Use of tissue culture to investigate the effect of methylation on methylene blue cytotoxicity

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ABSTRACT

Animal tissue culture has been in existence for over a century and, for much of that time, has played a subsidiary role to that of other life sciences. However, the discipline has expanded rapidly over recent years and is now firmly established as a science in its own right. The main purpose of this study was to research the historical and modern development of animal tissue culture and to gain practical expertise in some of the basic techniques which are employed in a working tissue culture laboratory.

Tissue culture techniques were used to study the effects of substitution on the efficacies of three well known, commercially available dyes, methylene blue, toluidine blue-O and Victoria blue-BO. These dyes, exploited traditionally in industry for their powers as photosensitizers, have now attracted interest as possible agents in clinical photodynamic therapy (PDT). PDT is a novel treatment for both microbial and malignant disease. It combines the application of a photosensitizing drug with red light irradiation, in the presence of molecular oxygen, to induce a cytotoxic response in target cells.

The major proportion of the study examined the effects of methylation on the photocytotoxicity and dark toxicity of the phenothiazinium dyes, methylene blue and toluidine blue-O, against the mammary tumour cell line, EMT-6. The toxicities of the dyes and several derivatives were assayed using standard tissue culture techniques and the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assay. Both 1-methyl methylene blue and 1,9-dimethyl methylene blue exhibited increased

photocytotoxicity, with concomitant higher dark toxicity, compared to methylene blue. A similar trend was found in the toluidine blue series, but with some variation. It would appear therefore, that, in terms of a clinical application, the methylated derivatives may hold greater promise than the parent compound itself.

In addition, the effect of amino substitution on the cellular uptake of the triarylmethane, Victoria blue-BO, was examined, also in the EMT-6 cell line. Victoria blue-BO and its derivative, MOVB, were extracted from cells using methanol and their intracellular concentrations determined spectrophotometrically at 612 nm and 622 nm respectively. Amino substitution reduced cellular uptake at lower concentrations, but at concentrations above 2.5 μM, uptake of MOVB was similar to that of the parent compound.

These results are important, but it must be remembered that, although animal tissue culture is a valuable tool for preliminary testing, cultured cells are not representative of similar cells *in vivo,* and cannot replace whole animal testing of potential drugs.

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DECLARATION

I declare that this Thesis has been composed by myself and that, while registered as a candidate for the degree of Master of Science by Research, I have not been a registered candidate for any other award.

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ABBREVIATIONS

AA.....azure A
AP.....acid phosphatase
BME.....Eagle's basal medium
BSS.....balanced salt solution
CVDE.....crystal violet dye elution
DPIBF.....1,3-diphenylisobenzofuran
DMAA.....1,9-dimethyl azure A
DMEM.....Dulbecco's modified Eagle's medium
DMMB.....1,9-dimethyl methylene blue
DMSO.....dimethyl sulfoxide
EBSS.....Earle's balanced salt solution
EDTA.....ethylenediaminetetraacetic acid
EtNBS.....5-ethylamino-9-diethylaminobenzo[*a*]phenothiazinium chloride
FCS.....foetal calf serum
'HAT' .....'complete' culture medium + hypoxanthine, aminopterin, thymidine
HBSS.....Hanks' balanced salt solution
HEPES.....*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid
HGPRT.....hypoxanthine phosphoribosyl transferase
HpD.....haematoporphyrin derivative
IC_{50}.....inhibitory concentration to achieve 50% of the maximum effect
IMS.....industrial methylated spirit
LD_{50}.....lethal dose required to achieve 50% of the maximum effect
LMB.....leucobase methylene blue
MAA.....9-methyl azure A
MB.....methylene blue
MMB.....1-methyl methylene blue
MOP.....morpholinopropane sulfonic acid
MOVB.....4-morpholino analogue of Victoria blue-BO
MTT.....3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-2*H*-tetrazolium bromid
NMB.....new methylene blue
NR.....neutral red
PBS.....phosphate-buffered saline
PBSA.....phosphate-buffered saline-Solution A
PDT.....photodynamic therapy
PEG.....poly (ethylene glycol)
PTFE.....poly (tetrafluorethylene)
PVC.....poly (vinyl chloride)
SDS.....sodium dodecylsulfate
SRB.....sulforhodamine B
TBO.....toluidine blue-O
TCA.....trichloroacetic acid
TPX.....thermanox
VBB.....Victoria blue-B
VBBO.....Victoria blue-BO
VBR.....Victoria blue-R

1. INTRODUCTION

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1.1 Project background

The primary aim of this study was to acquire practical experience of animal tissue culture and to assess the role of this methodology in scientific research. It was decided that this was best achieved by giving the project a particular direction, rather than it being merely a collection of single isolated procedures. Photodynamic therapy (PDT) is a novel approach to the treatment of malignant disease. It was proposed to study the effects of chemical substitution on the efficacies of several commercially-available photosensitizing dyes which have undergone some preliminary testing as potential PDT agents. Although the use of an ongoing project restricted the range of techniques which could be studied in the time available, it did, however, permit more valuable experience to be gained in the day to day running of a modern tissue culture laboratory.

The bulk of the work was to be devoted to the study of the effects of methylation on the cytotoxicity of phenothiazine photosensitizers. Both the photocytotoxicities and dark toxicities of methylene blue, azure A and several derivatives were tested against the murine mammary tumour cell line, EMT-6. Standard tissue culture techniques were used and, following incubation, the toxicities of the dyes assayed with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-2*H*-tetrazolium bromide (MTT).

In addition, a small proportion of the time available was to be used in support of an existing project, to study the effect of amino substitution on the cellular uptake of Victoria blue-BO and one of its derivatives, also in the EMT-6 cell line.

It was hoped that this work would form the basis of future research involving the PDT of cancer.

1.1.1 Cancer

The majority of cancers are caused by the progressive mutation of specific genes [Soloman *et al.*, 1991], the proto-oncogenes and tumour suppressor genes, which act reciprocally to control the growth and differentiation of normal cells [Weinberg,1985]. The fundamental event is damage to DNA and is likely to be a single mutation in a single cell [Bishop, 1987]. The first stage (initiation), whether induced or spontaneous, is, in itself, insufficient to cause the disease directly. The transformation to malignancy (promotion) is brought about by an accumulation of mutations involving the conversion of protooncogenes to oncogenes, and/or the deletion of tumour suppressor genes [Weinberg, 1991]. This multi-step process occurs, usually in a specific sequence, and often over a long period of time [Marx, 1989]. The succession of subtle changes in the behaviour of the cell favour progression towards malignancy, when the cell is able to overcome all restraining signals and become invasive.

1.1.2 Conventional cancer treatments

There have been few advances in cancer therapy over recent decades, and conventional treatments still remain surgery, chemotherapy and radiotherapy. Surgery is always a drastic measure, can be disfiguring, and there is a danger that microscopic malignancies may be released systemically and thence metastasise. Chemotherapy and radiotherapy exhibit limited specificity due to similarities between normal and malignant cells. The side-effects produced by cytotoxic drugs are often more disabling than the disease itself and so increase morbidity. In addition, malignant cells commonly develop specific resistances or multidrug resistance mechanisms which, in the latter case, overcome the effects of a range of structurally unrelated chemotherapeutic agents [Robinson, 1991].

1.1.3 Photodynamic therapy

Photodynamic therapy (PDT) is a relatively recent approach to the clinical treatment of certain solid tumours. The regime combines the application of a photosensitizing drug with red light irradiation to induce a cytotoxic response, though independently, neither drug nor light has any pharmacological effect [Henderson & Dougherty, 1992]. Inactive drug is administered systemically and allowed to accumulate in malignant tissue where it is subsequently activated by precisely targeted laser light, commonly delivered endoscopically or *via* fibre optics. Absorption of light by the photosensitizer transforms it into a transient electronically excited triplet state molecule which readily transfers the acquired energy either directly to a substrate (Type I Photochemical Reaction) or to ground state triplet oxygen (Type II Photochemical Reaction) according to availability [Foote, 1990] (Figure 1). In biological systems, a Type II mechanism with the formation of singlet oxygen is believed to predominate [Weishaupt *et al.*, 1976; Gomer & Razum, 1984], although other highly reactive species such as superoxide or hydroxyl radicals may also participate. The toxic free radical species formed by the action of singlet oxygen on such cellular components as amino acids and cholesterol, initiate a direct killing of the tumour cell. In addition, PDT causes the rapid occlusion of tumour blood vessels, depriving the malignant cells of nutrients and oxygen [Reed *et al.*, 1988]. Since PDT drugs require light activation, the use of fibre optic delivery systems allows tumours to be targeted specifically and ensures a localised response. The regime has a further advantage in that very rapid healing rates with considerably less scarring are obtained, compared with conventional laser ablation.

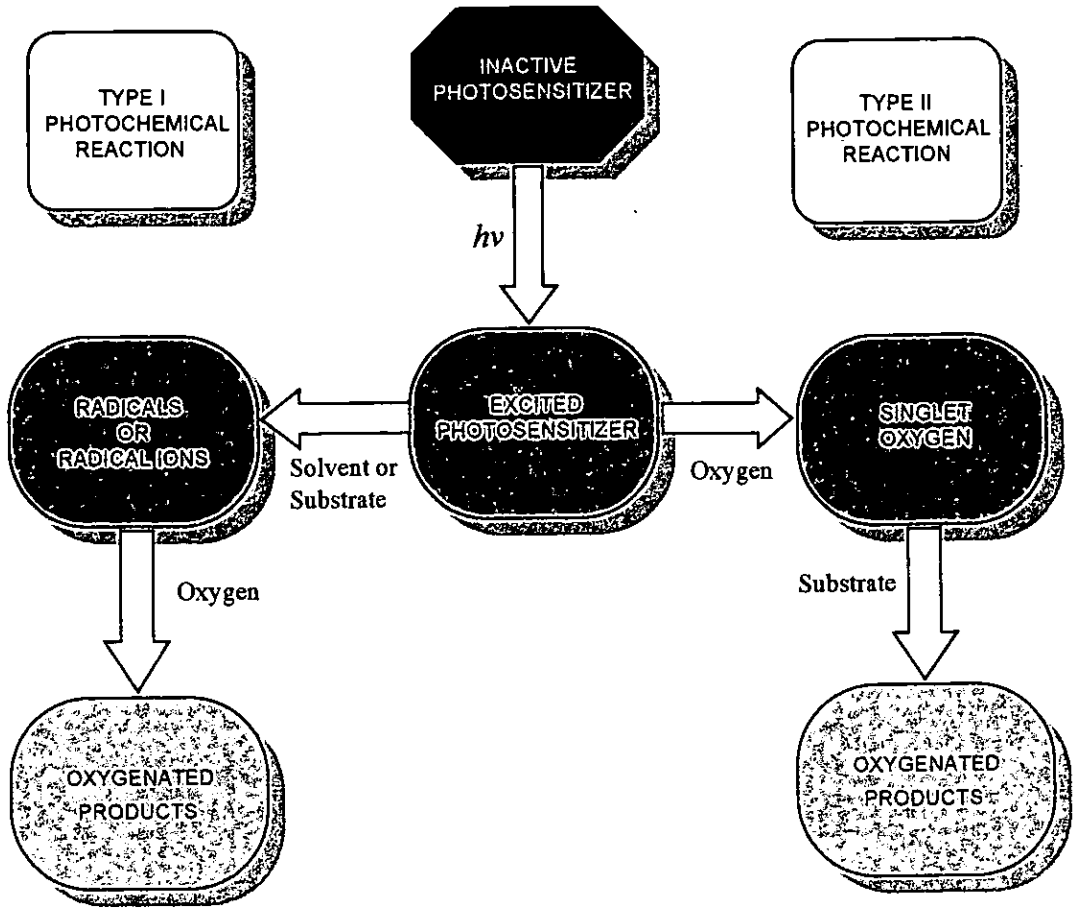


Figure 1. Chemical mechanisms of photodynamic action. (Adapted from Foote, 1990)

Until recently, the porphyrin-related molecules, haematoporphyrin derivative (HpD) and its purified counterpart, porfimer sodium (Photofrin®) have been used almost exclusively as agents in clinical PDT [Kessel & Thompson, 1987]. This virtual monopoly is surprising, considering there are several disadvantages associated with the use of Photofrin®, and given the vast array of both natural and synthetic compounds which could be considered as alternatives. Porphyrins are, in fact, natural molecules; in the metabolic disorder, porphyria, for example, a dysfunction of heme metabolism is responsible for an accumulation of phototoxic blood porphyrins in the skin which causes cellular damage when activated by visible or UV light. Unfortunately, the apparent selectivity of Photofrin for neoplastic tisssue is not as great as originally believed, and the drug is also found to accumulate in tissues high in reticuloendothelial cells, *eg.* liver, kidneys and adrenals [Bugelski *et al.*, 1981]. Its localisation and retention in the skin leads to prolonged skin photosensitization

necessitating that patients avoid exposure to bright light and sunlight for up to eight weeks following treatment [Dougherty *et al.*, 1990]. Furthermore, the maximal absorption band of porphyrins in the blue region of the visible spectrum (around 400 nm), is not aligned with the "therapeutic window" (600-800 nm) which permits deeper light penetration into tissue, and avoids problems of light scattering associated with shorter wavelength irradiation. Long wavelength light is of most use in the treatment of tumours since tissue penetration typically doubles between 630 and 730 nm (Svaasand, 1984; Wilson *et al.*, 1985). Consequently, porphyrins are illuminated in the near infra red (around 630 nm) where their light absorption properties are poor.

The need to address the limitations of current photosensitizers has compelled the search for more effective agents, with considerable efforts having been made to investigate existing compounds and to design and synthesise new analogues. Second generation photosensitizers for cancer therapy still tend to be porphyrin-related molecules with improved light absorption properties, such as the synthetically modified bacteriochlorins [Spikes *et al.*, 1993], or *de novo* porphyrin analogues, the phthalocyanines [Oleinick *et al.*, 1993]. However, more recently, the vital stains, such as crystal violet, methylene blue and Nile blue, which have been in use for over a century, have also attracted interest as potential photosensitizers in clinical PDT.

1.1.4 Methylene blue

The phenothiazinium photosensitizer methylene blue (MB) has a long tradition of use as a disinfectant and in pre-surgical vital staining and histology. Like many other cationic dyes, MB demonstrated *in vivo* cytotoxicity against animal tumours in the 1940s, prior to the advent of modern PDT [Lewis *et al.*, 1946; Riley, 1948]. More recently, the ability of the dye to intercalate with DNA has led to its widespread acceptance as a photosensitizer in the eradication of viruses (including HIV) from whole blood [Zeiler *et al.*, 1994].

Unlike porphyrins, MB and its analogues absorb light strongly within the therapeutic range (*i.e.* > 600 nm). In addition, it is possible that the rapid metabolism of such compounds by healthy tissue and greater tumour selectivity would avoid the prolonged skin sensitization which is problematic in conventional PDT (Section 1.1.3).

MB is reported to exert its photodynamic action mainly *via* the intermediacy of singlet oxygen (Section 1.1.3), but the dye is rapidly reduced *in vivo* to the neutral leucobase (LMB) by standard redox systems [Bongard *et al.*, 1995]. This, and an inherent (dark) toxicity associated with the photosensitizer, may explain the apparent dismissal of MB as an agent in mainstream PDT. Nevertheless, some success has been achieved using MB to treat inoperable oesophageal tumours [Orth *et al.*, 1995] and cancers of the bladder [Williams *et al.*, 1989]. In these circumstances, it is possible physically to direct the photosensitizer to the target site, thus minimalising systemic side effects.

1.1.5 Toluidine blue-O

Toluidine blue-O (TBO) is a closely related commercial analogue of MB which, too, has been widely used as a vital stain. TBO has proved particularly valuable in the diagnosis of oral disease since it will selectively stain oral cancers and various oral pathogens. Combined with its actions as a photosensitizer, this selectivity has been exploited further in modern dentistry to eradicate oral pathogens such as *Streptococcus mutans, Lactobacillus casei* (Wilson *et al.*, 1995) and some oral *Candida* strains which are commonly associated with AIDS patients. TBO has also demonstrated photocytotoxicity against methicillin-resistant *Staphylococcus aureas* (MRSA) (Wilson & Yianni, 1995) which is a major problem in post-operative situations such as biofilm infestation of implants, catheters, *etc.*. Another refractory strain of pathogens, *Helicobacter,* involved in gastric ulcers and cancer, is also susceptible to photodynamic treatment using TBO (Millson *et al.*, 1996).

1.1.6 Victoria blue-BO

The cationic triarylmethane, Victoria blue-BO (VBBO) is one of a series of dyes related to crystal violet, but which have a phenyl group replaced by naphthyl. Crystal violet itself has been widely used for many years in topical antimicrobial medicine (Browning, 1964). The Victoria blue dyes are well known in histology and cytochemistry where they are used in diagnosis and for the visualisation of fine structures. In contrast, the phototoxic properties of these dyes have been little studied, although VBBO and Victoria blue-R (VBR) have demonstrated photocytotoxic effects in the human squamous cell carcinoma (FaDu) and human melanoma cell lines [Wadwa *et al.*, 1988], whilst the phenylamino analogue, Victoria blue B (VBB) has shown antitumour activity in animal models [Riley, 1948].

Like the phenothiaziniums, the triarylmethanes also absorb light maximally in the 'therapeutic window' for PDT (Section 1.1.3), but it is believed that the flexibility of the ring structure of the compounds in solution probably results in the rapid deactivation of the excited state, and thus a low degree of photosensitizing power. However, once the dye structure is immobilised, an increase in photosensitizing activity ensues, as could occur when the dyes are bound to biomolecules within mitochondria [Oster, 1955]. As with MB, concomitant dark toxicity is a problem with VBBO which may yet be eradicated by modifications to the structure.

1.2 Animal tissue culture: a definition

Animal tissue culture has been in existence since the beginning of this century. At that time, the term "tissue culture" aptly described the first rudimentary attempts to culture cells from large undisaggregated fragments of tissue. Growth was sporadic, and dependent upon the migration and occasional mitoses of cells into the periphery. Today, cultures may be initiated from organs (organ culture), fragments of tissue (primary explant culture) or dispersed cells (cell culture) [Schaeffer, 1990] and are

roughly divided into two main types - primary and established (or 'continuous', or 'immortal') cell lines. The industry is now in a phase of specialisation, with a wide range of different tissues from different organisms able to be cultured using many sophisticated and refined techniques. The nutritional requirements and growth conditions of individual cell types *in vivo* are now established, and can be simulated *in vitro*. Well defined nutient media for feeding cell cultures have been formulated to meet the needs of many cell types. Tissue culture operations range from the culture of a single cell to large scale mass production at the commercial level. Strict regulations are in place to ensure that all work with human or animal tissue conforms to medical ethics or current animal experiment legislation.

1.2.1 Primary cultures

A primary cell culture is one initiated from cells taken directly from tissue (whole blood, for example), discrete organs (liver, heart, kidney, *etc.*) or whole organisms (chicken embryos). Almost any species may be used, depending upon the requirements of the experiment, although the choice may be limited by the availability of the tissue concerned. Human cell types such as lymphocytes from blood or tonsils, skin fibroblasts, umbilical cord and placental cells, are all readily available, but cell types from internal organs are generally very difficult to acquire. However, some of these may be obtained, with the patient's and surgeon's permission, from the margins of diseased or malignant tissue, during routine surgical procedures. Three main methods of isolation are used.

Organ culture aims to preserve the three-dimensional structure of the tissue *in vitro*. This is optimised by growing cultures at the liquid-gas interface on a raft, grid or gel. Organ cultures are particularly suitable for the study of growth metabolism, tissue differentiation and the effects of specific allergens. In primary explant culture, a tissue fragment is allowed to attach at a glass (or plastic)-liquid interface from where cells migrate in the plane of the solid substrate. Cell culture is the culture of dispersed cells

from an original source. The tissue or outgrowth from a primary explant is disaggregated, either mechanically or enzymatically, to form a cell suspension which may then be cultured as such, or as an adherent monolayer on a solid substrate.

Mechanical methods of disaggregation may involve the tissue being carefully chopped with scissors, then rinsed and pressed through sieves of varying mesh size, or forced through a syringe and needle. However, the favoured method is digestion by proteolytic enzymes, of which, trypsin (normally from pig pancreas), and pronase (protease from *Streptomyces griseus*) are most effective, but may cause damage to cells. To produce a highly viable cell suspension, the process should be a compromise to allow the enzyme to digest the intracellular matrices, but not the cells themselves. Alternatives are collagenase and dispase which are less harmful but may be less effective. In practice, cultures are found to grow best from suspensions which contain small aggregates rather than single, though possibly damaged cells.

Primary cultures are sometimes preferred to established cell lines because they bear the closest morphological resemblance to the parent tissue, and retain some diversity of cell type. In the absence of hormones, cells quickly lose their specialised functions and become increasingly less differentiated with each successive propagation. In fact, cells in culture generally adopt one of two forms and become either spindle-shaped ('fibroblast-like') or polygonal ('epithelial-like'). However, primary cultures are more difficult to propagate than cell lines and require extensive supplements such as attachment factors and collagen to support growth. Relatively few cells are obtained and these tend to grow discontinuously and have a finite life span. Contamination from agents affecting the animal at the time of dissection may be problematic.

1.2.2 Continuous cell lines

Once a primary culture has been subcultured (or 'passaged' or 'transferred') for the first time, it becomes a cell line. Subculture is essential once the culture has outgrown

the available area, and involves stripping the cells from their substrate and reseeding them at a lower density in fresh medium, and into new vessels to produce secondary cultures. Once subcultured, the culture undergoes an important transition from a very heterogeneous mixture of cell types to a more homogeneous cell line. While the growth fraction of the primary culture is likely to be variable, that of the cell line is usually high, and may be up to 80 % or more. Thompson in 1914 [cited in Whitaker, 1972] was amongst the first to distinguish between the organised, regulated growth of differentiated organs and tissues, and the disorganised, uncontrolled growth of undifferentiated cells.

Cell lines can generally be divided into two types- monolayer (adherent) cells or suspension (non-adherent) cells. The method of culture usually reflects the original source of the cells *in vivo*. Suspension cultures, for example, are reserved for cells, such as immune cells or their precursors, which would naturally circulate in the bloodstream, whilst monolayer cultures are suitable for cells derived from organs, and which are not motile in the body.

Cell lines, whether adherent or suspension, may be described as immortal or transformed. Transformed cell lines are derived from tumour cells, or somehow manipulated to produce cells with a novel, transformed phenotype. This may be by tranfection with oncogenes or treatment with carcinogens. Immortal cells, on the other hand, are not necessarily malignantly transformed, but are nevertheless changed to produce a continuously growing cell line.

