# STUDIES OF THE INDUCTION OF TUMOUR CELL TERMINAL DIFFERENTIATION

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## Summary

A wide variety of agents, at marginally sub-toxic concentrations, induce terminal differentiation of HL-60 human promyelocytic leukaemia cells to granulocyte- or monocyte-like cells. 170mM N-methylformamide induced >70% of HL-60 cells to differentiate to granulocytes while viablity remained at >70%. Commitment to differentiation did not occur until after a minimum of 24 hours of incubation with NMF.

Manipulation of growth conditions in the presence of NMF showed that inhibition of proliferative potential of the cells was required for the induction of differentiation, but at least one cell division was required for development of the mature phenotype.

The hypothesis that the induction of a stress response was important in the induction of differentiation by pharmacologic agents was tested. There was no evidence for the induction of gene amplification during commitment to differentiation with NMF, but there was evidence to support the involvement of the heat shock response in HL-60 cell differentiation. Six known inducers of heat shock protein (hsp) synthesis induced HL-60 cell differentiation: ethanol (213mM), sodium arsenite (6uM), lidocaine (3mM), procaine (5mM), cadmium (60uM), and hyperthermia (43.5°C for 1 hour). The extent of maximal differentiation induced by these agents was inversely correlated with the inhibition of normal protein synthesis; the "weak" inducers cadmium and hyperthermia (which induced <30% of cells to differentiate) inhibited protein synthesis more than the "strong" inducers such as NMF, ethanol, arsenite and local anaesthetics (which induced >50% differentiation).

Synthesis of hsps was unexpectedly repressed rather than induced during differentiation, but cellular levels of hsp70 increased concomitant with commitment to differentiation induced by NMF. The data support the hypothesis that a stress response, possibly involving heat shock proteins, is important in the induction of HL-60 cell differentiation by disparate pharmacologic agents.

Key words: differentiation; promyelocytic leukaemia; HL-60; stress; heat shock protein.

FOR MY PARENTS,

FOR ALL THEIR HELP

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| | |
|---|---|
| APL | Acute promyelocytic leukaemia |
| ara-A | Adenine arabinoside |
| ara-C | Cytosine arabinoside |
| ATP | Adenosine triphosphate |
| BFU | Burst-forming unit |
| CFU | Colony-forming unit |
| CHO | Chinese Hamster ovary cells |
| CSF | Colony-stimulating factor |
| DHFR | Dihydrofolate reductase |
| DM | Double minute chromosome |
| DMF | Dimethylformamide |
| DMSO | Dimethyl sulphoxide |
| DNA | Deoxyribonucleic acid |
| EGF | Epidermal growth factor |
| FACS | Fluorescence-activated cell sorting |
| GTP | Guanosine triphosphate |
| HCGF | Haematopoietic cell growth factor |
| HEPES | N-2-hydroxyethylpiperazine-N'-2-ethanesulphonate |
| HGPRT | Hypoxanthine/guanine phosphoribosyl transferase |
| HMBA | Hexamethylene bisacetamide |
| hsp | Heat shock protein |
| HSTF | Heat shock transcription factor |
| IL-2 | Interleukin-2 |
| IL-3 | Interleukin-3 |
| LAK | Lymphokine-activated killer cells |
| MEL | Murine erythroleukaemia cells |

| | |
|---|---|
| MGI | Macrophage/granulocyte inducer |
| mRNA | Messenger ribonucleic acid |
| Mw | Molecular weight |
| NMF | N-methylformamide |
| PBS | Phosphate-buffered saline |
| PDGF | Platelet-derived growth factor |
| RPMI | Roswell Park Memorial Institute medium |
| SDS | Sodium dodecylsulphate |
| TBS | Tris-buffered saline |
| TEMED | N,N,N',N'-tetramethylethylenediamine |
| TNF | Tumour necrosis factor |
| TPA | 12-O-tetradecanoylphorbol-13-acetate |
| TRIS | Tris(hydroxymethyl)aminomethane base |

# CHAPTER ONE

# INTRODUCTION

## 1.1 The search for new chemotherapeutic strategies

Cancer patients are often treated with chemotherapy following surgery, in cases where the tumour is inaccessible to surgery or has spread from the primary site. Chemotherapy has improved survival of some patients with a variety of malignancies, particularly leukaemias (Catovsky 1984), Hodgkin's and non-Hodgkins lymphomas (Longo and de Vita 1984), ovarian and cervical cancer (Sausville and Young 1984), breast cancer (Loprinzi and Carbone 1984), testicular cancer (Williams and Stoter 1984) and osteosarcoma (Bonadonna and Santoro 1984). Unfortunately, few responses have been achieved using chemotherapy for head and neck cancers (Taylor 1984), lung cancer (Hansen and Rørth 1984), colorectal and pancreatic carcinoma (Smith and Goldberg 1984), malignant melanoma (Rümke 1984) and soft tissue sarcomas (Bonadonna and Santoro 1984). Responses are most commonly achieved when combination chemotherapy is used, selecting agents to enhance or act synergistically with the other agents without enhancing toxicity (Capizzi et al 1977, Frei 1972, Schein et al 1976, Vogl et al 1979).

Classical antitumour agents rely upon cytodestruction of proliferating cells as the means of removing malignant cells from the body; unfortunately, normal tissues such as the bone marrow, gastrointestinal tract and basement membrane of the skin contain rapidly proliferating cells

which are often damaged by the cytotoxic chemotherapy, leading to side effects. Bone marrow toxicity - leukopaenia, thrombocytopaenia and anaemia - may be dose-limiting during chemotherapy, particularly with alkylating agents (nitrosoureas) and antimetabolites (methotrexate and 5-fluorouracil) (Dorr and Fritz 1980), while severe nausea and vomiting occurs in more than 80% of patients treated with agents such as cisplatin or nitrosoureas. Alopecia occurs with many chemotherapeutic regimes, but is reversible on cessation of the treatment. Other side effects include phlebitis, allergic responses and neurological, pulmonary, renal, hepatic, cardiac and dermatologic toxicity, depending on the drug, or combination, administered. Long term complications include skeletal alterations, testicular and ovarian suppression, and the inducement of new tumours, as many of the highly cytotoxic drugs used appear to be mutagenic and carcinogenic (Dorr and Fritz 1980).

Apart from the problem of classical cytotoxic chemotherapy causing toxicity to normal tissues, at any given time it is likely that only a proportion of the cells in a tumour will be proliferating actively. This is particularly true in solid tumours where there tends to be a small growth fraction with large numbers of quiescent cells which will be unaffected by antiproliferative chemotherapy, and which may be recruited into the proliferative pool once chemotherapy has ceased (Schabel

1969).

New strategies are required if chemotherapy is to overcome these problems; one approach would be to increase the selectivity of drugs for malignant cells by targeting the drug to the tumour. Agents such as adriamycin, methotrexate or vindesine could be attached to a monoclonal antibody which reacts with an antigen on the tumour, enabling the drug to accumulate at the target site (Olsnes 1981, Arnon and Sela 1982, Baldwin 1984). However, different monoclonals would be required for different tumours and raising antibodies specific for tumour cells is a time-consuming process. Also, tumours tend to be heterogeneous and not all cells in a give tumour might express the required antigen (Heppner 1984). It is important that the drug is not released from the complex before it reaches the target, and if the tumour is not well vascularised the antibody might not even reach the target. There are many such problems to overcome before monoclonal-drug complexes may be used routinely for therapy (Davis and Rao 1984).

Because chemotherapeutic agents cause such undesirable side effects, it has been proposed that biological response modifiers be used for cancer treatment instead of cytotoxic agents. The first of these tested were the interferons; a group of molecules with a key function in sensitizing natural killer cells in the body,

stimulating them to become cytotoxic towards malignant cells (Ortaldo et al 1983). Clinical trials have been carried out on various interferons. The most commonly reported side effect was a severe 'flu-like syndrome (Quesada and Gutterman 1983, Edelstein et al 1983), with myelosuppression and hepatotoxicity at high doses, and also central nervous system toxicity (Rohatiner et al 1983). Phase II trials reported very few responses (Weiss et al 1984), and it has been concluded that interferons alpha and beta have no significant antitumour effect. Interferon gamma, however, was more potent than the other interferon types against certain tumours in experimental systems, and has yet to be tested in the clinic (Fleischmann et al 1984).

Another biological response modifier, Interleukin-2 (IL-2), has recently been tested in clinical trial, both alone and with co-administration of lymphokine-activated killer (LAK) cells. Lymphocytes were obtained from the patient by leukapheresis, treated with IL-2 <u>in vitro</u> to generate LAK cells, then re-infused in conjunction with administration of IL-2. IL-2 alone induced 6 responses out of 46 patients, but there were severe side effects such as hypotension, fluid retention, cardio-pulmonary disorders and severe anaemia, with 3 treatment-related deaths. In combination with LAK cells, there were 25 responses out of 106 patients but again severe toxicity occurred (Rosenberg et al 1987). Using a different dosing

regime of IL-2 and LAK cells, West et al (1987) showed similar responses, with somewhat reduced toxicity, although still severe.

More recently, tumour necrosis factor (TNF) has been proposed for cancer therapy. TNF is a protein secreted by macrophages which produces haemorrhagic necrosis in tumours in experimental animals and cytolytic or cytostatic effects in tumour cells in culture, while untransformed cells are generally unaffected (Tsujimoto et al 1985, Fung et al 1985). TNF is currently in clinical trial, and any therapeutic activity has yet to be established.

Another alternative strategy for chemotherapy involves development of agents which, rather than being cytotoxic to cells, induce terminal differentiation or reversion of the malignant phenotype, resulting in mature end-stage cells which are no longer able to proliferate (Sartorelli 1985, Reiss et al 1986). This strategy has several potential disadvantages, such as the fact that even though rendered benign by the treatment, the existing tumour mass might be life threatening due to its position pressing on vital organs. Also, the agents used could possibly cause inappropriate differentiation of normal stem cells in the body, depleting stem cell pools necessary for self renewal of the bone marrow, for example. Other potential problems include the difficulty

in ensuring that all the malignant cells have been induced to differentiate terminally, and that the differentiation process is irreversible so that the cells could not revert to a malignant phenotype at a later date. The great advantage, though is that because the drugs would not act by inducing cytotoxicity but by "reforming" the malignant cells, this less aggressive therapy should have none of the problems of toxicity to normal tissues. This strategy relies upon the fact that, in general, malignant cells have failed to undergo full development and differentiation and have remained in an immature, proliferating state, as explained below.

## 1.2 Differentiation of malignant cells

### 1.2.1 Neoplasia is a disease of cell differentiation and is reversible

As early as 1874, the Italian Francisco Durante stated that "elements which have retained their anatomical embryonal characters in the adult organism, or have regained them through some chemico-physiologic deviation, represent, in my opinion, the generative elements of every tumor variety, and specifically those of a malignant nature" (Triolo 1965). Markert, in 1968, expanded on this idea and proposed that neoplasia was a disease of cell differentiation, in which normal gene activity was

misprogrammed by epigenetic mechanisms to produce a neoplastic pattern of metabolism in which all the individual components were normal. He showed that three characteristics were important for malignancy: persistent cell division, adhesive properties of the cell membrane which would allow the cell to migrate or metastasize, and specific patterns of cellular metabolism. In abnormal combinations or in excess these various normal cell properties would produce neoplasms.

Also in 1968, Potter introduced the theory that "oncogeny is blocked ontogeny", meaning that malignancy is due to a block in the normal pathway of cellular development and differentiation, which would explain the "embryonic" character of tumours noted by Durante. Normal cellular development involves a limited number of stem cells which proliferate in a controlled manner, some of the progeny remaining in the stem cell pool, while others undergo further divisions resulting in more highly differentiated cells which ultimately lose their proliferative potential. Usually the balance between self-renewal and differentiation is controlled so that a tissue is maintained in a steady state with sufficient newly differentiated cells being produced to replace those mature cells lost through senescence. Potter (1978) stated that "there are a multitude of stages of differentiation, prior to the terminal stage that, if prevented from advancing toward the terminal stage, will

cause a cell to retain its capacity to re-enter the proliferative cycle". Blocked ontogeny would lead to continuous proliferation of stem cells with no subsequent differentiation, so that all daughter cells also proliferate, resulting in a neoplasm. Potter proposed that the concept must include "partially blocked ontogeny" where a fraction of the tumour cell population undergoes extensive differentiation but does not progress to the normal organized terminal state.

In 1974, Pierce postulated that a tumour could be seen as a caricature of a normal tissue and might appear undifferentiated because of the predominance of undifferentiated proliferating stem cells in relationship to the number of cells that had differentiated and become benign. Many tumours actually contain differentiated, non-dividing cells as well as the stem cells, particularly prominent in the teratomas. These tumours were shown to represent a block in the development of a primordial germ cell, and the stem cells in the tumour (embryonal carcinoma cells) proliferated and produced a chaotic arrangement of many differentiated cell types, resembling bone, teeth, brain, skin cells, etc. (Pierce 1974, Markert 1968). These differentiated cells tended to divide at much reduced rates, or not at all, and were not malignant. Squamous cell carcinomas similarly were shown to be composed of areas of proliferating, undifferentiated cells, some of the progeny of which differentiated to form

"pearls" of non-malignant, non-proliferating, mature squamous cells (Pierce and Wallace 1971). These results suggest that malignancy is not an irreversible phenomenon.

In concordance with this, several authors showed that some malignant cells differentiated normally and became non-malignant if placed in a normal environment. Illmensee and Mintz (1976) introduced mouse embryonal carcinoma cells into genetically marked mouse blastocysts. Mosaic offspring were produced in which cells derived from the tumour cell had made substantial contributions to a wide range of somatic tissues in the animal, with no tumour formation. Gootwine et al (1982) injected mouse leukaemic cells into 10 day mouse embryos and produced healthy adult mice whose normal granulocytes contained a marker from the leukaemic cells. These results show that restoration of orderly gene expression was achieved simply by placing the malignant cells in a normal embryonic environment.

It has been suggested that normal cells contain potent tumour suppressor genes, which are either missing or not expressed in malignant cells. Hybrids of different human cancer cells with normal human fibroblasts or with differentiating epithelial keratinocytes were unable to produce tumours in nude mice, whereas the parental cancer cells were tumourigenic (Sabin 1981). Surprisingly, the hybrids were still transformed in that they underwent

infinite multiplication *in vitro* and showed loss of contact inhibition. Prolonged culture of these hybrids resulted in gradual loss of chromosomes and often gave rise to revertants which had regained their tumourigenicity. Hybrids of two parental cancers of different somatic cell origins were also non-tumourigenic, suggesting that the two cancers had different forms of genetic loss or impairment and that complementation occurred on hybridization to restore a more normal phenotype. The conclusion from these experiments was that certain chromosomes carry suppressor genes, and loss of this chromosome, or part of the chromosome, would lead to tumourigenicity. Weissman et al (1987) showed that introducing a normal human chromosome 11 into a Wilms' tumour cell line abolished tumourigenicity, while the cells remained transformed *in vitro*. Wilms' tumour cells have a deletion in chromosome 11, and these experiments suggest that chromosome 11 contains a tumour suppressor gene which is absent in Wilms' tumour cells.

Stanbridge (1985) discussed the relation between apparently dominant transforming oncogenes and the suppressor genes. Oncogenes are thought to arise from normal cellular genes (proto-oncogenes) by mutation, gene amplification or chromosome translocation, leading to their continuous expression. Oncogenes are generally thought to play roles in maintenance of cell proliferation in the malignant state, comparable to the functions of the

proto-oncogenes in normal cell proliferation (Varmus and Bishop 1986). The product of the sis oncogene is homologous to one chain of platelet-derived growth factor (PDGF) (Doolittle et al 1983); the erbB oncogene encodes a protein resembling part of the epidermal growth factor (EGF) receptor (Downward et al 1984); src, abl and fps oncogenes encode protein-tyrosine kinases (Hunter and Cooper 1985); ras oncoproteins are probably regulatory G-proteins in the adenyl cyclase system (Bar-Sagi and Feramisco 1985); and myc and fes encode nuclear, possibly DNA binding proteins (Evan and Hancock 1985, Holt et al 1986). These oncogenes have the ability to transform cells, resulting in sustained proliferation in vitro and the ability to form tumours in vivo. A single activated oncogene does not appear to be sufficient for complete transformation of normal cells, although cooperation between two oncogenes such as myc and ras will convert normal cells to malignant cells (Lane 1984, Land et al 1983). The cell hybridization experiments reviewed by Sabin (1981) however showed that tumourigenicity could be suppressed in cell hybrids, whilst expression of the oncogenes was maintained. Thus, oncogene expression alone is not sufficient for malignancy if the cells contain functioning tumour suppressor genes (Stanbridge 1985). Whether these suppressor genes have a role in reinstating the normal developmental control processes of the malignant cell, resulting in its differentiation to a more mature, non-proliferating cell type, is unclear.

Malignant cells can certainly be induced to differentiate and become non-malignant as a result. Several instances have been recorded of neuroblastomas which spontaneously differentiated into benign ganglion cells (Cushing and Wolbach 1927, Pierce and Wallace 1971, Fox et al 1959, Dyke and Mulkey 1967), and cells *in vitro* have been induced to differentiate with a variety of agents, as will now be discussed.

### 1.2.2 The induction of differentiation in vitro

Rat mammary cells have been induced to differentiate to alveolar -like cells by dimethylsulphoxide (DMSO) or prostaglandin $E_1$, with reduced tumourigenicity when injected back into mice (Rudland et al 1982); neuroblastoma cells were also induced to differentiate by DMSO into cells with neurite processes and an excitable membrane which also expressed such neuronal enzymes as acetylcholinesterase (Kimhi et al 1976). Treatment of colonic adenocarcinoma cells with DMSO, sodium butyrate or dimethylformamide (DMF) increased the doubling time of the cells and changed expression of certain cell surface antigens, but this was reversible on removal of the inducer, and did not reduce the tumourigenicity of the cells (Kim et al 1980, Hager et al 1980). Transformed mouse embryonal fibroblasts treated with DMF assumed a phenotype similar to the untransformed cell line, and

responded to normal growth controls (Marks et al 1985); differentiation was also induced in human melanoma cells with DMSO or phorbol esters, as characterized by inhibition of cell growth and melanin synthesis (Huberman et al 1979). F9 embryonal carcinoma cells were induced to differentiate to parietal ectoderm by retinoic acid and other agents (Strickland and Mahdavi 1978, Dean et al 1986), and a number of different murine and human leukaemia cells were also induced to differentiate terminally with a variety of agents (reviews Tsiftsoglou and Robinson 1985, Reiss et al 1986). It is clear that in some of these cell types the extent of differentiation induced was minimal, with the expression of antigens characteristic of more mature cells but with no cessation of cell proliferation or suppression of tumourigenicity; in other cases the cells ceased cell division and expressed different antigens but reverted to the original proliferating cell type as soon as the inducing agent was removed, while some cell types underwent full, irreversible, terminal differentiation.

### 1.2.3 Clinical trials of differentiation inducers

A variety of agents which will induce differentiation of cell lines *in vitro* have been proposed as possible chemotherapeutic agents, and the following have entered clinical trials:

Retinoic acid, an inducer of leukaemic cell differentiation in vitro (Breitman et al 1980), was tested on one patient with acute promyelocytic leukaemia (APL) (Daenen et al 1986), and after seven weeks of daily oral treatment the patient went into complete remission. After six months of treatment, however, there appeared to be the first signs of a relapse. The authors believed that the remission was due to induction of differentiation of the leukaemic cells rather than cytotoxicity, but there was no direct evidence for this. Nilsson (1984) also treated one APL patient with retinoic acid, and the proportion of bone marrow promyelocytes decreased to normal levels, after about 16 weeks. The patient was still in remission after one year of treatment. There were side effects of slight desquamation of the skin, and conjunctivitis. Another patient with APL was treated with etretinate (a retinoic acid analogue) in combination with actinomycin D (a combination which induced differentiation of the patient's bone marrow promyelocytes when treated in vitro), but there was no antitumour effect and the patient died after 15 days of treatment (Sampi et al 1985). Retinoic acid is currently being tested against a variety of other malignancies (Cheson et al 1986).

N-methylformamide (NMF), which induces differentiation of leukaemic cells in vitro (Tanaka et al 1975, Collins et al 1978), was first used in clinical trial in 1956 (Myers et al) after it was found to have

some activity against the sarcoma 180 in mice (Clarke et al 1953). It was tested against 5 patients with advanced carcinomas and sarcomas, and had no apparent antitumour effect whilst causing severe, though reversible hepatotoxicity. However, it was later discovered that NMF did not have any adverse effects on the compromised bone marrow of the mouse (Langdon et al 1985a), unlike most cytotoxic chemotherapy, indicating that NMF might act by a different mechanism to the classical antiproliferative drugs. As well as antitumour activity against the Sarcoma 180, NMF was found to be active against the M5076 sarcoma and the TLX5 lymphoma in mice (Gescher et al 1982), and in combination with cyclophosphamide an additive antitumour effect was observed against the M5076 (Langdon et al 1985b). NMF has since been used in a number of clinical trials. McVie et al (1984) showed two partial responses and two minimal responses out of 15 patients in phase I trial, with nausea and vomiting and some reversible hepatotoxicity as the side effects. Ettinger et al (1985) carried out a phase I trial in 35 patients and found the same side effects, including considerable malaise, but, encouragingly, there was no myelosuppression. In a subsequent phase II trial, hypophosphataemia was seen in 5 out of 7 patients (Sternberg and Yagoda 1985), accompanied by muscular aches, restlessness and insomnia. Another phase II trial was terminated early because of frequent and occasionally severe toxicity, particularly hepatic and gastrointestinal, and no responses were seen (Eisenhauer

et al 1986). Alternative schedules are currently being explored to determine whether the toxicity can be prevented.

Hexamethylene bisacetamide (HMBA) is also a potent inducer of differentiation *in vitro* (Collins et al 1980, Fibach et al 1977) and has recently entered clinical trials (Egorin et al 1987). The dose limiting toxicity was metabolic acidosis and neurotoxicity, with agitations, hallucinations, confusion and alteration of consciousness, which generally reverted to normal by 8 days after the end of treatment. One patient developed myocardial infarctions, and several developed transient renal insufficiency, with nausea and vomiting in many cases. Bone marrow toxicity was also apparent, and no objective tumour regressions were noted. This study was useful, however, in establishing the maximum tolerated dose and pharmacokinetics, and it requires further trials to determine whether HMBA will be useful for chemotherapy.

Cytosine arabinoside (ara-C), an inducer of human myeloid leukaemic cell differentiation *in vitro* (Griffin et al 1982), has been tested in many clinical trials, both alone and in combination. It induced some clinical responses in patients with acute non-lymphocytic leukaemia, but caused severe myelotoxicity, leading to some deaths (Cheson et al 1986).

Other inducers of differentiation that have been tested in the clinic include sodium butyrate (Miller et al 1985, Novogrodsky et al 1983), vitamin $D_3$ (Koeffler et al 1984) and aclacinomycin (Case et al 1985, Sakurai et al 1983), and all have shown limited antitumour activity whilst causing toxicity to the patient.

The clinical trials suggest that chemical agents which can induce differentiation *in vitro* cause just as many side effects as conventional chemotherapeutic agents, so any agents employed to induce differentiation *in vivo* will probably have to be molecules closely related to the natural inducers of differentiation, perhaps polypeptides acting specifically on a cellular receptor. In order to design such drugs, more needs to be known about the processes involved in differentiation induction.

## 1.3 Differentiation of leukaemic cells

### 1.3.1 The leukaemic cell system as a model

Many leukaemic cell lines can be induced to undergo full irreversible terminal differentiation *in vitro*, so that the cells cease proliferation and take on a new phenotype characteristic of mature peripheral blood cells, with altered antigen expression, changes in biochemical pathways and changes in cellular morphology. These cell

lines include HL-60, Friend MEL, U937, WEHI, etc., and will be discussed in more detail later. The haematopoietic cell system provides a good experimental model as differentiation processes can be studied in the normal cells as well as the malignant cell lines.

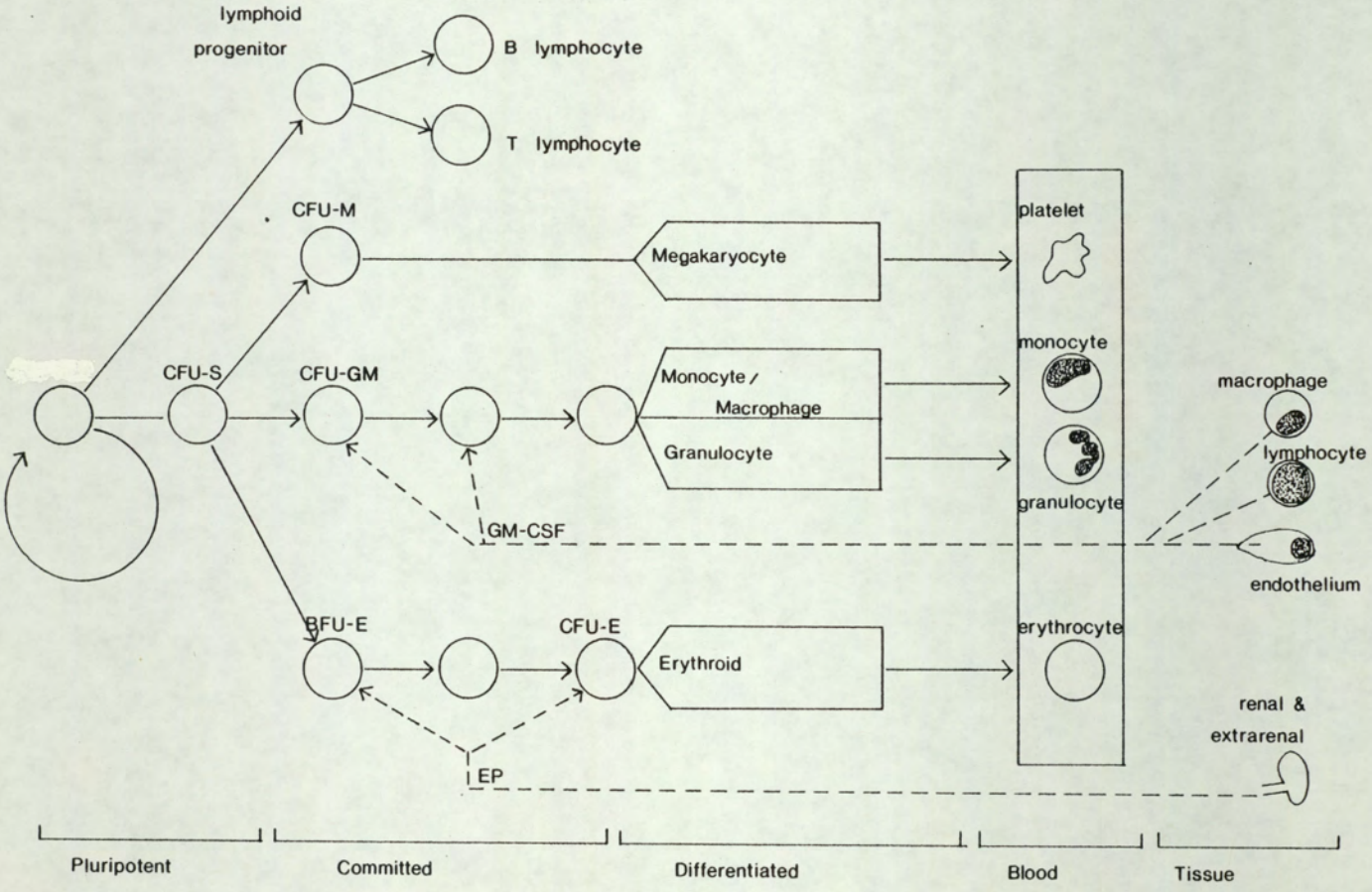
### 1.3.2 Normal haematopoiesis

Normal haematopoiesis occurs largely in the bone marrow. The most primitive stem cells found in the bone marrow are pluripotent; that is they have the potential to produce cells of lymphoid, myeloid, erythroid or megakaryocytic lineage (Thompson 1979, Greenwalt and Jamieson 1970). These cells divide and differentiate to produce stem cells with more restricted potentials, as outlined in Figure 1.1.

The stem cells known as lymphoid and myeloid colony-forming units (CFU-LM) give rise to CFU-S and lymphoid precursors, the CFU-S then gives rise to CFU-C (also known as CFU-GM, granulocytic and monocytic), CFU-M (megakaryocytic), and BFU-E (erythroid). The BFU-E divides to produce CFU-E cells which proliferate and differentiate to mature erythroid cells. This process is under the control of erythropoietin, which is produced mainly in the kidney, in response to a change in circulating $pO_2$ or $pCO_2$, and the new erythrocytes appear in the blood only one to two days after an increase in

FIGURE 1.1 Stem cells involved in normal haematopoiesis
(From Ruddon 1981, p90)

CFU = colony forming unit; BFU = burst forming unit; -LM = lymphoid and myeloid; -S = spleen; -GM = granulocte and macrophage; -M = megakaryocyte; -E = erythroid; GM-CSF = granulocyte/macrophage colony stimulating factor; EP = erythropoietin.



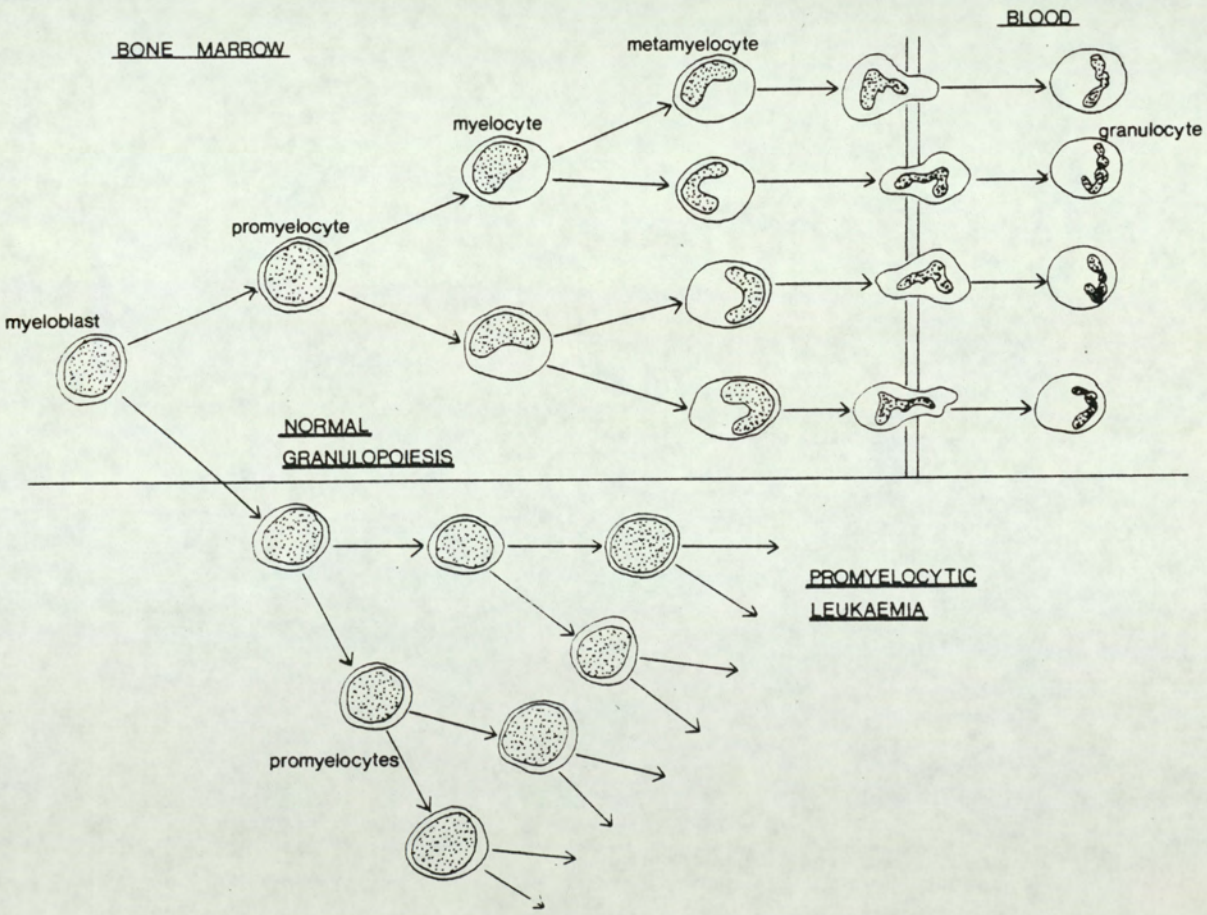
erythropoietin levels (Stohlman 1970).

The CFU-M cells are under the control of thrombopoietin, which stimulates them to divide and differentiate to megakaryocytes, which fragment to produce circulating platelets.

The CFU-GM cells are probably the precursors of both granulocytic and monocytic cells, are under control of the granulocyte-macrophage colony stimulating factor (GM-CSF), and divide to produce myeloblasts. The process by which monocytes are derived from myeloblasts is unclear, but the monocytic cells are released into the blood stream, from which they enter the tissues as macrophages (resulting either in reticulum cells fixed in certain tissues, or histiocytes which are mobile within the tissues) (Bessis 1973). The maturation process for granulocytes is much more clear. The myeloblast divides to produce promyelocytes, which divide in turn to produce myelocytes. The myelocytes then divide to produce metamyelocytes which subsequently mature and pass into the blood stream as granulocytes (either neutrophils, eosinophils or basophils). This sequence of events is shown in Figure 1.2.

The stages of granulocytic maturation can easily be followed as the nucleus changes shape dramatically from an oval to a concave then banded form, finally resulting

FIGURE 1.2 Differentiation of promyelocytes to granulocytes, compared to promyelocytic leukaemia



in segmentation of the nucleus in mature granulocytes.

It was originally thought impossible that both granulocytic and monocytic cells could be derived from the promyelocyte, as the two different pathways were thought to have diverged prior to this point. However, the HL-60 cell line, a promyelocytic leukaemia which can be induced to differentiate either to granulocytes or monocytes, has disproved this. It is no longer even so clear that the pathways shown in Figure 1.1 are correct, as Rose et al (1986) presented evidence that the progenitor cells committed to differentiation towards megakaryocytes, erythrocytes, neutrophils, monocytes, B cells and T cells are arranged in a linear sequence rather than a divergent tree. It is not easy to clarify this as the bone marrow stem cells are difficult to isolate and grow in culture.

### 1.3.3 Myeloid growth and differentiation factors

There are four different types of growth-inducing proteins which exert their effects on myeloid cells. They are called Colony Stimulating Factors (CSF, or CSA) or Macrophage-Granulocyte Inducers type 1 (MGI-1) (Sachs and Lotem 1984, Dexter 1984). MGI-1M induces proliferation and subsequent differentiation of cells giving rise to macrophages, MGI-1G (G-CSF)gives rise to granulocytes in a similar manner, MGI-1GM (GM-CSF) gives rise to both cell types, and the fourth class, known as Interleukin-3 (IL-3)

produces macrophages, granulocytes, eosinophils, mast cells, erythroid cells and megakaryocytes. This variation between the types of MGI-1 presumably means that they stimulate cells at different parts of the haematopoietic stem cell system, with IL-3 stimulating one of the earliest, pluripotent stem cells. IL-3 is known by various other names, including Haematopoietic Cell Growth Factor (HCGF), Mast Cell Growth Factor (MCGF), Burst Promoting Activity (BPA) and Persisting Cell-Stimulating Factor (PSF) (Metcalf 1985).

As well as the MGI-1 factors, there is a second class of proteins which exert their effects on myeloid cells - the Differentiation Factors (DF) or MGI-2 (Sachs and Lotem 1984). Normal myeloid precursor cells cultured with an MGI-1 endogenously produce MGI-2, so that cells stimulated to proliferate by MGI-1 will subsequently differentiate in response to endogenous MGI-2. There are again various types of MGI-2, and different types of MGI-1 may switch on synthesis of different MGI-2.

Dexter et al (1984) have reported some interesting findings on the mechanism of action of HCGF (IL-3) on a non-leukaemic myelomonocytic cell line, which was dependant on the factor for growth. HCGF directly increased glucose transport into the cell, which led to high cellular ATP levels from glycolysis. When HCGF was removed, glucose transport decreased dramatically, the

cells quickly became depleted in ATP and lost viability. The authors suggested that HCGF is normally produced by stromal cells in the bone marrow, in close proximity to developing haematopoietic cells. When the primitive cells are released or escape from this stromal cell environment, they will lose the source of HCGF, their ATP levels will quickly fall and they will be "programmed to die". It is possible that at this point the cells become responsive to circulating differentiation factors, or that part of the programmed cell death involves differentiation to normal mature terminal end stage cells. This suggests that a cellular stress, due to lack of ATP, may be partly responsible for terminal differentiation of these cells in vivo. The fact that HCGF is produced by the stromal cells and remains bound to the stromal cells or their extracellular matrix to maintain an HCGF-rich micro-environment has recently been confirmed by Gordon et al (1987), and the importance of this stromal micro-environment in haematopoiesis has also been reported (Zipori 1986).

The factors may be useful therapeutically if they can be produced in sufficient quantities. Recently, recombinant human GM-CSF has been produced from genetically engineered Chinese Hamster Ovary cells, and has been tested on neutropaenic AIDS patients. The GM-CSF elicited a dose-dependent increase in white blood cell count that more than corrected the leukopaenia at higher

doses (Clark and Kamen 1987). GM-CSF might therefore be effective in preventing or reversing leukopaenia induced by cytotoxic cancer chemotherapy, but whether it will be useful for inducing differentiation of leukaemia cells remains to be established.

### 1.3.4 Haematopoiesis and leukaemia

Leukaemic cells arise due to some abnormality of the normal haematopoietic process so that immature cells fail to differentiate and continue to proliferate, resulting in immature cells circulating in the blood. Many are found to have increased expression of oncogenes such as *abl*, *myc*, *ras* and *src* (McClain 1984). It is possible that many leukaemic cells endogenously produce their own MGI-1 which maintains proliferation, and that they do not produce MGI-2 which would induce differentiation. Alternatively, a cellular receptor for the MGI-1 may be modified in such a way that either it is permanently stimulated even in the absence of growth factor, or it has a higher avidity for the growth factor and thus responds to lower concentrations (Metcalf 1985). The HL-60 human promyelocytic leukaemia secretes a factor which stimulates its own growth and colony formation *in vitro*. This factor, known as autostimulatory activity (ASA) is similar to a human CSF (MGI-1) in that it will stimulate normal bone marrow cells, and has antibody cross-reactivity (Perkins et al 1984). WEHI-3 murine myelomonocytic

leukaemia cells also produce a growth factor which is probably IL-3 (Bazill et al 1983).

Clinically, leukaemias can be classified as either acute, which evolve and progress very quickly, or chronic, where the progression is very slow. Leukaemias are also classified according to their cell type, the most common ones being acute or chronic lymphoid leukaemias (with the appearance of lymphoblasts and lymphocytes respectively), and acute or chronic myeloid leukaemias (with the appearance of myeloblasts/promyelocytes and myelocytes/granulocytes respectively). There are also, more rarely, erythroleukaemias, megakaryocytic leukaemias, etc. Even among the myeloid leukaemias there are different types, depending on whether the cells resemble myeloblasts, promyelocytes, or more differentiated cells. Various cell lines have been derived from leukaemias, which can be induced to terminally differentiate in vitro.

The human K-562 cell line was derived from a patient with chronic myelogenous leukaemia in blast crisis and the cells were induced to differentiate in vitro to erythroid cells which synthesised haemoglobin when treated with butyrate or hemin (Anderson et al 1979). These cells have also been induced to differentiate into cells of the myeloid lineage (Lozzio et al 1981). Phorbol ester or retinoic acid treatment of U-937 human histiocytic lymphoma cells induced differentiation to macrophagic or

monocytic cells respectively, and the KG-1 cell line derived from an acute myelogenous leukaemia also differentiated to macrophage-like cells after exposure to phorbol esters (Hemmi and Breitman 1984). WEHI-3BD$^+$ murine myelomonocytic leukaemia cells were induced to differentiate to mature granulocytes after retinoic acid treatment, and granulocytes were also produced after treatment of M1 murine myeloid leukaemia cells with dexamethasone (Reiss et al 1986). The most extensively studied cell lines which will undergo differentiation in vitro are the Friend murine erythroleukaemia and the HL-60 human promyelocytic leukaemia, which will now be discussed in detail.

## 1.4 The Friend murine erythroleukaemia cell line

The Friend murine erythroleukaemia (MEL) is a cell line derived from viral transformation of an erythrocyte precursor. The resulting cells appear to be analogous to CFU-E cells, and will proliferate continuously in vitro (Friend et al 1971, Rifkind et al 1984). The cells can be induced to differentiate terminally to more mature erythrocyte-like cells which synthesise haemoglobin in response to a wide variety of agents, including DMSO, HMBA, actinomycin D, ouabain, prostaglandin E1, hypoxanthine, 6-thioguanine (Collins et al 1980), butyrate, UV irradiation, X-rays, azacytidine,

methylisoxanthine (Rifkind et al 1984), nicotinamide and related compounds (Terada et al 1979), L-ethionine (Christman et al 1977), and poly L-lysine (Supino et al 1986). Hemin was also able to induce differentiation of MEL cells in that it induced synthesis of haemoglobins, but the cells retained their proliferative potential so hemin did not induce true terminal differentiation (McMahon et al 1984). The phorbol esters were able to inhibit the induction of differentiation by DMSO (Yamasaki et al 1977), as was the local anaesthetic procaine (Bernstein et al 1976). Surprisingly, although butyrate induced differentiation at concentrations greater than 0.75mM, concentrations less than 0.75mM actually inhibited the induction of differentiation by HMBA (Corin et al 1986). Lithium chloride was also able to inhibit MEL cell differentiation induced by DMSO, (Gallicchio 1985) which is of interest as lithium decreased the numbers of committed erythroid stem cells (CFU-E, BFU-E) while increasing the granulocytic stem cell compartment when administered to patients. Lithium is thought to act <u>in vivo</u> by increasing circulating levels of GM-CSF, and has been proposed as adjuvant therapy for amelioration or prevention of myelosuppression caused by chemotherapy or radiotherapy (Steinbertz et al 1980).

Rovera and Surrey (1978) suggested that MEL cell differentiation inducers could be divided into three classes from studies of resistant clones, with resistance

to any inducer in one class also resulting in resistance to every other inducer in that class, but not necessarily to inducers in other classes. In fact, all the inducers apart from butyrate and hemin were designated as "class A" with butyrate as "class B" and hemin as "class C". These data suggest that the majority of MEL cell differentiation inducers act by a common pathway.

## 1.5 Characteristics of MEL cell differentiation

### 1.5.1 Possible actions of DMSO

In an attempt to determine the mechanism of induction of differentiation of MEL cells by DMSO, Friend and Freedman (1978) reviewed some of the possible effects of the solvent on cells of various types. Its effects included inhibition of some enzymes such as catalase, chymotrypsin and peroxidase (Rammler 1967), while it increased the activity of deoxyribonuclease (Monder 1967) and activated lysosomes which lead to the release of acid phosphatase (Misch and Misch 1969). DMSO also altered secondary structure of DNA and RNA (Reem and Friend 1975), altered oxygen uptake (Bajaj et al 1970), decreased membrane fluidity and permeability (Lyman et al 1976), inhibited $Na^+,K^+$-ATPase and stimulated the $Ca^{2+}$-transport ATPase (Burges et al 1969). In addition, the solvent inhibited DNA synthesis (Hervas and Gimenez-Martin 1973),

caused scission in DNA and alterations in folded genomes (Scher and Friend 1978, Terada et al 1978), and decreased overall thermostability of chromatin (Lapeyre and Bekhor 1974). How any of these effects could lead to the specific changes in gene expression required to cause cessation of cell proliferation and development of the differentiated phenotype is as yet unclear.

### 1.5.2 Commitment as a multi-step process

Gusella et al (1976) determined that MEL cells needed to be incubated with DMSO for at least 18 hours before a significant percentage became committed to differentiate, with only 20% of cells committed after 24 hours, rising to 90% after 48 hours. They proposed that two sequential programs were necessary for differentiation of these cells. The first program, the commitment program, required the presence of inducer, and included both a latent period in which irreversible commitment events did not occur and a subsequent period in which individual cells became committed to the second program in a stochastic manner. Conversion of a given cell from an uncommitted to a committed state occurred with a probability determined by culture conditions and genetic lineage of the cell. The second program did not require the presence of inducer, and exhibited concomitant accumulation of haemoglobin and decreased proliferative potential until the non-dividing, haemoglobin expressing

end-stage cell was produced.

Levenson and Housman (1979 and 1981a) found that if the cells were washed free of DMSO before they became committed, they retained a memory of the exposure so that commitment would proceed without a lag period if the inducer was added back to the cells. This memory could be erased if the cells were treated with cycloheximide (a protein synthesis inhibitor) or cordycepin (which inhibits transport of mRNA from the nucleus into the cytoplasm) during the period of withdrawal of inducer. Murate et al (1984) and Chen et al (1982) showed that dexamethasone could block expression of the differentiated phenotype in cells exposed to HMBA, but the cells were still able to become partially committed even in the presence of the steroid, and retained memory of their exposure to the inducer so that commitment occurred much faster than usual when dexamethasone was removed. They suggested that the inducer-dependent processes in commitment to differentiation were not steroid sensitive, but the later steps involved in cessation of cell proliferation and the expression of the globin genes were steroid sensitive. Dexamethasone is known to inhibit transcription and post-transcriptional modification of certain mRNAs (Beutler et al 1986) and so probably acted in MEL cells by preventing transcription. This suggested that the early dexamethasone-insensitive commitment events caused accumulation of a signal which would subsequently lead to

gene transcription, provided this was not inhibited by a steroid. Nomura's group also suggested that there were two distinct reactions in the induction of differentiation by DMSO; they induced differentiation indirectly by fusion of MEL cells that had been briefly treated with DMSO with non-erythroid cells (baby hamster kidney BHK cells or FM3A mouse mammary gland cells) that had been treated with UV (or other DNA damaging agent). One reaction was not specific for MEL cells and originated as a consequence of damaging the DNA; the other reaction was MEL cell-specific (Nomura and Oishi 1983, Kaneko et al 1984). They have since isolated a proteinaceous fraction, produced in MEL or non-erythroid cells in response to agents that affect DNA replication, which induced differentiation in MEL cells provided those cells had been briefly exposed to DMSO (Watanabe et al 1985, Nomura et al 1986), suggesting that one of the two distinct reactions in the induction of differentiation involved synthesis of a specific protein factor.

### 1.5.3 The effects on DNA and the cell cycle

Various laboratories have studied the relationship between the cell cycle and the induction of differentiation. In 1974, McClintock and Papaconstantinou varied the length of the MEL cell cycle by changing the growth conditions and determined that haemoglobin synthesis only occurred after the cells had undergone two

mitoses in the presence of the inducer DMSO. Levy et al (1975) synchronised MEL cells with thymidine and showed that DMSO must be present during S phase and possibly $G_2$+M for any induction of differentiation to occur, and decided that the cells needed to be incubated with the inducer for 24 to 30 hours before this critical S phase for a significant number of cells to differentiate. Tsiftsoglou and Sartorelli (1976) showed that DNA synthesis was indeed essential for the induction of differentiation to occur in response to DMSO, by demonstrating that stationary phase cells would not differentiate. However when separation of daughter cells (growing logarithmically) was prevented by colchicine or cytochalisin, the cells were still able to differentiate in response to DMSO because DNA synthesis occurred despite the cells being unable to undergo cytokinesis. These three reports therefore agreed that it was critical for cells to undergo S phase in the presence of DMSO for differentiation to be induced. Geller et al (1978) showed that one of the earliest events which occurred during commitment with DMSO was a prolongation of the $G_1$ phase. By gravity sedimentation they were able to separate cell samples synchronised in each phase of the cycle. They found that cells in S phase were apparently not as competent at inducing differentiation as cells in $G_1$ or $G_2$, in that such cells required incubation with the inducer for longer periods before commitment occurred. Together with the previous reports this suggested that the events critical to the induction process might take place

very early in S phase, near the $G_1$ boundary. Gambari et al (1979) found the same phenomenon when HMBA was used as the inducer, but in contrast, Leder et al (1975) determined that MEL cells could still differentiate in response to sodium butyrate when DNA synthesis and cell division were completely blocked by ara-C or hydroxurea. This is not very surprising, as butyrate is thought to act by a different mechanism to DMSO and HMBA (Rovera and Surrey 1978). To complicate matters, Levenson et al (1980) showed that commitment of MEL cells to differentiate with DMSO could after all occur in the absence of DNA synthesis, so it still remains unclear whether the cells do need to traverse S phase or not for commitment to differentiation to occur.

After synthesis, mammalian and bacterial DNA is extensively methylated on N-6 or C-5 of cytosine, by methyltranferase enzymes using S-adenosyl methionine. This DNA methylation is thought to play a role in the control of gene expression, with methylation of a critical residue in a gene necessary to render the gene inactive (Razin and Riggs 1980). L-ethionine is converted to S-adenosyl ethionine in the cell and acts as a competitive inhibitor of the methyltransferases, resulting in hypomethylation of the DNA. Christman et al (1977) treated MEL cells with L-ethionine and found that differentiation was induced, although the concentration required to induce differentiation was rather higher than

the concentration known to induce hypomethylation. They also showed that during induction of differentiation with DMSO or butyrate, the DNA was hypomethylated (Christman et al 1980), whereas no hypomethylation occurred in resistant cells treated with these agents. Whether any critical gene-activating residues were demethylated concomitant with this general hypomethylation of the genome is unclear. Tsiftsoglou et al (1984) showed that addition of N-6-methyladenosine to MEL cells prevented differentiation induction by DMSO, but suggested that this was not due to N-6-methyladenosine incorporation into DNA rendering it hypermethylated, but more likely due to an effect on RNA metabolism that is critical in commitment to differentiation.

McMahon et al (1984) showed that during the induction of differentiation of MEL cells with DMSO or HMBA, an activatable $Ca^{2+}$,$Mg^{2+}$-dependant endonuclease was induced. This occurred between 6 and 48 hours of incubation with inducer, and the endonuclease activity induced single strand breaks in the DNA, which were quickly religated. This endonuclease activity was not induced by hemin, which did not induce true terminal differentiation. Terada et al (1978) had previously noted a decrease in sedimentation rate in alkaline sucrose gradients of DNA from MEL cells treated for at least 24 hours with DMSO, and they proposed that this susceptibility to the denaturing effects of the alkali was

either due to single strand breaks in the DNA or changes in the integrity of association of the DNA with nuclear membranes, chromosomal proteins, etc., caused by the DMSO treatment. Scher and Friend (1978) and Scher et al (1986) showed further evidence that the alkaline lability of the DNA induced during commitment to differentiate was indeed due to single strand breaks. Similarly, in differentiating mouse epidermal keratinocytes, single strand breaks were observed which were thought to be a direct consequence of the induction of terminal differentiation (Hartley et al 1985), and Weisinger et al (1986) showed that purified MGI-2 differentiation-inducing factor induced nicks in purified SV-40 DNA *in vitro* and in the DNA of myeloid cells *in vivo*. The precise function of these DNA strand breaks in the terminal differentiation process remains unclear, but they may facilitate the changes in DNA folding which are required for activation of previously silent genes.

### 1.5.4 Changes in protein synthesis

It is clear that major changes occur in gene expression during differentiation, and Parker and Housman (1985) measured the rate of synthesis of cytoplasmic proteins during differentiation by two-dimensional gel electrophoresis. The total protein synthetic rate was reduced when the cells were induced using DMSO, but there were major changes in the relative amounts of various

cytoplasmic proteins, with synthesis of many proteins decreasing while several increased markedly. None of the proteins were identified, however, so their roles and relevance to the induction process are unclear.

### 1.5.5 Changes in glucose transport

Kasahara et al (1986) showed that facilitated D-glucose transport was decreased within 24 hours of treatment of MEL cells with DMSO, beginning with a decrease in the transport rate and followed by a decrease in the amount of protein identified as the glucose transporter. Most malignant cells have elevated glucose transport activity, so it is perhaps not surprising that the transport activity decreased as the cells reverted to a more normal phenotype. However it is possible that the decrease in glucose transport could act as a trigger for commitment (Dexter et al 1984).

### 1.5.6 Modulation of cellular oncogene expression

During DMSO-induced differentiation, Lachman and Skoultchi (1984) showed that there was a biphasic change in mRNA levels of the *myc* cellular oncogene, which decreased rapidly within 2 hours of addition of inducer, remained low until 12 hours then increased to control levels by 18 hours. Subsequently the *myc* RNA levels declined slowly as the cells developed the differentiated

phenotype. When MEL cells were transfected with a vector containing the myc gene under control of a viral promoter so that myc mRNA levels in the cell were maintained even in the presence of DMSO, differentiation induction by the inducer was inhibited, suggesting that myc gene expression plays a role in preventing the normal differentiation of the MEL cells (Coppola and Cole 1986, Prochownik and Kukowska 1986, Dmitrovsky et al 1986). In contrast, Sherman et al (1987) showed that there was no change in c-myc or c-myb expression when MEL cells were induced to differentiate with xylosadenosine and that myc and myb mRNA levels decreased during DMSO treatment of a DMSO-resistant MEL line, suggesting that decreased myc or myb expression were neither sufficient nor obligatory for the induction of differentiation in MEL cells. Other reports suggest that the myc gene is involved in cellular proliferation rather than differentiation anyway - in F9 teratocarcinoma cells c-myc mRNA levels decreased on differentiation induction by retinoic acid, but also decreased under conditions where the cells were growth arrested but did not differentiate (Dean et al 1986). Interestingly, during F9 cell differentiation, the rate of transcription of the myc gene was unchanged, but a change in post-transcriptional control led to a reduction in the half-life of the myc mRNA, which resulted in decreased levels of the myc mRNA (Dony et al 1985). Myc mRNA levels also decreased in growth arrested Daudi Burkitt's lymphoma cells (Einat et al 1985) and increased in

mitogen-stimulated thymocytes (Moore et al 1986) and fibroblasts, again due to changes in the half-life of the mRNA rather than any change in the rate of transcription (Blanchard et al 1985). The myc gene has also been implicated in the cell proliferation stimulated in the liver after hepatectomy in rats (Makino et al 1984), and in proliferation of cells in certain regions of developing human and mouse embryos (Pfeifer-Ohlsson et al 1985, Zimmerman et al 1986).

Unfortunately, none of these studies have actually measured the levels of myc protein, and it is possible that the amount of myc protein in the cells does not correlate with the mRNA levels. Recently, Kindy et al (1987) showed that in MEL and other cells the "non-coding" strand of the myc gene was transcribed to give "antisense" RNA species which may play a role in controlling the translation of the "sense" mRNA. During MEL cell differentiation, the transcription of the antisense mRNA decreased less than that of the sense mRNA during the first few hours, and the subsequent increase in transcription up to 18 hours occurred with different kinetics for the sense and antisense RNA. The independent control of the transcription of the two RNAs represents a powerful mechanism for post-transcriptional regulation of myc gene expression, so the levels of "sense" mRNA do not necessarily correlate with protein levels. These findings demonstrate that myc protein levels in the cells need to

be determined before defining a role for myc in the differentiation of MEL cells.

### 1.5.7 Changes in ion fluxes

Changes in ion fluxes across cell membranes are important in regulation of cell proliferation (Boynton et al 1982, Metcalfe et al 1980, Lopez-Rivas and Rozengurt 1983, Hesketh et al 1985, Moolenaar et al 1983) and have been extensively studied during differentiation of MEL cells. It was thought that an increase in intracellular free calcium was essential for commitment to differentiation. Chapman (1980) proposed that there was a critical ratio between extracellular and intracellular free calcium which would allow differentiation to proceed. Housman's group, however, proposed that an increase in calcium uptake was essential in the commitment process (Levenson et al 1980, Levenson and Housman 1981b and c, Bridges et al 1981). They suggested that this was caused by alteration of $Na^+/Ca^{2+}$ antiport due to increased intracellular $Na^+$ content as a result of DMSO inhibiting the sodium pump (Smith et al 1982). In contrast, Faletto and Macara (1985) measured intracellular free calcium and found a small decrease during commitment, with no change in the total intracellular calcium, rather than the expected increases.

Yeh et al (1983) suggested that the increase in

intracellular sodium observed was due to a block of the $Na^+,K^+$-ATPase, as the increase was ouabain inhibitable. A decrease in the $Na^+,K^+$-pump would be expected to result in a decrease in uptake of $^{86}Rb$ (analogous to potassium) and Mager and Bernstein (1978) and Schaeffer et al (1984) showed that $^{86}Rb$ transport was decreased during commitment. Surprisingly, though, the actual $Na^+,K^+$-ATPase activity did not change and uptake of sodium was also decreased, suggesting inhibition of $Na^+,K^+,2Cl^-$-cotransport and possibly sodium-dependent amino acid cotransport. In contrast to Yeh et al, Lannigan and Knauf (1985) found that intracellular sodium actually decreased after DMSO treatment, but again suggested that this was due to decrease in sodium influx by cotransport. They subsequently showed that the decreased sodium influx was due to decreased sodium-dependent amino acid uptake resulting from decreased protein synthesis (Lannigan et al 1986). Many of the above results are contradictory, and it remains unclear whether changes in ion fluxes play a role in the commitment of MEL cells to differentiate.

