in appearance,no evidence of growth.
	0+HITES	Cells attached,unhealthy appearance, no evidence of growth.
	1%F.C.S.	Attached cells growing as small closely packed colonies.
	1%+HITES	Good colony growth,large colonies,almost confluent.
	2%F.C.S.	Good colony growth,large colonies formed.
	2%F.C.S.+HITES	Large colonies formed,approaching confluency.
	10%F.C.S.+HITES	Good growth,some cells detaching.

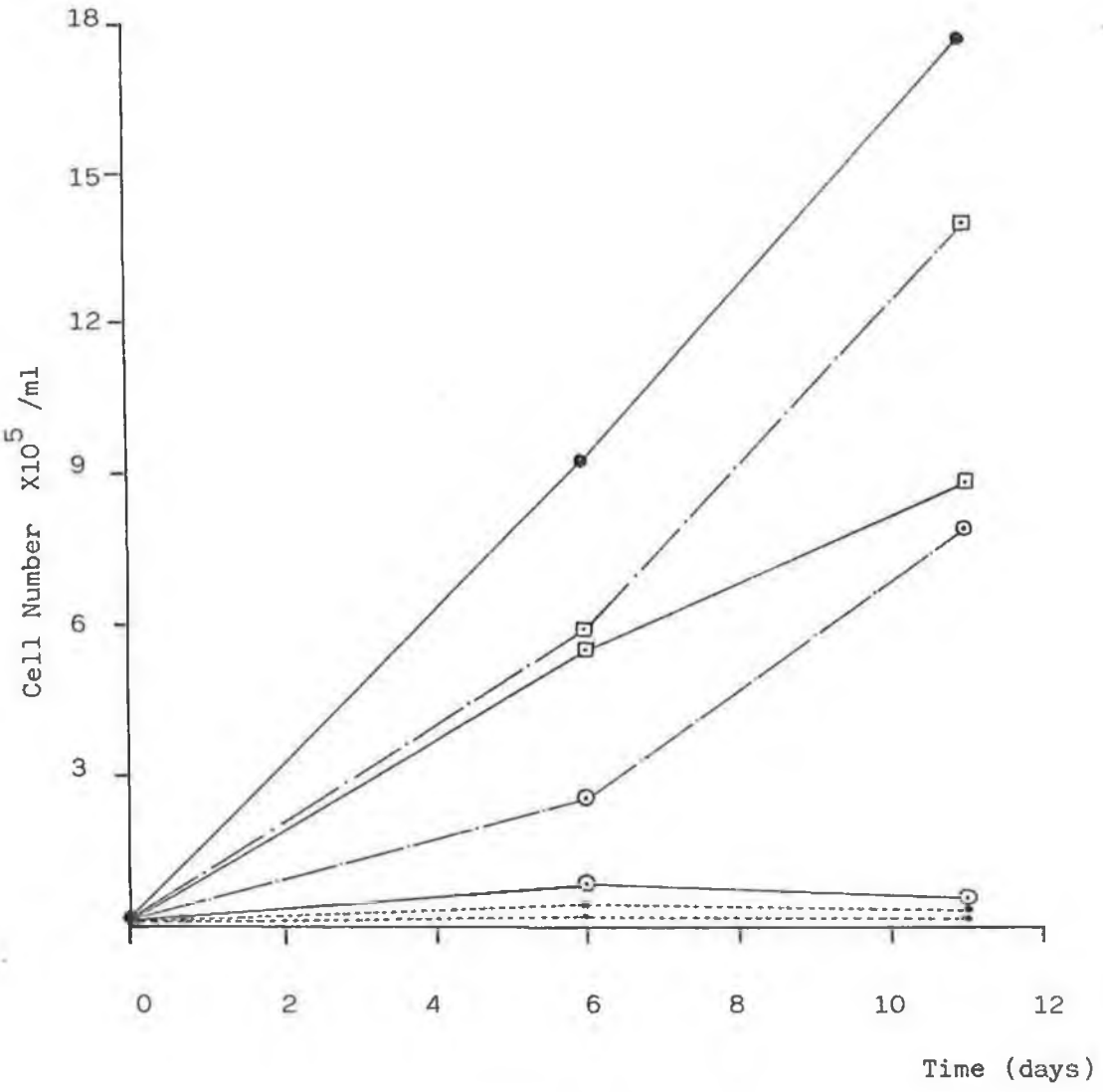


FIGURE 3.14

Effects of serum concentration on RPMI 2650 in the presence and absence of HITES supplements.

Key:

—●— 10% Serum control

⊡ 2% Serum

⊙ 1% Serum

—·— With HITES supplements

----▪---- HITES only

----●---- MEM only

At 1 X 10^5cells/flask, RPMI 2650 cells appeared to exhibit a growth requirement for serum which was not alleviated by the addition of HITES. In the presence of 1% and 2% serum, cells attached but growth was very poor. Where HITES, in addition to low serum levels, was examined, there was a marked increase in growth by comparison to cell growth at low serum levels without HITES supplementation. Reduced serum levels appeared to extend the lag phase of growth by comparison with growth in 10% serum which proceeded exponentially.

This experiment was repeated in monolayer using RPMI 2650 cells (passage level 33), in 1% serum with and without HITES and with a 10% serum control. The results are shown in Table 3.24. and Figure 3.15.

Cells cultured in 1% serum did not grow very well, however, when supplemented with HITES there was a marked increase in cell growth, on the basis of increasing cell number. The best growth was obtained in 10% serum. However, at day 12 there was a decrease in cell number, which may have been due to nutrient depletion of the medium, or cell senescence, since the decline in cell number was evident in medium containing 10% serum and in HITES supplemented medium containing 1% serum.

(B) Determination of the most potent component of HITES medium.

Pre-treated cells, passage level 35, were prepared in quadruplicate, in tissue culture phials, at 1 X 10^4cells/ml. HITES components were prepared at the concentrations previously used (Table 3.22.) and were added, with serum, to Eagle's MEM as follows:-

TABLE 3.24.

DESCRIPTION OF CELL GROWTH

Serum conc.	1% Serum	1%+HITES	10% F.C.S.
Day No.			
3	Cells attached, rounded,healthy, small colonies.	Attached,rounded healthy,larger colonies.	Attached,healthy numerous colonies
6	Small,infrequent, isolated colonies.	Numerous colonies.	Large spreading colonies.
9	Small,isolated, tightly packed colonies.	Large,spreading, closely packed colonies.	Large number of colonies.
12	Small colonies, few in number, dispersed.	Numerous large colonies, approaching confluency.	Many densely packed colonies.
15	Numerous colonies spreading, detaching.	Large and spreading,many detached cells.	Large colonies, many detaching cells.

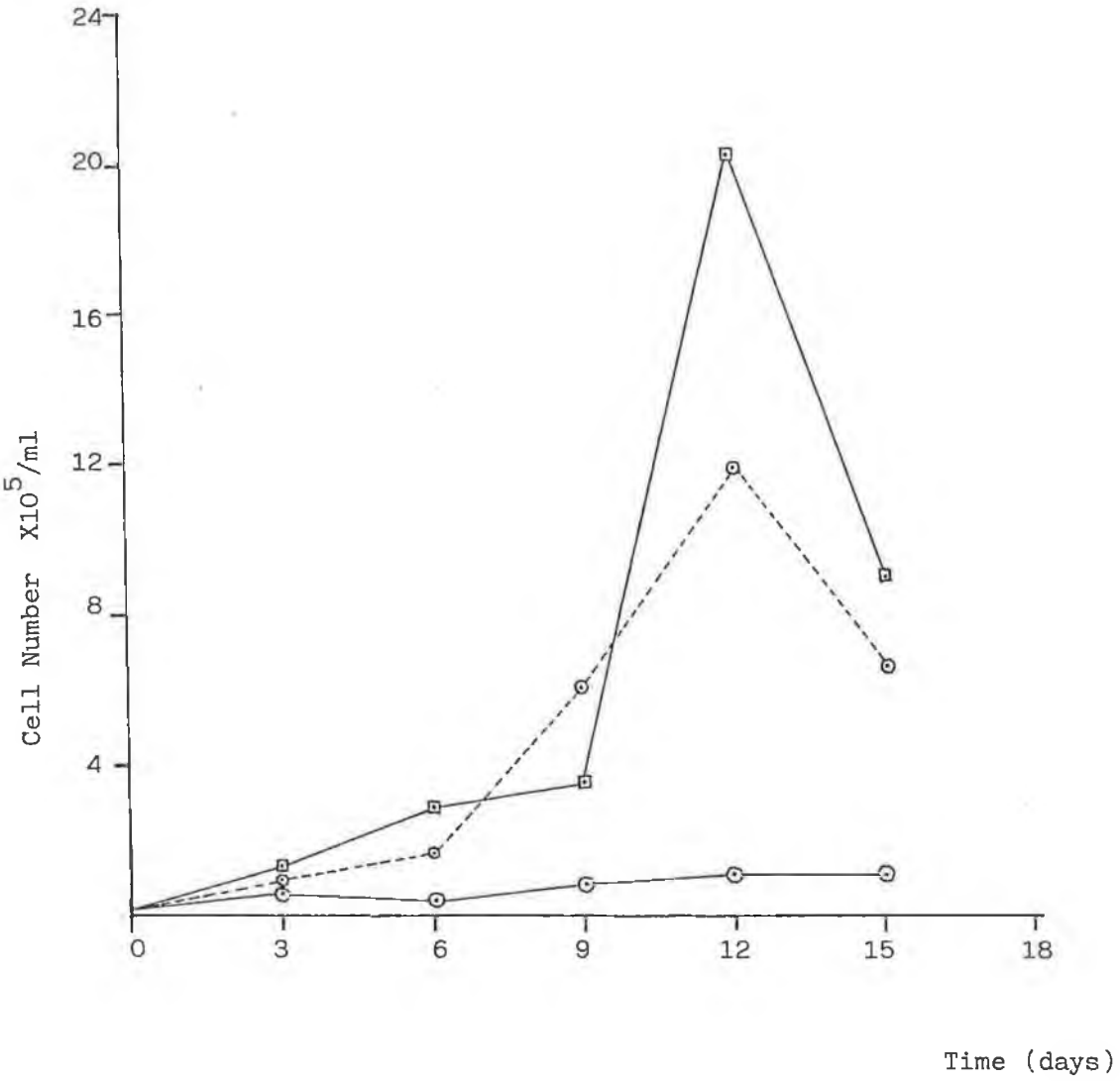


FIGURE 3.15

Time course of RPMI 2650 cells in 1% serum; ⊙ ; 1% serum + HITES, -----; and 10% serum, ⊡ , in monolayer culture.

(a) 1% serum - no HITES supplement.

(b) 1% serum + HITES - complete hormone supplementation.

(c) 1% serum + ITES - without hydrocortisone.

(d) 1% serum + HTES - without insulin.

(e) 1% serum +HIES - without transferrin.

(f) 1% serum + HITS - without estradiol.

(g) 1% serum + HITE - without selenium

(h) 10% serum - no HITES supplement.

After 10 days, the phials were examined using the Nikon Diaphot, and cells were harvested and counted (Section 2.9.). The results obtained are shown in Table 3.25. and Table 3.26.

The results obtained confirmed the previous finding that RPMI 2650 cells require serum at a minimum concentration of 1% for attachment (Section 3.9.(A)). Once the cells had attached, in 1% serum, a maximum of one doubling occurred, however, the cells remained shrivelled and did not grow further. When the entire components of HITES medium were included in medium containing 1% serum, the final cell count was greater than that obtained from medium containing 10% serum, however, cells in both instances were healthy and well spread out on the tissue culture surface.

As each individual component was deleted the following occurred:-

(1) Cell numbers increased by comparison with growth in medium containing 1% serum.

(2) Cell morphology approached the healthy appearance of cells in 10% serum supplemented medium.

(3) The effects of each individual component may be ranked in order of significance as

H > I > T > E > S,

TABLE 3.25

QUALITATIVE CHANGES IN SUPPLEMENTED MEDIUM

Media	Media Colour	Appearance of cells
(a) 1% F.C.S.	Dark Pink	Cells attached,shrivelled, few colonies.
(b) 1% F.C.S.+HITES	Dark Orange	Expansive,almost confluent colonies.
(c) 1% F.C.S.+ITES	Light Pink	> (a) but few colonies
(d) 1% F.C.S.+HTES	Light Pink	> (c)
(e) 1% F.C.S.+HIES	Light Pink	> (d)
(f) 1% F.C.S.+HITS	Light Pink	> (e)
(g) 1% F.C.S.+HITE	Pale Pink/Orange	Very good growth,not yet confluent.
(h) 10% F.C.S.	Pale Yellow/Orange	Good growth,colonies almost monolayer.

TABLE 3.26.

EFFECT OF MEDIUM SUPPLEMENTATION ON RPMI 2650 CELL NO.

Medium Supplement	Mean cell count/ml
(a) 1% F.C.S.	1.56×10^4
(b) 1% F.C.S.+HITES	1.24×10^6
(c) 1% F.C.S.+ITES	6.13×10^4
(d) 1% F.C.S.+HITES	7.37×10^4
(e) 1% F.C.S.+HIES	1.03×10^5
(f) 1% F.C.S.+HITS	1.12×10^5
(g) 1% F.C.S.+HITE	1.82×10^5
(h) 10% F.C.S.	0.99×10^6

Mean cell counts (n=2)

since the most deleterious effect was exhibited in cultures devoid of hydrocortisone. Since medium supplemented with all HITES constituents exhibited the greatest growth, the possibility that the constituents act synergistically exists.

(C) The effect of HITES medium on colony formation of RPMI 2650 cells in agarose.

HITES components (Table 3.22.) were included as 10X additives (Table 2.6.) in a clonogenic assay which contained 10% serum and feeder cells (Section 2.11.). Pre-treated RPMI 2650 cells (Section 2.11.) were included in the assay at 1 X 10^5 cells/35mm plate. The features of this experiment are shown in Table 3.27. and the results obtained are shown in Table 3.28. These results were analysed using ANOVA which showed that differences due to feeders were significant at the 5% level in the absence of HITES but were not significant when HITES components were included in the assay. ANOVA also showed that the addition of HITES components significantly decreased colony formation in the assay.

HITES components were included in an assay which already contained 10% F.C.S. Under these conditions the following may occur;

(1) The stimulatory effect of HITES components may be masked in the presence of high serum levels.

(2) Inhibition of colony formation may be due to an enrichment of certain factors which are cytotoxic at high concentrations and stimulatory at low levels.

The effects of HITES supplements on RPMI 2650 colony formation in agarose were further examined in the absence of feeder cells, at a range of serum and HITES concentrations. The features of this

TABLE 3.27

FEATURES OF HITES EXPERIMENT.

Serum Batch No.	20Q8833A
No. of Replicates:	3
Cell passage level:	32
Initial singlet conc:	95.25%
Initial doublet/clumps:	4.75%
Viability:	95%
Test cell conc:	$1X10^5$cells/35mm plate
Feeder cell concs:	0,$1X10^4$,$3X10^4$,$1X10^5$cells/35mm plate.
Assay incubation period:	14 days

TABLE 3.28

EFFECTS OF HITES COMPONENTS ON RPMI 2650 C.F.E.'s IN AGAROSE AT 10% F.C.S.

Feeder Cell Conc.	C.F.E.'s without HITES	C.F.E.'s with HITES
0	11.5 ± 2.47	2.98 ± 0.29
$1X10^4$	11.99 ± 0.65	3.41 ± 0.19
$3X10^4$	6.47 ± 0.79	2.86 ± 0.18
$1X10^5$	$6.08 \pm).36$	2.49 ± 0.13

Mean C.F.E.$\pm$S.E.M. (n=3)

TABLE 3.29

FEATURES OF HITES EXPERIMENT IN AGAROSE

Serum Batch No:	10F4049A
No.of Replicates:	3
Cell passage level:	47
Initial singlet conc:	95%
Initial doublet conc:	3%
Initial clump conc:	2%
Serum concs:	1%, 2%, 10%.
HITES concs:	1X, 3X, 5X.
Test cell concs:	$1X10^4$cells/35mm plate
Assay incubation period:	14 days

TABLE 3.30

EFFECT OF A RANGE OF HITES CONCENTRATIONS ON COLONY FORMATION OF RPMI 2650 CELLS.

HITES conc.	0	BSA	1X	3X	5X
Serum conc.					
10%	12.56±0.08	12.56±0.21	1.84±0.37	2.72±0.42	7.92±0.65
2%	2.64±0.12	2.24±0.21	2.24±0.42	2.24±0.21	1.92±0.48
1%	0.64±0.07	0.64±0.08	1.92±0.14	2±0.45	1.84±0.56

Mean C.F.E.±S.E.M. (n=3)

experiment are shown in Table 3.29. and the results are shown in Table 3.30.

3.10. T-CELL AND OTHER GROWTH FACTORS.

Human T-cell polyclone (Collaborative Research), prepared as conditioned medium from cultures of phytohemagglutinin-P stimulated human peripheral blood leukocytes, promotes proliferation of T-lymphocytes and contains TCGF (T-cell growth factor). In this experiment, effects of several concentrations of human T-cell polyclone, on the proliferation of RPMI 2650 cells in monolayer were examined. The features of this experiment are summarised in Table 3.31.

The TCGF concentrations required, were prepared from a stock TCGF solution (Section 2.15.) containing 1,150 half-maximal units of human T-cell growth factor activity per 10ml (Unit defined in Section 2.15), by dilution with serum-supplemented Eagle's MEM.

The results obtained are shown in Table 3.32. The effects of TCGF on colony formation of RPMI 2650 cells in monolayer may be summarised as follows:-

(1) By comparison with control cultures without human T-cell growth factor, the colony forming efficiencies obtained in the presence of high TCGF concentrations were reduced.

(2) Colony formation in 1% serum controls with TCGF was reduced three-fold by comparison to 10% serum controls without TCGF.

(3) Reduced serum levels of 1%, in the presence of TCGF, exhibited the greatest inhibitory effect on cell growth

TABLE 3.31

FEATURES OF TCGF EXPERIMENT.

Serum Batch No:	K223901A (309)
No.of Replicates:	3
Assay incubation period:	10 days
Cell passage number:	37
Viability:	92%
Cell concs.used:	$1X10^4$cells/35mm dish
TCGF conc:	10 and 20 half-maximal units of activity of human T-cell growth factor/ml.

TABLE 3.32.

EFFECTS OF HUMAN T-CELL POLYCLONE ON THE COLONY FORMATION OF RPMI 2650 CELLS IN MONOLAYER.

Human T-cell Polyclone conc.(½ maximal units of activity/ml).	1% Serum	10% Serum
0	15.54±0.1	54.2±0.33
10	1.76±0.36	45.28±2.09
20	0.32±0.02	21.12±1.03

Mean C.F.E.±S.E.M. (n=3)

These results were analysed using ANOVA (Section 2.19.), which found that differences in colony formation due to the serum concentration and human T-cell polyclone were significant at the 1% level.

and colony formation.

The use of human T-cell growth factor, at the concentrations examined, did not increase the colony formation of RPMI 2650 cells in monolayer culture. Possible methods of TCGF interaction with cells and foetal calf serum in culture will be considered in the discussion.

Other growth factors and their effects on colony formation of pre-treated RPMI 2650 cells (Section 2.11.) were examined under a variety of conditions in monolayer and agarose assays (Table 3.33.) The factors examined were prepared as described previously (Section 2.15.), at the required concentrations by dilution with phosphate buffered saline. The inclusion of phosphate buffered saline as a test factor allowed for possible phosphate buffered saline interference with cell growth. The results obtained are shown in Table 3.34.