As a general rule, cells from the primary cultures of post-natal animals die after only a few subcultivations. In contrast, those of embryonic origin may withstand prolonged subcultivation, retaining all the properties of normal somatic cells. This period is characteristically limited according to species; human embryonic cells will survive up to fifty subcultivations, mouse cells up to ten and cat cells up to forty, for example. At

the end of this period, the cells either die or enter a 'crisis' stage when their growth slows almost to the point of extinction. Once 'crisis' is overcome, the cells emerge with radically altered characteristics, and growth resumes, often exceeding its initial rate. The cell line is then capable of unlimited subcultivation, and is said to have become 'established' (or 'continuous' or 'immortal').

Established cell lines are readily available and easy to propagate. They have advantage in that they grow continuously and generate large numbers of cells over relatively few passages. Cells or cell lineages with the highest capacity for growth will predominate creating a uniform identity in the cell population. This allows adventitious contamination to be revealed and establishes the integrity of the cell line. A fully characterised cell line has advantage in quantitation and replicate sampling, but lacks the cell-cell and cell-matrix interactions which are found in organ cultures.

1.3 Origins of tissue culture

The earliest origins of tissue culture can be traced to the nineteenth century and began with the study of tissue and organ specimens which had been preserved in glass vessels. Possibly the first record of successful tissue culture was made in 1885 when Wilhelm Roux kept the medullary plate of a chick embryo alive in a warm saline solution for several days. This was followed in 1887 by Arnold [cited in Whitaker, 1972], who noted that, alderpith fragments, implanted subcutaneously into the peritoneal cavity of the frog, would migrate and survive for several hours when put into saline. It cannot be certain however, whether these and similar experiments, demonstrated actual survival, or merely delayed death, since the experimental conditions created at the time were not fully described, making the work difficult to repeat. However, an important step forward was made in 1898 when Ljunegren [cited in Whitaker, 1972] successfully transplanted human skin which had been kept alive

media are based on this early work to formulate solutions which would allow tissue to survive for short periods outside the host body.

As would be expected, medical science demanded that research remained focused on the use of warm-blooded animals which more closely resemble the human. At first, the fertilised hen's egg was the popular choice for its diversity of cell types, until genetically pure strains of mammals, particularly rodents, became available with the advent of experimental animal husbandry. Rodent tissue had advantage in that it produced continuous cell lines [Earle *et al.*, 1943] and a range of transplantable tumours. More sophisticated media were produced by modification of traditional formulae, making it possible to propagate tumour cells taken from malignant tissue samples of both animals and humans.

In 1952, Gey *et al.*, shifted interest to human tissue by propagating the now well established HeLa cell line from human cervical carcinoma tissue, thus demonstrating that human tumours could also give rise to continuous cell lines. This work was affirmed by the classical studies of Hayflick and Moorhead in 1961 who found that normal fibroblasts from the human foetus could be kept in serial cultivation for long periods of time without loss of integrity of the diploid karyotype. Prior to these studies, heteroploidy had been the major obstacle to the production of human virus vaccines using tissue culture techniques. Moreover, changes in chromosome number had previously been believed to be an unavoidable natural consequence or even the cause of the altered status of a primary culture to that of a cell line.

Of all the biological disciplines, probably virology has benefited more than any other from the development of animal tissue culture. Attempts to propagate viruses in culture can be traced almost to its inception, beginning in 1913, when Steinhardt *et al.* [cited in Whitaker, 1972] found that the activity of the *vaccinia* virus was retained for more than four weeks in fragments of rabbit cornea maintained in a drop of plasma. In

1932, Haagen and Theiler [cited in Whitaker, 1972] successfully cultured a modified Yellow Fever virus in chick embryo extract, this being followed in 1935 by Rivers and Ward [cited in Whitaker, 1972] who carried out *vaccinia* vaccinations using a strain of virus which had been propagated for several years *in vitro*. Many similar experiments were subsequently reported, strengthening the links between the two disciplines. A major breakthrough came in 1949 when Enders and Robbins successfully propagated the Lansing (type 2) strain of poliomyelitis virus in non-neural human embryonic tissue. This work made possible new methods for the detailed study of the biology of viruses. At the same time the discovery of antibiotics revolutionised tissue culture methodology and, consequently, since 1949, there has been an explosion of activity in this field which has benefited many areas of biological research.

1.4 Modern aspects of tissue culture

For many years, the developing role of tissue culture has been seen as subservient to that of other life sciences. In spite of this, tissue culture has established itself as a science in its own right, forging important links between many biological disciplines. It has provided the experimental tools for toxicology, genetics, embryology, cancer research, cell biology, immunology and virology, some of which have already been reviewed in Section 1.3. Much of this is owed to the need of medical research to understand the mechanisms of neoplasia and to produce antiviral vaccines. This has forced the technical improvements needed to develop reliable media and to control contamination, and led to the standardisation of conditions and cell lines. Tissue culture has lent itself to the understanding of DNA replication and transcription, protein synthesis, energy systems, drug metabolism, signal transduction, membrane processes, cell-cell communication, environmental interactions, cell products and secretion, and the genetic analysis of higher animals.

In medicine, tissue culture technology has been used routinely to measure the toxic effects of pharmaceutical compounds and environmental pollutants, to assay viral infections both qualitatively and quantitatively, and to reveal genetic disorders in the developing foetus by chromosomal analysis of cells derived by amniocentesis. It has become possible to propagate an individual's own cells in culture and use these to graft damaged areas of skin in burns patients, for instance. Similarly, a technique has been developed to implant normal foetal neurons into patients with Parkinson's Disease, though this remains an area of controversy on ethical and moral grounds.

Today, much of tissue culture remains devoted to mammalian and avian species. The culture of lower vertebrates and invertebrates has become a specialised area, useful for studying the molecular basis of development, and providing for the needs of agriculture, pest control and fish farming.

1.5 Modern techniques

1.5.1 Cytotoxicity and viability

Cytotoxicity and viability assays are now commonly used for the initial testing of new drugs, cosmetics, food additives, *etc.*. They are cheap, easily quantified and reproducible, and reduce the number of animal experiments which give rise to public concern.

An estimation of short-term viabilty can be obtained using dye exclusion tests on cells which have been subjected to potentially traumatic procedures such as primary disaggregation or freezing and thawing. Membrane damage, and therefore cell death, is indicated by the cellular uptake of a dye (such as trypan blue, erythrosin or napthalene black) to which cells would normally be impermeable [Kaltenbach *et al.*, 1958]. Conversely, it is possible to monitor the release of certain dyes or isotopes

(such as diacetyl fluorescein or ^{51}chromium) which would normally be taken up and retained by viable cells.

Short-term assays, though quick and easy to perform, will only reveal cells that are dead at the time of the assay. Cytotoxicity, however, is not necessarily defined as cell death, but may involve alterations in the metabolism of the cell, or changes in cell-cell signalling which might lead to a reduction in the capacity of the cell to proliferate. Thus, cells exposed to anti-cancer drugs or other toxic agents, frequently only demonstrate these changes several hours or days later, when the dead cells may have disappeared.

Long-term assays of viability for cytotoxicity testing are designed to measure cell survival after, rather than during, exposure to a particular toxic agent. Cytotoxicity testing, along with many other tissue culture applications, has been revolutionised by the development of the multiwell plate, which has facilitated fast, economical replicate sampling of cell cultures. Most popular is the 96-well microtitration plate, each well having 28-32 mm^2 growth area and capacity for 0.1 to 0.2 ml medium, and up to 10^5 cells (Figure 2).

The procedure involves microtitration plate cultures of cells in the rapid (logarithmic) phase of growth, being exposed to a toxin or cytotoxic drug at varying concentrations. The duration of exposure is determined both by the time required to inflict maximal damage, and by the stability of the compound to be assayed. Following removal of the agent, the cells are grown on over two to three more doubling times in order to reveal viable cells capable of proliferation as opposed to viable cells which cannot proliferate. Cell survival may then be determined directly by cell counts or by indirect methods such as isotope incorporation or colorimetric assay. Off these, the reduction of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) dye to formazan is a widely favoured method [Mosmann, 1983], the amount of MTT -

formazan produced being directly proportional to the number of surviving cells, and can be determined spectrophotometrically once solubilised in a suitable solvent.

1.5.2 Cell fusion

Cell fusion is a relatively simple procedure to fuse together the membranes of two adjacent cells. The best known fusion is between antibody-producing normal murine B-lymphocytes from an immunised mouse, and a murine myeloma cell line [Morgan & Darling, 1993]. The parent cells are incubated in the presence of a 'fusogen', commonly poly(ethylene glycol) (PEG), then incubated in 'HAT' medium (*ie.* complete culture medium containing hypoxanthine, aminopterin and thymidine) to select the hybrid cells. Aminopterin blocks *de novo* synthesis of nucleotides, whereas hypoxanthine and thymine are intermediate metabolites used in alternative bypass pathways. The myeloma cell line will die, since it is deficient in the enzyme, hypoxanthine phosphoribosyl transferase (HGPRT), which is required for the alternative pathway. The mouse lymphocytes die off naturally, but fused cells will remain viable because they have inherited the necessary enzymes from the lymphocyte parent to utilise the alternative pathway.

1.5.3 Transfection

The ability to introduce new genetic material into cultured mammalian cells has proved to be one of the most powerful tools for analysing eukaryotic gene expression. Current investigations include research into transcriptional control signals, gene modulation by hormonal and chemical regulators during differentiation, and the identification of cellular oncogenes implicated in carcinogenesis. Transfection is also a method of producing continuous cell lines, and for causing the overexpression of a particular gene, allowing purification of the product, or its large scale production for use as a drug. Human growth hormone, insulin and interferon have all been produced in this way.

DNA for transfection can be presented in several forms including cDNA clones, genomic DNA and genomic DNA clones. The DNA fragment and a suitable vector, usually a bacteriophage, retrovirus or plasmid, are restriction digested to give compatible ends so that, in the presence of ATP and DNA ligase, they join to become a single molecule which can then be used to transfect normal cells.

There are several methods of transfection although one of the earliest techniques, calcium phosphate co-precipitation, is still the method of choice in many circumstances. Alternative methods include DEAE-dextran and Polybrene (these both aim to increase the infectivity of viruses in cells by binding to both DNA and cell membranes), liposome-mediated gene transfer, microinjection (using a fine needle to introduce DNA directly into the target cell membrane) and single-stranded oligonucleotides which may be used to transiently introduce antisense sequences into cells [Morgan & Darling, 1993].

1.5.4 Cell cloning

Cell cloning produces cultures of identical cells derived from a single parent cell. Although this is achieved naturally to some extent through successive subcultivation, often by the time the clone has produced a usable number of cells, it may already be close to senescence. Cloning is a particularly valuable technique because it allows the isolation of a particular strain of cells having specific properties or markers. Nevertheless, over a period of time, genetic changes will occur during the growth of these clones, until the clonal identity of the culture is lost. In most cases, this is not important because, as cells are usually selected on the basis of a single characteristic, the suitability of the clone can easily be monitored throughout the assay. Cloning has been used to isolate specific biochemical mutants and cell strains with marker chromosomes, and is essential for cell fusion, immortalisation and transfection procedures.

The simplest method of cloning involves diluting a prepared suspension of cells, so that each vessel seeded will produce one colony of cells as a result of the divisions of a single cell [Puck & Marcus, 1955]. A large number of replicate cultures may be required to achieve this, but growth in those vessels with a single colony may be encouraged by frequently changing the nutient medium, and by trypsinisation to disperse the cells more evenly. Once confluent, the strain may then be handled as a cell line.

Alternative methods of cloning are soft agar cloning or cloning in Methocel [Buick *et al.*, 1979]. The cells are suspended in agar or Methocel, then plated out over an agar underlay, and allowed to set. In this case, a more concentrated suspension may be prepared, so that several colonies are contained, widely spaced, in each vessel. A small cylinder of agar over each colony is then removed using a sterile cloning ring (usually porcelain or stainless steel), leaving a well in the agar. This is trypsinised to detach the cells which can then be removed using a fine pasteur pipette and seeded into new vessels. Colonies of cells cloned directly into microtitration plates may be isolated by trypsinising individual wells.

1.6 Advantages of tissue culture

Tissue culture avoids the legal, moral and ethical questions of animal experimentation. It is an ideal system for studying the behaviour of animal cells free from normal homeostatic regulation and responses to stress during a whole animal experiment. Moreover, the physicochemical environment can be precisely controlled as regards pH, temperature, osmotic pressure and oxygen and carbon dioxide tensions.

Since cultured cell lines assume a degree of homogeneity under the selective pressure of culture conditions, identical experimental replicates are easily produced, reducing the need for statistical analysis of variance.

Reagents are used more economically when added directly to the cell *in vitro*: *in vivo* higher concentrations must be administered by systemic injection to compensate for losses through excretion and distribution to other tissues not under study.

1.7 Disadvantages of tissue culture

The environmental conditions required by cells in culture are complex, and require a high level of expertise and understanding. Despite very careful technique and monitoring, the risk of contamination, sometimes involving very serious losses, poses a constant threat. The expenditure of effort and materials generally, is enormous compared to the amount of tissue it generates. In fact, the cost of producing cultured cells is approximately ten times that of using animal tissue.

Although tissue culture is a valuable tool for preliminary testing, it is important not to extrapolate too far the results to similar cells *in vivo*. Cultured cells lack the nervous and endocrine components of homeostatic regulation, and the specific cell-cell interactions associated with the three-dimensional geometry of the whole animal. In addition, the loss of differentiation makes it difficult to relate the experimental results obtained from cultured cells to functional cells in the tissue from which they originated. Therefore, until more representative techniques are developed, the requirement for animals will continue, particularly for the preclinical trials of new pharmaceuticals.

2. THE CULTURE ENVIRONMENT

2. THE CULTURE ENVIRONMENT

The environment in which cultures are propagated *in vitro* is comprised of four main elements, precisely defined and regulated, which simulate the cells' natural surroundings *in vivo*. These are the substrate on or in which cells grow, the nutrient medium in which cells are bathed, the gas phase and the incubation temperature. Most importantly, these facilities must be provided and maintained, free from chemical toxins and microbial contamination, using rigorous aseptic technique. In addition, cells must be routinely subcultured in order to replenish the medium and to maintain the culture at an optimum density for growth. To preserve cells not in use and to safeguard against losses from contamination or incubator failure, it is important to be able to freeze healthy cultures at regular intervals, and retain them in liquid nitrogen storage until required.

2.1 Substrate

The type of substrate is dependent upon the characteristics of the cell line to be cultured, the yield required and the sampling regime employed. In the case of most vertebrate lines, the substrate is a solid artificial surface onto which cells adhere and proliferate as monolayers. Alternatively, cells may be grown as suspensions in either a semi-solid (*e.g.* collagen or agar gel [Macpherson & Montaquier, 1964]), or liquid phase [Nagaoka *et al.*, 1990], but these methods are reserved for specialised applications which are not described in this thesis.

A wide variety of artificial surfaces has been used to propagate monolayer cultures. Glass and plastic have proved most popular, but successes have also been achieved using cellophane [Sandström, 1965], cellulose [Savage & Bonney, 1978] and metals, including stainless steel [Birnie & Simons, 1967] and titanium [Litwin, 1973].

Glass has been traditionally favoured for its optical properties and because it carries a slight net negative charge which is found to support growth. However, its use has declined over recent years having been almost completely superceded by disposable, single-use plastic flasks and petri-dishes.

Polystyrene provides a flat, reproducible surface for cell culture with good optical quality. It is by far the cheapest and most common plastic substrate available, although others, including poly(vinyl chloride) (PVC), poly(carbonate), poly(tetrafluorethylene) (PTFE), melinex and thermanox (TPX) may also be used. Manufactured polystyrene is hydrophobic and thus unable to sustain cell growth. It must, therefore, be pretreated either by γ-irradiation, chemically, or with an electric ion discharge to create a wettable, charged surface. It is wise to bear in mind that quality may vary from one manufacturer to another. Popular disposable plastic culture vessels include the 25 cm^2 and 75 cm^2 flasks, 35 mm, 60 mm and 90 mm circular petri dishes, and a range of multiwell (microtitration) plates, of which the 96-well version is the most widely used (Figure 2).

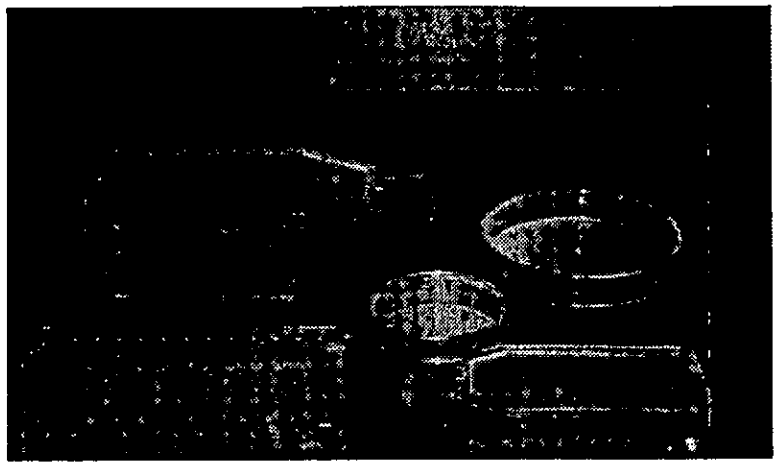


Figure 2. Disposable plastic culture vessels. Clockwise from top right: 90 mm and 35 mm petri dishes, 25 cm^2 flask, 96-well plate, 75 cm^2 flask.

2.2 Media

The function of a medium is to supply to the cell culture a balance of energy and nutrients *in vitro* as close as possible to that experienced *in vivo*, while at the same time, being sufficiently defined so as to permit a measure of control and standardisation.

2.2.1 Constituents of media

Prior to 1960, natural media based on amniotic fluids, embryo extracts, protein hydrolysates, lymph and other tissue fluids were in common use. However, although natural media closely mirror the cellular environment *in vivo*, they lack the standardisation and control vital for modern technology. On the other hand, completely defined media will only support the growth of a narrow range of cells.

Today, there is a vast number of commercial media available but, in essence, they are all comprised of a basal synthetic medium to which various supplements and a buffer are added. Despite its poor buffering capacity at physiological pH, a bicarbonate buffer is most frequently used, though *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid (HEPES) may be preferred since it is a more efficient buffer in the pH 7.2 - 7.6 range, and may be used in a closed system without exogenous carbon dioxide (Section 2.3.2).

The basal medium is a balanced salt solution (BSS) containing a variety of inorganic salts and glucose, with added vitamins, amino acids and other defined components (coenzymes, nucleic acids, hormones and growth factors, for example). A BSS is not capable of sustaining cell growth but will keep cells alive in the short-term, the only important factors being osmotic pressure, pH, a carbohydrate source and dissolved gases. A BSS is, therefore, useful for short incubations up to four hours, as a diluent for 'complete' media and as a washing or dissection medium for primary culture. The two most commonly used today are Earle's BSS (EBSS) which is strongly buffered with sodium bicarbonate in 5 % CO_2 and often used as a diluent for Eagle's media, and Hanks' BSS (HBSS) designed to equilibriate with air rather than CO_2, so having correspondingly less bicarbonate.

2.2.2 Types of media

Defined media are characterised by differences in their basic constituents and the concentrations in which these are present. One of the original defined media, Eagle's basal medium (BME) [Eagle, 1959], is a relatively simple formula commonly used for

monolayer cultures such as HeLa (human cervical) or primary cells. More complex media like RPMI 1640 [Moore *et al.*, 1967] and F12 [Ham, 1965] generally contain an increased number of amino acids, particularly non-essential types, and may be supplemented by minerals and various metabolites such as nucleosides, tricarboxylic acid cycle intermediates and lipids. Dulbecco's modified Eagle's medium (DMEM) [Dulbecco & Freeman, 1959] is a more complex medium based on BME and developed specifically to culture embryonic mouse cells. Like many other media, originally created for specialised cells, DMEM is now also used as an all-purpose medium for a wide range of mammalian lines, particularly adherent monolayer cultures. However, despite many years of extensive research, the choice of medium is still often empirical, and no all-purpose medium has yet been developed to meet the specific requirements of specialised lines.

2.2.3 Media preparation

The majority of basal nutrient media are available in three formulae - powder, ready made (× 1) liquid and (× 10) liquid concentrate. Different types are made up according to manufacturers' instructions and sterilised, then various sterile supplements added immediately prior to use.

Powder medium is the cheapest but most time-consuming to prepare, yet is more convenient when large quantities are required. It also requires a positive-pressure filter- sterilisation system unless autoclavable powder is being used.

Ready-to-use medium is the simplest though most expensive option, and still requires supplements to be added before use.

Liquid medium supplied as a 10 × concentrate is the most flexible method since it can be used when only small quantities are required, and does not require a positive pressure filter-sterilisation system. Again, sterile supplements must be added immediately prior to use.

2.2.4 Supplements

Most supplements are stored pre-sterilised at -20 °C and added to sterile 'incomplete' media when required. The most frequently used supplements are serum, L-glutamine and antibiotics.

Serum is the principal constituent of the majority of growth media, being typically supplemented at a concentration of 10 % v/v, but this may vary with cell type. Most sera are derived from a natural source, usually foetal calf, although newborn calf, donor horse and human may be preferred in some circumstances.

Serum is an important source of polypeptides, hormones, lipids and trace metals, and is vital for its growth-promoting properties. However, natural serum is associated with several disadvantages which have created the need for a chemically defined serum-like substitute, and led to the development of some serum-free media.

Natural serum is expensive with a huge demand which may eventually exceed supply. The product tends to vary from batch to batch [Olmsted,1967; Honn *et al.*, 1975], and a replacement supply must always closely resemble that used previously. Furthermore, despite rigorous testing, serum is still often a source of contamination, often viral, which cannot be eliminated by present methods of filtration. Despite its growth-promoting activities, serum also has inhibitory effects on cell growth, and it is often difficult to predict the results of these combined actions.

Unfortunately, serum-free media have not been as successful as was anticipated. Cell growth is never as vigorous as with natural sera, and fewer generations of finite cell lines are achieved. The supply of such media is limited and expensive, and must be highly specific for each cell type. All reagents must have a high degree of quality and purity, since serum-free media appear to lack the protective, detoxifying action which is associated with some serum proteins. Consequently, serum is still extensively used as a supplement, and most media today are a combination of natural and defined components.

L-glutamine is an essential amino acid for cells in culture. It is a major source of carbon and energy and provides precursors for biosynthesis and protein production. Glutamine is labile in solution and must be added to 'incomplete' medium immediately prior to use. At 4 °C, its half-life is nineteen days, so 'complete' medium can only be stored for up to two weeks.

