

*CYTOTOXICITY TESTING IN VITRO:
INVESTIGATION OF 5 MINIATURIZED,
COLORIMETRIC ASSAYS.*

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I declare that the work described in this thesis is entirely my own work, and has not been previously submitted for a degree at this or any other University.

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FOR MY MOTHER...

*Here's to:
".....the way forward...."*

ABSTRACT

Recent years have seen the development of miniaturized, colorimetric assays for quantification of cell growth, and their use in toxicity testing has grown. These methods offer significant advantages (speed, ease of performance, sensitivity and better use of resources) over previously used methods such as the Stem Cell Assay or determination of DNA, RNA or protein synthesis. At present many such assays are used in many different areas of toxicity testing. This thesis undertook to compare 5 such assays in detail, and assess their performance in terms of linearity, sensitivity, precision, reproducibility and ease of performance. An Acid Phosphatase (AP) assay, not previously applied to toxicity testing, was developed and validated against some commonly used assays (Neutral Red (NR), MTT, and staining with Crystal Violet and Sulforhodamine B), where it was found to offer the best combination of sensitivity, ease of performance, reproducibility and versatility. It was later applied to quantification of growth in serum batch testing, and in determination of cytotoxicity in screening for new drugs, assessment of Multiple Drug Resistance, and screening for toxicity in samples of environmental relevance, including industrial sludges and wastewaters. While results from the ecotoxicity tests were found to correlate with invertebrate tests, difficulties with sample preparation and absence of biotransformation ability were identified.

The MTT assay, exhaustively used in the literature, was found to be least favourable of all the methods compared in this work, due to its low sensitivity and technical difficulties. It was established that problems with reproducibility of these assays do exist, identifying the need to consider IC_{50} values as guide values only, and not as absolute values.

Activity in each of these assays was found to vary when cells were grown under different conditions, (AP levels per cell increased in starved cultures). The NR assay however, was less prone to variation in activity than any of the other methods. Investigation into differences in assay response to chemicals with mechanisms related to their own mechanism of action showed that after 6 days' exposure to a range of relevant chemicals, no bias in assay ability to detect toxicity was evident. Conditions where each of these assays underestimated cell death were identified; after 1 or 2 days exposure

to low concentrations of Vinblastine, the methods (and also trypan blue exclusion) did not register cell death in cells which were shown to be nonviable by cloning studies.

A number of factors which significantly influence the IC_{50} were identified. Increasing cell seeding density had a masking effect on drug toxicity, while increased drug exposure time and assay duration resulted in greater cell kill (as expected). Small variations in pretreatment regime and trypsinization time prior to drug exposure did not significantly affect cellular response to drug.

Attempts made to improve culture conditions for lung tumour biopsies resulted in identification of a number of factors which must be optimized before individualized chemosensitivity testing can have an impact on selection of treatment regimes, and ultimately prognosis.

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INTRODUCTION

1.1 Toxicology and toxicity testing of chemicals

In considering the wide area of toxicity testing, it is worthwhile to firstly familiarise ourselves with some important concepts and definitions.

A typical definition of toxicology is that used by Klaassen [1986], which defines toxicology as:

"..the study of the adverse effects of chemicals on living organisms".

Virtually every known compound has the potential to cause death due to toxicity if given in sufficient quantities. Similarly any chemical can be permitted to come in contact with a biological mechanism without producing an adverse effect, provided the concentration is below a minimal effective level [Loomis, 1978]. In toxicology therefore, it is not simply the characteristics of the compound itself, but the dose at which it is administered which is important in distinguishing between compounds that are known as safe and those regarded as toxic. We can sum this up by the following quote by Paracelsus (1493-1541), who is considered to be the father of the science of toxicology:

"All substances are poisons; there is none which is not a poison. The right dose differentiates a poison and a remedy".

Toxicology does not endeavour to determine whether a compound is toxic or not (since all compounds are potential toxins), but to determine the degree and nature of toxicity which the compound is capable of causing. To this end, toxicology involves investigations into mechanisms of actions of toxicants. Toxicity is a relative property of a chemical, and may be directly or indirectly desirable or undesirable as far as man is concerned, but always refers to a harmful effect on some biological mechanism [Loomis, 1978].

One of the key elements in toxicology is to determine the safety of a chemical. Safety itself cannot be demonstrated experimentally. Rather, the degree of unsafeness (hazard) associated with a chemical

can be demonstrated, and its safety ascertained from this [James, 1985]. Thus there are two important concepts here; safety and risk. They are inextricably linked. Risk is the probability that a substance will produce harm under specified conditions. Thus we note that toxicology is essentially a predictive science and is primarily about making risk assessments on compounds [Leslie and Johnson, 1987]. In order to do this we make use of many different systems to assess the toxicity of chemicals.

The currently accepted method for chemical toxicity testing (scientifically and legally) is by administration to animals (in vivo) [Ryan and Mc Sweeney, 1986]. Much effort however, is being channelled into the development and use of in vitro tests (experimental tests not involving whole animals).

The degree of toxicity elicited is dependent on a number of factors. Firstly it depends on the chemical and physical properties of the agent; secondly it depends on the exposure of the chemical to the organism, both in terms of dose and method of administration; thirdly, on the biochemical reactions that occur in the organism; and fourthly, on the susceptibility of the organism to the chemical. Susceptibility can vary among individuals as well as among species. This latter consideration means that precision is necessary in reporting data from a toxicological evaluation, and also implies that this data must not be taken out of context and applied indiscriminately to any situation.

Among the interactions that a chemical may take part in before producing a toxic response at the site of action, are protein binding, biotransformation and excretion.

We have already noted that exposure is an important phenomenon. It is a factor of the physical state of the agent itself (e.g. liquid, gas, particle size, solubility), and of the environment in which the chemical presents itself to the organism (air, water, food etc.,). Toxic agents can gain access to the body through a number of routes, including ingestion (the gastrointestinal tract), inhalation (by

lungs), topically (exposure to the skin) or by parenteral methods (injection). The route of administration can measurably influence the toxicity of the agent [Loomis, 1978]. Duration and frequency of exposure are usually used to define 4 different categories of exposure for experimental toxicology in vivo (outlined in more detail in Klaassen [1986]).

A single, short term exposure (normally for less than 24 hours) is known as an **acute** exposure, although repeated exposures may be given within the same 24 hour period for chemicals with only slight toxicity.

The other categories of exposure all refer to repeated administrations.

Sub-acute exposure is the repeated exposure to a chemical for one month or less.

Sub-chronic exposure is generally for less than 3 months, and **chronic** exposure is generally for more than 3 months. These repeated exposures can be by any route of administration, but are generally by the oral route (given in diet of the test animal).

The effects produced by a single exposure are generally quite different to those which result from repeated exposures, so in defining the toxicity of a chemical from toxicity testing results, the **frequency, duration and method of exposure** are all determining factors, and should always be reported.

It should be appreciated therefore, that characterization of the toxic effects of a chemical is not a simple matter. It becomes even further complicated when we consider what happens (toxicologically) when chemicals interact with each other. One of the following outcomes can occur:

Synergism, where the combined effect of the chemicals is greater than the sum of the individual effects of the chemicals.

Potentiation. This is what occurs when one substance, given alone does not effect toxicity, but when given in combination with another agent, makes the second agent much more toxic.

Antagonism occurs when a number of chemicals, administered together, interfere with the action of one another and results in reduced overall toxicity than if the agents were given singly.

Another aspect of the toxic response is **tolerance**, which is a state of decreased responsiveness to the toxicity of a chemical. It results from prior exposure to that, or a structurally related, chemical. It may be due to a decreased amount of toxicant reaching the site of action, to a reduced responsiveness of a tissue to the chemical, or to increased catabolism of the chemical.

In 1938 in the USA, a drug (elixir of sulfanilamide) was associated with the death of 107 people. This led to the Federal Food, Drug and Cosmetic act, which required submission of New Drug Applications to provide premarketing assurance, and permitted the FDA (Food and Drugs Administration) to inspect factories [Anello, 1984]. After the Thalidomide disaster in Europe in the early 1960's, world attention was drawn to the importance of toxicity testing. This culminated in the establishment of more formal toxicological evaluation procedures and prompted the U.S. Congress to ammend the Act mentioned above, to provide for premarketing approval by FDA and to require drug manufacturers to establish efficacy and safety of a product before marketing.

With a new substance, the customary starting point in a toxicologic evaluation uses lethality as an index. Measurement of lethality is precise, quantal and unequivocal. It provides a measure of comparison among many substances whose mechanism and site of action may be markedly different [Klaassen, 1986]. A key element must be careful, disciplined, detailed observation of the intact organism extending from the time of administration to death of the test organism. By histologic examination of major tissues and organs, lethal effects, target organs involved and a suggestion of mechanism of toxicity, may be obtained. Thus **Acute lethality tests** are the

first toxicity tests to be performed on a new chemical. The median lethal dose (LD_{50}) and other acute toxic effects are determined after one or more routes of administration in one or more species. The LD_{50} test has become a public issue because of increasing concern for the welfare and protection of laboratory animals, although many would argue that it is essential for characterizing the toxic effects of chemicals and thus determining their hazard to humans. When exposure is by air or by water (for example with fish), the dose received is usually not known, and thus the Lethal Concentration-50 (LC_{50}) is usually determined, that is the concentration of chemical in the air or water that causes death to 50% of the animals. When reporting an LC_{50} , it is imperative that the duration of exposure be indicated.

In LD_{50} determinations, the number of animals that die in a 14 day period after a single dosage, is ascertained. In addition to mortality and weight, periodic examination of test animals should be conducted for signs of intoxication, lethargy, behavioral modifications, morbidity etc. Acute dermal exposure or acute inhalation experiments are performed if there is a reasonable risk of such exposure occurring in routine use of the chemical.

Other acute tests such as skin and eye irritation testing are carried out and are usually performed in rabbits. Fur is removed and the skin is exposed to chemical for 4 hours. For ocular irritation, the chemical is instilled into one eye, the other serving as a control, and both eyes are examined at various times after application (Draize test) [Draize et al., 1944].

Subacute tests are typically performed at 4 different dosages given in the animals' feed. Repeated doses are performed for 14 days, after which clinical chemistry and histopathology are performed [Klaassen, 1986].

Subchronic exposure can last for different periods but 90 days is the most common test duration and is usually performed in rat and dog by the route intended. At least 3 doses are generally used. Observations include mortality, body weight changes, diet consumption, pharmacologic and toxicologic signs, hematology and blood chemistry measurements. Hematology and blood chemistry are usually performed prior to, during and at the end of exposure.

Then the agent can be filed with the FDA and if approved, clinical trials carried out in 3 phases can commence.

These studies in humans involve:

Phase I: clinical pharmacology which focuses on dose levels, pharmacokinetics and safety

Phase II: closely monitored clinical trials with an aim of establishing efficacy and identifying overt and unusual toxicities, and

Phase III: additional clinical trials aimed at better defining efficacy and identifying the less common adverse effects [Anello, 1984].

While these are being performed, chronic exposure of the animals to the test compound can be carried out in laboratory animals as well as additional specialized tests. They depend to a certain extent on the product (e.g., oral or paraenteral route of administration).

Chronic or long term exposure studies are performed similarly to the subchronic studies, except that the period of exposure is longer, and is dependent on the intended period of exposure in humans. For a planned short term use, a chronic exposure of 6 months might be sufficient, whereas for a food additive with the potential lifetime exposure in humans, then a chronic study up to 2 years in duration is likely to be required. These studies are often used to determine the carcinogenic potential of chemicals and are usually in rats and mice over the average lifetime of the species (2 years for a rat). Both gross and microscopic pathological examinations are made on all test animals, including those that die early.

1.2 The dose response relationship

In the measurement of the toxic response, the most fundamental concept to be understood is that of the **dose-response** relationship. In utilizing this relationship in toxicological studies, we are making a number of assumptions. These assumptions, considered more extensively in Klaassen [1986], are as follows:

1. The response obtained is due to the chemical administered
2. The response obtained is related to the dose given. This itself is based on 3 sub-assumptions:
 - a) the dose administered relates to the concentration at the reactive site
 - b) the concentration of the agent at the reactive site relates to the response (and the degree of response)
 - c) the response is produced by the interaction of the chemical with a molecular or receptor site(s)
3. There is a quantifiable method of measuring and a precise means of expressing the toxicity.

It is this latter point with which this thesis is mainly concerned.

1.3 In vivo versus in vitro toxicity testing

In vivo testing is considered to be the ultimate testing system for toxicity because it has a number of important merits lacking in in vitro systems. The most important of these are outlined below:

(a) The whole system can be examined:

- unlike in vitro tests, a complete range of parameters may be measured and the interrelationships between these parameters can be studied, e.g., hepatotoxicity, nephrotoxicity, neurotoxicity, immunotoxicity, teratogenicity, temperature changes, blood pressure etc.

(b) Secondary organ involvement can also be ascertained and side effects can be established.

(c) Organ specificity can be determined to identify target organs.

(d) Blood levels, distribution and excretion are important in determining the fate of the chemical when it enters the bloodstream, and the actual dose that is available at a site to exert its toxicity. This is also important in revealing information about bioavailability and bioaccumulation of compounds, which cannot be detected in vitro.

(e) Tumorigenicity can be established using in vivo systems.

(f) The presence of a functional metabolizing system which is involved in many biotransformation reactions to modify the toxicity of a chemical. The relevance of this must be appreciated when we consider that some chemicals (cyclophosphamide for example) are not toxic in themselves, but when modified by certain mixed function oxidase enzymes concentrated in the microsomes of cells (particularly in the liver), can exert toxic action [Shrivastav et al., 1980]. The inverse of this is also true; a compound known to be toxic can be detoxified by this system and then excreted from the animal. Some of the more important aspects of biotransformation are described below.

In biotransformation, two distinct types of reactions are involved. They are described as phase I and phase II reactions. Phase I reactions involve oxidation, reduction and hydrolysis, and serve to make the xenobiotic more water-soluble and thus more suitable for excretion. Phase II reactions are conjugation reactions which involve the substitution of different functional groups on the xenobiotic causing it to become more lipophobic (water soluble). Phase I

reactions may occasionally produce metabolites that are somewhat less water-soluble than the parent. However, such metabolites are presumably then more susceptible to phase II transformations, which then result in greater water solubility and excreatability [O'Flaherty, 1985].

In the phase I reactions, the enzyme system of most importance is the group of mono-oxygenases containing cytochrome P-450. Two enzymes are involved in this system which together insert one atom from molecular oxygen into the substrate, while the other oxygen atom, in combination with two protons, is reduced to water. The enzyme systems involved are: NADPH (Nicotinamide adenine dinucleotide phosphate) cytochrome P-450 reductase, and the cytochrome P-450 itself (which in fact, is not a single enzyme, but a family of isoenzymes with different but frequently overlapping specificities). These cytochrome P-450 enzymes have been found in all species of animals investigated and also in plants. Predominantly found in the liver endoplasmic reticulum, they have also been found in the endoplasmic reticulum of lung, intestinal tract wall, kidney, skin, brain and other tissues. While they mostly produce metabolites that are less toxic than the parent compound, the reverse can also be true. This is seen by the production of epoxides, particularly those of aromatic hydrocarbons, believed to be responsible for mutagenic or carcinogenic effects of these hydrocarbons [O'Flaherty, 1985].

Other reactions catalysed by monooxygenases containing cytochrome P-450 are; N-, O-, or S-dealkylation; aromatic and aliphatic hydroxylation; sulfoxidation; and desulphuration. Amine oxidase, epoxide hydratases and dehydrogenases are other important oxidases. Esterases and amidases, found in most tissues including the blood, are responsible for hydrolysis. These enzymes are fairly nonspecific with regard to their substrate requirements [O' Flaherty, 1985].

Conjugation (phase II) reactions are numerous, but perhaps the most important in humans is glucuronidation. The enzyme involved here is glucuronyl transferase, which synthesises glucuronides from uridine-5'-diphospho- α -D-glucuronic acid. The glucuronic acid can be conjugated to a number of compounds, including aliphatic and aromatic alcohols, mercaptans, certain acids, primary and secondary aliphatic and aromatic amines. Another important phase II reaction is

catalysed by the enzyme Glutathione S-transferase, which attaches a molecule of glutathione (a tripeptide) to the substrate by a thioether bond. Then two amino acids are lost from the conjugate. Finally, an acetyl group is added to form a mercapturic acid derivative. This reaction requires that the substrate carries an electrophilic carbon, so highly potent metabolites such as epoxides can be rendered harmless. Xenobiotics may also be conjugated to the amino acids glutamine and glycine, or they may be sulfated, methylated, or acetylated. The enzymes involved in these transformations are found in the endoplasmic reticulum, the mitochondria or the cytoplasm of the cell.

In vivo tests however are not completely satisfactory. There are a number of problems associated with this form of testing, including the following:

(a) Ethical considerations represent a factor becoming increasingly important due to by remonstrations by animal rights activist groups, and an increasing concern by the general public over cruelty to animals. Certainly some of the tests are deserving of such concern; the classical example being the Draize eye test for irritancy, already described in 1.1.

(b) Time:

toxicity trials using live animals can take a number of years to complete.

(c) High cost of testing:

maintenance of animals, personnel, etc., on a long term basis.

(d) Expertise/ specialized facilities:

in addition to cost, the technical facilities and expertise may just not be available.

(e) Statistical limitations:

due to the factors already mentioned, the trials are limited by the number of animals which can be used, but this has serious statistical limitations in terms of detecting low level incidence of effects.

(f) Extrapolation problems:

exact homology does not exist in the extent of toxicity of chemicals to different species; some are more susceptible to certain chemicals than others. This places limitations on the extrapolation of toxicity data from test animals to man.

(g) Individual variation:

variations in sex, age, weight, genetic background etc., all make it difficult to extrapolate from the data derived from a relatively small number of test individuals.

In recent years there has been increasing pressure on governments and industry to reduce the use of animals in research [Ryan and Mc Sweeney, 1986]. There has been with this, a concomitant demand for methods which will model human metabolism more precisely.

Some options for reducing animal useage, conveniently denoted as the 3 R's are outlined below:

Reduction of numbers of animals used

Refinement of tests to reduce animal suffering

Replacement of animal tests

These can be accomplished by:

a) simplification of test procedures and protocols to reduce numbers

of animals used; e.g., removal of LD₅₀ test. In practice the test is of little use, but it does use large numbers of animals. It may often be equally relevant to use an LD₁₀ test.

b) use of invertebrates or other lower-order animals instead of animals. A problem associated with these, is that often their physiology and metabolism are very different from man. However they may be useful indicators of toxicity in some cases, and in further instances they may be a more relevant test organism to use, e.g., for ecotoxicity testing, where these organisms ordinarily live in the ecosystem which will be affected, or for pesticides and fertilizers, whose effect on these organisms may indirectly effect the entire food web.

c) use of computer modelling and structure-activity analysis. This is a form of predictive toxicology and requires a large amount of information already available on the test chemical. Where no such information is available, such methods are of limited use.

d) in vitro (cell culture-based) tests, which have some advantages, for example

- * speed

- * reduced cost

- * more potential for replication

- * simplicity

- * potential for automation

- * tests using human tissue may be more relevant than some in vivo animal tests

However there are barriers to the development and use of in vitro tests because:

a) they are not technically advanced enough yet, to replace animal tests

b) even if the above is achieved, there are many legislative barriers which will prevent/ greatly delay the replacement of in vivo tests. An issue of extreme importance here is the lack of adequate validation of existing in vitro techniques, which is said to be more difficult than assay development itself [Ekwall et al., 1991].

It is probable that there will always be a need for in vivo tests, but their use could be substantially reduced [Goldberg, 1986].

1.4 Some important considerations in toxicity testing

In beginning a toxicity testing programme there are a number of important considerations which must be addressed before deciding on a programme to use.

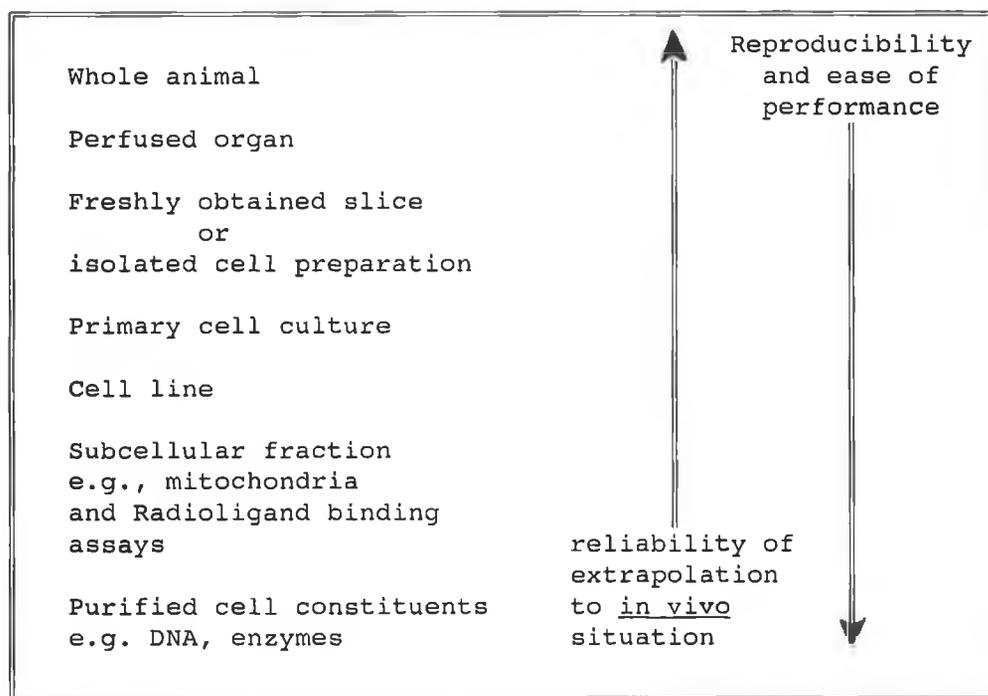
Probably the most common reason for wishing to carry out a toxicity test, is to demonstrate low toxicity of a new chemical or drug, or to demonstrate the efficacy of chemicals with a desired toxicity (e.g. pesticides, anticancer agents). In these cases it is necessary to consider and abide by existing legislation governing such procedures. The F.D.A., E.P.A (Environmental Protection Agency) and the O.E.C.D. (Organization for Economic Cooperation and Development) have specific rules and guidelines relating to this, and the E.C. (European Community) have a number of directives for member states concerning specific tests to be carried out. Some of the specific tests to be performed before the compound (new compound or reformulation of a previously existing compound) can be filed with the FDA, have been outlined already. While clinical trials are being carried out other tests are performed, such as chronic exposure tests, teratogenicity, reproductive toxicity, mutagenicity, tests relating to special routes of exposure, immunotoxicity etc. The specific tests performed (which have been approved by the governing authority) should always be stated.

Development of many important drugs and chemicals has often been inhibited because of the time span and the exorbitant cost involved in toxicity testing. By 1986, the International Register of Potentially Toxic Chemicals had acknowledged the existence of 63,000 chemicals in everyday use; 48,000 of which were used in commercially significant amounts. Of these, only 6,000 have been tested for toxicity [Ryan, 1987]. Testing of a typical new chemical costs between 500,000 and 1.5 million U.S. dollars; takes up to 2 or 3 years; and may entail the sacrifice of thousands of animals [Goldberg and Frazier, 1989]. Because of this, and because the process of testing itself is so slow, there is a huge backlog of chemicals for toxicity testing. The N.C.I. (National Cancer Institute U.S.A.) for example, has amassed a multitude of chemicals for testing. It is obvious that what is needed here, is an effective screen to determine the compounds of greatest potential, which can then be singled out for more intensive in vivo tests. For food additives and drugs, those showing excessive toxicity can be eliminated from further study, and in the search for efficacious anticancer agents, those showing significant toxicity to tumour cells can be examined more closely. It is here, in such screening systems that in vitro systems have much to offer.

Other applications of in vitro toxicity testing include chemosensitivity testing of tumour biopsies, quality control (e.g., water, drug batches), and environmental monitoring.

There are a very wide variety of toxicity testing systems available for use, ranging from isolated organs and tissues, through invertebrate and plant systems, to purified cell constituents. In choosing a system for toxicity testing it is important to realize the limitations of the different systems. The merits of a range of different systems are summarized in the table below, which has been modified from Bridges [1980].

Table 1.4 Range of toxicity testing systems and their merits



It is important to consider the use to which the chemical will be put. For example, in screening an easily biodegradable compound for use as a fungicide in fish farming; it may be more relevant to perform fish toxicity tests than toxicity tests using rats; the former being more likely to be exposed to the chemical. In such an instance, bioaccumulation studies may be appropriate. Thus the important message here is that, the appropriateness of each of the available systems should be established before one is chosen. It may be found that more than one system would better represent the eventual use of the test chemical.

Once the system has been chosen, the next parameter to consider is the endpoint to use. In deciding this, it is imperative to consider the use to which the compound is being put e.g. an anticancer agent, a shampoo, a food additive, or a pesticide. There would be little point for example, in testing for irritancy in evaluation of an anti-cancer agent; clearly this test would be more appropriate in evaluating a shampoo.

1.5 In vitro toxicity tests

In vitro toxicity tests may be short term or long term. Various in vitro short term tests have been developed and extended as potential replacements for whole-animal toxicity assay systems and as we have seen, speed, test reproducibility, increased sensitivity and economy are some of the advantages which these sorts of assays have to offer over in vivo assays. Some technical problems still need to be addressed, though, before they can be more fully used as replacements for whole-animal testing. In fact, - just as the results of animal testing are not fully representative of the human experience of toxic exposure, it is unlikely that in vitro cyto- or genotoxicological systems will ever provide toxicity data that will be totally equivalent to that derived from in vivo systems [Stark et al., 1986].

Some of the more usual types of in vitro toxicity tests will be briefly considered here.

1.6 Tissue specific toxicity tests

Tests on a particular tissue for a specific effect are performed when specific tissue or target organ toxicity is to be determined. Target organs are often not the site of highest concentration of the chemical, but are the organ most sensitive to the chemical. The Central Nervous System is most often the target organ, followed by liver, kidney, lung and then the skin. Muscle and bone are least often target organs [Klaassen, 1986]. For this type of testing, the end point used is often disruption of some normal biochemical function of the cell which is specific to that tissue or cell type. Some of the more usual tissue toxicities studied are outlined below.

Neurotoxicity:

Neurotoxins are diverse compounds that may be toxic to specific regions, cell types or functions within the nervous system. Some neurotoxins are capable of fairly selective injury and may damage only specific portions of the brain or peripheral nerves. Agents that disrupt neuronal transmission may be: blocking agents (prevent the continuation of the electrical impulse e.g., botulinum toxin),

depolarizing agents (depolarize the cell or eliminate the electrochemical gradient that normally exists within it, e.g., DDT), stimulants and depressants (increase or decrease the excitability of neurons), receptor antagonists (prevent the neurotransmitter from activating the receptor and initiating the impulse by binding to the postsynaptic receptors), anticholinesterase agents (e.g., organophosphate insecticides) or neuromuscular blocking agents [James, 1985]. Other agents may physically damage the myelin sheath or other parts of the nervous system.

Central nervous system (CNS) preparations which have been used for neurotoxicity assessment include brain slices, isolated nerve and glial cells, and brain nuclei, synaptosomes and vesicles [Walum et al., 1990]. Use of invertebrate preparations for in vitro work have also been found useful [Lee et al., 1986, 1987] because of their relative simplicity, and also because of their relevance to the many pesticides that have been designed to act on the insect nervous system.

Because neurones (the principle component of the CNS) have lost the ability to divide, problems with cell culture systems for neurotoxicity testing were apparent in the past [Dewar, 1983], however the last few years has seen the establishment of hundreds of cell lines originating from both normal and malignant brain tissue from different cell types - methods for such culture are given by Shahar et al., [1989]. Differentiation of embryonic nervous tissue in culture has often been found to mimic that of in vivo fairly closely [Walum et al., 1990], but because little neurophysiological work is performed by neurons and glia in vitro, lethality manifest in vivo by impairment of some active CNS process can not be detected with cultured cell systems [Goldberg, 1980]. Cultured neuronal cells have been shown to express more genetic information than several other cell types [Kaplan et al., 1982] so it has been suggested that cultured neurons are likely to react to a wider range of toxic agents than any other single cultured cell type [Walum et al., 1990]. Nardone [1983], Atterwill [1989] and Wallum et al., [1986] have all used batteries of different neural cultured cells as models to distinguish between neurotoxic and non-neurotoxic substances. Using neuroblastoma and glioma cells to determine toxic effects on oxidative energy metabolism and membrane integrity as well as cytotoxicity, results were compared

to effects seen in primary cultures of muscle heart and liver cells. Co-cultivation with hepatocytes was also used by Wallum in an attempt to discern the influence of liver metabolism on neurotoxicity. More complex in vitro systems have been used which retain more of the structural and functional complexity of the nervous system, such as organotypic cultures [Veronesi et al., 1983], reaggregate cultures [Atterwill, 1987] or mid-brain micromass cultures [Flint and Orton, 1984], which, in combination with general endpoints can generate toxicological data highly representative of the situation in vivo [Walum et al., 1990]. Endpoints such as energy metabolism, protein synthesis, receptor-second messenger and calcium homeostasis may be used [Walum et al., 1990]. Hansson [1986] has used nerve-glia co-cultures in an attempt to preserve some cell-cell interactions in vitro. Indeed practically all types of neural cell and tissue cultures have been used for screening for neurotoxicity and in attempting to elucidate the mechanisms of action of xenobiotics.

Haemototoxicity:

From the undifferentiated mesenchymal stem cells of the bone marrow, clones of cells differentiate and ultimately appear as mature (committed) cells with a specific purpose. Haemotoxic effects induced in the blood are hypoxia (effected by Carbon monoxide, nitrites, nitrates and aromatic amine compounds), hypotoxic hyposia (cyanide poisoning, hydrogen sulfide anion poisoning) and chemical induced-blood disorders) [James, 1985]. Besides being tested as human primary cells in standard cytotoxicity assays, as well as in the presence of subcellular metabolising fractions to better simulate the conditions of in vivo exposure, peripheral blood lymphocytes can also be examined with respect to their specific properties, such as their susceptibility to exogenous mitogens [Celotti and Bianchi, 1990]. They have been used extensively for cytotoxicity testing and genotoxicity testing.

Hepatotoxicity:

The liver is the major site of biotransformation of xenobiotics. It is therefore an important target organ, especially in relation to chemicals ingested orally or administered intraperitoneally, because

it is the first organ perfused by the blood containing the chemical [James, 1985]. In studying hepatotoxicity, it is important to be familiar with normal liver functions if liver injury is to be adequately detected. Differences in biokinetic behaviour among test compounds in different species is an important source of uncertainty, and thus the use of liver cell cultures from different species may improve understanding of such interspecies differences [Blaauboer et al., 1990].

The two types of tests generally used to screen for injury to liver are: (1) those that measure liver function, and (2) those that measure cellular damage - most often leakage of intracellular enzymes from the cells [James, 1985]. Complete organs or liver slices can be used for such studies; isolated hepatocytes however are often used. Hepatocytes retain most of the metabolic capabilities of the intact liver [Abraham et al., 1983; Tsuru et al., 1982], at least for a few days [Paine et al., 1982] and thus allow studies on metabolism of drugs and other new chemicals, to be undertaken. While hepatocyte cultures from rat, mouse, hamster etc., are frequently used, experience with the use of human liver cells in culture is more limited [Blaauboer et al., 1985,; Butterworth et al., 1989]. The human epithelial hepatoblastoma cell line Hep G2 has been widely used in hepatotoxicity testing, and although it has been found to be less sensitive than human hepatocyte primary cultures for detecting cytotoxic and genotoxic effects of 10 *N*-nitroso compounds [Campart et al., 1989] it has been found to possess parenchymal cell functions and to metabolise some procarcinogens [Eddy et al., 1987].

Lactate dehydrogenase (LDH) activity and LDH leakage are routinely used to measure integrity of hepatocytes. Oxygen consumption in freshly isolated hepatocytes, is often measured using an electrode, or protein synthesis may be measured. These endpoints will be considered in more detail later in this section. Changes in cell structure [Chenery et al., 1981] and observance of surface blebs are common techniques, as well as maintenance of such differentiated functions as cytochrome P₄₅₀ and other phase I and II enzyme activity.

Nephrotoxicity:

The unique structure and function of the kidneys make them particularly susceptible to xenobiotic-induced toxic injury [Smith *et al.*, 1987]. Known nephrotoxins like Cis-platinum and chloroform are used to set up models for nephrotoxicity testing. Other chemicals particularly toxic to the kidney are heavy metals such as cadmium, mercury and lead, agents causing obstructive uropathies, those producing pigment-induced nephropathies and therapeutic agents such as Gentamycin and Amphotericin B [Goodman, 1985; Bertelli *et al.*, 1991]. Isolated perfused kidneys, renal slices and explants [Tveito *et al.*, 1989], tubule and cell suspensions and cell cultures have been used to determine toxicity to this target organ. Perhaps the most commonly used cell lines for such studies are MDCK and LLC-PK1 (dog and pig kidney lines respectively). Alterations in three renal processes: glomerular filtration, tubular reabsorption and tubular secretion; are used to diagnose the functional state of kidneys in the clinical situation [Goodman, 1985]. Effects on biochemical functions such as gluconeogenesis, or changes in levels of xenobiotic metabolising enzymes are some *in vitro* tests often used as indicators of nephrotoxicity. Andreoli and Mallett [1991] have reviewed methods for assessment of oxidant injury in renal tubular epithelial cells *in vitro*.

Pulmonotoxicity:

The lungs are another particularly sensitive organ as they constantly filter air, polluted with chemicals and combustion products (e.g. carbon monoxide, sulfur oxides, photochemical oxidants and tobacco smoke). In fact, this organ has the greatest exposure to the environment than any of the other organs. Some chemicals become trapped in the respiratory tract and for this reason they exert their toxicity there, but others are specifically toxic for this organ (e.g. paraquat and 4-ipomeanol). Deposition of aerosols in the lung may be by impaction, sedimentation and diffusion. Mathematical and physical models can be used to predict where particles will deposit in the respiratory tract [Brown and Poole, 1983]. Mucociliary mechanisms and alveolar macrophages constitute the lung clearance mechanisms [Duffell, 1985]. Toxicant interaction with macrophages and phagocytic leukocytes may cause impairments of the immune system,

resulting in conditions such as silicoetuberculosis, release of inflammatory mediators and the production of free radicals by oxygen [Brown and Pool, 1983]. These free radicals may also cause direct oxidation of xenobiotics, leading to formation of active metabolites.

The lungs consist of at least 40 different cell types. This heterogeneity complicates any attempts to isolate pure cell populations and maintain them in culture [Poole and Brown, 1987]. Clara cells are probably the most widely studied of these cell types, at least for toxicological purposes [Devereux *et al.*, 1980, Jones *et al.*, 1982]. These are non-ciliated cells in which levels of mono-oxygenase activity are enhanced. A number of authors have found that the toxicity of various mineral dusts towards macrophages and macrophage-like cell lines correlated with the fibrogenic activity of those dusts in vivo [Brown and Poole, 1983]. The size of the fibres causing cell death in vitro is similar to those causing tumours in vivo, so the study of mineral dust cytotoxicity has been advocated as a preliminary screen for potentially pathogenic dusts [Brown *et al.*, 1978; Chamberlain and Brown, 1978; Davies, 1980]. The toxic effects of metals [Evans *et al.*, 1981] and asbestos [Rajan and Evans, 1973] on cultured human fetal lung have been reported. Mossman and colleagues [1981 (a) and (b)] used tracheal explants in studies of particulate toxicants. The epithelial type II cell line, A549, has been reported to have some functional drug metabolising ability [Wiebel *et al.*, 1980], and may be useful for studies in this area.

Dypbukt *et al.*, [1987], Grafstrom *et al.*, [1988] and many others have used cultured human bronchial epithelial cells to detect aldehyde and acrolein induced cytotoxicity to this tissue. Grafstrom *et al.*, [1990] used both bronchial epithelial and fibroblasts cells in culture to study general mechanisms of the toxicity of acrolein, and to determine whether it caused any effects regarded to be of importance for carcinogenesis. In these studies, the different variables investigated in acrolein-exposed bronchial cells included colony survival, membrane integrity, growth rate, low-molecular-weight thiol status, cytosolic concentration of free calcium, and various types of DNA damage. Ryrfeldt *et al.*, [1990] has reported the need to develop techniques for the isolation and culture of other lung cell types aside from those mentioned above, and also to improve the culturing techniques for those already isolated.

Dermatotoxicity:

Toxic responses of the skin may be manifest as irritant contact dermatitis, pigment disturbances, ulceration, neoplasms or follicular and acneiform dermatoses [Reitschel, 1985]. The key clinical test for dermatitis is the patch test, but in vitro systems have been used to mimic this response. Addo et al., [1982] used a Candida killing test, photohaemolysis (indicated substances which attack membranes), and photo-oxidation to assess the phototoxic potential of various fragrance materials (may give an indication of potential photoallergy). Less progress has been achieved in the use of in vitro tests for the detection of irritancy and potential sensitizers as for other areas [Marks, 1983]. Studies on whole skin in chambers in which the release of enzymes from the skin into the medium has been measured has been found unreliable, but adult human and porcine keratinocytes have been used successfully in the investigation of anthralin toxicity [Marks, 1983].

The Hen's Egg Test (HET) is well known as a basic test for embryotoxicity and for special aspects of systemic toxicity and immunopathology. It has been extended and standardized as the HET-chorioallantoic membrane (CAM) test for membrane irritation [Luepke and Kempler, 1986] in dermatotoxicity as well as ototoxicity. In such tests, good correlations have been observed between classifications based on HET-CAM observations (including electron microscopy) and reported data from Draize tests.

Examination of keratome slices of skin for release of enzymes, altered histochemistry and for utilization of radioisotope-labelled amino acids can detect weak irritants, but is of doubtful value for moderate irritants, and will detect corrosive substances only through their inhibition of all cell activities [Parish, 1986]. Fibroblast cultures, tested with *Clostridium perfringens* toxins and chemicals show similar limitations in detecting moderate or severe irritants, but can be made more relevant by overlaying with a layer of agar containing keratin.

Ocular toxicity:

The cornea and the conjunctiva are the portions of the eye directly exposed to external insults. The cornea must maintain its transparency in order to remain functional. This is thought to be achieved by the boundary layers of epithelium and endothelium [Maurice, 1961]. Damage to these boundary layers result in absorption of water and loss of transparency [Potts, 1986]. Thus the cornea is exquisitely more sensitive to corrosive substances than are other body tissues. Thus, corneal swelling studies and permeability measurements as well as changes in corneal thickness [Jacobs and Martens, 1990] are widely used to detect irritation caused by agents such as acids, bases, organic solvents, detergents and smog; and are frequently used in cosmetic screening systems. Many of the general endpoints for cytotoxicity testing such as Neutral Red Uptake and Release [Gettings and McEwen, 1990] and Glutamic Acid uptake inhibition [Dierickx, 1989] (considered later) are used for eye irritancy testing. The CAM and HET-CAM tests already mentioned are also used in assessment of eye irritation. An enucleated rabbit eye model has been described in which changes in corneal thickness are assessed in response to test materials applied to the ocular surface of the enucleated eyes [Price and Andrews, 1985], and a bovine eye model combined with the CAM assay [Weterings and Van Erp, 1987], as well as isolated ocular tissues [Muir, 1985] have been similarly used. Obviously these systems do not offer the same advantages as simpler in vitro tests. Assays of tissue repair are also widely used where corneal epithelial monolayer healing is measured (wound closure rates) after inflicting damage with a suitable probe or sharp instrument, in the presence of the test material [Jumblatt and Neufeld, 1985, 1986]. Assays of inflammation are also used whereupon the release of different known mediators of inflammation (e.g. histamine, prostaglandin) are determined [Frazier et al., 1987], however these assays should be used cautiously as the mediators measured to date are almost always present in measurable amounts, and their levels are easily changed even with the most innocuous stimulus [Wilcox and Bruner, 1990]. Another potentially useful system currently under evaluation is the ETYTEXTM System [Kelly, 1989; Dierickx and Gordon, 1990]. It is based on appearance of opacity in a

necessary for mutagenesis or carcinogenesis (or to detoxify a more toxic substance), an 'S9' mix, containing animal (usually rat) liver microsomes, is frequently added to the test. Such biotransformation systems and others, are used for many other in vitro mutagenicity tests [reviewed by Jenssen and Romert, 1990].

Other bacterial systems have been used to detect gene mutations including *Escherischia coli* [[Mohn et al., 1980]. A colorimetric assay (SOS chromotest) based on the primary cellular responses of this bacterium to genotoxic agents, has been developed [Hofnung and Quillardet, 1988]. Moulds such as *Neurospora crassa* [De Serres and Malling, 1971], and yeasts, commonly *Saccharomyces cervisiae* [Zimmerman, 1973], have also been used, but the permeability of the cell wall is a general problem, as it can restrict entry of certain chemicals into the cell. Also eukaryotic microorganisms usually require higher concentrations of chemicals for induction of mutations than do bacteria, probably because yeast and moulds exhibit more powerful detoxification mechanisms and have more DNA repair pathways [Dunkel, 1983].

DNA damage can also be measured by quantifying DNA repair. In tests for DNA repair, the effects of a compound are established by comparing its toxicity to two isogenic strains of bacteria, identical in all respects except for their ability to repair DNA lesions (one is DNA-repair deficient and the other is competent in repairing DNA lesions). *E. coli*, *S. typhimurium* and *Bacillus subtilis* [Kada et al., 1972] have all been used for such tests.

Similar tests are carried out in mammalian systems using cell lines (frequently V79, CHO and L5178Y cells [Kuroki et al., 1977; Hsie et al., 1979; Clive et al., 1979]. Metabolic activating systems have sometimes been added to these systems, either by S-9 as already described, or by co-culturing with intact cells (primary fibroblasts and hepatocytes have been used [Huberman and Sachs, 1974; Langenbach et al., 1978]). It has been suggested [Bigger et al., 1980], that mammalian cell mediated mutagenesis appears to be a better indicator of in vitro metabolic pathways than bacterial, and may also reflect the organ specificity of the chemicals tested.

Some genetic alterations are visible with the light microscope. Chromosomal aberrations observed in mammalian cells are characteristic of damage sustained in G₁ cells. This damage is translated into breakage/exchange figures prior to chromosome replication. Abberations can be detected on a wide range of mammalian cells. Chinese hamster cells have been widely used [Mutsuoka *et al.*, 1979] as they have short cell cycles and small numbers of large chromosomes. Three different types of cytogenetic change can be distinguished in cells exposed to chemical mutagens [Evans, 1983]: 1) alterations in chromosome structure 2) sister chromatid exchange, and 3) alterations in chromosome number.

Sister Chromatid Exchange (SCE's) [Perera *et al.*, 1989], are a special class of chromosomal aberrations which involve equal and symmetrical exchange between sister chromatids and hence do not result in an altered chromosome morphology and are not detectable unless grown for two cell cycles in bromodeoxyuridine and then stained.

DNA strand breakage can be measured by alkaline sucrose gradient centrifugation [Lett *et al.*, 1967] or by alkaline elution from membrane filters [Kohn, 1979]. Repair synthesis or unscheduled DNA synthesis can be measured by autoradiography or by liquid scintillation counting [Painter and Cleaver, 1969; Stich and San, 1970] but the former is preferred because it can distinguish repair synthesis from replicative DNA synthesis [IARC, 1980]. Development of a DNA-repair assay in primary rat hepatocyte cultures [Williams, 1976] has become important for this type of testing (rather than using cell lines), and it has been shown to be sensitive and reliable for chemicals that require metabolic activation [Probst *et al.*, 1980]. Human peripheral lymphocyte cultures exposed to organotin and other metal compounds have shown spindle-inhibition effects (assessed by chromosome length measurements) [Anderson and Ronne, 1990; Jensen *et al.*, 1990] in a dose-related and exposure time-related fashion.

In mammalian systems, in addition to the types of tests mentioned above, neoplastic transformation is also measured. This has much relevance in assessing chemicals for carcinogenic potential and is considered to be the most relevant in vitro model for carcinogenesis

[Dunkel, 1983]. Cells pile up in an irregular, criss-cross pattern representing a loss of growth inhibition and cell-cell orientation. They acquire other transformed characteristics with increasing passage, such as ability to grow in semi-solid medium and capacity to produce tumours after transplantation into syngenic or immunosuppressed animals. The Syrian hamster embryo clonal assay, in which primary or early passage cells are used as the target population, is one of such methods [Pienta *et al.*, 1977]. Continuous cell lines can be used for transformation also, and here, foci of morphologically transformed cells appear on a monolayer of normal cells.

1.8 Embryotoxicity/ Teratogenicity

Tremendous growth in development and use of in vitro teratogenicity tests have been made in recent years. These assays range from whole embryo cultures, derived from invertebrates and vertebrates, to cell and organ cultures [Bournias-Vardiabasis, 1990], and have been the subject of a number of reviews [Welsh, 1990; Brown, 1987]. It is generally perceived that teratogens can have a variety of effects on the developing embryo and at various organisational levels, and like other aspects of toxicity testing it is therefore appropriate to use more than one in vitro system.

The HET has already been dealt with, and its use in embryotoxicity mentioned.

Systems using lower animals, non-mammalian vertebrates and invertebrates are better able to indicate teratogenic hazard than isolated mammalian embryonic tissues or cells, because both adult and embryonic forms can be studied in vitro [Fabro *et al.*, 1982].

Many of the tests which follow are still undergoing validation however they are mentioned in order to give the reader an idea of the sorts of such tests available for use.

Chick embryo tests [Wilson, 1978] have been widely used and rank-order studies suggest [Jelinek and Rychter, 1979] that the teratogenic potential obtained is similar to that obtained from whole animal studies.

The frog embryo teratogenesis assay [Dumont et al., 1982] has also been used as a model for teratogenicity screening of industrial wastes for ecotoxicological assesment. Here, embryos from the blastula stage are examined regularly over a 4 day test exposure and tadpoles scored for motility, pigmentation, malformations in development as well as mortalities. Other amphibian tests have been used for ecological toxicity testing [Birge et al., 1983; Dumpert and Zietz, 1984; Fulton and Chambers, 1985].

Of the sub-vertebrate assays, the *Drosophila* embryo test has often been used [Schuler et al., 1982]. Several endpoints may be used in this assay including detection of interference with normal muscle and / or neuron differentiation, induction of heat shock (stress) proteins, and inhibition of normal neurotransmitter levels [Bournias-Vardiabasis, 1990]. The endpoint used by Schuler was the following: after controlled mating, eggs are deposited in nutritive medium containing the test agent and emerging adult flies are assessed for shape, size and colour of body parts. Obviously this involves specialist practice, which limits the tests' usefulness. Other disadvantages are that the test does not show a dose-response relationship, nor does it indicate teratogenic hazard.

A similar assay using the cricket *Acheta domesticus* has been useful in detecting the teratogenicity of impurities in complex mixtures of industrial chemicals [Walton, 1983]. Eggs are first developed in contaminated sand and then on moist filter parer. Emerging nymphs and cricket embryos when exposed to teratogen display a range of abnormalities associated with compound eyes, antennae, legs and head. This test has distinguished between specific teratogenic effects and general embryotoxic effects.

Another invertebrate teratogenicity assay uses the brine shrimp, *Artemia salina*. The increase in body length of nauplii larva after hatching from cysts, is measured and used as an index of

teratogenicity [Kerster and Schaeffer, 1983]. The test has been used with heavy metals and organic compounds but no attempts have been made to correlate the effects of these agents on naupliar development with mammalian data. Differences in larval development are apparent at 6h and 24 h LC₅₀'s. This data can then be used to distinguish general toxicants from those with a specific stage-dependent developmental effect [Sleet and Brendel, 1985].

Planarian tests using *Dugesia dorotocephala* [Best and Morita, 1982] examine the ability of surgical fragments of the flatworm to regenerate into an intact animal in the presence of the test agent. This procedure is assumed to model many of the differentiation and development processes that occur in embryogenesis. The endpoints of the test are quite defined: delayed regeneration of head, failure of the subject to regenerate all or part of the head process, or regeneration of an abnormal head or loss of normal morphology. There has been little validation of these tests so it is uncertain whether the tests are specifically indicative of developmental toxicity.

Hydra assays [Johnson et al., 1982] are specifically designed to estimate teratogenic hazard. *Hydra attenuata* adult polyps are exposed to test agent in defined medium, for up to 90 hours. Degeneration and eventual loss of tentacles, followed by a total loss of normal morphology and the assumption of a rounded shape, indicates a toxicological response. In a second part of the assay, dissociated adult cells are seeded in tissue culture wells in reaggregation medium containing the test agent; and reformation of adult polyps is monitored (over 90 hours). The toxic endpoint of the test is dissociation of the cell aggregates. This can occur at any stage of the developmental sequence. The ratio of the minimum effective concentration for the adult over the disaggregated hydra gives a teratogenic hazard index and again can indicate a specificity of action on the developing organism.

Sea urchin embryos have long been used in studies of developmental biology and have now been extended to include a test for teratogenicity. Hose [1985] described a system in which the sea urchin embryo is used in a combined embryotoxicity-genotoxicity test. Gametes and embryos are exposed to test agent in sea water. Fertilization success is monitored (after combination of sperm and

egg) by examining the eggs for the presence of a fertilization membrane. Embryos are allowed to develop for a further 48 h under test conditions and are then assessed for survival and abnormalities. Mitotic rates of isolated embryonic cells are used as an indicator of general embryonic health. Analysis of cytologic irregularities is also carried out, but again, this requires specialist knowledge. Only benzo(a)pyrene has been tested with this system so far.

Durston *et al.*, [1985] used a slime mould test to determine the lowest concentration of the test agent causing an effect on spore yield and morphogenesis of *Dictyostelium discoideum*, and the highest concentration at which no cell death was recorded.

1.9 Reproductive toxicity

The potential of using testicular cell cultures for investigating chemically induced testicular toxicity has been considered [Ng and Liu, 1990; Waalkes and Perantoni, 1988], but *in vitro* manipulation of gametes and embryos for evaluating the effect of chemicals on germ cell function and development potential has been more extensive. This can be measured by direct assessment of fertilization and embryo development, or indirect assessment by measuring physiological and biochemical parameters related to sperm and oocyte function. Sperm cell bioassays [Seibert and Gosch, 1990] can also give useful toxicity data on the cytotoxic potential of chemicals.

1.10 General cytotoxicity tests

In the broad context of cytotoxicity testing of chemicals and environmental pollutants, primary cell cultures and cell lines are widely used. Many different types of cells can be grown from different tissues and from organisms in widely different taxonomic groups. Human tissue may not always be available and it is important that the significance of using tissue from another species be realized. The American Type Culture Collection (A.T.C.C), has an extensive range of cell lines, any of which may be suitable for a cytotoxic evaluation in a particular case. Cultures of fish cells are an option that is often used, especially in relation to ecotoxicity testing. Early passage fish cells will obviously be more relevant than

using cell lines [Babich and Borenfreund, 1987], or a fish hepatoma cell line, such as the rainbow trout hepatoma line used by Babich et al., [1989], may offer some advantages over lines from other tissues if any slight xenobiotic metabolizing activity remains in these lines. Marine and freshwater algae and multicellular plants are very widely used for detection of marine and freshwater pollutants. Growth rates of phytoplankton species such as *Chlorella stigmatophora* [Nielsen and Clausen, 1984] and retardation of root and shoot tip development of multicellular plants (often duckweed and grass species) is commonly measured to give an indication of toxicity of heavy metals and other pollutants [Wong and Chui, 1990]. Invertebrate assays measuring cell kill for crustaceans such as *Artemia* and *Daphnia* are often used. The O.E.C.D. [1984] have defined guidelines for Ecotoxicity testing.

1.11 Endpoints for in vitro toxicity testing

The ideal endpoint should be based on events that are closely associated with the molecular events which result from exposure to the toxin. If this can not be achieved, it is important to use an endpoint which is unequivocal and clearly relevant to the toxic effect. This is not always straightforward, because although many endpoints are quantitative and precise, they are often indirect measures of toxicity. Neither is it the case that all direct measures of effects are related to the mechanism by which a substance exerts its toxicity, but they do allow a causal relationship to be drawn between the agent and its action.

As established already, the choice of an endpoint for in vitro cytotoxicity testing is very important. Many, different endpoints have been widely used (the literature abounds on this subject, particularly in relation to chemosensitivity testing). This thesis confines itself to the study of some of these methods for cytotoxicity testing. The pages which follow introduce the more important and widely used of these assays, and consider the more important aspects of them.

1.12 Assays for colony formation

Assessment of viability is the endpoint most often used in determination of cytotoxicity. While growth assays give a general indication of viability, the most relevant parameter for determining the viability of a cell is often considered to be retention of its replicative potential. Tests for colony formation are able to determine this; a cell which will replicate and form a new colony can be assured to be viable. These tests have been widely used in cytotoxicity testing studies, chiefly in the area of chemosensitivity testing.

The ability of malignant (and transformed normal) cells to grow independently of a substrate for anchorage is exploited in agar colony formation assays. The in vitro colony forming assay was developed by Puch and Marcus in 1955. In the assay, single-cell suspensions of mammalian cells were dispensed in semisolid medium. After an appropriate time, cells capable of sustained replication produced small colonies containing 50 or more progeny of the original cell. This technique has been developed and modified so that today it is a powerful experimental tool. It is widely employed for the detection and study of clonogenic cells from a variety of normal organs and experimental tumours. The types of assays used to study the anchorage independent colony forming population within a tissue are either soft agar, as described by Bradley and Metcalf [1966] or methylcellulose. These assays have been commonly used for the detection of stimulatory effects of substances [Mc Cullouch et al., 1974].

In 1977, Hamburger and Salmon, and later in 1978, Courtenay and Mills published papers describing the use of a clonogenic assay to determine the chemosensitivity of patients' cells to a range of anti-neoplastic agents. In the following 7 years, more than 700 publications described the general use of soft agar tumour colony forming assays for quantitation of cancer cell proliferation and anticancer drug effects in vitro [Human Tumor Cell Cloning Bibliography (1984)], while a paper by Bertelsen et al., [1984] reviewed the results of 1582 such assays with 258 clinical correlations.

Basically the assays involved culturing the tumour-isolated-cells in semi-solid medium in a 2 layer agar system together with the chemotherapeutic agent. Only what was termed the 'clonogenic cells' in the tumour cell population would form colonies in this system, so this was thought to be of relevance to the 'critical cells' in the tumour.

A particular advantage to this assay is that contaminating normal cells will not grow in the agar layer, so the possibility of misinterpretation of results from contaminating stromal cells is minimized.

Prospective and retrospective trials resulted in various interpretations to the usefulness of these assays. Some highly positive correlations between the test results and in vivo human tumour response were reported [Salmon *et al.*, 1978; Sarsody *et al.*, 1982; Kern *et al.*, 1983]. The tests were performed on a wide range of tumour tissues and the response obtained differed not only with the source of material and the laboratory involved, but with repeated work on the same tissues in the same laboratory. Generally though, it has been reported [Selby *et al.*, 1983] that the Human Tumour Stem Celling Assay (HTSCA) gives a 60-70% true positive and an 84-98% true negative correlation of chemosensitivity for a limited number of different tumours and Drugs [Salmon & Von Hoff, 1981 and Von Hoff *et al.*, 1981]. These figures are variable, depending upon the tissue type.

Despite the limitations of this system [Bertoncello *et al.*, 1982; Dendy, 1975], and even though the results required cautious interpretation, the system represented a big step forward in the area of cytotoxicity testing for chemosensitivity. It was felt that many of the technical problems could be overcome, so further work was carried out in this area [Bertoncello *et al.*, 1982; Alley and Lieber, 1984; Selby *et al.*, 1983; Twentyman, 1985]. Problems with low plating efficiency, clumping, long assay duration and technical cumbrousness still remained however.

These technical difficulties resulted in a de-popularization of the assay, and a reversion to other methods of cytotoxicity testing and development of new methods.

1.13 Dye exclusion assays

Viability dyes have been used extensively in the past for determination of cellular integrity and detection of cell death. Their use has been reported as far back as 1914 [Evans and Schulemann].

Among the dyes excluded by live cells which have been used are eosin, nigrosin, trypan blue, erythrosin B, fast green and naphthalene black [Bhuyan *et al.*, 1976]. Fluorescent dyes such as propidium iodide have also been used (often in combination with flow cytometry) [Pavlik *et al.*, 1985; Funa *et al.*, 1986]. Upon membrane damage they bind to nucleic acids and become highly fluorescent.

More recently, some workers have shown a preference for dye exclusion assays over other cytotoxicity assays. Yip and Auersperg [1972] found that out of a number of stains tested, Alcian Blue gave the most consistent results for cell viability for cell staining of fixed samples after different periods of time. Hanson *et al.*, [1989] also used this stain for work on leukemia and lymphoma cell lines. Hartman and Rieck [1989] found Janus green superior to other dyes used in dye exclusion for staining of damaged and dead endothelial cells.

Combinations of acridine orange and ethidium bromide have also been used to detect viability. Viable cells appear green (acridine orange), becoming orange on death (ethidium bromide) [Detta and Hitchcock, 1990].

Several pre-1983 studies used a range of these dyes with differing degrees of success. While some workers reported favourably on the use of such assays for chemosensitivity testing [Durkin *et al.*, 1979], many reported a poor correlation with other assays and with clinical trials in vivo. Many workers found that dye exclusion methods underestimated cell kill [Yugas *et al.*, 1974; Bhuyan *et al.*, 1976]. Roper and Drewinko [1976] demonstrated that reproductively dead cells may not take up dye. Other technical flaws were highlighted from which it was deduced that such methods were unsuitable for chemosensitivity testing of most tumours.

A study carried out by Weisenthal et al., [1983a] investigated some variables in the use of this assay. They reported that, provided the technical limitations of the assay are recognised, dye exclusion assays may be useful in chemosensitivity testing. Using a modification of a fast green staining method, results comparable to clonogenic assays were obtained. Later studies reported similar conclusions [Bird et al., 1986; De Vries et al., 1987], and so these assays have been somewhat redeemed. Some of the limitations and artifacts outlined by Weisenthal are outlined below.

The timing of the assay is important. It is imperative that a sufficient time has elapsed to allow membrane damage to occur and to prevent underestimation of cell kill. Also, after prolonged exposure to dyes, viable cells will begin to take up the dye.

Additionally, membrane damage may not always equate with loss of viability.

Notwithstanding these technical limitations, these type of assays have been suggested as useful methods for chemosensitivity testing, particularly in non-dividing or slow growing cells which may not be evaluable by other methods. A feature of the more widely accepted assays now, is that the cells are first fixed and can then be counterstained with haemotoxalin and eosin [Hanson et al., 1989], thus enabling normal and malignant cells to be distinguished. Apart from the theoretical and technical difficulties considered above, their major disadvantage is their labour intensiveness as the method cannot be automated.

1.14 Other tests for membrane integrity

Similar in principle to dye exclusion assays, chromium release assays, fluorescein diacetate uptake, and measurement of enzyme leakage have also been widely used for cytotoxicity testing [Van Rooljen, 1977; Neville, 1987]. Also widely used have been the release of other radiolabelled molecules from the damaged cell such as proline, uridine and selenomethionine [Saal et al., 1976; Van Rooljen, 1977]. They are all based on detection of loss of membrane integrity.

1.15 Dye inclusion

Dye inclusion assays used to indicate viability are simply the converse of dye exclusion. Fluorescein diacetate (an ester of the fluorochrome Fluorescein) or similar compounds are actively transported into cells and are cleaved by cellular esterases to yield an impermeable charged molecule, e.g. Fluorescein. Undamaged cells retain this highly fluorescent dye, whereas cells with damaged membranes do not, and fluoresce only weakly [Rotman and Papermaster, 1966]. Assessment of cytotoxicity is performed by microscopic enumeration of fluorescent cells. While the assay has been reported to be a sensitive assay for assessment of lysis of CK cells in intact layers, it is *subjective* and does not allow for quantification of the fluorescence [Norris *et al.*, 1985]. Neither can it be automated, so it is very time consuming.

1.16 Chromium release assays

Originally developed by Brunner [1968], the ^{51}Cr release assay is widely used for cytotoxicity studies [Russel, 1981; Pene *et al.*, 1986; Stanislawski *et al.*, 1989; Bonavida *et al.*, 1990] including immunological studies for determination of cytotoxic T cell activity against tumour target cells [de Bueger *et al.*, 1990]. Prelabelled cells are exposed to cytotoxic drugs, and covalent binding of labelled chromate to basic amino acids of intracellular proteins occurs. These labelled proteins leak out of the cell when the membrane is damaged, at a rate which is proportional to the amount of damage incurred. Leakage into media is determined by Gamma counting. Although it has been reported that high values of spontaneous chromium leakage occur and thus the time period over which the assay can be used should be restricted to approximately 4 hours, Rabinovitch *et al.*, [1990] have used the assay in a 5 day exposure to cytokines. In a study by De Bueger *et al.*, [1990], a modified form of a ^{51}Cr assay is reported, where spontaneous release values of adherent CK cells in the 4 hour assay were reduced to 15% of the total (Triton-X-100) values. Further work by Colsky and Peacock [1990] report that the addition of sodium pyruvate in these assays, greatly reduces the spontaneous release of ^{51}Cr for extended periods.

In one comparative study which evaluated ^{51}Cr release as an end-point for drug cytotoxicity testing, the method was found to be of no value [Roper and Drewinko, 1976]. Studies by Tzeng and Barth [1990] indicated that the clonogenic assay was capable of detecting differences in target cell sensitivity that were otherwise undetectable by the standard ^{51}Cr release assay.

1.17 Other release assays

A microcytotoxicity assay using [^{35}S]methionine developed by Akagi et al., [1985] is reported to have increased sensitivity over [^{51}Cr] release assays in terms of detecting cell number, due to increased incorporation into cells [Stedman and Campbell, 1989]. Other release assays which have been used are ^{125}I -UDR, [^3H]proline, [^{75}Se]selenomethionine and [^3H]uridine [Hofer and Swartzendruber, 1973; Gee et al., 1985; Saal et al., 1976; Van Rooljen, 1977].

1.18 Chromium uptake

Use of a ^{51}Cr -uptake assay (where labelled sodium chromate is taken up by viable but not dead cells) is reported to be more useful than the typical ^{51}Cr release assay [Neville, 1987] because such problems as spontaneous release do not occur. The assay was shown to be linear with cell number over the range 5×10^3 to 5×10^5 cells/well. Dead cells did not incorporate ^{51}Cr . This work also showed that inhibitors of DNA, RNA and protein synthesis did not inhibit ^{51}Cr -uptake. In a comparison of these two assays, the ^{51}Cr release assay failed to detect cytotoxicity in most of the cytotoxic preparations. Thomas et al., [1978] suggested that such a failure may be due to the short length of the assay, which would have been insufficient for release of the labelled chromium if minimal damage was done to the plasma membrane. Thus we see that this has similar limitations to the dye exclusion assays.

1.19 Enzyme leakage tests

Assessment of leakage of a wide variety of enzymes has been used for cytotoxicity testing endpoints. Lactate dehydrogenase (LDH) is a convenient marker because of the stability of the enzyme activity in the culture system. It is frequently used in hepatocyte cytotoxicity and viability studies [Mitchell and Acosta, 1981; McQueen and Williams, 1981; Tyson *et al.*, 1983], but is also generally used for assessing viability in cell lines [Ekwall and Acosta, 1982].

Typically the LDH activity in the culture medium (after exposure to the cytotoxic agent) is measured spectrofluorometrically by measuring the production of NAD^+ (or the disappearance of NADH) during the conversion of pyruvate to lactate [Bergmeyer, 1979; Gobran and Rooney, 1990].

In some assays, after conversion of lactate to pyruvate, the electron carrier phenazine methosulphate reduces a tetrazolium salt (INT) present in the medium, to a red coloured product by an irreversible reaction. There are some drawbacks; serum can contaminate the system with endogenous enzymes and mask low levels of leached enzymes, and the presence of phenol red in the medium will mask the appearance of coloured products of enzyme reactions. Also MEM (Minimum Essential Medium) lacks added pyruvate and therefore favours the lactate to pyruvate specific reaction.

LDH leakage has been compared to DNA leakage, as an endpoint in cytotoxicity testing [Skaanild and Clausen 1989] where it was found that the former gave lower LC_{50} values (concentration causing 50% lethality) than the latter. This was thought to be because the cells have to be more damaged to exhibit DNA leakage than for LDH to be lost. Thus DNA leakage would not be a sensitive endpoint for cell damage/ death.

Hexosaminidase leakage and glutamic oxaloacetic transaminase leakage [Anuford *et al.*, 1978; Salocks *et al.*, 1981; McQueen and Williams, 1981] have been used as indicators of cell viability, but they have not offered significant advantages over other methods.

The main drawback of all these assays however, is similar to that discussed for dye exclusion - namely their reliance on the premise that membrane integrity and cellular viability are closely linked. This is not always the case, at least in early stages of cell death; so these assays may not always be true/ sensitive indicators of viability. Thus we shall move on from these types of assays to other functional assays, that is, assays which measure some metabolic components necessary for growth.

1.20 ATP (Adenosine TriPhosphate) assays

ATP is a basic store of energy in living cells. Its intracellular concentration can be expected to be correlated with cellular viability because it rapidly disappears when cells die [Gronroos *et al.*, 1984; Kuzmits *et al.*, 1984; Maehara *et al.*, 1986]. It has been demonstrated to be a reliable and sensitive measure of cell viability for various cell lines and has been favourably compared with other cytotoxicity assays such as dye exclusion, [³H] thymidine incorporation, colony forming assays and a succinate dehydrogenase inhibition test [Kangas *et al.*, 1984; Kuzmits *et al.*, 1986; Garewal *et al.*, 1986; Maehara *et al.*, 1987]. More recently it has been adapted to solid human tumor specimens [Peng *et al.*, 1987; Perras *et al.*, 1990] to assess overall cell viability of cells grown in a soft agar cell culture method.

Measurement of ATP is often determined by the bioluminescence method. Light (560 nm) is generated by oxygenation of LH₂-AMP and its intensity is precisely dependent on the total number of ATP molecules. Measurements with luciferase-luciferin reagent are highly sensitive and can measure the ATP content of as few as 50 cells [Moyer and Henderson, 1983]. Other methods for direct ATP measurement are ³¹P NMR measurements and ³²P labeling of ATP, ADP and AMP pools.

Kangas *et al.* [1984], reported that intracellular ATP levels were significantly correlated with cellular viability and might be useful for in vitro chemosensitivity testing for malignant cells. Some investigators also reported use of intracellular ATP measurement for

chemosensitivity testing. In one study, Miura et al., [1988] investigated in vitro and in vivo intracellular ATP levels for chemosensitivity testing in rats, and found them to be significantly correlated. A highly positive correlation was also found for ATP levels and trypan blue determined viable cell numbers in 2 cell lines. In Miura's work, determination of ATP levels was preferred after a 72 hour drug incubation, rather than a 48 or 24 hour incubation, as a decrease in ATP levels was seen more clearly after 72 hours. Sevin et al., [1988] also demonstrated a direct relationship of ATP luminescence and viable cell count over the range 250 to 485,000 cells/ml. This group also evaluated the chemosensitivity of 56 tumour specimens using this assay, and correlated it to clinical response. They found a true positive correlation of 100% and a true negative correlation of 66.7% (thus yielding a sensitivity of 89.5% and a specificity of 100%).

Others however, [Lahio and Trump, 1974] showed only poor correlation between ATP levels and dye exclusion following injury to Ehrlich ascites cells by various mitochondrial and respiratory inhibitors, so good correlations have not been universal.

ATP normally cannot penetrate the intact plasma membrane, so ATP leakage can be used as an indicator of cellular membrane damage. In such methods 2 subsequent measurements are made on the same cell suspension, namely extracellular ATP and within a few minutes after addition of a detergent, total ATP [Andersson et al., 1990].

Measures of cellular function other than direct ATP measurements have been used to indicate cellular viability. Some of the more commonly used ones are dealt with in the following text.

1.21 Assays of respiration and glycolysis

Observations of aerobic glycolysis in the presence and absence of drugs provide information about the manner in which a drug may affect respiration; for example, stimulation of aerobic glycolysis may indicate either inhibition or uncoupling of respiration [Bickis and Quastel, 1965; Quastel and Bickis, 1959].

Tracers have been used for quantification of respiration and glycolysis since early days in chemosensitivity and other cytotoxicity testing.

Drug effects on RNA synthesis, aerobic glycolysis and aerobic glycolysis have each been shown to occur independently of each other in tumour slices, while an apparent correlation has been noted between energy-producing reactions and measured biosynthesis [Bickis *et al.*, 1965]. Further work showed that in glucose-containing media under aerobic conditions, the total ATP made available in the tumour tissue by respiration and glycolysis remains constant in the presence of respiratory or glycolytic inhibitors. Therefore *in vitro* inhibitors of biosynthesis, such as those mentioned above, are possibly due to more specific interactions than diminished availability of ATP.

The approach of using a tracer as an index of respiration and glycolysis, was semiautomated by Von Hoff *et al.*, [1985] to screen for antineoplastic agents. A modified BACTEC system (a technique for radiometrically measuring bacterial growth) was used on a variety of tumour cell lines. After drug exposure, the cell suspension (30-40,000 cells) was mixed with uniformly labelled [^{14}C]glucose and seeded into sterile, disposable 15 ml glass vials by injection through rubber aluminium caps. The vials were placed in the BACTEC system, which adjusts the atmosphere to 5% CO_2 and 95% air (37°C). Measurement of the release of $^{14}\text{CO}_2$ resulting from metabolism of [^{14}C]glucose was performed on days 3, 6 and 9 and the instrument directly converted disintegrations into growth index values. There was a clear linear relationship between the number of cells seeded and the production of $^{14}\text{CO}_2$ from [^{14}C] glucose. There was also a reasonably good correlation with the results obtained for the same compounds using a human tumour cloning assay, especially when a continuous drug exposure was used in both systems. This suggested that the inability to discriminate between reproductive cell death and drug induced delay in cell division (one of the theoretical limitations of a metabolic BACTEC assay) might only be of minor relevance when this technique is utilized for screening purposes [Scheithauer *et al.*, 1986].

Further work led to development of modified microassays for the determination of metabolically generated $^{14}\text{CO}_2$, such as that described by Gaines [1989]. The reaction, which measures decarboxylase activity, takes place in a microcentrifuge tube inside a 20ml scintillation vial that contains a centre well with a CO_2 -trapping agent, resulting in greater safety than other methods.

Assays for glucose consumption are numerous, but recent work has led to semiautomated, colorimetric, modifications of such assays. A procedure utilizing the coupled activities of glucose oxidase and horseradish peroxidase, was described by Blake and McLean [1988] which is not subject to the spectral interferences caused by phenol red and sera in tissue culture media, apparent in other assays. The product of an oxidative couple between 4-aminoantipyrine and N-ethyl-N-sulfopropyl-m-toluidine, which is a chromophore, was used to develop a fixed time assay for glucose in media samples. Only $10\mu\text{l}$ of media in a 1-ml assay volume was needed. Similar radiometric assays for glycerol and glycogen, which are also intrinsically linked to cellular and whole-animal energy balance, have been developed [Bradley and Kaslow, 1989], which are 10-100 times more sensitive than assays based on the detection of NADH by absorbance. They offer several practical advantages over spectral assays (detecting levels of 0.2 to $1.0\ \mu\text{M}$ NADH), and having better reproducibility than previous assays.

These assays are cumbersome to carry out; those involving the use of radiolabels are potentially hazardous and do not lend themselves to successful routine screening of cytotoxicity.

1.22 Determination of metabolites in culture medium

Excreted metabolites reflect the activity of certain metabolic processes in vitro. Metabolites such as lactic acid, Alanine, 2,2-Dihydroxyacetic acid, Maltol, Benzeneacetamide, Pyroglutamic acid ethyl ester, Pimelic acid, Phthalic acid, Citric acid, Cholesterol and Palmitic acid hydroxyethyl ester, have all been identified and quantified in biological material [Figenschou and

Marais, 1991] and in the culture medium of fibroblasts [Antoshechkin et al., 1988]. Analytical assays with bacterial luciferase have been developed for a great many metabolites [Hastings, 1978; Brodin and Agrew, 1977; Ugarova et al., 1988], and commercial kits are readily available for reactions based on soluble bacterial luciferase and NADH:FMN oxidoreductase, such as formation of the metabolites NADH, FMN, Formate, NAD, Glucose-6-phosphate and Glucose-1-phosphate. Improved sensitivity in detection of these metabolites is evident with further work in the area. Glucose-6-phosphate can now be assayed for in the ranges 1-100 and 1-1000 pmol with soluble and co-immobilized highly purified enzymes [Mc Elroy and DeLuca, 1983].

Some such metabolites have often been used as indicators of cellular activity in chemosensitivity testing. Familletti and Wardwell-Swanson [1988], have used quantification of the secondary cellular metabolite, lactic acid, to reflect not only cell growth, but also subtle changes in the cells' metabolism that might occur when exposed to lymphokines or other biological response modifiers. In a coupled oxidation of lactic acid, a tetrazolium dye present in the culture medium is reduced to a coloured formazan, where colour change and intensity are proportional to the concentration of lactic acid produced by assay cells. This colour is quantified photometrically in a 96-well plate reader. One of the advantages of this method is that it requires only 15 μ l of sample culture medium, so a time-dose response evaluation can be prepared without setting up multiple cultures of the same cell line. It has been used to detect the activity of interferon.

Highly specific micromethods for the enzymatic determination of radioactive [14 C]lactate have also been used [McCormick et al., 1991] by collecting released 14 CO₂ following the enzymatic decarboxylation of radiolabelled lactate, enabling picomoles of the latter to be precisely, easily and reproducibly measured in very small volumes or in micro samples. Disadvantages to such systems however, include unavoidable introduction of inhibitors of the enzyme, as many diverse biological ions and other compounds are culpable. Also, significant expense is incurred in determinations. Other enzymes have been employed to measure [14 C]lactate. Lactate oxidase from *Pediococcus* sp. (L-lactate: O₂-oxidoreductase), which forms pyruvate and H₂O₂,

can detect as little as 2.5 nmol of lactate after further peroxidase-catalyzed coupling of a sensitive chromogen to 4-aminoantipyrine [Bozimowski *et al.*, 1985].

Yamashoji *et al.*, [1989] used the production of H_2O_2 by intact cells (promoted by the presence of menadione) to determine viable mammalian cells. They found the concentration of H_2O_2 produced was proportional to the density of viable cells, determined by measurement of the chemiluminescence generated in the mixture of H_2O_2 , pyrene, and bis(2,4,6-trichlorophenyl)oxylate, needing an incubation time of only 10 minutes for determination. No extraction of NAD(P)H from cells was required and a stable chemiluminescent reagent can be used. While cell density of PC-3 cells was proportional to the production of H_2O_2 in the presence of menadione, the correlation was not linear over the whole cell density range tested (3 to 30×10^5 cells/ml). The minimum detectable cell density under the conditions used was only 3×10^5 cells/ml, however they suggest that lower cell densities in the range 10^4 to 10^5 cells/ml may be determined by using "the simple mixture" containing 0.25M sucrose, 5mM imidazole-intrate buffer (pH 7) and 0.1 mM menadione.

1.23 Determination of protein synthesis and nucleic acid synthesis

As early as 1966 and from then onwards, quantitative determinations of drug activity were being successfully carried out through the evaluation of interference on nucleic acid metabolism in different human neoplasms [Bickis *et al.*, 1966; Byefield *et al.*, 1971; Knock *et al.*, 1971]. Tisman *et al.* [1973] demonstrated a correlation between the antimetabolic activity in vitro and clinical results. A more recent attempt to evaluate the effects of antitumour drugs was based on the reduction of labeling index (the number of cells in mitosis) during 24 h incubation of cells in vitro [Thirwell *et al.*, 1976].

Horakova *et al.*, [1978] showed that total cell number in control cultures was directly proportional to the total cell protein or nucleic acids. However in treated cultures (exposure to 6-Thioguanine and Vermiculine), the relationship between these parameters was

disturbed. They felt that this was due to the unbalanced growth which was an integral part of the cytotoxic reaction of the studied agents. They concluded from their results that only direct cell enumeration was suitable for the detection of the cytotoxicity of such agents.

Knock *et al.*, [1978] however, found that assays for incorporation of thymidine into DNA, uridine into RNA and leucine into protein, reliably predicted clinical results. They also insisted on the value of these assays in providing an insight into mechanisms of drug activity. Chowdry and Neogy [1980] used incorporation of phenylalanine and uracil to establish the effect of a number of antitumour antibiotics on protein biosynthesis.

Overall, many workers used these methods effectively for chemosensitivity testing, and their usefulness was established. The methodology used however, was often quite involved and time consuming. Modifications to simplify these assays were sought. In a comparison of autoradiography and scintillation counting to determine incorporated radioactivity, Tanigwa *et al.*, [1982] found a strong correlation between the results of the two methods and colony growth, but favoured scintillation counting due to its technical ease.

Short-term radioincorporation assays were also developed [Volm and Matterson, 1976] and applied to chemosensitivity testing. This approach consists of exposing the cell suspension to drug for 1-2 hours and then to radioactive precursor for a short period (around 1 hour). Cultures were then washed, harvested and radioactivity was measured. Volm *et al* [1979, 1981] have used the short term assay (STA) extensively to detect proliferation-dependent and induced tumour-resistance to cytotoxic agents. They reported that if a rapidly proliferating tumour responds to Doxorubicin treatment in vitro, then in most cases a similar effect can be detected with other cytotoxic agents. In these short term assays sterility does not have to be maintained [Zirvi *et al.*, 1986]. Others used similar short term assays [Livingston *et al.*, 1980], with the use of a longer incubation time with radioisotope (18 hrs). Here, one million cells per tube were used so due to shortage of viable cells only 63% of 35 specimens could be tested. This indeed is an important drawback of these assays.

Although it was known that inhibition of incorporation into DNA and reduction of cell survival depended on both the **time of exposure** and the **concentration of drug in the cell**, the optimum time of exposure in the short-term assay had not been established. Discordance between the results in these assays and the clonogenic assay was felt to be due to the drug exposure time being too short, which prevented slow growing tumours from responding. Thus it was concluded by Zirvi *et al.*, [1986] that the short term assay was less sensitive than the clonogenic assay in measuring tumour chemosensitivity as far as drug dosage was concerned. Its possible predictive value for some agents (eg, BCNU, Cis-Platinum and Doxorubicin) was thought to be still relevant though. They suggested that further studies on the STA should focus on alkylating agents and other antimetabolites.

When compared to results from the HTSCA and clinical observations it was felt that the short drug exposure created a problem [Seeber and Schmidt, 1977] which Bazeed *et al.*, [1988] later confirmed - the incubation time for the test may be too short for a slowly growing tumour such as a renal cell carcinoma. Their kinetic studies not only showed that the uptake of [³H] thymidine and [³H] uridine is very low in the first 4 hours, but found that nearly 80 hours is the average time needed for the uptake to reach a maximum.

Freidman and Glaubiger [1982] further developed the HTSCA by utilizing a liquid top layer containing the cells and a soft agar bottom layer. Tumour growth was measured by [³H] thymidine incorporation into TCA precipitable material. Good correlations were obtained for cell number and colony number and in drug treated samples they got a 95% similarity. Many other workers obtained similar promising results [Tanigwa *et al.*, 1982; Rupinak *et al.*, 1983; Sondak *et al.*, 1984; Twentyman *et al.*, 1985; Jones *et al.*, 1985]. Unlike the HTSCA however this assay only takes 5 days, and has lower variances. Normal tissues did not incorporate significant amounts of [³H] thymidine into TCA-precipitable material, so it was reported that normal components plated did not interfere with assay results. Reaggregation is also avoided in this assay. In contrast to the HTSCA however, the growing tumour cells are not fixed, thus the assesment of individual colonies is not possible and the clonal origin of the aggregates is less certain. Overall the assay had a higher success rate and lower

false-negative rate than the HTSCA for chemosensitivity testing. In 1985 Kern *et al.*, developed a miniaturized version of this assay; the MINI assay. The assay was performed in 16mm cluster dishes instead of 35mm; using 15,000 cells per well instead of 50,000 and using agarose instead of agar. This development offered improved growth of breast and colon tumour cells from 41% and 47% respectively to 63% and 68%.

Further modifications of the 5 day test in semisolid medium were done [Akiyoshi, 1986; Wada *et al.*, 1988]. Originally, the labelled cell pellet (from centrifugation of the solubilized agar layer containing cells) was precipitated overnight. Instead now, the labelled cells were collected on a membrane filter sealed on the underside of the dish. This reduced the assay to a 4 day assay and removed the need for a precipitation procedure. There was still significant correlation between the 2 procedures.

A rapid ³⁵S-methionine uptake assay for drug sensitivity testing, using scintillation autofluorography to measure the endpoint was used by Darling and Thomas [1983], Freshney and Dendy [1983] and Thomas *et al.* [1985]. Cells were exposed to drug for 72 hours and then washed and allowed to recover for 3 - 5 days, after which ³⁵S-methionine incorporation into cells was measured. Some of this work [Freshney *et al.*, 1975] showed that prolonged exposure to drug, followed by recovery, were necessary to obtain stable ID₅₀ (inhibitory dose) values for most drugs, especially phase specific agents. They found that using this system, the ID₅₀ measured by clonogenicity and microtitration assay correlated well.

Overall, although good correlation was obtained with these assays and clonogenic assays, they were still cumbersome to carry out and hazardous by their use of radioactive material. They also required the use of a high cell number.

1.24 Determination of intracellular enzyme activity

Assays for other intracellular enzymes have historically been used to indicate cellular viability. Probably the most commonly used is that of lactate dehydrogenase. It has been found promising as an indirect measure of viable cell counts by Jauregui et al., [1981] and by Chao et al., [1988] in assessing the number of viable hepatocytes in monolayer cultures. In the latter study, the decline of intracellular LDH was observed with a concomitant detachment of the monolayers. Also this decline in intracellular LDH corresponded with the increase in extracellular LDH. Intracellular LDH was found to be more indicative of cytotoxic effects of a number of chemicals, than measurement of intracellular protein. Intracellular DNA showed a poor correlation with cytotoxic effects. The assay was determined to be procedure utilizing the coupled activities of glucose oxidase and in 2 mls. However, the number of viable cells thus determined, should be regarded as 'viable average equivalents', since partially viable/injured cells may retain part of their LDH content and remain attached to the monolayer cell culture. Ponsoda and Castell [1991] developed a semi-automated spectrophotometric assay for intracellular LDH in 96 well plates of adherent cells for cytotoxicity studies. The procedure involves 'in situ' homogenization of cells, followed by measurement of LDH activity with a colorimetric method based on the reduction of a tetrazolium salt to a violet formazan by the NADH formed by LDH. The assay was used to measure the dose-response loss of intracellular LDH after 24-h exposure of rat hepatocyte cultures to cytotoxins.

The reader may note that a feature of the assays recently considered is that they have been readily adapted to miniaturization and semiautomation. Such a process constitutes a great advance in the applicability of general growth and viability assays to cytotoxicity testing and should be especially borne in mind as these and the following endpoints for cytotoxicity testing are considered.

The lysosomal enzyme, Hexosaminidase, has been found to be a sensitive and simple microtitre assay for cell number and detection of

lymphokines and cell surface antigens [Landegren, 1984]. This assay has been found to have similar sensitivity to the thymidine incorporation assay, but to be more precise and less laborious.

Determination of Alkaline Phosphatase has also been used to detect proliferation. It has been combined with Fast Red to allow simultaneous detection of proliferation and cell surface markers [Murdoch *et al.*, 1990].

Another intracellular enzyme assay is that of Acid Phosphatase. In a paper by Connolly *et al.*, [1986], linear activity of the enzyme was demonstrated for cell number over the range 100 to 100,000 endothelial cells per well. This miniaturized, semiautomated, colorimetric assay was used to determine growth curves for endothelial cells in the presence and absence of endothelial cell growth factor from bovine hypothalamus and to monitor fractions during purification of growth factors. In the work described in this thesis, the assay was applied to cytotoxicity testing.

The Succinate Dehydrogenase inhibition test (SDI) was introduced for chemosensitivity testing based on the correlation of the Succinate Dehydrogenase assay using tetrazolium salt as a hydrogen acceptor with cell viability [Dallne, 1960] and has since been used to this end [Kondo *et al.*; Kondo, 1971]. In the development of this assay, it has been semiautomated and miniaturized [Mossman, 1983], and extensively compared to other endpoints for chemosensitivity testing (mostly HTSCA and radioincorporation assays). With its development (considered in 1.25) and the development of similar tests, the merits of semiautomated, colorimetric assays came to be realized, and was responsible for changing the emphasis of chemosensitivity testing. Presumably this also influenced developments in other areas of cytotoxicity testing. This thesis concerns itself with the development and use of these cytotoxicity assays.

1.25 Colorimetric assays

Developed in 1984 by Borenfreund and Puerner, the Neutral Red (NR) method quantifies surviving cells by incubation with the supravital dye, neutral red, followed by colorimetric analysis of the dye extracted from the lysosomes of viable cells. The assay has also been combined with a microscopic screen for morphological alterations. This assay has been used extensively for in vitro screening of chemicals, heavy metals and environmental compounds as well as a rapid chemosensitivity assay [Borenfreund *et al.*, 1990; Fiennes *et al.*, 1987; Zhang *et al.*, 1990; Flick and Gifford, 1984]. Because the rate of NR release is similar to the rate of uptake of the NR, an NR release assay has also been used similarly to the former assay [Reader *et al.*, 1989].

Improved sensitivity of assays for protein determination [Bradford, 1976] has been found with the use of a number of protein stains. Crystal violet has been used [Kramer and Carver, 1986; Matthews and Neale, 1987; Flick and Gifford, 1984; Bonnekoh *et al.*, 1989] for screening of cytotoxins, as has Naphthol yellow [Skehan and Friedman, 1985] methylene blue [Finlay *et al.*, 1984; Absher *et al.*, 1991], Comassie brilliant blue [Elson *et al.*, 1992] and Kenciad blue; the latter of which has been adopted as part of a general screening system by the FRAME (Fund for Replacement of Animals in Medical Experiments) multicentre project on cytotoxicology [Knox *et al.*, 1986]. Skehan *et al.*, [1990] compared the ability of 21 histologic dyes to measure cell density and cytotoxicity in 96-well microtitre plates. Each of the dyes bind electrostatically to macromolecular counterions in cells fixed with TCA, which allows their binding and solubilization to be controlled by pH. These workers found that 13 of the dyes stained well enough to provide an adequate basis for assay of cytotoxicity in 96 well plates, but overall they found the best performance with Sulforhodamine B (SRB). Indeed, because of its technical advantages, this assay has been adopted by the National Cancer Institute (NCI), USA in their in vitro drug-screening program [Rubenstein *et al.*, 1990].

Apart from the sensitivity, simplicity, safety, linearity with cell number, ability to be easily automated, and relative inexpense of these assays, a further advantage of the staining assays (SRB among

others) is their undestructive endpoint. Using a modification of the Neutral Red assay, Knocks and colleagues [1986] were able to read NR stained plates, de-stain and re-stain with the kenacid blue method. Plates were again de-stained and stored for re-staining at a later date if necessary.

By far the most extensively validated of these assays however, (especially in the field of chemosensitivity testing) is the MTT assay based on the SDI assay already mentioned. Mossman, in 1983 used this semiautomated, colorimetric assay to measure cytotoxicity, proliferation and activation in lymphocytes. The assay was reported to detect living but not dead cells and the signal generated was reported to be dependent on the degree of activation of the cells. As already suggested, the main reason for its popularity was that the plates could be read on a multiwell scanning spectrophotometer (ELISA reader) with a high degree of precision. No washing steps were needed and low cell numbers could be used, giving potential for more replicates to be carried out.

The basis of the assay is that MTT [3, (4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide] a soluble yellow dye, is metabolized by the enzyme succinate dehydrogenase in the mitochondria of metabolically active cells, to a dark blue insoluble formazan product. The precipitate formed can then be solubilized and colour development read on a plate reader. The enzyme reduction takes place via coupling at 2 points along the cytochrome oxidase system, however there is presently no convincing evidence that the mitochondria are the *only* site of MTT reduction in the intact cell [Jabbar et al., 1989].

In the years which followed, not only did this paper prompt extensive use and validation of this method, but also encouraged the development of other miniaturized, semi-automated, colorimetric assays (some of which have been mentioned already; e.g., the LDH 96 well assay by Ponsoda and Castell, 1991). Some technical problems involving non-dissolution of the formazan dye were overcome [Twentyman and Luscombe, 1987]. The assay was tested on an extensive range of human cell lines, and linear relationships of cell number versus formazan production was generally shown [Carmichael et al., 1987;

Wilson *et al.*, 1990; Campling *et al.*, 1991]. It was widely used for drug sensitivity testing [Alley *et al.*, 1986; Carmichael *et al.*, 1987, 1988; Finlay *et al.*, 1986; Park *et al.*, 1987., Twentyman and Luscombe, 1987; Fanning *et al.*, 1990; Coles, 1986]. Good correlations were observed when the assay was compared to clonogenic assays [Carmichael *et al.*, 1978; Chang and Gregory, 1987; Wasserman and Twentyman, 1988; Mc Hale and McHale, 1988; Shimoyama *et al.*, 1989], electronic cell counting [Finlay *et al.*, 1986; Twentyman and Luscombe, 1987] and dye exclusion assays [Carmichael *et al.*, 1987; Ruben and Neubauer, 1987; Pieters *et al.*, 1988; Twentyman *et al.*, 1989]. It was also used for radiosensitivity testing [Carmichael *et al.*, 1987], for analysis of terminal differentiation in cultures [Wiedermann *et al.*, 1990] and macrophage cytotoxicity activity [Ferrari *et al.*, 1990].

A modified form of the assay was adopted by the National Cancer Institute USA (NCI) as part of their screening system for chemosensitivity testing of new drugs on cell lines [Alley *et al.*, 1988, Ruben and Neubauer, 1987], however it has since been shown to compare less favourably with the SRB assay already mentioned.

The technique has been widely used for chemosensitivity testing of tissue samples. Among the cell types that have been tested are chronic [Twentyman *et al.*, 1989] and acute [Pieters *et al.*, 1988] lymphatic leukemia cells and leukemic blast cells [Campling *et al.*, 1988], acute myeloid leukemia [Sargent and Taylor, 1989], renal cell carcinoma [Mickisch *et al.*, 1990] and small cell lung cancer [Campling *et al.*, 1991]. Wilson *et al.*, [1990], reported that in a feasibility study for ovarian malignancies, in vitro results with the MTT assay mirrored those determined from clinical response rates. The importance of validating the assay for each cell type has been stated [Sargent *et al.*, 1989].

Carmichael *et al.*, [1988], reported that optimal assay duration for human lines should be a minimum of 4 days to allow for cell death and loss of dehydrogenase activity, with a maximum of 7 days to obviate the necessity for refeeding cultures. Campling *et al.*, though, [1991], stated that a good reproducible variation in drug effect between patients is obtained after only 48 hours incubation. Excellent

reproducibility of the assay has been reported by Sargent et al., [1989], though elsewhere [Carmichael et al., 1988], some inter-experimental variability has been noted.

Some drawbacks to the assay have been identified however. Contamination by metabolically active, non malignant cells capable of reducing MTT formazan can falsify results [Carmichael et al., 1987; Wilson et al., 1990] and this has been reported to limit its use. Note however, that this factor is common to all similar short-term assays. The other factor of importance which has been mentioned [Carmichael, 1987] is that the assay is a growth assay, and is therefore incapable of distinguishing between cytostatic and cytotoxic effects.

Other tetrazolium dyes were used in similar assays (XTT, INT, NBT) [Scudiero et al., 1988; Bernabei et al., 1989; Alley et al., 1988] but none has been as widely used in the area of chemosensitivity testing as the MTT assay. INT (p-iodonitrotetrazolium violet) is metabolized into a red formazan derivative, evaluable at 492nm. Linear relationships have been found for viable cell number and O.D. and its use has been established in chemosensitivity testing [Bernabei et al., 1989; Dal Pozzo et al., 1989; Santini et al., 1989].

So, all of these assays have been shown to be fast, reliable and simple. Also because they have been miniaturized, smaller cell numbers can be used (which is a very important factor with tumor biopsies). Certainly they offer more advantages than any of the assays previously used. What further merits could be possibly needed for effective toxicity assays - increased sensitivity perhaps?

1.26 Fluorometric assays

Van Lambalgen and Lelieveld [1987] developed the PIT method for drug sensitivity testing. After drug exposure a solution containing propidium iodide, ink and triton X-100 (PIT) was added to the cell culture. Triton X-100 lysed all cells, which were subsequently stained by the DNA specific fluorescing propidium iodide. The ink quenched all background fluorescence. Results showed a linear

relationship between cell number and fluorescence intensity and reproducible dose-response curves were obtained for 6 drugs tested. With DNA-enhanced fluorescence [Mc Caffrey *et al.*, 1988] using DAPI or Hoechst 33342, which intercalate the DNA at A-T bonds, similar relationships were shown. Here, the cells were first fixed, again allowing storage for determination at a later date. Fluorescein diacetate fluorescence was used by Larsson and Nyger [1989] and was found to be sensitive to viable cell number for cell lines and fresh leukemic samples. Another fluorescence - based viability assay that has been used is based on a double staining technique with BECF (2'-7'-biscarboxyethyl-5(6)-carboxyfluorescein) as a vital dye and propidium iodide [Leeder *et al.*, 1989]. A further combined dye is the combined Hoechst 33342 and fluorescein diacetate [Larsson and Nygren, 1989; Larsson, 1990] method. It has been successfully used for rapid chemosensitivity testing and also for detection of multiple drug resistance in acute lymphoblastic leukemia cell lines. Aside from DNA fluorometric stains, fluorogenic enzyme assays have been developed. Huschtscha *et al.*, [1989] developed a fluorogenic alkaline phosphatase assay which estimated low cell numbers for many cell types.