From Table 3.34. it may be seen that:-

(1) In agarose, the best colony forming efficiencies were obtained in the presence of epidermal growth factor.

(2) The maximum colony forming efficiencies in monolayer were obtained in the control cultures containing 10% F.C.S. without further growth factor supplementation.

(3) The presence of additional factors reduced colony formation in monolayer by comparison with control cultures. However, the colony forming efficiencies obtained were numerically very close. Differences in colony forming efficiencies were examined using ANOVA which showed that the differences observed in both monolayer and agarose assays were significant at the 1% level.

TABLE 3.33.

FEATURES OF CLONOGENIC ASSAYS WITH ADDITIONAL GROWTH FACTORS.

Serum Batch No:	10G2038A (309)
No.of Replicates:	3
Assay incubation period:	10 days
Cell passage number:	45
Viability:	95%
Initial single cell conc:	94%
Initial doublet conc:	6%
Initial clump conc:	0%
Cell conc. used:	$1X10^4$ cells/35mm plate
ADDITIONAL FACTORS USED:	
Hydrocortisone	10µg/ml
Epidermal growth factor	50ng/ml
Insulin	0.01i.u./ml
Non-essential amino acids	
Phosphate buffered saline	10%

TABLE 3.34.

THE EFFECTS OF ADDITIONAL GROWTH FACTORS ON THE C.F.E.'s OF RPMI 2650 cells.

Test Component	Agarose	Monolayer
Control (no additions)	$5.24^{\pm}0.20$	$54.2^{\pm}0.33$
EGF (50ng/ml)	$8.52^{\pm}0.23$	$39.24^{\pm}0.48$
Insulin (0.01i.u./ml)	$6.6^{\pm}0.21$	$41.48^{\pm}2.31$
Hydrocortisone (10µg/ml)	$6.6^{\pm}0.18$	$35.64^{\pm}1.44$
N.E.A.A.	$3.28^{\pm}0.29$	$39.44^{\pm}1.21$
P.B.S.(10%)	$6.84^{\pm}0.39$	$36.3^{\pm}1.18$

Mean C.F.E.$^{\pm}$S.E.M. (n=3)

3.11. FEEDER EFFECTS.

Clonogenic assay culture conditions were modified to include RPMI 2650 feeder cell layers, in an attempt to improve plating efficiencies in agarose, particularly where low cell densities were used (Section 4.3). The effects of both dividing 'live' RPMI feeders and non-dividing mitomycin c-treated feeders on colony formation in agarose were examined as follows:-

(A) Untreated 'live' RPMI 2650 feeder cells.

Pre-treated cells were prepared as monolayer feeder cells as described previously (Section 2.11.). Cells were tested in 0.3% agarose (Section 2.12)at a range of concentrations. Table 3.35 summarises the features, including cell concentrations, considered in this experiment.

The colony forming efficiencies obtained for the range of feeder cell and test-cell concentrations examined are shown in Figure 3.16. The results may be summarised as follows:-

(1) No colonies were observed in cultures containing feeder layers alone, indicating that the feeder layers did not contribute to the final colony forming efficiencies.

(2) Colony formation, in low density cultures of 1 X 10^3cells/plate and 1 X 10^4cells/plate, was stimulated in the presence of feeder layers. Feeder layers had the greater stimulatory effect on cultures of 1 X 10^3cells/plate which exhibited a three-fold increase in colony formation in the presence of feeders. By comparison,

TABLE 3.35.

FEATURES OF FEEDER LAYER EXPERIMENT.

Serum Batch No:	20Q8833A
No.of Replicates:	5
Assay incubation period:	14 days
Initial doublet conc:	5.85%
Initial clump conc:	2.65%
Viability:	95%
Feeder cell passage No:	33
Feeder cell concs:	0, $2X10^2$, $1X10^3$, $1X10^4$, $1X10^5$, $3X10^5$ cells/dish
Suspended cell passage No:	34
Suspended cell concs:	0, $1X10^3$, $1X10^4$, $1X10^5$ cells/dish

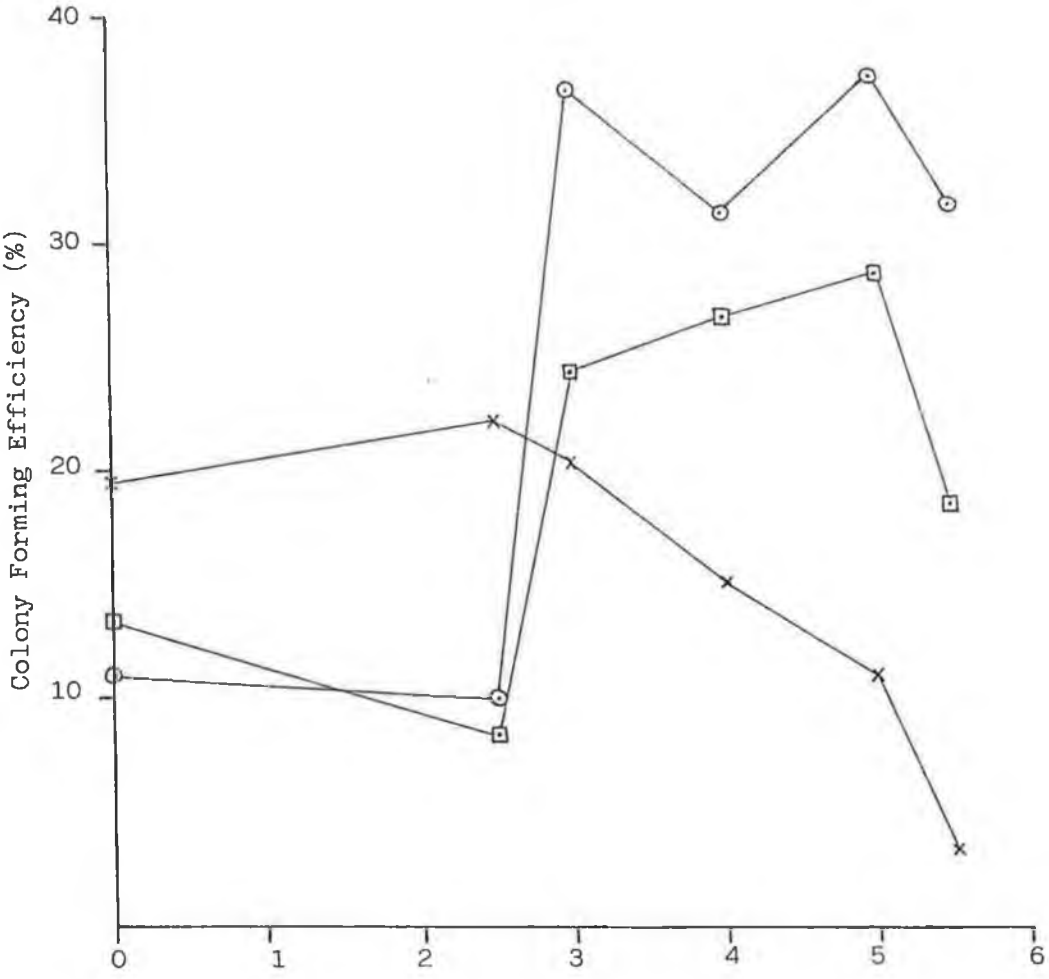


FIGURE 3.16

Effects of feeder cell concentration on colony formation of $1X10^3$, ⊙ , $1X10^4$, ⊡ , and $1X10^5$, X , RPMI cells/35mm plate in agarose.

feeders layers stimulated colony formation 2-fold in cultures of 1 X 10^4cells/plate.

(3) At the test-cell concentration of 1 X 10^5cells/plate, colony formation was inhibited in the presence of feeder layers. As the feeder cell concentration increased there was a corresponding decrease in colony formation.

(B) Non-dividing mitomycin C-treated feeder layers.

Pre-treated cells were prepared as live feeder cells and mitomycin C treated feeder cells (section 2.11.). The range of cell concentrations and experimental features considered are shown in Table 3.36.

After two weeks, monolayer feeder controls were examined microscopically. Mitomycin C-treated feeders set up at 1 X 10^4cells/plate had formed very few colonies; most of the cells had remained as single cells. Feeders prepared at 3 X 10^5cells/plate, after mitomycin c-treatment, formed small, evenly dispersed, colonies which were 25% confluent. Non-treated cells, by comparison, formed expansive colonies at 1 X 10^4cells/plate and confluent monolayers with cells piling up at 3 X 10^5cells/plate.

Table 3.37. shows the effects from two separate experiments, of mitomycin c-treated feeders and live feeders on colony formation in agarose. The effects of feeder layers on colony size in agarose are shown in Table 3.38.

Comparing the effects of mitomycin c-treated feeders with the effects of untreated feeders on colony size and colony forming

TABLE 3.36

EXPERIMENTAL FEATURES OF FEEDER EXPERIMENT.

Features	Run 1	Run 2
F.C.S. Batch No:	K223901A(309)	10F4049A
No.of Replicates:	3	3
Initial single cell conc:	84.4%	90.2%
Initial doublet conc:	7.4%	7.84%
Initial triplet conc:	2.4%	0
Initial clump conc:	0.9%	1.96%
Viability:	95.1%	100%
Feeder cell passage no:	36	38
Test cell passage no:	39	46
Feeder cell conc:	0, $1X10^4$, $3X10^5$ cells/35mm dish.	
Test cell conc:	$1X10^3$, $1X10^4$ cells/35mm dish.	
Assay incubation period:	14 days	

TABLE 3.37

EFFECTS OF MITOMYCIN C-TREATED AND NON-TREATED FEEDERS ON RPMI 2650 COLONY FORMATION IN AGAROSE.

(A) IN THE PRESENCE OF UNTREATED FEEDER CELLS

	Experiment 1.		Experiment 2.	
Test Layer conc. (cells/plate).	$1X10^4$	$1X10^3$	$1X10^4$	$1X10^3$
Feeder layer conc. (cells/plate).				
0	35.25 ± 2.57	3.2 ± 0.4	11.76 ± 1.39	11.61 ± 1.44
$1X10^4$	31.37 ± 3.3	8.4 ± 2.4	7.08 ± 1.43	19.5 ± 2.79
$3X10^5$	31.12 ± 3.05	15.2 ± 2.12	6.63 ± 1.48	19.99 ± 2.1

(B) IN THE PRESENCE OF MITOMYCIN C-TREATED FEEDERS

	Experiment 1.		Experiment 2.	
Test Layer conc. (cells/plate).	$1X10^4$	$1X10^3$	$1X10^4$	$1X10^3$
Feeder layer conc. (cells/plate).				
0	35.25 ± 2.57	3.2 ± 0.4	11.76 ± 1.39	11.61 ± 1.44
$1X10^4$	43.7 ± 2.31	9.6 ± 0	15.96 ± 0.8	14.01 ± 4.17
$3X10^5$	56.64 ± 1.86	40.8 ± 3.67	41.6 ± 1.05	38.01 ± 7.5

Mean C.F.E. $\pm$ S.E.M. (n=3).

TABLE 3.38

FREQUENCY OF COLONY SIZES IN AGAROSE IN THE PRESENCE OF DIVIDING AND NON-DIVIDING FEEDERS.

(A) UNTREATED FEEDER CELLS.

Feeder conc. (cells/plate)	0		$1X10^4$		$3X10^5$	
Test cell conc. (cells/plate).	$1X10^4$	$1X10^3$	$1X10^4$	$1X10^3$	$1X10^4$	$1X10^3$
Colony diameter µm						
40	20%	30%	8%	20%	64%	60%
40	70%	66%	70%	68%	36%	40%
60	10%	4%	20%	12%		
80			2%			

(B) MITOMYCIN C-TREATED FEEDERS.

Feeder conc. (cells/plate).	0		$1X10^4$		$3X10^5$	
Test cell conc. (cells/plate).	$1X10^4$	$1X10^3$	$1X10^4$	$1X10^3$	$1X10^4$	$1X10^3$
Colony diameter µm						
40	20%	30%	20%	18%	10%	10%
40	70%	66%	60%	62%	78%	80%
60	10%	4%	20%	20%	18%	10%

efficiencies the following conclusions may be made:-

(1) A direct correlation between feeder cell density and colony formation in the upper agarose layer was evident where mitomycin c-treated feeder layers were used; as the feeder cell density increased, the colony forming efficiency also increased.

(2) When live, dividing feeder cells were used, density-dependent effects were evident; high feeder densities inhibited growth in high density cultures and stimulated growth in low density cultures.

(3) Untreated feeder cells at high density (3×10^5 cells/plate), arrested colony growth by comparison with control cultures without feeders or cultures where mitomycin c-treated feeders were used.

Overall, the effects of live feeders on the plating efficiencies and colony size of RPMI 2650 cells, in agarose, seemed to be density-dependent, with interaction occurring between the feeder layer and test cells. Mitomycin c-treated feeders also exhibited density-dependent effects for the range of cell concentrations examined, however, the feeder layer - test cell interactions appeared to be altered.

Possible methods of interaction and the influence of interaction between feeder and test cells on colony size and number will be considered in the discussion.

3.12. INHIBITION.

Where high feeder cell concentrations and high concentrations of test cells were used together in the clonogenic assay (Section 3.11.), growth inhibition, in the form of reduced colony formation, was frequently encountered. Inhibition of colony formation may result from nutrient depletion in the presence of high cell concentrations, or, may be attributed to growth inhibiting factors produced by the cells under certain conditions.

In this experiment, an attempt was made to mimic the inhibition observed in agarose at high cell concentrations using a monolayer clonogenic assay and RPMI 2650 conditioned medium.

(A) Preliminary experiment: Preparation of conditioned medium.

Pre-treated RPMI 2650 cells (passage level 47; viability 98%), were prepared in duplicate 25cm^2 flasks at 2 X 10^5 and 2 X 10^6 cells/flask as described previously (Section 2.11.). After incubation at 37°C for 4 days,* the medium (Eagle's MEM + 10% F.C.S.) was discarded and the cells in the flasks were rinsed three times with P.B.S.. 5ml of fresh Eagle's MEM + 10% F.C.S. (Batch NO. k223901A(309)) was then added to the flasks. Controls were prepared by adding 5ml of fresh medium to 25cm^2 flasks without cells. At intervals of 24,48, and 96hr, 5ml volumes of medium were collected from duplicate flasks,pooled, centrifuged at 3,000 r.p.m. for 15 min. and filter-sterilized through a millex G.V. disposable filter (Table 2.1.).

* NOTE: At the time of collection of conditioned medium (24hr), flasks which had been prepared at 2 X 10^5 cells/flask

were subconfluent, whereas those which had been prepared at 2 X 10^6 cells/flask were confluent.

Table 3.39. outlines the colours of the conditioned and control media collected, as an indication of pH before adjustment to pH 7.5 (approx.) by the addition of 1.5N NaOH or 1.5N HCl.

Preparation of monolayer inhibition assay

RPMI 2650 cells, (Passage level 49), were pre-treated as described previously (Section 2.11.) and prepared at 1 X 10^4 cells/ 35mm dish, in Eagle's MEM + 10% F.C.S. After 24hr the medium was discarded and the dishes were washed with P.B.S. 2ml volumes of conditioned and control media were assayed in duplicate as follows:-

(i) Undiluted conditioned and control media.

(ii) Conditioned and control media diluted 1:1 with fresh medium + 10% F.C.S.

(iii) Fresh medium + 10% F.C.S. controls.

(iv) Conditioned and control media assayed in 34mm dishes in the absence of cells to ensure that cells suspended in conditioned media did not contribute to colony forming efficiences.

The cells were incubated at 37°C for 10 days and colonies formed were stained with May-Grünwald Giemsa and counted (Section 2.9.). The results obtained are shown in Table 3.40.

From Table 3.40. the following observations may be made:-

(i) Conditioned or control media did not contain cells which contributed to the final colony forming efficiencies.

(ii) Control cultures, containing fresh medium + 10% F.C.S. gave the highest colony forming efficiencies.

TABLE 3.39

PHENOL-RED INDICATION OF THE PH OF CONDITIONED AND CONTROL MEDIA

Cell conc/flask	$2X10^6$	$2X10^5$	0 (control).
Time (hrs.)			
24	Dark Orange	Red	Pink/Red
48	Dark Yellow	Light Red	Pink/Red
96	Yellow	Dark Yellow	Pink/Red

TABLE 3.40

COLONY FORMING EFFICIENCIES OBTAINED FOR PRELIMINARY INHIBITION EXPERIMENT.

Source of c.m.	$2X10^6$ cells/flask			$2X10^5$ cells/flask			o cells/flask		
Sampling interval (hrs)	24	48	96	24	48	96	24	48	96
c.m. Dilutions.									
Undiluted c.m.	1.98	1.44	1.08	3.18	2.88	2.26	4.56	2.76	4.98
c.m.diluted 1:1 with fresh medium +10% F.C.S.	5.58	4.86	4.56	7.68	7.20	5.04	8.46	6.24	6.84
Fresh medium control	8.96								
Control (c.m.-cells).	No colonies formed in the absence of cells.								

Mean C.F.E. (n=2).

c.m. = conditioned medium.

(iii) The control medium which was tested gave the highest overall C.F.E.'s of the media tested, however, colony formation was reduced by comparison with control cultures. This would suggest that a natural decomposition of media components occurs with time (See Section 4.).

(iv) The collection time of conditioned medium did not have a great effect on colony formation as C.F.E.'s declined very slightly between 24 and 96 hr.

(v) Conditioned medium from confluent cultures gave the poorest C.F.E.'s. By comparison conditioned medium from subconfluent cultures supported almost twice as many colonies.

(vi) Dilution of the conditioned or control media examined, with fresh medium + 10% F.C.S., resulted in the following overall increases in colony formation:-

- Approx. 3-fold increase in conditioned medium derived from confluent cultures.
- Approx 2½-fold increase in conditioned medium derived from subconfluent cultures.
- Approx. 1¾-fold increase in control medium.

(B) Effects of ultrafiltered conditioned media on colony formation.

In this experiment, the inhibitory factors which operated in the preliminary experiment were examined. The molecular weight characteristics of ultrafiltered conditioned and control media and the effects of different molecular weight fractions on colony formation of RPMI 2650 cells, were examined.

Preparation of conditioned media.

Conditioned medium was prepared as described previously (Section 3.12.(A)) from duplicate 75cm^2 flasks containing 25ml medium (Eagle's MEM + 10% F.C.S: Batch NO. 10A1130D) and 2 X 10^6 RPMI 2650, passage level 46, at intervals of 24 and 96 hr. Controls from 75cm^2 flasks without cells were also collected. Duplicate 25ml volumes of conditioned and control media were pooled, centrifuged at 3,000 r.p.m. for 15min, and filter-sterilized as described previously (Section 3.12.(A)). 10ml of each conditioned and control media were retained.

Ultrafiltration of conditioned media.

The control and conditioned media obtained were pH adjusted with 1.5N NaOH or 1.5N HCl to approximately 7.5 and ultrafiltered through a 5,000 molecular weight filter, to 20% of the initial volume (50ml) as described previously (Section 2.17.). The retentates and filtrates obtained from ultrafiltration were filter-sterilized before use.

Dilution of conditioned and control media for testing.