## 1.6 The HL-60 cell line

The HL-60 human promyelocytic leukaemia cell line is an acute leukaemia consisting of proliferating promyelocyte-like cells which differentiate terminally in

response to a wide variety of agents. These processes have been well documented, so this provides a useful model system in which to study the induction of differentiation.

The HL-60 cell line was isolated in 1977 from the peripheral blood leukocytes of an adult female with acute promyelocytic leukaemia (Collins et al 1977). In response to agents such as phorbol esters (Huberman and Callaham 1979, Rovera et al 1979) and 1,25-dihydroxyvitamin $D_3$ (Tanaka et al 1983) the cells ceased proliferation and differentiated to monocyte or macrophage-like cells, with characteristic cell morphology and monocytic non-specific esterase activity. These cells also developed the functional characteristics of mature cells and were able to phagocytose complement-coated yeast particles. In contrast, when HL-60 cells were treated with a range of agents including planar-polar solvents (Collins et al 1978), retinoic acid (Breitman et al 1980), hypoxanthine (Collins et al 1980), anthracycline antibiotics (Schwartz and Sartorelli 1982), chymotrypsin (Fibach et al 1985) and 8-bromo-cAMP (Fontana et al 1984), they differentiated into mature granulocyte-like cells, with the functional, biochemical and morphological markers characteristic of such cells. The cells underwent one or two divisions in the presence of the inducer before proliferation ceased, the nuclear to cytoplasmic ratio decreased and the nucleus began to segment. When stimulated to produce a

respiratory burst, the differentiating cells produced superoxide which could then reduce the dye nitrobluetetrazolium to purple formazan granules clearly visible by microscopy. They also ingested complement-coated yeast particles by phagocytosis, and gave a chemotactic response to N-formyl-methionyl-leucyl-phenylalanine (Burgess et al 1984). Several granulocytic cell surface antigens were also induced which were then recognised using specific antibody stains (reviewed by Tsiftsoglou and Robinson 1985). From the changed nuclear morphology it was seen that the cells progressed from promyelocytes to myelocytes and metamyelocytes, and some continued to mature banded and segmented neutrophils. Both the promyelocytes and the mature granulocytes expressed chloroacetate esterase and sudan black reactivity (Collins et al 1978). Myeloperoxidase activity decreased to 20% on differentiation to granulocytes or monocytes, whereas acid phosphatase, ß-glucuronidase and lysosome activity increased on differentiation (Ross 1985, Abita 1984).

Interferon-alpha A was found not to induce HL-60 cell differentiation, but it enhanced differentiation induced by phorbol esters, retinoic acid or DMSO in a synergistic manner (Grant et al 1985). In contrast, Ball et al (1986) showed that interferon gamma induced HL-60 cells to express markers of monocytic differentiation but true terminal differentiation did not occur as the cells

continued to proliferate. In combination with 1,25-dihydroxy vitamin $D_3$, however, terminal differentiation was induced to a much greater extent than with 1,25-dihydroxy vitamin $D_3$ alone.

Incubation of HL-60 cells with recombinant GM-CSF or with murine G-CSF induced differentiation as measured by increased expression of granulocytic and macrophage membrane antigens and suppression of clonogenicity. This induced differentiation was barely detectable after 1 week of culture with the CSF, and was optimal only after 2 to 3 weeks of culture, whereas other chemical differentiation inducers only required 3 to 4 days to induce optimal differentiation (Begley et al 1987).

Table 1.1 lists the various agents known to induce granulocytic or monocytic differentiation of HL-60 cells.

## 1.7 The mechanism of the induction of HL-60 cell differentiation

### 1.7.1 Characteristics of HL-60 cell differentiation

#### 1.7.1.1 Commitment to differentiation

When HL-60 cells were incubated with inducers such as retinoic acid or DMSO for 24 hours (approximately one cell doubling period) and were then washed free of the drug,

TABLE 1.1 INDUCERS OF HL-60 CELL DIFFERENTIATION

| Granulocytic inducers | References |
|---|---|
| acetamide | Langdon and Hickman 1987 |
| N-methylacetamide | " |
| N,N-dimethyacetamide | " |
| diethylacetamide | " |
| formamide | " |
| N-methylformamide | " |
| N,N-dimethylformamide | " |
| N-ethylformamide | " |
| diethylformamide | " |
| t-butylformamide | " |
| urea | " |
| 1,1-dimethylurea | " |
| 1,3-dimethylurea | " |
| tetramethylurea | " |
| acetone | " |
| methanol | " |
| ethanol | " |
| hexamethylene bisacetamide | Collins et al 1978 |
| triethylene glycol | " |
| dimethylsulphoxide | Collins et al 1980 |
| hydroxyurea | " |
| hypoxanthine | " |
| 6-thioguanine | " |
| 6-mercaptopurine | " |
| piperidone | Collins et al 1978 |
| 1-methyl-2-piperidone | " |
| 5-azacytidine | Bodner et al 1981 |
| 5,6-dihydro-5-azacytidine | " |
| 5-iodo-2'-deoxyuridine | " |
| 5-bromo-2'-deoxyuridine | " |
| 3-deazauridine | " |
| thymidine | " |
| pyrazofurin | " |
| virazole | " |
| tricyclic nucleoside (TCN) | " |
| TCN phosphate | " |
| puromycin aminonucleoside | " |
| tiazofurin | Sokoloski et al 1986 |
| selenazofurin | " |
| mycophenolic acid | " |
| ribavirin | " |
| cycloleucine | Grosso and Pitot 1984 |
| 3-aminobenzamide | " |
| theophylline | " |
| dibutyryl cyclic AMP | Hemmi and Breitman 1984 |
| 8-bromo-cyclic AMP | Fontana et al 1984 |
| retinoic acid | Breitman et al 1980 |
| actinomycin D | Collins et al 1980 |

TABLE 1.1 CONTINUED

| Granulocytic inducers | References |
|---|---|
| aclacinomycin A | Schwartz and Sart. 1982 |
| marcellomycin | " |
| tunicamycin | Nakayasu et al 1980 |
| vincristine | Collins et al 1980 |
| methotrexate | Bodner et al 1981 |
| W-13 naphthalene sulphonamide | Veigl et al 1986 |
| cholera toxin | Fontana et al 1984 |
| ethionine | Hemmi and Breitman 1984 |
| prostaglandin $E_2$ | " |
| antithymocyte globulin | Hunter et al 1985 |
| trypsin | Fibach et al 1985 |
| chymotrypsin | " |
| elastase | " |
| GM-CSF | Begley et al 1987 |

| Monocytic inducers | References |
|---|---|
| 12-O-tetradecanoylphorbol -13-acetate (TPA, PMA) | Rovera et al 1979 |
| mezerein | Grosso and Pitot 1984 |
| 1,25-dihydroxyvitamin $D_3$ | Tanaka et al 1983 |
| sodium butyrate | Boyd and Metcalf 1984 |
| cytosine arabinoside (ara-C) | Griffin et al 1982 |
| adenine arabinoside (ara-A) | Munroe et al 1984 |
| aphidicolin | Griffin et al 1982 |
| verapamil | Okazaki et al 1986 |

only a small proportion of the cells were committed and subsequently differentiated into granulocytes. When incubated for 48 hours with the inducer, however, the majority (usually 70 to 90%) of the cells were committed to differentiate even in the subsequent absence of inducer (Fibach et al 1982a and 1982b, Yen et al 1984, Yen 1985, Tarella et al 1982). The cells retained a memory of exposure to the inducer, similar to the situation in MEL cells (Yen et al 1984). Tarella et al (1982) suggested that commitment events occurred in a stochastic manner, as with MEL cells, so that commitment occurred randomly with a particular probability in the cell population exposed to the inducer.

Interestingly, commitment to monocytic differentiation with the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) occurred much faster than with granulocytic inducers. Fibach et al (1982a) showed that incubation of HL-60 cells with TPA for for as little as 4 hours committed a small but significant percentage (10%) of cells to differentiate, with 90% of the cells committed after 24 hours of exposure to the inducer. In contrast, after incubation for 24 hours with DMSO, only 10% were committed and 48 hours were required to commit >80% of cells to granulocytic differentiation. The authors showed that pre-incubating cells with DMSO enabled macrophage differentiation in the subsequent presence of TPA to occur even faster, suggesting that

early stages in HL-60 cell differentiation were probably common to the macrophage and granulocyte lineages. Yen et al (1987a) carried out similar experiments with retinoic acid and 1,25-dihydroxyvitamin $D_3$ - granulocytic and monocytic inducers, respectively - and concluded that commitment to differentiation was a two-step process. The early events in commitment were common to both pathways and later events were involved in the determination of the differentiation lineage.

1.7.1.2 The cell cycle dependence of induction

In order to determine which changes in the cell are important in the induction process and why the cells need to be incubated for 48 hours with granulocytic inducers to commit >70% of them to differentiate, it is important to discover whether the commitment events are in any way cell cycle related. There have been many studies on this subject, with often conflicting results. Tarella et al (1982) synchronised HL-60 cells using a thymidine block and exposed them to DMSO at different stages of the cell cycle, and found that the cells were equally sensitive to the inducer and required the same length of incubation to commit them to differentiate regardless of their cell cycle phase. They showed that the cells could still differentiate in response to DMSO in the total absence of cell division when blocked with the continued presence of thymidine (Ferrero et al 1982). The cells were also able

to differentiate with retinoic acid when cell division was blocked using either hydroxyurea or ara-C. Unfortunately, both ara-C and thymidine appeared to have some inducing effect of their own so that experiments combining such agents with inducers of differentiation such as retinoic acid are difficult to interpret. Hemmi and Breitman (1984) also reported that inhibition of cell proliferation with hydroxyurea did not effect retinoic acid -induced differentiation, nor did suppressing DNA synthesis with aphidicolin (Chou and Chervenick 1985). Rovera et al (1980) showed that the induction of monocytic differentiation with TPA did not require DNA synthesis and that many of the cells in the population did not undergo even one full cell cycle before arresting in $G_1$ and becoming differentiated. In contrast when the cells were induced to differentiate along the granulocytic pathway, they underwent one or two cell divisions in the presence of inducer before the cells lost their potential for self-renewal and developed the differentiated phenotype. At most the cells were able to undergo 3 to 5 cell divisions in the presence of inducer (Tsiftsoglou et al 1985, Schwartz et al 1983, Fibach et al 1982b and 1982c, Yen et al 1985). Several studies also indicated that the loss of self-renewal potential of the cells preceded the commitment of the cells to differentiate (Von Melchner and Hoffken 1985, Fibach et al 1982b and 1982c, Yen 1985), possibly by as much as 24 hours. In contrast to the studies which showed that commitment to differentiate with

DMSO did not occur at any particular cell cycle stage, Yen and Albright (1984) showed that cells were responsive to retinoic acid only when they reached S-phase of the cell cycle. This was explained by the fact that retinoic acid uptake into the cells was greatly enhanced in S-phase, so it is possible that the concentration of inducer required was not attained until the cells traversed S-phase. Studzinski et al (1985) suggested that the cells were sensitive to 1,25-dihydroxyvitamin $D_3$ in late $G_1$ or early S phase, while there seemed to be a "trigger" for butyrate differentiation in late $G_2$-M or very early $G_1$ (Boyd and Metcalf 1984). These latter inducers are more "physiological" than those such as DMSO and it is possible that the apparent cell cycle phase-dependent induction was due to cell cycle specific uptake of the agents, whereas simple small molecules such as the polar solvents simply diffuse into the cell without any specific transporter system.

1.7.1.3 Membrane changes

Membrane fluidity is thought to influence cell growth and differentiation and the fluidity of membrane lipids did indeed decrease on treatment of HL-60 cells with DMSO, but this decrease was first observed on day three following exposure, coincident with morphological and cytochemical differentiation rather than preceding it (Ip and Cooper 1980). The decreased fluidity was therefore a

characteristic of the differentiated cells rather than a perturbation of fluidity by the polar solvent itself. Cooper et al (1981) however showed that synthesis of sterols and phospholipids, which would normally be incorporated into the cell membranes, was inhibited very early during the commitment of HL-60 cells to differentiate.

Induction of differentiation of HL-60 cells to either granulocytes or macrophages resulted in decreased production of glycosaminoglycans, large polyanionic carbohydrate molecules associated with the cell membrane. This decreased synthesis was maximal on day 4, when the majority of cells had differentiated, and although glycosaminoglycans have been implicated in the regulation of haematopoiesis there was no evidence that this decreased synthesis was associated with the commitment process (Luikart and Sackrison 1986).

Many cell surface proteins exist in the form of glycoproteins, with large oligosaccharides linked to asparagine residues on the protein. Sokoloski et al (1986) showed that inhibitors of IMP dehydrogenase, such as tiazofurin, induced HL-60 cell differentiation and concomitantly reduced the synthesis of the oligosaccharide precursors for the glycoproteins, due to depletion of the cellular GTP pool. This inhibition of synthesis occurred within 8 hours of exposure to the inducer during the

commitment period and before any significant differentiation was observed. Differentiation induction by tiazofurin was inhibited by addition of guanosine to the cells to maintain the GTP levels. The authors suggested that, if specific populations of plasma membrane glycoproteins were critical to replicative and differentiative processes, inhibition of oligosaccharide synthesis might indirectly alter the biosynthesis of glycoproteins in a manner which initiated or enhanced the development of a program of terminal cell differentiation. Tunicamycin also inhibited glycoprotein synthesis by preventing synthesis of the oligosaccharide precursors, and induced differentiation (Nakayasu et al 1980). Addition of the oligosaccharide precursors to the cells actually prevented tunicamycin-induced differentiation, and the authors proposed that glycosylation of cell proteins was important in maintaining the undifferentiated state in these cells. Morin and Sartorelli (1984) showed that the anthracycline antibiotics marcellomycin and aclacinomycin also induced HL-60 cell differentiation, and within 3 to 6 hours of exposure the synthesis of the asparagine-linked oligosaccharides was reduced. However, during DMSO-induced differentiation there was no measurable change in oligosaccharide synthesis, so while inhibition of glycoprotein synthesis may be important in commitment to differentiation by some inducers, it is clearly not of universal importance in the commitment process. Concomitant with the reduction of

oligosaccharide synthesis in HL-60 cells treated with anthracyclines, there was a decrease in activity of one specific glycoprotein, the transferrin receptor, which transports iron into cells via the iron-carrying serum glycoprotein transferrin. Reduced activity of this receptor also occurred during the induction of differentiation with other agents, and in other cells types such as K562 and M1. A very rapid loss in transferrin receptor activity was also reported during TPA-induced monocytic differentiation of HL-60 cells, and in this case the down regulation was caused by internalization of many of the receptors from the cell surface to an internal pool, probably regulated by phosphorylation of the receptor (Abita 1984). Whether reduction in transferrin receptor activity is directly related to the induction of differentiation or whether it is simply caused by inhibition of cell proliferation is unclear.

1.7.1.4 Changes in folate and nucleoside metabolism

Sirotnak et al (1986) reported that within 2 hours of the addition of DMSO, HMBA or retinoic acid to HL-60 cells there was a decline in folate influx, as measured by the use of [$^{3}$H]-methotrexate. There was also a decline in intracellular dihydrofolate reductase (DHFR) levels, and the decline was reversible if the inducer was removed before the cells were committed to differentiate.

Methotrexate, an inhibitor of DHFR, also induced HL-60 cell differentiation, as did various nucleosides thought to interfere with de novo purine or pyrimidine synthesis or with conversion among the pyrimidines (Bodner et al 1981). Decreased folate levels and DHFR activity caused decreased de novo synthesis of thymidylate and purines, resulting in reduced incorporation into DNA and decreased DNA replication, leading to growth arrest. How inhibition of these metabolic pathways could lead to the changes in gene expression resulting in differentiation is unclear.

Changes have also been reported in nucleoside transport during HL-60 differentiation; adenosine and 2'-deoxyadenosine transport decreased after 24 hours incubation with DMF, falling to 5% of that in untreated cells after 5 days, whereas transport of purine bases adenine, guanine and hypoxanthine was virtually unchanged (Chen et al 1986). The changes in nucleoside transport were probably the result of decreased proliferative potential and would occur in other cells in which proliferation was inhibited regardless of whether differentiation was induced.

1.7.1.5 Modulation of protein kinase activity and protein phosphorylation

Changes in protein kinase activity have been observed during HL-60 cell differentiation. Fontana et al (1986)

showed that activities of both cAMP-dependent protein kinase and the phospholipid-sensitive, calcium-dependent protein kinase (protein kinase C) were increased in retinoic acid-treated cells induced to differentiate for 4 days. Zylber-Katz and Glazer (1985) showed that during retinoic acid- or DMSO-induced differentiation, protein kinase C activity increased slowly from day 1 to a maximum on day 7, concomitant with the expression of the mature phenotype. Whether these kinases play a role in the commitment events or are simply associated with the altered growth state and functions of the mature cells has yet to be elucidated. Protein kinase C is usually activated in the cell by $Ca^{2+}$ and diacylglycerol (one of the products released from inositol phosphate breakdown as a consequence of activation of growth factor receptors), and is thought to be involved in stimulation of cell division (Joseph 1984), so why the activity should increase in the differentiating cells which are growth arrested is unclear.

More extensive studies have been carried out during TPA-induced macrophagic differentiation. TPA is known to activate protein kinase C by mimicking the action of diacylglycerol (Castagna et al 1982). Yamamoto et al (1985) showed that protein kinase C activity increased during differentiation, but 1-oleoyl-2-acetyl glycerol (OAG) also activated protein kinase C without the induction of differentiation. They suggested that protein

kinase C activation was not the sole event sufficient for differentiation induction. Merrill et al (1986) demonstrated that the protein kinase C inhibitor sphinganine blocked TPA-induced differentiation and concluded that protein kinase C activation was necessary for differentiation. They noted that while OAG did not induce differentiation, another synthetic diacylglycerol - dioctanoyl glycerol - was an effective inducer. In contrast, Zylber-Katz et al (1986) showed that while a short treatment with TPA activated protein kinase C, after 48 hours incubation with TPA protein kinase C was down-regulated and became undetectable. They induced macrophagic differentiation with a combination of TPA and retinoic acid exposures, or with 1,25-dihydroxyvitamin $D_3$ (Zylber-Katz and Glazer 1985) and demonstrated that this down-regulation was not obligatory for the induction of macrophagic differentiation. In 1985, Kraft and Berkow reported that another activator of protein kinase C, bryostatin, did not induce differentiation and actually inhibited differentiation induction by TPA, and they concluded that the induction of differentiation by TPA must occur by a more complex mechanism than previously thought.

Kreutter et al (1985) again illustrated that TPA activated protein kinase C during differentiation, and showed that 14 cellular proteins were phosphorylated as a result. OAG, which did not induce differentiation but

activated protein kinase C, only induced phosphorylation of 9 of these 14 proteins, suggesting that the extra 5 proteins phosphorylated in TPA-treated cells were phosphorylated by a mechanism other than protein kinase C. Zylber-Katz and Glazer (1985) discovered that proteins of 37 and 38 kD were phosphorylated during HL-60 cell differentiation to granulocytes or macrophages, while a 21 kD protein was phosphorylated only during granulocytic differentiation. Two nuclear matrix proteins of 33 and 80 kD were phosphorylated within minutes of HL-60 cell exposure to TPA (McFarlane 1986) and it was suggested that the 33 kD protein might be the eukarytic initiation factor 2-alpha, or one of the sub-units of RNA polymerase II. A 17 kD cytosolic protein, prosolin, was rapidly phosphorylated within 1 hour of TPA treatment then subsequently dephosphorylated (Braverman et al 1986), and the authors suggested that this was a response to the signal for cessation of cell proliferation by TPA and was probably independent of changes leading to differentiation. DNA topoisomerase II is also known to be a substrate for phosphorylation by protein kinase C, resulting in activation of the topoisomerase (Sahyoun et al 1986), which is an enzyme which catalyses a number of topological isomerization reactions in the DNA, such as knotting and unknotting, relaxation of superhelical twists, etc., and thus may be involved not only in DNA replication but in exposing regions of the DNA for gene expression (Tewey et al 1984). Activation of

topoisomerase by protein kinase C was suggested to play a role in HL-60 cell differentiation, as topoisomerase inhibitors such as novobiocin blocked TPA-induced differentiation. However, Morin et al (1987) showed that dioctanoylglycerol, which activated protein kinase C but did not induce HL-60 cell differentiation, was able to activate topoisomerase II. This suggested that topoisomerase activation was dissociable from the induction of differentiation.

1.7.1.6 The involvement of calcium ions and calmodulin

There is contradictory evidence as to whether calcium and calmodulin are involved in the induction process. Calcium ions are necessary for phosphorylation reactions catalyzed by protein kinase C, and $Ca^{2+}$ also binds with very high affinity to calmodulin, which then regulates such cellular processes as cell division, phagocytosis and phosphorylation (Veigl et al 1984). Naphthalene sulphonamide calmodulin antagonists induced very limited HL-60 differentiation, but they augmented differentiation induced by retinoic acid, DMSO, etc., which suggests there may be a role for calmodulin in the induction process (Veigl et al 1986). In contrast, Matsui et al (1985) reported that calmodulin and microfilament-dependent processes might be involved in the proliferation of HL-60 cells but not in the induction of differentiation, as calmodulin antagonists brought about growth arrest but did

not inhibit differentiation induced by 1,25-dihydroxy vitamin $D_3$. Okazaki et al (1986) showed that both calcium-free medium and verapamil enhanced HL-60 cell differentiation after DMSO, retinoic acid or 1,25-dihydroxy vitamin $D_3$ treatment, and they suggested that this enhancing effect was related to inhibition of calcium mobilization in the cells, perhaps inhibiting the ability of the cells to proliferate. Yen et al (1986a) showed that induction of differentiation with retinoic acid was accompanied by an increase in the relative abundances of certain calcium-binding proteins, whose activities were probably regulated by cytosolic calcium. The functions of these are yet to be elucidated. They also demonstrated, however, that there was no difference in calmodulin levels in untreated or retinoic acid-treated HL-60 cells, and that differentiation was not inhibited by calmodulin antagonists or calcium channel blockers such as verapamil, which argued against a role for calmodulin or calcium flux in the control of HL-60 cell differentiation (Yen et al 1986c).

1.7.1.7 Changes in ion fluxes

Using DMSO as an inducer, several authors reported that within six hours of addition of the drug there was a decline in the rate of ouabain-sensitive $^{86}Rb$ transport into the cell (a measure of $K^+$ transport through the $Na^+,K^+$-ATPase), however efflux from the cell also

decreased and there was no change in the intracellular concentrations of either sodium or potassium as a result (Ladoux et al 1984 and Gargus et al 1984). Ladoux et al (1987) discovered that during differentiation of HL-60 cells with either retinoic acid or DMSO cell alkalinization occurred, so that $pH_i$ increased from 7.03 in untreated cells to 7.37 after 5 days with inducer. The pH increased to 7.25 by day 1, when few cells had actually differentiated, so this was an early event during the commitment process. The increase in $pH_i$ was shown to be due to increased activity of the $Na^+/H^+$ exchange system, but it was not clear how this change in pH could affect gene expression and trigger the induction of differentiation.

1.7.1.8 Modulation of cellular oncogene expression

The expression of cellular oncogene products was regulated in HL-60 cells during differentiation. After 48 hours incubation with DMSO the activity of the src gene product - $pp60^{c\text{-}src}$ - was elevated and reached a maximum of ten fold above the control levels by day 3. This increase was not however due to an increase in the expression of the gene as the mRNA levels remained unchanged, but was due either to an increase in activity of the protein itself or an increase in the number of molecules (Barnekow and Gessler 1986). The $pp60^{c\text{-}src}$ is a protein-tyrosine kinase, an activity which is also found

in many growth factor receptors (Hunter and Cooper 1985). How this activity relates to the control of cell proliferation or differentiation is unclear, as the substrates phosphorylated by the $pp60^{c\text{-}src}$ are unknown, as yet.

The c-myc gene was shown to be amplified in the HL-60 cell line, with between 4 and 30-fold amplification of the gene depending on the sub-line studied, and the levels of c-myc mRNA were increased compared to levels found in normal leukocytes, correlated to the gene copy number (Graham et al 1985). During granulocytic or monocytic differentiation with DMSO, TPA, theophylline, butyrate, 3-aminobenzamide, mezerein or cycloleucine, the levels of myc mRNA gradually fell until the mRNA was virtually undetectable by day 5, when the majority of cells had differentiated (Grosso and Pitot 1984). This decrease in mRNA levels was found to be due to decreased transcription of the gene (Grosso and Pitot 1985a) and the inactivation of transcription correlated with loss of one $S_1$-nuclease sensitive site on the myc gene, suggesting the involvement of a conformational change in the chromatin (Grosso and Pitot 1985b). Filmus and Buick (1985) showed that myc mRNA levels began to decline dramatically by day 1 of incubation with DMSO and reached a minimum after about 3 days, in parallel with the loss of clonogenicity of the cells as they differentiated. Inhibition of proliferation without differentiation of the cells was achieved by

culture of the cells in reduced serum, and although c-myc mRNA levels decreased, the fall was not as dramatic as that observed during differentiation. This suggested that the inhibition of myc gene transcription during differentiation was not just due to inhibition of proliferative potential. A similar decrease in myc mRNA levels occurred after treatment with 1,25-dihydroxyvitamin $D_3$ (Simpson et al 1987). Yen and Guernsey (1986) showed some conflicting results, in that although myc mRNA levels ultimately decreased on day 6 of induction of differentiation by retinoic acid, prior to that time there was an increase in the myc RNA levels. They argued that as the decreased expression occurred so late, reduction of the myc mRNA levels could not be causative in the terminal differentiation process. More recently, Yen et al (1987b) showed that the early increase in myc mRNA levels coincided with precommitment memory, so perhaps the early induction of myc mRNA is important in controlling commitment.

When HL-60 cells were induced to differentiate to monocytes with TPA, the c-fos gene was rapidly induced and high levels of c-fos mRNA accumulated within 1 hour (Mitchell et al 1985). The authors envisaged a role for the c-fos protein in the monocytic differentiation pathway but not in the granulocytic pathway, as no c-fos induction was observed during DMSO-induced differentiation. Müller et al (1985) confirmed that c-fos mRNA levels increased

after TPA treatment, and also showed that levels of the protein increased. Calabretta (1986), however, treated HL-60 cells with TPA and retinal (a protein kinase C inhibitor) and showed that while the induction of c-fos mRNA was abolished, macrophage differentiation was still induced. C-fos mRNA was also induced by OAG treatment of the cells which activated protein kinase C but did not induce differentiation, so Calabretta suggested that c-fos mRNA induction was related to protein kinase C activation but was not necessary for TPA-induced differentiation.

Sariban et al (1985) showed that decreased c-myc and increased c-fos mRNA levels were followed by increased expression of c-fms mRNA during monocytic but not granulocytic differentiation. They suggested that as c-fms mRNA was also detectable in normal peripheral blood monocytes, this gene product may play a role in monocytic differentiation.

1.7.1.9 Alterations in cellular mRNA and protein levels

There are other genes, apart from the oncogenes whose expression is altered on induction of differentiation in HL-60 cells; R.H. Chou et al (1984) identified a 60 kD nucleosomal protein which was induced during differentiation of HL-60 cells with retinoic acid, reaching a maximum level by day 6. This protein was not induced in a sub-line resistant to differentiation

induction by retinoic acid, but neither was it induced during differentiation of HL-60 cells with DMSO, so while it might be important in retinoic acid-induced differentiation it is not generally involved in the induction of myeloid differentiation. Concannon et al (1985) isolated cDNA clones from more than 20 genes whose expression was strongly regulated by induction with phorbol esters, and found that when the cDNA genes were re-introduced into the cell, their expression was regulated in the same way as the endogenous genes when an inducer was added to the cell. They have not yet been able to isolate the regulatory elements involved in this process, or determine the function(s) of these genes. The cDNA clones isolated from mRNAs strongly regulated during differentiation were probed, and it was found that 50% of these clones contained repeat sequences compared to less than 5% of clones from an unselected clone library (C-C. Chou et al 1984, Davis et al 1987). Those genes containing repeat sequences may be involved in regulating transcription, but their precise functions are unclear. Both these studies however concentrated on genes whose expression was altered at a time when the cells had already attained their differentiated phenotype, rather than genes regulated very early in the induction process, so these were unlikely to have causative roles in the induction of differentiation.

1.7.1.10 The induction of DNA single strand breaks

An interesting study by Farzaneh et al (1985) showed that between 12 and 24 hours of incubation of HL-60 cells with DMSO a large number of single strand breaks were formed in the DNA, which had religated and were undetectable by 48 hours. These breaks were formed despite a proficient DNA repair mechanism, and were thought to be important in the differentiation induction process, as had previously been suggested for MEL cell differentiation. They may occur as a result of gene amplification, or part of a genomic rearrangement, or as a result of topoisomerase action relaxing regions of the chromatin, and may be intimately involved in programming the changes in gene expression necessary for the induction of differentiation. Till, in 1982, suggested that gene rearrangement might be frequent during early differentiation of many cell types, mainly affecting genes with regulatory functions, and rearrangement of genes within or between chromosomes would presumably require strand breaks to allow the movement to occur.

1.7.1.11 Changes in nuclear structure

Yen et al (1985) showed changes in the nuclear structure of HL-60 cells, as measured by narrow angle light scatter from single cells on a flow cytometer, occurring after 24 hours incubation with retinoic acid,

before any significant differentiation had occurred. This change was shown by scanning electron microscopy to be a morphological change occurring in the nuclear membrane, from a smooth to a dimpled or pitted structure. Changes in the nuclear matrix could potentially lead to changes in post-transcriptional modification of hnRNA and its export to the cytoplasm as mRNA, resulting in regulation of proteins translated in the cell. Specific functions for the change in nuclear morphology are unknown, as yet.

### 1.7.2 Relationship between the induction of differentiation and cytotoxicity

Because such a wide range of agents induce the HL-60 cell to differentiate into granulocytes it is difficult to envisage a common mechanism whereby all the inducers could bring about the same changes in gene expression necessary to cause the cessation of cell proliferation and expression of the mature phenotype.

Langdon and Hickman (1987) studied the induction of HL-60 cell differentiation by a variety of alkylformamides, alkylacetamides, alkylureas and alcohols. All the agents tested induced differentiation, suggesting that there were no structural requirements for activity in these polar molecules. For each inducer, the concentration which induced optimal differentiation (the maximum percentage of cells expressing markers of mature

cells whilst maintaining viability >70%) was found to be marginally below a concentration which was cytotoxic to the cells. The cytotoxic concentration was that concentration which prevented a single doubling of the cells and reduced viability by >70% over a four day period. Table 1.2 displays the concentrations required for the induction of differentiation and cytotoxicity for these polar solvents.

It was noted that increasing the concentration marginally above that which induced differentiation resulted in cytotoxicity with a variety of other HL-60 differentiation inducers, such as DMSO (Bunce et al 1983), HMBA and analogues (Haces et al 1987), Ara-A (Munroe et al 1984), antithymocyte globulin (Hunter et al 1985) and tunicamycin (Nakayasu et al 1980). Supino et al (1986) induced differentiation of MEL cells with poly-L-lysine molecules of different sizes and showed that at concentrations higher than those which induced differentiation, the molecules were toxic to the cells. Reuben et al (1978) also noted that this was the case for MEL cell differentiation by a range of polar solvents, and suggested that "whatever the changes are that occur when murine erythroleukemia cells are cultured with inducer, these changes are cytotoxic when the inducer is present at concentrations slightly greater than those at which inducing activity is optimized. This suggests that cellular changes associated with induction are damaging to

TABLE 1.2 CONCENTRATIONS OF POLAR SOLVENTS REQUIRED TO INDUCE DIFFERENTIATION AND CYTOTOXICITY IN HL-60 CELLS

| # | Inducer | conc. D* (mM) | conc. C* (mM) | Mw |
|---|---|---|---|---|
| 1 | methanol | 625 | 1000 | 32 |
| 2 | formamide | 400 | 600 | 45 |
| 3 | ethanol | 213 | 261 | 46 |
| 4 | acetone | 138 | 207 | 58 |
| 5 | acetamide | 250 | 300 | 59 |
| 6 | N-methylformamide | 180 | 300 | 59 |
| 7 | urea | 300 | 400 | 60 |
| 8 | N,N-dimethylformamide | 100 | 150 | 73 |
| 9 | N-ethylformamide | 100 | 150 | 73 |
| 10 | N-methylacetamide | 25 | 50 | 73 |
| 11 | N,N-dimethylacetamide | 20 | 25 | 87 |
| 12 | 1,1-dimethylurea | 50 | 100 | 88 |
| 13 | 1,3-dimethylurea | 25 | 50 | 88 |
| 14 | t-butylformamide | 40 | 50 | 101 |
| 15 | diethylformamide | 25 | 50 | 101 |
| 16 | diethylacetamide | 10 | 20 | 115 |
| 17 | tetramethylurea | 5 | 10 | 116 |

(Figures from Langdon and Hickman 1987)

* Conc. D = the concentration required to induce optimal differentiation, as measured on day 4, whilst maintaining viability at >70%.

* Conc. C = the concentration required to cause cytotoxicity; prevention of one cell doubling and significant reduction of cell viability by day 4.

the cell if too extensive". These results suggested that some form of sub-cytotoxic threat or stress was involved in the induction of HL-60 differentiation, and that almost any cytotoxic agent, used at sub-cytotoxic concentrations, would induce differentiation.

Ishiguro and Sartorelli (1985) suggested that cytotoxicity and differentiation were in fact separable events. They studied the induction of differentiation by 6-thioguanine in wild type HL-60 cells and in mutants lacking hypoxanthine-guanine phosphoribosyltransferase, and demonstrated that 6-thioguanine was partially converted to TGMP in wild type cells, and that TGMP was responsible for the cytotoxicity observed after 6-thioguanine administration, whereas 6-thioguanine was responsible for the differentiation inducing effects. The cytotoxicity was much reduced by co-administering hypoxanthine or inosine to prevent the conversion of 6-thioguanine to TGMP. In the mutant $HGPRT^-$ cells, 6-thioguanine was not converted to the nucleoside TGMP and 6-thioguanine was a much more effective differentiation inducer, with less cytotoxicity than in wild type cells. The authors concluded that the processes of cytotoxicity and the induction of differentiation by 6-thioguanine were separable phenomena. However, prevention of the conversion of 6-thioguanine to TGMP did not totally abolish cytotoxicity, suggesting that 6-thioguanine itself was cytotoxic when given in sufficient concentrations,

although less toxic than TGMP. This suggests that while some agents may be cytotoxic without inducing differentiation, those that induce differentiation will by cytotoxic at concentrations above the optimal inducing concentration.

The fact that such a wide variety of agents are able to induce HL-60 cell differentiation suggests that the commitment of the cells to differentiate must be a very basic adaptive response to a variety of threats or stresses.

### 1.7.3 Dependence of differentiation-inducing concentration on molecular weight

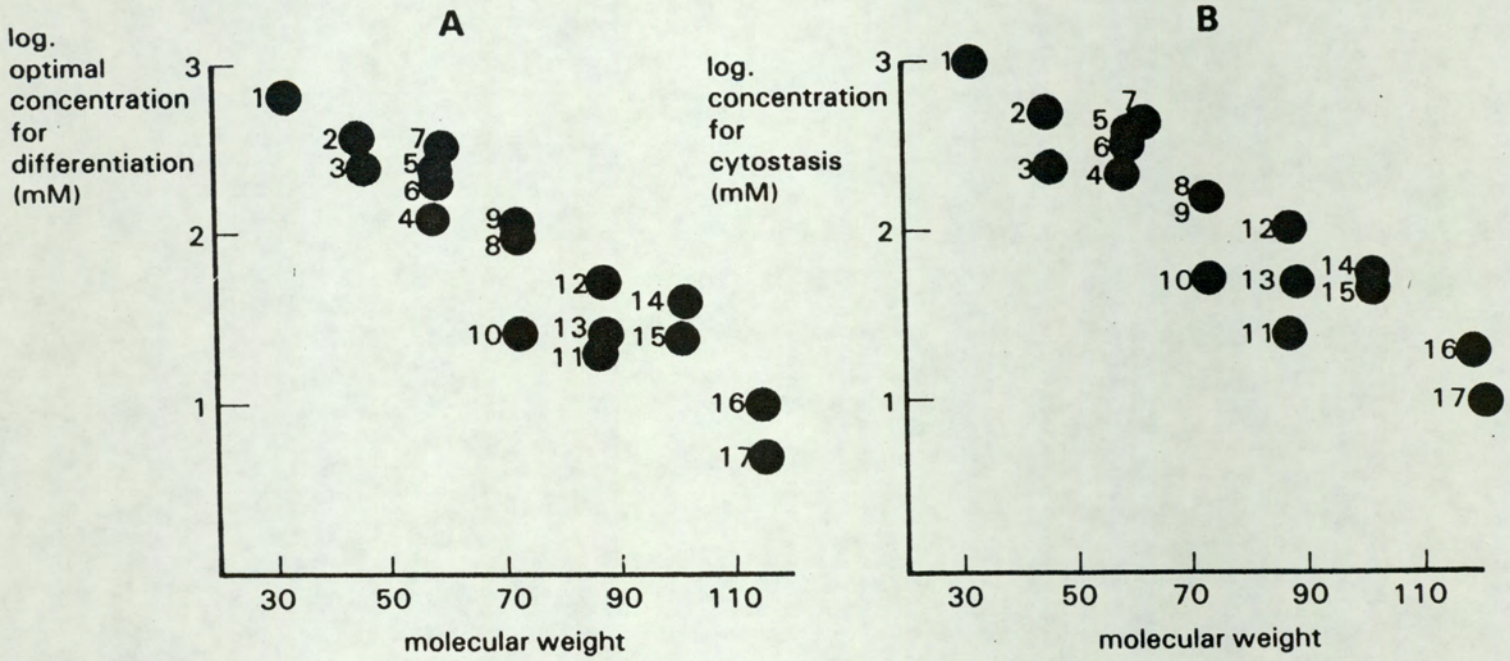
As well as determining the relationship between differentiation and cytotoxicity, Langdon and Hickman (1987) showed an interesting dependence of the concentration required to induce HL-60 cell differentiation and the molecular weight of the inducer. For the range of polar solvents studied, there were no structural requirements for differentiation-inducing activity, and the optimum concentration was proportional to the molecular weight of the molecule, so that the larger the molecule the lower the concentration required. When the logarithm of the optimum concentration for the induction of differentiation was plotted against the molecular weight, a linear relationship resulted (log.

concentration = -0.02288 x Mw +3.573) with a correlation coefficient of -0.937 (see Figure 1.3A). Interestingly, when the logarithm of the concentration of each agent which induced cytotoxicity was plotted against the molecular weight, a linear relationship was again apparent, with the line virtually parallel but slightly higher than that for differentiation inducing concentrations (concentration = -0.02126 x Mw + 3.658, correlation coefficient -0.935) (see Figure 1.3B).

The relationship between concentration and molecular weight for these polar solvents could be connected to the partition coefficients of the molecules, which would affect intracellular accumulation of the polar solvents, but when the logarithm of the differentiation inducing concentration was plotted against the octanol/water partition coefficient, a correlation of only -0.6654 was obtained, suggesting that the partition coefficient is not responsible for the molecular size-dependency of the induction of differentiation (Langdon and Hickman 1987).

Supino et al (1986) showed a similar molecular weight dependency for concentrations of poly-L-lysine molecules required to induce MEL cell differentiation, so that larger poly-L-lysine molecules were required in smaller concentrations. Such a relationship might be expected for a series of one molecular type, such as the poly-L-lysines, and it is perhaps not surprising for the

FIGURE 1.3 Relationship between the molecular weights of polar solvents and the concentrations required to induce differentiation or cytotoxicity in HL-60 cells
(From Langdon and Hickman 1987)




**Key:**

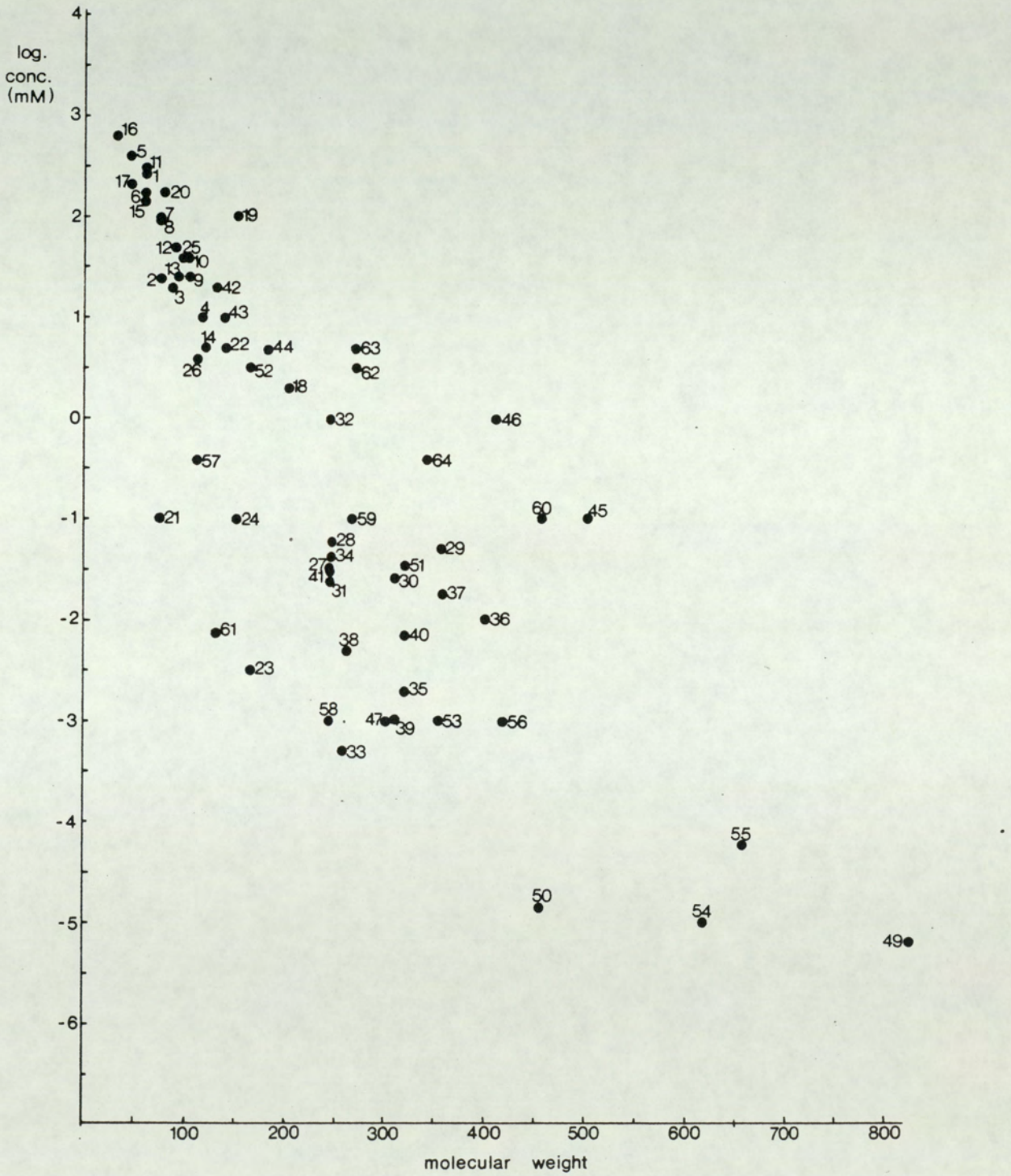
1,methanol;2,formamide;3,ethanol;4,acetone;5,acetamide;
6,N-methylformamide;7,urea;8,N,N-dimethylformamide;9,N-ethylformamide;
10,N-methylacetamide;11,N,N-dimethylacetamide;12,1,1-dimethylurea;
13,1,3-dimethylurea;14,t-butylformamide;15,diethylformamide;
16,diethylacetamide;17,tetramethylurea.

range of closely related polar solvents studied by Langdon and Hickman. However, compilation of optimum differentiation inducing concentrations for 64 different HL-60 differentiation inducers shows the same molecular weight dependency of concentration, with molecules as diverse as HMBA, purine and pyrimidine analogues and retinoic acid (see Figure 1.4. The figures for concentrations and molecular weights of these compounds are tabulated in Appendix 1). Despite the fact that the concentrations plotted were determined in different laboratories under different experimental conditions and with different sub lines of HL-60 cells, there was a correlation coefficient of -0.8593 when 59 of the molecules were included, or -0.887 with 52 of the inducers.

Several molecules do not fit this line; trypsin and elastase have molecular weights of 24,000 and 25,000, and induce differentiation at 40uM and 25uM, respectively (Fibach et al 1985), but whereas most differentiation inducers probably act intracellularly, it is possible that these serine proteases act at the cell surface - Utsunomiya and Nakanishi (1986) showed that trypsin and other serine proteases at less than 20uM triggered transmembrane signalling and resulted in $Ca^{2+}$ release from intracellular stores in cytotoxic T cells.

Other inducers which do not fit the line could

FIGURE 1.4 Relationship between the molecular weights of inducers of differentiation and the concentrations required to induce optimal differentiation in HL-60 cells



KEY TO FIGURE 1.4

1. acetamide
2. N-methylacetamide
3. dimethylacetamide
4. diethylacetamide
5. formamide
6. N-methylformamide
7. dimethylformamide
8. N-ethylformamide
9. diethylformamide
10. t-butylformamide
11. urea
12. 1,1-dimethylurea
13. 1,3-dimethylurea
14. tetramethylurea
15. acetone
16. methanol
17. ethanol
18. hexamethylene bisacetamide
19. triethylene glycol
20. dimethylsulphoxide
21. hydroxyurea
22. hypoxanthine
23. 6-thioguanine
24. 6-mercaptopurine
25. piperidone
26. 1-methyl-2-piperidone
27. 5-azacytidine
28. 5,6-dihydro-5-azacytidine
29. 5-iodo-2'deoxyuridine
30. 5-bromo-2'-deoxyuridine
31. 3-deazauridine
32. thymidine
33. pyrazofurin
34. virazole
35. tricyclic nucleoside
36. TCN phosphate
37. puromycin aminonucleoside
38. tiazofurin
39. selenazofurin
40. mycophenolic acid
41. ribavirin
42. cycloleucine
43. 3-aminobenzamide
44. theophylline
45. dibutyryl cAMP
46. 8-bromo cAMP
47. retinoic acid
48. actinomycin D
49. vincristine
50. methotrexate
51. W-13
52. ethionine
53. prostaglandin E2
54. TPA
55. mezerein
56. 1,25-dihydroxy vitamin D
57. sodium butyrate
58. ara-C
59. ara-A
60. verapamil
61. sodium arsenite
62. lidocaine HCl
63. procaine HCl
64. 5,5-dimethyl-hydantoin

possibly be metabolised intracellularly to an active species with a molecular weight which would bring that inducer onto the line.

It is apparent that there is a cut-off point around a molecular weight of 400, so that all molecules above this molecular weight are active at concentrations between 1 and 50 nM. Even a molecule as large as actinomycin D (molecular weight >1500) is active at 3nM, whereas its expected activity from the line would be in the order of 1 x $10^{-24}$M.

This demonstrates that the larger a molecule is, up to a certain size, the lower the concentration that would be required to induce HL-60 differentiation, regardless of its molecular structure. It is possible that these "foreign" molecules administered to the cells disrupt some as yet undefined intracellular structure, with larger molecules causing more disruption and thus requiring lower concentrations to induce the critical amount of disorder necessary to trigger the changes in gene expression leading to differentiation. In this way, all differentiation inducers could be causing intracellular damage, leading to a stress response from the cell. There are various ways in which cells are known to respond to stress, either dampening the damage that the stress causes in some way, or undergoing changes so that the cells become resistant to that stress. The mechanisms include

amplification of genes resulting in overexpression of the gene products, or the induction of transcription of specific genes whose products are important in protecting the cells from the stress, such as the heat shock genes. These will be discussed in more detail below.

### 1.7.4 Gene amplification

Gene amplification represents an adaptive cell response to changes in microenvironment and to genotoxic stress and often results in drug resistance. Agents known to induce gene amplification include methotrexate, cadmium, pyrazofurin, hypoxanthine plus aminopterin and thymidine (HAT), and possibly adriamycin, vincristine, actinomycin D, colchicine, etc. (Stark and Wahl 1984).

When Chinese Hamster Ovary (CHO) cells were treated with methotrexate, clones of cells emerged which had become resistant to cytotoxicity induced by methotrexate and had increased levels of the protein DHFR, which is the cellular target for methotrexate. This increased level of protein was due to an increase in the number of copies of the DHFR gene per cell - the DHFR gene was amplified (Schimke 1984). Amplification of other genes has been observed when cells were placed under different selective pressure, including the genes for metallothionein, CAD, hydroxymethylglutaryl CoA reductase, adenosine deaminase, ornithine decarboxylase and glutamine synthetase (Schimke

et al 1985). Amplification of a gene, named mdr1, has been correlated with the development of multidrug resistance as well as resistance to single agents (Roninson et al 1984, Shen et al 1986, Scotto et al 1986). This is of interest as when Friend cells are rendered resistant to the induction of differentiation by one agent they often are concomitantly resistant to many other inducers of differentiation; another form of multidrug resistance (Rovera and Surrey 1978).

It was originally thought that treatment of cells, for example with methotrexate would lead specifically to amplification of the target gene (in this case DHFR), with no effect on other genes, but more recently it has been suggested that large amounts of DNA were actually over-replicated in an unstable fashion, and that some of this DNA subsequently became incorporated into the chromosomes. The selective pressure of methotrexate would only allow the growth of those clones which happened to have incorporated extra copies of the DHFR gene into their genome, resulting in a population of cells which appeared to have specifically amplified the DHFR gene (Mariani and Schimke 1984).

In 1981, Woodcock and Cooper showed that ara-A and ara-C induced gene amplification, as did cycloheximide. They suggested that transient inhibition of DNA synthesis, either directly or indirectly, would result in double

replication of segments of DNA during a single, interrupted S phase of the cell cycle. This would occur because freezing of the replication forks would occur temporarily, and on release from the DNA synthesis block, replication would start again so that those parts of the DNA already replicated would be copied a second time. Heilbronn et al (1985) showed that various DNA polymerase inhibitors and other DNA synthesis inhibitors prevented gene amplification when present at high concentrations, but when added transiently or at lower concentrations they actually induced gene amplification, suggesting that partial or transient inhibition of DNA synthesis was sufficient to cause over-replication of DNA. Transient hypoxia of CHO cells inhibited DNA synthesis and resulted in >60% of cells in S phase at the time of hypoxia undergoing over-replication of DNA and resulted in an increased frequency of DHFR gene amplification and methotrexate resistance (Rice et al 1986a). Johnston et al (1986) showed that cells blocked at the $G_1$-S boundary synthesised increased levels of the DHFR protein and other early S-phase specific proteins, and proposed that this excess of S-phase specific proteins might be responsible for the over-replication of the DNA that subsequently occurred when the cells were released from drug inhibition.

Several authors reported that hydroxyurea induced gene amplification and chromosomal aberrations. Hill and

Schimke (1985) suggested that hydroxyurea caused over-replication of the DNA and that recombination of this extra DNA gave rise to chromosomal aberrations as well as gene amplification. In contrast, Hahn et al (1986) suggested that hydroxyurea directly caused chromosome aberrations (fragmentation, rearrangement, etc.) and that asymmetric segregation of genes into daughter cells resulted in amplified genes in certain cells without over-replication of any DNA.

Varshavsky (1981a) stated that "replicon misfiring" (or extra illegitimate DNA replication) resulted in gene amplification, and that substances termed "firones" could induce this replicon misfiring. He suggested that tumour promoters such as TPA could act as firones, and TPA has since been shown to increase the frequency of methotrexate-resistant or cadmium-resistant cells due to amplified DHFR or CAD genes, respectively (Varshavsky 1981b, Bojan et al 1983, Herschman 1985).

Many of the agents shown to induce gene amplification, such as methotrexate, TPA, pyrazofurin, and actinomycin D, are known to induce HL-60 cell differentiation, and one common feature of HL-60 cell differentiation inducers is that they act at concentrations only marginally below cytotoxic , which would suggest that at concentrations which induced differentiation some inhibition of DNA synthesis would

occur, whether by direct or indirect mechanisms. In 1984, Schimke stated that in cells such as CHO, "enhancement of gene amplification occurs at doses.....that produce only 10 to 30% killing", i.e. at marginally cytotoxic doses. It seems plausible therefore that one early effect of HL-60 cell differentiation inducers would be to cause over-replication of DNA, leading to gene amplification. Gene amplification could presumably lead to major changes in gene expression resulting in cell differentiation. Yen et al (1986b) very recently reinforced this theory, when they showed that hydroxyurea induced a precommitment state in HL-60 cells which subsequently differentiated much faster than normal on exposure to retinoic acid. They showed that these hydroxyurea-treated HL-60 cells had an enriched number of a group of small chromosomes and suggested that myeloid differentiation was caused by amplification of gene(s) carried on the over-replicated chromosomes. They did not however report whether amplification of these genes occurred when differentiation was induced with retinoic acid alone. Strom and Dorfman (1976) showed that during normal differentiation of chick cartilage there appeared to be amplification of certain moderately repetitive sequences, so there may even be a role for gene amplification in normal cellular differentiation.

### 1.7.5 The heat shock response

#### 1.7.5.1 Evolutionary conservation of the heat shock response

When cells are subjected to temperatures above those which are physiological, they synthesise a family of "heat shock proteins" (hsps) which are thought to play a role in protecting that cell from the damaging effects of the hyperthermic treatment. The heat shock response is detectable in almost every cell type in organisms from bacteria to man, and including insects, plants, birds and mammals, and the hsps which are synthesised are remarkably similar between the species. The 70kD hsp, known as hsp70, has a very highly conserved amino acid sequence; the human protein is 73% identical to that from Drosophila, and 50% identical to the product of the E.coli dnaK gene, which is heat-inducible (Lindquist 1986). Kelley and Schlesinger (1982) isolated antibodies to hsp70 and hsp89 (an 89kD protein) from chicken embryo fibroblasts and found that the antibodies cross-reacted with heat-inducible proteins of similar sizes from most adult chicken tissues. The anti-hsp70 antibody also reacted with hsp from yeast, slime mold, dinoflagellate, corn seedling roots, worm, Drosophila fruit fly, Xenopus frog, mouse and human, while the anti-hsp89 antibody reacted with hsp from Drosophila, Xenopus, mouse and human. The fact that the proteins are so highly conserved

suggests that they play a vital role in the cell, presumably protecting it from adverse environmental conditions, not only hyperthermia.

1.7.5.2 Mammalian heat shock proteins

Most mammalian species produce heat shock proteins with molecular weights around 70, 80, 90, 100 and 110 kD when incubated at temperatures around 43°C (6°C above physiological) (Welch et al 1982). There are also usually several smaller proteins between 20 and 40kD, which do not necessarily contain methionine and so are not detected by the usual methods of labelling proteins with [$^{35}$-S]-methionine. There is actually a family of hsp70-like proteins with molecular weights varying between 68 and 73 kD, and with different isoelectric points, several of which are heat inducible, several are not heat inducible but are induced by glucose deprivation - "glucose regulated proteins" - and some of the hsp70-like proteins are expressed under normal conditions and are not heat-inducible, known as "heat shock cognates" (Pelham 1986). Using two-dimensional electrophoresis a variety of minor heat shock proteins have been detected in addition to the 5 or so major hsps; Maytin et al (1985) detected a total of 68 different proteins which were heat inducible in primary thymocytes.

1.7.5.3 Inducers of heat shock protein synthesis

Hyperthermia is not the only treatment which induces hsp synthesis in cells; they can also be induced when cells are allowed to recover from anoxia or when Drosophila cells are treated with oxidising agents such as hydrogen peroxide or with gamma rays (Love et al 1985). Arsenite, zinc, copper and cadmium induced synthesis of hsps 100, 90, 72 and 32 in human cells, and calcium ionophore A23187 and amino acid analogue canavanine induced all but the hsp34 (Caltabiano et al 1986). Other cell types respond in a similar way to these agents (Carr and de Pomerai 1985, Bensaude and Morange 1983, Kelley and Schlesinger 1978, Jones and Findly 1986, Burdon et al 1982, Atkinson et al 1983), although Welch (1985) showed that treatment of quiescent rat embryo fibroblasts with phorbol ester, A23187 or serum did not induce hsp synthesis but did increase phosphorylation of several isoforms of hsp28. In contrast to the other heavy metals, lead did not induce hsps but did induce synthesis of some glucose-regulated proteins in rat epithelial cells (Shelton et al 1986). Local anaesthetics such as lidocaine were shown by Hahn et al (1985) to induce hsp70 and other hsps. Ethanol induced several hsps in yeast (Plesset et al 1982) and Chinese hamster fibroblasts (Li and Werb 1982, Li 1983); 5-azacytidine, coumarin, diphenylhydantoin and pentobarbitol all induced 22kD hsps in Drosophila embryonic cells, but none of the larger

proteins (Buzin and Bournias-Vardiabasis 1984). Treatment of rats with hepatocarcinogens such as 2-acetylaminofluorene induced synthesis of hsp83 and to a lesser extent hsp70 in the liver, and the same hsps were induced when liver cells proliferated during regeneration after partial hepatectomy, coincident with increased c-Ha-ras and c-myc expression (Carr et al 1986). A 32kD hsp was synthesised in BALB/c3T3 cells after incubation with phorbol ester, N-methyl-N'-nitro-N-nitrosoguanidine, L-ascorbic acid and 3,3'-diaminobenzidine, but none of the larger hsps were induced (Hiwasa and Sakiyama 1986). Morange et al (1986) showed that pretreatment of mouse cells with interferon enhanced the accumulation of hsps when the cells were subsequently mildly stressed with a brief heat or arsenite treatment, so interferon lowered the threshold at which the cells responded to stress. Interferon also reduced the inhibition of normal protein synthesis which usually accompanies such stresses.