Although some of these fluorescent dyes have been used in the past and have been dismissed earlier in this chapter, they are given greater consideration in this text because here they have been modified to give quantitative results for cell number. Additionally they have been semi-automated which at this stage in the development of assays for cytotoxicity testing, we have seen to be an important advantage. These fluorometric assays are reported to have increased sensitivity over other semi-automated micro assays which would appear to be a further advantage.

1.27 Aim of this project

The need for in vitro toxicity tests has been well established, and the variety of different systems for toxicity testing have been alluded to here. The wide range of endpoints which have been used for cytotoxicity testing has been considered, but we have seen that some of the systems and some of the endpoints used have not been extensively validated. This creates a problem in ensuring the widespread acceptance and use of in vitro cytotoxicity testing methods. Indeed, it has been pointed out [Balls and Bridges, 1984; Ekwall, 1983], that validation of the currently used methods is the key to extending the use of in vitro methods.

With this in mind, the aim of this project was to validate and compare a number of in vitro cell culture assays, primarily for use as chemosensitivity tests, but also for their application to other areas. Because we have been perhaps 'overcome' with the merits of miniaturized, semiautomated, colorimetric, assays, 5 of such assays were chosen for comparison purposes, including an acid phosphatase growth assay, for prospective application to toxicity testing. The emerging importance of the MTT assay in chemosensitivity and immunological work, warranted the inclusion of this assay in the study for further scrutiny. The neutral red assay, which is probably the most widely used cell culture cytotoxicity assay for environmental screening, was also added to this list, along with a simple crystal violet dye elution assay. The final assay to be considered was the SRB assay, which has assumed much importance in the National Cancer Institute's screening program.

At the commencement of this work, a popular instrument for endpoint determination in this laboratory and in other laboratories throughout Europe, [Ryan and Mc Sweeney, 1986] was an image analyser, so it was considered pertinent to relate some of this work on assay performance, back to the performance of the technique of image analysis.

It was proposed firstly, to define the limits of sensitivity and linearity for these assays; and determine if this varied for different cell lines. Precision and reproducibility, two very

important factors in the eventual success of an assay, were also to be considered. Because these assays have widely differing end points, their suitability to cytotoxicity testing in general was to be questioned by examining their relationship to cell viability. A further interest in characterizing these assays, was to investigate the ability of these assays to suggest the mechanism of toxicity of a chemical (by comparing the sensitivity of each of the assays after exposure of target cells to chemicals with mechanisms of action relevant to the various endpoints). Previous work has demonstrated that the MTT assay at least, is not consistent in its ability to distinguish cellular viability [Jabbar et al., 1989]. Thus the aim of this work was also to consider the ability of the different assays to distinguish viability under different circumstances (e.g., after drug treatment of different durations), by determining the replicative potential of the cells (after cloning).

Chemosensitivity testing has been the area to which the assays described in this thesis have been most often used, so it would be pertinent to consider the application of these assays to this end. As we have seen, this area is fraught with technical hurdles which must be overcome before significant advances can be made. Probably the biggest stumbling block is poor tumour biopsy growth in culture. Any improvements in this area would contribute substantially to this end, as would the identification of factors adversely affecting tumour culture growth. Because of this, it was also decided to attempt to culture some primary lung tumor tissue and perhaps gain an insight to factors which need to be improved before chemosensitivity testing of these tumors can improve.

Resulting from the scrutiny of these assays, it was proposed to choose one or more assay from this panel, on the grounds of performance relative to the others, and identify areas where it could be applied (aside from chemosensitivity testing); for example environmental monitoring.

MATERIALS AND METHODS

2.1 Safety

All cells and biological samples were handled in a class II vertical laminar air flow cabinet. No infected tissue samples were processed, and in dealing with primary tissue, latex gloves were worn at all times. All materials which had been in contact with cells were autoclaved before disposal. Stock anticancer drugs were prepared in a fume cupboard. All subsequent drug work was performed in a Cytoguard safety laminar air flow cabinet, regularly validated by contract engineers. All materials which had been in contact with drugs were transferred to a fume hood and rinsed thoroughly in running water leading to a main drain. Copious amounts of water were emptied down the drain after drug rinsings to aid dilution. (Obviously all cell-contaminated drug waste was autoclaved before this step). Mutagenic and other dangerous chemicals (e.g., MTT) were weighed out on an analytical balance in a fume hood. For all these manipulations, latex gloves and a face mask were always worn.

2.2 Maintenance of sterility

Because animal cells in culture have such slow doubling times relative to micro-organisms, it is imperative that sterility be at a premium at all times. All materials were autoclaved before use (121°C for 20 minutes), except for unstable reagents, which were filtered through a 0.22 μm filter (Millex-GV, SLGV025BS). All sterile work was carried out in a class 2 vertical-flow laminar air flow cabinet adhering strictly and rigorously to aseptic technique.

2.3 Growth surface

Cells were grown in polystyrene tissue culture grade flasks (25cm² and 75cm²) (Costar 3055, 3375) and cluster dishes (6, 24 and 96 well) (Costar 3056, 3524, 3599) electrostatically treated to allow cell attachment. When larger cell numbers were required, cells were grown in glass roller bottles in 75 mls of medium. Nonadherent cells (small cell carcinomas) were sometimes grown in spinner flasks, which were first coated with Dimethyldichlorosilane solution (BDH 33164) to discourage cell attachment to the surfaces of the flask. Spinners were rinsed well after this treatment.

2.4 Media preparation

2.4.1 Glassware

All glassware used was first thoroughly washed by steeping (30 minutes - 2 hours) in a 1% solution of RBS (R. Borghraef) and then scrubbing with bottle brushes. They were washed thoroughly with tap water before 3 separate rinses in distilled water and one final rinse in ultrapure water. Caps were washed separately to prevent metals from leaching onto glassware.

2.4.2 Ultrapure water

High quality purity water is critical for cell culture. This was satisfied by firstly passing the water through two prefilters to remove ionic and non-ionic solutes and then through two ion-exchange filters, a carbon filter and a 0.22 μm cellulose acetate filter. The

reagent grade water which resulted was continuously monitored by an on-line conductivity meter and was used only when the resistance was between 10-18 megaOhms/cm.

2.4.3 Bottles

Stock media was prepared in 500ml bottles and transferred to sterile 100 ml bottles for routine use. Bottles for media preparation and use were kept solely for this purpose and stored separately from the stock of bottles used for collecting waste. All bottles were checked regularly for the presence of a rubber seal in the cap and for the absence of fractures.

2.4.4 Liquid media

To 450 mls of sterile ultrapure water the following constituents were added:

Constituent	Supplier	Catalogue no	concentration /100 mls	volume mls
10 x MEM	Gibco	042-01430M		50.0
10 x DMEM	Gibco	042-02501M		50.0
1M Hepes	Sigma	H9136	23.83 g	10.0
7.5% NaHCO ₃	Riedel-de Haen	31437	7.5 g	4.5
1M NaOH	BDH	30167	6.0 g	*
to MEM only:				
NEAA'	Gibco	043-1140H		5.0

* For MEM approximately 3.0 mls were needed to pH to 7.45 - 7.55, whereas for DMEM approximately 5.5 mls were needed.

' = Non Essential Amino Acids

(Hepes, NaHCO₃, and NaOH solutions were autoclaved before use; all other constituents were sterilized during manufacture).

2.4.5 Powdered media

Hams F12 powdered media (Gibco BRL 074-01700N) was added to 5L of sterile ultrapure water in an erlenmeyer flask and mixed thoroughly. 100 mls 1M Hepes buffer was added and the contents mixed thoroughly. The pH was adjusted to 7.45 - 7.55 with 5N NaOH. Using a peristaltic pump (Braun FE411) at a flow rate of 90 mls/min (the rate was set at 10 mls/ min initially and was gradually adjusted to this maximum level) and a length of autoclaved teflon tubing to which a 0.22µm high volume millipore bell filter (Gelman Sciences 12158) was attached; the medium was filtered into sterile 500 ml bottles.

2.4.6 pH

All media was adjusted to 7.45 - 7.55 pH units with sterile 1N NaOH or 1.5N HCl (BDH 28507) before use.

2.4.7 Sterility checks

An aliquot of medium from each labelled bottle was transferred into a sterile universal and incubated at 37°C for at least 4 days to show up any bacterial contamination. Blood agar plates (Medlabs), Thioglycollate broth (Oxoid, CM173) and Sabourand broth (Oxoid, CM143) were also inoculated with medium to determine the presence of microorganisms. Only media shown to be free from microbiological contamination was used.

2.4.8 L-Glutamine

After sterility checking of medium and before its use, 1 ml of 10mM L-Glutamine (Gibco 043-4030H) was added to 100 mls of medium.

2.4.9 Serum

Before use, serum was added to sterility checked media. Generally cells were grown in 5% serum. Because serum contains a host of undefined constituents, and because of the huge variability in the presence, amount and activity of these constituents from one batch of serum to another, its capacity for supporting growth can consequently vary substantially. It was therefore essential to screen serum batches before use to identify suitable batches. Suitable serum for growth was dictated by supply, performance (in terms of growth promoting ability) and cost. It was important that the same batch of serum be used for the whole duration of experiments to be compared. The serum routinely used for cell growth throughout this work was Foetal Calf Serum (FCS) (Sera Labs, batches 801117, 801111, 801017). The experimentation involved in batch testing and selection of these sera is described in 2.35 and 2.42.8.3

2.4.10 Antibiotics

Cells were routinely grown in antibiotic-free medium.

2.5 Incubators

Incubators were set at 37°C and were regularly checked with a thermometer. They were connected to an emergency electricity supply which switched over to an electrical generator in cases of power-cuts. CO₂ incubator regulators were set to provide a 5% CO₂ atmosphere from a CO₂ cylinder on a changeover unit. This unit switched to a standby cylinder, when the original cylinder became empty.

2.6 Thawing frozen stocks

To re-start a culture from stocks frozen in liquid nitrogen, a vial was carefully removed from storage using visor and gloves, and this transaction was entered in the catalogue system. The name and passage number of the cell line were carefully double checked. The vial was quickly thawed in a 37°C waterbath, resuspended in 5 mls of medium in a sterile universal and centrifuged (1000 rpm/ 5 mins) to remove the

cells from the 10% DMSO (DiMethylSulphOxide) solution in which they were frozen. Cells were then resuspended in fresh medium (10% serum), assessed for viability using the trypan blue method (see below) and seeded in a culture flask at a density of $\geq 10^6$ cells. The following day the culture was fed with fresh medium (10% serum).

2.7 Feeding cells

As cells metabolize in culture, they produce lactic acid which lowers the pH of the medium and turns the phenol red indicator present in the medium from red to orange/ yellow. This visually indicates that the medium is spent and must be replaced to enable further cell growth. Spent medium was poured into a waste-bottle and replaced by fresh medium (7 mls/ 25cm² flask; 20 mls/ 75cm² flask). Flasks were incubated at 37°C.

Different cell lines require different culture media, and the medium routinely used for culture of the different cell lines used here, is tabulated below:

cell line	cell type	growth medium
RPMI-2650	Nasal, squamous cell carcinoma	MEM
SK-LU-1	Lung, adenocarcinoma	D/HF12
SK-MES-1	Lung, squamous cell carcinoma	D/HF12
Calu 3	Lung, squamous cell carcinoma	D/HF12
DLKP	Lung, squamous cell carcinoma	D/HF12
DLRP	Lung, squamous cell carcinoma	D/HF12
SCC-9	Tongue, squamous cell carcinoma	D/HF12*
Hep-2	Cervix, squamous cell carcinoma	D/HF12
MDCK	Normal canine kidney, epithelial	MEM
NRK	Normal rat kidney, fibroblastic	DMEM

- where D/HF12 refers to equal volumes of DMEM/ Hams F12

* SCC-9 culture medium contained 0.4 µg/ml Hydrocortisone (Sigma H4001).

All of the above were routinely grown in 5% FCS except for Calu-3 which was grown in 10% FCS.

All of these lines were obtained from the ATCC (American Type Culture Collection) except for DLKP and DLRP which were established in this laboratory [Law *et al.*, in print].

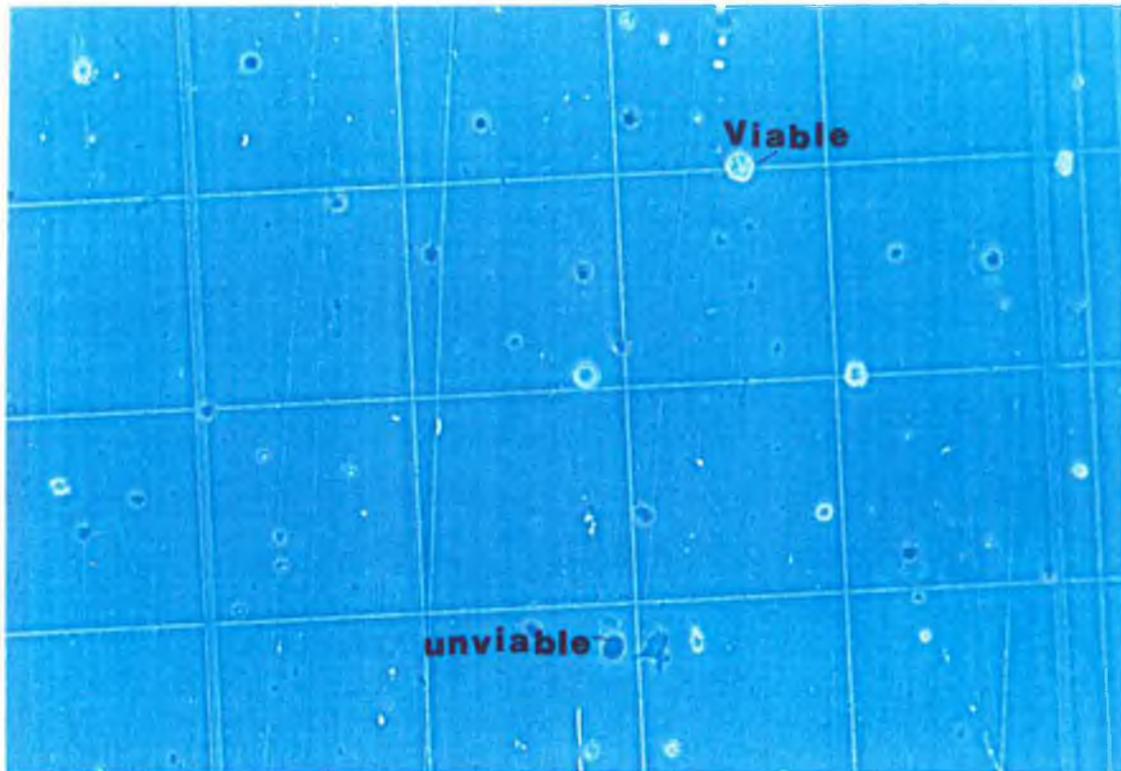
2.8 Subculturing

Anchorage dependent cells require a cell surface for attachment and growth. Once a cell monolayer becomes confluent, cells will generally become contact inhibited and will enter a stationary phase of growth. Some cells (Hep-2, for example) will continue to grow in piled up layers and form multilayer colonies. For a culture to remain in exponential phase though, it must be supplied with fresh medium and adequate free surface for attachment. To ensure this, cells are regularly subcultured (passaged) and seeded at lower densities in fresh medium. Cells were generally passaged twice weekly, but this depended on their growth characteristics. Very slow growing cells (for example, Calu-3) were passaged less often, whereas rapidly growing cells (MDCK, Hep-2) necessitated more frequent passaging. The frequency, of course, depended on the initial seeding density in the flask.

To passage the cells, medium was removed to a waste bottle and flasks were rinsed with PBS (Phosphate Buffered Saline, Oxoid BR14a; 1 tablet per 100 mls water before autoclaving). This was to remove any traces of FCS in the medium, as FCS contains a trypsin inactivator. Then Trypsin Verseine solution (TV) (0.25% Trypsin; Gibco, 043-05090H; and 0.022% Ethylene Diamine Tetrachloroacetic Acid (EDTA); Sigma ED2SS; in PBS) was added to flasks (2.5 mls/ 25cm² flask; 7.5 mls/ 75cm² flask) and incubated at 37°C for 10 - 15 minutes or until a single cell suspension was obtained. Then 5 mls of medium was added to the flask, and the liquid (containing cells) was removed to a universal (Greiner, 201175) and centrifuged at 1000 rpm for 5 minutes. The supernatant was poured off into a waste bottle and the pellet resuspended initially in 1 ml of medium with a Gilson pipette and then in 5 mls of medium. A cell count was performed (see below) and the required number of flasks were seeded at the desired density.

2.9 Counting cells

A 1:6 dilution of trypan blue (Gibco 043-05250H) in cell suspension was made in an eppendorf and after 5 minutes, 100 µl of the mixed suspension was placed on a haemocytometer. Each of the 4 large (16 small) squares on each arm of the hatched cross was counted and the average count obtained. Viable cells remained unstained as they excluded the dye, and those with a damaged cell membrane (generally equated with 'dead' cells) took up the dye and stained blue/brown. This is illustrated in the photograph below.



Photograph 2.1: Photomicrograph of a haemocytometer counting chamber (x 100), showing 'viable' cells excluding Trypan blue, while 'dead' cells are stained (blue/ brown) by the dye.

2.10 Freezing cells

It is imperative that cells for freezing should be free of all contamination and in good condition. It is preferable to freeze down cells at a high cell density to ensure an adequate viable cell seed upon thawing. Because cell size differs greatly, it is not relevant to report a universal cell number to freeze per vial. Instead no less than one 'almost' confluent 25cm² flask, or whenever possible one 75cm² flask was frozen in each vial.

Cells for freezing were trypsinized in the normal manner, and the cell pellet was resuspended in 0.75 mls for each vial to be frozen. An equal volume of 10% DMSO in medium was added dropwise very slowly, and with intermittent shaking. The suspension was then transferred into a cryovial (Greiner 122278) labelled clearly with the cell line, passage number and date, and placed in the vapour phase of the liquid nitrogen container. After 3 hours it was placed in the liquid phase and then catalogued. The freezers themselves were regularly checked and replenished with liquid nitrogen so that the level always remained well above the vial containers.

2.11 Prevention of contamination

Adherence to aseptic technique will prevent contamination by fungi, yeast and bacteria. Contamination, when it occurs can generally be spotted by careful microscopic examination. Contamination by mycoplasma, or cross contamination with other cell lines however, is generally not easily observed.

2.11.1 Mycoplasma

Contamination by mycoplasma may be expected if the cells in the culture change in their morphology (become more granular or 'raggy looking') or growth characteristics (slowing in growth rate) accompanied sometimes by sudden drop in pH of the medium. However the best way of monitoring the cultures is to regularly screen for the presence of these bacteria. Cultures were regularly tested for the presence of mycoplasma by the procedure outlined below and those found to be mycoplasma contaminated were immediately eliminated and laminar flow cabinets thoroughly cleaned or fumigated.

22 mm² cover slips were washed in 2% RBS, rinsed in ultrapure water and then in 70% IMS (Industrial Methylated Spirits, Lennox UN No. 1170) and dried in lint-free cloth before autoclaving in a glass petridish. Using a sterile forceps, one slide was placed into each 30mm³ culture dish.

The indicator cells used were low passage NRK which were known to be mycoplasma free. 1 ml of a 5×10^2 /ml cell suspension was added to each cover slip and incubated at 5% CO₂ overnight.

1ml culture medium (known to be antibiotic free) which had been in contact with cells for no less than 3 days, was added to the cover slips (2 replicates per culture). Control dishes had only fresh medium added.

After cells grew to approximately 50% confluence (3-4 days), slides were washed twice with PBS, once with PBS/ Carnoys reagent (1:1) and fixed in Carnoys for 10 minutes. Slides were air dried, washed twice

with ultrapure water and stained for 10 minutes with 2 mls dilute Hoescht (1/20,000 in water). Because Hoescht is light sensitive, these manipulations were carried out in petridishes covered in tinfoil.

Slides were washed 3 times in ultrapure water and mounted in glycerol mounting medium, pH 5.5, and examined under x40 and oil immersion lens on a fluroscent microscope using the UV filter. The phase contrast condenser was used to check cell morphology.

Cells appearing positive for mycoplasma presence had extranuclear fluorescence.

2.11.2 Cross contamination by other cell lines

Provided certain rules to eliminate contact among cell lines are adhered to, there should be no reason why cross contamination should occur, however, due to human error it is better to monitor cell lines for cross contamination. This was done by DNA fingerprinting.

Procedure followed in routine cell cutlure to prevent cross contamination of cell lines:

When freezing and thawing cell stocks, the entries on the label, catalogue card, and culture flask were double checked to ensure that the name of the cell line was clearly and correctly entered.

A separate waste bottle, 100ml bottle of medium and vial of trypsin was kept (and labelled so) for each cell line.

At any time, only one cell line was handled in the laminar flow cabinet. All universals containing cell suspension were clearly labelled with the name of the cell line, especially when centrifuging.

At no time was a pipette which had contained cells, placed into a medium bottle.

After working with each cell line, everything was removed from the laminar flow cabinet and swabbed thoroughly with 70% IMS. A clearing time of 15 minutes was left before the cabinet was swabbed down and used again.

Only in a clear laminar airflow cabinet was stock medium dispensed into freshly autoclaved bottles.

All flasks were clearly labelled with the name of the cell line, kept in discrete places in the incubator, and checked before any manipulations with the flask were carried out.

Any unlabelled medium or cell suspension were discarded.

2.12 Pretreatment of cells for experiments

To ensure that cells plated out in experiments would begin to grow in a uniform manner, it was imperative that all cells used were in exponential phase of growth when plating. Thus cells were always 'pretreated' 2 days before being used in the experiment, by seeding at a predetermined density which would result in a subconfluent and actively dividing culture on the day of the proposed experiment. The day before the experiment, cultures were again fed. Optimal seeding density for pretreatment was determined by observation of the growth rate in routine culture and are given below.

Table 2.12 Pretreatment cell seeding density used for various cell lines

cell line	pretreatment density used (cells/ 75cm ² flask)
RPMI-2650	6×10^6
SK-LU-1	9×10^5
SK-MES-1	1.5×10^6
Calu-3	9×10^6
DLKP	1×10^6
DLRP	1.5×10^6
SCC-9	1.2×10^6
Hep-2	2.5×10^6
MDCK	1.4×10^6
NRK	9×10^5

2.13 Preparation of cell suspension for experiments

In preparation of the cell suspension, the most important factor to be accommodated was achievement of a homogeneous single cell suspension. This however, was helped by previous pretreatment of the cells which meant that they were easily trypsinized and so formed a single cell suspension relatively easily. In resuspending the cell pellet, it was imperative to do so firstly in a minute amount of medium, and this was done with a Gilson tip. Gradually more medium was added and the

suspension was taken up and down using the pipette tip. The entire cell suspension was mixed thoroughly in this manner before counting and while making the appropriate dilutions.

2.14 Plating of cell suspension

The appropriate aliquot was dispensed down the side and into the well of the miniaturized dish. Bacterial petri-dishes were used to dispense the medium into the 96 well dish. Plates were gently, but thoroughly moved in all directions to disperse the cells in the wells and allow formation of an even monolayer. This was particularly important in 24 well plates to be read by image analysis.

2.15 Assay duration

Plates were incubated in a 37°C incubator at 5% CO₂, which, along with the Hepes and Sodium bicarbonate in the culture medium, formed the buffering system. Dishes were monitored regularly by microscopic examination throughout the incubation period, and were taken down before the highest density wells reached confluence.

2.16 Storage of cytotoxic agents

Drugs were reconstituted either in 0.9% NaCl, or in PBS (if no vial of NaCl accompanied the drug), diluted (1/100) in PBS and further diluted in medium before aliquoting in cryovials and freezing at -20°C. Vials were thawed and frozen no more than 3 times, after which a fresh vial was used. Cis Platinum (Eli-Lilli) was stored at room temperature, as recommended. Controls for the drug diluent were included in toxicity experiments.

2.17 Toxicity tests

Cells were plated at required density and allowed to attach. 24 hours later, double strength drug dilutions were added to the wells to cover the desired range of toxicity. For 24 well plates, 0.5 mls were added; for 96 well dishes 100 µl/ well was added. After an additional 6 days, plates were processed using the relevant endpoint. For 8 hour and 24 hour exposure experiments, drug medium was removed after the relevant exposure time, the cultures rinsed in PBS and replaced with fresh culture medium and the incubation allowed to continue for the remainder of the 7 day period.

2.18 Cell counting experiments to determine assay linearity and sensitivity

For experiments to determine linearity and sensitivity of the various methods, cells were plated at densities in the range 100 - 100,000 cells/ 96 well. Either 1 or 4 days later, cells were trypsinized and counted both by Coulter counter and by haemocytometer. On replicate plates colorimetric assays were performed. To perform cell counts, medium was removed and wells were rinsed in PBS. Then 50 μ l of double strength TV was added to each well. When trypsinization was complete, cells were taken up and down with the pipette tip to ensure a single cell suspension and the contents of 8 replicate wells were pooled in an eppendorf. A further 50 μ l of TV was added to the wells to remove any remaining cells, and this was also pooled and added to the original suspension. Haemocytometer counts were performed on trypan blue - exposed cells in the normal manner. For Coulter counts, a 1:20 dilution was performed in Isoton Balanced Electrolyte Solution (Coulter Electronics Ltd.,) and counted on a Coulter counter (Coulter Electronics Ltd, model DN) (blanked on Isoton). Cell counts/ ml were performed and these were transposed to cell number per 96 well.

2.19 Pipetting

All multiwell pipetting was carried out using an electronic multiwell pipette (Rainin EDP plus) using the appropriate pipette tips (Rainin RT 96). This generally gave low Coefficients of variation (< 1% for a single manipulation).

2.20 Monolayer staining with crystal violet

Medium was removed from plates by gentle flicking of medium into a container, and plates were rinsed twice with PBS (1ml for 24 well plate, 2 mls for 6 well plate). Plates were then fixed for 5 minutes in Formalin (BDH 101113) and then stained for 10 minutes in 0.25% aqueous Crystal Violet (Gurr) filtered before use in Wattman no. 1). Plates were rinsed 4 times in tap water, dried with paper to prevent water stains and then allowed to dry thoroughly in a 37°C incubator.

2.21 Image analysis

Using an AMS 40-10 image analyser, the optimum reading conditions were chosen for the set of plates to be read, and were carefully noted. These instrument settings were reproduced, as far as was possible each time plates were being read. Generally detection level had to be varied each time, especially when reading different cell lines. This parameter was chosen to compromise between what the instrument actually picked up and what was present on the plate. Because of flickering images, a voltage stabilizer had to be used and the instrument switched on overnight before being used. The surface area (mm²) occupied by the culture was determined by the instrument and used to represent growth.

2.22 Colorimetric assays

Each of the colorimetric assays described here were read on a Titretex multiskan plus ELISA plate reader using the 620nm filter as a reference wavelength. This subtracted interfering absorbance (from plastics, cells etc.,) from the total optical density and presented the results as absorbance at the test wavelength only.

All incubation periods were at 37°C and 5% CO₂, except where otherwise stated.

2.23 Crystal violet dye elution (CVDE)

After removal of medium, 96 well plates were rinsed with 100µl PBS/well and stained with 100µl 0.25% (g/ 100mls) Crystal Violet for 10 minutes. Plates were rinsed as outlined above. When dry, 100µl/well of 33% Glacial Acetic Acid (BDH 10001) (v/v) was added and the contents of each well were mixed before reading at 570nm. A typical assay is shown in the photograph below.



Photograph 2.2: A typical toxicity assay determined by the CVDE assay (increasing drug concentration from top to bottom).

2.24 Neutral Red (NR) assay

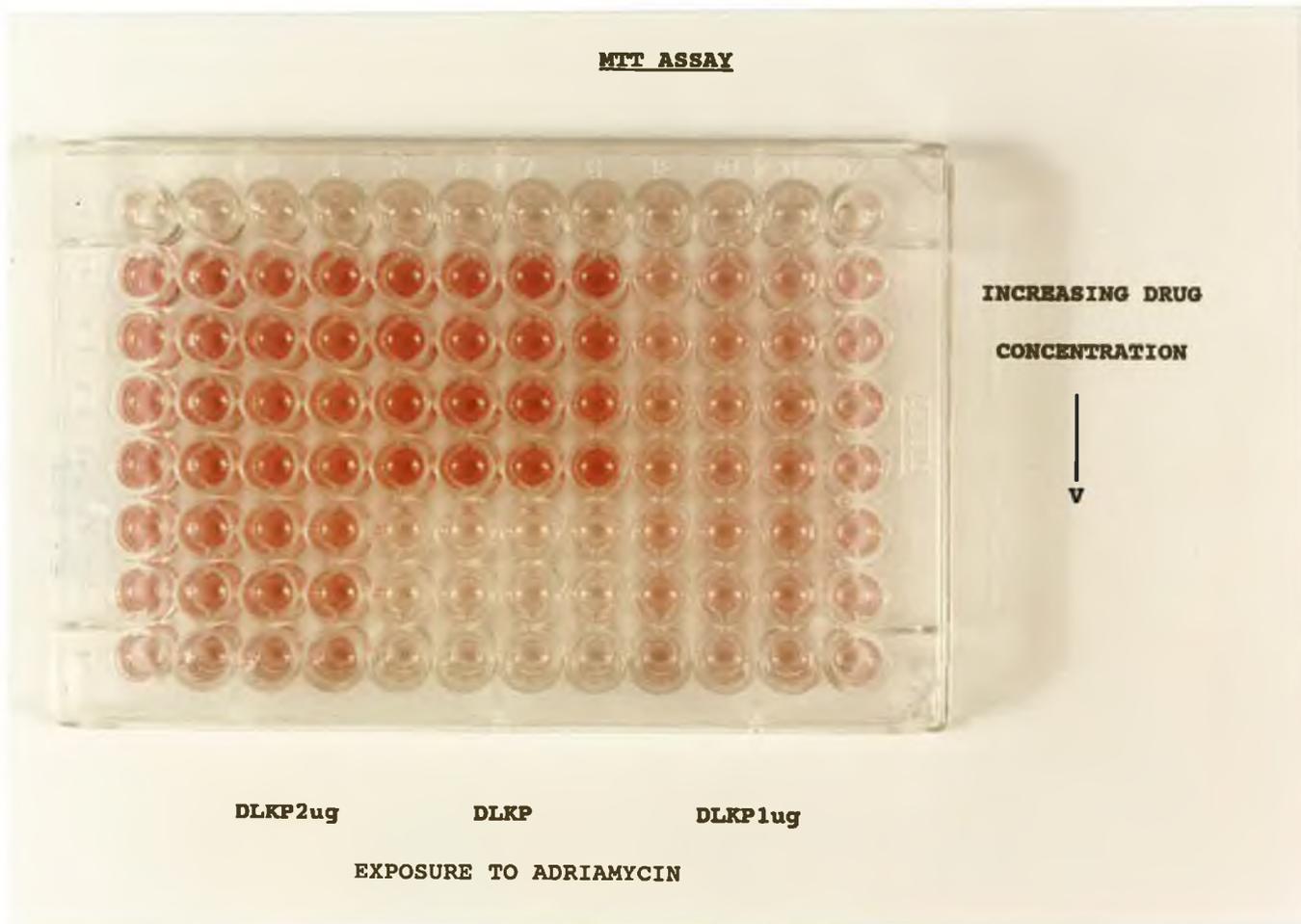
A 1:80 dilution of (stock) 0.4% Neutral Red (Difco, 50040) was prepared in culture medium, giving a final concentration of 50 μ g/ml. This was centrifuged at 1,500 x g for 10 minutes before use to remove insoluble crystals and the supernatant removed and used. Stock solutions were stored for up to 8 months protected from light.

Medium was removed from plates which were rinsed in PBS (200 μ l/well) before addition of the neutral red solution (prepared as above). After 3 hours incubation, neutral red was removed and wells were rinsed with 50 μ l of Formol-Calcium mixture (10 mls of 40% Formaldehyde, BDH 101113, and 10 mls of anhydrous Calcium Chloride; Sigma C7902; in 80 mls of ultrapure water). Then 200 μ l/well of Acetic Acid - Ethanol mixture was added (1% glacial acetic acid in 50% ethanol (mls/ 100 mls), before plates were shaken and read at 570nm.

Photograph 2.3: A typical toxicity test determined by the NR endpoint

2.25 MTT assay

20 μ l/ well of 5mg/ml MTT [3(4,5-Dimethylthiazol-2-yl) 2,5- diphenyl tetrazolium bromide] (Sigma, M-2128) was added before incubation for 4 hours. Then medium was *carefully* removed by pipetting (so as not to disturb the formazan crystals) and 100 μ l/ well DMSO was added and aspirated repeatedly to give a uniform colour before reading at 570nm.



Photograph 4: A typical toxicity test determined by MTT endpoint

2.26 MTT assay/ SDS modification

MTT was added to wells as in above, then after 4 hours incubation, 50 μ l of 25% SDS at pH 2.0 was added directly on top of medium. This pH is critical since the formazan colour is lost when the pH is too acid and the crystals do not dissolve if the pH is too high. Plates were incubated overnight to dissolve the formazan before reading at 570nm.

2.27 Sulforhodamine B (SRB) assay

Cells were first fixed by layering 50 μ l of 50% TCA (Trichloric acetic low, Reidel-de-Haen 27242) directly on top of incubation medium continued for a further hour at 2 $^{\circ}$ C. Then wells were rinsed 5 times with tap water to remove solutes and low molecular weight metabolites, flicked dry and then dried thoroughly. Cells were then stained with 200 μ l/ well of SRB (Sigma, S-9012) (0.4% in 1% acetic acid) for 30 minutes and rinsed 4 times in 1% glacial acetic acid. When dry 200 μ l/well of 10mM Tris buffer (Sigma T-8524; pH 10.5) was added to release unbound dye. After mixing well, plates were read at 570nm.



Photograph 2.5: A typical toxicity test determined by SRB.
(increasing drug concentration from top to bottom).

2.28 Acid phosphatase (AP) assay

Wells were rinsed in 100 μ l PBS after removal of medium. Then 100 μ l substrate - containing buffer was added to each well (10mM p-nitrophenyl phosphate; sigma C-104; added just before use to 0.1% Triton X-100; BDH; in 0.1M Sodium Acetate; Sigma S-2889; pH 5.5). Incubation proceeded for the standard time of 2 hours (or the test time indicated in the particular results) after which time plates were either read at 405nm, reincubated for a further time, or 'stopped' with addition of 50 μ l/well of 1N NaOH to cause an electrophilic shift in the P-Nitrophenyl chromophore and thus develop the yellow colour.



Photograph 2.28: A typical toxicity test determined by APNaOH.
(increasing drug concentration from top to bottom).

2.29 Relationship between assay performance and growth status of cell

Hep-2 cells, after pretreatment, were seeded in 24 well dishes at the following densities; Culture A - 2.5×10^3 / well, Culture B - 1×10^4 cells/ well, and Culture C - 2.5×10^4 cells/ well. One plate was set up with medium containing no cells to be used for blanking colorimetric assays. After culturing for 11 days Culture A was subconfluent, Culture B was confluent and Culture C was superconfluent and in very bad condition. Plates were centrifuged (1,000 rpm for 5 mins) and medium was carefully removed, wells rinsed in 500 μ l PBS and then carefully trypsinized with 250 μ l/ well 2X TV for 10 minutes and cells resuspended and mixed with a further 250 μ l of medium containing FCS, and a Trypan Blue count performed on 50 μ l of well mixed cell suspension. Triplicate wells were counted for each culture and a mean Trypan Blue cell count obtained. For each culture cell suspensions of 500, 700 and 1100 apparently viable (trypan blue excluding) cells/ ml were prepared and quadruplicate replicates were plated for cloning in fresh 24 well dishes. Cells were well shaken and dispersed in the dish before incubating for 10 days until colonies had formed from single cells.

Concurrent with trypsinization from the 24 wells, triplicate wells of each dish were used to perform AP2h, APNaOH, NR, CVDE, SRB and CV image analysis assays, simply by scaling up the volumes used in the 96 well dish by a factor of 6.25 (surface area of 96 well dish is 0.32cm²; that of 24 well dish is 2cm²). The final solution for colorimetric analysis was transferred to a 96 well plate in 100 μ l aliquots and read as described above; wells containing no cells were used in the normal way to prepare blanks.

Colonies arising from cloning of single cells from original plates were stained and counted manually and by image analysis after 7 days growth. Differences in viability among cultures A, B, and C were determined from counts of trypan blue excluding cells/ well, OD in colorimetric assays and colony number after cloning.

2.30 Ability of colorimetric assays to assess cell viability after treatment with cytotoxic agents

This experiment was first carried out in 24 well dishes, in a similar manner to the experiment described above, except that different drug concentrations were added to a single density of cells. 10^4 Hep-2 cells were seeded in ten 24 well dishes in a volume of 710 μ l. 24 hours later, 625 μ l of 2x 0.005 μ g/ml Vinblastine was added to one third of the wells, 625 μ l of 2x 0.006 μ g/ml Adriamycin added to one third of wells and 625 μ g/ml of control medium (no drug) added to the remainder of the wells. Again wells containing no cells were included for colorimetric blanks. Each day, beginning one day and finishing 5 days after drug addition, trypan blue counts were prepared from triplicate wells, cells were cloned exactly as described in 2.29, and AP2h, APNaOH and MTT assays were simultaneously carried out. Results were similarly compared as described in 2.29.

In scaling the experiment up to 25cm² flasks to improve accuracy on haemocytometer counts, cell densities and assay volumes were similarly scaled up by a factor of 78.125 over 96 well dishes (12.5 times over 24 well dishes), so cells were plated at 1.25×10^5 / flask in a volume of 7.8 mls, and a similar volume of 2x drug concentration

were added to flasks (to give final concentrations of 0, 0.005, 0.00075, and 0.001 μ g/ml Vinblastine for analysis after 2, 3 and 4 days exposure, and 0, 0.0002, 0.0004, 0.00075 μ g/ml Vinblastine for analysis after 6 days exposure). After 2,3,4 and 6 days exposure to drug triplicate flasks were trypsinized and counted in trypan blue. This time, cell suspensions for cloning were prepared and plated separately for two of these three replicates, for each drug point. Again cells were plated at 700, 900 and 1100 cells/ 24 well to give a range of growth for cloning. Each of the colorimetric assays was carried out exactly as described for the miniaturized version, except that volumes were scaled up as already indicated, and again aliquots were transferred into 96 well dishes for colorimetric analysis.

After colonies had formed from cloned cells (10 days), plates were stained as usual and analysed manually and by image analysis for colony formation. Results were compared as already described in 2.29.

2.31 Relationship between endpoint and mechanism of action

Preliminary experiments were first conducted on all of the 11 test chemicals from which concentrations giving a span of 0 to 100% cell kill were chosen. Leaving one row of wells per plate with medium only (no cells), 100 μ l of 2×10^5 RPMI-2650 cells/ well were plated in 96 well dishes and incubated for 24 hours, after which concentrations of each chemical were added. After an incubation time of 6 days, AP2h, MTT, NR and CVDE were performed as previously described on replicate plates for each of the chemicals. Radioincorporation assays were performed as described below. Dose response graphs were prepared in the usual manner.

2.32 Determination of DNA synthesis

24 hours before endpoint determination, 10 μ Ci/ml (3.7×10^5 Bq/ml) [3 H]thymidine (sp. activity 25 Ci/mmol) was added to each well of the plate. After incorporation of the [3 H]thymidine (and after microscopic analysis) the plate was washed twice with cold PBS, followed by addition of 5% cold TCA for 20 minutes at 4°C. After a further washing with 5% TCA, the plates were washed thoroughly with ethanol to remove all the TCA. The cells were solubilized by addition of 200 μ l solubilization buffer at 37°C for 1 minute. After extensive mixing with a pipette, 50 μ l aliquot samples were placed in 4 ml scintillation cocktail [Ecolite 882475] in a scintillation vial [Beckman], and counted for 1 minute on a scintillation counter [Beckman LS 7500]. Test results in cpm (counts per minute) were compared to control values to calculate % kill values.

Solubilization buffer:

(500ml) NaOH, 2g (final concentration 0.1M) Na₂CO₃, 10g (final concentration 2%) Dissolved in 500ml of distilled water. Just prior to use 1% SDS was added (10ml of a 10% stock/ 100 ml medium).

2.33 Determination of RNA synthesis

1 hour before assay termination, $10\mu\text{Ci}$ ($3.7 \times 10^5 \text{ Bq/ml}$) [^3H]uridine (sp. activity 5 Ci/mmol , 185 Bq/mmol) was added to plates, and plates were incubated at 37°C . Plates were washed once with PBS on ice and then 3 times with cold 5% TCA. Then $100 \mu\text{l}$ of cold 5% TCA was added and plates were heated for 1 hour in an 80°C water bath to hydrolyse the RNA. $50\mu\text{l}$ samples from each well were placed in 4 ml scintillation fluid and these were read for 1 minute on a Beckman scintillation counter.

2.34 Determination of protein synthesis

Determination of protein synthesis by incorporation of [^3H]leucine was carried out as above except that the 1 hour 80°C incubation period was omitted. Instead of a 20 min TCA treatment step the precipitates were washed with ethanol and solubilized as in 2.32.

2.35 Miniaturized serum batch testing

Cells were seeded into 96 well plates in $100\mu\text{l}$ serum free medium. Serum samples for testing were prepared at 2x desired concentration in culture medium (minus FCS) and $100\mu\text{l}$ / well added to the cells. Before the cells became confluent the plate was removed from the incubator and the extent of growth determined using the miniaturized endpoints described earlier.

2.36 Culture of drug-resistant lines

DLKPA (already adapted to growth in $0.75 \mu\text{g/ml}$ Adriamycin in this laboratory) was cultured in DMEM/HF12 in the presence of $0.75 \mu\text{g/ml}$ of Adriamycin and gradually increased to concentrations of 1 and $2\mu\text{g/ml}$ before use in assays. Cells were grown in the relevant concentration of drug for at least 2 weeks prior to assay. For toxicity testing experiments, cells were seeded in 96-well dishes (4×10^3 / well for variants; 3×10^3 for parent cell line (due to higher doubling rate)). 24 hours later, drug concentrations were added (4 replicates/ drug concentration) and experiment continued for a further 6 days. AP2h, APNaOH, MTT, NR, CVDE and SRB assays were then performed and drug toxicity profiles determined.

2.37 Toxicity testing of landfill leachates

Leachates were collected from a landfill site (by a Local Authority) at 3 regular monitoring stations. They were centrifuged to remove any solids, pH adjusted to 7.45 - 7.55 and then filter sterilized before use. Concentrations prepared in culture medium, were tested in the range 56% - 0.032% (mls/ 100 mls) on a monolayer of MDCK cells which had attached for 24 hours (passage numbers 102 - 107; 5×10^3 / 96-well) for 72 hours. Tap and uptrapure water were used as controls at equivalent concentrations. Osmolarity readings for all samples were obtained using a Osmomat 0300 Osmometer. IC_{50} and Toxic Unit values were obtained as described in Appendix 1.

2.38 Toxicity testing of chemical sludges

Sludges were obtained from EOLAS in Shannon. Leachates were generated by preparing a 10% w/v solution in ultrapure water and shaking on a shaking table for 8 hours. After settling for 8 hours (photograph 7) pH was then adjusted to 7.45 - 7.55 and samples were filtered through Wattman no. 41 and filter sterilized before testing on MDCK as described in 2.37.



Photograph 2.38: Two very different industrial sludges after leachate generation and settling for 8 hours.

2.39 Toxicity testing of industrial wastewaters

5 wastewater samples were obtained and their pH adjusted to 7.45 - 7.55 before filter sterilizing. They were tested as above on MDCK using the AP assay.

2.40 Analysis of results

No less than 3, and typically 4 replicate wells were used in the 24 well system, while 8 replicate wells were used when using 96 well plates. The mean and standard error of the mean (SEM) were calculated from replicates for each point of a graph (see Appendix 1). Drug treated wells were compared to control wells and % survival was calculated. Graphs were plotted using the software package Sigmaplot, and IC_{50} values (values showing concentrations which have a specific effect; in this case loss of AP activity; on 50% of the population) were calculated. Error bars are shown in all graphs.

2.41 Primary culture

2.41.1 Sample collection

Samples were collected from resection and immediately placed in collection medium (1:1 DMEM/Hams F12, 10% FCS, 1% L-Glutamine, 1% Fungizone; 500 μ g/ 100 mls (Gibco 043-05290D), 500 U/ ml Penicillin, 0.5 mg/ml Streptomycin (combined; Gibco, 043-05070H) at 2°C, and transported to the laboratory in a cooler bag with ice.

2.41.2 Sample storage

When multiple samples were obtained, samples were kept at 2°C until processing could be carried out.

2.41.3 Sample dissection

Necrotic tissue, blood vessels, fatty and connective tissue were removed from the sample with scissors and scalpels in a petridish (Greiner 633171). Then the sample was rinsed a number of times in PBS and chopped finely with crossed scalpels.

2.41.4 Explants

For explant culture, firm tissue pieces $\leq 1\text{mm}^2$ were placed in a 25cm^2 culture flask coated with culture medium using a forceps. At least 20 tissue explants were placed in the flask, each approximately 0.5 cm apart. Then the flask was left to stand upright in the laminar flow cabinet with the lid loose for 30 minutes, to allow the explants to attach to the substratum. Then 4 mls of culture medium was very carefully added to the flask, taking care not to dislodge any of the explants. After 24 hours another 4 mls of medium was added and the culture monitored and fed as necessary thereafter.

2.41.5 Enzymatic disaggregation

Any further tissue was dissected with crossed scalpels to pieces of 2mm^2 size and placed in a flat-bottomed 60 ml universal (Sterilin 125AP) with 200 units of collagenase (Sigma C6885) and 0.2% Protease

neutrale (dispase) (Bohringer Mannheim GmbH 165859) in 10 mls of medium. A sterile magnetic stirring bar was included and the universal placed at gentle stirring speed in a 37°C warm room. At 30 minute intervals throughout the digestion, an aliquot of the suspension was checked microscopically for cell clumps released from the extracellular matrix. When most of the tissue had been disaggregated and numerous free clumps of cells were observed, the suspension was allowed to gravity sediment. The supernatant was removed from undisaggregated tissue clumps and again gravity sedimented to allow clumps of cells to sediment. This lower fraction was usually enriched with epithelial cell clumps, while the upper fraction was enriched with single epithelial and fibroblastic cells. The fractions were then centrifuged (1000 rpm for 5 minutes) and resuspended in fresh medium. 25cm² flasks were then seeded at a density of 10⁶ cells, or as high a density as could be obtained for small samples. Cultures seeded at high densities of epithelial cells stood a better chance of overcoming fibroblast overgrowth, and of growing up in culture. Clumps of cells were preferable for culturing than single cells for the same reasons.

2.41.6 Dissection medium (DM)

The culture medium in the petri-dish from which the explants and tissue for enzymatic disaggregation were removed, was usually heavily enriched with cells. This was centrifuged, washed in PBS and then resuspended in culture medium.

2.42.7 Isolation of blood cells

In the case of heavily bloodstained samples, these were first rinsed vigorously several times in a universal of PBS and then processed as normal. The processed sample suspension in culture medium, was then layered on top of 15 mls of Ficoll (Pharmacia) in a universal, using a pasteur pipette. Two distinct phases were formed. After centrifugation at 3500 x g for 10 minutes, the top layer of medium, which contained no cells, was removed to waste. The epithelial cells were found at the interface between the two phases but some were also present in the Ficoll layer itself. The erythrocytes formed a pellet at the bottom. The fractions containing epithelial cells were isolated with a pasteur pipette and centrifuged in 5 mls of culture medium. The cells were then processed as normal

2.42.8 Primary culture assays

Most of the samples received for processing, generated a very small number of epithelial cells, not sufficient to allow testing of different variables. Where possible, cells were plated into 24 well dishes and a number of variables were tested as described below.

2.42.8.1 Density assays

Cell suspensions covering the relevant density range were prepared and 1ml of suspension was plated per well.

2.42.8.2 Basal media assays

A single cell suspension was prepared and 0.5 ml added to each well. Then 0.5 ml of the relevant basal medium was added. The following day, or when cells had attached, medium was carefully removed from wells with a pasteur pipette and replaced by the relevant fresh medium. The reason for avoiding preparation of a number of cell suspensions for each of the different media was to avoid the error which this would introduced by this technique. Because a cell suspension isolated from tissue can not be expected to be fully homogeneous, such an error would be significant.

2.42.8.3 Serum batch tests

The cell suspension was prepared in medium minus serum and 0.5 mls were added to each well. Then 0.5 mls of medium plus test serum was added to the wells. Because cell density per ml has now been diluted 1:2 and simultaneously, the serum concentration (%) has also been halved, double strength of each of these preparations were used to overcome this dilution factor.

All plates were carefully and thoroughly shaken to distribute the cell suspension evenly by slowly but vigourously moving the plates in alternate directions.

Plate exteriors were swabbed with 70% IMS and incubated at 37°C/ 5% CO₂, and taken down before wells with most growth had reached confluence. They were stained with crystal violet and read by image analysis.

2.42.9 Maintenance of primary culture

The culture was checked regularly and fed as medium was utilized. Cultures, when confluent, were passaged with trypsin (0.25%) in the normal manner. Trypsin was preferred to TV because of its gentler action. Monitoring of cultures was felt to be a very important process, as fibroblast overgrowth; which was often a limiting factor in the success of the cultures; could be observed and sometimes prevented.

2.42.10 Fibroblast removal

This was carried out in two ways:

2.42.10.1 Trypsin/ EDTA

Cultures were exposed to lower strength TV (0.04% Trypsin, 0.01% EDTA) at 2°C for a few minutes. Fibroblasts most often became detached quicker than the rest of the culture and were removed. However this method only worked well if epithelial cells were in colonies and fibroblasts were in single cells or in sheets. If epithelial cells were present only as single cells or tiny colonies, then these cells were equally vulnerable to this trypsinization procedure.

2.42.10.2 Geneticin treatment

Fibroblast experiments:

For a number of fibroblast cultures, Geneticin dose-response curves were constructed. Fibroblast cells were plated in 24 well dishes at a concentration of 5×10^2 cells/ ml and allowed to grow. When the

culture was actively growing (2 days after setting up) concentrations of Geneticin (G 418 Sulphate, Gibco 066-1811) in the range 0 - 500 $\mu\text{g}/\text{ml}$ were added in culture medium. Again because of the dilution factor due to the medium already present in the wells, double strength of the final desired concentration were prepared. Each day for 4 consecutive days, a set of plates were removed and stained with crystal violet and a kill curve was prepared.

For routine use thereafter, 200 $\mu\text{g}/\text{ml}$ of this agent was added to growing cultures in culture medium. After 2 days, medium was removed and cultures were rinsed with PBS before feeding with fresh medium.

RESULTS 1:

ASSAY COMPARISON

3.1 Image Analysis

3.1.1 Cell distribution on the plate

It was established that cell distribution on the plate is an extremely important phenomenon when reading plates by image analysis. The result obtained depends upon the surface topography of the monolayer, thus, even though the same number of cells may be present, if they are not spread evenly over the plate, then totally different readings may be obtained.

3.1.2 Staining

Image analysis relies greatly on effective cell staining. Only then can colonies be distinguished from their background, and their image quantified by computer analysis. If wells are stained for too long, then plastic will also be stained, which will then be imaged and quantified, giving erroneous results. A similar situation has been found to arise when stained wells are improperly rinsed and dried; water marks resulting from this will also be detected by the instrument.

3.1.3 Instrument settings

It was found that two very important settings on the instrument are the light and detector sensitivity settings. If either of these were changed, the image being detected and processed changed accordingly. It was obvious that the absolute values resulting from reading a single plate at different detector sensitivities varied, however it was not clear whether the observed trend would also be altered. By reading a number of stained toxicity assays at different settings, and preparing dose response data, it was found however that altering the detector sensitivity could in fact result in significantly different overall results. Some of the observed results are shown in the table below. It is obvious that choosing an unsuitable setting would give improper results (for example if the detection level was much too high or too low to pick up growth); note however that both settings chosen here could be described as two distinct, but suitable settings for the plate analysed.

Table 3.1.3 Effect of varying image analyser detector sensitivity, on the outcome of toxicity curves of RPMI-2650 exposure to Vinblastine

Drug conc. $\mu\text{g/ml}$	Plate 1	Plate 2	Plate 3	Plate 4	Plate 5	Plate 6
Detector sensitivity = 35 colony area (% of control)						
0.00	100 \pm 31	100 \pm 8	100 \pm 32	100 \pm 8	100 \pm 10	
0.005	109 32	77 12	145 39	72 10	93 17	
0.01	92 21	69 69	43 36	45 5	48 3	
0.05	76 16	60 14	46 28	14 2	18 3	
0.1	87 74	14 4	61 7	11 2	8 1	
0.5	55 -	37 7	27 20	2 1	2 1	
1.0	61 1	9 4	19 5	1 2	2 0	
5.0	33 23	4 3	11 11	1 0	1 0	
Detector sensitivity = 57 colony area (% of control)						
0.0	100 \pm 61	100 \pm 11	100 \pm 27	100 \pm 10	100 \pm 10	100 \pm 4
0.005	162 48	71 11	80 41	57 2	66 16	78 21
0.01	153 31	42 36	40 28	38 4	30 2	41 2
0.05	112 24	44 8	32 20	9 1	5 1	7 1
0.1	153 11	9 1	51 8	5 1	2 0	3 0
0.5	145 83	23 4	24 13	1 1	0 0	0 0
1.0	161 77	7 9	25 8	1 0	0 0	0 0
5.0	119 45	2 1	20 12	1 0	0 0	0 0

Plates 1 to 6 in the tables above, represent toxicity experiments with 1 to 6 days' cell growth in drug.

Changing the detector sensitivity, does in fact, alter the final outcome of the results, and IC_{50} values estimated from these two different sets of results, would be substantially different. Plate 1 had in fact, very little growth present, so there is a vast difference in the observed results after reading at the two different sensitivities. This of course is largely due to low amount of cell

growth on the plate, however it may be concluded from this, that increasing detector sensitivity on low density plates does not make the plate any more suitable for reading; in fact it has detected no cell kill (when some was visible to the eye) and has resulted in enormous SEM values.

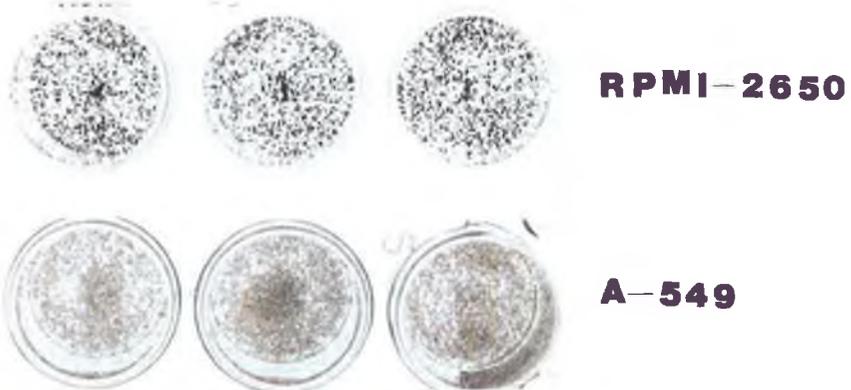
On plates 4 and 5, significant differences in % survival, arising from the two different readings are evident, when SEM values are taken into consideration.

It must then be concluded that detector sensitivity is a parameter which deserves special attention, and it must not be indiscriminately altered without expecting consequent alteration in observed results. The practical significance of this information means that for this model of image analyser at least (AMS 40-10), as much as is possible, constant settings should be used throughout a section of work, or a control stained plate should be used to set the instrument settings appropriately, before each set of plates are read. Obviously if a group of plates are read together, they will be more appropriate for direct comparisons of absolute values, than plates which were read on different occasions. As a consequence of such observations, settings were defined for each cell line, and these were adhered to in all experimental work thereafter.

3.1.4 Applicability to different cell lines

It has been made apparent that there may be problems in defining an appropriate detector sensitivity for a stained plate. In fact, it was generally observed that the most appropriate detector sensitivity (visually) for the plate gave a computerized image which did not correspond well with the actual stained image. This was more of a problem with some cell lines than others. It was determined that dense, discrete, clearly stained colonies were reasonably well imaged by the instrument, while diffuse, sprawling, poorly stained colonies, or complete monolayers, were very poorly imaged, no matter which instrument settings were used.

It was determined thus, that some cell lines were more appropriate for image analysis determination, due to their growth characteristics, than others. The following photograph, which shows growth characteristics of a whole range of cell lines, illustrates this point.



Photograph 3.1 Variation in stained growth characteristics of a range of cell lines

Some of the cell lines (RPMI-2650 and SCC-9, for example) form discrete tight colonies, and are hence suitable for image analysis growth determination, while those forming diffuse monolayers (e.g. A549), are poorly estimated on the image analyser.

3.1.5 Ease of performance/ technical limitations

While the method is relatively easy to perform (plates are rinsed, stained with crystal violet, rinsed, dried and analysed by the instrument) the technical difficulties involved with reading plates on this model are often significant.

- * Some cell lines are unsuitable for analysis
- * Lines suitable for analysis, but with unevenly distributed growth will not be accurately determined
- * Clearly stained plates of cell lines suitable for analysis with touching colonies may not be accurately determined
- * Poorly stained plates, or plates with 'water marks' from improper drying may not be accurately determined
- * It is important to try and use a constant detector sensitivity for a group of plates read together, however a single setting may not be appropriate for numerous plates
- * A single detector sensitivity setting may not give an appropriate image for plates in which there is a wide span of cell growth, for example, in toxicity tests
- * Plates with extensive cell kill will result in much cell debris, some of which can remain on the plate after rinsing, and may be determined by the instrument as additional colony growth

3.2 MTT assay

3.2.1 Optimization

3.2.1.1 Formazan solubilization

In the original method (Mossman, 1983), acid isopropanol was used to solubilize the insoluble formazan precipitate formed. It was found in the course of this work however, that this resulted in formation of a dense precipitate (presumably from precipitation of serum proteins) which strongly interfered with absorbance readings. Many slight modifications of this procedure were tried, including exclusion of serum from the MTT incubation medium and rinsing before solvent addition, however these additional manipulations still gave unsatisfactory results; some formazan was lost upon rinsing, and the remainder would not fully dissolve in the acid isopropanol.

Solvents used by other workers for this purpose included DMSO (Freshney and Plum, 1988; personal communication; Twentyman and Luscombe, 1987), so this solvent was used in this work and results were very satisfactory; there was no need to change the culture medium on addition of MTT, no rinse was needed after incubation with MTT and most pertinently, complete solubilization was quickly and uniformly achieved. Thereafter this solvent was used for the assay.

3.2.1.2 MTT Concentration

Presumably the concentration of MTT used in the assay influences the results obtained, therefore the effect of using different MTT concentrations was investigated. The results for RPMI-2650 and Hep-2 are shown below.

Figure 3.2.1 (a) shows that increasing MTT concentration results in a corresponding increase in O.D. in an approximately linear manner from 0.1 to 4 mg/ml MTT. At 5 mg/ml no further increase in O.D. was obtained. Thus the optimum concentration for use is somewhere between 4 and 5 mg/ml MTT for RPMI-2650 under these conditions.

In Figure 3.2.1 (b), the relationship between MTT concentration and O.D. is shown for another cell line, Hep-2, for the concentration range 1 - 7mg/ml MTT over a range of cell densities. The plots are not linear; the most significant increase in OD is gained when the MTT concentration is increased from 1 to 3 mg/ml. Thereafter further increase to 7mg/ml gives a relatively smaller increase in OD. The higher the cell density per well, the greater the slope of the plot from 1 to 3 mg/ml. At the highest cell density (just under 1×10^6 cells/well), optimum absorbance has been reached at 3mg/ml MTT; for all the other cell densities, any concentration between 3 and 7mg/ml could be used, as there is little difference between these points. Taking 5mg/ml as the midway point, and considering that most of the literature reports use of this concentration, for comparative purposes, 5mg/ml MTT was used routinely thereafter.

3.2.1.3 Storage of MTT

It has been reported in the literature that a single stock of MTT working solution may be stored at 2°C and used for a number of weeks. Some workers (C. Lowry; personal communication) have found it necessary to make up a fresh working solution of MTT each day; this is

obviously more cumbersome, particularly as special handling is required when dealing with the agent due to its mutagenic properties. Thus the importance of making up a fresh stock was considered. The performance of an 8-week-old stock was compared to that of a freshly prepared stock.

Figure 3.2.1 (c) shows that there is little difference in the absorbance values between the old and the fresh MTT solutions; the error bars overlap for most points. There appears to be only a slight loss of activity when the MTT is stored for this period of time. In routine work thus, the solution was stored in a light resistant bottle at 2°C for periods up to 6 weeks, after which a new stock was used.

3.2.1.4 Outer wells of 96-well plate

Figure 3.2.1 (d) shows that there is no significant difference in results when the outer wells of the 96-well dish are included in the experimental results, thus outer wells were always used in the work which follows.

3.2.2 Relationship between cell number and MTT O.D

To establish the relationship between cell number and O.D in the MTT assay, cells were plated at densities in the range 100 - 100,000 cells/ 96 well. After 1 day and again after 4 days growth, cells were trypsinized and counted both by Coulter counter and haemocytometer. MTT assays were performed on replicate plates and cell number was plotted against O.D. over the entire range of cell densities. These experiments were performed on 6 epithelial cell lines and 1 fibroblastic cell line in order to establish the relationship for a range of cell lines. Experiments were repeated once for RPMI-2650, SKMES-1 and Hep-2 and twice for DLKP. Results from one repeat experiment have been shown in the figures and tables which follow, for SK-MES-1, Hep-2 and DLKP. Graphs of cell number versus O.D. have been shown for growth after 1 day and growth after 4 days in culture. The method of obtaining cell counts (*i.e.*, haemocytometer or Coulter counter) is indicated below each graph.

Counts from Haemocytometer and Coulter counter did not always agree. Some data points were missing for the haemocytometer counts because the limitations of the method did not allow low counts to be performed, and frequently resulted in high SEM values.

From these graphs, shown in Figures 3.2.2 (a) - (n), the minimum detectable cell density, extent of the linear range, and the sensitivity of the assay have been determined for each of the cell lines. The minimum detectable cell number has been empirically defined as the cell number corresponding to 0.02 OD units. The linear portion of the graph is indicated in each figure where it is clear (the range is ambiguous for those marked *). The slope of the linear portion of each graph has been calculated (see appendix A) to obtain a value for sensitivity. The cell number to which we refer in both graphs and tables, is actual cell number, and not the cell number which was originally plated.

The tables summarizing these results are reproduced in the following pages.

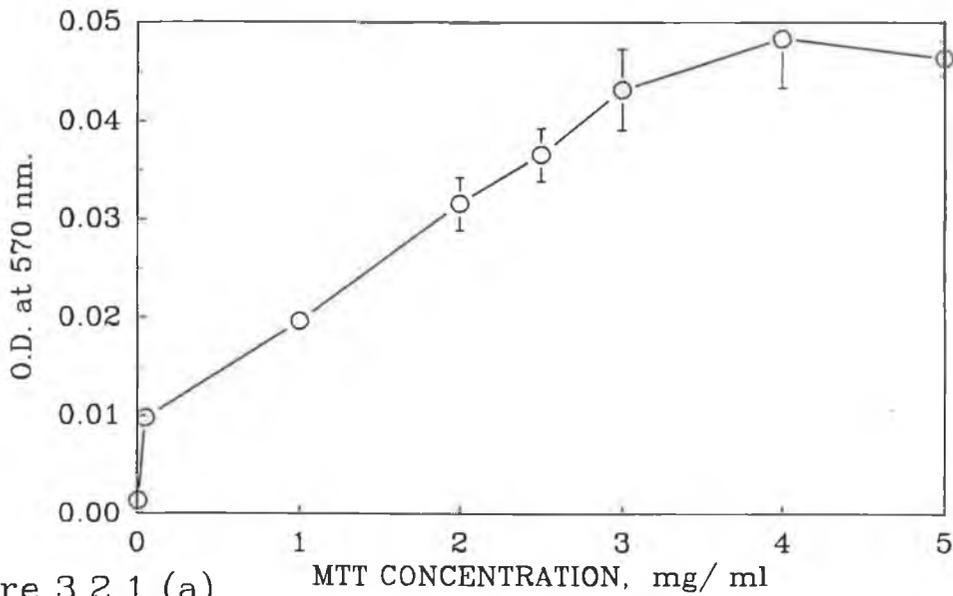


Figure 3.2.1 (a) MTT CONCENTRATION, mg/ ml
 Determination of optimum MTT concentration for RPMI-2650
 10,000 cells/ well were seeded and plates incubated for 2 days before assay determination

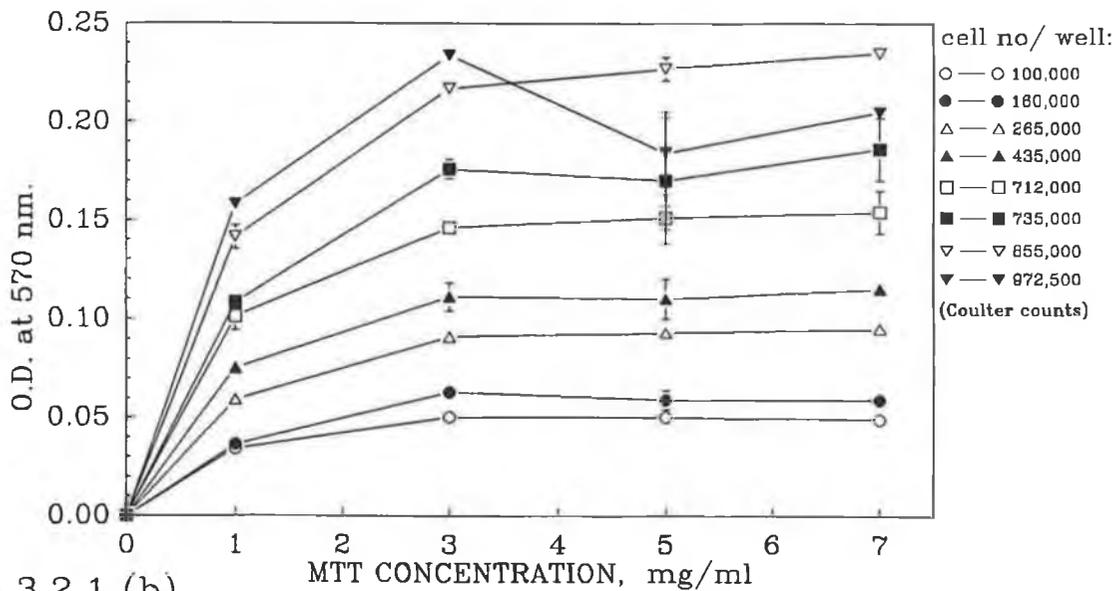


Figure 3.2.1 (b) MTT CONCENTRATION, mg/ml
 Determination of optimum concentration for Hep-2 cells.
 Cells seeded at densities indicated were grown in 96 well dishes for 6 day
 MTT assays were performed and cells from replicate plate trypsinized and counted.

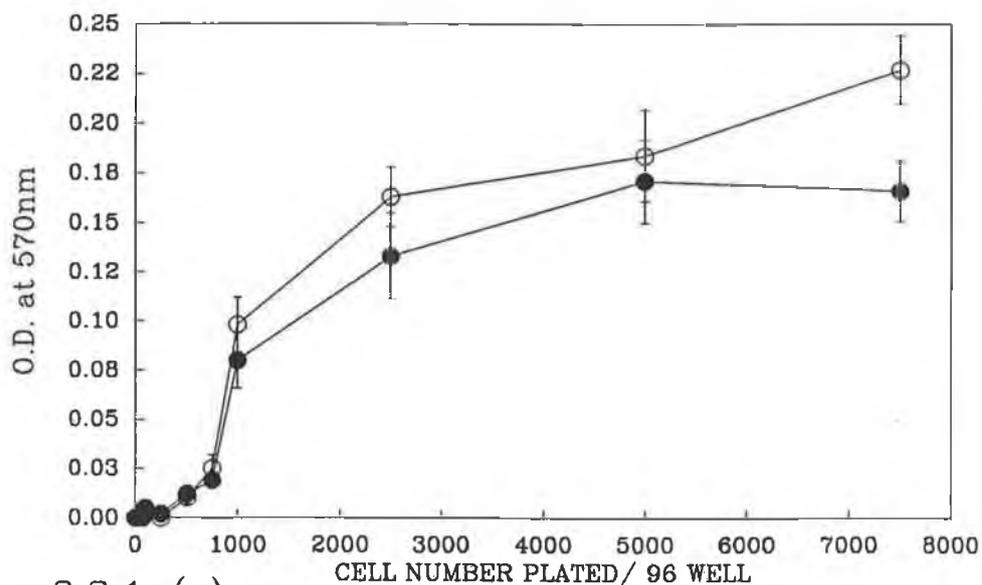


Figure 3.2.1. (c)
Activity of a freshly prepared MTT solution (○—○) and an 8-week old solution (●—●) with varying numbers of SCC-9 cells.

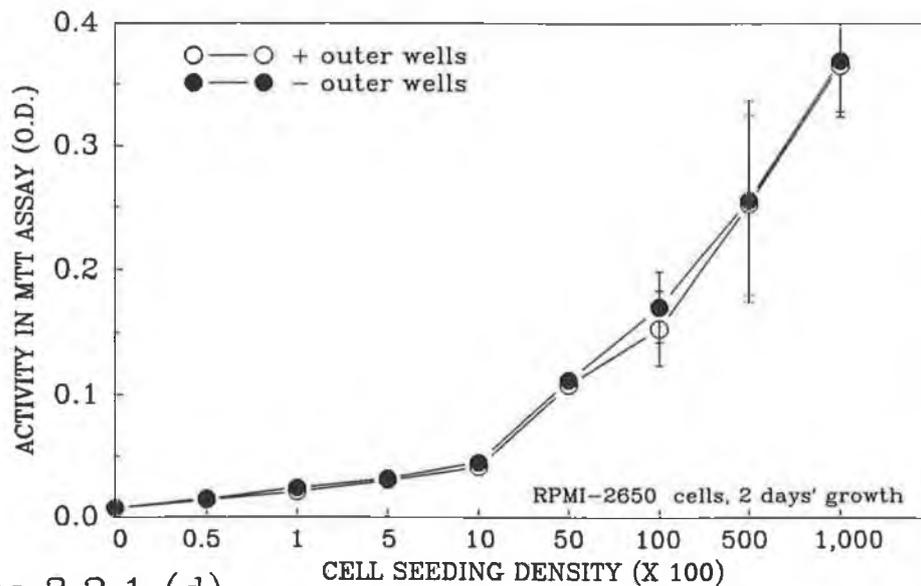


Figure 3.2.1 (d)
Effect of including/ excluding outer wells of 96 well plate from results

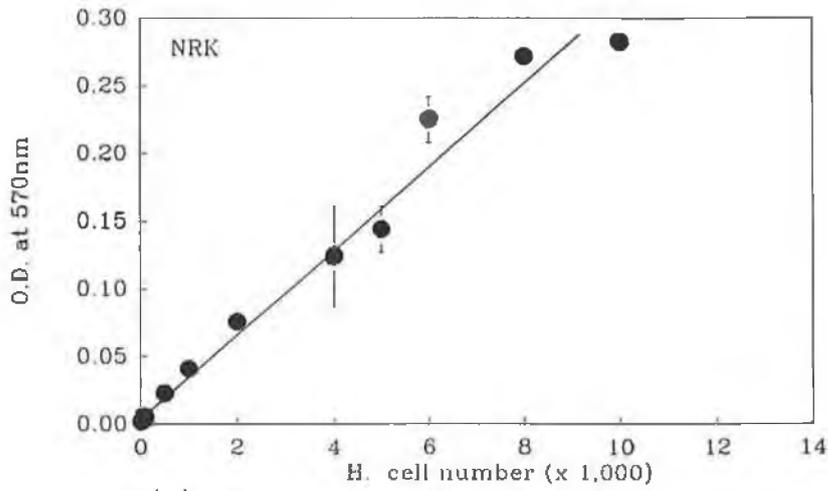


Figure 3.2.2 (a)

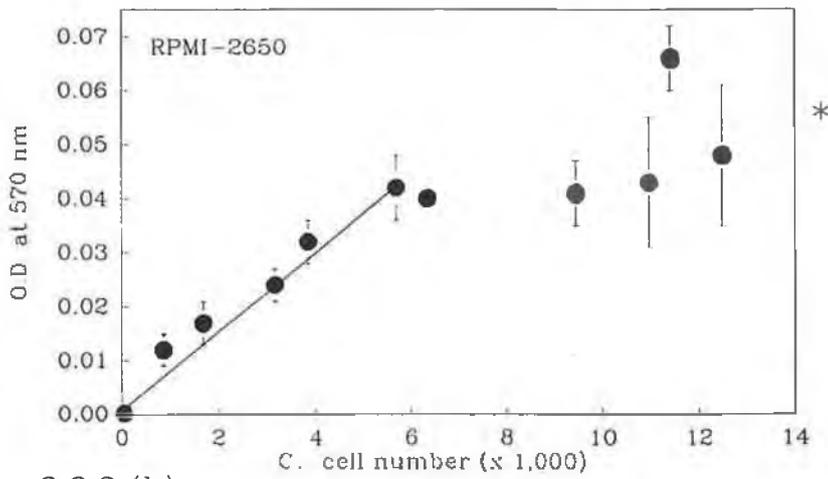


Figure 3.2.2 (b)

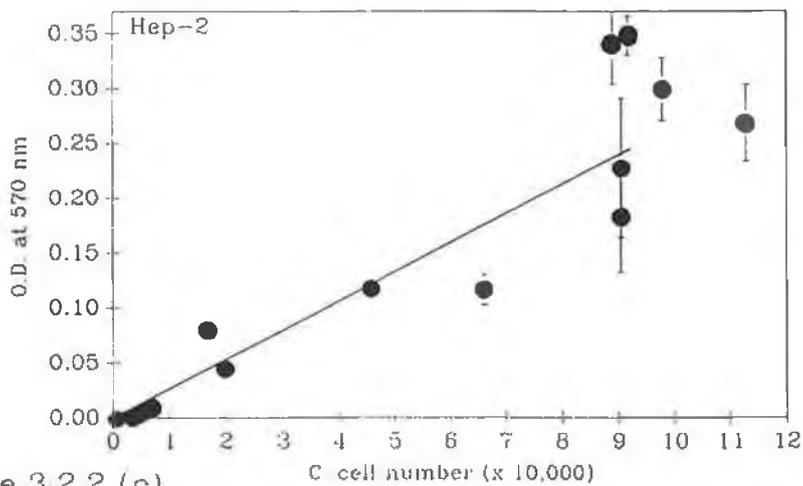


Figure 3.2.2 (c)

Figures 3.2.2 (a) - (c):

Relationship between cell number and Optical Density in MTT assay for the cell line indicated, after 1 day's growth.

H. represents haemocytometer counts. C. represents coulter counts.

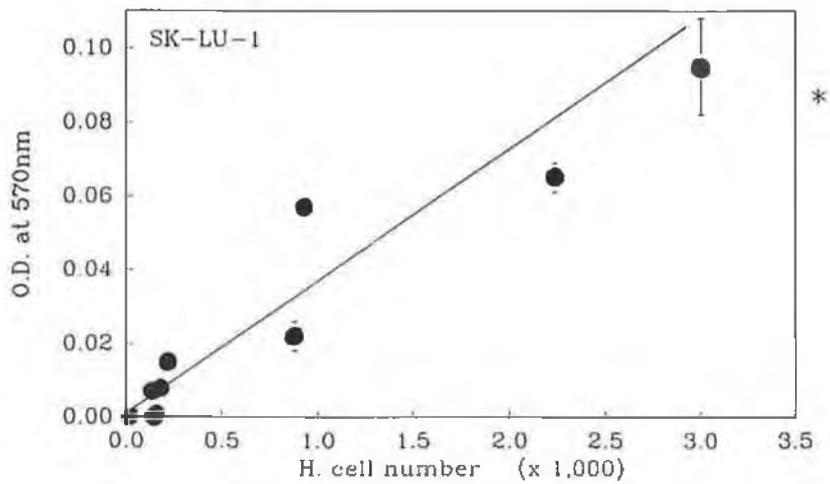


Figure 3.2.2 (d)

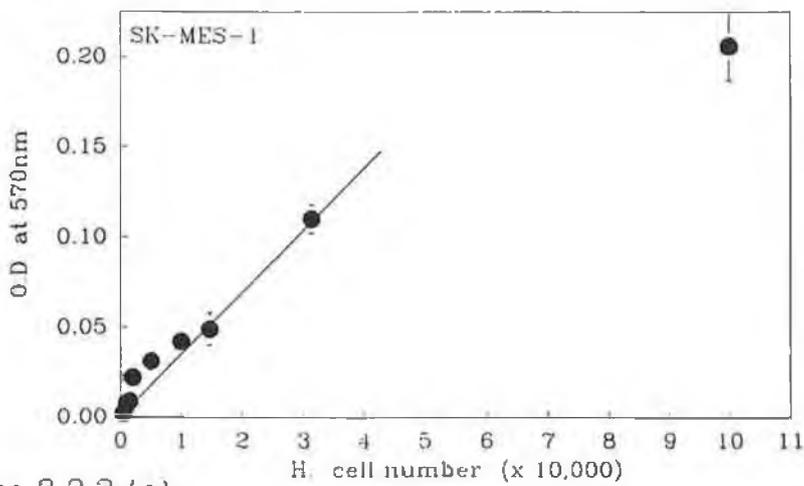


Figure 3.2.2 (e)

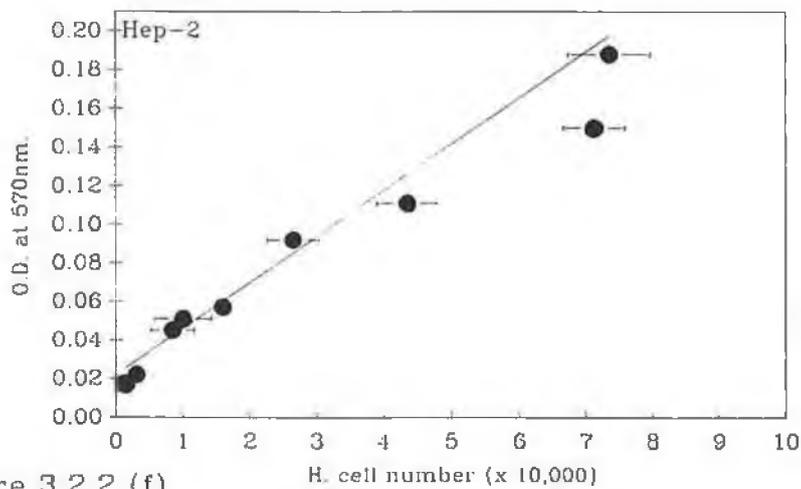


Figure 3.2.2 (f)

Figures 3.2.2 (d) - (f).

Relationship between cell number and Optical Density in MIT assay for the cell line indicated, after 1 day's growth.

H. represents haemocytometer counts; C. represents coulter counts.

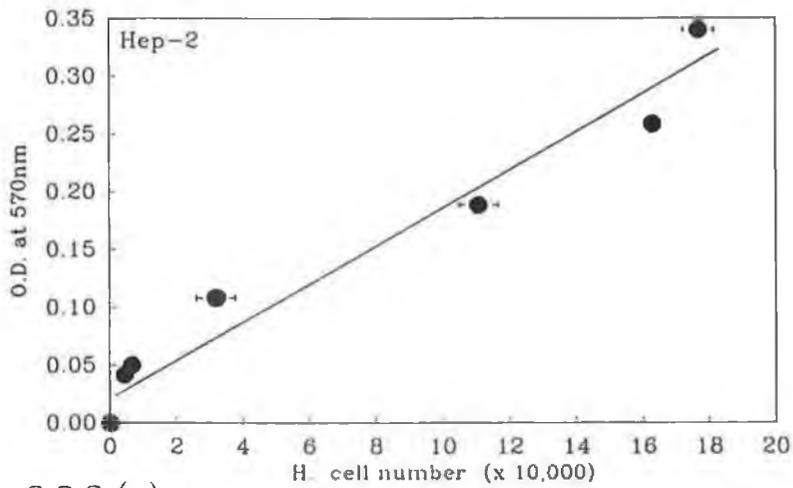


Figure 3.2.2 (g)

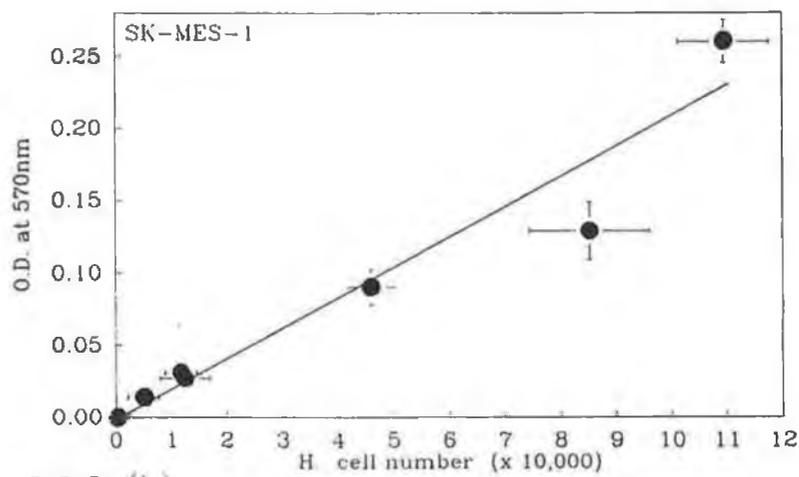


Figure 3.2.2. (h)

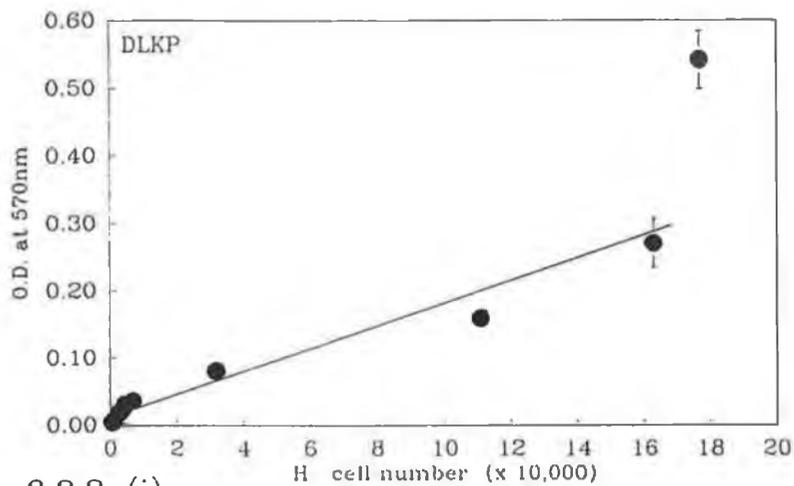


Figure 3.2.2. (i)

Figures 3.2.2 (g) - (i):

Relationship between cell number and Optical Density in MTT assay for the cell line indicated, after 1 day's growth.

H. represents haemocytometer counts. C. represents coulter counts.

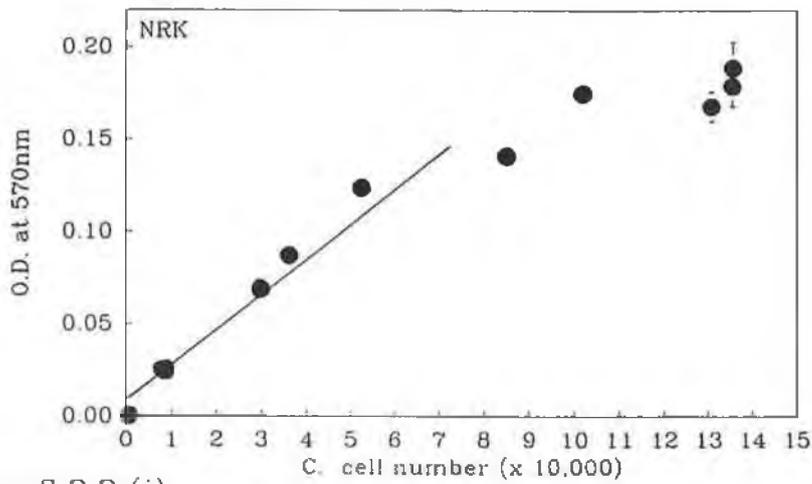


Figure 3.2.2 (j)

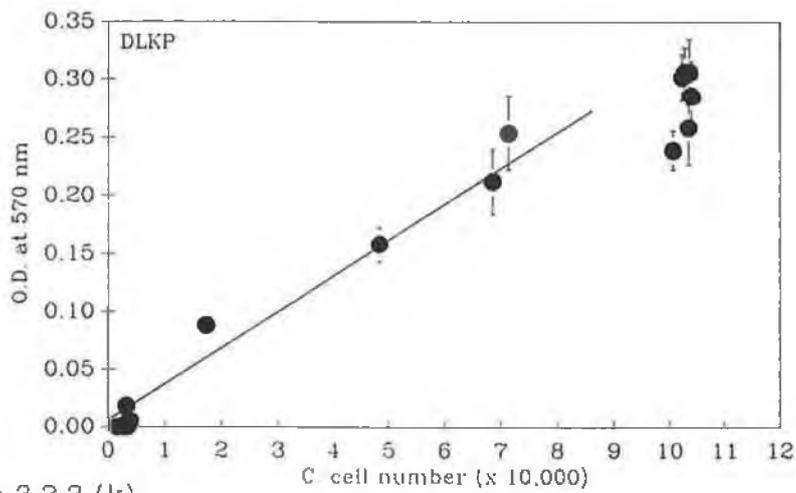


Figure 3.2.2 (k)

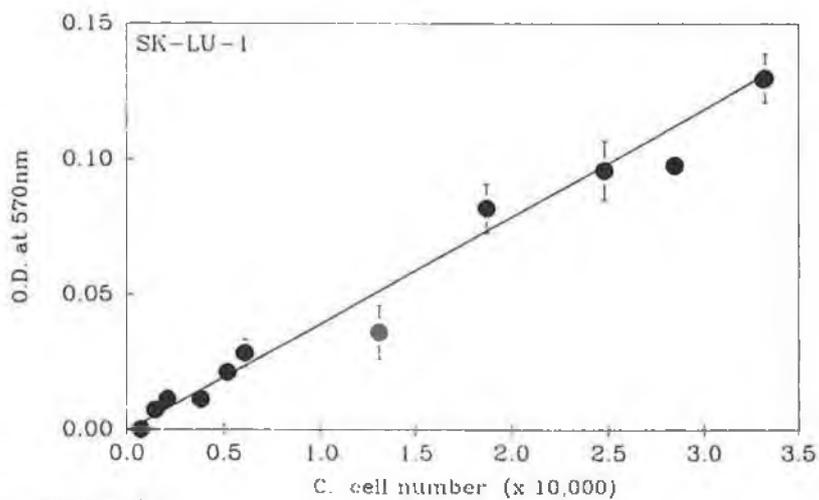


Figure 3.2.2 (l)

Figures 3.2.2 (j) – (l):

Relationship between cell number and Optical Density in MTT assay for the cell line indicated, after 4 days' growth.

H. represents haemocytometer counts; C represents coulter counts.

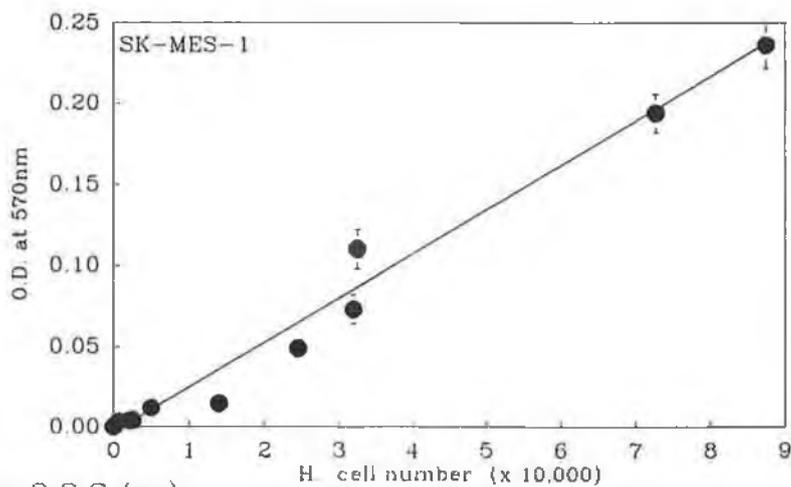


Figure 3.2.2 (m)

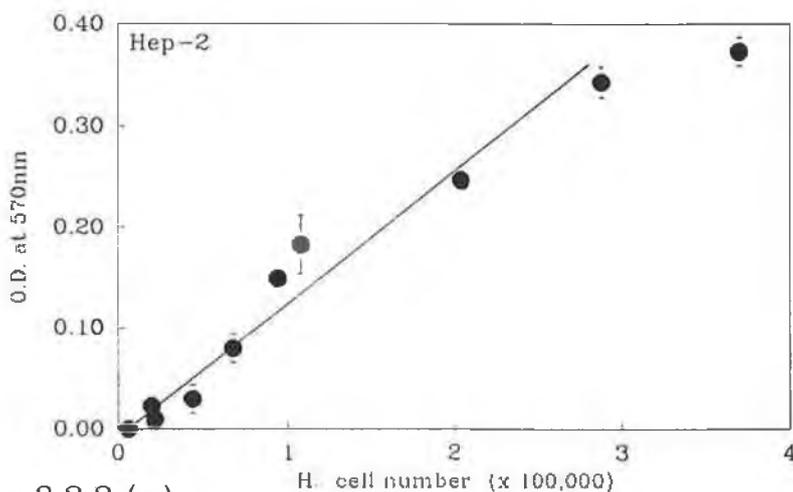


Figure 3.2.2 (n)

Figures 3.2.2 (m), (n):

Relationship between cell number and Optical Density in MTT assay for the cell line indicated, after 4 days' growth.

H. represents haemocytometer counts, C. represents coulter counts.

3.2.3 Detection level

Table 3.2.3 Minimum detectable cell number for the MTT assay after 1 and 4 days growth in culture (cell number corresponding to 0.02 OD units)

cell line	cell number/ 96 well	
	day 1	day 4
RPMI-2650	31,000	7,500
NRK	5,000	12,000
DLKP	9,500	5,400
repeat	13,000	-
Hep-2	9,500	18,750
repeat	10,500	-
SK-LU-1	6,750	4,750
SK-MES-1	5,250	6,750
repeat	9,000	-

For the cell lines tested the assay was not capable of detecting fewer than 5,000 cells/ 96 well. Indeed the figure was much worse for most cell lines, e.g. RPMI-2650, for which cell numbers less than 31,000 per well could not be detected after 1 days' growth. The threshold of detection varied after culture for 1 day and 4 days, but were particularly different for RPMI-2650 and NRK. For the other cell lines, the discrepancy was not as great after the two different culture periods. This may indicate differing MTT metabolizing activity, for similar numbers of some cells under different growth conditions.

3.2.4 Linearity

Table 3.2.4 Highest cell number to which the MTT assay remained linear

cell line	cell number ($\times 10^4$) / 96 well	
	day 1	day 4
RPMI-2650	≥ 6	*
NRK	≥ 8	≥ 10
DLKP repeat	≥ 9 16	≥ 12 -
Hep-2 repeat	≥ 8 ≥ 16	≥ 38 -
SK-LU-1	*	-
SK-MES-1 repeat	≥ 4 ≥ 8.5	≥ 9 -

* = difficult to determine

For every cell line, and under both sets of experimental conditions (1 and 4 days' growth), increasing cell number gives a concomitant increase in OD, right up to high cell densities. This relationship is linear for some part of every graph, although deviation occurs to a greater extent in some of the graphs than others (notably SK-LU-1 after 1 day in culture, Figure 3.2.2 (d)). For some cell lines the upper limit of linearity has been reached at high cell densities (e.g. NRK, Figures 3.2.2 (a) and (j)), but for others this was not the case. For logistical reasons, it was not possible to test higher densities of some cell lines (e.g. SK-LU-1 cells are large and slow growing - the densities required could not be cultured within the available time and without scaling up the size of the growth vessels considerably).

Differences in the extent of linearity also occurs when cells are grown under different conditions, but overall the trend among cell lines is similar. For RPMI-2650 for example the linear range is very short, whereas for Hep-2, the range is considerably more extensive.