RPMI 2650 cells, passage level 47, were pre-treated as described previously (Section 2.11.) and prepared at 1 X 10^4 cells/35mm dish, in Eagle's MEM + 10% F.C.S., as a monolayer assay (Section 3.12.(A)). Conditioned and control media were prepared as follows:-

(1) Non-ultrafiltered conditioned and control media with unadjusted pH, tested undiluted and 1:1 with fresh Eagle's MEM + 10% F.C.S.

(2) Non-ultrafiltered conditioned and control media with adjusted pH values, tested undiluted and 1:1 with fresh medium containing 10% F.C.S.

(3) The undiluted retentate (obtained after ultrafiltration) and 1:1 with fresh medium + F.C.S., from conditioned and control media.

(4) The filtrate obtained from control and conditioned media, after ultrafiltrations, undiluted and 1:1 with fresh medium.

(5) The retentate from ultrafiltered conditioned and control media rediluted with fresh medium + F.C.S. to 1/5 and 1/10 the original concentration.

(6) Fresh Eagle's MEM + 10% F.C.S. controls.

2ml volumes of medium to be tested were added to each 35mm plate containing 1 X 10^4 cells. After 10 days the colonies formed were stained (Section 2.13.) and counted.

The results are shown in Table 3.41.

From Table 3.41. the following observations may be made:-

- 24hr and 96hr control filtrates did not support colony formation. Where the filtrate was diluted 1:1 with fresh medium + 10% F.C.S. growth was observed in the 96hr control but not in the 24hr control.
- Adjusting the pH of the conditioned and control media obtained to 7.5 enhanced colony formation.
- The retentate from 24hr RPMI conditioned medium gave the highest colony forming efficiencies of the media tested even when compared with control cultures containing fresh medium + 10% F.C.S.

TABLE 3.41

C.F.E.'s OBTAINED FROM ULTRAFILTERED CONTROL AND CONDITIONED MEDIA

Source of c.m.	Control	Control	$2X10^6$ cells/ flask	$2X10^6$ cells/ flask
Sampling intervals (hr).	24	96	24	96
c.m. dilutions.				
Undiluted, non-U.F. unadjusted pH.	10.32 ± 0.77	10.6 ± 0.91	10.54 ± 0.99	9.58 ± 0.84
1:1 fresh medium Non-U.F. unadjusted pH	18.45 ± 1.04	10.87 ± 0.55	16.83 ± 0.27	10.49 ± 0.07
Undiluted 1:1 fresh medium, non-U.F. pH adjusted.	22.87 ± 0.12	12.86	17.04 ± 4.08	11.55 ± 1.7
Diluted 1:1 fresh medium, non-U.F. pH adjusted.	20.98 ± 0.58	10.2 ± 1.27	20.04 ± 1.27	11.14 ± 2.5
Retentate, undiluted	9.29 ± 0.75	16.32 ± 0.24	24.24 ± 1.3	11.16 ± 0.17
Retentate, diluted 1:1	7.47 ± 0.03	12.48 ± 0.19	16.82 ± 0.94	10.03 ± 0.05
Filtrate, undiluted	-	-	5.42 ± 0.02	4.51 ± 0.05
Filtrate diluted 1:1	-	-	13.54 ± 1.15	6.07 ± 0.84
Retentate 1/5	11.42 ± 1.16	11.472	9.36	12.84 ± 0.31
Retentate 1/10	8.02 ± 1.88	0.864 ± 0.19	16.22 ± 1.92	10.76 ± 0.39
Controls	18.55 ± 1.14	12.07 ± 0.45	18.55 ± 1.14	12.07 ± 0.45

Mean C.F.E. $\pm$ S.E.M. (n=3). U.F. = Ultrafiltered.

- The retentate from 96hr RPMI conditioned medium inhibited growth by comparison with 96hr control conditioned medium and 24hr conditioned medium.
- Overall, colony formation in 96hr conditioned medium was lower than colony formation in 24hr conditioned medium.

(C) Stability of conditioned medium with time.

Conditioned and control media were prepared as described previously (Section 3.12.(A)) and stored at 4°C for 14 days before testing in monolayer (Section 3.12.(A)) for stability and reproducibility with respect to the inhibitory effects exhibited in the preliminary experiment. The results are shown in Table 3.42.

After 2 weeks storage at 4°C, conditioned and control media remained stable and the inhibitory effects exhibited in the preliminary experiment were reproduced. However, colony formation overall was reduced by comparison with the C.F.E.'s obtained in the preliminary experiment. Possible causes of this day to day variation will be discussed in Section 4.

(D) The effects of 96hr conditioned, ultrafiltered medium on colony formation.

96hr control and conditioned media were prepared, ultrafiltered and tested in monolayer in an attempt to determine whether the effects obtained from ultrafiltered media were reproducible.

The results obtained are shown in Table 3.43. and may be summarised as follows:-

TABLE 3.42

C.F.E.'s OBTAINED FOR CONDITIONED MEDIA STABILITY/ REPRODUCIBILITY EXPERIMENT.

Source of c.m.	$2X10^6$ cells/flask			$2X10^5$ cells/flask			Control		
Sampling interval (hrs).	24	48	72	24	48	72	24	48	72
c.m. dilution									
Undiluted	1.57	1.32	0.75	2.4	1.02	1.34	2.59	2.48	2.68
Diluted 1:1 fresh medium containing 10% F.C.S.	2.99	3.07	3.14	3.37	2.96	3.11	3.37	3.26	3.44
Control (fresh medium).	3.68								
Control (c.m. in the absence of cells) *	No colonies formed in the absence of cells								

Mean C.F.E. (n=2)

* Proves that no cells were carried over in conditioned medium.

TABLE 3.43

C.F.E.'s OBTAINED FROM ULTRAFILTERED CONTROL AND CONDITIONED MEDIA.

Source of c.m.	Controls	$2X10^6$ cells/flask
Sampling interval (hrs.)	96	96
C.M. dilution.		
Undiluted,non-U.F. unadjusted pH.	10.68	12.5
1:1 fresh medium+F.C.S. non-U.F. unadjusted pH.	10.72	12.98
Undiluted,non-U.F. pH adjusted.	11.75	8.9
Diluted 1:1 fresh medium +F.C.S.,non-U.F.,pH adjusted	11.45	9.2
Retentate,undiluted	12.42	14.7
Retentate, diluted 1:1	10.78	12.59
Filtrate,undiluted	0.2	0.6
Filtrate diluted 1:1	7.2	11
Retentate 1/5	12.1	10.8
Retentate 1/10	10	14.3
Controls	14.9	14.9

Mean C.F.E. (n=2)

- By comparison with control cultures, the undiluted retentate obtained from 96hr conditioned medium inhibited growth.
- The filtrates obtained from both 96hr control and conditioned medium did not support colony formation.
- Adjusting the pH of conditioned medium increased colony formation by comparison with pH unadjusted conditioned media and pH adjusted controls.
- Dilution of both conditioned and control retentates resulted in a decrease in colony formation.

Overall, the results from Table 3.43. correspond with the results obtained for 96hr control and conditioned medium obtained in Table 3.41. and appear to be inconclusive with respect to inhibition.

(E) Effects of 96hr C.M.prepared in the absence of serum, on colony formation.

The presence of foetal calf serum in conditioned medium may have masked the activity of any inhibiting factor which may have been produced by the cells. Another source of interferance may have been the test cells themselves, which were set up 24hr before testing in Eagle's MEM +10% F.C.S., and may have contained sufficient endogenous supplies of various growth factors to overcome the effects of externally supplied inhibitory factors.

In this experiment, 96hr RPMI 2650 conditioned medium was prepared from Eagle's MEM without F.C.S., and ultrafiltered and tested as described in Sections 3.12(A) and 3.12.(B). The results obtained are shown in Table 3.44. and may be outlined as follows:-

TABLE 3.44

C.F.E.'s OBTAINED FROM ULTRAFILTERED CONTROL AND CONDITIONED MEDIA IN THE ABSENCE OF F.C.S.

Source of c.m.	Control	$2X10^6$ cells/flask
Sampling interval (hrs.)	96	96
C.M. dilution.		
Undiluted retentate	8	20.5
Retentate 1:1 fresh medium + F.C.S.	15.7	18.4
Filtrate	0.45	0.3
Filtrate 1:1 fresh medium	0.5	0.35
⅓ retentate: filtrate	5.2	0.6
Filtrate: MEM (1:1)	0.75	0.25
1/5 retentate: MEM	5.6	19.2
1/10 retentate: MEM	5.9	13.6
Control without F.C.S.	0.6	0.5
Control with 10% F.C.S.	18.7	17.9

Mean C.F.E. (n=2)

(1) Cells did not form colonies in control cultures containing Eagle's MEM without F.C.S.

(2) The filtrates obtained from control and conditioned medium did not support colony formation.

(3) The highest colony forming efficiencies were obtained from the retentates of ultrafiltered 96hr conditioned media. Control retentates yielded poorer C.F.E.'s which would suggest that the conditioned medium contained a cell produced growth factor which was greater than 5,000 D.

Summarising the results obtained from these experiments it would appear that:-

- C.M. from high density cultures inhibit colony formation.
- Cells produce a growth promoting factor. The activity of the cell produced growth promoting factor is best exhibited in the absence of serum.
- The growth promoting factor or factors examined are greater than 5,000 D.
- The growth inhibition demonstrated is probably due to nutrient depletion of the medium by the presence of cells (Table 3.43. dilution of retentate with filtrate, particularly supports this).
- One set of data (Table 3.41. 24hr VS 96hr) indicated the possibility of a high molecular weight inhibitor. This was not borne out by subsequent experiments, however, which were consistent with an inhibitor in the low molecular weight area (Table 3.44. dilution of retentate with filtrate).

3.13. THE CELL CYCLE.

Cells used in agarose clonogenic and monolayer assays were pre-treated (Section 2.11.) 48hr before use, in an attempt to standardise assay conditions. However, colony forming efficiencies obtained in agarose were generally low (Section 3.11.). In this experiment, the association between low colony forming efficiencies in agarose and different times after seeding was examined. This data, in conjunction with cell counts, would give some indication whether or not cell cycle stage is related to colony forming efficiency in agarose.

Pre-treated RPMI 2650 cells (Section 2.11.) were prepared in replicate $25cm^2$ flasks. Duplicate flasks were trypsinized, counted and suspended in agarose (Section 2.12.) at different intervals and subsequent colony formation was recorded after 12 days incubation in a 5% CO_2 atmosphere at 37°C.

Table 3.45. shows the features of this experiment and the results are shown in Table 3.46. In all experiments it may be seen that an overall increase in cell growth, with respect to cell number, occurred between 24 and 66hr. However, colony formation in agarose and monolayer did not appear to be associated with any particular phase of the cell cycle.

From Table 3.46. it may be seen that an overall increase in cell growth with respect to cell number, occurred in all experiments between 24 and 66hrs. However, colony formation in agarose and monolayer did not appear to be associated with any particular phase of the cell cycle.

TABLE 3.45

FEATURES OF THE CELL CYCLE EXPERIMENT.

Experiment	1	2	3
F.C.S. Batch No.	K223901A(309)	K223901A(309)	10F4049A
Cell passage no.	44	46	46
Stock cell conc./ $25cm^2$ flask	$5X10^5$	$5X10^5$	$5X10^5$
% Viability	92.46	94.45	93.79
Assay conc./ 35mm dish.	$1X10^4$	$1X10^4$	$1X10^4$

TABLE 3.46.

INCREASING CELL NUMBER, SINGLE CELL STATUS OF CELL SUSPENSIONS AND COLONY FORMING EFFICIENCIES OBTAINED FROM RPMI 2650 CELLS SAMPLED OVER A 66 HR. PERIOD.

EXPERIMENT NO. 1 (AGAROSE)

Sampling time (hrs)	Cells/flask	% Viability	% Doublets	% Clumps	% Triplets	C.F.E. (mean).
24	$10.13X10^5$	90.8	3.15	0	0	10.79 ± 0.17
40	$15.23X10^5$	92.5	1.3	0	0.4	11.09 ± 0.08
42	$16.35X10^5$	90.8	1.29	0	0	10.15 ± 0.21
45	$19.58X10^5$	90.3	5.55	0	0.67	5.17 ± 0.65
48	$21.68X10^5$	96	1.3	0	0.4	4.72 ± 0.48
63	$37.2X10^5$	95.75	1.95	0	0	2 ± 0.45
66	$46.2X10^5$	93.67	3.4	0.1	0	-

Mean C.F.E. $\pm$ S.E.M. (n=4) Mean cell no./flask (n=2)

EXPERIMENT NO. 2. (AGAROSE)

Sampling time (hrs)	Cells/flask	% Viability	% Doublets	% Clumps	% Triplets	C.F.E. (mean).
24	$10.8X10^5$	93.74	4.06	0	0	7.68 ± 0.79
36	$19.7X10^5$	92.68	2.29	0	0	12.57 ± 0.65
39	$22.9X10^5$	97.43	1.45	0	0	12.17 ± 0.78
42	$24.2X10^5$	95.95	1.98	0	0	10.95 ± 0.39
45	$38.6X10^5$	93.89	1.32	0	0	7.27 ± 0.45
48	$32.7X10^5$	100	0	0	0	8.45 ± 0.57
60	$49.5X10^5$	95.24	2.14	0.95	0	9.27 ± 0.41

. Mean C.F.E. $\pm$ S.E.M. (n=4) Mean cell no./flask (n=2)

TABLE 3.46. (cont.).

EXPERIMENT NO. 3 (AGAROSE)

Sampling time (hrs)	Cells/flask	% Viability	% Doublets	% Clumps	% Triplets	C.F.E. (mean)
24	$6.6X10^5$	94.36	2.28	0	1.09	11.8 ± 0.79
40	$10.8X10^5$	89.92	5.82	0.56	2.13	16.54 ± 0.8
43	$19.2X10^5$	92.09	3.51	0.56	0.44	17.03 ± 0.9
47	$16.2X10^5$	93.55	3.02	0.42	0	15.46 ± 0.6
50	$20.2X10^5$	94.66	4.39	0	0.64	15.86 ± 0.7
61	$29.3X10^5$	93.42	3.68	0.79	0	13.93 ± 0.2
64	$30.1X10^5$	89.05	5.47	1.99	0	13.92 ± 0.59

Mean C.F.E $\pm$ S.E.M. (n=4) Mean cell no./flask (n=2)

EXPERIMENT No. 3. (MONOLAYER).

Sampling time (hrs)	Cells/flask	C.F.E.(mean)
24	$6.6X10^5$	17.45 ± 0.93
40	$10.8X10^5$	17.77 ± 0.97
43	$19.2X10^5$	14.7 ± 1.27
47	$16.2X10^5$	13.74 ± 0.85
50	$20.2X10^5$	14.59 ± 0.45
61	$29.3X10^5$	14.38 ± 0.73
64	$30.1X10^5$	14.62 ± 0.35

Mean C.F.E. $\pm$ S.E.M. (n=4) Mean cell no/flask (n=2)

3.14. ANCHORAGE - INDEPENDENT GROWTH OF NORMAL HUMAN FIBROBLASTS.

The transition of a normal cell to a transformed one is accompanied by a host of phenotypic changes. One such change is the ability of the transformed cell to grow unattached to a solid substratum, a property that has been termed 'anchorage-independent' growth.

Peehl and Stanbridge (1981) reported that normal human fibroblasts and other supposedly anchorage-independent cells are capable of anchorage-independent growth under appropriate conditions. In this experiment, an attempt was made to culture normal fibroblasts as anchorage-independent clones, using the method described by Peehl and Stanbridge (1981).

Pre-treated fibroblasts and RPMI 2650 cells were prepared as described in Section 2.11. Three types of semi-solid support were used (Section 2.10.); Sigma type II agarose, Dow methylcellulose 400cps and Fluka methylcellulose. Anchorage-independent fibroblasts were cultured in DMEM (Table 2.4.) supplemented with 20% F.C.S. and 10μg/ml hydrocortisone (Section 2.15.). RPMI 2650 cells were cultured in Eagle's MEM + 10% F.C.S. (Table 2.3.). Other features considered in this experiment are shown in Table 3.47.

Monolayer fibroblast controls were maintained and monitored visually (without counting), as a check on growth after passaging. The results obtained are shown in Table 3.48. Monolayer controls which were monitored exhibited the usual pattern of growth. Normal human fibroblasts cultured in agarose or methylcellulose did not grow as anchorage-independent clones. A further attempt to clone

TABLE 3.47

FEATURES OF ANCHORAGE-INDEPENDENT EXPERIMENT.

Serum batch	10F4049A	
No.of replicates	3	
Assay incubation period:	14 days	
Cell type	RPMI 2650	Human Fibroblasts
Cell passage no.	45	5
Medium used	Eagle's MEM,10% F.C.S.	DMEM, 20% F.C.S.
Initial single cell conc.	95.5%	97%
Initial doublet conc.	4.5%	3%
Initial clump conc.	0%	0%
Viability	94%	97%
Cell conc.used	$1X10^4$cells/35mm plate	$1X10^4$cells/35mm plate

TABLE 3.48

C.F.E.'s OF NORMAL HUMAN FIBROBLASTS AND RPMI 2650 CELLS GROWN AS ANCHORAGE-INDEPENDENT CLONES.

Cell type	RPMI 2650	Normal fibroblasts
Semi-solid support		
Sigma type II agarose	5.52 ± 0.8	No colonies formed
Dow methocel	3.08 ± 0.24	No colonies formed
Fluka methocel	7.96 ± 0.12	No colonies formed

Mean C.F.E. $\pm$ S.E.M. (n=3)

These results were shown to be significantly different at the 1% level.95% Confidence intervals showed that Fluka methocel produced significantly higher colony forming efficiencies than either Dow methocel or Sigma type II agarose.

Colony size distribution curves in the different semi-solid supports examined are shown in Figure 3.17. Fluka methocel has the widest range of colony sizes by comparison with Dow methocel and Sigma type II agarose. The significance of colony size distribution will be discussed in Section 4.

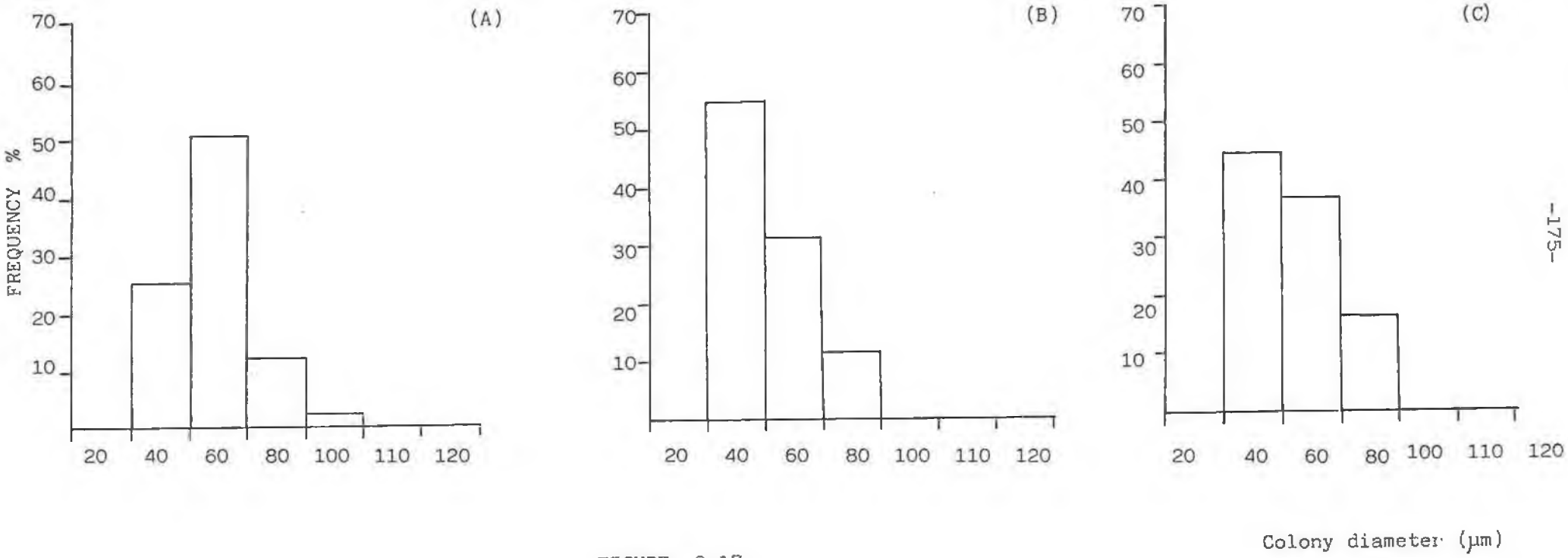


FIGURE 3.17.