Another stimulus for hsp synthesis was found by Kingston et al (1984), who showed that the product of the myc oncogene was able to activate the promoter of the Drosophila hsp70 gene. This might explain why hsp70 was synthesised in regenerating liver where the myc gene was also expressed (Carr et al 1986). The product of the adenovirus E1A gene, which activates expression of the early adenovirus genes, also induced hsp70 synthesis in adenovirus infected HeLa cells (Nevins 1982), and a cell

line which allowed a high level of uninduced hsp synthesis also was able to express early adenovirus genes in the absence of the E1A gene (Imperiale et al 1984), suggesting that the hsp70 gene and the early adenovirus genes may be controlled by very similar mechanisms.

In yeast, McClanahan and McEntee (1986) found a novel set of genes which were activated by heat shock, and also activated by DNA damaging agents such as 4-nitroquinoline-1-oxide, although DNA damage did not activate expression of hsp70 or any of the other previously described hsps, and heat shock did not activate any other DNA damage-responsive genes. Whether such a class of heat- and DNA damage-inducible genes exists in mammalian cells remains to be determined. Schaefer et al (1987) determined that hsp70 mRNA levels increased in human colon adenocarcinoma cells treated with BCNU or CCNU (chloroethylnitrosoureas with both chloroethylating and carbamoylating activity), but not by treatment with other nitrosoureas which had either chloroethylating or carbamoylating activity but not both. This would appear to suggest that DNA damage and cross linking could induce hsp70 gene expression, but BCNU and CCNU react with cellular proteins etc., as well as DNA.

1.7.5.4. Functions of heat shock proteins

The hsps are thought to play a role in protecting the cell from damage caused by the stress which induces their synthesis, but precisely how they may do this is unclear. It was thought that it was these proteins which conferred thermotolerance on cells, but this seems increasingly unlikely, as explained later.

Lindquist's group studied Drosophila and yeast hsp26 and found that the protein did not appear to be necessary for thermotolerance in either proliferating or quiescent cells or for resistance to ethanol, for spore development in the yeast or for germination or thermoresistance of the spores (Petko and Lindquist 1986). Inhibition of the hsp26 also did not affect the synthesis of any other protein during heat shock or recovery (McGarry and Lindquist 1986), so as yet no function has been assigned for hsp26.

hsp90 was shown to associate in Rous sarcoma virus transformed cells with the viral encoded polypeptide $pp60^{v\text{-}src}$ in the absence of heat shock, forming complexes with a 50kD protein as well (Yonemoto et al 1982, Ziemiecki et al 1986), and the resulting complexes did not have the tyrosine kinase activity characteristic of the active $pp60^{src}$. The hsp90 also complexed in the same manner with the transforming proteins of two different

avian sarcoma viruses. The $pp60^{src}$ contains a small hydrophobic region which is necessary for attachment of the protein to the plasma membrane where it is active, and hsp90 may interact with this region on newly synthesised $pp60^{src}$ as it is shuttled to the membrane, preventing aggregation of $pp60^{src}$ in the cytoplasm. The steroid receptors of non-transformed cells are oligomeric and contain a non-hormone binding sub unit of 90kD, which was recently found to be identical to hsp90 (Catelli et al 1985). Following activation with a steroid, the hsp90 was released from the receptor, and it was thought that this interaction of hsp90 with the steroid binding sub-unit plays a role in maintaining the receptor in its inactive form and in preventing aggregation of the receptor. The hsp90 was also found to be involved in actin binding (Nishida et al 1986). It bound optimally to F-actin under physiological conditions, and was dissociated from the F-actin by the binding of tropomyosin. hsp90 also bound to calmodulin in the presence of calcium but not in the absence of calcium. Nishida et al suggested that hsp90 acts as a carrier protein, transporting $pp60^{src}$, steroid receptor or other protein along actin filaments. Its role in heat shocked cells, however remains unclear.

Hsp70 has been shown to exist in the cell as a dimer with four molecules of fatty acid (palmitate and stearate) per dimer and the authors suggested that the fatty acid may be important in the function of the protein (Guidon

and Hightower 1986). Ungewickel (1985) showed that hsp70 was the "uncoating ATPase" in unstressed mammalian cells; a protein which catalysed ATP-dependent dissociation of clathrin triskelia from clathrin-coated vesicles of the plasma membrane. Hsp70 is usually mostly found in the cytoplasm, but on heat shock or other stress it localised in the nucleus (Lewis and Pelham 1985, Pelham et al 1985, Velazquez and Lindquist 1984), binding first to some nuclear components and then to the nucleoli. The hsp70 could be released from the nucleolus by ATP, and it was suggested that hsp70 helps to solubilize aggregates of proteins and ribonucleoproteins that form after heat shock. Pinhasi-Kimhi et al (1986) found that hsp70 associated with p53 transforming protein in transformed cells, stabilising the p53 and presumably aiding in cellular transformation. A cognate of hsp70 (known as grp78 or BiP) was found to bind to nascent immunoglobulin heavy chains in the endoplasmic reticulum of pre-B cells (Munro and Pelham 1986). This was thought to prevent aggregation of the heavy chains until they were able to react with light chains and form active antibody molecules. Hsp70 therefore seems to have a similar role to hsp90 in preventing undesirable aggregation of proteins, and as heat could partially denature cellular proteins causing them to aggregate, higher levels of the hsp70 would be required to be able to bind to all the aggregates, leading to stimulated hsp70 synthesis in heat shocked cells. Interestingly, Harrison et al (1987)

mapped the multiple copies of the hsp70 gene in human cells and found that several hsp70 genes resided at known fragile sites on chromosomes 6 and 14, and the same region of chromosome 6 is involved in translocations and alterations seen in acute leukaemia and melanoma. It is possible therefore that hsp70 genes could be altered as a result of such chromosomal changes, and may play a role in transformation.

Hsp110 in mammalian cells localised in the nucleolus along with hsp70 on heat shock, but in actively proliferating, unstressed cells it could also be seen in the nucleolus, and its distribution changed when the cells reached confluency (Shyy et al 1986, Welch and Suhan 1985). Its function has not yet been determined.

1.7.5.5 Thermotolerance and relation to heat shock proteins

When a cell is subjected to a sub-toxic heat shock and allowed to recover at physiological temperature, if it is subsequently subjected to a normally lethal heat shock, the cells will be resistant and are said to be thermotolerant. The acquisition of thermotolerance occurs concomitantly with the synthesis of heat shock proteins, and the presence of hsps was thought to be responsible for this thermotolerance. Thermotolerance can be induced by pretreatments other than a mild heat shock; arsenite and

other known hsp inducers also induce thermotolerance, and thermotolerance is also accompanied by resistance to such agents as arsenite. Synthesis of hsp70 and hsp87 induced by arsenite or ethanol correlated well with the development of thermotolerance (Li and Werb 1982, Li 1983), and Laszlo and Li (1985) found that heat-resistant variants of Chinese hamster cells had elevated levels of hsp70 under normal growth conditions, which were increased even more on heat treatment, suggesting that hsp70 plays a role in protecting the cells from damage inflicted by high temperatures. Ohtsuka et al (1986) showed that a cell line that did not express hsp70 constitutively, and only produced small amounts after heat shock, could not become thermotolerant, while a cell line producing the protein constitutively could become thermotolerant, even in the presence of cycloheximide which prevented synthesis of new hsp70 induced by heat. They suggested that the presence of existing hsp70 was sufficient to cause thermotolerance. Landry and Chrétien (1983) showed that acquisition of thermotolerance paralleled the synthesis of hsps, and the decay of thermotolerance closely followed the degradation kinetics of the newly synthesised hsps, but they suggested that while the expression of hsps and thermotolerance were most likely regulated by some interrelated mechanisms, hsp synthesis did not appear to be either sufficient or necessary for thermotolerance. Other authors have also discounted the involvement of hsps in thermotolerance: Widelitz et al (1986) showed that cycloheximide prevented

hsp synthesis while allowing build-up of high levels of hsp70 mRNA, but thermotolerance still developed. Cavicchioli and Watson (1986) found that the decay of thermotolerance occurred while there were still very high hsp levels in yeast cells, and that thermotolerance could be restored by a very brief heat treatment that had no further effect on hsp synthesis. In L1210 cells, gradual heating to 42°C produced cells which were thermotolerant without inducing any hsp70 synthesis (Burns et al 1986). Normally, heat shock is accompanied by inhibition of normal protein synthesis, and in thermotolerant cells this inhibition does not occur when the cells are heated, due to some stabilisation of the translational machinery. Hallberg (1986) showed that the presence of hsps was not essential for this stabilisation. Surprisingly, however, cycloheximide and puromycin treatment of cells, to inhibit protein or mRNA synthesis, rendered them thermotolerant (Lee and Dewey 1986), but the acquisition of thermotolerance did not temporally follow the inhibition of protein or mRNA synthesis, suggesting that these drugs might confer thermotolerance by some mechanism not related to protein synthesis inhibition. This casts doubt on many of the results showing that hsp synthesis is not important in thermotolerance development, as most of the studies used cycloheximide to inhibit hsp synthesis.

Rice et al (1986b) demonstrated that CHO cells in S and $G_2$ phases of the cell cycle were more heat sensitive

than cells in $G_1$, but thermotolerance was induced with the same kinetics regardless of their cell cycle phase, while the induced expression of hsps differed between cell cycle phases. Fox et al (1985) showed essentially the same results for HeLa cells.

Hatayama et al (1986) showed that treatment of HeLa cells with sodium butyrate or dibutyryl cAMP rendered the cells thermotolerant, but the drug treatments neither induced hsp synthesis themselves nor enhanced the induction of hsp synthesis when the cells were heated. The authors suggested that there was a factor other than heat shock proteins or cell culture conditions which could be responsible for thermoresistance in HeLa cells.

Thermotolerance can be induced by many agents other than heat and arsenite treatment. Hahn et al (1985) showed that aliphatic alcohols (ethanol to octanol), local anaesthetics such as lidocaine, and polar solvents such as DMSO and DMF all induced thermotolerance in Chinese hamster cells. They found that the concentrations of alcohols required were similar to threshold doses for cytotoxicity, strikingly similar to the situation for the induction of HL-60 cell differentiation outlined previously. Surprisingly, while cells needed to be incubated with alcohols or local anaesthetics for 1 hour only to induce thermotolerance, the polar solvents required at least 12 hours incubation. Whether this shows

an alternative mechanism for thermotolerance induction is unclear. Brenner et al (1981) also showed that DMF induced thermotolerance in mouse mammary adenocarcinoma cells, after 4 or 5 days incubation with the solvent, but they suggested that this was due to phenotypic changes occurring in the cells rather than direct effects of the solvent.

Hahn et al (1985) showed that while thermotolerance was induced by a range of agents, a heat shock also induced tolerance to the local anaesthetics and alcohols, suggesting that thermotolerance is actually a form of multidrug resistance. In accordance with this, Li and Hahn (1978) showed that heat or ethanol induced tolerance to adriamycin, and stresses that induced glucose regulated proteins (such as anoxia or 2-deoxyglucose) also induced resistance to adriamycin (Gessner et al 1987).

1.7.5.6 Modulation of heat shock protein synthesis with development

When murine bone marrow was exposed to hyperthermia, pluripotential CFU-S cells were sensitive to the heat shock, but committed myeloid cells (CFU-GM) were even more sensitive. Mature granulocytes and monocytes were more resistant to the toxic effects of the heat (Elkon et al 1984). CFU-GM cells could develop thermotolerance *in vitro* or *in vivo* (accompanied by the synthesis of hsp70

and hsp87), but this decayed after 24 hours, much faster than in most cell types (Mivechi and Li 1985). CFU-E cells were more resistant to heat than BFU-E, and CFU-E developed thermotolerance in a similar manner to CFU-GM. BFU-E, however could not develop thermotolerance (Mivechi and Li 1986). It seems that as the bone marrow precursors mature they develop the ability to become thermotolerant; this may be related to their ability to synthesis heat shock proteins.

In 1983, Bensaude et al discovered that activation of the embryonic genome at the late two-cell stage of mouse development was preceded by induced synthesis of hsp68 and hsp70. They suggested that the hsps might be involved in activating the embryonic genome, and this could explain how heat shock or ethanol treatment of mouse oocytes induced parthenogenetic activation of the oocytes. The group showed that mouse embryonal carcinoma cells synthesised high levels of hsp70 and hsp89 in the absence of stress, and that these carcinoma cells and early embryo cells were not able to induce synthesis of hsps over the constitutive levels when heat-shocked. Induction of differentiation of the carcinoma cells by retinoic acid restored the heat-inducibility, as did development of the embryo to the blastocyst stage (Morange et al 1984, Bensaude and Morange 1983). Wittig et al (1983) also showed this inability to induce heat shock protein synthesis in undifferentiated teratocarcinoma and early

embryo cells, and confirmed that induction of the proteins could occur after differentiation.

Kurtz et al (1986) showed that during ascospore development in yeast, hsp26 and hsp89 were strongly induced in the absence of stress, whereas hsp70 was neither induced nor inducible but two hsp70 cognates were synthesised. They suggested that the hsps and their related cognate proteins may have important functions in development as well as in response to stress. Dictyostelium was induced to differentiate by stress such as amino acid starvation, and this was accompanied by hsp synthesis, suggesting a link between the two phenomena (Zuker et al 1983), and hsp70 was synthesised during haemin-induced differentiation of K562 human erythroid leukaemia cells (Singh and Yu 1984), although they did not measure hsp70 in K562 cells undergoing true terminal differentiation with any other agent. In contrast to this, Raaphorst et al (1984) reported that heat shock of Friend MEL cells did not induce differentiation, and induction of differentiation by DMSO was inhibited by heat shock, suggesting that hsps might play an inhibitory role in differentiation of these cells. There appears to be some contradiction therefore, as to whether hsps play any role in the induction of differentiation, dependent on the cell type studied.

1.7.5.7 Control of hsp synthesis

When Drosophila or mammalian cells were subjected to heat shock, a mechanism of translational control was induced which promoted the translation of heat shock mRNA while repressing translation of pre-existing mRNAs, presumably by disrupting existing polysomes and sequestering the mRNAs (Lindquist 1981). Transcription of mRNA for the heat shock proteins also occurred very quickly, while normal mRNA transcription was repressed, probably related to changes in histone methylation (Desrosiers and Tanguay 1985). There is controversy, however, about how the induction of the heat shock response is triggered. It was thought that a decrease in intracellular pH could occur, leading to activation of the heat shock genes (Weitzel et al 1985), but it has since been found that the changes in pH and intracellular calcium observed during heat shock were not responsible for hsp induction (Drummond et al 1986). Rowe et al (1986) showed that inhibition of topoisomerase II induced hsp synthesis, but the role of this enzyme has yet to be established. The cellular levels of the tripeptide glutathione were shown to be an important factor in the intrinsic heat sensitivity of a cell line, but it was not apparently involved in the development of thermotolerance or stimulation of hsp synthesis (Shrieve et al 1986, Freeman et al 1985, Lilly et al 1986). Glucose and energy metabolism were also thought to play a role in the heat

shock response (Kasambalides and Lanks 1985, Warren et al 1986, Lanks et al 1986), but thermotolerance and hsp synthesis were not triggered exclusively as a result of adverse effects on energy metabolism (Landry et al 1986).

Polyadenylylated nucleotides (such as diadenosine 5',5'''-$P^1$,$P^4$-tetraphosphate, $Ap_4A$) were implicated in the heat shock response and have been called "alarmones" by Varshavsky (1983). They were thought to be synthesised in response to specific metabolic stress (Lee et al 1983a). Lee et al (1983b) suggested that these dinucleotides might be the trigger for heat shock protein induction, and found that levels of alarmones increased when bacterial cells were subjected to heat shock or to ethanol. Segal and Le Pecq (1986) measured alarmone levels in eukaryotic cells (NIH 3T3 and Chinese hamster lung) and found that contrary to the situation in bacteria, there was no induction of alarmone synthesis when cells were heat shocked or subjected to other stresses, but levels did increase when cells reached confluency, associated with contact inhibition of cell growth. In contrast. Guédon et al (1986) measured alarmone levels in frog oocytes and cultured hepatoma cells and found that they did accumulate after heat shock, but only under conditions leading to irreversible damage and ultimate cell death. Synthesis of heat shock proteins was not always accompanied by increased alarmone levels, so they concluded that these alarmones did not act as a trigger for heat shock protein

synthesis in eukaryotic cells.

Recently, Colbert and Young (1987) detected mRNA for hsps in non-heat shocked lymphocytes. These mRNAs were not however translated until the cells were heat shocked. The authors suggested that translation of these mRNAs was inhibited in unstressed cells by the binding of small RNA molecules with complementary sequences to regions of the mRNA; these antisense RNA molecules were probably transcribed from the "non-coding" strand of the DNA in the hsp gene. Heat shock was thought to disrupt the mRNA:RNA hybrids and allow translation of the heat shock proteins. If this is the case, it represents a new mechanism for the translational control of gene expression.

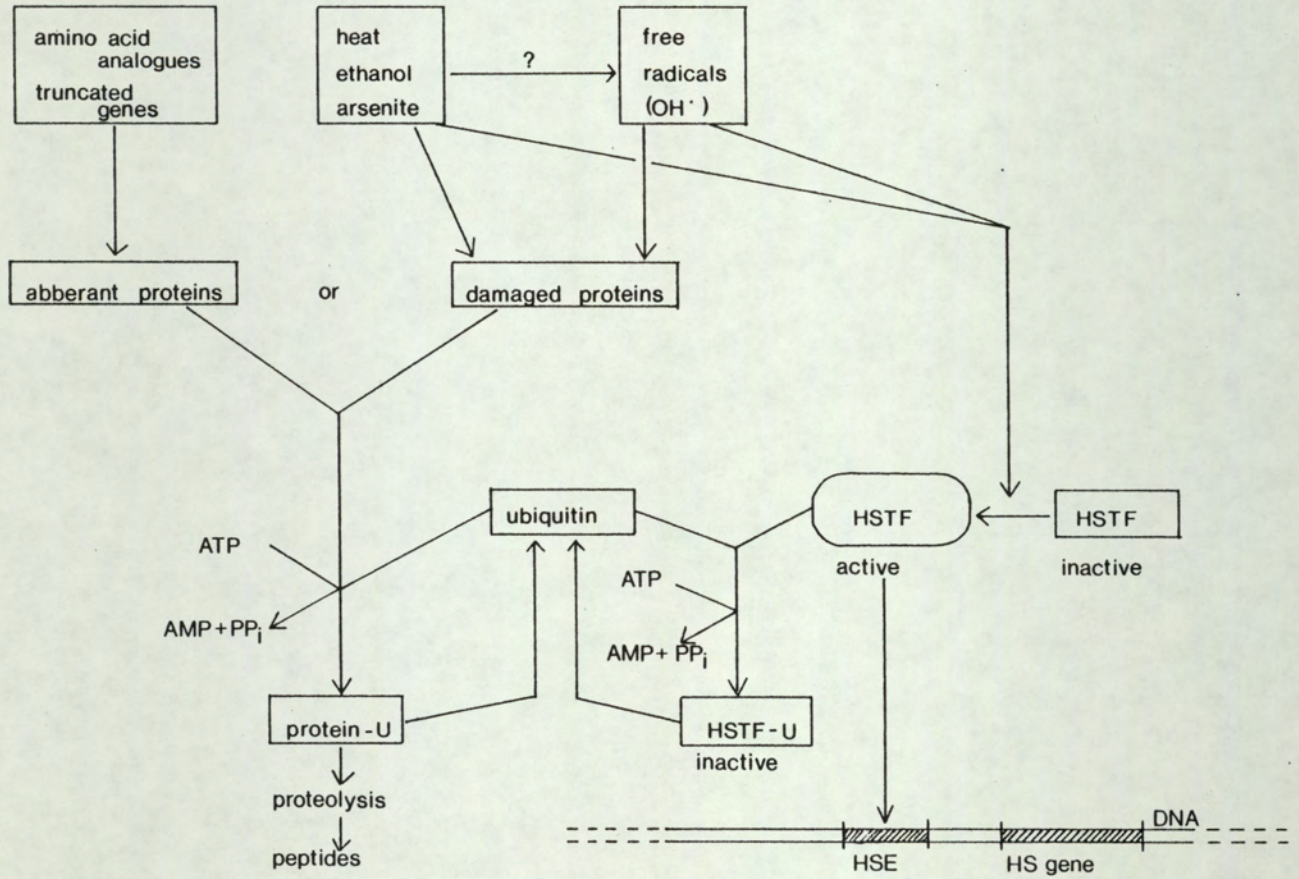
The most widely favoured view of the transcriptional control of heat shock gene expression involves the ubiquitin system. It is known that heat shock genes have a "consensus sequence" in the nontranscribed region upstream from the transcribed region of the gene (Voellmy et al 1985), and this consensus sequence was thought to bind protein factor(s) involved in activation of the gene (Cartwright and Elgin 1986). This "heat shock transcription factor" (HSTF) has since been identified and shown to bind to the "heat shock element" (consensus sequence) during stimulation of the heat shock response. Munro and Pelham (1985) suggested that the binding of the HSTF was regulated by ubiquitin - a small protein found in

all cells, which is involved in degradation of abnormal or damaged proteins. This led to a proposal that heat and other stresses damage cellular proteins, which are then degraded using ubiquitin, and the pool of ubiquitin becomes depleted. The HSTF is normally ubiquitinated in a dynamic equilibrium with ubiquitin continuously being removed and replaced and is unable to bind to DNA, but when the ubiquitin pool is depleted, loss of ubiquitin from the HSTF causes a conformational change and the active HSTF binds to the heat shock element on the DNA, activating transcription of the heat shock genes (Burdon 1986, Tanguay 1983). This proposed scheme is shown in Figure 1.5.

This theory suggests that any agent capable of causing intracellular accumulation of damaged or abnormal proteins would induce heat shock protein synthesis. Heat is known to denature proteins by partial unfolding of the tertiary structure; amino acid analogues such as canavanine would be incorporated into proteins which would be inactive, possibly folding aberrantly; arsenite would bind to sulphydryl groups disrupting bonding, and ethanol could disrupt hydrophobic bonding by changing the polarity of the environment within the protein. Ananthan et al (1986) carried out some very elegant experiments in which they microinjected either native or partially denatured protein into frog oocytes, and measured the induction of the heat shock response using a co-injected gene construct

FIGURE 1.5 Possible mechanism for the induction of heat shock gene expression triggered by damaged cellular proteins
(From Burdon 1986)

HSTF = heat shock transcription factor
HSE = heat shock element
U = ubiquitin



with an hsp promoter. Injecting native protein into the cell did not activate the gene, but injecting denatured protein activated the heat shock promoter, suggesting that damaged proteins are indeed the trigger for induction of the heat shock response.

Many inducers of HL-60 cell differentiation are known to bind to certain target proteins in the cell, and this binding might perturb the protein structure enough for the cell to recognize it as damaged. In this way it is conceivable that HL-60 cell differentiation inducers could induce heat shock gene expression. The hsps, thought to have a role in normal cellular development, could then be involved in initiating the program of gene expression necessary for cell differentiation.

## 1.8 The aim of these studies

The aim of these studies was first to characterise the process of the induction of differentiation of HL-60 cells by studying changes in cell cycle distributions and the expression of various markers of differentiation in response to different agents, and to determine whether cell division was necessary in order for the cells to differentiate.

The second aim was to investigate whether a type of

stress response was involved in commitment of the cells to differentiate. It is plausible that many, if not all, of the known inducers of HL-60 cell terminal differentiation could induce gene amplification as the concentrations which induced differentiation also perturbed DNA replication. If this gene amplification involved only those genes normally synthesised at the very beginning of S phase, for example, it is possible that a specific set of genes could be amplified whose expression could trigger differentiation of the cells. It was proposed that the DNA content of the cells, and the appearance of the chromosomes, should be studied for any evidence of gene amplification.

An alternative hypothesis was that most, if not all, of the inducers of HL-60 cell differentiation could cause protein damage and thus induce heat shock protein synthesis in the cells. The heat shock proteins might then be able to trigger the cascade of changes in gene expression necessary for differentiation of the cells. To investigate this it was proposed that various known inducers of heat shock protein synthesis in other cell types would be tested to determine whether they could induce HL-60 cell differentiation, and heat shock protein synthesis would be studied in the cells during the induction of differentiation by various agents.

# CHAPTER TWO

# MATERIALS

## 2.1 SOURCES

### 2.1.1 Purchased from Sigma Chemical Company Ltd., Poole, Dorset

alpha-naphthyl acetate
amido black (naphthol blue black)
ammonium persulphate
bovine serum albumin
bromophenol blue
cadmium sulphate
4-chloro-1-naphthol
colchicine
coomassie brilliant blue R
ethylene glycol monomethyl ether
L-glutamic acid
glycerol
glycine
high molecular weight protein markers
hydrogen peroxide (30%)
iodoacetamide
lead nitrate
lidocaine
methyl green
nitrobluetetrazolium
nonidet P-40

pararosaniline

phenylmethylsulphonylfluoride

procaine hydrochloride

propidium iodide

protein A-peroxidase

ribonuclease A type 1-A

sodium azide

sodium nitrate

12-O-tetradecanoylphorbol-13-acetate

N,N,N',N'-tetramethylethylenediamine

toluidine blue

tris(hydroxymethyl)aminomethane base

triton X-100

2.1.2 Purchased from Fisons Scientific Equipment, Loughborough, Leics.

acetic acid

acrylamide

dimethylsulphoxide

formaldehyde solution (40%)

hydrochloric acid

orthophosphoric acid

potassium chloride

sodium hydroxide

trichloroacetic acid

trisodium citrate

urea

2.1.3. Purchased from BDH Chemicals Ltd., Poole, Dorset

acetone

disodium hydrogen orthophosphate

ethanol

Giemsa's stain - improved R66 (Gurr)

magnesium chloride

ß-mercaptoethanol

methanol

N,N'-methylenebisacrylamide

potassium dihydrogen orthophosphate

sodium arsenite

sodium carbonate

sodium chloride

sodium dodecylsulphate

trypan blue (Gurr)

XAM mountant (Gurr)

2.1.4 Other purchases

Aldrich Chemical Company Ltd., Gillingham, Dorset

disodium hydrogen orthophosphate dodecahydrate

N-methylformamide

xylene

Sterilin Ltd., Feltham

25ml plastic universal tubes

7ml plastic bijoux bottles

sterile plastic pipettes

Oxoid Ltd., Basingstoke, Hants.

complement fixation buffer tablets

phosphate buffered saline tablets

Gibco Ltd., Paisley, Scotland

RPMI-1640 tissue culture medium with L-glutamine and 25mM HEPES

leucine-free RPMI-1640

methionine-free RPMI-1640

foetal calf serum

Nunc cryotubes

Nunc 25ml plastic tissue culture flasks

Nunc 250ml plastic tissue culture flasks

Becton Dickinson Labware, Oxnard, CA., USA

Falcon 2054 plastic tubes with lids

BioMedical Laboratory Supplies, Birmingham

1.5ml microfuge tubes

0.4ml microfuge tubes

New England Nuclear, DuPont (UK) Ltd., Southampton

L-[$^{35}$S]-methionine, >800Ci/mmol

L-[U-$^{14}$C]-leucine, 342mCi/mmol

May and Baker Ltd., Manchester

Luma Gel Scintillation fluid

Coulter Electronics Ltd., Luton, Beds.

Isoton balanced electrolyte solution

Pharmacia Ltd., Milton Keynes, Bucks

Pharmalyte ampholines, pH 5-7 and pH 3-10

Bio-Rad Laboratories Ltd., Watford, Herts.

Protean gel electrophoresis apparatus

blotting filter paper

trans-blot membrane - 0.45um nitrocellulose

Kodak Ltd., Hemel Hempstead, Herts

D-19 developer

Kodafix

LKB Instruments Ltd., Croydon, Surrey

agarose-M

Whatman Labsales Ltd., Maidstone, Kent

3MM CHR chromatography paper

Ceaverken AB, Strängnäs, Sweden

CEA Singul non-screen medical X-ray film X-RP

### 2.1.5 Gifts

Human plasma was kindly provided by the Blood Transfusion Centre, University of Birmingham.

Procion rubine MX-B was a gift from ICI Organics Division, Cheshire.

Rabbit anti-human hsp70 antibody was generously donated by Dr Hugh Pelham, MRC Centre, Cambridge.

## 2.2 SOLUTIONS

### 2.2.1 Phosphate buffered saline

| | |
|---|---|
| Oxoid PBS tablets | 5 |
| Distilled water | to 500ml |

This gave a solution equivalent to 0.8g sodium chloride, 0.2g potassium chloride, 1.15g disodium hydrogen orthophosphate dihydrate, 0.2g potassium dihydrogen orthophosphate per litre, pH 7.4.

### 2.2.2 Complement fixation buffer

| | |
|---|---|
| Oxoid CFB tablets | 5 |
| Distilled water | to 500ml |

This gave a solution equivalent to 0.575g barbitone, 0.185g soluble barbitone, 8.5g sodium chloride, 0.168g magnesium chloride, 0.028g calcium chloride per litre, pH 7.2.

### 2.2.3 Buffer for ribonuclease solution

| | |
|---|---|
| disodium hydrogen orthophosphate dodecahydrate | 0.742g |
| disodium hydrogen orthophosphate, anhydrous | 1.689g |
| hydrochloric acid, dropwise | to pH7.0 |
| distilled water | to 100ml |

This 0.14M phosphate buffer was used to dissolve ribonuclease A (type 1-A from bovine pancreas, 5X crystallized, protease and salt free, 75 kunitz/mg) to give a 1mg/ml solution.

2.2.4 Formol acetone fixative

| | |
|---|---|
| potassium dihydrogen orthophosphate | 0.1g |
| disodium hydrogen orthophosphate | 0.02g |
| distilled water | 30ml |
| acetone | 45ml |
| formaldehyde solution (40%) | 25ml |

2.2.5 Hypotonic 0.075M potassium chloride

| | |
|---|---|
| potassium chloride | 5.59g |
| distilled water | to 1000ml |

2.2.6 Reticulocyte standard buffer

| | |
|---|---|
| tris(hydroxymethyl)aminomethane base | 1.211g |
| sodium chloride | 0.584g |
| magnesium chloride | 0.143g |
| hydrochloric acid, dropwise | to pH 7.4 |
| distilled water | to 1000ml |

2.2.7 Separating gel buffer for electrophoresis

| | |
|---|---|
| tris(hydroxymethyl)aminomethane base | 22.71g |
| sodium dodecylsulphate | 0.5g |
| hydrochloric acid, dropwise | to pH 8.8 |
| distilled water | to 500ml |

2.2.8 Stacking gel buffer for electrophoresis

| | |
|---|---|
| tris(hydroxymethyl)aminomethane base | 7.569g |
| sodium dodecylsulphate | 0.5g |
| hydrochloric acid, dropwise | to pH 6.8 |
| distilled water | to 500ml |

2.2.9 Sodium dodecylsulphate sample buffer (2X concentration)

| | |
|---|---|
| tris(hydroxymethyl)aminomethane base | 1.514g |
| sodium dodecylsulphate | 4.0g |
| hydrochloric acid, dropwise | to pH 6.8 |
| glycerol | 20ml |
| distilled water | to 100ml |

just prior to use:

| | |
|---|---|
| ß-mercaptoethanol | 10ml |
| bromophenol blue | 0.002g |

For a 1X concentrated solution, one volume of the above buffer was mixed with one volume of distilled water.

2.2.10 Electrolyte buffer

| | |
|---|---|
| tris(hydroxymethyl)aminomethane base | 15.14g |
| glycine | 72.07g |
| sodium dodecylsulphate | 5.0g |
| distilled water | to 5 litres |

The pH of this solution was 8.3

2.2.11 Blotting transfer buffer

| | |
|---|---|
| tris(hydroxymethyl)aminomethane base | 15.14g |
| glycine | 72.07g |
| methanol | 1 litre |
| distilled water | to 5 litres |

The pH of this solution was 8.3.

2.2.12 Tris buffered saline

| | |
|---|---|
| tris(hydroxymethyl)aminomethane base | 1.211g |
| sodium chloride | 9.0g |
| hydrochloric acid, dropwise | to pH 7.4 |
| distilled water | to 1000ml |

2.2.13 Tris buffer for peroxidase reaction

| | |
|---|---|
| tris(hydroxymethyl)aminomethane base | 0.606g |
| hydrochloric acid, dropwise | to pH 7.4 |
| distilled water | to 500ml |

2.2.14 30% acrylamide for isoelectric focusing

| | |
|---|---|
| acrylamide | 30.0g |
| N,N'-methylenebisacrylamide | 1.8g |
| distilled water | to 100ml |

This solution was stored at 4°C.

2.2.15 Gel overlay buffer

| | |
|---|---|
| urea | 9.6g |
| distilled water | to 20ml |

This solution was divided into 2ml aliquots and stored at -20°C.

2.2.16 Cell lysis buffer

| | |
|---|---|
| urea | 5.7g |
| nonidet P-40 | 0.2g |
| ampholines pH 5-7 | 0.16ml |
| ampholines pH 3-10 | 0.04ml |

| | |
|---|---|
| ß-mercaptoethanol | 0.5ml |
| distilled water | to 10ml |

This solution was divided into 0.5ml aliquots and stored at -20°C.

2.2.17 Isoelectric focusing cathode buffer

| | |
|---|---|
| sodium hydroxide | 4.0g |
| distilled water | to 500ml |

2.2.18 Isoelectric focusing anode buffer

| | |
|---|---|
| orthophosphoric acid | 0.32ml |
| distilled water | to 500ml |

# CHAPTER THREE

# METHODS

## 3.1 Assays of cell growth and differentiation

### 3.1.1 HL-60 cell culture

HL-60 human promyelocytic leukaemia cells were provided by Mr Tim Ward, Paterson Laboratories, Christie Hospital and Holt Radium Institute, Manchester. They were grown by serial subculture every 3 or 4 days in 50 or 250 ml plastic tissue culture flasks with RPMI-1640 medium containing L-glutamine and 25mM HEPES, supplemented with 10% foetal calf serum (virus and mycoplasma screened), under aseptic conditions in a Flow Laboratories Gelaire BSB3 laminar flow cabinet. Each flask was gassed with a mixture of 5% $CO_2$ in air then incubated at 37°C. The cells were maintained in logarithmic phase of growth between $5 \times 10^4$ and $1.5 \times 10^6$ per ml; the cell number was determined with a Coulter Laboratories ZM or ZB1 electronic cell counter.

### 3.1.2 Storage of cells in liquid nitrogen

Cells grown in logarithmic phase were centrifuged for 5 min at 1200 rpm (250 g) in a Heraeus Labofuge 6000, then resuspended at $1 \times 10^7$ per ml with medium containing 10% foetal calf serum plus 10% dimethylsulphoxide, before measuring 1 ml aliquots into 8 cryotubes and freezing

slowly (at 2.3°C per min) on ring C of a Linde BF-5 biological freezer in liquid nitrogen. The cells could then be stored indefinitely at this temperature and were resurrected by thawing one cryotube and diluting the cells into 15ml of fresh medium. After 5 - 7 days the cells were usually growing logarithmically.

### 3.1.3 Induction of differentiation

Cells were diluted to 1 x $10^5$ per ml with fresh medium and the appropriate volume of drug was added using a P1000 or P100 Gilson pipetman. After gassing the flasks were incubated at 37°C and assays of differentiation were carried out daily or on day four after addition of inducer. The volume of polar solvent (such as N-methylformamide, dimethylsulphoxide or ethanol) added to the cells was calculated as follows:

$$\frac{\text{conc (mM)}}{1000} \times \frac{\text{vol. in flask (ml)}}{1000} \times Mw \times 1/p = \text{vol. (ml)}$$

where Mw is the molecular weight of the solvent and p is the density. Other inducers of differentiation were made up in solution as follows:

a) Sodium arsenite

0.013g of $NaAsO_2$ (Mw 129.91) were dissolved in 5 ml of distilled water and this 20mM solution was filtered

through a Flowpore 0.2um filter to sterilize it before further diluting with sterilized distilled water to give 10, 5 and 1mM solutions. These solutions were then added directly to the cells (60ul of 1mM solution giving a final concentration in 10ml of cells of 6uM).

b) Lidocaine hydrochloride

2.343g of lidocaine (Mw 234.3) were dissolved in 1ml of conc. hydrochloric acid (10N), then made up to 10ml with distilled water, to give a 1M solution of lidocaine hydrochloride. This was then sterilized using a Flowpore 0.2um filter before adding to cells. 100ul of the 1M solution added to 10ml of cells gave a final concentration of 10mM.

c) Procaine hydrochloride

2.728g of procaine hydrochloride (Mw 272.8) were dissolved to 10ml with distilled water to give a 1M solution, which was sterilized by filtration before adding to the cells. 100ul of this solution added to 10ml of cells gave a final concentration of 10mM.

d) Cadmium sulphate

0.128g of $3CdSO_4.8H_2O$ (formula weight 769.5) were dissolved in 10ml of sterilized water to give a 50mM

solution with respect to cadmium. This solution was filtered, then further diluted with sterile water. 100ul of a 10mM solution added to 10ml of cells gave a final solution of 100uM cadmium.

e) Lead glutamate

According to Shelton et al (1986), lead glutamate is able to accumulate intracellularly. A solution of 100mM lead glutamate was prepared by dissolving 0.331g of lead nitrate ($Pb(NO_3)_2$, molecular weight 331) and 0.147g of L-glutamic acid (molecular weight 147) in 10ml of sterilized water. The crystals dissolved slowly over a period of 12 hours. The solution was filtered through a 0.2um Flowpore filter, then further diluted with sterile water. 50ul of 100mM lead glutamate added to 10ml of cells gave a final concentration of 0.5mM.

### 3.1.4 Assay of cell viability by the exclusion of trypan blue

$1 \times 10^5$ cells were centrifuged in a 1.5ml microcentrifuge tube for 20 secs at 11,600 g using a Beckman microfuge B, and most of the supernatent was removed. To the cell pellet was then added 5ul of 0.1% trypan blue dye in phosphate-buffered saline (pH 7.4), and this was mixed using a Fisons whirlimixer, before

pipetting a drop onto a microscope slide and viewing with 10x eyepiece and 40x objective on either a Zeiss or Nikon Optiphot microscope. Non-viable cells had taken up the dye and appeared blue in colour whereas viable cells were able to exclude the dye and remained colourless. At least 300 cells were counted per sample, and the percentage of viable cells calculated.

### 3.1.5 Assay of cell differentiation by the reduction of nitrobluetetrazolium

A solution of nitrobluetetrazolium (NBT) was made up as follows:

To 10ml of phosphate-buffered saline, pH7.4, was added 10mg of NBT powder to give a saturated solution. An aliquot of 100ug of 12-O-tetradecanoylphorbol-13-acetate (TPA, stored frozen in glass vials) was dissolved in 100ul of acetone, then 50ul of this was added to the NBT solution.

$1 \times 10^5$ cells were centrifuged at 11,600g for 20 secs, and the supernatent was removed. 0.5ml of the NBT solution was added to the cell pellet which was then mixed and incubated in a shaking water bath at 37°C for 30 to 45 mins. The cells were centrifuged again and most of the supernatent was removed before pipetting a drop onto a microscope slide and viewing with a 40x objective. Cells which had differentiated to either granulocytic or monocytic cells were stimulated by the TPA to produce an

oxidative burst yielding superoxide which then reduced the NBT dye to blue/black formazan granules, causing the cells to appear purple. Undifferentiated cells remained colourless. At least 300 cells were scored and the percentage of differentiated cells calculated.

### 3.1.6 Determination of the capacity for phagocytosis

#### a)Preparation of complement-coated, red-dyed yeast particles

Yeast particles were prepared according to the method of Shaala et al (1979) as follows:
Several grams of baker's yeast were washed twice with phosphate buffered saline (PBS), by centrifugation in a Heraeus Labofuge 6000 for 5 mins at 5000 rpm, then resuspended in 50ml of PBS and autoclaved. The yeast were washed 7 or 8 times with PBS until the supernatent remained clear, then incubated at 37°C for 2 hours in 25ml of PBS with 0.17ml of ß-mercaptoethanol. After washing once the yeast were alkylated by resuspension in 50ml of 0.02M iodoacetamide in PBS (pH7.2) and incubation at room temperature for 2 hours, checking that the pH remained at 7.2. The yeast were subsequently washed three times, autoclaved and washed 7 more times until the supernatent remained clear. The number of yeast particles per ml were determined using a Weber haemocytometer. They were resuspended in 0.9% saline at $2 \times 10^9$ per ml, giving a

total of 20ml. 120ml of saline containing 260mg of procion rubine dye was then added and incubated at room temperature for 3 hours. The excess dye was removed by washing, and the red-dyed yeast were resuspended at 2 x $10^9$ per ml with 20mg sodium azide in 20ml of PBS, before storing at 4°C.

To ensure efficient uptake into phagocytosing cells the alkylated, red-dyed yeast were then complement coated:

2ml of the red-dyed yeast suspension (approx. 5 x $10^9$ yeast) were added to 8ml of complement fixation buffer and 2ml of rabbit serum, then incubated for 30 mins at 37°C. After washing twice with RPMI-1640 medium and resuspending in a total of 20ml of medium (to approx. 5 x $10^8$ per ml), the yeast were aliquoted into 0.5ml portions and stored frozen at -20°C.

b) <u>Assay for phagocytosis</u>

1 x $10^5$ HL-60 cells were centrifuged at 11,600g for 20 secs, and the supernatent was removed. The cell pellet was mixed with 50ul of a 1:4 mixture of RPMI-1640 medium and hepatitis-free AB+ human plasma, and 15ul of the prepared yeast were added. After mixing thoroughly the cells were incubated at 37°C for 1 hour, then centrifuged and most of the supernatent was removed. 4ul of 0.1% toluidine blue in saline was added to counterstain any yeast remaining outside the cells, a drop was placed on a

microscope slide and the cells were viewed using a 40x objective. Cells which had differentiated to either granulocytic or monocytic cells were able to take up the yeast by phagocytosis; these cells contained one or more red yeast particles. At least 300 cells were scored and the percentage of cells capable of phagocytosis was calculated.

### 3.1.7 Stain for monocytic non-specific esterases

This was carried out essentially according to the method of Yam et al (1971). 2.5ml of 2% alpha-naphthyl acetate in ethylene glycol monomethyl ether were added to 45ml of phosphate buffered saline, followed by 3ml of hexazotized pararosaniline (a 1:1 mixture of 4% sodium nitrate and 4% pararosaniline in 2M HCl). This solution was adjusted to pH 5.8 - 6.2 with 1M sodium hydroxide, and the staining solution was filtered before use.

1 x $10^5$ cells were centrifuged at 11,600g, most of the supernatent was removed, and a drop of the remaining cell pellet was placed on a microscope slide and smeared. The cell smear was air dried, then fixed with buffered formol acetone (7.35mM $KH_2PO_4$, 1.4mM $Na_2HPO_4$ in 45% acetone, 10% formaldehyde) for 30 secs and washed with distilled water. The slide was air dried again then incubated in the freshly prepared staining solution for 2 hours at 37°C. After washing with distilled water the slide was counterstained with 1% methyl green in distilled

water for 30 secs then washed again and dried before viewing with a 40x objective.

Cells which had differentiated to monocyte-like cells expressed non-specific esterases active at pH 5.8 - 6.2, and these cleaved the naphthyl acetate to naphthol, which then reacted with the hexazotized pararosaniline to give an azo dye, resulting in red staining of the cells. Cells which did not express the esterases stained green with the counterstain. At least 300 cells were counted and the percentage of esterase-positive cells was calculated.

### 3.1.8 Staining with Wolbach Giemsa to reveal nuclear morphology

This staining technique utilized the variation of Giemsa's stain recommended by Wolbach, in which the staining solution was made slightly alkaline with sodium bicarbonate (Cramer et al 1973).

$1 \times 10^5$ cells were centrifuged at 11,600g and most of the supernatent was removed, then a drop of the remaining cell pellet was placed on a microscope slide and smeared. The smear was air dried, fixed with 3% acetic acid in absolute ethanol, washed with water and then stained in a solution of Giemsa's stain (1ml of Gurr improved R66 Giemsa's stain, 1.25ml of methanol, 4 drops of 0.5% aqueous $Na_2CO_3$, plus 40ml of distilled water). The stain was changed for a fresh solution after 30 mins and again

after 60 mins, then left overnight. The slides were differentiated by immersion in 95% ethanol, checking under the microscope every few minutes until the nuclei of the cells were clearly distinguished from the cytoplasm. The cells were dehydrated in absolute ethanol, then xylene, and mounted in XAM or DPX mountant.

## 3.2 Preparation of cells for flow cytometric cell cycle analysis

A modification of the method of Gray and Coffino (1979) was used. To analyse 20,000 individual cells on the flow cytometer, at least 1ml containing $5 \times 10^5$ cells were required. Sufficient cells were centrifuged in a Heraeus Labofuge 6000 at 1200 rpm for 5 mins, the supernatent was removed and the pellet was resuspended in 10ml of phosphate buffered saline, pH 7.4. After centrifugation the pellet was resuspended in 1ml of 70% ethanol, and the fixed cells were then stored in the dark at 4°C until required. They could be stored for at least one month without adverse effects.

The cells were stained no more than 12 hours before analysis, as the fluorescent signal became reduced and perturbed with time. The 1ml samples were centrifuged at 11,600g for 20 secs and the fixative was removed before resuspending the pellets each in 1ml of 1mg/ml ribonuclease A in 0.14M phosphate buffer and incubating at

37°C for at least 30 mins. The cells were centrifuged again and each pellet was resuspended in 1ml of 50ug/ml propidium iodide in 0.1% sodium citrate, 0.1% Triton X-100 before being transferred into Falcon 2054 tubes. Flow cytometric analysis was then carried out using a Becton Dickinson 440 Fluorescence Activated Cell Sorter with Consort 40 computer and Tektronix 4612 printer, in the Dept. of Anatomy, Birmingham University, (used with permission of A. Vaughan and technical assistance from A. Milner). Forward light scatter (related to cell size) and fluorescence (540nm excitation and 625nm emission for propidium iodide, related to DNA content per cell) were determined from each individual cell passing through a laser beam. The results were plotted as a histogram of the number of cells against fluorescence or light scatter. From the fluorescence histogram, the cell cycle phase of the cells could be determined. The left hand peak on the histogram corresponded to cells with a diploid DNA content (in $G_0$ or $G_1$ phase), the central plateau corresponded to cells synthesizing DNA in S phase, and the right hand peak represented tetraploid cells in $G_2$ and M phases. The percentage of cells in each cell cycle phase could be determined from the area under the peaks.

## 3.3 Determination of the time course of commitment to differentiation

### 3.3.1 Measurement of commitment to differentiation to granulocytes after treatment with NMF

Cells grown in logarithmic phase were diluted to 1 x $10^5$ per ml with fresh RPMI-1640 containing 10% foetal calf serum, with 10ml in at least 14 flasks. In some experiments an extra 10ml were prepared per flask, and this was removed for FACS analysis at the time point. N-methylformamide (NMF) was added to all but two of the flasks to give a concentration of 170mM, and the cells were gassed and incubated at 37°C. At time points from 2 to 72 hours, one NMF-treated flask was removed from the incubator, and the NMF was removed from the cells by washing three times by centrifugation in a Heraeus Labofuge for 5 mins at 1200 rpm and resuspension in 10ml of fresh medium without NMF. The cells were finally resuspended in 10ml, gassed and returned to the incubator. At 72 hours from the start of the experiment assays of differentiation were carried out on all the samples to determine the length of incubation with NMF required to irreversibly commit a significant proportion of the cells to differentiate.

### 3.3.2 Measurement of uptake of [$^{14}$C]-NMF into HL-60 cells

The results were very variable when uptake of labelled NMF was measured at 37°C or at 4°C, so the experiment was carried out at room temperature (23°C). Cells in the logarithmic phase of growth were suspended in 15ml of RPMI-1640 with 10% foetal calf serum to a concentration of 2 x $10^6$ cells per ml, gassed and left to equilibrate at room temperature for at least 30 mins. [$^{14}$C]-($CH_3$)-NMF was synthesised by M.D.Threadgill with an activity of 2mCi/g, 1uCi/ul. 15uCi were added to the cell suspension, along with 135.45ul of unlabelled NMF to give a final concentration of 170mM NMF, at 1uCi/ml of cells. At various time points from 1 to 35 mins, 3 x 0.5ml of cells were taken from the sample, centrifuged at 11,600 g for 30 secs and washed once with 0.5ml of ice cold 0.9% saline. The cell pellets were dissolved overnight with 100ul of 1% Triton X-100 in water, then each sample was mixed with 5ml of Luma Gel scintillation fluid. The amount of [$^{14}$C]-NMF in the cells was determined using a Packard Tri Carb 2000CA liquid scintillation counter.

At the end of the experiment, one 0.5ml sample of cells was centrifuged and washed as usual, then the cell pellet was suspended in 20ml of Isoton solution and the cell number was determined using a ZB1 Coulter counter, to ensure that the pellet contained 1 x $10^6$ cells.

## 3.4 Induction of differentiation under different proliferative conditions

### 3.4.1 Induction of differentiation in serum-deprived cells and cells at saturation density

Saturation density was achieved by growth of the cells without replenishment of medium so that they exhausted the nutrients in the medium and virtually ceased cell division. The cell density at this point was usually approximately 2 x $10^6$ per ml. Some cells were maintained in the old medium and incubated with or without 170mM NMF. Other cells at saturation density were centrifuged once and resuspended at the same density in fresh 10% foetal calf serum in RPMI-1640, with or without NMF.

Serum deprivation was achieved using either cells at saturation density or at lower concentrations. The cells were centrifuged and washed three times with RPMI-1640 without serum, and resuspended in serum-free RPMI-1640 with or without 170mM NMF. In some experiments either 0.2% foetal calf serum or 0.5% bovine serum albumin were added to the serum-free medium to maintain viability without stimulating cell proliferation.

Assays of differentiation were carried out several days after the initiation of these experiments, and some samples were prepared for FACS analysis.

### 3.4.2 Induction of differentiation whilst diluting and refeeding cells in the continuous presence of NMF

Cells in the logarithmic phase of growth were diluted to 1 x $10^5$ per ml with fresh RPMI-1640 containing 10% foetal calf serum. NMF was added to 150ml of the cells to give a concentration of either 100 or 170mM, and 50ml of cells were left untreated. Cell counts and assays of differentiation were carried out at intervals for up to 17 days, and every few days a sample of the NMF-treated cells was diluted to 1 x $10^5$ per ml to try to maintain logarithmic growth. NMF was added to either 170 or 100mM to the medium with which the cells were diluted, to maintain a constant concentration of inducer whilst diluting and refeeding the cells. Some cells of the original sample were allowed to grow to a plateau without dilution, others were diluted once, while further samples were diluted serially several times before allowing them to plateau.

## 3.5 Preparation of cell chromosomes for microscopy

0.1ml of a solution of 0.02% colchicine in distilled water was added per ml of cell suspension. The cells were incubated at 37°C for 10 mins to block those cells in which mitosis was taking place so as to disrupt the microtubules and allow release of the chromosomes. After

centrifugation in glass tubes in a Heraeus labofuge 6000 for 5 mins at 1200 rpm (250 g), the supernatent was removed. A total of 3ml of hypotonic 0.075M KCl was added gradually to each of the cell pellets whilst mixing, and the samples were incubated at 37°C for 15 mins or more, to cause swelling of the cells. They were again centrifuged and the supernatent was removed, then each cell pellet was carefully resuspended by adding 3ml of fixative (3:1 methanol: glacial acetic acid) dropwise whilst mixing to prevent the cells from clumping. After several minutes the cells were centrifuged at 250 g and the supernatent was removed before resuspending the pellet in fresh fixative, after which the cells could be stored at 4°C until required.

To prepare spreads of chromosomes for microscopy, the cells were centrifuged at 250 g and resuspended in 1ml of fixative to give a concentrated cell suspension. Clean microscope slides were cooled by placing in a deep-freezer for 15 mins prior to use, so that when removed they became covered in condensation. Drops of cell suspension were then dropped from a height of one metre onto these slides using a pasteur pipette. This process burst the cells and spread out the chromosomes. The slides were left to dry then stained for 1 hour with Giemsa solution (1:10 dilution of Gurr improved R66 Giemsa's stain in water) before washing with distilled water for 1 to 10 minutes. The slides were rinsed in acetone then 1:1 acetone:xylene and finally xylene before mounting a coverslip using XAM

mountant. The slides were viewed with a 10x eyepiece and either 40x or 100x objective, and photographed using Kodak EPY-50 film (50 ASA, tungsten).

### 3.6 Heat Shock of HL-60 cells

Cells in the logarithmic phase of growth were diluted with fresh medium to 1 x $10^5$/ml for assays of differentiation, or were used at higher densities for measurement of heat shock protein synthesis. If the cells had been diluted they were incubated for at least 30 mins at 37°C before heat shock. The flasks were sealed around the neck with adhesive tape and were immersed in a Mercia Scientific or Grant JB1 water bath previously set and equilibrated at the required temperature from 39 to 46°C. A lid was placed on the water bath and the flasks were left for various periods of time. The temperature in the baths fluctuated by no more than ±0.5°C. After the required period of time the flasks were removed from the bath and cooled for 5 mins at room temperature before returning to 37°C. Assays of differentiation were carried out after 4 days. For measurements of protein synthesis the cells were allowed to recover for 2 hours at 37°C after heating before labelling the proteins.

## 3.7 Isolation of cell proteins

### 3.7.1 Labelling of cell proteins with [$^{35}$S]-methionine

Cells were treated with drug or heat shocked as required so that 10ml of cells at >5 x $10^5$/ml were obtained from each treatment. After counting the cell density in each sample, 5 x $10^6$ cells were removed from each flask and washed twice with 5ml of methionine-free RPMI-1640 medium at 37°C. Each cell pellet was then resuspended in 2ml of methionine-free RPMI-1640 supplemented with 10% dialysed foetal calf serum, and to each sample was added 20 or 40uCi of L-[$^{35}$S]-methionine (specific activity 1114Ci/mmol, 1mCi in 0.081ml). The samples were gassed with 5% $CO_2$ in air and incubated in a shaking water bath at 37°C for 2 hours. The samples were washed twice with 5ml of methionine-free RPMI-1640, then once with 5ml of reticulocyte standard buffer (0.01M Tris HCl, pH 7.4, 10mM NaCl, 1.5mM $MgCl_2$) containing 20ug/ml protease inhibitor phenylmethylsulphonylfluoride. Each cell pellet was then resuspended in 1ml of reticulocyte standard buffer with protease inhibitor and sonicated for 3 x 10 secs, cooling on ice in between times. A 10ul aliquot was removed from each sample and mixed with 5ml of Luma Gel scintillation fluid before counting on a Packard Tri Carb 2000CA scintillation counter. The remainder of each sample was stored at -20°C until required.

### 3.7.2 Labelling of cell proteins with [$^{14}$C]-leucine

Cells were labelled with 3uCi of L-[U-$^{14}$C]-leucine (specific activity 342 Ci/mmol, 50uCi in 1ml) per sample in leucine-free RPMI-1640, using the same method as for [$^{35}$-S]-methionine (Section 3.7.1).

## 3.8 Separation and identification of cell proteins

### 3.8.1 Sodium dodecylsulphate polyacrylamide gel electrophoresis

a) Gel preparation

Gel electrophoresis was carried out essentially according to the method of Laemmli (1970).

Polyacrylamide gels were poured between 16 x 18cm glass plates, spaced 1.5mm apart, in a Bio-rad Protean 16cm gel system. 100ml of a separating gel solution (7.5% acrylamide and 0.2% N-N'-methylenebisacrylamide in 0.375M Tris HCl, pH 8.8, containing 0.1% sodium dodecylsulphate) was degassed under a vacuum for at least 10 mins. 250ul of freshly made 10% ammonium persulphate solution were added to the gel solution followed by 250ul of N,N,N',N'-tetramethylethylenediamine (TEMED). After

mixing, the gel was poured between the glass plates to within 4cm from the top. Any bubbles were quickly removed and the separating gel was left to polymerise for at least one hour.