The antibiotics gentamycin, penicillin and streptomycin are routinely added to cell culture by many laboratories, although laboratories often prefer to limit their use, since they may conceal low-level contaminations. Penicillin at 100 units ml^{-1} is effective against gram positive bacteria, while streptomycin (100 μg ml^{-1}) and gentamycin (50 μg ml^{-1}) will eliminate both gram positive and gram negative types. Gentamycin is a convenient antibiotic since it is broad-spectrum and will also eradicate mycoplasma (Morgan & Darling, 1993).

2.2.5 pH

Most cell lines grow well at or around pH 7.4. Phenol red is commonly added to culture media as an indicator, turning yellow at pH 6.5, orange at pH 7.0, red at pH 7.4, through to purple at pH 7 - 8.

Most culture media have their pH adjusted before sterilisation by slowly adding dropwise either 1 M HCl or 1 M NaOH to the stirred solution. Powder media are usually adjusted to 0.1 - 0.2 points lower than the required pH (about pH 7.0) before filtration because the positive-pressure system removes CO_2 from the medium thus raising the pH. Autoclavable media are adjusted to pH 4.5 during sterilisation in order to maintain the stability of the solution. Sterile buffering solutions are added following the procedure and the pH finally readjusted to pH 7.0 - 7.2.

2.3 The gas phase

The major constituents of the gas phase are oxygen and carbon dioxide.

2.3.1 Oxygen

Oxygen requirements vary with cell type but normal atmospheric oxygen tensions are sufficient for most cell cultures. In fact, some systems, such as human tumour cells [Courtenay *et al.*, 1978] and human embryonic lung fibroblasts [Balin *et al.*, 1976] grow better at oxygen tensions lower than atmospheric. In contrast, organ cultures, particularly late-stage embryonic, newborn or adult cultures require up to 95 % oxygen in the gas phase [Trowell, 1959; De Ridder & Mareel, 1978]. This is more likely to be due to slower rates of diffusion into organ cultures than into dispersed cells, rather than differences in individual cellular requirements.

2.3.2 Carbon dioxide

The by-products of cellular metabolism tend to lower the pH of culture media. A bicarbonate / CO_2 buffering system is used to regulate pH, with CO_2 commonly being supplied to the incubator and bicarbonate (as sodium hydrogen carbonate) added to the medium. Since the net result of increasing atmospheric CO_2 is to lower the pH, this must be countered by increasing the bicarbonate concentration, thereby bringing the culture medium back to pH 7.4. It is possible to vary the amount of bicarbonate at different oxygen tensions in order to achieve the desired pH, although most media are supplied with specific manufacturers' recommendations.

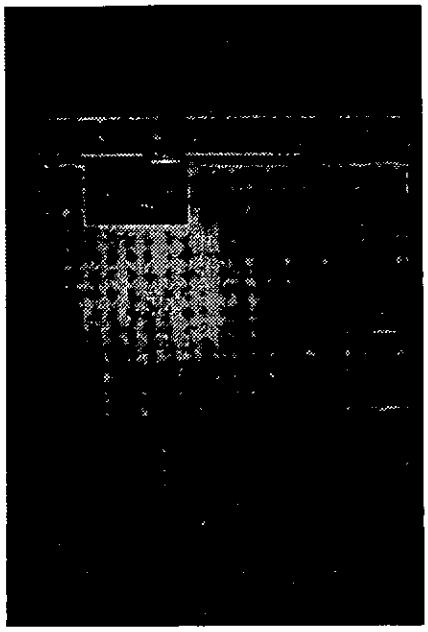


Figure 3. The CO_2 incubator. The monitor at the top left, which includes an alarm, registers humidity, current temperature and CO_2 level.

CO_2 is usually supplied to cultures as a 95 % air : 5 % CO_2 mixture in a CO_2 incubator (Figure 3). Most CO_2 incubators are factory-calibrated for 5 % CO_2 in air in high humidity. A water reservoir is used to provide a highly humidified atmosphere which helps to prevent evaporation of the medium. Once the gas supply is connected and the incubator set to 5 % CO_2, a gas regulator is opened to supply CO_2 at the recommended pressure (Figure 4). Alternatively, 'dry' non-gassed incubators which use HEPES or morpholinopropane sulfonic acid (MOPS) are sometimes preferred because they eliminate the risk of contamination from the water tray.

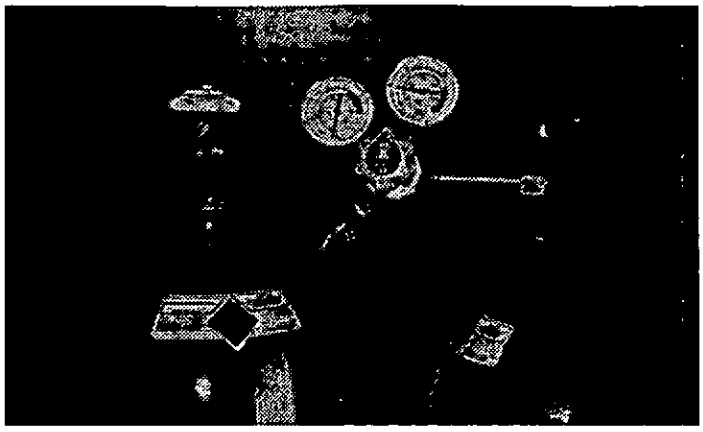


Figure 4. The gas regulator.

2.4 Incubation temperature

The incubation temperature for a particular cell type in culture is dependent upon the animal species from which the cells were obtained and the area of the body in which the cells would normally be found. Cells from the skin or testis, for instance, are naturally found at a temperature lower than those taken from internal regions of the body. Most incubators are set to 37 °C, although a safety factor is sometimes incorporated and the cells incubated at slightly lower than average body heat to allow for errors in regulation. Cultured cells will survive sharp drops in temperature but cannot tolerate more than 2 °C above normal for long, and soon die at 40 °C.

2.5 Subculturing of cells

Growth patterns for cells in culture tend to show an initial lag phase where little or no growth occurs, followed by an exponential or logarithmic increase in cell concentration. Eventually, after a few days, there is a 'plateau' with no further

increase, or even a decline in cell growth. The time interval from inoculation of the culture to 'plateau' varies with cell type, but growth characteristics can be determined by setting up a growth curve for individual strains.

A decline in the rate of cell proliferation is generally due to exhaustion of the medium or lack of availability of the substrate. In the case of adherent cells, this is at the point of "confluence" when the dividing cells have formed a continuous monolayer over the base of the flask into which they were inoculated.

Cells should be subcultured and reseeded at a lower density whilst still in the exponential phase of growth, and when they are approximately 80 % confluent. For cells in suspension, this is relatively straightforward involving simple dilution of the culture. Adherent monolayers, on the other hand, must first be subjected to a trypsin digest which causes the cells to become rounded and enter suspension. They can then be diluted and reseeded to the required density. Trypsinisation is a traumatic procedure and must only be continued just long enough to detach the cells from their substratum. At this point, medium must be added to prevent further enzymatic digestion of the cell culture itself.

The process of trypsinisation is often referred to as "passaging", each trypsinisation corresponding to single passage and, consequently, passage number is indicative of the age of a cell stock. Cultures should be discarded after ten to fifteen passages, since ageing cell populations are likely to incur mutations which bring about changes in cell characteristics.

2.6 Scaling up production

The most popular culture vessels are the 25 cm^2 and 75 cm^2 flasks (Section 2.1, Figure 2) which respectively hold 10 ml and 50 ml of culture medium. The total range of sizes available includes the very small (suitable for 100 µl culture medium) to the very large flask with a 225 cm^2 growing area. However, culture volumes over 50 ml often lead to problems with the efficiency of gas exchange and equilibration, particularly in suspension cultures, which tend to settle in high concentrations at the bottom of the flask. There are a number of methods available with which to scale up production.

2.6.1 Roller bottles

Roller bottles are used to scale up the culture of adherent cells which are able to proliferate over the total curved surface area of the vessel. Only a small volume of medium is required to provide a shallow covering over the cells, which are then rotated at 2 r.p.h. in specialised CO_2 incubators with attachments to turn the bottles along the long axis.

2.6.2 Spinner cultures

Spinner cultures consist of a straight-sided glass flask with a central teflon paddle which is used in conjunction with a magnetic stirrer to agitate suspension cultures. This improves gas exchange, and allows the scale up of production without overcrowding of the cells.

2.6.3 Microcarrier beads

Dextran or glass-based microcarrier beads are available in a range of densities and sizes, and are used with spinner culture flasks, though, in this case, to provide an increased surface area for the culture of adherent cells. This makes it possible to grow large volumes of cells, again without overcrowding. The culture medium may need to be replaced regularly when culturing cells at such high densities, and it is a good idea to add HEPES to the medium to stabilise the pH.

2.7 Cryopreservation

New cell lines and cell strains should be stored frozen as large master "seed" stocks held in reserve, from which secondary "using" stocks may be generated. This using stock should also be frozen and thawed as required. Once depleted, the using stock may be replenished from the seed stock. When the seed stock itself becomes depleted, it should also be replenished, and with the minimum increase in passage number from the first freezing .

Cryopreservation is the storage of cells at very low temperatures. Ideally, liquid nitrogen should be used, since minimal deterioration is observed in cultures stored at temperatures below -180 °C [Green *et al.*, 1967]. Heavy gloves and eye protection must be worn when handling liquid nitrogen, since its extreme low temperatures will cause burning to exposed skin. Alternatively, if liquid nitrogen is not available, cultures may be stored in a conventional freezer at -70 °C although some deterioration may occur.

Cell cultures for cryopreservation should not be confluent or overgrown, but growing in the exponential phase and free from contamination. Healthy cells are suspended at a high concentration in a sterile solution of medium containing an increased supplement of serum and to which a cryopreservative has been added. The cryopreservative may be either dimethyl sulfoxide (DMSO) or glycerol but, in either case, its function is to reduce the water content of cells, thus preventing the formation of ice crystals which would otherwise lyze cells by damaging the membranes. DMSO is a powerful solvent and must, therefore, be treated with extreme caution. It readily and quickly diffuses across membrane bilayers due to its small size and lipid-solubility. DMSO will penetrate not only natural membranes including skin, but also synthetic membranes such as rubber gloves, carrying with it potentially toxic substances which happen to be in use at the time.

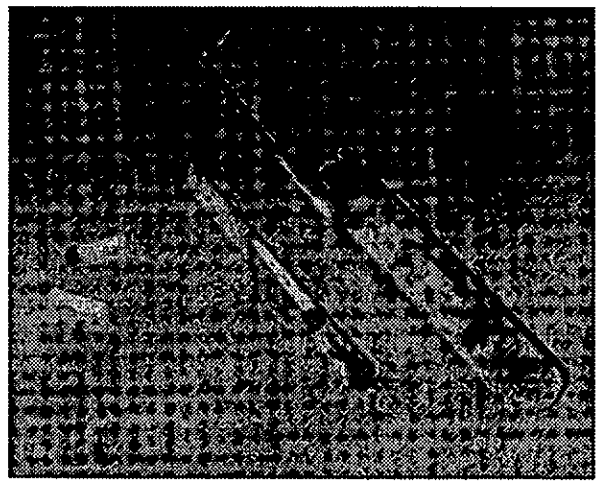


Figure 5. Vessels for cryopreservation. Left to right: cryotubes, freezing straw, freezing canister.

Cells suspended in freezing medium are added in 1 ml aliquots to cold sterile cryotubes. These may be packed into cardboard freezing straws or a polystyrene box,

then frozen slowly, initially in a conventional freezer and ideally at 1 °C per minute. [Leibo & Mazur, 1971; Harris & Griffiths, 1977]. The containers are finally transferred to freezing canisters (Figure 5) and lowered gently into liquid nitrogen (Figure 6). Freezing straws or polystyrene permit cells to be cooled at the required rate in the nitrogen vapour which forms in the upper part of the storage tank.

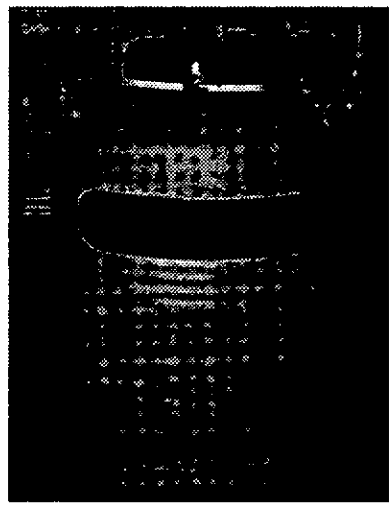


Figure 6. Liquid nitrogen storage.

2.7.1 Reviving cells

Cryotubes of cells to be thawed must be lifted carefully from liquid nitrogen storage and thawed as quickly as possible in a 37 °C water bath. For safety, this may be placed within a closed container, since some tubes may have inspired liquid nitrogen during storage, and will explode when exposed to a sudden increase in temperature. Trypan blue, or another suitable agent, can be used to test cell viability, as not all cells will survive the trauma of freezing and revival (Sections 1.5.1 & 3.8).

2.7.2 Transporting frozen cells

Frozen cryotubes of cells, correctly packaged in solid carbon dioxide or thick polystyrene foam, will remain frozen for up to three days. It is thus possible to send cultures, clearly labelled as to their contents, by post, or preferably *via* a carrier, to other laboratories, although customs must be informed of any international mailings.

2.8 Aseptic technique

Fundamental to successful tissue culture is the provision of a sterile barrier between the pure culture and opportunistic microorganisms in the environment. Aseptic technique is a combination of procedures, based largely on common sense and experience, designed to both protect the operator and reduce the likelihood of contamination, without severely compromising the user's ability to carry out routine manipulations. The technique should be applied as a series of steps (detailed in Sections 2.9 & 2.10), in both the general preparation and immediate handling of cell culture. Although the omission of one or more of the procedures will not necessarily give rise to infection, in the event that it does occur, the point of breakdown will be more easily traced if the regime has been adhered to routinely.

2.9 Laboratory environment

Modern tissue culture laboratories are designed to minimise the entry of microorganisms, yet laboratory personnel must be constantly vigilant in order to maintain a sterile working environment.

The main working area, usually containing the laminar flow cabinet, should be devoted exclusively to sterile activities, and situated in a quiet part of the laboratory with little traffic. Despite hot weather and working equipment, windows and doors should remain closed at all times to minimalise air flow which is likely to carry contaminants into the room. Some local regulatory authorities require that tissue culture laboratories have two entry doors, far enough apart to create an ante-room, which can be used to store specialised tissue culture clothing (Figure 7). Where a two-door entry system is in operation, it should be possible, with careful observation, to ensure that the tissue culture suite is always completely isolated from other disciplines within the building. No microbial or animal work should be undertaken in the laboratory.

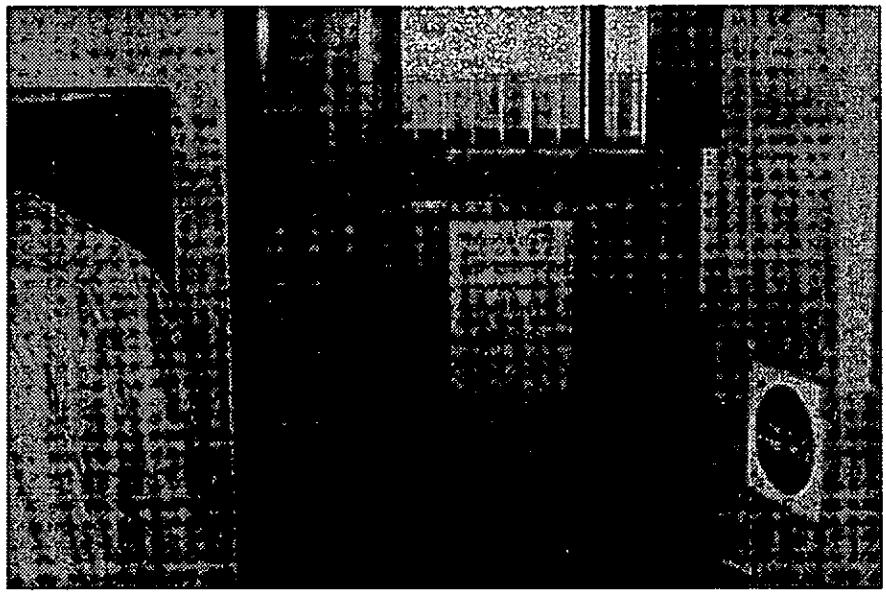


Figure 7. A two-door entry system creates an ante-room where tissue culture clothing can be exchanged for outdoor wear

2.9.1 Personal hygiene

Laboratory coats for tissue culture are designed with elasticated cuffs and a high collar, and should be regularly washed and sterilised. They should be retained in the laboratory and worn in place of outdoor clothing or standard laboratory coats on entry into the room (Section 2.9). Long hair should be tied back.

Immediately prior to the handling of the culture, handwashing with a disinfectant soap will remove dry skin and some microorganisms, though surgical gloves frequently swabbed with 70 % v/v ethanol offer the best protection for both operator and culture. These should be stretched to cover the cuffs which are likely to harbour microorganisms.

Face masks and safety goggles must be worn when handling toxic substances or when the operator is suffering from infection.

2.9.2 Work surfaces

The tissue culture laboratory should be dust and clutter free, particularly in the working area, its surfaces regularly cleaned with detergent, then swabbed with 70 % v/v ethanol or 70 % v/v industrial methylated spirit (IMS) to sterilise. Equipment not in use should be stored in cupboards housed beneath the work surfaces (Figure 8).

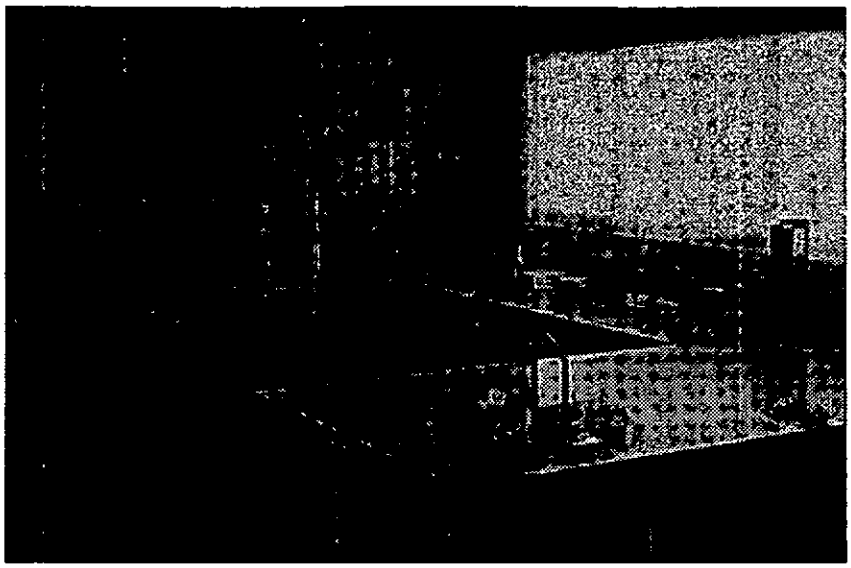


Figure 8. A typical tissue culture laboratory.

The laminar flow cabinet, though not strictly essential for some non-human cell lines, is a regular feature of modern tissue culture laboratories, and is the focal point of the working area. There are several types of cabinet, the Class II system being the most common, and the type in which all the sterile manipulations described in this thesis were performed. The Class II affords protection to both culture and operator and provides the minimum level of containment necessary when handling human material. Non-sterile air is drawn in through the top of the hood, and also inwards and downwards past the operator, before being recirculated through a sterilising filter and down over the culture (Figure 9).

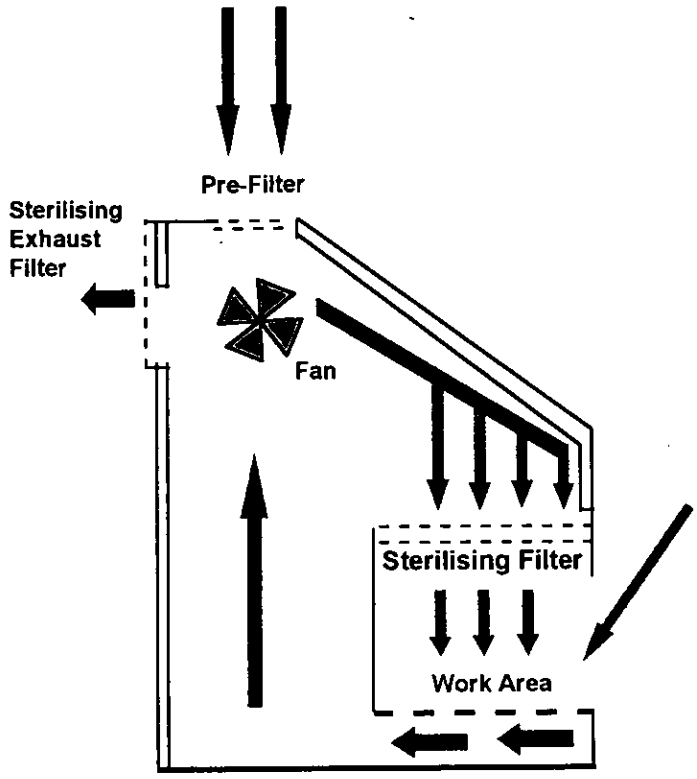


Figure 9. The class II recirculating cabinet showing flow of sterile (➡) and non-sterile (➡) air. (Adapted from Morgan & Darling, 1993)

The cabinet should be switched on, the protective cover removed, and the motor left running. After a few minutes, when the air flow has stabilised, all the interior surfaces of the cabinet, excluding the filter area, should be swabbed liberally with 70 % v/v ethanol or 70 % IMS. Only essential items should be brought into the cabinet. Once work is completed, the cabinet must be emptied, and reswabbed, then the cover replaced and the motor switched off. To help minimise contamination, the UV lamp may be switched on out of working hours, remembering that eye protection must be worn whenever entering a room which is emitting UV light.

2.9.3 Cleaning Equipment

To reduce the risk of chemical contamination, glassware for tissue culture should be kept separate from that used for other purposes. Although cells are usually propagated in pretreated sterile disposable flasks or petri-dishes, it is, however, still possible for cultures to come into contact with toxic chemicals leached, for instance, from metal instruments, rubber stoppers and tubing, plastic tops and glassware. It is therefore essential, that all equipment is vigorously cleaned and rinsed before being sterilised for reuse. Soiled equipment should not be allowed to dry out, but left to soak, overnight, if possible, in a solution of sodium hyperchlorite or Decon, to remove potential biohazards and prevent contamination.

Cleaning agents should be powerful enough to remove traces of toxic substances and proteinaceous deposits, without disturbing the natural properties of the surface. Modern detergents, such as Decon, rinse off easily after use. Copious amounts of tap water, then deionised or distilled water, should be used to thoroughly rinse equipment before it is allowed to dry.