RPMI 2650 colony size distribution in (A) Fluka Methocel, (B) Dow Methocel, and (C) Sigma Type II agarose.

normal fibroblasts in semi-solid medium was made using a higher fibroblast concentration of 1 X 10^6 cells/plate, however, fibroblasts did not form colonies even at the higher concentrations which were examined. By comparison, RPMI 2650 tumor cells (Section 2.7.), formed colonies in the three semi-solid supports examined; the highest C.F.E.'s were found in Fluka methylcellulose and the lowest in Dow methylcellulose (Table 3.48.).

3.15. OUABAIN AND THIOGUANINE.

The active transport of Na^+ and K^+ across the plasma membrane mediated by $Na^+ - K^+ -$ ATPase can be inhibited specifically by the cardiac gylcoside ouabain. Ouabain is toxic to mammalian cells in culture, the degree of sensitivity varying with species.

In this experiment, a range of ouabain concentrations were prepared as described in Section 2.18 and included in both monolayer and agarose assays containing pre-treated RPMI 2650 cells (Section 2.11.). Table 3.49. shows the features considered in this experiment and the results obtained are shown in Table 3.50.

From Table 3.50. it may be seen that

- In agarose, ouabain concentrations greater than 10^{-8}M inhibited RPMI 2650 colony formation. By comparison, ouabain concentrations greater than 2.5 X 10^{-8}M inhibited RPMI colony formation in monolayer.
- Colony forming efficiencies obtained in agarose were lower than those obtained in monolayer, even in the absence of ouabain.

Thioguanine, an analog of guanine which is incorporated in DNA and subsequently disrupts protein synthesis, was also included in both

TABLE 3.49

FEATURES OF THE OUABAIN EXPERIMENT.

Feature	Experiment 1	Experiment 2	Experiment 3
F.C.S. batch	20Q8833A	10Q20485	10F4049A
Cell passage no.	45	64	49
% Viability	94	92	91
% Single cells	92	88	93
% Doublets	6.2	5.6	6.0
% Clumps	3.8	3.6	4.0
Cell densities	$1X10^4$ cells/ 35mm plate	$1X10^4$, $3X10^3$ cells/dish	$1X10^4$ cells/ dish
No.of replicates	3	3	3
Incubation period	14 days	14 days	14 days

TABLE 3.50

C.F.E.'s OBTAINED FROM OUABAIN EXPERIMENT.

Experiment No.	1	2		3	
Assay type.	Monolayer	Monolayer	Agarose	Monolayer	Agarose
Initial cell density/dish	$1X10^4$	$1X10^4$	$1X10^4$ $3X10^3$	$1X10^4$	$1X10^4$
Ouabain conc.(M)					
0	$63.33^{+}3.27$	$60.88^{+}4.03$	$20.12^{+}2.84$ $30.47^{+}2.87$	$17.52^{+}0.9$	$9.92^{+}0.5$
10^{-9}	$40.83^{+}3.07$	$46.24^{+}3.35$	$1.68^{+}0.28$ $1.44^{+}0.59$	$15.26^{+}0.5$	$3.4^{+}0.9$
10^{-8}	$25.63^{+}5.8$	$25.92^{+}0.14$	$0.32^{+}0.21$ $0.17^{+}0.02$	$10.95^{+}1.1$	$0.57^{+}0.75$
$2.5X10^{-8}$	$6.88^{+}0.36$	$4.98^{+}0$	No growth No growth	$6.95^{+}0.4$	No growth
$5X10^{-8}$	No growth	No growth	- -	No growth	-
10^{-7}	-	-	- -	-	-
$5X10^{-7}$	-	-	- -	-	-
10^{-6}	-	-	- -	-	-

Mean C.F.E. $\pm$ S.E.M. (n=3).

TABLE 3.51.

FEATURES OF THIOGUANINE EXPERIMENT.

Feature	Experiment 1	Experiment 2
F.C.S. Batch	10F4049A	10F4049A
Cell Passage No.	52	45
% Viability	95	96
% Single cells	97.42	95
% Doublets	2.11	3
% Clumps	0.2	2
Cell densities	$1X10^4$	$1X10^4$
No.of replicates	3	3
Incubation period	12 days	10 days

TABLE 3.52.

FEATURES OF THE THIOGUANINE EXPERIMENT.

Experiment No.	1		2	
Assay Type	Monolayer	Agarose	Monolayer	Agarose
Initial cell density/dish	$1X10^4$	$1X10^4$	$1X10^4$	$1X10^4$
Thioguanine conc.µm.				
0	$26.98^{+}0.24$	$12.83^{+}0.19$	$16.23^{+}0.31$	$9.92^{+}0.5$
10	$15.6^{+}0.46$	$6.95^{+}0.15$	$7.11^{+}0.61$	$5.37^{+}0.25$
20	$7.68^{+}0.12$	$2.49^{+}0.11$	$3.28^{+}0.42$	$2.24^{+}0.14$
30	$2.45^{+}0.09$	$0.97^{+}0.61$	$2.03^{+}0.21$	$1.07^{+}0.02$
40	$0.89^{+}0.05$	$0.43^{+}0.2$	$1.56^{+}0.02$	$0.8^{+}0.09$
50	No growth	No growth	No growth	No growth

Mean C.F.E. $\pm$ S.E.M. (n=3)

monolayer and agarose assays (Section 2.18.). The features of this experiment are shown in Table 3.51. From Table 3.52., which shows the results of this experiment, the following observations may be made:-

- Colony formation in agarose was lower than in monolayer even in the absence of thioguanine.
- 50μM thioguanine inhibited colony formation in both agarose and monolayer assays, by comparison ouabain inhibition was exhibited at different ouabain concentrations in agarose and monolayer.

The significance of these results will be considered in the discussion.

SECTION 4.

DISCUSSION.

DISCUSSION

4.1. LIMITATIONS OF THE CLONOGENIC ASSAY

Much attention has been focused on the culture of human tumours in *in vivo* and *in vitro* assays. These assays are used for drug screening and sensitivity testing of tumours of individual patients. Experiments with tumour cell lines support the hypothesis that colony formation is the most significant end point for measuring the effect of drugs on tumour cells (Roper & Drewinko, 1976; Courtenay, 1976; Rupniak *et al.*, 1983). The *in vitro* double layer soft-agar colony-forming assay, applied to fresh human tumours, seemed promising after the first report of Hamburger and Salmon (1977). However, wider application of this technique has been hampered by many limitations, which subsequently became apparent (Selby *et al.*, 1983).

In vitro colony-forming assays with fresh human tumour specimens have many practical and theoretical problems. The paramount problem encountered by most workers using semi-solid media for cell cultivation is the poor cloning ability of primary tumours. Colony formation is achieved in less than half of the tumours (Von Hoff, 1983) and with few exceptions (Tveit *et al.*, 1982), the plating efficiencies (P.E.'s) obtained are in the range 0.001 - 0.1%. A P.E. of 0.01% implies that only 1 out of 10^4 cells is forming a colony.

Given the small amount of surgical material available for culture in many clinical situations, and the poor cell yield obtained even after enzymatic treatment of solid tumour specimens, this is a serious problem. Furthermore, in order to obtain statistically significant data, colony formation of the order of, say, 30 colonies per plate

(minimum) is desirable. When we consider that several drugs at a range of concentrations, in triplicate must be tested to obtain useful data, the scale of the problem becomes apparent. (For example, to test 4 drugs at 5 concentrations in triplicate, with control, means 63 plates. If controls have 30 colonies per plate, and P.E. is 1 in 10^4, then 3 X 10^5 cells per plate are needed. The total cell number required for this relatively simple test would be, therefore, about 2 X 10^7 cells. This sort of cell number is not readily obtainable from clinical samples). The failure to grow *in vitro* may be partly explained by inadequate culture conditions. Several ways to optimize culture conditions have been investigated including the application of a low oxygen concentration, the replenishment of the medium at weekly intervals, the addition of feeder cells or cell free ascites fluid. Neverthless, *in vitro* assays are frequently hampered by insufficient growth.

Among the technical problems associated with the clonogenic assay is that of obtaining a viable single cell suspension. Single cell suspensions from well-established *in vitro* propagated human tumour cell lines can be readily prepared and generally proliferate well *in vitro*. Clonal growth of such cells in semi-solid culture media has been used to study various aspects of tumour cell proliferation and sensitivity to anticancer agents. In contrast, preparation of pure single-cell suspensions from fresh human solid malignant neoplasms is difficult and such cell suspensions generally proliferate poorly in soft agar or agarose cultures. Agrez *et al.* (1982) have reported that almost all of the observed growth appears to result from the enlargement of seeded small cell aggregates rather than from the clonal expansion of single cells. A few clumps of 10 cells in a total population of 10^5 cells (i.e. 1 in 10,000 cells approx.) are very

difficult to detect but when the plating efficiency may be as low as 1 colony per 10^4 cells, such clumps may become of overwhelming importance. Counting colonies on the day following plating does not completely answer the question as it does not account for the influence of clumps on the interpretation of sensitivity data (see below).

While the ability to grow colonies from primary tumour cell suspensions or explants in semi-solid culture media has great potential, one major problem has been the creation of an environment that gives tumour cells a selective advantage over normal cells. For cell types other than those of the hematopoietic system (see section 1.), anchorage-independent growth in culture was initially thought to be an acquired character of malignantly transformed cells (MacPherson and Montagnier, 1964; Shin et al., 1975). Several normal diploid cell types , however, have been found to grow well in semi-solid medium (Bertoncello et al., 1982; Laug et al., 1980; Horowitz and Dorfman, 1970). Normal human fibroblasts, for example, form large colonies at high efficiency in semi-solid medium if high serum concentrations and hydrocortisone are provided (Peehl and Stanbridge, 1981). The failure of many normal cells to demonstrate anchorage-independent growth may be due to inadequate culture conditions. Thus, the creation of an optimal growth environment for tumour cells may also increase the clonogenicity of normal cells, further decreasing the specificity of the assay for tumour cells.

Several approaches to optimization of culture conditions in the assay have been investigated and will subsequently be discussed in relation to the results obtained from this investigation.

4.2 DISAGGREGATION, REAGGREGATION AND COLONY SIZE

The study of human tumour cell colonies in soft agar (including survival studies of tumours after irradiation and chemotherapy), requires improvements in the techniques for disaggregating solid tumours (Courtenay et al., 1978; Hamburger and Salmon, 1977; Salmon et al., 1978; Rasey and Nelson, 1980). The two basic approaches for obtaining a single cell suspension are the mechanical separation of cells and the enzymatic treatment of tumour tissue (Grabstein and Cohen, 1965; Russel et al., 1976; Pretlow et al., 1977; Pavelic et al., 1980; Slocum et al., 1980; Agrez et al., 1982; Engleholm et al., 1985). In these experiments it is necessary to obtain a large number of freely suspended tumour cells by the disaggregation of solid tumour tissue. The disaggregation methods must produce a high yield of viable tumour cells and a suspension representative of the cell population(s) of the tumour. With these criteria in mind, the effects of different enzyme mixtures (Clynes, 1981; Kruse and Patterson, 1973; Matsumura, 1975) on the enzymatic dissociation of RPMI 2650 monolayers (Section 3.2.) were examined.

Solid tumours are difficult to dissociate enzymatically to good single-cell suspensions(Slocum et al., 1981; Pavelic et al., 1980). However, good single cell suspensions can be readily derived from pleural or pericardial effusion by mechanical methods, with relatively little damage. Differences in single cell yield between malignant melanoma, sarcoma, breast and pulmonary carcinoma have been reported by Pavelic et al., (1980), and Slocum et al., (1981), using either enzymatic or mechanical dissaggregation techniques. Mechanical disaggregation, however, is generally regarded as being unsatisfactory

with a view to cell yield and biological viability i.e. the clonogenic yield and the tumourigenicity of the disaggregated cells. Engleholm et al., (1985) has shown that combined mechanical and enzymatic methods are appropriate for the disaggregation of a number of human solid tumours. Tumour tissue is initially minced finely with razor blades to $\sim 1mm^3$ fragments which are then incubated with an enzyme or enzyme mixture. The choice of enzyme depends on the nature and amount of available tissue. Engleholm et al., (1985) compare long term trysinization at 4°C with repeated short term typsinization at 37°C. Long-term exposure to trypsin is cytotoxic for living cells in vitro (Grabstein and Cohen, 1965; Hodges et al., 1973). However, the penetration of trypsin is temperature dependent and negligible at 4°C whereas the enzyme still has some effect on the cell surface at this temperature (Hodges et al., 1973). This may explain why long-term trypsinization at 4°C was found (Engleholm et al., 1985) to result in a higher yield of clonogenic cells than short-term incubation at 37°C.

In the experiments described earlier (Section 3.2.) 2 human tumour cell lines RPMI 2650 (a nasal carcinoma) and HT29 (a colon adenocarcinoma) in monolayer culture were used as models to compare different disaggregation methods. (I am aware of the limitations inherent in this test system, since it provides a 2 - dimensional system in contrast to the 3 - dimensional surgical samples. However, the cell lines provided a readily available, reproducible source of materials for initial studies).

Short-term trypsinization of RPMI 2650 and HT29 monolayers at 37°C frequently produced poor single cell suspensions with reduced viability, which may be explained by the cytotoxic nature of trypsin. Dead cells may subsequently release "sticky" components (e.g.DNA)

which, in turn, cause cells to form clumps and clusters in suspension. Thus, the single cell nature of a cell suspension may be governed by the susceptibility of the cells to the cytotoxic effects of the enzymatic disaggregation procedure which is used. As mentioned previously, the enzyme preparation desired would produce a good single cell suspension, with viability between 90% and 100%, in the shortest time possible. Collagenase II(0.02%)-DNase I (0.02%) produced the best, viable single-cell suspension of RPMI 2650 and HT29 cells after incubation for 1 hr. However, a considerably shorter incubation period is desirable, since the use of enzymes in the disaggregation of cells may result in changes in biological properties. Trypsin (0.25%) - EDTA (0.02%) produced the best viable single-cell suspension of RPMI 2650 cells after 15min. incubation and was adopted for routine use for these reasons. By comparison, HT29 monolayers were not readily dissociated with trypsin (0.25%) -EDTA (0.02%) but after 20 min. incubation Dispase (1.2 units/ml) produced the best single cell suspensions of HT29 cells. Overall, HT29 cells and RPMI 2650 cells reacted differently to the various enzyme preparations examined. This reflects the difficulties frequently encountered when a universal enzymatic approach is applied to fresh biopsy material.

The efficiency of several enzyme mixtures containing collagenase (collagenase II (0.02%) - DNase I (0.02%) and collagenase II (0.04%)-pronase (0.1mg/ml) - DNase (0.04mg/ml) were examined. As the collagenase concentration was increased it was found that the incubation period decreased. The chemosensitivity of tumour cells may be dependent on the procedure applied (Rasey and Nelson, 1980), for example, trypsin is known to destroy membrane bound immunoglobulin (Russell et al., 1976). The development of suitable disaggregation

techniques may demand increased incubation periods as milder treatments, for example (as described above) reducing the enzyme concentration or using temperature dependent enzymes at sub-optimal conditions, are chosen (Engleholm et al., 1985).

Thus far, it has proved extremely difficult to prepare good viable single cell suspensions from RPMI 2650 monolayers (Section 3.2.). The problem is further compounded by the apparent ability of the cells to spontaneously adhere and reaggregate to form clumps and cell clusters (Section 3.3.). Reaggregation of cells was first observed by Holtfreter (1931) in amphibian embryonic eggs which were disaggregated in a Ca- and Mg-free salt solution. After dissociation the separated cells were mixed and random adhesion of the cells was observed followed by a sorting of like-cells. Moscona (1952) demonstrated the ability of mammalian and avian cells to recognise and adhere to each other.

RPMI 2650 cells and normal human fibroblasts were found to reaggregate spontaneously in vitro (section 3.3). However, by comparison with normal fibroblasts, RPMI 2650 cells had a higher reaggregation rate. Wright et al., (1977) examined the rate of spontaneous aggregation of various cell types suspended with EDTA. Their experiments indicated that the rate of spontaneous aggregation is increased in cells that have lost anchorage dependence and become tumourigenic. This would suggest that the membrane property of cell-to-cell adhesion may be used as a marker for tumourigenicity. However, Wright et al., (1977) used EDTA suspended cells whereas our study used trypsinized RPMI 2650 and fibroblasts. Cassiman and Bernfield (1975) have shown that trypsinization decreases the cell-to-cell adhesion of normal or transformed cells and also renders both cell types equally adhesive. Thus, the real significance of experiments

using trypsinized cells is difficult to assess.

Spontaneous aggregation of cells in suspension is important with respect to the clonogenic assay, since aggregates or clusters of cells appear as colonies in agar and can effect both the character of the cultures and the results obtained from chemosensitivity testing (Wright et al., 1977; Agrez et al., 1982; Rockwell, 1985). Many of these effects have been studied extensively using established cell lines and primary explants from animal tumours (systems in which good single-cell suspensions can be obtained, and cells can be cloned with high efficiencies). Rockwell (1985) describes the artifacts and problems which may be encountered when "clumpy" suspensions are used to generate "cell" survival curves. Using data obtained by Mate et al., (1984), which defines the survival of partially committed granulocyte/monocyte progenitor cells in suspensions prepared from the bone marrow of irradiated BALB/cKaRw mice, a database was defined. By comparing the results from "clumpy" suspensions with this "control", it was possible to identify the major influences of clumps and clusters in the clonogenic assay. They are as follows:- (using Rockwell's terminology of "multiplicity" to describe more than one cell per colony forming unit).

(a) The effects of multiplicity on exponential survival curves.

If the colony-forming units plated during cloning studies are not single cells, the measured survival curve will be different from the single-cell survival curve. For example, if there are 2 clonogenic cells in a colony-forming unit, it would be necessary to kill both cells to prevent the formation of a colony. Survival curves measured for colony-forming units composed of clusters

containing 2 or more clonogenic cells will have "shoulders" at low doses even when the single cell survival curves lack shoulders, because at low doses it would be extremely improbable that all the cells in a cluster would be killed (see figure 4.1.).