After removing the water from the top of the gel, a plastic comb with 15 teeth was slotted into the gap between the plates, to make wells at least 1cm deep. 100ml of a stacking gel solution (3% acrylamide and 0.08% N,N'-methylenebisacrylamide in 0.125M Tris HCl, pH 6.8 containing 0.1% sodium dodecylsulphate) was degassed and 250ul of ammonium persulphate were added followed by 250ul of TEMED. The gel solution was mixed thoroughly and poured on top of the separating gel filling the gaps around the teeth of the comb until it was level with the top of the glass plates. After removing any air bubbles the gel was left to polymerise for at least one hour.

Gels prepared this way could be stored at 4°C for several days covered with polypropylene film.

b) Preparation of molecular weight markers

1ml of 1X concentrated SDS sample buffer (62.5mM Tris HCl pH 6.8, 2% sodium dodecylsulphate, 10% glycerol, 5% ß-mercaptoethanol) was added to 3mg of high molecular weight markers (Sigma code SDS-6H), and mixed to dissolve the proteins. 20ul aliquots were pipetted into 0.4ml tubes and frozen until required. The kit contained proteins as follows:

| | |
|---|---|
| carbonic anhydrase | Mw 29,000 |
| ovalbumin | 45,000 |
| bovine serum albumin | 66,000 |
| phosphorylase B (sub unit) | 97,400 |
| ß-galactosidase (sub unit) | 116,000 |
| myosin (sub unit) | 205,000 |

c) Loading and running of gels

Radiolabelled cell samples previously prepared and stored frozen were thawed and 100ul from each were mixed with 100ul of 2X concentrated SDS sample buffer (125mM Tris HCl pH 6.8, 4% sodium dodecylsulphate, 20% glycerol, 10% ß-mercaptoethanol with 0.002% bromophenol blue). An aliquot of 20ul of molecular weight markers was also thawed and 20ul of the sample buffer added. The samples were then immersed in a boiling water bath for 2 to 3 mins to denature the proteins.

The 15 wells in the top of the prepared stacking gel were filled with electrolyte buffer (25mM Tris, pH 8.3, 192mM glycine, 0.1% sodium dodecylsulphate) and 60ul of protein sample were loaded under the buffer in each well, with 10ul of molecular weight markers in one well per gel.

The gel apparatus was immersed in a tank with an upper reservoir both filled with electrolyte buffer. Electrophoresis was then carried out at a constant current

of 50 to 70mA until the bromophenol blue dye front was within 2cm of the bottom of the gel (usually 5 hours). The gels were removed from the plates and either fixed for at least 10 mins in 50% trichloroacetic acid (TCA), until the blue dye front turned yellow, or used immediately for blotting onto nitrocellulose.

d) Staining of gels with Coomassie blue

The TCA fixative was poured off the gel and replaced with a solution of 0.25% Coomassie Brilliant Blue R in 45% methanol, 9% acetic acid. The gel was agitated slowly for one hour in the staining solution then the stain was poured off (and salvaged for re-use). The gel was rinsed carefully under the tap to remove excess dye before destaining overnight in several changes of 7% acetic acid, 50% methanol until the background of the gel was almost colourless and bands of protein were visible. The destaining solution was salvaged for re-use by mixing with charcoal and leaving to decolourise for several days before filtration.

Once destained sufficiently the gel could be stored for one or two days in 7% acetic acid before drying it and carrying out autoradiography.

e) Drying and autoradiography of gels

One ml of glycerol was added to the destain or storage solution and the gel was left to soak in this for 15 mins to soften the gel and prevent cracking. The gel was then dried under vacuum onto Whatman 3MM Chr chromatography paper using a Bio-rad model 224 gel slab dryer.

A CEA Singul X-RP non-screen medical X-ray film was exposed to the dried gel in the dark for 2 to 3 days. The autoradiograph was then developed by incubation for 5 min in Kodak D-19 developer followed by Kodafix solution and washed in distilled water for 30 mins.

### 3.8.2 Transfer of proteins to nitrocellulose by Western blotting

a) Western blotting

Proteins separated by SDS polyacrylamide gel electrophoresis were transferred to nitrocellulose paper immediately after electophoresis, essentially according to the method of Towbin et al (1979). The gel was aligned on a sheet of wet 0.45um nitrocellulose (Bio-rad trans-blot transfer membrane) and sandwiched between filter paper and foam rubber in a blotting cassette. The cassette was slotted into a Bio-rad blotting cell containing blotting

transfer buffer (25mM Tris pH 8.3, 192mM glycine, 20% methanol), and electrophoresis was carried out for at least 16 hours at 50V, ensuring that the electrodes were attached so that the negatively charged SDS-proteins migrated from the gel onto the nitrocellulose. Once electrophoresis was complete the nitrocellulose was either stained with amido black or probed with a specific antibody, and/or autoradiographed. The blots could be stored at any stage in the dark at -20°C.

b) Staining of nitrocellulose blots with amido black

A nitrocellulose blot was incubated with gentle agitation for 1 hour with a 0.1% solution of amido black (naphthol blue black) in 45% methanol, 10% acetic acid. It was then destained by incubation for 30 mins or more in 90% methanol, 2% acetic acid, until the background was pale and bands of protein were clearly visible.

c) Autoradiography of nitrocellulose blots

A nitrocellulose blot was dried thoroughly and an X-ray film was exposed as outlined for dried polyacrylamide gels (Section 3.8.1.e).

d) Probing of nitrocellulose blots with anti-hsp70 antibody

Nitrocellulose blots were soaked in 3% bovine serum albumin in Tris buffered saline (TBS: 0.9% NaCl, 10mM Tris HCl pH 7.4) for 2 hours to saturate any additional protein binding sites, then washed three times in TBS before incubating with a 1:200 dilution of rabbit anti-hsp70 antiserum (Lewis and Pelham 1985) (diluted with 3% bovine serum albumin, 20% foetal calf serum in TBS) for 24 hours. The blots were washed three times with TBS, then incubated for 3 hours with 5ug protein A-peroxidase (a 200ul aliquot of stock solution) in 20ml of 3% bovine serum albumin in TBS, and washed again with TBS.

The protein A-peroxidase stock solution was prepared by dissolving a 0.5mg sample in 1ml of TBS, then aliquoting 50ul into small vials. These samples were made up to 1ml with TBS (giving a concentration of 25ug/ml), and stored at -20°C.

After incubation with protein A-peroxidase the blot was incubated for 10 minutes or more with a colour reaction solution, prepared as follows: 200ul of 30% hydrogen peroxide were added to 50ml of 10mM Tris HCl pH 7.4. Several grams of 4-chloro-1-naphthol were dissolved in a small volume of methanol and this was added to the peroxide solution, which turned cloudy. When nitrocellulose blots were incubated with this solution, purple bands appeared where a reaction with the anti-hsp70

antibody/protein A peroxidase complex had occurred. Once the reaction was complete, the blots were washed in water and stored in the dark at -20°C to prevent discolouration.

### 3.8.3 Two-dimensional gel electrophoresis

#### a) Isoelectric focusing gel preparation

Two-dimensional gel electrophoresis was carried out essentially according to the method of O'Farrell (1975).

To 6ml of water were added 8.25g urea, 2ml of a 30% acrylamide stock, 0.5ml of pH 5-8 pharmalyte ampholines and 0.25ml of pH 3-10 pharmalyte ampholines. The mixture was incubated at 37°C to dissolve the urea then degassed under vacuum and warmed slightly again. 0.3ml of warmed Nonidet P-40 was added, followed by 70ul of 10% ammonium persulphate and 10ul of TEMED. The gel solution was quickly sucked up to the 0.4ml level of 1ml glass serological pipettes which had previously been cut off at the 0.1ml mark. The filled pipettes were embedded in plasticine and the gel was left to set for at least one hour.

The tops of the gels were washed with water, then shaken dry and the tubes were then inserted in the isoelectric focusing apparatus. The two reservoirs were clamped together so that the tips of the tube gels were suspended in the anode buffer (0.01M $H_3PO_4$) filling the

bottom tank, ensuring that any air bubbles on the ends of the gels were removed. The top of each gel was covered with 160ul of gel overlay buffer (8M urea), the upper reservoir was filled with cathode buffer (0.2M NaOH) and electrophoresis was carried out at 200V for 1 hour to set up a pH gradient by equilibration of the ampholines down the gels. The cathode buffer and gel overlay buffer were then removed from the top of each gel.

b) Isoelectric focusing of samples

[$^{35}$S]-methionine or [$^{14}$C]-leucine labelled cell samples previously prepared were thawed and 100ul of each sample were added to 233ul of cell lysis buffer (9.5M urea, 2% Nonidet P-40, 1.6% pH 5-7 ampholines, 0.4% pH 3-10 ampholines; 5% ß-mercaptoethanol). The samples were left to stand for 30 mins or more, then 100ul samples were loaded onto the isoelectric focusing gels. At least one gel was left as a blank for determination of the pH gradient after electrophoresis. 120ul of gel overlay buffer were carefully added to the top of each sample, and electrophoresis was carried out for at least 16 hours at 400V.

After electrophoresis each gel was extruded into 5ml of 1X SDS sample buffer (as used for polyacrylamide gel electrophoresis above), and frozen until required. The gels left as blanks were cut into 1cm lengths, each piece being placed in 5ml of distilled water and left for

several hours before measuring the pH of the solutions. This gave a measure of the pH gradient down the gels.

c) The second dimension - SDS polyacrylamide gel electrophoresis

A separating gel (7.5% acrylamide) was prepared as previously described for one- dimensional electrophoresis, and the gel was poured to the top of the glass plates. Once this gel had set, the upper reservoir was attached and a 3% stacking gel was poured through this onto the top of the separating gel. An aliquot of 1% agarose (1g of agarose-M dissolved in 100ml 1X SDS sample buffer, then stored at 4°C) was melted in a boiling water bath, and 1ml was poured into the groove in the top reservoir, on top of the stacking gel. An isoelectric focusing gel previously incubated for several hours in SDS sample buffer was tipped into the groove and embedded in the agarose gel. A control sample was always run in parallel with a treated sample to enable comparison. Electrophoresis was carried out at 60mA until the dye front was 1cm from the bottom of the gel, and the gel was removed, stained, dried and autoradiographed for approximately 10 days.

# CHAPTER FOUR

# RESULTS

## 4.1 Characterization of differentiation induced by N-methylformamide

Incubation of HL-60 cells with N-methylformamide (NMF) was found to induce terminal differentiation, as previously determined by Collins et al (1978) and Langdon and Hickman (1987) (Table 4.1 and Figure 4.1).

The untreated cell population divided approximately 3.5 times over the four day period studied, suggesting a population doubling time of 27 hours; increasing concentrations of NMF inhibited cell proliferation, with total inhibition at 250mM NMF. Viability decreased significantly at concentrations greater than 180mM, with 100% cell kill at 300mM. The extent of cell differentiation induced was concentration-dependent, with less than 10% of cells induced to differentiate at 50mM, increasing to a maximum of >80% of cells differentiated after treatment with 170mM NMF. At concentrations above 170mM, the percentage of cells induced to differentiate decreased, concomitant with the decreased viability.

The optimum concentration of NMF for the induction of differentiation (the concentration at which maximal differentiation was induced whilst the viability remained above 70%) was found to be 170mM. This was in concordance

TABLE 4.1

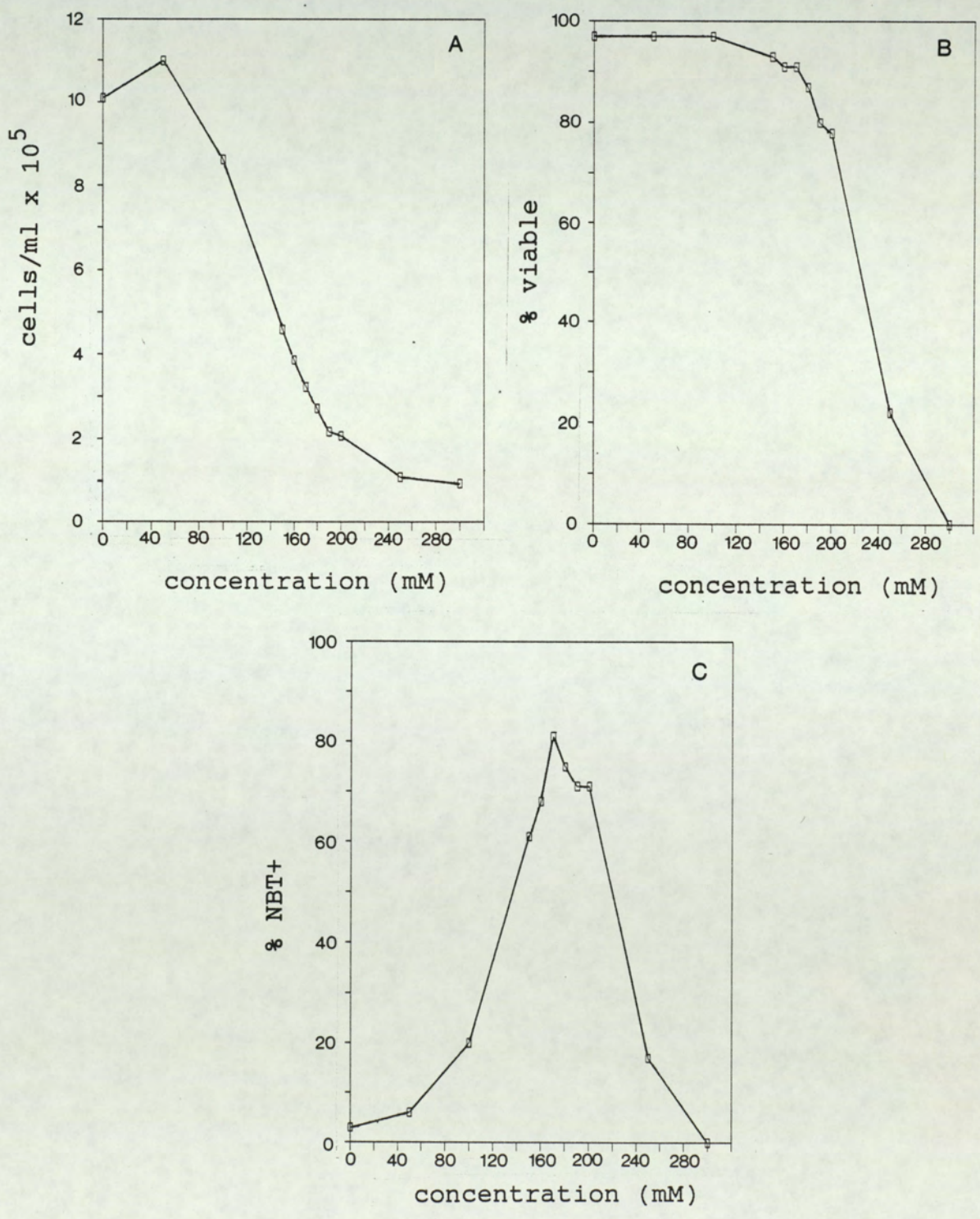
The effect of N-methylformamide on HL-60 cells, measured on day 4

| Conc. (mM) | cells/ml $x10^5$ | % viable | % NBT+ * |
|---|---|---|---|
| 0 | 10.10 ± 0.28 | 96.60 ± 1.68 | 2.93 ± 1.62 |
| 50 | 11.00 ± 0.32 | 96.71 ± 1.60 | 6.30 ± 3.46 |
| 100 | 8.62 ± 1.73 | 97.32 ± 1.29 | 19.98 ± 10.69 |
| 150 | 4.60 ± 1.18 | 93.13 ± 2.81 | 60.65 ± 12.15 |
| 160 | 3.87 ± 1.07 | 91.05 ± 2.73 | 68.08 ± 15.92 |
| 170 | 3.23 ± 0.75 | 90.91 ± 3.26 | 81.49 ± 12.19 |
| 180 | 2.73 ± 0.75 | 86.58 ± 3.90 | 74.77 ± 10.18 |
| 190 | 2.17 ± 0.43 | 79.69 ± 5.42 | 71.20 ± 8.21 |
| 200 | 2.07 ± 0.33 | 77.59 ± 8.29 | 71.01 ± 15.46 |
| 250 | 1.09 ± 0.32 | 21.72 ± 20.56 | 17.13 ± 14.71 |
| 300 | 9.40 ± 0.57 | 0 ± 0 | 0 ± 0 |

* All points represent the mean ± S.D. of six experiments.

Figure 4.1 The induction of differentiation of HL-60 cells treated with NMF for 4 days.

Cells were incubated with different NMF concentrations and cell growth (A), viability (B) and NBT reduction (C) were determined on day 4. Each point represents the average of 6 experiments.



with the value determined by Langdon and Hickman (1987), and the results showed, as expected, that optimal differentiation was induced by a concentration which was marginally below that which was cytotoxic.

In further experiments, this optimum concentration of NMF was used to determine the temporal relationship between the addition of inducer and the expression of markers of differentiation (Table 4.2, Figure 4.2). NMF had very little effect on HL-60 cell growth after 1 day, but from day 2 cell proliferation was inhibited so that 2.25 doublings of the population occurred by day 4, compared to 3.5 in the untreated population. The cell viability decreased to 83% after 4 days; the number of cells capable of reducing NBT increased to 86% on day 4 and the number of cells with the ability to ingest yeast by phagocytosis increased to 76% after 4 days (Figure 4.3).

Monocytic non-specific esterase activity was determined in the cells treated with NMF; it was undetectable in >90% of the cells over the four day period studied. This confirmed that the differentiated cells were not monocytic. Their granulocytic appearance was corroborated by staining with Wolbach Giemsa to reveal the nuclear morphology. Many of the cells had reniform nuclei, characteristic of metamyeloctes, or the banded nuclei of more mature granulocytes, which were not seen in

TABLE 4.2

The effect of 170mM N-methylformamide on HL-60 cells over a 4 day period

170mM NMF

| Day | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| cell/ml x $10^5$ | 1.5 ± 0.3* | 2.1 ± 0.4 | 2.3 ± 0.4 | 2.6 ± 0.3 |
| %viable | 88.4 ± 7.6 | 88.7 ± 5.4 | 84.4 ± 8.4 | 86.3 ± 8.8 |
| %NBT+ | 2.9 ± 2.2 | 49.7 ± 20.8 | 79.0 ± 9.2 | 82.5 ± 10.4 |
| %phago | 8.1 ± 7.0 | 59.9 ± 15.6 | 70.4 ± 10.1 | 75.6 ± 11.8 |
| %NSE+ | 0 ± 0 | 0.2 ± 0.3 | 6.4 ± 8.6 | 0.3 ± 0.2 |
| %Morph | 1.9 | 40.9 | 59.1 | 72.3 |

Control

| Day | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| cell/ml x $10^5$ | 1.4 ± 0.3 | 2.9 ± 0.6 | 5.9 ± 2.0 | 11.5 ± 2.0 |
| %viable | 90.4 ± 6.7 | 91.2 ± 6.5 | 94.6 ± 4.3 | 96.1 ± 2.0 |
| %NBT+ | 3.9 ± 3.1 | 3.0 ± 2.3 | 1.8 ± 0.7 | 1.5 ± 0.8 |
| %phago | 1.2 ± 0.1 | 0.8 ± 0.9 | 0.9 ± 0.6 | 1.6 ± 1.1 |
| %NSE+ | 0.4 ± 0.1 | 0.3 ± 0.5 | 0.2 ± 0.3 | 0.4 ± 0.4 |
| %morph | 1.0 | 0 | 0.9 | 1.3 |

* All figures except morphology represent mean ± S.D. for 6 experiments.

% phago = cells able to phagocytose yeast particles.

% morph = cells with a nuclear morphology characteristic of myelocytes, metamyelocytes, or banded granulocytes.

Figure 4.2 The induction of differentiation of HL-60 cells treated with 170mM NMF over a 4 day period.

Cells were incubated with (□) or without (+) 170mM NMF and cell growth (A) and viability (B) were assessed daily. Differentiation was also assessed (C) by the measurement of NBT reduction ( ■ ), phagocytosis (+), non-specific esterase activity (◆) and nuclear morphology (Δ) in the cells treated with NMF. Each point represents the average of 6 experiments.

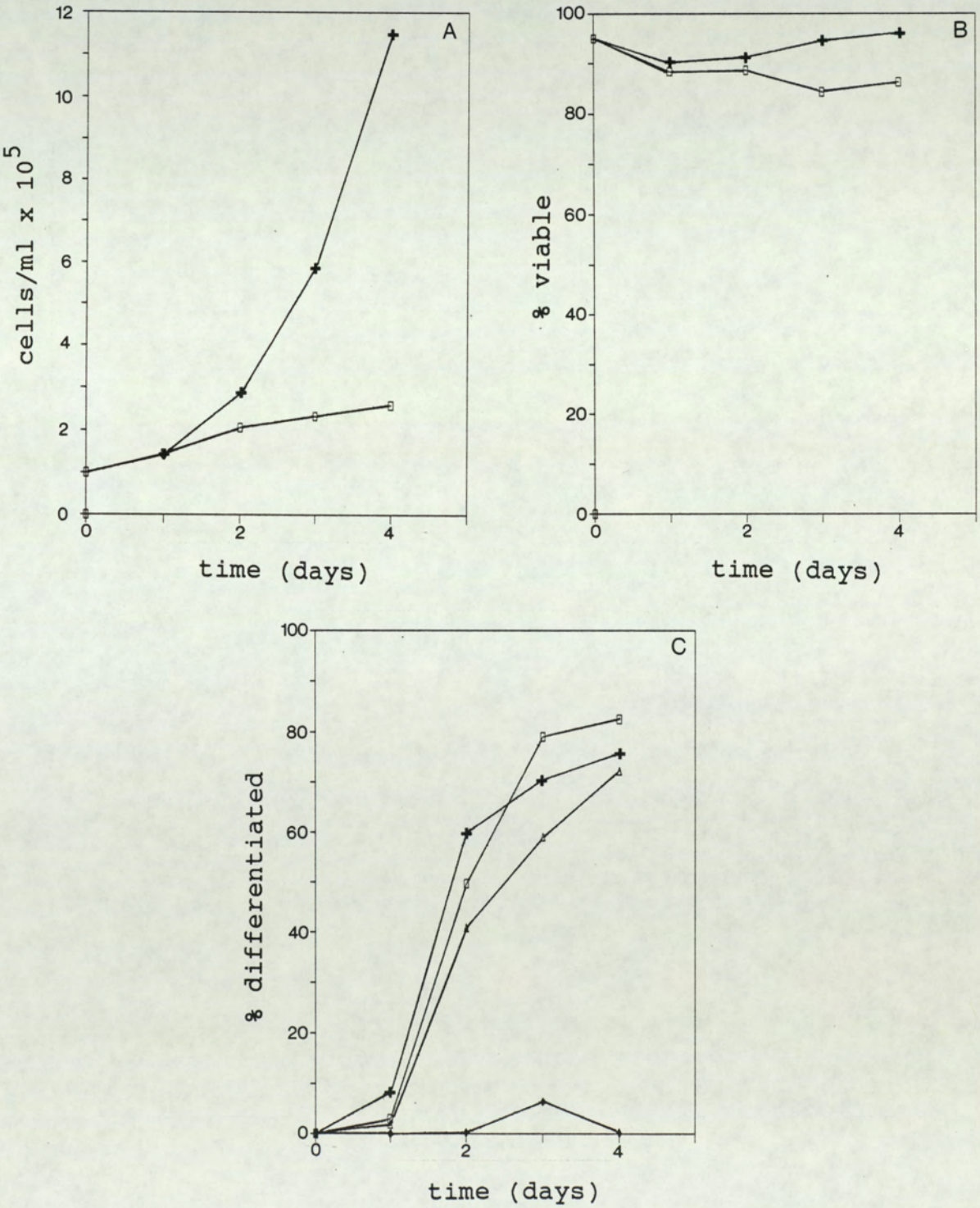
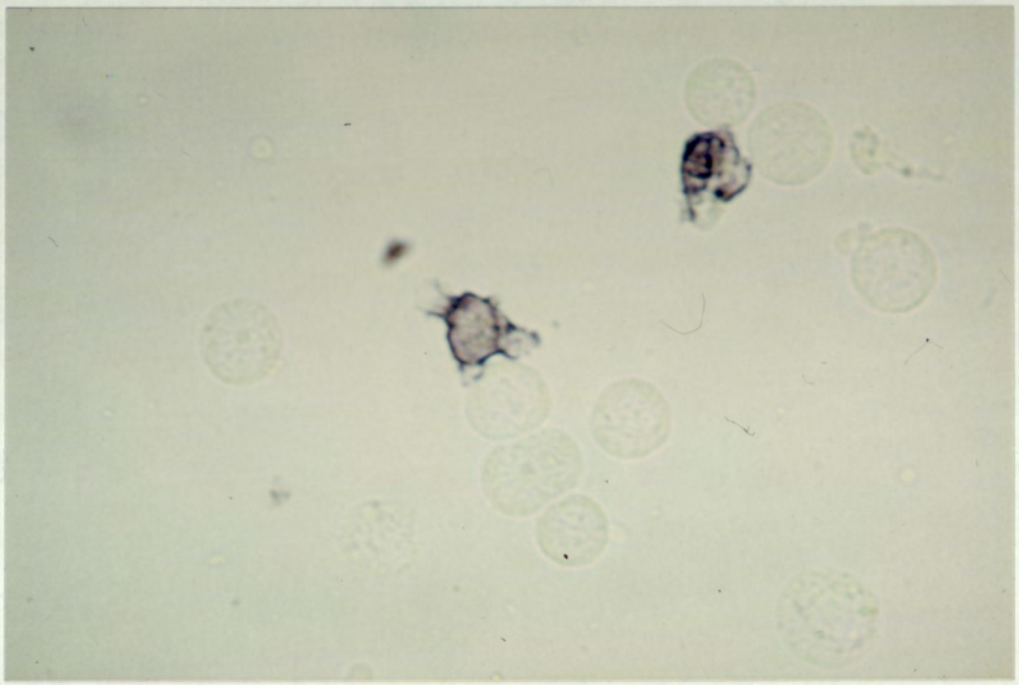


Figure 4.3 Expression of the markers of differentiation in HL-60 cells.

A/ Nitrobluetetrazolium reduction; positive cells stained purple. B/ Ability to phagocytose particles; positive cells contain red dyed yeast.

A



B

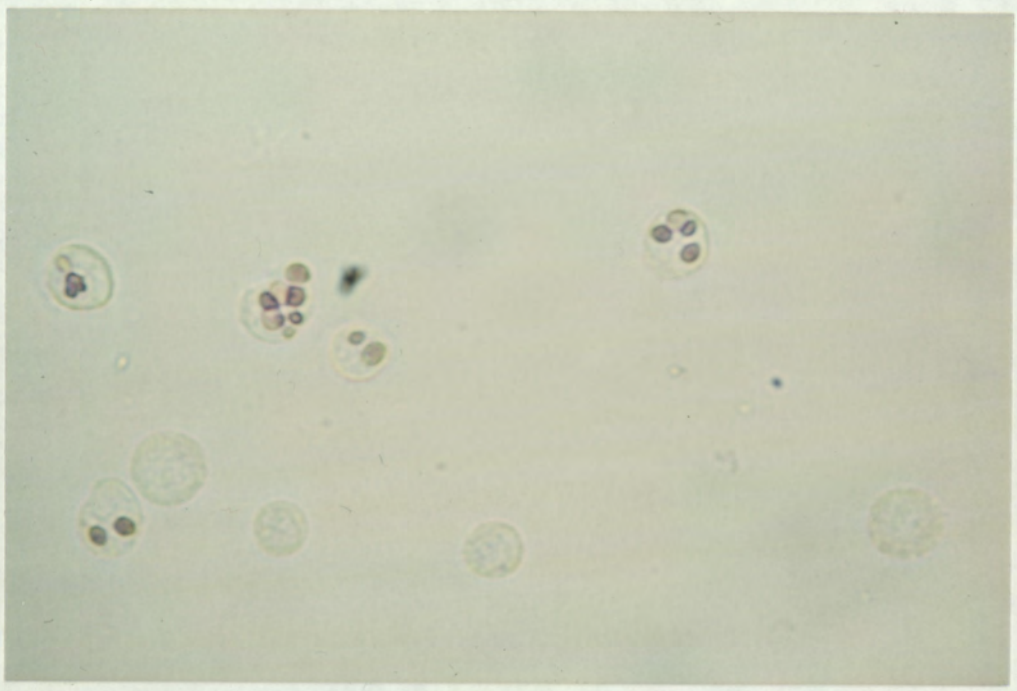
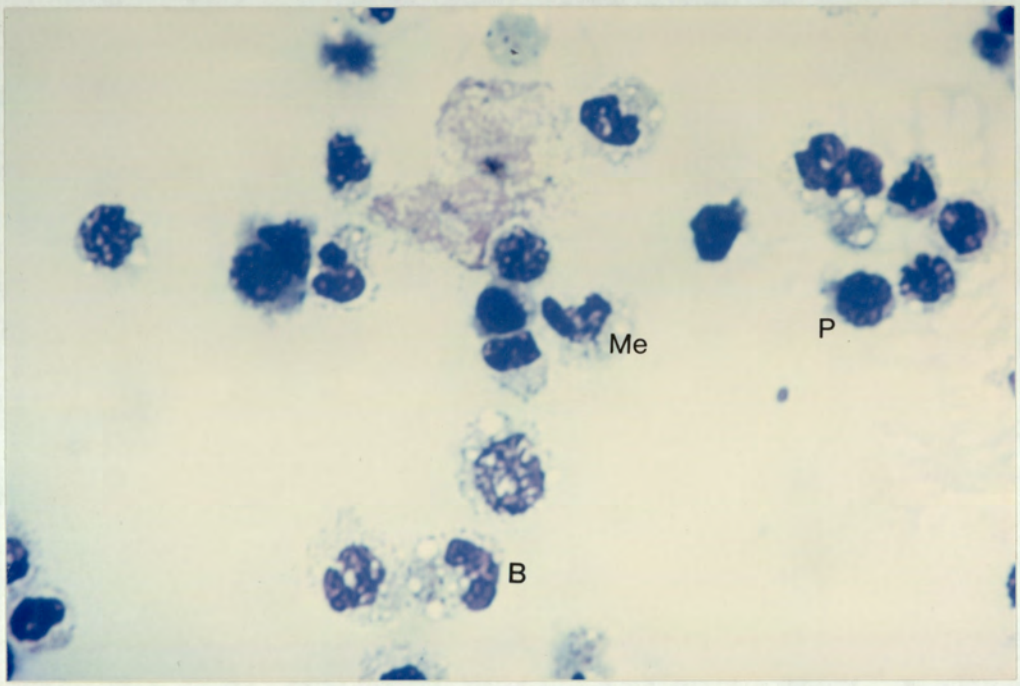
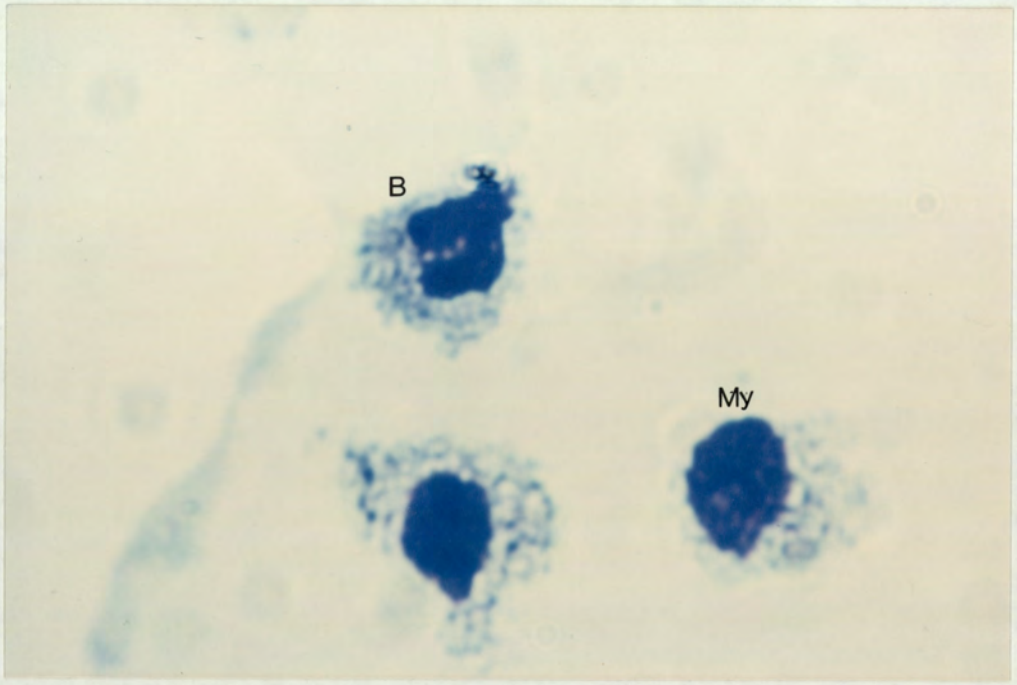


Figure 4.4 The nuclear morphology of HL-60 cells treated with 170mM NMF for four days.

Cells were stained with Wolbach Giemsa. Promyelocyte (P), myelocyte (My), metamyelocyte (Me), banded granulocyte (B).



the untreated promyelocytic cell sample (Figure 4.4). On day 4, as many as 72% of the cells had nuclei which were visibly more mature than those of the promyelocytes. There were very few cells visible with truly lobulated nuclei even after four days of treatment, but it is probable that these most mature cells require rather longer than four days to develop from promyelocytes.

## 4.2 Commitment of cells to differentiation with N-methylformamide

The above experiments showed that at least two days of incubation with NMF were required to induce detectable increases in the various markers of cell differentiation. It was thought possible, however, that commitment to differentiation occurred more rapidly than the expression of these markers. Experiments were therefore performed to determine the length of incubation with 170mM NMF required to commit the cells to differentiate even in the subsequent absence of inducer (Table 4.3 and Figure 4.5). The extent of differentiation was assessed 72 hours after the start of the experiment, and it was shown that no significant commitment occurred during the first 12 hours of incubation with NMF. From 24 hours onward there was an almost linear increase in the percentage of cells committed to differentiate, reaching a maximum after 56 hours of 79%, reminiscent of the phenomenon described by

TABLE 4.3

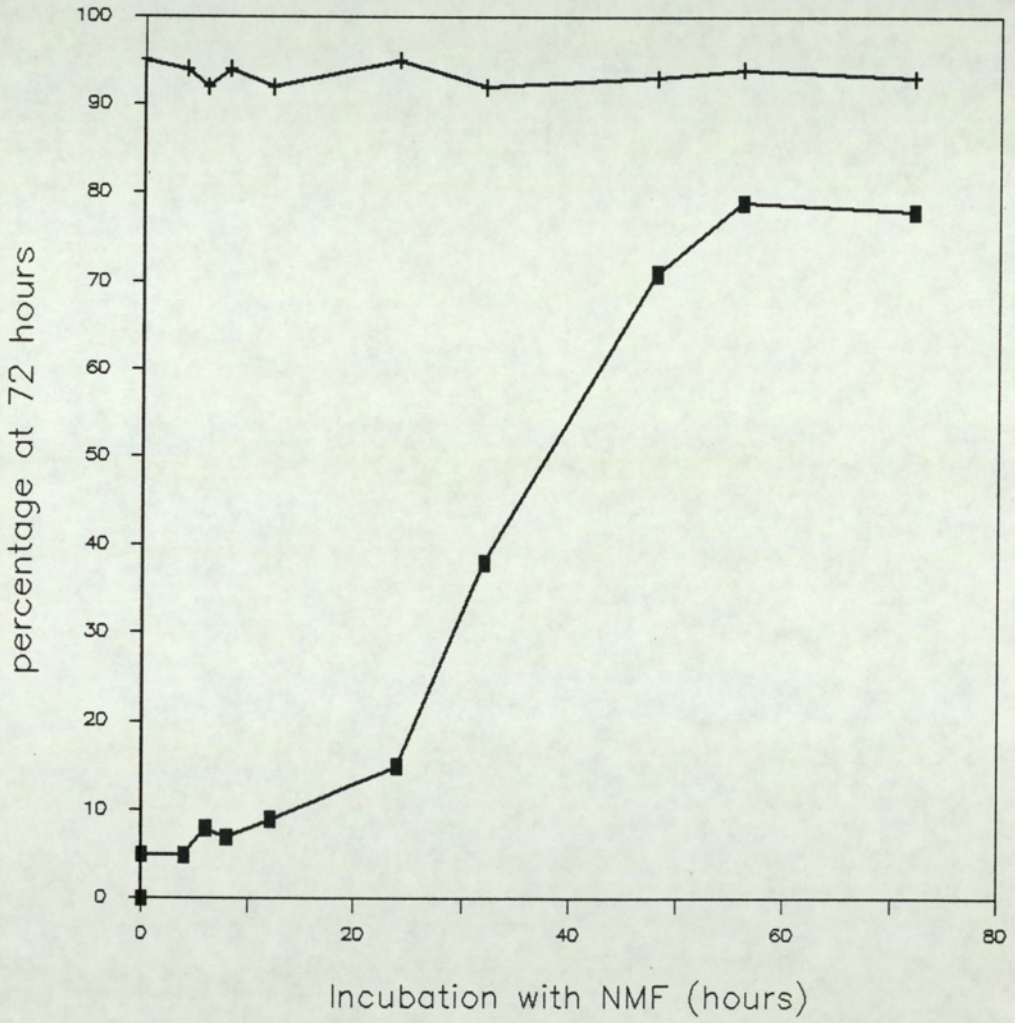
Commitment to differentiation with 170mM N-methylformamide

| Incubation with NMF before resuspension (hours) | % viable after 72 hours | % NBT+ after 72 hours |
|---|---|---|
| 0 | 94.90 ± 1.90 | 4.58 ± 2.37* |
| 4 | 94.20 ± 2.15 | 5.14 ± 4.83 |
| 6 | 91.90 ± 4.67 | 7.75 ± 0.07 |
| 8 | 94.15 ± 3.46 | 6.85 ± 1.06 |
| 12 | 91.55 ± 2.47 | 8.90 ± 0.42 |
| 24 | 95.17 ± 0.41 | 15.34 ± 5.46 |
| 32 | 92.16 ± 1.65 | 37.89 ± 9.37 |
| 48 | 93.41 ± 3.05 | 71.43 ± 10.26 |
| 56 | 93.98 ± 1.53 | 78.70 ± 8.79 |
| 72 | 93.22 ± 2.30 | 77.73 ± 11.51 |

* All points represent the mean ± S.D. of 3 experiments

Figure 4.5 Commitment of HL-60 cells to differentiation with 170mM NMF.

Cell viability (+) and differentiation (■) were assessed 72 hours after the start of the experiment. Each point represents the average of 3 experiments.

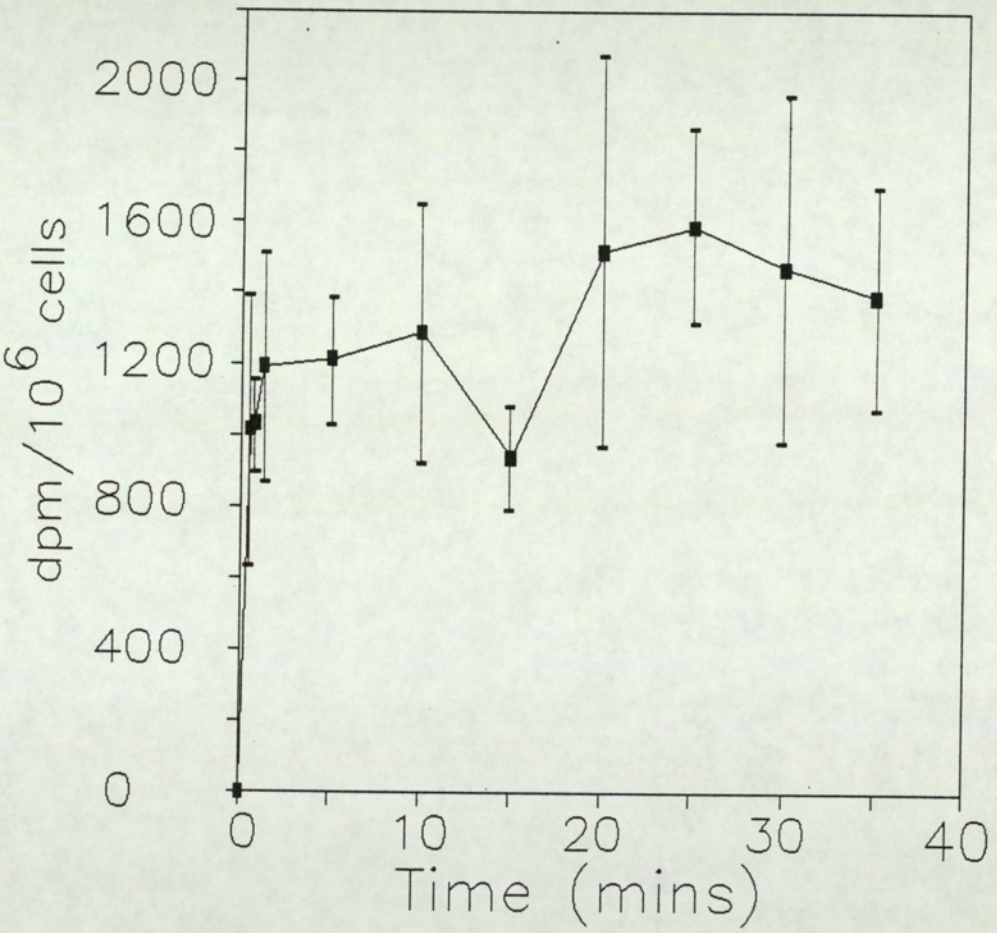


Von Melchner and Höffken (1985): "the probability of each cell to differentiate .... increases with each generation in the presence of a chemical inducer". The minimum commitment period of 24 hours was similar to that previously defined for retinoic acid or DMSO (Fibach et al 1982a and 1982b, Tarella et al 1982, Yen et al 1984 and Yen 1985). The apparent "lag phase" of 24 hours before any significant commitment occurred with NMF was probably not due to a factor as simple as transport of the inducer into the cells. NMF and other polar solvents were thought to enter the cells rapidly after addition to the medium, and using [$^{14}$C]-NMF to measure intracellular concentrations of NMF this was indeed found to be the case. Within 2 minutes the radiolabelled NMF had entered the cells and reached a plateau level at approximately 1400 dpm (Figure 4.6).

The NMF concentration in the medium was 170 umoles/ml and 1 uCi/ml, so it was calculated that 1400 dpm represented $1.072 \times 10^{-7}$ moles of NMF per $10^6$ cells. An average volume of $9.2 \times 10^{-13}$ litres per HL-60 cell was determined using a Coulter electronic cell counter, and the intracellular concentration of NMF was thus calculated to be 117mM; 70% of the NMF concentration in the extracellular medium. NMF therefore underwent rapid diffusion into the cells until an equilibrium was reached. Because of the rapid diffusion, it was unlikely that the 24 hour lag period before commitment represented the time

Figure 4.6 Uptake of $^{14}$C-NMF into HL-60 cells.

Each point represents the average of triplicate points from three experiments.



required to accumulate intracellular levels of NMF to a threshold above which differentiation was initiated.

The 24 hour lag period was approximately the same length as the time required for the cells to undergo one cell cycle, and it is possible that commitment only occurred after the cells had undergone at least one full cell division cycle in the presence of inducer. This would suggest a "restriction point" in one phase of the cell cycle, at which commitment to differentiation can occur (discussed in section 5.6). Experiments in section 4.3 were performed to determine whether the induction of differentiation by NMF was cell cycle-dependent.

At each time point during the assays for commitment to differentiation, samples were prepared for cell cycle analysis by FACS. Figure 4.7 shows the cell size during NMF treatment: the average cell size in the population decreased from 24 hours onward, reaching a minimum after 56 hours at approximately 80% of the size of the untreated cells. This is not unexpected as normal granulocytes are usually 12 - 15um in diameter, compared to 15 - 24um for promyelocytes (Laszlo and Rundles 1977).

Significant changes in the cell cycle distribution were also observed during commitment of the cells to differentiate. Table 4.4 shows the percentages of cells in each phase of the cell cycle, calculated from the area

Figure 4.7 Commitment of HL-60 cells to differentiation with 170mM NMF. Histograms of cell size, determined by flow cytometry.

Samples were taken after incubation with NMF for 0, 4, 24, 32, 48, 56 and 72 hours.

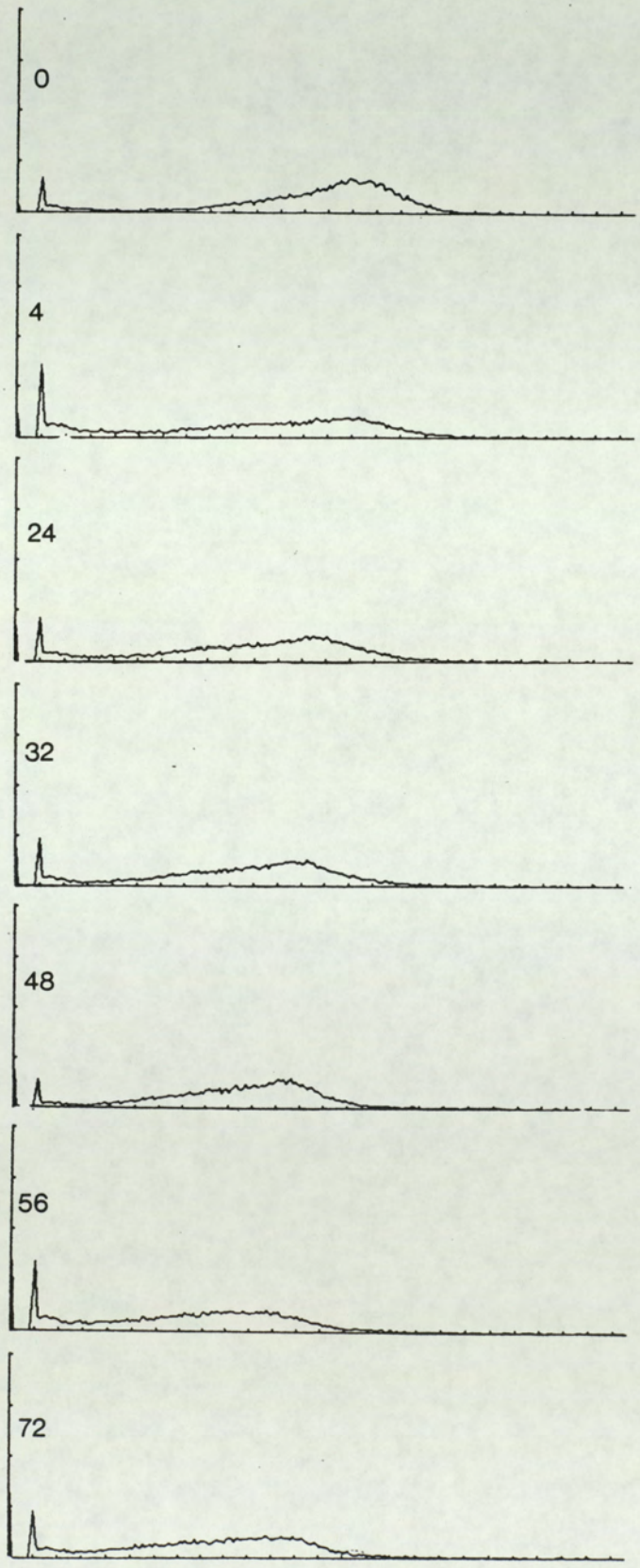


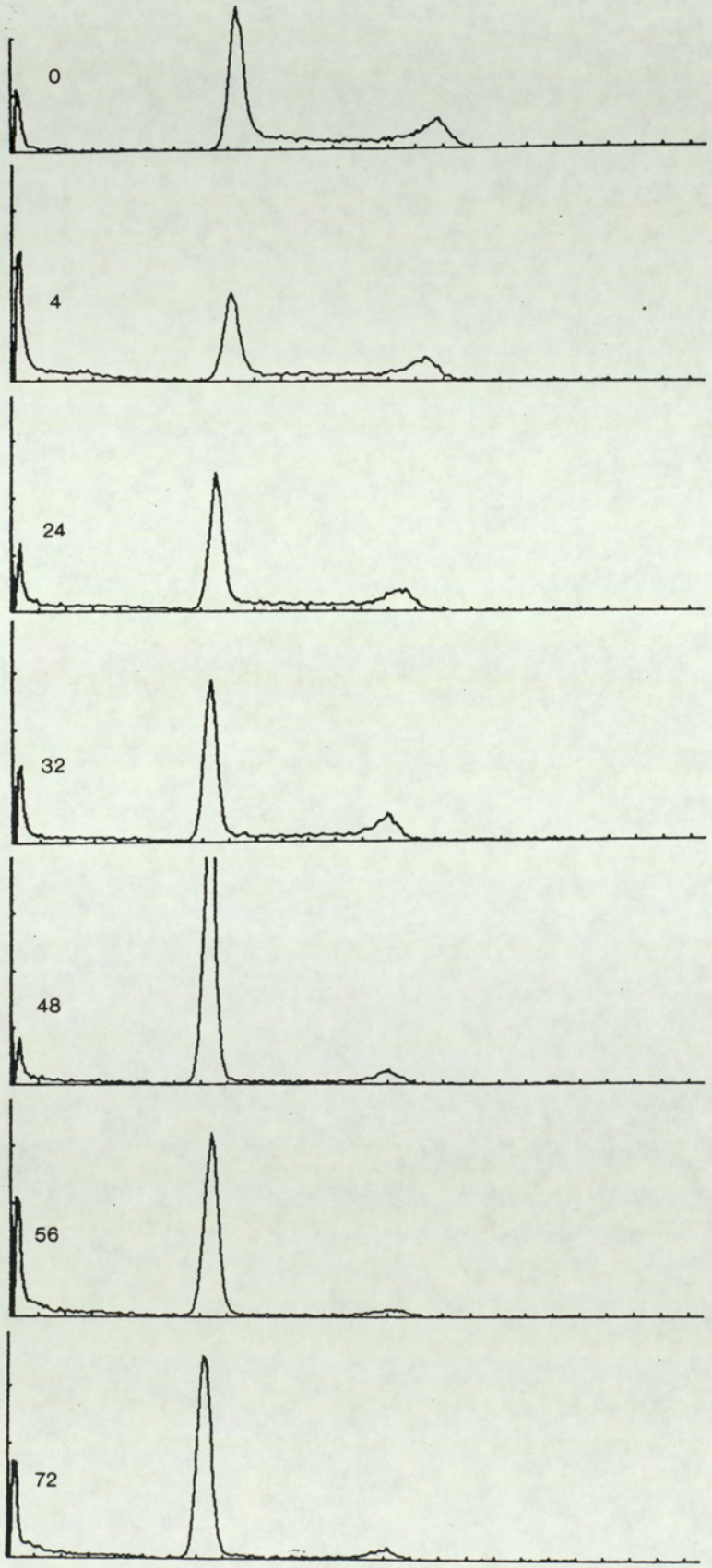
TABLE 4.4

The percentage of cells in each phase of the cell cycle during commitment to differentiation with 170mM N-methylformamide

| Time (hours) | % of cells in $G_1$ | % of cells in S | % of cells in $G_2$ + M |
|---|---|---|---|
| 0 | 54 | 26 | 20 |
| 4 | 49 | 26 | 25 |
| 24 | 57 | 23 | 20 |
| 32 | 64 | 17 | 20 |
| 48 | 89 | 3 | 8 |
| 56 | 89 | 3 | 8 |
| 72 | 91 | 1 | 8 |

Figure 4.8 Commitment of HL-60 cells to differentiation with 170mM NMF. Histograms of cell DNA content, determined by flow cytometry.

Samples were taken after incubation with NMF for 0, 4, 24, 32, 48, 56 and 72 hours.



under the peaks of the FACS histograms presented in Figure 4.8. The proportion of cells in S phase decreased from 24 hours onward, resulting in less than 1% of cells residing in S phase after 72 hours. The proportion of cells in $G_2$+M also decreased from 32 hours onward, but there remained 8% of cells in these phases even after 72 hours of incubation with NMF. In accordance with these decreases in cells in S and $G_2$+M, the proportion of cells in $G_1$ (or $G_0$) increased from 24 hours onward. This "$G_1$ block" during terminal differentiation was expected as most cells in the body which are differentiated terminally reside in $G_1$, with the exception of a small percentage of cells in tissues such as mouse ear epidermis, which are arrested in $G_2$ (Prescott 1976, Schwartz et al 1984).

There was a close temporal relationship between the onset and progression of the $G_1$ block and the commitment of the cells to differentiate, which suggested that inhibition of proliferative potential of the cells was an important, and perhaps even causative, event in the commitment process. In view of the evidence that many agents induced HL-60 cell differentiation at concentrations marginally below those which were cytotoxic, it was proposed that inhibition of the proliferative potential of the cells by a variety of means was sufficient to induce differentiation of HL-60 cells. This hypothesis will be discussed in more detail in section 5.2.

## 4.3 The induction of differentiation of cells in different proliferative states

### 4.3.1 The induction of differentiation of cells deprived of serum

HL-60 cells were deprived of serum and 170mM NMF was added to half of the sample. Table 4.5 and Figure 4.9 demonstrate that cell proliferation was inhibited in the cells deprived of serum, with only 0.5 population doublings over the four day period of study. Viability remained at >70% and surprisingly, in the experiment shown, 9.4% of the cells differentiated in the absence of NMF. Four experiments were performed at different initial cell densities, and the average values for viability and differentiation are shown at the bottom of Table 4.5. It is clear that a small but significant percentage of the cells were always induced to differentiate by deprivation of serum in the absence of any other inducer (average 14.72% on day two). It was suggested that the increased percentage of differentiated cells was due to selective killing of the non-differentiated cells, leaving a population enriched in the small numbers of differentiated cells present at the start of the experiment. However, in the experiment shown in Figure 4.9, the absolute number of differentiated cells increased from $3.60 \times 10^4$ per ml at the start to $1.61 \times 10^5$ by day two, and to $1.79 \times 10^5$ on

TABLE 4.5

Deprivation of serum from HL-60 cells, in the presence or absence of 170mM N-methylformamide

Figures in A represent a single experiment; in B, the mean ± S.D. of 4 experiments at different initial cell densities.

A.

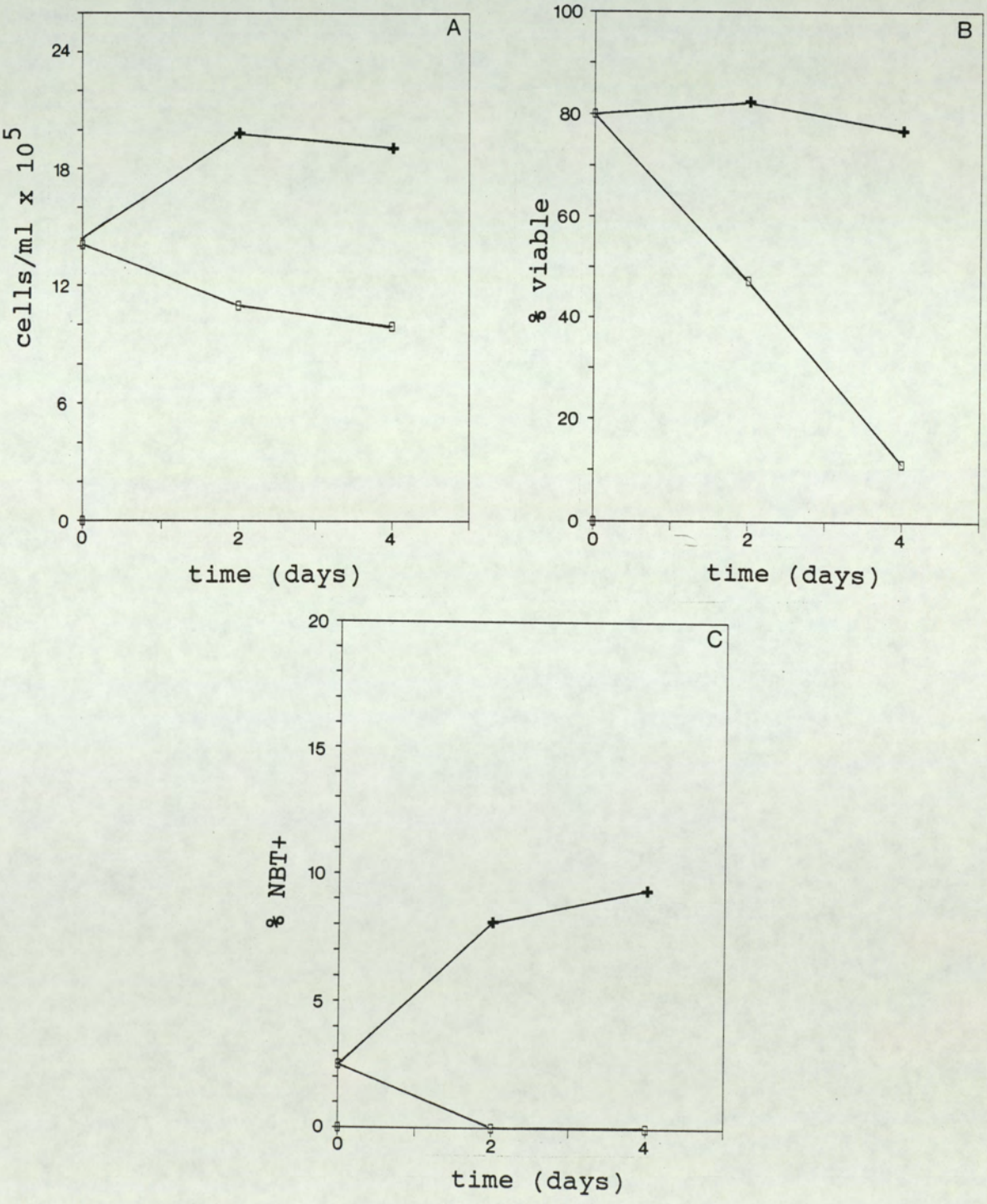
| Time (days) | | 0 | 2 | 4 |
|---|---|---|---|---|
| -NMF | cells/ml $x10^5$ | 14.40 | 19.80 | 19.10 |
| | % viable | 80.00 | 82.18 | 76.83 |
| | % NBT+ | 2.50 | 8.11 | 9.36 |
| +NMF | cells/ml $x\ 10^5$ | 14.10 | 11.00 | 9.96 |
| | % viable | 80.00 | 47.25 | 11.20 |
| | % NBT+ | 2.50 | 0.00 | 0.00 |

B.

| | Day 2 | | Day 4 | |
|---|---|---|---|---|
| | % viable | % NBT+ | % viable | % NBT+ |
| -NMF | 86.0 ± 3.6 | 14.7 ± 5.8 | 70.5 ± 8.9 | 11.4 ± 2.9 |
| +NMF | 60.2 ± 13.7 | 8.1 ± 7.1 | 8.1 ± 4.4 | 0.0 ± 0.0 |

Figure 4.9 Deprivation of serum from HL-60 cells, in the presence or absence of 170mM NMF.