3.2.5 Sensitivity

Table 3.2.5 Sensitivity of the MTT assay over the linear graph

cell line	change in OD/ 10,000 cells	
	day 1	day 4
RPMI-2650	0.007	*
NRK	0.036	0.019
DLKP	0.020	0.034
repeat	0.019	-
Hep-2	0.022	0.010
repeat	0.016	-
SK-LU-1	0.030	0.037
SK-MES-1	0.035	0.028
repeat	0.018	-
SCC-9	*	*

* = difficult to determine

The sensitivity of the assay, indicated by the slopes of the lines, is quite low, and again this varies with different cell lines. It is however, consistently greater, for SK-LU-1 for example, than for Hep-2. Thus there is a greater increase in OD for SK-LU-1 than there is for Hep-2 for the same increase in cell number. This of course is related to the growth rate and growth characteristics of the individual cell line. Again, the sensitivity differs slightly in repeat experiments and when cells were cultured under different conditions. There seems to be a correlation between the data in Tables 3.2.3 and 3.2.5, both of which measure sensitivity in a slightly different way.

3.2.6 Ease of performance/ technical flaws

There are no rinsing steps involved in the MTT method, either before or after MTT addition; so the number of actual steps involved is very small. Thus the assay is very easy to perform. However, one of the steps (removal of assay medium after MTT metabolism) is technically difficult, as the insoluble formazan salt crystals formed on the surface of the cells (Figure 3.2) may be very easily detached and lost with the waste medium. The plate can not be 'flicked' to remove waste medium; it must be removed with a multiwell pipette, yet regardless of how carefully this step is performed, touching the bottom of the dish with the pipette tip will result in some loss of formazan. Practical measures such as fitting syringe needles with a small bore, to the tip of pipette tips themselves (to reduce surface contact between pipette tip and monolayer), and use of an automatic plate washer, were not successful.

The photographs which follow illustrate formazan crystal formation on the outside of RPMI 2650 cells.

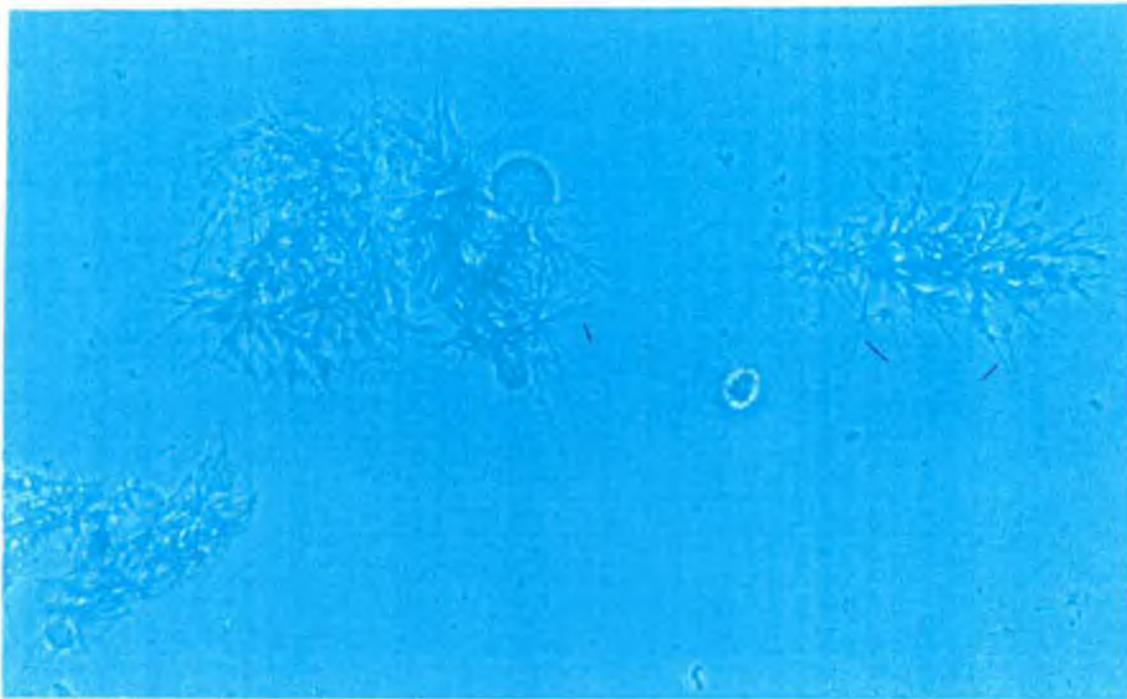
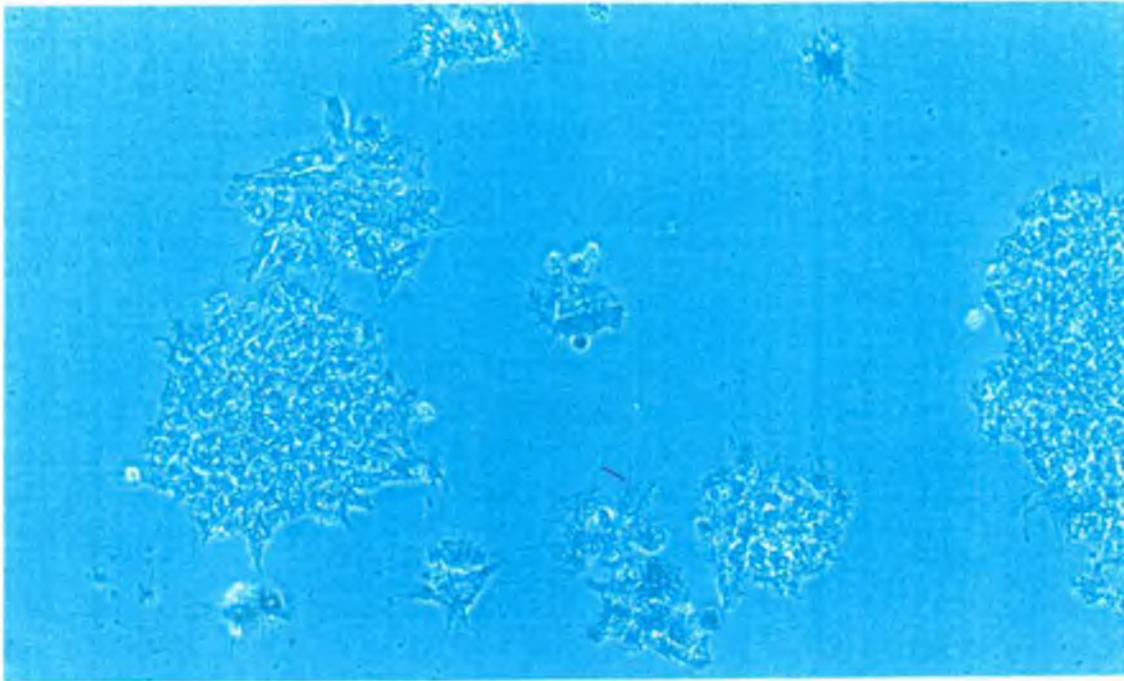


Figure 3.2: Extracellular formazan crystal formation on RPMI-2650 cells in the MTT assay. These "hair-like" protrusions (↖) are easily disturbed during medium removal.

3.2.7 Applicability to a range of cell lines

Notwithstanding the technical difficulty of removal of medium, the MTT assay was equally applicable to each of the cell lines tested.

3.3 MTT assay/ SDS modification

Given the technical flaws of the MTT assay just outlined, one particular modification of the procedure seemed an attractive alternative. In the alternative procedure (Tada et al., 1986), removal of the incubation medium does not take place; formazan solubilization takes place by addition of 100 μ l 10% SDS solution to the wells on top of the incubation medium. Not only does this obviate the 'medium removal' step, but it should eliminate the errors associated with handling of the plates (as the formazan crystals are prone to detachment).

This section investigates the performance of this assay compared to the original method.

3.3.1 Relationship between cell number and O.D. in SDS modification of the MTT assay

The relationship between cell number and O.D. has been determined for this assay as outlined in section 3.2.2., but was only performed after growth for 1 day in culture. Simultaneous to cell counting, MTT/SDS assays were performed on replicate plates on SK-MES-1, DLKP and Hep-2 (including repeat) cell lines. The results have been graphed and are shown in Figures 3.3.1 (a) to (d). The cell numbers referred to, relate to actual cell number counted and not cell number seeded. The lowest detectable cell number, linear range and slope of the linear graph, were calculated, and the results appear in the tables which follow.

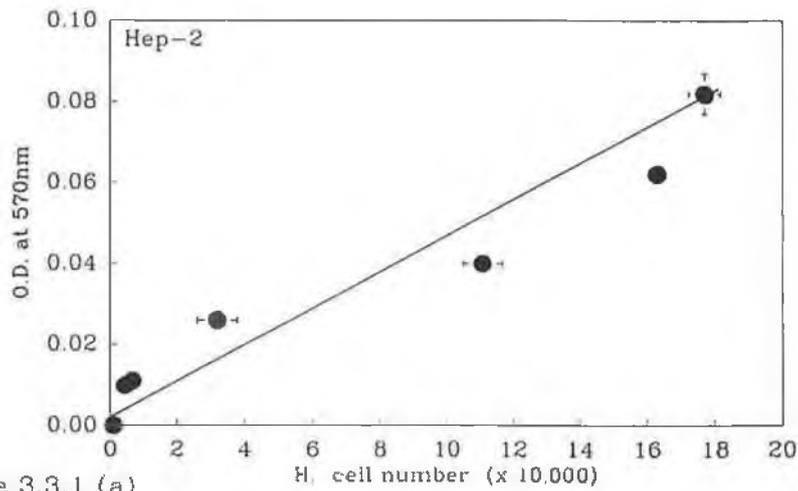


Figure 3.3.1 (a)

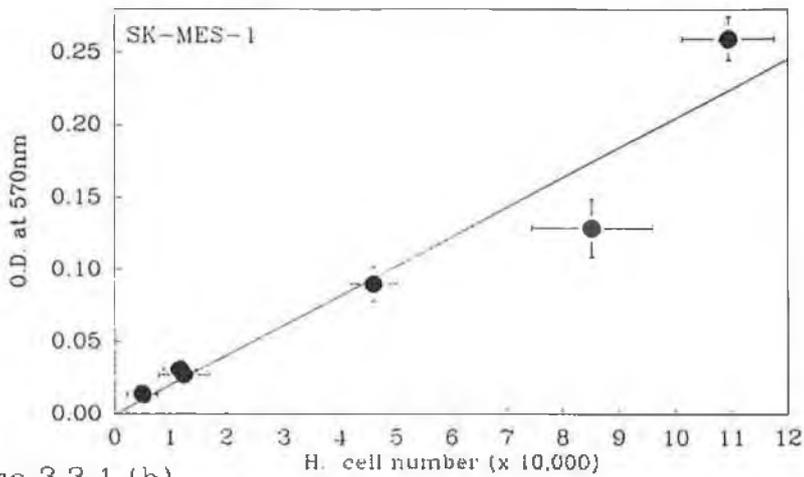


Figure 3.3.1 (b)

Figures 3.3.1(a) - (b):

Relationship between cell number and Optical Density in MTT/SDS assay for the cell line indicated, after 1 day's growth.

H. represents haemocytometer counts, C. represents coulter counts.

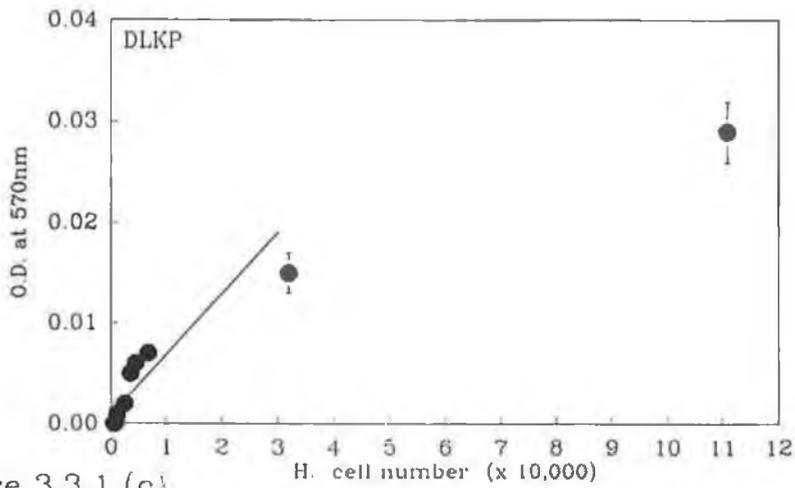


Figure 3.3.1 (c)

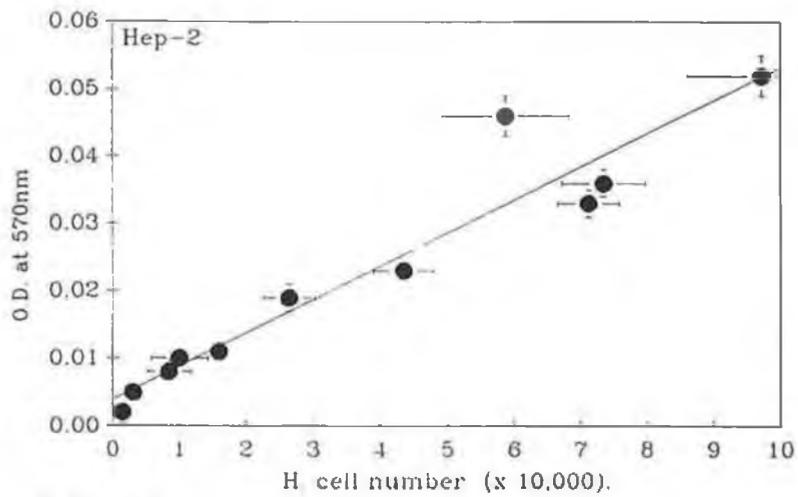


Figure 3.3.1 (d)

Figure 3.3.1 (c) – (d):

Relationship between cell number and Optical Density in MTT/SDS assay for the cell line indicated, after 1 day's growth.

H. represents haemocytometer counts; C. represents coulter counts.

3.3.2 Detection level

Table 3.3.2 Lowest detectable cell number for the MTT/SDS assay after 1 days growth (cell number corresponding to 0.02 OD units)

cell line	cell number/ 96 well
DLKP	57,000
Hep-2 repeat	40,000 29,000
SK-MES-1	66,000

Overall, the lower limit of detection corresponds to quite high cell densities (66,000 cells for SK-MES-1, compared to 9,000 cells for the MTT assay), so the assay was not capable of detecting low cell numbers and certainly it was a disimprovement on the standard MTT assay.

3.3.3 Linearity

Table 3.3.3 Highest cell number to which the MTT/ SDS remained linear (after 1 days growth)

cell line	linear to: cell number/(x 10 ⁴) 96 well
DLKP	≥ 16
Hep-2 repeat	≥ 9 ≥ 18
SK-MES-1	≥ 11

The assay is linear to high cell numbers (equivalent to, or slightly higher than the standard method).

3.3.4 Sensitivity

Table 3.3.4 Sensitivity of the MTT/SDS method over the linear graph (after 1 days' growth)

cell line	change in OD/ 10,000 cells
DLKP	0.003
Hep-2	0.005
repeat	0.004
SK-MES-1	0.003

The extremely poor sensitivity of this modification can be seen from the very low slopes of the lines. Thus a substantial increase in cell number gave only a very small increase in corresponding OD.

The low OD readings may be explained by the increased volume into which the formazan is dissolved; thus it is more dilute, resulting in a lower absorbance reading. It may also be that the SDS has a lower solubilizing power than DMSO. Another explanation may be that the phenol red in the medium is causing spectral interferences which are resulting in lower readings overall.

Using this procedure, 100,000 Hep-2 cells will only produce a reading of 0.040 absorbance units, whereas with the standard method, a value of 0.170 absorbance units was reached. Comparing the slopes of the lines for the 2 methods, we see that the SDS modification is 6 times less sensitive for SK-MES-1 and 4 times less sensitive for Hep-2. It is clear thus, that the sensitivity is extremely low.

3.3.5 Ease of performance/ technical flaws

The SDS modification of the MTT assay is particularly easy to perform, and does not encounter the difficulties of medium removal discussed for the MTT assay.

3.3.6 Application to a range of cell lines

This assay is particularly appropriate for all cell lines; even non-adherent ones, because there are no rinsing or medium-removal steps in which cells can be lost, however its very low sensitivity for each of the cell lines tested, limits its usefulness.

3.4 Crystal Violet Dye Elution (CVDE) assay

3.4.1 Plate rinsing

If the dye is improperly rinsed from the plates, results will be inaccurate. Crystal violet is an aqueous stain so superfluous washings will lessen the intensity of staining. It is therefore important to properly rinse the plate without resulting in de-staining. It was found that 4 separate rinses in tap water was the minimum number needed for adequate washing, thus this procedure was routinely used for all assays.

3.4.2 Relationship between cell number and O.D. in CVDE assay

These experiments were performed as described in the materials and methods, and as outlined again in section 3.2.2. The CVDE assay was performed on replicate plates after 1 day and again after 4 days growth. Additional replicate plates were trypsinized and counted on these days by haemocytometer and Coulter counter. Repeat experiments were performed once for DLKP and SK-MES-1, and twice for Hep-2. The experiment was carried out on a total of 7 cell lines (6 epithelial and one fibroblastic), and cell counts were plotted against O.D. Figures 3.4.2 (a) to (i) illustrate the data pertaining to 1 day's growth, while the graphs in Figures 3.4.2 (j) to (n) relate to 4 days' growth. These include graphs of repeat experiments for Hep-2, SK-MES-1 and DLKP, the data for which is also given in the tables.

The minimum detectable cell number, the linear range of the graph, and the slope of this linear portion have been calculated and are shown in the tables which follow the graphs.

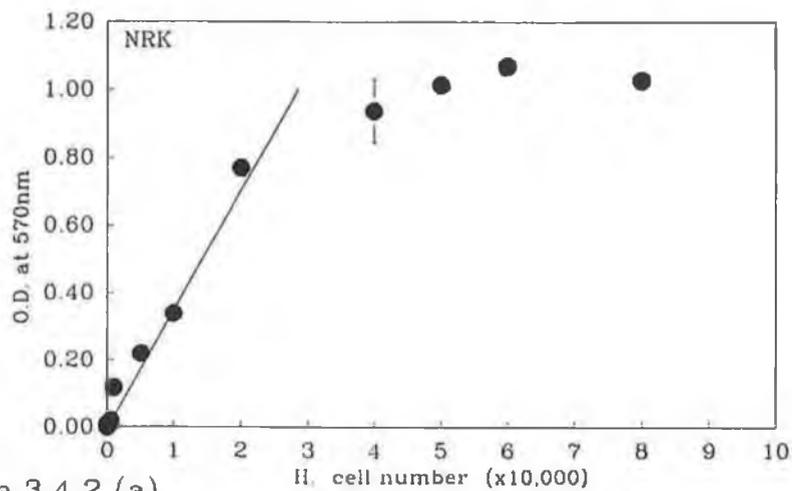


Figure 3.4.2 (a)

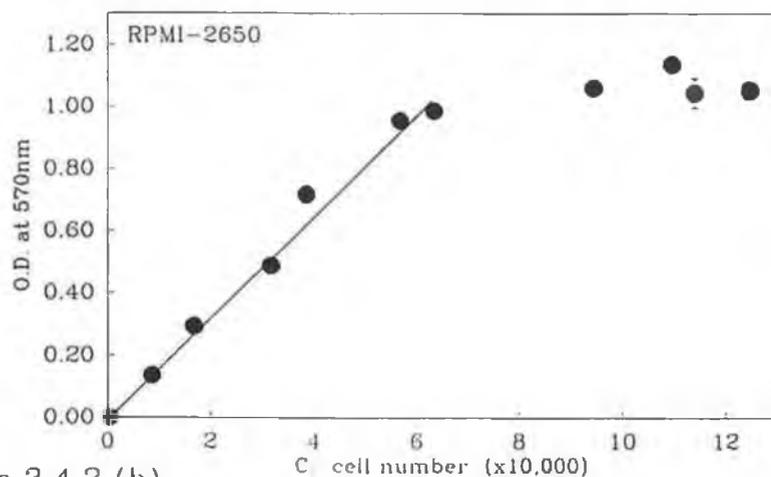


Figure 3.4.2 (b)

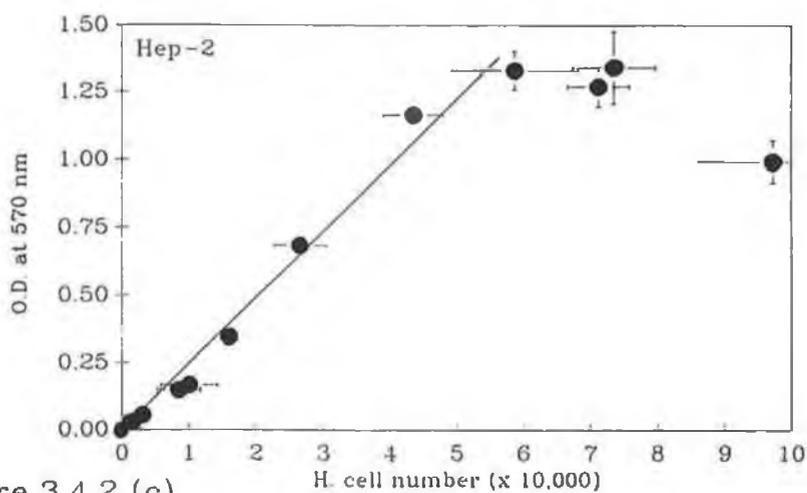


Figure 3.4.2 (c)

Figures 3.4.2 (a) - (c):

Relationship between cell number and Optical Density in CVDE assay for the cell line indicated, after 1 day's growth.

H. represents haemocytometer counts, C. represents coulter counts.

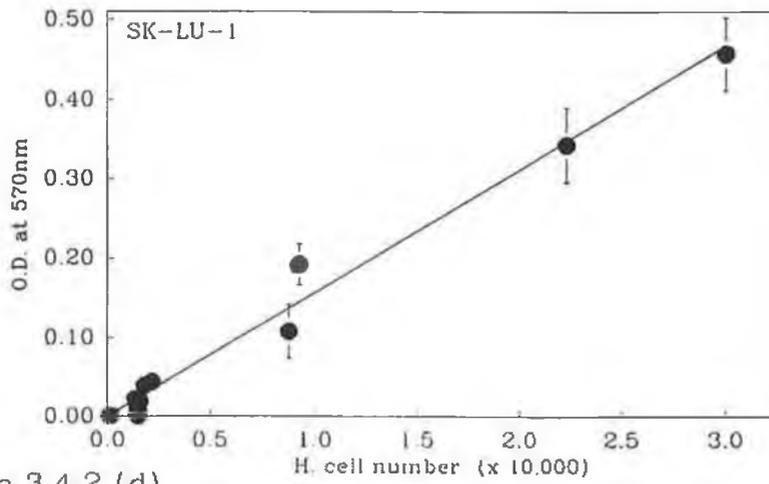


Figure 3.4.2 (d)

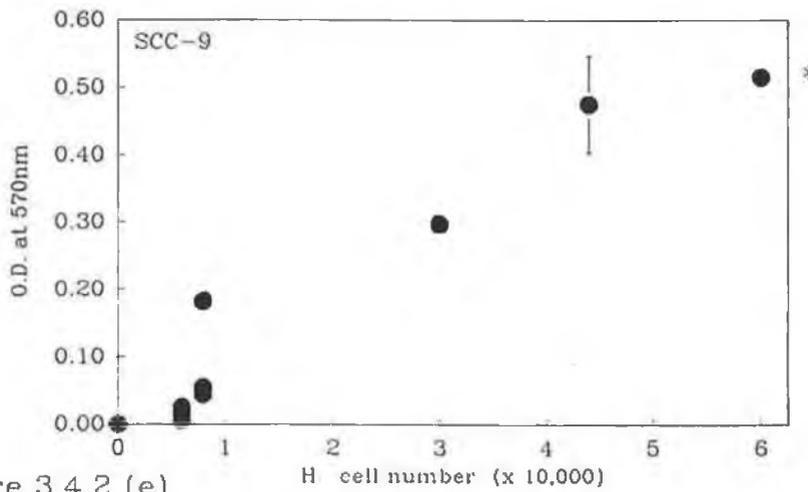


Figure 3.4.2 (e)

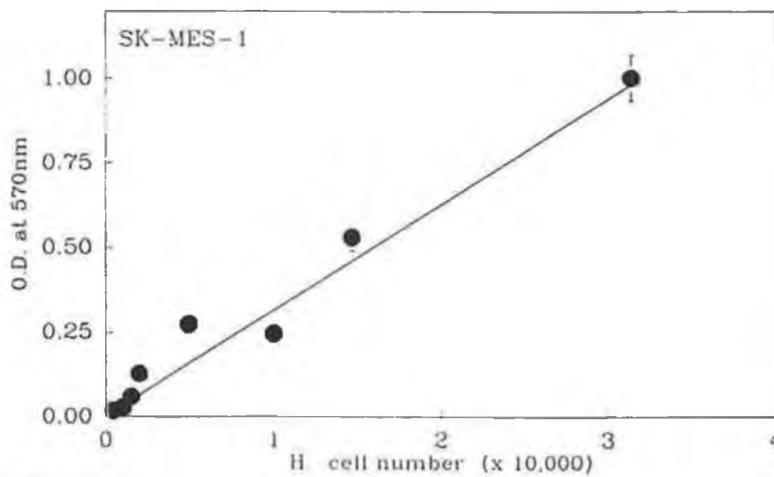


Figure 3.4.2 (f)

Figures 3.4.2 (d) – (f):

Relationship between cell number and Optical Density in CVDE assay for the cell line indicated, after 1 day's growth.

H. represents haemocytometer counts, C represents coulter counts.

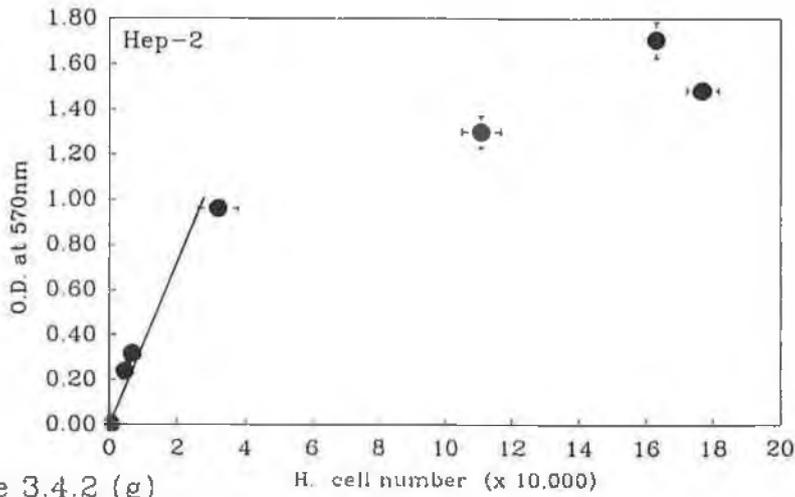


Figure 3.4.2 (g)

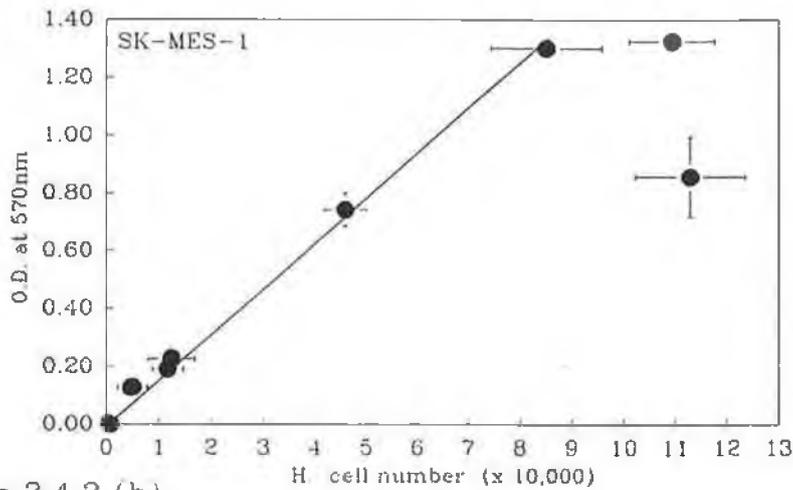


Figure 3.4.2 (h)

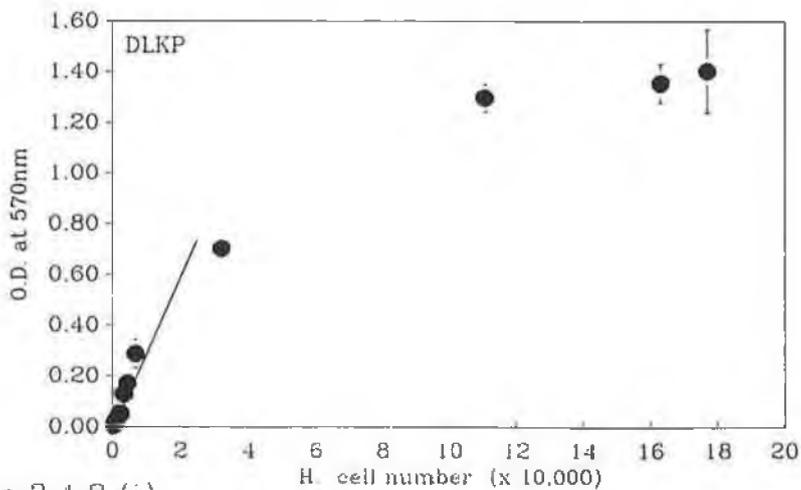


Figure 3.4.2 (i)

Figures 3.4.2 (g) – (i):

Relationship between cell number and Optical Density in CVDE assay for the cell line indicated, after 1 day's growth.

H. represents haemocytometer counts; C represents coulter counts.

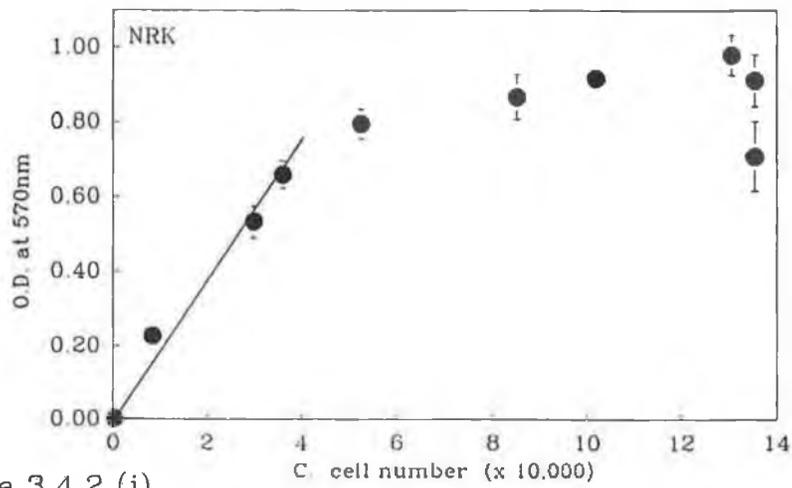


Figure 3.4.2 (j)

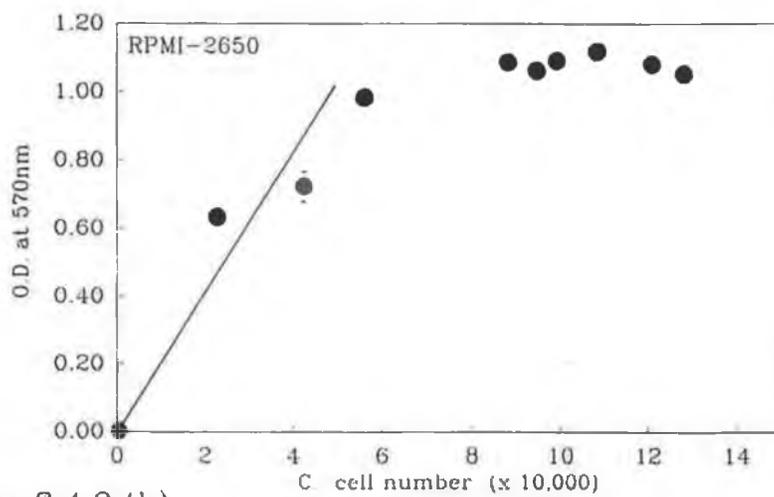


Figure 3.4.2 (k)

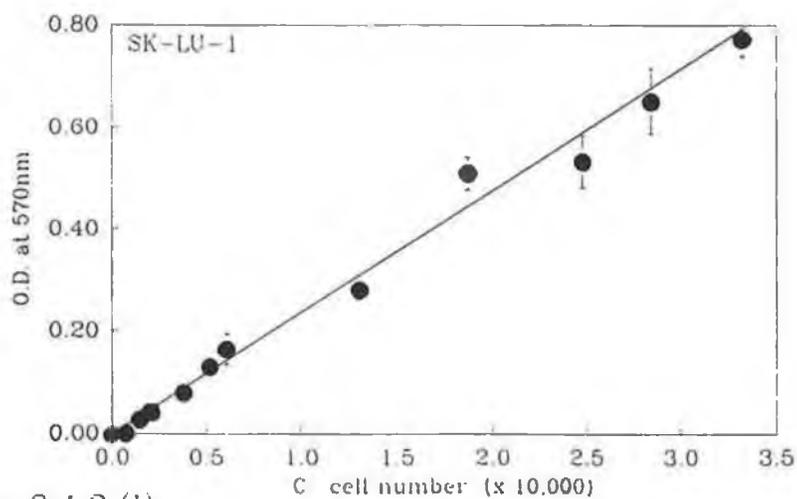


Figure 3.4.2 (l)

Figures 3.4.2 (j) - (l):

Relationship between cell number and Optical Density in CVDE assay for the cell line indicated, after 4 days' growth.

H. represents haemocytometer counts. C represents coulter counts.

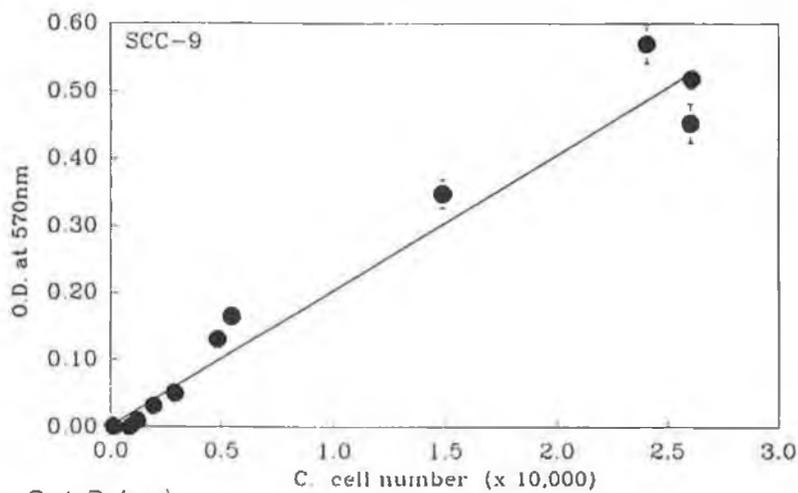


Figure 3.4.2 (m)

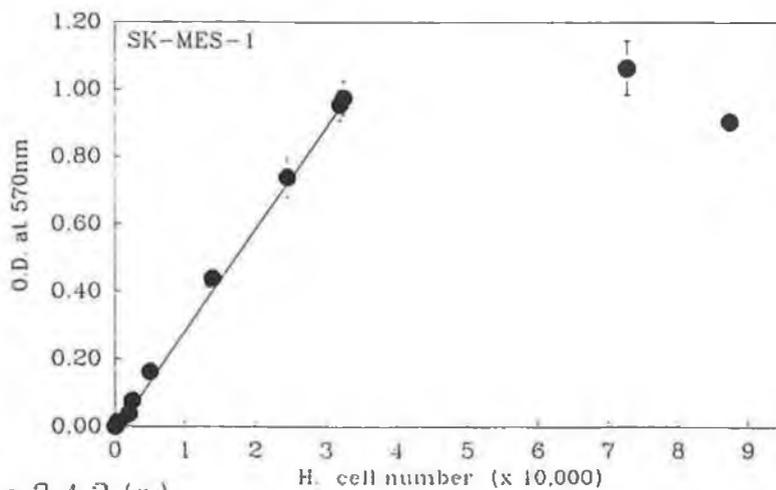


Figure 3.4.2 (n)

Figures 3.4.2 (m), (n):

Relationship between cell number and Optical Density in CVDE assay for the cell line indicated, after 4 days' growth.

H. represents haemocytometer counts. C represents coulter counts

3.4.3 Detection level

Table 3.4.3 Minimum detectable cell number for the CVDE assay after 1 and 4 days growth, (corresponding to 0.02 OD units)

cell line	cell number/ 96 well	
	day 1	day 4
RPMI-2650	1,750	2,000
NRK	750	1,200
DLKP	600	-
Hep-2	750	-
repeat	1,000	-
SK-LU-1	1,300	750
SK-MES-1	700	875
repeat	1,700	-
SCC-9	1,850	1,000

The CVDE assay detected relatively low numbers of cells. For many cell lines, it was capable of detecting 1,000 cells/ 96 well, or fewer.

3.4.4 Linear range

Table 3.4.4 Highest cell number to which the CVDE assay was linear

cell line	linear to: cell number ($\times 10^4$) / 96 well	
	day 1	day 4
RPMI-2650	7	9.5
NRK	6	5
DLKP	4	-
Hep-2	5.5	-
repeat	≥ 4	-
SK-LU-1	≥ 3.5	≥ 3.5
SK-MES-1	≥ 3	≥ 3
repeat	≥ 8	-
SCC-9	*	≥ 2.5

* = difficult to determine

For at least some part of the graph, there is a linear relationship of cell number and O.D. This was easily observed with all of the cell lines, except SCC-9, for which this relationship was not easily determined. While the assay was more sensitive at detecting lower cell numbers of DLKP than RPMI-2650, the linear range was shorter for DLKP than RPMI-2650.

Overall the assay became non-linear at moderate cell densities, after which there was no further increase, or indeed a slight decrease, in absorbance. Certainly the assay was not linear up to 100,000 cells/96 well for any of the cell lines tested; for many cell lines in fact, it did not even remain linear for 50,000 cells/ well. Thus, accompanying the increased sensitivity of this assay over previous assays, there is a concomitant decrease in linearity.

3.4.5 Sensitivity

Table 3.4.5 Sensitivity of CVDE assay over the linear range

cell line	change in OD/ 10,000 cells	
	day 1	day 4
RPMI-2650	0.160	0.088
NRK	0.188	0.169
DLKP	0.347	-
SK-LU-1	0.122	0.227
Hep-2	0.258	-
repeat	0.273	-
SK-MES-1	0.305	0.304
repeat	0.147	-
SCC-9	0.086	0.193

* = difficult to determine

By and large, the slopes of the lines are substantial, indicating good sensitivity. Again values differ somewhat for the graphs of 1 and 4 days growth, and also for experiments carried out on different days.

3.4.6 Ease of performance/ technical flaws

The CVDE assay is simple to perform. The dye solution should be filtered before use. The lack of an incubation period of a few hours is also an advantage (staining takes only 10 minutes), however the plate must be fully dried before addition of solvent for dye release. If wished, the plate may be fixed in 10% Formalin previous to staining, whereupon it may be rinsed, dried and restained if the results need to be verified. The delay while the plates dry may be a

disadvantage if the results are needed rapidly, but it may be an advantage if time is short - they may be stored dry, in the dark, until there is sufficient time to process them.

3.4.7 Applicability to a range of cell lines

Similar to the other assays, there appeared to be no difference in the performance of this assay among different cell lines.

3.5 Neutral Red (NR) assay

3.5.1 Optimization

3.5.1.1 Effect of serum in incubation medium

Dye was not taken up into cells if the medium did not include serum, thus for all assays, complete medium including 5% FCS was used.

3.5.1.2 Removal of crystals from dye medium

In the original method [Borenfreund and Puerner, 1984] the authors used a procedure in which the working solution of NR in fresh medium was prepared and incubated in the fridge overnight before use (to accommodate precipitation of dye crystals). Just before use, the solution would then be centrifuged at 5,000 rpm for 10 minutes. Omission of this step would be of benefit for the laboratory worker.

On a number of assays performed simultaneously using NR solution prepared in different ways it was determined that the latter step was much more important than the former; incomplete centrifugation did not result in removal of all of the NR precipitate which customarily appeared in the solution. These precipitates in the incubation medium remained on the cells after removal of excess dye and interfered with the readings; they were observed microscopically as large crystals sitting on the outside of the cells. Thorough centrifugation of the solution followed by careful handling to ensure that pelleted precipitate was not resuspended, was sufficient to ensure that this interference did not materialize.

Careful observation of the cultures indicated that there was no difference between medium that had been prepared the night previously and that which had not; the precipitate was removed to the same extent upon centrifuging either way. It was therefore concluded that this incubation step was not necessary provided that care was taken in the steps which followed, so this step was omitted from the procedure.

3.5.2 Relationship between cell number and O.D. in NR assay

Cells were trypsinized and counted by haemocytometer and Coulter counter after 1, and again after 4 days' growth, as described in the materials and methods section. NR assays were performed on replicate plates for each of 7 cell lines. Figures 3.5.2 (a) to (j) show the relationship between actual cell count and O.D. after 1 day's growth, while Figures 3.5.2 (k) to (q) show the relationship after culturing for 4 days. This relationship was defined by determining the lowest detectable cell number for each cell line, the linear range of each graph, and finally the sensitivity of the assay for each cell line (slope of the linear portion). The following tables show this information.

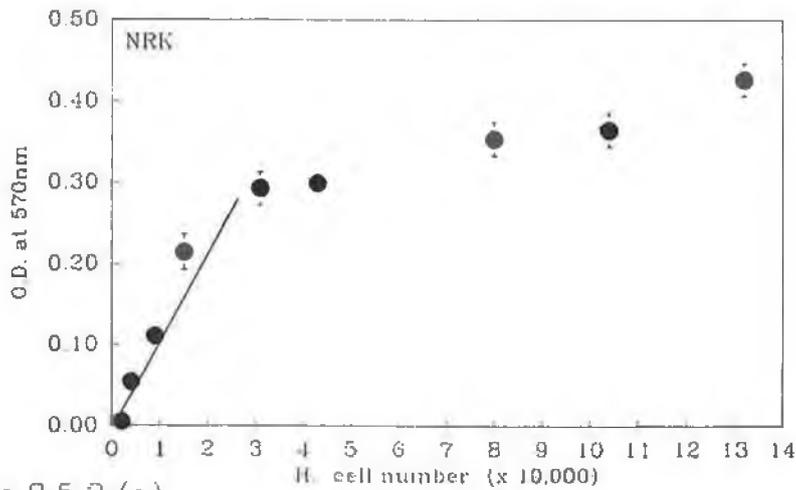


Figure 3.5.2 (a)

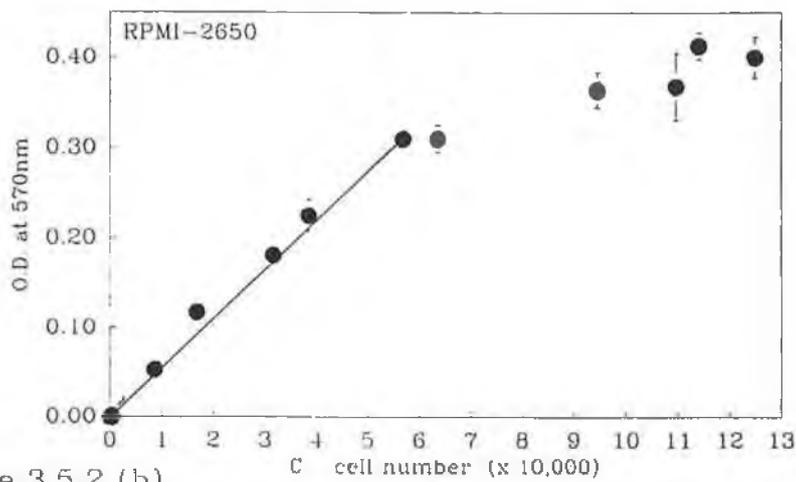


Figure 3.5.2 (b)

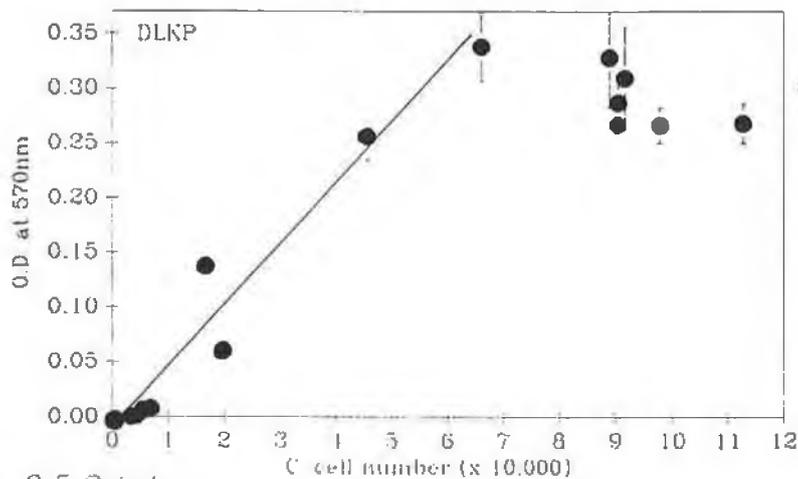


Figure 3.5.2 (c)

Figures 3.5.2 (a) - (c):

Relationship between cell number and Optical Density in NR assay for the cell line indicated, after 1 day's growth

H. represents haemocytometer counts, C represents coulter counts.

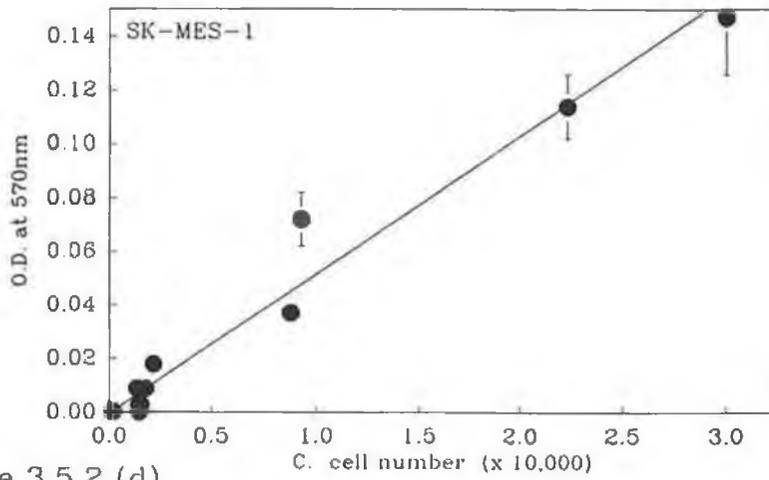


Figure 3.5.2 (d)

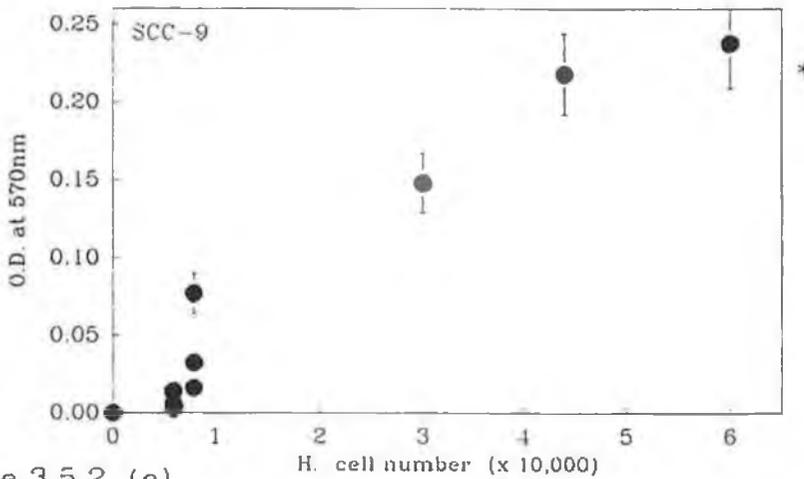


Figure 3.5.2. (e)

Figures 3.5.2 (d), (e):

Relationship between cell number and Optical Density in NR assay for the cell line indicated, after 1 day's growth

H. represents haemocytometer counts, C represents coulter counts.

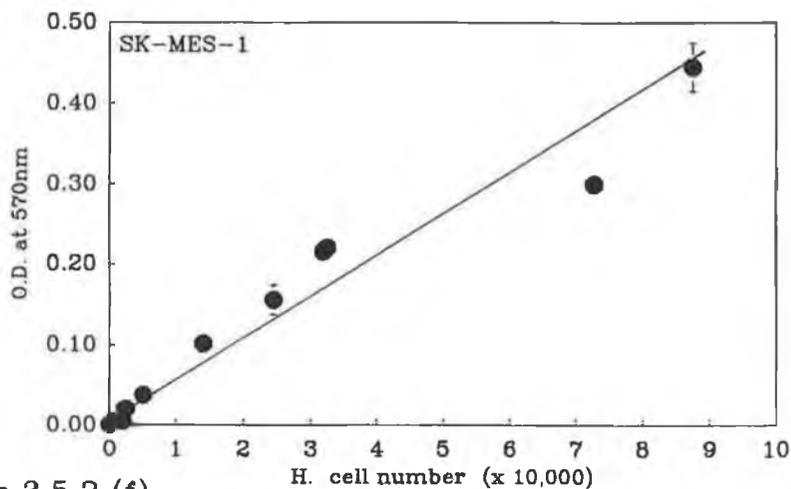


Figure 3.5.2 (f)

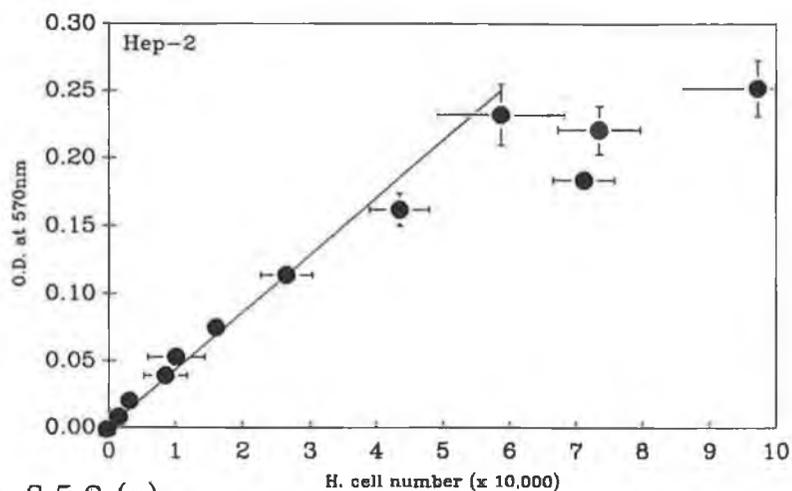


Figure 3.5.2 (g)

Figures 3.5.2 (f), (g):

Relationship between cell number and Optical Density in NR assay for the cell line indicated, after 1 day's growth

H. represents haemocytometer counts; C. represents coulter counts.

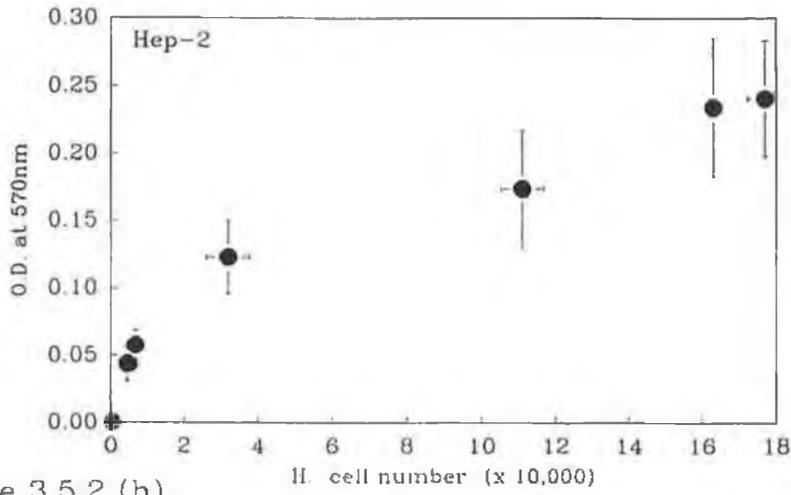


Figure 3.5.2 (h)

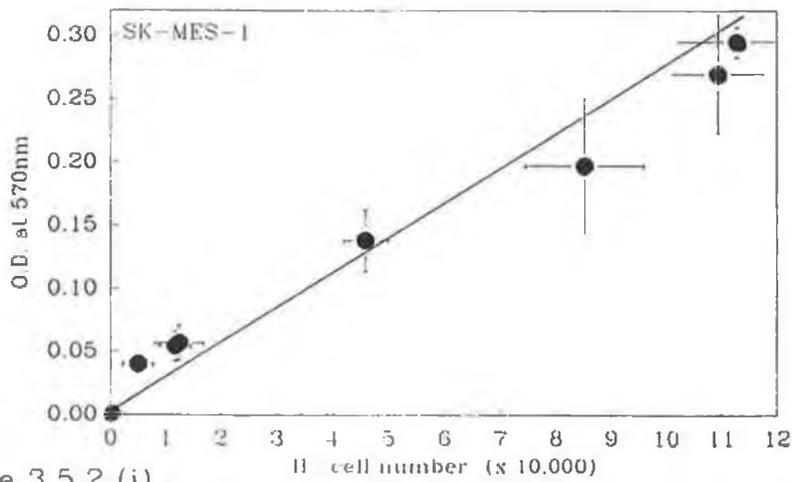


Figure 3.5.2 (i)

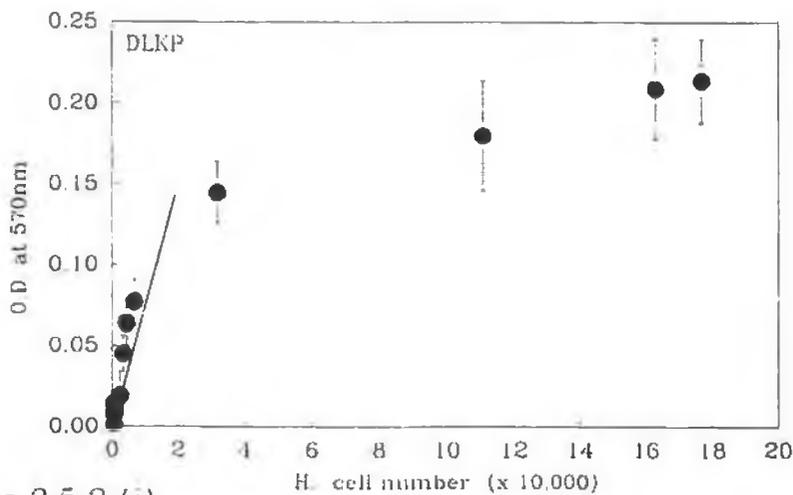


Figure 3.5.2 (j)

Figures 3.5.2 (h) - (j):

Relationship between cell number and Optical Density in NR assay for the cell line indicated, after 1 day's growth

H. represents haemocytometer counts, C. represents coulter counts.

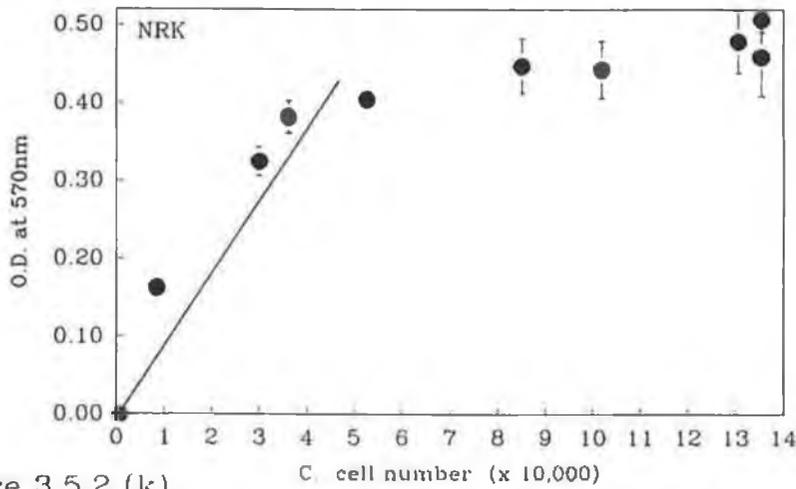


Figure 3.5.2 (k)

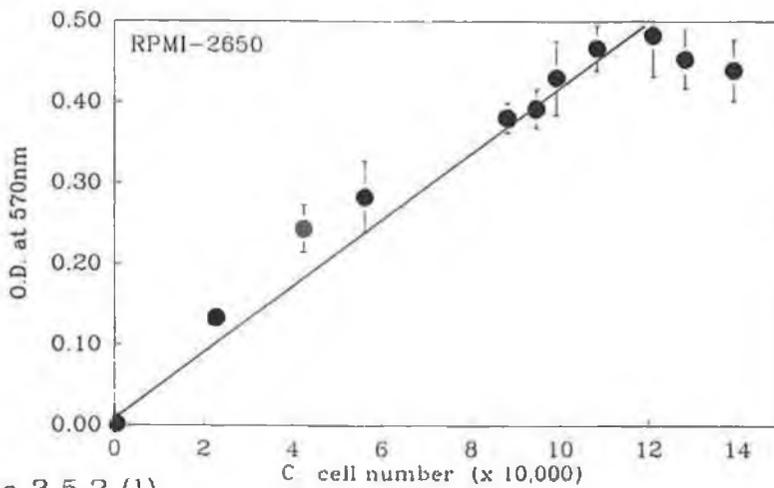


Figure 3.5.2 (l)

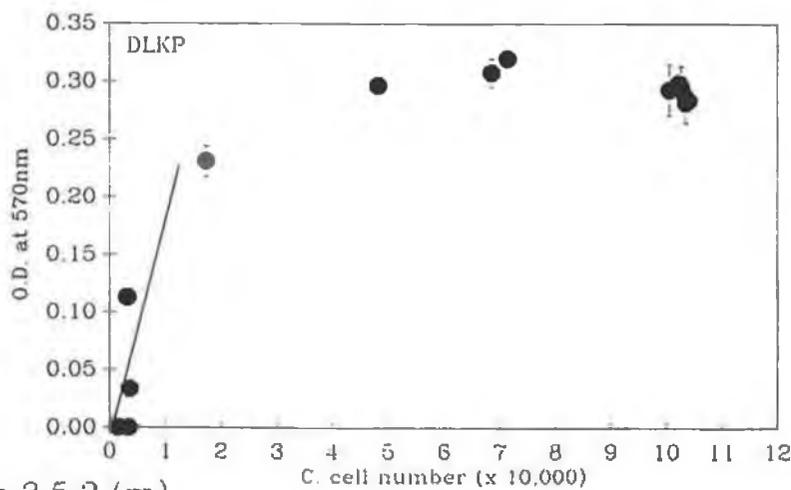


Figure 3.5.2 (m)

Figures 3.5.2 (k) –(m):

Relationship between cell number and Optical Density in NR assay for the cell line indicated, after 4 days' growth.

H. represents haemocytometer counts; C. represents coulter counts.

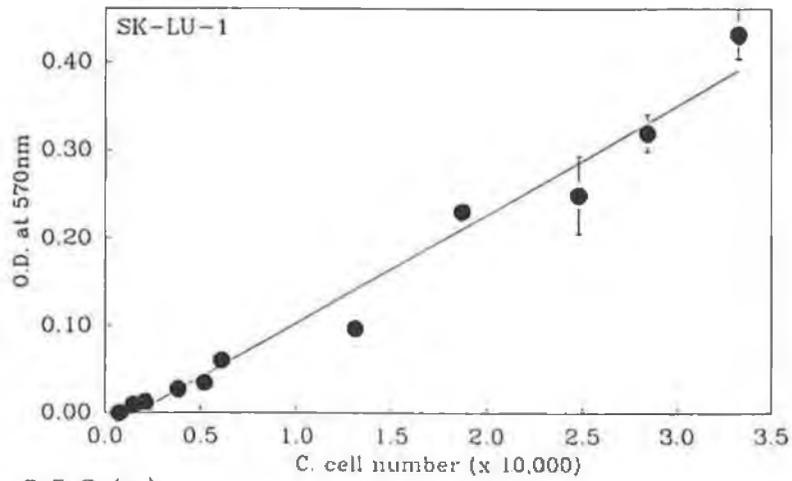


Figure 3.5.2 (n)

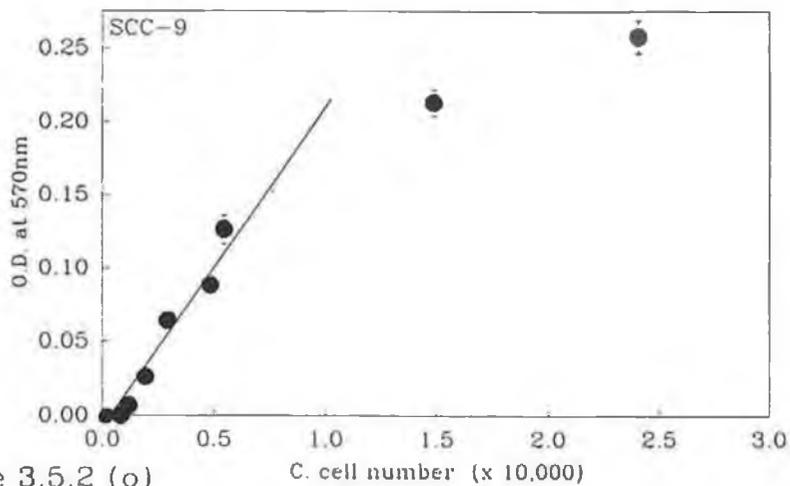


Figure 3.5.2 (o)

Figures 3.5.2 (n), (o):

Relationship between cell number and Optical Density in NR assay for the cell line indicated, after 4 days' growth.

H. represents haemocytometer counts, C. represents coulter counts.

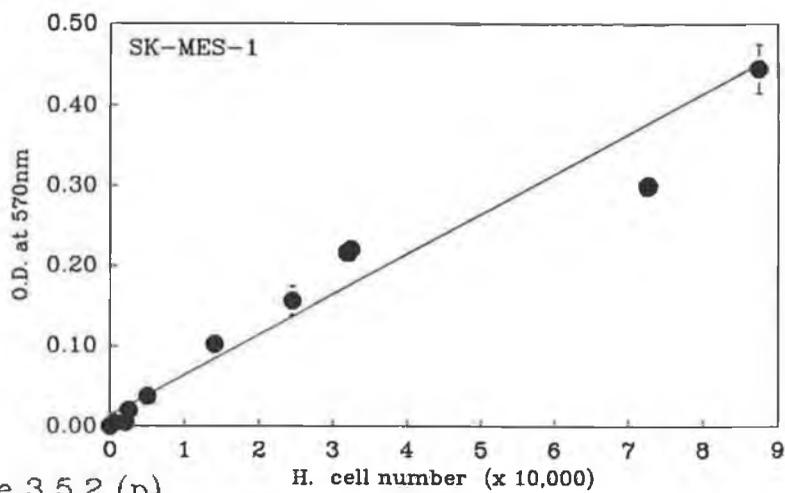


Figure 3.5.2 (p)

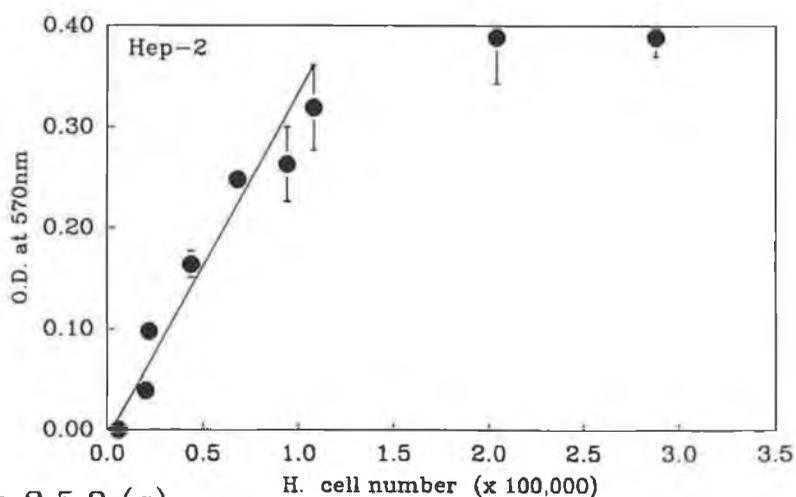


Figure 3.5.2 (q)

Figures 3.5.2 (p), (q):

Relationship between cell number and Optical Density in NR assay for the cell line indicated, after 4 days' growth.

H. represents haemocytometer counts; C. represents coulter counts.

3.5.3 Limit of detection

Table 3.5.3 Lowest detectable cell number for the NR assay after 1 and 4 days growth (cell number corresponding to 0.02 OD units)

cell line	cell number / 96 well	
	1 day	4 days
RPMI-2650	3,500	3,750
NRK	2,000	3,500
DLKP	2,500	1,250
repeat	2,750	-
Hep-2	8,000	5,500
repeat	5,000	-
SK-LU-1	3,500	1,750
SK-MES-1	3,300	-
repeat	8,000	4,500
SCC-9	*	1,100

* = difficult to determine

The NR assay is moderately sensitive to low cell numbers of each of the cell lines; in fact it can detect cell numbers fewer than 5,000 cells/ well in many cases. However it was not able to detect cell numbers less than 1,000 cells/ well.

3.5.4 Linearity

Table 3.5.4 Highest cell number to which the NR assay was linear

cell line	linear to: cell number ($\times 10^4$) / 96 well	
	1 day	4 days
RPMI-2650	5.5	11
NRK	3.5	3.5
DLKP repeat	\geq 6.5 2 - 3	15
SK-LU-1	\geq 3.5	\geq 3.5
Hep-2 repeat	\geq 9 *	\geq 10 -
SK-MES-1 repeat	\geq 3.5 \geq 11	\geq 9 -
SCC-9	*	\geq 2

* = difficult to determine

The linear range is easily distinguishable from the Figures for the various cell lines (except for SCC-9).

Again differences in the extent of linearity are apparent for different cell lines. For some cell lines the assay is linear for a substantial cell number (e.g., Hep-2; linear over a range of $\geq 90,000$ cells, whereas for others, it is linear over only a short range of cell densities.

The variation in the linear range between experiments for 1 and 4 day's culture were particularly great for DLKP.

3.5.5 Sensitivity

Table 3.5.5 Sensitivity of the NR assay for various cell lines (slope of the linear graph)

cell line	change in OD/ 10,000 cells	
	1 day	4 days
RPMI-2650	0.045	0.031
NRK	0.096	0.064
DLKP	0.061	0.162
repeat	0.110	
SK-LU-1	0.050	0.124
Hep-2	0.030	0.034
repeat	0.039	
SK-MES-1	0.058	-
repeat	0.024	0.049
SCC-9	*	0.176

* = difficult to determine

At a glance it is clear that the slopes are moderately high, and again they vary for different cell lines. While there is very substantial variation on lines plotted from experiments carried out on different days, we can see for example, that the slope of all Hep-2 graphs is much less than that of DLKP graphs. Overall, the NR assay was not of very high sensitivity.

3.5.6 Ease of performance/ technical flaws

While simple to perform, there are many steps involved in the NR assay. Medium is removed, the wells are rinsed, dye is added to the wells for 4 hours, removed, rinsed in Formal Calcium to remove unincorporated dye, and then solvent added to release bound dye. In addition the dye must be diluted in culture medium, and centrifuged for 5 minutes before use, to remove dye precipitates which might interfere with the assay, thus the assay is more cumbersome to perform than the other assays.

3.5.7 Applicability to a range of cell lines

This assay was not found to be inappropriate for any of the cell lines tested.

3.6 Sulforhodamine B (SRB) assay

This assay, now adopted by the National Cancer Institute (NCI) drug screening program, is a protein binding dye assay. Cells are first fixed in TCA before staining with this intense pink dye. Bound dye is released by addition of Tris buffer at pH 10.5.

3.6.1 Relationship between cell number and O.D. in SRB assay

As described in the materials and methods section, cells covering a range of densities, were trypsinized and counted from 96 well plates after 1 day's growth. On replicate plates the SRB assay was performed as also described in materials and methods. Figures 3.6.1 (a) - (d) show plots of actual cell number from haemocytometer counts, for DLKP, SK-MES-1 and Hep-2 (including repeat). Lowest detectable cell number, range of linearity and sensitivity have been calculated from these graphs and the tables which follow display this information.

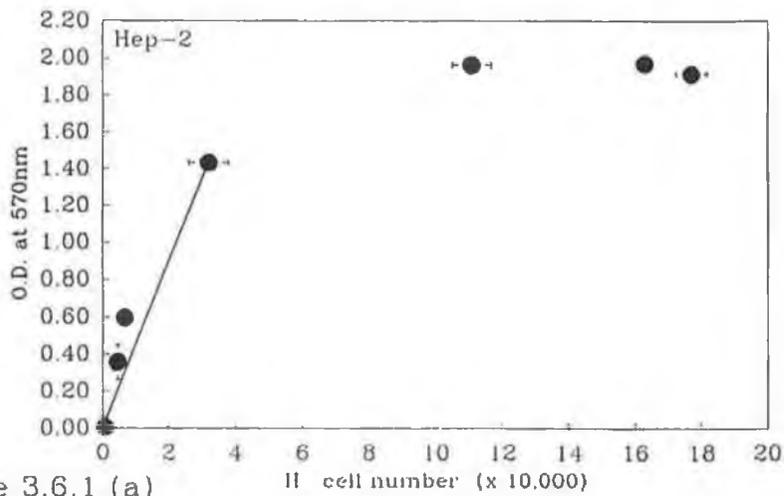


Figure 3.6.1 (a)

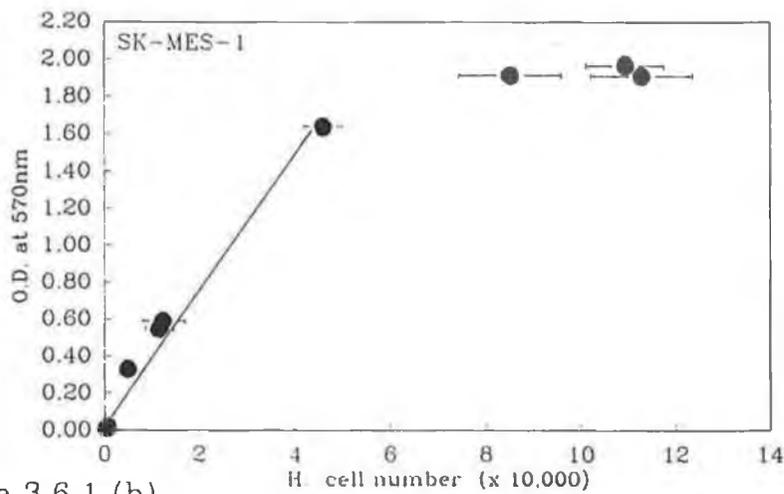


Figure 3.6.1 (b)

Figures 3.6.2 (a), (b):

Relationship between cell number and Optical Density in SRB assay for the cell line indicated, after 1 day's growth.

H represents haemocytometer counts, C represents coulter counts.

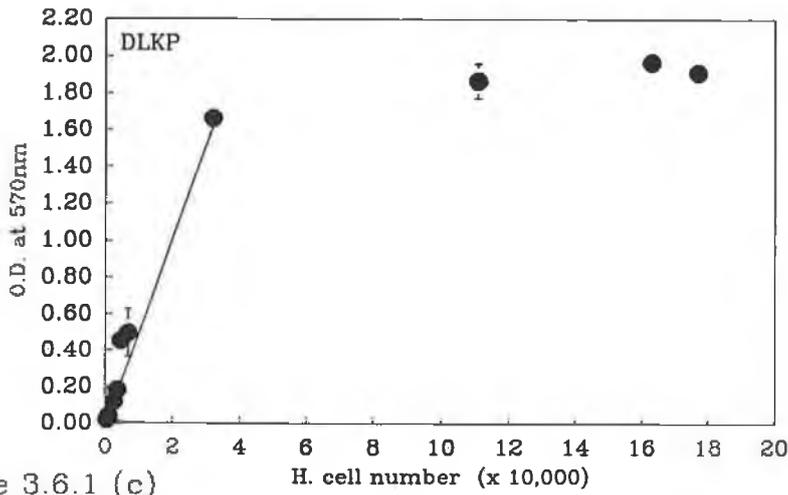


Figure 3.6.1 (c)

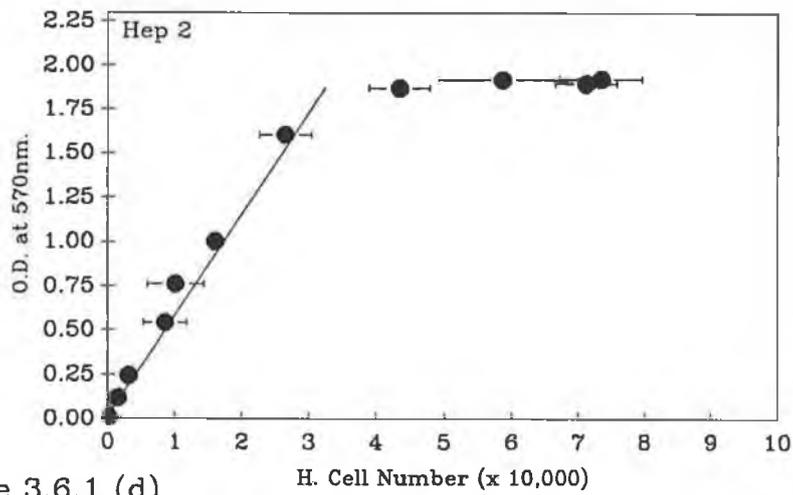


Figure 3.6.1 (d)

Figures 3.6.2 (c), (d)

Relationship between cell number and Optical Density in SRB assay for the cell line indicated, after 1 day's growth.

H. represents haemocytometer counts; C. represents coulter counts.

3.6.2 Detection level

Table 3.6.2 Lowest detectable cell number for the SRB assay after 1 days' growth (cell number corresponding to 0.02 OD units)

cell line	cell number/ 96 well
DLKP	300
Hep-2	300
repeat	400
SK-MES-1	500
SCC-9	750

The assay is extremely sensitive to low cell number, being capable of detecting as few as 300 DLKP cells/ 96 well; and certainly fewer than 1,000 cells /well for each of the lines tested.

3.6.3 Linear range

Table 3.6.3 Highest cell number to which the SRB assay is linear (after 1 day's growth)

cell line	linear to; cell number ($\times 10^4$) / 96 well
DLKP	2.75
Hep-2	3 - 4
SK-MES-1	5
SCC-9	1.5

Although the cell number detected is very low, the assay does not remain linear to high cell densities. At cell numbers greater than 40,000 DLKP cells per 96 well, the assay is no longer linear and can not give an accurate estimate of cell number. For SCC-9 (which are particularly large cells), linearity is only maintained to 15,000 cells per 96 well. This deviation from linearity can be seen quite dramatically by a brief glance at the Figures on the previous page.

3.6.4 Sensitivity

Table 3.6.4 Sensitivity of the SRB assay for various cell lines after 1 days' growth

cell line	change in OD/ 10,000 cells
DLKP	0.562
Hep-2	0.537 0.433
SK-MES-1	0.358
SCC-9	0.960

The assay is very sensitive. The slope of the SCC-9 plot, is very steep; for every increase of 10,000 cells, there was an increase of almost 1 OD unit; however the range of this linear portion was not very extensive, spanning only approximately 15,000 cells.

3.6.5 Ease of performance/ technical flaws

Again this assay is simple to perform, but there are quite a number of steps involved. The first step is a fixing step, with cold Trichloroacetic acid (TCA) which continues at 4°C for an hour. Thereafter the plate is rinsed and dried, stained for 30 minutes, rinsed and dried, and then unbound dye is released by addition of Tris buffer. Like the CVDE assay, the steps where the plate is being dried, may be disadvantageous if the results are required immediately, or may offer an advantage as they may be stored and read at a later time.

3.6.6 Applicability to a range of cell lines

As with the other assays, the assay appeared to be equally suitable for a range of cell lines. Unlike the other assays, addition of TCA resulted in loosely adherent cells (DLKP for example) being fixed also, so additionally, this may make the assay appropriate for cell lines growing in suspension, such as Small Cell Lung Carcinoma cell lines.

3.7 Acid Phosphatase (AP) assay

3.7.1 Optimization

3.7.1.1 Storage of buffer for AP assay

A short experiment was conducted to determine if the stability of the incubation buffer was a problem. A two week-old stock of buffer containing substrate (stored at 2°C) was compared to a freshly made up stock, in a 96 well AP toxicity experiment. The results were compared to those obtained by image analysis, and are shown in Figure 3.7.1.1.

It can be seen that the freshly prepared AP buffer gives results similar to those from image analysis, however the results with the old buffer are completely erroneous; negligible cell kill has been detected, so the assay did not work at all. This is obviously due to hydrolysis of the substrate in the buffer.

It is obvious thus, that the substrate-containing buffer is not stable for this length of time. Thereafter, the substrate was always added to the sodium acetate/ triton X-100 buffer immediately before use.

3.7.1.2 pH of incubation buffer

Although the original method of Connolly *et al.*, [1986] uses an acetate buffer at pH 5.5, it has been stated [Hollander, 1971] that the optimum pH for acid phosphatase with an acetate buffer is pH 4.9. Thus the aim of this experiment was to determine if pH 4.9 would be preferential to pH 5.5. Cell number versus AP activity (OD units) has been plotted for these 2 buffers (Figure 3.7.1 (b)). Both pH's were used over an entire cell density range of SCC-9. While there was little difference in the results of a toxicity assay using these 2 different buffers, the OD was significantly higher at the higher pH than at pH 4.9. Thus acetate buffer adjusted to pH 5.5 was confidently used for all assays thereafter, as in the method of Connolly *et al.*

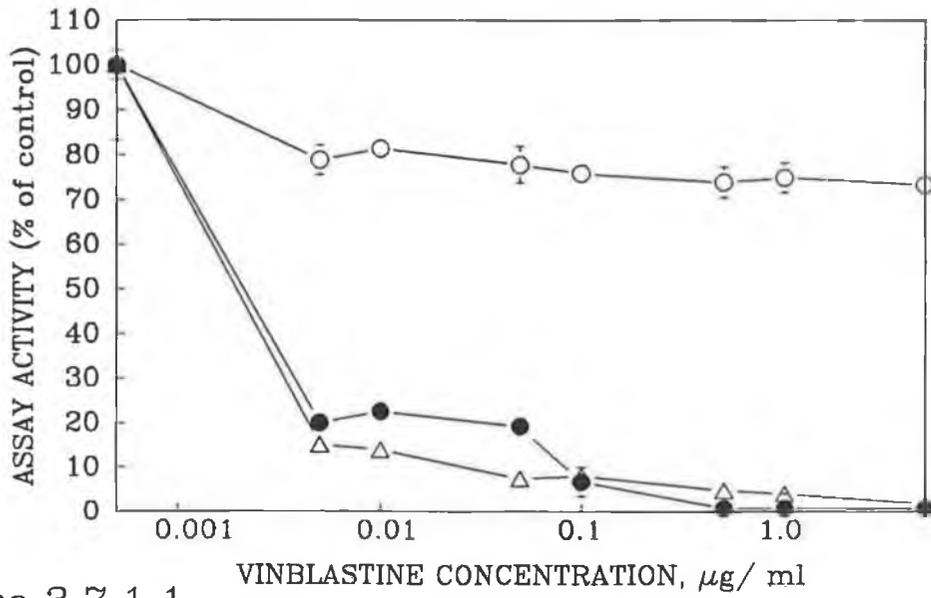


Figure 3.7.1.1

Activity of fresh (●—●) and 2-week old stock (○—○) of AP buffer in a toxicity experiment on RPMI-2650 cells, compared to IA (△—△).

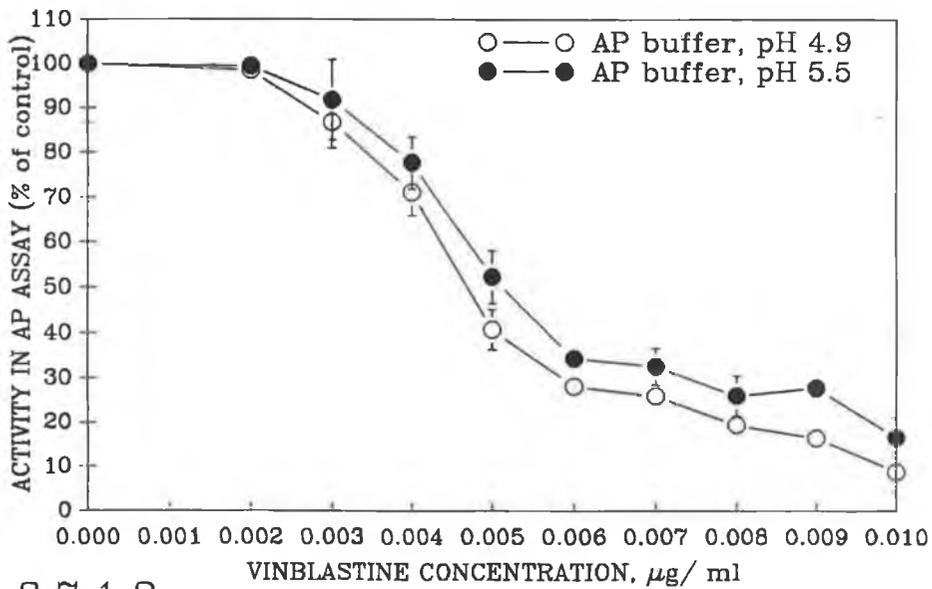


Figure 3.7.1.2

Effect of buffer pH on AP activity in toxicity assay with SCC-9 cells.

3.7.1.3 Elimination of bubbles before reading

Because the incubation buffer contained Triton X-100, there was a strong tendency for bubble formation which may have interfered with absorbance readings.

A number of plates were read initially and then after bubbles had been removed (by bursting with a syringe needle). It was apparent that the presence of bubbles on the plate caused some degree of interference while the plate is being read. Accordingly, as a matter of practice, bubbles, when present, were burst before the plate was read.

3.7.2 Relationship between of cell number and O.D in AP assay

This relationship was determined in exactly the same manner as it was for the previous assays, and is described in detail in the Materials and Methods section. Briefly, cells seeded at a 3-log range of densities, were cultured for 1 day. Replicate plates were cultured for 4 days and on both sets of plates, cells were trypsinized and counted by Haemocytometer and by Coulter counter. AP assays were performed on replicate plates on each of these days and read immediately after 2 hours incubation. Actual cell number counted was plotted against O.D. to compose the Figures 3.7.2 (a) to (j) for data relating to 1 day's culture, and Figures 3.7.2 (k) to (q) for 4 day's culture.

Lowest detectable cell number, range of cell numbers over which the assay was linear, and sensitivity of the assay were determined, and these are condensed in the Figures which follow.

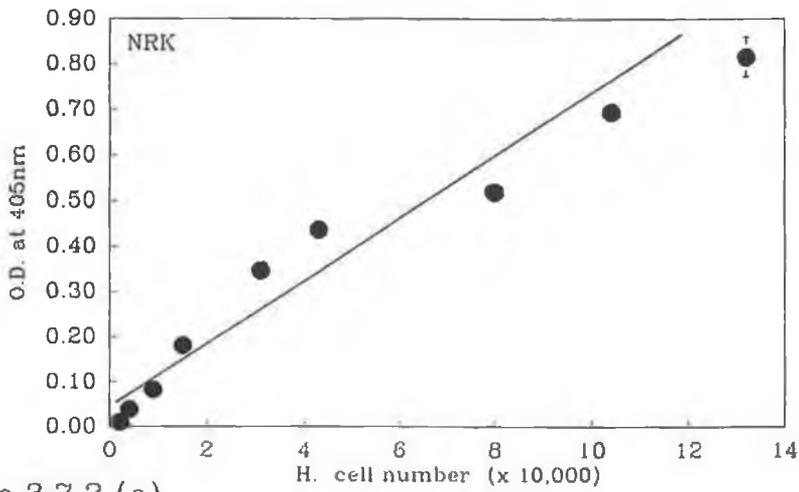


Figure 3.7.2 (a)

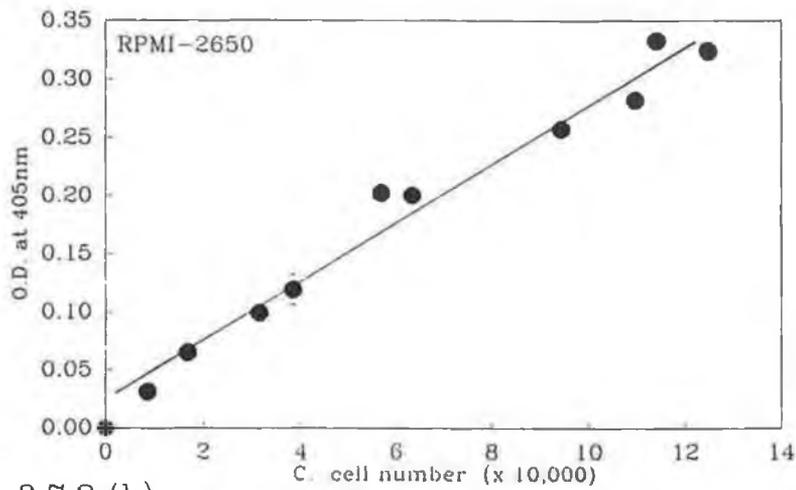


Figure 3.7.2 (b)

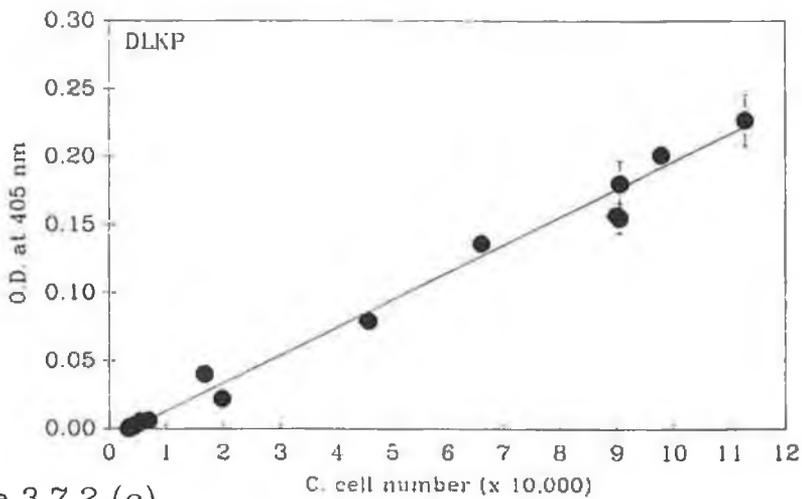


Figure 3.7.2 (c)

Figures 3.7.2 (a) - (c):

Relationship between cell number and Optical Density in AP2h assay for the cell line indicated, after 1 day's growth.

H. represents haemocytometer counts; C represents coulter counts.

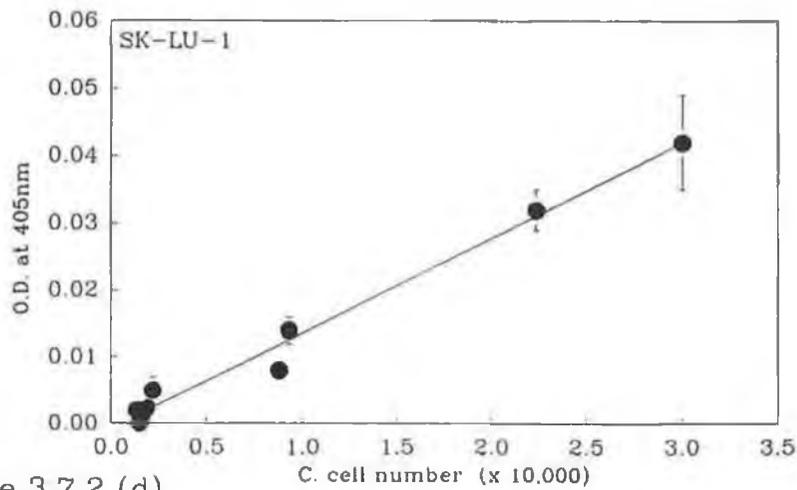


Figure 3.7.2 (d)

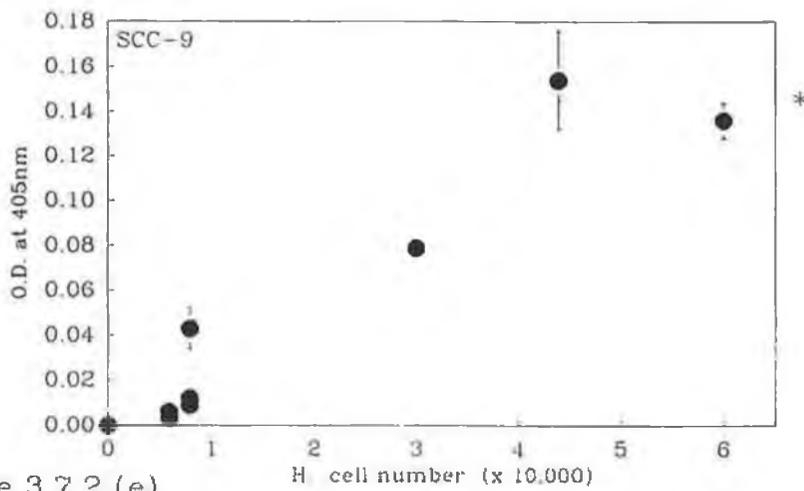


Figure 3.7.2 (e)

Figures 3.7.2 (d) – (e):

Relationship between cell number and Optical Density in AP2h assay for the cell line indicated, after 1 day's growth.

H. represents haemocytometer counts, C represents coulter counts.

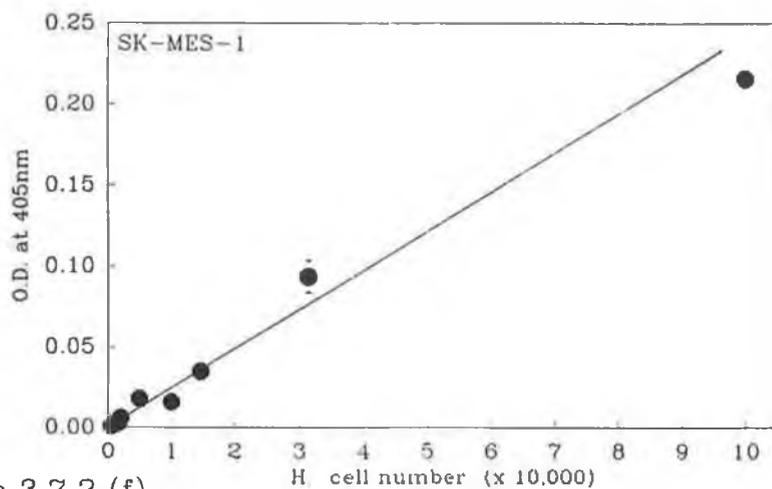


Figure 3.7.2 (f)

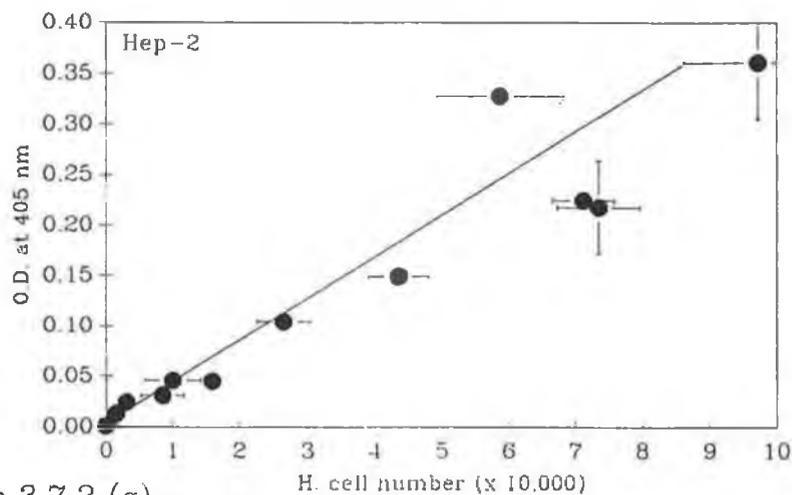


Figure 3.7.2 (g)

Figures 3.7.2 (f) – (g):

Relationship between cell number and Optical Density in AP2h assay for the cell line indicated, after 1 day's growth.