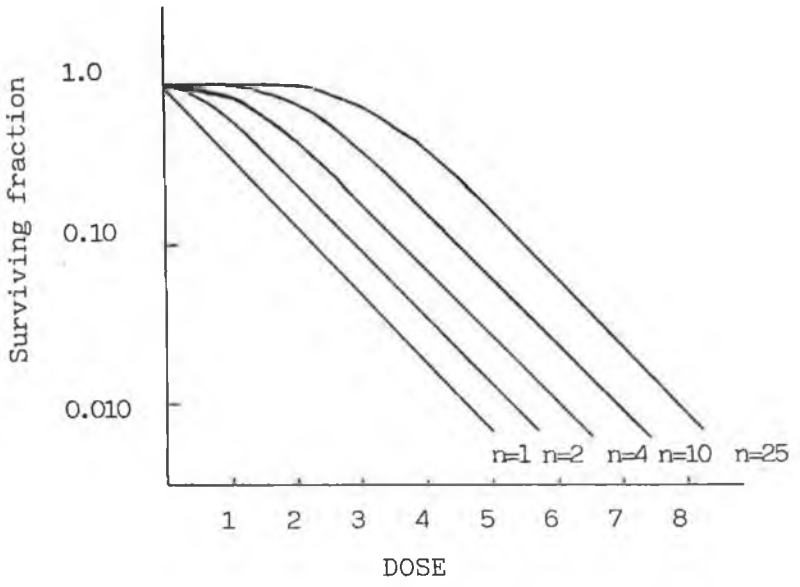
(b) The effects of multiplicity on curvilinear survival curves.

Exponential single-cell survival curves are often observed when normal hematopoietic cells, leukemic cells, or lymphoma cells are treated with radiation or alkylating agents. When cells of other origins are treated with these agents, the single-cell survival curves often have shoulders, which are thought to reflect the ability of the cells to accumulate a certain sublethal injury. If clusters are present the size of the shoulder would be further increased because of the effect of multiplicity. A survival curve measured for a mixed population of single cells and multicell aggregates would be curvilinear at low doses and would plateau at high doses (fig. 4.2.). In order to correct the measured survival curve to yield the single-cell survival curve, one would need to define in detail the distribution of the clonogenic cells among the single cells and clusters; it is generally impossible to obtain this information.

(c) Abortive clones.

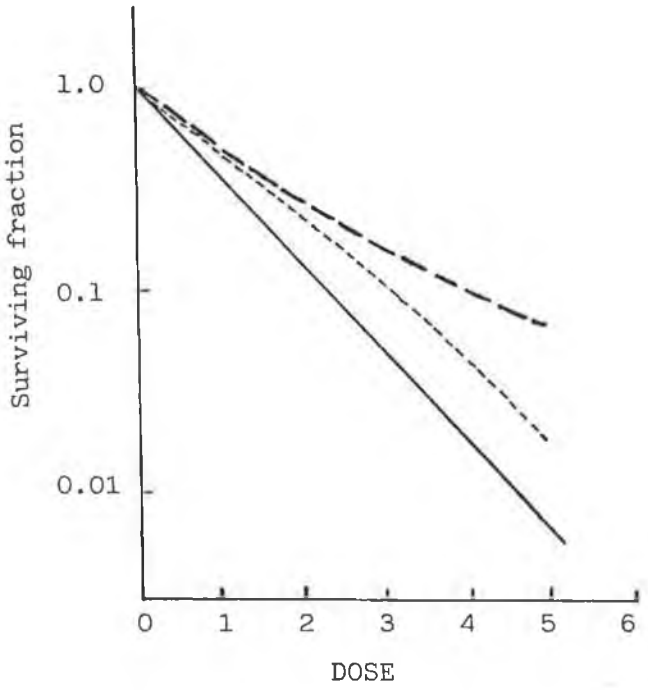
Many agents used in the treatment of cancer do not kill cells immediately. Instead, dying cells remain intact and indistinguishable from surviving cells and may even

FIGURE 4.1.



Effect of multiplicity on a survival curve, The single-cell survival curve is labeled n=1. Curves marked n=2,n=4,n=10,and n=25 are calculated survival curves for clusters containing 2,4,10,or 25 clonogenic cells, each having the exponential single-cell survival curve shown for n=1.(Rockwell,1985).

FIGURE 4.2.



Effect of a small proportion of clumps and clusters on a curvilinear survival curve. This example is similar to that in Figure 4.1 except that the single cell survival curve (——) has a shoulder. ----,survival curve for mixed cell populations of single cells,clusters,and clumps, if clumps are scored as colonies;———, survival curve obtained if clumps are excluded with complete accuracy (Rockwell, 1985).

proliferate relatively normally for one, 2, or several, divisions. If clusters are present in the cultures, even very limited proliferation potential among the dying cells may create problems, as abortive proliferation may expand nonclonogenic clusters so that they are artifactually scored as colonies. For example, if a cluster of 20 cells included 4 cells which were capable of very limited proliferation (e.g. 3 divisions), the clusters would grow to 40 cells and would be scored as a colony, even though none of the cells were clonogenic.

(d) Changes in cell volume.

Many treatments alter the volumes of living and dying cells. For example, radiation produces a dose-dependent increase in cell size. The alkylating agents, the mitomycins, hydroxyurea and many other drugs alter the cell size (Taylor and Bleehen, 1978). If cell volumes increase after treatment, tightly packed clusters of cells might increase in size sufficiently to be scored as colonies, without increasing in cell number.

(e) Microenvironmental effects.

Cell grown as "spheroids", small balls of closely packed cells (Freyer and Sutherland, 1980; Thomas et al., 1982), have different sensitivities to radiation and drugs than do single cells. Part of this difference reflects differences in cell viability, cell proliferation, and repair capacity related to cell-cell contact. In addition, the metabolic activity of the cells produces gradients of

nutrients, metabolites, catabolites, pH, O_2 etc., between the edge and center of the spheroid; these produce gradients in cell viability, cell proliferation, cellular metabolism, and drug sensitivity within the unit (Freyer and Sutherland, 1980). Gradients in environment, metabolism, and proliferation undoubtedly occur within clumps and clusters unintentionally plated from tumours, just as they do in spheroids deliberately prepared from human and animal tumour cells,and would similarly alter the response of the clumps to treatment.

(f) Effects of contact with host cells

Cell suspensions prepared from tumours contain a variety of normal cells (e.g. lymphocytes, macrophages, fibroblasts) as well as malignant cells (Asano et al., 1981; Courtenay et al., 1976; Hamburger et al., 1983). Many of these can grow in vitro and some can interact with and alter the viability, clonogenicity, and / or behaviour of malignant cells (Asano et al., 1981; Hamburger et al., 1983). Interactions with nearby normal cells could cause tumour cells in clumps to behave differently from isolated tumour cells,and may actually increase the number of colonies arising from the clusters.

(g) Drug penetration.

Many drugs, including such commonly used agents as Adriamycin and methotrexate, do not penetrate into the center of spheroids or into intact tumour tissue (Durand, 1981). Because of this, cells in the center

of clumps of cells or chunks of tissue may never be exposed to cytotoxic concentrations of drugs which are added to the culture medium; these sequestered cells would survive treatment of the cultures with regimens which would be lethal if the cells were actually exposed to the treatment. In such cases, a survival curve with a plateau at high doses may not indicate that some cells are resistant; rather , it may indicate that some cells were untreated. It is worth pointing out, however, that in this regard spheroids may give a better indication than single cells of in vivo effects; the problem may be that in such cases colony formation is not an easily measurable end-point.

With the mechanical and / or enzymatic methods commonly used by many laboratories for disaggregating human tumours into cell suspensions, it has not been possible to obtain preparations which are composed exclusively of single tumour cells. In an attempt to determine the contribution of cell clumps and clusters to final colony growth, RPMI 2650 cell preparations containing single cells and cell clusters of various sizes were plated into agarose. Some clusters no doubt were undisrupted monolayer fragments and other clusters probably re-aggregates of cells formed after the initial monolayer disaggregation. RPMI 2650 single cells, doublets and clumps all appear to contribute (in varying degrees) to final colony growth (Section 3.4.). Single cells and clumps, in both good single cell and "clumpy" cell suspensions, were consistently found to contribute to final colony forming efficiencies to a greater extent than RPMI cell doublets. However, these "clumpy" RPMI 2650 cell suspensions were found to be improved by syringing

through a narrow-gauge microlance. This resulted in a reduction in the number of clumps present, however, it also reduced the proportions of single cells, doublets and clumps which contributed to final colony forming efficiencies. Some workers studying colony formation by primary human tumour cells have claimed that cell aggregates present the day after plating do not have an impact on the final evaluation of colony formation because the aggregates tend to lyse or at least undergo changes which make them unrecognizable as colonies. However, our results did not support this hypothesis since, single cells, doublets and clumps of RMPI 2650 cells were found to grow in agarose (Figure 3.5.) as easily identifiable colonies which contributed to final colony forming efficiencies. Clumps present in unsyringed suspensions ceased growing after 6 days incubation, whereas clumps from syringed suspensions increased in size throughout the 12 day period examined and were indistinguishable from colonies formed by the growth and division of single cells. Agrez et al., (1982) have also shown that clumps, clusters and aggregates in the clonogenic assay contribute to final colony scores. They also suggest that virtually all clusters of cells which appear to be colonies arising from clonal growth of single cells arise from small clusters of cells plated in the agar, since tumour cell aggregates initially plated are often identical in appearance to images subsequently identified as proliferating colonies.

As mentioned previously (Section 3.4.), a correction is sometimes used for clumps in clonogenic assays. In some cases, cultures are examined a few hours after plating, and clumps of plated material which would meet the criteria to be scored as colonies are enumerated and the artifactual "colony counts" are then subtracted from the "colony numbers" obtained when the experimental plates are counted.

A correction of this nature was found to be useful in some instances where the effects of additives in the assay (for example, ouabain and thioguanine) were not examined. However, this correction does not address any of the problems associated with multiplicity or any of the other potential artifacts created by the clumps or by smaller clusters in the cultures. Estimates of the proportion of colonies in untreated cultures that are actually clumps range from a few percentages to > 50% of total colony growth in the soft agar assays (Agrez et al., 1982; Bertoncello et al., 1982; Kirkels et al., 1983). This factor is often a major limitation on the use of these assays to measure cell survival.

A critical point in the test is the definition of in vitro tumour cell growth, which has often unfortunately, been based on variable criteria without much rationale: 5-30 colonies (Cowan and Von Hoff, 1983; Salmon, 1980)after 7-29 days in culture (Alberts et al., 1980; Williams et al., 1983) constitutes growth. The size threshold of a "colony", is currently accepted as growth of 50 or more tumour cells (Fan et al., 1984). This criterion is technically difficult to fulfill since cells from many tumour types grow in very close proximity during growth and the number of individual cells in a colony is not readily measurable. Many workers use the diameter of the growth to define a colony (Agrez et al., 1982), but the size difference between tumour cells of various origins makes it difficult to set a standard size for all colony types. It is not uncommon for primary human tumour cells in culture to take 60-80 hrs. for one division even under the best in vitro conditions. Considering in this assay, the vigorous treatments and poorly defined growth conditions including the lack of an adequate feeding/perfusion mechanism, a cluster of 10-20 tumour cells (approximately 30μm in diameter) developed from a single seeding

cell could theoretically represent a "true" tumour colony having prolonged doubling time and / or cells ceasing to divide owing to exhaustion of nutrients. As long as soft agar is used as a selective condition for tumour-cell growth, a cell that ceases growth after three to four divisions could be just as "clonogenic" as one which divides six times (64 cells). By lowering the size threshold of a colony more information may be obtained from the soft-agar growth of clinical human tumours. However, before chemosensitivity data obtained in different laboratories may be directly compared a strictly standardized approach to colony sizing is required, particularly if the wide range of colony size thresholds are considered; for example Endresen et al., (1985) consider colonies to be >30 cells; Engleholm et al., (1985) > 50 cells; Hill and Whelan (1983) > 50 cells; Hamburger et al., (1983) > 30 cells; Agrez et al., (1982) 25-50 cells; Hamburger and Salmon (1977) >40 cells.

4.3. PHYSICAL AND CHEMICAL ENVIRONMENT

Many solid tumours fail to proliferate in vitro. A low potential for growth and low plating efficiencies have been rationalized on the basis of a model in which only a small fraction of the total tumour cell population (the so-called "stem-cell" population) is capable of repopulating the tumour (i.e. "self-renewal"). Cells that do proliferate in the in vitro test environment have been presumed to include a small fraction of tumour cells that maintain the growth and spread of the tumour (Sikic and Taber, 1981). Nevertheless, not all tumour cells that proliferate in primary culture continue to have the capacity for self-renewal after re-culturing (Buick and MacKillop, 1981; Bertoncello et al., 1982; Thomson et al., 1982).

Both low plating efficiencies and the loss of the capacity for self-renewal may at least in part reflect a somewhat inadequate nutritional environment for perpetual tumour cell growth, as has been suggested (Hamburger et al., 1981). This premise is supported by the reports that plating efficiencies can be increased when the tissue culture nutritional environment is augmented by the addition of insulin (MacKintosh et al., 1981), epidermal growth factor (Hamburger et al., 1981; La Rocca and Rheinwald, 1985) or multihormonal supplementation (Bettger et al., 1981).

The in vitro environment consists essentially of liquid nutrient media and agents that render the media semi-solid so that only anchorage-independent growth can occur. The growth related effects of several agents that are used to make liquid media semi-solid were examined using RPMI 2650 cells. The effects of agar (Bacto-agar), different agaroses (LE, ME, HEEO, Seaplaque and Seaprep) and methylcelluloses (Fluka and Dow) on colony size and formation were examined (Section 3.7.). The major observations made are as follows:-

(a) Optimum colony formation was obtained using low gelling temperature agaroses, when the temperature was adjusted to account for individual gelling properties.

(b) Decreasing the concentration of the semi-solid support to 0.15% did not significantly increase colony formation.

(c) Colony forming efficiencies in agar and Sigma type II agarose, which was routinely used, were comparable.

(d) Methylcellulose did not promote RPMI 2650 colony formation dramatically.

Similar findings have been reported by other investigators:

- Pavlik et al., (1983a) using human cell lines as models, representing colon, ovarian, and cervical cancers, indicate that good growth was observed in low melting agaroses (Seaplaque and Seaprep 15/45). Optimal growth in these semi-solid agents was observed for the ovarian cell line only, thus showing the ability of different cell lines to demonstrate different growth responses to agents used to make media semi-solid.

- By decreasing the agarose concentration from 0.3% to 0.18%, Whelan and Hill (1981) demonstrated an increase in colony forming efficiencies in four out of six cell lines tested. By comparison, our study concluded that a reduction in the semi-solid support concentration did not increase colony formation of RPMI 2650 cells.

- Agar is a mixture of both neutral agarose and ionic agaropectin, which can contain various impurities (i.e. Na, Mg, Pb, As, sugars etc.,). As a consequence, the opportunity exists for a cell population to respond to both the agent regarded as responsible for gelation and to a host of undefined components which may inhibit colony formation (Dixon et al., 1981). Agarose is agar with most of the large charged molecules removed and is generally thought to be less selective for tumour cells than agar (MacPherson, 1973). However, RPMI 2650 colony formation in agar and Sigma type II agarose, which has a low ionic sulfate content (Table 2.5.), were comparable. Pavlik et al., (1983a) report similar comparisons between colony growth in agar and agarose, however, the comparisons were dependent on the cell line examined.

- The inclusion of methylcellulose,rather than agar or agarose, in media has been found to significantly increase the growth of a human urinary transitional cell line (Colo 232, Pavlik et al., 1983b), and a cervical cell line (Pavlik et al., 1983a).

However, RPMI 2650 colony formation was not dramatically enhanced by the use of methylcellulose as a semi-solid support. Pavlik et al., (1983a) reported that some of the cell lines which were examined exhibited sub-optimal growth in methylcellulose; it is possible that the use of methylcellulose also presented a sub-optimal environment for RPMI 2650 colony formation. Overall, it appears that different tumour cell lines can demonstrate different growth responses to agents used to make media semi-solid so that generalisations cannot be made.

There are several potential sources of variation for any given assay that requires the multiplication of cells. The nutritional state of cells before transfer and assay is an obvious one which can be avoided by a medium change a day before transferring the cells (Section 2.11.). Since this precaution is not universally observed, prior medium depletion may have contributed to apparent heterogeneity in some studies. During the preparation of semi-solid media, the quality of water used will determine which additional micro-elements, whether stimulatory or inhibitory, are present. Ultrapure water, (prepared by the Millipore Milli-Ro 60 and Milli-Q water purification systems), used in the preparation of semi-solid media, yielded higher RPMI 2650 colony forming efficiencies than reverse osmosis or tap water. Growth inhibition using reverse osmosis and tap water may be due to the presence of ions and / or toxins e.g. fluorine and chlorine, which are removed in the preparation of ultrapure water. Larger colonies were generally observed (Section 3.5.) using reverse osmosis and tap water, however, this may be a reflection of increased cell volume, due to the presence of micro-elements and toxins (Rockwell, 1985), rather than an actual increase in cell growth and division. In the description of their method, Courtenay and Mills

(1978) used distilled water as a solvent for agar but more recently Courtenay (1983) describes using PBS for this purpose. Using the Courtenay (1978) and Hamburger and Salmon (1977) assays, Walls and Twentyman (1985) compared colony formation of cells in PBS and distilled water. This report suggests that an isotonic agar solution is advantageous for plating cells in the Courtenay method as agar prepared in PBS produced higher plating efficiencies than agar prepared in distilled water. In the Hamburger and Salmon (1977) method, there was no clear advantage of using PBS to prepare agarose, however, this finding was not investigated further in this study. It appears that fresh ultrapure water is the best type of water to use in the assay, since it is of uniform quality and helps to diminish assay variation.

In the majority of cell culture systems, the gas phase used has been air containing various concentrations of carbon dioxide. In vivo, however, most tumour cells are known to exist under low oxygen tension because of the nature of their vascular supply and distance from the nearest blood vessel (Thomlinson and Gray, 1955). Physiological concentration of oxygen has been reported to enhance clonal growth of some cell lines (Richter et al., 1972; Taylor et al., 1978; Tveit et al., 1981).

Low oxygen concentration may not be beneficial for all culture systems; for example, colony formation of Chinese hamster cells decreased with hypoxia(Born et al., 1976). Tveit et al., (1981a; 1981b) showed that when cloning melanoma xenografts 5% O_2 produces higher plating efficiencies than does 20% O_2. Gupta and Eberle (1984) demonstrated that although there was great variation between the plating efficiencies of cellsfrom the various human cell lines tested, the optimal concentration could be as low as 0.1% These reports

suggest that the most effective O_2 tension for improving the plating efficiency of cells in the clonogenic assay is in the range of 0.1%-5%. Endresen *et al.*, (1985), Sridhar *et al.*, (1983), Walls and Twentyman, (1985) and Joyce and Vincent (1983) have all reported on the advantage of reduced oxygen tension in growth of human tumour cells in semi-solid cultures.

Our data (section 3.6) are in accord with these findings and show that RPMI 2650 colony size and colony forming efficiencies were improved under hypoxic conditions by comparison with cells grown in high (20%) oxygen concentrations. However, it appears that RPMI require some oxygen, since colony formation was inhibited in 10% CO_2/N_2 (Richter, 1973) and pure N_2 gas. Gassing with O_2 - free N_2 produced plating efficiencies for RPMI 2650 cells (in agarose) which were better than those obtained when 5% O_2 was used. However, the colonies formed in agarose were very small and RPMI 2650 monolayer growth was totally inhibited in O_2 - free N_2, which suggests that the agarose had trapped O_2 which was sufficient for an initial spurt of growth but inadequate for a further increase in colony size.