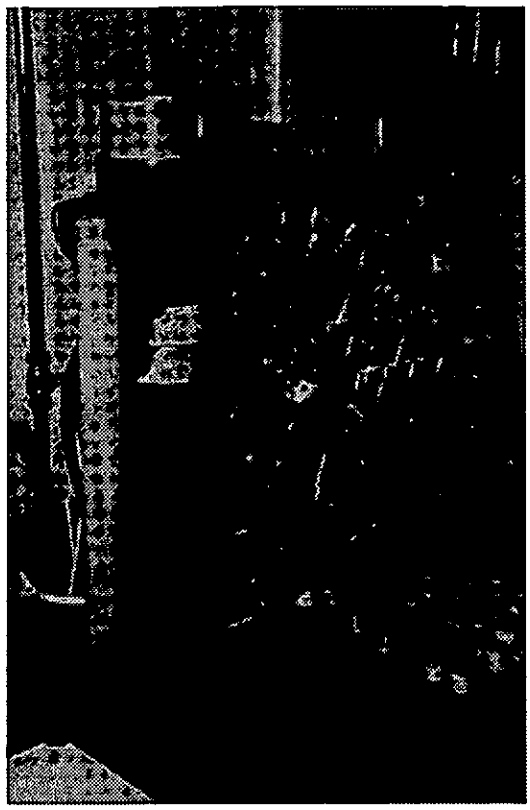


Figure 10. Pipette washing syphon and a range of drying glassware.

Glass pipettes should be collected separately from other glassware and placed, tips downwards, directly into plastic disposal cylinders containing a sterilising agent. When work is complete, cotton wool plugs must be removed, and pipettes left to soak in a Decon solution overnight, tips upwards, in a pipette washing syphon. Subsequently, pipettes should be rinsed for a minimum of four hours in hot water, by the syphoning action of the pipette washer (Figure 10). When dry, pipettes can be replugged with cotton wool and placed, tips downwards, into metal canisters, ready for sterilisation.

2.9.4 Sterilisation

Sterilisation is the final preparatory procedure which, if performed correctly, kills microorganisms and eliminates resistant spores.

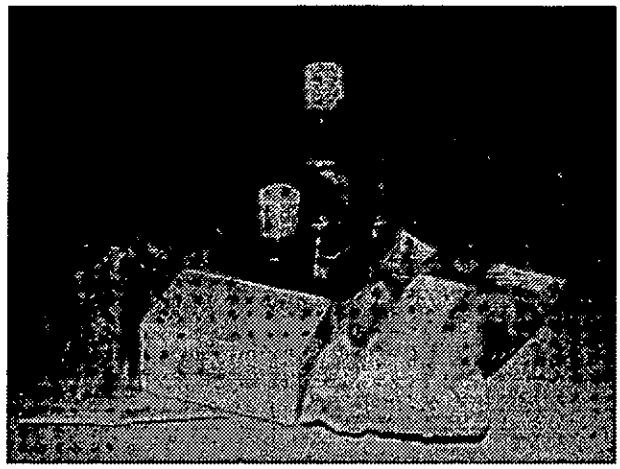


Figure 11. Items to be sterilised.

Most equipment is sterilised in autoclaves or hot-air ovens, although other methods are available for items which cannot withstand high temperatures. Autoclave tape is attached to the apparatus to be sterilised in order to identify items which have been through the sterilisation process and as an added seal to pipette canisters, boxes of micropipette tips, etc. (Figure 11). The tape, which is initially plain, produces dark stripes on exposure to high temperatures (Figure 12). Items which cannot be sealed may be packaged in special autoclave envelopes or bags. The caps of glass items, particularly bottles, are best loosened during the procedure to prevent explosion. They should be retightened afterwards and the bottle neck sealed with tape.

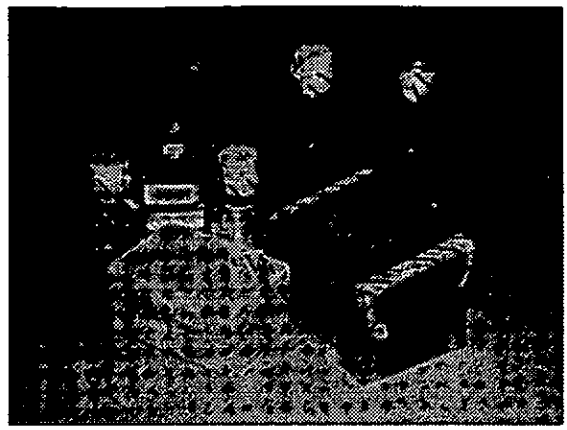


Figure 12. Items following sterilisation.

Hot-air ovens sterilise by dry heat, but their use is limited mainly to some glassware, pipettes and beakers, for instance. Dry heat kills microorganisms less efficiently than moist heat, so a minimum temperature of 160 °C for one hour is required.

Autoclaves sterilise *via* a combination of steam, temperature and pressure, and range from small bench-top models to large capacity, free-standing machines. Moist heat denatures proteins efficiently at a temperature of 121 °C and pressure of 103.5 kPa for fifteen minutes although these may need to be increased to eliminate resistant fungal spores. All items suitable for hot-air sterilisation are naturally capable of being autoclaved, along with some fluids (phosphate-buffered saline (PBS), some, but not most media, for instance), perishable items, some plastics (micropipette tips, centrifuge tubes), metals and rubber.

Certain items of equipment, such as some plastics, which cannot withstand the high temperatures of autoclaving or dry-heat, may be sterilised by immersion in 70 % v/v ethanol for thirty minutes, then dried under UV light in the laminar flow cabinet.

Figure 13. Media filtration.

Media and other solutions are usually sterilised through disposable filters, although some autoclavable 'incomplete' media are commercially available. Filters, which come in a range of sizes, are attached directly to the collection vessel connected to a pump *via* rubber tubing in the laminar flow cabinet (Figure 13). For smaller volumes, it is more economical to sterilise solutions through a disposable plastic syringe attached to a presterilised syringe filter unit (Figure 14).

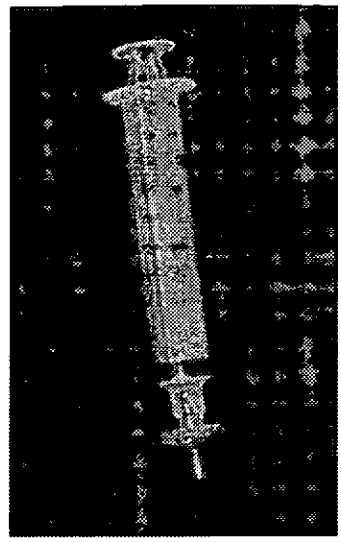


Figure 14. The plastic disposable syringe and syringe filter unit.

2.9.5 Apparatus

All existing apparatus (incubator, centrifuges, weighing scales, microscopes, *etc.*) should be routinely cleaned and kept clear of debris. The incubator should be periodically dismantled, then thoroughly cleaned and sterilised. The water tray must be regularly cleaned out and refilled with fresh water containing an antimicrobial agent such as copper sulphate (Figure 15). Microscopes must be handled particularly carefully; only special lens tissue should be used to gently clean the lens.

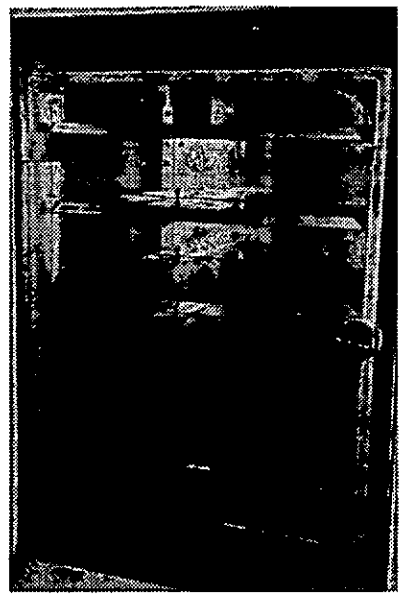


Figure 15. The interior of a humidified CO_2 incubator with (bottom) the water tray.

2.10 Handling

Once the laminar flow cabinet is running and sterilised, essential equipment required throughout the experiment (pipette canisters, pipette fillers, bottles of media, marker pens, waste beaker), may reside within the cabinet, but are best placed to either side when not in use.

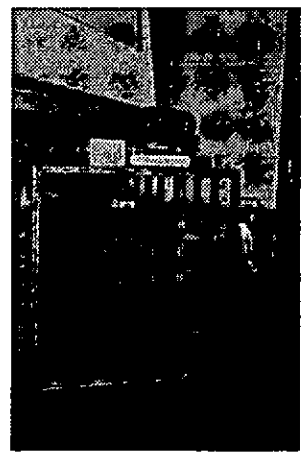


Figure 16. The laminar flow area

Packages of sterile equipment, taped bottles of media and micropipette tip boxes, should be opened and later resealed within the cabinet. Once sufficient items have been selected, resealed packages may be removed from the cabinet to a convenient position within reach of the operator (usually a shelved trolley). Used items which will not be required again should also be removed (Figure 16). Clutter restricts air flow and reduces the working area, thus increasing the risk of contamination. Items should be swabbed, well-spaced and arranged for easy access, so that it is never necessary to reach over one item to get at another (Figure 17). This interrupts the stream of sterile air and increases the risk of contamination from gloves and sleeves. Absence of clutter enables manipulations to be carried out towards the back of the cabinet avoiding the non-sterile area at the front.

Figure 17. A well set out laminar flow cabinet.

It is good general practice, before beginning work, to remove all sealant tape and to loosen all lids and bottle-caps, thereby facilitating smooth control of movements

during manipulations. In addition, the operator should continue to swab down the area during work, particularly following any spillage.

2.10.1 Attaching pipettes

Canisters of glass pipettes usually contain a range of sizes (1 ml, 2 ml, 5 ml and 10 ml), depending upon requirements. The canister should not be too full so as to make it difficult to slide the pipettes over one another once the canister is tilted. The operator must avoid touching the inside of the canister, but should tilt it slightly downwards so that a single pipette may be selected, once the bulk has moved partially out of the can. The end (never the body or the tip) of the pipette should be grasped to attach it to an automatic or manual filler (Figure 18).

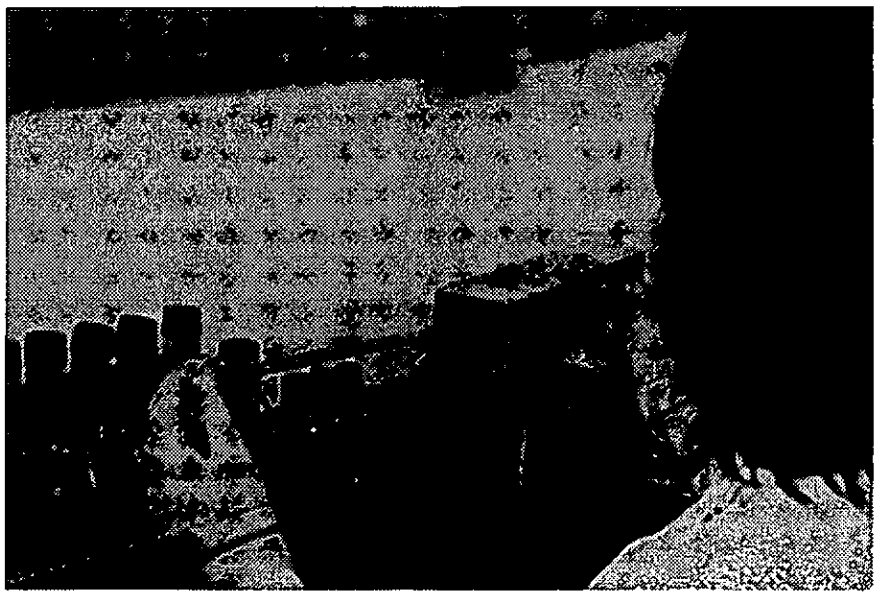


Figure 18. Selecting a pipette from a pipette canister.

Sterile disposable pipettes are best accessed by pulling back the wrapping, once the top has been torn, thus avoiding contact with the body and tip of the pipette. These can then be attached as above.

(a) *(b)*

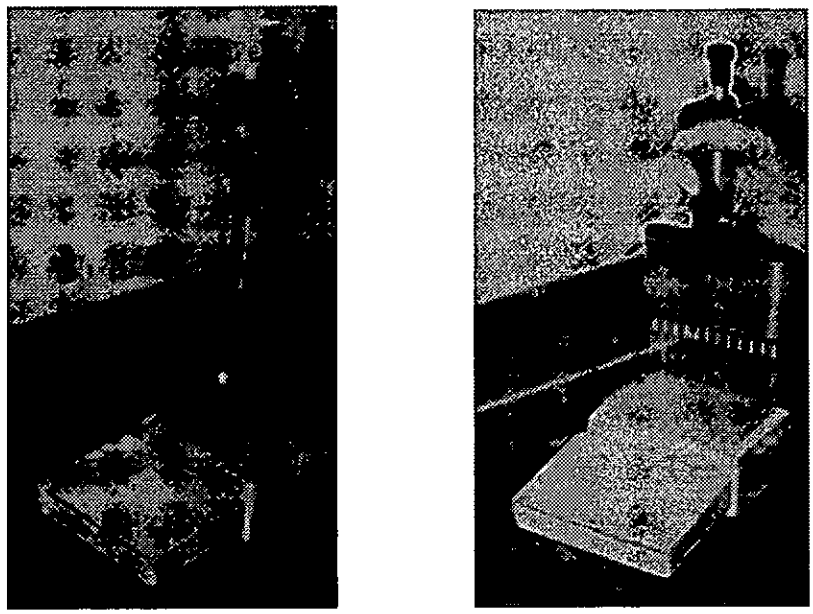


Figure 19. Attaching pipettes *(a)* single pipette *(b)* multipipette.

To attach plastic micropipette tips from a rack, either to a single or multipipette, the lid should be lifted, and the pipette directed vertically, then pressed firmly, into the tip(s) before it (they) are withdrawn (Figure 19).

2.10.2 Bottles and flasks

When withdrawing an aliquot of liquid from a sterile bottle or flask, caps should not be put down on the work surface, but held between fore- and middle fingers, so that the interior of the cap faces away from the bottle when the operator takes hold of the body of the bottle (Figure 20). Should it be necessary (during media filtration, for

instance), caps may be placed, internal face upwards, at the back of the cabinet. The operator must avoid holding the neck of the vessel, and not allow the pipette to come into contact with any external surface. Once liquid has been withdrawn, the cap should be replaced (though not tightened) and the vessel set aside.

Figure 20. Withdrawing liquid from a bottle.

2.11 Contamination

Unfortunately, despite even the most rigorous aseptic technique and routine use of antibiotics, all tissue culture laboratories will experience some level of contamination from time to time. This may be confined to a single culture, to a whole experiment or, at worst, consume the entire laboratory stock.

2.11.1 Identifying Contamination

The incidence and extent of contamination will be minimised by regular inspection of cell cultures. This may be by naked eye, by simply holding culture flasks up against a natural background light, or by microscope. A standard microscope is essential for counting cells, but the inverted microscope is invaluable for visualising cell cultures *in vitro* (Figure 21).

(a) *(b)*

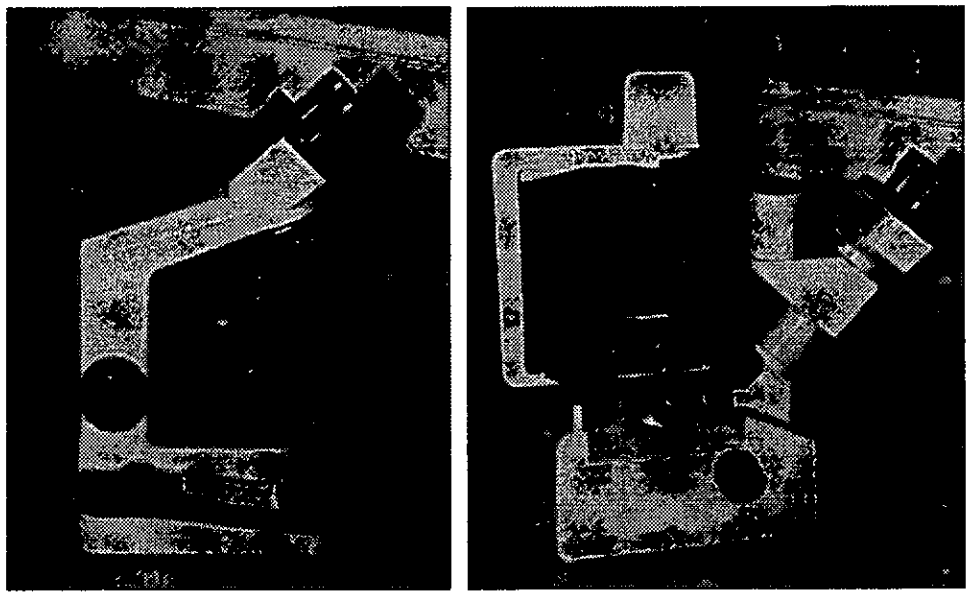


Figure 21. Microscopes. *(a)* standard microscope *(b)* inverted microscope.

In most cases, the type of infection is not particularly important, unless it becomes frequently recurrent. It is, therefore, important to record the date and details of each incident including the operator's name and likely source.

2.11.2 Microbial contamination

Bacteria are not visible to the naked eye, but may be detected microscopically. On low-power (× 100), extracellular spaces may appear granular, and there may be some

clumping of bacteria around cultured cells. At high power (× 400), individual types may be identified. Cocci types are round and appear to be in Brownian motion, whereas rod types, which are more motile, may make the culture appear to shimmer. The source of bacterial infection is believed to be usually human.

Fungi are easily identified at the macroscopic level. At this stage, the long microscopic hyphal growths give rise to large spore-producing structures which grow on the surface of the culture medium to give a fluffy, often blue-grey appearance. The main source of fungal contamination tends to be atmospheric spores.

Yeast infections are similar to fungus, but appear more uniform; the round or ovoid bodies grow and spread to give the appearance of a branched string of pearls. Again, the likely source of contamination is airborne particles.

Mycoplasma are the most problematic of microbial contaminants since they are difficult to detect and do not necessarily bring about either macro- or microscopic changes in affected cells or culture media. Smaller than bacteria and lacking a cell wall, they survive inside infected cells. Many mycoplasma infections are slow-growing and bring about subtle changes in the viral sensitivity and metabolism of cultures without destroying host cells. Undetected, this invalidates the results of diagnostic tests and research experiments.

Mycoplasma infections may be indicated by a decline in the rate of cell proliferation, the appearance of vacuoles, or excess extracellular matrix in suspect cultures. In

addition, certain mycoplasma produce acidic metabolites from the fermentation of glucose, which lower pH and turn culture media yellow. Generally, however, confirmation is by time-consuming diagnostic tests or commercial kits (such as Mycotrim), which are now available for cell lines.

The simplest and most reliable method of detection is *via* Hoechst 33258 [Chen, 1977]. This fluorescent dye binds specifically to DNA. Since other microorganisms also contain DNA, the test cannot confirm mycoplasma directly. If mycoplasma are present, their DNA will fluoresce as a filamentous pattern on cell surfaces or in the culture medium. More specific tests can detect mycoplasma-specific enzymes, such as, arginine deiminase or nucleoside phosphorylase [Schneider & Stanbridge, 1975; Levine & Becker, 1977].

Alternatively, the organism can be cultured and seeded into mycoplasma broth, then grown on for six days and plated out onto special nutrient agar. The colonies which form after eight days can be identified by their characteristic size and shape. [Taylor-Robinson, 1978; Hay, 1992].

The mode of mycoplasma infection is uncertain. Possible sources include, despite suppliers' "mycoplasma-free" guarantees, trypsin and bovine serum, laboratory personnel and original tissue specimens. Infected cell cultures themselves pose a major threat since the concentration of mycoplasmas is high in infected cultures. Handling of these cultures easily leads to droplet formation which, even when dried, can survive for up to ten days in laminar air flow.

2.11.3 Cross-contamination

If more than one cell line is being cultured in the same laboratory, it is not too difficult to contaminate one cell line with another. Even if only a few cells of a fast-growing line are introduced into a slow-growing culture, the faster growing line will rapidly supplant the other. In fact, transfection is a useful experimental tool, and unexpected transformations brought about by accidental cross-contaminations have occasionally been reported in the literature. Cross-contamination is, however, a serious problem and is not always apparent, since cells of different lines often share similar morphology. Although good general indicators are any sudden change in morphology or growth rate, confirmation of cross-contamination must be obtained by DNA fingerprinting [Stacey *et al.*, 1992], karyotype [Nelson-Rees & Flandermeyer, 1977], or isoenzyme analysis [O'Brien *et al.*, 1977, 1980].

Cross-contamination may arise as a result of sharing media or other solutions amongst operators, or allowing insufficient time to elapse between handling different cultures.

2.11.4 Curing contamination

The simplest remedy for contamination is to discard affected cultures and initiate new ones from frozen stock. For most bacterial infections, and microscopic fungal infections which have not reached the spore-producing stage, this may be sufficient. However, at the macroscopic level, fungi and yeast are not so easy to contain since their spores spread rapidly through air leading to widespread contamination of the incubator, or even the entire laboratory. In this case, the area should be thoroughly disinfected and affected equipment dismantled, the individual components bagged

separately and autoclaved. Media may be checked for contamination by incubation at 37 °C for two to four days. If contamination persists, then fumigation must be considered.

Fumigation, using formaldehyde, is carried out as a last resort against contamination, and also as a pre-requisite to servicing the laminar flow cabinet. Formaldehyde, which is highly toxic in both liquid and vapour form, must only be handled in accordance with strict safety precautions. Breathing apparatus is recommended throughout the procedure. Fumigation is carried out overnight by placing 50 ml potassium permanganate solution (10 % w/v potassium permanganate in formaldehyde solution) within the contaminated area. The mixture will effervesce and give off formic acid vapour so the area must be immediately sealed and access strictly prohibited. Following incubation, the contents of the beaker can be flushed down a fume cupboard sink by copious volumes of water. To remove any remaining traces of formaldehyde, a 50 ml beaker containing ammonium carbonate (5 g) is left in the area for a further two to four hours.

In cases of mycoplasma infections, if a cell line is deemed irreplaceable, the only option may be to attempt to decontaminate it. This should be done only if absolutely necessary and by an experienced operator in strict quarantine conditions. Some strains of mycoplasma are inhibited by certain antibiotics including kanamycin, gentamycin and tylosin [Friend *et al.*, 1966], and several other agents which may be effective in some cases. Infected cultures are rinsed five times with phosphate buffered saline-

3. MATERIALS & METHODS

3. MATERIALS & METHODS

All the experiments and procedures described in this chapter were carried out in a specialist tissue culture laboratory using, unless otherwise specified, aseptic technique throughout. Only reagents of tissue culture quality were purchased.