H. represents haemocytometer counts; C. represents coulter counts.

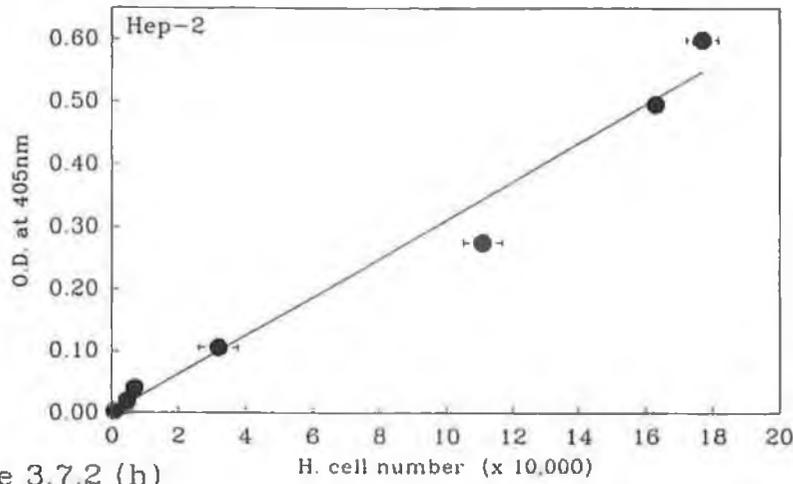


Figure 3.7.2 (h)

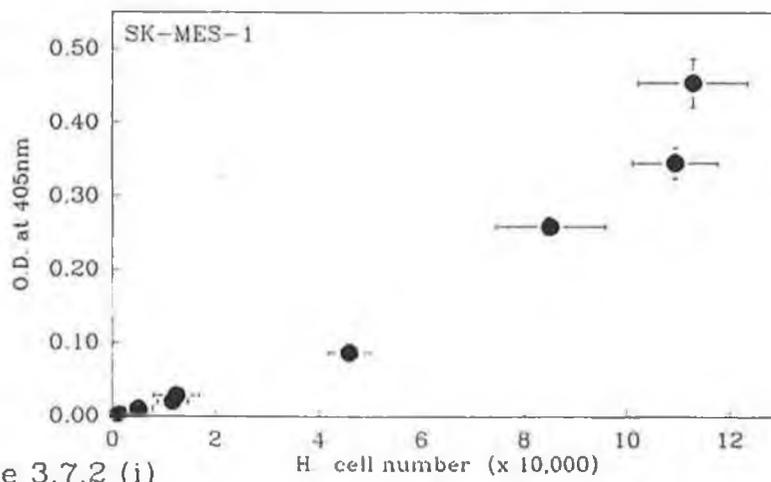


Figure 3.7.2 (i)

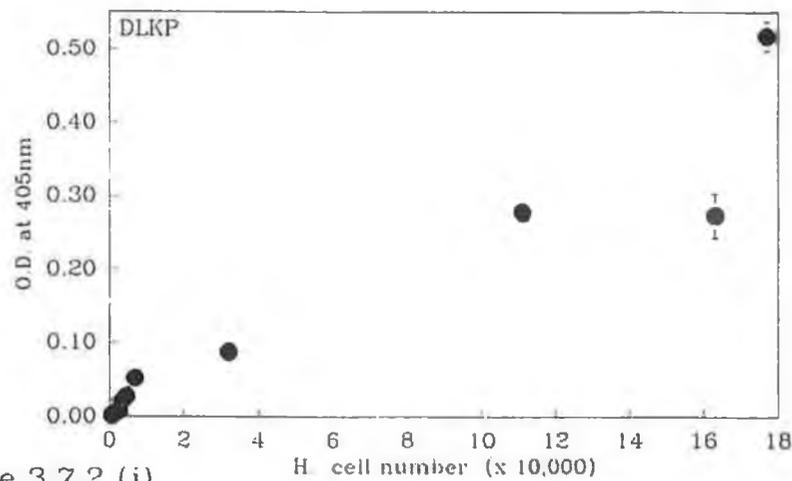


Figure 3.7.2 (j)

Figures 3.7.2 (h) - (j)

Relationship between cell number and Optical Density in AP2h assay for the cell line indicated, after 1 day's growth.

H. represents haemocytometer counts. C. represents couller counts.

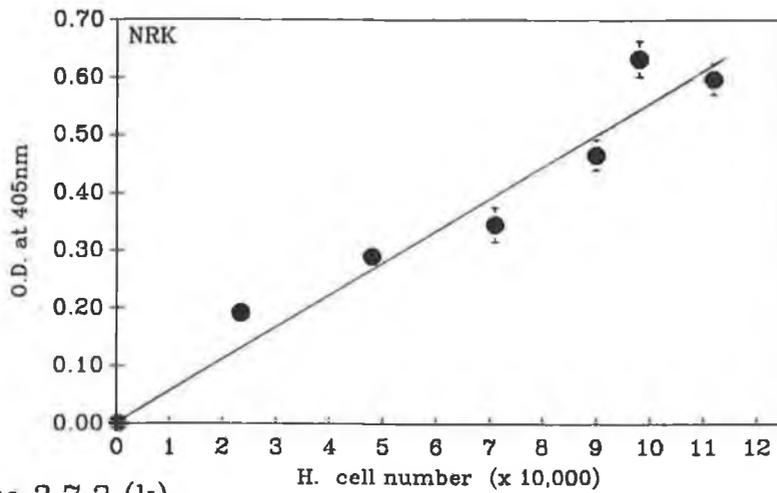


Figure 3.7.2 (k)

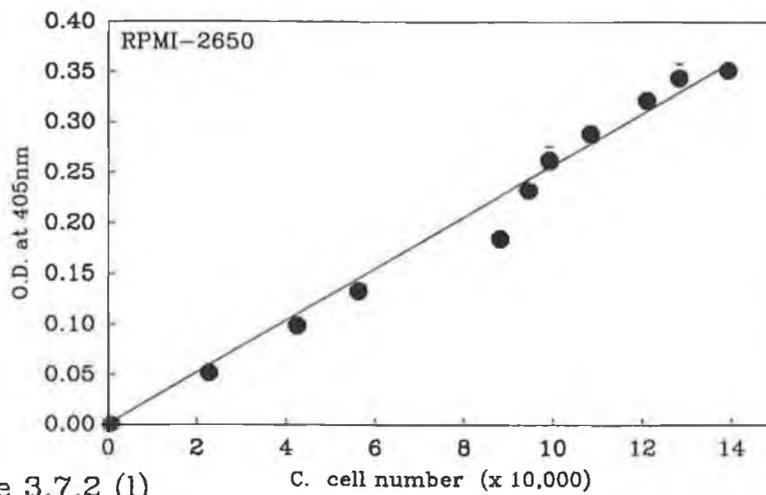


Figure 3.7.2 (l)

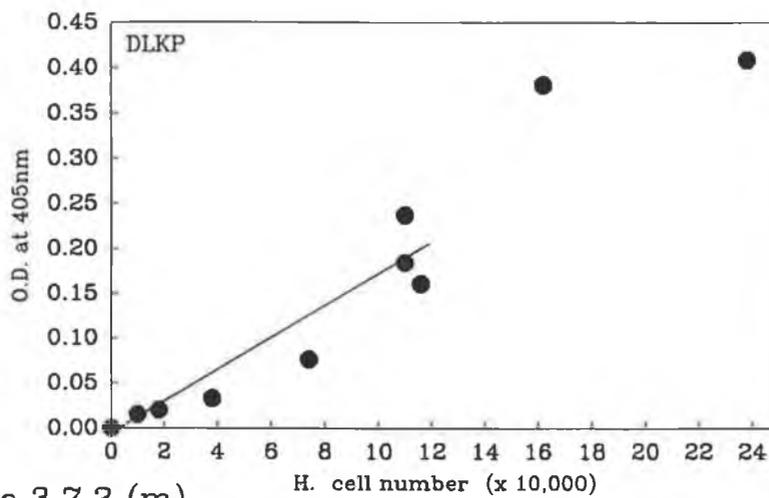


Figure 3.7.2 (m)

Figures 3.7.2 (k) – (m):

Relationship between cell number and Optical Density in AP2h assay for the cell line indicated, after 4 days' growth.

H. represents haemocytometer count; C. represents counter count.

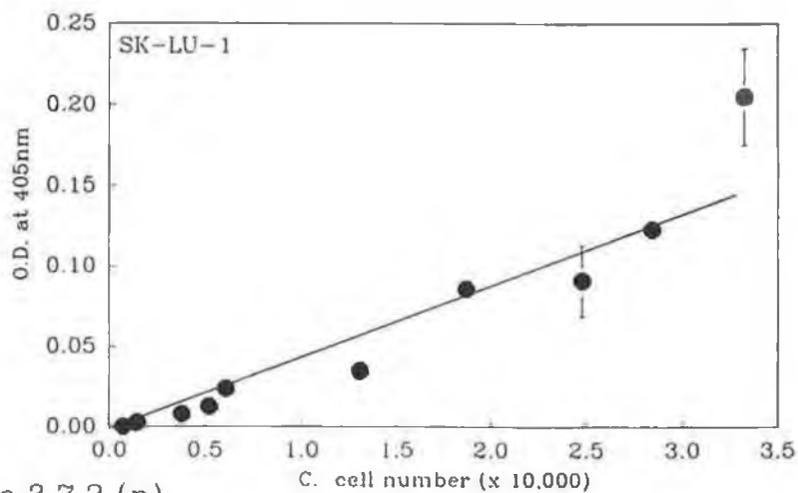


Figure 3.7.2 (n)

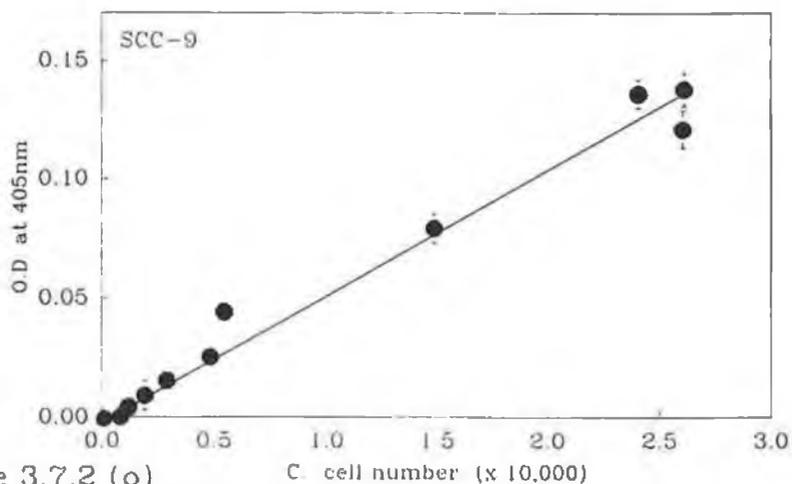


Figure 3.7.2 (o)

Figures 3.7.2 (n) - (o):

Relationship between cell number and Optical Density in AP2h assay for the cell line indicated. after 4 days' growth.

H. represents haemocytometer count, C. represents counter count

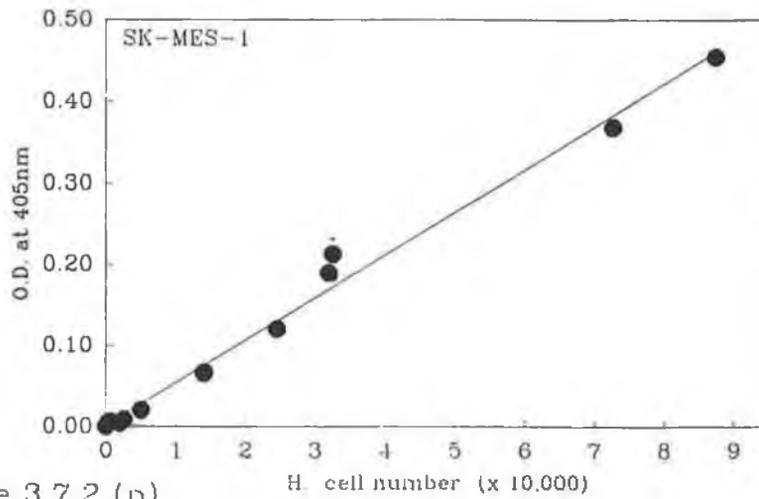


Figure 3.7.2 (p)

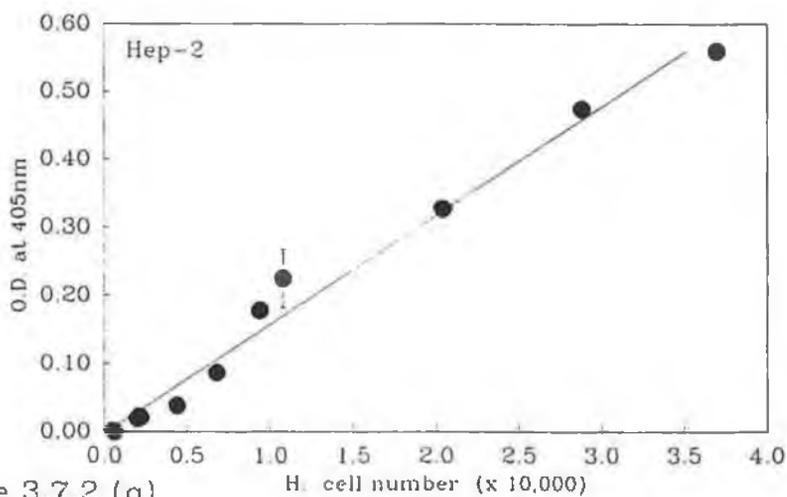


Figure 3.7.2 (q)

Figures 3.7.2 (p), (q):

Relationship between cell number and Optical Density in AP2h assay for the cell line indicated, after 4 days' growth.

H. represents haemocytometer count; C. represents counter count.

3.7.3 Detection level

Table 3.7.3 Lowest detectable cell number with the AP2h assay (cell number corresponding to 0.02 OD units)

cell line	cell number/ 96 well	
	1 day	4 days
RPMI-2650	7,500	6,500
NRK	2,500	3,000
DLKP	7,500	4,500
repeat	16,000	-
Hep-2	8,000	11,500
repeat	9,500	-
SK-LU-1	14,000	3,750
SK-MES-1	7,750	4,000
repeat	6,000	
SCC-9	*	3,250

The limit of detection corresponds to reasonably small cell numbers, but overall was not very sensitive to low cell numbers. In most cases it has detected fewer than 10,000 cells/ well, but it was generally not capable of detecting fewer than 2,500 cells/ well.

3.7.4 Linear range

Table 3.7.4 Highest cell number to which the AP2h assay was linear

cell line	linear to: cell number ($\times 10^4$) /96 well	
	1 day	4 days
RPMI-2650	≈ 12	≈ 13
NRK	≈ 12	≈ 10
DLKP	≈ 11	≈ 11
repeat	≈ 11	-
Hep-2	≈ 8	≈ 38
repeat	≈ 18	-
SK-LU-1	≈ 3	≈ 3.5
SK-MES-1	≈ 10	≈ 9
repeat	≈ 11	-
SCC-9	*	*

We can see that the AP2h assay remained linear up to high cell numbers for many cell lines. This can be easily seen from a quick glance at the Figures on the previous page. In many cases, the linear range extends throughout the entire range of cell densities, and does not level off as seen for some of the other assays.

3.7.5 Sensitivity

Table 3.7.5 Sensitivity of the AP2h assay for various cell lines (slope of the linear graph)

cell line	change in OD/ 10,000 cells	
	1 day	4 days
RPMI-2650	0.023	0.027
NRK	0.072	0.058
DLKP	0.024	0.042
repeat	0.027	-
Hep-2	0.029	0.016
repeat	0.022	-
SK-LU-1	0.014	0.052
SK-MES-1	0.022	0.050
repeat	0.030	-
SCC-9	*	0.054

* = difficult to determine from graph

Sensitivity of the AP assay, like the other assays has been determined by calculating the actual slope of the linear portion of each graph. Overall the assay was not very sensitive.

It is interesting to note that there is sometimes a great difference in sensitivity of the assay when growth after 1 day and growth after 4 days' culture are compared. This may indicate that the AP activity per cell number is not constant and may vary under different conditions.

3.7.6 Ease of performance/ technical flaws

The AP2h assay is simple to perform, only involving one rinse and addition of one compound. Once the incubation period has been carried out, the plate can be read directly without any further step. Bubbles were generally burst with a syringe needle as a matter of course (refer back to section 3.7.1.3), but these were not always present, and the exercise only took a few seconds. Whilst the substrate must be added to the buffer just before use, this may be pre-weighed, aliquoted and frozen in vials ready for use to speed up the step.

3.7.7 Applicability to a range of cell lines

Similar to the other assays, the AP assay was found to be appropriate for use, for all the cell lines tested.

3.8 Effect of increased incubation time on AP assay activity

The merits of a 2 hour AP assay have been determined and from brief comparisons to other assays (dealt with extensively in section 3.10) it appears that the assay is convenient and is linear to substantially high cell numbers, but an improvement in sensitivity would be particularly beneficial for its use in quantification of cell number. If the sensitivity of the assay could be increased without any decrease in its range of linearity, then this would be a very significant improvement in the assay.

Thus, let us now consider the effect of increasing the incubation period of the assay, and determine the effect this has on the characteristics of the AP assay.

3.8.1 Minimum detectable cell number

The lowest cell number which can be detected ($OD \geq 0.02$) decreases as incubation time of the AP assay progresses; that is, the assay becomes more sensitive at detecting low numbers of cells with increased incubation time. This was universally the case. The relationship of this increased sensitivity to low cell numbers, with OD, was approximately linear over the time ranges tested, and this is illustrated for DLKP, RPMI-2650, and SK-MES-1 in Figures 3.8.2 (a) to (d). When the assay duration was increased from 2 hours to 3.75 hours for example, 2,000 as opposed to 9,500 DLKP cells could be detected.

3.8.2 Linearity

The linearity of the AP assay has been compared over a time range for a number of cell lines and some typical data has been presented in the graphs shown. It can be seen that increasing incubation time, from 0.5 to 3 or 4 hours, results in no substantial change in linearity of cell number versus O.D.

Graphs of RPMI-2650 and SCC-9 show no change in linearity for up to 5 and 7 hour assay times respectively. After 6 hours incubation of SK-MES-1 cells though (Figure 3.8.2 (d)), the linear range under these conditions, has been reduced from 4.5×10^4 cells to 2×10^4 cells so this is an important factor to be borne in mind for this cell line.

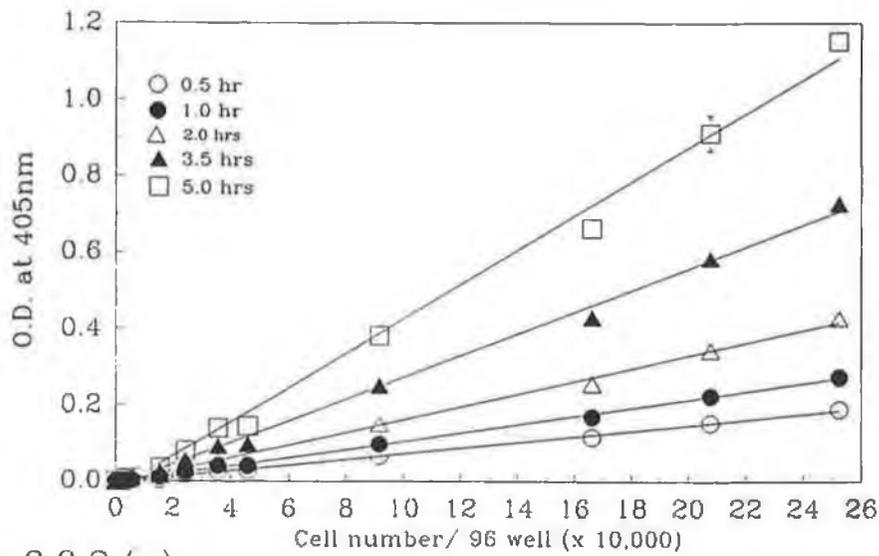


Figure 3.8.2 (a)

Relationship between RPMI-2650 cell number and AP activity for increasing assay duration (growth after 4 days' in culture).

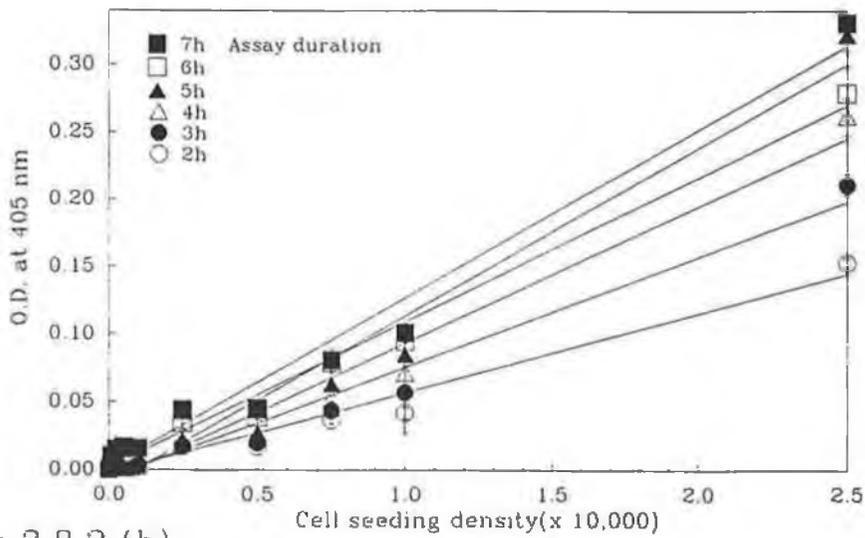


Figure 3.8.2 (b)

Relationship between SCC-9 cell number and AP activity for increasing assay duration (growth after 1 day in culture).

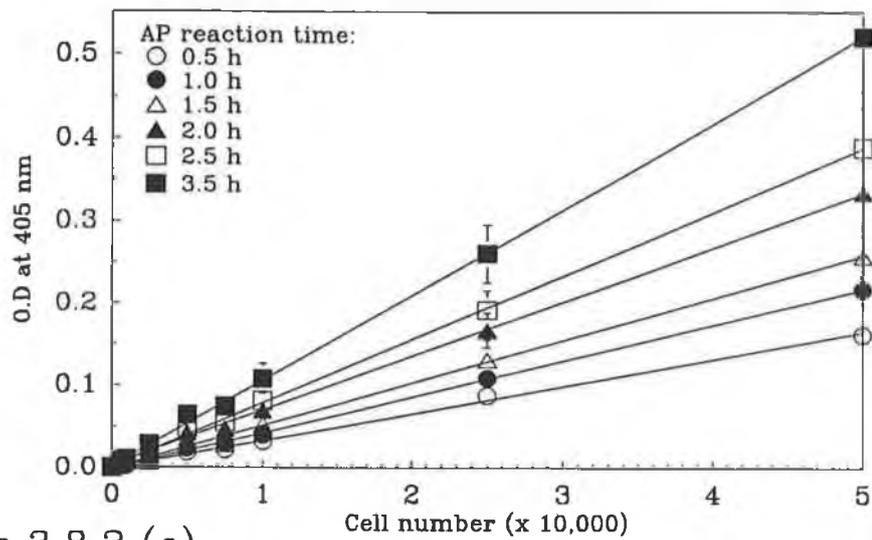


Figure 3.8.2 (c)

Relationship between DLKP cell number and AP activity for increasing assay duration (growth after 1 day in culture).--

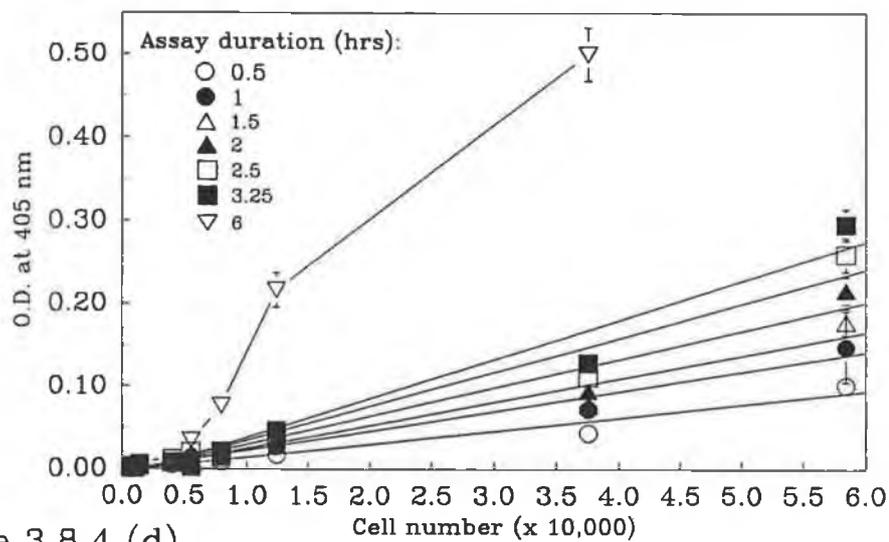


Figure 3.8.4 (d)

Relationship between SK-MES-1 cell number and AP activity for increasing assay duration (growth after 1 day in culture).

3.8.3 Sensitivity

Although increased assay duration shows no change in linearity for assay times of 4 hours at least, there is a substantial change in sensitivity with increased incubation. In all cases, increased sensitivity results from longer assay duration in a constant fashion. The Table below shows that for RPMI-2650 after 4 days' growth in culture, the sensitivity of the assay increased approximately linearly as a result of increased incubation time to 5 hours at least.

Table 3.8.3 Change in sensitivity of the AP assay for RPMI-2650 with increasing incubation time (after 4 days in culture)

Time (hours)	change in OD/ change in 10,000 cells
1	0.031
2	0.055
3.5	0.099
5	0.145

Figure 3.8.3 shows the linear relationship between assay duration and O.D. over the range 0 to 5 hours for RPMI-2650, over a complete range of cell densities.

Overall it appeared that while increased incubation time improved assay sensitivity, the linearity did not generally suffer, as the assay was still linear to high cell densities.

Use of a standard assay time of 4 hours (the same time as an MTT assay) would be a beneficial step in improving the assay as the relationship between assay duration and AP activity was found to be linear up to 4 hours at least, for a range of cell lines.

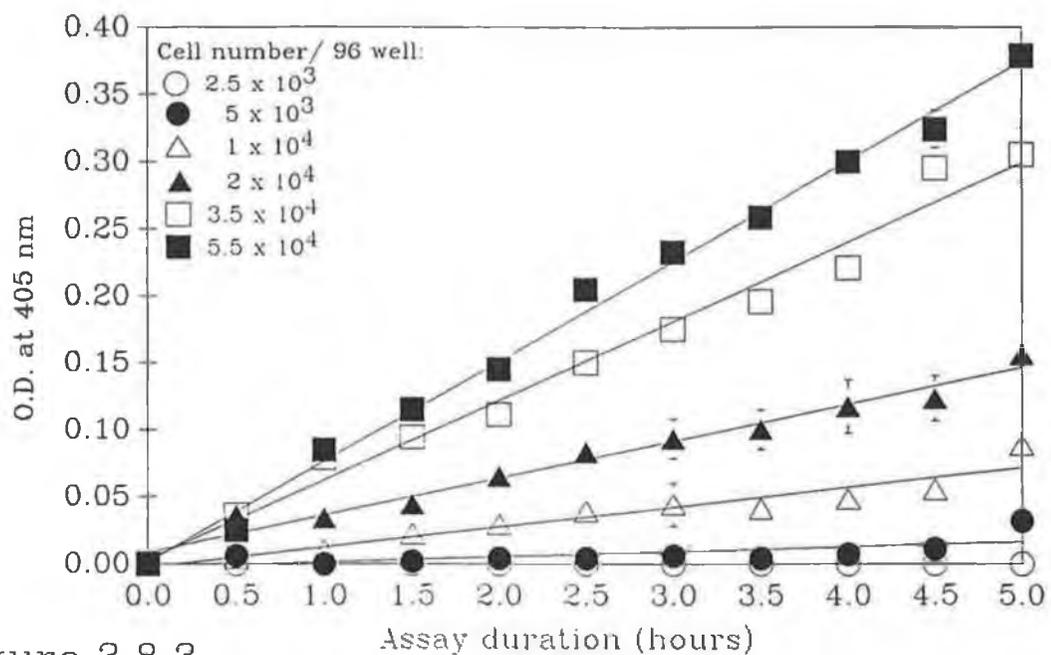


Figure 3.8.3

Relationship between assay duration and AP activity for a range of RPMI-2650 cell densities (growth after 1 day in culture; cell number by Coulter counts).

3.9 Effect of NaOH addition on AP assay

In the AP method by Connolly et al., [1986], the endpoint is determined after 10 μ l NaOH has been added to the wells. In practice, this has often resulted in a strong yellow colour with absorbance readings greater than the sensitivity of the instrument. This section is concerned with the effect of addition of NaOH on the performance of the assay.

3.9.1 Relationship between cell number and OD in the APNaOH assay

This relationship was determined in exactly the same manner as it was for the AP2h assay (section 3.7). After 2 hours standard incubation time, 10 μ l 1N NaOH was added to the plates to effect colour development. Figures 3.9.1 (a) to (d) show the relationship between cell number and OD after addition of 10 μ l 1N NaOH to an AP2h plate (for DLKP, DK-MES-1 and Hep-2 (including repeat)). The extent of the linear range of the plots, minimum detectable cell number (> 0.02 OD units) and sensitivity of the linear region of the graph were determined and are shown in Tables 3.9.2 - 3.9.4.

3.9.2 Minimum detectable cell number

Addition of NaOH caused a dramatic increase in the sensitivity of the method to low numbers of cells, compared to that detectable in the AP2h assay.

Table 3.9.2 Lowest detectable cell number for the APNaOH assay after 1 day's growth (cell number corresponding to 0.02 OD units)

cell line	cell number/ 96 well
DLKP	200
Hep-2	250
repeat	275
SK-MES-1	500

This NaOH step means that 25 times, 24 times and 20 times fewer cells can be detected for DLKP, Hep-2 and SK-MES-1 respectively, than is possible with the AP2h assay; thus leaving it highly sensitive to low cell numbers.

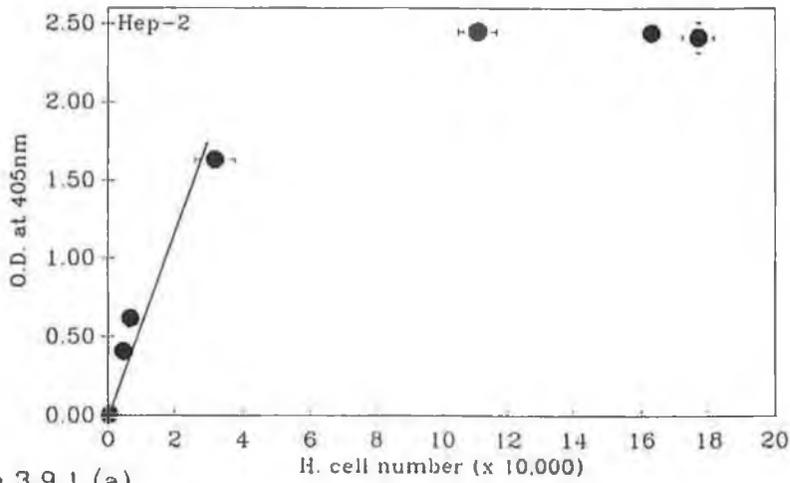


Figure 3.9.1 (a)

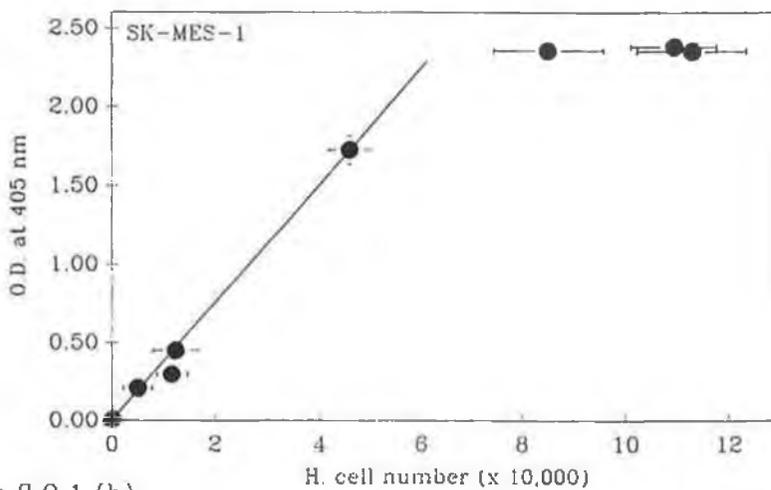


Figure 3.9.1 (b)

Figures 3.9.1 (a), (b):

Relationship between cell number and Optical Density in APNaOH assay for the cell line indicated, after 1 day's growth.

H. represents haemocytometer counts, C represents coulter counts.

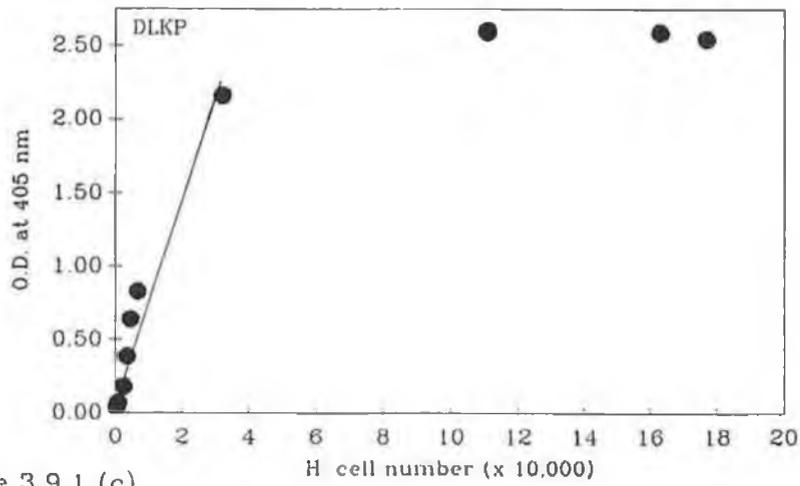


Figure 3.9.1 (c)

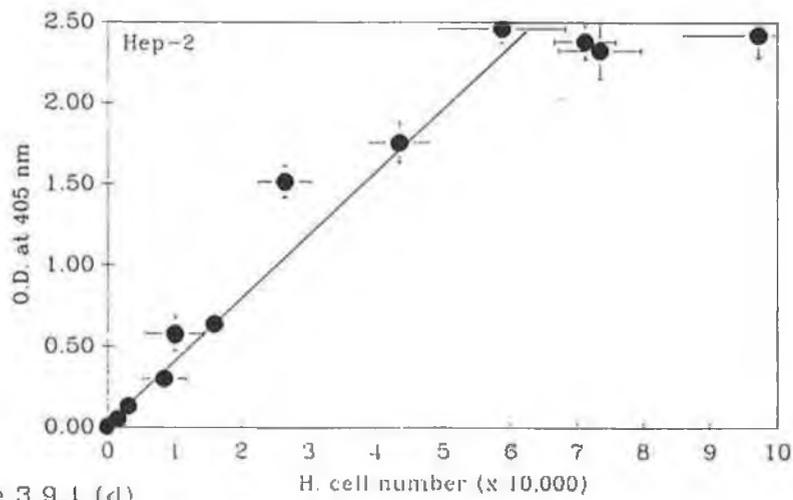


Figure 3.9.1 (d)

Figures 3.9.1 (c), (d):

Relationship between cell number and Optical Density in APNaOH assay for the cell line indicated, after 1 day's growth.

H. represents haemocytometer counts, C. represents coulter counts.

3.9.3 Linear range

Although there is still a linear relationship with cell number and OD, it's range has been decreased immensely. This is easily seen when the figures below are compared to those in table 3.7.4.

Table 3.9.3 Highest number to which the APNaOH assay remains linear (after 1 day's growth in culture)

cell line	cell number (x 10,000)/ 96 well
DLKP	2.75
Hep-2 repeat	≥ 5 3
SK-MES-1	5

The linear range for DLKP had been shortened from $\geq 11 \times 10^4$ to 2.75×10^4 ; from $\geq 18 \times 10^4$ to 3×10^4 cells/ well for Hep-2, and approximately halved for SK-MES-1 when the NaOH is added after 2h incubation.

3.9.4 Sensitivity

The increase in sensitivity proffered as a result of NaOH addition has been very substantial, as implied by the lowest detectable cell numbers shown in the relevant Table (3.9.2).

Table 3.9.4 Sensitivity of the APNaOH assay for various cell lines after 1 days growth

cell line	change in OD change in 10,000 cells
DLKP	0.725
Hep-2 repeat	0.500 0.683
SK-MES-1	0.395

Sensitivity of the APNaOH assay over the 2h assay has been augmented approximately 27 times for DLKP, 17 - 31 times for Hep-2 and 13 times for SK-MES-1.

3.9.5 Ease of performance/ technical flaws

Addition of NaOH provides an additional step over those outlined for the 2h assay, but the assay still remains simple and quick to perform.

3.9.6 Addition of different volumes and concentrations of NaOH

It was thought that by decreasing the concentration of NaOH, linearity might be increased somewhat, so consequently, a wide range of NaOH concentrations were used on replicate plates of differing cell numbers. As in all similar previous experiments, cells were trypsinized and counted by haemocytometer and Coulter counter, and actual cell number per well was plated against O.D. Ten concentrations of NaOH in the range 1N down to 0.02N NaOH were tested for cell lines Hep-2 and SK-MES-1. No change whatsoever in linearity or sensitivity of the assay was evident.

Use of yet again lower concentrations, may result in reduced sensitivity, with a concomitant increase in linearity, but this remains to be seen.

Differences in volume addition (e.g., 50 μ l instead of 10 μ l NaOH) were also considered, as accuracy would be more achievable on addition of larger volumes with a multiwell pipette. There was also no significant difference in linearity or sensitivity when the volume of NaOH was increased to 50 μ l.

In conclusion then, addition of 50 μ l NaOH can be used for a very substantial increase in the sensitivity of the AP2h assay, giving it similar sensitivity (greater in fact) than the SRB assay. However the investigator is advised to note that there will of course be a concomitant decrease in the linearity of this assay.

The practical significance of this point, and those made in the previous section about longer incubation times for the assay is summarized by the following conclusion/ recommendation. The AP assay should be briefly examined after the standard assay time of 2 hours. If there is significant colour development (visible to the eye), then it is acceptable to read the plate immediately. If there is slight but unsubstantial colour development, then the assay may be re-incubated for a further time (say a further two hours) and evaluated then. If there is no colour development at all (for example if there is only very poor cell growth evident) then it would be of greatest benefit to add 50 μ l of NaOH immediately after the 2 hour incubation. With either of these alternatives, it may be enticing for the investigator to first read the plate at the standard 2 hour assay time, and then perform the alternative treatment, so that a backup measure of the original data is available for double checking any obvious trends.

3.10 Comparison of the characteristics of the assays

In the previous sections of this chapter, lowest limit of detection, the extent of linearity and the sensitivity of each of the methods was established for a number of cell lines and was presented separately in table form. The results for these parameters are now given in a form which will allow intercomparison among the assays. The results shown below are directly comparable among the assays, as each of the assays were carried out on the same day, on the same culture, and using the same experimental procedures (*i.e.*, where Coulter counts have been used, they have been used for all of the assays, and similarly with Haemocytometer counts).

As in each of the previous sections, the Figures presented refer to actual cell number counted, and not to the original cell number which was seeded.

Coulter and haemocytometer counts did not always agree. Some data points were missing for the haemocytometer counts because the limitations of the method did not allow low cell number to be performed and frequently resulted in high SEM values.

It is important to emphasize that the values given in the Tables are not constants, so while absolute values for the different parameters (particularly linearity) have varied slightly when experiments were carried out on different days, the trends shown among the different assays were still retained, and the values in the Tables which follow, can be taken as approximate values - however the trends are real.

The performance of these assays shall now be compared, firstly in terms of lower detection limit.

3.10.1 Minimum detectable cell number

Table 3.10.1 (a) Comparison of lowest detectable cell number for each assay (after 1 day's growth)

Cell line	cell number /96 well						
	MTT	MTT/SDS	AP2h	APNaOH	NR	CVDE	SRB
SCC-9	*	-	*	-	*	1,850	-
NRK	5,000	-	2,500	-	2,000	750	-
DLKP	9,500	-	7,500	-	2,500	-	-
repeat	13,000	57,000	16,000	200	2,750	600	300
SK-LU-1	6,750	-	14,000	-	3,500	1,300	-
Hep-2	9,500	40,000	8,000	250	8,000	750	300
repeat	10,500	29,000	9,500	275	5,000	1,000	400
SKMES-1	5,250	-	7,750	-	3,300	700	-
	9,000	66,000	6,000	500	8,000	1,700	500
RPMI-2650	31,000	-	7,500	-	3,500	1,750	-

Table 3.10.1 (b) Comparison of lowest detectable cell number for each assay (after 4 days' growth)

Cell line	cell number /96 well						
	MTT	MTT/SDS	AP2h	APNaOH	NR	CVDE	SRB
SCC-9	*	-	3,250	-	1,100	1,000	-
NRK	12,000	-	3,000	-	3,500	1,200	-
DLKP	5,400	-	4,500	-	1,250	-	-
SK-LU-1	4,750	-	3,750	-	1,750	750	-
Hep-2	18,750	-	11,500	-	5,500	-	-
SKMES-1	6,750	-	4,000	-	4,500	875	-
RPMI-2650	7,500	-	6,000	-	3,750	2,000	-

The extremely poor sensitivity of the MTT/SDS assay to low cell number, has already been noted, and we can see this clearly from this Table; it required 4 times as many DLKP cells to be present, to evoke a threshold response, than it took for the standard MTT assay (and

almost 100 times more than for the CVDE assay!), while it required 7 times as many SK-MES-1 cells to be detected with this assay than with the MTT assay.

The MTT and AP2h assay were often comparable in terms of sensitivity to low cell number; for some cell lines the MTT assay detected slightly lower numbers, but more often, it was the AP2h assay which was the more sensitive to these cell numbers. The AP2h assay was particularly better, in this respect, for NRK and RPMI-2650.

The NR assay appeared next in rank order of detecting low cell numbers. It was sometimes able to detect cell numbers only very slightly lower than, or similar to the AP2h assay, but on most occasions it was able to detect about 3 times fewer cells than the AP assay. The CVDE assay was much more sensitive to detection of low cell numbers than the NR assay or the other assays already mentioned. It detected 2 to 4 times fewer cells than the NR assay.

The superior performance of the SRB assay in this comparison is obvious from the Table, however the APNaOH was even more sensitive again to low cell numbers. SRB and APNaOH were capable of detecting very low cell numbers - certainly fewer than 500 cells per well. In using Hep-2 cells in an experiment similar to the ones described here, it might be expected that the SRB assay would detect 2.5 times fewer cells than the CVDE assay, approximately 15 times fewer than NR, 12 - 20 times fewer than AP2h, 30 times fewer than MTT and at least 75 times fewer cells/ well than the MTT/SDS modification. If instead the APNaOH assay was used, then these figures would be further increased by a factor of 1.3!

These findings are mirrored somewhat by Tables 3.10.2 which show the sensitivity of the various assays over the linear portion of the graph.

3.10.2 Sensitivity of various methods

Table 3.10.2 (a) Sensitivity of various methods after 1 day's growth for a range of cell lines

Cell line	change in OD/change in 10,000 cells						
	MTT	MTT/SDS	AP2h	APNaOH	NR	CVDE	SRB
SCC-9	*	-	*	-	*	0.086	*
NRK	0.036	-	0.072	-	0.096	0.188	-
DLKP	0.020	-	0.024	-	0.061	-	-
	0.019	0.003	0.027	0.725	0.110	0.347	0.562
SK-LU-1	0.030	-	0.014	-	0.050	0.122	-
Hep-2	0.022	0.005	0.029	0.500	0.030	0.258	0.537
	0.016	0.004	0.022	0.685	0.039	0.273	0.433
SKMES-1	0.035	-	0.022	-	0.058	0.305	-
	0.018	0.003	0.030	0.395	0.024	0.147	0.358
RPMI-2650	0.007	-	0.023	-	0.045	0.160	-

Table 3.10.2 (b) Sensitivity of various methods after 4 days growth for a range of cell lines

Cell line	change in OD/change in 10,000 cells						
	MTT	MTT/SDS	AP2h	APNaOH	NR	CVDE	SRB
SCC-9	*	-	0.054	-	0.176	0.193	-
NRK	0.019	-	0.058	-	0.064	0.169	-
DLKP	0.034	-	0.042	-	0.162	-	-
SK-LU-1	0.037	-	0.052	-	0.124	0.227	-
Hep-2	0.010	-	0.016	-	0.034	-	-
SKMES-1	0.028	-	0.050	-	0.049	0.304	-
RPMI-2650	*	-	0.027	-	0.031	0.088	-

It is obvious that there is a very great difference in sensitivity among the methods. For some of the cell lines the distinction among the assays is extreme; take DLKP for instance, where the APNaOH assay is >240 times more sensitive than the MTT/SDS assay, so again attention is drawn to the MTT/SDS method, which is much less sensitive than all of the other assays. In fact this assay is extremely insensitive.

While the MTT assay is clearly an improvement on this, it too is quite insensitive; certainly less sensitive than the other methods. The remainder of the assays are in the following order of increasing sensitivity: AP2h, NR, DE, SRB and APNaOH. APNaOH is significantly more sensitive than SRB for the three cell lines tested above, and is considerably more sensitive than each of the other assays, giving a very great increase in OD per increase in cell number.

3.10.3 Linear range

Table 3.10.3 (a) Highest cell number to which the assay is linear (after 1 days growth)

Cell line	cell number ($\times 10^4$) /96 well						
	MTT	MTT/SDS	AP2h	APNaOH	NR	CVDE	SRB
SCC-9	*	-	*	-	*	*	*
NRK	≥ 8	-	≥ 12	-	3.5	6	-
DLKP	≥ 9	-	≥ 11	-	≥ 6.5	-	-
repeat	16	≥ 16	≥ 11	2.75	2 - 3	4	3
SK-LU-1	*	-	≥ 3	-	≥ 3.5	≥ 3.5	-
Hep-2	≥ 8 ≥ 16	≥ 9 ≥ 18	≥ 8 ≥ 18	≥ 5 3	≥ 9 *	5.5 ≥ 4	2.75 3 - 4
SKMES-1	≥ 4 ≥ 8.5	- ≥ 11	≥ 10 ≥ 11	- 5	≥ 3.5 ≥ 11	≥ 3 8	- 5
RPMI-2650	≥ 6	-	≥ 12	-	5.5	7	-

* = not easily determined from graphs

Table 3.10.3 (c) Highest cell number to which the assay is linear (after 4 days growth)

Cell line	cell number ($\times 10^4$) /96 well						
	MTT	MTT/SDS	AP2h	APNaOH	NR	CVDE	SRB
SCC-9	*	-	*	-	≥ 2	≥ 2.5	*
NRK	≥ 10	-	≥ 10	-	3.5	5	-
DLKP	≥ 12	-	≥ 11	-	15	-	-
SK-LU-1	-	-	≥ 3.5	-	≥ 3.5	≥ 3.5	-
Hep-2	≥ 38	-	≥ 38	-	≥ 10	-	-
SKMES-1	≥ 9	-	≥ 9	-	≥ 9	≥ 3	-
RPMI-2650	*	-	≥ 13	-	11	9.5	-

* = not easily determined from graphs

Approximate values only could be determined from the graphs as data points were often far apart at the top of the linear range, or sometimes the top of the linear range had not been reached over the density range tested.

Not only were there differences among the cell lines, but there were clear trends among the assays. Another quick glance at the Figures for each of the cell lines can illustrate this very clearly, but this can be quantified by the data above.

Generally it is the case that the higher the sensitivity, the shorter the linear range; and it is clear that the assays demonstrated as having high sensitivity (APNaOH, SRB, and CVDE; and in that order) have shorter linear ranges. Conversely, when an assay is linear over a longer range, then the sensitivity will be decreased.

The MTT/SDS assay is linear to greater cell numbers than the MTT assay, giving it the greatest range of linearity. The AP2h assay is linear to cell numbers as great as, or even greater than the MTT assay. At the other end of the scale, are the SRB and APNaOH assays which are linear only to quite low cell numbers. In between fall the NR and CVDE assays. The NR assay is sometimes (but not always) linear to greater cell numbers than the CVDE assay.

On a general note, SCC-9 did not show as clear a relationship of cell number and OD as any of the other cell lines. Generally it was difficult to determine this relationship from any of the graphs, and this may have been related to the experimental procedure, as low cell numbers had to be used to demonstrate the relationship because of the difficulty of growing the high densities of these large, slow-growing cells required for the experiment, combined with the difficulty in counting these low cell numbers manually.

In many cases thus, linearity and sensitivity were clearly inversely related. Assays such as APNaOH, SRB, and CVDE, result in a relatively high O.D. per cell, and as a consequence are sensitive to low cell number. Because of this high O.D. though, they become non linear at moderate to high cell densities. The NR assay seems to be the exception, in that it is neither very sensitive nor is it linear to high cell numbers.

The remainder of the assays (AP, MTT and SDS/ MTT) although they are linear over a greater range of cell densities, suffer from a consequential lack of sensitivity which would make them inadequate for some work. It has been shown though, that the sensitivity of the AP assay can be substantially increased by increasing the assay duration up to 4 or 6 hours, while still retaining its favourable range of linearity. Also, addition of NaOH can vastly improve the sensitivity of the assay making it more sensitive than any of the other assays, but, similar to SRB, and CVDE, there is a consequential decrease in linearity at high numbers.

3.10.4 Precision

The task of quantifying the precision of the various endpoints was in fact quite difficult as results and trends varied from experiment to experiment. Overall values for coefficient of variation (CVar) for a very wide range of experiments (using sampling points between 0.02 and 1.6 OD units) are shown in Figure 3.10.4 (a). An attempt to get an overall picture of the precision involved with each of the endpoints determines NR, APNaOH, AP2h and MTT to be more precise (and in that order), having CVar values between 6 - 8%, than the CVDE assay (CVar = 12%). In such a comparison, the SRB seemed to be the least precise of all the methods, giving inter-replicate variations averaging at

22% of mean values. When precision for individual cell lines is considered however, the picture is completely altered for different cell lines. Figures 3.10.4 (b) - (d) illustrate the differences that can be seen among cell lines and endpoints with regard to precision. SRB was clearly the most precise assay for SK-MES-1, and the CVDE assay performed well in this comparison, however for DLKP the situation is quite the inverse - SRB giving CVar values of 20%, together with CVDE, NR and MTT. Yet again for Hep2, it is apparent that the APNaOH assay is less precise than it is for other cell lines (these trends have been confirmed by other sets of data).

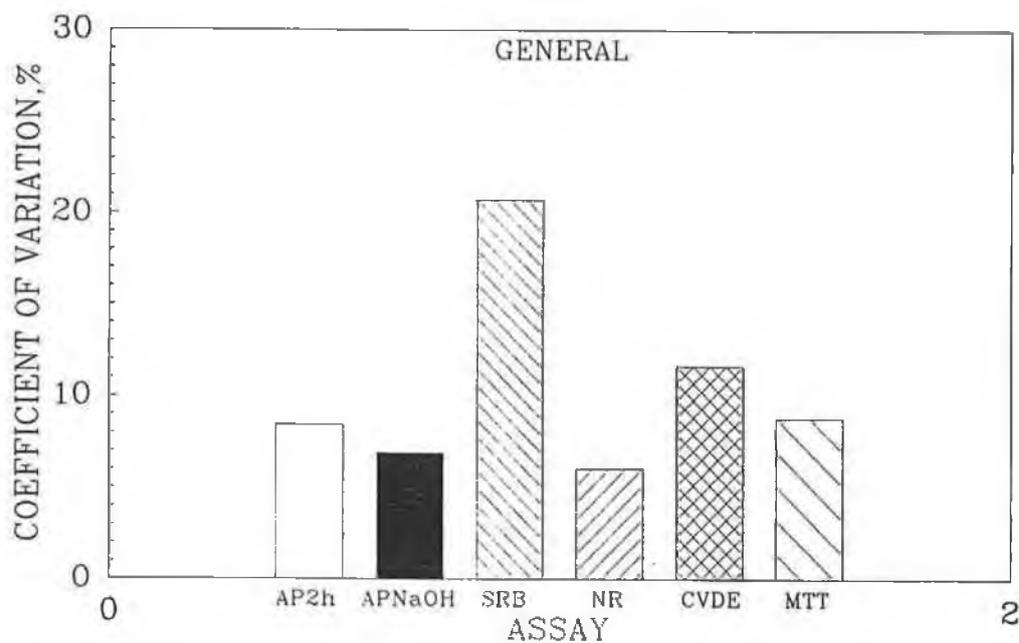


Figure 3.10.4 (a):

Overall comparison of precision of each of the endpoints over a large pool of experiments.

Values represent the averaged Co-efficient of variation (%) from these experiments.

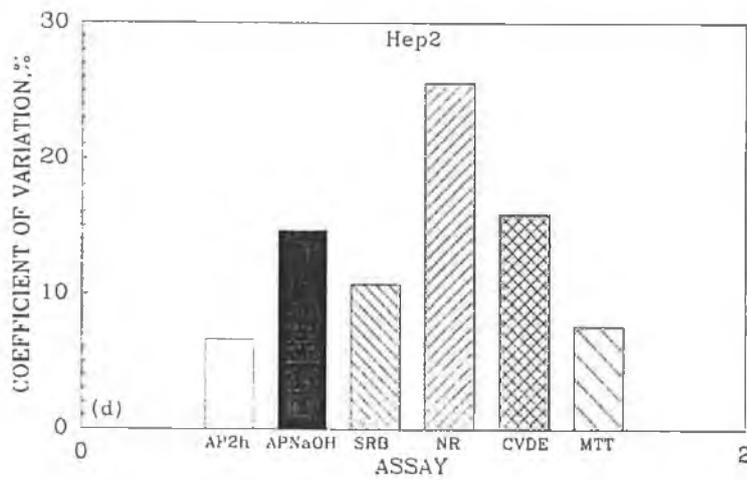
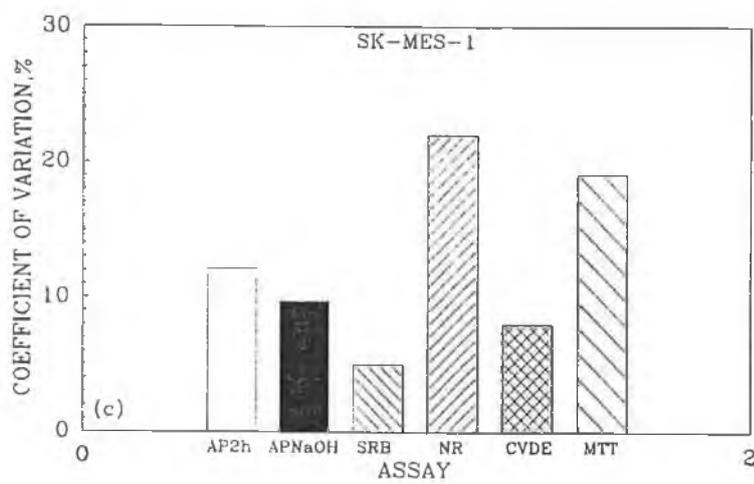
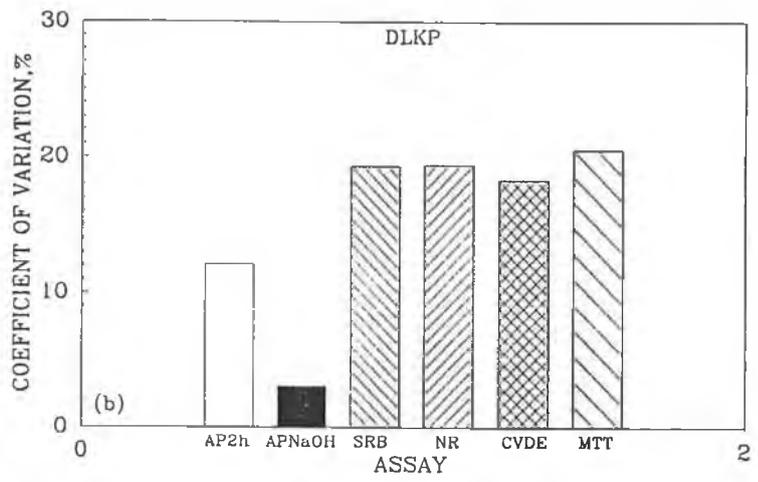


Figure 3.10.4 (b) - (d).

Precision of various endpoints for linearity experiments with 3 cell lines. Values represent averaged Co-efficients of variation for points within 0.02 - 1.60 O D units.

3.10.5 Reproducibility

Three separate toxicity assays performed in immediate succession gave rise to the reproducibility data used here. The data is shown for each of the assays in Figures 3.10.5 (a) - (j). Figure 3.10.5 (a) - (c) show how different versions of the AP assay compare in the expression of the dose response relationship with Vinblastine. In the first and the third experiment (run) shown, the values correlate well for AP2h, AP15h and APNaOH. In the second experiment however, there is significant difference between them, especially for the APNaOH experiment which differs by at least 15% for the entire range of the relationship.

The reproducibility of the APNaOH assay when the 3 runs were compared 3.10.6 (f)) was very slightly worse than that from the unstopped reaction (Figures 3.10.5 (d), (e)) and was similar to the CVDE assay (Figure 3.10.5 (g)) (repeats were within 15 - 20% of each other for the most part of the graph - at the latter end of this scale for APNaOH and CVDE). For the NR assay, repeatability was slightly greater than the AP assay. Repeatability was less good (at 25% difference) for SRB (Figures 3.10.6 (h)) and even higher at 30% for MTT (Figure 3.10.5 (i)) which in this case was also extremely imprecise. In fact, taking the precision data into account, the actual MTT graph could be anywhere between two points which are '60%' (using the units on the Y axis) apart.

With respect to the actual IC_{50} values given by each of the endpoints, they have been calculated (as shown in appendix A) and are shown in the table which follows the graph.

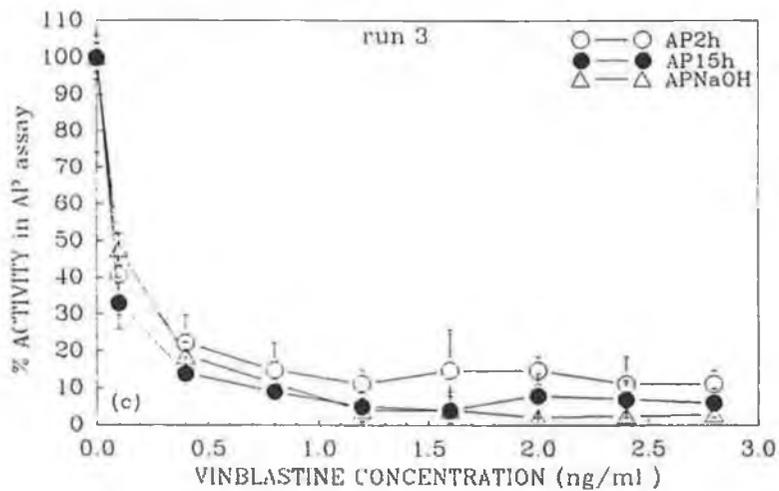
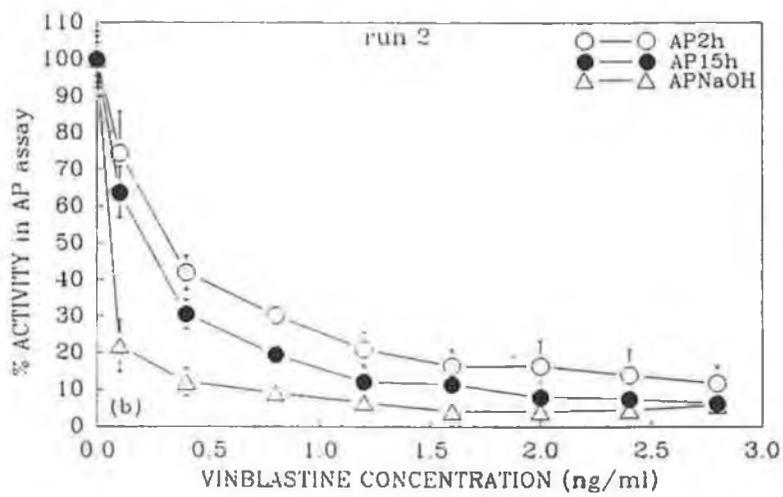
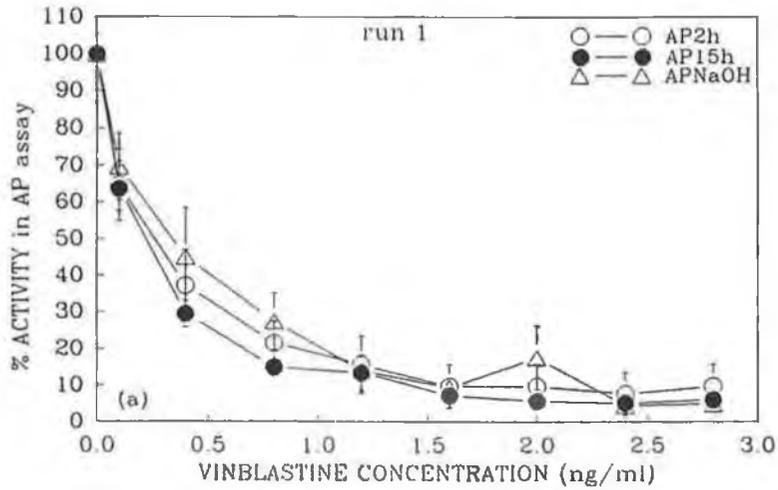


Figure 3.10.5 (a) - (c)

Comparison of AP2h, AP15h and APNaOH in determining response of Hep-2 cells to Vinblastine (after 6 days' continuous exposure).

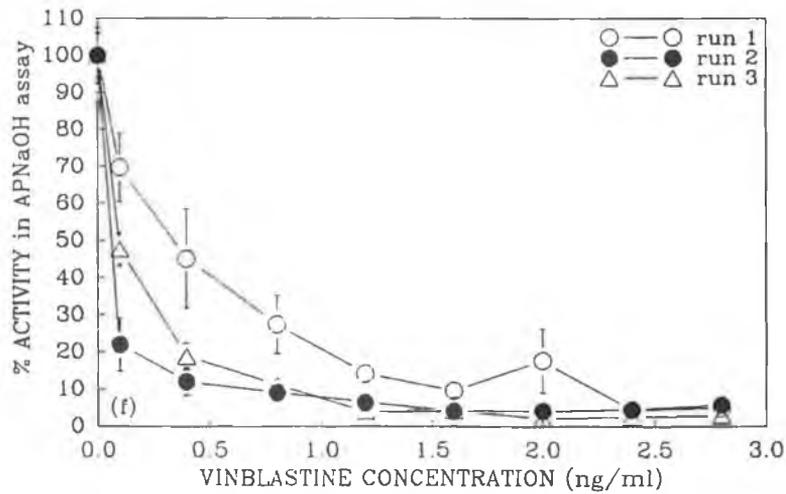
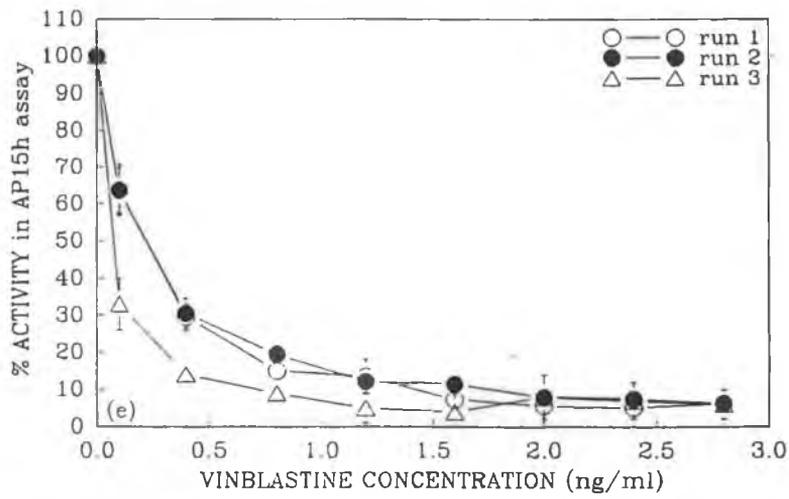
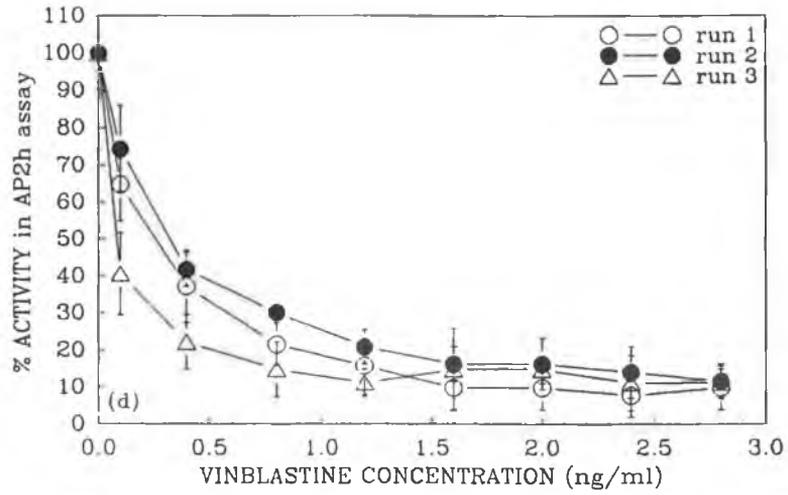


Figure 3.10.5 (d) - (f):

Comparison of repeatability of AP assay (3 modifications) in a Vinblastine toxicity experiment with Hep-2 cells. (after 6 days' continuous exposure).

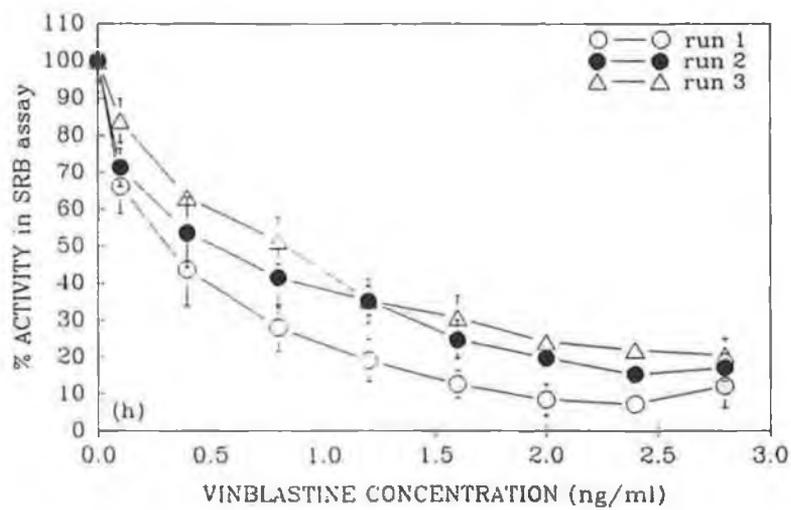
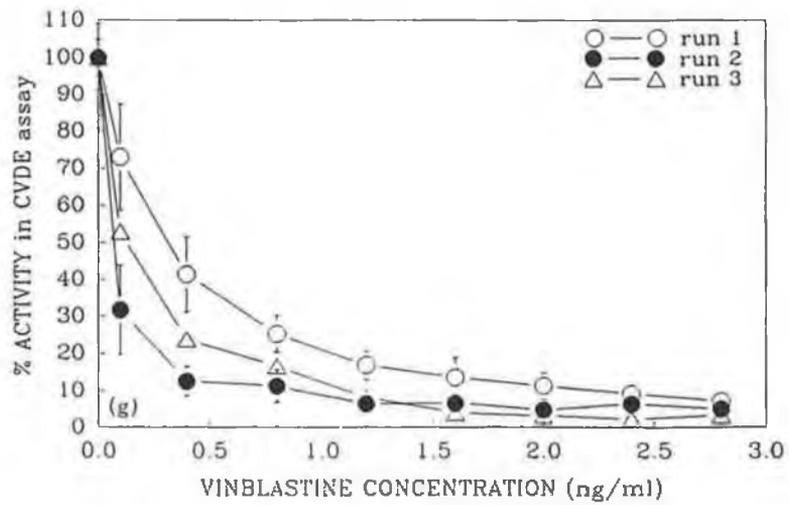


Figure 3 10.5 (g), (h):

Comparison of repeatability of (g) CVDE and (h) SRB assays in a Vinblastine toxicity experiment with Hep-2 cells. (after 6 days' continuous exposure)

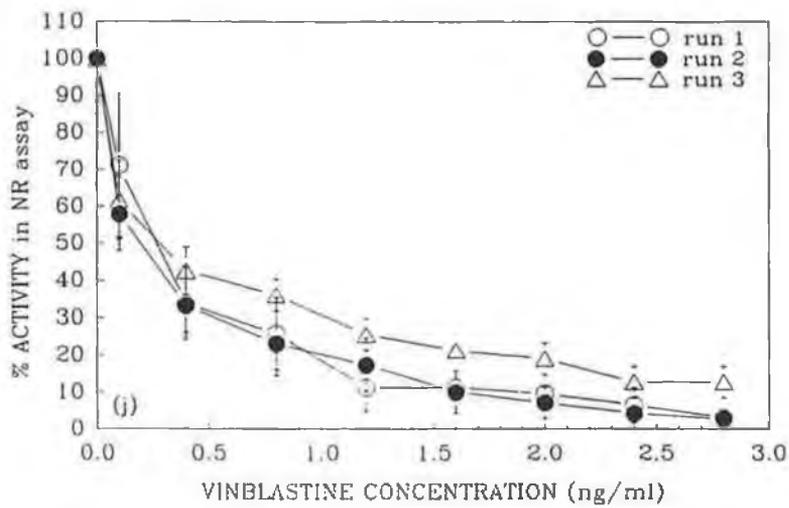
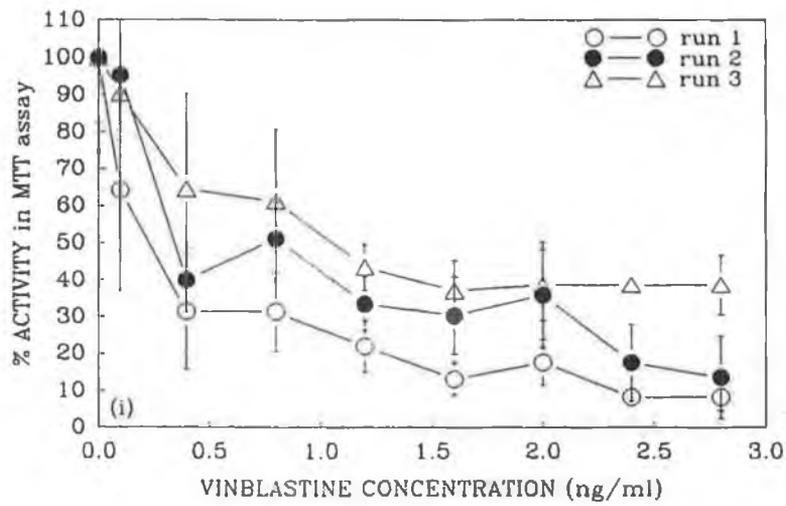


Figure 3.10.5 (i) - (j)

Comparison of repeatability of (i) MTT, and (j) NR assays in a Vinblastine toxicity experiment with Hep-2 cells. (after 6 days' continuous exposure)

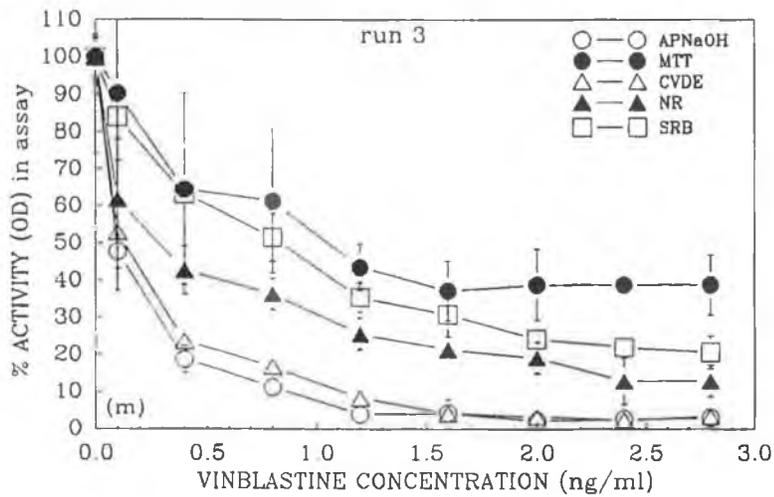
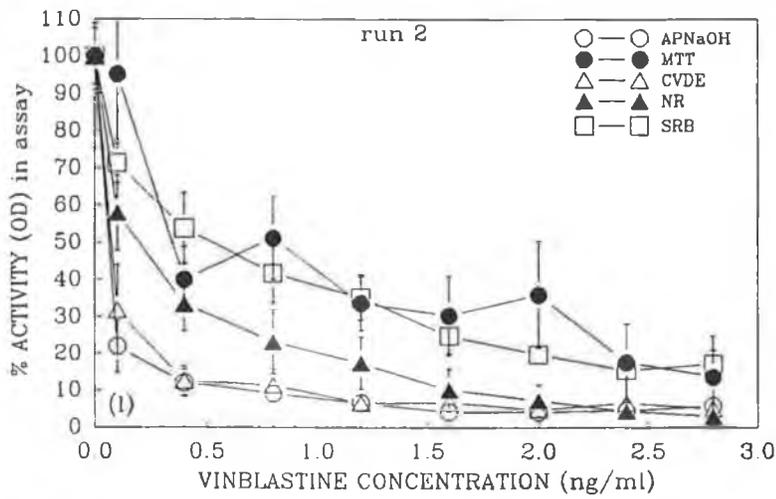
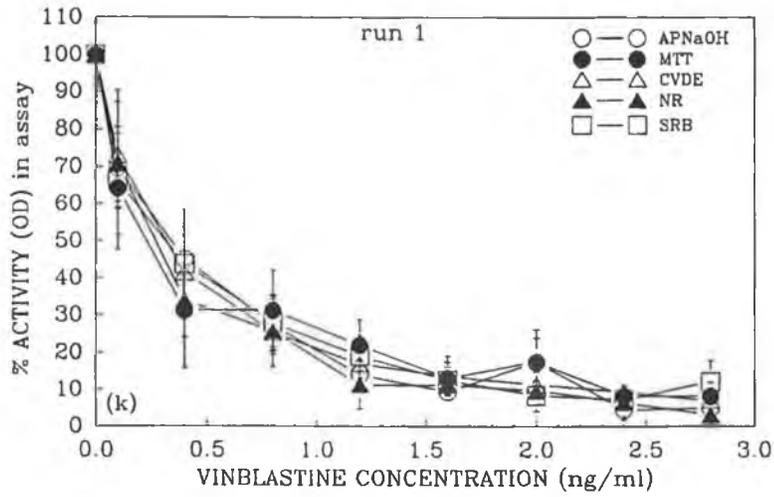


Figure 3.10.5 (k) - (m):

Comparison of repeatability of each of the miniaturized assays in a Vinblastine toxicity experiment with Hep-2 cells. (after 6 days' continuous exposure).

Table 3.10.5 Reproducibility of the IC₅₀ of Vinblastine to Hep-2 cells (5,000 cells/ 96 well) after 6 days drug exposure

Endpoint	Run 1		Run 2		Run 3	
	IC ₅₀ (ng/ ml Vinblastine)					
	±		±		±	
AP2h	0.261	0.078	0.333	0.046	0.075	0.056
AP15h	0.220	0.049	0.220	0.049	0.072	0.029
APNaOH	0.377	0.174	0.058	0.015	0.094	0.029
CVDE	0.339	0.087	0.058	0.045	0.116	0.049
MTT	0.203	0.151	* 0.333	0.058	1.067	0.130
			* 0.816	0.232		
NR	0.290	0.073	0.202	0.130	0.290	0.073
SRB	0.307	0.087	0.528	0.220	0.870	0.259

* MTT graph crossed the 50% inhibition line at 2 points in run 2.

To summarize these results, the range between which the IC₅₀ has fallen, for the 3 repeats of the Vinblastine toxicity experiment is shown in the table below. The first column shows how different the results can be, if SEM values are taken into account.

Table 3.10.5 (b) Range of IC₅₀ values in repeat determinations for various endpoints (under the conditions described above, for Hep-2 cells)

Values in ng/ml Vinblastine (x 10)

Endpoint	Range of IC ₅₀ values incorporating SEM's	Range of <u>mean</u> IC ₅₀ values
AP2h	0.019 - 0.379	0.075 - 0.333
AP15h	0.043 - 0.269	0.072 - 0.220
APNaOH	0.043 - 0.551	0.054 - 0.377
CVDE	0.013 - 0.426	0.058 - 0.339
NR	0.072 - 0.363	0.202 - 0.290
SRB	0.220 - 1.029	0.307 - 0.870
MTT	0.052 - 1.197	0.203 - 1.067

The individual IC_{50} values determined for Vinblastine exposure to Hep-2 cells under the conditions described, has varied from 0.054 ng/ml to 1.067 ng/ml when mean results from different endpoints and separate experiments are considered. The NR assay has most consistently determined the IC_{50} value. CVDE and APNaOH assays were both slightly less reproducible in determining the IC_{50} . Very poor reproducibility however was evident in the SRB assay (values spanning 6.4 times as much as NR) and the MTT assay (which indicated the IC_{50} value to fall between a range 9.8 times wider than that of the range given by NR).

For the complete graphs, including the 50% inhibition level, NR appears to be more reproducible, followed by CVDE and APNaOH. It is clear that SRB is less reproducible than the assays mentioned, followed then by MTT which was least reproducible of all.

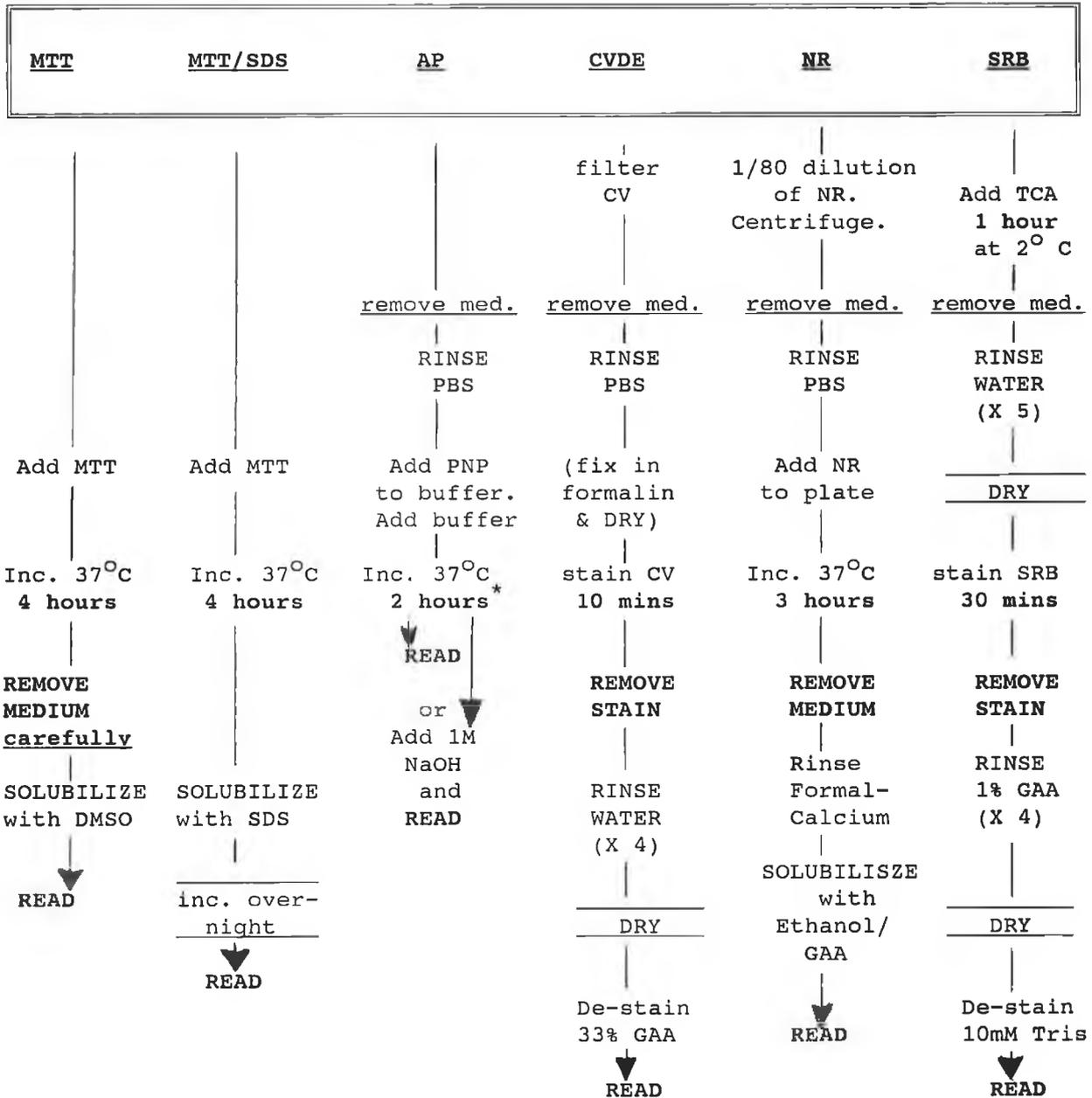
3.10.6 Ease of performance

Overall, each of the assays were easy to perform and did not suffer from technical difficulties, with the exception of the MTT assay, where medium removal without disturbing the formazan product, was difficult. The Figure which follows, is a schematic diagram of the steps involved in each of the assays, and serves to illustrate the ease of performance when each of the assays are compared.

We can see that the SRB and the NR assays together involve the greatest number of steps. If the technical difficulty of removing the waste medium without disturbing the formazan product in the MTT assay, could be overcome, then the MTT assay would indeed be very simple to perform, as there are no rinsing steps whatsoever in this assay. However, as the assay stands, the difficulty remains, limiting its simplicity. The MTT/SDS assay overcomes this problem (but as we have already seen is of limited usefulness because of its low sensitivity). The CVDE assay has one less step than the SRB assay and NR assay. Next to the MTT assay, the AP2h assay is the quickest and simplest to perform, and as it does not have the difficulties of the MTT assay, it may be favoured for this reason.

3.10.7 Applicability to a range of cell lines

The same degree of difficulties with assay quantification was not found with any of these endpoints as was experienced with the IA assay for some cell lines; however the precision involved did seem to differ among cell lines and this has been attributed to growth characteristics. The TCA precipitation step in the SRB assay seemed to result in greater variability among results for the cell line DLKP for which many of the cells are loosely adherent.



* longer if increased sensitivity is required.

☐ = Incubation times

Figure 3.10.6 Schematic diagram of the major steps involved in each of the methods compared

3.11 Relationship between assay activity and status of cell

A pertinent question at this stage, is whether or not the activity per cell in each of the assays, changes under different physiological conditions. It has already been noted that, in some cases, AP activity per cell differed from one to four days growth in culture. It would be relevant then, to determine if assay activity per cell differed when cells were cultured under different conditions. This of course would have repercussions on the suitability of these assays for toxicity testing.

To determine this experimentally, Hep-2 cells were plated out in 24 well dishes at three very different seeding densities and cultured for 11 days. The seeding densities used are given below.

Culture A = 2.5×10^3 cells/ well
Culture B = 1×10^4 cells/ well, and
Culture C = 2.5×10^4 cells/ well

After this time, cells at the lowest cell density were sub confluent, cells in the middle range were confluent, and cells at the highest density were over confluent, peeling off and in very bad condition. Thus, each culture represented cells in different stages of growth.

Cells were trypsinized and Trypan blue haemocytometer cell counts were performed on the wells as described in materials and methods. To determine the true viability of these cells, they were set up for re-growth at various clonal densities (700, 900 and 1100 cells/ 24 well). After a further 7 days growth, colony number was counted manually and compared to colony counts and area measurements as determined by image analysis.

Simultaneous with counting by haemocytometer on day 11, AP, MTT, NR, SRB and CVDE assays were carried out on replicate 24 well dishes; 3 replicate wells of each culture for each method. Further wells were stained with crystal violet and read by image analysis.

The results are presented in the Tables below. Table 3.11.1 shows the true viability of the cells from each of the cultures after a similar number of cells were set up for cloning.

Table 3.11.1 Colony formation of 700 cells deemed viable by trypan blue, for each of the cultures

Determination			
	Culture A	Culture B	Culture C
Colony count (manual)	49 ± 9	49 ± 9	66 ± 8
Colony count (IA)	6 ± 4	20 ± 11	14 ± 4
Colony area, mm ² (IA)	0.031 ± 0.030	0.181 ± 0.172	0.112 ± 0.045

Apparent differences in colony forming ability among the three different cultures of cells is not statistically significant, thus it can be concluded that under the conditions of the experiment, trypan blue viability counts have equated with true viability.

It may also be seen that image analysis colony counting did not accurately quantify colony number; and although it reiterated the same trend, the SEM values make the counts unreliable. The result when colony area was quantified by image analysis was extremely different from colony counting data, and the errors were unacceptably high. Thus this method should not be interchangeably used with colony counting (even though colony sizes were quite regular), and IA analysis overall using the AMS-40-10 was not as accurate as manual counting.

It should be concluded therefore that in this instance, image analysis would be entirely inappropriate for quantification of such cloning experiments.

Now let us examine the performance of each of the colorimetric assays in its ability to assess viability under the circumstances of different cell growth. Table 3.11.2 takes the Trypan blue counts at face value and compares them to the results from each of the growth assays performed on the same day.

Table 3.11.2

Relationship between viable cell number (TB determined, confirmed by cloning) and various measures of cell growth, for different cell cultures

Determination	Culture A	Culture B	Culture C
Trypan blue cell count* $\times 10^4$	15.80 \pm 4.08	27.88 \pm 6.15	2.70 \pm 1.55
Colony area, mm ² (IA)	37.21 \pm 4.80	89.11 \pm 18.42	17.83 \pm 7.64
AP2h O.D.	0.036 \pm 0.003	0.088 \pm 0.003	0.057 \pm 0.006
APNaOH O.D.	0.713 \pm 0.022	1.273 \pm 0.060	1.068 \pm 0.042
MTT O.D.	0.036 \pm 0.006	0.077 \pm 0.015	0.033 \pm 0.014
NR O.D.	0.065 \pm 0.002	0.110 \pm 0.004	0.009 \pm 0.003
CVDE O.D.	0.294 \pm 0.039	0.547 \pm 0.109	0.218 \pm 0.064
SRB O.D.	1.426 \pm 0.124	1.980 \pm 0.028	1.269 \pm 0.010

* It was not possible to count dead cells by the Trypan blue method, because only debris was present.

It is clear that after 11 days in culture, the resultant cell number from culture B, which had a seeding density of 10^4 cells/well, is 1.76 times that of culture A (seeded at 25,000 cells/well). At a seeding density of 5×10^4 cells/well (culture C, which was 20 times greater than culture A), there were 5.8 times less cells than the number present in culture A. Thus cells have stopped growing and have detached from the surface.

Image analysis (growth area) determined that there was 2.7 times more growth on the middle density plate, and half as much growth on the highest density plate. Thus this technique is obviously appropriate for the first two densities, but was clearly unable to give a reliable indication of viability in the highest density plate.

AP2h, APNaOH, MTT and CVDE assays all indicated approximately twice as much growth in the middle density plate as there was in the lowest, and the same as, or half as much growth again on the highest density plate as the lowest (when SEM values are considered). This latter result does not give a good indication of viable cell number present, as determined by direct counting. The SRB assay detected greater difference among the three densities than did any of the other assays. NR assay results bore greatest resemblance to those from the viable haemocytometer counts.

To answer the question in hand as to whether the assay activity per viable cell varies under these different culture conditions, the activity (in terms of O.D. units) per 10,000 viable cells have been tabulated below for the assays.

Because the error on the haemocytometer count is so large for the highest seeding density (the % error for the other two counts is similar to each other), the range of values for each of the assays has been given for this count.

Table 3.11.3 Relative optical density of various colorimetric assays per 10,000 viable cells

Determination	O.D. per 10,000 cells		
	A	B	C
			range
AP2h	0.0023 ± 0.0002	0.0030 ± 0.0001	0.0134 to 0.0496 ±0.0014 ±0.0052
APNaOH	0.0451 ± 0.0014	0.0457 ± 0.0022	0.2520 0.9287 ±0.0099 ±0.0366
MTT	0.0023 ± 0.0004	0.0028 ± 0.0005	0.0078 0.0287 ±0.0033 ±0.0122
NR	0.0041 ± 0.0001	0.0039 ± 0.0001	0.0021 0.0078 ±0.0007 ±0.0003
CVDE	0.0186 ± 0.0025	0.0196 ± 0.0039	0.0513 0.1896 ±0.0151 ±0.0577
SRB	0.0803 ± 0.0078	0.0710 ± 0.0001	0.3355 1.2400 ±0.0292 ±0.1078

It may be seen that for all of the assays, except SRB, the activity of the parameter being measured is similar per cell for the first two cultures; but there is some variation for the superconfluent

culture. AP activity per viable cell number is much higher in the superconfluent culture than it is per viable cell in the other cultures. The same is true of the CVDE assay. Enzyme activity in the MTT assay appears to be slightly higher in the last column (even when errors on haemocytometer counts in the first two columns are considered), but not to the same extent as the other assays already mentioned. NR is the most consistent in its activity; when related to cell number, the activity is quite constant in all culture conditions.

The relative O.D. per cell obtained in the two lower density cultures differs slightly, but that obtained for the superconfluent culture is exceedingly different. In fact, the SRB assay has performed very badly, relative to the other colorimetric assays and to cell counts, in this experiment.

Unfortunately, time did not permit a repeat of this experiment, but even this single experiment seemed to answer the question in hand.

In conclusion then, it does appear that when cells are in a substantially different state of growth, the activity relating to different endpoints per cell may change significantly. In this case, cells in poor condition had greater AP activity, while two cell cultures, both still growing (slowly, due to limited nutrients, and to limited surface for attachment for the confluent culture) at different cell densities, had similar activity per viable cell. Similar variation in results from different cultures occurred for the CVDE assay, and less so for the MTT assay. There was no difference in the O.D. per viable cell under different conditions for the NR assay. The O.D. per cell in these three cultures differed most for the SRB assay. This conclusion confirms the assumption made in sections addressing sensitivity of the assays (particularly section 3.7) that a standard curve for cell number vs O.D. under the same experimental conditions must be completed on the same day as the colorimetric determination and under precisely the same conditions, if O.D. must be related to actual cell number. However these results also show that cells in different conditions can have vastly different enzyme activity (or simply O.D. measurements unrelated to enzyme activity, as in the case of CVDE), so if comparing cell number under such different growth conditions, it may not be entirely relevant to relate O.D. values back to O.D. values from control cells, to obtain values for % kill or % stimulation.

The only exception to these constraints might be the NR assay, which gave a good indication of viability indiscriminate of the stage of growth of the cell.

3.12 Relationship of endpoint to assessment of cell viability after exposure to anticancer agents

In comparing the endpoints and in making conclusions about their performance, it is important to consider any differences which may exist between the ability of each of the assays to differentiate between viable and non-viable cells. Only then can the true value of these methods as toxicity tests or generalized indicators of growth be assessed.

To assess the true relationship between viability and each of these methods after exposure to anticancer agents, the replicative potential of each cell must be determined by cloning. If colony formation occurs, then the cell has not lost its replicative potential, and is therefore still viable.

To investigate this experimentally, the assays were scaled up from 96 well (0.32mm²) to 24 well dishes (2 cm²), to enable sufficient cell growth for trypsinizing and replating the cells. Each day for 5 consecutive days, and beginning one day after continuous exposure to one concentration each of Adriamycin and Vinblastine, Hep-2 cells were trypsinized from 3 replicate wells, counted by haemocytometer after exposure to trypan blue, and the remainder of the cells were diluted and plated (in 24 well dishes) at clonal densities. Three different plating densities (500, 700 and 1,000 cells/ well) were used, to ensure at least one optimum density for counting. On replicate wells of the original 24 well dish, MTT and AP activity were determined (all as described in materials and methods).

After 11 days growth, the dishes with replated cells were stained with crystal violet and analysed both manually and by image analysis. All these results are presented in the table below. The first column contains the direct trypan blue haemocytometer counts. The three columns which follow this, relate to the colony growth after 700 of these trypan blue-determined viable cells were replated, for each of the cultures (i.e., control, Vinblastine-treated and Adriamycin - treated), and these 3 columns may be directly compared with each other to determine any differences in manual and image analysis reading of the plates. The remaining 3 columns are the results of colorimetric endpoints performed simultaneous to haemocytometer counts.