Cell growth (A), viability (B) and NBT reduction (C) were assessed on days 2 and 4 after deprivation of serum in the presence (□) or absence (+) of 170mM NMF.



day four. This shows that deprivation of serum actually induced some cells to differentiate, and this reinforced the idea that simple inhibition of the proliferative potential of the cells could induce differentiation.

Removal of serum from the HL-60 cells enforced a major inhibition of proliferative potential; the increase in cell number from 14.1 x $10^5$/ml to 19.8 x $10^5$/ml (shown in figure 4.9) represented a doubling of only 19% of the cell population. This may be important in explaining why only a small percentage of cells were induced to differentiate after deprivation of serum (discussed in section 5.2).

Serum deprivation was originally performed as an alternative to using a thymidine block - to induce $G_1$ arrest in the cells, so that it could then be determined whether the cells were able to differentiate in response to NMF even when proliferation was prevented. Unfortunately, addition of NMF to the cells deprived of serum caused marked cytotoxicity even though neither deprivation of serum alone nor NMF alone reduced viability to less than 70%. By day four, cells deprived of serum and NMF-treated were only 11% viable, and as a result, no induction of differentiation was observed (Figure 4.9). This cytotoxicity was easily observed in the cell cycle analyses shown in Figures 4.10 and 4.11 as a dramatic increase in the amount of debris in NMF-treated cells

Figure 4.10 The effect of deprivation of serum on the cell cycle. Histograms of DNA content, determined by flow cytometry.

Cells were deprived of serum for 0, 1, 2 or 3 days.

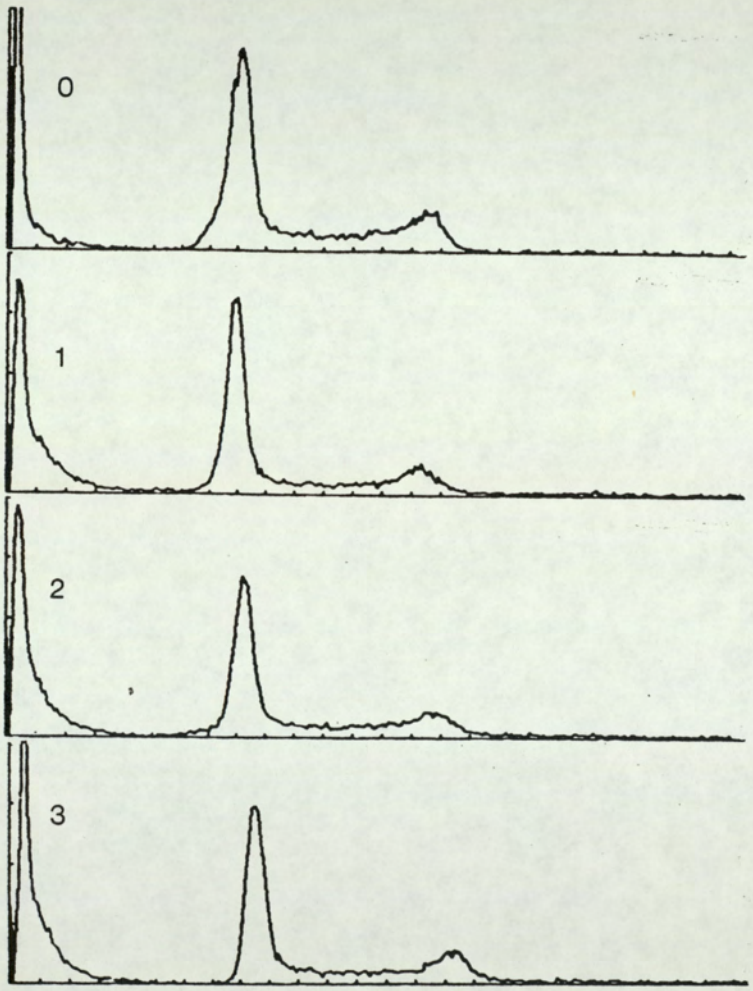
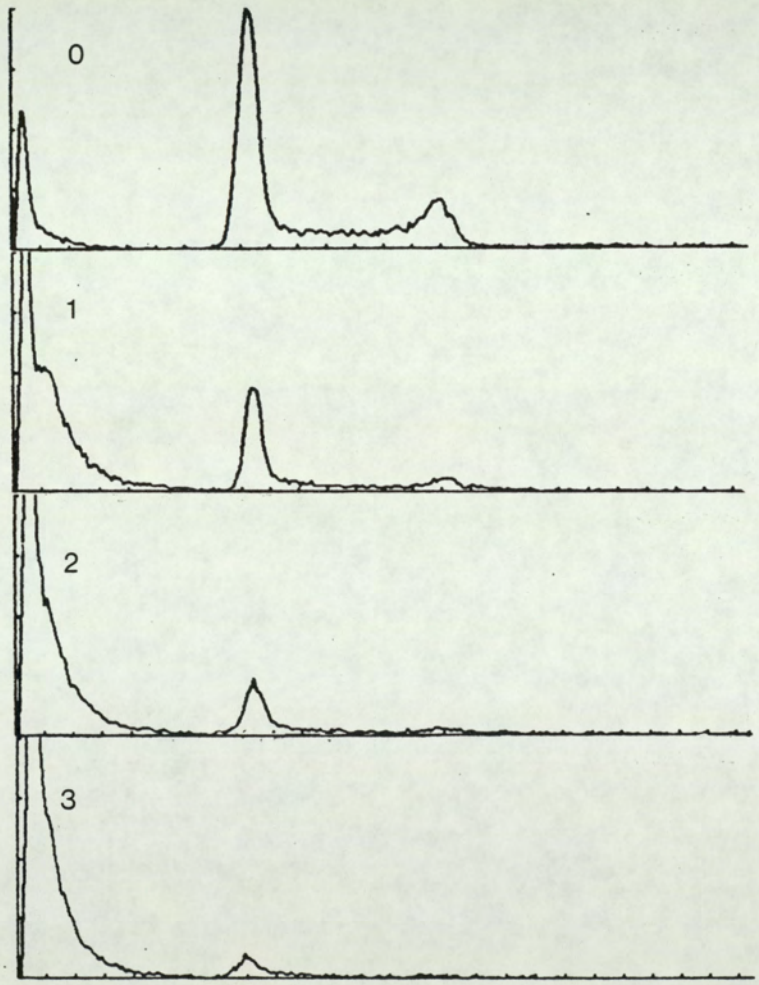


Figure 4.11 The effect of deprivation of serum and treatment with 170mM NMF on the cell cycle. Histograms of DNA content, determined by flow cytometry.

Cells were deprived of serum in the presence of 170mM NMF for 0, 1, 2 or 3 days.



which were deprived of serum.

In the experiment shown, cells only treated by deprivation of serum did not undergo any major changes in cell cycle distribution, although cell proliferation was blocked. The cells in this experiment had been grown to a plateau at high cell density prior to being deprived of serum. On other occasions, when cells were deprived of serum from populations growing logarithmically, there was a reduction of the numbers of cells in S and $G_2$+M and an increase in $G_1$. This difference probably relates to a phenomenon noted by Yen et al (1977) - that cells grown to plateau and inhibited due to high cell density did not undergo $G_1$ arrest; the cell cycle distribution remained similar to that in log phase cells, despite the fact that no increase in cell number occurred. On the other hand, cells deprived of nutrients such as serum at lower density, accumulated in $G_1$. Yen and co-workers suggested that the cells apparently blocked by high density were proliferating continuously but that cell death balanced this, leaving a constant cell number. However, no non-viable cells were detectable so the cells must have disintegrated very rapidly after death. There is an alternative hypothesis to the theory of continued proliferation at plateau balanced by rapid cell death; the cells at the plateau phase of growth might arrest at all phases of the cell cycle as they approached the plateau, rather than progressing to $G_1$ first.

4.3.2 The induction of differentiation of cells at the plateau phase of cell growth

Deprivation of serum prevented proliferation of the cells, but under these conditions viability was not maintained when NMF was added. This technique was therefore not suitable for the determination of whether the induction of differentiation by NMF could occur in the absence of cell proliferation (Section 4.3.1). Growth of the cells to the plateau phase of cell growth, where they had reached saturation density, was found to be a more suitable technique.

Three experiments were performed, all of which demonstrated the same results, but as the plateau was attained at different cell densities in each case the results could not be averaged. Table 4.6 and Figure 4.12 show the results from a typical experiment and demonstrate that cells maintained at the plateau in the old, exhausted medium did not undergo any cell division, and although viability decreased to 26% on day four there was no induction of differentiation. Addition of 170mM NMF to a sample of the same cells had no effect on the cell proliferation or viability, and no differentiation was induced.

In contrast, when the cells were resuspended at the same high initial density in fresh medium, the cells with

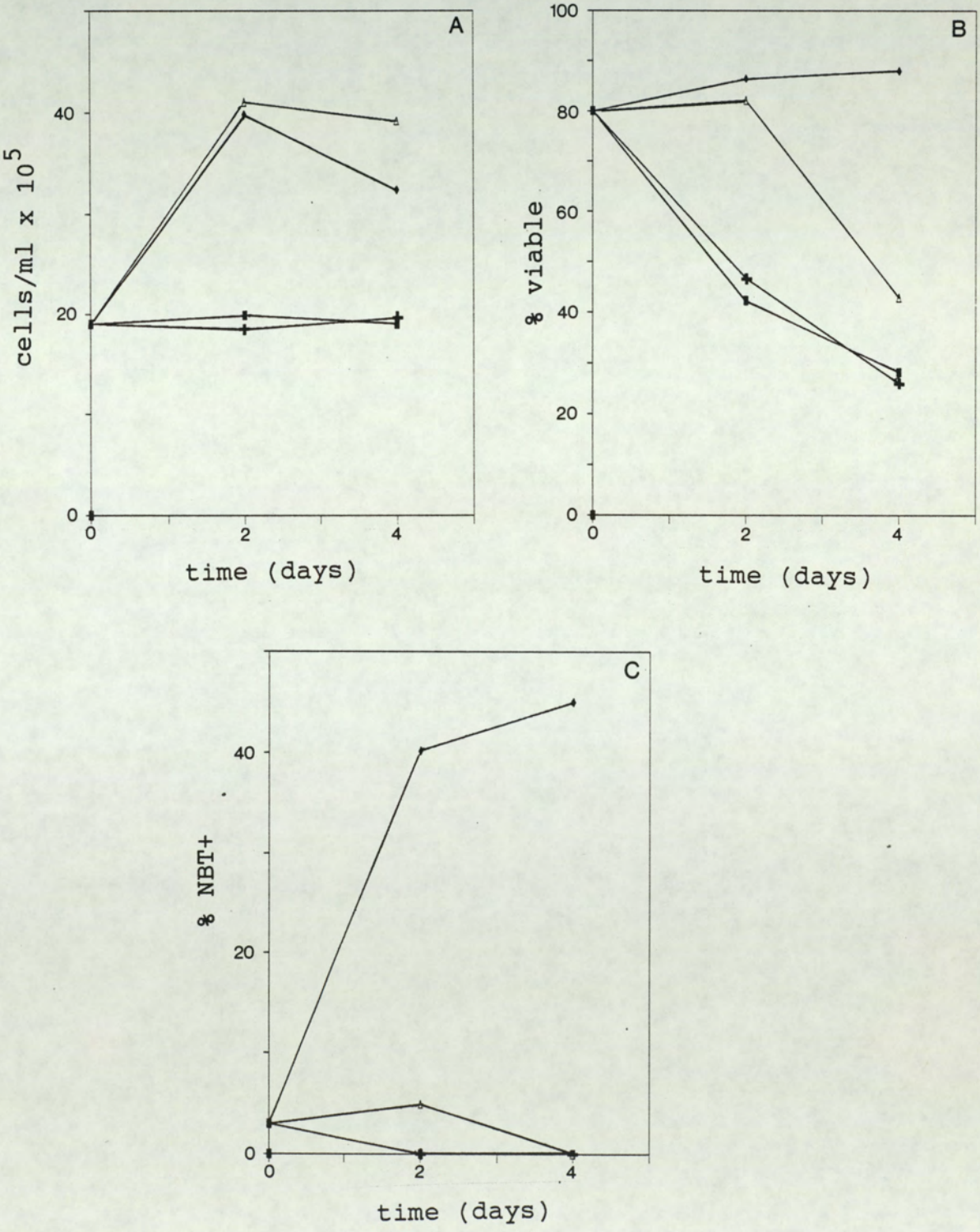
TABLE 4.6

HL-60 cells at saturation density treated with or without 170mM N-methylformamide

| Treatment | | Old medium | Old medium + NMF |
|---|---|---|---|
| Day 0 | cells/ml x $10^5$ | 19.00 | 19.00 |
| | % viable | 80.00 | 80.00 |
| | % NBT+ | 3.00 | 3.00 |
| Day 2 | cells/ml x $10^5$ | 18.50 | 19.90 |
| | % viable | 46.67 | 42.29 |
| | % NBT+ | 0.00 | 0.00 |
| Day 4 | cells/ml x $10^5$ | 19.60 | 19.00 |
| | % viable | 26.05 | 28.26 |
| | % NBT+ | 0.00 | 0.00 |
| Treatment | | Fresh medium | Fresh medium + NMF |
| Day 0 | cells/ml x $10^5$ | 19.00 | 19.00 |
| | % viable | 80.00 | 80.00 |
| | % NBT+ | 3.00 | 3.00 |
| Day 2 | cells/ml x $10^5$ | 41.10 | 39.80 |
| | % viable | 82.20 | 86.49 |
| | % NBT+ | 4.91 | 40.17 |
| Day 4 | cells/ml x $10^5$ | 39.20 | 32.40 |
| | % viable | 42.94 | 88.00 |
| | % NBT+ | 0.00 | 44.98 |

Figure 4.12 The effect of 170mM NMF on HL-60 cells at a plateau phase of growth.

Cell growth (A), viability (B) and NBT reduction (C) were assessed on days 2 and 4 after the cells had reached saturation density at a plateau phase of cell growth. The cells were maintained in the old medium with (■) or without (+) 170mM NMF, or were resuspended in fresh medium with (◆) or without (△) 170mM NMF.



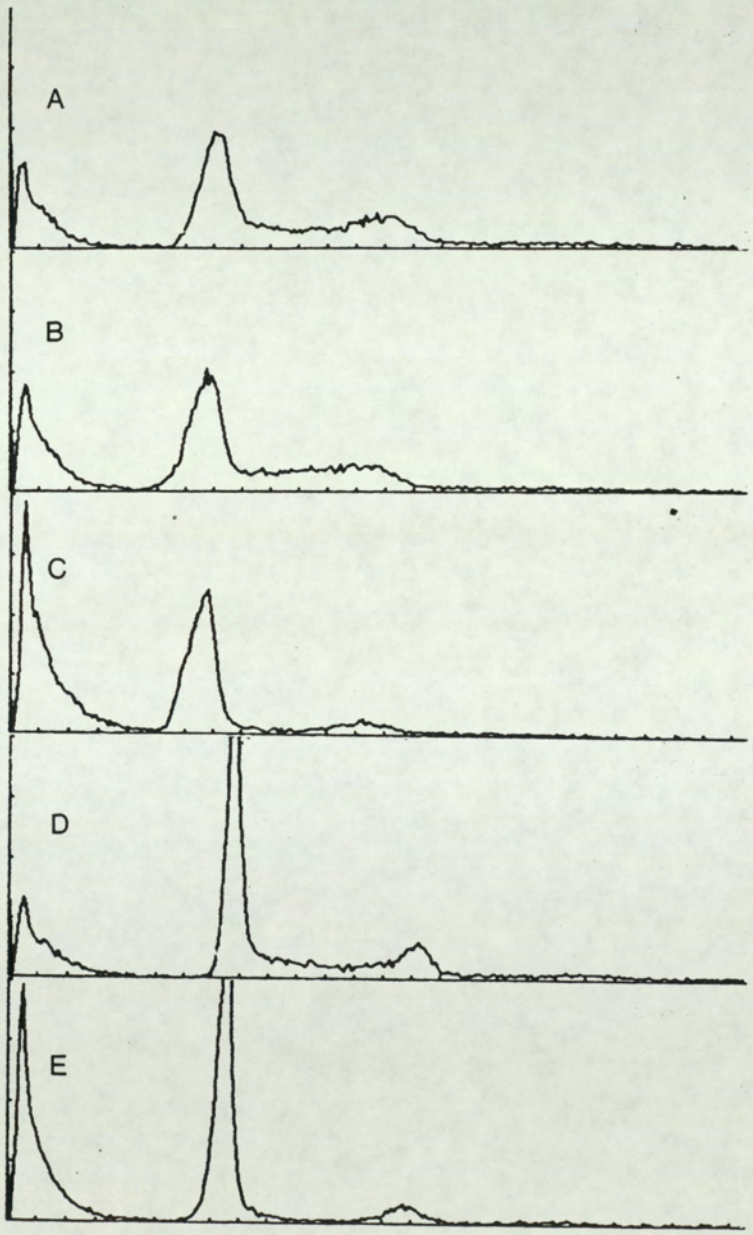
or without NMF underwent one population doubling by day two before reaching a plateau again. Those cells without NMF did not undergo any differentiation, but 45% of those with NMF differentiated by day four.

The cell cycle analyses are presented in Figure 4.13. Growth of the cells to a plateau phase of cell growth did not induce $G_1$ arrest, with 25.4 and 20.3% of cells in the S and $G_2$+M phases, respectively. The $G_1$ peak (54.3% of cells) was very broad, whereas in cells growing logarithmically the $G_1$ peak was narrow (see Figure 4.8). Addition of NMF to these cells reduced the numbers in the S and $G_2$+M phases to 7.3 and 10.4% respectively, and restored the narrow shape of the $G_1$ peak (82.3% of cells), despite the fact that no changes in viability or differentiation were induced. When the cells were resuspended in fresh medium, a cell cycle distribution similar to that expected for a population of cells growing logarithmically was restored ($G_1$ = 58.0%, S = 24.5%, $G_2$+M = 17.5%), and concomitant addition of NMF caused $G_1$ arrest expected as the cells differentiated ($G_1$ = 85.1%, S = 3.7%, $G_2$+M = 11.2%).

It is possible that in the old, exhausted medium there was either an accumulation of a differentiation-inhibiting factor synthesized by the cells, or a depletion in some factor required by the cells for differentiation. It seems more likely that the total

Figure 4.13 Cell cycle analysis of cells at a plateau phase of growth in the presence or absence of 170mM NMF. Histograms of DNA content, determined by flow cytometry.

Cells were grown to a plateau phase of growth on day 0 (A); some were maintained in the old medium for 2 days in the absence (B) or presence (C) of 170mM NMF; others were resuspended in fresh medium and serum for 2 days in the absence (D) or presence (E) of 170mM NMF.



block in cell proliferation prevented NMF-induced differentiation, whereas allowing the cells to divide in fresh medium allowed differentiation in response to NMF. This is in agreement with the hypothesis that the cells are required to undergo at least one division in the presence of inducer to be able to differentiate.

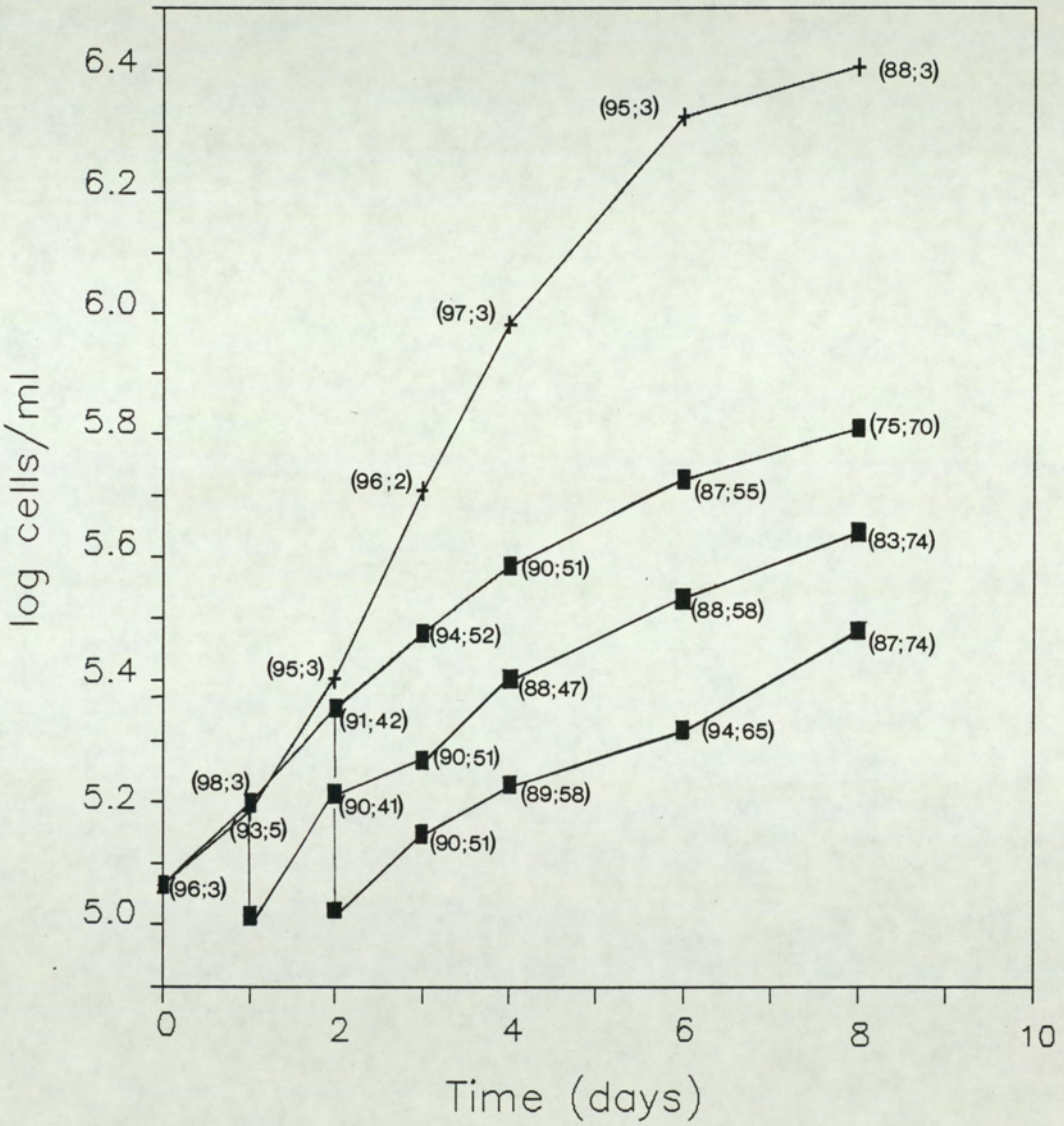
The fact that the cells did not differentiate as they approached plateau in the absence of other inducer suggests that simple inhibition of proliferative potential of the cells was not sufficient to induce differentiation. The induction of differentiation therefore probably requires two steps, one of which is associated with the inhibition of the proliferative potential of the cells.

### 4.3.3 The induction of differentiation whilst maintaining proliferation by dilution and refeeding of the cells

To determine the effect of maintaining proliferative potential in the presence of an inducer of differentiation, HL-60 cells were incubated with or without 170mM NMF at 1 x $10^5$ cells per ml. On days 1 and 2 samples were removed and diluted back to 1 x $10^5$ cells per ml with fresh medium and serum, maintaining the NMF concentration at 170mM (Figure 4.14). In the fraction of cells which were not diluted after the start of the

Figure 4.14 The effect of refeeding HL-60 cells with fresh medium and serum in the continued presence of 170mM NMF.

Cell growth, viability and NBT reduction were determined every one or two days while the cells were incubated in the presence (■) or absence (+) of 170mM NMF. The figures in parentheses represent % viable and % NBT+ cells, respectively.



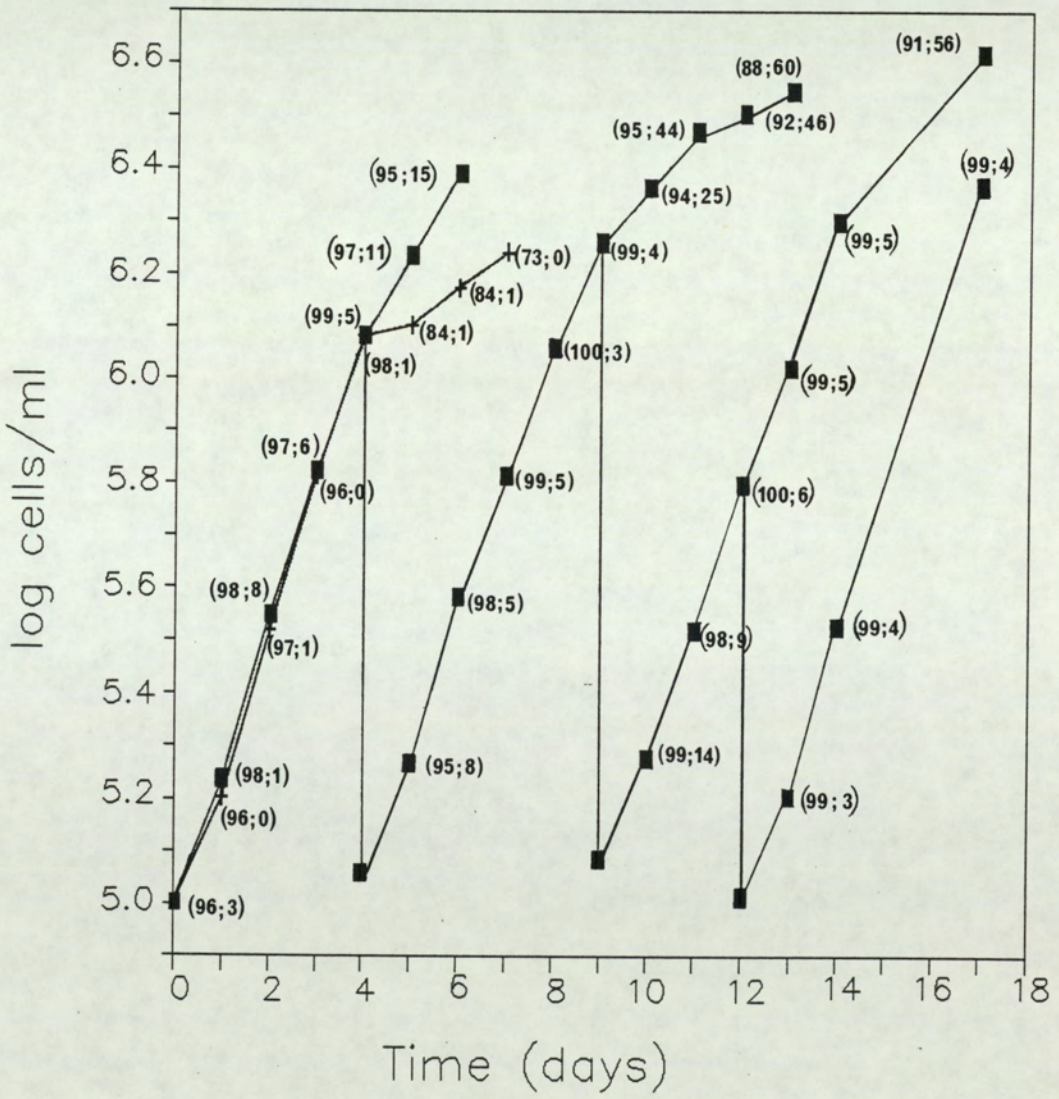
experiment, inhibition of proliferation and significant differentiation were first evident on day two after the addition of NMF, as expected from the results in Section 4.1. The percentage of differentiated cells increased from 42% on day 2 to 70% on day 8, when the cell population had undergone 2.5 doublings in the presence of NMF compared to 4.25 in the untreated population.

Inhibition of proliferation also occurred from day 2 onward in the samples which were diluted in the presence of 170mM NMF, regardless of whether refeeding was performed on day 1 or day 2. Furthermore, differentiation was induced in the diluted cells with the same time course as in the undiluted cells, so that 74% of the cells were NBT positive by day 8, and the population of cells had undergone 2.5 doublings. Thus, even in the presence of fresh medium and serum, growth inhibition occurred concomitant with the induction of differentiation by 170mM NMF. This suggested that the inhibition of cell proliferation during differentiation of HL-60 cells was directly related to the induction of terminal differentiation rather than an effect of depletion of some essential factor in the medium.

In contrast to the experiments with 170mM NMF, it was found that dilution and refeeding of cells treated with 100mM NMF had a profound effect on the extent of cell differentiation and proliferation (Figure 4.15). 100mM

Figure 4.15 The effect of refeeding HL-60 cells with fresh medium and serum in the continued presence of 100mM NMF.

Cell growth, viability and NBT reduction were determined every one or two days while the cells were incubated in the presence ( ■ ) or absence (+) of 100mM NMF. Figures in parentheses represent % viable and % NBT+ cells respectively.



NMF did not cause inhibition of cell proliferation and unless diluted the cells proliferated to reach a plateau of cell growth at the same stage as untreated cells after 7 or 8 days and approximately 4.5 population doublings. As long as the cells continued to proliferate, less than 10% of the cells were NBT positive. However, when the cells exhausted the medium and reached a plateau phase of growth, as many as 60% of those treated with 100mM NMF differentiated to become NBT positive. If the cells were diluted and refed to maintain logarithmic growth in the presence of 100mM NMF, differentiation did not occur until the cells were finally allowed to reach a plateau phase of growth. By serial refeeding of the cells in the presence of 100mM NMF, at least 14 doublings of the population were achieved over a period of 17 days, without significant differentiation, and only when allowed to plateau did 56% of the cells differentiate.

These data suggested that two signals were required for the induction of differentiation by NMF, one of which was the inhibition of proliferative potential. The marginally toxic concentration of 170mM NMF was able to provide both signals, whereas 100mM NMF could only provide one, but the second signal could be provided by the cessation of cellular proliferation induced by growth to a plateau phase. This is discussed in Section 5.3

## 4.4 Investigations on the induction of gene amplification

### 4.4.1 Analysis of cellular DNA content by flow cytometry

Gene amplification is thought to arise as a result of either direct or indirect inhibition of DNA replication (Woodcock and Cooper 1981), as outlined in Section 1.7.4, and as a result, over-replication of genes tends to occur after treatment of cells with marginally cytotoxic concentrations of agents (Schimke 1984), as a form of stress response. These conditions are therefore very similar to those at which differentiation is induced in HL-60 cells, and several known inducers of gene amplification are known to induce differentiation of HL-60 cells. When DNA replication is inhibited, it tends to be the genes normally replicated early during S phase which become over-replicated; if the genes responsible for the induction of HL-60 cell differentiation were among this sub-set of genes replicated in early S, they would probably become amplified and their subsequent over-expression could induce differentiation. Furthermore, Yen et al (1986b) demonstrated the appearance of an enriched number of a group of small chromosomes after the induction of a precommitment state in HL-60 cells by hydroxyurea. This suggested that the amplification of genes might play a role in the induction of HL-60 cell differentiation.

In light of this evidence, cellular levels of DNA were measured during the commitment of HL-60 cells to differentiate with NMF. Schimke's group demonstrated that an increased cellular DNA content caused by gene amplification in a variety of cells was detectable by FACS analysis, revealed as a population of cells with more than the normal tetraploid $G_2$ DNA content (Hill and Schimke 1985, Schimke et al 1985, Johnston et al 1986, Rice et al 1986a). Study of the FACS profiles obtained during commitment with NMF (Figure 4.8), however, did not show any evidence of HL-60 cells with an increased DNA content. If any gene amplification occurred therefore, the amount of DNA over-replicated was too low to be detected by flow cytometry.

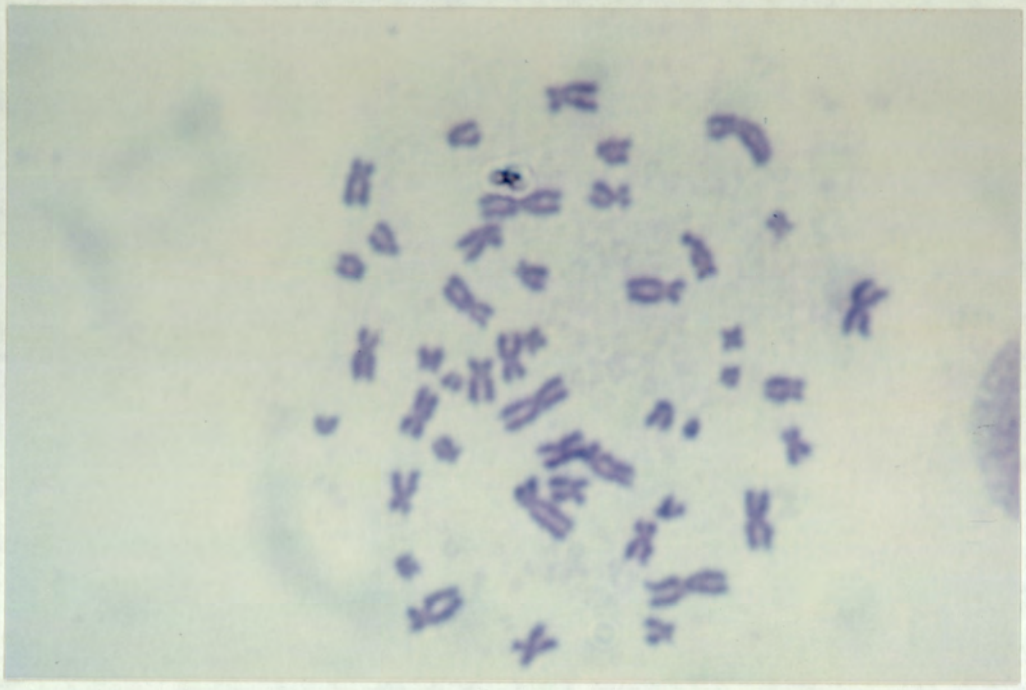
### 4.4.2 Analysis of cell chromosomes for over-replicated DNA or other aberrations

Another method of detecting gene amplification is to study the cell chromosomes; amplified genes often exist on double minute chromosomes, which are detectable on stained slides. Cell chromosomes were therefore prepared and stained from cells during the induction of differentiation with 170mM NMF. At least ten chromosome spreads were studied per treatment, and typical examples are shown in Figure 4.16. In no samples (whether treated with 170mM NMF for 0, 1, 2, 4, 6, 8, 24 or 48 hours) were there any obvious chromosome breaks or double minute chromosomes

Figure 4.16 Chromosomes of HL-60 cells during commitment to differentiation with 170mM NMF.

Chromosomes were isolated from cells treated with 170mM NMF for 0, 2, 4, 6, 8 or 24 hours.

0



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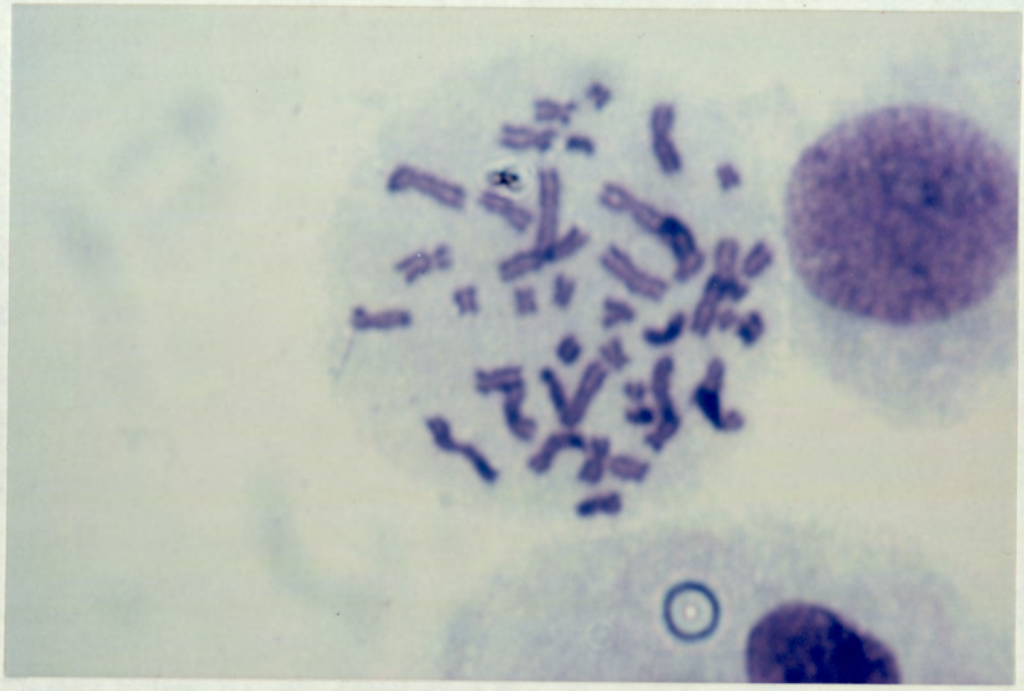
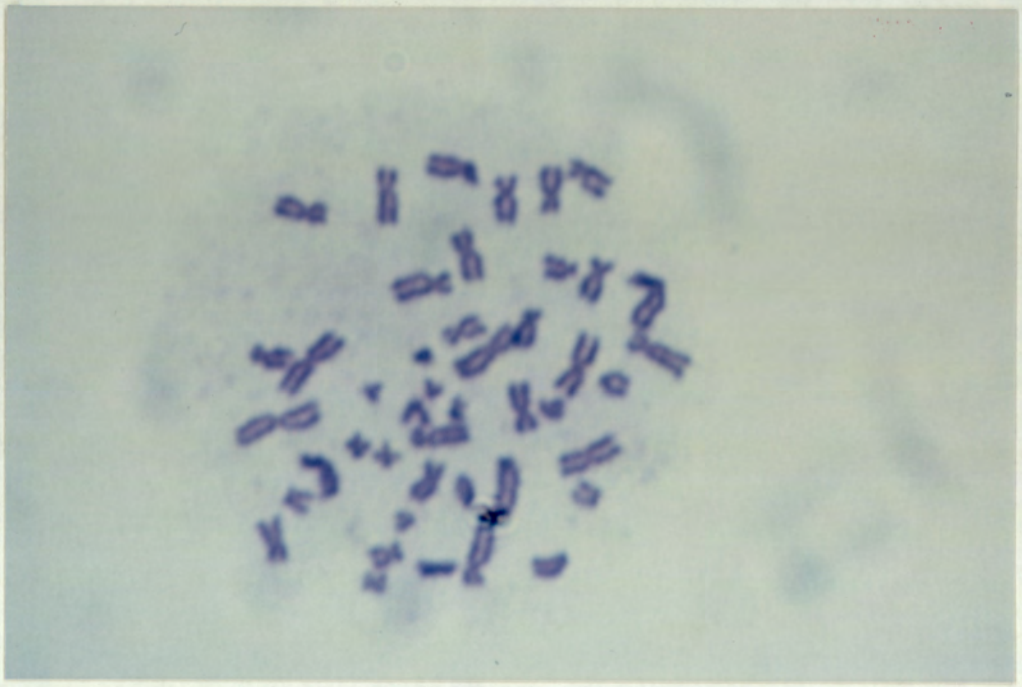


Figure 4.16 continued

4



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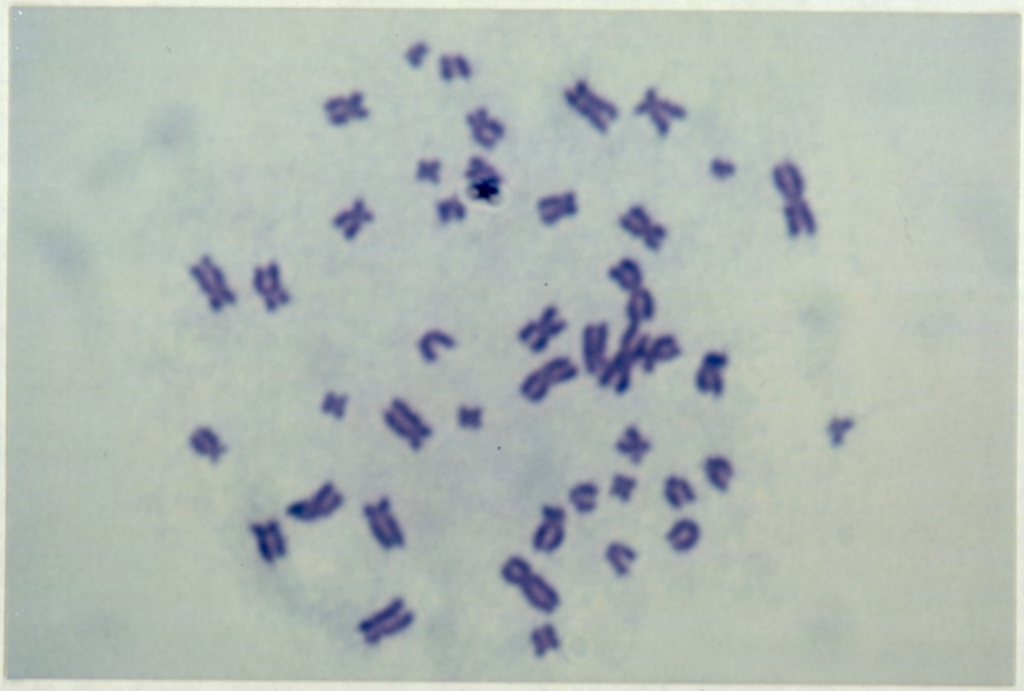

Figure 4.16 continued

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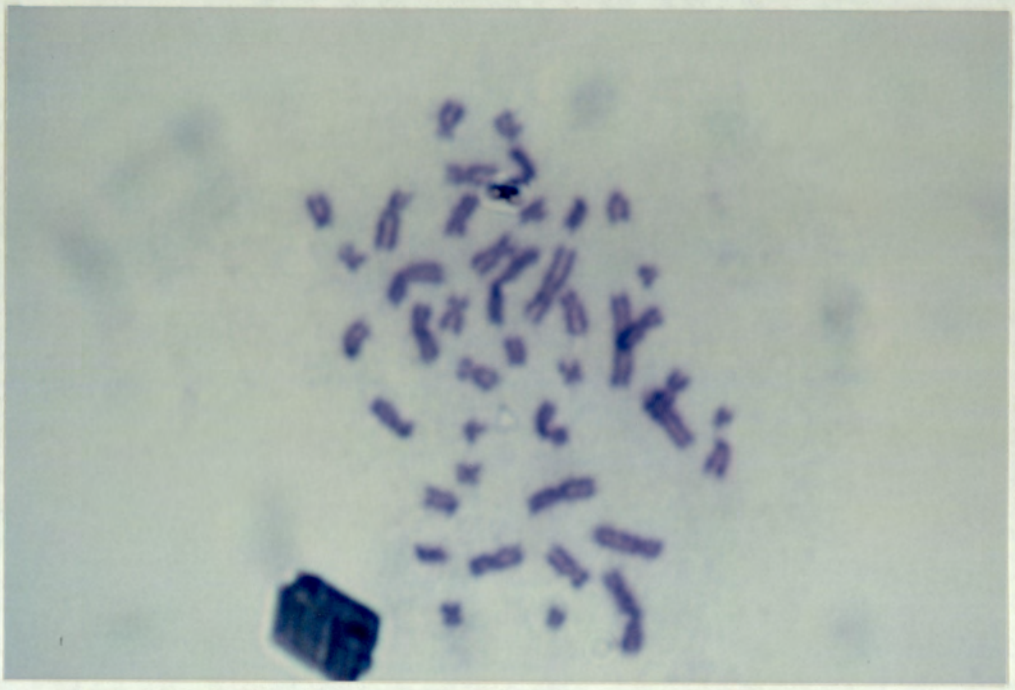

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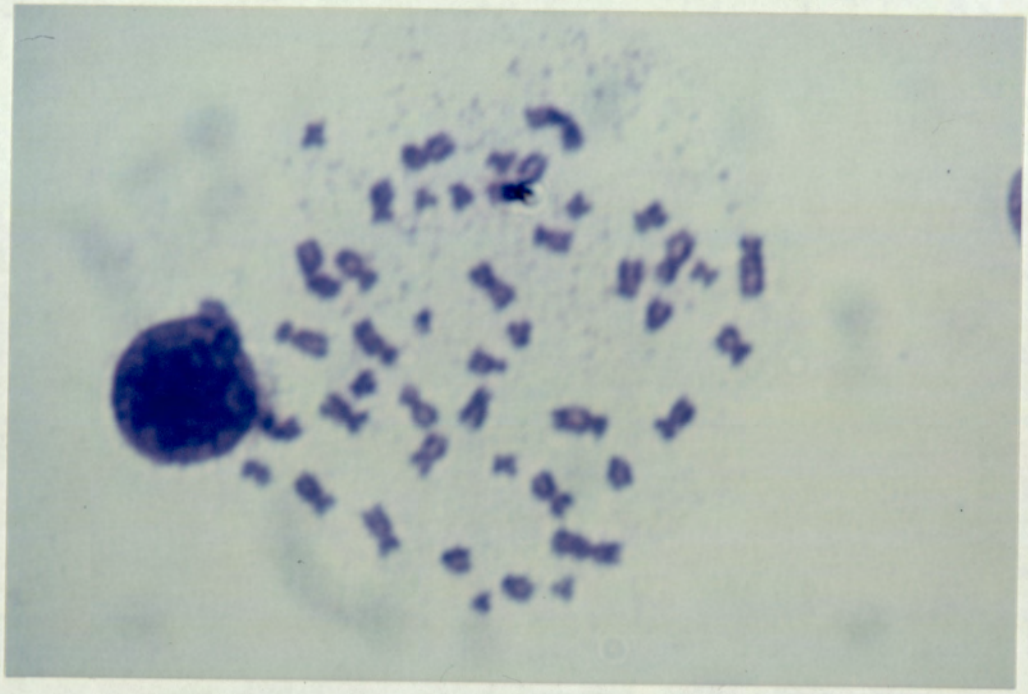


(DM); the type of abberations noted by Hill and Schimke (1985) in cell types in which gene amplification had occurred.

These Giemsa stained chromosomes were unfortunately not very clearly defined by the staining technique. The clarity might have been improved by treatment of the chromosomes with trypsin before staining; a technique which reveals well-defined banding patterns on the chromosomes and allows detection of structural rearrangements (Seabright 1972) as well as identification of the chromosomes. However, the staining technique used in the above experiments would have detected any DM or major chromosome breaks produced during NMF treatment of the cells.

There was no obvious induction of DM or other gross chromosomal abberations in treated HL-60 cells, so in the absence of any direct evidence for the occurrence of gene amplification during commitment of HL-60 cells to differentiate with NMF, it was decided to study whether the heat shock response was induced during commitment.

## 4.5 The induction of differentiation by agents known to induce a stress response

### 4.5.1 Ethanol ($CH_3CH_2OH$)

Many cell types have been shown to respond to stress such as hyperthermia or treatment with agents such as ethanol, by inducing the synthesis of heat shock proteins, as discussed in section 1.7.5. These proteins tend to be induced after marginally toxic treatments and are thought to play a role in protecting the cells from the stress. The proteins have also been implicated in activation of the embryonic mouse genome and in ascospore development in yeast, suggesting a role for the hsps in normal cellular development (see section 1.7.5.6). In light of this evidence it was thought that the heat shock proteins might play a role in the induction of HL-60 cell differentiation. In order to study whether there was a relationship between the induction of hsp synthesis and the induction of HL-60 cell differentiation, agents which were known to be hsp inducers in other cells were tested to determine whether they would induce HL-60 cell differentiation.

Ethanol induced hsp synthesis in Chinese hamster fibroblasts after treatment for 1 hour with 1M or 6% (v/v) of the solvent (Li and Werb 1982, Li 1983), and in yeast

after incubation for 20 mins with 1.55M, but not after a 1 hour treatment with 170mM (Plesset et al 1982).

Langdon and Hickman (1987) showed that ethanol induced differentiation of HL-60 cells at an optimum concentration of 213mM. To confirm this, HL-60 cells were incubated with 213mM ethanol for four days, and differentiation was assessed daily, as shown in Table 4.7 and Figure 4.17. The drug had no significant effects on cell growth, viability or differentiation after 1 day, but from day 2 cell proliferation was decreased compared to that of the untreated cells; after 4 days a total of 1.2 divisions had taken place compared to 3.5 in the untreated population.

Viability remained above 80% during the four day period, and the percentage of NBT positive cells increased to 48% by day 2, and continued to increase to 73% on day 4. The percentage of NBT positive cells was closely paralleled by the percentage of cells which were able to phagocytose yeast, reaching 70% by day 4. Surprisingly, as ethanol was previously considered to induce granulocytic differentiation, the percentage of cells expressing monocytic non-specific esterases (Figure 4.18) increased after 2 days of treatment, reaching a maximum of 25% of cells on day 3. The percentage of cells expressing detectable levels of the non-specific esterases was only half of the percentage of cells expressing the other

TABLE 4.7

The effect of 213mM ethanol on HL-60 cells over a four day period

213mM Ethanol

| day | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| cells/ml x $10^5$ | 1.4 ± 0.1 | 1.8 ± 0.3 | 2.2 ± 0.4 | 2.3 ± 0.5 * |
| % viable | 85.3 ± 11.7 | 84.9 ± 12.5 | 82.8 ± 14.4 | 82.1 ± 12.8 |
| % NBT+ | 5.5 ± 6.1 | 47.7 ± 3.1 | 67.1 ± 5.4 | 72.5 ± 11.0 |
| % phago | 5.9 ± 6.1 | 48.0 ± 7.3 | 60.4 ± 4.0 | 69.2 ± 11.1 |
| % NSE+ | 0.0 ± 0.0 | 8.0 ± 6.2 | 24.8 ± 7.2 | 21.8 ± 4.7 |

Control

| day | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| cells/ml x $10^5$ | 1.5 ± 0.2 | 3.2 ± 0.4 | 7.2 ± 1.5 | 12.0 ± 0.3 |
| % viable | 90.3 ± 7.4 | 90.8 ± 7.4 | 95.6 ± 2.1 | 95.5 ± 2.5 |
| % NBT+ | 4.6 ± 4.8 | 2.4 ± 0.6 | 1.7 ± 1.0 | 1.5 ± 0.8 |
| % phago | 1.1 ± 0.1 | 1.2 ± 1.1 | 1.0 ± 0.7 | 1.1 ± 0.7 |
| % NSE | 0.4 ± 0.1 | 0.3 ± 0.5 | 0.2 ± 0.3 | 0.4 ± 0.4 |

* All figures represent mean ± S.D. of 3 experiments

Figure 4.17 The induction of differentiation of HL-60 cells treated with 213mM ethanol over a 4 day period.

Cells were incubated in the presence (□) or absence (+) of 213mM ethanol and cell growth (A) and viability (B) were assessed daily. Differentiation was also assessed (C) by the reduction of NBT ( □ ), phagocytosis ( + ) and non-specific esterase activity ( ♦ ) in the presence of 213mM ethanol. Each point represents the average of 3 experiments.

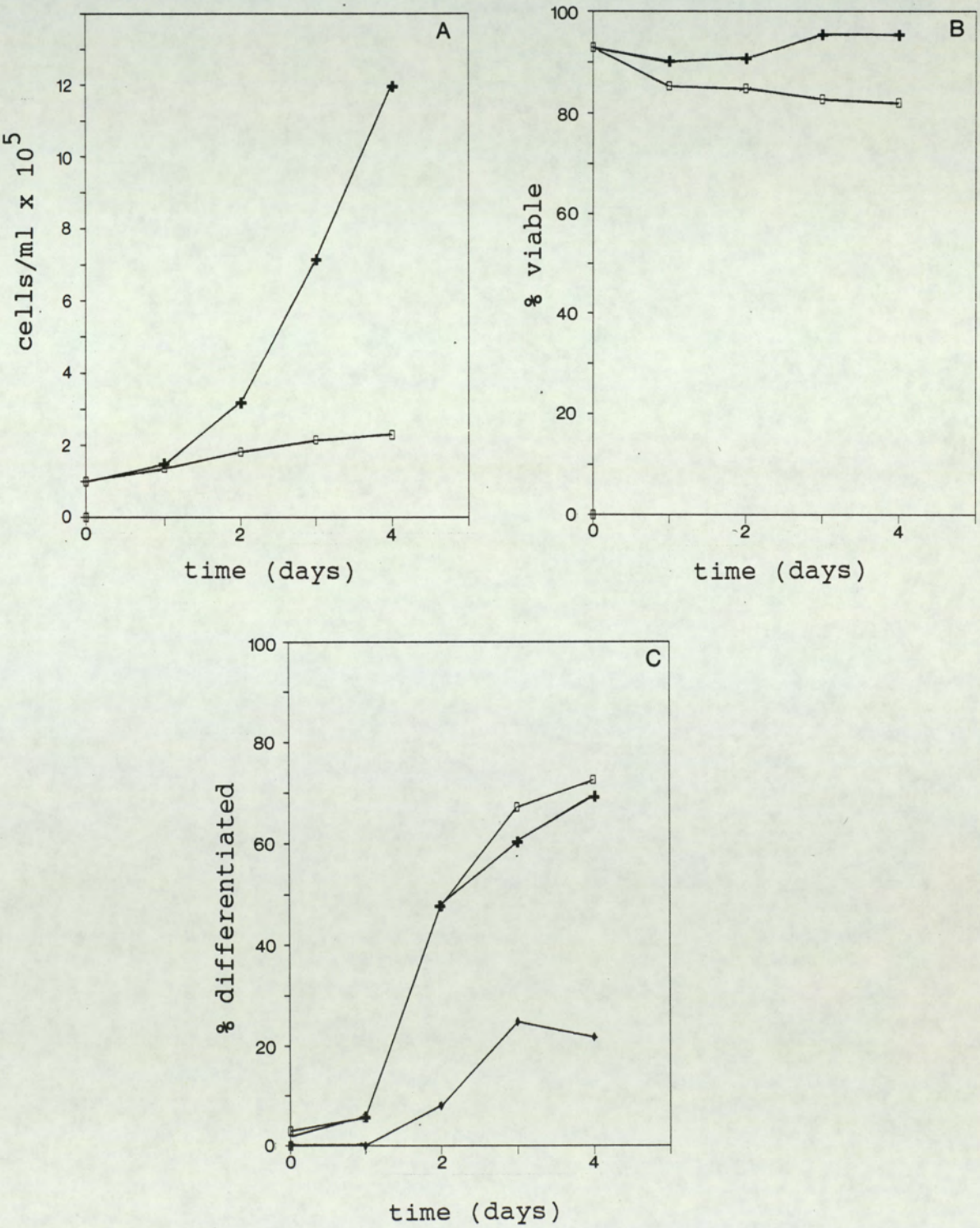
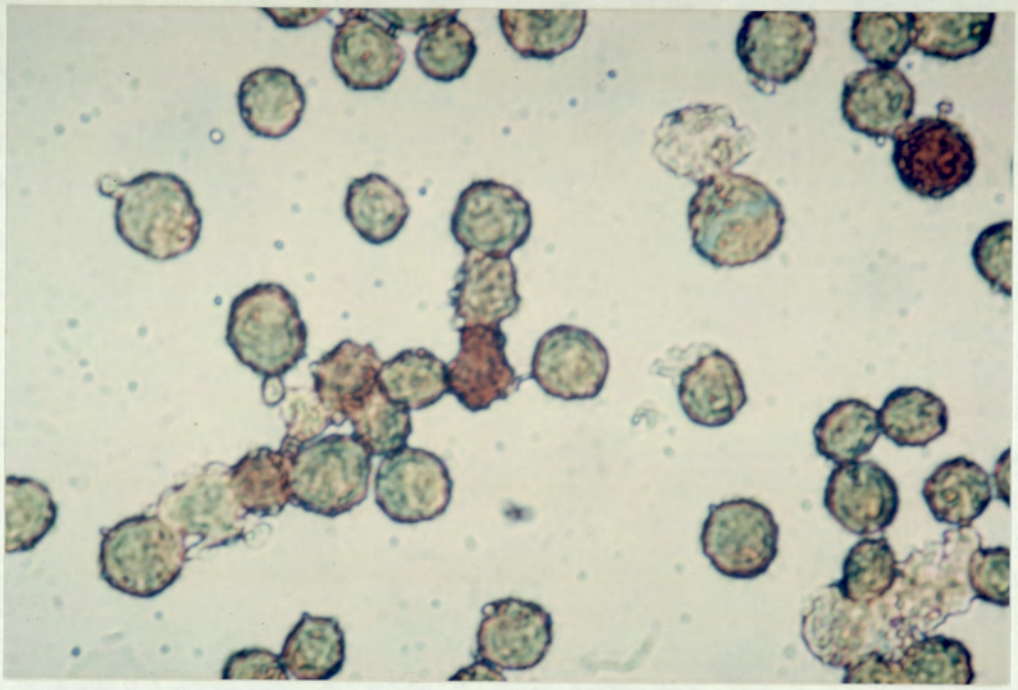


Figure 4.18 Expression of the markers of differentiation in HL-60 cells. Monocytic non-specific esterase activity.