3.1 Preparation of Dulbecco's phosphate-buffered saline-solution A (PBSA)

A 10× stock concentrate of PBS was obtained by dissolving 8 g NaCl, 0.2 g KCl, 1.14 g Na_2HPO_4 and 0.2 g KH_2PO_4 (all Sigma) in distilled water and making up the volume to 100 ml. The solution having a pH of 7.4 was used as an indication of accuracy. The stock solution was stored unsterilised in 10 ml aliquots at - 20 °C, but thereafter used diluted one in ten for all the experimental work described in this thesis.

PBSA or phosphate-buffered saline (PBS) was used in the preparation of trypsin solution (Section 3.2.2), for rinsing cells (Section 3.11.1) and for making up MTT solution (Section 3.10.4).

3.2 Preparation of trypsin solution

3.2.1 Stock trypsin

Trypsin from porcine pancreas, specified as negative for porcine parcovirus after γ-irradiation, was purchased from Sigma and stored at 4 °C. When required, 2.5 g trypsin was weighed out in a fume cupboard and made up to 100 ml with diluted PBS

(Section 3.1), then stored unsterilised as stock in 2 ml aliquots at - 20 °C. Trypsin activity = 1200 BAEE units / mg solid, chymotrypsin activity = 2.6 BTEE units / mg solid.

3.2.2 Trypsin solution (0.25 % v/v) in PBS and 0.5 % w/v ethylenediaminetetraacetic acid (EDTA)

100 ml of 0.5 % w/v EDTA made up in PBS (Section 3.1) was added to a thawed 2 ml aliquot of stock trypsin to give a 0.25 % w/v trypsin solution in PBS and 0.5 % w/v EDTA. This was then filter sterilised into a sterile universal using a 10 ml disposable syringe attached to a 0.2 μm filter unit. The solution was stored at 4 °C for up to two weeks.

3.3 Preparation of RPMI 1640 medium from 10× RPMI 1640 liquid stock

3.3.1 200 mM stock L-glutamine

2.92 g L-glutamine (Sigma) was made up to 100 ml with distilled water. This solution was filter sterilised into sterile universals in 5 ml aliquots using a 10 ml disposable syringe attached to a 0.2 μm filter unit. These were then stored at - 20 °C ready for use.

3.3.2 Foetal calf serum

Sterile foetal calf serum (FCS), mycoplasma-tested by the manufacturer, was purchased from M. B. Meldrum Ltd. and stored in 56.6 ml aliquots at - 20 °C ready for use.

3.3.3 Antibiotic

Sterile antibiotic solution (10,000 units penicillin ml^{-1} and 10 mg streptomycin ml^{-1} in 0.9 % w/v NaCl) was purchased from Sigma and stored in 5 ml aliquots at - 20 °C.

3.3.4 'Incomplete' medium

100 ml stock 10× RPMI 1640 (from Gibco) and 2 g $NaHCO_3$ (Sigma) were dispensed into a 1 L volumetric flask and made up to one litre with distilled water. The solution was stirred continuously and the pH adjusted to 7.1 - 7.3 by carefully adding 1 M NaOH dropwise. This 'incomplete' medium was filter sterilised into two 500 ml glass media bottles through a 500 ml filter unit (Becton Dickinson) attached to a vacuum pump in the laminar flow cabinet. The bottles were dated and labelled as 'incomplete' medium and stored at 4 °C for up to three months.

3.3.5 'Complete' medium

One aliquot each of L-glutamine (final concentration = 2 mM) (Section 3.3.1), antibiotic (final concentration approximately, penicillin = 100 units ml^{-1} : streptomycin = 100 μg ml^{-1}) (Section 3.3.3) and FCS (final concentration, 10 % v/v) (Section 3.3.2) were thawed and added to a 500 ml bottle of 'incomplete' medium (Section 3.3.4). The reagents were left to equilibriate by diffusion. 'Complete' bottles were labelled as such, dated and stored at 4 °C for up to two weeks.

3.4 Subculture of the EMT-6 cell line

The murine mammary tumour cell line, EMT-6, was originally obtained from Zeneca Pharmaceuticals, Macclesfield, Cheshire. Cultures of cells were maintained in a CO_2 incubator (Napco, Model 5410) at 37 °C, 5 % CO_2 : 95 % air, 100 % humidity and in 'complete' RPMI 1640 medium (Section 3.3.5). Cultures were routinely subcultured every two to three days, usually in 25 cm^2 flasks, as detailed below.

3.4.1 Trypsinisation to detach adherent monolayer cultures

Culture flasks of cells in the 'log' phase of growth and approximately eighty per cent confluent (Section 2.5) were stood on end and their caps loosened. The medium was discarded into chloros, and 0.5 ml trypsin solution (Section 3.2.2) per 10 ml medium was added to each flask. The caps were re-tightened and each flask rotated gently to evenly distribute the trypsin solution over the monolayer. The flasks were left for three to six minutes to allow the trypsin to detach the monolayer from its substrate. At this point, fresh 'complete' medium (5 ml for each 0.5 ml trypsin) (Section 3.3.5) was added to prevent any further enzymatic digestion of the cells themselves. The cell suspension was pipetted up and down several times to ensure complete detachment of the cells and to disperse any clumps.

3.4.2 Routine maintenance

Cell cultures were routinely grown in 25 cm^2 disposable plastic tissue culture flasks supplied by Becton Dickinson. In this case, following trypsinisation (Section 3.4.1), 1 ml of cell suspension was added to 9 ml of fresh 'complete' medium (Section 3.3.5) in

a new flask and mixed. A duplicate was prepared, then both flasks placed in the CO_2 incubator and their caps loosened.

3.5 Freezing cells for liquid nitrogen storage

3.5.1 Preparation of freezing medium

5 ml FCS (Section 3.3.2) was added to 45 ml RPMI 'complete' medium (Section 3.3.5) to obtain a 20 % v/v FCS supplemented freezing medium. 5 ml dimethyl sulfoxide (DMSO) (from Sigma) was prewarmed to 37 °C and added to 45 ml of freezing medium in the fume cupboard. The solution was mixed well and filter sterilised into sterile universals using a 10 ml syringe attached to a 0.2 μm filter unit. The solution was stored at 4 °C for up to two weeks or at -20 °C for up to three months.

3.5.2 Freezing down cells

Cultures of cells in 25 cm^2 culture flasks and in the log phase of growth (Section 2.5) were dissociated using trypsin (Section 3.4.1) and the cell suspension from each flask pipetted into separate centrifuge tubes, then spun at 150 *g* for five minutes. The supernatants were discarded into chloros and each pellet resuspended in approximately 3 ml of freezing medium (Section 3.5.1).

Cell suspensions were pipetted in 1 ml aliquots into sterile freezing cryotubes, each tube having been labelled and dated. The cryotubes were placed into cardboard freezing straws taped at the bottom and loosely plugged at the top with cotton wool. The tape was pierced several times to allow liquid nitrogen vapour to access the

cryotubes. The tubes were stored for twenty minutes at -20 °C, followed by twenty minutes at -70 °C and then finally transferred to liquid nitrogen storage (-180 °C).

3.6 Reviving cells from liquid nitrogen storage

Vials of frozen cells were removed from liquid nitrogen storage and thawed quickly in warm water. Each 1 ml aliquot of cell suspension was added to 10 ml RPMI 1640 'complete' medium (Section 3.3.5) in a sterile centrifuge tube and centifuged at 150 *g* for five minutes. The supernatant was discarded into chloros and the cell pellet gently resuspended in 1 ml fresh medium. This cell suspension was added to a further 7 ml of medium in a 25 cm^2 culture flask. Flasks were placed in the CO_2 incubator and their caps loosened. The cultures were inspected regularly by microscope and subcultured (Section 3.4) once approximately eighty per cent confluent (Section 2.5). At least two routine subcultures were performed before the cells were used experimentally.

3.7 Counting cell numbers in suspension

3.7.1 Obtaining a sample of cell suspension

A monolayer of cells in the log phase of growth (Section 2.5) and contained in a 25 cm^2 culture flask was dissociated using trypsin (Section 3.4.1). Then, using a 10 ml syringe fitted with a wide bore needle, the cell suspension was drawn up and expelled back into the flask several times in order to thoroughly disperse the cells and eliminate clumps. Finally, a small sample of suspension was taken up into the syringe for counting purposes, as described below (Section 3.7.4). Aseptic technique was not required for the remainder of the procedure.

3.7.2 Loading the samples onto a haemocytometer

An improved Neubauer haemocytometer (Figure 23) was taken and a coverslip, slightly moistened at the edges, secured over the counting grids by applying even pressure until "Newton's Rings" were observed. With the needle removed, the syringe was touched to either edge of the coverslip allowing a few microlitres of cell suspension to fill the counting chambers by capillary action. The cells were allowed to settle before counting.

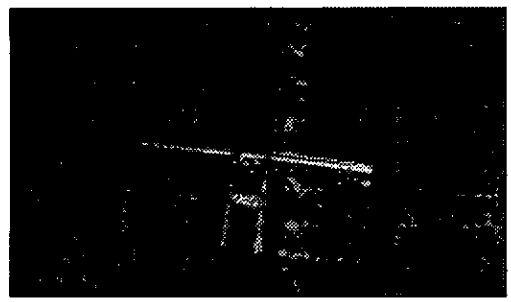


Figure 23. The improved Neubauer haemocytometer and (top) coverslip.

3.7.3 Setting up a microscope

A standard laboratory microscope (Figure 21) was switched on and the condenser fully racked up with the diaphragm open. The haemocytometer containing the samples was placed on the microscope stage and the low power (×10) objective lowered until it rested just above the surface of the coverslip. The objective was then raised until the grid lines of the counting chamber came into focus. The iris diaphragm was closed down and the condenser adjusted to focus sufficient light on the sample to highlight the cells against their background. Live cells are white and appear to "fluoresce" so more definition is achieved by reducing the light intensity in order to create a darker

background. The two condenser centering screws were used to centre the slide and bring the central counting square into view.

3.7.4 Counting cells

All cells in the central large square were counted except those touching the outer tramlines on the bottom or right-hand side of the grid (Figure 24). In order to verify the count, the procedure was repeated using the remaining counting chamber. If a large discrepancy existed between the two counts, cells in any of the four outer squares of the grid were counted and a mean value obtained.

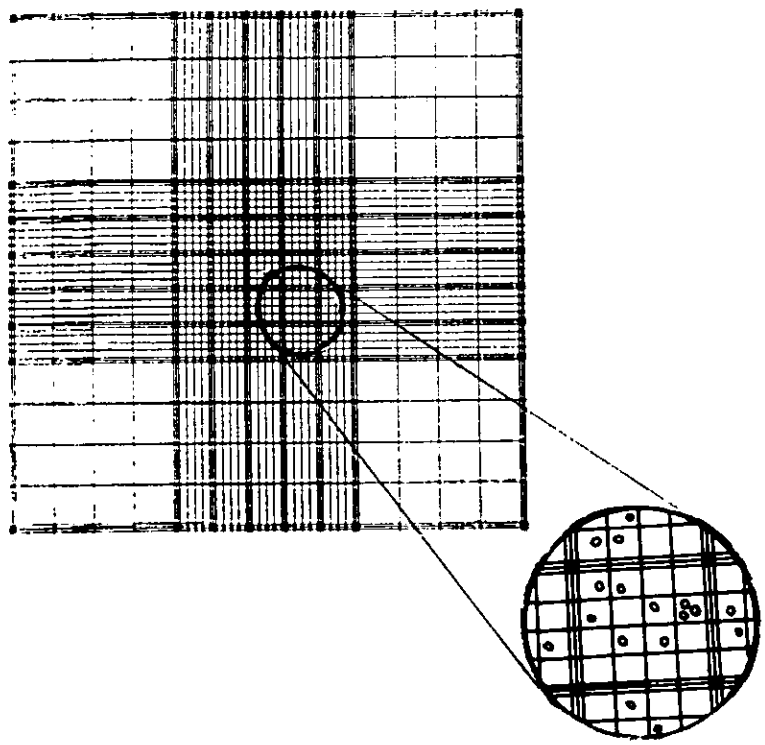


Figure 24. The counting chamber of the improved Neubauer haemocytometer. Any of five areas may be counted, *ie.* the central square (having 25 small squares within an area of 0.1 mm^2) or the four corner squares (each having 16 small squares within an area of 0.1 mm^2).(Adapted from Morgan & Darling, 1993)

The space between the bottom of the coverslip and the top of the slide measures 0.1 mm when the coverslip is correctly in place over the haemocytometer. Each of the five squares (*ie.* the four corners and the central square) encloses 1 mm^2. The volume above each square is, therefore, 1 mm × 1 mm × 0.1 mm = 0.1 mm^3 (or 0.1 µl). Thus, once the cell count has been obtained for a particular square, the total number of cells in 1 ml of suspension is this value × 10^4.

3.8 Trypan blue test of cell viabilty

200 µl of cell suspension (Section 3.7.1) and 50 µl of 0.4 % v/v trypan blue dye were mixed and left to stand for approximately ten minutes. Live (colourless) and dead (blue) cells were counted (Section 3.7.4). The percentage of living cells in the culture was calculated.

3.9 Setting up a growth curve

A cryotube of EMT-6 cells was taken from liquid nitrogen storage and thawed (Section 3.6). Using 25 cm^2 culture flasks throughout, the culture was routinely subcultured (Section 3.4) every two days on three successive occasions to obtain fifteen 25 cm^2 culture flasks of cells, *ie.* three cultures for each of five days. Three cultures were dissociated using trypsin (Section 3.4.1) and counted (Section 3.7.4) at the same time of day for five consecutive days. Growth curves were constructed on standard graph paper. Log_{10} cell number was plotted on the *y*-axis, and time elapsed from initiation of the culture on the *x*-axis. Each point represented the mean of three cell counts.

3.10 Cytotoxicity testing using animal tissue culture techniques

3.10.1 Seeding cells

A culture of cells in the 'log' phase of growth and approximately eighty per cent confluent (Section 2.5) was dissociated using trypsin (Section 3.4.1) and counted (Section 3.7.4), and the mean cell number used to calculate the dilution factor required to obtain a cell suspension of 5×10^3 cells ml^{-1}. The suspension was diluted accordingly in RPMI 1640 'complete' medium (Section 3.3.5) to give a final volume of approximately 170 ml. This was sufficient to seed eight 96-well microtitration plates (Becton Dickinson) with 200 µl of cell suspension to each well at a final concentration of 10^3 cells per well. The cell suspension was shaken continuously whilst being added to the microtitration plates from a plastic "boat" using an automatic multipipette with twelve disposable plastic tips. The plates were placed in the CO_2 incubator for two days in preparation for drug testing (Section 3.10.2).

3.10.2 Drug testing

Methylene blue (MB) 1,9-dimethyl methylene blue (DMMB), toluidine blue O (TBO) and azure A (AA) were purchased from Aldrich (Gillingham, UK.) and were recrystallised from methanol prior to use. The various derivatives were synthesised by the Department of Chemistry, University of Central Lancashire, Preston. Drug purity was assured by HPLC, proton NMR elemental analysis and chromatography. Drug dilutions were made in four 96-well microtitration plates according to the scheme illustrated in Figure 25. [Stock solutions of MB, MMB, AA, TBO, MAA and DMAA: light experiments = 200 µM, dark experiments = 800 µM, NMB and DMMB: light experiments = 6.25 µM, dark experiments = 25 µM]. Using a Gilson pipette, 300 µl

stock solution Drug A (the parent compound) was added to wells A 1-5, similarly 300 µl stock solution Drug B (the methylated derivative) added to wells A 6-10 and 300 µl RPMI 1640 'complete' medium (Section 3.3.5) to wells A 11 and 12 as a control. Since the parent compounds are well characterised, these were used as a reference to allow comparison between experiments.

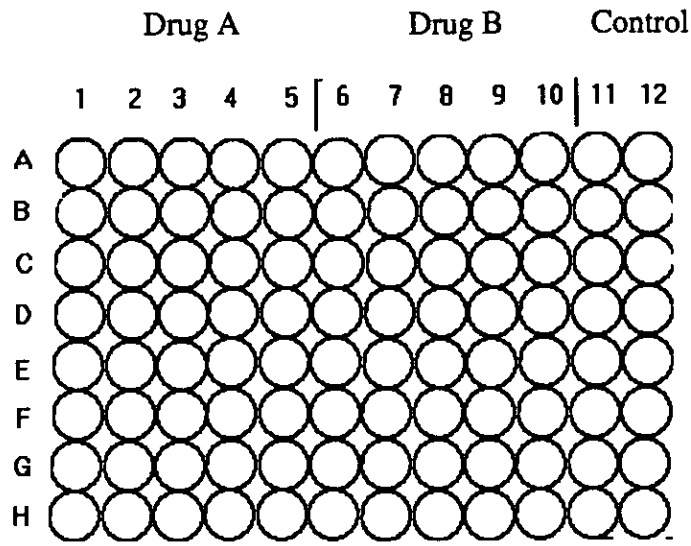


Figure 25. Schematic illustration for additions to microtitration plates.

Using a multipipette with twelve disposable tips, 150 µl 'complete' RPMI 1640 medium was added to all other wells. Next, again using the multipipette, 150 µl of solution was transferred from wells A1-12 to wells B1-12 and mixed up and down several times, avoiding the formation of any air bubbles. Similarly, 150 µl of this

solution was transferred from wells B1-12 to C1-12 and mixed. The procedure was repeated through to wells H1-12, then the tips discarded into chloros. Next, using the multipipette and twelve new tips, starting at the lowest concentration (wells H1-12), 50 µl from each well of diluted drugs was transferred across to wells H1-12 of 96-well microtitration plates containing cell cultures at approximately twenty per cent confluence which had been previously incubated for three days. The various drug dilutions were mixed gently with the cell suspensions, it being possible to add drugs to two microtitration plates of cells from each microtitration plate of drugs. The procedure was repeated though to wells A1-12, then the cell plates replaced in the CO_2 incubator. The cells were incubated in the presence of drugs for three hours, preparatory for the next stage (Section 3.10.3).

3.10.3 Drug test conditions

Medium and drug were aspirated and rinsed with 200 µl RPMI 1640 medium, this was also aspirated, then 200 µl of fresh medium added. Since, in this case, both the photocytotoxicities and dark toxicities were being measured, half the plates were illuminated for thirty minutes and the remaining duplicate plates kept in darkness for thirty minutes. Light was provided by a bank of fluorescent tubes with maximum emission in the 600-700 nm region and a fluence rate of 14.4 mW cm^{-2}. The light source alone had previously been shown to produce zero toxicity, on EMT-6 cells. No increase in temperature was noted during illumination. The plates were replaced in the CO_2 incubator and the cells grown on for a further three days before testing for viability with MTT.

3.10.4 Preparation of MTT

Dessicated MTT was purchased from Sigma and the stock bottle stored in a sealed container at 4 °C. When required, 0.5 g MTT was made up to 100 ml with diluted PBS (Section 3.1). The solution was mixed thoroughly using a magnetic stirrer, then sonicated for a few seconds to ensure that the powder was completely dissolved. The solution was filter sterilised into a sterile 100 ml bottle through a 125 ml filter unit (Becton Dickinson) attached to a vacuum pump in the laminar flow cabinet. MTT deteriorates when exposed to light so unused solution was stored, wrapped in aluminium foil, at 4 °C.

MTT is carcinogenic and may give rise to inheritable genetic change. The product, particularly in solid form, should be handled with extreme caution. To prevent inhalation of fine particles, the powder should be weighed out only on an enclosed balance and preferably in a fume cupboard, wearing a face mask, safety goggles and two pairs of rubber gloves.

3.10.5 Toxicity testing

25 µl MTT (5 mg ml^{-1}) (Section 3.10.4) was added to each well of prepared microtitration plates (Sections 3.10.1-3.10.3) using the multipipette and disposable tips. The plates were returned to the CO_2 incubator for five hours. The remainder of the procedure was carried out under non-sterile conditions.

The medium and MTT were aspirated using a pulled pasteur pipette attached to a vacuum line, taking great care not to disturb the formazan crystals, leaving

approximately 30 µl in each well. 200µl DMSO was then added to each well to solubilise the crystals. The plates were shaken for ten minutes and the absorbances read on a plate reader (Anthos HT111, measuring filter 540 nm: reference filter 620 nm). The various toxicities were calculated as a percentage of control. All data are expressed as mean ± standard error of the mean (SEM). Data were compared using student's t-test and values with $P = < 0.05$ were taken as significant.

3.11 Determination of cellular uptake using animal tissue culture techniques

Victoria blue-BO was purchased from Aldrich and used without further purification. The 4-morpholino analogue, MOVB, was synthesised by the Department of Chemistry, University of Central Lancashire, Preston, following an established procedure (Barker *et al.,* 1961). Purity was assured by HPLC, proton NMR and elemental analysis.

3.11.1 Determination of the most efficient extraction solvent

Cell cultures were grown to one hundred per cent confluence over three days in four 25 cm^2 flasks. The medium was poured off into chloros and replaced by 10 ml of 5 µM VBBO in 'complete' RPMI 1640 medium. The cultures were incubated in the presence of drug for three hours. Medium and drug were discarded into chloros and

the cells rinsed twice with 10 ml PBS. The cells from each flask were dissociated using trypsin (Section 3.4.1). The remainder of the procedure was carried out under non-sterile conditions. The cell suspensions from each flask were centrifuged at 150 *g* for ten minutes and the supernatants discarded into chloros. The pellets were twice mixed in 2 ml PBS (Section 3.1) to resuspend and rinse the cells. The cell suspensions were again centrifuged at 150 *g* for ten minutes and the supernatants discarded into chloros. 2 ml of either 2 % w/v trichloroacetic acid (TCA) or 2 % w/v sodium dodecylsulfate (SDS) or butan-2-ol or methanol (HPLC grade) was added to each of the four cell pellets, mixed and left for ten minutes. The cell suspensions were centrifuged at 2000 *g* for thirty minutes. The absorbances of the supernatants were read spectrophotometrically at 612 nm. Waste solutions were disposed of into sealed glass waste vessels.

3.11.2 Calibration curves for VBBO and MOVB

Calibration curves for VBBO and MOVB were prepared using 0-10 μM dilutions of each drug in methanol and measuring absorbances spectrophotometrically at 612 nm (VBBO) and 622 nm (MOVB).

3.11.3 Uptake experiments

Cell cultures in 20 ml 'complete' RPMI 1640 medium (Section 3.3.5) were grown to confluence over three days in 75 cm^2 culture flasks. The medium was poured off into chloros and replaced by doubling dilutions of each drug (0-10 μM) in RPMI

'complete' medium. The cultures were incubated in the presence of drugs for three hours. Medium and drugs were discarded into chloros and the cells rinsed twice with 20 ml PBS (Section 3.1). The cells from each flask were dissociated using trypsin (Section 3.4.1) and counted (Section 3.7). The remainder of the procedure was carried out under non-sterile conditions.