Table 3.12.1 Relationship of AP, MTT and Trypan blue exclusion to viability (as determined by clonogenicity)

(on following page)

	viable count $\times 10^4$	growth after cloning:			AP2h	APNaOH	MTT	
		colony count (manual)	colony count (IA)	colony area mm^2 (IA)				
1	O	2.96 \pm 1.66	204 \pm 20	142 \pm 7	5.14 \pm 1.53	.008 \pm .004	0.185 \pm .034	.007 \pm .004
	V	2.80 \pm 1.3	124 \pm 18	60 \pm 5	1.11 \pm 0.24	.008 \pm .006	0.159 \pm .044	.016 \pm .001
	A	2.63 \pm 1.48	207 \pm 26	73 \pm 14	1.01 \pm 0.27	.010 \pm .006	0.215 \pm 0.028	.013 \pm .002
2	O	6.60 \pm 2.25	194 \pm 20	88 \pm 17	0.82 \pm 0.11	.017 \pm .012	0.315 \pm .017	.025 \pm .003
	V	2.95 \pm 1.18	157 \pm 13	83 \pm 26	0.98 \pm 0.19	.012 \pm .003	0.294 \pm .022	.025 \pm .003
	A	6.39 \pm 2.58	194 \pm 14	112 \pm 13	1.41 \pm 0.22	.018 \pm .003	0.413 \pm .048	.025 \pm .003
3	O	5.30 \pm 1.84	282 \pm 25	118 \pm 28	1.05 \pm 0.28	.050 \pm .005	0.514 \pm .105	.023 \pm .002
	V	1.73 \pm 0.97	246 \pm 5	147 \pm 8	2.70 \pm 0.67	.039 \pm .004	0.424 \pm .115	.013 \pm .003
	A	4.60 \pm 2.14	228 \pm 38	183 \pm 15	4.87 \pm 2.17	.053 \pm .004	0.562 \pm .046	.023 \pm .002
4	O	6.32 \pm 1.44	349 \pm 29	238 \pm 33	4.46 \pm 0.99	.067 \pm .029	1.034 \pm .125	.026 \pm .002
	V	2.54 \pm 0.79	285 \pm 15	157 \pm 12	2.23 \pm 0.82	.037 \pm .007	0.681 \pm .080	.018 \pm .003
	A	6.16 \pm 1.27	282 \pm 26	227 \pm 19	5.44 \pm 2.03	.055 \pm .008	1.000 \pm .066	.029 \pm .002
5	O	7.57 \pm 1.69	386 \pm 33	249 \pm 22	13.16 \pm 1.53	.065 \pm .002	1.017 \pm .091	.036 \pm .007
	V	1.93 \pm 1.08	424 \pm 14	260 \pm 23	9.47 \pm 1.78	.047 \pm .002	0.644 \pm .024	.030 \pm .005
	A	5.31 \pm 1.26	438 \pm 33	176 \pm 100	7.91 \pm 2.41	.070 \pm .005	1.039 \pm .093	.040 \pm .006

(All data is given as Mean \pm S.E.M.)

where O = untreated by drug
V = 0.005 $\mu\text{g/ml}$ Vinblastine, and
A = 0.006 $\mu\text{g/ml}$ Adriamycin

Values on left margin refer to DAYS OF EXPOSURE TO DRUG

Let us first consider the second, third and fourth columns which relate to true viability, and compare them to trypan blue counts.

If it were the case that trypan blue underestimated cell kill from exposure to drug, (as has been well documented under certain circumstances in the literature) then the drug treated cell suspension from a trypan blue count, upon cloning, would contain a smaller number of viable cells than expected, and thus the plate, after cloning, would exhibit a smaller number of colonies than expected (*i.e.*, smaller number than the control wells). This effect has not been observed in most cases. When the SEM values are considered, there is little difference between control and drug treated wells for each of the days, or if there is a significant difference, it is showing a greater number of colonies in drug treated plates; this may be explained by the extensive SEM values observed on the haemocytometer counts. The exception to this is after 1 day's treatment with Vinblastine - here the number of colony forming cells which arose when the same number of drug treated and control cells were plated, is significantly lower than the number of colonies formed on control plates.

We can now consider the other columns, all of which relate to growth of the cells upon exposure to drug. We can see clearly that cell number in control flasks has increased by a factor of 2.5 over the 5 days of the experiment. We can also see by cell counts and by enzyme activity (in both AP and MTT assays), that Adriamycin did not elicit significant cell kill at this concentration over the entire duration of the experiment; Vinblastine on the other hand did.

To enable examination of these relationships more easily, the Table above has been transposed into a separate table, given on the following page, which expresses each of the values as a % of its own control (*i.e.*, untreated with drug) value.

Table 3.12.2 Relationship of AP, MTT and Trypan blue exclusion to viability (as determined by clonogenicity)

(on following page)

where 0 = untreated by drug
V = 0.005 μ g/ml Vinblastine, and
A = 0.006 μ g/ml Adriamycin

Values on left margin refer to DAYS OF EXPOSURE TO DRUG.

Note again that the second, third and fourth columns refer to cloning after a similar number of control and drug treated cells were counted by trypan blue haemocytometry and replated for growth.

It can be seen that for day five, there is no significant difference between clonal growth on control and drug - treated wells, so the trypan blue count has correctly differentiated between viable and non-viable cells.

On day four, there is a slight difference between control and drug - treated plates; that is, approximately 10% of the cells, indicated to be viable by trypan blue, were incapable of replication. Given the high SEM's for the trypan blue counts themselves though, this discrepancy could be accounted for in the original plating.

On day three and day two, there is no significant difference between Adriamycin clonogenicity and the clonogenicities of the control cells. There may be a slight difference for Vinblastine - treated cells, but again, it is possible that this is due to the errors in original counting (especially as the error on the Vinblastine count is quite different to the other two counts on both days).

On day one of drug treatment however, there appears to be a substantial difference between the Vinblastine treated cells and the control cells. As errors on the original trypan blue haemocytometer counts are similar for all three counts, and the difference in cloning efficiency is significant, it appears that the trypan blue method is not accurately detecting dead cells - it has determined that there has been relatively little cell kill, whereas approximately 40% of these 'viable' cells have been irreparably damaged and will not form colonies.

From these two tables then, now consider the performance of the colorimetric assays.

At day one, when it appears that there is significant cell kill not being picked up by trypan blue, neither the AP2h nor the MTT assay detect cell kill either (in fact the MTT results indicate significant stimulation!). The APNaOH modification indicates some slight kill, but this is not statistically significant. Referring to Table 3.11.1, we see that the OD values for the AP2h and MTT assays are extremely low; in fact the assays are not sensitive to the amount of cell growth present. The APNaOH assay is sensitive to this level of growth, and indeed is indicating a better ability to differentiate between non-viable and viable cells than is the trypan blue method.

On day two, the MTT and AP2h assays are still insensitive (having low O.D. values); MTT does not detect any cell kill at all, whereas AP2h and APNaOH do detect some kill (note that the errors on the APNaOH assay are low). Direct counting shows that there is in fact, some cell kill. All three colorimetric methods agree with counts and cloning data in reporting no cell kill from Adriamycin.

Now consider day three, in which there is significant cell kill by Vinblastine (and remember that cell kill may even have been underestimated by the trypan blue count). All three colorimetric assays detect cell kill, MTT to a greater extent than AP.

On days 4 and 5, all three colorimetric assays detect cell kill induced by Vinblastine (and not by Adriamycin) to more or less the same degree. On day 5, the errors for the MTT assay are greater than the AP assays.

To sum up the main disclosures of the experiment, firstly the SEM's on the haemocytometer counts are too large to allow a more thorough analysis of the results.

The concentration of Adriamycin used ($0.006\mu\text{g/ml}$) did not result in significant Hep-2 cell kill, even after 5 days. At one days exposure of Hep-2 cells to $0.005\mu\text{g/ml}$ Vinblastine, the trypan blue method appears to underestimate cell kill.

Thereafter, and for all concentrations of Adriamycin, this method does not appear to be inconsistent with indications of viability from cloning experiments; however some suggestions of inconsistency cannot be properly evaluated because of high variations in haemocytometer counts.

The MTT assay is inappropriate for use for the first two days of the assay; the AP_{2h} assay, for the first day; because of insensitivity. Thereafter all three assays are relatively close in their assessment of viability. Again because of large errors, it is not possible to produce quantitative data on the accuracy of the methods in detecting true viability, but it appears that the APNaOH assay is more sensitive than trypan blue, at detecting loss of viability after one days exposure to Vinblastine.

It is clear that the method of counting by haemocytometer puts the limiting constraint on determining an answer to this question. In an attempt to defray the technical errors incurred by using this method, the assay was further scaled up into 25cm² flasks and repeated. It is described below.

Hep-2 cells were set up in 25cm² flasks at an equivalent cell density to area ratio as in the previous experiment (all described in section 2.30) and continuously exposed to drug. It was decided to concentrate solely on Vinblastine and to include three concentrations of the drug.

This assay was technically an extremely difficult assay to carry out, as it was so large (700 small flasks were set up for the experiment) and strictly dependent on timing. It was set up three times in total, but because of the possibility of so many technical hitches, only results from the last run could be used. It was necessary to avail of technical assistance in performing the cell counts (due to timing of the other parts of the experiment) and this is gratefully acknowledged in the Acknowledgements.

Triplicate flasks were set up for each point. After 2 days', 3 days', 4 days' and 6 days' exposure to drug, cells from drug - treated (3 drug concentrations) and control flasks were trypsinized and counted by the trypan blue haemocytometer counting method. Cells from two out of the three replicate flasks were set up separately for a cloning

assay at 3 separate cell densities. This was to ensure that at least one of the cell densities would have optimum colony growth for counting. After 7 days these plates were stained and colonies were counted manually.

One days' exposure to Vinblastine was specifically excluded from the experiment as it was felt that cell number would still be too low and that results would then be statistically limiting. To overcome this, but yet to observe an early drug effect possibly unidentified by trypan blue, three concentrations of Vinblastine were used.

For this experiment, a new batch of Vinblastine was used. On rangefinding experiments with the new batch, the activity was much greater, thus unfortunately the results from the first and second assay cannot be directly compared. Much lower concentrations were then needed to give the same extent of cell kill. This discrepancy must be attributed to storage of the drug, for though it was aliquotted into vials and stored at -20°C , the stocks were 2 years old.

Simultaneous to counting, AP, NR, MTT, DE and SRB assays were carried out in flasks on each of the drug concentrations (3 replicates per drug concentration for each assay on each day) as described in the materials and methods.

Cell counts, colony forming efficiencies of trypsinized cells and activity determined by each of the colorimetric assays was calculated for each day, and the results are summarized in the tables which follow.

Table 3.12.3 Relationship between trypan blue count and true viability

Vinblastine concentration $\mu\text{g/ml}$	Viable cell count (Trypan Blue) $\times 10^4$				Colony formation after 1 week			
two days' exposure to drug								
	count, $\times 10^4$		% survival		colonies		% control	
0	106.0 \pm 6.9		100.0 \pm 6.6		54 \pm 13		100.0 \pm 24.1	
0.0005	50.0	5.6	47.2	5.3	-		-	
0.00075	23.7	5.6	22.3	5.3	30	5	56.0	9.3
0.001	22.0	5.0	20.7	4.7	32	10	59.4	18.5
three days' exposure to drug								
0	177.0 \pm 28.9		100.0 \pm 16.4		56 \pm 6		100.0 \pm 10.7	
0.0005	87.9	16.3	49.7	9.2	58	15	103.6	26.8
0.00075	22.9	2.7	12.9	1.5	62	16	110.7	28.6
0.001	22.5	0.8	12.7	0.4	59	18	105.4	32.1
four days' exposure to drug								
0	250.0 \pm 29.6		100.0 \pm 11.8		110 \pm 30		100.0 \pm 27.3	
0.0005	112.5	27.8	45.0	11.1	132	8	120.0	7.3
0.00075	32.5	8.9	13.0	3.6	-		-	
0.001	18.2	4.9	7.3	2.0	79	31	71.8	28.2
six days' exposure to drug								
0	116.5 \pm 38.8		100.0 \pm 33.3		-		-	
0.0002	103.8	23.6	89.1	20.3				
0.0004	33.4	0.4	28.7	0.3				
0.00075	11.8	18.4	10.1	15.8				

The first thing to note from the table is that significant cell kill does occur for the Vinblastine concentrations chosen, and at the higher drug concentrations used, the extent of cell kill increases slightly with time. At 0.005 $\mu\text{g/ml}$ Vinblastine though, approximately 50% of the population has been killed after 2 days' exposure; longer incubation (up to 4 days) with this concentration of drug does not elicit further cell kill. The errors for haemocytometer counts for 2, 3 and 4 days' exposure are relatively low (often $< 10\%$), but for the sixth day are much more significant. In expressing the cloning data, one of the three densities plated out was chosen, and actual colony number was expressed as a percentage of controls (for the above data, the plating density used was 700 cells/well).

The main conclusion that may be drawn from the above experiment is that on days 3 and 4 there was no significant difference in cloning efficiency (after replating) from 'viable' drug - treated cells (as

defined by trypan blue exclusion) and viable control cells. Thus trypan blue, under those conditions, does not appear to be underestimating cell kill.

For day two though, there is a difference between drug - treated cloning efficiencies and control cloning efficiencies. At 0.00075 μ g/ml Vinblastine, Trypan blue has underestimated cell kill by up to 40%; for the higher drug concentration the difference is not statistically significant though a similar trend is seen.

This finding mirrors the finding of the last experiment where early in drug exposure, trypan blue has underestimated cell kill.

Unfortunately a dilution error in the control plate for day 6 prevents the cloning data from being analysed. Likewise was the case for the lowest drug concentration on day 2. The experiment still allows comparisons to be made among the colorimetric assays however.

Where it has been shown that these trypan blue counts are an accurate measure of viability in determining the relationship between the colorimetric assays and cell viability under the conditions of this experiment, it is now appropriate to compare the colorimetric results to actual trypan blue viability counts. These results are presented overleaf.

Table 3.13.4

Effect of Vinblastine on Hep-2 viability:
comparison of miniaturized endpoints

VBL. ($\mu\text{g/ml}$)	AP2h	APNaOH	MTT	NR	DE	SRB
DAY 2						
0	.059 \pm .001	1.801 \pm .085	.071 \pm .003	.140 \pm .009	.436 \pm .016	.718 \pm .017
0.0005	.047 \pm .003	.984 \pm .015	.027 \pm .011	.087 \pm .002	.218 \pm .015	.310 \pm .029
0.00075	.036 \pm .001	.476 \pm .050	.016 \pm .006	.040 \pm .006	.067 \pm .034	.093 \pm .067
0.001	.042 \pm .010	.329 \pm .084	.013 \pm .005	.034 \pm .007	.077 \pm .012	.060 \pm .025
DAY 3						
0	.041 \pm .004	1.687 \pm .036	.111 \pm .003	.212 \pm .001	.718 \pm .006 \pm
0.0005	.016 \pm .010	.866 \pm .066	.071 \pm .002	.113 \pm .007	.269 \pm .011	.664 \pm .090
0.00075	.007 \pm .007	.350 \pm .010	.028 \pm .012	.028 \pm .007	.084 \pm .033	.114 \pm .048
0.001	.000 \pm .005	.125 \pm .018	.031 \pm .003	.022 \pm .010	.074 \pm .028	.120 \pm .048
DAY 4						
0	.071 \pm .008	2.332 \pm .039	.157 \pm .003	.147 \pm .007	1.086 \pm .045	1.962 \pm .022
0.0005	.048 \pm .013	1.385 \pm .113	.107 \pm .006	.088 \pm .002	0.544 \pm .021	1.425 \pm .137
0.00075	.015 \pm .006	.337 \pm .086	.036 \pm .009	.047 \pm .016	0.133 \pm .060	.119 \pm .055
0.001	.001 \pm .002	.195 \pm .020	.031 \pm .007	.021 \pm .011	0.120 \pm .027	.186 \pm .133
DAY 6						
0	.077 \pm .001	2.709 \pm .080	.133 \pm .003	.256 \pm .017	.823 \pm .018	.994 \pm .003
0.0002	.042 \pm .002	2.131 \pm .221	.107 \pm .003	.179 \pm .014	.528 \pm .026	.527 \pm .013
0.0004	.014 \pm .007	.486 \pm .153	.040 \pm .005	.035 \pm .004	.134 \pm .003	.097 \pm .005
0.00075	.007 \pm .003	.069 \pm .016	.010 \pm .005	.007 \pm .001	.023 \pm .007	.009 \pm .001

where VBL. = Vinblastine

It is clear that the AP2h O.D. values are very low - even lower than the MTT values. All three drug concentrations from 2-day exposure, and the last three concentrations for the other days, have particularly low O.D values; lower, in fact than their sensitivity range. For all the other methods O.D. values are acceptable, as are SEM values.

Haemocytometer counts for control wells show that cell number increases from 2 days to a maximum at 4 days (2.36 times that of day 2 counts). CVDE elution and SRB (possibly) show this trend, but the other assays, by their O.D. values, generally indicate increasing growth as time progresses.

Unlike the last experiment, the O.D. values for all of the assays are significant, and thus the interpretation of the results can be more reliable. Also the errors in the haemocytometer counts are lower due to a greater cell number.

In comparing the performance of the assays in their ability to distinguish between viable and non-viable cells, consider the following table, where the results have been represented as percentage of control values.

Table 3.12.5 Relationship of various colorimetric assays to cell viability after drug treatment

All figures represent % survival from control figures

TB Viable count		AP2h	APNaOH	MTT	NR	CVDE	SRB
	DAY 2						
	±	±	±	±	±	±	±
A	100 6	100 2	100 5	100 4	100 6	100 2	100 4
B	47 5	79 5	55 1	38 7	62 1	42 4	50 3
* C	22 5	61 6	26 3	23 8	29 4	13 9	15 8
D	21 5	71 17	18 5	18 7	24 5	8 4	18 3
	DAY 3						
	±	±	±	±	±	±	
A	100 16	100 10	100 2	100 3	100 1	100 1	-
B	50 9	39 24	51 4	64 2	53 3	38 2	
C	13 2	17 17	21 1	25 11	13 3	12 5	
D	13 1	0 12	7 1	28 1	10 5	10 4	
	DAY 4						
	±	±	±	±	±	±	±
A	100 12	100 11	100 2	100 2	100 5	100 4	100 1
B	45 11	68 18	59 5	68 4	60 1	50 2	73 7
C	13 4	21 8	16 4	23 6	25 6	12 6	6 3
D	7 2	1 1	8 1	20 4	21 5	11 3	9 7
	DAY 6						
	±	±	±	±	±	±	±
A	100 33	100 1	100 3	100 2	100 7	100 2	100 0
E	89 20	54 3	79 8	80 2	70 5	64 2	53 1
F	29 0	18 3	18 6	30 4	14 2	16 0	10 1
G	10 16	9 4	3 0	8 4	3 0	3 1	1 0

where:

* = Trypan Blue count (TB) has underestimated cell kill by ≥ 12%

A = no drug (control)

B = 0.0005 µg/ml Vinblastine

E = 0.0002 µg/ml Vinblastine

C = 0.00075 µg/ml Vinblastine

F = 0.0004 µg/ml Vinblastine

D = 0.001 µg/ml Vinblastine

G = 0.00075 µg/ml Vinblastine

On day 2 at the lowest drug concentration, it can be noted that compared to Trypan blue counts (which also may have underestimated cell kill, as cloning data for this concentration could not be used), NR has underestimated cell kill, by at least 10%. AP2h for all three

drug concentrations has underestimated cell kill by 32%, 40% and at least 33% (for increasing concentration of drug). As the Trypan Blue count has been underestimated (by up to 12%) for 0.00075 μ g/ml, this means that APNaOH, MTT, NR, CVDE and SRB have also underestimated actual cell kill.

Each of the other assays for the other drug concentrations correlate quite well with actual viable cell number, with the exception of MTT which slightly but consistently underestimated cell kill on many occasions throughout the experiment.

On day 3, AP2h has high SEM values (12 - 24%), as does MTT for the middle drug concentration (\pm 11%). For 0.0005 and 0.001 μ g/ml Vinblastine, MTT slightly underestimates cell kill, as does APNaOH for 0.0005 μ g/ml.

On day four, for all three drug concentrations, NR underestimates cell kill (by between 10 and 15%); MTT performs similarly for the first and third drug dilution. The SRB assay disagrees somewhat with viable counts for the two lowest drug concentrations; initially it appreciably underestimates cell kill, but for 0.00075 μ g/ml Vinblastine it overestimates cell kill by about 6% - these discrepancies may simply be due to overlapping SEM values.

Because there is no cloning data for Day 6, it is not possible here to relate trypan blue counts to actual viable cell number. It is likely that the correlation is good for the 2 highest drug concentrations, as this was the case for the previous two days, however this may not be so for the lower drug concentration, as only low cell kill has occurred. Also the high SEM errors on the haemocytometer counts make interpretation of the results difficult. What can be said is that MTT has detected less cell kill than the other colorimetric assays at the two lowest drug concentrations. SRB and CVDE tended to declare higher cell kill than the other methods (particularly the former), especially at lower drug concentrations, where it is possible that cell kill has been overestimated by the Trypan Blue reference; thus, these assays may relate more closely to true viability than MTT, AP2h, APNaOH and even NR.

In conclusion then, it appears that in the early stages of drug exposure and at low concentrations of drug, there is a discrepancy between trypan blue viability determinations and true cell viability. In such instances trypan blue has underestimated cell kill as have each of the methods used here. At all other times, trypan blue was an accurate indicator of cell viability and apart from MTT which was very slightly but consistently different, each of the methods correlated quite well with TB results.

3.13 Relationship between endpoint and mechanism of action

While the work shown thus far has given a good indication of the relative performances of these assays, it has not indicated how well each of these assays responds to toxicity induced by agents of differing mechanisms of action. Because the assays measure different cellular functions, it may be reasonable to expect that each one may be more/ less sensitive than the others for a drug with a **particular mechanism of action related to that function.**

To investigate this possibility, a panel of chemicals were chosen, each having a different mechanism of toxicity.

RPMT-2650 cells were exposed to each of the chemicals listed below over an entire range of concentrations in a continuous exposure regime for 6 days, and toxicity was determined using the endpoints listed.

The chemicals tested were:

Vinblastine (VBL),
Mitomycin C (MMC),
5-Fluorouracil (5FU),
Hydroxyurea (HU),
Actinomycin D (ACT),
Cycloheximide (CHX),
2,4-DinitroPhenol (DNP),
Triton-X-114 (TX),
L-Tartaric Acid (TA),
Tunicamycin (TMC) and
Cordycepin (CCP).

The endpoints investigated were:

MTT,
AP2h,
NR,
CVDE,
DNA synthesis (incorporation of [³H] thymidine)
RNA synthesis (incorporation of [³H] uridine) and
protein synthesis (incorporation of [³H] leucine)

The chemical concentrations were chosen, after initial rangefinding experiments, to produce dose response curves within the range of cell kill. Each of the assays mentioned was then carried out on a replicate plate for each chemical. Six replicate wells were used for each chemical concentration. For comparison purposes, it was felt prudent to perform all of the assays on the same day, even though the work-load was extremely large. The radioincorporation assays for [³H] thymidine, uridine and leucine were also performed in 96 well dishes under identical experimental conditions, to enable a confident comparison to be made.

For each of the assays the dose-response curves produced are presented here in Figures 3.13.1 to 3.13.7.

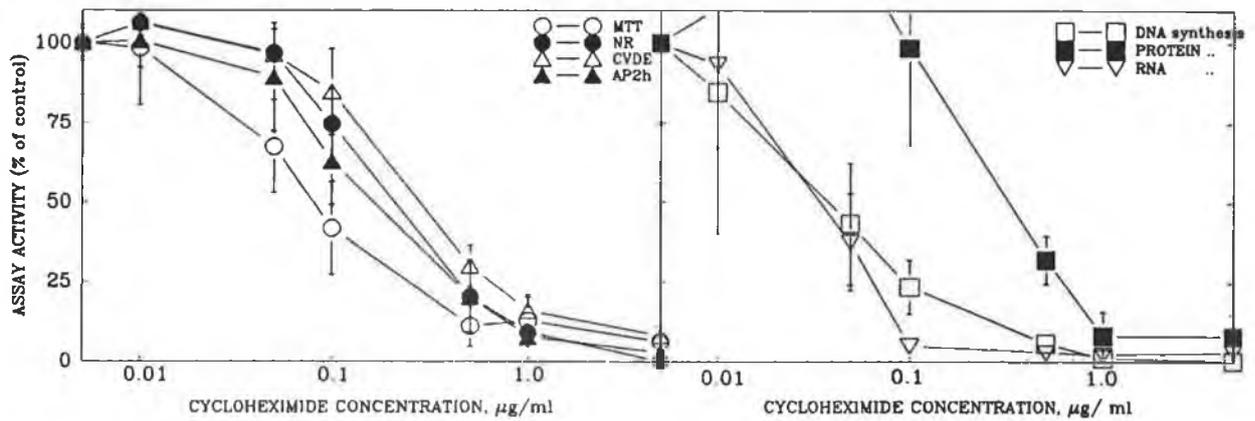


Figure 3.13.1 (a)

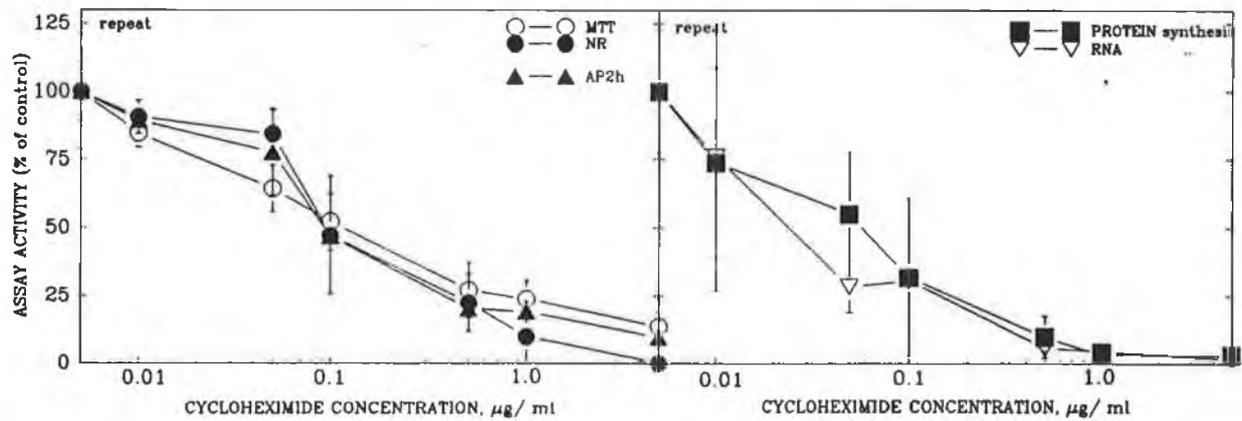


Figure 3.13.1 (b)

Figures 3.13.1 (a), (b):

Comparison of performance of various assays in detecting toxicity of Cycloheximide to RPMI-2650 cells.

(6-day exposure to chemical)

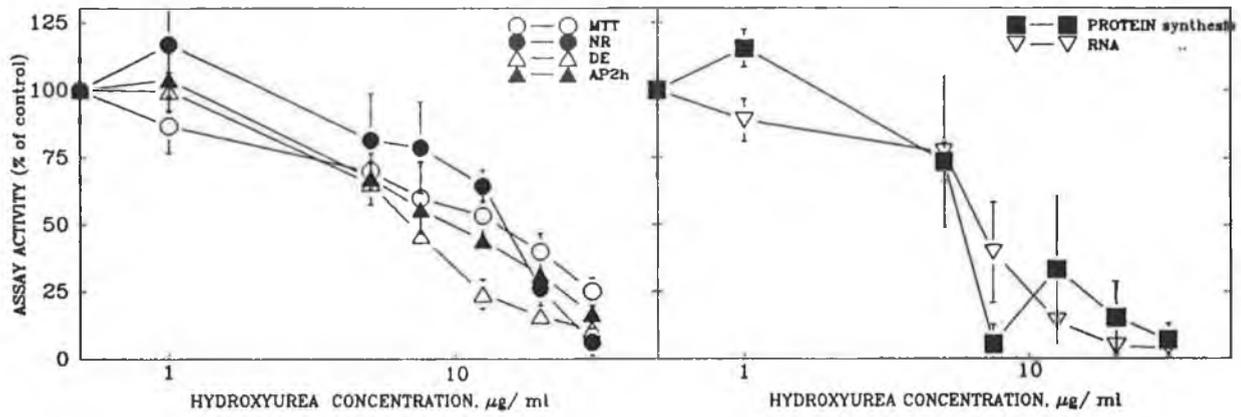


Figure 3.13.2 (a)

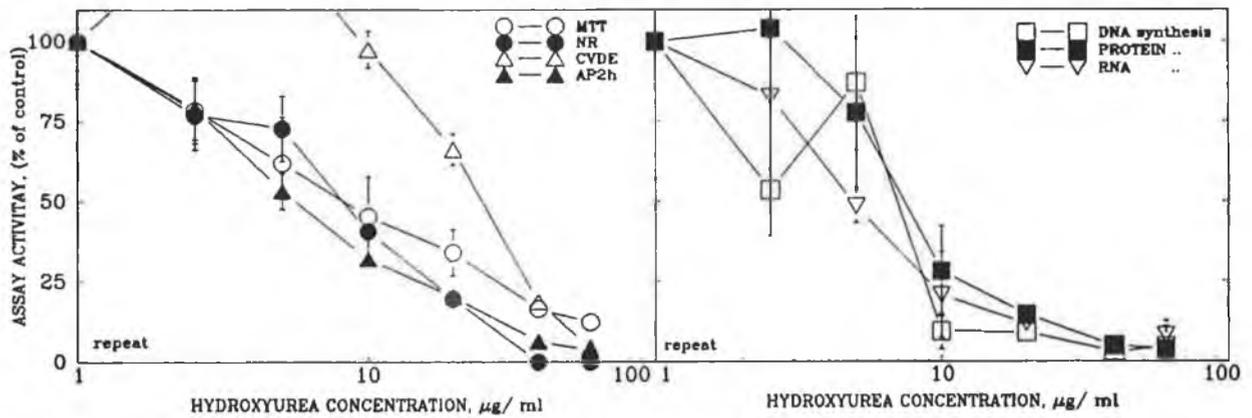


Figure 3.13.2 (b)

Figures 3.13.2 (a), (b):

Comparison of performance of various assays in detecting toxicity of Hydroxyurea to RPMI-2650 cells.

(6-day exposure to chemical)

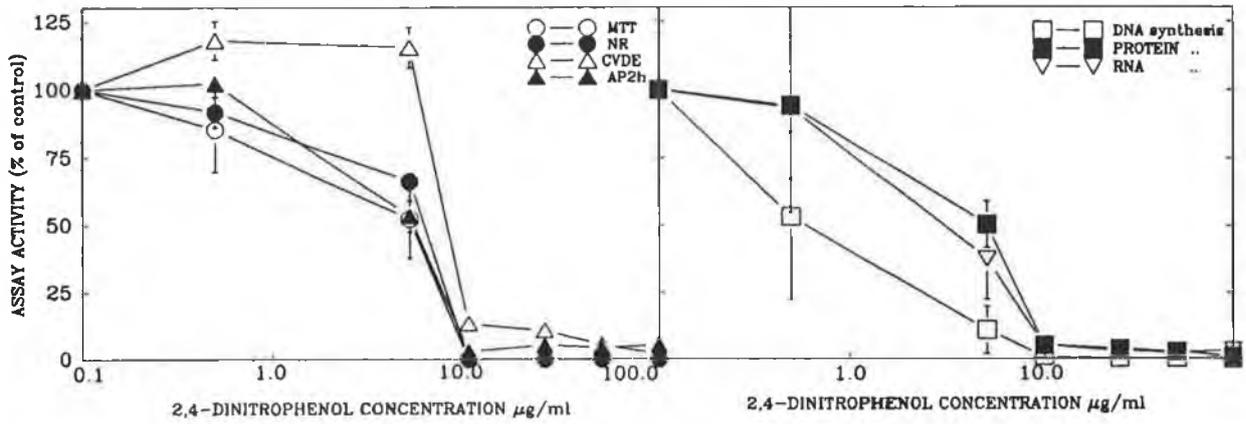


Figure 3.13.3 (a)

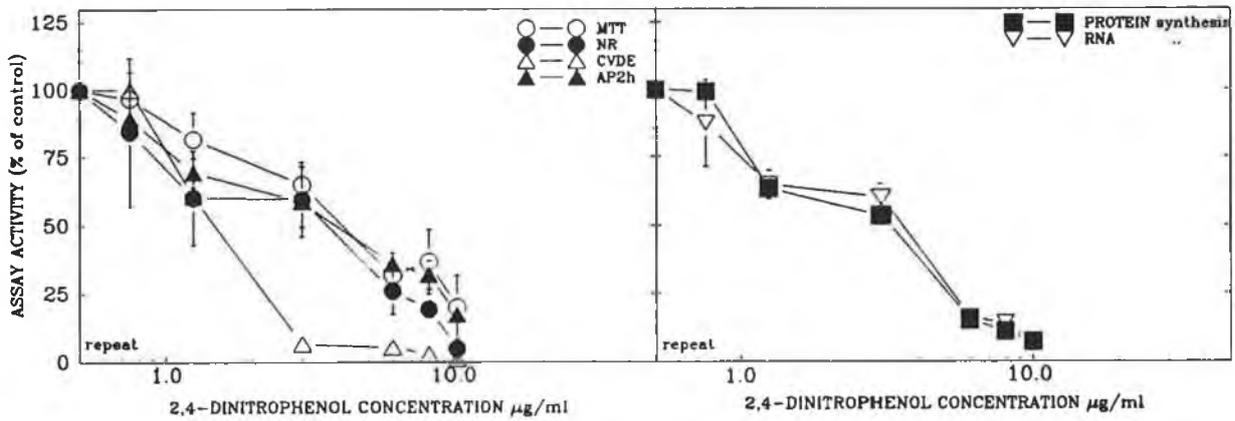


Figure 3.13.3 (b)

Figures 3.13.3 (a), (b):

Comparison of performance of various assays in detecting toxicity of 2,4-Dinitrophenol to RPMI-2650 cells. (6-day exposure to chemical).

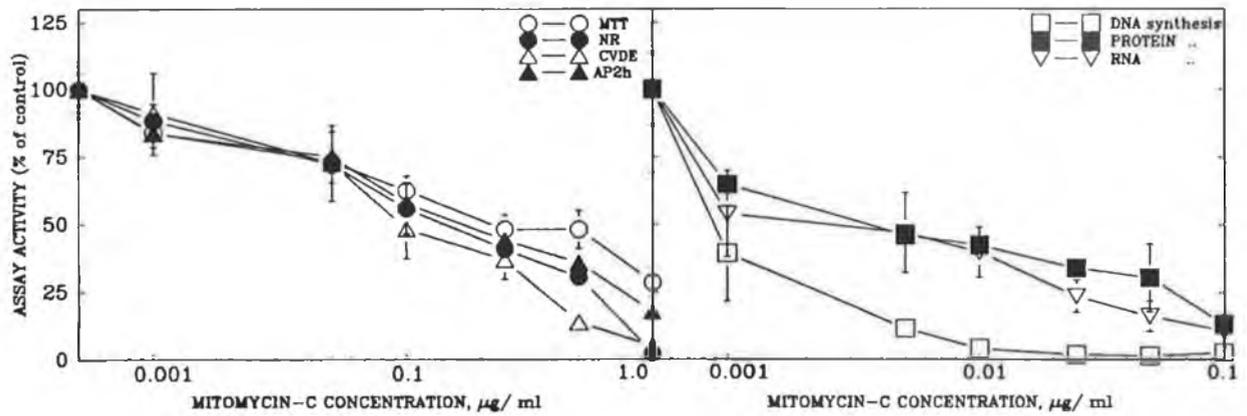


Figure 3.13.4:
 Comparison of performance of various assays in detecting toxicity of Mitomycin-C to RPMI-2650 cells. (6-day exposure to chemical).

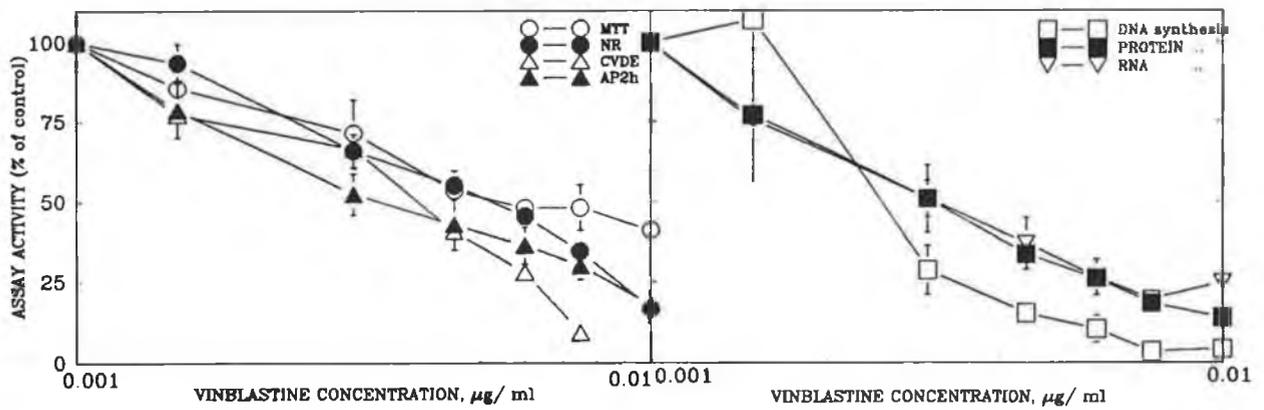


Figure 3.13.5:
 Comparison of performance of various assays in detecting toxicity of Vinblastine to RPMI-2650 cells. (6-day exposure to chemical).

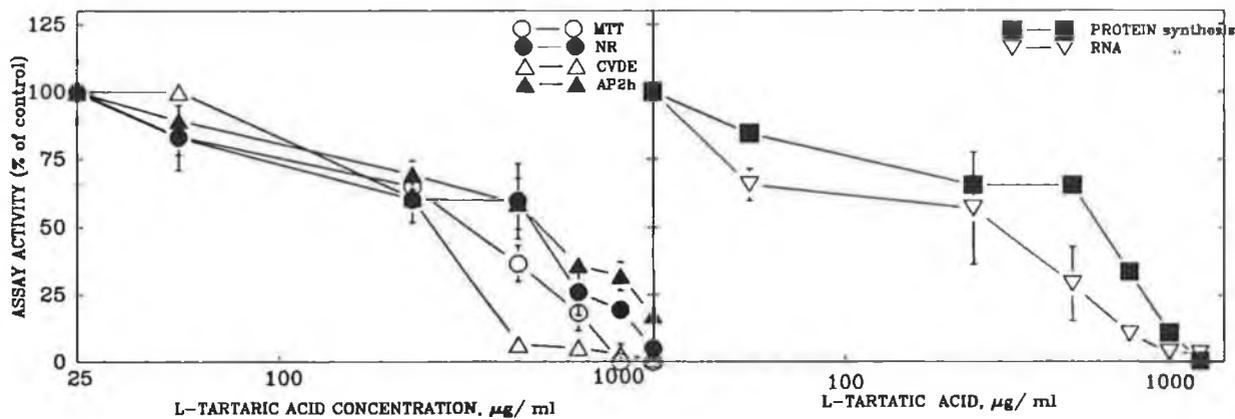


Figure 3.13.6: Comparison of performance of various assays in detecting toxicity of L-Tartaric acid to RPMI-2650 cells. (6-day exposure to chemical).

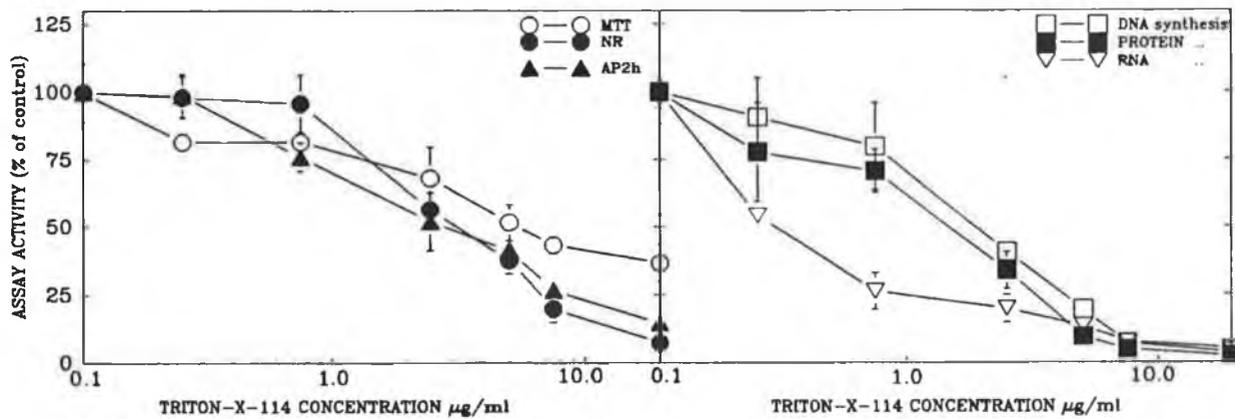


Figure 3.13.7: Comparison of performance of various assays in detecting toxicity of Triton-X-114 to RPMI-2650 cells. (6-day exposure to chemical).

There is a clear distinction in terms of the toxicity of the agents to RPMI-2650 as determined by these endpoints.

In some of the experiments, SEM values for the radioincorporation assays were extremely large; in fact some were quite unacceptable. The other major point to note from repeat experiments, was that the CVDE assay (much more than the other colorimetric assays) was very inconsistent in its ability to show a response to a chemical. This effect was observed for Hydroxyurea, DNP, Mitomycin C and L-tartaric acid, where it was seen that in one experiment the CVDE assay was extremely insensitive to cell kill (initially at least) and was responding completely differently to the other assays; whereas in the repeat experiment, it was closer to the other experiments or was fact much more sensitive in detecting cell kill. The other assays, are not nearly as inconsistent. This is illustrated in Figures 3.13.2 and 3.13.3 (a) and (b).

Next, let us look more closely at the response obtained for individual chemicals.

Cycloheximide:

Because this is an inhibitor of protein synthesis, it might be expected that the leucine incorporation assay would detect toxicity at lower doses of cycloheximide more quickly than the other assays. In fact this effect is not seen. Among the colorimetric assays, there does appear to be a trend, consistent through the repeat; MTT being slightly more sensitive in detecting toxicity, followed by AP2h and then NR. When SEM's are considered however, the difference is not appreciable. Because SEM values for all the radioincorporation assays are very high, in both experiments (including repeat) it is difficult to discriminate between these assays. However, it does seem that the uridine incorporation assay is the most sensitive of all assays. Certainly it can not be said that the leucine incorporation assay shows more sensitivity than all of the other assays.

Hydroxyurea:

This is a specific inhibitor of DNA synthesis in vivo; RNA and protein synthesis are not affected; thus it might be expected that the thymidine incorporation assay for DNA synthesis would be more sensitive than the other assays. On analysis of the graphs, it appears that the assays for DNA, RNA and protein synthesis may be slightly more sensitive than the other assays. The thymidine incorporation assay is actually slightly more sensitive in detecting toxicity, but unfortunately the SEM values for this assay on run 2 made those results unusable.

2,5-Dinitrophenol:

DNP is an uncoupling agent, and thus inhibits oxidative phosphorylation. It would therefore be expected to affect mitochondrial MTT reduction by succinate dehydrogenase more quickly than affecting activity in the other assays. In the first run of the experiment, the MTT assay appears to be very slightly more sensitive than the other assays, but in the second run this is not the case. In fact it appears from the first experiment, that inhibition of DNA synthesis is most sensitive of all. It must be concluded overall

then, that when the SEM values are considered, the MTT assay is no more specific for detecting toxicity due to DNP than any of the other methods.

Mitomycin C:

This anticancer drug is a DNA alkylating agent so particular attention will be paid to the thymidine incorporation assay. Indeed it does appear that this assay is more sensitive than each of the other assays (the effect is apparent in the repeat experiment also). Closely following, in terms of sensitivity to the chemical, are the uridine and then the leucine assays, all being more sensitive than any of the colorimetric assays.

Vinblastine:

This vinca alkaloid ultimately inhibits DNA synthesis, so again note the response of the thymidine incorporation assay. It is clear (from both experiments) that the thymidine incorporation assay is again more sensitive to this drug than are the other assays, followed by AP2h. The other assays all have simultaneous sensitivities.

L-Tartaric acid

The L- isomer of this chemical is reported to be a specific inhibitor of Acid Phosphatase activity. In fact the AP assay is no more sensitive to detecting the inhibitory effect of this chemical than any of the other assays. In this case, unlike the previous two chemicals, there is no great difference among all the assays (even the radioincorporation assays). As noted already, the 'sensitive' response of the CVDE assay was the inverse of the result obtained in the repeat experiment, and tended to be a trait of this assay.

Triton-X-114:

This surfactant is a membrane damaging agent. AP and NR reactions occur in the lysosomes, so both should rely on an intact lysosomal membrane (perhaps less so for AP; this is considered more fully in the discussion). MTT reactions, which occur in the mitochondria, may also be dependent on an intact mitochondrial membrane. Indeed the AP assay appears to be very slightly more sensitive to this chemical than the other colorimetric assays (the effect is repeatable), however it seems that the radioincorporation assays are slightly more sensitive again, particularly uridine incorporation, which is significantly more sensitive than all of the other assays.

Actinomycin D:

This agent, which affects RNA synthesis, was highly toxic to cells, even at ng/ml concentrations.

Tunicamycin and Cordycepin:

Although Tunicamycin (0.01 - 200 $\mu\text{g}/\text{ml}$) and Cordycepin (0.0001 - 500 $\mu\text{g}/\text{ml}$) were tested in these experiments, none of the concentrations used produced any significant cell kill, so the results are not useful (-it is possible that the stocks were not active).

It should be noted that the AP2h assay, has shown a slightly increased sensitivity in detecting toxicity to Vinblastine, Triton-X-114 and Hydroxyurea than either MTT or NR.

Overall, it must be concluded that the colorimetric assays do not give a clear indication of the mechanism of toxicity of any of these chemicals, not generally expressing most sensitivity when expected to. The same has been the case for the leucine incorporation assay. Thymidine incorporation, when moderate SEM's allowed analysis, was generally more sensitive to chemicals exerting their action on DNA. Perhaps if effects are measured after shorter periods of drug exposure, mechanism-specific effects might be more prominent.

3.14 Application of growth assays to chemosensitivity testing: factors affecting the IC₅₀

Problems with poor reproducibility have been determined during the course of this work (see section 3.10.5), thus a number of factors which might affect this were investigated.

3.14.1 Pretreatment regime

For experimental work it is imperative to ensure that cells are in the correct phase of growth. Cells still in lag phase, or having left the exponential phase and entered into stationary phase are not suitable for use in assays. It is necessary to first subculture the cells into fresh medium so that they will be actively dividing when they are removed for use in an experiment (hereafter termed 'pretreatment'). This experiment attempted to determine how critical the pretreatment density was in affecting the results.

RPMI-2650 cells in logarithmic phase growth were trypsinized and set up at a range of seeding densities. The following day, the cells were fed as indicated, with fresh medium. Two days after setting up, each flask was trypsinized, and a 6-day, 96 well, Vinblastine toxicity assay performed on each one as described in section 2.

Figures 3.14 (a) and 3.14 (b) illustrate the results obtained from an AP assay carried out on each of the plates.

The graphs show that there is no particular trend as to the effect of pretreatment density on response to this drug. The dose response curves did not fall in an order bearing any correlation with its seeding density, and given the interexperimental variation of the assay (discussed in 3.10.5 for each of the assays in turn) and taking the SEM values into account, there was no significant effect noted on toxic response.

Note that although there were 13.6 times more cells in the highest density pretreatment flask than in the lowest, all the cells were in good condition under microscopic scrutiny, and none were totally confluent. Thus the main conclusion from this experiment was that as long as the cells are subconfluent, appearing healthy and in good condition, the pretreatment regime will not largely affect the cells subsequent performance in a toxicity assay of this sort.

Also note that elimination of a 'feed' after one days growth (at 1×10^6 cells/ flask) has no significant effect on the cells performance in a toxicity assay.

3.14.2 Length of exposure to trypsin

Trypsin, an extracellular enzyme which cleaves peptide bonds on the carboxyl side of lysine or argenine residues, is used to release cells from the biological glue of the extracellular matrix which cells in culture secrete for themselves. In exposing cells to trypsin for anything other than a short period of time, the cell can become damaged and thus its physiological response to agents can be altered. Agents, such as trypsin, affecting the membrane permeability of cells may also affect the drug sensitivity of the cell.

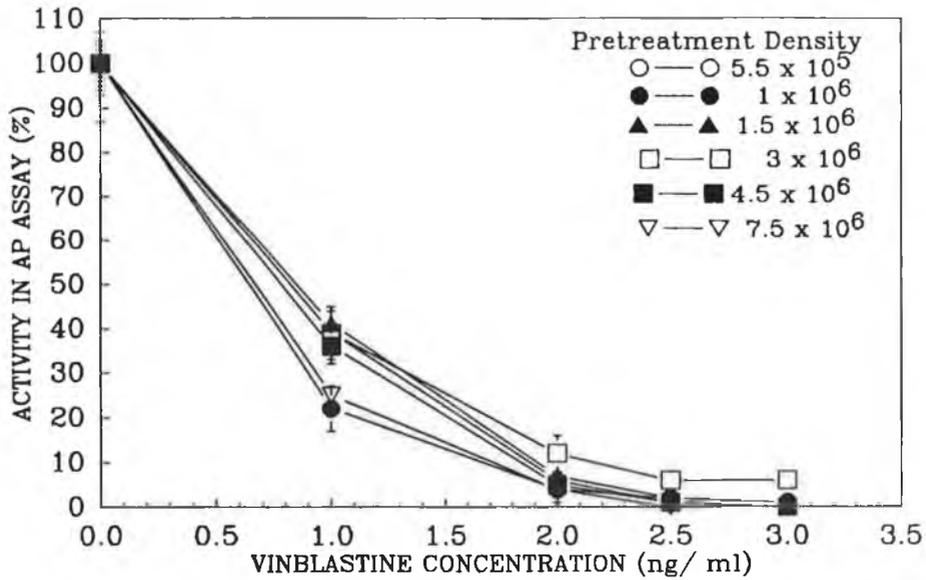


Figure 3.14.1 (a)

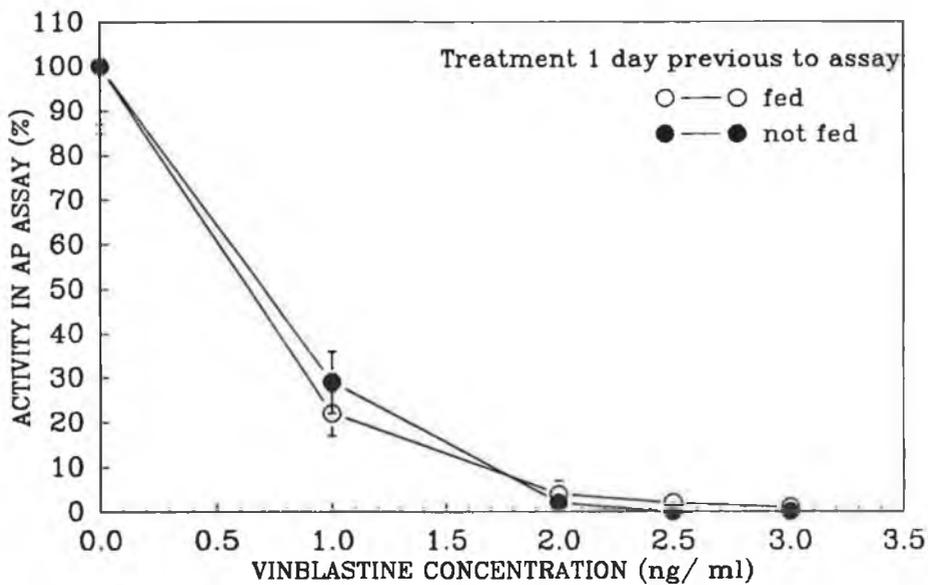


Figure 3.14.1 (b)

Figures 3.14.1 (a), (b):

Effect of different pretreatment regimes on outcome of toxicity experiment when (a) various seeding densities of RPMI-2650 and (b) different feeding regimes, were used prior to a 6 day exposure to Vinblastine.

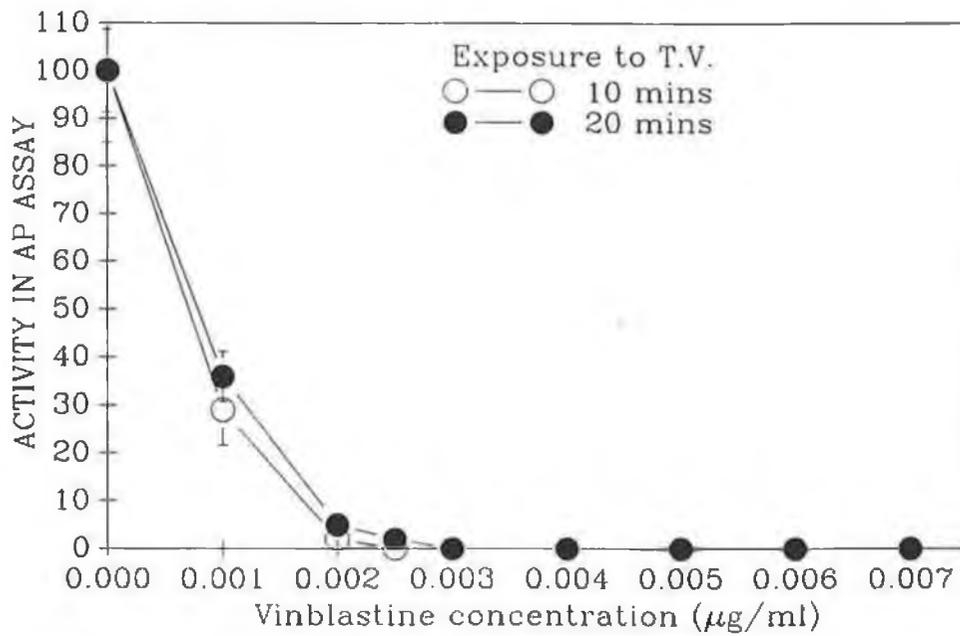


Figure 3.14.2

Effect of duration of exposure of RPMI-2650 cells to T.V. prior to toxicity experiment, on the outcome of the experiment.

In an attempt to identify factors causing intraexperiment variation, this experiment aimed to determine if relatively minor increases in exposure time to trypsin, would have a significant effect on the observed response to Vinblastine.

Figure 3.14.2 shows that there is no significant difference in drug sensitivity, after the exposure time during preparation of cells for assay is increased from 10 to 20 minutes. In experiments described in this thesis, trypsinization never exceeded 20 minutes duration.

3.14.3 Effect of cell density on drug sensitivity

When using different cell seeding densities, one should not necessarily expect that the response to drug should be the same. This would have implications for comparing assays which used different plating densities (e.g., assays set up at different densities in different size dishes). The following experiments were designed to investigate density effects, if any, on a range of cell lines, in toxicity experiments.

A clear density - dependent effect is noted in Figure 3.14.3 (a) and (b) with Navelbine and Vinblastine on RPMI-2650 using the AP assay. Plating increasingly higher numbers of cells introduces a masking effect on the efficacy of the drug. The effect was established with all of the assays and is shown here for NR, MTT, CVDE, and IA.

To demonstrate this phenomenon more extensively, similar experiments were carried out using Hep-2. The results are shown in Figure 3.14.3 (c).

3.14.4 Effect of drug exposure regime on drug sensitivity

Presumably the drug exposure regime will be a determining factor in the observed response of the cells to the drug. One of the goals of research into in vitro chemosensitivity testing, is to design procedures which will bear relevance to exposure of the drug to the tissues in vivo. Thus the success of the in vitro assay will rely heavily on a good exposure regime. The relationship between drug exposure and toxic effect was investigated here in a number of simple systems.

Figures 3.14.4 (a) and (b) show that, as expected, observed toxicity is related to duration of exposure. As exposure is increased from 6 to 24 hours, significantly many more cells were killed, and this effect is further increased at an exposure time of 7 days. At the drug concentration 0.01 μ g/ml Mitomycin C, for example, 30% cell kill occurs after a 6 hour incubation time, whereas cell kill has increased to 55% at 24 hours, and 80% at 7 days incubation with drug. The graphs reproduced here demonstrate this effect for 5-Fluorouracil and Mitomycin C, but the same effect was established for other classes of drugs, including Cis-Platinum, Adriamycin and Vinblastine. Table 3.14.4 also summarizes this.

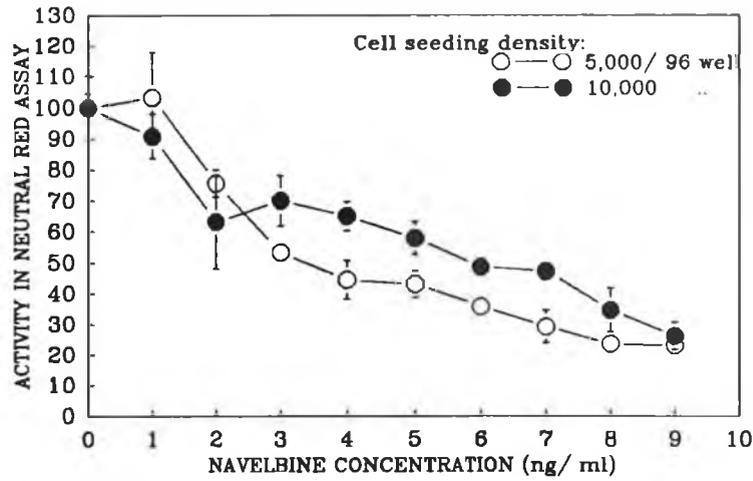


Figure 3.14.3 (a)

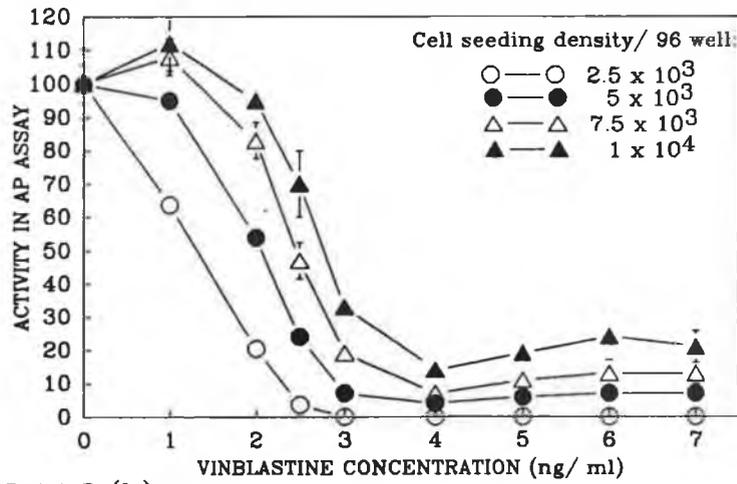


Figure 3.14.3 (b)

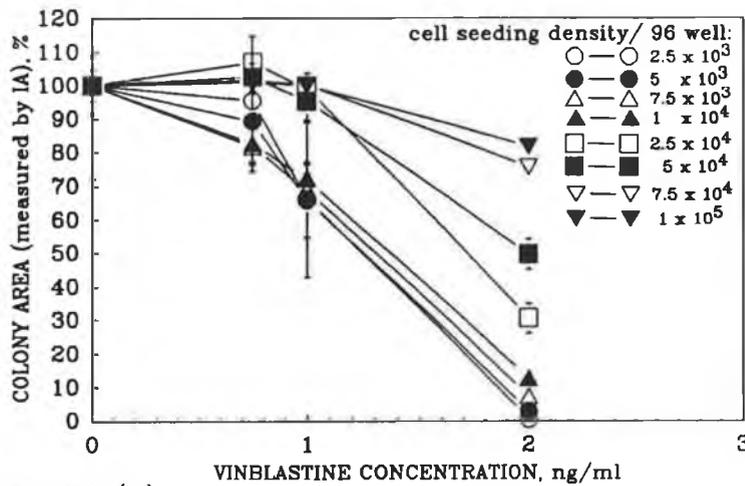


Figure 3.14.3 (c)

Figures 3.14.3 (a) – (c):

Effect of cell seeding density on drug efficacy as assessed by:
 (a) NR, (b) AP (both for RPMI-2650 cells) and (c) IA for Hep-2 cells.
 Toxicity tests were performed as 6 day continuous exposures.

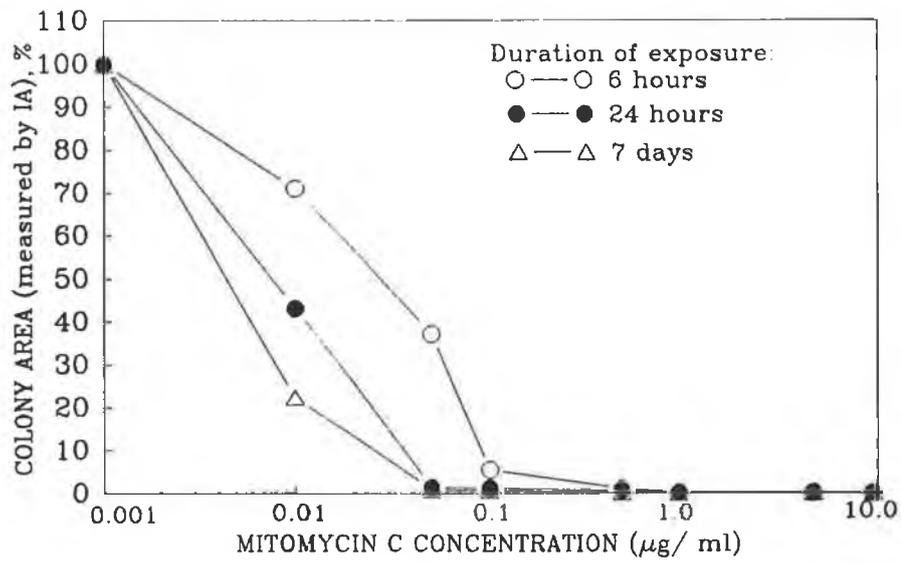


Figure 3.14.4 (a)

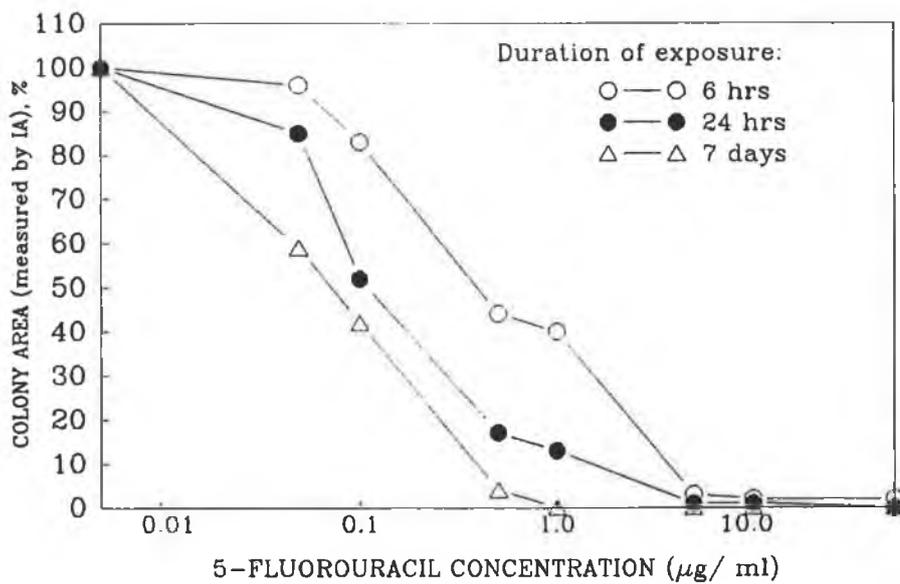


Figure 3.14.4 (b)

Figures 3.14.4 (a), (b):

Effect of different drug exposure regimes on drug efficacy for RPMI-2650 cells.

Table 3.14.4 Effect of drug exposure on IC₅₀ for RPMI-2650 cells

Drug exposure time	IC ₅₀ Mitomycin C	IC ₅₀ 5-Fluorouracil
7 days	0.007 ± 0.015	0.070 ± 0.020
24 hours	0.010 0.015	0.100 0.020
6 hours	0.036 0.015	0.440 0.020

25,000 RPMI-2650 cells seeded per well (24-well dish)

3.14.5 Duration of toxicity assay

Closely linked with the last parameter is the duration of the toxicity assay itself. It would be expected that assay duration after or including drug exposure time would have some effect on the IC₅₀. In the previous experiment, two of the plots show short exposure times of 6 and 24 hours, after which drug was removed and the assay was allowed to continue for a total of 7 days. This of course was to allow the toxic effect to be manifest by quantifiable cell number.

The experiments represented by the table below shows a time course toxicity assay on RPMI-2650 cells. Replicate plates were exposed to drug in the normal manner with a continuous exposure regime. Beginning at one day continuous exposure to drug, and continuing for six days, a single plate was removed and stained each day, and read by image analysis. The effect of the drug on the cell population in terms of cell killing can be observed as it occurs.

Table 3.14.5 Effect of assay duration on IC₅₀ for Vinblastine exposure to RPMI-2650 (determined by IA)

Days in culture with drug	approximate IC ₅₀ µg/ml Vinblastine
1	2.5
2	0.225
3	0.06
4	0.0875
5	0.00975

As the assay duration increases, the IC₅₀ decreases; that is, the same concentration of drug becomes more potent to the cells. This is in accordance with the outcome which would be expected on assimilation of the results from experiment 3.14.4. Thus the time at which the endpoint is determined will directly, and very significantly affect the observed results.

In the early days of the experiment, cell number was low, as cells had not divided much. In the latter stages of the experiment, cell number had increased significantly in control wells and in wells with low levels of drug where growth was only partially affected. This of course will have consequences in deciding when to determine the endpoint of the assay.

Obviously the critical factor in deciding when to terminate the assay will be not allowing the control wells to reach confluence. Aside from this, the factors mentioned here should be considered.

Thus it should be decided for each individual case when the assay should be taken off. This was routinely done in these experiments. Take for example SK-LU-1, shown here in Figure 3.13.5. The OD of the control wells throughout the duration of the experiment were plotted when 5×10^3 cells/ 96 well were plated, for a toxicity assay. It can be seen that after 6 days growth, the level of growth has actually decreased. This is because cells have become confluent and ceased to grow. Cells may then have dislodged from the plates. Thus taking this plate off at day 7 would lead to erroneous results, as wells had become confluent and control wells have stopped growing while drug treated wells had continued to grow, thus the IC_{50} would be overestimated.

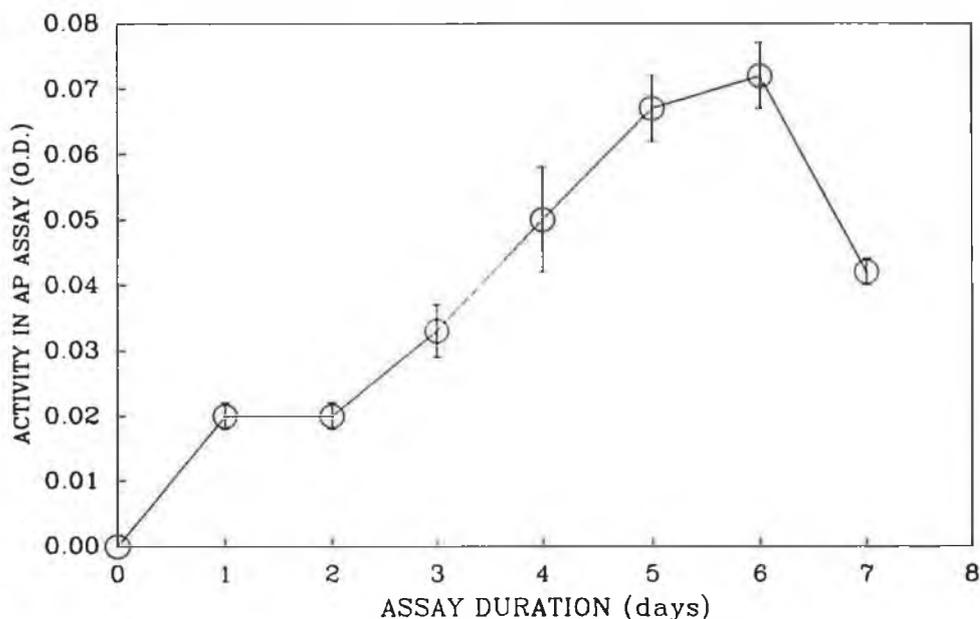


Figure 3.14.5

Activity of control wells (no drug) in AP assay with time when 5,000 SK-LU-1 cells were plated on day₀.

RESULTS 2:

APPLICATIONS OF ASSAYS

4.1 Serum batch testing with miniaturized, colorimetric endpoints

4.1.1 General considerations of serum batch testing

Serum batch testing is an inconvenient but necessary requirement for effective cell culture, to exclude bad serum batches from prospective use and to identify samples supporting good growth. The high cost of serum makes this consideration more important than ever.

Where a range of cell lines are being cultured, it is important to consider the optimal batch for each cell line, as different cell lines have different preferences for serum batches. Figures 4.1 (a) to 4.1 (c) exemplify how a given serum batch can be a good growth promoter for one cell line, and yet effect poor cell growth with another cell line. All of the batches tested were Foetal Calf Serum (FCS). Serum batch 801066 is the optimal batch for DLKP, followed by batch 801051; however these 2 batches are the least optimal for RPMI-2650, and in the reverse order also. In fact, the results of serum preference for RPMI-2650 are quite the inverse of those from DLPK. SKMES-1 displays yet another different profile for serum batch preference, with batches 901020 and 801117 giving the most growth.

For serum batch testing, it is crucial to test at more than one serum concentration, as the observed effect sometimes changes at different serum levels. If possible, testing should be done at 3 serum concentrations; low, moderate and high (3%, 5% and 10%, for example), but if this is not possible then one of the test concentrations can be excluded. To illustrate this point, again consider Figures 4.1 (a) to (c).

Whilst 1% serum for SKMES-1 results in little growth all round, some differences in serum performance are noted with this concentration for the other two cell lines. With RPMI-2650, it is particularly interesting to note the degree of growth stimulation apparent with batch 801066. The importance of considering multiple serum concentrations is evident when we observe the growth resulting from higher concentrations of this serum. Contrary to what might be expected with increasing serum concentration, no increase in growth was noted. In fact, growth has been inhibited at 5% serum, though at 10%, the inhibition is less marked. A similar phenomenon occurs at 5% serum batch 801117.

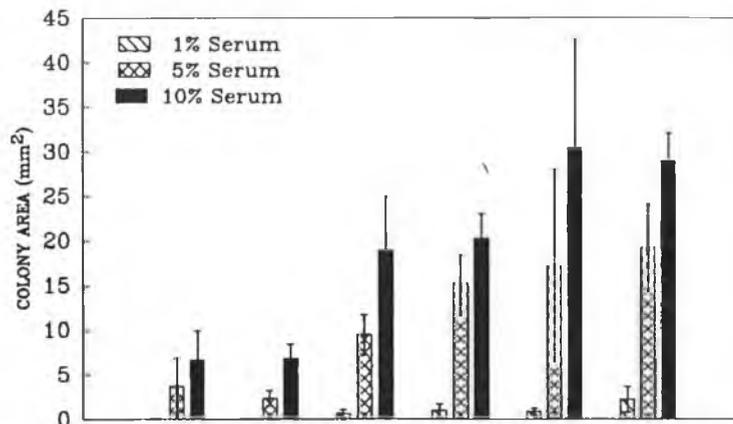


Figure 4.1.1 (a)

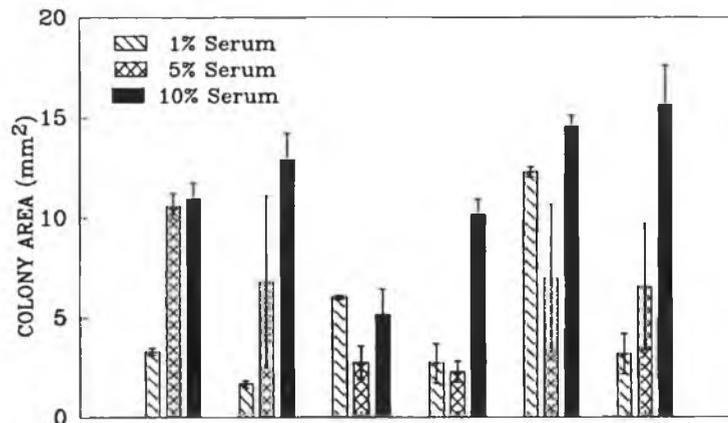


Figure 4.1.1 (b)

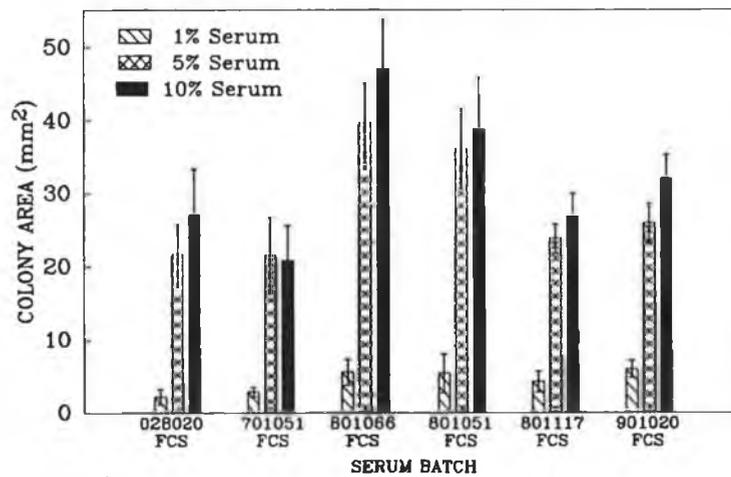


Figure 4.1.1 (c)

Figures 4.1.1 (a) – (c):

Effect of different FCS batches on cell growth:

- (a) 5,000 SK-MES-1 cells, (b) 5,000 RPMI-2650 cells and
- (c) 10,000 DLKP cells, after 7 day's growth.

Considering the aforementioned variables which need to be addressed, in addition to other factors (such as cell density) which may affect serum batch performance, it must be appreciated then, how easily the size of the experiment can amplify. If several cell lines have to be tested, for, say 15 batches of serum, then this represents an immense amount of incidental work.

Previous work in this laboratory relied on preparing growth curves and monolayer experiments in 24 well dishes (analysed by IA) to distinguish between good and poor batches of serum, but difficulties with IA (previously discussed) especially with some cell lines which did not form concise colonies, and plates in which the colonies were not well distributed, resulted in inaccurate results with the monolayer method. Not only did this mean that there was an urgent need for improved accuracy of quantification in this system, but clearly the procedure would benefit from miniaturization and semi-automation.

This section considers the problems of serum batch testing and deals with the application of one of such miniaturized, semi-automated assays (AP) to serum batch testing, and then assesses its performance.

The graphs which follow illustrate the performance of 11 different batches of serum on a range of different cell lines at some different cell densities. Results from the AP assay are compared with results obtained using other assays.

4.1.2 Practical/ technical significance of batch testing using the AP assay

Changing from a previously used monolayer system of batch testing (analysis of crystal violet stained cells in 24 well dishes by image analysis) to the AP assay was not a major task. Adaptation of the procedure required few modifications. Obviously the smaller surface area and volume of 96 well plates meant that cell loading density and volumes had to be adjusted. Volume was reduced from 0.5ml cells + 0.5ml double-strength serum containing medium, to 0.1 ml of each. Cell density was reduced; some preliminary work was needed to establish the extent for each cell line. Because less media and cells were used, more replicates could be set up, or more variables could be tested. The time saved during setting up and reading of the assays was considerable. The tedium of carefully trying to establish an evenly spread monolayer was removed and the importance of having a single cell suspension was lessened. Any subjectivity relating to quantification of results (by choosing a detection level and other settings on the image analyser for optimum performance) was relinquished.

4.1.3 Significance of cell density

Cell density is extremely important in determining the success of the image analysis assay due to cell distribution phenomena, but let us just see how critical it is when using a different testing system such as a miniaturized, colorimetric assay.

Figures 4.1.3 (a) to (e) demonstrate the effect of cell density on response to serum batch for DLKP cells. As cell seeding density increases, so too does the O.D. This is summarized in the Table below.

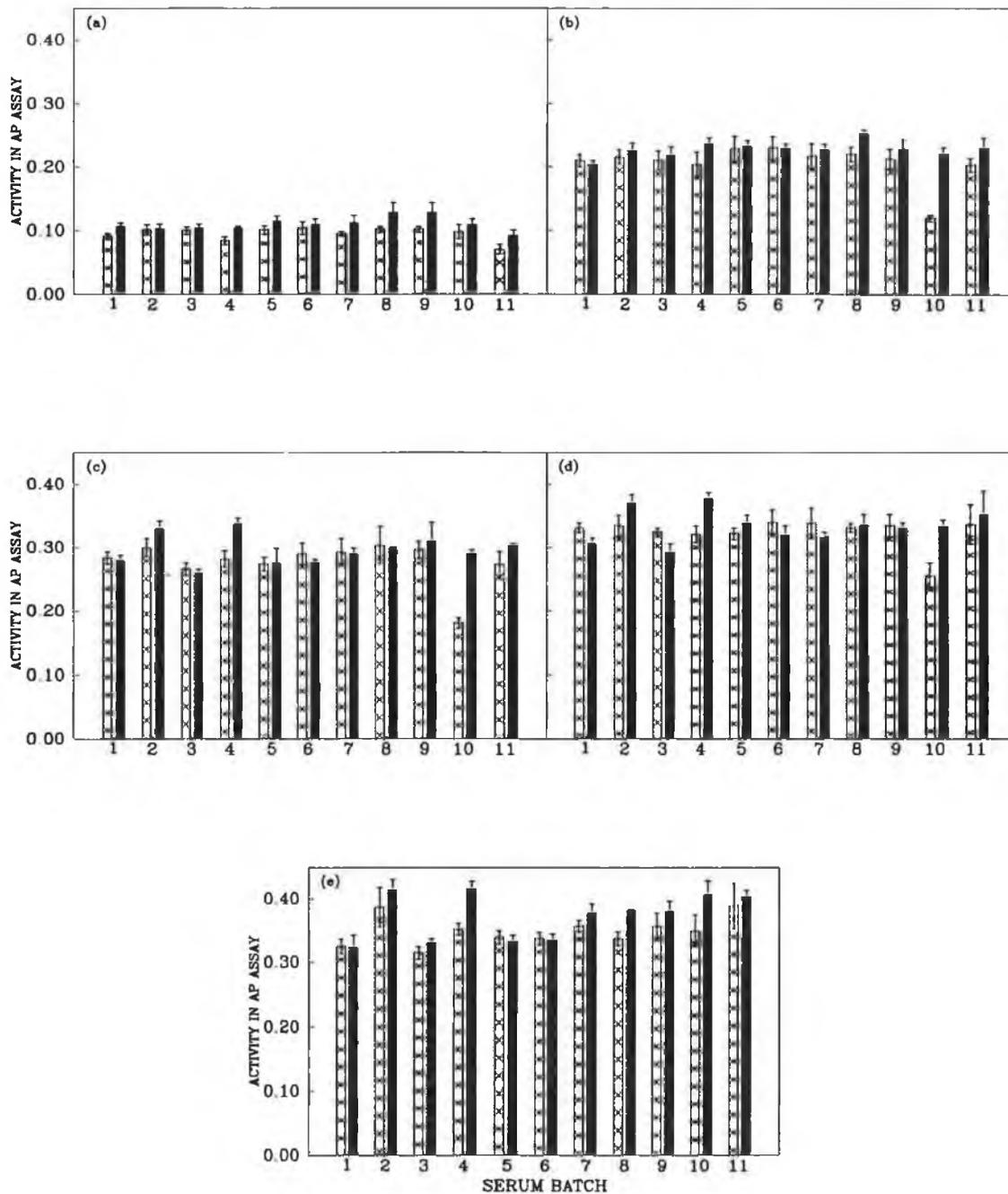
Table 4.1.3 Relationship of cell seeding density to O.D. (DLKP)

Viable cell number seeded (/96 well)	approximate optical density* (Absorbance Units)
1 x 10 ³	0.1
2.5 x 10 ³	0.2 - 0.25
5 x 10 ³	0.3
7.5 x 10 ³	0.35
1 x 10 ⁴	0.3 - 0.4

* after 6 days in culture

Optical density increases as a consequence of cell plating density after 7 days in culture, but not in a linear fashion. Significant increase in O.D. was achieved with increasing plating density up to 7.5 x 10³ cells per well, but at 1 x 10⁴ cells/well, no further increase was noted. This response is due to confluent conditions being reached at seeding densities greater than 7.5 x 10³/ well.

Whilst increasing the cell seeding density generally gave a significant increase in O.D., doubling the serum concentration from 5 to 10% made very little difference to the extent of cell growth in the case of most of the sera. This was the case for all cell densities tested.



Figures 4.1.3 (a) – (e):

Effect of DLKP cell seeding density on response to serum batch:

(a) = 1,000 cells, (b) = 2,500 cells, (c) = 5,000 cells, (d) = 7,500 cells

and (e) = 10,000 cells/ 96 well (growth after 6 days' culture).

▨ = 5% serum; ■ = 10% serum .

All the serum batches tested resulted in good cell growth; there were no extremes in batch performance, which makes it more difficult to analyse the findings; however, some differences are apparent. All batches were FCS, except for batch 2 (Donor Calf Serum (DCS)) and batch 4 (Donor Horse Serum (DH)).

Figure 4.1.3 (a) shows that at 10^3 cells/well, batches 11 and 4 support least growth, but for batch 4, this effect is only noticeable at the lower concentration of serum. At 2.5×10^3 cells however (Figure 4.1.3 (b)), batches 11 and 4 are much improved and now it is batch 10 which gives low cell growth at the low serum concentration; all the other batches showing little difference in performance. Moving on to 5×10^3 cells/well, again batch 10 is poorer than the others (at low serum concentration); this time though, batches 4 and 2 seem to be slightly better than the rest of the batches at high serum concentration (Figure 4.1.3 (c)). This trend is also maintained for 7.5×10^3 cells/well, but at the higher density of 10^4 cells/well the situation changes again. Here, batch 10, which has generally been the poorest of all the batches at other cell densities tested, now performs as well as any of the other batches, and significantly better than batches 1, 3, 5 and 6 (Figure 4.1.3 (e)).

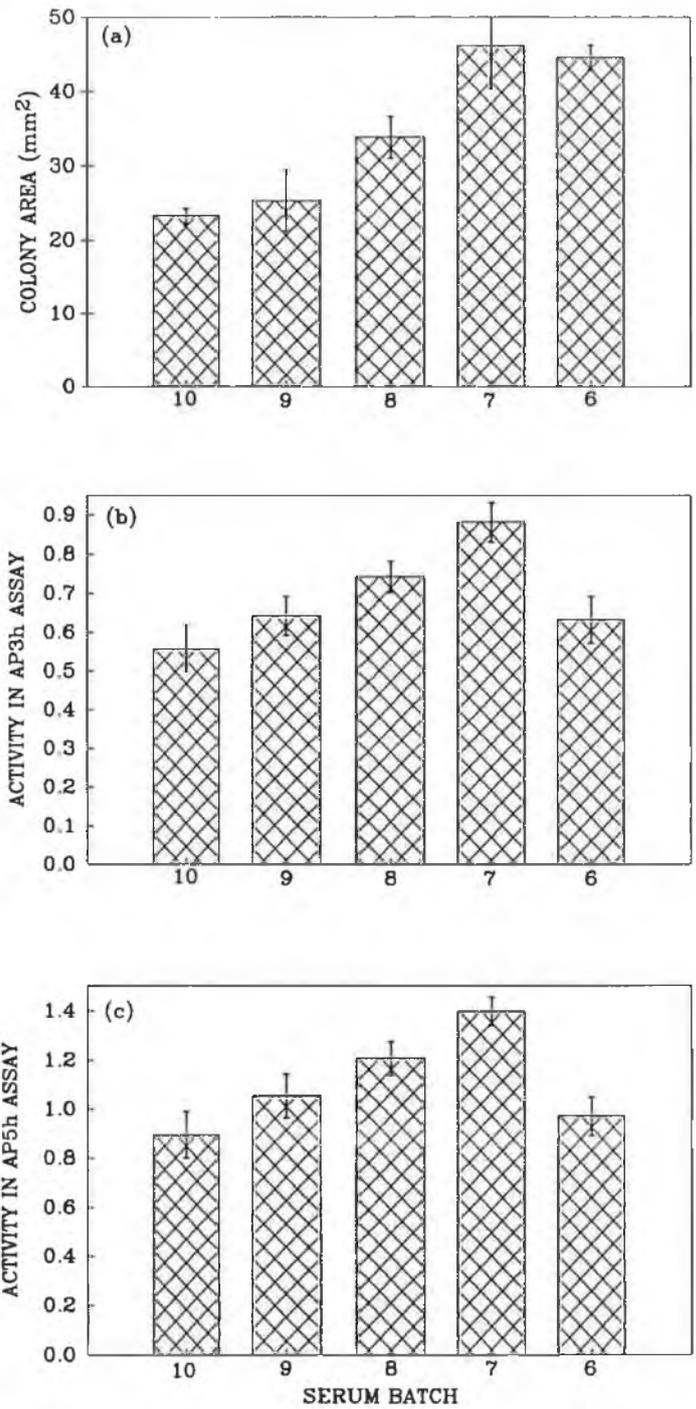
Thus it is evident that plating density can significantly affect the results observed in a miniaturized colorimetric assay system.

4.1.4 Comparison of AP assay with IA

In comparing serum batch testing results obtained from using the AP assay with that seen in a monolayer assay quantified by image analysis, comparable results were found for both assays. Figure 4.1.4 (a) shows similar response to the range of FCS batches tested (at a concentration of 5%) on NRK cells when quantified by IA, to that observed after testing by the AP method in the two graphs below it. Figure 4.1.4 (b) shows AP response after a 3 hour incubation time, while Figure 4.1.4 (c) shows the same experiment after a 5 hour incubation time. It can be seen that the response is very similar after both AP incubation times.

At 10% serum it is clear that both AP(3h) (Figure 4.1.4 (d)) and IA(4d) (Figure 4.1.4 (e)) have detected similar cellular response to batches 6, 7 and 8, while batches 9 and 10 have been poorer at promoting NRK growth. Additionally it was noted that the IA assay, when allowed to grow for an extra day (5 days) (Figure 4.1.4 (f)) has resulted in a very different response to that seen a day earlier. After 5 days growth, no differences whatsoever were detected in batch performance.

Having shown that the AP assay produced results similar to those obtained by the IA method, it was then applied to screening the same panel of 11 sera over a wide range of cell types to indicate serum performance.

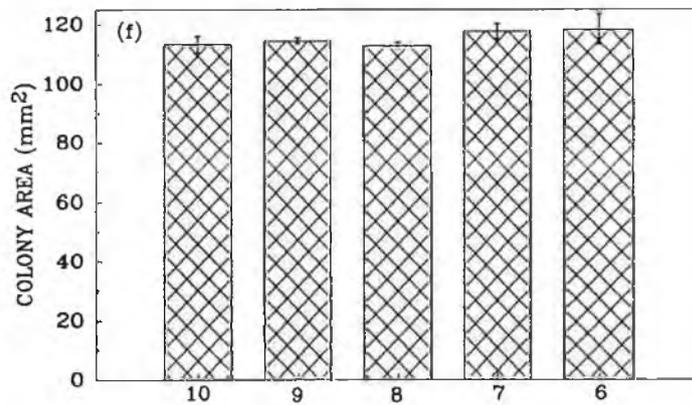
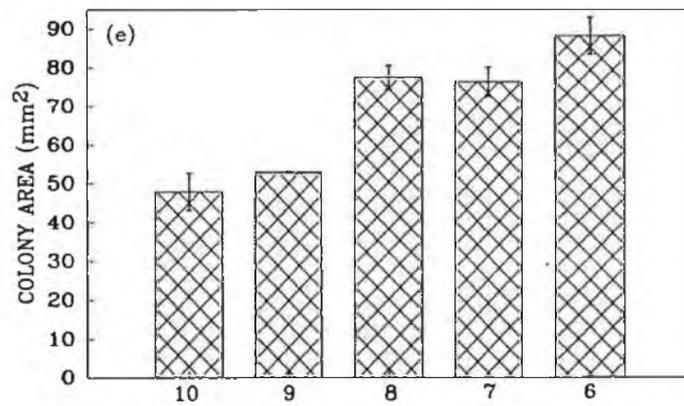
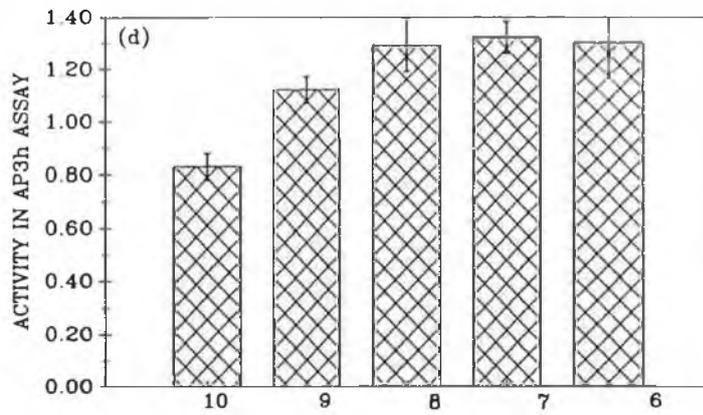


Figures 4.1.4 (a) – (c):

Comparison of activity of serum batches in IA and AP assays.

NRK growth after 5 days in culture, at 5% serum:

(a) = IA, (b) = AP3h, (c) = AP5h.



Figures 4.1.4 (d) - (f):

Comparison of activity of serum batches in IA and AP assays.

NRK growth after 4 and 5 days growth with 10% serum:

(d) = AP3h, (e) = IA after 4 days, (f) = IA after 5 days' growth.

4.1.5 Differential response of cell lines to serum batches: can the AP assay detect this?

Seven cell lines; SK-LU-1, SK-MES-1, Calu 3, DLRP, DLKP, RPMI-2650, and NRK; were tested for their serum batch preference with the panel of 11 batches of serum. Five of these cell lines were of lung origin. They were tested at seeding densities of 5×10^3 and 10^4 cells/ well and at serum concentrations of 5 and 10%. The paragraphs which follow are a synopsis of the main trends observed for each cell line.

SKLU-1 (Lung adenocarcinoma; Figure 4.1.5.1 (a) and (b))

Batches 4 (DH) and 10 (FCS) were significantly worse than the other batches. This trend was noted at both serum concentrations (5% and 10%).

SKMES-1 (Lung squamous cell carcinoma; Figures 4.1.5.2 (a) and (b))

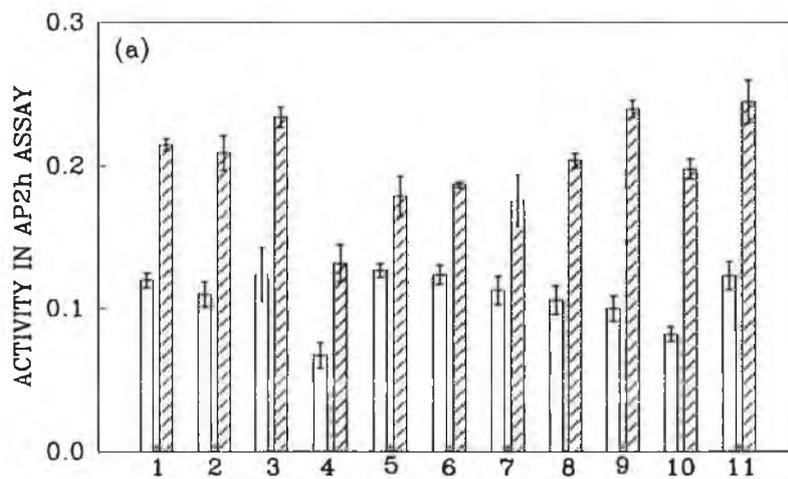
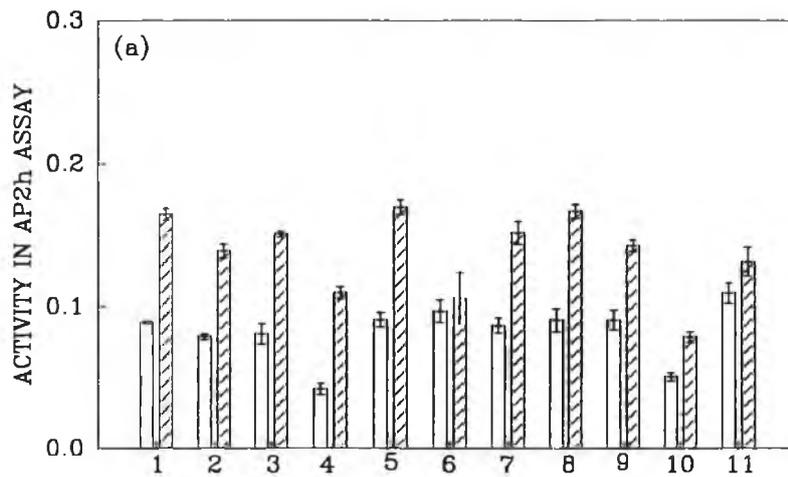
Batch 10 was consistently the worst. Batches 8 and 5 were slightly better than the other batches, followed by batches 1 and 11. All others were very close together. This trend was maintained at both cell densities and at both serum concentrations.

CALU 3 (lung squamous cell carcinoma; Figures 4.1.5.3 (a) and (b))

Doubling serum concentration to 10% did not result in a very significant increase in growth, (particularly for batches 2 and 10) however, doubling cell seeding density to 10^4 cells/ well resulted in greater than double the amount of growth at the end of the experiment. At 5% serum, batch 1 was the worst growth promoter, but this improved at 10% serum, surpassing batches 4, 10, and less significantly, 11. Under all conditions, batch 9 gave the best performance in terms of stimulating cell growth.