Positive cells stained red due to alpha-naphthyl acetate esterase activity; negative cells were counterstained green.

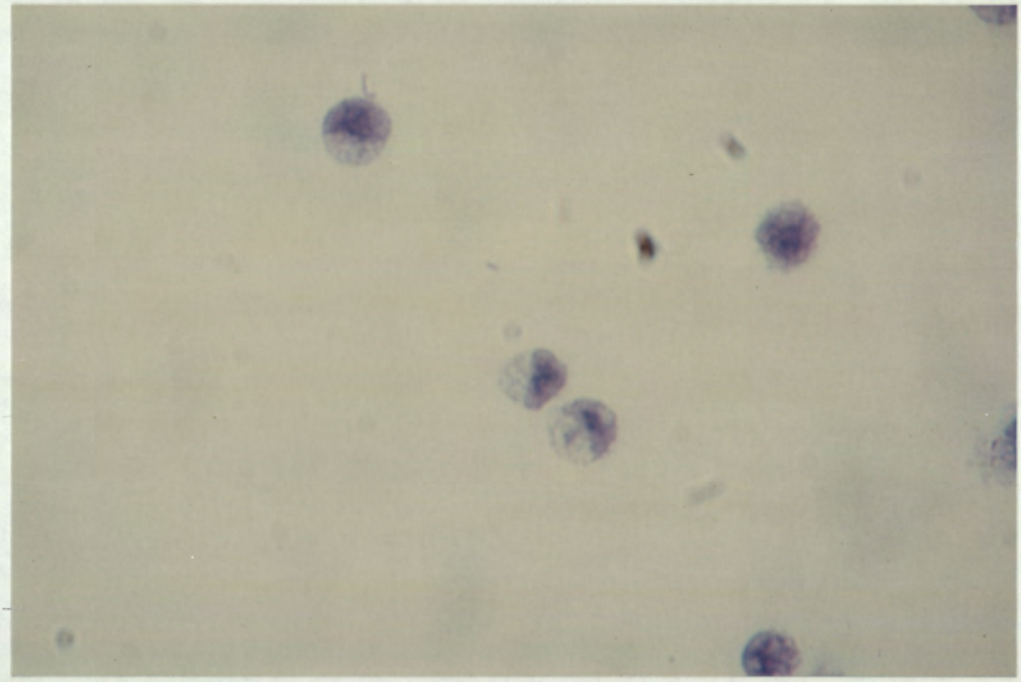


markers of differentiation (NBT reduction and the ability to phagocytose), and it was thought that ethanol might have induced two different populations of differentiated cells, one population of granulocytic cells, and one population of cells with monocytic characteristics.

The nuclear morphology of the cells was studied after Giemsa staining (Figure 4.19); there were very few cells visible with banded nuclei, although many had reniform or ovoid nuclei much smaller than those of the promyelocyte, suggestive of differentiated cells. Since little is known about the cells intermediate between the promyelocyte and the monocyte, it was difficult to determine whether the cells had nuclei characteristic of cells differentiating to granulocytes or to monocytes. An attempt was made to stain the cells with two different monoclonal antibodies specific for human granulocytes and monocytes, respectively, but staining could not be achieved of a strong enough nature to be able to decide the lineage of the cells which differentiated in response to ethanol. Interestingly, Zinzar et al (1987) described HL-60 cells induced to differentiate with sequential combinations of TPA and DMSO or TPA and retinoic acid. These cells expressed non-specific esterases but had nuclear indentation and lobulation more characteristic of granulocytes, suggesting a type of cell intermediate between monocytes and granulocytes.

Figure 4.19 The nuclear morphology of HL-60 cells treated for 4 days with 213mM ethanol.

Cells were stained with Wolbach Giemsa.



It was clear, despite the uncertainty of the mature cell type, that ethanol was a strong inducer of terminal differentiation in HL-60 cells. Other known inducers of the heat shock response were then tested in HL-60 cultures.

4.5.2 Sodium arsenite ($NaAsO_2$)

HL-60 cells were incubated with various concentrations of sodium arsenite, a known inducer of heat shock protein synthesis in many other cell types (Li and Werb 1982, Atkinson et al 1983, Bensaude and Morange 1983, Caltabiano et al 1986). 1uM Sodium arsenite had very little effect on HL-60 cell growth, viability or differentiation, whereas 15uM arsenite totally inhibited cell proliferation and reduced viability to 24% on day 4. In between these concentrations, differentiation was induced (Table 4.8 and Figure 4.20). The optimum concentration of arsenite for the induction of differentiation was found to be 6uM, and at this concentration viability remained at greater than 85% with 36% of the cells becoming NBT positive by day 4.

Having established the optimum concentration, differentiation was assessed in cells incubated with 6uM arsenite over a 4 day period (Table 4.9 and Figure 4.21). Growth inhibition commenced after 1 day, and continued so that after 4 days the population had doubled 1.25 times,

TABLE 4.8

The effect of sodium arsenite on HL-60 cells, measured on day 4

| conc (uM) | cells/ml $x10^5$ | % viable | % NBT+ * |
|---|---|---|---|
| 0 | 10.40 ± 2.30 | 95.37 ± 3.00 | 1.66 ± 1.05 |
| 1 | 8.43 ± 1.28 | 96.39 ± 1.96 | 4.23 ± 3.26 |
| 4 | 4.60 ± 0.61 | 92.96 ± 3.12 | 16.69 ± 5.55 |
| 5 | 3.82 ± 0.45 | 91.55 ± 3.47 | 31.75 ± 6.54 |
| 6 | 3.37 ± 1.05 | 86.91 ± 6.74 | 35.99 ± 12.27 |
| 7 | 2.91 ± 0.84 | 81.35 ± 9.75 | 34.00 ± 11.80 |
| 8 | 2.62 ± 1.45 | 76.10 ± 18.67 | 29.07 ± 3.64 |
| 10 | 2.18 ± 1.27 | 63.21 ± 24.00 | 24.01 ± 9.28 |
| 15 | 1.08 ± 0.51 | 24.03 ± 13.07 | 4.76 ± 3.30 |

* Figures represent mean ± S.D. of 5 experiments

Figure 4.20 The induction of differentiation of HL-60 cells treated with sodium arsenite for 4 days.

Cells were incubated with different concentrations of sodium arsenite; cell growth (A), viability (B) and NBT reduction (C) were assessed on day 4. Each point represents the average of 5 experiments.

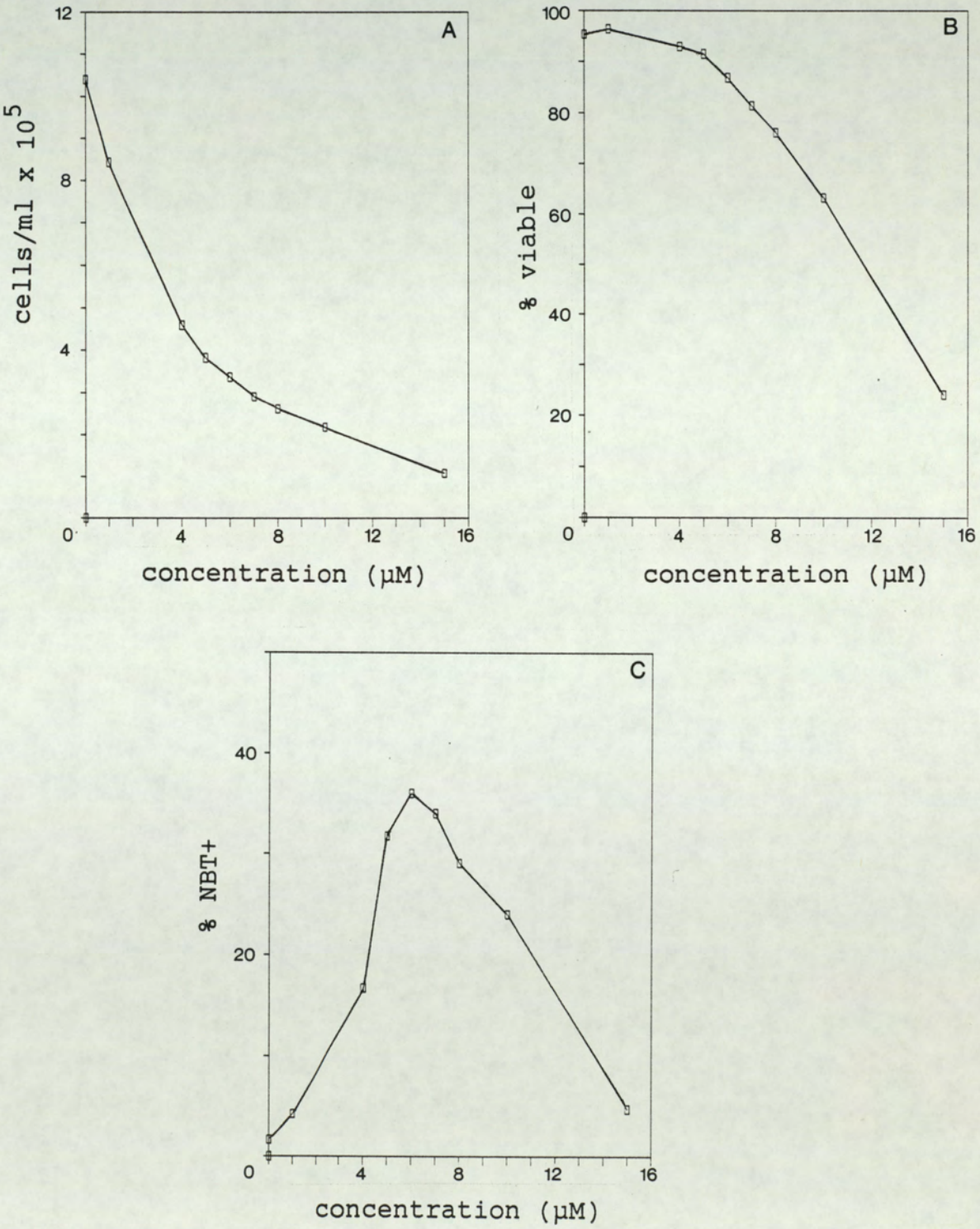


TABLE 4.9

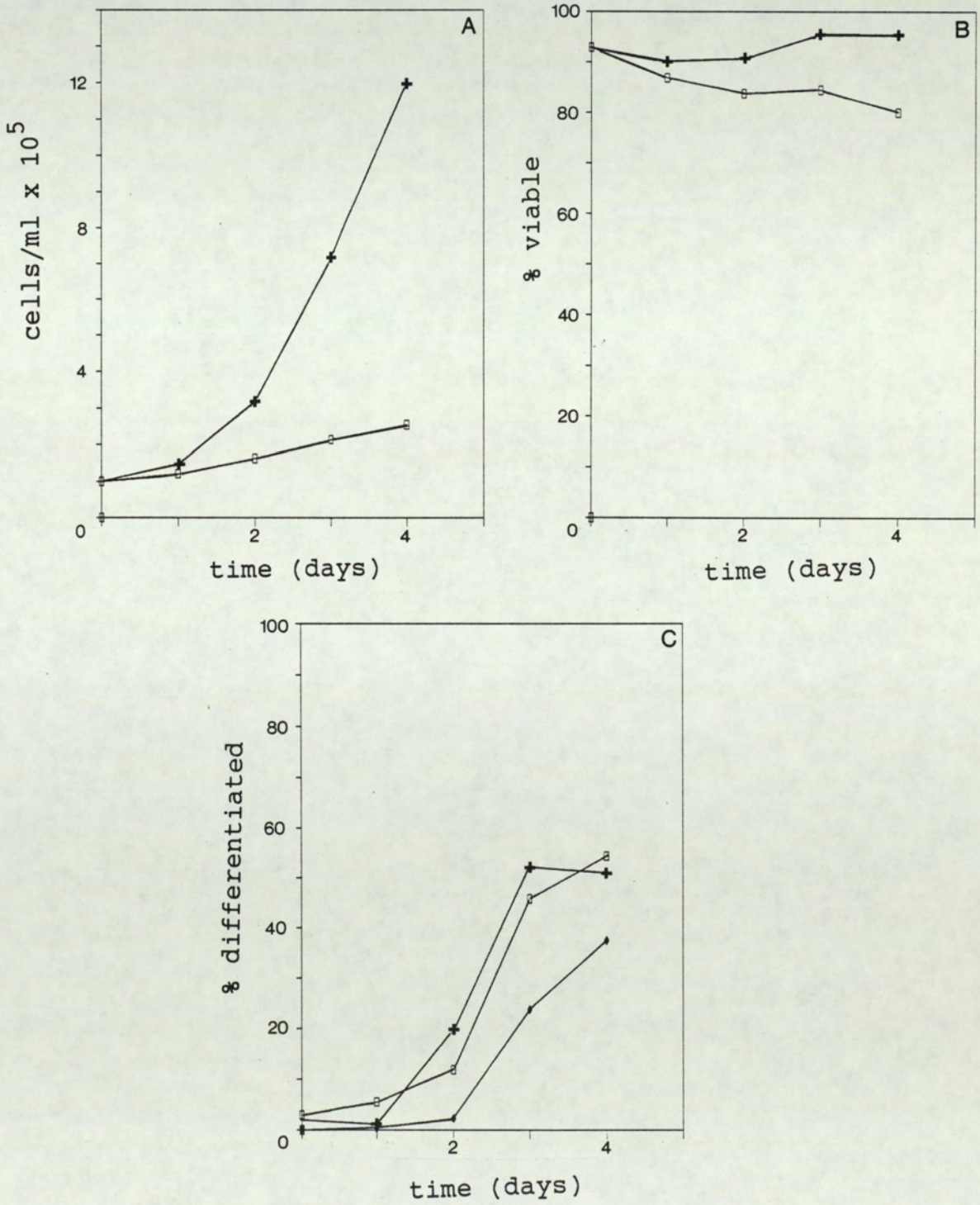
The effect of 6uM sodium arsenite on HL-60 cells over a four day period

| Day | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| cells/ml $x10^5$ | 1.2 ± 0.3 | 1.6 ± 0.2 | 2.1 ± 0.2 | 2.6 ± 0.4 * |
| % viable | 86.9 ± 8.0 | 83.7 ± 7.6 | 84.5 ± 8.2 | 79.9 ± 6.0 |
| % NBT+ | 5.5 ± 4.7 | 11.9 ± 4.7 | 45.7 ± 6.4 | 54.4 ± 6.7 |
| % phago | 1.2 ± 1.2 | 19.7 ± 7.5 | 52.1 ± 6.6 | 51.0 ± 10.3 |
| % NSE+ | 0.4 ± 0.1 | 2.3 ± 0.4 | 23.8 ± 13.9 | 37.4 ± 16.2 |

* Figures represent mean ± S.D. of 3 experiments. Figures for untreated cells are in Table 4.7.

Figure 4.21 The induction of differentiation of HL-60 cells treated with 6uM sodium arsenite over a 4 day period.

Cells were incubated in the presence (□) or absence (+) of 6uM sodium arsenite; cell growth (A) and viability (B) were assessed daily. Differentiation was also assessed (C) by NBT reduction ( □ ), phagocytosis ( + ) and non-specific esterase activity ( ♦ ) in the cells incubated with 6uM sodium arsenite. Each point represents the average of 3 experiments.



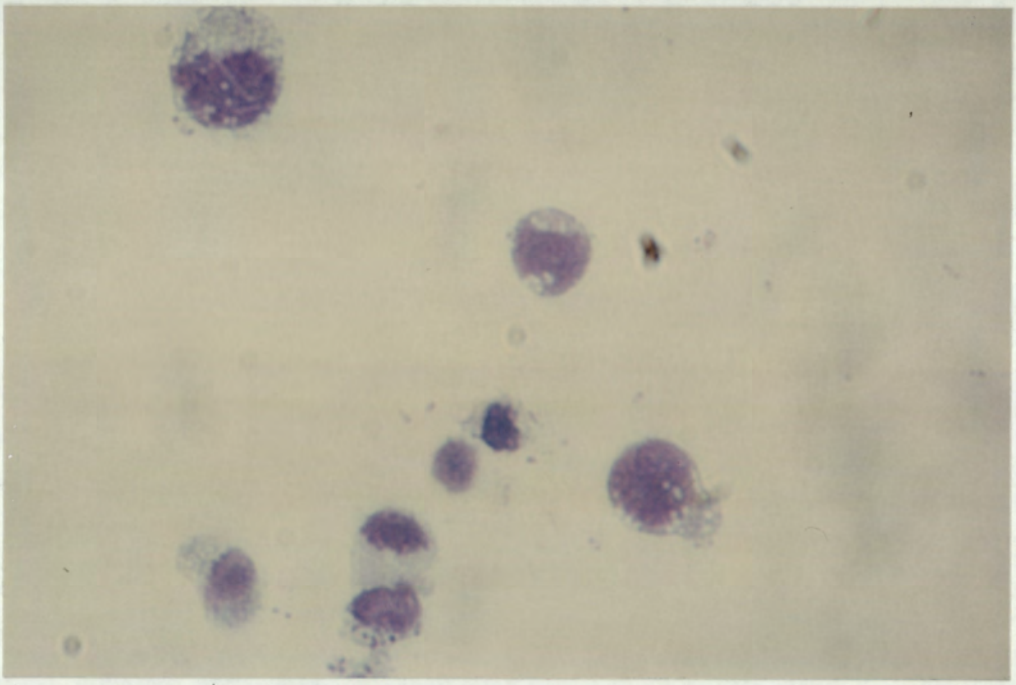
compared to 3.5 times in the untreated population. Viability remained above 80% even after 4 days of treatment; the percentage of NBT positive cells increased from day 2 onward to reach a maximum of 54% by day four, closely parallelled by the percentage of cells which were able to phagocytose yeast. Non-specific esterase activity increased on days three and four, suggesting that the differentiated cells were monocytic in character.

The nuclear morphology was studied in cells stained with Giemsa; it was apparent that many cells had matured beyond the promyelocyte, as evidenced by reduction in the nuclear to cytoplasmic ratio and the oval or reniform shape of the nuclei. None of the cells had matured to banded granulocytes (Figure 4.22). The differentiated cells did not adhere to the culture flasks or attain the flattened morphology characteristic of macrophages (including those macrophages produced by differentiation of HL-60 cells in response to the phorbol ester TPA, as outlined by Abita in 1984). The cells which differentiated after arsenite treatment were similar to the non-adherent monocytes which resulted from HL-60 cell differentiation in response to ara-C (Ross 1985a).

These experiments demonstrated that a well known inducer of heat shock protein synthesis, sodium arsenite, was also an inducer of HL-60 cell differentiation. Arsenite induced heat shock protein synthesis in many cell

Figure 4.22 The nuclear morphology of HL-60 cells treated for 4 days with 6uM sodium arsenite.

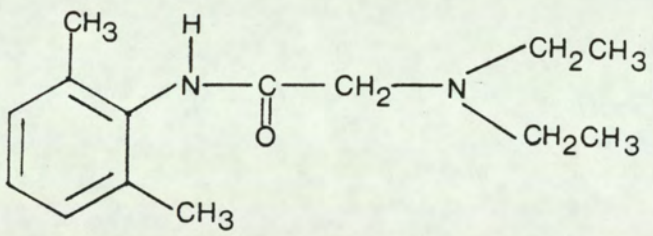
Cells were stained with Wolbach Giemsa.



types after incubation for one or two hours at concentrations of 25 to 250uM (Li and Werb 1982, Li 1983, Atkinson et al 1983, Bensaude and Morange 1983), but Caltabiano et al (1986) showed that hsp synthesis was also induced after incubation of cells for 8 hours with concentrations as low as 6uM arsenite, which was the concentration found to induce HL-60 cell differentiation.

### 4.5.3 Lidocaine hydrochloride

2-(diethylamino)-N-(2,6-dimethylphenyl)acetamide




The local anaesthetic lidocaine was shown by Hahn et al (1985) to induce synthesis of hsps 70, 87 and 110 in Chinese hamster cells, after incubation of the cells for 2 hours with 14mM lidocaine. To determine whether it would induce differentiation in HL-60 cells, lidocaine hydrochloride was added to the cells at concentrations between 1 and 5mM, (Table 4.10 and Figure 4.23). 1mM lidocaine reduced cell growth to about 50% of that in untreated cells, and induced 12% of cells to differentiate by day four. 5mM lidocaine, on the other hand, completely inhibited cell proliferation and reduced viability to 24%. Between these concentrations, significant differentiation

TABLE 4.10

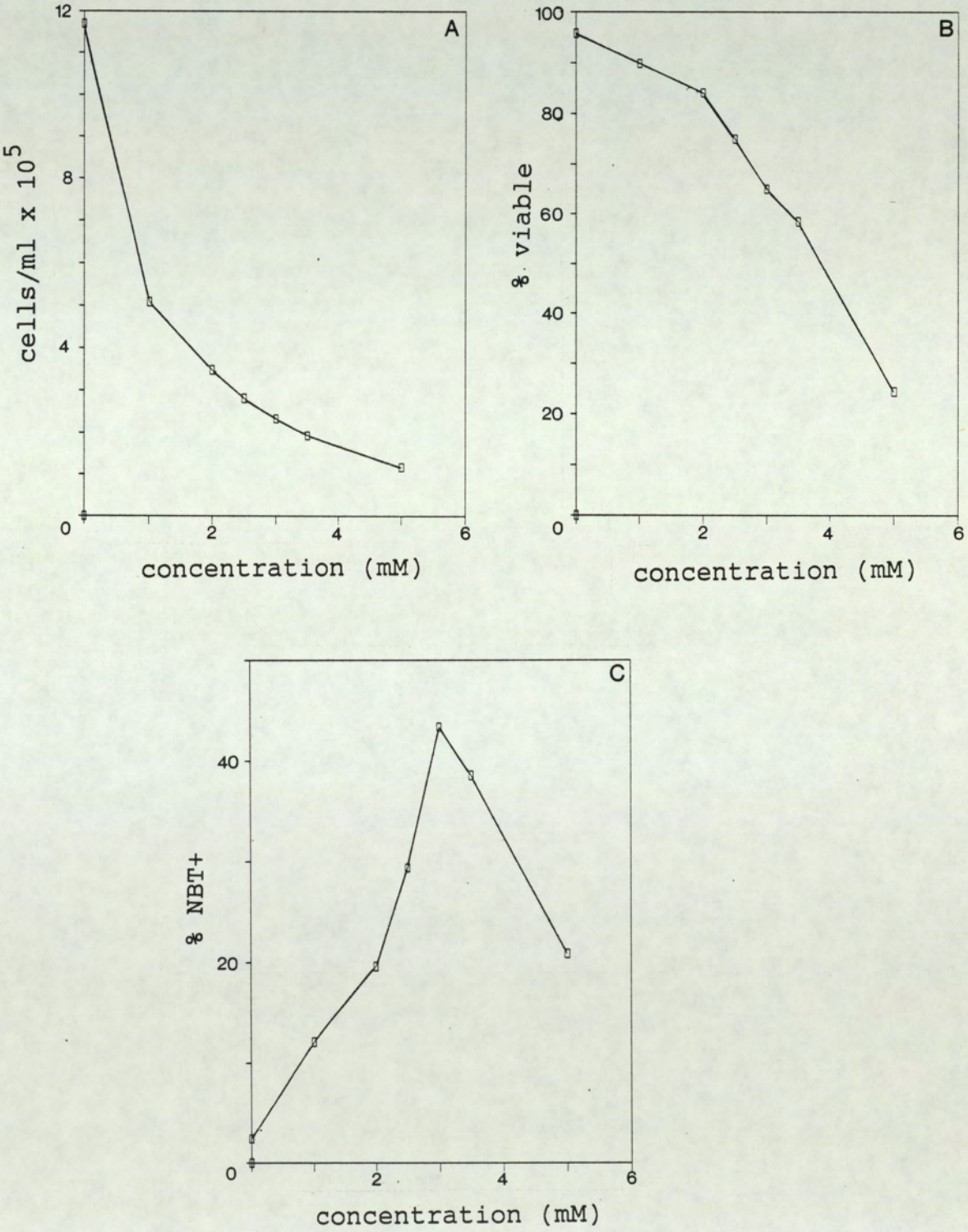
The effect of lidocaine hydrochloride on HL-60 cells, measured on day 4

| conc (mM) | cell/ml x $10^5$ | % viable | % NBT+ |
|---|---|---|---|
| 0 | 11.70 ± 2.10 | 95.84 ± 2.16 | 2.41 ± 1.05 * |
| 1 | 5.09 ± 1.76 | 89.91 ± 5.56 | 12.11 ± 10.41 |
| 2 | 3.48 ± 1.07 | 84.11 ± 4.30 | 19.61 ± 7.94 |
| 2.5 | 2.81 ± 1.15 | 74.96 ± 10.67 | 29.36 ± 11.46 |
| 3 | 2.33 ± 0.93 | 64.92 ± 14.83 | 43.37 ± 9.31 |
| 3.5 | 1.93 ± 0.94 | 58.44 ± 16.82 | 38.55 ± 14.83 |
| 5 | 1.17 ± 0.54 | 24.40 ± 16.98 | 20.87 ± 22.43 |

* Figures represent mean ± S.D. of 7 experiments.

Figure 4.23 The induction of differentiation of HL-60 cells treated with lidocaine hydrochloride for 4 days.

Cells were incubated with different concentrations of lidocaine hydrochloride; cell growth (A), viability (B) and NBT reduction (C) were determined on day 4. Each point represents the average of 7 experiments.



was induced. The optimum concentration was found to be 3mM lidocaine, which allowed the cells to double 1.2 times in the four days compared to 3.5 doublings of the untreated cells. Viability was reduced to 65% by day four, and 43% of the cells became NBT positive.

Differentiation was assessed daily during incubation of the cells with this optimum concentration of 3mM lidocaine hydrochloride (Table 4.11 and Figure 4.24). Over the four day period the cells underwent 1.35 population doublings; viability decreased to slightly over 80%. The percentage of cells capable of reducing NBT increased from day 1 onward to reach 53% on day 4, and 52% of the cells were able to phagocytose yeast. The percentage of cells expressing non-specific esterases increased to 45% on day 4, suggesting that these cells, like those induced to differentiate with arsenite, had some monocytic characteristics.

The nuclear morphology was studied in cells stained with Giemsa; it was apparent that no banded granulocytes were present, but many of the cells had a reduced nuclear to cytoplasmic ratio and slightly reniform nuclei - indicative of differentiation from promyelocytes to myelocytes and metamyelocytes, or similar cells (Figure 4.25).

These experiments showed that yet another known

TABLE 4.11

The effect of 3mM lidocaine hydrochloride on HL-60 cells over a four day period

| Day | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| cells/ml $x10^5$ | 1.4 ± 0.3 | 1.7 ± 0.4 | 2.3 ± 0.8 | 2.8 ± 1.2 * |
| % viable | 87.3 ± 4.2 | 81.7 ± 5.0 | 81.8 ± 7.4 | 82.6 ± 7.7 |
| % NBT+ | 6.1 ± 4.0 | 20.5 ± 7.1 | 46.5 ± 10.4 | 53.5 ± 2.3 |
| % phago. | 2.8 ± 2.3 | 27.1 ± 4.8 | 42.6 ± 5.7 | 51.5 ± 2.8 |
| % NSE+ | 0.3 ± 0.2 | 8.8 ± 4.1 | 21.3 ± 9.8 | 44.9 ± 5.0 |

* Figures represent mean ± S.D. for 3 experiments. Values for untreated cells are in Table 4.7.

Figure 4.24 The induction of differentiation of HL-60 cells treated with 3mM lidocaine hydrochloride over a 4 day period.

Cells were incubated in the presence (□) or absence (+) of 3mM lidocaine hydrochloride; cell growth (A) and viability (B) were assessed daily. Differentiation was also assessed (C) by NBT reduction (□), phagocytosis (+) and non-specific esterase activity (♦) in cells incubated with 3mM lidocaine hydrochloride. Each point represents the average of 3 experiments.

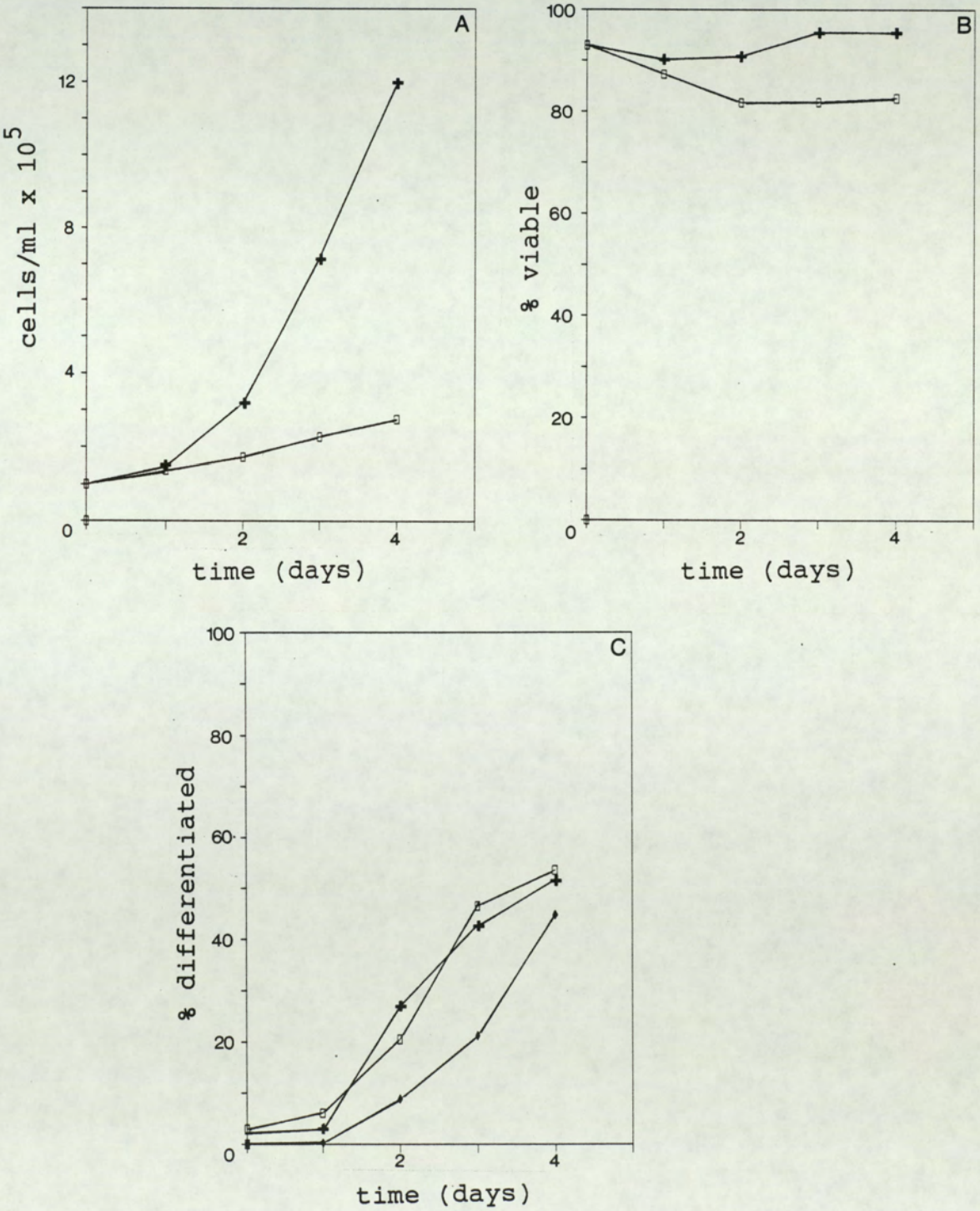
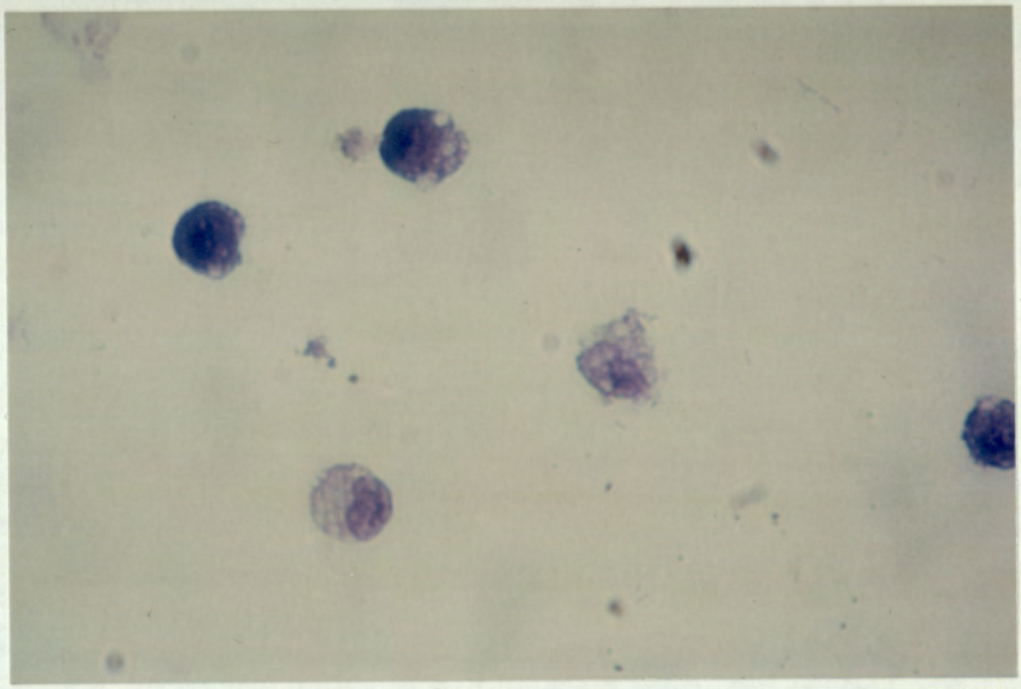


Figure 4.25 The nuclear morphology of HL-60 cells treated with 3mM lidocaine hydrochloride for 4 days.

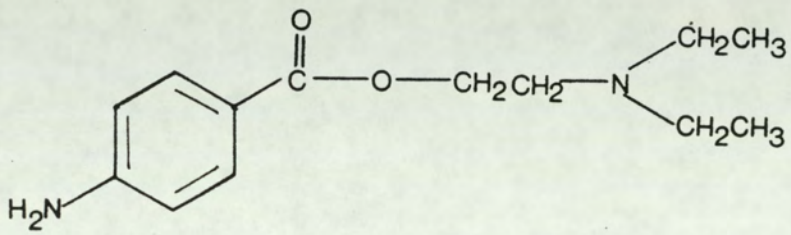
Cells were stained with Wolbach Giemsa.



inducer of heat shock protein synthesis, with a structure totally dissimilar to those previously tested, was able to induce differentiation of HL-60 cells.

### 4.5.4 Procaine hydrochloride

4-aminobenzoic acid 2-(diethylamino) ethyl ester




Procaine hydrochloride was tested as an inducer of hsp synthesis in Chinese hamster cells by Hahn et al (1985) but they did not detect the synthesis of hsps after 2 hour exposure to 31mM procaine. This was surprising because these authors found that lidocaine (which is structurally very similar to procaine) induced hsp70 synthesis. In HL-60 cells (see section 4.7.5), both procaine and lidocaine apparently induced hsp70 synthesis.

HL-60 cells were treated with procaine hydrochloride, to compare the effects of this local anaesthetic with the effects of lidocaine. 3mM procaine hydrochloride inhibited cell proliferation by approximately 60%, and induced 17% of cells to differentiate; 10mM procaine totally inhibited cell growth and reduced viability to

less than 10% on day four. Between these two concentrations, significant differentiation was induced; the optimum concentration was 5mM (Table 4.12, Figure 4.26). At this concentration, the cell population doubled once during the four day period of study, compared to 3.5 doublings in the untreated cells. Cell viability was reduced to 71% by day four, and the proportion of NBT positive cells was 46%.

HL-60 cells were incubated with 5mM procaine hydrochloride for four days and differentiation was assessed daily: the cells underwent 1.25 doublings in the four days and viability remained at greater than 80%. The percentage of NBT positive cells increased from day two to reach a maximum on day four of 55%, concomitant with an increase to 53% in the proportion of cells able to phagocytose yeast. (Table 4.13 and Figure 4.27). Non-specific esterase activity in the cells increased so that 50% stained positive on day four. This suggested that, like sodium arsenite and lidocaine, procaine induced monocyte-like differentiation of the cells. However, similar to the latter agents, procaine-treated cells did not adhere to the culture flasks or attain a morphology characteristic of macrophages.

The nuclear morphology was studied in Giemsa stained cells; it was clear again that no banded granulocytes were visible, but many of the cells had small oval or reniform

TABLE 4.12

The effect of procaine hydrochloride on HL-60 cells, measured on day 4

| conc (mM) | cells/ml x $10^5$ | % viable | % NBT+ |
|---|---|---|---|
| 0 | 12.30 ± 0.43 | 92.99 ± 6.23 | 1.45 ± 0.59 * |
| 3 | 4.87 ± 1.24 | 87.31 ± 9.38 | 16.97 ± 13.39 |
| 4 | 3.60 ± 1.91 | 78.19 ± 12.41 | 35.71 ± 13.12 |
| 5 | 1.92 ± 0.53 | 71.32 ± 10.51 | 46.32 ± 17.57 |
| 6 | 1.93 ± 0.37 | 65.64 ± 14.12 | 41.92 ± 24.02 |
| 7 | 1.56 ± 0.28 | 46.81 ± 19.31 | 21.04 ± 24.98 |
| 8 | 1.41 ± 0.06 | 35.21 ± 17.93 | 13.01 ± 16.21 |
| 10 | 1.04 ± 0.08 | 8.34 ± 9.38 | 0.23 ± 0.47 |

* Figures represent mean ± S.D. of 4 experiments.

Figure 4.26 The induction of differentiation of HL-60 cells treated with procaine hydrochloride for 4 days.

Cells were incubated with different concentrations of procaine hydrochloride; cell growth (A), viability (B) and NBT reduction (C) were determined on day 4. Each point represents the average of 4 experiments.

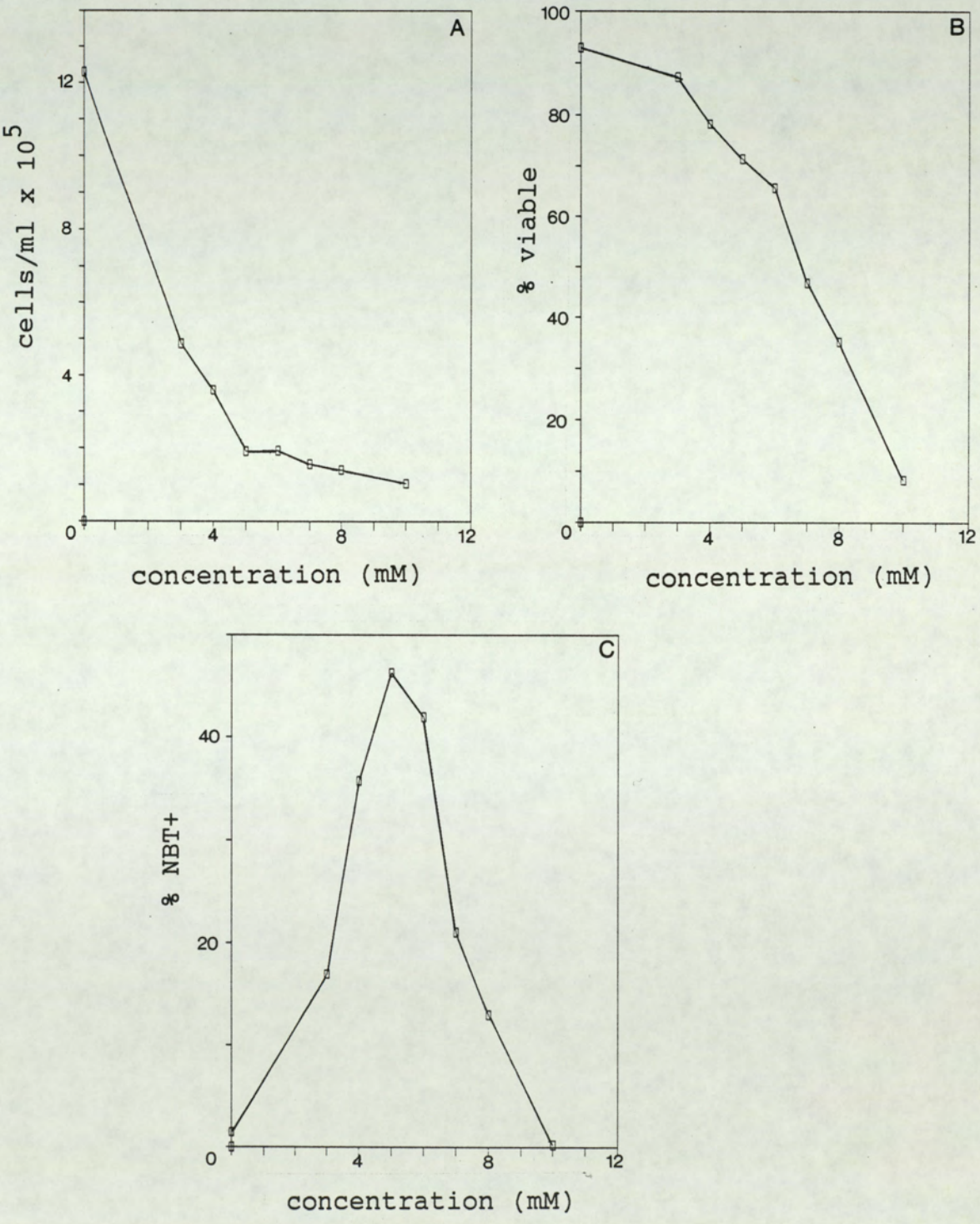


TABLE 4.13

The effect of 5mM procaine hydrochloride on HL-60 cells over a four day period

| Day | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| cells/ml x $10^5$ | 1.3 ± 0.2 | 1.8 ± 0.5 | 2.3 ± 0.8 | 2.5 ± 0.9 * |
| % viable | 87.3 ± 6.5 | 85.9 ± 7.3 | 84.1 ± 4.4 | 83.7 ± 6.6 |
| % NBT+ | 5.0 ± 5.4 | 20.9 ± 4.2 | 50.1 ± 9.1 | 55.4 ± 3.5 |
| % phago. | 3.1 ± 1.0 | 30.6 ± 7.1 | 46.4 ± 4.7 | 53.4 ± 3.0 |
| % NSE+ | 0.9 ± 0.1 | 6.7 ± 4.2 | 22.7 ± 11.1 | 49.4 ± 4.1 |

* Figures represent mean ± S.D. for 3 experiments. Values for untreated cells are in Table 4.7.

Figure 4.27 The induction of differentiation of HL-60 cells treated with 5mM procaine hydrochloride over a 4 day period.

Cells were incubated in the presence (□) or absence (+) of 5mM procaine hydrochloride; cell growth (A) and viability (B) were assessed daily. Differentiation was also assessed (C) by NBT reduction (□), phagocytosis (+) and non-specific esterase activity (♦) in cells incubated with 5mM procaine hydrochloride. Each point represents the average of 3 experiments.

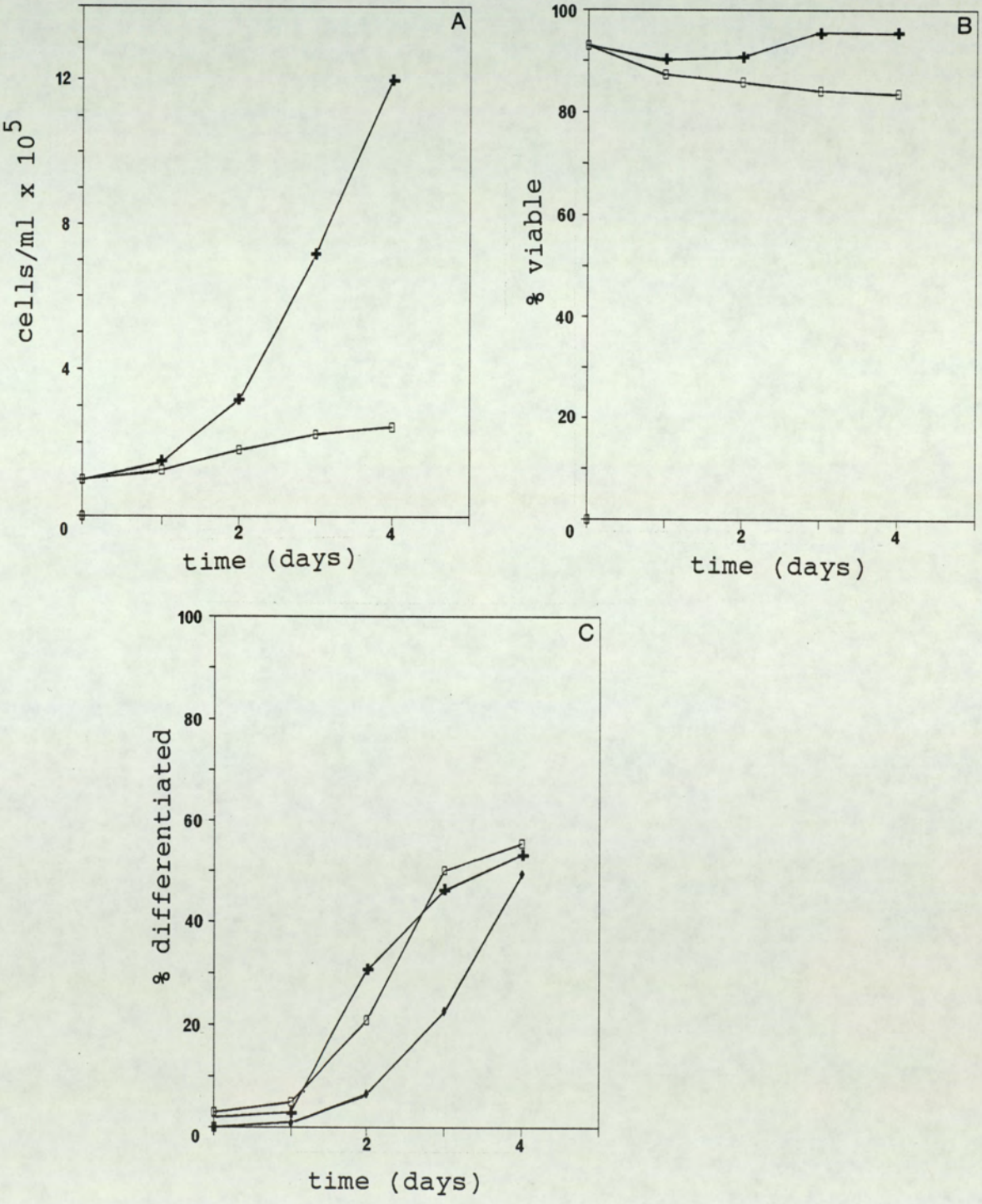
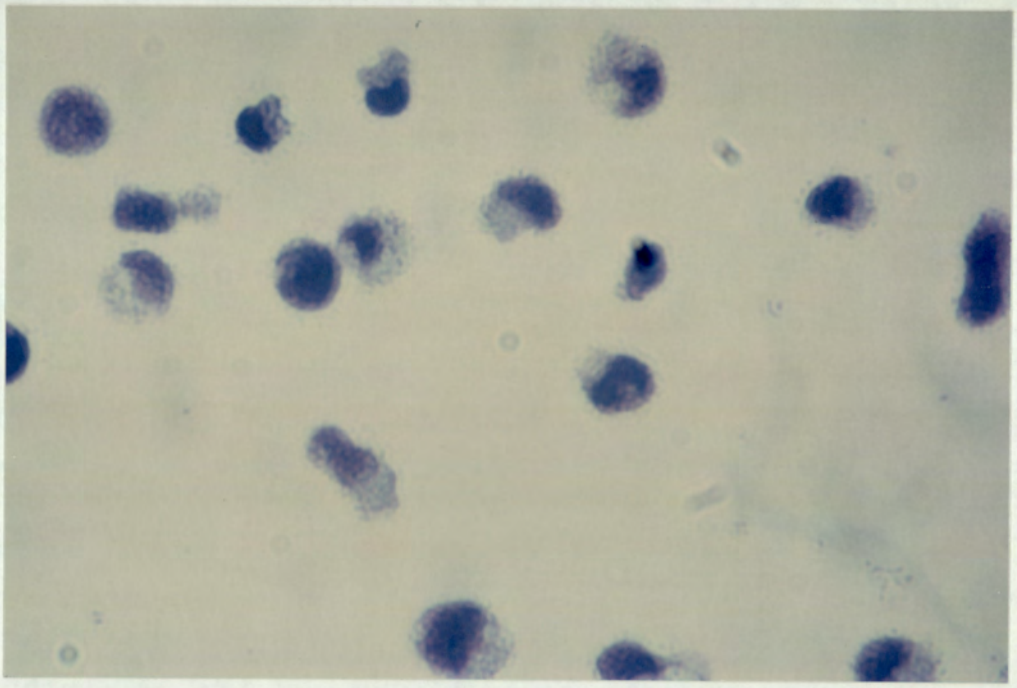


Figure 4.28 The nuclear morphology of HL-60 cells treated with 5mM procaine hydrochloride for 4 days.

Cells were stained with Wolbach Giemsa.



nuclei characteristic of cells which had differentiated from promyelocytes to myelocytes and metamyelocytes or perhaps their equivalents in the pathway from promyelocytes to monocytes (Figure 4.28).

### 4.5.5 Cadmium sulphate ($3CdSO_4.8H_2O$)

10uM cadmium was shown by Caltabiano et al (1986) to induce synthesis of hsps 72, 90, 100, 32 and 34 in melanoma cells after incubation for 8 hours. HL-60 cells were incubated with cadmium sulphate at various concentrations to determine whether differentiation could be induced (Table 4.14 and Figure 4.29). Concentrations above 40uM inhibited cell growth so that no doubling occurred over a four day period with 80uM cadmium. Only 12% of the cells were viable after four days at this highest concentration. Incubation with 60uM cadmium caused partial inhibition of cell proliferation so that 1.75 doublings occurred in four days, and viability fell gradually to 75%. At this concentration, the maximum attainable differentiation was induced, but this only reached an average of 14% after four days.

Cadmium is thought to have adverse effects on normal protein synthesis whilst inducing hsp synthesis (Burdon et al 1982), and this may be important in explaining why only a small percentage of cells were able to differentiate.

TABLE 4.14

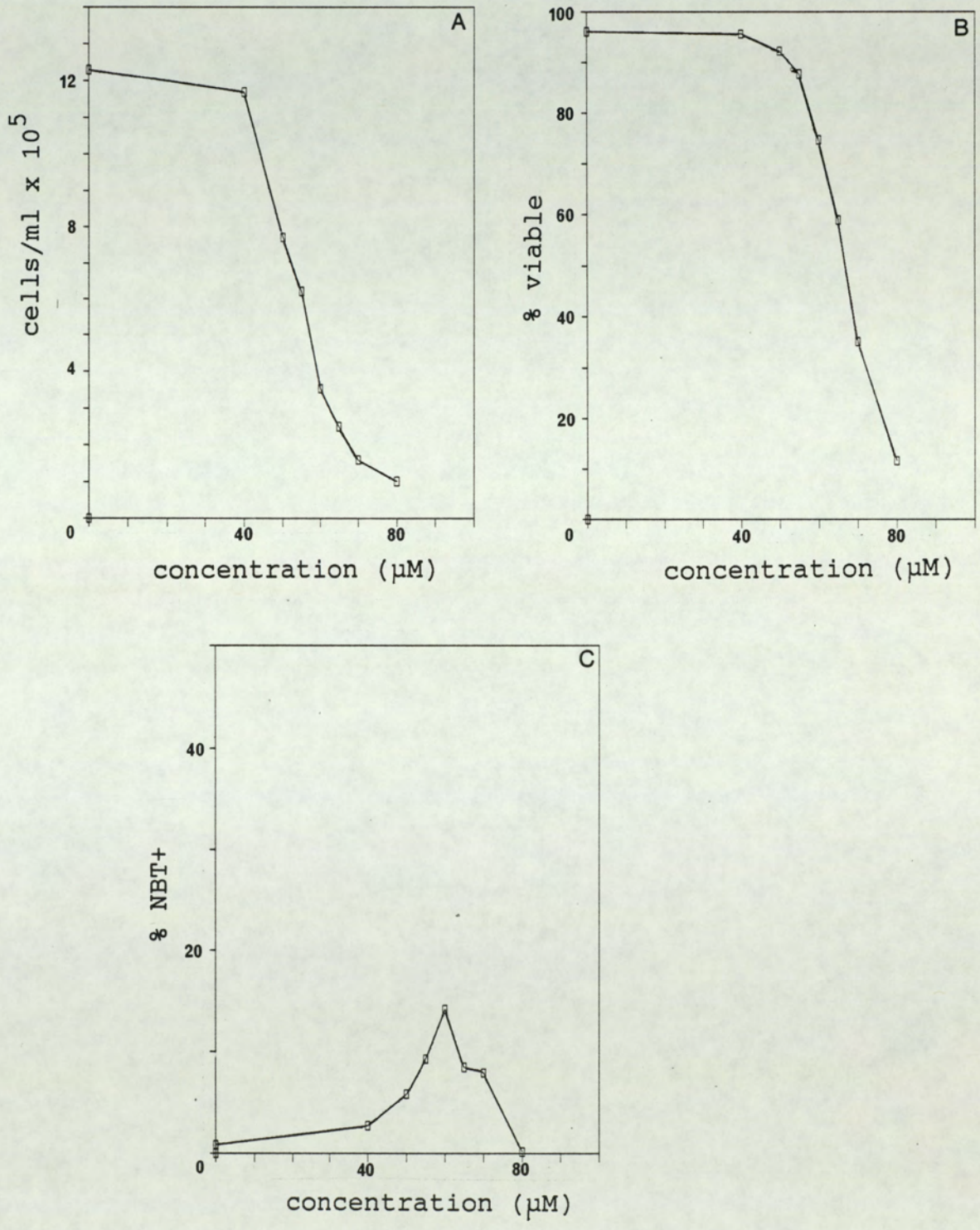
The effect of cadmium sulphate on HL-60 cells, measured on day 4

| conc (uM) | cells/ml x $10^5$ | % viable | % NBT+ |
|---|---|---|---|
| 0 | 12.30 ± 2.70 | 96.14 ± 1.64 | 0.87 ± 0.56 * |
| 40 | 11.70 ± 2.30 | 95.69 ± 2.74 | 2.74 ± 1.82 |
| 50 | 7.71 ± 2.21 | 92.28 ± 3.95 | 5.84 ± 1.61 |
| 55 | 6.21 ± 2.50 | 87.70 ± 7.42 | 9.30 ± 3.63 |
| 60 | 3.54 ± 1.62 | 74.70 ± 15.86 | 14.19 ± 11.34 |
| 65 | 2.51 ± 2.10 | 58.88 ± 19.33 | 8.47 ± 6.44 |
| 70 | 1.60 ± 0.96 | 35.07 ± 23.73 | 7.91 ± 12.15 |
| 80 | 1.02 ± 0.13 | 11.64 ± 3.31 | 0.16 ± 0.39 |

* Figures represent mean ± S.D. of 6 experiments.

Figure 4.29 The induction of differentiation of HL-60 cells treated with cadmium sulphate for 4 days.