The factors controlling the ability of human tumour cells to form colonies in soft agar are poorly understood. Recently, several groups have demonstrated that factors contained in serum stimulate the growth of cells in soft agar. Cowan *et al.*, (1982) demonstrated that platelet lysates enhanced the growth of cells from 47% of epithelial and mesodermally-derived tumours in soft agar. O'Neill et al, (1979) reported that anchorage-dependent NIL - 8 hamster fibroblasts exhibited serum concentration dependent growth in soft agarose and that the rate of growth of attached and suspended cells was approximately the same when the concentration of foetal bovine serum (FBS) was raised to 66%. Peehl and Stanbridge (1981) found that normal human fibroblasts grew when suspended in methylcellulose

with 20% FBS, but not in medium with 10% FBS. However, since serum is poorly defined, its inclusion in the agar assay introduces further undesirable variation. Current research in tissue culture is tending toward more defined media which are either serum-free (Barnes and Sato, 1980; Simms et al., 1980; Taketazu et al., 1984) or serum-supplemented media (Simms et al., 1980; Hamburger et al., 1983; Carney et al., 1981). Hamburger et al., (1983) examined the effects of serum concentration on colony formation of different tumour cells in soft agar and found that cells were unable to form colonies in the absence of serum. Similarly, RPMI 2650 cells did not form colonies in agarose in the absence of serum and grew poorly in 1% serum. The threshold concentration of serum required for RPMI 2650 growth appeared to be 2%. A plateau was reached in the serum dose-response curve between 15% and 20% serum. Inhibitory effects were observed at 15% and 20% serum when lower cell concentrations were used, which would suggest that serum contains factors whose effects are dependent on assay conditions e.g. cell density. Serum supplies a number of growth factors and nutrients that stimulate the proliferation of human tumour cell lines in monolayer culture. Growth factor supplements have been found to either replace or reduce the serum requirement (Hamburger et al., 1983; Simms et al., 1980; Carney et al., 1981). The addition of HITES to monolayer RPMI 2650 cultures increased the number of colonies at serum levels of 1% and 2% and reduced the threshold concentration of serum. However, serum was still required since colonies did not grow without serum even when supplemented with HITES. Hamburger et al.,(1983) examined the ability of ITS to reduce serum requirements; they also suggest that many cell lines also require the addition of hydrocortisone and estradiol to serum-free media to allow proliferation. RPMI 2650 cells were found to be most dependent on the presence of hydrocortisone for

monolayer colony formation (Section 3.9.).

The inclusion of HITES in the agarose assay did not reduce the serum requirement: in fact HITES inhibited colony formation, except at 1% serum. Our inability to demonstrate anchorage-independent growth of RPMI cells in the absence of serum or at low serum concentrations may have been due to inadequate culture conditions. The problems of creating an optimal nutritional environment for the growth of different cells in suspension have been widely considered (Ham and McKeehan, 1979; La Rocca and Rheinwald, 1985; Swart and Lowenberg, 1984; Endresen et al., 1985; Taketazu et al., 1984). In general, growth appears to be limited by inadequate nutritional or hormonal environments. It is likely that serum, in addition to supplying TGF's and other unknown polypeptides required for anchorage-independent growth, supplies hormones and other nutrients which enable RPMI 2650 cells to grow in agar. HITES medium has been developed by Carney et al., (1981) for small cell lung cancer cells and may be unsuitable for RPMI 2650 anchorage-independent growth.

Other growth factors were tested (Section 3.10.) in an attempt to improve colony formation of RPMI 2650 cells in agarose. The following factors were tested:- TCGF, EGF, insulin and hydrocortisone, however, EGF was the only factor found to improve colony forming efficiencies in agarose. EGF has been found to stimulate proliferation of epithelial cells in serum-free media and to enhance colony formation by human tumour cells in soft agar (Todaro et al., 1981; La Rocca and Rheinwald, 1985; Hamburger et al., 1981; Pathak et al., 1982). The stimulatory effect of EGF on RPMI 2650 colony formation was low by comparison with other reports, for example, Pathak et al., (1982) found that the addition of EGF could increase cloning efficiency by 50%. However,

since they used EGF in low serum concentrations and this study used 10% FCS, it appears likely that any major stimulatory effect elicited by EGF would be masked in high serum concentrations.

RPMI 2650 cells have lower plating efficiencies in agar than in monolayer. Some human lines such as HT-1080 fibrosarcoma show a linear relationship between cells plated and colonies formed in agar (M. Clynes, unpublished results). Several other lines, including RPMI 2650 carcinoma, display linear behaviour down to a certain critical density, below which cloning efficiency rapidly falls to zero (Section 3.11). There are several possible explanations for this observation, including the presence in the medium of toxic substances (e.g. Metal ions, peroxides, superoxides), or damage to the cells during dilution at low density. However, since the cut-off point of RPMI 2650 cells was approximately 10-fold higher in agarose than in monolayer and varied considerably from day to day, the failure of RPMI 2650 cells to exhibit growth at low densities may have been caused by inadequate culture conditions.

It has been known for many years that the use of lethally irradiated feeder cells or of medium conditioned by the growth of cells can stimulate growth of cells plated at low density in culture. Hamburger et al., (1981) tested fibroblast feeder layers for their colony stimulating activity with moderate success. Courtenay et al., (1976) and Whelan and Hill (1983) also used highly irradiated cells (HR) which were always of the same type as the live cells being tested (Walls and Twentyman, 1985).

Using unirradiated feeder layers of RPMI 2650 cells, grown under the hard agar base layer (Section 3.11.), low density cultures of RPMI 2650 cells were stimulated to form colonies. When higher cell

densities were plated in the presence of feeders, growth inhibition was observed (McManus and Clynes, 1983). When mitomycin C-treated feeders were used, colony formation was stimulated, even at high cell densities in both the feeder and test cell layers. The survival, proliferation and the development of colony-forming cells *in vitro* requires the continuous presence of specific growth regulatory molecules (Dexter *et al.*, 1984). These factors can be:

(a) produced by cells distant from the target cells and reach them by the circulating plasma (endocrine);

(b) produced by stromal cells and be transmitted locally to stimulate the targets bearing receptors for this factor (paracrine); or

(c) produced by the target cells themselves (autocrine).

It has been postulated that the malignant phenotype of certain cells may be the result of an autocrine stimulation (Todaro and De Larco, 1978); this is discussed in more detail below. The feeder layer stimulation of RPMI 2650 cells may also be due, at least in part, to production by the cells of substances which enhance growth and / or survival of the same cell type. Preliminary experiments by Margaret Dooley in this laboratory have shown that this growth promoting factor or factors, is greater than 5,000 daltons M.W. (as determined by ultrafiltration).

Where high feeder cell concentrations and high concentrations of test cells were used together in the agarose assays colony formation was inhibited (Section 3.12.). Density-dependent inhibition exhibited by RPMI 2650 cells in the assay was probably due to nutrient depletion, however, there was some indication that a high molecular weight inhibitor was responsible for poor colony formation. Fryling *et al.*, (1985), Harel *et al.*, (1984), Iwata *et al.*, (1985), and Wells

and Mallucci (1983), have investigated the effects of inhibitory diffusible factors on cell growth. Growth inhibitory activity has been found in conditioned medium of 3T3 cells (Steck et al., 1979), and more recently, activity to inhibit growth has been recovered in a fraction from 3T3 conditioned medium containing two proteins with M.W. of 11k and 13k daltons (Steck et al., 1982). Tucker et al., (1984) have recently shown that purified growth inhibitor from BSC-1 cells and TGF-β from platelets have similar biological activities. Both of these molecules can stimulate or inhibit growth depending on the experimental conditions. It appears that the action of autocrine growth factors released by RPMI 2650 feeder cells may also be dependent on culture conditions, in particular, the cell density used (Hays et al., 1985).

Autocrine secretion of growth factors is a concept which is emerging as a unifying theme in the search for the molecular and cellular basis of malignant transformation. This search has resulted in the merging of the study of oncogenes and peptide growth factors, as seen in the recent discoveries that oncogenes can confer growth factor autonomy on cancer cells (Doolittle et al., 1983; Waterfield et al., 1983; Downward et al., 1984). The relatively autonomous nature of malignant cells has been known for many years; that is, they require fewer exogenous growth factors for optimal growth and multiplication than do their normal counterparts (Peehl and Stanbridge, 1981). To explain this phenomenon, it was suggested that cells could become malignant by the endogenous production of polypeptide growth factors acting on their producer cells via functional external receptors, allowing phenotypic expression of the peptide by the same cell that produces it (Sporn and Todaro, 1980). This process has been termed 'autocrine secretion'.

Many types of tumour cells release polypeptide growth factors into their conditioned medium when grown in cell culture and these same tumour cells often possess functional receptors for the released peptide. The peptide growth factors that function via an autocrine mechanism in cancer cells include type α transforming frowth factor (TGF - α), peptides related to platelet-derived growth factor (PDGF), bombesin and type β transforming growth factor (TGF - β). The action of each of these four peptides is mediated by a distinct membrane receptor, which in turn activates a post-receptor signalling mechanism and leads eventually to a mitogenic response.

The autocrine action of a growth factor in a cancer cell was first described in rodent cells transformed by either Moloney (De Larco and Todaro, 1978)or Kirsten (Ozanne et al., 1980) murine sarcoma viruses (Mo-MSV and Ki-MSV, respectively). The first peptides identified as participating in this transformation (Roberts et al., 1983) are structurally related to (but distinct from) epidermal growth factor (EGF) and are now called type α TGF's. The only known receptor for these peptides is the EGF receptor and all of their effects are thought to be mediated through this locus. The signalling pathways activated by an autocrine peptide need not evoke a positive growth response, as seen most strikingly in the response of cells to TGF-β. This peptide was characterized initially by its ability to stimulate the growth of non-neoplastic fibroblasts as colonies in soft agar (Roberts et al., 1983). However, TGF-β can also be a potent inhibitor of the growth of many cells, which suggests that in certain cells endogenous TGF-β plays a role in cell-cycle regulation. Thus, the autocrine hypothesis may now be extended to include the concept that malignant transformation may be the result not only of excessive production, expression and action of

positive autocrine growth factors, but also of the failure of cells to synthesize, express or respond to specific negative growth factors they normally release to control their own growth. If such mechanisms turn out to be involved frequently in human cancers, there may be therapeutic possibilities including treatment with antagonists of positive autocrine growth factors.

4.4. NATURE OF ANCHORAGE - INDEPENDENT GROWTH

Although it remains debatable whether or not growth in semi-solid medium will prove to be a good correlate of tumourigenicity, normal human fibroblasts are considered to be entirely anchorage dependent for growth in vitro. Attempts to culture normal human fibroblasts (Section 3.14.) in agarose or methylcellulose failed, even when elevated serum concentrations (20%) and hydrocortisone (10μg/ml) were included in the assay. These results are contrary to those reported by Peehl and Stanbridge (1981) under the same conditions of increased serum and medium supplementation. At present, the reasons why normal human fibroblasts were incapable of growing under the conditions examined are unknown. It appears that, in some way, the assay used by Peehl and Stanbridge (1981) may have been less selective for tumour cells only, by comparison with the assay currently used in this laboratory. It is possible that different properties of serum batches provided a contributory factor to the discrepancy.

The nature of anchorage-independent growth and factors which influence anchorage-independent growth are poorly understood. Having considered the influence of environmental parameters (e.g. semi-solid support, feeders and hypoxic conditions) on RPMI 2650 colony formation

in agarose, this study examined the contribution of cell cycle stage to colony formation. However, colony formation in agarose and monolayer did not appear to be influenced by cell cycle stage (Section 3.13.). Cell cycle analysis may not prove useful in determining the nature of anchorage-independent growth.

In an attempt to gain some insight into the nature of anchorage-independent growth at the molecular level several experiments were conducted using the cardiac glycoside ouabain and the guanine analog thioguanine. Ouabain effects the membrane Na^+ - K^+ ATPase which actively pumps Na^+ out and K^+ into the cell against their concentration gradients. The Na^+ and K^+ gradients maintained by the Na^+ - K^+ pump are responsible not only for maintaining the cells' membrane potential, but also for controlling cell volume and for driving the active transport of sugars and amino acids. The active transport of the Na^+ - K^+ - ATPase in controlling cell volumes is shown by the fact that animal cells swell and burst if they are treated with ouabain which inhibits the Na^+ - K^+ - ATPase (Figure 4.3). 5×10^{-8}M ouabain was found to inhibit RPMI 2650 colony formation in monolayer (Section 3.15.), however, a lower ouabain concentration (2.5×10^{-8}M) was found to inhibit colony formation in agarose. However, no difference in sensitivity was seen between monolayer and agarose conditions with 6 - thioguanine, which acts by interference with intracellular reactions rather than at the cell surface. RPMI 2650 colony formation was inhibited in both monolayer and agarose assays at a concentration of 50μM thioguanine, which would suggest that cell surface receptors are responsible for the differences in ouabain sensitivity exhibited between monolayer and agarose assays.

In order to explain these results, we propose the following hypothesis:

(1) The surface area of a cell is greater when extended on

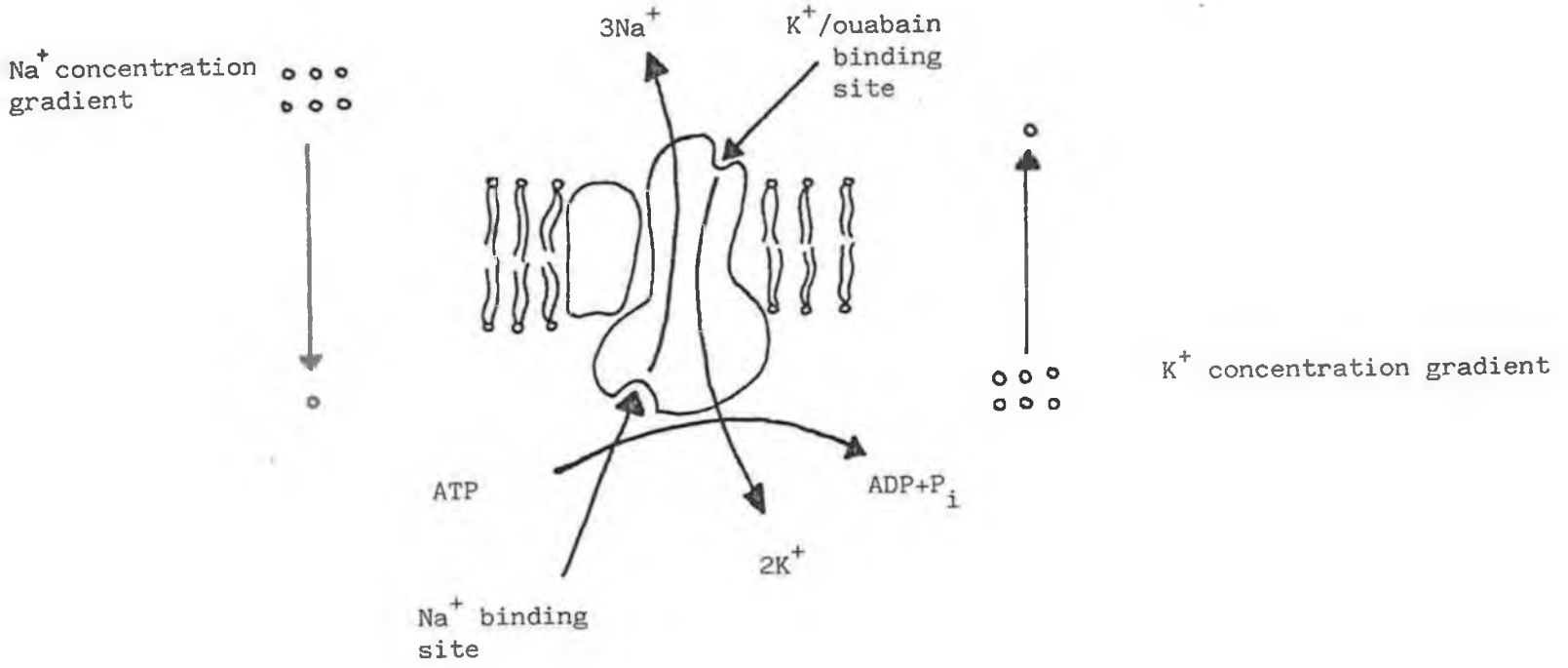


FIGURE 4.3.

SODIUM - POTASSIUM ATPase PUMP

a monolayer surface, although the cell volume remains constant. (A simple geometrical model for this is presented in Table 4.1).

(2) In order to maintain all surface functions, we propose that the cell maintains a constant number of important surface molecules e.g. receptors, $Na^+ - K^+$ - ATPases per unit area, irrespective of total surface area.

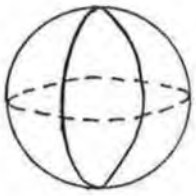
So a constant number of receptors and ATPase exist per unit of membrane area. In both agarose and monolayer the same percentage of ATPase will be inhibited at a given drug concentration. However, a definite number of ATPase receptors e.g. 50,000 must Be active in order to maintain the Na^+ and K^+ levels within the cell (N.B. cell volume is identical in monolayer and agarose).

Since according to our hypothesis cells in agarose have fewer surface receptors (e.g. 100,000) than cells in monolayer (e.g.1,000,000) then 75% binding of a toxin such as ouabain would leave 250,000 receptors free in monolayer and 25,000 in agarose. This would result in cell death in agarose if 50,000 receptors are required for maintanance of the Na^+/K^+ pump, and only 25,000 are available. This hypothesis (i.e. fewer total surface growth factor receptors per cell in agarose suspension) could provide a basis for understanding the nature of anchorage - independent growth: our proposal is that a certain number of receptors per cell need to be stimulated in order to initiate cell growth. At a given growth factor concentration, a certain proportion of receptors will be stimulated, if receptor number per unit area remains constant. This hypothesis is consistent with a number of results presented here e.g. high serum requirement

TABLE 4.1

Cell surface area in agarose and monolayer.

AGAROSE

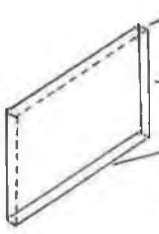


Sphere, radius r

Surface area = $4\ \pi\ r^2$

Volume = $4/3\ \pi\ r^3$

MONOLAYER



Volume = LXBXH

Surface Area = 2(LXB)+2(BXH)
+2(LXH)

Approx. area of exposed surface = (LXB)+(BXH)+(LXH)

Mathematical Model

AGAROSE			MONOLAYER				
Radius (μm)	Surface area (μm^2)	Volume (μm^3)	L (μm)	B (μm)	H (μm)	Exposed S.A. (μm^2)	Volume (μm^3)
6	452.16	904.32	12	12	6.28	294.72	904.32
			20	20	2.26	490.4	904.32
			40	20	1.13	867.8	904.32
			30	30	1.005	960.3	904.32

* Approx. radius of RPMI 2650 cells in agarose.

(3.8) and high cut-off point (3.11.) of RPMI 2650 cells in agarose.

It appears that anchorage-independent growth may be dependent on the number of surface receptors available for the uptake of nutrients and stimulation by growth factors. It is possible that tumour cells grow in agarose because they have more growth factor receptors than normal cells. Alternatively some tumour cells may have an equal number of receptors to normal cells but may have a reduced requirement for a particular growth factor. Altering culture conditions of the assay may allow normal cells to grow in agar but also reduces the specificity of the assay for tumour cells. Altering the culture conditions which are suitable for tumour cells but unsuitable for normal cells may bypass fundamental properties which are characteristic of tumour cells e.g. the ability of tumour cells to propagate in stricter environmental conditions. Since cloning efficiencies are generally higher in monolayer than agarose assays, it is possible that the criteria governing monolayer and agarose growth may also govern normal and tumour cell growth. Future investigations may well consider the differences in cell surface receptors between normal and tumour cells and also the regeneration times of surface receptors after trypsinization.