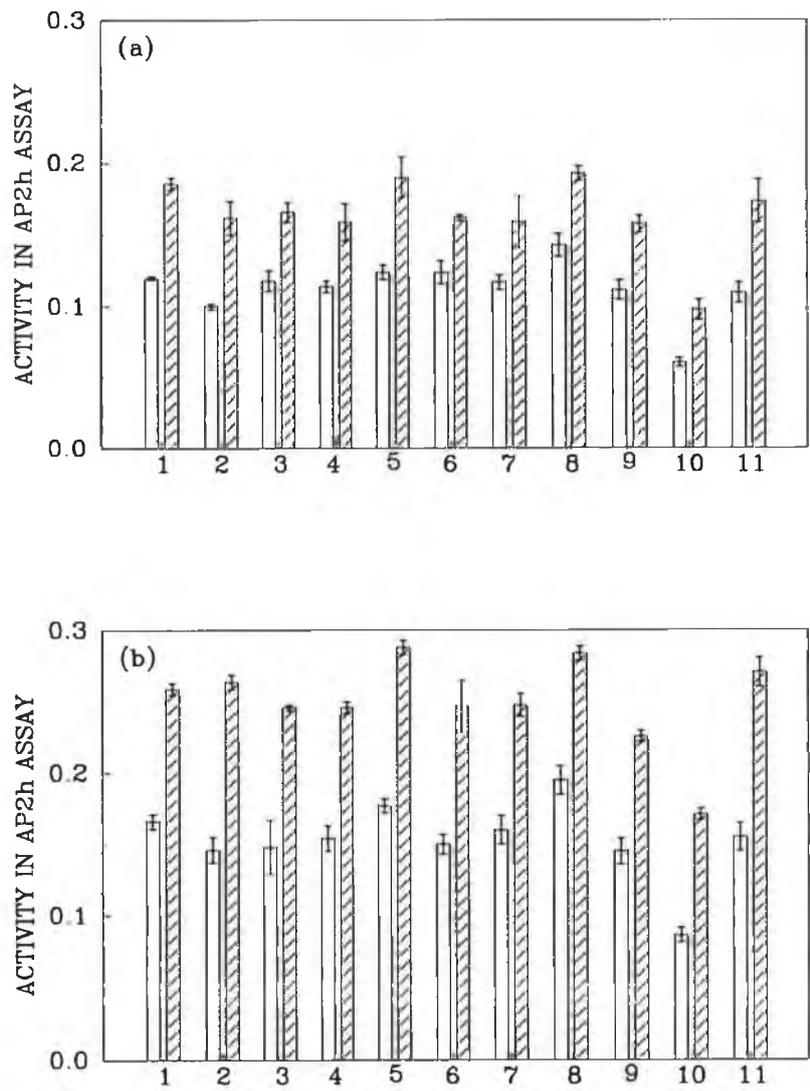
DLRP (lung squamous cell carcinoma; Figures 4.1.5.4 (a) and (b))

At low cell density, augmenting the serum concentration to 10% made little difference to growth; at higher plating density the increase was more beneficial. For this cell line also, batch 10 was the poorest growth promoter. Batches 1, 8 and 11 were slightly better than the remaining batches.



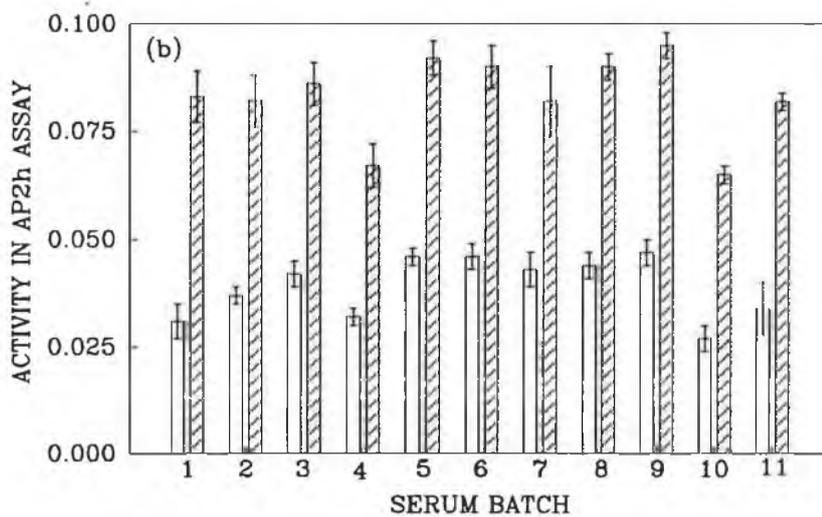
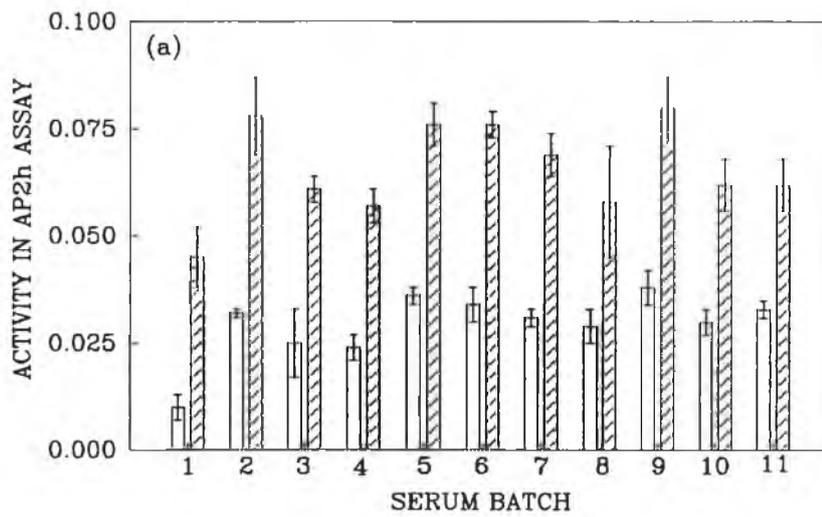
Figures 4.1.5.1 (a), (b):

Effect of different serum batches on cell growth (assayed by AP2h activity). SK-LU-1 cells after 6 days' growth at seeding densities of \square 5,000 and hatched 10,000 cells/ 96 well. (a) = 5% serum, (b) = 10% serum.



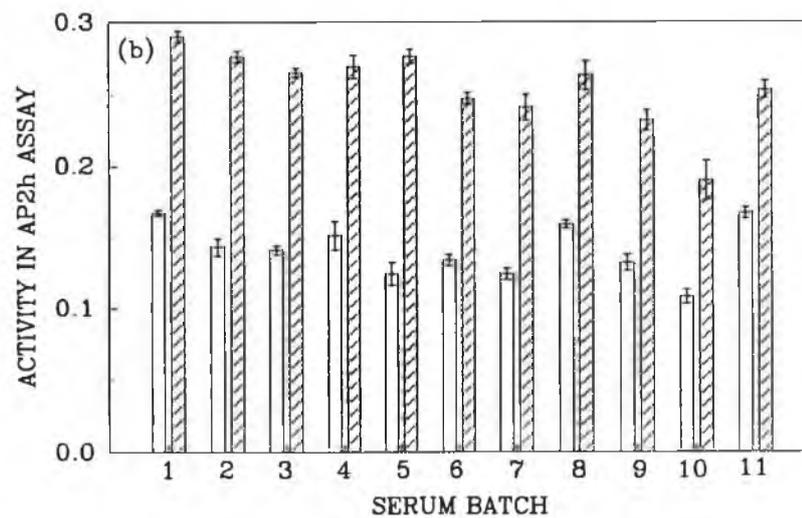
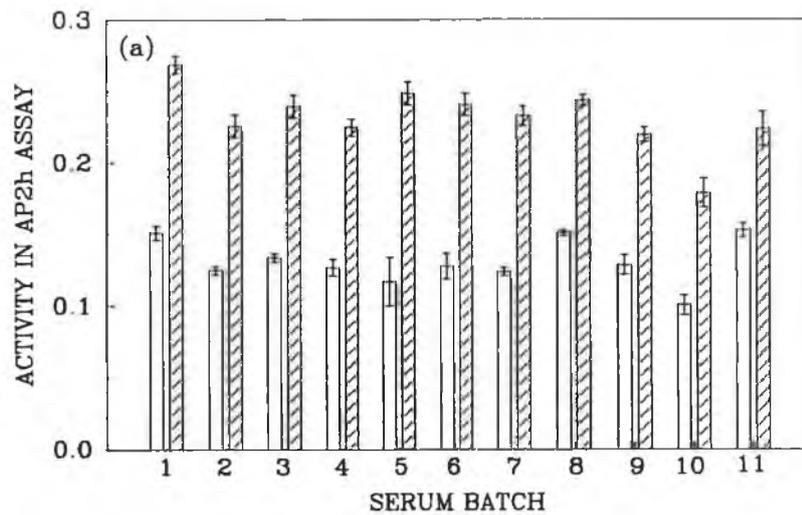
Figures 4.1.5.2 (a), (b):

Effect of different serum batches on cell growth (assayed by AP2h activity). SK-MES-1 cells after 6 days growth at seeding densities of \square 5,000 and hatched 10,000 cells/ 96 well. (a) = 5% serum, (b) = 10% serum.



Figures 4.1.5.3 (a), (b):

Effect of different serum batches on cell growth (assayed by AP2h activity). Calu-3 after 6 days growth at seeding densities of 5,000 and 10,000 cells/ 96 well. (a) = 5% serum, (b) = 10% serum.



Figures 4.1.5.4 (a), (b):

Effect of different serum batches on cell growth (assayed by AP2h activity).DLRP cells after 6 days growth at seeding densities of 5,000 and 10,000 cells/ 96 well. (a) = 5% serum, (b) = 10% serum.

DLKP (lung squamous cell carcinoma; Figures 4.1.3 (c) and (d))

This cell line has a higher growth rate than has Calu 3 or DLKP; note that increasing the cell density from 5×10^3 to 10^4 cells did not result in the same observed improvement in growth as seen by the previous two cell lines. At high cell density, batches 2 (DC) and 4 (DH) were slightly better than the other sera. At low cell density, batch 8, which was good for many of the other cell lines, was the worst for this cell line.

NRK (normal rat kidney fibroblasts; Figures 4.1.5.5 (a) and (b))

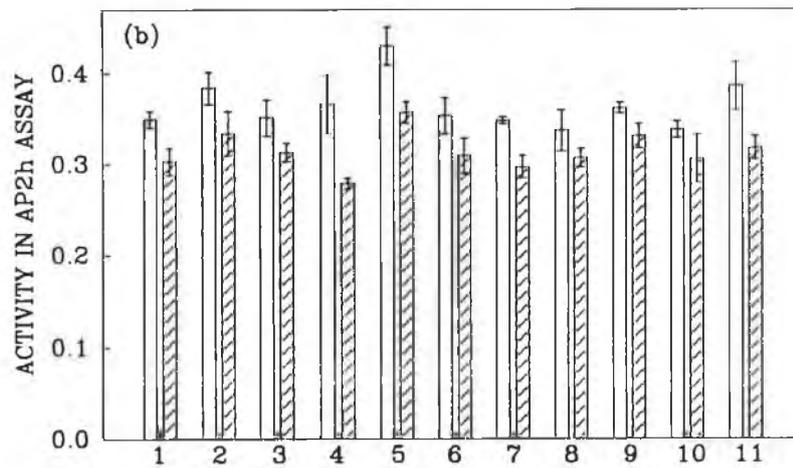
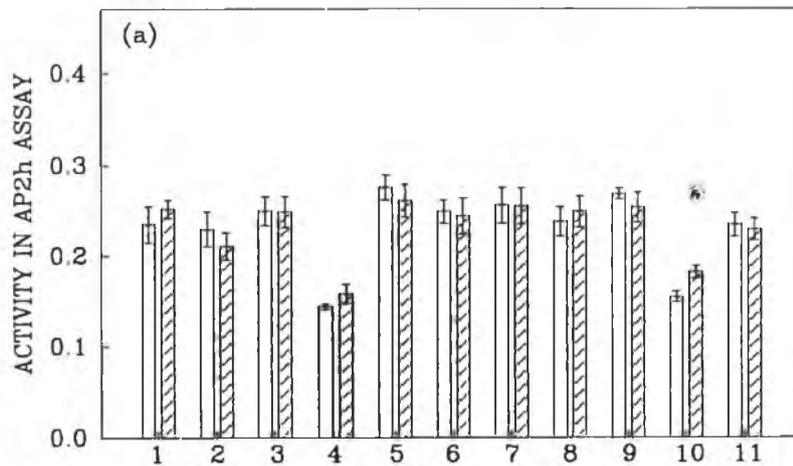
Increasing the cell density to 10^4 cells/ well gave similar, or indeed even lower OD readings than the lower cell density did. Batches 4 and 10 gave lowest growth, especially at 5% serum. All the other batches were quite similar, except for batch 5, which was marginally better than the others.

RPMI-2650 (nasal squamous cell carcinoma; Figures 4.1.5.7 (a) and (b))

Again batches 8 and 5 were slightly better than the other batches, while batch 10 was the worst, followed by batches 2, 4 and 9.

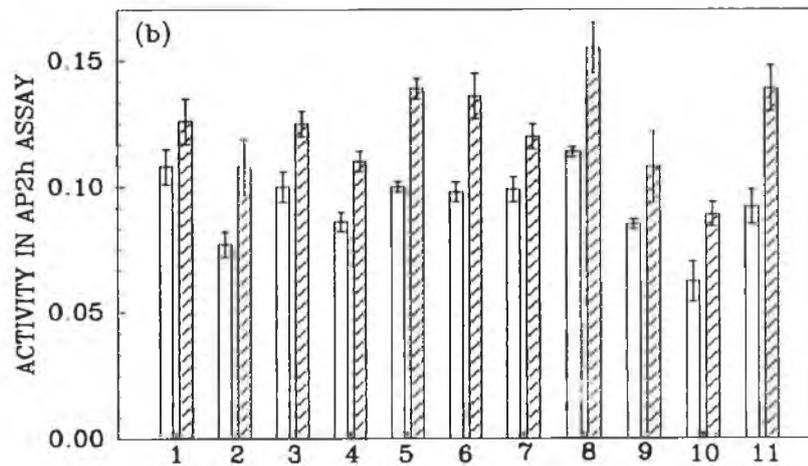
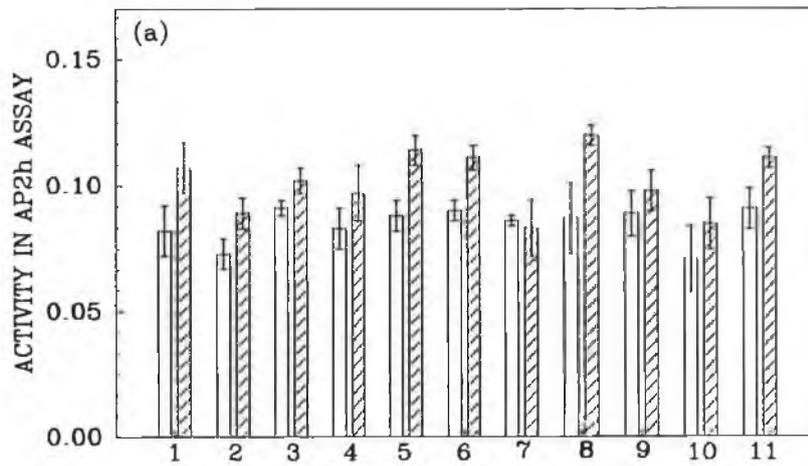
Synopsis:

The cell lines have behaved differently in response to different serum batches. The AP assay has detected differences in responses.



Figures 4.1.5.5 (a), (b):

Effect of different serum batches on cell growth (assayed by AP2h activity). NRK cells after 6 days growth at seeding densities of \square 5,000 and hatched 10,000 cells/ 96 well. (a) = 5% serum, (b) = 10% serum.



Figures 4.1.5.6 (a), (b):

Effect of different serum batches on cell growth (assayed by AP2h activity). RPMI-2650 cells after 6 days growth at seeding densities of \square 5,000 and hatched 10,000 cells/ 96 well. (a) = 5% serum, (b) = 10% serum.

4.1.6 Comparison of the performance of the AP assay to other miniaturized, colorimetric assays

Using the cell line RPMI-2650, the results obtained with the AP assay were compared to the NR, MTT and CVDE assays. This was carried out with 10^4 cells/ well and at the same two serum concentrations; 5% and 10%. Figures 4.1.6.1 to 4.1.6.2 illustrate these results.

The O.D. values obtained for all of the assays were similar; 10^4 cells/ well (after 7 days growth) produced values in the range of 0.1 to 0.15 O.D. units. As already noted above, the AP assay detected poor growth with batch 10, followed then by 2, 4 and 9. This result was also obtained with the NR assay; the difference between these 'poor' batches and the rest of them is more marked with the NR assay though. However the SEM values were much bigger for this assay than for the AP assay ((c) Figures). A similar situation resulted when the MTT assay was used; error bars were significantly bigger ((b) Figures). The results from this assay are very similar to AP - while batch 10 is clearly seen as the worst, the differential between the others is less marked. Both systems identify significantly poorer growth at 5% batch 7, which is improved at 10% of this serum. The other 2 assays did not show this trend. The CVDE assay ((d) Figures) shows the same trends that have been mentioned already, but the assay is accompanied by small error bars (similar to the AP assay).

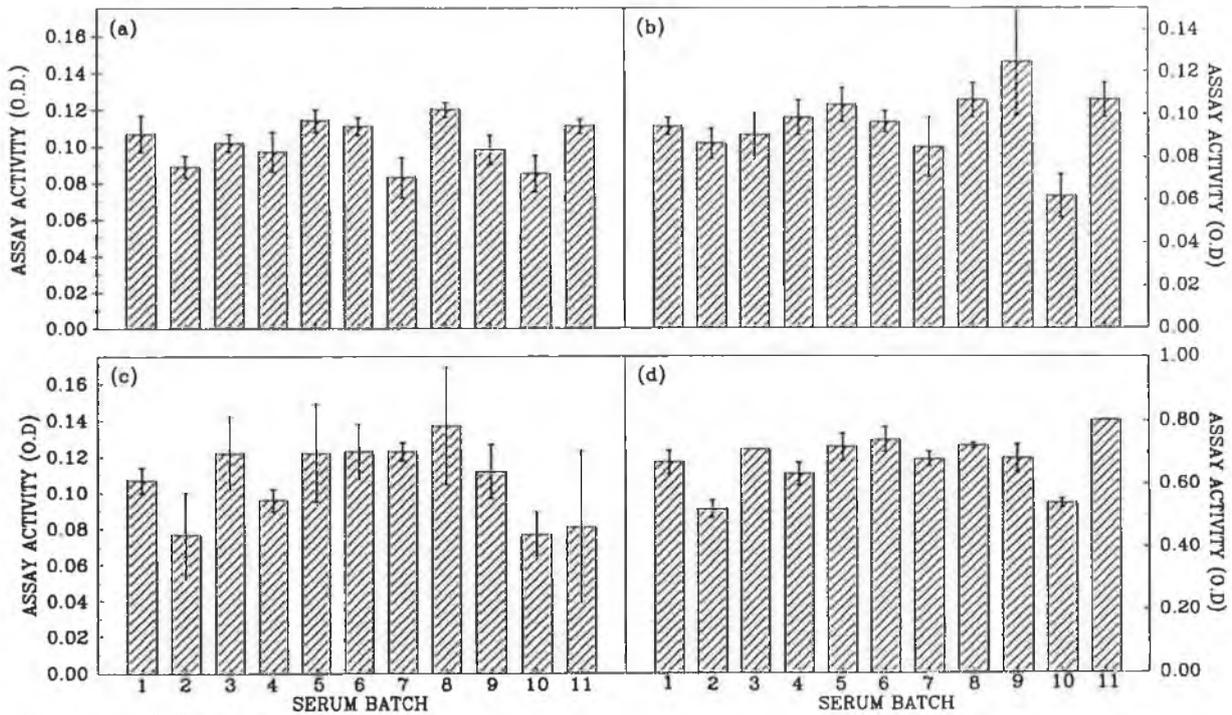


Figure 4.1.6.1

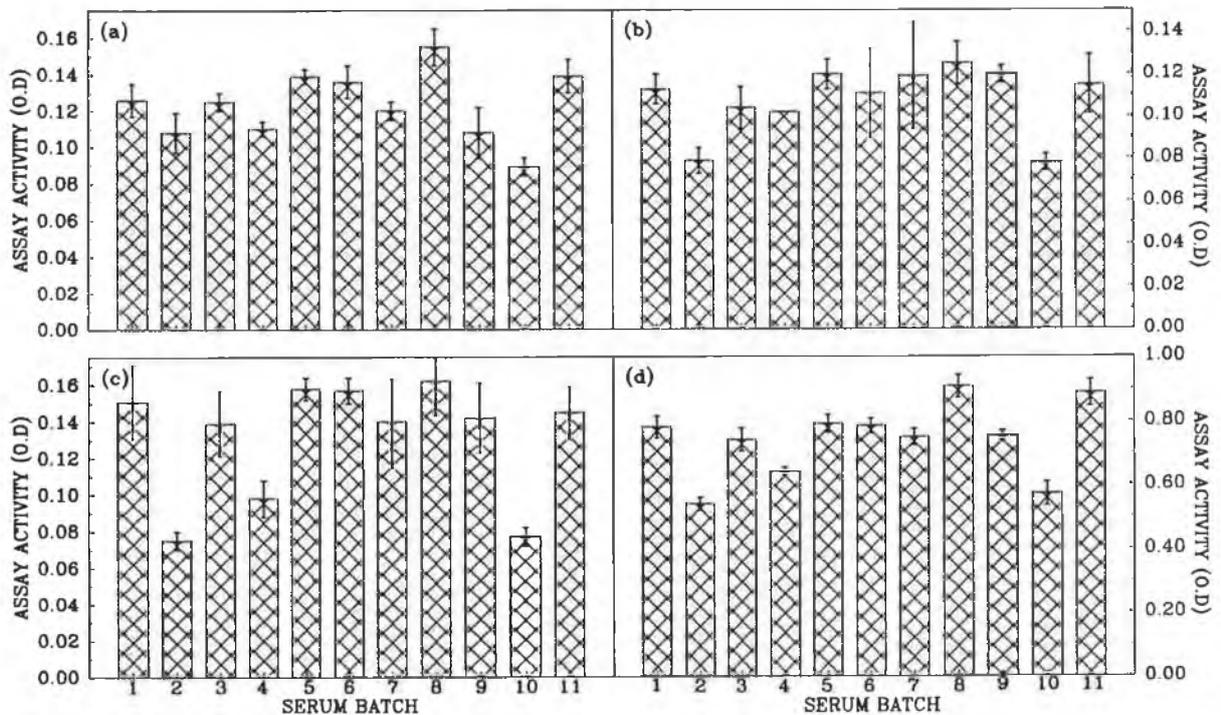


Figure 4.1.6.2

Figures 4.1.6.1 and 4.1.6.2:

Comparison of different endpoints in assessment of serum batch activity on RPMI-2650 cells (10,000 cells/ 96 well after 6 days growth): (a) = AP2h, (b) = MTT, (c) = NR, (d) = CVDE, = 5% serum, = 10% serum.

4.2 Toxicity testing of industrial sludges

For many industries the waste treatment system used, aims to produce an effluent with as high a quality as is economically and technically possible. This is brought about by removal of solids and oxidizable material from the waste stream to produce an effluent of lower polluting potential. This however, results in a sludge which must then be disposed of. Disposal is often by landfilling (considered in 4.3). Sludges should be demonstrated to be suitable for landfilling or spreading on land, by their low toxicity.

This section describes how a number of industrial sludges were assessed for their suitability for landfilling, and how the results obtained by the AP assay compared to in vivo toxicity testing with one invertebrate species.

Eight authentic sludge samples were obtained from EOLAS, Shannon. They were designated, samples A - H, and the chemical nature or toxicity of the samples was not disclosed (to circumvent biased testing/ results, and also for reasons of confidentiality). A leachate was generated from each of the samples as described in section 2.38 and samples, after preparation were tested on MDCK cells for 72 hours. AP activity was used to determine cell cytotoxicity. IC₅₀ values were determined in the usual manner and results were expressed as Toxic Units (TU), where 1 TU = 1000/ IC₅₀. In vivo tests were performed on 2 species of invertebrates by EOLAS at Shannon in addition to a bacterial Microtox test. All of the procedures have been described in section 2. The Table below compares these results to the AP test results obtained in this work.

Table 4.2 Toxicity of some industrial sludges in various toxicity testing systems (IC₅₀, TU's)

Sludge sample	AP assay	Microtox assay	<i>D.magna</i>	<i>A.salina</i>
A (unidentified)	5	.	nt	.
B (pharmaceutical)	5.5	12	9	.
C (pharmaceutical)	22	.	36	.
D (metal ore extract)	<2	3.9-9	7	.
E (pharmaceutical)	<=2	.	1.7	.
F (circuit board wash)	<=2	.	nt	.
* G (dry cleaning sludge)	770	.	13,157	.
H (harbour sediment)	<2	<1	nt	<1

where nt = not toxic

Unfortunately only some of the EOLAS results were available for comparison purposes. Rainbow trout studies were also carried out but the results could not be made available for comparison.

Bear in mind that the values will vary depending on experimental factors - so in routine testing, an internal control should always be used and the same experimental conditions adhered to.

However it is certainly clear from above that the only sample showing real toxicity in the EOLAS tests is sample G (*) - the dry cleaning sludge sample. Sample C, a pharmaceutical sludge showed slight toxicity. In the AP results, the same general trend was seen. Sample C showed slight toxicity and sample G was very toxic to the cells. Although the same trend was found with the AP assay, the sensitivity in terms of TU's was lower for the AP assay - 20 times less so for sample G than for the bacterial Microtox assay. The same rank order was seen for the invertebrate and cell culture tests - only sludge G would have been rejected for landfilling.

4.3 Toxicity assessment of Landfill Leachates

In Ireland, most of the domestic and industrial waste produced is disposed of by 'landfilling' (considered in detail in the general discussion). A characteristic feature of these landfill sites is the production of a 'leachate' which may find its way into ground and surface water and cause serious pollution problems. Leachates are often high in heavy metals and other toxic compounds. Constant monitoring of groundwater is not practical due to lack of resources needed to carry out comprehensive chemical monitoring schemes.

If a simple, inexpensive biological monitoring system were available, to determine the total toxicity of the leachate or the groundwater, then this would go a long way to helping in the correct management of a landfill site.

This section describes attempts to apply the AP assay to such an end.

Heavy metals, which may be significantly present in landfill leachates, are toxic in their own right (do not require bioactivation) and are particularly toxic to the kidney [Goodman, 1985], thus it was felt appropriate to use the kidney cell line MDCK as the target for toxicity assessment.

To determine the ability of the AP assay to assess chemical toxicity of heavy metals to MDCK cells, some standard solutions of Cadmium oxide, Nickel nitrate and Lead nitrate were prepared and tested on cultures in a 5 day continuous exposure system (as described in Materials and Methods).

The dose /response relationships (which, when repeated resulted in excellent reproducibility) are shown in the the Figure 4.3.1.

It is clear that Cadmium oxide is much more toxic to MDCK than either of the other metals, having an approximate IC_{50} (under the conditions of this work) of $11.4 \mu M$. Nickel nitrate is next toxic to the cells with an approximate IC_{50} of $23.6 \mu M$, and least toxic of the three is Lead nitrate which has resulted in approximately 65% cell survival based on AP levels at $155 \mu M$. Having seen that the culture system and the assay are capable of detecting some toxicity to the type of compounds expected to be found in a landfill leachate, it is now appropriate to test some leachate samples themselves.

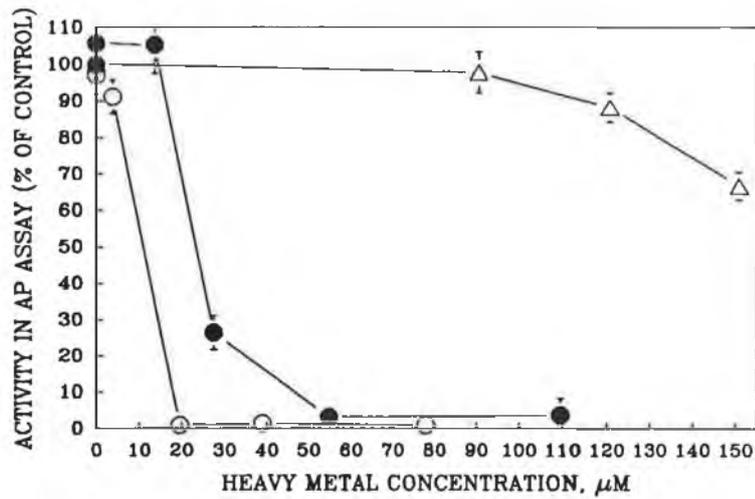


Figure 4.3.1:

Toxicity of heavy metal salts to MDCK cells (5,000 cells/ 96 well seeded, 5-day continuous exposure) using AP endpoint.

○ = Cadmium oxide, ● = Nickel Nitrate, △ = Lead Nitrate.

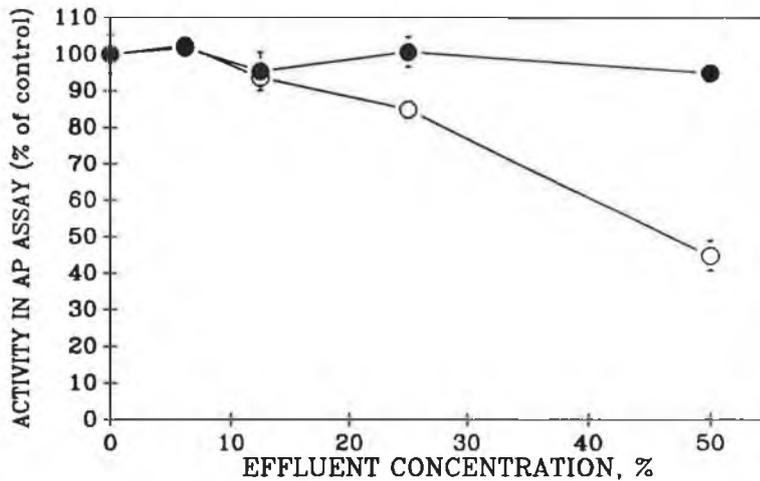


Figure 4.3.2:

Toxicity of ○ metalworks effluent and ● landfill leachate to MDCK cells (5,000 cells/ 96 well seeded, 5-day continuous exposure).

Endpoint: AP activity.

Further preliminary work was carried out with MDCK in the culture system alluded to above (and described in detail in Materials and Methods), with a range of effluents and a landfill leachate obtained from a Local Authority (LA). Because of the problems considered in the general discussion in relation to obtaining authentic, sufficiently toxic samples for testing, no backup analytical data was available for these samples (and due to severe time constraints could not be undertaken as part of this thesis), so this data has not been shown. However, it is seen in Figure 4.3.2, that even with low dilutions of effluent (up to approximately 12% effluent in medium) there has been some cell kill. The significance of this in a practical situation will be discussed more fully in the following chapter. We note that the landfill leachate did not exert significant toxicity in to the cells in culture, even at concentrations of 50% leachate in culture medium.

Three further leachate samples were obtained from a LA sanitary landfill site. The site is a well run and regularly monitored site. While it was hoped to obtain leachates of varying potency it seemed unlikely that very toxic sample would be obtained. Samples were labeled 1-3 and identified by this label alone. When the results from AP testing were surrendered to the LA, analytical data pertaining to the samples was then obtained and retrospective analysis was carried out. The leachates were pH'ed to 7.45 - 7.55 and filter sterilized before testing on an MDCK culture (set up 24 hours previously) for 72 hours. TU (Toxic Units) values were calculated from IC_{50} s and these have been compared to the chemical characteristics of the wastes in the table below.

Table 4.3 Relationship between chemical analysis of leachates and performance in AP assay

Parameter	Leachate sample		
	1	2	3
pH	7.35	6 - 8	6 - 8
conductivity	20,000	20,000	500 - 3,000
BOD	> 800	> 800	5 - 200
COD	5,000	5,000	10 - 500
NH ₄ ⁺	2,400	5 - 100	5 - 1000
Cl	3,900	50 - 100	20 - 200
susp. solids		10 - 500	10 - 500
Zn	2.2	<0.2	<0.2
Na	2,000	30 - 150	30 - 150
K	1470	10 - 100	10 - 100
Ca		100 - 500	100 - 250
NO ₃ ⁻	36	1 - 10	1 - 10
NO ₂ ²⁺		<0.2	<0.2
SO ₄ ²⁺	8	5 - 100	50 - 500
total hardness	131	500 - 600	50 - 1,000
Fe		1 - 10	50 - 600
Mn	8.6	1 - 10	1 - 10
Pb		<1	1 - 10
Cu		<0.1	<0.1
Cd		<0.1	<0.1
Ni		<0.1	<0.1
alkalinity	10,000	50 - 1,000	
D.O.	0.7		
temperature	15.3		
Cu	0.15		
Cd	0.05		
AP toxicity	7.5	<=2	<=1

Units: AP toxicity in Toxic Units
 pH in pH units
 Conductivity in mOhms/kg
 Temperature in °C
 All other parameters in ppm

Where relevant these terms have been explained in the discussion.

We note from chemical analysis (by the Local Authority) that none of the samples are extremely potent. Metal concentrations are low for all of the leachates, thus on retrospective consideration it was not expected to observe toxicity similar to that in Figure 4.3.1, in this toxicity screen.

Sample 1 is quite high in nitrogen; nitrate levels are enriched over the other two samples, but most of this nitrogen is in the form of ammonia. COD levels, which would probably give the best indication of potency from all of the parameters listed here, are not terribly high.

A leachate having a COD of 10's or 100's of thousands (ppm) would be considered to be very potent; substantially less than this would not be considered so. Thus, we note that none of the three leachates appear to be particularly toxic. If some distinction had to be made among them, it would have to be said that leachate no. 1 was the most potent, having higher ammonia, nitrate, and zinc levels than the others. The sodium and potassium levels are also higher. The results obtained from the AP assay are in good agreement with this assesment. All 3 leachates were non-toxic, however the rank order of potency from this assay was the same as that observed from the analytical data; sample 1 was somewhat more potent than the other 2; samples 1 & 2 were close together, with sample 3 (which had the lowest BOD and COD) being very slightly less toxic than sample 2.

Overall, it was disappointing that no particularly noxious leachates were available; as more could have been extruded from such results, nevertheless it would appear that this assay may be very useful in routine screening programmes, as the trend observed from extensive chemical analysis of the leachates was shown also with the AP results.

4.4 Screening for efficacy of new drugs

This section describes the use of AP, NR, MTT and IA to detect cytotoxic activity using a novel anti-cancer agent. The investigational drug used was Navelbine (Vinorelbine) [developed by Pierre Fabre Research Centre] which at the time of this study [1989] was undergoing Phase II clinical trials, and had given promising results to date, encompassing low neurotoxicity. It is a vinca alkaloid, but differs from the other compounds in this class, and has a very high affinity for Tubulin. Initial experimental toxicological findings were interpreted to suggest that Navelbine provided higher inhibition of tubulin polymerization than Vinblastine, and consequently was suggested to provide a better toxicity ratio and improved therapeutic index, particularly for non small cell lung carcinomas [unpublished company report].

In order to investigate this, the efficacy of Navelbine was compared to Vinblastine for the non small cell lung cell lines DLKP, DLRP (both squamous) and SK-LU-1 (adenocarcinoma), and for a number of cell lines from other sites. The structures of these drugs are given in Appendix B.

The lines were tested first in an image analysis monolayer system and then tested with the AP, NR, CVDE and MTT assays. Some of these image analysis results are condensed and presented in the table below (assays were repeated 4 times, so the data is extensive and cannot all be reproduced here) together with results obtained in some of the colorimetric assays.

7 - day continuous exposure assays were used and were carried out exactly as described in section 2.

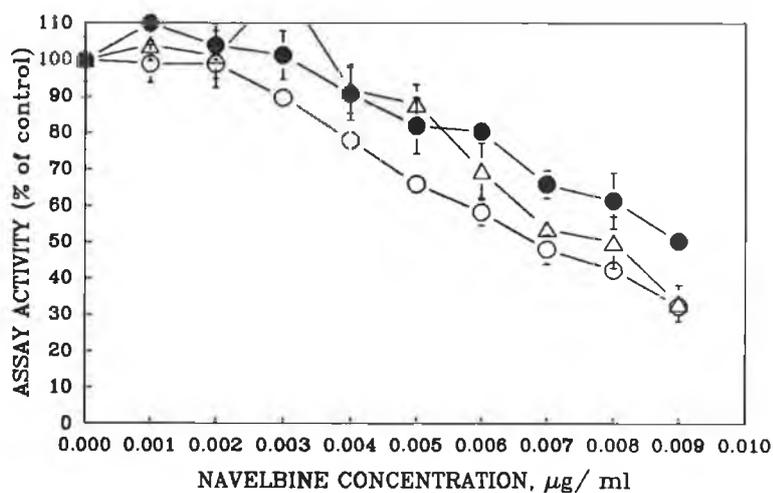


Figure 4.4.1:

Toxicity of Navelbine to SK-MES-1 cells after 6-day continuous exposure. ○ = AP, ● = NR, △ = CVDE.

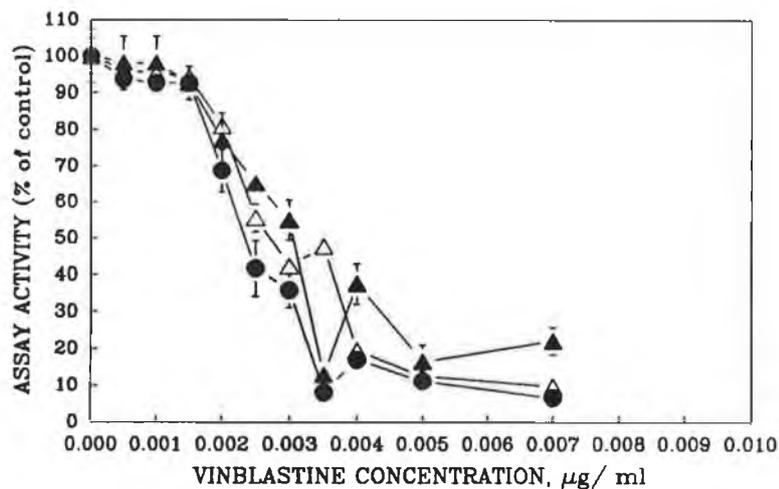


Figure 4.4.2:

Toxicity of Vinblastine to sk-mes-1 cells, after 6-day continuous exposure. ● = NR, △ = CVDE, ▲ = MTT.

Table 4.4.1 Toxicity of Navelbine and Vinblastine to DLKP, SKMES-1 and SCC-9 cell lines

drug conc. $\mu\text{g/ml}$	DLKP		SKMES-1		SCC-9	
	Assay activity (% of control)					
	Navelb	Vinbl	Navelb	Vinbl	Nabelb	Vinbl
0.000	100±22	100±30	100± 8	100± 4	100±18	100±19
0.005	-	75 11	-	-	-	-
0.00075	95 10	54 3	-	87 10	94 11	79 24
0.001	71 14	50 7	90 9	81 8	72 7	3 0
0.0025	16 5	0 0	77 4	7 5	8 1	2 0
0.005	1 0	0 0	11 19	0 0	4 0	3 0
0.0075	1 2	0 0	5 2	0 0	2 0	4 0

Image Analysis results

Table 4.4.2 Toxicity of Navelbine and Vinblastine to RPMI-2650

drug conc. $\mu\text{g/ml}$	Navelbine			Vinblastine		
	Assay activity (% of control)					
	IA	MTT	AP	IA	MTT	AP
0.000	100±12	100±15	100±2	100±33	100±15	100±5
0.00075	112 26	75 10	99 8	-	66 14	109 4
0.001	92 18	73 8	84 1	64 21	48 10	52 4
0.0025	40 2	35 4	37 8	25 11	4 3	5 1
0.005	15 7	10 3	18 3	20 12	4 1	6 2
0.0075	1 0	5 3	5 3	0 0	-	-
0.01	0 0	1 1	0 0	0 0	1 1	2 1

Table 4.4.3 Toxicity of Navelbine and Vinblastine to Hep-2

drug conc. $\mu\text{g/ml}$	Navelbine				Vinblastine			
	Assay activity (% of control)							
	IA	MTT	AP	NR	IA	MTT	AP	NR
0.000	100±2	100±3	100±4	100±8	100±13	100±8	100±10	100± 6
0.001	99 2	83 6	71 4	85 5	35 2	61 6	34 9	36 11
0.0025	87 5	72 7	23 8	62 6	0 0	3 1	1 0	5 1
0.005	56 2	28 7	1 3	16 3	0 0	4 2	1 0	2 1
0.0075	11 1	11 6	0 0	5 1	0 0	0 0	0 0	4 1
0.01	1 1	5 1	0 0	3 1	0 0	0 0	0 0	0 0

The above tables show that there is certainly a difference in toxicity between the two drugs. Navelbine, which was expected to be as toxic or more toxic than Vinblastine, resulted in significantly less cell kill than Vinblastine. The results have been given in $\mu\text{g/ml}$, but as

Navelbine has a higher molecular mass than Vinblastine (see appendix B), the results are even less efficacious for Navelbine when they are compared in terms of molarity (see Table 4.4.4). Each of the endpoints used were capable of detecting this difference in efficacy, however the methodology was simpler and quicker, and the resources used were less when the miniaturized assays were used instead of IA. This trend was similar for all of the cell lines tested, even for the lung lines.

When differential toxicities to the cell lines were compared, it appears that SCC-9 was more sensitive to both drugs, and SK-LU-1 more resistant. This is seen in the table below where the approximate IC₅₀ values for both drugs are summarized.

Table 4.4.4 Approximate relative IC₅₀s of Navelbine and Vinblastine for some human cell lines determined by AP activity

cell line (all human)	Vinblastine	Navelbine
	IC ₅₀ (nM)	
lung squamous carcinomas		
SK-MES-1	2.2	6.4
DLKP	2.7	4.2
DLRP	2.7	6.4
lung adenocarcinoma		
SK-LU-1	9.9	>> 20.0
non-lung carcinomas		
RPMI-2650	1.6	6.4
SCC-9	1.9	3.2

Broadly the assays agree on the extent of toxicity, although some differences exist. With RPMI-2650, the SEM values were much lower for the MTT and AP assays than for IA. Analysis of all the dose response curves produced for each of the cell lines with the various assays, shows broadly similar results for each of the assays. Repeat assays qualified this further. Figures 4.4.1 and 4.4.2 are typical examples of the results obtained.

In conclusion, the miniaturized endpoints assessed would form a suitable part of a prescreening programme for establishment of efficacy of new drugs, and for ranking their toxicity alongside other clinically proven drugs.

4.5 Rapid assessment of Multiple Drug Resistance

Multiple Drug Resistance (MDR) of tumours is a clinically important phenomenon, the adverse effects of which could be diminished in some cases by alteration in drug therapy choice if early detection of the phenomenon was possible. Here too, the availability of a simple, rapid, sensitive method for assessment of MDR is of importance. Such methods can be investigated in the laboratory by assessing their ability to detect drug sensitivity in cell lines with increased resistance to specific chemotherapeutic drugs.

This section describes how the AP assay performs as a new alternative to rapid assessment of MDR, and compares it to the performance of MTT, NR, SRB and DE.

Variant cell lines were cultured in growth medium with Adriamycin, as described in section 2.36. 96 well plates were seeded 24 hours before drug addition at densities of 4×10^3 cells/well (3×10^3 /well for the parent line, due to a higher doubling rate). Cells were exposed to various concentrations of the drugs Adriamycin, Vinblastine, 5-Fluorouracil and Cis-Platinum. After 6 days continuous exposure, an AP assay was carried out and IC_{50} values were obtained from dose/response curves for all of the drugs. They are presented in the table below.

Table 4.5.1 Approximate IC_{50} 's of 4 drugs to DLKP and 2 variant cell lines under the conditions described

Cell line	Drug (μM)			
	Adriamycin	Vinblastine	Cis-Platin	5-Fluorour.
DLKP $2\mu g/ml$	7,600 \pm 1,000	25 \pm 1	900 \pm 30	8.5 \pm 3.0
DLKP $1\mu g/ml$	13,000 \pm 500	67 \pm 2	155 \pm 20	44 \pm 12
DLKP	750 \pm 50	0.275 \pm 0.02	640 \pm 15	15 \pm 8

Table 4.5.1 shows that the variant cell lines are at least 10-fold resistant to Adriamycin and almost 100-fold resistant to Vinblastine. There is significant variation in resistance of the three cell lines to Cis Platinum and 5-Fluorouracil, but these differences show no particular trend. Thus the cross resistance pattern can be seen for the Vinca Alkaloid, Vinblastine, as well as the drug for which it was selected against, but cross resistance has not included drugs with mechanisms of action like that of Cis Platinum and 5-Fluorouracil.

While the assay can easily detect the significant difference between the parent and variant cell lines, it does not detect the increased resistance of the variant cultured in $2\mu\text{g/ml}$ Adriamycin over that cultured in $1\mu\text{g/ml}$ ($7,600$ or $13,000\ \mu\text{M}$ respectively).

To determine if this was applicable only to the AP assay, the same experiment was carried out on the other colorimetric assays; MTT, CVDE, NR and SRB. The dose-response curves from these experiments are given in the following pages (Figure 4.5.1 - 4.5.6) for each of the 4 drugs.

It was speculated that the problem with the AP2h assay identified in the table above might be due to insensitivity, as the OD was in fact quite low; so the APNaOH assay, with its much increased sensitivity was performed. We can see by comparing Figures 4.5.1 and 4.5.2 that, while the result with the parent cell line is still the same, we can now detect the increased resistance of DLKP2 over DLKP1 for both the relevant drugs (Adriamycin and Vinblastine). Thus it appears that as expected, the problem was due to low sensitivity, since this is the only difference between the two procedures.

Let us now consider the other assays, and investigate if the problem recurred elsewhere.

Using the MTT assay, Figure 4.5.3 demonstrates that again upon exposure to Adriamycin and Vinblastine, the parent cell line, DLKP, is much more sensitive to drug than are the variants, DLKP1 and DLKP2. In the case of the antimetabolite 5-Fluorouracil, and for Cis-platinum, there is however, no difference in drug sensitivity, that is, the variants have not spontaneously acquired resistance to these drugs as has occurred with Vinblastine. While the MTT assay, like the AP2h assay, is clearly able to demonstrate the difference in sensitivity between parent and variant cell lines, it is less well able to do so among the variants themselves. From the figure, it would appear that DLKP2 is slightly more resistant to Adriamycin and Vinblastine than the variant selected in the lower concentration of drug- DLKP1, however the error bars from SEM values are quite large in many cases; much more significant in fact than either the AP2h or APNaOH assays.

Figure 4.5.5, which illustrates the results obtained by the CVDE assay shows the same ability of the assay for detecting the much greater sensitivity of the parent line over the variants. Here however, a slight increase in the resistance of DLKP2 has been detected over DLKP1 for Vinblastine, and a substantial difference for Adriamycin.

The same has been reported for the effect of Vinblastine on the variants in the NR assay (Figure 4.5.4), while no difference is perceived between the two lines when the Adriamycin graph is studied.

DLKP, the cell line used for these studies, was chosen not for its growth characteristics, but because it was the only cell line adapted for growth in higher concentrations of Adriamycin which was available for use at the time of this work. In fact, the cell line has an undesirable characteristic for screening work of this type; as many of the cells attach only loosely to the substratum, and some in fact grow in suspension.

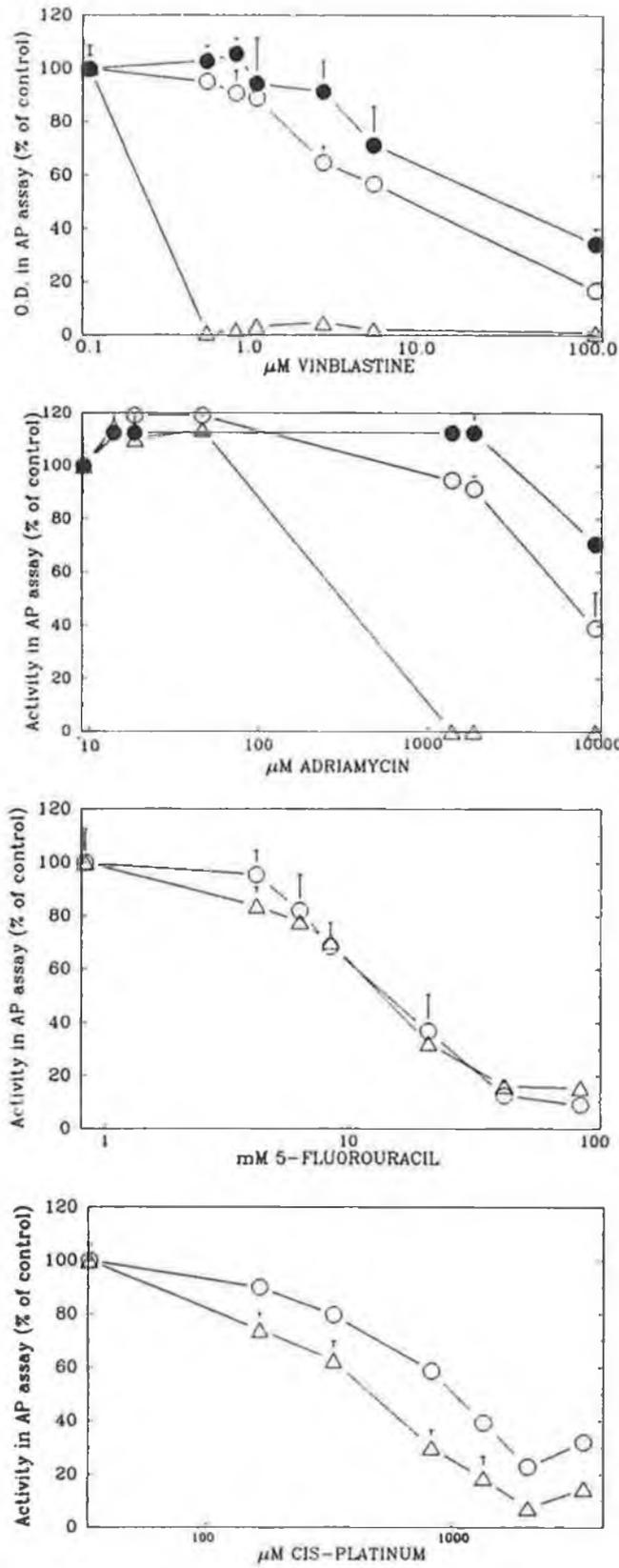


Figure 4.5.1:

Assessment of Multiple Drug Resistance in DLKP (Δ) and 2 variants of this line cultured in $1 \mu\text{g}/\text{ml}$ (\bullet) and $2 \mu\text{g}/\text{ml}$ (\circ) Adriamycin using the AP2h assay

(For clarity, error bars shown only on one side of each plot)

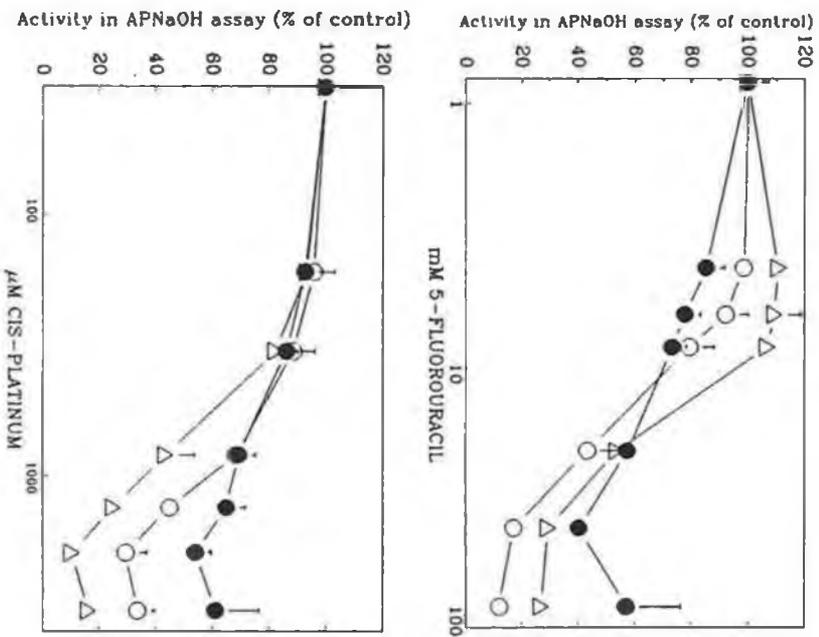
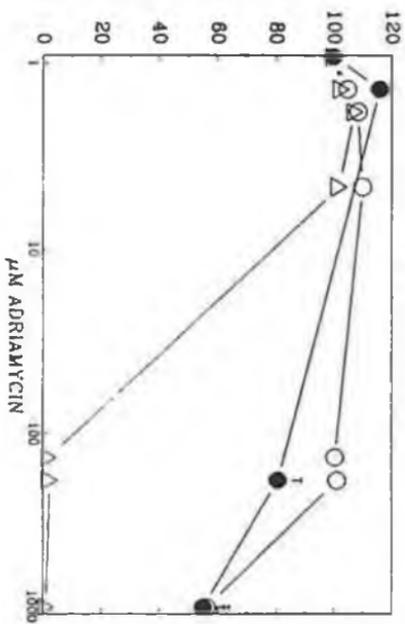


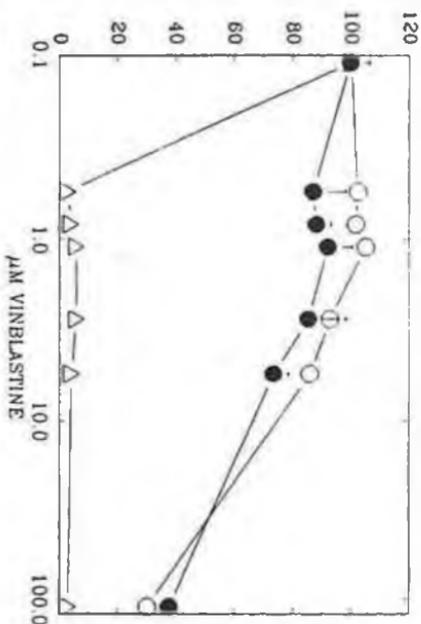
Figure 4.5.2:

Assessment of Multiple Drug Resistance in DLKP (\blacklozenge) and 2 variants of this line cultured in $1 \mu\text{g}/\text{ml}$ (\bullet) and $2 \mu\text{g}/\text{ml}$ (\circ) Adriamycin using the APNaOH assay
 (For clarity, error bars shown only on one side of each plot)

Activity in APNaOH assay (% of control)



Activity in APNaOH assay (% of control)



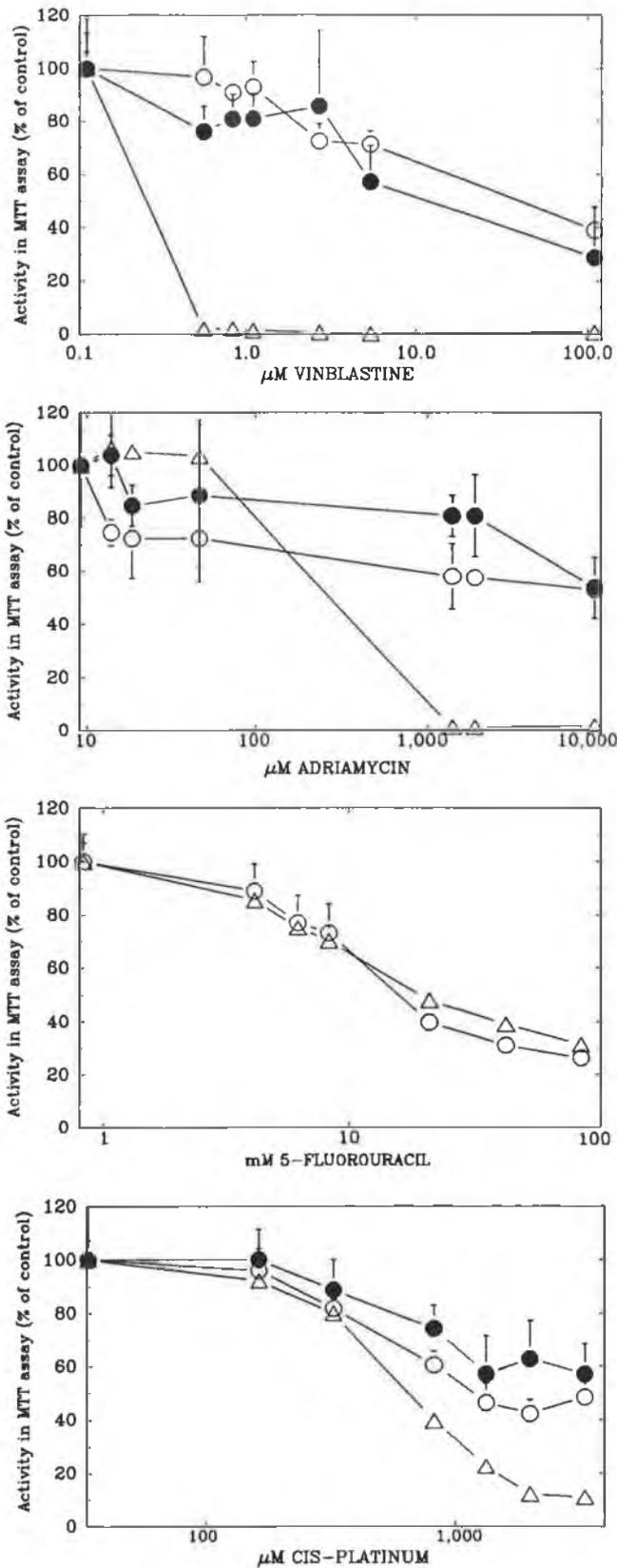


Figure 4.5.3:

Assessment of Multiple Drug Resistance in DLKP (Δ) and 2 variants of this line cultured in $1 \mu\text{g}/\text{ml}$ (\bullet) and $2 \mu\text{g}/\text{ml}$ (\circ) Adriamycin using the MTT assay

(For clarity, error bars shown only on one side of each plot)

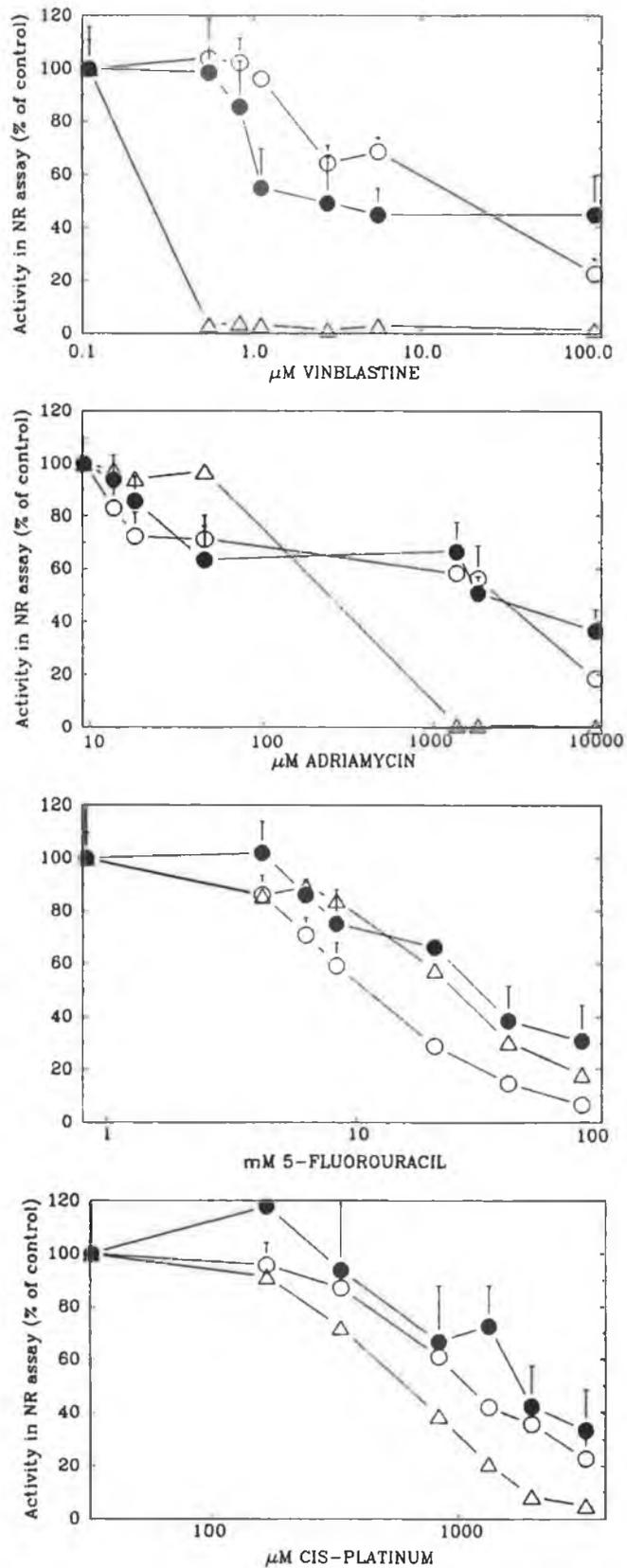


Figure 4.5.4:

Assessment of Multiple Drug Resistance in DLKP (Δ) and 2 variants of this line cultured in $1 \mu\text{g/ml}$ (\bullet) and $2 \mu\text{g/ml}$ (\circ) Adriamycin using the NR assay

(For clarity, error bars shown only on one side of each plot)

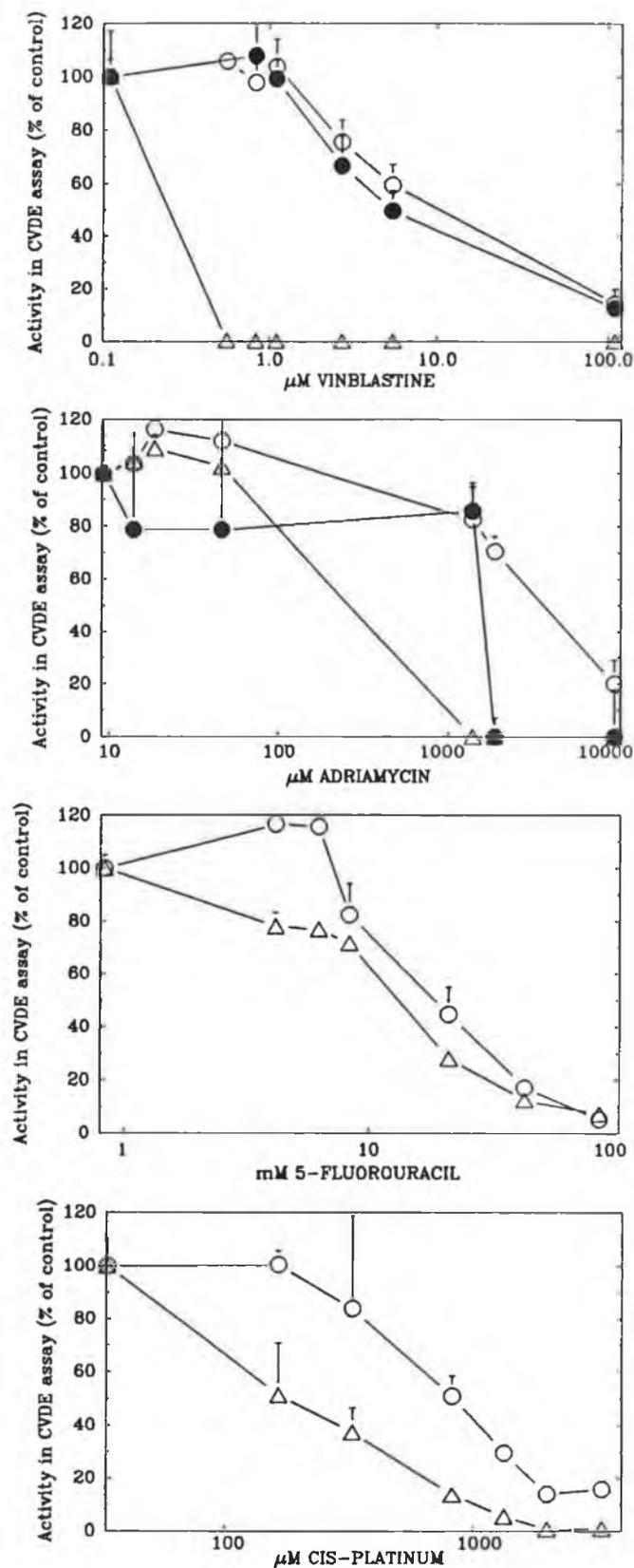


Figure 4.5.5:

Assessment of Multiple Drug Resistance in DLKP (Δ) and 2 variants of this line cultured in 1 $\mu\text{g}/\text{ml}$ (\bullet) and 2 $\mu\text{g}/\text{ml}$ (\circ) Adriamycin using the CVDE assay.

(For clarity, error bars shown only on one side of each plot).

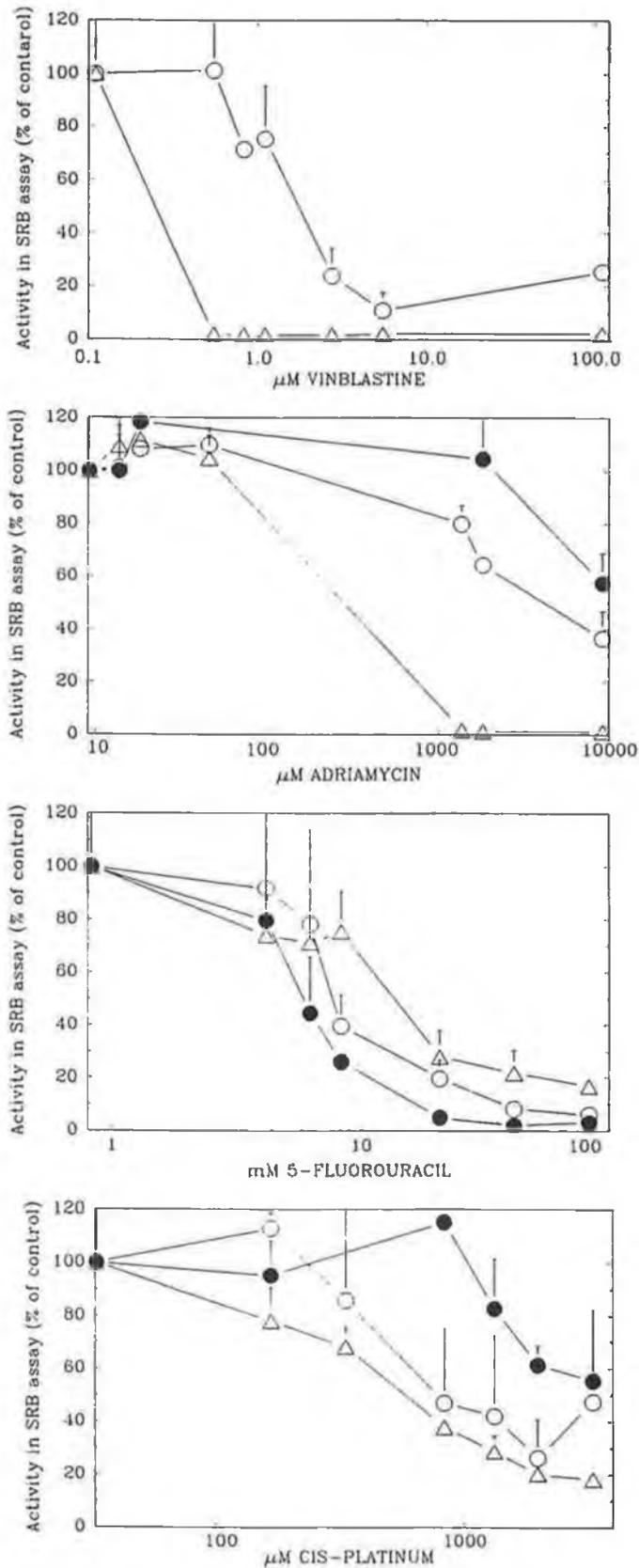


Figure 4.5.6:

Assessment of Multiple Drug Resistance in DLKP (Δ) and 2 variants of this line cultured in 1 $\mu\text{g}/\text{ml}$ (\bullet) and 2 $\mu\text{g}/\text{ml}$ (\circ) Adriamycin using the SRB assay

(For clarity, error bars shown only on one side of each plot).

The TCA addition step of the SRB assay, described and discussed in section 3.8.5, was thought to have been an advantage when assaying loosely adherent cell lines such as DLKP, so similar, or slightly better results (in terms of precision/ accuracy) would have been expected with this assay. In fact it is surprising to note the very high SEM values and low OD results. A practical explanation for this may be that the plates were fixed as usual, and stored dry for a number of days in the dark before staining took place. Perhaps the assay is not suitable for storing and later analysis, as has been suggested (Skehan et al., 1990). The low OD readings and the high standard deviations for DLKP1 in the Vinblastine graph rendered the results unacceptable, so they have been omitted from the graph (Figure 5.4.6). The assay was not able to detect the higher resistance to drug of DLKP2 over DLKP1, and had unacceptably high SEM values for all of the drugs tested.

In summary, there was a problem with low precision of the SRB assay and low sensitivity, which should not be the case, according to results in earlier sections. This may have been due to storage of the plates after fixing and before staining, as there was little time to perform this task at the appropriate time (due to attention being given to the other assays); perhaps this should not be done in future. The AP2h assay was insensitive to the growth of the variant cell lines (which grow slower than the parent line) and was not able to distinguish the difference in drug sensitivity between the two, but this was not a problem when NaOH was added to increase sensitivity. With both procedures, SEM values were lower than for the other assays, giving more precise results. Precision was more of a problem with MTT, NR and CVDE assays.

Slight differences in sensitivity resulting in the correct rank order of resistance of the two variants was given by APNaOH, CVDE and NR assays, however given the greater precision and simplicity of the APNaOH assay, this was preferred for use as a convenient and rapid screen for detecting cross resistance patterns and assessing the level of resistance of selected variants.

RESULTS 3:

PRIMARY CULTURE OF BRONCHIAL TUMOURS

5 Primary culture of bronchial tumours

5.1 Collection and culture of samples

Eighty four samples were collected and processed during the course of this project. Of these samples, 82 were from the thoracic region, the other two were accessory skin samples. One was a normal lung biopsy. Of the prospective tumour samples received, 25.9% were bronchoscopy scrapings, 29.6% were mediastynal nodes, 21% were lobectomies and 7.4% were pleural effusions. Blebectomies or other lung biopsies constituted 7.5% of samples, and the remaining 8.6% were oesophageal samples (4.9%), or thoracic, non-lung samples (3.7%). This breakdown is shown in Table 5.1.

Table 5.1 Breakdown of total number of samples cultured

SAMPLE TYPE	NUMBER
Bronchoscopies (Bronc)	21
Mediastynoscopies (Med)	24
Lobectomies (Lob)	18
Blebectomies (Biop)	1
Other Lung Biopsies (Biop)	5
Pleural Effusions (PE)	6
Oesophageal (Oes)	3
Other Non-Lung, thoracic	3
Normal Lung	1
Normal Skin	2
TOTAL NUMBER	84

Bronchoscopy samples were obtained when the surgeon used a fibre optic bronchoscope to remove tiny scraps of tissue from the bronchus. Biopsies of mediastynal nodes gave rise to mediastynoscopy samples. Both are the result of investigative surgery to determine the presence of malignant tissue. These samples together with those from other operations such as removal of a lobe (lobectomy) or bleb of the lung (blebectomy) were the samples most often obtained.

5.2 Growth of sample subsets in culture

The extent of growth from each of these samples is summarised in Table 5.2.1. 'Growth' is broken down into: growth of fibroblast cells only; initial attachment of epithelial cells, but no further growth; and differing degrees of successful epithelial growth. For 37% of samples cultured, there was no cell attachment or growth whatsoever. For a further 29.6%, there was no epithelial cell growth - only growth of fibroblasts. The number of samples for which epithelial cell attachment occurred, but no further growth took place was 16.1%. This leaves us with only 17.3% of samples for which some degree of epithelial cell proliferation took place. 7.4% of samples had initial limited growth, while a further 6.2% resulted in significant growth and proliferation. In 3.7% of samples, extensive epithelial cell

growth occurred and was maintained for a period of at least a number of weeks in culture. However none of the cultures proliferated to such an extent or for long enough to produce a permanent cell line.

Table 5.2.1 Growth of thoracic samples in culture

NG	Fib	Att	I	Sig	Ext	CL
30	24	13	6	5	3	0

Table 5.2.2 Success of sample in culture according to sample origin

	Bronc	Med	Lob	Biop	PE	Oes	Other
NG	9	7	7	3	3	-	2
Fib	6	7	7	2	-	2	-
Att	5	6	-	1	-	-	-
I	1	-	1	-	3	1	-
Sig	-	4	1	-	-	-	-
Ext	-	-	2	-	-	1	-
CL	-	-	-	-	-	-	-
	21	24	18	6	6	4	2

Where:

- NG = No cell attachment or growth
- Fib = No attachment or growth of epithelial cells - fibroblast growth only
- Att = Attachment of epithelial cells, but no subsequent growth
- I = Initial epithelial growth
- Sig = Significant epithelial growth
- Ext = Extensive epithelial growth
- CL = Permanent cell line established

Table 5.2.2 categorizes sample growth performance in culture, with respect to the origin of the sample. 95.2% of bronchoscopy samples did not produce any cell growth in culture, and the remainder only gave initial growth - i.e. growth sustained for a few days only. With the mediastinoscopy samples, a huge proportion again did not give any epithelial cell growth - this time 83.3%. The remaining 16.7% though, grew significantly for at least 10 days. Excluding the lobectomies, none of the other biopsy samples effected any epithelial cell growth.

Of a total of 18 lobectomy samples, 77.7% did not result in any epithelial cell growth. Of the 22.2% that did, 5.5% produced significant growth, and another 11% produced extensive epithelial growth, for a number of weeks. This status was achieved also by 16.7% of the pleural fluid samples processed, accompanied by a further 33.3% giving initial growth; the remainder had no growth or attachment. Of the remaining subset of samples showing even limited success in culture, the oesophageal samples behaved quite well. 25% did afford extensive epithelial growth; another 25% grew initially but did not continue, and the remaining 50% were unsuccessful.

In essence, growth from bronchoscopy samples was extremely poor. This was only slightly better for mediastinoscopy samples. Lobectomy samples, pleural fluids and oesophageal samples were the most successful in culture.

Teasing this out further we can make some observations from the sample quality and relate this back to growth in culture.

5.3 Sample Quality

The general appearance of each sample was noted before culture. Some samples were just too small to produce an adequate number of cells for the culture to 'take off'. Some were severely necrotic or heavily bloodstained. These samples generally did not produce epithelial growth. It appears that visually 'poor' samples behaved worse in culture than visually 'good' samples.

Out of a total of 25 samples which were characterized as:

- * necrotic,
- * presence of numerous blood vessels, or
- * composed of significant amounts of adipose or cartilage tissue,

23 resulted in no epithelial attachment due either to absence of sufficient epithelial cells, or inhibition of attachment due to settling out of blood cells and cell debris. One of these 25 samples exhibited initial attachment but no further growth of epithelial cells, while a single sample displayed some epithelial growth which was very limited.

5.4 Success of various processing methods

**Table 5.4 Epithelial growth from solid tissue:
Analysis of effective methods of processing**

Number of samples..	Explants	Enzymatic	Dissection medium	Collection medium
set up	13	8	11	5
success	7	6	10	4

Note 1: number of ways a sample was processed was dependent on sample size and texture

Note 2: some samples developed cell growth in more than one of the categories above

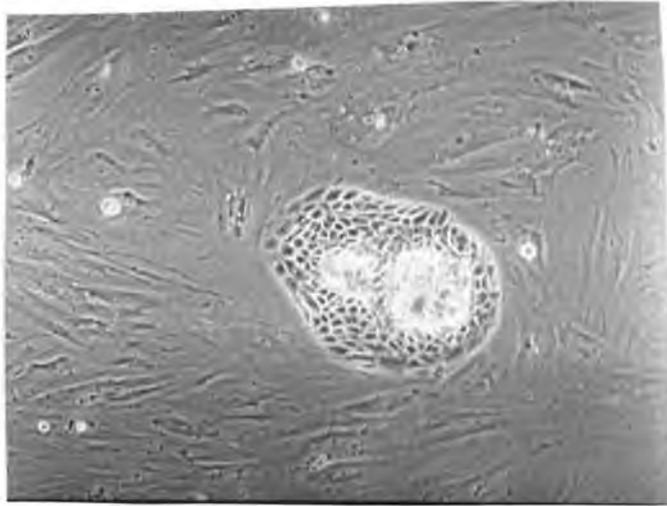
Table 5.4 shows the frequency of success (represented by epithelial growth or attachment) for each of the different methods from the total of 22 samples that grew (to any extent). It must be appreciated that the sample size and quality dictated the method(s) which could be used (refer to section 2); it was not possible to set up a fragment of the sample for each of these methods. Thus it is not appropriate to calculate success rates for each of the methods based on the above figures.

5.5 Collection medium as a source of cells

If the sample was particularly friable (prone to dissociate easily into smaller particles) and tiny fragments had detached from the main specimen, then this collection medium (CM) was used as an additional source of cells. The medium was agitated repeatedly in the universal to dislodge as many fragments as possible from the main specimen. If heavily bloodstained, it was then necessary to spin the suspension on a Ficoll gradient after rinsing in PBS. Although epithelial growth did result from this fraction in a number of cases, most often, resilient blood cells remained to contaminate the culture.

5.6 Explant growth

Only in the case of samples which were easily dissected did explants prove to be successful. In other words, samples which were stringy and difficult to cleave, or which did not produce cleanly cut edges were not successful and thus were not routinely set up by this procedure. Explants from such samples did not attach very well. Generally it appeared that the smaller the explant, the better chance it had of attaching and of remaining attached to the growth surface; thus the greater the chance of growth occurring from these explants. Unless several explants in the flask resulted in outgrowth, the culture was unlikely to be a success. Growth from an isolated explant, even if extensive, led to localised confluency (if, for example, the leading edge was near the edge of the flask, or near other unproductive explants, growth would thus be confined). Then the cells in the middle of the outgrowth would begin to senesce. In these cases, passaging did nothing to alleviate the situation as the cells were difficult to trypsinize, and did not respond well to replating (often necessarily at too low a cell density). Additionally, during this procedure fibroblasts would be removed and re-seeded, quickly benefitting from the rearrangement in location and permeating the monolayer of epithelial growth. Photograph 5.6 shows epithelial outgrowth from a lung tumour explant.



Photograph 5.6: Epithelial cell outgrowth from a lung primary culture (x 100). Note that cells are small and tightly packed. These cells were very slow to grow.

5.7 Dissection medium as a source of cells

The medium in the petri-dish, in which the dissection took place was termed the 'dissection medium' and was often found to be rich in cells. Particularly in the case of friable tumours, and with large (usually lobectomy) samples, cutting of the tumour to produce fragments suitable for explants or for enzymatic disaggregation, yielded a medium abundant in tiny clumps of epithelial cells. These clumps were more successful at attaching and growing than were single cells (Photograph 5.7). Also, damage to the cells was minimized as they were not exposed to enzyme treatment.

Photograph 5.7: Epithelial colonies arising from clumps of cells isolated from 'dissection medium'. Note absence of fibroblasts at this stage (4 days in culture; magnification x 100).

5.8 Enzymatic disaggregation

Frequently the fragments were not disaggregated at the end of a 2 - 3 hour incubation with Collaganase/ Dispase. Further incubation with enzyme did little to improve disaggregation, but instead caused disintegration of the cells which had been released. Sometimes the suspension formed a slimy mass (indicating DNA damage) even though the bulk of the tissue pieces remained un-disaggregated. In an effort to improve this performance, the sample pieces were dissected into pieces as small as possible, however the results did not improve.

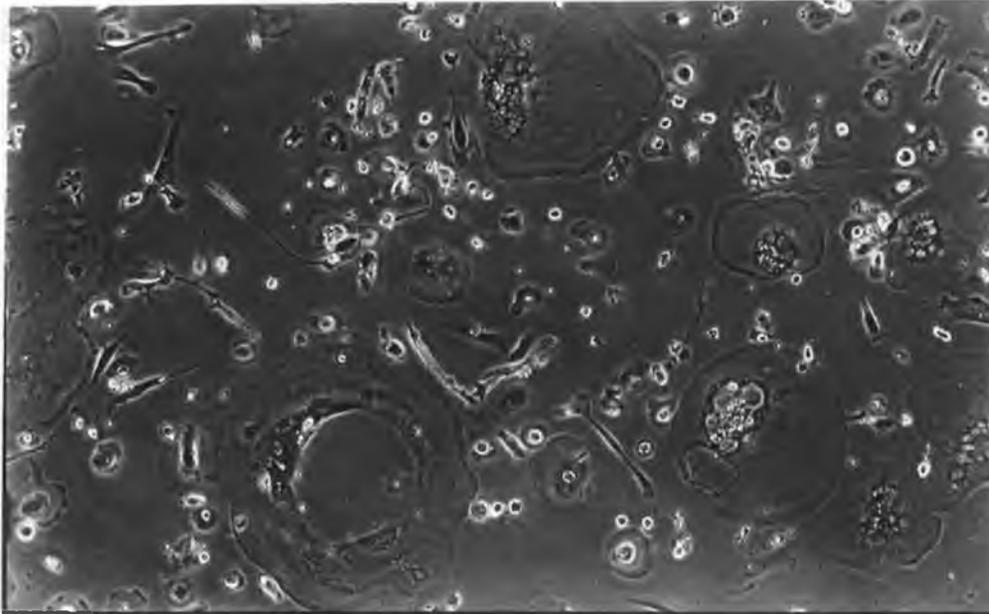
Because clumps of epithelial cells performed exceptionally better in culture than did single cells, it was attempted to design disaggregation practices to isolate epithelial clumps from the suspension in preference to single cells.

Cultures derived from enzymatic disaggregation characteristically had a greater problem with fibroblast growth in the culture. Initiation of fibroblast overgrowth was much more rapid after enzymatic treatment than was the case with cultures derived from the other procedures.

5.9 Blood cells and cell debris

Excessive numbers of blood cells in the cell suspension or extensive debris (resulting from dissociation of necrotic tissue) resulted in a film forming on the culture surface. This film either prevented cell attachment, or, if cells had already attached, it coated the cells and fixed upon them. Rinsing with PBS was ineffective at removing much of this film, and consequent deterioration of the culture occurred. Cells became grainy and vacuolated, stopped growing and then died (see photograph 5.7).

Photograph 5.9: Cells isolated from a malignant pleural effusion after 8 weeks in culture. Cell cytoplasm has expanded dramatically, become vacuolated, darkened and granular as the cells terminally differentiate (magnification x 100).



While centrifugation of the cell suspension with Ficoll generally pelleted the erythrocytes, leucocytes and lymphocytes tended to appear together with the epithelial cells, and thus remained in the culture.

Figure 5.11 (b) shows that a similar number of epithelial cells in 2 different cell suspensions prepared from the same specimen, result in differing amounts of growth. Suspension *, which was isolated from the collection medium (see section 2.41) contained much more blood cells and cell debris than the other cell suspension (a purer cell suspension isolated from the dissection medium (DM) which had been rinsed several times), and subsequently resulted in less growth. This would appear to enforce the constantly observed phenomenon that blood cell contamination inhibited the attachment and growth of epithelial cells.

5.10 Fate of epithelial cultures

Table 5.10 shows the manner in which the epithelial cultures were lost.

Table 5.10 Fate of epithelial cultures

Differentiation \senescence	Fibroblast overgrowth	Both diff. and fibroblasts	Fungal contamination
5	12	4	1

54.5% perished exclusively due to overgrowth by fibroblasts, even though extensive efforts were made to rescue the epithelial cell population, both by treatment with Trypsin/ EDTA at room temperature, and by exposure to Geneticin. However in 18.2% of cases, the cultures stopped growing and fibroblasts, which had not been prevalent, then took over the culture. Thus, in total, fibroblast overgrowth was involved in the demise of 72.7% of cultures.

The second most common reason for failure of the culture was differentiation. The cells, bright and irregular in profile, began to flatten out more, become much darker and become vacuolated. With this change in appearance, the growth rate decreased and cells stopped dividing. No longer could mitotic cells, bright and slightly detached from the surface, be seen. The cells appeared very granular. Some cultures developed cells with grossly enlarged cytoplasm, which contained multiple nuclei. In these cells fibres stretching from the nucleus out to the cell membrane could be seen. In some samples, as the cells darkened they seemed to compact together, as the nucleus and cytoplasm began to shrink. They would then shrivel up, before they finally detached from the surface.

This phenomenon occurred independently in 22.7% of epithelial cultures and, accompanied by fibroblast overgrowth, resulted in loss of the culture in a further 18.2% of cases.

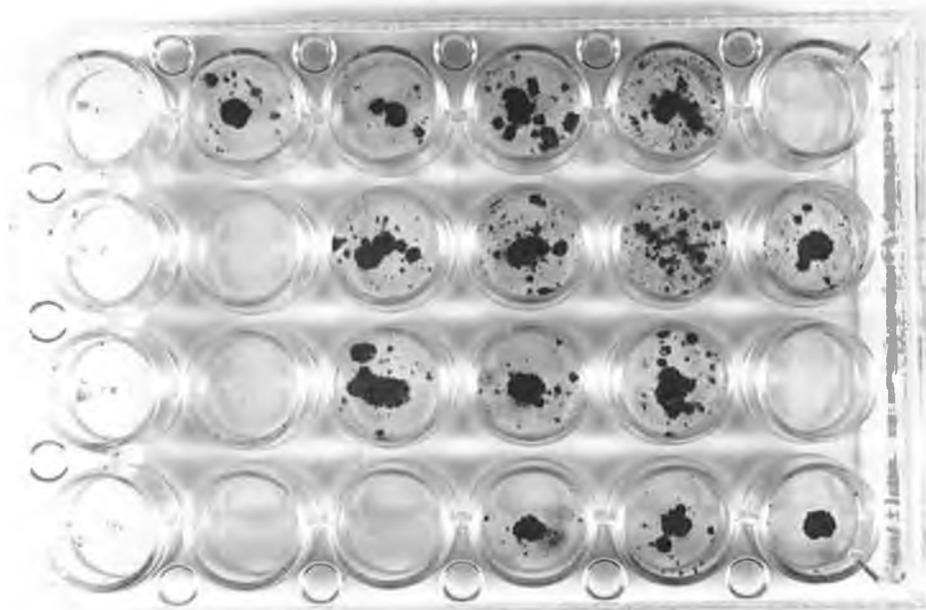
Microbial contamination was usually not a significant problem, as, generally the sample was immediately placed in a universal of culture medium containing antibiotics and fungizone. However, occasionally delays were incurred while the specimen was dissected from the complete biopsy; or was not placed immediately into the sterile collection vessel. In culture, some samples, which did not attach or grow, had evidence of bacterial contamination. One epithelial culture had low level fungal contamination, which was not controlled by fungizone, and it eventually spoiled the culture.

5.11 Effect of cell density on culture proliferation

In the case of 3 samples, a sufficient number of cells were obtained to support investigations into cell seeding density. Cells from the primary cell suspension were seeded directly into 24 well cluster dishes and the extent of growth assessed by image analysis after 7 days in culture (photograph 5.11). The results from this work are represented in Figures 5.11 (a) - 5.11 (c) As the experiments represent growth of cells *directly* from the primary cell suspension,

it must be appreciated that the high SEM value arises from the presence of clumps, for which it was not always possible to obtain a single cell suspension without damaging the cells.

CRYSTAL VIOLET STAINING



J.L. PRIMARY EPITHELIAL COLONIES

EFFECT OF PLATING DIFFERENT CELL DENSITIES

Photograph 5.11: Effect of different cell seeding densities on establishment of a lung primary culture.

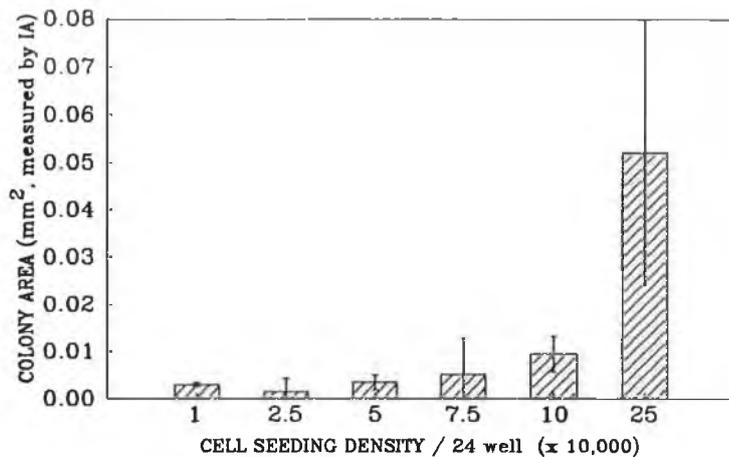
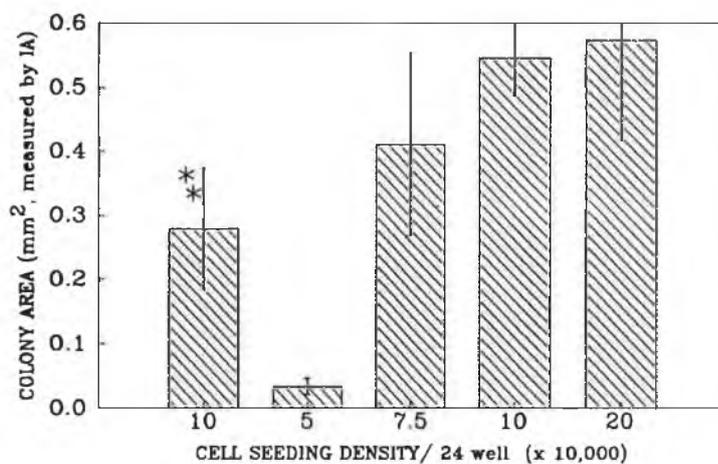


Figure 5.11 (a)



** from CM; others from DM.

Figure 5.11 (b)

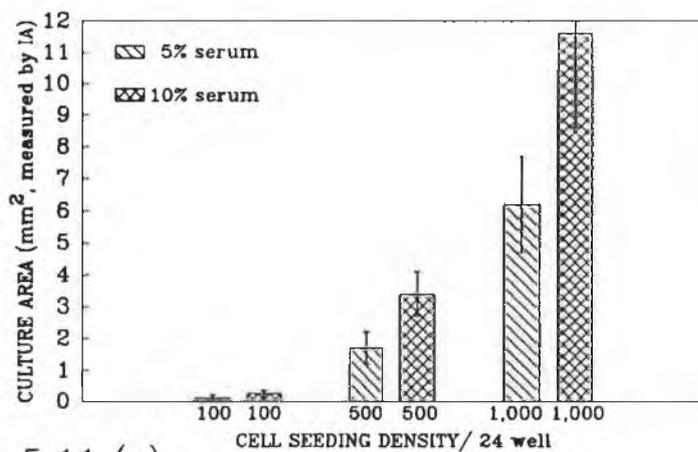


Figure 5.11 (c)

Figures 5.11 (a) – (c):

Effect of cell seeding density on establishment of (a) and (b) lung primary tumour cultures and (c) a lung fibroblast culture.

Figure 5.11 (a) shows that a marked increase in growth is obtained when the cell seeding density is increased from 1×10^5 cells/ well to 2.5×10^5 cells/ well. Densities below 1×10^5 cells/ well do not result in significant cell growth.

Figure 5.11 (b) also illustrates the importance of an adequate seeding density in a sample from a different patient. Two different cell suspensions were prepared for this experiment; suspension ** was isolated from the collection medium and contained many blood cells and cell debris, whilst the remaining cells were isolated from the dissection medium and gave a 'cleaner' cell suspension. We note that two vastly different amounts of growth were obtained from plating a similar number of cells isolated at the two different stages, so we gather from this that the procedure used for cell isolation is extremely important in determining the potential growth of the cells, and also that the purity of the cell suspension (and lack of contaminating blood cells and cell debris) is important in encouraging cell growth. As in the previous experiment, the Figure shows a similarly extended increase when the cell loading is increased from 5×10^4 to 7.5×10^4 cells/ well. Above this seeding density, the increase in growth is less marked. Doubling the starting density from 1×10^5 to 2×10^5 cells per well gives no significant difference in cell growth, so the maximum beneficial cell loading has been reached at between 7.5×10^4 and 1×10^5 cells per well.

The third graph, Figure 5.11 (c), shows the effect of cell seeding density on a lung fibroblast culture (passage 1), in combination with the effect of high and moderate serum levels. Doubling the serum concentration to 10% results in an approximately linear increase in stimulation for different cell loadings. Increasing the cell density from 100 to 500 cells gives an expected increase in growth performance. Doubling the cell density from 5×10^2 to 1×10^3 cells/ well however, results in a corresponding increase in growth which is much more than double. It is obvious that the Fibroblast culture is less density dependant than the epithelial cultures, as significant growth was apparent at a seeding density of only 500 cells/ well.