Cells were incubated with different concentrations of cadmium sulphate; cell growth (A), viability (B) and NBT reduction (C) were determined on day 4. Each point represents the average of 6 experiments.



This idea will be discussed in more detail later (section 5.7).

### 4.5.6 Lead glutamate

Concentrations of lead glutamate between 1uM and 1mM had no effect on cell growth, viability or differentiation. Unfortunately lead glutamate was found not to be a suitable compound for addition to cell cultures (in contrast to the findings of Shelton et al 1986). At concentrations as low as 0.1mM in the culture medium, the lead glutamate visibly precipitated out, presumably due to the lead complexing with sulphur-containing amino acids in the medium. A solution of lead nitrate similarly precipitated immediately on addition to the medium. Because of this precipitation it was unlikely that any lead would have entered the cells, so the experiments were discontinued.

It is clear from the experiments in section 4.5 that a variety of agents known to induce heat shock protein synthesis in other cells types were capable of inducing terminal differentiation in HL-60 cells. The agents all induced optimum differentiation at concentrations only marginally below cytotoxic, as expected if some form of

stress response was important in the commitment of the cells to differentiate. This evidence suggested that the heat shock response was involved in HL-60 cell differentiation; it was then determined whether heat shock itself could induce HL-60 cells to differentiate.

## 4.6 The induction of differentiation by heat shock

Preliminary experiments were performed by Steven Turner in this laboratory to determine whether hyperthermia at temperatures between 39.5°C and 45°C was toxic to HL-60 cells. Figure 4.30 shows that incubation at 39.5°C continuously for up to 4 days had very little effect on cell growth or viability; neither did incubation at 40.5°C. Hyperthermia at 41.5°C for 2 days inhibited cell proliferation and caused viability to decrease to less than 70%, but during continued incubation for up to 7 days the cells re-commenced proliferation and viability began to increase again. Continuous incubation at 42.0°C totally inhibited cell proliferation and viability decreased to less than 10% by day four. In none of these studies was differentiation induced to a level more than 3% above that of untreated cells. HL-60 cells were then incubated for shorter periods of time at 42.0°C; viability gradually decreased after 5 hours of hyperthermia, to reach 75% viable after 30 hours of heat (Figure 4.31). Toxicity was increased when the temperature was increased

Figure 4.30 The effect on HL-60 cells of continuous heat shock at temperatures between 39.5°C and 42.0°C.

Cells were incubated at 37°C (□), 39.5°C (+), 40.5°C (♦), 41.5°C (△) and 42.0°C (×); cell growth (A), viability (B) and NBT reduction (C) were assessed for up to 7 days.

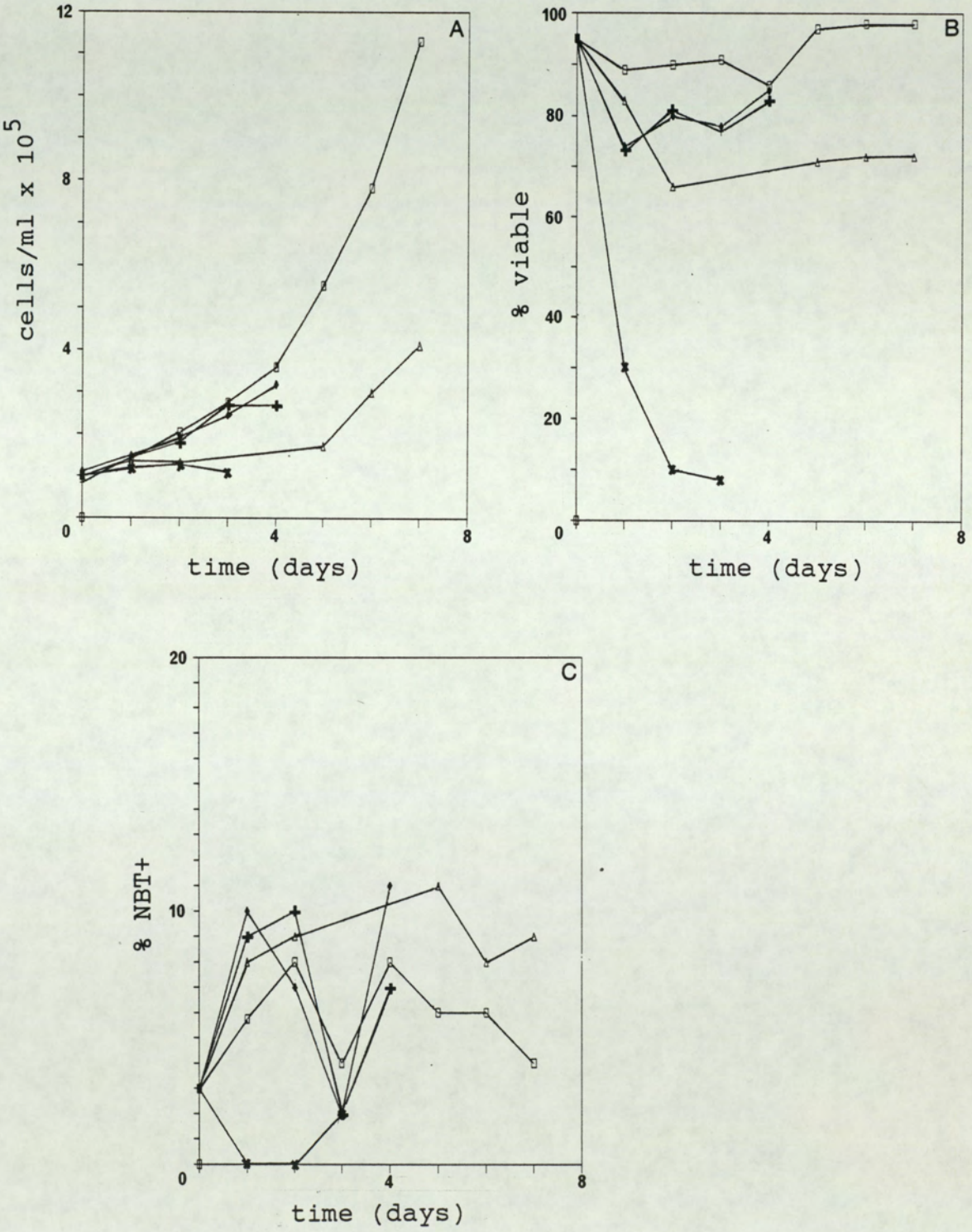
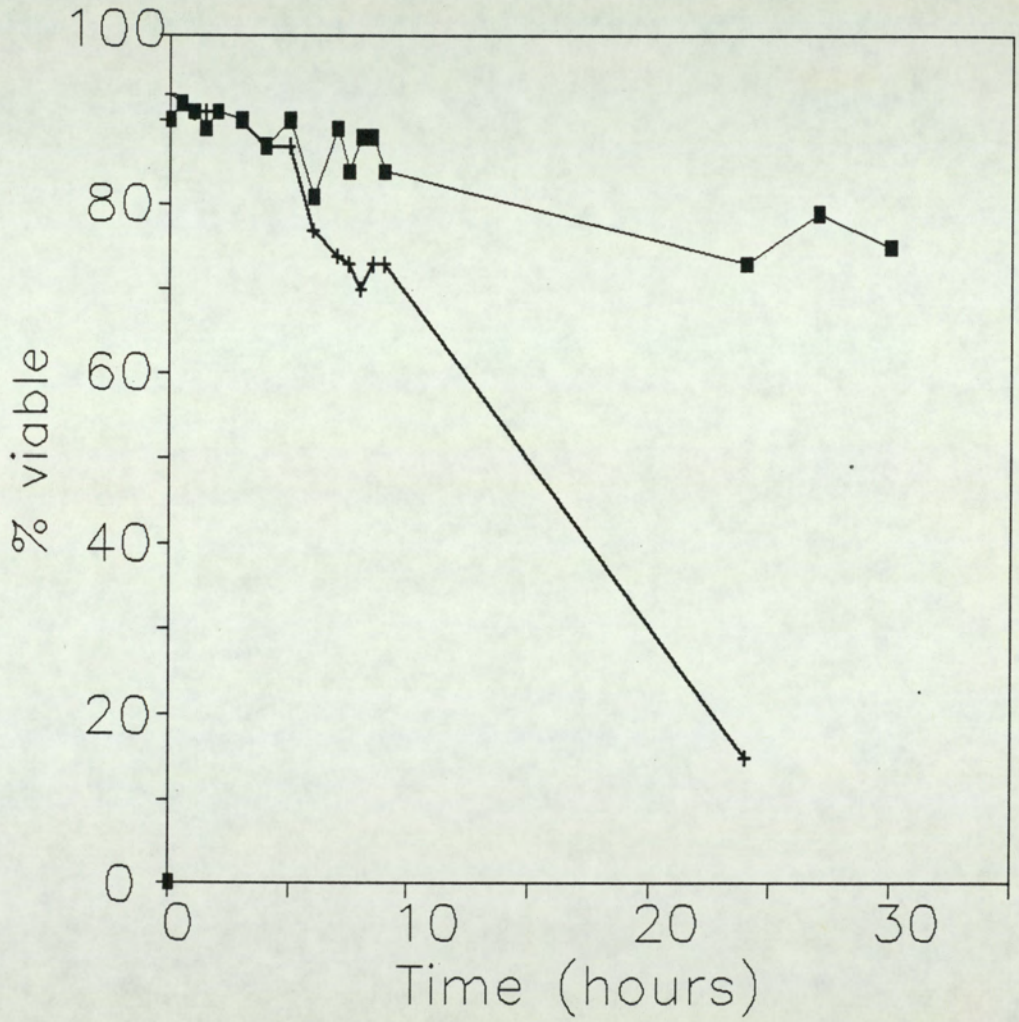


Figure 4.31 The effect on HL-60 cells of brief heat shock at 42.0°C or 43.0°C.

Cells were incubated at 42.0°C (■) or 43.0°C (+) and viability was determined over a period up to 27 hours.



to 43.0°C, and only 24 hours incubation at this temperature reduced viability to 15% (Figure 4.31).

These preliminary experiments showed that heat shock at temperatures above 42°C was toxic to the cells. To determine whether differentiation could be induced, HL-60 cells were incubated for short periods at 43.5°C, then cooled and incubated at 37°C for a total of 4 days, to allow development of the differentiated phenotype. Table 4.15 and Figure 4.32 show the average of six experiments. It was clear that 20 minutes of heat shock at this temperature had no effect on cell growth, viability or differentiation, but cell growth was inhibited as the length of heat shock was increased. 80 minutes at 43.5°C prevented any proliferation over the four day period and reduced viability to 34%, without inducing differentiation.

Differentiation was induced by a 60 minute heat shock at this temperature, with an average of 20% of cells capable of reducing NBT after 4 days. The extent of differentiation was rather variable, but was always significantly higher than that in untreated cells. Growth in the cells heated for 60 mins was inhibited so that by day four they had undergone 1.5 doublings compared to 3.5 in the untreated cells, and viability was decreased to 72%.

TABLE 4.15

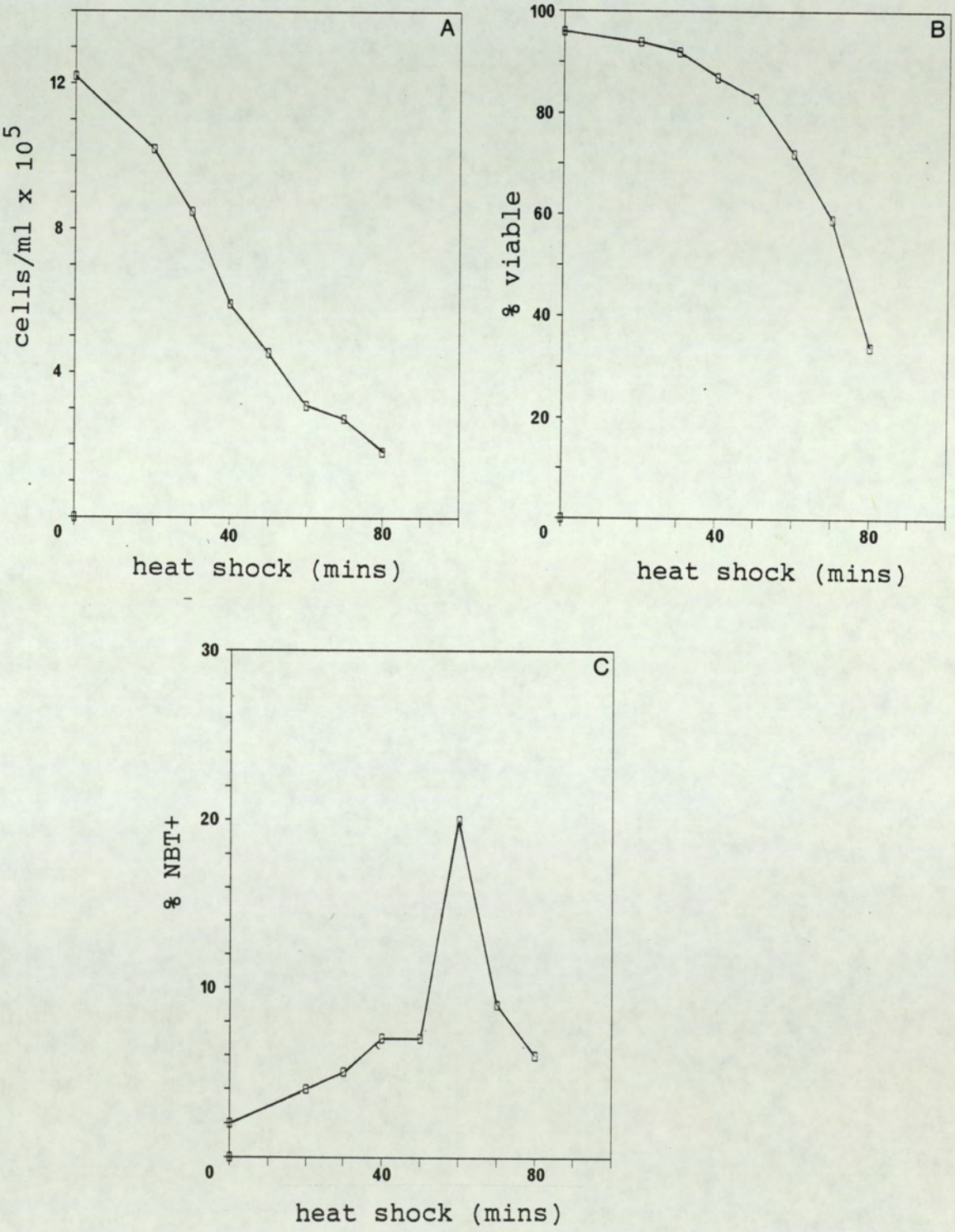
The effect on HL-60 cells of heat shock at 43.5°C for short periods, followed by incubation at 37°C for a total of four days

| Time at 43.5°C (mins) | cells/ml x $10^5$ | % viable | % NBT+ |
|---|---|---|---|
| 0 | 12.20 ± 2.50 | 95.83 ± 1.50 | 2.21 ± 1.57 * |
| 20 | 10.20 ± 2.10 | 93.69 ± 2.14 | 3.75 ± 2.45 |
| 30 | 8.48 ± 1.24 | 92.18 ± 2.25 | 5.44 ± 2.85 |
| 40 | 5.91 ± 0.84 | 86.62 ± 1.81 | 7.20 ± 4.52 |
| 50 | 4.55 ± 1.88 | 83.18 ± 1.97 | 7.20 ± 1.23 |
| 60 | 3.09 ± 1.02 | 71.71 ± 13.09 | 20.06 ± 7.29 |
| 70 | 2.73 ± 1.63 | 59.37 ± 30.52 | 8.97 ± 2.59 |
| 80 | 1.81 ± 0.68 | 34.12 ± 23.64 | 5.70 ± 0.60 |

* Figures represent mean ± S.D. of 6 experiments.

Figure 4.32 The induction of differentiation of HL-60 cells heat shocked at 43.5°C.

Cells were heated at 43.5°C for the periods shown, cooled and returned to 37°C for 4 days, when cell growth (A), viability (B) and NBT reduction (C) were determined. Each point represents the average of 6 experiments.



It is striking that only one hour of the "stress" was required to commit the cells to differentiate when chemical inducers required at least 24 hours. Heat shock was, however, a "weak" inducer of differentiation because only 20% of the cells were induced to differentiate. This is discussed in more detail in sections 5.5 to 5.7.

As expected, the induction of differentiation in HL-60 cells by heat occurred at a hyperthermic dose which was only marginally below cytotoxic, and heat shock at temperatures which did not induce cytotoxicity did not induce differentiation. This reinforced the idea that a stress response, in particular the heat shock response, is important in the commitment of HL-60 cells to differentiate.

The logical progression from these experiments was to determine whether heat shock proteins were actually synthesized during the induction of HL-60 cell differentiation by various agents.

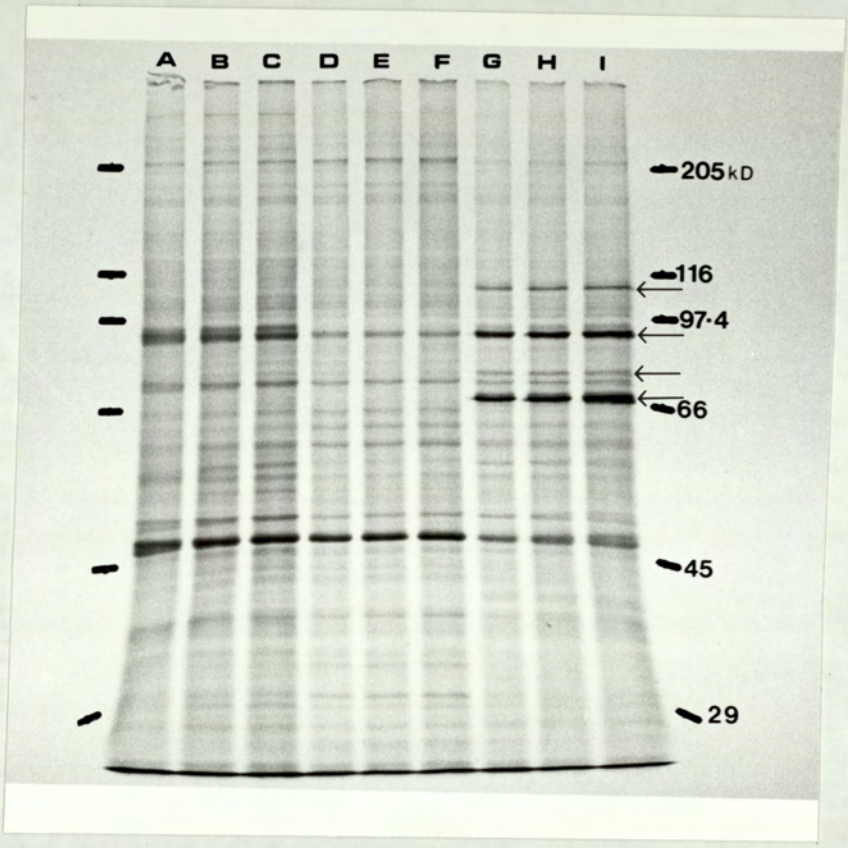
## 4.7 Analysis by electrophoresis of heat shock protein synthesis during the induced differentiation of HL-60 cells

### 4.7.1 Analysis of protein synthesis after incubation for 24 hours with 170mM NMF

A preliminary experiment was performed using samples prepared from untreated cells, cells treated for 24 hours with 170mM NMF, and cells heat shocked at 43.5°C for 1 hour then returned to 37°C for a 2 hour recovery period. The cell proteins which were synthesized were labelled using $^{35}$S-methionine, then equal cell numbers were loaded per lane of an SDS-polyacrylamide gel. Because treatment might induce large changes in the total amount of protein in the cells, it was thought to be valid to use samples of equal cell numbers rather than equal amounts of protein. The autoradiograph of the gel clearly shows bands of proteins of different molecular weights (Figure 4.33). The synthesis of four heat shock proteins was induced in the heated cells (marked with arrows); the molecular weights were determined after plotting a standard curve of the logarithm of molecular weight against distance migrated for the molecular weight standards. The heat shock proteins synthesized had apparent molecular weights of 68, 76, 87 and 107 kD, corresponding to hsps 70, 80, 90 and 110 respectively, as previously described in other

Figure 4.33 Protein synthesis in HL-60 cells after a 24 hour incubation with 170mM NMF, or a heat shock.

Autoradiograph of $^{35}$S-methionine-labelled proteins separated by SDS-polyacrylamide gel electrophoresis. Cells were untreated (A - C), treated for 24 hours with 170mM NMF (D - F) or heat shocked for 1 hour at 43.5°C and allowed to recover for 2 hours at 37°C (G - I).



mammalian cells by Welch et al (1982).

The autoradiograph was scanned using an LKB 2202 Ultroscan Laser Densitometer with a GelScan XL program, to determine the density of the bands of proteins of interest. The rates of synthesis of the various proteins in NMF or heat-treated cells were then calculated and compared to the synthesis in the untreated cells. Synthesis of actin (apparent Mw 45 kD) increased by about 4% in NMF treated cells, and decreased by 50% in heated cells, compared to controls. Synthesis of hsp70 was negligible in NMF-treated and control cells, and increased 50-fold after heat shock. Synthesis of hsp80 was also negligible in NMF-treated and control cells, and increased 3-fold after heat shock. In contrast, hsp90 was synthesized in control cells; NMF-treated cells synthesized hsp90 at half this rate, and heated cells synthesized hsp90 at twice the rate of the untreated cells. Hsp110 was undetectable in control and NMF-treated cells, and increased at least 16-fold after heat shock.

This experiment showed that 24 hour treatment with 170mM NMF did not induce synthesis of any major heat shock proteins; their synthesis was in fact slightly inhibited compared to that in untreated cells.

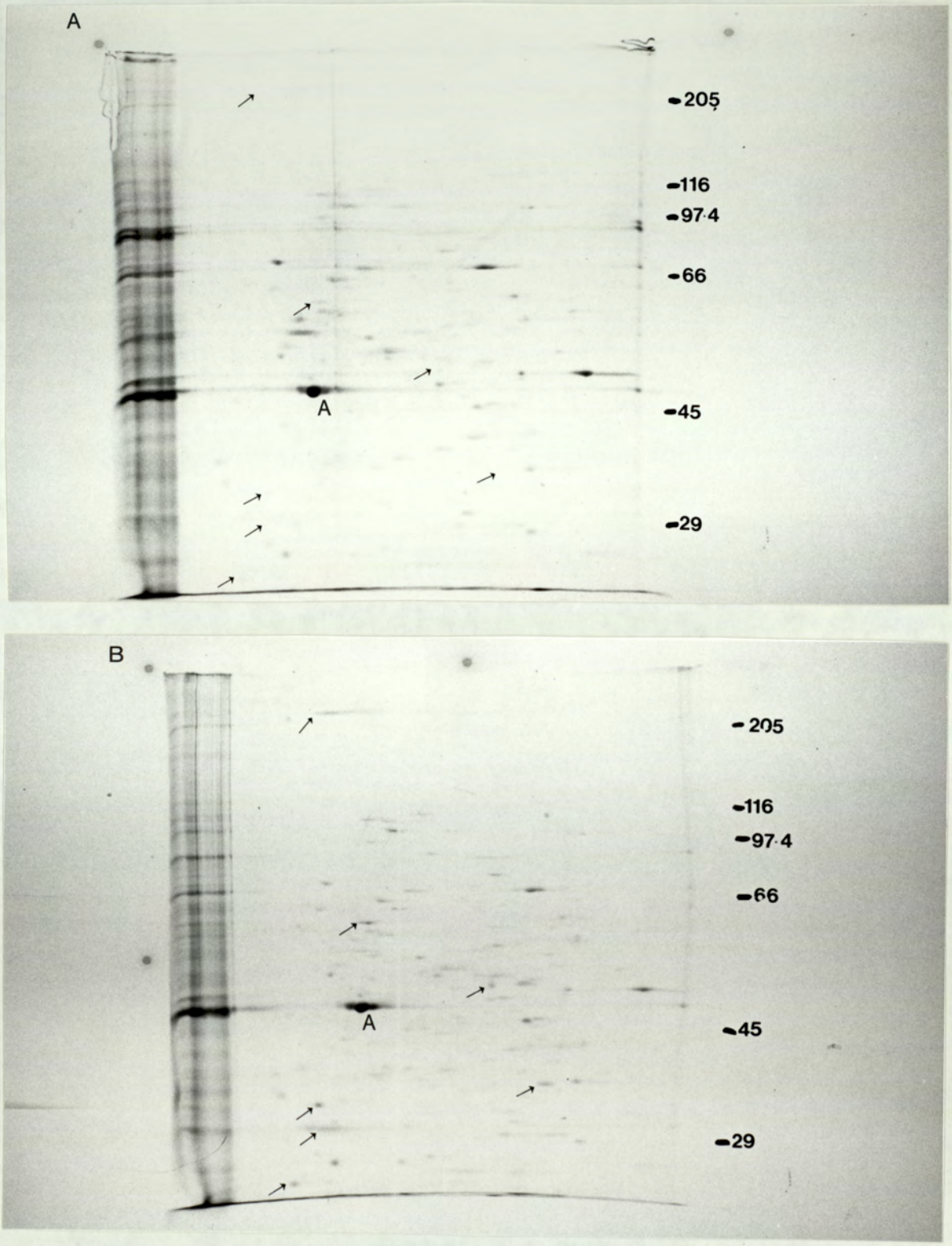
### 4.7.2 Analysis by two-dimensional electrophoresis of proteins synthesized after incubation for 24 hours with 170mM NMF

To confirm that hsp synthesis was not induced after 24 hour incubation of HL-60 cells with 170mM NMF, two-dimensional gel electrophoresis was performed on untreated cells and cells treated with NMF. Autoradiography of the gels (Figure 4.34) showed that there were few differences in the protein patterns between the two samples, although synthetic rates of many proteins were reduced in the NMF-treated cells. However, there were 7 proteins whose synthesis was apparently enhanced after NMF treatment (marked with arrows). These had apparent molecular weights of 195 - 251, 63 - 74, 50 - 55, 36, 32, 30, and 23 - 27 kD. The uncertainty of the molecular weights arose from the technical problems of ensuring good separation of the molecular weight markers in one dimension at the edge of the two-dimensional gel.

The protein of molecular weight 63 - 74 kD might represent hsp70, as other reports have shown that hsp70 tends to have a similar pI to actin (marked A on the autoradiograph) and therefore lies vertically above actin on two-dimensional gels (Singh and Yu 1984, Hatayama et al 1986, Bensaude and Morange 1983). However, if this protein was hsp70, the amount of induced protein synthesis

Figure 4.34 Protein synthesis in HL-60 cells after a 24 hour incubation with 170mM NMF.

Autoradiograph of $^{35}$S-methionine-labelled proteins separated by two-dimensional gel electrophoresis. Cells were untreated (A), or incubated with 170mM NMF for 24 hours (B). Proteins induced by NMF treatment are marked with arrows, actin is marked "A".



was very small, so further investigations were carried out to determine whether a large induction of hsp70 synthesis could be induced under different conditions.

### 4.7.3 Investigation of protein synthesis after 12 or 24 hour incubation with different concentrations of NMF or ethanol

HL-60 cells were heated as before or treated with 120 or 170mM NMF or 167mM ethanol for 12 or 24 hours (Figure 4.35). Heat shock of the cells induced synthesis of heat shock proteins with apparent molecular weights of 70, 78.5, 89 and 112 kD, corresponding to those observed previously (Section 4.7.1). This autoradiograph was scanned and the peak heights of any interesting proteins were measured from the gel scan, shown in Table 4.16. It is clear that hsp70 synthesis was not induced by any treatment except heat itself; neither were hsp80 or hsp110. Hsp90 was induced slightly by ethanol as well as by heat, although not by NMF.

There were several proteins which were not heat inducible, whose synthesis was induced after 12 and 24 hour incubation with either concentration of NMF or ethanol (apparent molecular weights 38, 47 (actin), 60 and 214 kD). Proteins with molecular weights of 36, 73 and 292 kD were induced after 24 hour incubations with NMF or ethanol, and a protein of 101kD was induced by ethanol

Figure 4.35 Protein synthesis in HL-60 cells after treatment for 12 or 24 hours with 120 or 170mM NMF, or 167mM ethanol.

Autoradiograph of $^{35}$S-methionine-labelled proteins separated by SDS-polyacrylamide gel electrophoresis. Cells were untreated (A) or treated with 120mM NMF for 12 hours (B); 120mM NMF for 24 hours (C); 170mM NMF for 12 hours (D); 170mM NMF for 24 hours (E); 167mM ethanol for 12 hours (F); 167mM ethanol for 24 hours (G); or heated at 43.5°C for 1 hour and allowed to recover at 37°C for 2 hours (H).

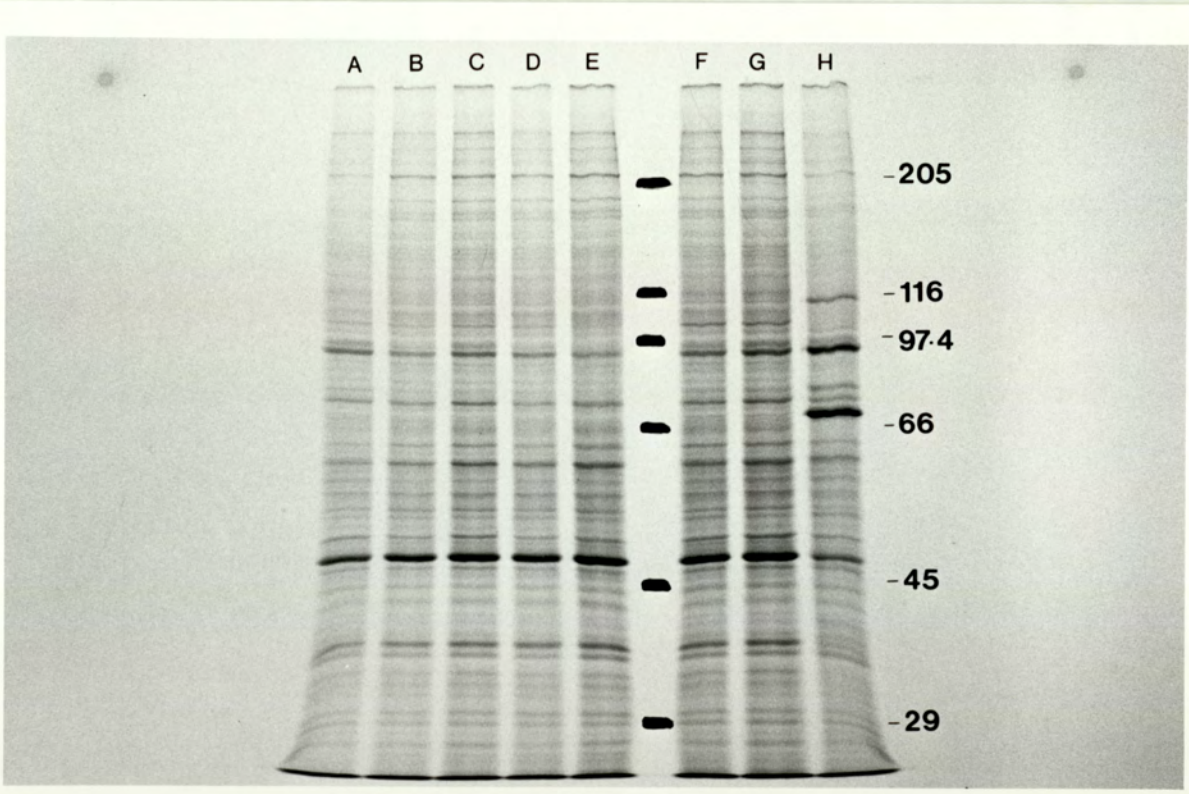


TABLE 4.16

Relative rates of protein synthesis, detected by laser densitometry of autoradiograph of $^{35}$S-methionine-labelled proteins

| | | NMF | | | | ethanol | | |
|---|---|---|---|---|---|---|---|---|
| | | 120mM | | 170mM | | 167mM | | heat |
| Mw | none | 12h | 24h | 12h | 24h | 12h | 24h | 1h |
| 36 | 0.10 | 0.12 | 0.19 | 0.12 | 0.22 | 0.18 | 0.19 | 0.13 |
| 38 | 0.18 | 0.24 | 0.29 | 0.27 | 0.38 | 0.32 | 0.35 | 0.11 |
| 47[a] | 0.61 | 0.83 | 0.98 | 0.87 | 0.95 | 0.88 | 0.96 | 0.40 |
| 60 | 0.15 | 0.21 | 0.37 | 0.22 | 0.33 | 0.26 | 0.34 | 0.18 |
| 70[b] | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.10 |
| 73 | 0.17 | 0.15 | 0.31 | 0.14 | 0.20 | 0.27 | 0.32 | 0.15 |
| 79[c] | 0.09 | 0.03 | 0.05 | 0.02 | 0.03 | 0.10 | 0.10 | 0.17 |
| 89[d] | 0.33 | 0.21 | 0.35 | 0.25 | 0.20 | 0.38 | 0.49 | 0.68 |
| 101 | 0.11 | 0.09 | 0.17 | 0.09 | 0.10 | 0.25 | 0.25 | 0.03 |
| 112[e] | 0.03 | 0.05 | 0.07 | 0.05 | 0.07 | 0.08 | 0.09 | 0.25 |
| 214 | 0.08 | 0.17 | 0.23 | 0.16 | 0.24 | 0.21 | 0.23 | 0.07 |
| 292 | 0.07 | 0.07 | 0.15 | 0.07 | 0.15 | 0.13 | 0.20 | 0.03 |

a = actin, b = hsp70, c = hsp80, d = hsp 90, e = hsp110

after 12 and 24 hours, but not by NMF. None of these proteins were identified, so their role in the induction of differentiation was unclear.

### 4.7.4 Measurement of hsp70 after 24 hour incubation with 170mM NMF

The cellular levels of a particular protein are not controlled entirely by its rate of synthesis, however: the amount of a protein in a cell is determined by the balance between the rate of synthesis and the rate of degradation. A stable protein which is degraded very slowly will be present in high levels in the cell even if its rate of synthesis is slow; in contrast a labile protein with a short half-life will be present in very small amounts in the cell unless the rate of synthesis is very high. The rate of turnover of a protein depends not only on the protein structure, but on the metabolic state of the cell and whether the protein has undergone covalent modifications or binding to some cellular structure (Rivett 1986).

Because the rate of synthesis of a protein does not necessarily correlate with the cellular levels of the protein, it was important to study the cellular levels of hsp70 as well as its rate of synthesis. Three cell samples (untreated, incubated for 24 hours with 170mM NMF, and heat shocked) were separated by electrophoresis as usual; the proteins were transferred onto nitrocellulose

by Western blotting, then probed with a mouse antiserum to human hsp70 (Lewis and Pelham 1985), followed by protein A peroxidase. The peroxidase colour reaction revealed large amounts of hsp70 in the heat shocked cells, and a low but detectable amount in the untreated cells, with slightly more in the NMF-treated cells (Figure 4.36).

Autoradiography of the blot for the same length of time as a duplicate gel revealed that autoradiography was much more efficient from the blot than from the gel (Figure 4.37). The autoradiographs showed that hsp70 synthesis was elevated as expected in heat shocked cells, but was not elevated in the NMF treated cells. Heat shocked cells also synthesized hsps 80, 90, 100 and 110 (apparent molecular weights 77, 89, 105 and 114 kD respectively), but none were visible in the NMF treated cells.

The antibody staining intimated that there might be slightly higher levels of hsp70 in the cells after 24 hours incubation with 170mM NMF compared to controls, despite the fact that synthesis was not elevated at this time. It was proposed that hsp70 synthesis might have been elevated at some point earlier than 24 hours, and that elevated levels of the protein remained after the rate of synthesis had declined again. Experiments to test this hypothesis are outlined below (Section 4.7.6)

Figure 4.36 Hsp70 levels in HL-60 cells after 24 hour incubation with 170mM NMF, or heat shock.

Nitrocellulose blot of proteins separated by SDS-polyacrylamide gel electrophoresis, probed with anti-hsp70, then protein A-peroxidase. Cells were untreated (C); incubated for 24 hours with 170mM NMF (N); or heated at 43.5°C for 1 hour and allowed to recover at 37°C for 2 hours (H).

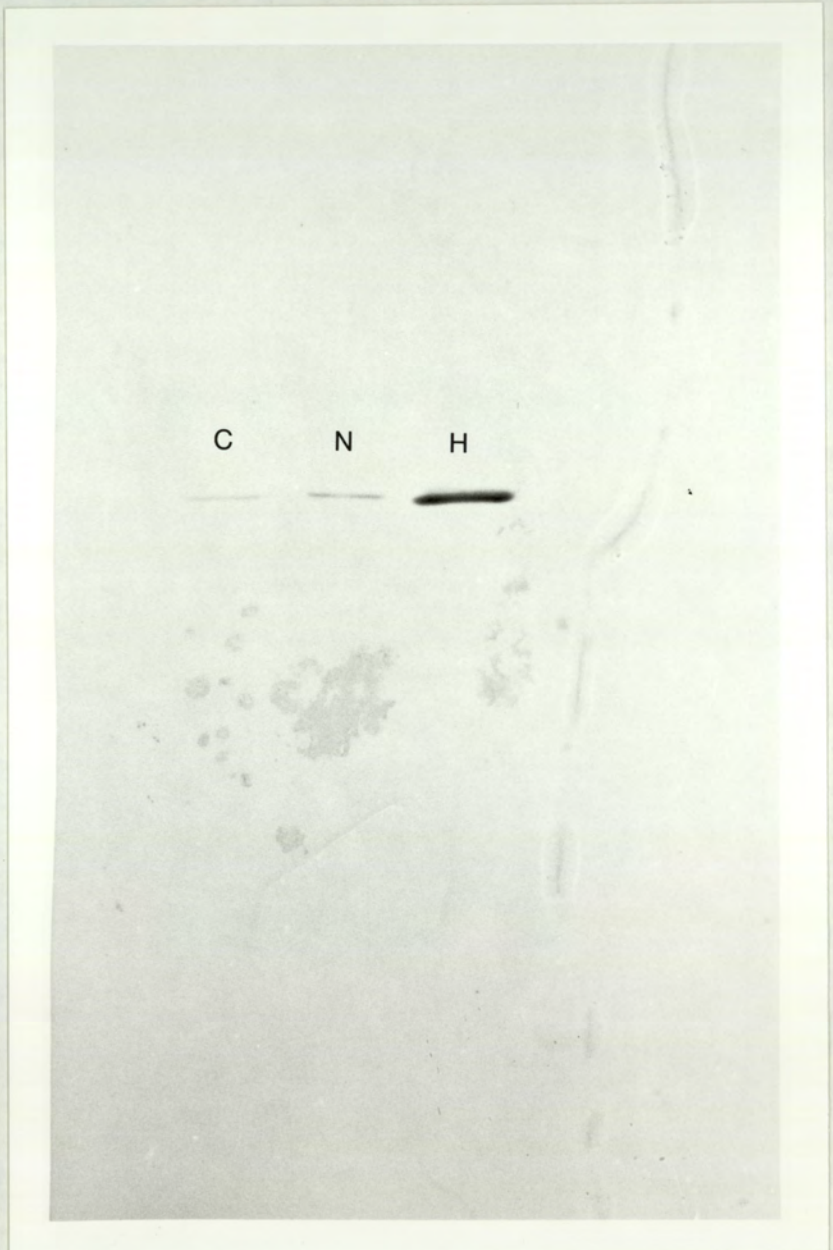
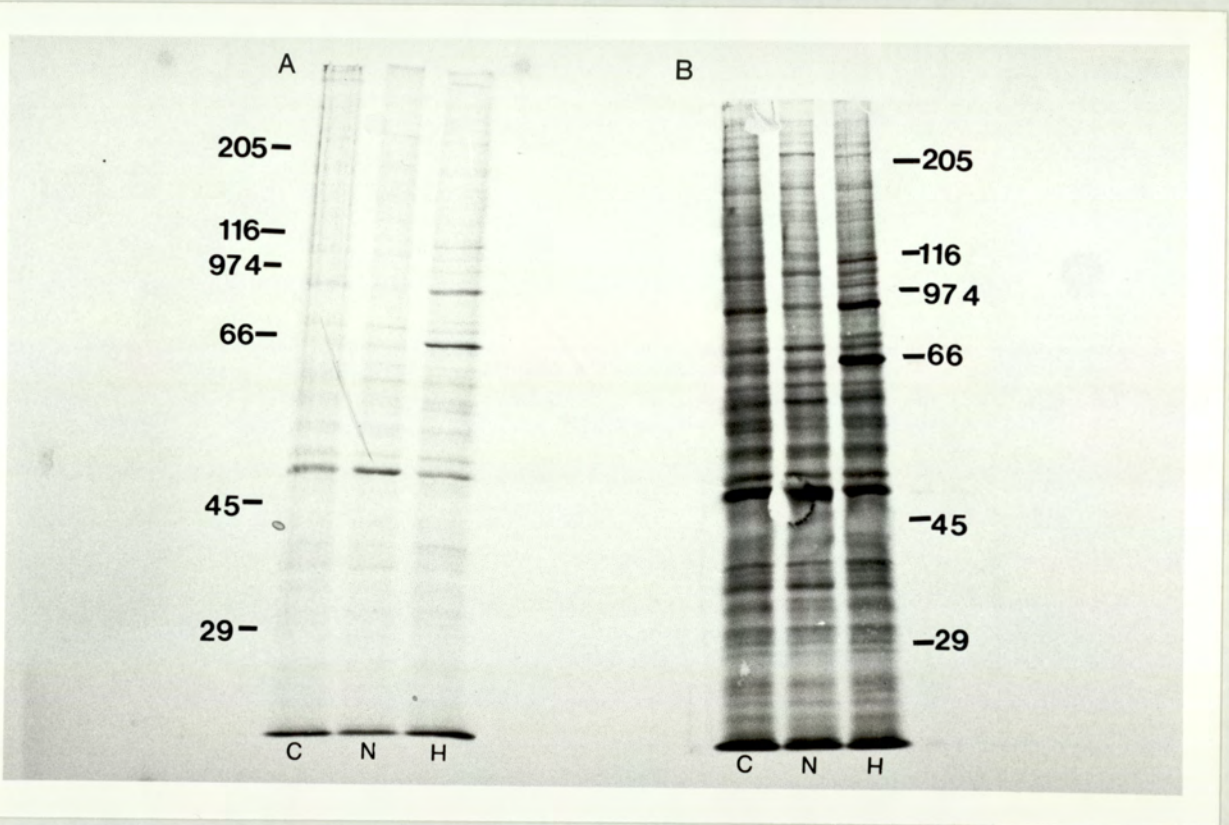


Figure 4.37 Hsp synthesis in HL-60 cells after 24 hour incubation with 170mM NMF, or heat shock.

A/ Autoradiograph of $^{35}$S-methionine-labelled proteins on dried SDS polyacrylamide gel. B/ Autoradiograph of nitrocellulose blot from duplicate gel. Cells were untreated (C); incubated with 170mM NMF for 24 hours (N); or heated for 1 hour at 43.5°C and allowed to recover at 37°C for 2 hours (H)



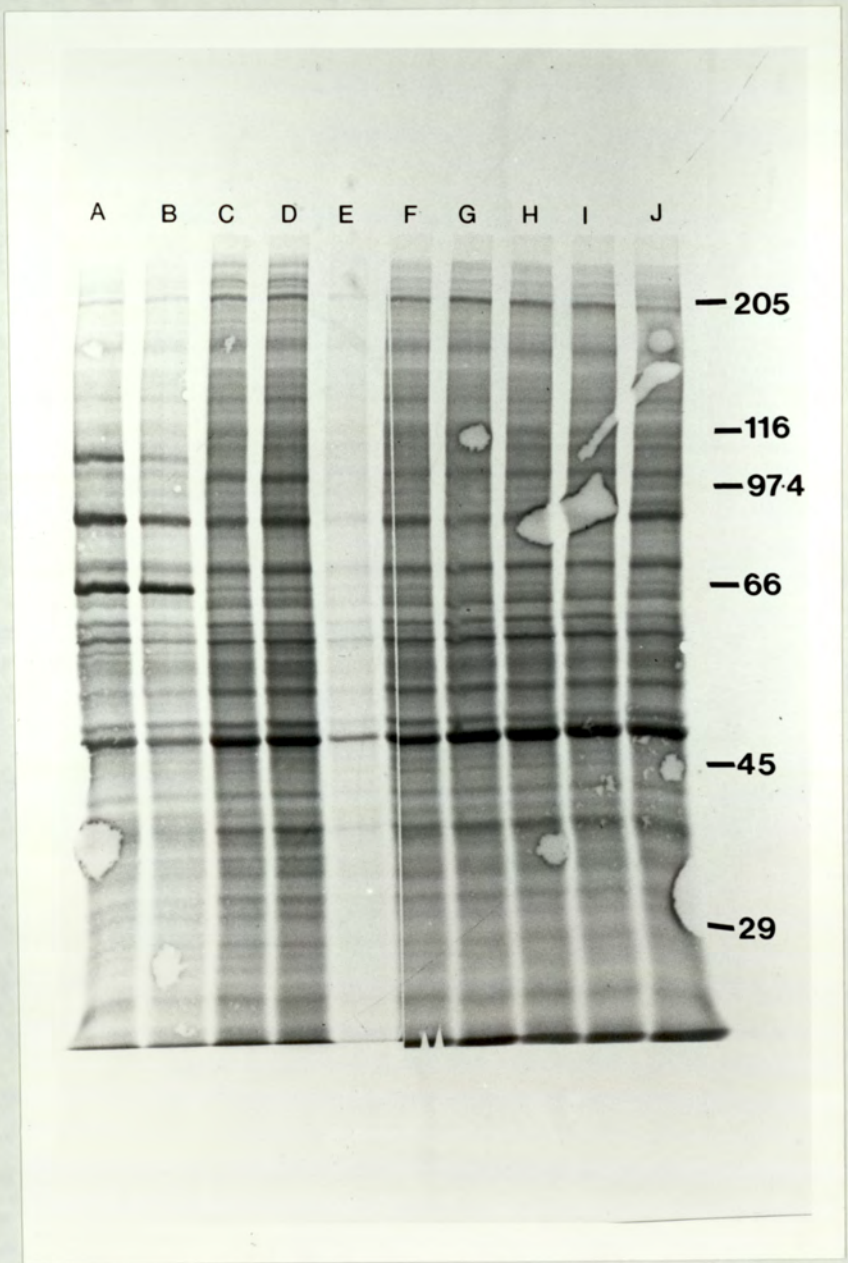
4.7.5 Analysis of hsp synthesis during the induction of differentiation by various agents

In order to determine whether the agents used in section 4.5 induced hsp synthesis during the induction of differentiation, cells were incubated for a period of 24 hours with the optimum concentration of each agent for the induction of differentiation. The proteins were then labelled with $^{35}$S-methionine (Figure 4.38). The autoradiograph of the nitrocellulose blot showed an induction of the synthesis of the major hsps in cells heated for 1 hour either at 43.5°C or 45°C. Hsp70 synthesis appeared to be slightly elevated in procaine- and lidocaine-treated cells, but not in any of the other samples, and there was no induced synthesis of any other hsps with any of the drugs.

Cadmium sulphate, a weak inducer of differentiation (with a maximum of 14% NBT positive cells on day 4), radically inhibited all protein synthesis. It is possible that cadmium only induced a small number of cells to differentiate because they could not synthesize the proteins required for the differentiated phenotype. Neither NMF, ethanol, DMSO, arsenite, procaine nor lidocaine, which were stronger inducers of differentiation (with more than 50% of cells NBT positive on day 4), had any adverse effects on the synthesis of normal cellular

Figure 4.38 Protein synthesis in HL-60 cells after treatment for 24 hours with a variety of agents at the optimum concentration for the induction of differentiation.

Autoradiograph of nitrocellulose blot of $^{35}$S-methionine-labelled proteins separated by SDS-polyacrylamide gel electrophoresis. Cells were heated for 1 hour at 45.0°C (A) or 43.5°C (B) then allowed to recover at 37°C for 2 hours, or were treated for 24 hours with 5mM procaine hydrochloride (C); 3mM lidocaine hydrochloride (D); 60uM cadmium sulphate (E); 6uM sodium arsenite (F); 160mM dimethylsulphoxide (G); 213mM ethanol (H); 170mM NMF (I); or were left untreated (J).



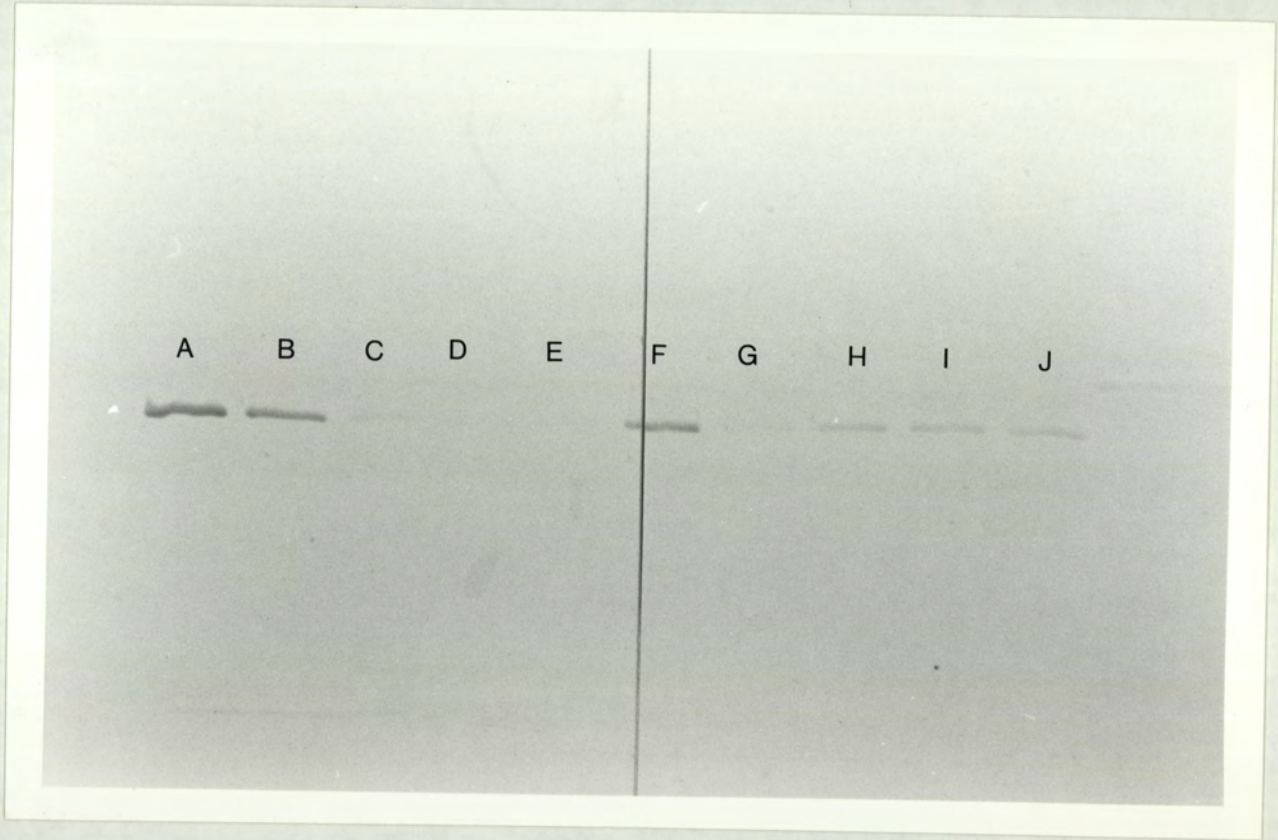
proteins after the 24 hour incubations.

When the nitrocellulose blot was probed with anti-hsp70, some interesting results were observed (Figure 4.39): hsp70 was detectable at low levels in untreated, NMF-, and ethanol-treated cells, but was virtually undetectable in DMSO-, cadmium-, lidocaine- or procaine-treated cells. This was surprising as lidocaine and procaine had an enhanced rate of synthesis of hsp70 (as determined from the autoradiograph). Furthermore, hsp70 was present in arsenite-treated cells at high levels comparable to those in the heat shocked cells, whereas the rate of hsp70 synthesis was not enhanced in the autoradiograph of arsenite-treated cells.

This experiment was important in that it demonstrated the lack of correlation between the rate of synthesis of a protein and its cellular level. There are several possible explanations for the high levels of hsp70 in arsenite treated cells in the absence of enhanced synthesis. Proteins were labelled 24 hours after the addition of arsenite to the cells; it is possible that hsp70 synthesis was enhanced prior to this point and that as high levels of the protein built up synthesis declined again. Hsps are known to repress their own synthesis (Yost and Lindquist 1986). Another possibility is that arsenite had no effect on hsp70 synthesis at any stage, but stabilized the protein in some way so that it was not

Figure 4.39 The levels of hsp70 in HL-60 cells after treatment for 24 hours with a variety of agents at the optimum concentration for the induction of differentiation.

Nitrocellulose blot of proteins separated by SDS-polyacrylamide gel electrophoresis, probed with anti-hsp70 and protein A-peroxidase. Cells were heated for 1 hour at 45.0°C (A) or 43.5°C (B) and allowed to recover at 37°C for 2 hours; or were treated for 24 hours with 5mM procaine hydrochloride (C); 3mM lidocaine hydrochloride (D); 60uM cadmium sulphate (E); 6uM sodium arsenite (F); 160mM dimethylsulphoxide (G); 213mM ethanol (H); 170mM NMF (I); or were left untreated (J).



degraded at the usual rate and hence built up to high levels.

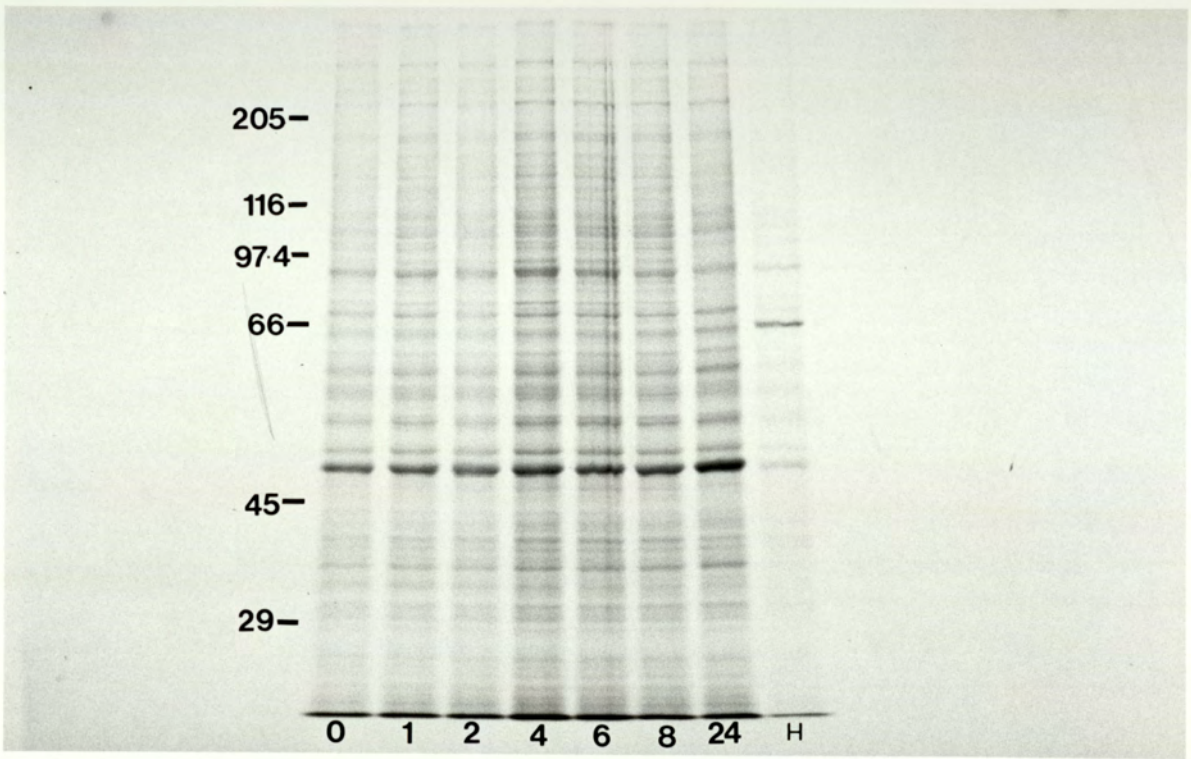
The conclusion from this experiment was that neither synthesis nor cellular levels of hsp70 were consistently elevated after 24 hours of incubation with known inducers of differentiation. The 24 hour incubation was chosen as it was the minimum period of exposure to inducer that resulted in commitment of a significant number of cells to differentiate. However, the possibility remained that either the synthesis or levels of hsp70 were enhanced either prior to this during the precommitment period, or after this during the stochastic increase in the percentage of committed cells.