The cell suspensions from each flask were centrifuged at 150 *g* for ten minutes and the supernatants discarded into chloros. The pellets were twice mixed in 2 ml PBS (Section 3.1) to resuspend and rinse the cells. The cell suspensions were again centrifuged at 150 *g* for ten minutes and the supernatants discarded into chloros. 1 ml methanol was added to each cell pellet, mixed and left for ten minutes. The cell suspensions were then centrifuged at 2000 *g* for thirty minutes. The absorbances of the supernatants were read spectrophotometrically at 612 nm (VBBO) and 622 nm (MOVB). Waste solutions were disposed of into sealed glass waste vessels.

The dye concentrations of the various supernatants were determined using the "Minitab" computer package, by plotting the calibration curve data for each drug and entering the absorbance value of the unknown. Cellular uptake of dye (picomoles /10^3 cells) was plotted against dye incubation concentration (μM). All data are expressed as mean ± standard error of the mean (SEM).

4. RESULTS

4. RESULTS

4.1 Maintenance of the EMT-6 cell line

The EMT-6 cell line responded well to routine subculture and freezing and thawing regimes.

4.1.1 Cell counts

Following subculture (Sections 2.5 & 3.4), cell densities ranged from 9.2×10^4 cells cm^{-2} to 3.7×10^5 cells cm^{-2}, although these were extremes. Most counts were around a mean value of 1.8×10^5 cells cm^{-2}.

4.1.2 Cell viability

Since living cells fluoresce (Section 3.7.3), cell viability was generally checked by naked eye or microscopically (Section 2.11.1) rather than using trypan blue exclusion (Section1.5.1) as routine. However, when this test was performed, cell viability was calculated at >90 %.

4.1.3 Microbial contamination

During the course of the work only one incidence of visible microbial contamination occurred. The microbes were rod-shaped and motile and identified as bacterial (Section 2.12.2). The infection was confined to two 25 cm^2 plastic culture flasks and one set of eight 96-well microtitration plates which had been prepared for toxicity

testing. The problem was eradicated by immediate disposal of the affected cultures and swabbing down the incubator thoroughly with 70 % v/v ethanol. Part-used bottles of medium and trypsin solution were poured away in accordance with COSHH regulations. Autoclaving of the incubator components or fumigation was not required.

Cross-contamination (Section 2.12.3) was never considered as a problem since no other cell lines were in use for the duration of the project.

4.1.4 Other problems

A cause for concern arose following the Christmas vacation of 1995 when the cells were revived from liquid nitrogen storage (Section 3.6) in preparation for resuming experimental work. The cell counts obtained at this time were very low ($<1.1 \times 10^5$ cells cm^{-2}) and the morphological characteristics of the cultures appeared to change. The cells which had previously been rounded with uniform coverage of the flask, sometimes appeared elongated with random areas of growth, particularly in the central areas of the flasks.

The cell counts did eventually recover, but the cause of the problem was never identified, and it was never certain whether or not it had been overcome. Consequently, new cultures were initiated from frozen stock (Section 3.6). The cell stock has since been screened for mycoplasma and was found to be completely clear of this type of infection (Burrow, 1997).

4.2 Growth characteristics of the EMT-6 cell line

EMT-6 cells were seeded into 25 cm^2 flasks at 1.1×10^5 cells cm^{-2} on Day 0. Up to Day 1, the graph shows an initial "lag" phase when little growth occurred. From Day 1 to Day 3, growth increased in a logarithmic manner (the "exponential" phase). After Day 3, growth ceased and, in fact, the graph shows a sharp decline in cell number up to Day 4 which continued, but levelled, up to Day 5 (Figure 26).

4.3 Effect of methylation on the cytotoxicity of phenothiazinium dyes

Both the photocytotoxicity and dark toxicity of MB were increased by successive methylation, *ie.* DMMB>MMB>MB in both cases (Figures 27, 28, 29). and the light-dark differential at the LD_{50} value was at a maximum for dimethyl substitution, *ie.* DMMB>MMB> MB (17.2 *cf.* 11.9 and 6.3 respectively, Table 1). Similarly, the percentage dark toxicity at the LD_{90} value was minimised by a single methyl substitution, *ie.* MMB<DMMB<MB (14.2 % *cf.* 21.3 % and 27.9 % respectively). The results for NMB (Figure 30) are comparable with those for DMMB. These data are summarised in Table 1.

As regards the toluidine blue series (Figures 31-34), though not all the relevant values could be calculated from this set of experiments, the cytotoxicities of the parent compound were similarly altered by various stages of methylation. When the results for DMAA are omitted, the pattern of increased photo- and dark toxicity with

successive methylation is preserved for the other three photosensitizers *ie.* MAA>TBO>AA. The photocytotoxicity of DMAA is greater than that of AA but less than that of TBO and MAA *ie.* AA<DMAA<TBO<MAA. In this study, the dark toxicity associated with DMAA appears to be negligible.

	Dose (μM)	% Light toxicity	% Dark toxicity	Light : Dark toxicity
MB	18.7	50	7.9	6.3
MMB	2.20	50	4.2	11.9
DMMB	0.09	50	2.9	17.2
MB	37.7	90	27.9	3.2
MMB	4.80	90	14.2	6.3
DMMB	0.27	90	21.3	4.2
AA	19.2	50	16.8	3..0
TBO	15.7	50	13.1	3.8
MAA	4.1	50	11.3	4.4
DMAA	18.0	50	1.6	31.3
NMB	0.39	50	5.0	10.0
TBO	35.9	90	47.1	1.9
MAA	9.0	90	11.3	8.0
DMAA	36.5	90	0.5	180.0
NMB	0.61	90	6.8	13.2

Table 1. Toxicity data and light:dark ratios for the phenothiazinium photosensitizers used in this study.

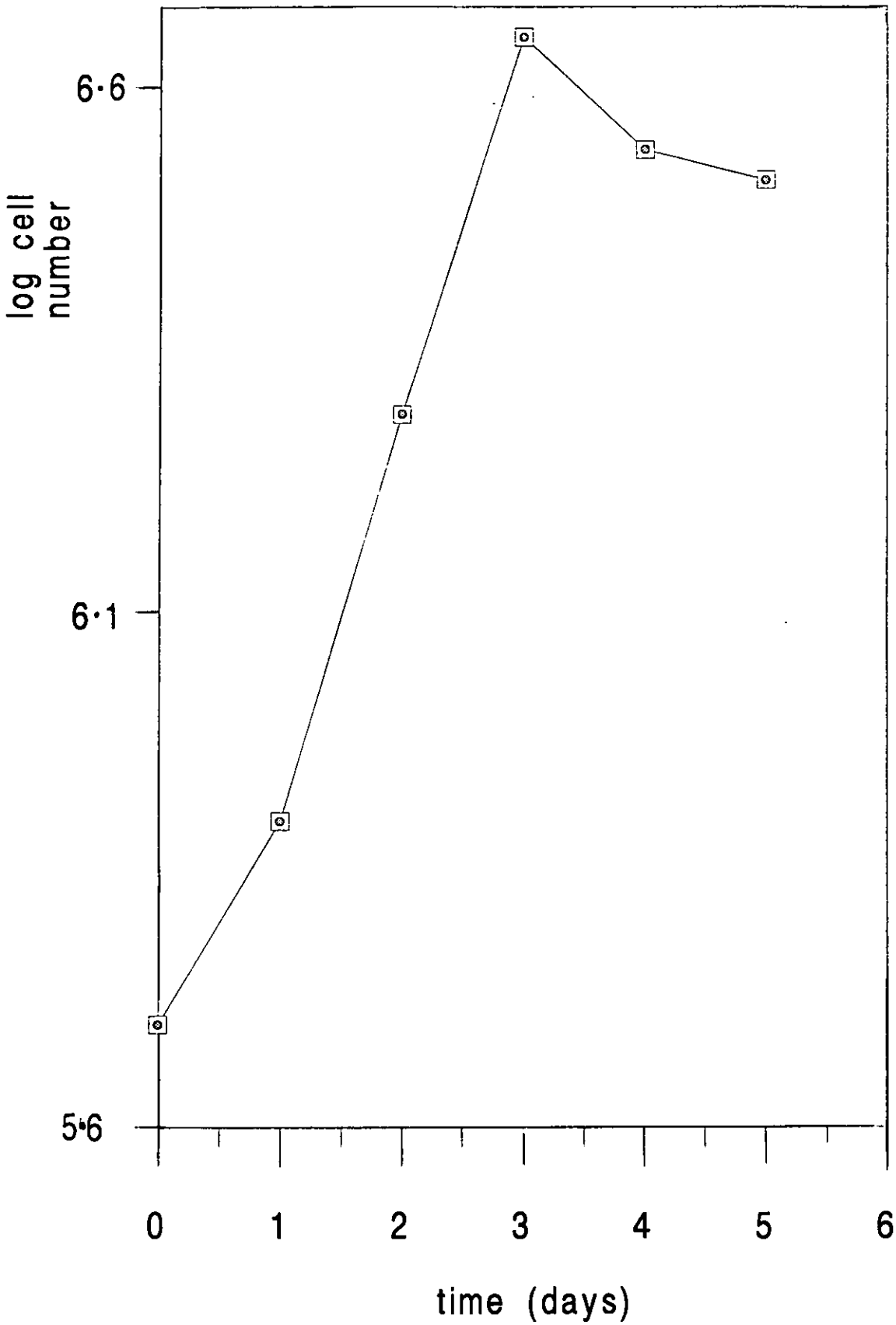


Figure 26. Growth characteristics of the EMT-6 cell line. Each point is the mean of a single experiment performed in triplicate. SEMs were less than 5 %.

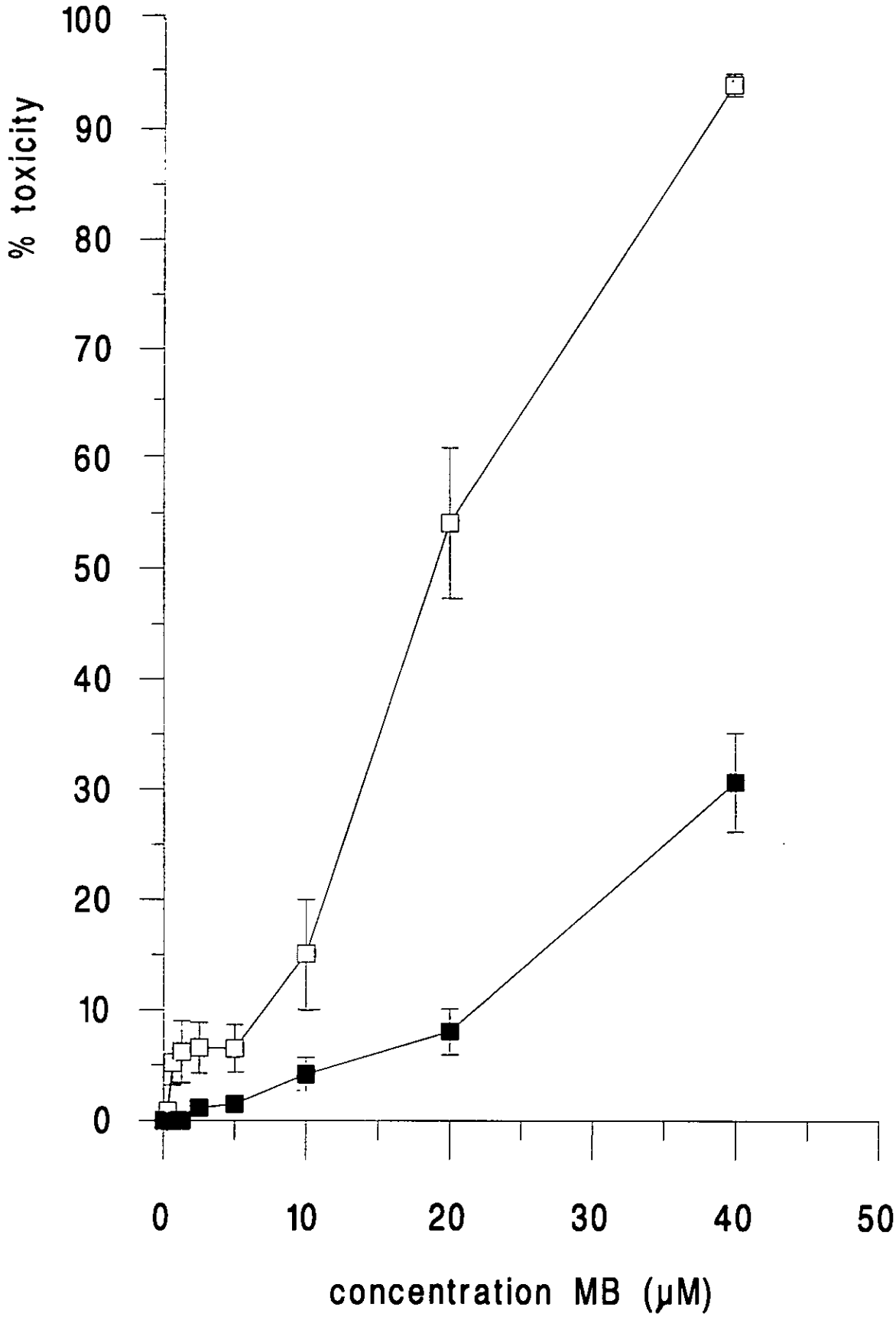


Figure 27. Photocytotoxicity (□) and dark toxicity (■) of methylene blue against the EMT-6 cell line. Each point is the mean of at least 14 experiments. Error bars represent SEMs.

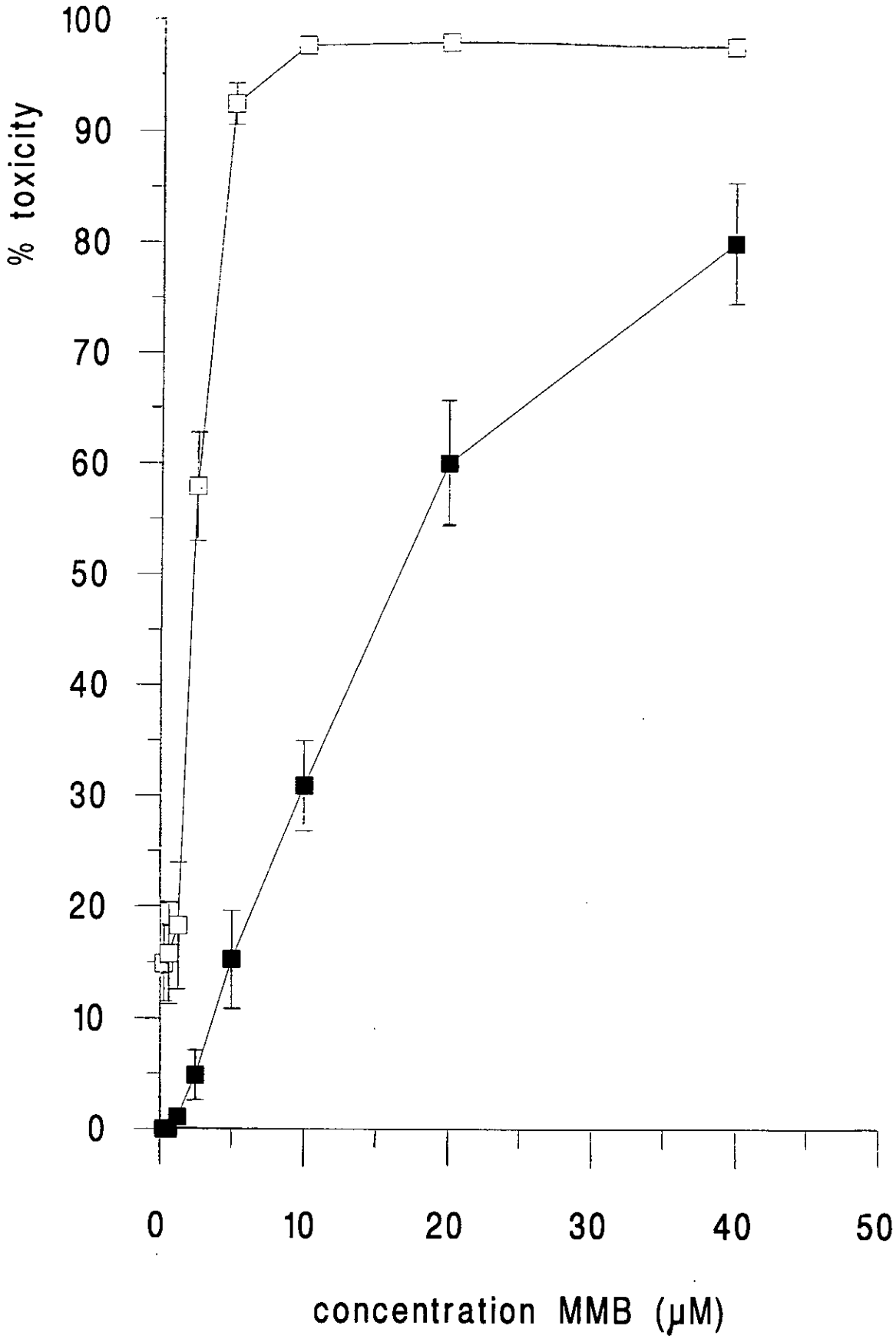


Figure 28. Photocytotoxicity (⊥) and dark toxicity (■) of 1-methyl methylene blue against the EMT-6 cell line. Each point is the mean of at least 9 experiments. Error bars represent SEMs.

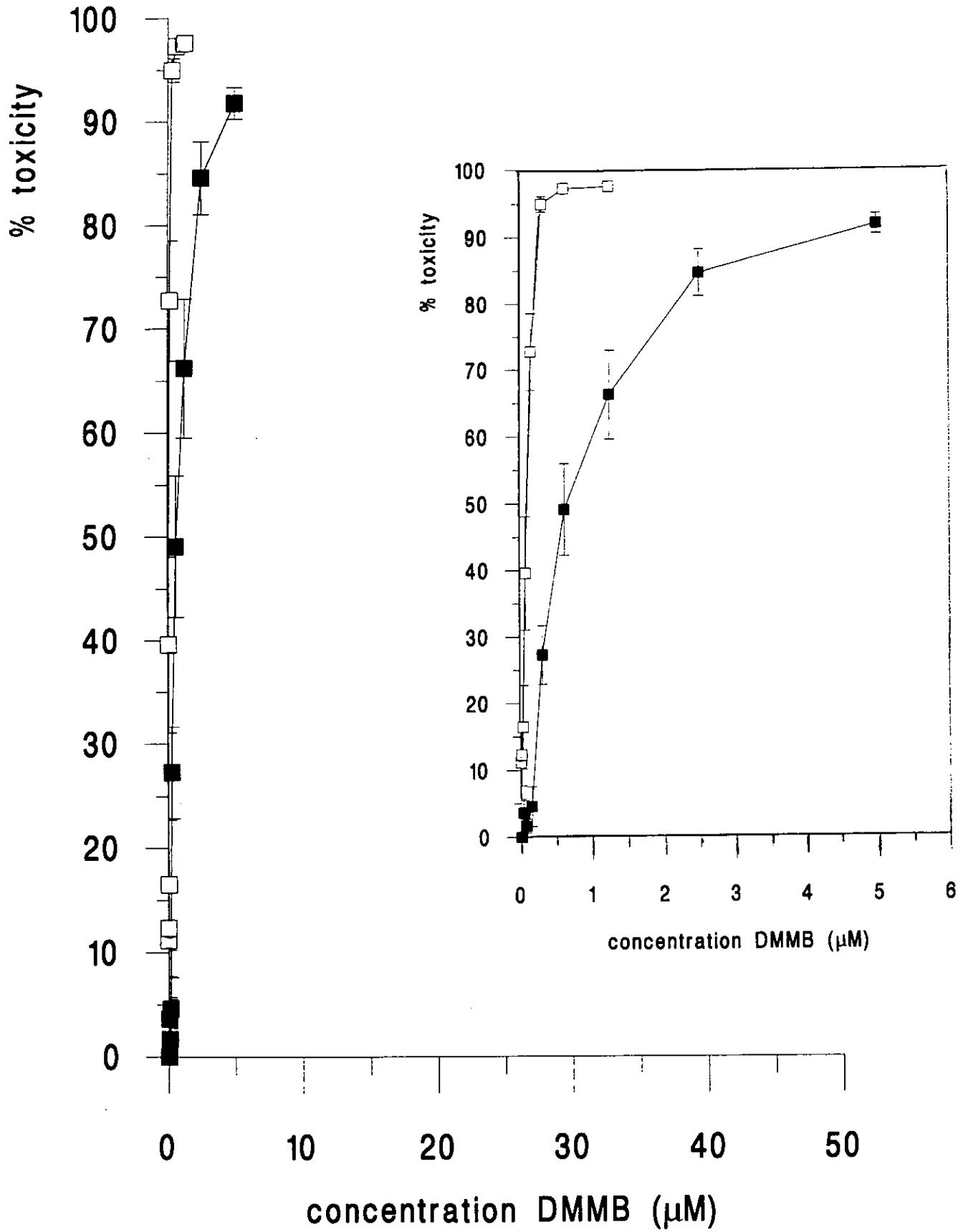


Figure 29. Photocytotoxicity (_) and dark toxicity (■) of 1,9-dimethyl methylene blue against the EMT-6 cell line. Each point is the mean of at least 8 experiments. Error bars represent SEMs. Insert shows enlargement of responses at low concentrations.