5.12 Effect of basal media on culture growth

This variable was briefly investigated and some results are shown in Figure 5.12 (a) and 5.12 (b). In graph 5.12 (a), a mixture of Hams F12 and DMEM (1:1), which was routinely used in this laboratory, encouraged less attachment and initial growth than did some of the other media tested. MEM and Hams F12 (1:1) gave significantly better growth. Again the large variations among replicates are attributed to the presence of clumps (since this was a primary cell suspension).

It was observed however that each of the media, or combinations of media tested differentially encouraged epithelial or fibroblast growth to some extent. MEM/Hams F12 encouraged best epithelial growth, with relatively fewer fibroblasts, and Hams F12 alone also encouraged more epithelial than fibroblastic cell growth. At the other end of the scale, RPMI-1640 promoted mostly fibroblastic growth. The remaining media (MEM, DMEM/Hams F12 and DMEM) resulted in mixtures of the two cell types (with relatively few epithelial cell colonies in total).

Figure 5.12 (b) shows that Hams F12 alone (in the presence of a batch of Newborn Foetal Calf Serum (NBCS)) promoted significantly less growth than did a mixture (1:1) of DMEM and Hams F12. With the other serum batch this is less obvious, but still apparent.

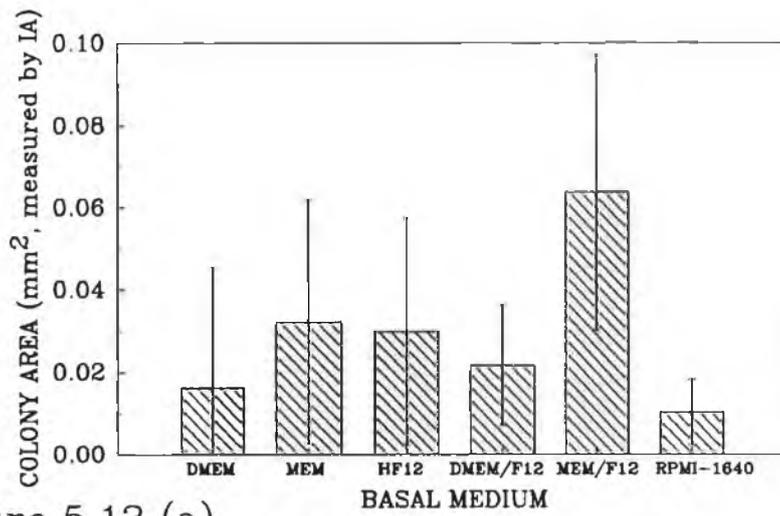


Figure 5.12 (a)

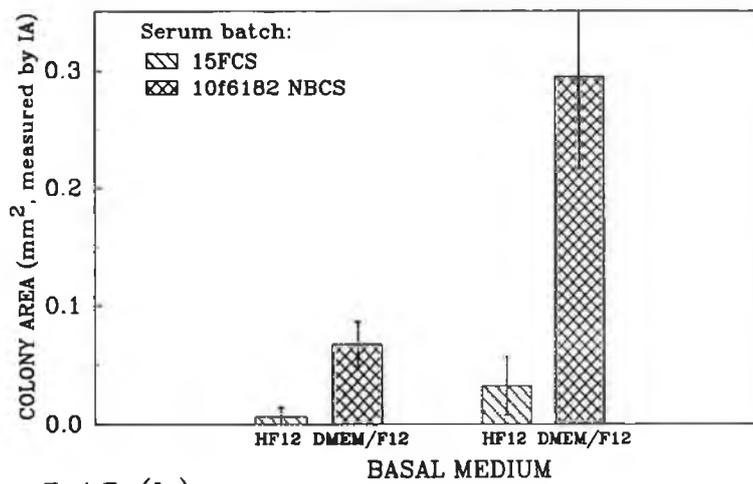


Figure 5.12 (b)

Figures 5.12 (a) and (b):

Effect of different basal media on establishment of two primary lung tumour cultures.

5.13 Effect of different serum batches on culture growth

In addition to Figure 5.12 (c), Figures 5.13 (a) through to 5.13.4 demonstrate the huge variation in growth that different batches of serum can provide. This is particularly obvious in Figure 5.13 (a) where batch 10F Foetal Calf Serum (FCS) promotes 5 times more growth than do batches 15FCS and 10F6182 NBS, while it promotes approximately 33 times more growth than does NuFCS.

This huge stimulatory effect of batch 10F FCS is less marked in two separate experiments with cells from another patient (experiment shown in Figure 5.13 (b) is a preliminary test, that shown in Figure 5.13.3 is a more extensive test from the same patient), however the poor performance of batch NuFCS is also noted for this patient's cells. This may be due to the presence of some growth inhibitor(s) in this serum batch. In the extended experiment (Figures 5.13.3), at the lower serum concentrations of 2% and 5% slightly lower growth is again observed with batch 701 FCS than was observed with the majority of batches. Batches such as 10f6182 NBS and 10G1676 FCS were also identified to be poor batches. As a result of this work, half of the culture stocks from this patient were changed from the general laboratory serum batch (15 FCS) to batch 30F7079 NBS, however all stocks (then in culture for 4 weeks) soon began to terminally differentiate and eventually died off. This was a significant finding because the 30F7079 NBS was much cheaper than the FCS batches.

For many of the serum batches, augmenting the concentration to 10% resulted in substantial stimulation over growth at 2% or 5%, so this was some encouragement for continuing to use the higher serum concentration for routine work, which was already the general practice.

The experiment represented in Figure 5.13.2 was prepared from a primary cell suspension which was quite unhomogeneous due to accompanying blood cells and tiny tissue clumps from which the cells had been expelled (by enzyme treatment); thus the SEM values are particularly high. However, we still observe considerable variation in growth stimulation distinctly arising from different batches of serum. Yet again, Nu FCS was a relatively poor batch, but for batches 10G2368, 30F7075 and 10Q4280 (all NBS), the performance was even worse.

A total of 12 batches of serum were tested on the tumour sample in Figure 5.13.4. The range of growth induced by the different batches was quite remarkable. Batch 33 (DHS) was the worst of this group tested, generating less than one quarter the extent of growth as that seen with batch 41 (FCS). Some of this patient's autologous serum was also tested, and while this promoted good growth, perhaps surprisingly it was not the best of all the batches tested.

Because the dynamics of serum batch testing in this laboratory (which dictated the batches of serum available for testing) were no match for the intermittent and disjointed appearance of a suitable sample for these tests, unfortunately the same batches were not always available to test on successive cultures. However, what the results clearly illustrate, is the benefit of carrying out a screening test to choose an appropriate batch of serum, as the effect of this parameter on culture growth has been shown to be of great significance.

Unfortunately also, this work could not be followed up because of the lack of appropriate samples (large, non-necrotic) available for investigation, and, after an attempt was exerted to obtain appropriate samples from many different sites, lack of time.

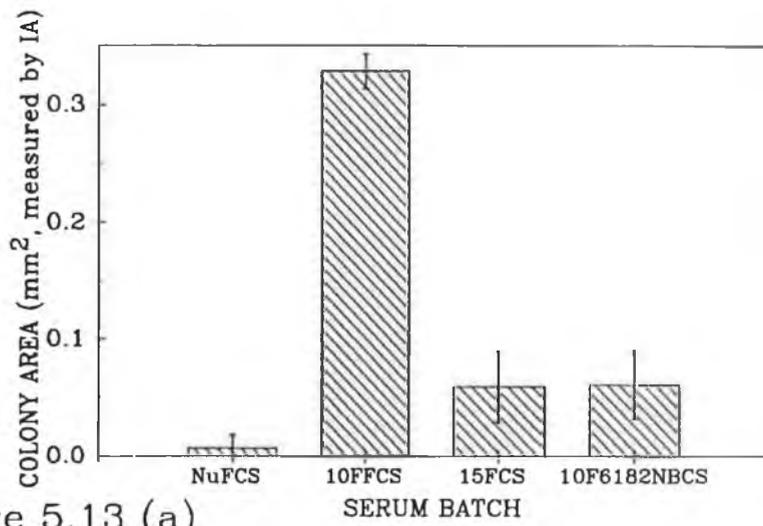


Figure 5.13 (a)

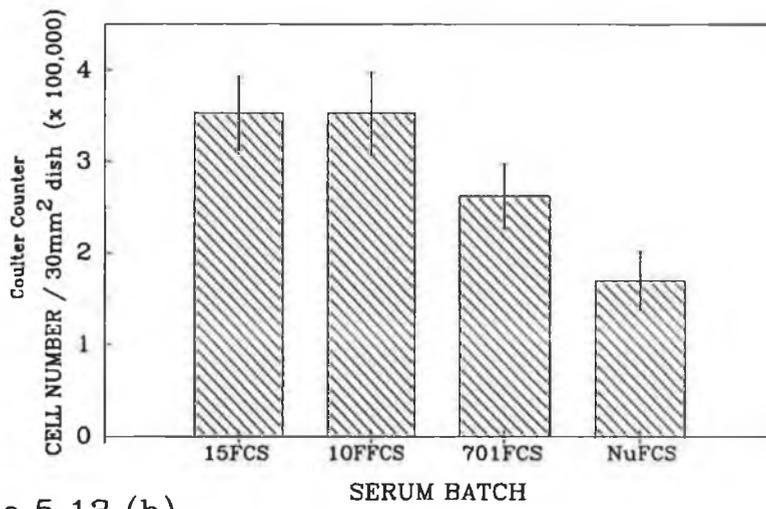


Figure 5.13 (b)

Figures 5.13 (a) and (b):

Effect of different serum batches (10% in medium)
on establishment of two lung tumour primary cultures

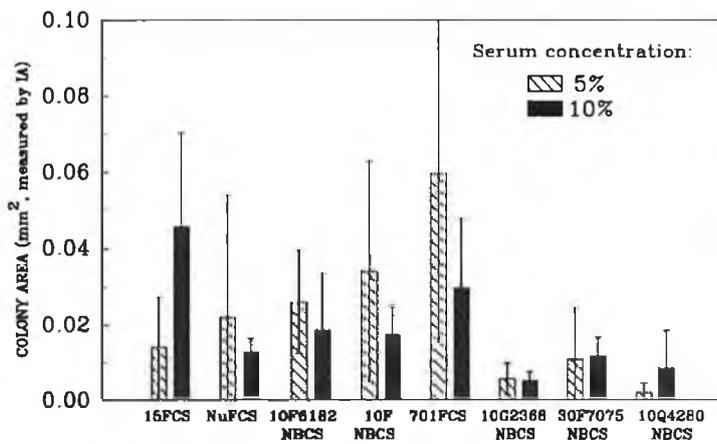


Figure 5.13.2

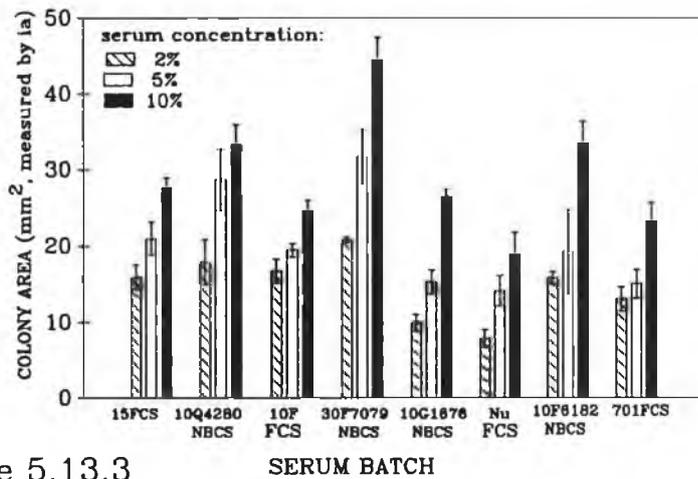


Figure 5.13.3

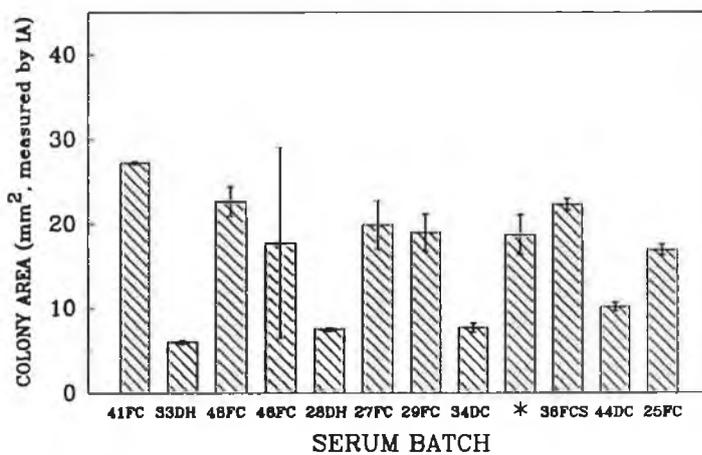


Figure 5.13.4

(* - patient's autologous serum)

Figures 5.13.2 – 5.13.4:

Effect of serum batch variation on growth of a lung primary tumour (5.13.4) and cells from a malignant pleural effusion (5.13.2 and 5.13.3).

5.14 Effect of Growth Factors on culture growth

In the same sample as referred to above, there were sufficient cells to allow preliminary investigation into the effect of some growth factors on the growth of the culture. Figure 5.14 demonstrates the results. Growth resulting from a growth factor cocktail (Transferrin (T), Insulin (I), EGF (E), Selenium (S), and Hydrocortisone (H) to batch 25FCS did not result in a significant increase in growth, however addition of H alone gave a very significant increase (x2) in growth.

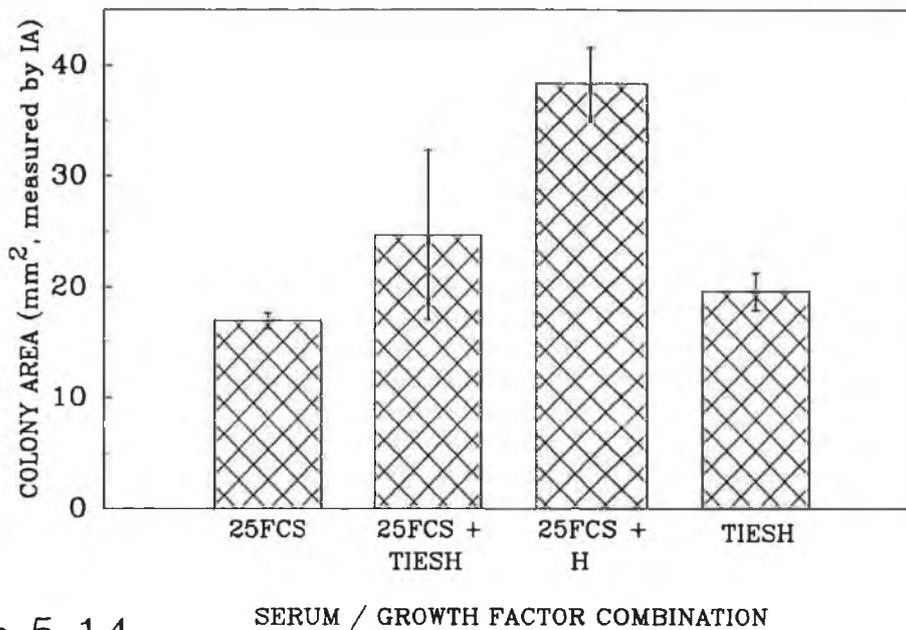


Figure 5.14

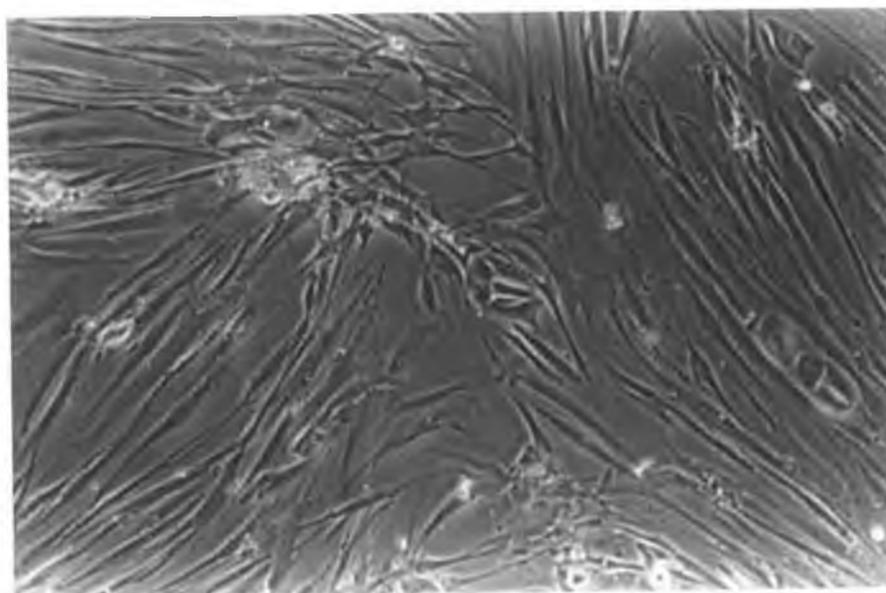
Figure 5.14:

Effect of some growth factors on establishment of a lung tumour primary culture.

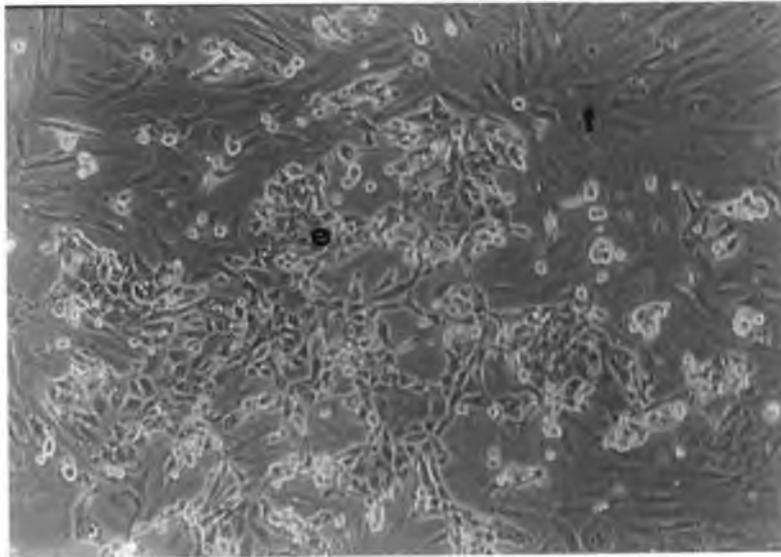
Where: T. = 5 $\mu\text{g/ml}$ Transferrin
 I. = 1 $\mu\text{g/ml}$ Insulin
 E. = 10ng/ml EGF
 S. = 1×10^{-8} M Selenium
 H = 0.4 $\mu\text{g/ml}$ Hydrocortisone

5.15 Significance of fibroblast growth

Table 5.10 has already established the importance of fibroblast overgrowth in epithelial cultures, where more than half of the cultures were finally overcome by fibroblast growth. The fibroblasts grew much more quickly than did the epithelial cells, and so were able to overcome the culture in a very short time. When this happened they spread on top of the epithelial colonies and overwhelmed them. They metabolized the medium very rapidly and were quite unfastidious in their growth requirements. They could readily 'take off' from single cells or small isolated associations of cells (they did not form colonies, but aspired to form regular monolayer formations continually). They generally grew in 'sheets' across the culture (Photograph 5.15 (a)) and growth from even a relatively small initial number was extremely fast. If unchecked, the culture could be completely overcome by these cells in only a few days. Their morphology, in contrast to epithelial cells, are illustrated in Photograph 5.15 (b).



Photograph 5.15 (a): 'Sheet'-like culture-morphology of fibroblasts isolated from a primary lung tumour (magnification x 100).



Photograph 5.15 (b): Mixed culture of epithelial (e) and fibroblast cells (f) from a thoracic primary culture. This culture was soon overcome by fibroblast growth despite removal of fibroblasts with 0.04% EDTA/ 0.01% Trypsin (magnification x 100).

Throughout the life of each of the cultures, fibroblasts were targeted for removal or destruction respectively, by either exposure to Trypsin /EDTA, or Geneticin. Although limited success was achieved periodically, eventually the number of epithelial colonies were outnumbered by the fibroblast monolayer. Photographs 5.15 (a) shows a primary culture which is being overcome by fibroblasts.

5.15.1 Treatment with Trypsin and EDTA

A number of variations on the use of Trypsin/ EDTA solutions were attempted. Three such alternatives and their relative degree of success are outlined below.

Table 5.15.1 Fibroblast removal by Trypsin and EDTA

Procedure	Temperature	Time (mins)	Success Rating
0.02% EDTA	R.T.	5	poor
0.02% EDTA, followed by 0.02% EDTA + 0.01% Trypsin	R.T. 37°C	5 5	poor
0.04% EDTA + 0.01% Trypsin	2°C	10	moderate

Previous work in this laboratory with primary skin carcinomas (S. Mc Donnell, unpublished thesis, 1987) had determined the first two methods outlined in the table to be of use. This work however, showed that no differential effect was achieved as regards removal of fibroblasts without disturbing epithelial cells or vice versa; all of the cells became unattached simultaneously. This may be due to inherent differences between the different tissue types, the epithelial cells from the skin cultures attaching more tightly to the substrata than the lung epithelial cells. This will be considered more fully in the general discussion.

In further attempts to separate the two cell types, the cell suspension was added to a culture flask and the rate of attachment was observed. While it typically appeared that the fibroblasts tended to attach more quickly than the epithelial cells, any attempts to remove the remaining unattached cells and replat them did not meet with success. There was always a sufficient proportion of fibroblasts remaining to ensure an adequate seeding density for subsequent overgrowth in the new flask. Endeavours to separate the two cell types using cell separation media (as described in Materials and Methods) were unsuccessful. Even a small number of contaminating fibroblasts was usually enough to cause overgrowth in the culture once again.

The latter of the three methods shown in the table above, seemed to give better results. Trypsin/ EDTA solution at refrigerator temperature (2°C) and checking the culture very frequently under the microscope, a differential removal effect was often seen. Typically the fibroblasts disattached first. The spindle morphology of the fibroblast was readily attacked by the Trypsin/ EDTA solution and the

polar regions of the cell could be readily seen to peel-off. Often the fibroblast monolayer would remain in a remarkably intact sheet. The epithelial cells, were generally slower to detach.

The success of this method, however, was dependent on the arrangement of the fibroblasts and epithelial cells in culture. If the epithelial cells appeared in tight colonies with 'sheets' of fibroblasts on the extremities of the colony, then the method would be more likely to remove the fibroblasts and leave the epithelial colonies intact. If on the other hand, the epithelial cells appeared as single, tiny colonies, or loose associations of fibroblasts and epithelial cells, then the method was less likely to remove fibroblasts without also removing the epithelial cells.

5.15.2 Treatment with Geneticin

In the latter case, treatment with Geneticin was preferred. Previous work in this laboratory (S. Mc Donnell, 1987; unpublished thesis) indicated highly successful differential toxicity to fibroblasts versus established epithelial cell lines at a concentration of 100 μ g/ml Geneticin in culture medium for 2 days, however; this method was found to be largely unsuccessful in this work where primary epithelial cell cultures, rather than established lines were used. Fibroblasts still remained after the 2 day incubation period and then had to be removed with 0.02% EDTA or 0.04% EDTA/ 0.01% Trypsin. Thus the problem still remained. Thus it seemed pertinent to carry out Geneticin toxicity studies with primary and secondary fibroblast cultures.

Figures 5.15.2 (a) to 5.15.2 (d) show toxicity of some different cultures (A - F) of lung fibroblasts (passage 2 - 3) to different concentrations of Geneticin for different periods of time. The dose/response relationship is clear, and is also clearly related to time of exposure. Generally, 200 to 250 μ g/ml Geneticin for 3 days was sufficient to kill 80 to 100% of fibroblasts. Concentrations and exposure times less than this resulted in correspondingly less cell kill, but the magnitude varied among the different cultures.

Plates were not easily read by IA due to the diffuse growth characteristics, so CV dye was eluted from the 24 well dishes and read as in the CVDE assay.

The variation in efficacy of Geneticin is seen in Figure 5.15.2 (d) as shown in 6 different lung fibroblast cultures (passage 3). thus it is obvious that a single concentration of Geneticin for a specific exposure period may not be universally used on every cell culture. Further work with epithelial samples alone, and then combinations of epithelial and fibroblast cells should suggest a range of concentrations/ times which are most appropriate for eliciting differential toxicity to fibroblasts.

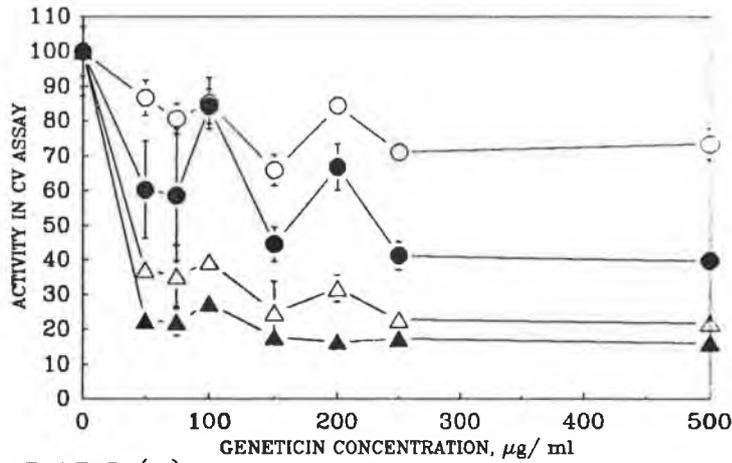


Figure 5.15.2 (a)

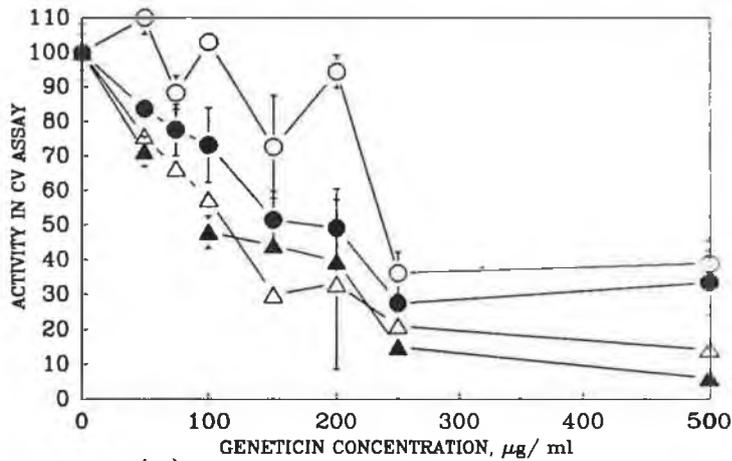


Figure 5.15.2 (b)

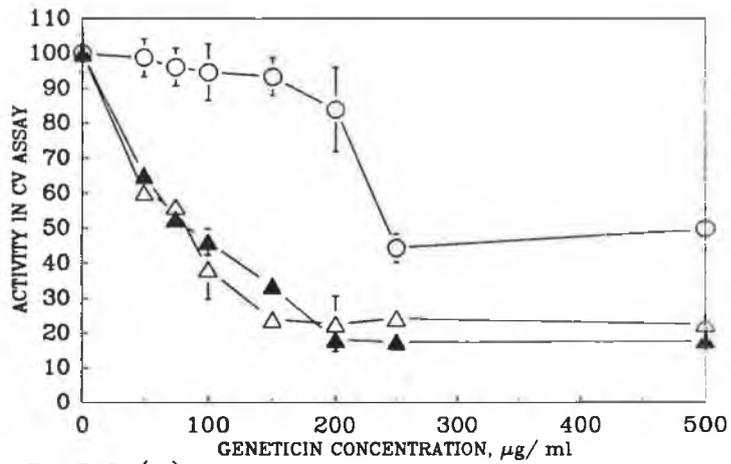


Figure 5.15.2 (c)

Figures 5.15.2 (a) – (c):

Effect of Geneticin treatment on 3 lung fibroblast cultures (p2).

○ = 1 day, ● = 2 days', △ = 3 days', ▲ = 4 days' treatment with Geneticin.

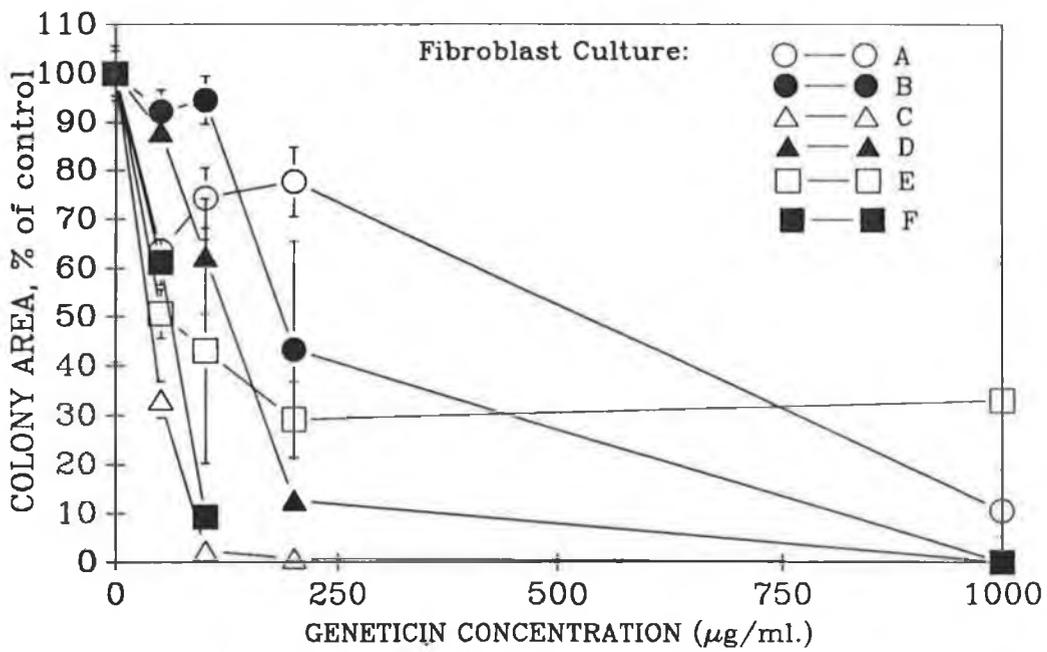


Figure 5.15.2 (d)

Toxicity of Geneticin to a number of lung fibroblast cultures (p3)

Endpoint determined after 2 days' exposure to Geneticin.

DISCUSSION

6.1 In vitro toxicity testing

The relevance of in vitro tests in screening programmes, and the need for their continued development and validation has by now been accepted. This has come from exhaustive interest and research into this area in the last 10 years. Many new in vitro methods have been developed since then, including the NR assay developed in the Rockefeller institute for the Revlon cosmetic company. Many other methods have been extensively validated against animal studies and against in vitro tests already established. In selecting a system for use as an in vitro toxicity test, one must question very closely the characteristics which must be expressed for evaluation of the toxic insult. If the effect of differentiated functions is to be measured, then the system chosen must clearly express these differentiated functions in the absence of the test chemical. If a chemical is preferentially toxic to the liver, then the tissue used should be liver. Additionally, there is little point in developing a model system involving the use of a tissue that is not sensitive to the test chemical. Thus one must choose a system (and of course a relevant endpoint) which is capable of detecting the desired effect, whether it be carcinogenicity, mutagenicity, teratogenicity, irritancy, modification of a particular organ function, or more broadly, cytotoxicity. In essence, for most studies, some knowledge of the mechanism of action and the target organ toxicity is preferred before embarking on a toxicity testing schedule. In attempting to elucidate the mechanism of action of chemicals, both in vivo and in vitro tests have been of use in the past.

6.2 Use of cell cultures for cytotoxicity testing

For general cytotoxicity screening, cell cultures (both established and freshly isolated cells) have been widely used. Cells used have been from very many species, from different tissues, and from histologically different cell types. While the complexity of biochemical functions evident in organ cultures may not be evident in cell cultures, the relative simplicity involved in using cell cultures together with the much improved reproducibility, offer important advantages over other more complex in vitro systems. An important impetus to using animal cell cultures as opposed to other commonly used toxicity testing systems such as algae, spermatozoa,

and invertebrate tests, is the possibility of using human derived material, which diminishes the uncertainty of extrapolation from other species back to humans. This uncertainty, when extrapolating results from other mammals such as rats and mice to potential effects in humans, has already been introduced, and encompasses general biological differences as well as differences in biotransformation abilities. These differences in biotransformation abilities have been described [Sipes and Gandolfi, 1986] as quantitative or qualitative; the former resulting from differences in enzyme levels or natural inhibitors which might adjust the balance of reverse enzyme reactions or the extent of competing reactions, thought to be restricted to phase I reactions; while the latter are apparently related to phase II reactions. Variability in phase I reactions are explained by differences in profiles of cytochrome P-450 isoenzymes, while those in phase II reactions are associated with defective enzymes in certain species, unique species reactions and other evolutionary developments. Quantitative variations are more apparent and result from differences in the animals' ability to synthesize and (or) activate the cofactor, transferase and conjugating agent involved, and from differences in the nature of the xenobiotic itself.

To illustrate this point, some differences in a number of bioactivation reactions from different species are shown in the table below, which has been modified [Sipes and Gandolfi, 1986] from Jakoby [1980].

Table 6.2 Species defects in xenobiotic metabolism

Reaction	Defective species
Aliphatic amine N- hydroxylation	Rat, marmoset
Arylacetamide N- hydroxylation	Guinea pig
Arylamine N- acetylation	Dog
Glucuronidation	Cat, lion, lynx, Gunn rat
Sulfation	Pig, opossum, brachymorphic mice
Hippuric acid formation	African fruit bat
Mercapturic acid formation	Guinea pig

From this we must appreciate that considerable differences may exist in the biotransformation abilities among different species of mammals, which may result in a metabolite more or less toxic than that formed in humans. If such variability exists among mammals, even more variation would be expected when invertebrates are compared to mammals, and more specifically to humans.

6.3 Selection of cell type for cytotoxicity testing

The number and diversity of tissues or cell types which can now be successfully cultured is most extensive, and the choice of tissue will to some extent be dictated by what is available to the investigator. Cell banks such as the American Type Culture Collection (ATCC) supply an extensive range of established cell lines from different species, including a large number of human tumour cell lines, and cells from a large variety of tissues.

Selection of a target cell line for cytotoxicity evaluation from a range of different tissues may appear a daunting task. Although the growth characteristics of the cell line will have to be considered (e.g., monolayer culture or suspension culture, rapid doubling time, high cloning efficiency etc.,) cells from the organ/ tissue which is most sensitive to the chemical should be chosen. All human cells have similar basal functions [Ekwall, 1983] but organ-specific functions which vary among different cell types may be necessary for certain studies, so a cell line expressing these functions may be selected with this in mind. In areas where little is known of the mechanism of action of the test chemical, it may be prudent to use two cell lines of differing cell type, e.g., fibroblasts and hepatocytes. It may be of some assurance to the investigator in such a dilemma, that in work by Babich *et al.*, [1987], the rank order of sensitivity of the chemicals to the cell lines remained the same, even though differences in sensitivity of cell lines from different tissues were apparent in the cytotoxicity testing of a number of polycyclic aromatic hydrocarbons.

6.4 In vitro biotransformation of xenobiotics

One of the biggest problems with using cell cultures as opposed to intact organisms or organs is the loss of biotransformation ability when cells are cultured. Although some cell lines have been reported to maintain some mixed function oxidase and other enzyme activity, in general this faculty deteriorates quickly during culture. Some established cell lines reported to have functional drug metabolizing activity are A-549 [Wibel *et al.*, 1980], alveolar type II cells [Devereux and Fouts, 1980] and NCI-H322 and NCI-H358 [Falzon *et al.*, 1986]. Primary hepatocytes have been reported to retain their drug metabolizing activity for limited periods. Acosta *et al.*, [1978] have maintained differentiated liver functions for as long as 20 days and Gomes-Lechon *et al.*, [1988] have found the use of hepatocytes maintained in culture (as opposed to isolated hepatocytes) to be a good model for cytotoxicity testing, as they can allow the design of experiments with longer xenobiotic exposure times. However, it has long been known that the cytochrome P-450 content of liver-derived

cell cultures can be quite different from that of liver in vivo, and cautions have been given with respect to extrapolations between these two systems [Owens and Nebert, 1975].

Other methods of addressing the problem of biotransformation when using such metabolically incompetent cell lines are as follows:

* Prior induction of cell lines to augment enzyme levels before drug exposure (chemicals such as 5-6-bensoflavone, phenobarbital, benz(a)anthracene or pregnenolone-16 α -carbonitrile have been used for this purpose [Swierenga *et al.*, 1983])

* Addition of crude tissue (usually liver) homogenate, supernatant from centrifugation of crude homogenate (S-9 fraction), or a microsomal preparation consisting of S-9, NADPH and glucose-6 phosphate.

- obviously the metabolic properties of these various subcellular systems will not be identical; loss of soluble glutathione transferases, sulfotransferases and cofactors are evident with the microsomal preparation [Dipple and Bigger, 1983]

Problems with toxicity in the absence of the test chemical have been encountered when using microsomal preparations, and thus exposure times with addition of S-9 are short (often in the order of 3 to 5 hours) whereas comparative experiments using freshly isolated or cryopreserved hepatocytes have used exposure times up to 2 days [Swierenga, *et al.*, 1983]. It is imperative therefore to use the necessary and relevant controls for experiments including microsomal preparations.

* Co-culturing the target cells with cells of greater drug metabolizing ability, often hepatocytes [Langenbach *et al.*, 1978].

Comparison of toxicological data among laboratories even where a broadly similar activating system has been used, must be carried out with caution. Differences in species, genetic strain, age, weight, sex, diet and nutritional status of the animal used to prepare the

microsomal preparation, all serve to introduce variability into the performance of the preparation, and thus may alter results quite significantly. In fact significant inter-individual variability of activity and induction of cytochrome P-450 and UDP-glucuronosyl transferases has been identified [J.P. Cano 1990, personal communication] in human liver microsomes and primary cultures of human hepatocytes. Whether or not the animal has been induced prior to isolation of the tissue for its microsomal fraction, and the actual inducing agent involved, can measurably affect the results obtained.

Inducing agents may be drugs, chemicals or natural compounds such as ethanol. The most widely studied are phenobarbital and the polycyclic aromatic hydrocarbons (benzo(a)pyrene and 3-methylcholanthrene). Although it was first thought that it was only the cytochrome P-450 dependent monooxygenases which could be induced, it is now known that certain conjugating enzymes can also be induced [Sipes and Gandolfi, 1986]. Although the enzymes or isoenzymes induced by different chemicals may be similar, the morphologic and biochemical effects in the liver can be different. Thus the choice of an inducer for biotransformation is another variable which must be taken into account; it may be that the one chosen for use does not affect a particular reaction, so this must not be presumed for any inducer. This is clearly exemplified when some characteristics of phenobarbital and polycyclic hydrocarbons are compared in the table below.

Table 6.4 **Characteristics of some hepatic effects of**
phenobarbital and polycyclic aromatic hydrocarbons

Characteristic	Phenobarbital	Polycyclic hydrocarbons
Onset of effects	8 - 12 hours	3 - 6 hours
Time of maximum effect	3 - 5 days	24 - 48 hours
Persistence of induction	5 - 7 days	5 - 12 days
Liver enlargement	marked	none
Protein synthesis	marked increase	small increase
Enzyme components:		
Cytochrome P-450	increase	no effect
Cytochrome P-488	no effect	increase
NADPH-cytochrome-c reductase	increase	no effect
Substrate specificity:		
N-demethylation	increase	no effect
Reductive dehalogenation	increase	no effect
Glutathione conjugation	small increase	small increase
Cytosolic receptor	none identified	identified

modified from Sipes and Gandolfi [1986].

Other major classes of inducing agents include halogenated pesticides (DDT, aldrin, hexachlorobenzene, robenzene, lindane, chordane); polychlorinated and polybrominated biphenyls; steroids and related compounds and chlorinated dioxins (2,3,7,8-tetrachlorodibenzo-p-dioxin

(TCDD)). Some of these produce a spectrum of induction similar to phenobarbital or the polycyclic aromatic hydrocarbons, whereas some induce the synthesis of other forms of cytochrome P-450.

Use of different concentrations of S-9 fraction in the S-9 mix, and different co-factors, may also result in qualitative as well as quantitative differences in results, so this should also be given careful consideration.

It would appear, therefore that an investigator should choose the cells and culture system with due consideration of the variabilities discussed, optimize the conditions if necessary, and stick to this standard procedure as tightly as possible in subsequent and comparative work.

6.5 Choice of end point for cytotoxicity testing

This then brings us to discuss the choice of endpoint for the cytotoxicity system.

Many different end points were considered in the opening section of this thesis. While there will be advantages and disadvantages to any endpoint, and in theory some end points are more applicable to certain types of experiments, there are often severe technical disadvantages which preclude their use.

Measurement of a very specific or differentiated cell function or pathway may be used to assess the effect of xenobiotics on cells. Certain cellular functions affected by the chemical may not cause manifestation of cell death [Gomez-Lechon *et al.*, 1988]. However the relevance of these sort of effects must be questioned for the particular investigation involved; most often it is only the direct cytotoxic effects on the cells which it is necessary to measure. Generally then, such effects will ultimately be manifested in cellular death; and in cytotoxicity determinations, the most commonly used measure of toxicity is impairment of cell growth.

In determination of impairment of cell growth a distinction must be made between cytostatic and cytotoxic effects. Cytostatic effects may only affect the growth rate of the cell, or may reversibly damage the

cell, resulting in only a temporary impairment of cell growth. Cytotoxic effects on the other hand result in irreversible damage to the cell. The question of the ability of different assays to distinguish between these effects and its relevance will be addressed later.

How can we measure cell growth? Cell growth is an increase in biomass (cell number x mean cell mass) and can be achieved by an increase in either of these individual components. In practice, regulatory factors keep the cell mass within strongly defined limits, so 'growth' mainly refers to proliferation; *i.e.*, an increase in cell number. To measure inhibition of growth induced by cytotoxic agents, we can use methods of direct cell counting by haemocytometer. Electronic particle counters are not able to distinguish between viable and non-viable cells, and are thus of no value in this sort of assessment. Dye exclusion and inclusion methods can be used to distinguish viable from non-viable cells, so these methods [Bhuyan *et al.*, 1976; Funa *et al.*, 1986; Hartman and Riech, 1989; Weisenthal *et al.*, 1983a; Detta and Hitchcock, 1990] have been incorporated with haemocytometer and flow cytometer measurements. These and other methods (*e.g.*, cytoplasmic leakage of radiolabelled molecules and enzymes) are based on detection of membrane integrity, and thus may not always be true indicators of cell viability.

Many methods have been used for measurement of cellular viability, but the critical parameter in relation to cell viability, is retention of the replicative potential of a cell. Viable cells have the ability to self propagate, whereas dead cells have lost this ability. Thus if we place cells in an environment conducive to cell replication, and they form colonies, then there can be no doubt as to their viability. However this method is not without theoretical problems, as conditions may not always be optimal for cellular colony formation, and so absence of colonies after re-growth might be due to this factor, and not in fact to loss of viability. In cytotoxicity testing, use of a relevant control can overcome this problem and so colony formation efficiency has been widely used for cytotoxicity testing, particularly for chemosensitivity testing. On closer scrutiny of assays for clonogenicity, there appear to be a number of problems. Problems with

low plating efficiencies [Hill and Wheelan, 1983; Selby *et al.*, 1983], unsuitability of some tumour types [Von Hoff *et al.*, 1981, Selby *et al.*, 1983], clumping of cells (leading to false negative results) [Agrez *et al.*, 1982; Rockwell, 1984], requirement of large numbers of cells, long assay duration (typically 14 - 21 days), extensive time taken to evaluate the result (manual microscopic counting); all served to reduce the applicability of the assay.

Other measures of growth such as measurement of synthesis of cellular macromolecules (protein, DNA, RNA) and amino acids have been used as end points for cellular survival but these assays are generally tedious to carry out and many involve the use of radioisotopes which has associated hazards. In cytotoxicity screening programmes, the requirements for an appropriate end points are as follows:

- * fast
- * reliable
- * accurate
- * sensitive
- * simple
- * requiring only small numbers of cells, and amounts of reagents
- * non hazardous
- * applicable to a wide range of cell types
- * not dependent on obtaining a single cell suspension
- * technically easy to quantitate

Many if not all of these requirements are met by semi-automated, miniaturized, colorimetric assays. Some of these methods have been used for toxicity testing to a greater extent than others. The MTT assay for example [Mossman, 1983], and the Neutral Red assay [Borenfreund and Puerner, 1984] have been widely used for cytotoxicological studies, however, there have been few studies comparing the characteristics of these different assays and their potential applicability to different situations.

This work aimed to carry out an extensive study of 5 of such assays, in an attempt to distinguish any differences in their performance and perhaps in their relevance for some current and potential applications.

6.6 Image analysis as a method for growth analysis

Computerized analysis of stained cells is becoming more prominent in determination of growth and cytotoxicity. Baker *et al.*, [1986] and Rotman *et al.*, [1989] have successfully used computerized image processing to determine cellular viability and cell kill. Some techniques are now able to assess damage to individual cells [Tillmann *et al.*, 1989]. In using image analysers to such an end it is possible to obtain readings for cell number and colony area. It is important that the most relevant of these parameters be used for the investigation. It is possible that cell and colony size will be altered when grown under different conditions, and when exposed to different drugs and mitogens; Jabbar *et al.*, [1989] for example, found that cell size increased in cultures treated with interferon. Thus in comparing colony size from test wells to control wells, erroneous results may be obtained. Good resolution may not be obtained if colonies are very close together or partially joined - certainly this was a problem with the instrument (AMS 40-10) used in this work, but other more sophisticated (and expensive) models are available. Other technical problems encountered when using this instrument included inability to properly analyse some cell lines, a critical requirement for evenly spread (and evenly stained) cultures, and problems when using the same settings for a range of growth. An additional disadvantage to this method is that relatively large numbers of cells are required, as assays are carried out in 24 well dishes.

In comparison to miniaturized, semiautomated, colorimetric assays, Image Analysis does not compare favourably as a method for quantification of cell growth, particularly if cell lines are to be tested which do not form tight, discrete colonies, if available cell number is low, if ranges of cell growth are to be analysed, and if the laboratory is to be equipped with only basic or low cost instrumentation.

6.7 Optimization of the MTT assay

Since its development in a miniaturized, semiautomated form, the MTT assay has been very widely used, not only for chemosensitivity testing but for other areas of cytotoxicity testing as well as growth studies (e.g., growth factor stimulation [Nakanishi et al., 1988]). Section 3.2.1 described the optimization of the assay for use with RPMI-2650 and Hep-2 cell lines. Use of DMSO [Plumb and Freshney, 1989, personal communication] was found superior to acid - isopropanol - the solvent used in the original paper [Mossmann, 1983], and this finding agreed with that reported by Alley et al., [1986]. Addition of acid isopropanol resulted in the formation of a dense precipitate, even after medium removal, and this was felt to be caused by acid denaturation of residual serum proteins from the medium. This did not occur when DMSO was used as a solvent. Carmichael et al., [1987] found use of mineral oil also acceptable as a solvent, but in comparative experiments, Twentyman and Luscombe [1987] preferred DMSO to either acid propanol or mineral oil, and reported that even the 10-20 μ l of medium remaining per well greatly impeded solubilization of formazan when using the latter two solvents.

Optimal concentration of MTT (20 μ l/ well) for use was found to be between 4 and 5 mg/ml for RPMI-2650, and between 3 and 7 mg/ml for Hep-2 (section 3.2.1.2) so 20 μ l of 5mg/ml MTT was selected for routine use (0.1mg/well); this concentration has been widely found to be optimal for many cell lines [Twentyman and Luscombe, 1987; Edmonson et al., 1988; Scudiero et al., 1988; and others].

Differences of opinion as to the stability of MTT stock solution is evident in the literature. Many investigators use freshly prepared and filtered MTT stock [Leprince et al., 1989], but other investigators have found it acceptable to use stocks which have not been freshly prepared. Pieters et al., [1988] used frozen (-20 $^{\circ}$ C) stock; Green et al., [1984] reported stability of the stock solution at 4 $^{\circ}$ C in a darkened tube for at least several days, while under these conditions it was considered acceptable for use when stored for a maximum of 3 weeks [Ford et al., 1989], 1 month [Alley et al., 1988;

Mickisch, 1990], 'more than a month' [Tada *et al.*, 1986]; 1 -1½ months [Kotnik and Fleischmann, 1990 and at least 6 weeks [Twentyman and Luscombe, 1987].

The ability to store the solution for some length of time would be an advantage, as preparation of this solution, and filtering through a 0.45µm filter immediately prior to use, introduces an extra measure of cumbersome to the procedure. This is particularly so because the compound is mutagenic, and thus requires special handling procedures. The results of an experiment described in this thesis show no significant loss in activity after 8 weeks storage of the solution at 4°C in the dark (Figure 3.2.1 (c)).

6.8 Relationship of cell number with OD in MTT assay

Succinate dehydrogenase, an enzyme involved in MTT reduction to formazan, is part of the Trichloroacetic cycle, where it catalyses the oxidation of succinate to fumarate. Its reducible coenzyme FAD functions as a hydrogen acceptor in this reaction [Lenninger, 1970]. The reduced enzyme can then donate electrons to various artificial electron acceptors, *e.g.*, tetrazolium salts and other reducible dyes, whereupon they undergo a change in their absorption spectrum (MTT changes from yellow to purple). The reaction involved in conversion of MTT tetrazolium salt to formazan can be seen in appendix C. Tetrazolium salts have long been used to quantitate cellular reductive capacity [Vistica *et al.*, 1991] and to approximate cell growth.

The Figures shown in 3.2.2, illustrate a linear relationship of cell number and MTT reduction (OD at 570nm), for every cell line tested under different culture conditions (1 and 4 days growth), the range of which varies for the different graphs. Similar relationships have been shown by many other workers for many cell lines [Mossman, 1983; Green *et al.*, 1984; Gerlier and Thomasset, 1986; Carmichael *et al.*, 1987; Edmonson *et al.*, 1988; Romijin *et al.*, 1988; Mc Hale and Mc Hale, 1988; Campling *et al.*, 1988,]. The most significant work on this area [Alley *et al.*, 1988] (as part of the NCI feasibility assessment for preclinical screening of anticancer drugs) demonstrated suitable colorimetric profiles of cell number and OD, for 30 lung cell lines and 76 lines from other sources. Linear relationships of cell

number and OD have also been shown for primary cell suspensions of tumour cells [Pieters *et al.*, 1988; Sargent and Taylor; 1989, Shimoyama *et al.*, 1989; Twentyman *et al.*, 1989; Mickisch *et al.*, 1990; Wilson *et al.*, 1990; Campling *et al.*, 1991].

Sensitivity has also varied greatly among cell lines. Such differences in extent of linearity and sensitivity among cell lines, have been obtained in much of the work referenced above. Ford *et al.*, [1989] reported that the individual variation around the ability of cell lines to metabolize MTT can be considerable, and in this work, a non-linear relationship of cell number and OD was obtained for some cell lines (while the relationship was linear with a uridine incorporation assay).

In comparing the performance of the assay for NRK and Hep-2 cells, we see that there has been greater sensitivity for NRK, but a shorter linear range. This observation has not been limited to MTT, but is seen for each of the other assays. In considering why this may be so, we must examine the growth characteristics of the different cell lines.

The relationship of cell number and OD, which determines differences in linearity and sensitivity, is obviously related to growth. Cells actively growing will reduce the MTT to a greater extent than cells which are not. This has been shown by Mossman [1983] and others. In a recent investigation of selected parameters affecting formazan production, prompted by differences in MTT reduction among cell lines [Vistica *et al.*, 1991], MTT reduction in cell lines was found to correlate with the D-glucose content of the medium. MTT reduction in cells lines which metabolized D-glucose extensively (SN12K1, HOP 62, and HT 29; with doubling times of 18, 29 and 32 hours respectively) was more extensive than in other cell lines.

Differences in growth characteristics (*e.g.*, contact inhibition of cells such as NRK) as well as inherent differences in activity of MTT reducing enzymes among cell lines will also lead to differences in sensitivity of the assay. This may be due to differences in enzyme levels among cell lines, and/ or differences in the specific activity of such enzymes.

Overall the linear range of the assay was considerable, but the sensitivity of the assay was felt to be quite low, and particularly so for some cell lines (e.g., RPMI-2650). While in general, workers report good sensitivity of this assay, there have also been reports of low sensitivity [Carmichael *et al.*, 1978, Alley *et al.*, 1988; Ford *et al.*, 1989], and there have been some recommendations that the assay be further optimized and modified to increase sensitivity. Alley *et al.* [1988] identified a number of cell lines (several fibroblasts and certain Small Cell Lung Carcinomas) for which he considered the assay to be relatively insensitive. In some cases low sensitivity has been attributed to technical difficulties (e.g., cell and formazan crystal loss during washing and aspiration) [Ford *et al.*, 1989].

The results from this work showed clearly that that the assay suffered from low sensitivity which must be greatly improved if it is to be of widespread use in cytotoxicity testing. This may be brought about by increased incubation time, as found beneficial in some work - Alley *et al.*, [1988] used incubation times up to 8 hours for problem cell lines with some improvement in sensitivity. Use of a reference wavelength of 690nm, rather than the 620 nm filter used here and in other work [Twentyman and Luscombe, 1987], has been reported to increase sensitivity [Denizot and Lang, 1986]. Exclusive use of spectrophotometric grades of DMSO has been reported to produce stable 'background' absorbance levels for up to 2 hours following solvent application, whereas use of nonspectrophotometric DMSO preparations or DMSO preexposed to air are accompanied by ever-increasing levels of 'background' absorbance [Alley *et al.*, 1988], and will thus lessen sensitivity. The wavelength used for colour quantification has varied, and while 570nm, (the filter used in this work) has been found acceptable by other workers [Wilson *et al.*, 1990], the absorption maximum for MTT in DMSO is at 553nm (570nm in acid - isopropanol); so, although many workers resort to using the nearest fixed filter available, (as was the case in this work), it may be necessary if not critical, in the light of this low sensitivity, to use the specific filter for the appropriate solvent being used.

The sensitivity of the MTT/SDS assay was found to be extremely poor, much worse in fact than the usual method. This is contrary to that described by others [Tada *et al.*, 1986; Eliason *et al.*, 1990], who found similar sensitivity for the method using SDS as the solvent. In

such work, cell numbers (KB-8-5 cells; MDR cervical carcinoma) less than 100/ well were capable of being detected, even when the wavelength used was 540nm [Eliason *et al.*, 1990] (when the optimum for SDS was shown to be 570nm [Tada *et al.*, 1986]). It is possible that this significant discrepancy in sensitivity may be explained by technical errors involved in this work; however all critical points (such as critical pH dependence for dissolving formazan and maintaining colour) were addressed and followed. It is unlikely that this huge difference in sensitivity can be accounted for by differential MTT reduction among cell lines (between the cell lines used in the published work, and those used here). Other workers, such as Nikis and Otto [1990] reported low solubilizing power and colour stability using this method, and their results indicated poor sensitivity, similar to that reported here.

Due to the poor sensitivity of the MTT assay determined by this work, in future work it is recommended that the factors discussed here which may effect improvement in assay sensitivity, are considered and adopted if found useful.

6.9 Technical problems of the MTT assay and applicability to different cell lines

Some of the work published in the last 4 years has reported significant technical difficulties with the assay. Many of them relate to the use of acid - isopropanol as a solvent. Some investigators (Ford *et al.*, 1989, for example) observed fewer difficulties when they switched to using DMSO. Nikis and Otto [1990] have recognised the need (experienced in this work) for optimizing the MTT assay. They recount technical problems with formazan solubilization, and compare various modifications made by different workers. DMF-SDS solubilization buffer (Hanson *et al.*, 1989) was found to be superior to other buffers, but it was laborious, as manual mixing or overnight incubation is required to achieve complete solubilization. Sonication has been suggested as a measure to relieve this.

Other difficulties are low optical densities, and thus low sensitivity. Investigators using a washing step report the loss of cells and formazan crystals (this step also increases the time involved for the assay), but if no such step is involved, residual medium can interfere with the absorption spectrum of MTT in DMSO and cause a drift in it. Therefore complete removal of residual medium is preferred. The procedure used in most of the work described, is to invert and 'flick' the plate, to assist rapid medium removal. This eliminates the need for medium removal with a pipette and is a time and labour saving step, however, it was found throughout the duration of this work that such a step resulted in loss of formazan crystals. Use of an automatic platewasher did not relieve this situation. Some papers report the need for 'careful' removal of medium, but on the whole, the difficulty has not been extensively aired. I feel that this is a significant technical difficulty with the assay, and one which needs to be addressed if the assay is to be improved.

Other reported difficulties are primarily related to 'problem cell lines'; those producing low MTT reduction and giving particularly low sensitivity [Alley *et al.*, 1988], and floating cell lines. Although centrifugation is used to prevent cell loss, it is still considered a problem. Practical experience of flicking plates with non-adherent cells to effect medium removal in this work, even after centrifugation (and use of poly-lysine coated plates), was still a problem after careful formazan removal with a pipette, and resulted in extensive cell and formazan loss.

Difficulties due to chemical reduction of MTT by chemicals present in the medium; or reduction by contaminating microorganisms has also been reported.

Overall the assay has been reported to be useful for growth determinations on many, many cell lines [Alley *et al.*, 1988 has shown its suitability for 106 cell lines; others have shown this for other lines]. Cells not determined as suitable (due to low sensitivity) have been mostly floating cell lines (Small Cell Lung Carcinomas), but also some fibroblasts.

6.10 Relationship between cell number and OD in CVDE assay

In optimizing cell densities for L-M cells in a bioassay for Tumor Necrosis Factor, Kramer and Carver [1986] showed resultant OD values over a short range of cell densities (3×10^4 to 6×10^4 cells/well) which was linear under some conditions (M199/0.5% Peepitone) and non linear under different conditions (EMEM/5% FBS). Work by Bonnekoh *et al.*, [1989] illustrated the increased uptake of CV from a cell culture, with time, but did not specifically measure the relationship between cell number and dye uptake. Indeed many other workers have used this method for growth related assays, but few have examined this relationship.

The relationship between cell number and crystal violet uptake shown in Figures 3.4.2 (a) - (m) for the panel of cell lines used in this study, was again found to be linear at least over some part of the range of cell numbers tested for every cell line. At high cell densities, the relationship deviated from linearity substantially (see graphs 3.4.2 (j) and 3.4.2 (k)), while the relationship remained linear for other assays at these cell numbers (see e.g., graph 3.7.2 (l)). As with each of the other assays, the linearity and sensitivity of the assay varied among cell lines.

In an extensive study by Skehen *et al.*, [1990] comparing 21 dyes for quantitative assessment of cell number, 13 were found to be acceptable for this use; Crystal Violet was not one of them. It, along with azure C, Coomassie brilliant blue, ethidium bromide, methyl green, naphthol yellow S, propidium iodide and pyronin B, either stained cells too lightly to be useful or stained different cells with widely varying intensity. In another comparative study of a number of dyes, Scragg and Ferreira [1991] compared the sensitivity of 4 dyes to fibroblast cells, and found Coomassie brilliant blue to be the least sensitive, followed by Methylene blue, Crystal violet and Toluidine blue. Loss of linearity at 8×10^4 cells/96 well was found with CV and Toluidine blue. Coomassie blue was found preferable because of its moderate sensitivity, increased linearity, and low interexperimental variation relative to the other methods. The staining intensity observed here was found to be much improved over

the MTT, AP2h and NR assays, and variations in activity among cell lines was found to be no worse in the CVDE assay, than for these assays.

Kueng et al., [1989] found a linear correlation of cell number and OD for the CVDE assay with sensitivity to $\leq 2 \times 10^3$ cells. Sensitivity decreased when the pH of the dye solution was decreased from pH 6 downwards. This figure correlates well with figures obtained in this work, where 2×10^3 SCC-9 cells were detected, 1×10^3 Hep-2, SK-MES-1 and RPMI-2650 cells were detected, and as few as 500 NRK and DLKP cells were detected. Kueng and colleagues found a significant decrease in OD when cells were not dried prior to staining - wet cells took up less dye than dried cells.

6.11 Technical problems of the CVDE assay and applicability to a range of cell lines

No significant technical problems were encountered with the assay- it was simple to perform, and it was equally applicable to all of the cell lines tested.

6.12 Relationship between cell number and OD in the NR assay

The NR assay, semiautomated and miniaturized by Borenfreund and Puerner [1984] is based on the incorporation, by lysosomes in living mammalian cells, of this supravital dye [Zhang et al., 1990]. Dead cells however, have been stained (albeit more diffusely) by NR [Allison and Young, 1973; Dingle and Barnett; 1969; Hammond et al., 1980] so an unequivocal relationship with cellular viability should not be assumed. Hammond and colleagues however, have found the method to be 'at least' as sensitive a measure of cell viability as trypan blue.

Figures 3.5.2 (a) - (p) illustrate the relationship between increasing cell number and OD for 7 cell lines; some under the different culture conditions of 1 and 4 days growth. For some part of the graph at least, there is a linear relationship between NR uptake and cell number. Like the other assays discussed, the extent of linearity and sensitivity differs among cell lines, and this is summarized in Tables

3.5.3 and 3.5.4. Linear relationships similar to this have been shown by other workers for different cell lines; 3T3 mouse fibroblasts [Borenfreund and Puerner; 1984], colonic cancer lines (HT29 and EC), human renal carcinoma (GYL), pancreatic cancer (GER), breast cancer (MDA 157) [Fiennes *et al.*, 1987], and human and rat hepatocytes [Zhang *et al.*, 1990].

Fiennes *et al.*, [1987] observed differences in the relationship of cell number and NR activity among the cell lines tested, and some deviations from linearity among cell lines. This is similar to what is reported here. SCC9 for example, after 1 days growth, did not show a linear relationship at the lower cell densities tested. Differences in sensitivity were also noted among the cell lines tested in Fiennes' study (tenfold in one case). This too is apparent from the figures presented here; the assay is much more sensitive to numbers of NRK for example, than to SK-MES-1. Explanations as to why this might be, have been discussed already (section 6.8) in the context of the MTT assay.

In general the assay was much more sensitive to cell number for the various cell lines tested than the MTT assay or the AP2h assay, while it was less sensitive to cell number than the CVDE, SRB and APNaOH assays.

6.13 Technical problems and applicability to a range of lines

The assay had a substantial number of steps and was slightly more tedious to perform than the other assays, but was equally applicable to different cell lines.

6.14 Relationship between cell number & OD in the SRB assay

This assay was only recently developed for miniaturized quantitation of cell number [Skehan *et al.*, 1990], where it was found to be superior to 20 other dyes when tested on A-2780 ovarian, HT-20 colon and UO-31 renal, tumour cell lines. It was subsequently examined

more closely with additional cell lines covering a wide variety of tissue types. Intense staining of cells, lack of wide differential staining among cell lines, and low signal-to-noise ratio, were the characteristics contributing to its selection. Loss of linearity at high cell densities described here (Figures 3.6.1 (a) - (c)), was similarly described in the paper by Skehan. This loss of linearity was more pronounced for SRB than NR assay, and only slightly different from CV. It is explained by the high OD values obtained at these values, which were all greater than 1.6 OD units, and above the sensitivity of the platereader. Another explanation is given by Finlay *et al.*, [1984] (who used methylene blue in the same manner), who suggests that this deviation from linearity at high cell density is due to a decrease in the amount of stain bound per cell, because of a possible decrease in size as cultures approach saturation density in the wells and cells accumulate in G₁ phase.

Topley *et al.*, [1991] also found the method to be more sensitive than methylene blue.

In this work, the SRB assay was found to be extremely sensitive - much more sensitive to cell number than the MTT, AP2h and NR assay, and somewhat more sensitive than the CVDE assay. It had similar ranges of linearity and sensitivity to the APNaOH assay. It was capable of detecting 400 Hep-2 and SK-MES-1 cells under the conditions used in this work, and 300 DLKP cells, compared to 5,000 DLKP cells for AP2h and 10,000 for MTT. It is thus a highly sensitive assay for low cell numbers. The sensitivity available with this assay appears to be greater than that described in Skehan's work (quantitative at 2,500 cells/well, and semiquantitative at 1,000 cells/well).

6.15 Technical problems and applicability to a range of cell lines

The method had an equal number of steps to that of the NR assay, and was thus slightly more tedious than the other methods, but no technical difficulties were evident, and was applicable to all the cell lines tested.

6.16 Relationship of cell number with AP activity

Acid phosphatases (orthophosphoric monoester phosphohydrolase) are ubiquitous throughout nature, where they catalyse hydrolysis of a variety of orthophosphate esters as well as transphosphorylation reactions. They are distinguishable from alkaline phosphatases, not only by their low pH optimum (5.5, as opposed to 8.8 - 9.0), but by differences in ability to cleave a number of substrates [Hollander, 1971]. Four electrophoretic bands of AP activity have been separated [Beckman *et al.*, 1969], and their distribution among different tissues has been noted. In the work by Beckman it was found that cultured cells showed a different pattern of activity of the 4 bands (designated A, B, C, D), than was apparent in the original tissue. Also the presence in 4/7 cell cultures, of the C component, which was present in many foetal tissues, was suggested to represent dedifferentiation to a primitive state.

In assaying for acid phosphatase activity, most investigators have used *p*-nitrophenyl or α -naphthyl phosphate as substrate [Hollander, 1971] to spectrophotometrically determine the activity of the enzyme. The substrate used in this work was *p*-nitrophenyl phosphate, and the assay was essentially carried out as described by Connolly *et al.* [1986]. In this work, Connolly described the use of a semi-automated, miniaturized assay for quantitating small numbers of endothelial cells, and reported sensitivity to as few as 100 endothelial cells. No other significant work has been carried out using this method, so the work reported here serves as an important contribution to this area.

The high sensitivity of the assay, reported by Connolly, was also experienced in this work. Sensitivity was even greater than that found with the SRB assay. This was linked with a consequent lowering of the range of linearity of the assay, as was experienced with the other assays, to cell numbers of up to 20,000 for DLKP and Hep-2.

This however, meant that cell numbers greater than this were unevaluable when using the AP assay as described by Connolly, as it often resulted in a deep yellow colour, the intensity of which was often beyond the range of sensitivity of the microplate

spectrophotometer. This of course was due to the addition of NaOH, which caused an electrophillic rearrangement in the *p*-nitrophenyl released from the substrate by AP, and caused its colour to intensify. During the course of this work, it was found that there was often adequate colour present without this NaOH step, to effect a substantial spectrophotometric reading, so the relationship of cell number and OD in the standard 2 - hour AP assay without NaOH addition (AP2h), was investigated in section 3.7.

As was noted for the other assays, and as was established for the APNaOH assay there was a linear response of cell number and AP activity. Again, this was seen to vary with the cell line involved, in terms of lowest detectable cell number, linear range, and sensitivity along this linear range. The AP2h assay was not particularly sensitive but it gave a detectable (>0.02 OD units) increase in OD with every increase of 10,000 cells, and was linear up to high cell densities.

Extending the incubation period from the standard 2 hour time however, resulted in a substantial improvement in the lowest detectable cell number and an increase in sensitivity; activity was linear up to 5 hours at least, for a range of cell lines (section 3.9.2). This assay still remained linear to high cell densities, so this improvement in sensitivity has not resulted in a substantial loss of the extensive linear range of the AP assay.

The practical significance of this, is that an experimental plate, if not sufficiently developed after the 2 hour incubation (this can be determined visually), can be re-incubated for a further number of hours until sufficient colour has developed. Alternatively, at the end of the 2 hour incubation period, NaOH can be added for greatly improved sensitivity. If very low cell numbers are used in the experiment the NaOH modification would therefore be appropriate, but where linearity to high cell densities is required (e.g., if high cell numbers are present), then the AP assay as described in this work may be more relevant.

6.17 Technical problems of the AP assay, and applicability to a range of cell lines

No technical problems were encountered with the assay. It was simple to perform and involved a small number of steps. Obviously addition of NaOH was an extra step in the method, but even this considered the assay was still quick and easy to perform and it was equally applicable to the range of cell lines tested.

6.18 Comparison of the performance of the methods

The advantages offered by semiautomated, miniaturized, colorimetric assays for growth quantitation with the features of speed, simplicity, sensitivity, reliability, and cost - effectiveness have been widely publicised [Mossman, 1983; Finlay *et al.*, 1984; Carmichael *et al.*, 1987; Margis and Borojevick, 1989]. Their miniaturization means not only that smaller numbers of cells are needed, but that more replicates of expensive components such as growth factors, can be included. Individual methods have been shown to correlate precisely with cell number, as indicated in the previous sections. Correlations too, of cell quantitation for different applications, have been made with some such assays (mostly MTT and clonogenic assays, radioincorporation assays and dye exclusion tests) [Carmichael *et al.*, 1987; Carmichael *et al.*, 1988; Ferrari *et al.*, 1990 and many others], and in many of these studies good correlations have been achieved.

No extensive intercomparison study of these commonly-used semiautomated, miniaturized assays has been carried out, except for a study with one cell type (primary fibroblasts) where MTT, NR and Hexosaminidase activity were compared [Givens *et al.*, 1990], so the work described in this thesis represents a reference point for further work in this area.

This work has established that APNaOH, the protein staining assays, and NR were more sensitive than MTT, although linearity for the high sensitivity assays is subsequently decreased. Similar findings were reported by Givens *et al.*, [1990] and also by Alley *et al.*, [1988] who

compared MTT to a cellular protein microplate assay using methylene blue. The latter group found that while the protein microplate assay exhibited greater sensitivity to lower cell density than the MTT assay, the former did not accurately measure the mass of overly confluent wells (e.g., inoculation densities > 1250 cells/well on day 8). In other words, they reported better linearity and poorer sensitivity of the MTT assay, compared to the protein stain. This agrees well with what was reported here when MTT was compared to CV and SRB. In their work, Givens *et al.*, [1990] described the poor sensitivity of MTT noted in this work, and reported the inability of the assay to detect cell number accurately below 10,000 cells/well. Romijin *et al.*, [1988] reported that the MTT assay was superior to DNA measurement for sensitivity and accuracy when both were used to assess cell number, but the converse was found by Ford *et al.*, [1989] when RNA synthesis was compared to MTT cleavage. In measuring numbers of parasites, Kiderlin and Haye [1990] found similar dose response relationships of cell number and DNA synthesis/ MTT, but concluded that the DNA synthesis assay was more sensitive at low cell densities and less sensitive at high cell densities.

Other tetrazolium assays have been used, but none seem to provide better sensitivity or significant advantages over the MTT assay. INT (*p*-iodonitrotetrazolium violet) was linear over 1.5×10^3 to 1×10^5 cells [Bernabei *et al.*, 1989], but overnight reincubation was required to give a substantial colour for determination, indicating sensitivity problems yet again. Scudiero *et al.*, [1988] found that XTT offered advantages over MTT in that it was water soluble and was non-toxic, however low sensitivity was apparent after the standard 4 hour incubation time used for MTT. An incubation period of 96 hours was needed to obtain OD values as high as those obtained in 4 hours with MTT, so this limited the applicability of this method.

Given the low sensitivity of the MTT assay and its technical problems already discussed, protein assays would seem to be more successful for quantification of cell number. Some work on intercomparison of a number of such stains has been done. Comassie brilliant blue has been used by Margis and Borojevic [1989] to detect 10^4 cells in microplate cultures, and by Laughton [1984] to detect fewer than 1,000 cells per well. Methylene blue was linear with cell numbers up to 5×10^4 in the hands of Finlay *et al.*, [1984] and sensitive to cell numbers

around 1,000 cells/ well, and this method was found to be well correlated with thymidine incorporation for quantification of cell number [Absher *et al.*, 1991]. Skehan *et al.*, [1990] carried out a validation programme on 21 histological dyes used to quantify cell number and found that SRB compared favourably with several fluorescence assays in terms of sensitivity and was superior to those of both the Lowry and Bradford assays and 20 other visible dyes (including CV). The results reported here agree with the superior sensitivity of the SRB assay when compared to CV and when compared to sensitivity indicated in the published work for other protein dyes.

Problems with loss of linearity at high cell densities, due to high OD readings, may occur with intensely staining protein dyes, however a helpful way round this was suggested by Kueng *et al.*, [1989] who destained such plates, and restained them with a dye solution (CV) of a lower pH, which was found to stain less intensely. Presumably less concentrated dye solution could be used either. Dilution of the dye from the excessively coloured test plate, or reading at a suboptimal wavelength has been suggested [Skehan *et al.*, 1990].

However, the problem with protein dyes is that they stain live and dead cells indiscriminately, unlike MTT and similar assays which elicit some response from the cells, and are reported to stain only living, metabolically active cells.

The AP assay is a potentially better indicator of viability than protein stains, and in this study it was found to be capable of detecting very low cell numbers (200 DLKP cells); even lower than that detected by SRB. Other workers apart from Connolly *et al.*, [1986] have not used the APNaOH assay and few have used the SRB assay so there is no previous work to compare this finding to. A method similar to the AP assay, using hexosaminidase (another lysosomal enzyme) has been used though [Landegren, 1984], and it too was found to be very sensitive to cell number (0.05 OD units = 800 - 6,000 cells, depending on cells). In a later study comparing this enzyme activity to MTT and NR [Givens *et al.*, 1990] the hexosaminidase assay was consistently more sensitive to low cell number than NR or MTT, and this corroborates the findings reported in this work. A fluorescent alkaline phosphatase assay has also been used in a similar way for

counting cells 'in situ' [Huschtscha et al., 1989] but it was not as sensitive as the AP assay, detecting only $10^4 - 5 \times 10^5$ cells/ well. Other semiautomated fluorometric assays have been used however, and appear to have extremely good sensitivity to cell number. A fluorometric DNA assay described by Mc Caffrey et al., [1988] appears to be able to detect 100 cells/ well. A BCECF (2'-7'-biscarboxyethyl-5(6)-carboxyfluorescein) assay used for quantifying cell number was found to be 3-fold more sensitive, >10-fold faster, and required 1/10-1/100 the cell numbers needed for the MTT assay [Leeder et al., 1989]. A disadvantage to this assay however, is that it required the use of a vacuum filtration plate for cell measurement. FDA (Fluoroscene Diacetate) fluorescence was linearly related to living cell number from < 100 cells to 100,000 cells/ well [Larsson et al., 1990] so this too was an extremely sensitive assay. For each of these assays though, a fluorometric platereader is required, so this limits their application to some extent. The APNaOH assay, is almost as sensitive as these assays, and does not require the use of this instrument.

Another high sensitivity assay used in quantitation of cell numbers is the ATP-Bioluminescence assay. While it can detect cell numbers ranging from 250 to 485,000 cells/ ml [Sevin et al., 1988] this assay has not been semiautomated and miniaturized, so it does not offer the same advantages as any of the colorimetric assays mentioned.

In comparison of precision data for the various methods, there was little consistency from experiment to experiment in terms of identifying the extent and rank order of precision among the endpoints. On closer examination, it appeared that the performance was in some way related to the cell line tested, as different rank orders of assay precision were apparent for some cell lines (Figures 4.10.4 (a) - (c)). Work by the NCI has also noted differences in CVar's with different cell lines [Paull et al., 1988 (b)]. SRB, which had given low CVar values (in the region of 5%) for SK-MES-1 and moderately low values (c. 10%) for Hep-2, was very imprecise for replicate wells of DLKP cells. The CVDE assay also showed a similar tendency. If this trend does exist, as is indicated here, then it is most likely related to the morphology and growth pattern of the cell lines in question. DLKP, for example, expresses a subpopulation of

cells which are loosely adherent [Law et al., in print], so the monolayer surface is undulated. The tendency of cells to pile up is also greater for Hep2, than is for SK-MES-1, which grow in very flat squames - thus endpoints which involve staining the surface of the monolayer, might be expected to express greater variability in results than those relying on an intracellular mechanism. While the MTT is enzyme related, the product of the reaction is formed on the exterior of the cell (an operation more susceptible to disturbance by flicking and pipette manipulations in loosely adherent cells than those on planar surfaces). Indeed it has often obvious when microscopically observing cell colonies which have been stained by CV or SRB, how unevenly distributed the dye is on the plate.

Generally speaking the assays were quite precise, with CVar values in the range of 7 to 15%, however, this was significantly better or worse for assays on different occasions (values have reached up to 40%, especially for CV, SRB and less often for MTT).

Whole graph reproducibility was slightly better for NR than for others, followed by AP, CV, SRB and lastly MTT (the latter two suffered from poor reproducibility). While the same trend was shown for reproducibility of IC_{50} values determined from the whole graphs, the distinction among the assays was greater. NR was significantly better at concisely determining IC_{50} for Vinblastine to Hep-2 under the conditions specified, than were the other assays. The values within which the IC_{50} fell during the 3 runs of this experiment, represent 'middle of the range' IC_{50} values for all of the endpoints.

This brings to light a number of important points which should be kept in mind when performing toxicity assays. Firstly the reproducibility of the assays may be poor, and this may vary over different parts of the curve. In comparing the toxicity of different drugs thus, the range of variability which generally accompanies the graph should also be considered. In selecting a single value to express toxicity, a certain danger is inherent, in that it ignores what happens for the rest of the graph. Other more useful ways of expressing such data may be by using the Area Under the Curve method (AUC), which takes into account the shape of the whole relationship including the slope of the

different regions [Park *et al.*, 1987; Houde and Arora, 1990; Campling *et al.*, 1991]. If the reproducibility of the IC_{50} value is well established, then it does serve as a useful index of toxicity. If however this factor is ignored then a misconstrued situation arises where the value is taken and precisely compared to other similarly ildefined values for other drugs, giving reportedly precise, but erroneous values. In these comparisons (which abound in the literature), neither does it happen that precision figures are taken into account. Frequently in the literature, IC_{50} values are given as absolute values without consideration of the variability involved. Indeed precision data has been blatantly disregarded in much published work, accompanied by phrases such as 'error bars have been omitted for clarity [Ford *et al.*,; 1989]. It is felt that it would be much more accurate to give the IC_{50} (or equivalent values) as a range within which the value most likely falls, based on reproducibility and precision data; for it can be seen that the range of values in which it falls can be very dispersed, especially if different methods are used to establish these values (see Figures 4.10.5 (l), (m)).

It is also interesting to observe that in the two repeats of the Vinblastine toxicity experiment where there were significant differences among the dose-response graphs determined by different endpoints, the same rank-order has been maintained - AP and CVDE being the most sensitive at detecting toxicity, followed then by NR, SRB and lastly by MTT. This prompted further investigations into the possibility that there may generally be significant differences in ability to detect toxicity among such endpoints, and this point is further discussed elsewhere in this thesis.

To return to indices of expression of toxicity, it is felt that values describing toxicity to 70% or more of the population (*e.g.*, IC_{70} , IC_{90}) are too inaccurate for use in toxicity comparisons using the type of assays described in this thesis: firstly because sensitivity is questionable at the low cell numbers corresponding to these values for many of the endpoints, and secondly because this end of the graph generally tails off for some time, giving a span of drug concentrations which could correspond to these values (this is probably due to the survival of one or more subpopulations of cells which continue to grow at increasing drug concentrations).

In terms of speed, simplicity, ease of performance and sensitivity, the APNaOH assay was superior to all of the other assays tested, and is recommended here as the method for use for growth quantification. We have seen that a paradox exists in the range of available miniaturized, colorimetric assays for quantification of cell number; MTT and other similar assays are not sensitive enough, while often protein assays such as SRB are so sensitive as to be non-linear at some high test-densities. Some suggestions have been made to overcome this problem, but the versatility of the AP assay means that it is less prone to causing this problem. In situations where a range of cell numbers must be quantified, which exceed the linear range of the assay, the AP2h or extended duration assay can be used alternatively, as it has a longer linear range of cell number and OD.

The caveat of using this assay, however is that the precise relationship of cell number and OD varies under different culture conditions, so in cases where quantification of exact numbers of cells is important (instead of comparison of test wells to control wells), a standard curve of cell number and AP activity must be performed at the same time as the test plate, and under the exact same conditions. This not only applies to this assay but each of the assays considered here [Herz, 1989], as none are direct measures of cell number.

The NR assay however, has also performed well in the comparison undertaken, expressing enhanced precision and reproducibility over the other endpoints. It is more sensitive than the AP2h assay, but sometimes not terribly more sensitive, and its linear range is very significantly shorter than the AP2h assay. In situations where sensitivity to low cell numbers is not required (assays for stimulation, e.g., serum batch tests, growth factor assays), the NR assay would be very suitable. Overall these two assays represent the best performance under the headings outlined and are recommended to be more useful than the remaining endpoints.

The ability of the AP assay, and other assays, to accurately distinguish differences between viable and nonviable cells is an important question which must be properly addressed, and will now be considered.

6.19 Relationship between assay activity and condition of cell

In the work described in this thesis, the AP assay was found to exhibit a number of advantages over other similar assays. It was found to be a simple, sensitive, and versatile determinant of cell number. Given however, that AP activity (and activity in other assays) was found to vary when cells were grown under different conditions, it was considered important to quantify how great this variation could be, under differing culture conditions. When cells were grown at three very different cell densities resulting in subconfluent, confluent and superconfluent cultures, AP activity per cell (as well as activity per cell in the other end points) varied greatly among the three cultures.

There is a general difficulty, in assays of this type, to positively distinguish the dead cells from the live cells. Trypan blue and other dye exclusion assays have generally been used as an indicator of cell viability [Yahas *et al.*, 1974; Yip and Auesperg, 1972; Hanson *et al.*, 1989]; however the correlation between dye exclusion and viability has not always been absolute [Roper and Drewinko, 1976; Bhuyan 1976]. Recently-dead cells may not exclude dye, and if left in contact with the dye for extended periods, viable cells too will take up the dye. It may therefore be inaccurate to state that cells unstained with trypan blue are viable, all that can be said is that their membrane is still intact.

In order to determine the viability status of the test cells in this experiment, and provide a reference point from which the assay's ability to determine viability could be evaluated; similar numbers of trypan blue excluding (presumably viable) cells were plated at low density for clonogenicity. After colony formation had occurred, it was possible to determine the colony forming potential of each culture; from which it was ascertained that there was no significant difference in colony forming ability (and thus numbers of viable cells) in the cells plated from the three cultures. It is possible that Trypan blue dye exclusion has wrongly estimated cell viability, but if this was the case, it did so to a similar extent in all three cultures. Thus an assay which could be used for accurately determining numbers of viable cells, should deliver similar readings for the three cultures.

In performing the counts, it was not possible to obtain a count for dead cells, because any cells other than those excluding Trypan blue had disintegrated and were only represented by debris in the superconfluent culture. According to trypan blue counts, the number of intact unviable cells present in each of the cultures was negligible, thus all of cells were 'viable' (as can best be determined by trypan blue). The activity per 'viable' cell was given for each of the assays in Table 3.11.3. It was seen that activity, or staining intensity was increased for all assays, except NR, in the confluent culture.

In fact, the only assay which fulfilled this criterion during this experiment, was the NR assay, which maintained a constant activity per cell for the three cultures. Thus this assay may be the only reliable indicator of viability under these conditions. All of the other assays had increased activity in the superconfluent culture. The high SEM values pertaining to this count leaves greater uncertainty on the values per cell in this culture, however activity in culture C for each of the assays (excluding NR) may have been as high as 10 times that experienced in cultures A and B.

The high AP activity may be related to the fact that the cells in culture C were starved of nutrients and in very poor condition. Lysosomal enzymes are involved with cell destruction and disintegration, so it is conceivable that cells close to death as these were (cells were peeling off and in very poor condition), would have increased activity. Indeed it has been shown by Cristofalo [1972] that in cultured cells, lysosomal enzyme levels often rise with age, and lysosomes may become more numerous. These rises may have some physiological importance (perhaps in removing accumulating aberrant proteins, as suggested by Bradley and Schmike [1975]). This may similarly occur with cells stressed, as in culture C.

It may be that total lysosomal enzyme levels increased under these conditions, or that selective induction occurred, but the latter is less likely as there are few known instances (one example is the hormonal induction of rat kidney β -glucuronidase [Dean and Barrett, 1976]). Most reported changes in lysosomal enzyme levels in tissues in vivo are small and quite often, as under several starvation regimes, total lysosomal enzyme activities per organ are unchanged

and so there may be little change in synthesis or degradation of lysosomal enzymes. In vitro however, large increases in lysosomal enzyme activity detected in limb bone rediments and macrophages were found (these tended to be accompanied by secretion of lysosomal enzymes into the culture medium). In macrophages, phagocytosis of many digestible substrates causes the induction of lysosomal enzymes in a concentration-dependent manner [Dean and Barrett, 1976].

The augmented activity observed in the starved culture C, may have been due to an increased amount of enzyme, or due to increased activity of the same amount of enzyme per cell. To determine this would necessitate further experimental work where the rate of enzyme activity would be measured.

Succinate dehydrogenase (involved in MTT reduction) on the other hand, is most active in metabolizing cells and is decreased in stationary phase cultures, or those in which levels of glucose in the medium are low [Alley et al., 1988]. Because of this it might have been expected that activity of this enzyme per cell would have been lower than the activity seen in the more actively growing cultures (A and B). An explanation as to why an increase was seen in the confluent culture might be that enzyme, still active from dead cells, remained in the culture medium to metabolize the MTT to formazan. In the case of the AP assay the washing step would be likely to remove any cell debris (and presumably associated enzyme) but in the MTT assay, there is no washing step, and the medium removal step occurred **after** the MTT had been reduced, thus any active enzyme from dead cells would contribute to the reaction, and overestimate cell viability. This would seem to concur with the report by Jabbar et al., [1989] in which the MTT assay was found to overestimate the growth inhibitory effects of interferon, and cell survival was overestimated.

With regard to the protein stains it is evident that cell debris was present on the cultures, and was stained also. This may account for the increased intensity of staining, as such stains do not distinguish between live and dead cells, and cell debris [Laughton, 1984].

NR uptake on the other hand is dependent on active uptake into lysosomes. Four main mechanisms of uptake of materials into lysosomes have been discussed by Dean and Barrett [1976]: phagocytosis (particulate matter), pinocytosis (soluble and adsorbed matter), autophagy (intracellular materials), and permeation.

Many low molecular weight compounds including drugs, dyes and carcinogens rapidly penetrate the lysosomal membrane in uncharged forms and are accumulated, probably by protonation of bases and hydrophobic interactions [Dean and Barrett, 1976]. NR may be accumulated in this manner. Hammond [1980] was unable to identify which of these mechanisms are involved in uptake/release and transport of NR, but felt that it could be a combination of endocytosis and diffusion.

This mechanism of uptake is obviously not affected in the same manner as intracellular lysosomal enzyme activity is. NR is taken up extremely rapidly (as early as 30 seconds after exposure), and in macrophages has been shown to be secreted with similar kinetics to its incorporation [Hammond, 1980]. Contrary to that discussed for AP above, NR uptake has been shown to be unaffected by the duration of macrophage culture prior to exposure to dye [Hammond, 1980] and this would concur with the results obtained in this work.

While the experimental work described here would benefit from a repeat to confirm these results (time did not allow this), a distinct difference in the activity of the assays was shown in cultures grown under different conditions. Activity in the NR assay was constant in cells in the different conditions, but this was not the case for each of the other assays. Thus the use of any of the assays described in this thesis, other than NR, as a method of determining cell viability under the different culture conditions used here, would have resulted in erroneous results. This report agrees with some observations reported in the literature, discussed above. This serves to confirm cautions given for similar assays [Alkaline Phosphatase, Herz, 1989] of the possible effects of changes in specific enzyme activity during growth and the necessity to complete a standard curve of each cell line under the precise conditions of the experiment, if the exact relationship between cell number and activity is required. A similar

caution has been given with protein stains, in that they do not distinguish between intracellular and extracellular protein [Laughton, 1984].

6.20 Relationship between assay activity and cell viability after exposure to anticancer agent

We have seen that cells grown at different densities or for different periods of time [Cristofalo, 1972] can vary greatly in their expression of intracellular enzyme activity, or in the capacity of the culture to bind a protein dye. We should therefore expect that cultures of cells in the presence of different growth modifying agents (*e.g.*, antineoplastic drugs and other cytotoxic compounds) may affect the performance of some growth assays. Tests for membrane integrity, such as ⁵¹Cr release, LDH leakage and dye exclusion assays, have been shown in the past to give poor correlations with cell viability after exposure to cytotoxic agents [Roper and Drewinko, 1976; 1979]. In proffering the assays described here, and particularly the AP assay (which has been found to have several advantages over the other assays described here), as useful indicators of cell survival in cytotoxicity assays, it is important to determine if similar limitations exist.

In investigative work such as this, it is important to have a reference method for which actual viability can be measured. Clonogenic assays (as used here) provide an unequivocal criterion for measuring viability in a proliferating culture of cells [Roper and Drewinko, 1976]. In this experiment, test cells were trypsinized (after the test exposure) and replated under optimum conditions for colony forming ability. Because control cells which had not been exposed to drug, were treated in a similar way, differences in colony forming ability were deemed to be due to drug treatment, and thus viability differences between the two cultures were quantitated (by differences in resultant colony number). One possible pitfall with this method is the trypsinization step needed to carry out the determination. It is conceivable that antineoplastic agents may have caused cellular damage, and subsequent exposure to trypsin may have had a synergistic effect and caused the ultimate death of the

cell. Because the success of the method relies greatly on the presence of a distinct single cell population, the time of exposure to trypsin is longer than in usual subculture procedures. In order to minimize the damage that may have been caused by trypsinization, cells were exposed to trypsin for the minimum amount of time necessary to obtain a single cell suspension. An additional caution about the results of these experiments is that high SEM values were evident on haemocytometer counts, and this introduces greater uncertainty to the results - a limitation of haemocytometer counting with small numbers of cells.

Using replicative potential (clonogenicity) as the ultimate test for viability, differences between control and drug treated cells were attributed to drug effect. When similar numbers of 'viable' cells (as determined by trypan blue exclusion) from control and drug treated cells gave similar clonogenicity results, it was concluded that the trypan blue exclusion method had assessed cell viability accurately. When significant differences occurred, the difference was attributed to inaccurate assessment of cell viability by trypan blue exclusion.

Because some problems with IA growth analysis were outlined earlier in the results section, manual counting of colonies was performed and compared to IA assessment. IA colony counting did not accurately determine colony numbers, and gave significantly different results when control and drug treated plates were compared (Table 3.12.2). These differences were even more pronounced when colony area was used as the measure of growth. Based on these results thus, analysis for colony number by IA would not be recommended under these conditions.

After 5 days of drug treatment, Trypan blue (TB) had assessed viability accurately; however at drug exposure times earlier than this, the relationship was not absolutely accurate. After 1 days exposure to drug, 40% of TB determined viable cells were not able to form colonies, thus they had been irreparably damaged, but their membranes were still intact. The results for 2,3 and 4 days are more difficult to determine because of high SEM values in counts, however there is some indication of further underestimation of cell death. This inability of TB to accurately determine non-viable cells at early stages in drug exposure was reiterated in the second experiment on day 2 at low drug concentration. Similar results have been observed by

other workers under similar conditions [Yuhua, 1974; Bhuyan, *et al.*, 1976]. Weisenthal *et al.* [1983] has discussed findings such as these [1983], and his data strongly implies that some lethally damaged cells may not take up a stain until several days after exposure to a cytotoxic drug. Provided this limitation is realized then, TB (and other dye exclusion tests) may be successfully used to indicate drug induced cell death after sufficient time has elapsed to allow toxicity to be manifest by membrane damage, but if used shortly after drug treatment, will underestimate cell kill.

Regarding the colorimetric assays themselves, problems with poor correlation of AP2h were felt to relate to low sensitivity, as low OD values were encountered (particularly on day 2 of exposure, Table 3.12.5) By using the APNaOH assay this was not as apparent. At concentration C on day 2, APNaOH, NR and SRB assays have estimated cell survival to be similar to (or slightly higher than) TB, and thus they have similarly underestimated cell kill (in the region of at least 12% but possibly up to 40%). Thereafter the results with these assays were close to the TB results, and cannot be said to differ very significantly from viability determined by cloning. Vinblastine acts by inhibiting polymerization of tubulin and halting cells in mitosis [Bender, 1986]. Thus it could take time substantially in excess of one doubling time for damage to lysosomes and cell membranes to be exerted. After this time, while the cell might be lethally damaged, AP activity may not have been affected, and would not thus reflect this damage, whereas cells taken and plated for replicative capacity would be unable to continue replicating.

MTT gave results which were slightly, but consistently higher than TB counts for days 3 and 4, and thus may be said to overestimate cell viability slightly on these days. This may be due to the procedure discussed in 6.19, whereby MTT is added directly to the medium without washing, so any enzyme remaining active from dead cells is included in the reaction. This on the other hand does not happen with the AP assay due to the washing step involved. It may also be due to the finding that cells growing in medium with reduced pH (control cells) reduce MTT to a lesser extent than cells growing in a higher pH (growth inhibited cells) as this has been used to explain why the MTT assay has underestimated the growth inhibitory effects of interferon [Jabbar *et al.*, 1989]. Either way, this artefact could be overcome

by introducing a washing step and changing the medium before the MTT assay is carried out. It is recommended here that such a step be introduced into the assay, to overcome such problems, as it the basis of the assay *i.e.*, inhibition of MTT reducing enzymes (particularly succinate dehydrogenase) may potentially be a more sensitive indicator of cytotoxicity than lysosomal damage or inhibition of protein binding capacity.