4.5. CONCLUSION.

A major problem in cancer chemotherapy, and in other kinds of drug treatment, is the large and unpredictable variation in response of different patients to the same drug treatment. By analogy with antibiotic sensitivity tests in bacteria, it should be possible to grow a patient's tumour cells in culture, and by testing response to drug treatment *in vitro* to predict sensitivity or resistance of

the tumour *in vivo*.

Since SaLmon and his colleagues reported on the application of the clonogenic assay in predicting *in vivo* responses to chemotherapy, considerable advances in development, improvement and application of the clonogenic assay have occured. This assay has been applied to preclinical screening of new antineoplastic agents, cytogenetic analysis of human tumour specimens, and to the identification of growth factors and hormones for different tumour types.

Despite these major advances in the application and solving of technical problems associated with the assay, major problems continue to exist, the foremost being the over-all poor growth of most tumour specimens in the assay such that *in vitro* chemosensitivity data can be obtained in only 30 - 40% of specimens. Indeed, because only this fraction grow, it is important to demonstrate that growth itself in the assay is not an independent prognostic factor.

Further, pharmacologic (including metabolic and pharmacokinetic) considerations will have to be investigated for each drug such that *in vitro* studies of drug exposure, and drug/drug interaction will mimic the clinical situation. Constant critical analysis of this, and other assays will no doubt lead to improvements in particular their use as tools for biologic studies. Many problems remain to be solved before this assay can be of routine clinical use, including failure of some tumour cells to grow in soft agar (Bertoncello *et al*, 1982), difficulties in obtaining a viable single-cell suspension from solid tumours, false positive colony formation by yeast contaminants (Clynes and Moriarty, 1982), and tumour cell

heterogeneity including sensitivity differences between the primary tumour and metastic deposits (Schlag and Schreml, 1982). Progress in overcoming these problems is being made, however, and the inherent attractiveness of such systems warrants further research.

The work described in this thesis makes some contribution to the technical improvements needed to make such assays clinically applicable. Presently, insufficient data on prospective trials evaluating *in vitro* assay for prediction of clinical responses is available. These assays may be most useful in identitying which antineoplastic agents not to give as most studies reflect a predictive accuracy of *in vivo* resistance greater than 90%; further randomized studies will clarify this point. Until major improvements in growth rate of tumour specimens occur these randomized trials will be difficult to complete, and therefore, *in vitro* tests of cytotoxicity should at present be considered as research interest and not for widespread clinical application.

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BIBLIOGRAPHY

Agrez, M.V., Kovach, J.S. Lieber, M.M. (1982)

Br. J. Cancer 46: 880 - 887.

"Cell aggregates in the soft agar 'human tumour stem-cell assay'".

Alberts, D.S., Chen, H.S.G., Soehnlen, B., Salmon, S.E., Surwit, E.A., Young, L., Moon, T.E. (1980)

Lancet 2: 340-342.

"In vitro clonogenic assay for predicting response of ovarian cancer to chemotherapy".

Alley, M.C., Uhl, C.B., Lieber, M.M. (1982)

Life Sciences 31: 3071-3078.

"Improved detection of drug cytotoxicity in the soft agar colony formation assay through use of a metabolizable tetrazolium salt."

Asano, S., Mandel, T.E. (1981)

JNCI 67: 25-32.

"Colonies formed in agar from Human Breast Cancer and their identification as T-Lymphocytes".

Barnes, D., Sato, G. (1980)

Analyt. Biochem 102:255-270.

"Methods for the growth of cultured cells in serum-free medium."

Barnes, D., Van der Bosch, L., Masui, H., Miyazaki, K, Sato, G. (1981)

Methods Enzymol: 79: 369-399.

"The culture of human tumour cells in serum free medium."

Bertoncello, I., Bradley, T.R., Campbell, J.J., McDonald, I.A.McLeish, G.R., Quinn, M.A., Rome, R., Hodgson, G.S. (1982).

Br. J. Cancer 45: 803-811.

"Limitations of the clonal agar assay for the assessment of primary human ovarian tumour biopsies".

Bettger, W.J., Boyce, S.T., Walthall, B.J., Ham, R.G. (1981)

Proc. Natl. Acad. Sci., U.S.A. 78: 5588-5592.

"Rapid clonal growth and serial passage of human diploid fibroblasts in a lipid-enriched synthetic medium supplemented with epidermal growth factor, insulin, and dexamethasone".

Borek, C. (1980)

Nature (Lond.) 283: 776-778

"X-ray-induced in vitro neoplastic transformation of human diploid cells."

Born, R., Hug, O., Trott, K.R. (1976)

Int. J. Radiation Oncol. Biol. Phys., 1 : 687-697.

"The effect of prolonged hypoxia on growth and viability of chinese hamster cells."

Buick, R.N., Fry, S.E., Salmon, S.E. (1980)

Cancer 45: 1238-1242.

"Application of in vitro soft agar techniques for growth of tumour cells to the study of colon cancer."

Buick, R.N., Mackillop, W.J. (1981)

Br. J. Cancer 44: 349-355.

"Measurement of self-renewal in culture of clonogenic cells from human ovarian carcinoma."

Calvo, F., Carney, D.N., Brower, J.H., Minna, J.D. (1983)

Br. J. Cancer 48: 683-688.

"Hormone supplemented media for cloning breast cancer: increased colony formation without alteration of chemosensitivity."

Carney, D.N., Winkler, C.F. (Personnel Communication).

"In vitro assays of chemotherapeutic sensitivity."

Carney, D.N. (1981)

Personal Communication.

"The primary cloning and selective in vitro growth of small cell lung cancer."

Carney, D.N., Gazdar, A.F., Minna, J.D., (1980)

Cancer Research 40: 1820-1923.

"Positive correlation between histological tumour involvement and generation of tumour cell colonies in agarose in specimens taken directly from patients with small-cell carcinoma of the lung."

Carney, D.N., Bunn, P.A., Gazdar, A.F., Pagan, J.A., Minna, J.D. (1981)

Proc. Natl. Acad. Sci. U.S.A. 78: 3185-3189.

"Selective growth in serum-free hormone supplemented medium of tumour cells obtained by Biopsy from patients with small cell carcinoma of the lung."

Carney, D.N., Gazdar, A.F., Bunn, P.A.Jr., Minna, J.D. (1981)

Small cell lung cancer : 79-94.

Ed: Oldham, Burn.

"In vitro cloning of small cell carcinoma of the lung."

Cassiman, J.J., Bernfield, M.R. (1975)

Exp. Cell Res. 91: 31-35.

Chen, T.R. (1977)

Exp. cell res. 104: 255.

"In situ detection of mycoplasm contamination in cell cultures by fluorescent HOechst 33258 stain."

Clynes, M.M., Smyth, H., O'Kennedy, R., Corrigan, A. (1980)

"Separation of viable from nonviable cells using a discontinuous density gradient."

Clynes, M.M. (1981) Irish Association for Cancer research (abstracts).
"Comparison of the activity of some proteolytic enzymes in disaggregation of cell monolayers and of tumours."

Clynes, M.M., Moriarty, M.J. (1982)
Ir. J. Med. Sci. 151: 132.
"Yeast contamination as a source of false positive results in clonogenic tumour stem cell assays."

Colburn,N.H.,Bruegge,W.F.U.,Bates,J.R.,Gray,R.H.,Rossen,J.D.,Kelsey,W.H., Shimada,T. (1978)
Cancer research 38: 624-634.
"Correlation of anchorage independent growth with tumourigenicity of clinically transformed mouse epidermal cells."

Courtenay, V.D. (1976)
Br. J. Cancer 34: 39.
"A soft agar colony assay for Lewis lung tumour and B16 melanoma taken directly from the mouse."

Courtenay, V.D., Smith, I.E., Peckham, M. J., Steel, G.G. (1976)
Nature (Lond.), 263: 771-772.
"In vitro and in vivo radiosensitivity of human tumour cells obtained from a pancreatic xenograft."

Courtenay, V.D., Selby, P.J., Smith, I.E., Mills, J., Peckham, M.J.(1977)
Brit. J. Cancer, 38: 77-81.
"Growth of human tumour cell colonies from biopsies using two soft-agar techniques."

Courtenay, V.D. Mills, J. (1978)
Br. J. Cancer 37: 264-268.
"An in vitro colony assay for Human tumours grown in immune-suppressed mice and tested in vivo with cytotoxic agents.

Courtenay, V.D. (1983).

"Primary cultures in tumours."

In: "Human tumour drug sensitivity testing in vitro".

Eds Dendy and Hill: p 103. Academic Press, London.

Cowan, D.H. (1982)

Stem cells I ; 288-289.

"Stimulation of human tumour colony formation by platelet derived growth factors."

Cowan, J.D., Von Hoff, D.D. (1982)

In cancer chemotherapy, Muggia, F.M. (ed) p. 103. Niyhoff: Boston.

"The human tumour cloning assay: an in vitro assay for anti-tumour activity in solid tumours."

De Larco, J.E., Todaro, G.T. (1978)

Proc. Natl. Acad. Sci. U.S.A. 75 : 4001-4005.

"Growth factors from murine sarcoma virus-transformed cells."

Dexter, T.M. (1984)

Nature, 309 : 746-747.

"The message in the medium."

Dixon, R.A., Linch, D., Baines, P., Rosendaal, M. (1981)

Exp. cell res. 131 : 478-450.

"Autoclaved agar contains an Inhibitor of granulocyte-macrophage colony growth in vitro".

Dodson

Int. J. Cancer 32: 211 - 217.

"Fibronectin and anchorage-independent and anchorage dependent growth of benign and malignant cell-lines".

Doolittle, R.F., Hunkapiller, M.W., Hood, L.E., Devare, S.G., Robbins, K.C., Aaronson, S.A., Antoniades, H.N. (1983)
Science (Wash.D.C.) 221: 275–276.
"Simian sarcoma virus onc gene, v-sis, is derived from the gene (or genes) encoding a platelet derived growth factor".

Dow
Cancer research 42: 5662–5264.
"Clonogenic assay for Wilm's tumour: Improved technique for obtaining single cell suspensions and evidence for tumour cell specificity."

Downward, J., Yarden, Y., Mayes, E., Scrace, G., Totty, N.,Stockwell, P., Ullrich, A., Schlessinger, J., Waterfield, M.D. (1984).
Nature (Lond.) 307: 521–527.
"Close similarity of epidermal growth factor receptor and v erb B oncogene protein sequences."

Durand, R.E. (1981)
Cancer res. 41: 3495-3498.
"Flow cytometry studies of intracellular adriamycin in multicell spheroids in vitro".

Easty, G.E., Easty, D.M., Ambrose, E.J. (1960)
Exp. Cell Res. 19: 539-548.
"Studies of cellular adhesiveness."

Endresen, L., Treit, K.M., Rugstad, H.E., Pihl, R. (1985)
Br. J. Cancer 51: 843-852.
"Chemosensitivity measurements of human tumour cells by soft agar assays are influenced by the culture conditions."

Engleholm, S.A., Spang Thomsen, M., Brunner, N., Nohr, I., Vindelou, L.L. (1985)
Br. J. Cancer 51: 93–98
"Disaggregation of human solid tumours by combined mechanical and enzymatic methods."

Fan, D., Morgan, L.R., Schneider, C., Blank, H., Fan, S. (1984)

Cancer res. clin. oncol. 109: 23-28.

"Co-operative evaluation of human tumour chemosensitivity in the soft-agar assay and its clinical correlations."

Freedman, V.H., Shin, S. (1974)

Cell 3: 355-399.

"Cellular tumorigenicity in nude mice: correlation with cell growth in semi-solid medium."

Freshney, R.I. Hart, E. (1982)

Br. J. Cancer 46: 463.

"Clonogenicity of human glia in suspension."

Freyer, J.P., Sutherland, R.M. (1980)

Cancer research 40 : 3956-3965.

"Selective dissociation and characterisation of cells from different regions of multicell tumour spheroids."

Fryling, C.M., IWata, K.K., Johnson, P.A., Knott, W.B., Todaro, G.I. (1985)

Cancer research 45: 2695-2699.

"Two distinct tumour cell growth-inhibiting factors from a human rhabdomyosarcoma cell line."

Gallimore, P.H., McDougall, J.K., Chen, L.B. (1977)

Cell 10: 669 678.

Gazdar, A.F., Carney, D.N., Minna, D. (1983).

Semin Oncol 1 : 3-19.

"The biology of non-small cell lung cancer."

Grabstein, G., Cohen, J. (1965)

Science 150: 626.

"Collagenase: Effect on the morphogenesis of embryonic salivary epithelium in vitro".

Gupta, V., Eberle, R. (1984)

Br. J. Cancer 49: 587.

"Modulation of tumour cell colony growth in soft agar by oxygen and its mechanism".

Gupta, V., Krishan, A. (1982).

Cancer research 42: 1005-1007.

"Effects of oxygen concentration on the growth and drug sensitivity of human melanoma cells in soft-agar clonogenic assay."

Gupta, V., A., Zubrod, C.G. (1982)

Cancer research 43: 2560-2564.

"Correlation of in vitro clonogenic assay data with in vivo growth delays and cell cycle changes of a human melanoma xenograft."

Halton, D.M., Peterson, W.D., Bharati, H. (1983)

In vitro 19 : 16-24.

"Cell culture quality control by rapid isoenzymatic characterisation."

Ham, R.G. Mckeehan, W.L. (1979).

In Jakoby, W.B., Pastan, I.H. (eds) : "Methods in enzymology, Volume LVIII, cell culture. "Academic press, New York, pp. 44-93.

"Media and growth requirements."

Hamburger, A., Salmon, S.E. (1977)

J. Clin Invest. 60: 846 - 854.

"Primary bioassay of human myeloma stem cells.'

Hamburger, A.W., Salmon, S.E. (1977)

Science 197: 461-463.

"Primary bioassay of human Tumour stem cells."

Hamburger, A.W., Kim, M.B., Salmon, S.E. (1979)

J.Cell Physiol 98: 371-377.

"The nature of cells generating human myeloma colonies in vitro."

Hamburger, A.W. (1981)

JNCI 66: 981-988.

"Use of in vitro tests in predictive cancer chemotherapy."

Hamburger, A.W., White, C.P. (1981)

Stem cells 1 : 209-223.

"Interaction between Macrophages and human tumour clonogenic cells."

Hamburger, A.W., White, C.P., Brown, B.W. (1981)

JNCI 67: 825-830.

"Effect of epidermal growth factor on proliferation of human tumour cells in soft agar."

Hamburger, A.W., White, C.P., Tencer, K. (1981).

J. Nat 'l Cancer Inst. 68 (6) : 945-948.

"Effect of enzymatic disaggregation on proliferation of human tumour cells in soft agar."

Hamburger, A.W., White, C.P., Dunn, F.E. (1983)

Br. J. Cancer 48: 675-682.

"Modulation of tumour colony development over time in soft-agar culture."

Hamburger, A.W., Dunn, F.E., Tencer, K.L. (1983)

JNCI 70: 157-160.

"Separation on Percoll density gradients of cells derived from malignant ascites of mice."

Harel, L., Chatelain, G., Golde, A. (1984)

J. Cellular-physiology 119: 101-106.

"Density - dependent inhibition of growth: Inhibitory diffusible factors from 3T3 - and Rous Sarcoma virus (RSV) - transformed 3T3 cells."

Hays, E.F., Bessho, M., Kitada, S. (1985)

In vitro 21: 266-270.

"Conditions affecting clonal growth of lymphoma cells in a semi-solid matrix."

Heppner, G. (1984)

Cancer research 44: 2259 2265.

"Tumour heterogeneity."

Hill, B.T., Whelan, R.D.H. (1983)

Cell Biol. Int. Rep. 7: 617.

"Attempts to optimize colony-forming efficiencies using three different survival assays and a range of human tumour continuous cell lines."

Hodges, G.M., Livingston, D.C., Franks, L.M. (1973)

J. Cell Sci. 12: 887.

"The localisation of trypsin in cultured mammalian cells."

Holtfreter, J. (1931)

Arch. EntwGesch. 124: 404

"Uber die Aufzucht isolierter Teile des Amphibienkeimes."

Horowitz, A.L., Dorfman, A. (1970)

J. Cell Biol. 45: 434-438.

"The growth of cartilage cells in soft agar and liquid suspension."

Hug, V., Spitzer, G., Drewinko, B., Blumenschein, G.R. (1983)

Cancer research 43: 210-213.

"Effect of Diethylaminoethyl - dextran on colony formation of human tumour cells in semisolid suspension cultures."

Hynes, R.O. (1974)

Cell 1: 147-156.

"Role of cell surface alterations in cell transformation. The importance of proteases and cell surface proteins."

Hynes R.O. (1976) Biochim. Biophys. Acta. 458: 73-107.
"Cell surface proteins and malignant transformation."

Inoue S., Ottenbreit., M.J. (1978)

Blood 51: 195-205.

Heterogeneity of human colony-forming cells."

Iwata, K.K., Fryling, C.M., Knott, W.B., Todaro, G.J. (1985)

Cancer research 45: 2689-2694.

"Isolation of tumour cell growth-inhibiting factors from a human rhabdomyosarcoma cell line."

Joyce, R.M., Vincent, P.C. (1983)

Br. J. Cancer 48: 385.

"Advantage of reduced oxygen tension in growth of human melanomas in semi-solid cultures: Quantitative analysis."

Kennedy, K.A., Siegfried, J.M., Sartorelli, A.C., Tritton, T.B.(1983)

Cancer research 43: 54-59.

"Effects of anthracyclines on oxygenated and hypoxic tumour cells."

Kern, D.H., Campbell, M.A., Cochran, A., Burk, M.W., Morton, D.L. (1982)

Int. J. Cancer 30: 725 - 729.

"Cloning of human solid tumours in soft agar."

Kirkels, W.J., Pelgrim, O.E., Debruyne, F.M.J., Vooijs, G.P., Herman, C.J. (1982). Am, J. Clin. Pathol. 78: 690-694.

"Soft agar culture of human transitional cell carcinoma colonies from urine."

Kirkels, F.M.J., Pelgrim, O.E., Hoogenboom, A.M.M., Aalders, M.W., Debruyne, M.S., Vooijs, G.P., Herman, C.J. (1983).

Int. J. Cancer 32: 399-406.

"Patterns of tumour colony development over time in soft agar culture.

Kitten, C.M., Von Hoff, D.D., Bennett, Jr., E.V., Grover, F.L. (1982)
J. Thorc. cardiovasc. Surg. 83: 363-371.
"Growth of lung cancer in a human tumour clonogenic system."

Kruse, P., Patterson, M.K. (1973)
"Tissue culture, techniques and applications".
New York, Academic Press.

La Rocca, P.J., Rheinwald, J.G. (1985)
In vitro 21: 67-72.
"Anchorage-independent growth of normal human mesothelial cells: A sensitive bioassay for EGF which discloses the absence of this factor in fetal calf serum."

Laug, W.E., Tokes, Z.A., Benedict, W.F., Sorgente, N. (1980)
J. Cell Biology 84: 281-293.
"Anchorage-independent growth and plasminogen activator production by bovine endothelial cells."