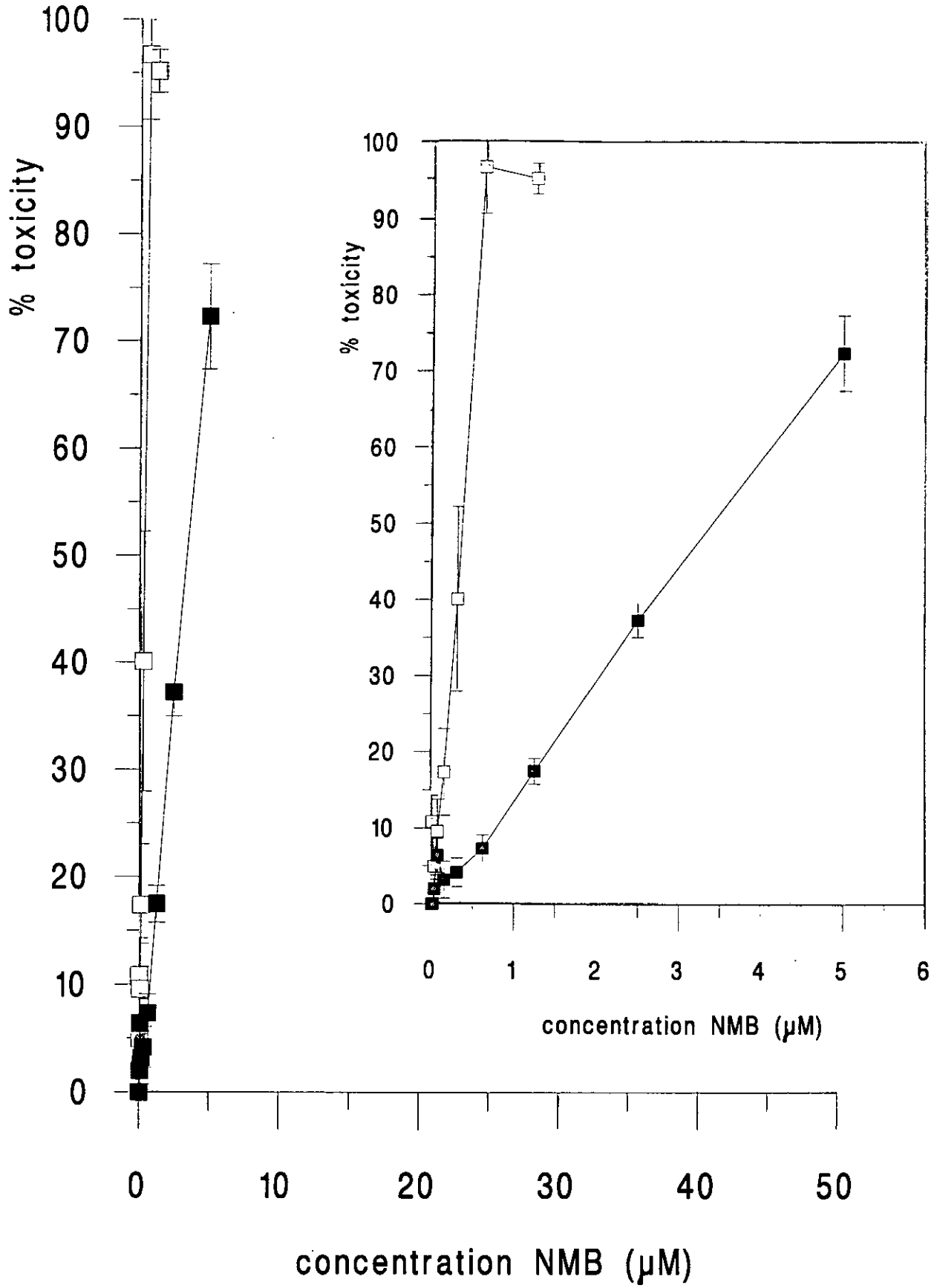


Figure 30. Photocytotoxicity (□) and dark toxicity (■) of new methylene blue N against the EMT-6 cell line. Each point is the mean of 8 experiments. Error bars represent SEMs. Insert shows enlargement of responses at low concentrations.

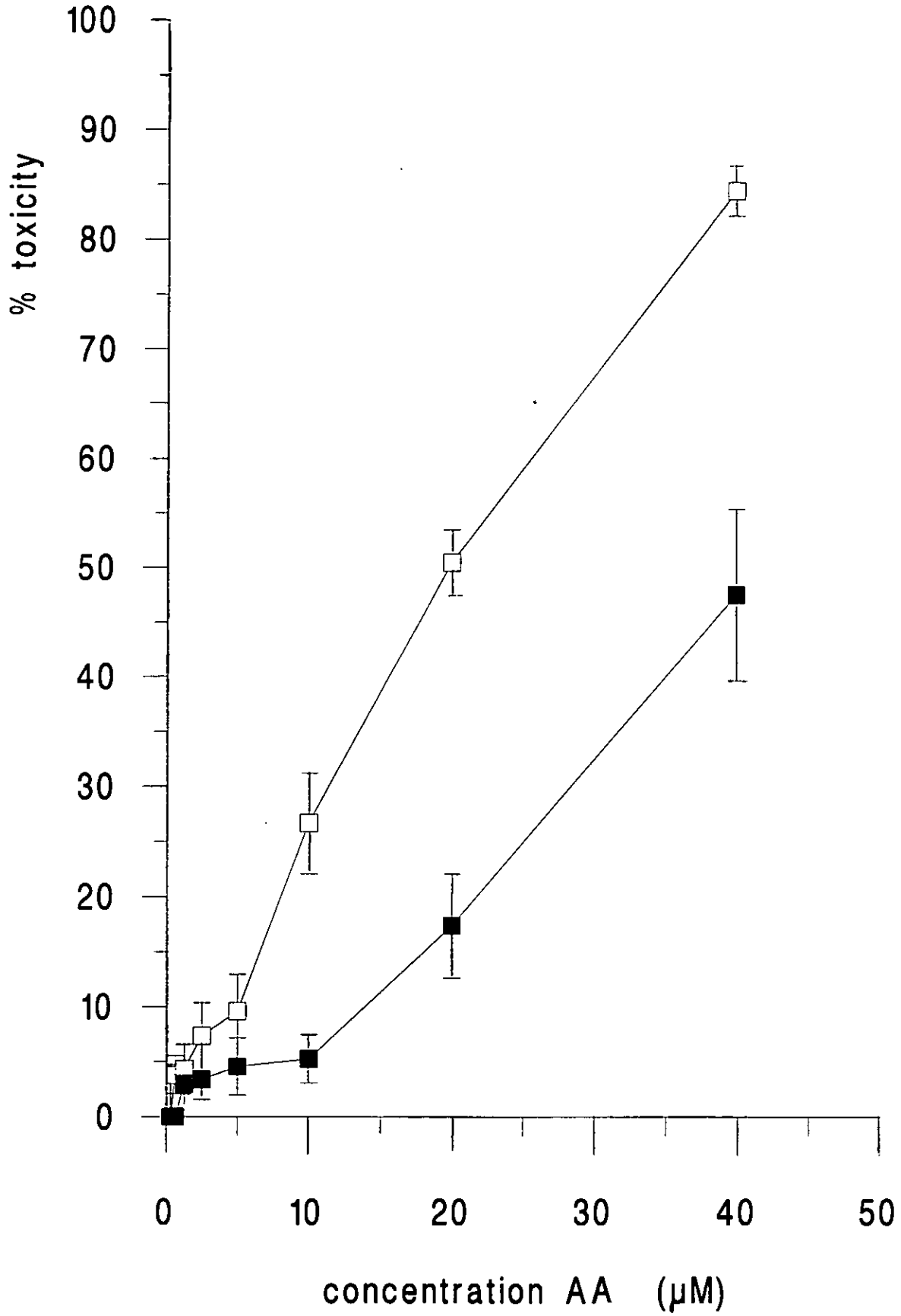


Figure 31. Photocytotoxicity (□) and dark toxicity (■) of azure A against the EMT-6 cell line. Each point is the mean of 10 experiments. Error bars represent SEMs.

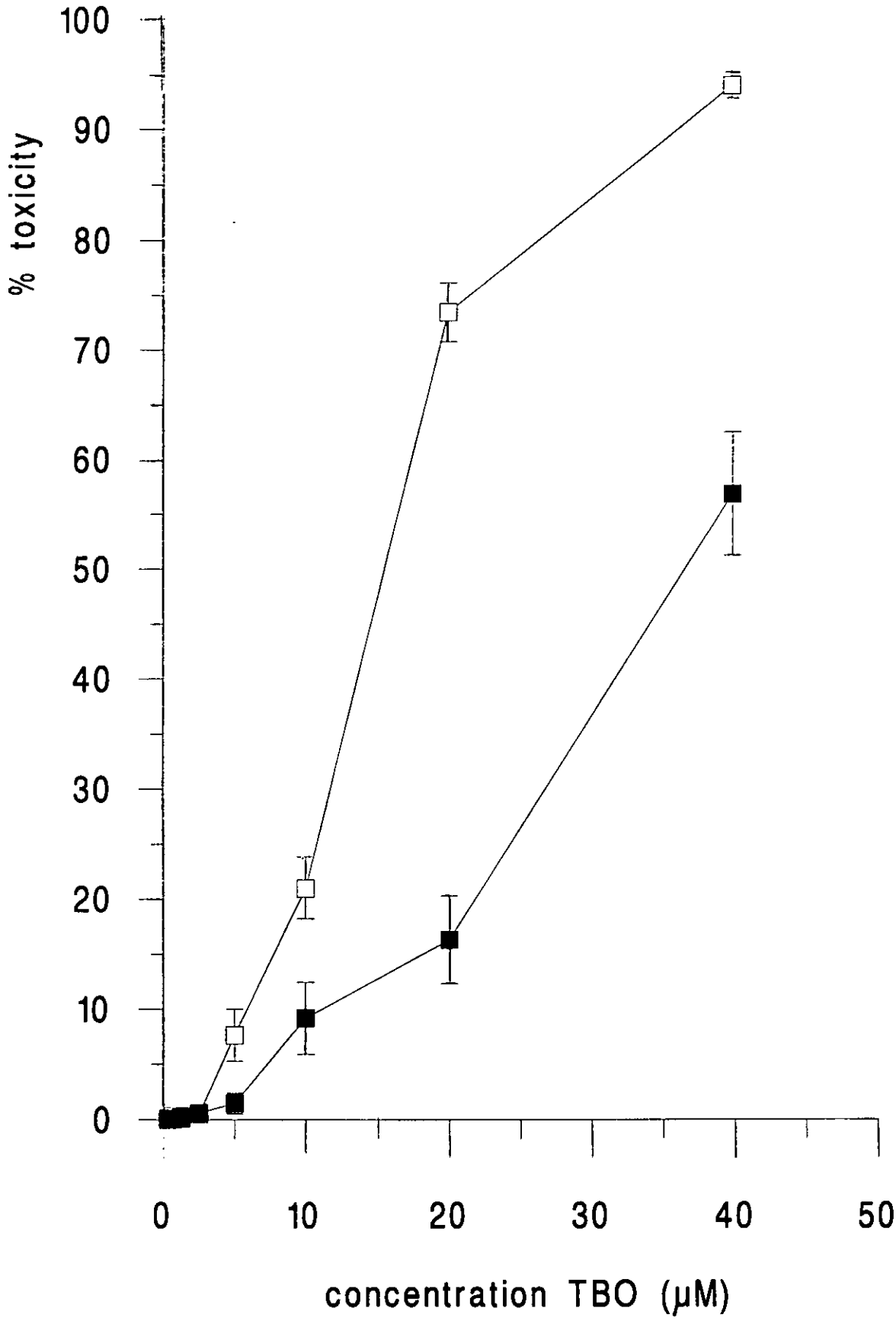


Figure 32. Photocytotoxicity (□) and dark toxicity (■) of toluidine blue against the EMT-6 cell line. Each point is the mean of at least 17 experiments. Error bars represent SEMs.

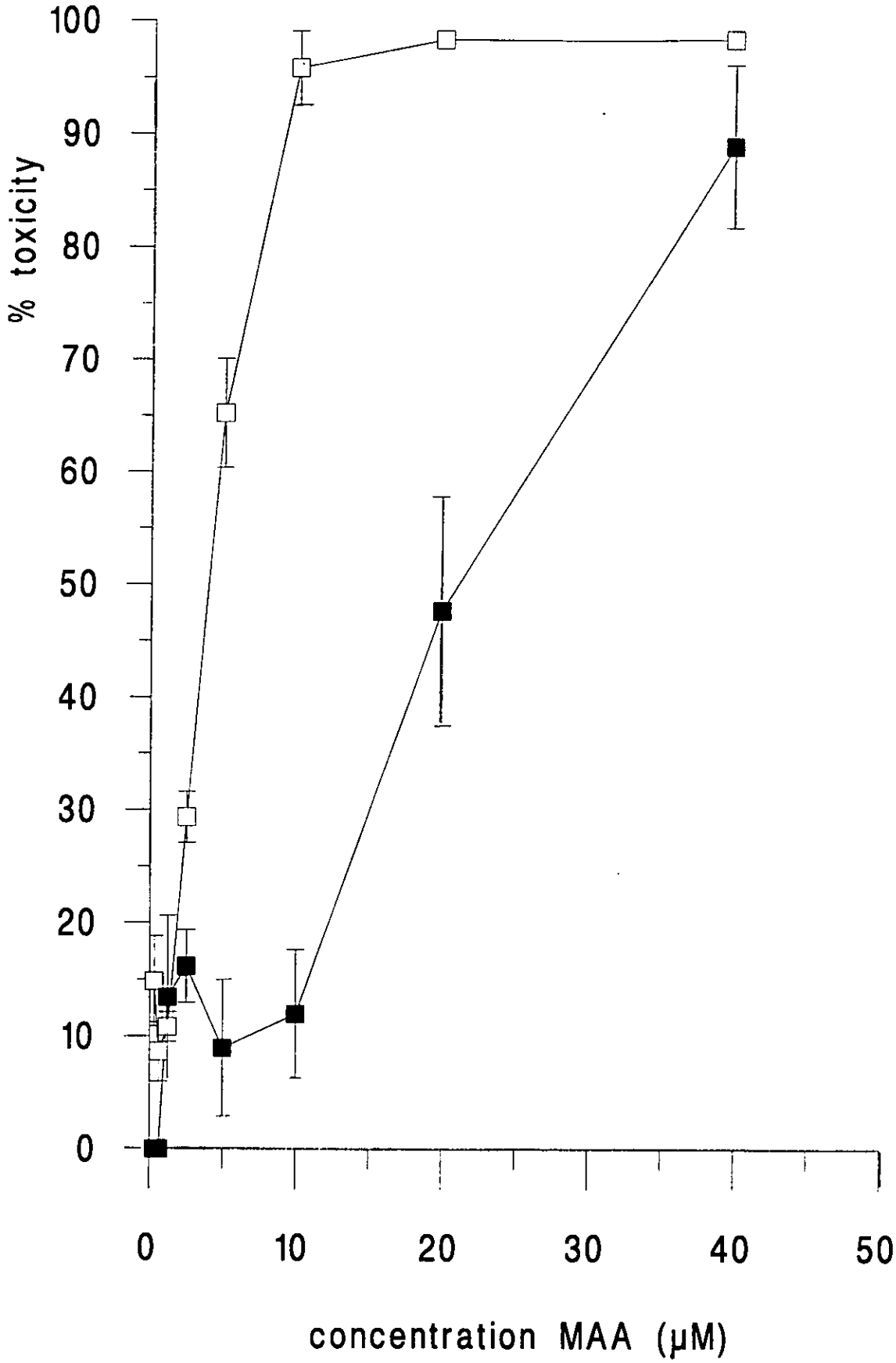


Figure 33. Photocytotoxicity (□) and dark toxicity (■) of 9-methyl azure A against the EMT-6 cell line. Each point is the mean of 3 experiments. Error bars represent SEMs.

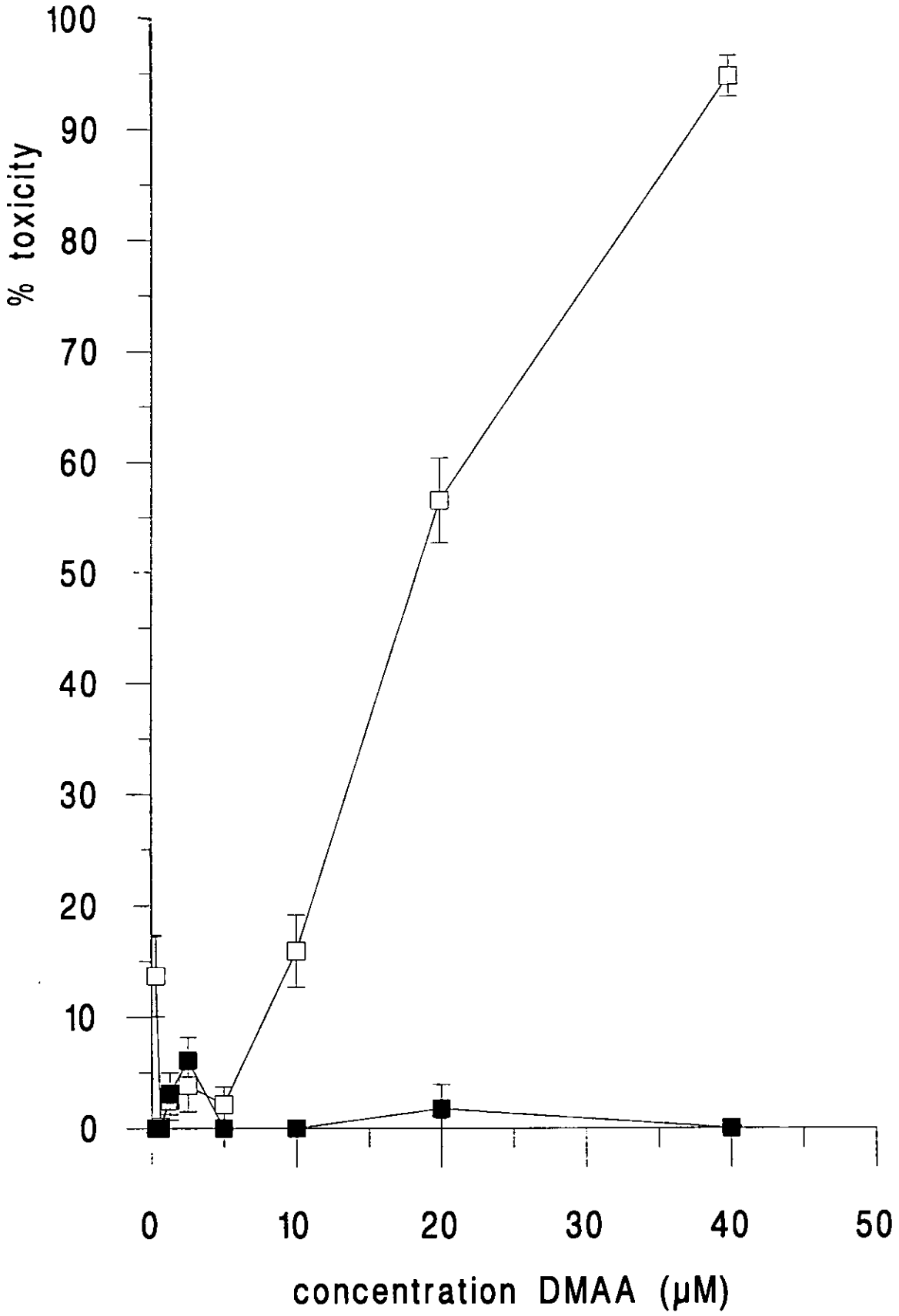


Figure 34. Photocytotoxicity (□) and dark toxicity (■) of 1,9-dimethyl azure A against the EMT-6 cell line. Each point is the mean of 6 experiments. Error bars represent SEMs.

4.4 Effect of amino substitution on cellular uptake of the triarylmethane, Victoria blue-BO

VBBO gave a good correlation between drug concentration and cellular uptake after three hours' incubation, with uptake increasing steadily from 0.002 picomoles per 1000 cells at 0.156 μM to 0.150 picomoles per 1000 cells at 10 μM (Figure 35).

In contrast, the situation for MOVB (Figure 36) is less clear. Cellular uptake of the drug appeared to be improved at concentrations less than 1.25 μM, with the greatest increase in rate being seen between 1.25 μM (0.001 picomoles per 1000 cells) and 2.5 μM (0.007 picomoles per 1000 cells). Between 2.5 μM and 5 μM (0.015 picomoles per 1000 cells), the rate of increase slowed, appearing to level, but thereafter increased again, reaching an uptake of 0.154 picomoles per 1000 cells (comparable to that of VBBO) at a concentration of 10 μM. This suggests a biphasic mode of uptake, though a linear relationship between concentration and uptake is still possible, when allowances for error are taken into concentration, and given that the experiment was carried out only three times. More experimental work is needed in order to clarify the situation.

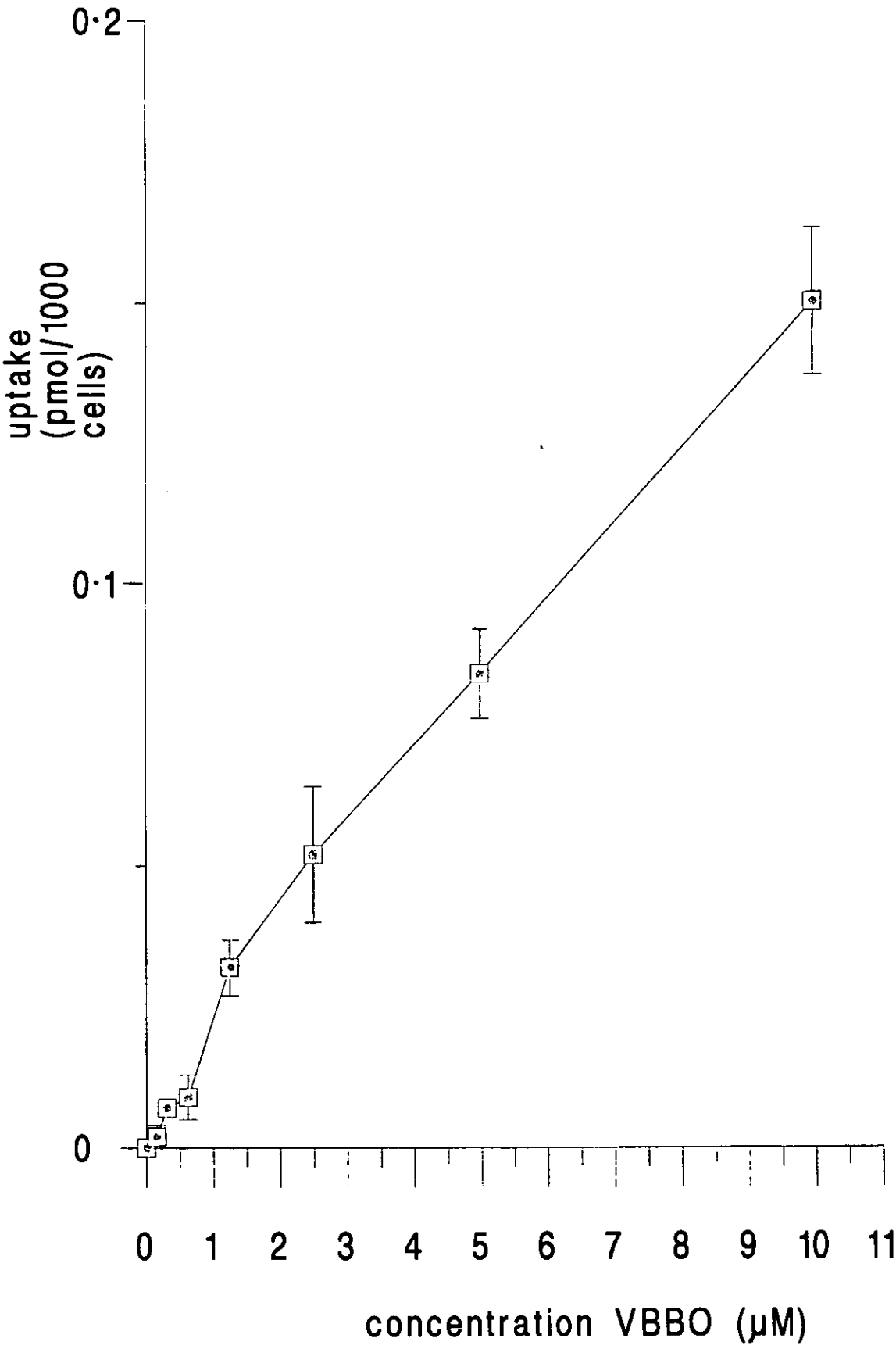


Figure 35. Cellular uptake of Victoria blue-BO. Each point is the mean of 5 experiments. Error bars represent SEMs.