### 4.7.6 Analysis of synthesis and cellular levels of hsp70 during precommitment and commitment with 170mM NMF

To investigate whether synthesis or cellular levels of hsp70 were elevated at some time other than 24 hours after the addition of the inducer of differentiation, cells were labelled during the precommitment period - at 1, 2, 4, 6, 8 or 24 hours after the addition of 170mM NMF (Figure 4.40). In this experiment, the heat shock was more inhibitory to protein synthesis than usual, and only hsp70 synthesis was induced, with virtually no other proteins synthesized at detectable levels. In none of the NMF-treated samples was synthesis of hsp70 or other hsp

Figure 4.40 The effect on protein synthesis of incubation of HL-60 cells with 170mM NMF for 1 to 24 hours.

Autoradiography of nitrocellulose blot of $^{35}$S-methionine-labelled proteins separated by SDS-polyacrylamide gel electrophoresis. Cells were incubated with 170mM NMF for 0, 1, 2, 4, 6, 8 and 24 hours, or heat shocked for 1 hour at 43.5°C and allowed to recover for 2 hours at 37°C (H).



apparent. This experiment was repeated with different cell samples and again no hsp70 synthesis was detectable during NMF treatment up to 24 hours. When probed with anti-hsp70 there was no detectable hsp70 in any of the samples.

This experiment showed that neither the rate of synthesis nor the levels of hsp70 were enhanced during the 24 hour pre-commitment period prior to the induction of differentiation with NMF.

In view of this evidence, it was proposed that during the induction of differentiation with NMF, the 24 hour precommitment period consisted of a build up of a certain cellular lesion, perhaps damaged protein, and that once a threshold was reached, synthesis of hsps was triggered and the cells became committed to differentiate. If this was the case, hsp70 synthesis would be expected to be elevated between 24 and 48 hours of incubation with NMF, during the stochastic increase in the numbers of committed cells.

To test this hypothesis, protein synthesis and hsp70 levels were analysed in cells treated for between 2 and 54 hours with 170mM NMF. In this experiment the cells were labelled with $^{14}$C-leucine instead of $^{35}$S-methionine, in case any methionine-free proteins which had not previously been detected were important. Autoradiography revealed that the rate of hsp70 synthesis decreased as the length

of incubation with NMF increased up to 54 hours, but synthesis of the other hsps was not affected by 170mM NMF treatment (Figure 4.41). The relative rates of hsp70 synthesis were determined by laser densitometry of the autoradiograph, and the figures are shown in Table 4.17. The rate of synthesis of hsp70 decreased almost 5-fold after 54 hours incubation with NMF, with the decrease beginning as early as 2 hours.

Probing the blot of this gel with anti-hsp70 revealed, surprisingly, that cellular levels of hsp70 increased with the length of incubation with 170mM NMF (Figure 4.42). The relative amounts of hsp70 in the samples were measured by laser densitometry of the stained blot (Table 4.17); the figures obtained confirmed the results seen with the naked eye. Up to 20 hours of incubation with NMF there was no detectable increase in hsp70 levels compared to untreated cells (concordant with previous experiments), but at 30 and 44 hours there was an increase (more than 30-fold) in hsp70 levels, decreasing slightly again after 54 hours. A repeat of this experiment, using different cell samples, showed a similar increase in hsp70 levels, but the levels apparently remained constant between 44 and 54 hours hours in this case. It would be useful to repeat the experiment using more time points, to determine whether the increase in hsp70 levels was indeed transient.

Figure 4.41 Protein synthesis in HL-60 cells after treatment with 170mM NMF for 2 to 54 hours, or with high concentrations of NMF or ethanol for 1 hour.

Autoradiograph of nitrocellulose blot of $^{14}$C-leucine-labelled proteins separated by SDS-polyacrylamide gel electrophoresis. Cells were untreated (A) or incubated with 170mM NMF for 2 hours (B); 6 hours (C); 20 hours (D); 30 hours (E); 44 hours (F); or 54 hours (G); incubated for one hour with 6% NMF (H) or 6% ethanol (I); or heated at 43.5°C for 1 hour and allowed to recover at 37°C for 2 hours (J).

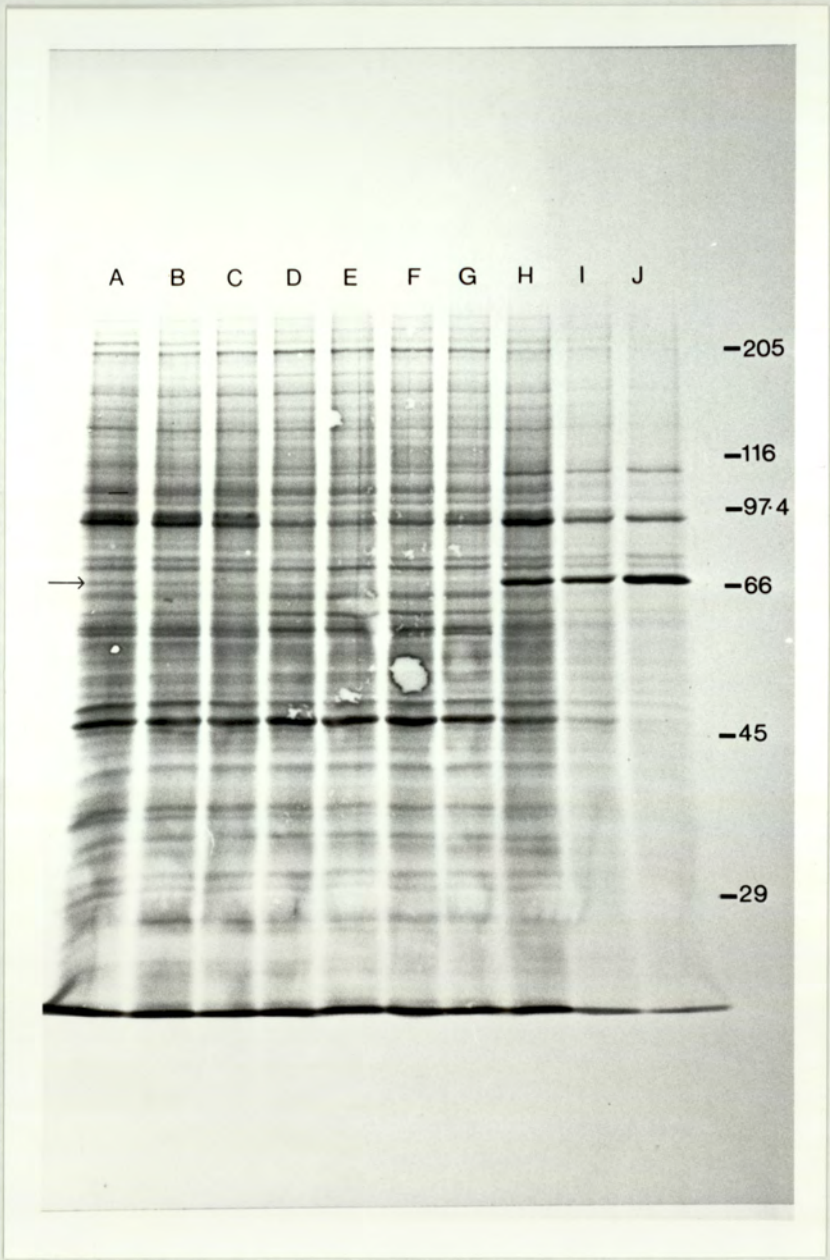


Figure 4.42 The levels of hsp70 after treatment of HL-60 cells with 170mM NMF for 2 to 54 hours, or with high concentrations of NMF or ethanol for 1 hour.

Nitrocellulose blot of proteins separated by SDS-polyacrylamide gel electrophoresis, probed with anti-hsp70 and protein A-peroxidase. Cells were untreated (A) or incubated with 170mM NMF for 2 hours (B); 6 hours (C); 20 hours (D); 30 hours (E); 44 hours (F); or 54 hours (G); incubated for one hour with 6% NMF (H) or 6% ethanol (I); or heated at 43.5°C for 1 hour and allowed to recover at 37°C for 2 hours (J).

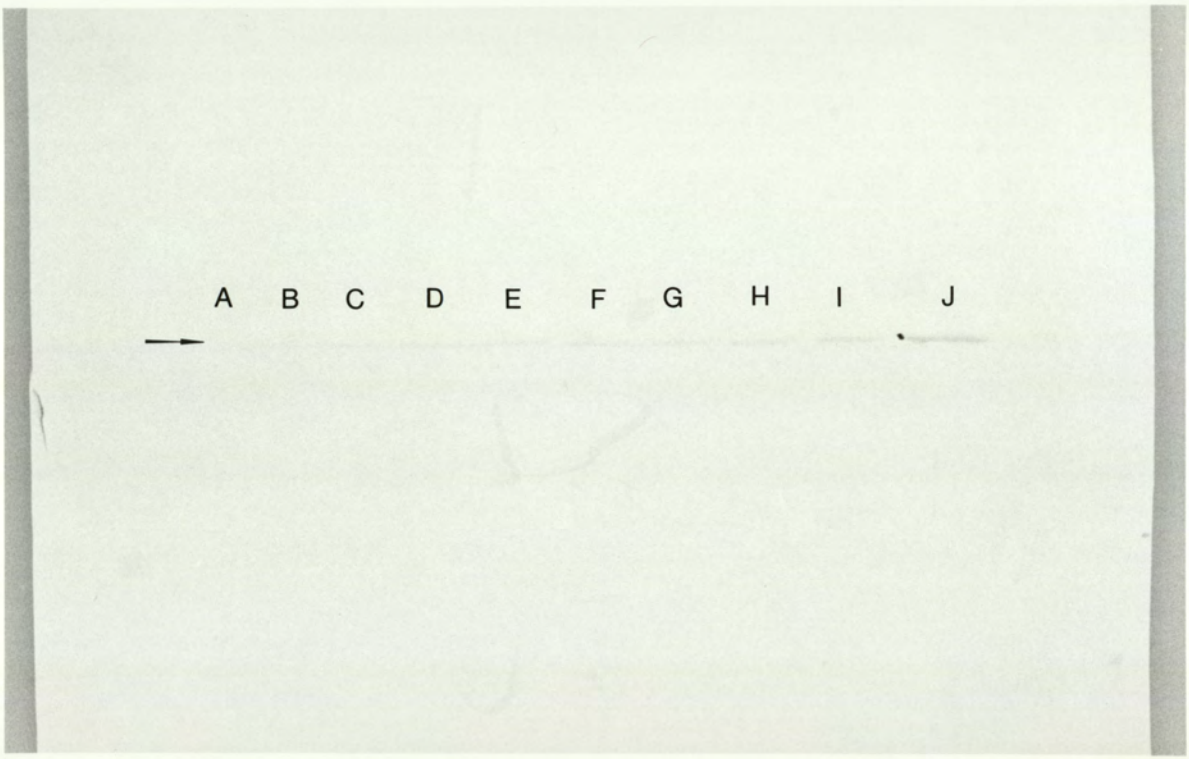


TABLE 4.17

The relative rate of hsp70 synthesis and relative amount of hsp70 in treated HL-60 cells, measured by laser densitometry.

| treatment | synth. rate* | hsp70 level* |
|---|---|---|
| heat | 717 | 220 |
| 6% ethanol, 1 hour | 380 | 57 |
| 6% NMF, 1 hour | 361 | 52 |
| 170mM NMF, 54 hours | 11 | 10 |
| 170mM NMF, 44 hours | 14 | 37 |
| 170mM NMF, 30 hours | 18 | 30 |
| 170mM NMF, 20 hours | 22 | 6 |
| 170mM NMF, 6 hours | 23 | 0 |
| 170mM NMF, 2 hours | 37 | 0 |
| control | 51 | 0 |

* The relative rate of hsp70 synthesis was determined from the density of the band on the autoradiograph of the nitrocellulose blot.

The relative amount of hsp70 was determined from the density of the peroxidase stain on the nitrocellulose blot probed with anti-hsp70.

The increase in hsp70 levels was temporally related to the commitment of cells to differentiate in response to NMF, as outlined in section 4.2. However, while the levels of the protein increased 30-fold after 44 hours, the rate of synthesis was decreased nearly 4-fold. This was a similar situation to that discussed for arsenite (Section 4.7.5). With NMF, however, many time points had been studied which ruled out the possibility that a transient stimulation of hsp70 synthesis had been missed. This would suggest that treatment of HL-60 cells with 170mM NMF stabilized hsp70 and reduced its rate of proteolysis.

### 4.7.7 Investigation of hsp synthesis in response to high concentrations of NMF and ethanol

To determine whether high concentrations of ethanol or NMF were capable of inducing hsp synthesis, cells were treated with 6% ethanol or 6% NMF for 1 hour before labelling the proteins with [$^{14}$C]-leucine. These were the conditions used by Li and Werb (1982) when they reported that ethanol induced hsp synthesis in Chinese hamster fibroblasts. Under these conditions, ethanol and NMF both induced the synthesis of hsps 70, 90 and 110 (Figure 4.41 and Table 4.17). Hsp 70 synthesis was elevated by ethanol or NMF more than 7-fold compared to the rate in untreated cells; heat shock increased the rate of hsp70 synthesis more than 12-fold.

Analysis of the probed blot of this gel revealed that hsp70 levels were elevated at least 50-fold over control levels in the cells treated with 6% ethanol or NMF, while heat shock induced an increase of hsp70 at least 200-fold (Figure 4.42 and Table 4.17).

This experiment showed that NMF was capable of inducing the full heat shock response, provided a high concentration (6% or 1M) was administered for a short period of time. The mechanism by which 1M NMF can induce hsp synthesis, and how this relates to the increase in hsp70 levels observed by 170mM NMF, will be discussed later in section 5.9.

# CHAPTER FIVE

# DISCUSSION

## 5.1 The induction of differentiation with NMF

NMF induced optimal differentiation at a concentration of 170mM, which was marginally below a cytotoxic concentration, in accordance with the findings of Langdon and Hickman (1987). Significant differentiation was first apparent 2 days after the addition of 170mM NMF, and more than 80% of the cells underwent granulocytic differentiation after incubation for 4 days. The cells gained the ability to produce superoxide and were able to reduce the dye nitroblue tetrazolium as a consequence of this; they also became able to phagocytose yeast particles. As the cells differentiated they accumulated in the $G_1$ (or $G_0$) phase of the cell cycle, and the cell size decreased concomitant with a change in the nuclear morphology indicative of differentiation from promyelocytes to myelocytes, metamyelocytes and banded granulocytes (Section 4.1).

## 5.2 The requirement for inhibition of proliferative potential

Manipulation of the growth conditions, to alter the proliferative state of the HL-60 cells, in the presence or absence of the inducer NMF, revealed that differentiation was only induced when the proliferative potential of the cells was compromised (Section 4.3). However, the cells

needed to be able to undergo one or two divisions before proliferation ceased completely, in order to develop the mature phenotype. This requirement was determined from studies in which NMF was added to cells at a plateau phase of growth so that they were incapable of division. This technique was preferable to those used by other groups who blocked cell division with thymidine or hydroxyurea prior to the addition of inducer such as DMSO or retinoic acid (Ferrero et al 1982, Hemmi and Breitman 1984). These reports suggested that cell division was not required for the induction of differentiation in HL-60 cells, but the results are questionable since thymidine and hydroxyurea were weak inducers of differentiation themselves (see section 1.7.1.2).

Fibach et al (1982b) explained this phenomenon rather well when they stated that "the potential for self-renewal was found to be irreversibly lost early during differentiation of HL-60 cells to either granulocyte or macrophage, before the cells had actually switched off their replicative mechanism, and were still able to complete 2 or 3 additional cell cycles."

Removal of serum from the cell cultures induced differentiation, but only in 15% of the cells. Serum deprivation inhibited proliferation to such an extent that less than 20% of the population were able to divide once after removal of the serum, so less than 20% of the cells

would be expected to differentiate. Other possible explanations for the low efficiency of the induction of differentiation after deprivation of serum will be discussed in section 5.7.

The requirement for at least one cell division for the development of the differentiated phenotype was not surprising; normal promyelocytes undergo division in order to give rise to more differentiated myelocytes and metamyelocytes. During normal granulopoiesis the cells do not lose their ability to proliferate until the metamyelocyte stage is reached, and the subsequent maturation to banded and segmented granulocytes occurs in the absence of proliferation (Bessis 1973).

Fibach et al (1982c) and Von Melchner and Höffken (1985) have indicated, from experiments in which HL-60 cells were cloned after treatment with inducer, that the loss of self-renewal potential of the cells preceded their commitment to differentiation. Yen et al (1986a) also stated that growth arrest was a prerequisite for phenotypic differentiation. Neither group explained why this inhibition should be necessary to trigger the cells to differentiate.

It is possible that whilst proliferating logarithmically the momentum of repeated cell division prevents the expression of those genes required for

differentiation, and only when division is inhibited can these genes be expressed. It is also possible that the inhibition of cell division by the sub-toxic effects of 170mM NMF or by starvation of growth factors and nutrients (when deprived of serum), results in the cells being "stressed" so that the amplification of genes or the expression of stress genes are induced. Over-expression of these genes could then induce differentiation, as discussed later.

## 5.3 The induction of differentiation as a two-step process

There were apparently two signals required for the induction of differentiation, one of which was directly related to the inhibition of proliferative potential of the cells. 100mM NMF by itself (which did not inhibit cell proliferation) did not induce significant differentiation, but in combination with a limitation of the proliferative potential (imposed by allowing the cells to grow to a plateau phase of growth at high density), differentiation was induced in more than 60% of the cells. 170mM NMF inhibited the proliferative potential of the cells because it was marginally cytotoxic, and this concentration of NMF also induced the subsequent events required for the induction of differentiation. Inhibition of the proliferative potential alone was not sufficient to induce differentiation; despite the fact that deprivation of serum induced differentiation, cells grown until they

reached the plateau phase of growth did not differentiate (Section 4.3).

This two-step hypothesis has been propounded by various other groups. Yen et al (1986a) showed that di-cis isomers of retinoic acid were able to complete early events leading to growth arrest but were unable to complete the later events leading to phenotypic differentiation which occurred with all other isomers of retinoic acid studied. Thus the two events were separable although the late differentiation-inducing events did not occur unless preceded by the events leading to growth arrest. Both Fibach et al (1982b) and Von Melchner and Höffken (1985) showed that the early loss of self-renewal potential was followed by events leading to irreversible commitment to differentiation, which reinforced the idea that the induction of terminal differentiation in HL-60 cells is a two-step process.

Yen subsequently showed (1987a) that the first step associated with growth arrest occurred whether the cells were treated with a granulocytic inducer (retinoic acid) or a monocytic inducer (1,25-dihydroxy vitamin $D_3$), and only during the second step of commitment was the lineage determined.

Although two steps were apparently necessary for commitment, it is possible that one "signal" was provided

by the inducers of differentiation, but that the cell was only competent to respond to this signal when proliferation was impaired. It was proposed that the signal for the induction of differentiation was the modification or damage of a cellular target, perhaps protein. In proliferating cells, the damage might be repaired or removed very quickly; in cells with impaired proliferative potential the damage might accumulate and induce a stress response, resulting in the induction of differentiation. The induction of stress responses by damaged proteins has already been described in section 1.7.5.7, and will be discussed further below.

The involvement of two steps in commitment of cells to differentiate is not apparently confined to HL-60 cells. Nomura's group studied commitment to differentiation in Friend MEL cells, and determined by cell fusion experiments that commitment involved two distinct reactions (described in section 1.5.2). One of the reactions was MEL cell-specific, but the other reaction could be induced in other cell types, and was a consequence of the inhibition of DNA replication. This reaction was associated with a component found in the proteinaceous fraction extracted from the cell, suggesting that the inhibition of DNA synthesis resulted either in synthesis of a specific protein factor, or in modification of a protein already expressed (Nomura and Oishi 1983, Kaneko et al 1984, Watanabe et al 1985, Nomura and Oishi

et al 1986). It is possible that the commitment of HL-60 cells to differentiation also involves the same proteinaceous factor.

In view of the relationship between the inhibition of proliferative potential and the induction of differentiation, several studies were performed to determined whether the induction of a stress response was correlated with commitment of HL-60 cells to differentiate.

5.4 The lack of correlation between gene amplification and the induction of differentiation

Gene amplification occurs in cells under similar conditions to those which resulted in the induction of differentiation of HL-60 cells (discussed in section 1.7.4). Genes which are normally replicated early during S phase are the most likely to become amplified after partial inhibition of DNA synthesis (Woodcock and Cooper 1981). If the genes which controlled the differentiation of HL-60 cells were among those "early S" genes, then these genes might be preferentially amplified during the inhibition of proliferation imposed by NMF. Over-expression of these amplified genes would then result in differentiation of the cells. However, neither flow cytometric analysis of cellular DNA content nor study of chromosomes revealed any evidence for the induction of

gene amplification concomitant with the induction of HL-60 cell differentiation by NMF (Section 4.4).

There were no major chromosome breaks or double minute chromosomes (DM) visible in the HL-60 cells either before or after treatment with NMF. In contrast, Au et al (1983) and Schwartsmann et al (1987) reported that untreated HL-60 cells contained abundant DM, and that a sub-line resistant to the induction of differentiation did not contain any DM. The fact that these reports showed DM in untreated cells, presumably containing amplified genes, does not rule out the possibility that further gene amplification occurred during the induction of differentiation (resulting in over-expression of genes critical for commitment to differentiation). If the resistant cells described in these reports lacked the capability to amplify genes (evidenced by the lack of DM in untreated cells), they would be unable to amplify the critical genes for differentiation. However, neither of the reports studied chromosomes during treatment of the cells with inducers of differentiation.

It is unclear why no DM were detectable in the untreated cells described in section 4.4.2. Comparison of the photographs with those provided by Au et al (1983) confirmed that there were no stained spots similar to those identified as DM by Au and colleagues. Both studies used standard Giemsa staining techniques, so this would

not account for the disparity. However, there may have been inherent differences in the sub-lines of HL-60 cells used; the cells had been grown in different laboratories for many years since isolation from the patient and may have undergone considerable adaptation. This could have resulted in the development of DM in the cells used by Au et al.

Yen et al (1986b) showed some evidence that supported the involvement of gene amplification in the induction of differentiation: hydroxyurea induced a precommitment state in HL-60 cells, and the development of this state was accompanied by the appearance of extra copies of a group of small chromosomes. However, Yen did not mention whether the same chromosomes were amplified during commitment with such agents as retinoic acid; it is possible that this gene amplification induced by hydroxyurea was coincidental and did not play a role in the induction of differentiation. The studies using NMF suggested that the amplification of genes was not required for the induction of differentiation in HL-60 cells.

## 5.5 The induction of differentiation by known inducers of heat shock protein synthesis

The induction of heat shock protein synthesis is a stress response that occurs in virtually all cell types in response to hyperthermia or treatment with diverse agents

(see section 1.7.5). The hsps are thought to protect the cells from the toxic effects of the stress which induces their synthesis. Heat shock gene expression is induced under similar conditions to those which cause HL-60 cell differentiation, so experiments were performed to determine whether there was a link between the two phenomena.

Ethanol, arsenite, lidocaine and cadmium all induced differentiation of HL-60 cells when added to the cells at concentrations which were marginally cytotoxic (Section 4.5). These agents were all known inducers of hsp synthesis in other cell types (Li and Werb 1982, Atkinson et al 1983, Hahn et al 1985, Caltabiano et al 1986) Procaine was tested to determine whether it would induce HL-60 cell differentiation because this local anaesthetic was said not to induce hsp synthesis, in contrast to the closely related molecule lidocaine (Hahn et al 1985). However, procaine induced HL-60 cell differentiation in the same percentage of cells as did lidocaine, although at a slightly greater concentration (5mM compared to 3mM for lidocaine).

Cadmium was a weak inducer of differentiation; only 14% of cells differentiated after 4 days at the optimum concentration. Ethanol, sodium arsenite, lidocaine and procaine were stronger inducers of differentiation, and each induced more than 50% of cells to differentiate. The

difference in efficiency between inducers of differentiation will be discussed later. The cells induced to differentiate by ethanol, arsenite or the local anaesthetics expressed non-specific esterases, although they did not flatten and adhere to the culture flasks, suggesting that these cells were monocyte-like rather than macrophagic.

The fact that these agents induced monocytic differentiation was surprising because cells induced to differentiate by NMF were granulocytic and did not express the non-specific esterases. Also, out of the large numbers of agents previously shown to induce HL-60 cell differentiation, only 8 were shown to induce macrophagic or monocytic differentiation: TPA, mezerein, 1,25-dihydroxy vitamin $D_3$, ara-A, ara-C, sodium butyrate, aphidicolin and verapamil (see Table 1.1). Many of the previous studies on inducers of HL-60 cell differentiation did not measure markers of monocytic differentiation such as non-specific esterase activity, and the assumption was often made that the cells induced to differentiate were granulocytic because they did not adhere as macrophages. It is therefore possible that more detailed study of some of these agents would reveal that they also induced monocytic differentiation. Previous studies by Yen et al (1987a) had determined that the early events in commitment were common to both granulocytic and monocytic pathways, but it is unclear what differences there are between NMF

and ethanol, for example, that could subsequently cause the cells to differentiate along different pathways.

In addition to the discovery that these agents induced HL-60 cell differentiation, it was determined that a marginally toxic heat shock at 43.5°C for 60 mins induced differentiation. This was very exciting because it was the first example of a physical rather than chemical treatment which could induce HL-60 cell differentiation.

The fact that heat (as well as other known hsp inducers) could induce HL-60 cell differentiation implied that there was a link between the heat shock response and HL-60 cell differentiation.

It was surprising that this one hour exposure to heat was effective in the induction of differentiation, because the chemical inducers required incubation with the cells for 24 hours or more before significant commitment occurred. Heat shock was therefore a very potent inducer of differentiation in terms of exposure periods, but it was not an efficient inducer because only 20% of the cells differentiated.

## 5.6 Commitment to differentiation

Commitment to differentiation with NMF did not occur until the cells had been incubated for a minimum of 24 hours with 170mM NMF, and after 24 hours there was an almost linear increase in the percentage of cells committed to differentiate (Section 4.2). It had previously been determined that HL-60 cells also required incubation for a minimum of 24 hours before commitment occurred in response to DMSO or retinoic acid (Fibach et al 1982a and 1982b, Yen et al 1985, Yen 1985, Tarella et al 1982), whereas commitment occurred more rapidly in response to TPA (Fibach et al 1982a).

Yen and Albright (1984) suggested that HL-60 cells required at least 24 hours incubation with retinoic acid before commitment occurred because retinoic acid uptake was limited to S phase of the cell cycle; in contrast NMF was found to diffuse rapidly into HL-60 cells, to reach an equilibrium within 5 minutes. Thus, the uptake of the inducer was not responsible for the 24 hour precommitment period which occurred during NMF-induced differentiation.

The length of the HL-60 cell cycle was approximately 24 hours, the equivalent of the precommitment period, which suggested that there was a restriction point for commitment in a certain phase of the cell cycle. A restriction point has been previously suggested by Boyd

and Metcalf (1984), Studzinski et al (1985) and Yen and Albright (1984), as an explanation for the cell cycle phase-dependent induction of differentiation by retinoic acid and 1,25-dihyroxy vitamin $D_3$. However this model would not account for the evidence reported by Tarella et al (1982), which showed that the induction of differentiation by DMSO was not cell cycle related.

This restriction point model is also not consistent with the idea that the early steps in commitment are common to both granulocytic and monocytic lineages, because commitment with TPA occurred from 4 hours onward compared to 24 hours with the other inducers (Fibach et al 1982a, Yen et al 1987a). Furthermore, the fact that only one hour of heat shock was required to induce differentiation (Section 4.6) apparently rules out the existence of a restriction point in one phase of the cell cycle. If the 20% of cells that differentiated after heat shock became committed because during that 1 hour they were in the particular phase of the cell cycle required for commitment, the same 20% of cells should be committed during the first hour of incubation with NMF.

An alternative explanation for the 24 hour precommitment period required with inducers of differentiation such as NMF is that the inducers caused a gradual accumulation of a cellular lesion which reached a critical level only after 24 hours, and then initiated a

stress response which resulted in commitment of the cells to differentiate. Heat shock and agents such as TPA could induce the putative damage more rapidly than NMF, resulting in more rapid commitment of the cells to differentiate.

## 5.7 The efficiency of inducers of differentiation

More than 70% of cells were induced to differentiate with NMF but, intriguingly, there was always approximately 20% of cells which did not differentiate in response to this concentration of NMF. Whatever agent was employed to induce differentiation, there was always a fraction of cells that did not differentiate. This fraction of cells was not thought to be a stably resistant sub-population, and some evidence for this was provided by Boyd and Metcalf (1984). They isolated the few clones of cells which did not differentiate in response to butyrate, and re-cloned these cells in butyrate. They found the same heterogeneity of response as in the original sample, suggesting that those cells which did not differentiate in the original sample were not resistant to the inducer of differentiation.

There was a great variation in the efficiency of the inducers of differentiation: NMF induced differentiation in more than 70% of cells at the optimum concentration; ethanol also induced more than 70%; and arsenite,

lidocaine and procaine each induced more than 50% of the cells to differentiate. These agents could all be classified as "strong" inducers of HL-60 cell differentiation. In contrast, cadmium only induced 14%, heat shock induced 20% and serum deprivation induced 15% of the cells to differentiate. These were classified as "weak" or "partial" inducers of differentiation. This variation in the efficiency of inducers has been noted before: Langdon and Hickman (1987) reported that while acetone, DMF and diethylacetamide induced more than 70% of the cells to differentiate, even at the optimal concentrations formamide could induce no more than 40%, and urea only induced 11%.

A suggestion was made that agents such as formamide were metabolized rapidly in the cells so that the intracellular concentration was not maintained for long enough for commitment of the majority of the cells to occur. However, this could not account for the weak induction of differentiation by heat shock.

The extent of differentiation induced by serum deprivation correlated with the fraction of the population of cells that were able to undergo one division (see section 5.2). However, proliferation was not inhibited to the same extent during the induction of differentiation with other weak inducers; the population of cadmium-treated cells underwent 1.75 doublings and heat

shocked cells underwent 1.5 doublings after 4 days. This was similar to the extent of proliferation in cultures treated with stronger inducers of differentiation such as procaine. Treatment with cadmium or hyperthermia therefore allowed the majority of the cell population to undergo at least one cell division, so differentiation was not limited by the replication required for development of the differentiated phenotype.

It was proposed that heat and other weak inducers of differentiation had adverse effects on normal protein synthesis in the cells, which prevented many cells from differentiating even though they had received the signals for commitment. The induction of heat shock protein synthesis in many cell types by heat is accompanied by severe repression in the expression of normal genes (i.e. genes active prior to treatment) (Ashburner and Bonner 1979, Desrosiers and Tanguay 1985). Whether or not the induction of hsp synthesis plays a role in commitment to differentiation (as discussed later), the cells would still need to synthesize normal proteins in order to develop the mature phenotype; in heat shocked cells inhibition of the synthesis of these normal proteins might prevent many cells from being induced to differentiate. Other more efficient inducers of differentiation could induce hsp synthesis without such drastic effects on normal protein synthesis, and thus allow more cells to differentiate. In accordance with this it was determined

that heat shock and cadmium (at the optimum concentration for the induction of differentiation) severely repressed normal protein synthesis in HL-60 cells. The rate of normal protein synthesis was largely unaffected by treatment at the optimum concentration for the induction of differentiation of either NMF, ethanol, DMSO, arsenite, lidocaine or procaine - the strong inducers of differentiation. Thus the efficiency of inducers of differentiation was inversely correlated with the extent of inhibition of normal protein synthesis. It remains to be determined whether inhibition of protein synthesis occurs during commitment with other weak inducers of differentiation such as urea or serum deprivation.

In relation to this theory, interferon (alpha and ß) did not induce hsp synthesis by itself in mouse cells, but enhanced the effects of heat or arsenite on hsp synthesis. Also, interferon reduced the inhibition of normal protein synthesis that occurred after heat shock (Morange et al 1986). In HL-60 cells, interferon-alpha dramatically increased the extent of differentiation induced by DMSO, TPA or retinoic acid (Grant et al (1985), and this might be related to the ability of interferon to enhance heat shock and normal protein synthesis.

## 5.8 Heat shock proteins and HL-60 cell differentiation

Heat shock of HL-60 cells for 1 hour at 43.5°C induced the synthesis of 3 major heat shock proteins; hsp70, 90, 110, and to a lesser extent hsp80, as previously detected in other mammalian cell types after heat shock (Welch et al 1982) (Section 4.7).

Since hyperthermia and at least 5 other known inducers of heat shock protein synthesis were able to induce HL-60 cell differentiation, it was proposed that the heat shock response played a role in the induction of differentiation. It was expected, therefore, that heat shock protein synthesis, particularly hsp70 synthesis, would be induced during the commitment of HL-60 cells to differentiate with 170mM NMF. Hsp70 in particular was thought to be the most likely hsp to play a role in HL-60 cell differentiation because of its apparent involvement in embryogenesis and normal cell development (discussed in section 1.7.5.6). However, 170mM NMF did not induce the synthesis of any heat shock proteins during the commitment of HL-60 cells to differentiation.

In untreated cells there was a low level of hsp70 synthesis and, contrary to expectations, this was specifically inhibited the longer the cells were incubated with 170mM NMF. This repression began very early during the precommitment period; one of the earliest changes in

the repertoire of protein synthesis. The rate of hsp70 synthesis decreased by 28% after the first two hours of incubation with NMF, and by 50% after 6 hours. Thereafter the rate of synthesis continued to decrease so that it was almost 5-fold less than in untreated cells after 54 hours of incubation with the inducer of differentiation.

Measurement of the total cellular levels of hsp70 using an antiserum revealed some surprising results; despite the fact that the rate of hsp70 synthesis was repressed during commitment, the amount of hsp70 in the cells increased. The increase was first apparent after 20 to 30 hours of incubation with 170mM NMF, and appeared to be temporally related to the commitment of the cells to differentiation.

The onset of the $G_1$ arrest that occurred during differentiation also followed the same time course as commitment, and it is possible that the increased levels of hsp70 were a result of $G_1$ arrest of the cells. However, several reports suggest that hsp70 levels are actually higher in proliferating cells than in cells in $G_1$ or $G_0$ (Rice et al 1986b, Carr et al 1986, Milarski and Morimoto 1986). Kaczmarek et al (1987) determined that hsp70 mRNA levels were higher in quiescent ($G_0$) peripheral blood mononuclear cells than when the cells were stimulated to proliferate, but they did not measure the rate of protein synthesis or the cellular levels of hsp70

and it is quite possible that the protein level did not correlate with the mRNA level. It therefore seems unlikely that in HL-60 cells the increase in hsp70 levels accompanying commitment to differentiation was a result of $G_1$ arrest of the cells.

These results clearly demonstrated that the rate of synthesis of a protein does not always correlate with the cellular levels of that protein; the half life of the protein is a more important determinant of the amount of the protein in the cell at any one time.

It was proposed that during commitment to differentiation with NMF, the half life of the hsp70 was increased so that the levels in the cell accumulated despite the decreased rate of translation. This was thought to be an effect of the NMF either directly stabilizing the hsp70 molecules, or damaging other cellular proteins so that they competed with hsp70 for the cells degradative enzymes and resulted in hsp70 being spared. The mechanism whereby NMF could stabilize or damage proteins will be discussed later.

Incubation of HL-60 cells for 24 hours with DMSO, ethanol, arsenite, cadmium, lidocaine or procaine did not cause any consistent changes in hsp synthesis or levels of hsp70 protein. Lidocaine and procaine slightly induced hsp70 synthesis, but the cellular levels of hsp70 were

lower than in untreated cells; arsenite did not cause elevated hsp70 synthesis at this time, but the arsenite treated-cells contained high levels of hsp70 comparable to the amounts found in cells that had been heat shocked. These data again indicated that it is important to measure the cellular levels of the protein rather than the rate of synthesis. It would be useful to study these inducers of differentiation at a time later than 24 hours to determine whether they all caused hsp70 to accumulate in the cells as commitment occurred, as was the case with NMF. It would also be interesting to test other inducers of differentiation such as retinoic acid, methotrexate, TPA, dibutyryl cAMP and sodium butyrate to determine whether they caused elevation of cellular hsp70 levels. Burdon (1986) reported that the latter 3 inducers were unable to induce hsp synthesis in HeLa cells, but very few concentrations of each agent were tested and only the rates of synthesis rather than the cellular levels of the hsps were measured.

Treatment of HL-60 cells for 1 hour with 6% ethanol induced the synthesis of hsps70, 90 and 110, which confirmed the results of Li and Werb (1982), who reported that ethanol induced hsp synthesis in Chinese hamster fibroblasts. Treatment of HL-60 cells for 1 hour with the same concentration of NMF (6% or 1M) also induced the synthesis of hsps 70, 90 and 110. This was the first demonstration that a polar solvent other than ethanol was

capable of the induction hsp synthesis, and casts doubt on the report by Burdon (1986), which reported that DMSO was unable to induce hsp synthesis. This study only measured hsp synthesis in HeLa cells after an overnight incubation with 1.5% DMSO (Burdon et al 1982), and at higher DMSO concentrations hsp synthesis would probably be induced.

## 5.9 The proposal that inducers of HL-60 cell differentiation induce conformational changes in proteins; relationship to the induction of a stress response

The proteolytic system in cells is very complex and there are at least 3 major types of proteases: lysosomal (cathepsins), extracellular (trypsin, chymotrypsin, etc.) and non-lysosomal. The non-lysosomal proteases are sub-divided into at least 3 groups: metalloproteinases (enkephalinase, meprin, etc.), $Ca^{2+}$-dependent cysteine endopeptidases (calpains, of which there are at least 2 types), and ATP-dependent proteases, of which there are again 2 types - a vanadate-sensitive system and the ubiquitin-associated system. Denatured or aberrant proteins are usually cleaved by the ATP-dependent proteases (Khairallah et al 1985).

Cells perform surveillance of their proteins; they recognize mutant or damaged proteins and mark them for degradation. However, "normal" proteins have a finite

life, and each protein type has a characteristic half-life (which may vary from 15 seconds to more than 20 hours) determined in part by such factors as molecular weight, hydrophobicity, isoelectric point. A very interesting phenomenon was observed by Bachmair et al (1986); the N-terminal amino acid residue of a protein regulates its half-life. For example, proteins with arginine at the N-terminal have half-lives of several minutes, while alanine at the N-terminal confers stability so that the protein has a half-life of 20 hours or more. It is thought that a surveillance protein exists which recognizes the N-terminal residue and causes modification of the protein so that it is marked for degradation. Such modifications as dissociation of cofactors, phosphorylation, methylation, acetylation, ribosylation, carbamylation, deamination, glycosylation, oxidation and ubiquitination apparently act as markers for degradation (Rivett 1986). Whether a protein is damaged or has reached the end of its natural life, it will become marked so that it is recognized by the proteolytic enzymes.

NMF and other agents probably induce hsp synthesis by damaging cellular proteins, as described in section 1.7.5.7. Some evidence for the link between the heat shock response and damaged protein was provided by Ananthan et al (1986), who showed that microinjection of damaged protein into oocytes induced activation of the heat shock genes. The damaged protein probably disrupts

the equilibrium in the ubiquitin-dependent proteolytic system, which results in activation of the heat shock transcription factor and expression of the heat shock genes (Munro and Pelham 1985).

Protein damage in HL-60 cells could be caused by the direct interaction of the inducer (such as NMF) with proteins, leading to unfolding and conformational changes which would cause the proteins to be marked for degradation. It was thought that interaction with proteins was responsible for the increase in the half-life of hsp70, which might in turn be responsible for commitment to differentiation. It is possible that low concentrations of agent (such as 170mM NMF) damaged a certain amount of cellular proteins which then competed with hsp70 for the proteolytic enzymes, resulting in higher levels of hsp70 in the cells. An alternative possibility is that 170mM NMF induced a degree of conformational changes in hsp70 itself which caused it to be stabilized. In contrast, it is proposed that a high concentration of agent (such as 6% NMF) caused damage to larger amounts of cellular proteins which saturated the degradative pathways, depleted the ubiquitin pool and activated the heat shock transcription factor, leading to expression of the heat shock genes in accordance with the mechanism proposed by Munro and Pelham (1985). The fact that high concentrations of NMF induced hsp synthesis confirmed that this hypothesis is tenable.

How could NMF and other polar solvents interact with proteins to cause conformational changes?

Such solvents have profound effects on the "structure" of cellular water by interference with hydrogen bonding between water molecules (Kennedy and Symons 1987) and could presumably interact with hydrogen bonding in proteins in a similar manner, causing changes in protein folding. Some recent studies on anaesthesia and similar phenomena induced by small molecules have provided evidence that these molecules are able to interact directly with proteins:

The traditional view of anaesthesia was that halothane, local anaesthetics and alcohols caused direct perturbation of membrane lipids, and indeed large concentrations of alcohols did induce structural rearrangements in artificial lipid bilayers (Paterson et al 1972). However, the assumption that membrane lipids were the locus of pharmacologic action of these agents was mostly based on the fact that pharmacologic activity correlated with lipophilicity of the molecules (Hansch and Glave 1970, Jain et al 1975, Babich and Borenfreund 1987). A more lipophilic molecule will also interact more readily with hydrophobic regions of proteins, and Evers et al (1987) have recently correlated anaesthesia to the interaction of the drug with a saturable binding site in

the brain, probably protein in nature. Franks and Lieb (1986) showed that chemicals such as ethanol, acetone and chloroform were able to inhibit purified Firefly luciferase (and other enzymes) *in vitro* at concentrations identical to those which induced anaesthesia *in vivo*. The inhibitory activity was dependent on lipophilicity of the agent and on the molecular size. There was a cut-off point above a certain size, indicating that the pocket in the protein for binding of these molecules had a finite volume and could bind a single large molecule or several smaller molecules. This correlation between activity and size, up to a cut-off point, was strikingly similar to that observed for the inducers of HL-60 cell differentiation (discussed in section 1.7.3), suggesting that protein(s) may be the target for the initiation of events leading to commitment to differentiation.

Ethanol was shown to induce changes in the native conformation of purified ubiquitin such that a large amount of helical folding was induced (Wilkinson and Mayer 1986). Alcohols with larger molar volumes also induced conformational changes, but at lower concentrations - again a correlation between size and activity. Presumably alcohols and related molecules such as NMF could cause conformational changes in a similar manner in other, larger, proteins.

How could other inducers of differentiation cause conformational changes in proteins?

Heat shock is known to cause unfolding of protein tertiary structure (Pfeil 1987), and arsenite is a thiol-reactive agent which reacts with sulphydryl groups on cysteine residues in proteins (Lanks 1986). Hsp70 contains approximately 6 cysteine residues (Lindquist 1986), and so could be directly altered by arsenite. Heavy metals similar to cadmium have been shown to induce cross-linking of proteins (Wedrychowski et al 1986). Another inducer of hsp synthesis and a weak inducer of HL-60 cell differentiation, adriamycin, is thought to interact with the enzyme topoisomerase II (Ross 1985b), and other inducers of HL-60 cell differentiation have known protein targets in the cell. Examples are TPA and protein kinase C (Castagna et al 1982); methotrexate and dihydrofolate reductase (Matthews et al 1977); tiazofurin and inosine 5' phosphate dehydrogenase (Sokoloski et al 1986). In the process of inhibiting the activity of the target enzymes, these drugs would induce conformational changes in the protein.

To confirm the hypothesis that protein damage is responsible for the induction of HL-60 cell differentiation, it would be necessary to study protein conformation. This is not possible using SDS-polyacrylamide gels as all proteins are denatured and

unfolded by the SDS before separation on the gel. It might be possible to detect changes in conformation of a protein using non-denaturing gels or by immunoprecipitation of the protein. If the protein (e.g. hsp70) had an altered conformation due to NMF treatment, it is possible that it would no longer be recognized by a specific monoclonal antibody to that protein. This technique was successful in detecting changes in the conformation of p53 (Milner 1984), but requires a number of different monoclonal antibodies which react with different epitopes on the protein in question.

## 5.10 The complex mechanisms controlling the activity of heat shock proteins

Heat shock gene expression is controlled at many levels. Transcriptional control is common, so that mRNA synthesis is induced when the cells are stressed (Desrosiers and Tanguay 1985). However, at least in lymphocytes, it has been shown that heat shock mRNA was present in unstressed cells, and its translation was apparently prevented by the binding of complementary RNAs to the messages (Colbert and Young 1987). The translation of the mRNA was then stimulated when the cells were stressed, and translational control has also been noted in other cell systems (Lindquist 1981). As discovered in the experiments with NMF, the cellular levels of hsp can also

be regulated by factors other than the rate of synthesis; preventing the degradation of the protein (by indirect means or by direct stabilization of the protein) would cause the protein to accumulate in the cell. It is therefore necessary, in order to obtain a clear picture of the expression and functions of heat shock proteins in any system, to measure the rates of synthesis and amounts of the mRNA as well as the protein.

The amount of functioning protein may also be regulated by intracellular compartmentalization. Hsp70 is usually present in the cytoplasm of unstressed cells, and translocation to the nucleus occurred in various cell types after heat shock or other stress (Lewis and Pelham 1985, Pelham et al 1985, Velazquez and Lindquist 1984). This presumably indicates a change in the function of the hsp70 after stress. It is possible that translocation of hsp70 occurred during the induction of HL-60 cell differentiation, but this was not detected because the whole repertoire of cell proteins were analysed by electrophoresis. Such changes in subcellular distribution could be detected either by immunohistochemical techniques or by subcellular fractionation prior to separation of labelled proteins by electrophoresis. A cytoplasmic-to-nuclear translocation of hsp70 may be able to control gene expression, by binding of the hsp70 to nuclear proteins which would otherwise bind to the DNA.

There is an added complication, since Pelham (1986) determined that there are at least 3 members of the hsp70 family - hsp70, hsx70 and hsc70 (cognate). Hsp70, which is hardly detectable in unstressed cells, is highly heat-inducible; hsx70, which is also quite heat-inducible is expressed at significant levels in unstressed cells, and is regulated in a cell cycle-dependent manner. Hsc70 is only slightly heat-inducible and is found in higher levels in growing cells than in resting cells. The 3 types are antigenically similar and are indistinguishable in many experiments. The antiserum used in the experiments on HL-60 cells was apparently more specific for the hsp70 than the cognates (Lewis and Pelham 1985), but the protein referred to as hsp70 may actually be the sum of the 3 different proteins; it is possible that the "hsp70" whose levels increased during NMF-induced differentiation was not the same protein as was dramatically induced by heat shock or high NMF or ethanol concentrations. However, the hsp70 proteins seem to be functionally similar.

## 5.11 Speculation on the function of hsp70 in the induction of differentiation of HL-60 cells

Hsp70 apparently has several functions, but they all depend on the ability of the hsp70 to bind to other proteins (described in section 1.7.5.4). In unstressed cells, hsp70 (or a very similar cognate) acts as an

uncoating ATPase; it catalyses the dissociation of clathrin triskelia from clathrin-coated vesicles of the plasma membrane (Ungewickel 1985). Hsp70 has also been found to bind to and stabilize p53, a protein involved in the control of cell proliferation (Pinhasi-Kimhi et al 1986, Hinds et al 1987). Hsp70 cognates bind to nascent immunoglobulin chains, preventing their inappropriate aggregation (Munro and Pelham 1986), and in heat shocked nucleoli the hsp70 helps to solubilize aggregates of proteins and ribonucleoproteins (Velazquez and Lindquist 1984, Pelham et al 1985, Munro and Pelham 1985). In general, hsp70 appears to bind to other proteins in order either to stabilize the proteins or to prevent the inappropriate binding of the protein.

How could hsp70 induce the expression of those genes required for the induction of HL-60 cell differentiation? Hsp70 could bind to proteins involved in the regulation of gene expression, and prevent the binding of the proteins to their target site, allowing expression of the program of genes required for differentiation of the cells.

5.12 Is the induction of a stress response relevant to the induction of differentiation in other cells types?

Many agents which induce HL-60 cell differentiation at marginally sub-toxic concentrations also induce Friend MEL cell differentiation at very similar concentrations

(Collins et al 1980), but some inducers of HL-60 differentiation such as TPA (Yamasaki et al 1977) and procaine (Bernstein et al 1976) actually inhibit Friend cell differentiation. This response to different agents is perhaps not surprising as erythroid and myeloid precursors respond to different effectors in vivo (see section 1.3.2), but it is possible that those agents which do induce Friend cell differentiation do so by inducing the same stress response as in HL-60 cells.

Raaphorst et al (1984) stated that "the process of induction of heat shock proteins in mammalian cells does not appear to be related to the process of differentiation in MEL cells". However this statement was based on the fact that differentiation was not induced when MEL cells were subjected to hyperthermia. The authors did not test any temperature between 42 and 45°C, so it is possible that differentiation would be induced if the cells were heated at 43.5°C (the temperature which induced differentiation of HL-60 cells). Raaphorst and co-workers also stated that hyperthermia inhibited the induction of differentiation by DMSO in MEL cells. Cell survival was markedly reduced by the combination of DMSO and heat, so it was not surprising that such a toxic combination of treatments induced less differentiation than DMSO alone. A similar combination of hyperthermia and NMF in HL-60 cells also resulted in toxicity and reduced cell differentiation. Further experiments therefore need to be

performed to determine whether heat shock induces the same response in MEL cells as it does in HL-60 cells.

The synthesis of heat shock proteins has been studied in K562 cells after the induction of erythroid differentiation by haemin (Singh and Yu 1984). 40 hours after the addition of haemin, when most of the cells expressed haemoglobin, the synthesis of hsp70 was elevated. The authors also found the protein expressed in a fraction of human bone marrow cells consisting mostly of erythroblasts, but not in less mature erythroid cells. Thus the hsp70 might play a role in normal erythroid maturation. It is unfortunate that they did not measure hsp70 synthesis earlier during the induction of differentiation to determine whether it could possibly have a causative role. In relation to this study, Davis et al (1986) showed that vesicles were shed from reticulocytes during the final stages of erythroid maturation. These vesicles contained transferrin receptors which were no longer required by the cells, and also contained high levels of hsp70, presumably functioning as the clathrin-uncoating ATPase. The hsp70 in K562 cells might therefore be involved in the expulsion of unwanted transferrin receptors from the cells rather than playing a role in the protection of the cell from stress.

## 5.13 Conclusion

These studies suggest that the induction of a stress response may be important in the induction of terminal differentiation of HL-60 human promyelocytic leukaemia cells. A large number of agents induced HL-60 cell differentiation at concentrations which were marginally sub-toxic and which therefore inhibited the proliferative potential of the cells. It was expected that, at these concentrations, the agents would induce a stress response in the cells which could then control the changes in gene expression required for terminal differentiation. It was determined that although the synthesis of hsp70 was specifically repressed very early during commitment to differentiation with NMF, the cellular levels of hsp70 increased. NMF was thought to stabilize the hsp70 by interaction with proteins, which would lead to conformational changes. Many other inducers of HL-60 cell differentiation could similarly interact with proteins and cause conformational changes, inducing the same stress response in the cells.

It remains to be determined whether this hypothesis is applicable to other cell types; the relationship between a stress response and the induction of terminal differentiation may be confined to leukaemic cell lines such as the HL-60 and the Friend MEL. However, the hypothesis provides a potential explanation for a unifying

mechanism whereby a large number of disparate agents are able to induce differentiation of HL-60 cells.

APPENDIX ONE Molecular weight and optimum concentration for the induction of HL-60 cell differentiation for a variety of agents

| # | Inducer | Mw | Conc. (mM) | Reference |
|---|---|---|---|---|
| 1 | acetamide | 59 | 250 | Langdon & Hickman 87 |
| 2 | N-methylacetamide | 73 | 25 | " |
| 3 | dimethylacetamide | 87 | 20 | " |
| 4 | diethylacetamide | 115 | 10 | " |
| 5 | formamide | 45 | 400 | " |
| 6 | N-methylformamide | 59 | 170 | " |
| 7 | dimethylformamide | 73 | 100 | " |
| 8 | N-ethylformamide | 73 | 100 | " |
| 9 | diethylformamide | 101 | 25 | " |
| 10 | t-butylformamide | 101 | 40 | " |
| 11 | urea | 60 | 300 | " |
| 12 | 1,1-dimethylurea | 88 | 50 | " |
| 13 | 1,3-dimethylurea | 88 | 25 | " |
| 14 | tetramethylurea | 116 | 5 | " |
| 15 | acetone | 58 | 138 | " |
| 16 | methanol | 32 | 625 | " |
| 17 | ethanol | 46 | 213 | " |
| 18 | hexamethylene bisacetamide | 200 | 2 | Collins et al 1980 |
| 19 | triethylene glycol | 150 | 100 | Collins et al 1978 |
| 20 | dimethylsulphoxide | 78 | 180 | Collins et al 1980 |
| 21 | hydroxyurea | 76 | 0.1 | " |
| 22 | hypoxanthine | 136 | 5 | " |
| 23 | 6-thioguanine | 167 | $3x10^{-3}$ | " |
| 24 | 6-mercaptopurine | 152 | 0.1 | " |
| 25 | piperidone | 95 | 7.5 | Collins et al 1978 |
| 26 | 1-methyl-2-piperidone | 109 | 4 | " |
| 27 | 5-azacytidine | 244 | $3.2x10^{-2}$ | Bodner et al 1981 |
| 28 | 5,6-dihydro-5-azacytidine | 246 | $6x10^{-2}$ | " |
| 29 | 5-iodo-2'-deoxy uridine | 355 | $5x10^{-2}$ | " |
| 30 | 5-bromo-2'-deoxy uridine | 308 | $2.5x10^{-2}$ | " |
| 31 | 3-deazauridine | 243 | $2.5x10^{-2}$ | " |
| 32 | thymidine | 242 | 1 | " |
| 33 | pyrazofurin | 259 | $5x10^{-4}$ | " |
| 34 | virazole | 244 | $4x10^{-2}$ | " |
| 35 | tricyclic nucleoside (TCN) | 320 | $2x10^{-3}$ | " |
| 36 | TCN phosphate | 398 | $1x10^{-2}$ | " |
| 37 | puromycin amino-nucleoside | 357 | $1.7x10^{-2}$ | " |
| 38 | tiazofurin | 260 | $5x10^{-3}$ | Sokoloski et al 81 |
| 39 | selenazofurin | 307 | $1x10^{-3}$ | " |
| 40 | mycophenolic acid | 320 | $7x10^{-3}$ | " |
| 41 | ribavirin | 244 | $3x10^{-2}$ | " |

APPENDIX ONE CONTINUED

| # | Inducer | Mw | Conc (mM) | Reference |
|---|---|---|---|---|
| 42 | cycloleucine | 129 | 20 | Grosso & Pitot 1984 |
| 43 | 3-aminobenzamide | 136 | 10 | " |
| 44 | theophylline | 180 | 5 | " |
| 45 | dibutyryl cAMP | 503 | 0.1 | Hemmi & Breitman 1984 |
| 46 | 8-bromo cAMP | 409 | 1 | Fontana et al 1984 |
| 47 | retinoic acid | 300 | $1x10^{-3}$ | Breitman et al 1980 |
| 48 | actinomycin D | 1526 | $3.3x10^{-6}$ | Collins et al 1980 |
| 49 | vincristine | 825 | $6x10^{-6}$ | " |
| 50 | methotrexate | 455 | $1.4x10^{-5}$ | Bodner et al 1981 |
| 51 | W-13 | 313 | $3.5x10^{-2}$ | Veigl et al 1986 |
| 52 | ethionine | 162 | 3.2 | Hemmi & Breitman 1984 |
| 53 | prostaglandin E2 | 353 | $1x10^{-3}$ | " |
| 54 | TPA | 617 | $1x10^{-5}$ | Kreutter et al 1985 |
| 55 | mezerein | 655 | $6x10^{-5}$ | Grosso & Pitot 1984 |
| 56 | 1,25-dihydroxy vitamin D | 417 | $1x10^{-3}$ | Studzinski et al 85 |
| 57 | sodium butyrate | 110 | 0.4 | Boyd & Metcalf 1984 |
| 58 | ara-C | 243 | $1x10^{-3}$ | Ross 1985 |
| 59 | ara-A | 267 | 0.1 | Munroe et al 1984 |
| 60 | verapamil | 455 | 0.1 | Okazaki et al 1986 |
| 61 | Na arsenite | 130 | $6x10^{-3}$ | Richards & Hickman |
| 62 | lidocaine HCl | 271 | 3 | " |
| 63 | procaine HCl | 273 | 5 | " |
| 64 | 5,5-dimethyl-hydantoin | 338 | 0.4 | Haces et al 1987 |
| 65 | trypsin | 24kD | $4x10^{-2}$ | Fibach et al 1985 |
| 66 | elastase | 25kD | $2.5x10^{-2}$ | " |
| 67 | chymotrypsin | - | $5x10^{-2}$ | " |
| 68 | cholera toxin | - | $1x10^{-5}$ | Fontana et al 1984 |
| 69 | aclacinomycin A | - | $4x10^{-5}$ | Schwartz 1982 |
| 70 | marcellomycin | - | $4x10^{-5}$ | " |
| 71 | tunicamycin | - | $3x10^{-3}$ | " |
| 72 | antithymocyte globulin | - | - | Hunter et al 1985 |
| 73 | GM-CSF | - | - | Begley et al 1985 |
| 74 | aphidicolin | - | - | Griffin et al 1982 |

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