NR gives slightly higher values for cell viability throughout the assay, and so does not correlate absolutely with TB determined (and clonogenicity confirmed) viability but this may be slight when SEM values are considered. It is thought that NR is taken up by lysosomes by a combination of endocytosis and diffusion, and thus these processes would not be immediately affected by cell stoppage in mitosis, and thus may not be expected to correlate absolutely with clonogenicity, however it should be no less sensitive to cell death than dye exclusion. This indeed has been indicated by Hammond *et al.*, [1980]. NR has been found to stain dead cells [Allison and Young, 1973; Dingle and Barnett, 1969] although the pattern of staining of damaged cells is diffuse nuclear staining rather than particulate cytoplasmic staining [Hammond *et al.*, 1980], so it may be expected that this might happen in drug damaged cells which are dead.

CVDE and SRB correlated quite closely with TB determined cell viability but on many occasions they tended to overestimate cell kill slightly. They consistently indicated greater cell kill than the other colorimetric assays. Protein stains have been reported to be relatively insensitive in some cytotoxicity studies, as protein levels have not always been found to correlate with cell number [Salzaman, 1959]. In addition, secretion of matrix proteins by some cell lines may add to absorbance values, giving higher estimates of cell number, or relative proportions of various proteins within cells might vary with cell number [Laughton, 1984] or upon exposure to a growth modifying agent. It might have been expected thus that these assays would underestimate cell death because dead cells still present and partially attached to the surface would also be stained, however this was not the case. It therefore appears that synthesis of some important (in terms of biomass) proteins may have been affected by drug activity, and this inhibition was shown up more quickly with the protein stains than by dye exclusion or the enzyme assays. Tubulin

assembly into microtubules is inhibited by Vinblastine and other vinca alkaloids [Lenninger, 1970], and as tubulin is an important protein for cell structure this inhibition may have been detected more quickly by protein binding assays.

Overall the methods correlated quite closely with TB counts, and appear to have underestimated cell kill on the occasions with low drug exposure or short exposure time, when TB has done so. MTT appears to have underestimated cell kill slightly more than the others and reasons for this have been discussed, while the possibility that the protein staining assays have overestimated cell kill has also been addressed.

6.21 Relationship of end point performance to mechanism of action of cytotoxin

The ability of endpoints based on different principles, to differentially detect cell death induced by cytotoxins has already been shown [Roper and Drewinko, 1976, 1979; Bhuyan 1976]. When using incorporation of radiolabelled nucleic acid precursor assays, concerns have been expressed that incorporation of nucleic acid precursors can occur without completion of the cell cycle, and hence may not reflect actual cell replication [Absher *et al.*, 1991]. Differences in these endpoint's performance related to particular drugs, or different classes of drugs, have also been discussed. Drugs for example that influence nucleoside uptake and alter intracellular nucleoside precursor pools can result in changes in isotope incorporation which do not reflect actual nucleic acid synthesis [Maurer, 1981]. Horakova *et al.*, [1978] has found that measurement of cell protein, nucleic acids and glucose consumption were not directly proportional to cell number when cultures were treated with cytotoxic agents [6-Thioguanine and Vermiculine] due to unbalanced growth induced by the cytotoxic agents. Similar results were obtained using other drugs including 6-mercaptopurine [Horakova, 1974(a)], dactylarin [Horakova 1974(b)] and various inhibitors of DNA and RNA synthesis [Cohen and Studinzinski, 1967]. From the 1978 study it was concluded that only direct cell enumeration was suitable for the detection of agents' cytotoxicity.

It is conceivable that similar disruptions of normal biochemical reactions relating to MTT reduction, AP activity, NR uptake and binding of protein stains may occur when cells are exposed to different classes of drugs. In fact the results from the previous section have indicated the possibility of this, with respect to protein stains and Vinblastine toxicity. This would denote an assay to be more or less sensitive to a given class of chemicals and would have implications for chemosensitivity testing. To investigate this relationship, cells were exposed to chemicals with widely varying mechanisms of action. In the choice of chemicals, at least one chemical was chosen with a mechanism of action particularly relating to each of the assays. The chemicals chosen and the assay system in which they were more likely to have most activity, are given below.

Table 6.21

Mechanism of action of a panel of chemicals, and assay in which they were expected to have maximum effect

agent	known mechanism of action-inhibition of:	relevant assay
Vinblastine	DNA, RNA synthesis (blocks cells in mitosis) [Dawson <i>et al.</i> , 1986]	thymidine and uridine incorp.
Mitomycin C	DNA synthesis (interchelates DNA) [Dawson <i>et al.</i> , 1986]	thymidine incorp.
5-Fluorouracil	DNA and RNA synthesis (antimetabolite) [Dawson <i>et al.</i> , 1986]	thymidine and uridine incorporat.
2,4-DNP	oxidative phosphorylation (uncoupling agent) [Lenninger, 1970]	MTT assay
Cordycepin	RNA synthesis [Dawson <i>et al.</i> , 1986]	uridine incorp.
Tunicamycin	Glycosylation [Alberts <i>et al.</i> , 1983]	
Actinomycin D	RNA synthesis (blocks DNA transcription) [Lenninger 1970]	uridine incorpor.
Cycloheximide	Protein synthesis (blocks ribosomal peptidyl transferase, Alberts <i>et al.</i> , '83)	leucine incorp.
Triton-X-114	membrane disruption [Alberts, 1983]	AP, NR, MTT
L-(+)Tartaric acid	Acid Phosphatase activity [Dawson <i>et al.</i> , 1986]	AP
Hydroxyurea	DNA synthesis (RNA and protein synthesis not affected) [Dawson <i>et al.</i> , 1986]	thymidine incorp.

While the high SEM values obtained with the radioincorporation assays prevented extensive analysis of results, it appeared that the thymidine incorporation assay was indeed more sensitive to agents acting directly on DNA, than were any of the other endpoints. With the colorimetric assays however, this was not the general finding - the chemicals expected to have specific impact on each of the colorimetric assays, did not appear to induce a significantly different response in these assays, at least not in a consistent fashion. The increased sensitivity of the AP2h assay for Vinblastine toxicity suggested in the previous section, was slightly apparent in one run of the experiment, but this effect was not reproducible. This implies that these assays do not generally give an indication of the mechanism of action of the chemical, because they were not differentially toxic in the colorimetric assays. It also means that the assays are not biased towards particular chemicals with a mechanism of action related to the endpoint being measured, and they are thus of equal relevance as indicators of cell viability for any of these cytotoxic agents.

These findings also query the reproducibility of these assays; as it was concluded that none of the colorimetric assays were consistently more or less sensitive to a particular class of chemicals than another. The order in which these methods responded was not constant, and the SEM values for each assay overlapped to give a wide range in which the IC_{50} might appear. This would imply that the assays were not able to detect small differences in toxicity (within this range of overlap, in which any endpoint was likely to give a result).

In similar studies (using only a small number of the endpoints above) varying results have been obtained. Triglia *et al.*, [1990] examined the cytotoxicity of various classes of test agents on a human three-dimensional physiologic skin model, but only examined the differential toxicity with a single assay - the NR assay, so this does not allow comparison. Mickey *et al.*, [1990] used thymidine incorporation to detect antiproliferative effects of steroids, and MTT to detect cytotoxicity and found that in only 1 of 22 steroids (on one out of 4 cell lines) was there a significant difference between the results observed in the two assays - in most cases the dose -

response curves were similar when using the two endpoints. Ford et al., [1989] found great differences in IC₅₀ values obtained by MTT and [³H]-uridine incorporation with Doxorubicin and Vindesine with some cell lines, but that agreement was much closer for other cell lines. In this work, similar problems with inter-assay variation for uridine incorporation assays were found, and this varied with the cell line and with the drug, while similar findings were reported by Park et al., [1987]. Givens and co-workers [1990] have previously compared the largest number of colorimetric assays in this aspect, when they compared MTT, NR and Hexosaminidase assays against Coulter counting. In this work they found very substantial differences in the ability of these assays to determine Mithramycin induced cytotoxicity (NR and coulter count ID₅₀ values were much lower than values obtained by MTT and Hexosaminidase).

Overall, the results of this investigation imply one of two things - either the endpoints are not differentially sensitive to the agents tested after the exposure used, or they are differentially sensitive to a small extent, but poor reproducibility and low sensitivity does not allow this effect to be seen (perhaps it is a combination of both). Further experiments with shorter exposure times might further elucidate this investigation. From low observed OD values in each of the colorimetric assays tested here (AP2h, MTT, NR and CVDE), it is believed that low sensitivity is a significant problem. Further discussions on reproducibility of these assays will help to determine if this is so.

6.22 Factors affecting IC₅₀

It has been widely shown that cells in different stages of growth or under different conditions behave differently in bioassays [Freshney, 1987]. This is no less true for toxicity assays. The work described here has investigated a number of factors which might affect the IC₅₀ value. The increased cell kill observed when duration of exposure to drug was increased (3.14.4 and 3.14.5) is probably very obvious and is well documented [Barlogie et al., 1976].

Other factors, not quite as obvious, were also investigated. The importance of using cell cultures in logarithmic phase has also been stated [Freshney, 1987] but the influence of slightly different pretreatment regimes has not been so widely studied. The results from the work carried out here demonstrated that the relatively minor modifications in seeding density and feeding regime used for the pretreatment regime, had no predictable outcome on the results of the toxicity experiment. While there were differences in the IC_{50} values obtained, these differences did not follow a particular trend, and it appeared that results were simply attributable to random variation around mean values. While this answers the question in hand as to a possible definite effect of increasing cell seeding densities (it was concluded that there was no trend, so use of any of the pretreatment regimes resulted in equally relevant results), it does return again to the question of reproducibility of these assays. While many of the points on these graphs overlapped with many others, when the SEM values were considered individual graphs (representing individual assays carried out separately) spanned a range of 30% of the Y axis, which would lead to substantial differences in IC_{50} values.

The effect of exposure to trypsin for short variations in time (10 or 20 minutes trypsinization periods) was determined to be not significantly important when carrying out a toxicity assay with RPMI-2650. Presumably this would hold true for short variations in trypsinization times for other cell lines also, however this would not be presumed for longer periods of time. It has been shown by Barranco *et al.*, [1980] for example that immediately after a 5-minute exposure to 0.025% trypsin, chinese hamster ovary cells treated with anticancer drugs exhibited changes in drug sensitivity (depending on the drug). In this thesis however, the cells were allowed to attach fully (for 24 hours) before addition of drug, so the same differences in sensitivity (4 to 50 fold) would not have been expected. In fact Barranco *et al.*, determined that by the 12th hour post trypsinization, the drug survival responses had returned to values obtained with untrypsinized cells. This however, would be an important finding to bear in mind in short term drug exposure assays (such as that described by Rosanda *et al.*, 1985) if recently trypsinized cells are used.

The effect of increasing cell seeding density on drug toxicity was clearly demonstrated in this work. It was shown that increasing cell density resulted in reduced cell toxicity for the same drug concentration. It would appear that there are three obvious possibilities as to why this may be so; differences in drug uptake, differences in drug action when inside the cell, and differences in drug efflux. It may of course be a combination of these three factors. The most likely explanation is probably reduced drug uptake in the high cell density plates, due to masking effects at increasing cell density. This may be a physical phenomenon - if cells are more dilute and spread out, they will have greater surface area exposed to drug, and may therefore have increased drug uptake over cells which are crowded together. Cells seeded at different densities will obviously have different proportions of cells in different stages of growth. Cell surface and plasma membrane proteins have been shown to increase in quantity as cells progress from G1 to G2 cells [Pasternak *et al.*, 1974]. It is possible therefore, that the differences existing between the cell membranes or surface components of dividing and nondividing cells may affect the binding and transport of some drugs into the cells and ultimately cause rate limiting differential survival responses [Barraanco *et al.*, 1980]. Similar cell density effects have been shown by Pelletier *et al.*, [1990] who partly attributed the phenomenon to a general alteration of passive diffusion across the cell membrane (sodium chromate, analine and glucose uptake being affected in the same way).

Different exposure regimes were shown to effect differing degrees of cell kill, but in a concentration and time dependent manner. For the purposes of the work shown in this thesis, a continuous exposure was used, however this may not always be relevant (discussed further in 6.31). Duration of experiment with continuous exposure, was seen also to demonstrate the time dependent relationship of drug exposure to cells. Another factor which should be considered in this context would be the time taken for the drug to effect cell kill *detectable by the endpoint*. After 1 days exposure to drug, it is possible that the cells were lethally damaged, but not discernably so by image analysis. After 5 days growth however, the total maximum effect would be more likely to have been achieved, and thus lethally damaged

cells would be expected to portray all the characteristics of dead cells, including detachment from the culture, and thus measurable (relative to control cultures) by staining and reading with IA.

Other factors which are important in IC_{50} determinations are pH and serum [Nissen and Tanneberger, 1980] and obviously the cell line used [Paull et al., 1991].

6.23 Serum batch testing with miniaturised endpoints

Serum batch performance is often assessed by constructing growth curves, or by plating cells into flasks or multiwell dishes and counting cell numbers at the end of the test period [Jayme et al., 1988]. Both of these methods involve trypsinization of the cells and counting by haemocytometer or Coulter counter. Either way (but particularly with the haemocytometer) is extremely labour intensive and tedious. Attempts to simplify this procedure have been to stain the cultures (in multiwell plates) and count them by eye (still very time consuming) or score plates according to the extent of growth they express. The latter technique is semi-quantitative only and is sufficient for vastly different batches of serum, but is inadequate for batches of serum with more subtle differences in growth promotion abilities. Image analysis has further attempted to ease the burden on 'serum batch testers' by computerized quantification of stained growth surfaces, however this method leaves a lot to be desired. Its pitfalls have been identified in section 3.1.

We can verify some of these drawbacks from Figures 4.1 (a) - (c), in which growth has been analysed in this manner. Chief among the drawbacks are the gross error bars that accompany the graphs. These arose, not from plating errors and inaccuracy, but from suboptimal spreading of the cells upon the culture surface, the significance of which has already been stated. Values for replicate wells may consequently be reported to be significantly different (even though visually the total growth may be seen to be quite similar), and concomitantly, serum batches giving similar growth may produce extensively different absolute values, depending on the way in which they are spread and how the light plays on them.

This is attested by the relatively smaller error bars for RPMI-2650 and DLKP; both of which form colonies which are more successfully processed by image analysis (particularly RPMI-2650); compared to the high bars observed for SKMES-1 which quickly forms a monolayer.

Additional to problems of quantification of results, is the amount of resources (in terms of serum, medium, plastics and time) which are depleted in setting up serum batch testing experiments. Replicate wells must then be reduced to a minimum. In the case of primary or low passage cultures, low available cell number is another factor to consider; so it may not be possible to test all the required variables in the batch testing system.

Thus the case for applying a miniaturized, semiautomated assay to this procedure is made. In comparative experiments with IA assays, the AP method clearly gave similar results (Figures 4.1.4), so it was applied to a wider panel of sera and cell lines in an attempt to differentiate among the batches.

At the most basic level, it was clear that different cell lines had differences in their preference for sera - this is seen with batch 8, which was good for many cell lines, but appeared to be the worst of the panel for DLKP at low cell density. It was thought that similar trends in serum preference might be shared by cell lines from the same tissue type (hence the reason for testing 5 lung lines), however this conjecture did not seem to be any more true for the lung cell lines than for lines from any other tissue type.

Overall the screening system used did determine two consistently poor batches of serum, relative to the others. Batch 4, which was from donor horses, was recurrently poor for many of the lines, as was batch 10 FCS. Selection of serum batch for routine work (on a large scale) must be a compromise between serum performance and price, and generally FCS are more expensive than other sera (particularly DC, NBC and DH), so it was important to have identified these poor batches. Another significant finding was that batch 2, which was a Donor Calf batch (and was significantly cheaper than the FCS batches) was equally as good as many of the FCS batches for most of the cell lines.

The importance of cell density in this exercise was plainly established by the work shown in Figure 4.1.3. It appears that at very high cell densities there is less differential effect among the sera. At 'critical' cell densities - close to the cut-off point below which cells will not continue to grow - a good batch of serum will inspire growth above critical densities which will then continue to give a very substantial differential between it and the other serum batches. At high cell densities the cells are better able to take-off and grow, so will be less dependent on the presence of a good serum batch to aid them with this. Additionally, at high cell densities, there is less time before the wells become confluent and stop growing - giving poor sera a chance to catch up with good sera if the experiment is not taken down in time.

Thus the recommendation from this work is that cell lines are tested for serum batch preference at low cell density (preferably densities just above the cut-off seeding density at which they refuse to grow).

Comparison of the AP assay results to that of three of the other miniaturized methods (MTT, NR and CVDE) did result in similar trends. With the NR assay, the differential between good and poor batches was greater than that seen previously with the AP assay, which of course is desirable, however the SEM values were substantially greater for the NR assay, as was the case with the MTT assay (thought to be attributed to the technical difficulties of the assay; already discussed). Low variability among replicates was seen with the CVDE assay.

Overall, the AP assay gave results comparable to the other miniaturized assays (as well as comparable to NR), indicating that any of these methods would be suitable for serum batch testing. Lower precision on MTT and NR methods favours the AP or CVDE methods. In a comprehensive screen involving numerous cell lines and sera, two consistently poor sera were identified and a good quality Donor Horse serum (which offered substantial monetary advantages over other FCS batches) was identified. Similar cautions that accompany other methods for batch testing (and indeed other growth assays) such as use

of low cell loading, apply to these systems, nevertheless the method has been shown to be suited to this application, and to offer significant benefits over previously used monolayer/ IA screening.

6.24 Application to environmental monitoring

Pollution may be typically defined as the causation of a change in the natural environment which results in harm to a biological system. Effects of pollution are as diverse as the pollutants which cause them, which themselves are extensive. Pollution is often classified in terms of where the impact arises, i.e., water pollution, air pollution, land impact or social/ economic impact. In the case of water pollution, a simple classification of pollutant effects in water systems would distinguish between biodegradable compounds exerting their effect in the form of an oxygen demand, particulates which result in silting-up and physical effects on ecological life, and toxicants which exert a direct toxic action on ecological life. Sources of these pollutants include domestic and municipal activities, agriculture and industry.

Chemical monitoring of emissions of pollutant material is helpful, in fact necessary, to characterize waste and effluent produced by different sources, however chemical monitoring alone is not sufficient to characterize and indeed identify pollutants. While organic pollution is easily predicted by such tests as BOD (Biochemical Oxygen Demand), prediction of pollution by toxicants, or identification of a toxic pollution incident may not be realized if the particular agent(s) in question are not specifically analyzed for. In order to increase confidence in chemical monitoring, increasingly larger numbers of discrete tests may have to be carried out, and thus the cost and the time involved for processing a single sample is also increased. Generally then, the number of parameters tested is minimized, and thus testing of the critical pollutant may be missed. Use of biological monitoring on the other hand gives a result relating to the combined effects of the pollutant and can give an index of total toxicity by the use of a single test. Biological monitoring is often carried out at the site of pollution (or prospective pollution)

where the organisms already present in the ecosystem are monitored to give an indication of the health of the system, however this approach is often limited by the diversity of ecology at the site due to natural factors. Use of ecotoxicity tests on the other hand offer advantages of being able to screen such effluents before they are disposed of, and can thus aid in the prevention of toxic pollution, instead of simply identifying it after it has occurred. Such tests additionally are not dependent on the natural ecology present at the site, as sensitive indicator organisms can be chosen for use. Use of cell cultures have been used in the past for ecotoxicity testing of complex mixtures, and have been used in quality control assessment in the water industry and for raw materials. Miniaturized, semiautomated assays offer further advantages in terms of resources and time saved, and are thus less expensive to carry out once the basic equipment needed has been acquired. Endpoints such as NR and Kenacid Blue have been used for such purposes. The results described in this thesis show the use of the AP assay in screening for toxicity in a number of environmentally related samples.

6.25 Screening for cyto-toxicity in industrial sludges

Sludges are generated by wastewater treatment systems which result in removal of solids from the effluent. This is accomplished by a combination of primary and secondary treatment, and in some cases tertiary treatment. Primary treatment is the mechanical removal of grit, fats and large solids already in the effluent and is essential to the effective maintainance of a secondary treatment system. Secondary treatment is a biological treatment system, where specialized populations of micro-organisms use the nutrients in the wastewater as a food substrate, and in so doing, reduce the suspended solids, the organic content, and thus the polluting potential of the wastewater. Secondary treatment is commonly by Activated Sludge, Trickle Filter (both aerobic), Oxidation Lagoon (aerobic/anaerobic) or Anaerobic Fermentation [Hammer, 1977]; but all are based on the same principle of encouraging micro-organisms to break down the offending organic matter. Tertiary treatment, utilized only for high quality effluent, or for a wastewater high in nutrients such as phosphate and nitrates, may also result in the removal of colloidal material by precipitation and the use of

polyelectrolytes. From all these treatments, the quality of the effluent is improved as solids are removed from the waste stream. The resultant solids constitute sludges and are often problematic in terms of disposal because of their high polluting potential. The possibility generally exists that toxic elements are adsorbed to solids and are then concentrated in the sludge. Thus toxicity testing of sludges is an important environmental control parameter.

In this country, toxicity testing of industrial sludges is often a requirement before local authorities will accept sludges for landfilling. The systems available for such testing are carried out by EOLAS at Shannon and are carried out as fish (rainbow trout) toxicity tests and invertebrate tests. Apart from the huge expense involved in performing such tests, the available facilities for performing such tests are not adequate to facilitate the amount of testing to be done. Hence the availability of a cost-effective, rapid screen for toxicity that is correlated with toxicity tests already in use, would be a significant advancement to the Irish position in environmental monitoring.

It is felt that a miniaturized, semiautomated cell culture assay, as described here, would provide such a system for monitoring of toxicity in sludges for disposal. The AP assay was chosen for use because of its convenience and sensitivity - it is more sensitive than any of the other assays, and more convenient to carry out than the NR and SRB assays. Sensitivity is of the utmost importance when testing the low numbers of cells present in toxin treated cells, so it is appropriate to use the AP assay in preference to other assays described in this work.

In comparison of data from an algal bioassay [Rachlin et al., 1983] with a cell culture bioassay, Mochida [1986] found that the latter was more sensitive in determining aquatic toxicity. Similar findings in relation to the cell culture assay were reported by Kfir and Prozesky [1982] and Richardson et al., [1977] when a *Daphnia* bioassay was used. In the results presented here, AP has differentiated between the sludges similarly to the *Daphnia magna* test, but in expressing the results in terms of toxic units, has been less sensitive. It is thought that this was due to pretreatment of the wastes prior to cytotoxicity testing with the cell culture. Some of

the leachate samples were high in suspended solids (see photograph 2.38), so it was necessary to filter and centrifuge the samples before adding to the cell culture. This was particularly obvious with sample G which was extremely high in very small dissolved and suspended solids - thus much of the toxicity may have been lost before testing. It would be important therefore to investigate other methods of extracting leachates from samples, and cleaning up 'dirty' samples before assaying in cell cultures, without loss of the specific toxicity (considered later in more detail). Alternatively/ additionally, the difference in sensitivity may be attributed to the different systems. The cell culture system used here did not incorporate any method for biotransformation of xenobiotics, so this may account for some loss of sensitivity. While this factor had been appreciated from the onset of this work, it was outside the scope of this work to address this matter. Future work however, would benefit greatly from considering this factor. Addition of S9 mixes, co-culturing with hepatocytes or cell lines retaining some metabolic activity do attempt to address the problem, however such approaches as transfection of cytochrome P-450 cDNA from metabolically induced cells, into target cells, offers greater relevance, as the biotransformed compound will be generated inside the target cell and closer to the site of action [Dogra et al., 1990]. Significant work in this area is being carried out at present, in this and other laboratories.

Nevertheless, significant toxicity was identified by the method as it stands, and in utilizing the results determined here, it may be appropriate to set different acceptable limits to the toxicity (in terms of toxic units) when accepting/ rejecting sludges for landfilling depending on the screening method used. This should be done by comparing the AP assay to OECD recommended bioassays for ecotoxicity testing, and ascertaining the relationship between these for a number of standardized compounds of varying toxicity with varying methods of action. Obviously in such work, comparison of Toxic Units must be carried out with caution, because this expression, like the IC50 is not a 'stand alone' figure and depends strongly on the experimental parameters which were used to obtain this information. In routine screening with any of the endpoints investigated on cell cultures exposed to test samples, optimum conditions would first have to be set up for the cells in use

(including seeding density, exposure time and assay duration) and the endpoint to be used, and these would have to be strictly adhered to for all subsequent work to allow meaningful comparisons to be drawn, or reference to predetermined limits - otherwise, this would be just a meaningless figure.

With a little more work on pretreatment of samples for testing in cell culture systems, it is felt that the AP assay could replace some of the more expensive tests (e.g., fish tests) or be used to pre-screen samples for the more expensive tests. Sludges not having significant toxicity could be eliminated from further screening, if suitable comparative trials showed a very low incidence of false negatives.

6.25 Screening for toxicity in landfill leachates

Disposal of domestic and industrial solid waste is a major environmental problem. In the consumerist society in which we live, the amount of waste generated is constantly increasing.

Some typical figures available for the composition of refuse collected in the Dublin Corporation area (1977/78) are as follows:

Table 6.25 Typical composition of Dublin refuse (1977/'78)

Category	Average %
Paper	35
Plastic	12
Vegetable and Perishable matter	33
Glass	8
Rags	3
Metals	3
Ashes/ Fines	5
Others	1

From: Cabot, 1985

It can be seen that even from domestic refuse alone, metals and ashes constitute 8% of the waste.

Use of options such as recovery and recycling of municipal and industrial wastes, are only slowly being accepted and require a huge commitment from the general public, as well as centralized, well - manned sorting stations and increased expenditure [Chynhoweth, 1978]. Incineration, which can be an important option, requires huge

initial capital expenditure, and is receiving bad press at the moment [Mac Dubhghaill, 1991] due to production of dioxins and other toxic chemicals through incomplete combustion. Thus the only feasible alternative for use in Ireland at the moment is landfilling, which also represents the opportunity to generate methane in high volumes from emplaced wastes [Forster and Wase, 1987]. Much of our solid waste (both municipal and industrial) is disposed of in this way. Uncontrolled tipping of solid waste was reported [EEC, 1984] to account for 35% of the total wastes disposed of in Ireland (substantially greater than the proportion disposed of in this way in other countries), and of wastes arising from the manufacturing sector, 44.3% (276,000 tonnes/ yr) has been disposed of by landfilling; 17% of which is special waste with hazardous properties [Cabot, 1985].

A landfill site, as distinct from a 'tiphead' or 'dump', is where the compressed waste is emptied into discrete cells in the ground. Each cell is separated from the next by a layer of soil, and the complete site is covered with soil and grassed over when the area has been filled. Water trickling down through the soil and into the individual cells will saturate the waste and cause a leachate to be generated. This leachate will pass through the entire site and down into the underlying bedrock and can then pollute the groundwater in any aquifers underlying the site. This constitutes a major environmental problem, because, once contaminated, there is virtually nothing that can be done to save the aquifer; groundwater not having the same self cleansing abilities as surface waters [Daly and Wright, 1981]. The Water Pollution Advisory Council of Ireland [1983] have reported that the large scale pollution of groundwaters has mainly arisen from the land filling of sewage and industrial waste sludges or the spreading of agricultural wastes on land, so we can see that the area of landfilling is an important source of groundwater pollution.

There are 2 major types of landfill sites; 'dilute and disperse' and 'containment' sites [Kharbanda and Stallworthy, 1990]. Dilute and disperse sites rely on the topography and geology of the site to effect suitable control over the leachate. Suitable sites for dilute and disperse landfills have a thick layer of glacial material, with a high percentage of clays (or other material with a high cation

exchange capacity) overlaying a bedrock which is unfissured and has a low permeability. This will allow attenuation of the leachate before it reaches the saturated zone. In addition, the groundwater underlying these sites should not be significant, e.g., a low conducting aquifer. Sites which do not have these characteristics should be made into containment sites. In these sites an impermeable material covers the bottom and sides of the pit (either a naturally occurring layer or a polymerized material is laid down). The tip is then covered over with similar material when landfilling is complete. The object of such engineering is to prevent further leachate generation (a certain amount will take place anyway due to disposal of saturated materials), and collect that which does, by a series of drains and pumps. This can then be isolated and treated on site. The cost of a properly run containment site is prohibitive, so we find that in Ireland, due to insufficient funding, the local authorities generally 'make do with' the dilute and disperse sites even in areas where the geology is totally unsuitable and where maximal pollution of groundwater can occur. These sites [Cabot, 1985] are often disused quarries, in low-lying waterlogged land or in areas which can be reclaimed from the sea or estuaries.

Leachate from landfill sites can frequently be highly noxious. The leachate is normally highly concentrated and can have a COD (chemical oxygen demand) of several hundred thousand ppm. Difficulties with landfilling also occurs because the composition of the waste is not known. Legislation to prevent uncontrolled landfilling of toxic and dangerous waste exists [The European Communities (Toxic and Dangerous Waste) Regulations, 1982], however controlled landfilling of such wastes is often considered as a management option by co-disposal with household wastes and building site rubble. This relies on the properties of the inert material to attenuate the toxic waste, so that some chemical and microbiological oxidation of the waste can occur. In practice then, the possibility of pollution by toxic elements in leachates is a real worry. Because groundwater involves laminar flow, dissolved chemicals will follow groundwater and form distinct plumes, which may be up to several miles downstream of the pollution source [Went, 1989]. These plumes move very slowly and thus the pollutants tend to remain unchanged, where they may later effect harm if abstracted by wells or in their supplying baseflow to a river.

Frequent analysis of a few well - planned monitoring boreholes in addition to local wells and surface waters would provide much information which would be helpful in the management of these sites and help to identify contamination plumes, however this does not optimally occur, again, due to lack of resources.

BOD (biological oxygen demand) is not generally an effective measure of the polluting potential of landfill leachates because resilient components may not be easily oxidised by microorganisms and because of toxic effects on microflora due to chemical components in the waste. COD determined by oxidation by a strong chemical oxidant (potassium permanganate [OECD, 1984]) is generally a more useful parameter in characterizing the strength of a leachate.

In analysis of these samples, the operator would optimally need to know what parameters to test for, and even though a comprehensive series of tests may be carried out, the critical toxic component may not be analysed for, and thus a wrong judgement made about the quality of the water (and thus the polluting potential of the leachate and the management of the landfill site).

Use of biological monitoring for total effects of toxicity would offer several advantages in this field, and while this has been recognized theoretically for many applications, biological monitoring programmes are often technically specialized (as alluded to in the introduction), extremely time consuming and expensive, so such options are not open for general use. The use of cell culture screening systems may be more appropriate than other biological monitoring systems, can give a rapid indication of toxicity (48 hours) and may be much more cost-effective than other such systems, particularly fish toxicity tests. We can see that such a system, using MDCK cells has demonstrated toxicity effected by heavy metal salts, and by a number of industrial waste waters. Similar results have been shown by Jenssen and Syversen (1991) for Nickel using a fibroblast culture system, however exposure was only for 24 hours so IC_{50} 's were obviously much higher ($286.7 \pm 4.7 \mu M$) than those determined after 5 days exposure here.

The AP cell culture assay used here has determined toxicity of a metalworks effluent at concentrations greater than 12.5% effluent in the medium, but detected no significant toxicity with 50% addition of a landfill leachate. It has shown differential toxicity for three landfill leachates, and determined leachate 1 to be most toxic, however it can be seen (retrospectively) from the chemical data relating to the leachates that they do not appear to have significant toxic properties, having low COD and low heavy metal levels (indeed metal levels are similar to levels allowable by surface water criteria for public water supplies [Hammer, 1977]). However heavy metals, ammonia and nitrate are elevated in sample 1, and this may have accounted for the increased toxicity of leachate 1 to the cells in culture.

In considering this section it is important to highlight the difficulties which were involved in obtaining the samples for this work. Because environmental protection is becoming such an important and sensitive public concern, industries and local authorities are highly reluctant to distribute samples of effluent and waste, because they feel that if in the wrong hands it could result in prosecution (if the waste is above licence limits) or, in the case of local authorities, loss of votes at election time. These difficulties were only appreciated after extensive efforts to obtain samples from a wide variety of industries and local authorities, and the temptation for a prospective co-operator to donate only the cleanest samples, is now particularly realized.

Further work with chemically defined effluents of differing ranges of toxicity would enhance this work, but as yet it appears that the assay would be suitable for routine monitoring of leachates from landfill sites. This would be particularly useful for inclusion in groundwater protection policies - samples from strategically placed monitoring boreholes could be screened regularly with a single test, which would give an overall indication of toxicity of relevance to humans. Formation of noxious leachates could then be easily detected, and the plume of the pollutant could easily be traced using toxicity to cultured cells as an index. With assays of low sensitivity, high cell numbers would be needed to enable detection after exposure, and thus higher concentrations of toxin would necessarily have to be present in the sample. Alternatively, low

cell numbers could be used with low concentrations of toxin, but then the incubation time would have to be increased to enable surviving cells in wells expressing cell kill to replicate to well above detectable levels. The advantage of using the highly sensitive acid phosphatase endpoint for such studies is that a lower number of cells could be used, thus lowering the effective concentrations of toxin necessary to be present (see 3.14), and thus shortening the time necessary before a result could be reached. If this were standard practice in all of the landfill sites in the country, then this would be a good quality control system for groundwater toxic pollution by landfill sites, and would go a long way towards protecting the public from such pollutants arising from this source, and would alert the managers to problems as they occurred. Use of a miniaturized, semiautomated system such as this reduces the costs of performing such a test and makes it more practical for use as a monitoring system.

6.27 Potential applications for cell cultures in environmental toxicity testing

Some possibilities for using miniaturized, semiautomated, colorimetric cell culture assays have been considered here, and indications from experimental results would point to their usefulness in these areas. One of the disadvantages of using the system described here is that it will only detect acute effects - to detect sublethal effects requires the use of a modified system with different endpoints. In characterizing wastes for disposal, sublethal effects as well as acute effects may be important. This may be particularly important in identifying carcinogens. Thus in screening wastes for disposal, toxicity test systems should ideally cover different parameters of toxicity. The AP assay has been recommended here as the method of choice for cytotoxicity testing of such samples. In a detailed screening programme, where a second endpoint is being used, the NR assay would be recommended based on the results of this work. Using these two assays together would give a better representation of cytotoxicity when different classes of compounds would be encountered (even though no significant difference was noticed for the chemicals chosen for this work). While the NR

assay is not particularly sensitive, it has given more constant results in detecting viability, when cells were grown in different culture conditions during this work.

To obtain a better indication of the characteristics of the waste, it would be useful to test for cytotoxicity (for example with the system described in this thesis), mutagenicity, and carcinogenicity (by morphogenesis of cells in culture) with in vitro tests. The Ames test is commonly used for such mutagenicity testing, but it may be much more relevant to use a human cell culture assay for this.

An important aspect of classical biomonitoring studies for pollution is that these tests allow detection of bioaccumulation of pollutants, which is an important biological phenomenon - cytotoxicity tests by their nature do not. This however may be addressed in some way by monitoring the sediments in a freshwater or marine ecosystem. Ultimately sediments act as a sink for pollutants in aqueous systems as they act as adsorbants, binding a wide range of organic and inorganic compounds. In fast flowing rivers they may be continually resuspended, while in deep ocean sediments and lakes they will settle out. There are three main ways [Samoiloff, 1990] by which toxic materials bound to sediments may enter biological systems. The first is by contact between a target organism and the sediment, and absorption of contaminant through the integument. The second route is by ingestion of sediments by target organism and desorption of the contaminant in the digestive system into the general circulation of the host organism. The third route is by desorption of the contaminant from the sediment to the water column, and uptake to the target organism via the water. *In situ* toxicity may be considered to be a direct consequence of the desorption capacity of the sediment as well as a consequence of the actual toxicity of the contaminants. While detection of toxic chemicals bound to sediment suggests that there is a toxic potential of the sediment, chemical analysis alone is insufficient to provide a realistic appraisal of the toxicity of those sediments [Samoiloff, 1990]. Work by Samoiloff [1990] comparing chemical to biological analysis of sediments, showed discrepancies in the ability of these two systems to detect toxicity. The biological availability of the toxic contaminant is a factor which is not determinable by chemical tests alone. Ecological studies of natural populations of organisms present can determine

this, however preparation of sediment leachates to be tested on cell cultures may also give an indication of the potential biological toxicity of the sediments. Elutriation of loosely bound contaminants to yield an aqueous material for testing has been widely used, but chemical extraction is the only method that gives an evaluation of the entire range of toxic contaminants [Samoiloff, 1990] - these extracts can then be bioassayed.

Rapid cell culture screening may be potentially used in quality control of industrial effluents, not only for the final effluent but at different stages through effluent treatment. Activated sludge treatment systems may become poisoned due to contaminants in the effluent which kill off the microflora responsible for oxidation of the organic matter. Microbial or algal toxicity tests may be more easily used than cell cultures for such screening, however if a cell culture system is already being used for screening final effluent quality, it would also be appropriate to use it for screening the influent to the activated sludge chamber. In such a case a positive and negative control influent for toxicity would have to be used, as the wastes would be expected to be quite 'dirty' and not generally suitable for cell culture screening.

Another potential use for the methods described here is in screening for algal toxins in red tides. These toxins are produced by dinoflagellates which bloom when water temperatures heat up and surface turbulence is reduced so that mixing throughout the water column is minimized. *Gonyaulax* species and *Gymnodinium breve* are most often associated with production of these toxins [Wood, 1968] and when these algae are filter - fed upon by molluscs, toxin levels can bioaccumulate to very high levels which can be detrimental when ingested by humans. Depending on the toxin, one of two reactions is evoked; Paralytic Shellfish Poisoning (PSP) which paralyses muscular contractions and inhibits conduction along nerves [Evans, 1970], causing brain damage and death very soon after ingestion of toxic shellfish, or a less severe form of poisoning which causes diarrhoea after ingestion (Diarrhoetic Shellfish Poisoning (DSP)). For many years, the only method used to test for these toxins was by feeding test shellfish to rats or mice and observing toxicity in the form of

diarrhoea or death [APHA, 1970]. More recently chromatographic methods have been developed to separate these toxins, as chemically some have been identified as being virtually identical to saxitoxin [Evans, 1970] and ocadiac acid, however development of reliable techniques has been slow, due to the presence of a number of toxic components in active extracts [Risk *et al.*, 1979]. Recent work has used HPLC methods successfully for this task [Min. Agriculture and Food, 1990] but such methods may involve complex purification steps. Cell culture assays have been used to screen for these compounds [Aune, 1987] and it is suggested here that cell culture assays using the endpoints described here could be more widely used for this application, in centres where in vivo tests are still used (e.g., in the Fisheries Research Centre in this country).

Although outbreaks of PSP are not common in this part of the world, some incidences have been reported close to these waters, such as an incidence in Northumbria in 1968 [Eavns, 1970]. In the Southeast of Ireland however, outbreaks of DSP do occur. During the course of this work when the application of these assays was being investigated, no toxic shellfish were sampled from Irish waters, so it was not possible to put this application into practice.

More than 20 genera from the phylum Pyrrhopyta contain organisms which produce potent poisons [Risk *et al.*, 1979]. Toxins similar to those discussed are also produced by blue green algae. *Lyngbya majuscula* for example, is the causative agent of a severe contact dermatitis known to affect bathers at beaches in the Pacific. The active constituents of extracts from these organisms (aplysiatoxin, debromaplysiatoxin and lyngbyatoxin) have been shown to be tumour promoters in vivo. In other countries, including Ireland, death of cattle and other agricultural animals from other cyanobacterial toxins is not an unusual occurrence [L. Brava, personal communication] when they drink from fresh water contaminated by blooms of toxic microorganisms. Use of the assays described in this thesis would be an appropriate and relevant way for agricultural authorities to screen for such toxins over critical period when the microorganisms bloom.

Monitoring for toxicity on Tributyltin (TBT), which is carried out regularly by analytical methods in the UK, could also benefit from miniaturized cell culture cytotoxicity testing. Some UK marine sites showed elevated levels of microgram / litre values (*i.e.*, higher than concentrations known to affect survival of larval fish, crustacea and molluscs) while discharges from dry dock areas were found to exceed 40 µg/l TBT [Ministry of Agriculture, Fisheries and Food, 1990]. Zucker *et al.*, [1989] in examining cytotoxic effects of TBT and other trialkyltins on murine erythroleukemic cells, demonstrated cytotoxicity at above 1µM TBT (0.29 µg/ml), thus it should be feasible to use the assays described in this thesis to detect toxicity from the TBT levels experienced in contaminated sites.

In monitoring for other contaminants such as organochlorine pesticides and PCBs, analysis of body tissue of aquatic organisms may be used to provide samples with levels concentrated over those found in the water column. Eels, because of their high levels of fatty tissue, have been used by the UK Ministry of Agriculture, Fisheries and Food [1990] for this purpose, providing results from some sites which were much greater than those encountered previously in fish tissue. Again use of a miniaturized cell culture assay could conceivably be used for monitoring extracts from such samples to denote cytotoxicity (and perhaps other parameters such as mutagenicity/ carcinogenicity) to human tissues.

Cell culture assays have been well used in the past for cytotoxicity testing in quality control of surgical equipment [Wilsnack, 1976] and dental material [Wennberg, 1986]. More recently they have been used in the water industry to test for cytotoxicity in leachates from water exposed to fittings in plumbing [Ashworth and Colbourne, 1981], and in detecting the presence of toxins in water for re-use. Because Ireland's water supplies are more abundant than other European countries, at the present time this application may not be as important as in other countries, however in treatment of water for drinking purposes, water quality may be monitored by using cell culture assays similar to those already used *e.g.*, Fauris *et al.*, [1985], and the assays described in this work would be relevant to such an end.

6.28 Problems with using cell cultures in screening of environmental compounds

Problems with screening samples of environmental importance relate to the 'dirty' nature of many such samples. Animal cell cultures in vitro are quite fastidious and often difficult to grow even in the presence of favourable conditions (this is easily seen from the section on primary culture), so addition of a waste sample high in dissolved and suspended solids may cause significant differences in osmolarity from culture medium, and other differences which may not relate to toxicity in in vivo systems, or indeed less fastidious in vitro systems such as microorganisms, and this may result in cell death unrelated to the toxic contaminants present in the medium. Clean-up procedures, similar to those used in chromatography analysis may be used to deal with this (some such methods are described by Birkholz [1982] and Samoiloff *et al.*, [1983]), but this of course increases the complexity of the procedure and makes it less attractive for use as a screening method. In relatively clean samples (e.g., water samples) the actual sample may be used to make up the growth medium by dilution of concentrated medium or by addition to powdered medium [Hunt *et al.*, 1986]. In water samples where the contaminant level may be very low, samples may be concentrated by a range of methods [Jolley, 1981]. Concentrates of treated hospital wastewater appeared to be more toxic in studies by Cody *et al.*, [1979], than concentrates of tap water samples, so this approach would appear to offer potential to many areas. Of course it is important to use the relevant controls for such tests, because salts and other molecules may also be concentrated, and these may also affect the growth of the cells.

In screening effluent samples throughout treatment processes, another factor of importance is time to when results are available. In such cases it is imperative that a result is obtained rapidly so that effluent holding is kept to a minimum (as holding effluent may also create odour problems additionally). In order to detect low levels of toxicity, small seeding densities of cells should be used due to masking effects at higher cell densities, thus an assay displaying good sensitivity at low cell numbers should be used. Continuous exposure assays would give greatest demonstration of effect over

short periods, thus a 24 - 48 hour continuous exposure is suggested for use for this application (it may not be suitable to hold effluent for longer periods).

6.29 Screening of new drugs

Screening of new drugs is generally carried out to demonstrate low toxicity of a chemical for potential therapeutic use. While this is in progress though, cytotoxic agents may be identified which may then be considered for use as antineoplastic agents. The merits of using in vitro methods for toxicity testing of new agents have been well recognized [Goldberg and Frazer, 1989] and have been discussed more thoroughly in the introduction. The number of antineoplastic agents in routine therapeutic use is indeed quite small, and significant moves are being made in the discovery of new agents. Strategies for preclinical discovery and development of such agents, have, in the last 30 years, been based largely on testing of agents in mice bearing transplantable leukemias and solid tumours derived from a limited number of murine and human sources [Alley et al., 1988]. This screening system however, has been largely unsuccessful in the discovery of new agents with activity against human solid tumours [Marsoni and Wittes, 1984].

With the development of the concept of tumour cell heterogeneity [Fidler and Kripke, 1977; Heppner, 1984], the realization came that more than one patient's tumour of a particular histological type of solid tumour should be represented in a tumour screen [Von hoff et al., 1985]. To address this problem the NCI sponsored a program using a human tumour cloning system as a screen for new neoplastics [Shoemaker et al., 1985]. There are many theoretical reasons why cloning techniques are the most biologically attractive method for screening. Prime amongst these is that stem cells in tumours represent those cells capable of sustained replication and are thus the critical cells in the tumour [Steele, 1977]. In addition, it has been said that accurate assessment of cell survival requires the use of a clonogenic assay [Hill and Dendy, 1983]; other growth assays may only measure cytostatic effects rather than cytotoxic effects.

However, the stem cells in the tumour may not equate with the colony-forming cells in a clonogenic assay - in placing cells in a totally foreign and artificial environment (e.g., extremely low densities, no cell-cell interaction, totally different nutrient and oxygen levels to that found in the tumour), this in itself can lead to a distortion of cell behaviour [Susumu and Ottenbreit, 1978]. More importantly, it may be that the stem cells are not the only critical cells in the tumour; they may be the ones most sensitive to the drug, and it may be that other cells remain to form resistant sub-populations. It has been shown that 90% of clonogenic cells can be killed by CCNU without causing a significant delay in tumour growth [Stephens and Peacock, 1977]. There are also many technical problems associated with clonogenic assays, e.g., low plating efficiency [Hill and Wheelan, 1983], unsuitability of some tumour types to colony formation [Von Hoff et al., 1981; Selby et al., 1983], clumping, and difficulties with distinguishing clumps from colonies at assay time [Agrez et al., 1982], but of most practical importance is the length of time taken from assay beginning to end [14 -21 days] and the extreme labour intensiveness involved in reading the plates (manual counting).

The feasibility of implementing an alternative approach has been investigated by the National Cancer Institute, U.S.A. This new approach uses combined in vitro/in vivo screening for selective cytotoxicity among panels of human tumour cell lines derived from a broad spectrum of human solid tumours, and simpler, quicker endpoints for assessment of drug efficacy [Alley et al., 1988].

The goal of the program is to evaluate experimental agents against groups of cell line panels, each representing a major clinical category of human malignancy. Each panel (e.g., lung, colon, melanoma, renal, ovarian, and central nervous system) contains multiple, representative human tumor cell lines. Agents showing differential or selective patterns of in vitro growth inhibition are evaluated subsequently in athymic mice bearing the same human tumour cell lines found sensitive in vitro. Simultaneous testing of new drugs in both tumour and normal tissues has also been suggested [Gosalvez et al., 1976] as a consideration to detect agents with selective toxicity against tumour cells.

In the work presented here it has been shown that some simple in vitro systems have been capable of demonstrating the efficacy of Navelbine, a novel vinca alkaloid. The toxicity of this compound was compared to Vinblastine, but contrary to expectations, it was not as toxic as Vinblastine itself. Navelbine may be an important agent because of its potential high toxicity, a possible absence of cross-resistance to Vincristine [Maral *et al.*, 1984] and its relatively low toxicity in cancer patients. In this study however, it did not show enhanced toxicity to non small cell lung carcinoma cell lines, as might have been expected. This was felt to be a direct attribute of the drug itself, and not a defect in the system used for toxicity screening, as a differential toxicity effect among cell lines was seen both with Vinblastine and Navelbine, especially with SK-LU-1, which was considerably more resistant to both agents than were the other cell lines (SCC-9 was more sensitive). The pharmacokinetics of Navelbine have not yet been fully elucidated though, and it is possible that the active metabolite(s) may show the specificity for non small cell lung carcinomas expected. Unfortunately our collaborators working on this aspect of the drug [Rhamani *et al.*, 1987] could not supply such metabolites at the time of this study.

The rationale for using cell lines in screening for new drugs has been extensively considered [Boyd, 1986]. Use of fresh human tumour cells for primary drug screening is theoretically more acceptable, but the technical problems involved in this are extensive (see section 5), so use of a wide variety of human tumour cell lines representing many different forms of human cancer offer an attractive alternative [Boyd, 1989].

The fact that the small number of carefully chosen cell lines in this study has demonstrated differential drug efficacy to different cell types is encouraging, and it emphasises the importance of including a wide range of cell types from different tissues, as is the current NCI strategy (at least 60 human tumour cell lines are now being used). The miniaturized assays used in this work were also found not only to be capable of detecting the differential sensitivities mentioned above, but offered all the advantages associated with such endpoints, alluded to previously, which are of particular importance in the screening of new drugs because of the scale of such projects (the NCI screen

300-400 chemicals per week!, [Kerkvliet, 1990]). The different endpoints have given results that are not absolutely the same, (and reasons for this have been discussed already) but the trend was always the same, so any one assay allows the main findings to be seen.

Overall, these assays have clearly shown the cytotoxicity of Navelbine to a range of human tumour cell lines, and thus demonstrate the effectiveness of these endpoints in preclinical screening for new antitumour agents.

6.30 Rapid assessment of Multiple Drug Resistance

Multiple Drug Resistance (MDR) is a phenomenon by which cells spontaneously develop resistance to a range of structurally unrelated drugs. It is thought to be associated with altered membrane permeability, resulting in a decreased drug uptake and/or efflux of drug [Inba and Johnson, 1977; Kessel and Wheeler, 1984]. This emergence of resistance is the major cause of death in small cell lung cancer, breast cancer, ovarian cancer, acute leukemia, and others [Kaye, 1988]. It is of importance in the treatment of human cancers, as those initially responding to chemotherapy may suddenly, and for no apparent reason, acquire resistance, not only to the drug used for treatment, but to a host of other drugs which are then rendered useless in the management of the patient. Some cancers (colon carcinoma, for example) are inherently resistant to several agents. While a wide range of chemotherapeutic agents are rendered inactive by occurrence of this phenomenon, not all are. Resistance to Anthracycline Antibiotics, Vinca Alkaloids, Epipodophyllotoxins and Actinomycin D occurs, but this does not happen with Alkylating Agents. This means that if a patient's tumour could be demonstrated as MDR positive or negative, the treatment regime could then be designed around alternative drugs, which would not normally be chosen, but which would not be affected by MDR.

Extensive work is taking place in this whole area. To design experimental protocols for rapid, routine demonstration of MDR in

tumors, a complete understanding of the mechanisms involved must be acquired. In the study of these mechanisms, experimental establishment of MDR lines from sensitive lines, is a valuable tool.

The lines are grown in medium containing low levels of one of the agents known to be involved in MDR (most often the Anthracycline Antibiotic, Adriamycin (Doxorubicin)). Gradually subpopulations of cells adapt to growing in this level of drug, and when this happens, the drug concentration is increased. Thus the resistant cells are constantly selected for, and gradually a population of cells will result which is many orders of magnitude more resistant to the drug, than the original (parent) cell line. The cell line may then be demonstrated to be cross-resistant to other drugs which it may never have been in contact with before.

Studies on variant cell lines, in comparison with the parent lines, can then demonstrate any differences, which will be valuable in understanding the mechanisms involved and in identifying markers which will aid in the diagnosis of MDR tumours.

In this whole area, initial ability to demonstrate MDR will rely on toxicity testing of selected agents. Regular monitoring too, of variant cell lines will be an essential procedure, for which use of a rapid, sensitive toxicity assay will be essential. Circumvention of MDR can be carried out by using various compounds including calcium channel blockers, e.g., Verapamil and calmodulin antagonists [Tsuru *et al.*, 1982; Kessel and Wilberding, 1985]. The ability to screen for such efficacious compounds, and for their performance in complex combined therapy regimes will also be largely dependent on the availability of such an assay. Endpoints such as cell counting or scoring will be prohibitive to such a goal, whereas miniaturization would bring certain advantages to the system.

The work presented here shows that the APNaOH assay, as with the other colorimetric assays, was a convenient and rapid screen for detecting vastly enhanced resistance in cells expressing the MDR phenotype, over the parent cell line. They also detected the cross resistance of variant cell lines to other agents involved in MDR (Vinblastine), and the continued susceptibility to 5-Fluorouracil and Cis-Platinum.

However, the endpoints were less well able to discriminate the difference in Adriamycin and Vinblastine sensitivity between the two variant cell lines. This was particularly a problem with MTT and AP2h assays, and is felt to relate to low sensitivity, as the problem was not as evident when the APNaOH assay was used. While Eliason *et al.*, [1990] used MTT to demonstrate resistance modification of Verapamil and Quinidine in Vincristine resistance, the MDR line used was not tested at different levels of resistance, and so this difficulty was not encountered. Problems relating to SRB inability to detect this difference in resistance, may relate to technical problems in this experiment rather than theoretical problems, as OD values were exceptionally low, and this was felt to be due to storing plates after fixing, and before staining, or to incomplete fixing of cells.

In cell culture assays of this type it is important to carry out the determination while control cells are subconfluent (see section 3.14). Depending on the cell type, growth characteristics and cell size, the cell number corresponding to 90% confluency will vary. In 96 well dishes though, the number will be quite small. Toxicity assays aim to result in a range of cell kill from 0% up to 100%, so many wells will have very few cells present. We have already established the sensitivity of the endpoints described here, and in general, for the cell lines examined here, there should be no significant problem with loss of linearity at the cell densities which would be reached in such a toxicity assay. There may be however, significant problems with sensitivity in detecting the low cell numbers which would be evident after 50% cell kill. Take the MTT assay for instance. In a toxicity assay using RPMI-2650, 10,000 cells seeded on day 0, has resulted in 90% confluency in control wells 7 days later. These wells will have doubled approximately 4 times during this period, so there will be in the region of 80,000 cells/well present in control plates. The MTT assay in this work has not been sensitive to fewer than 23,000 cells/well; so at drug concentrations giving 30% kill, the assay will be insensitive, and will be of low sensitivity for drug concentrations giving 50% kill. Thus in expressing IC_{50} values using the MTT assay (and AP2h obviously) these assays may not give an accurate account of cell survival. This problem may or may not be apparent with the NR assay, and should not be a problem with CVDE, APNaOH and SRB methods.

Another factor for concern is the variation associated with these results. While the AP assay is relatively precise, variations incurred with the other assays were substantially greater (see Figures 4.5.1 - 4.5.6). The combination of low precision and low sensitivity together may make a significant impact on the final results from a chemosensitivity assay, particularly in relation to the MTT assay, and this may explain, partially at least, why the MTT was unable to detect the increased drug resistance of DLKP2 over DLKP1.

An interesting observation from these results and other chemosensitivity work carried out during this research programme was an increase in growth noted with small concentrations of anticancer agents, followed by cell kill at higher concentrations. While this finding has not often been particularly significant when SEM values were considered, it has been noticed time and again, especially in the case of Adriamycin (see Figures 4.5.1 - 4.5.6). We can see that slight stimulation has occurred when the AP, SRB and CVDE endpoints were used after Adriamycin addition up to $50\mu\text{M}$, but this has not been evident when MTT and NR assays were used. Other reports in the literature have shown slight increases in DNA synthesis at subtoxic doses of the drug, and Vichi and Tritton [1989] have clearly demonstrated this effect in a number of cell lines at concentrations about one order of magnitude lower than those causing antiproliferative effects when growth medium in cultures at high density has been used up. The results obtained using the endpoints for protein detection and AP activity would agree with this report. MTT and NR however, did not show this effect. The concentrations used to observe the stimulatory effect in the work by Vichi and Tritton were much lower than the concentrations giving the effect in the work presented here, so what is observed in these experiments is obviously the threshold where Adriamycin stops being stimulatory and begins to exert its toxicity.

The discrepancy seen by the two assays may corroborate the points discussed in relation to the ability of the endpoints to exhibit differential sensitivity to agents of differing mechanism of action; - it may be that mitochondrial and lysosomal - function damage have occurred at these Adriamycin concentrations whereas the cellular functions measured by the other endpoints haven't yet been impaired, or it may simply be that the discrepancy is due to assay insensitivity

(a 10% increase in growth is not detected as well in MTT assay as in other assays). Perhaps again it is a combination of both, for Mimnaugh *et al.*, [1985] have shown Adriamycin-induced mitochondrial lipid peroxidation at lower levels than were needed for other lipid peroxidation (50 - 100 μ M), or other harmful cellular events.

Overall, any one of the methods was acceptable for detecting gross differences in resistance/ sensitivity to chemotherapeutic drugs, but MTT and AP2h assays were not able to determine small differences in resistance (1 μ g/ml - 2 μ g/ml). On this point, it is felt that it is inappropriate to describe results from resistance experiments as giving '2- fold' resistance or '20- fold' difference for example, without giving the increase in terms of drug concentration, as this depends very much on the method used to detect this difference in sensitivity. An increased resistance from 0.01 to 0.1 μ g/ml is a tenfold difference in resistance, but so is an increase of 100 to 1,000 μ g/ml; but the ability to detect these differences may not be equal for a particular endpoint.

The APNaOH assay has been the most useful of the endpoints here, as it has detected the difference in drug resistance between DLKP1 and DLKP2, has had better precision than any of the other assays, and as has been pointed out previously, was easier to perform.

6.31 Usefulness of miniaturized assays for chemosensitivity testing

Certainly miniaturized, semiautomated, colorimetric assays (MSCA) seem to offer numerous advantages over other methods for chemosensitivity testing used in the past, particularly in comparison with the human tumour stem cell assay (HTSCA). These include the following:

(1) Improved plating efficiency:

The biggest problem with the HTCSEA is low plating efficiencies for most tumour types, resulting in low numbers of evaluable samples despite attempts to improve this [Hill and Wheelan, 1983]. In monolayer assays, including MSCA, plating efficiencies are obviously improved.

(2) Less problems with clumping:

Unlike other assays including MSCA, the HTCSEA requires that single cell suspensions are plated but in practice this is difficult to achieve without sacrificing cell viability. Agarez et al., [1982] identified an important source of error in clonogenic assays to be counting of existing aggregates of cells which had enlarged during the course of the experiment, as true colonies. Re-aggregation of isolated cells under assay conditions was also considered to be a problem, as were artifacts such as cell volume changes [Rockwell, 1984].

(3) Simpler assay outcome evaluation:

Manual counting of colonies in the HTCSEA was more than tedious despite attempts to automate this by computerized image analysis and staining clumps by incubation with MTT [Alley and Lieber, 1984], which still presented problems. MSCA are also simpler to set up and perform than many of the other available endpoints already discussed.

(4) Less hazardous:

MSCA do not involve the use of radioisotopes and are thus obviously less hazardous than the metabolic assays already described which involve radioisotope use.

(5) Quicker to perform:

Using the assays described in this thesis, a chemosensitivity profile may be obtained relatively shortly (approximately 4 days) after testing. This is obviously much faster to perform than the HTCSEA

(which takes 2 - 3 weeks) and of equal duration to many of the other assays discussed (shorter drug exposure periods may result in inaccurate determination of cell viability [see Weisenthal, 1983]).

(6) Fewer numbers of cells needed:

Because the assays are miniaturized, fewer cells are needed, so greater numbers of replicates, drugs and drug concentrations may be tested than may be tested with most of the assays already considered. Although Capillary Tumour Systems were developed [Von Hoff *et al.*, 1983, 1990] which improved tumour growth and resulted in fewer cells being needed, the miniaturized assays considered here still represent an improvement over this.

These and other related technical problems are reviewed by Arbuck *et al.*, [1985] and Shoemaker *et al.*, [1985].

The question as to the relevance of clonogenic as opposed to non-clonogenic assays remains unclear though. The presence in the tumour, of distinctly different subpopulations of different cell lineage has by now been generally accepted, and to say that the clonogenic cells are the most relevant tumour cells to assess for chemosensitivity is highly questionable [Weisenthal 1984, 1985]. In vivo tumours are a mixture of dividing and temporal/ non-dividing (G_0 cells) [Steel, 1977]. It has often been speculated that it is the failure to affect G_0 cells which is partly responsible for ineffectiveness of chemotherapy in human neoplasms [Drewinko *et al.*, 1981] therefore the relevance of testing the total cell population (including G_0 cells) and not only the clonogenic cells, is seen. Even in this respect, it is still presumptuous to think that the clonogenic cells truly represent stem cells at all [Suda and Ogawa 1983]. A further rebuke to the clonogenic assay system is the requirement for using single cells, which result in loss of normal cell-cell interactions [Miller *et al.*, 1981] and while such interactions occurring in non-clonogenic assays are unlikely to mimic those in vivo accurately, they are more likely to represent the in vivo situation more closely. The emerging picture is that clonogenic assays seem to be of less relevance than originally suspected, and the use of non clonogenic assays such as MSCA described here may be more relevant.

A significant problem related to the use of non-clonogenic assays however is the contribution which normal cells present in the tumour will make to the overall response. Continuing developments in generating media specific for each tumour type may alleviate this problem, but cell characterization techniques will be needed to confirm the identity of the tumour cell population. Dietel (1989, personal communication) has considered that the presence of 20% fibroblasts in monolayer chemosensitivity assays does not significantly affect test outcomes, but minimization of normal cell growth is theoretically essential to the success of the experiment.

The findings of this thesis would seem to indicate that sensitivity to cell number may be a problem when MSCA are used in toxicity testing. While this is more serious for some endpoints more than others, any attempt to increase sensitivity in these endpoints should be encouraged. My feeling is that the MTT assay should be replaced with other similar assays of higher sensitivity, such as the APNaOH, SRB or NR assays (after suitable validation with previously used assays such as HTCSA and Thymidine incorporation assays) which involve fewer technical difficulties. Development of fluorometric assays with increased sensitivity still, would also be seen as advantageous. It would then be appropriate to use other methods of expression of results, such as area under the curve (AUC), which tells more about the total response than simply IC_{50} values - it is felt that the low sensitivity of some of the assays at low cell number (high cell kill) may not give an accurate indication of the lower part of the dose response graph, and thus may not reliably contribute to this figure at present. Obviously the relationship of cell viability could be further established under different circumstances, however it seems from this work that the AP assay is no worse than the MTT assay in this respect, and the NR is possibly an improvement on these. Certainly all are no worse than trypan blue dye exclusion assays, so this type of assay, because of its technical cumbersome would seem to offer no real advantages over MSCA.

Other aspects of chemosensitivity testing systems as they stand which require improvement are the drug exposure regimes in use. It would seem that a single continuous dose is not sufficiently similar to the in vivo system as to be appropriate. Calculation of drug concentrations to give exposure to cells similar to the peak plasma

levels in the patient, is obviously more relevant and should perhaps be implemented on a wide scale. Some workers [Darling and Thomas, 1983] have expressed the viewpoint that 'recovery' assays - where drug is removed after a certain amount of cell kill has occurred, and remaining cells (which will be of extreme relevance in the patient) are monitored for recovery ability - may be much more appropriate than those which simply measure cytotoxicity after a single exposure. Such assays are more likely to distinguish between cytotoxicity and cytostaticity (not distinguishable by many endpoints) as the distinction is certainly of relevance in vivo for cells which will remain and repopulate the tumour.

In monolayer assays, another possible area for artefacts is viable cell loss during washing procedures - especially for loosely adherent cells. This would therefore favour the use of endpoints with fewer washing steps and manipulation steps (in this regard the AP assay is preferred to the NR and SRB assays).

Of course the overriding factor as to whether one specific in vitro sensitivity method is superior to another depends on which provides the best degree of clinical correlation [Elson et al., 1982], however the number of relevant clinical trials carried out have been small. The need for trials utilizing MSCA is hereby established.

6.32 Relevance of chemosensitivity testing

General opinion seems to be that individualized chemosensitivity testing can improve the success rate of therapy as opposed to empirical regimes. It is apparent from the literature that once one chemotherapeutic treatment has failed, the chances of another treatment being successful are extremely low [Mattern and Volm, 1982]. There is therefore a clear need for a system that would at least indicate treatments that are doomed to failure [Double, 1975]. In two prospective clinical trials, Von Hoff et al., [1990] using a capillary cloning system and Gazdar et al., [1990] using the Weisenthal dye exclusion assay reported superior clinical responses with chemotherapy regimens based on in vitro drug sensitivity testing compared with those based on clinical experience (21% versus 3% response rate in Von Hoff study; 25% versus 7% in Gazdar study for in vitro versus

clinician respectively). While the results did not appear to be statistically significant (due to low numbers both of evaluable tumours and of patients for which results could be compared) they clearly indicated that in vitro drug sensitivity testing does offer promise in this area. Two areas which prevent in vitro chemosensitivity testing from improving patient prognosis are the lack of ineffective drugs and drug regimes with differing mechanisms of action to combat multiple drug resistance, and poor tumour growth in vitro. These still remain to prevent tangible improvements in patient prognosis accruing from drug sensitivity testing. In vitro tests, particularly those considered to be particularly useful from the work in this thesis, can positively affect the search for new anticancer agents because of the increased speed these systems offer (so that the rate at which the huge backlog of new agents for testing can be screened is improved). Significant improvements in in vitro tumour cell growth is however needed before the predictive drug testing will make a difference to many patients - any attempts in this area will be beneficial to eventual improvement in prognosis for patients receiving chemotherapy (and radiotherapy).

6.33 Primary culture of Bronchial Tumours

Each year in Ireland, more than 1,500 patients die from lung cancer, thus it represents around 25% of deaths from cancer in this country. The World Health Organisation [1981] has recognized four main histological types of lung cancer, with differing patterns of clinical and biological behaviour and various prognostic and therapeutic implications. These distinctions are as follows: Small Cell Carcinomas, Squamous Cell Carcinomas, Adenocarcinomas and Large Cell Carcinomas. Since the former are relatively responsive to therapy [Duchesne *et al.*, 1987], these tumours are less likely to be presented for surgery. The object of the work described here was to identify factors important in establishing a primary lung cultures, (worthy of further investigation) thus it did not lend itself to follow-up experiments due to inadequacy of tissue samples and lack of time available for this work. By its nature, primary culture is a time consuming, and often poorly rewarding area in terms of generatable data, nevertheless it is an extremely important area of study in order to further understand the biology of the

cancer cell. Difficulties in establishment of cell lines from lung tumour tissue are universal - Duchesne *et al.*, [1987] for example, reported no success at culturing primary non-small-cell tumours in vitro from 41 tissue specimens, while 10 such cell lines were established from xenografts. No cell lines were generated from the work described in this thesis, nevertheless some important observations were made, and these are discussed in the following pages.

In a primary culture programme, it is essential to have complete co-operation and enthusiasm from the participating surgeon, who is familiar with the aims of the study, and who will only supply tissue which is relevant to the programme. For the most-part of this programme, collection of samples necessitated attending the operation and receiving the sample at the precise moment of tissue excision. This ensured that the microbiological status of the sample could be directly controlled, which was essential for subsequent work and ensured that a sample was collected. While this meant that a great amount of time was spent waiting for samples, it was generally accepted that this was a necessary and worthwhile procedure.

Limited success was attained after attempting to culture 84 thoracic samples, mostly lung. Because lung tissue comprises more than 40 different cell types, and includes large amounts of cartilage, elastic fibres, smooth muscle fibres, the presence of significant numbers of stromal cells was therefore expected. It is therefore inherently more difficult to culture epithelial lung tumours than other tumour types, for example brain.

At surgery, a portion of tissue was resected, and divided up leaving some for culturing and some for cytologic analysis in the hospital laboratory. Such a step is important to ensure that the histology of the sample can be confirmed at a later date.

Mediastinoscopy and Bronchoscopy samples generally resulted from investigative surgery, to enable determination and characterization of malignant tissue by histological analysis.

In Figures 5.11 (a) and (b), the remarkable importance of seeding density could be seen for a lung tumour primary culture. It was seen that in a 24 well dish, 10,000 - 100,000 cells (which would be expected to produce excessive growth in a short time, for many of lung cell lines) did not result in significant cell growth after 7 days. For the tumour sample shown, 250,000 cells seeded in a single well were necessary to significantly encourage cell growth. Using with epithelial primary cultures, Middleton [1976, 1977] demonstrated that pigmented retina epithelial cells lacking contacts with other cells, spread poorly on the substratum, but spreading was promoted when the isolated cells formed stable lateral contacts with other cells. This may help to explain the poor growth and spreading observed here with cells at low density.

It is also clear from work with normal and malignant cell lines that a certain cut-off seeding density is present, below which cells will refuse to grow, and above which they will grow successfully. This may be due to secretion of autocrine growth factors into the culture medium [Todaro and De Larco, 1978] which may be diluted to ineffective levels if not being produced in high enough levels. A further relationship between growth factors and cell density has been shown by Centrella [et al., 1987] with respect to the growth stimulatory effect of Transforming Growth Factor type β (TGF β) on osteoblast cells. In confluent cells, DNA synthesis was progressively enhanced by treatment with 0.15 - 15ng/ml TGF β after 23 hours treatment, whereas in subconfluent cells, 15ng/ml was less than maximal, and in sparse cell cultures, this concentration was inhibitory. Such a relationship, or similar conflicting relationships regarding cell densities, may exist with other growth factors present in serum, explaining why the effect of increasing cell seeding density does not simply result in a linear response of cell proliferation.

Density dependent growth observations have led to the use of feeder layers of cells to stimulate monolayer primary cultures as well as growth in agar [Mc Manus and Clynes, 1984]. Differences in growth factor production as well as numbers of growth factor receptors in cells from different tumour tissue, will obviously mean that cut-off densities needed for establishment of a culture will vary with individual samples. However, repeated observations of density

dependence during this work led to the belief that significant growth would generally not occur unless very high cell numbers (at least 7.5×10^4 cells/ 24 well dish) were seeded. Generally it was found that mediastinal and bronchoscopy samples were too small to allow isolation of sufficient numbers of cells to establish the culture. This observation led to setting up such samples in smaller dishes as opposed to 25cm² flasks (30 mm dishes and 24- well cluster dishes), however growth was still modest. It is possible that 96 well dishes would improve their growth somewhat.

For one third of the samples processed however, there was no attachment to the substratum. This may have been due to requirements for basement membrane proteins such as fibrin, collagen and laminin which mediate the substrate binding and cytoplasmic spreading of anchorage dependent cells, and have been found to be important in promoting the attachment, growth and differentiation of diverse cell types in vitro [Gummer *et al.*, 1990, Daneker *et al.*, 1989]. This has led to commercial formulations of such products (which are very expensive). Attachment factors such as laminin have intrinsic mitogenic properties and thus also function as growth factors [product information on Biomatrix Products, Universal Biologicals Ltd., 1988]. Whilst lack of time prevented investigation of these points, they have been raised to explain why many of the tumour samples may have failed to attach initially - clearly this area needs more work when culturing lung tumour tissue. Obviously there is a great possibility that in some (if not many) of the samples in question, the cells were dead (trypan blue exclusion counts would not be sufficient to demonstrate this, as cells which had only just died would be most likely to have intact membranes still).

In many of the cultures, fibroblast growth was favoured over epithelial growth. In one third of cultures, fibroblast growth occurred even though no epithelial cells grew. This may be due to the isolation procedures used, or to inadequacy of the culture medium which selectively encouraged fibroblast growth. In almost all cases when epithelial cell growth did occur, fibroblast cells (even if present in very low numbers) eventually overgrew the cultures. It is possible that the fibroblasts are being activated or stimulated to grow when cultured in vitro with epithelial cells. Shkrapp [1990]

reported the in vitro growth stimulation of neoplastic lung epithelial cells by lung fibroblast-conditioned medium, however it may also be possible that they produce an inhibitor for epithelial growth. It is also possible that the epithelial cells present produce paracrine factors which stimulate the growth of fibroblast cell types.

Fibroblasts are relatively easy cells to culture in vitro, as the lower plating densities needed have demonstrated (Figure 5.11.c) - thus it seems that the nutritional balance of the culture medium being used is suitable for these cells, but is lacking where epithelial cells are concerned. Investigation into the effect of some different basal media on the growth of these two cell types did show significant differences in the extent of epithelial cell growth. Additionally some differential growth between the two cell types was observed. This work was only preliminary but pointed to the need to investigate this factor more fully. A combination of Hams F12 and MEM (50%) followed then by Hams F12 alone encouraged greater epithelial cell growth than fibroblast growth. RPMI-1640 however preferentially encouraged fibroblast growth.

In 3.7% of samples significant cell growth did occur, however cell growth did not continue long enough for cell lines to be established. In some cases again, fibroblast growth overtook that of the epithelial culture, however in some cases the culture simply stopped proliferating. Cells became darker and more granular and began to differentiate. While controversy still exists around the relationship between differentiation and proliferation, it has been suggested that these are two independent parameters which may have unrelated prognostic significance [Dische et al., 1989]. Nevertheless the inverse association between the two has been often observed [Foulds, 1969] (and thus has led to the use of grading techniques for determining prognosis [Kemp and Hendrickson, 1982]) - cell division usually stops when signs of specialized structures or functions become apparent in cells that have been dividing at less than the maximum rate [Berril, 1963]. It is not yet fully known what makes cells which are growing in culture begin to differentiate. Serum has been shown to cause terminal squamous differentiation of normal human bronchial epithelial cells, the primary factor involved

(for normal human bronchial epithelial cells) being reported by Masui *et al.*, [1986] to be TGF β . High concentrations of extracellular calcium and vitamin D₃ has also been shown to be important in induction of differentiation of squamous cells [Lechner *et al.*, 1983, 1984], so the use of low calcium media is important. Miyashita *et al.*, [1989] have suggested that while an increase in ionized cytosolic calcium is associated with serum-induced squamous differentiation, serum factors in addition to TGF β mediate such changes. Thus the importance of the culture medium, including serum, is obvious; not only in affecting growth rate but in regulating differentiation - it is thought to be the most crucial variable in vitro irrespective of the tissue culture system used [Hodges, 1976]. Barnes *et al.*, [1980, 1984] postulated that each differentiated cell type has its own unique growth factor requirements, and that differentiated tumour cells have nutritional requirements similar to those of their normal counterparts, and Gazdar and Oie [1986] has found the former postulation to be true for the major forms of lung cancer. These workers recognize that the growth requirements for squamous cell carcinoma are complex and not fully defined, and part of the problem lies in a failure to identify all of the complex requirements for permitting replication of squamous cells while avoiding terminal differentiation.

The extreme importance of serum on primary lung growth was demonstrated in this work for a number of tumour samples. Not only does the concentration of serum drastically effect cell growth (10% serum was needed for substantial growth) but the influence of different batches was unquestionably shown. Thus the need for screening serum batches on a number of primary samples was seen. The recurrent poor performance of NuFCS was noted in 3 separate samples when compared to some FCS batches (Figures 5.13 (a) and (b), 5.13.4), but the differing effect of 15FCS compared to 10FFCS is seen in the two different samples in Figures 5.13 (a) and 3.13 (b). The possibility of using an 'indicator' cell line to screen for good and bad serum batches of relevance was considered, and in screening 5 lung cell lines over an entire range of serum batches (see section 4.1), the similar response of Calu 3 (a very slow growing adenocarcinoma) to the primary culture tested in Figure 5.13.4 was observed. If a significant relationship could be shown to exist, in

terms of serum preference for this cell line and a number of primary lung samples, then serum screening with this cell line would help to eliminate bad batches for primary culture work. It may also highlight batches which would be specifically good for primary culture of similar tissues (note that the response of Calu-3 was significantly different to the other lung lines - and the batches offering better growth differed - thus choice of a 'good' serum batch for cell line growth may not be effective for primary culture.

Supplementation of batch 25FCS serum with added growth factors did not result in increased growth, however the addition of hydrocortisone to 10% serum (Figure 5.14) did result in significant stimulation over growth as seen alone. It is therefore clear that the relationships involved in growth factor stimulation are highly complex. Either T.I.E.S are inhibitory in combination or these growth factors are competing for binding sites with H. The importance of added growth factors was also found by Gazdar and Oie [1986], who reported that in addition to Insulin, Transferrin and a steroid hormone, serum, cholera toxin, low Ca^{++} and feeder layers were required for lung squamous cell culture growth.

Relatively few cell lines have been established from the primary carcinomas of many organs, and many of the cell lines which are established are from metastases [Leibovitz, 1986], thus it would appear that with the present culture conditions only some tissue samples are capable of forming cell lines. Additionally the heterogeneity of tumours, which has been well established [Holozinger *et al.*, 1980] suggests that not all cells in the tumour are capable of continually renewing themselves, and it may only be a certain subpopulation of cells (the clonogenic cells, which may equate with the stem cells *in vivo*) which may be capable of forming a cell line. For chemosensitivity testing, establishment of cell lines is not important, but obtaining adequate cells for testing is. In such work it is imperative that the cell types present in the culture can be identified so that chemosensitivity testing of the tumour cells only can occur. Immunocytochemistry techniques may be used to distinguish different cell types, however distinguishing between normal and malignant epithelial cells is more difficult. Cytological indications, similar to those used in histological techniques for identifying malignant cells, such as increased numbers of nucleoli

and increased incidence of abnormal mitoses may be used [Gilvarry *et al.*, 1989]. Flow cytometry technology with sorting facilities can create invaluable opportunities in this area if such is available to the investigator for use.

6.34 Success of various processing methods

Culture media enriched with cell clumplets from mechanical disaggregation was found to generate the greatest numbers of samples with epithelial cell growth. In comparative work where tissue pieces were disaggregated by mechanical and enzymatic methods, more fibroblast growth was present in the enzymatically treated culture. This too was noted by Leibovitz [1986] and Dietel *et al.*, [1987]. Blood and lymph cell contamination was thought to be a significant problem in many of the cultures, as these cells settled on the substratum and prevented cell attachment. Cells which did attach appeared granular and did not proliferate, compared to counterpart cultures with fewer blood cells present. This may have been due to physical factors such as settling, or due to toxins or growth inhibitors released by blood and lymphatic cells. Impairment of macrophage function can be achieved by addition of tumour conditioned medium (CM) [Otu *et al.*, 1977], however there are several reports that tumours can stimulate macrophages [Meltzer *et al.*, 1977, Mantovani, 1978]. Cocultivation of tumour cells and macrophages therefore, may stimulate the latter cell type [Kaplan and Seljelid, 1977]. Thus the importance of cell separation techniques was considered to be extremely important, and significant efforts in this area would be worthwhile. The presence of dead and dying cells is also inhibitory to viable cell growth, due to the release of lysosomal enzymes (this led to the development of detoxification media for sample transport and growth [Leibovitz, 1975]). Discontinuous density gradients have been found useful for separation of red blood cells, most hemopoietic cells, dead cells and debris [Leibovitz 1985], however this area needs further work. Techniques for separation of stromal cells would be most welcome. Utilization of differential substrate affinities for attachment have been used to address this method [Riddle, personal communication; see Steinberg, 1970].

6.35 Fibroblast removal

In comparison of a number of modifications of differential trypsinization for removal of Fibroblasts, use of 0.04% EDTA and 0.01% Trypsin at 2°C for 10 minutes showed most success, however this was determined to be of use only when epithelial cells were present in tightly growing colonies, and not as single cells or disperse colonies invaded by fibroblast growth. Toxicity of Geneticin to fibroblast cells was demonstrated in a time dependant, dose response manner. It was established that little cell kill occurred after a single day's exposure to the drug - at least 2 day exposure was recommended. In addition, concentrations greater than 250 μ g/ ml did not elicit any further cell kill after 2 or more days exposure to Geneticin. The differential toxicity of this agent to a number of passage 3 fibroblast cultures was also seen, so this must be borne in mind. Generally though, use of 200 μ g/ ml Geneticin for 2 days in a number of mixed primary cultures (subsequent to this work) did not result in good success, so the problem remained to a large extent. It is thought that prevention of fibroblast growth by cell separation techniques during sample preparation, and use of low calcium medium would offer a better approach to control of fibroblast growth in these cultures.

7. CONCLUSIONS

For each cell line shown there was a linear relationship between cell number and OD for each of the assays. This however should first be established for each cell line and for the endpoint to be used, as a linear relationship may not be assumed (this was not as clear with SCC-9 cells for example as with the other lines shown here).

The AP assay was found to offer the best combination of sensitivity, linearity, flexibility, ease of performance, reproducibility and precision, of all the assays.

The CVDE assay was easy to perform and combined moderate sensitivity with linearity. In some repeat experiments however it was much less reproducible than the AP, NR or MTT assays. If results are required immediately, then the time lag while plates are being dried after rinsing is disadvantageous, but if plates are to be stored and read later, this may be advantageous.

The NR assay was reasonably insensitive to low cell numbers (and was less precise (greater variation in replicate wells) than some of the other methods) but was more reproducible (as regards inter-assay variability) than the other assays and was least prone to variation in assay activity when cells in different phases of growth were tested.

While the SRB assay was almost as sensitive as the APNaOH method, it involved many more steps, and appeared to be less reproducible and precise.

The MTT assay, although simple and quick to perform was technically cumbersome because the formazan crystals are prone to easy disturbance, resulting in lower precision than the other assays. It was also relatively insensitive. The SDS modification of this assay which was particularly easy to perform, and overcame the technical difficulty of the MTT assay, was substantially less sensitive than the normal method, and thus was found to be of limited value.

Differences in activity per cell were apparent for each of the assays described, when cells are grown under different conditions. It is therefore vital to construct standard curves of cell number versus OD for these assays under the precise conditions of the experiment, if absolute values of cell numbers are required.

In 6-day continuous exposure experiments, the assays described in this thesis were found to be equally relevant in assessment of toxicity for a range of chemicals with mechanisms of action related to their action. They were also found to correlate well with viability determined by clonogenicity after 4 or 5 days exposure to Vinblastine (at the specific concentrations described). Up to 2 days after drug exposure however, none of the miniaturized, colorimetric assays described, accurately assessed cell viability but overestimated it as did the Trypan Blue exclusion method.

Small variations in cell pretreatment regime and trypsinization duration directly before a toxicity experiment did not result in substantial differences in the outcome of the toxicity experiment. Cell seeding density, drug exposure regime and duration of assay however, directly affected the subsequently determined IC_{50} .

Cell seeding density, basal medium, serum batch and added growth factors all have substantial effect on establishment of a primary lung tumour culture.

The miniaturized, colorimetric assays described here were successfully used as rapid indicators of serum batch activity, multiple drug resistance and efficacy of new drugs.

The assays described in this work have numerous potential applications in environmental toxicity testing, including those shown in this thesis.

8. RECOMMENDATIONS

Further work on cell separation, attachment factors, optimization of basal medium and supplementation with growth factors is essential before the assays described here can be make an successful impact on chemosensitivity testing of lung tumour samples.

Problems of fibroblast overgrowth and cell differentiation are inhibitory to culture success, and need to be further addressed.

In using cell culture assays for environmental toxicity testing, substantial work on sample pretreatment and biotransformation is required.

the 1990s, the number of people in the UK who are aged 65 and over has increased from 10.5 million to 13.5 million, and the number of people aged 75 and over has increased from 4.5 million to 6.5 million (Office for National Statistics 2000).

There is a growing awareness of the need to address the needs of older people, and the need to ensure that the health care system is able to meet the needs of older people. The Department of Health (2000) has published a strategy for older people, which sets out the government's commitment to older people and the need to ensure that the health care system is able to meet the needs of older people.

The strategy for older people (Department of Health 2000) sets out the government's commitment to older people and the need to ensure that the health care system is able to meet the needs of older people. The strategy is based on the following principles:

- Older people should be able to live independently and actively in their own homes.
- Older people should be able to access the services they need to live independently and actively in their own homes.
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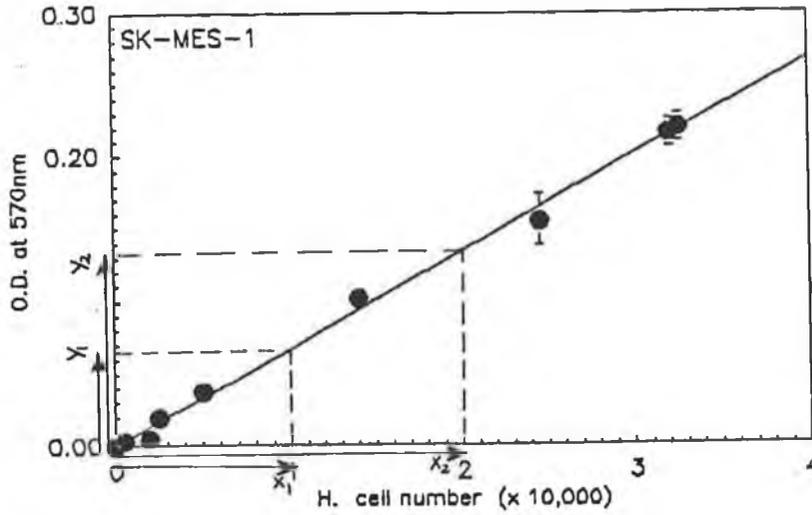
APPENDICES

APPENDIX A.

Calculations

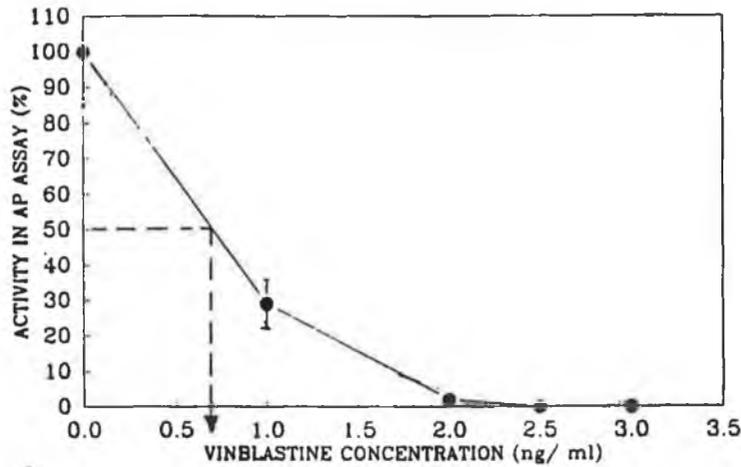
(a) Sensitivity:

Calculate slope of linear portion of line



$$\frac{Y_2 - Y_1}{X_2 - X_1} = \frac{39 - 19.5}{70 - 35} = \frac{19.5}{35} = 0.557$$

(b) IC₅₀:



IC₅₀ = 0.7 ng/ml

(c) Toxicity, in Toxic Units (TU's) = 1000 / IC₅₀ = 1,428.6

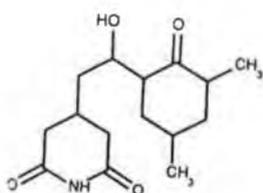
(d) SEM formula:

$$\sigma \approx \sqrt{\frac{\sum_{i=1}^n (x_i - \bar{x})^2}{n(n-1)}}$$

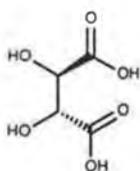
(e) Coefficient of Variation: = mean/ SEM

APPENDIX B

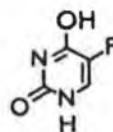
Molecular structures of compounds used in toxicity studies



Cycloheximide



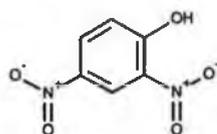
L-Tartaric Acid



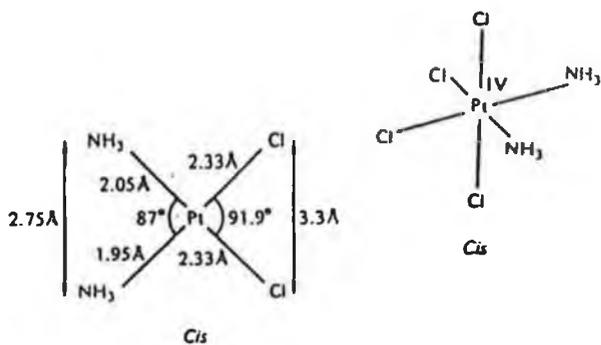
5-Fluorouracil



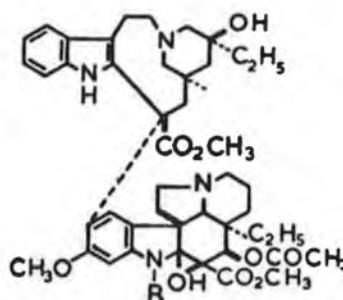
Hydroxyurea



2,4-Dinitrophenol

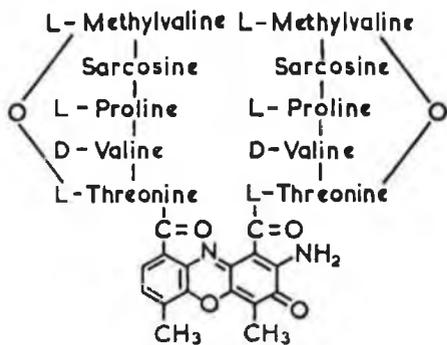


Cis-Platium

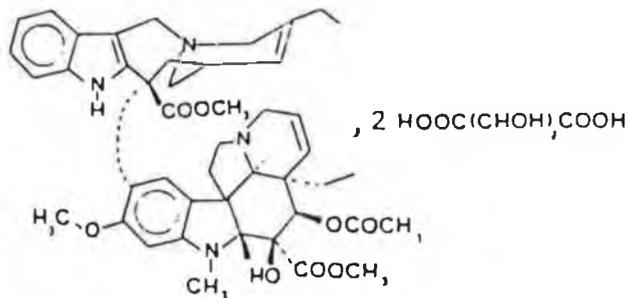


R = CH₃

Vinblastine



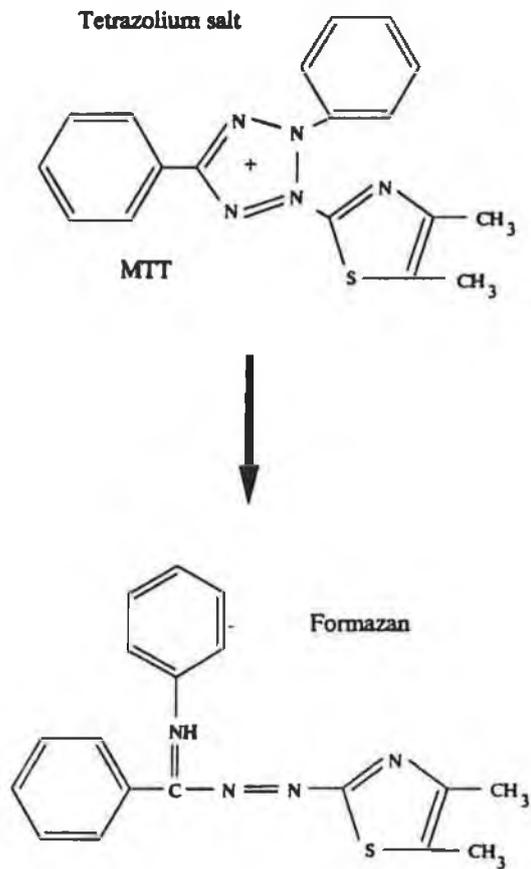
Actinomycin D



Navelbine

APPENDIX C

Conversion of MTT to MTT formazan:



APPENDIX D

Abbreviations

AP	Acid Phosphatase
A.T.C.C	American Type Culture Collection
B.O.D.	Biological Oxygen Demand
C.M.	Collection Medium
C.O.D.	Chemical Oxygen Demand
CVar	Coefficient of Variation
CVDE	Crystal Violet Dye Elution
D.C.S.	Donor Calf Serum
D.H.S.	Donor Horse Serum
D.M.	Dissection Medium
DMEM	Duelbeccos modification of MEM
D.M.S.O.	Dimethyl Sulphoxide
E.C.	European Community
EC ₅₀	Effective Concentration for 50% of population
E.D.T.A.	Ethylene diamino tetra-acetic acid
F.C.S.	Foetal Calf Serum
F.D.A.	Food and Drugs Administration
F.R.A.M.E.	Fund for the Replacement of Animals in experimental testing
G.A.A.	Glacial Acetic Acid
H.E.T.	Hens Egg Test
H.T.S.C.A.	Human Tumour Stem Cell Assay
IA	Image Analysis
IC ₅₀	Inhibitory Concentration to 50% of population
ID ₅₀	Inhibitory Dose to 50% of population
L.A.	Local Authority
MDR	Multiple Drug Resistance
MEM	Minimal Essential Medium
MTT	3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
MTT/SDS	SDS modification of MTT assay
N.E.A.A.	Nonessential amino acids
NR	Neutral Red
NBCS	New Born Calf Serum
O.D.	Optical Density
O.E.C.D.	Organisation for Economic Co-operation and Development
P.B.S.	Phosphate Buffered Saline
r.p.m.	Revolutions per minute
S.E.M	Standard error of the mean

<i>SDS</i>	<i>Sodium Dodecyl Sulfate</i>
<i>SRB</i>	<i>Sulforhodamine B</i>
<i>S.T.A.</i>	<i>Short Term Assay</i>
<i>TU</i>	<i>Toxic Unit</i>
<i>T.V.</i>	<i>Trypsin Versene</i>