Llyod, K.O., Travossos, L.R., Takahashi, T., Old, L.J. (1979)
J. Natl. Cancer Inst. 63: 623.
"Cell surface glycoproteins of human tumour cell lines: Unusual characteristics of malignant melanoma."

Mate, T.P., Rockwell, S., Fischer, J.J. (1984)
Int. J. Radiat, Oncol., 19: 791-797.
"Improved radiosensitivity in rodent tumours treated with an oxygenated perfluorochemical emulsion."

MacPherson, I. (1978).
Tissue culture-methods and applications.
In Kruse, P.F., Patterson, M.K. (eds): New York, Academic Press, pp. 276-280.
"Soft agar techniques."

MacPherson, I., Montagnier, L. (1964)
Virology 23: 291-294.
"Agar suspension culture for the selective assay of cells transformed by polyoma virus."

Marshall, C.J. (1979)

J. Cell Sci. 39: 319 327

Matsumura, T., Yamanaka, T., Hashizume, S., Irie, Y., Nitta, K. (1975)

Japan. J. exp. med. 45: 377-382.

"Tissue dispersion, cell harvest and fluid suspension culture by the use of bacterial neutral protease."

Mavligit, G.M., Gutterman, J.U., Hersh, E.M. (1983)

Immunological communications 2(s): 463-472.

"Separation of viable from non-viable tumour cells using Ficoll-density solution."

Mckintosh, F.R., Evans, T.L., Sikic, B.I. (1981)

Cancer Chemother. Pharmacol. 6: 205.

"Methodologic problems in clonogenic assays of spontaneous tumours."

McManus, V., Clynes, M. (1984)

Biochem. SOc. Trans.,

"Stimulation by feeder layers of human tumour cell colony formation in agar."

Meyskens, Jr., F.L., Soehnlen, B.J., Saxe, D.E., Casey, W.J., Salmon, S.E. (1981)

Stem cells 1: 61-62.

"In vitro clonal assay for human metastatic melanoma cells."

Meyskens, Jr., F.L., Moon, T.E., Dana, B., Gilmartin, E., Casey, W.J., Chen, H.S.G., Franks, D.H., Young, L., Salmon, S.E. (1981)

Br. J. Cancer 44: 787-797.

"Quantitation of drug sensitivity by human metastatic melanoma colony-forming units."

Meyskens, F.L., Thomson, S.P., Moon T.E. (1983)

Br. J. Cancer 48: 863-868.

"Potential biological explanation of stimulation of colony growth in semi-solid agar by cytotoxic agents."

Moore, G.E., Sandberg, A.A. (1964)

Cancer 17: 170.

Montagnier, L. (1968)

C.R. Acad. Sci., D. 267: 921-924.

"Corrélation entre la transformation des cellule BHk. 21 et leur résistance aux polysaccharised acides au milieu gélifié."

Montesano, R., Drevon, C., Kuroki, T., Vincient, L.S., Handleman, S., Sandford, K.K., Defeo, D., Weinstein, I.B. (1977).

J. Nat. Cancer Inst. 59: 1651-1657.

"Test for malignant transformation of rat living cells in culture. Cytology, growth in soft-agar, and production of plasminogen activator."

Moscona, A.A. (1952)

Exp. cell res. 3: 535.

"Cell suspension from organ rudiments of chick embryos."

Neugut, A.I., Weinstein, I.B. (1979)

In vitro 15: 351.

"Use of agarose in the determination of anchorage-independent growth."

Nicolson, G.L. (1976)

Biochim Biophys. Acta. 458: 1-72.

"Trans-membrane control of the receptors on normal and tumour cells. II. Surface changes associated with transformation and malignancy."

Niell, H.B., Soloway, M.S., Wood, C.A., McCallum, L.W., Webster, K.(1983)

Cancer 52: 619-625.

"The use of a tumour colony forming assay in predicting chemotherapeutic drug response in murine bladder cancer."

O'Neill, C.H., Riddle, P.N., Jordan, P.W. (1979).
Cell 16, 909-918.
"The relation between surface area and anchorage dependence of growth in hamster and mouse fibroblasts."

Ozanne, B., Fulton, R.J., Kaplan, P.L. (1980)
J. cell physiol. 105: 163-180.
"Kirsten murine sarcoma virus transformed cell lines and a spontaneously transformed rat cell line produce transforming factors."

Pathak, M.A., Matrisian, L.M., Magun, B.E., Salmon, S.E. (1982)
Int. J. Cancer 30: 745-750.
"Effect of epidermal growth factor on clonogenic growth of primary human tumour cells."

Pavelic Z.P., Slocum, H.K., Rustum, Y.M., Creaven, P.J., Karakousis,C., Takita, H. (1980).
Cancer research 40: 2160-2164.
"Colony growth in soft agar of human melanoma, sarcoma, and lung carcinoma cells disaggregated by mechanical and enzymatic methods."

(a) Pavlik, E.J. Kenady, D.E., Van Nagell, J.R., Keaton, K., Donaldson, E.S., Hanson, M.B., Flanigan, R.C. (1983)
In vitro 19: 538-545.
"The proliferation of human tumour cell lines in the presence of different agars, agaroses, and methylcellulose."

(b) Pavlik, E.J., Flanigan, R.C., Van Nagell, J.R., Swanson, L., Keaton, K., Kenady, D.E. (1983).
Journal of Urology 129 : 1254-1257.
"The effect of different agars, agaroses and methylcellulose on the in vitro proliferation of a human urinary transitional cell carcinoma cell line."

Peehl, D.M., Stanbridge, E.J. (1981)
Proc. Natl. Acad. Sci. 78, No. 5: 3053-3057.
"Anchorage independent growth of normal human fibroblasts."

Pretlow, T.P., Glover, G.L., Pretlow, T.G. (1977)

Cancer res. 37: 578.

"Separation of lymphocytes and mast cells from the furth transplantable most cell tumour in as isokinetic gradient of ficoll in tissue culture medium.

Rasey, J.S., Nelson, N.J. (1980)

Br. J. Cancer 41: 217.

"Response of an in vivo - in vitro tumour to X-rays and cytotoxic drugs: effect of tumour disaggregation methods on cell survival."

Reddy, J.K., Rao, M.S., Warren, J.R., Minnick, O.T. (1979)

Exp. cell Res. 120: 55-61.

"Concanavalin A agglutinability and surface microvilli of dissociated normal and neoplastic pancreatic acinar cells of the rat."

Richter, A., Sandford, K.K., Evans, V.J. (1972).

J. Natl. Cancer Inst. 49: 1705.

"Influence of oxygen and culture media on plating efficiency of some mammalian tissue cells."

Richter, A. (1973)

"Low oxygen tension technique."

In: Tissue culture - methods and applications.

Ed. Kruse, P.F., Patterson, M.K. Academic Press.

Roberts, A.B., Frolik, C.A., Anzano, M.A., Sporn, M.B. (1983)

Fed. Proc., 42: 2621-2626.

"Transforming growth factors from neoplastic and non-neoplastic tissues."

Rockwell, S. (1985)

Cancer res. 45: 1602-1607.

"Effects of clumps and clusters on survival measurements with clonogenic assays."

Roper, P.R., Drewinko, B. (1976).

Cancer res. 36: 2132-2188.

"Comparison of in vitro methods to determine drug-induced cell lethality."

Rosenblum, M.L., Kasquez, D.A. Hoshino, T., Wilson, C.B. (1978)
Cancer 41: 2305-2314.
"Development of a clonogenic cell assay for human brain tumours."

Rosenblum, M.L., Dougherty, D.V., Barger, G.R., Levin, V.A. Gerosa, M., Reese, C., Davis, R.L. Wilson, C.B. (1982)
Lancet 885-887.
"Age-related chemosensitivity of stem cells from human malignant Brain tumours."

Rupniak, H.T., Dennis L.Y., Hill, B.T. (1983)
Tumori 69: 37-42.
"An intercomparison of in vitro assay for assessing cytotoxicity after a 24hour exposure to anti-cancer drugs.

Russell, S.W., Doe, W.F., Hoskins, R.G., Cochrane, C.G. (1976).
Int. J. Cancer 18: 322.
"Inflammatory cells in solid murine neoplasms. I. Tumour disaggregation and identification of constituent inflammary cells."

Salmon, S.E., Hamburger, A.W., Soehnlen, B., Durie, B.G.M., Alberts, D.S. (1978)
N.E.J.M. 298: 1321-1327.
"Quantitation of differential sensitivity of human tumour stem cells to Anticancer drugs."

Salmon, S.E., Liu, R. (1979)
Br. J. Cancer 39: 779-781.
"Direct wet staining of tumour or hematopoetic colonies in agar culture."

Salmon, S.E. (1980).

In cloning of human tumour stem cells, Salmon, S.E. (ed) p-291
Alan R. Liss : New York.

"Application of the human tumour stem cell assay to new drug evaluation and screening."

Salmon, S.E., Von Hoff, D.D. (1981).

Semin. Oncol. VIII: 377-385.

"In vitro evaluation of anticancer drugs with the human tumour stem cell assay."

Salmon, S.E. (1984)

Cancer treat. Rep. Vol.

"The human tumour cloning assay and chemosensitivity testing."

Schlag, P., Schreml, W. (1982)

Cancer research 42: 4086-4089.

Heterogeneity in growth pattern and drug sensitivity of primary tumour and metastases in the human tumour colony forming assay."

Selby, P., Buick, R.N., Tannock, I. (1983)

N.E.J.M. 308: 129-134.

"A critical appraisal of the human tumour stem cell assay."

Shin, S.I., Freedman, V.H., Risser, R; Pollack, R. (1975)

Proc. Natl. Acad. Sci. U.S.A. 72: 4435-4439.

"Tumourigenicity of virus-transformed cells in nude mice is correlated specifically with anchorage-independent growth in vitro."

Sikic, B.I., Taber, R.L. (1981)

Cancer chemother. Pharmacol. 6: 201-203.

"Human tumour clonogenic assays."

Silinskas, K.C., Kateley, S.A., Tower, J.E., Maher, V.M.
McCormick, J.J. (1981)

Cancer res. 41: 1620-1627.

"Induction of anchorage-independent growth in human fibroblasts by propane sultone."

Simms, E., Gazdar, A.F. Abrams, P.G., Minna, J.D., (1980)
Cancer res. 40: 4356 4364.
"Growth of human small cell (oat cell) carcinoma of the lung in serum free growth factor supplemented medium."

Slocum, H.K., Pavelic, Z.P., Rustum, Y.M., Creaven, P.J., Karakousis, C., Takita, H., Greco, W.R. (1981)
Cancer res. 41: 1428 1434.
"Characterisation of cells obtained by mechanical and enzymatic means from human melanoma, sarcoma, and lung tumours."

Sporn, M.B., Todaro, G.J. (1980)
N. Engl. J. Med. 303: 878 880.
"Autocrine secretion and malignant transformation."

Sridhar, K.S., Plasse, T.F., Holland, J.F., Shapiro, M., Ohnuma, T. (1983)
Cancer research 43: 4629 4631.
"Effects of physiological oxygen concentration on human tumour colony growth in soft agar."

Stanbridge, E.J., Wilkinson, J. (1978)
Proc. Natl. Acad. Sci. USA 75: 1466 1469
"Analysis of malignancy in human cells: malignant and transformed phenotypes are under separate genetic control."

Steck, P.A., Voss, P.G., Wang, J.L. (1979)
J. Cell Biol. 83: 562 575.
"Growth control in cultured 3T3 fibroblasts. Assays of cell proliferation and demonstration of a growth inhibitory activity."

Steck, P.A., Blenis, J., Voss, P.G., Wang, J.L. (1982).
J. Cell Biology 92: 523 575.
"Growth control in cultured 3T3 fibroblasts. Molecular properties of a fraction enriched in growth inhibitory activity."

Steiner, T., McGarrity, G. (1983).

In vitro 19: 672.

"Mycoplasmal infection of insect cell cultures."

Styles, J.A. (1977)

Br. J. Cancer 36: 558.

"A method for detecting carcinogenic organic chemicals using mammalian cells in culture."

Sutherland, B.M., Cimino, J.S., Shih, A.G., Oliver, R.P. (1980)

Cancer res. 40: 1934-1939.

"Ultraviolet light-induced transformation of human cells to anchorage-independent growth."

Sutherland, C.M., Mather, F.J., Carter, R.D., Cerise, E.J., Krementz, E.T. (1983)

Surgery 94: 370-375.

"Breast cancer as analyzed by the human tumour stem-cell assay."

Swart, K., Lowenberg, B. (1984)

Cancer research 44: 657-660.

"Feeder cell requirements for leukemia cell colony formation in cultures supplemented with phytohemagglutinin

Taylor I.W., Bleehen, N.M. (1978)

Br. J. Cancer, 38: 143-147.

"Rozoxane - induced polyploidy."

Taylor, W.G., Camelier, R.F., Sandford, K.F. (1978)

J. Cell Physiol. 95: 33-40.

"Density - dependent effects of oxygen on the growth of mammalian fibroblasts in culture."

Taketazu, F., Kubota, K., Kajigaya, S., Shionoya, S., Motoyoshi, K., Saito,M.,Takaku,F.,Miura,Y. (1984).
Cancer research 44: 531-535.
"Clonal growth of human acute myeloid leukemia cells (ML-1 and HL-60) in serum-free agar medium."

Thomas, D.G.T., Darling, J.L., Morgen, D., Freshney, Hart, E., Celin, F. (1982)
(Abstract) Stem Cells 1: 291.
"Chemosensitivity of human Glioma cultures measured by Isotope Uptake Correlates with Cloning and Clinical response."

Thomlinson, R.H., Gray, L.H. (1955)
Br. J. Cancer 9: 539 - 549.
"The histological structure of some lung cancers and the possible implication for radiotherapy."

Thomson, S.P., Meyskens, F.L. Jr. (1982)
Cancer research 42: 4606-4613.
"Method for measurement of self-renewal capacity of clonogenic cells from biopsies of metastatic human malignant melanoma."

Tilgen, W., Boukamp, P., Breitkreutz., D., Dzarlieva, R.T., Engstner, M., Haag, D., Fusenig, N.E. (1983)
Cancer research 43: 5995-6011.
"Preservation of morphological, functional, and karyotypic traits during long-term culture and in vivo passage of two human skin squamous cell carcinomas."

Todaro, G.J., DeLarco, J.E. (1978)
Cancer res. 38: 4147 - 4154.
"Growth factors produced by sarcoma virus-transformed cells".

Todaro, G.J., DeLarco, J.E., Fryling, C., Johnson, P.A., Sporn, M.B. (1981)
J. Supramolecular structure cellular Bio. 15: 287 - 301.
"Transforming growth factors (TGFs) : Properties and possible mechanisms of action."

(a) Tveit, K.M., Endresen, L., Rugstad, H.E., Fodstad, O., Pihl, A. (1981)
Br. J. Cancer 44: 539 - 544.
"Comparison of two soft-agar methods for assaying chemosensitivity of human tumours in vitro: Malignant melanomas."

(b) Tveit, K.M., Fodstad O., Pihl, A. (1981)
Int. J. Cancer 28: 329.
"Cultivation of human melanomas in soft agar. Factors influencing plating efficiency and chemosensitivity."

Tveit, K.M., Fodstad, O., Lotsberg, J., Vaagem S., Pihl, A. (1982)
Int. J. Cancer 29: 533 - 538.
"Colony growth and chemosensitivity in vitro of human melanoma biopsies. Relationship to clinical parameters."

Tucker, R.F., Shipley, G.D., Moore, H.L. (1984)
Science (Wash. D.C.) 226: 705 - 707.
"Growth inhibitor from BSC - 1 cells closely related to platelet type β transforming growth factor."

Uitendaal, M.P., Hubers, H.A.J.M., McVie, J.E., Pinedo,H.E. (1983)
Br. J. Cancer 48: 55 - 59.
"Human tumour clonogenicity in agar is improved by cell-free ascites "

Vaage, J., Agarwal, S. (1979)
Cancer res. 39: 1511.
"Endogenous tumour growth factor indicated by increased ornithine decarboxylase activity in malignant cells treated with host serum or ascites fluid."

Van Beek, W.P., Glimelius, B., Nilson, K., Emmelot, P. (1978)
Cancer Lett: 5: 311 - 317.
"Changed cell surface glycoproteins in human glioma and osteosarcoma cells."

Von Hoff, D.D., Casper, J., Bradley, E., Trent, J.M., Hodach, A., Reichert, C., Makuch, R., Allman, A., (1980).
Cancer research 40: 3591 - 3597.
"Direct cloning of human neuroblastoma cells in soft agar culture."

Von Hoff, D.D., Casper, J., Bradley, E., Sandbach, J., Jones, D., Makuch, R., (1981).
Am. J. Med. 70: 1027 - 1032.
"Association between human tumour colony forming assay results and response of an individual patient's tumour to chemotherapy."

Von Hoff, D.D., Chen, H.S.G. Salmon, S.E., Surwit, E.A., Young, L., Moon, T.E., Meyskens, Hr., F.L. (1982).
Am. J. Med. 70: 1027 - 1032.
"Association between human tumour colony-forming assay results and response of an individual patient's tumour to chemotherapy."

Von Hoff, D.D. (1983)
N.E.J.M. 308 : 154.
"Send this patient's tumour for culture and sensitivity."

Walls, G.A., Twentyman, P.R. (1985).
Br. J. Cancer 52; 505 - 513.
"Cloning of human lung cancer cells."

Warren, L. Buck, C.A., Tuszynski, G.P. (1978)
Biochim. Biophys. Acta. 516 : 97.
Glycopeptide changes and malignant transformation. A possible role for carbohydrate in malignant behaviour."

Waterfield, M.D., Scrace, G.T., Whittle, N., Stroobant, P., Johnsson, A., Wasteson, A., Westermark, B., Heldin, C., Huang, J.S., Deidel, T.F.(1983).
Nature (Lond.) 304: 35—39.
"Platelet derived growth factor is structurally related to the putative transforming portein p28 sis of simian sarcoma virus."

Wells, V., Mallucci, L. (19 83)

J. Cellular Physiology

"Properties of a cell growth inhibitor produced by mouse embryo fibroblasts."

Whelan, R.D.H.,Hill, B.T. (1981)

Cell Biol. Int. Reports 5: 1137 - 1142.

"The influence of agarose concentration on the cloning efficiency of a series of established human cell lines."

Williams, T.J.,Lieber, M.M., Podratz, K.C., Malkasian, G.D. (1983).

Am. J. Obstet. Gynecol. 145 : 940-947.

"Soft agar colony formation assay for in vitro testing of sensitivity to chemotherapy of gynecologic malignancies."

Willingham, M.C., Pastan, I. (1975)

Proc. Natl.Acad. Sci. U.S.A. 72: 1263 - 1267.

"Cyclic AMP modulates microvillus formation and agglutinability in transformed and normal mouse fibroblasts."

Woodruff, M.F.A. (1982).

Br. J. Cancer 47: 589 - 594.

"Cellular heterogeneity in tumours."

Wright, T.C., Ukena, T.E., Campbell, R., Karnovsky, M.J. (1977)

Proc.Natl. Acad. Sci. U.S.A. 74: 258 - 262.

"Rates of aggregation, loss of anchorage dependence, and tumourigenicity of cultured cells."

Zimmerman, R.J., Little J.B. (1983).

Cancer Research 43: 2176 - 2182.

"Characterisation of a quantitative assay for the in vitro transformation of normal diploid fibroblasts to anchorage independence by chemical carcinogens."

APPENDIX