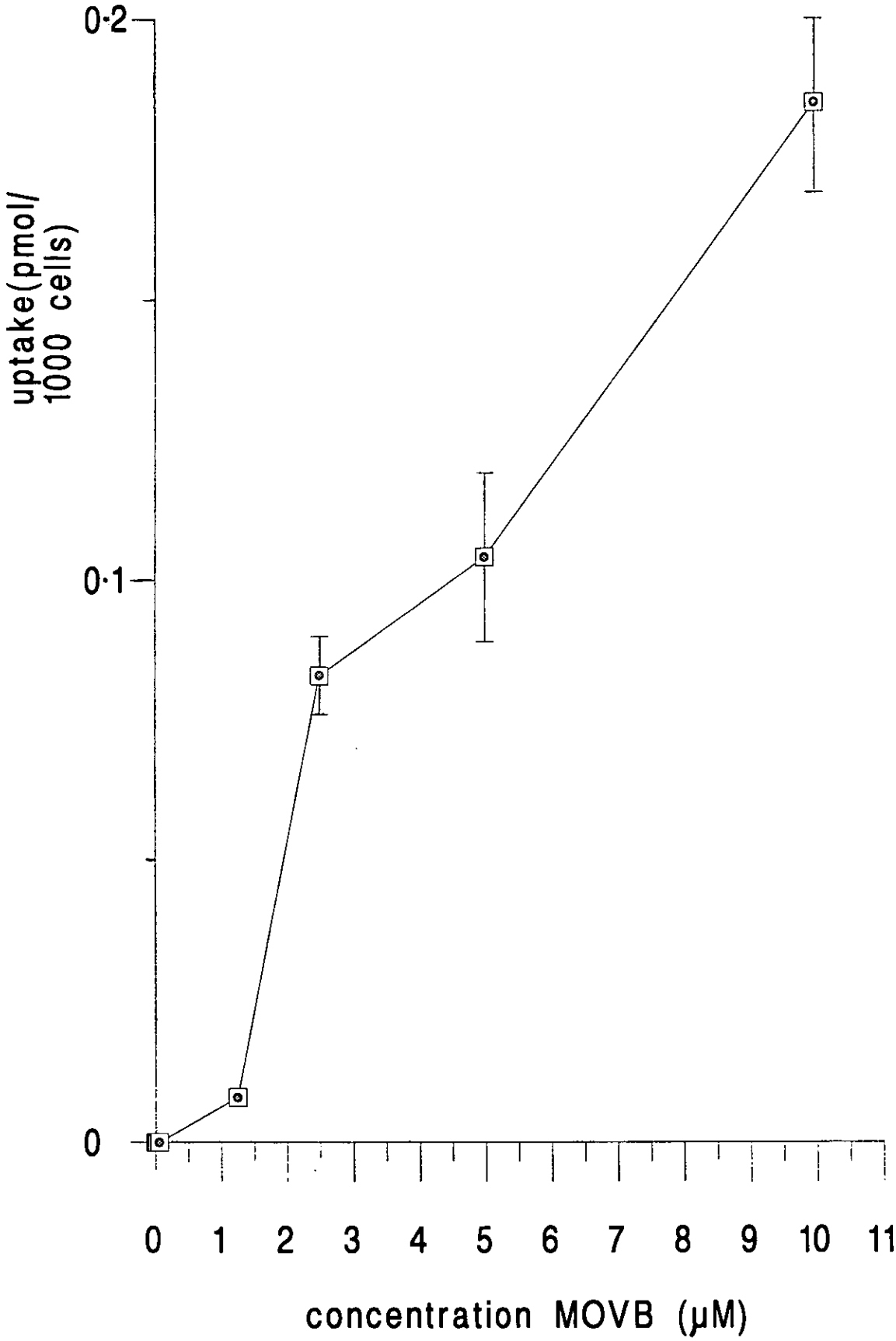


Figure 36. Cellular uptake of MOVB. Each point is the mean of 3 experiments. Error bars represent SEMs.

5. DISCUSSION

5. DISCUSSION

5.1 Project aims

The main objective of this project was that it should serve as a basic training in the running of a modern tissue culture laboratory (Section 1.1). The emphasis was on forward planning, preparation and, in particular, the need to provide a sterile growth environment for cultured cells. The work was given a focus by using tissue culture techniques to study the cytotoxicity of the phenothiaziniums methylene blue (MB) and toluidine blue-O (TBO), and the cellular uptake of Victoria blue-BO (VBBO). MB, TBO and VBBO are cationic dyes which may have potential as photosensitizers in clinical photodynamic therapy (PDT). This research topic established the routine necessary to acquire practical expertise in the manipulation of cell cultures, and to be able to recognise and to tackle typical problems which may occur.

5.2 Cell culture

EMT-6 is a robust cell line which can withstand fairly rough handling. It is easy to propagate, producing confluent monolayer cultures for experimental work over a relatively short period of time (*ie.* two to three days) (Section 4.2). This makes it an ideal experimental tool for fast, easy, replicate testing such as that involved in this project. With hindsight, when preparing growth curves it would have been better to have seeded the cells at a much lower density (Section 3.9). Morgan and Darling (1993) recommend 4×10^2 cells cm^{-2}. As a consequence of seeding such a high

number of cells, the sharp decline in cell number at Day 3 (Figure 26) is likely to be due to overgrowth of the culture and detachment of cells. These cells would ultimately be poured away with the medium prior to counting. In these experiments, reproducible results were obtained using tissue culture techniques and the MTT cytotoxicity assay, clearly demonstrating that methylated phenothiazine derivatives are toxic to EMT-6 cells under defined conditions, and that both the light and dark toxicities of MB and TBO are enhanced by methylation. Since the system is free from the homeostatic regulation and experimental stress found in the whole animal (Section 1.6), the toxicity of a particular agent can be related to its direct effect on the isolated cell.

Once cytotoxicity is indicated, tissue culture techniques may be used further to investigate the possible mechanisms of action of a particular agent. In this case, methods of extraction were employed to determine the cellular uptake of VBBO and its derivative, MOVB, which had been incubated at varying concentrations with cultured cells. Although, in this instance, only a few experiments were carried out, such methods provide additional information and define the nature of toxicity more clearly.

Tissue culture techniques can, in fact, be used in conjunction with confocal microscopy, to pinpoint precisely the movement and location of a drug within the cell [Neyfakh, 1987; Bucana *et al.*, 1990]. The advent of scanning confocal microscopy has permitted the once impossible resolution of images at high magnification by eliminating the flare from out of focus planes above and below that of the object.

Using fluorescent probes and their various patterns of distribution and staining intensities, it is now possible to record intracellular activity with these systems.

These studies clearly demonstrate that tissue culture, generally, is a successful method for studying the effects of particular agents on isolated cells without the need for animal experimentation. The results obtained gave a useful preliminary indication of the efficacy of the various photosensitizers but, for several reasons outlined below, these methods are inadequate for the complete testing of potential pharmaceuticals.

Cultured cells lack the metabolic regulation found in the whole organism which ultimately determines the distribution of a drug in the body. Drug molecules are altered biochemically by Phase I and Phase II reactions which occur mainly in the liver [Rang & Dale, 1991]. Phase I reactions usually consist of oxidation, reduction or hydrolysis, whereby the reactivity and sometimes the toxicity of the parent drug are increased. Phase II reactions involve conjugation which normally results in an inactive compound. Both processes tend to decrease the lipophilicity of the drug thus increasing the rate of renal elimination. Obviously, the mode of administration is of importance; a drug injected systemically would be subjected to these regulatory mechanisms to a greater extent than one which had been, for instance, applied topically to the skin.

Established cell lines tend to possess a high degree of homogeneity and have been changed in some way to produce immortal characteristics (Section 1.2.2). In contrast, tissue to be targeted by PDT is typically a mixture of tumour cells and several

populations of normal cells each having a specialised function. Experiments using these cell types do not, therefore, clarify the specificity of a toxic agent and may not predict accurately the effect it would have on normal cells. In conventional cancer therapies, lack of specificity is a cause of side effects (Section 1.1.2), so there would be added advantage in developing a photosensitizer which is selective for tumour tissue.

Adherent monolayer cultures lack the three-dimensional geometry of cells *in vivo* and, therefore, lack the cell-cell and cell-matrix interactions and signalling capacity which are important in the whole animal. As an alternative, organ cultures (Section 1.2.1) have advantage in that they retain the undifferentiated characteristics of that tissue but they are, in fact, slow-growing and proliferate only from the periphery, and usually only from embryonic tissue. They cannot, therefore, be propagated and fresh explants are required for each experiment. Consequently, several attempts have been made to culture cells in three-dimensional systems using, for example, hollow fibres [Knazek *et al.*, 1972], cellulose sponge [Leighton *et al.*, 1968] and perfused filter wells [McCall *et al.*, 1981]. Cells will grow up to several cells deep in these systems suggesting an analogy with the cellular environment *in vivo*.

As regards the experiments in this particular study, EMT-6 is a murine tumour cell line and it would be unwise to suggest that effects demonstrated in mouse cells would necessarily be repeated in human tumour cells. Problems of slowed growth rate and morphological change (Section 4.1.4) may have contributed to experimental error, but this is unlikely since severely affected cultures were discarded immediately. On the

single occasion that this occurred, the problem was rectified without its source being identified, but these features are sometimes associated with mycoplasma infection (Section 2.12.2). The cell stocks used in this work have subsequently been screened and found to be clear of mycoplasma, thus eliminating this possibility [Burrow, 1997] (Section 4.1.4). Alternatively, contributory factors may have been the thawing procedure, calibration of the new incubator or variations arising in a new batch of tissue culture flasks.

Until the 1980s, the main strategies for the discovery and development of new anticancer drugs involved the testing of potential agents in mice-bearing transplantable leukaemias, or solid tumours from a limited number of murine and human sources. This all changed in the early 1980s with the introduction of a new U.S. drug screening programme using multiple panels of human solid tumour cell lines. The microcultures, in conjuction with a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-2*H*-tetrazolium bromide (MTT)-based colorimetric assay developed by Mosmann [1983], made feasible the rapid, reproducible, initial-stage *in vitro* testing of promising new compounds [Alley *et al.*, 1988].

The MTT assay was used for all the cytotoxicity assays described in this thesis (Section 3.10.5). MTT is a pale yellow, water-soluble tetrazolium dye, which is reduced by live, but not dead, cells to dark purple, formazan crystals. MTT is cleaved by the mitochondria of metabolically active cells, such that the amount of formazan produced is directly proportional to the surviving cell number over a wide range, using a homogeneous cell population. The formazan product is not completely soluble in the

culture medium, so a solvent, in this case DMSO, is used to dissolve the crystals, thus producing a homogeneous colour suitable for spectrophotometric measurement of optical density.

An advantage of the MTT assay is speed of processing. Since neither the substrate nor the medium is found to interfere with measurement of the product [Mosmann, 1983], removal and washing procedures are not required, further reducing the time and minimalising variability between samples. A rapid, visual assessment can be made at the end of the assay, and this is found to correlate well with the printed data from the plate reader.

However, several parameters are reported to influence the specific activity of MTT. Significant variation in the magnitude of MTT reduction has been observed between some cell lines, and a decline in the rate of MTT reduction is apparent with increasing culture age [Vistica *et al.*, 1991]. The amount of MTT in the incubation medium is also important for maximal formazan production, and also tends to differ between cell lines. The absorption maxima of MTT-formazan varies with cell number and with pH, having a wavelength of 560-570 nm at low cell density or high pH, but two absorption maxima at high cell density or low pH, one at 510 nm and a second at 570 nm. At 570 nm, absorption measurements underestimate MTT-formazan and hence high cell numbers, incorporating experimental error into the results [Plumb *et al.*, 1989].

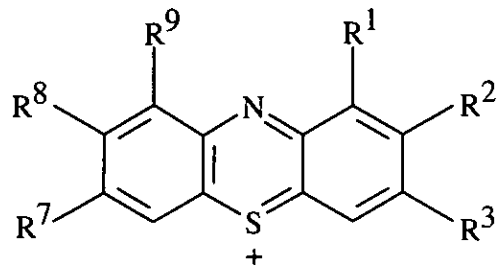
Several alternative colorimetric assays are now available though, in essence, they are all based on the principle of adding a suitable substrate to microcultures of cells following incubation with drug, resulting in the development of a coloured product which can then be measured on an ELISA plate reader. Some of these (acid phosphatase (AP), crystal violet dye elution (CVDE), neutral red (NR) and sulforhodamine B (SRB)) have been compared directly with MTT [Martin & Clynes, 1993]. It was found that all five assays gave a good correlation with viable cell number three to four days following drug treatment, but underestimated cell death after only two days. This was because for two days some cells, although lethally damaged, still retained some lysosomal or mitochondrial activity. It was also found that the various assays have different detection limits, different linear ranges and different sensitivities. AP, SRB and CVDE are more sensitive (in that order), but have a shorter linear range. Of all the methods, MTT is the least sensitive, but has the greatest range of linearity. Furthermore, the exact rank order of the assay varied from one cell line to another. However, the toxicity profiles of the methods were similar for various individual chemicals.

These results provoke question as regards the now widely used MTT-based assays which are less sensitive than other methods available. Assays such as AP also have advantage in that they fix the product to be assayed, whereas the precision of measurements may be compromised in the MTT assay due to possible disturbance of the formazan crystals during aspiration.

5.3 Experimental results

5.3.1 Effect of methylation on the cytotoxicity of phenothiazinium dyes

As regards the methylene blue series, it is apparent from Figures 27-29 that the methylated derivatives, MMB and DMMB, which have methyl substituents on the phenothiazinium ring at positions 1- / 1- and 9- respectively (Figure 37), are more toxic against EMT-6 cells under dark conditions than is the parent compound, MB. It is also obvious that the associated phototoxic effects are far greater. In terms of clinical application, the greater the ratio of light : dark toxicity, the more beneficial the drug. The IC_{50} for methylene blue on illumination is 18.7 μM (Table 1). At this concentration, the dark toxicity is 7.9 %. Thus the toxicity ratio (light : dark) here is 50 : 7.9 = 6.3. The corresponding values for MMB and DMMB are 11.9 and 17.2 respectively (Table 1). However, a more useful clinical indication is given by the IC_{90}. For example, the IC_{90} value for DMMB on illumination is 0.27 μM, and at this concentration the dark toxicity corresponds to a cell kill of 21.3 %, giving a ratio of 4.2. The corresponding ratios for MMB and MB are 6.3 and 3.2 respectively. Both of the methylated derivatives, therefore, appear to have greater clinical promise than methylene blue itself, with the monomethylated derivative giving the greater differential at high phototoxicity levels.



	R^1	R^2	R^3	R^7	R^8	R^9
Methylene blue (MB)	H	H	NMe_2	NMe_2	H	H
Methyl methylene blue (MMB)	Me	H	NMe_2	NMe_2	H	H
Dimethyl methylene blue (DMMB)	Me	H	NMe_2	NMe_2	H	Me
New methylene blue (NMB)	H	Me	NHEt	NHEt	Me	H

Figure 37. The chemical structures of the methylene blue derivatives used in this study.

The IC_{50} values for the MB derivatives (Table 1) are similar to those exhibited by the benzo[*a*]phenothiazinium compounds investigated by Cincotta *et al.* [1994]. For example, against EMT-6 cells, the promising benzophenothiazine photosensitizer, 5-ethylaminobenzo[*a*]phenothiazinium chloride (EtNBS) gave an IC_{50} of 0.1 μM. This is comparable with the value of 0.09 μM obtained in the present work for DMMB. However, it is difficult to compare the two directly because of the different light doses employed and the possibility of cell line variation. Although the dark toxicity for EtNBS is reportedly much lower for a 0.5 μM dose (6 % compared to 41 % for DMMB), no IC_{90} figure was given for the phototoxicity [Cincotta, *et al.*, 1994]. The higher dark toxicities and phototoxicities of the methylated derivatives may be explained by several factors.

Phenothiazinium photosensitizers, such as methylene blue, are prone to cellular reduction. Indeed this may be advantageous in the clinic if, as has been reported for EtNBS, the rate of reduction is greater in healthy tissues than in tumours [Cincotta, *et al.*, 1994]. This would have the effect of increasing tumour selectivity and decreasing the probability of skin photosensitization (Section 1.1.3). The *in vitro* reduction of MB, MMB and DMMB has been examined in aqueous media using a gold microdisc electrode using the method of Svetlicec *et al.* [1987] (data not shown, personal communication, M. Wainwright). The rates of reduction of the three photosensitizers followed the order:

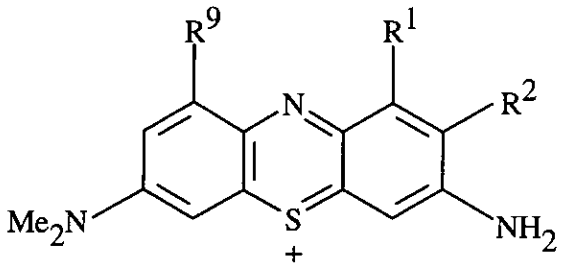
MB>MMB>DMMB

Thus, from a cellular point of view, it can be argued that both MMB and DMMB will be present in their oxidised (cationic) forms for a longer period of time than MB, and therefore that there will be higher concentrations of the photoactive form of the methylated derivatives present. The increased stability to reduction, at least in the *in vitro* electrochemical system used, may be explained by the weak electron-releasing effect of the methyl groups. This would make the chromophore more electron rich and thus less amenable to reduction. In cell culture this could contribute to the increased levels both of dark toxicity and phototoxicity exhibited by the methylated derivatives.

Methylation of the phenothiazinium chromophore resulted in considerable increases in the lipophilicity of the system (data not shown, personal communication, M.

Wainwright). Both MMB and DMMB have positive log P values, whereas that for MB is negative. It has been shown previously in fibroblasts that vital stains bearing a unipositive charge and having $0 < \log P < 5$ tend to localise in the mitochondria [Rashid & Horobin, 1990]. Methylene blue is thought to localise in the cell nucleus [Tuite & Kelly, 1993]. This could indicate that a different cellular localisation pattern for the methylated derivatives is responsible for their greater observed cytotoxicities. Increased levels of photosensitizers in the cell due to lower reduction rates could also explain the higher dark toxicities associated with the methylated derivatives.

The standard DPIBF oxidation test showed that methylation of MB increases the efficiency of singlet oxygen production (data not shown, personal communication, M. Wainwright). Taken with the increased resistance to reduction and the possibility of more critical intracellular localisation, this may explain the greater efficacy of the methylated derivatives against EMT6 cells.



	R^1	R^2	R^9
Toluidine blue O (TBO)	H	Me	H
Azure A (AA)	H	H	H
Methyl azure A (MAA)	H	H	Me
Dimethyl azure A (DMAA)	Me	H	Me

Figure 38. The chemical structures of the toluidine blue derivatives used in this study.

In order to address the effect of chromophore methylation in the toluidine blue series (Figure 38), azure A (Figure 31) was taken as the lead compound since it has no chromophoric methyl group. Methylation in the adjacent position to the amino group, as in TBO (Figure 32), appears to give a slight improvement in the photodynamic action of the compound against EMT-6 cells. Methylation either in position 1 and/or 9, as in MAA (Figure 33) and DMAA (Figure 34) respectively, greatly improved the photokilling effect and, in the case of DMAA, gave a large light:dark toxicity differential at 90 % light toxicity ($LD_{90} = 90$) (Table 1). In terms of photocytotoxicity, this reflects the effect of 1/9 methylation in the MB system.

It is difficult to compare the behaviour of new methylene blue (NMB) with the other photosensitizers, as its substitution pattern is quite different (Figure 37). However, NMB also exhibited a very high light:dark ratio (*ie.* 13 at light LD_{90}) (Table 1; Figure 30).

In terms of the relationship between the position of methylation and phototoxicity, it is known that coplanarity between the amino group and the remainder of the chromophore is required for efficient photosensitizing efficiency. While it is possible that the close proximity of the methyl and amino groups in TBO compared to that in MAA and DMAA would lead to steric interference and the twisting out of the plane of the amino moiety, this effect would be heightened in NMB which has much bulkier ethylamino groups in positions 3- and 7-. The increases in toxicity on methylation must therefore also be due to other factors such as relative uptake, degree of reduction, site of action *etc.*.

5.3.2 Effect of amino substitution on the cellular uptake of Victoria blue-BO

The experiments show that amino substitution at the 4-position of the naphthyl moiety of VBBO has limited effect on cellular uptake. Weak data from few experimental repeats and incorporating large margins of error, show that uptake of MOVB (Figure 35) with respect to concentration, generally resembles that of the parent compound, VBBO (Figures 36), particularly at concentrations above 2.5 μM.

Triarylmethane dyes, such as VBBO, are well known to react with water to form non-planar, neutral carbinol compounds. It is also possible, within the Victoria blue series,

for the secondary amino group attached to the naphthyl residue to become deprotonated, again giving a neutral compound. Although incapable of photoactivation, this still exerts some influence on pharmacological activity and its distribution.

Cellular uptake is expected to be influenced to some extent by the lipophilicity of the compound under investigation. VBBO is highly lipophilic (log P = +3.5) whereas MOVB is much less so (log P = +0.9), and its poor uptake at low concentrations may be due, in part, to a decrease in hydrophobic partitioning (data not shown, personal communication, M. Wainwright).

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6. REFERENCES

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7. PRESENTATIONS OF WORK

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1. THE EFFECT OF METHYLATION ON THE CYTOTOXICITY OF PHENOTHIAZINIUM DYES

Presented at :

The Applied Biology Research Seminar - 20/04/96

The Lancashire Centre for Medical Studies - 20/04/96

12th International Congress on Photobiology, Vienna. - 01/09/96-06/09/96

2. INCREASED CYTOTOXICITY AND PHOTOTOXICITY IN THE METHYLENE BLUE SERIES *via* CHROMOPHORE METHYLATION

Mark Wainwright, David A Phoenix, Lesley Rice and Jack Waring

Paper in preparation/submitted.