ALTERATION OF THE SMALL CELL LUNG CANCER PHENOTYPE
AND ITS EFFECT ON CHEMOSENSITIVITY.

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ABBREVIATIONS.

BLI	Bombesin-Like Immunoreactivity
BSA	Bovine Serum Albumin
CKBB	Creatine Kinase BB Isoenzyme
DbcAMP	N^6-2'-O-dibutyryl adenosine 3':5'-cyclic monphosphate, sodium salt
DDC	DOPA Decarboxylase
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DOPA	Dihydroxyphenylalanine
EDTA	Ethylene diamine tetra-acetic acid
FCS	Foetal calf serum
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethane sulfonic acid
HMBA	N,N'-Hexamethylene bisacetamide
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NaBut	N-Butyric acid, sodium salt
nSCLC	Non small cell lung cancer
RIA	Radioimmunoassay
RNA	Ribonucleic acid
RPMI 1640	Roswell Park Memorial Institute medium 1640
SCLC	Small cell lung cancer
TRIS	Tris-(hydroxymethyl)-aminomethane
PA	Plasminogen activator
PBS	Phosphate buffered saline

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SUMMARY.

Small cell lung cancer (SCLC) accounts for approximately 25% of all lung cancers. It is distinguished from other lung cancers by the presence of neurosecretory granules, frequent peptide secretion, high levels of L-DOPA decarboxylase (DDC), creatine kinase BB isoenzyme (CKBB), neurone specific enolase (NSE) and bombesin-like immunoreactivity (BLI). It is a particularly aggressive neoplasm which has the potential for early metastatic dissemination, and by the time of diagnosis metastatic spread is often extensive. Consequently, surgical removal of the tumour is in most cases unlikely to result in a cure from the disease and chemotherapy is the most important treatment modality for SCLC. The eventual goal in the treatment of SCLC is to develop effective systemic therapy that will produce high response rates and a high percentage of long term disease-free survivors (cures).

The cell of origin for SCLC has not been identified. The expression of certain specific characteristics by SCLC cells has suggested that SCLC may have a separate cell of origin from the other forms of lung cancer, collectively called non-small cell lung cancer (nonSCLC). The presence of dense core granules and the production of amine and peptide hormones have suggested that SCLC may originate from the endocrine cell of the lung, the K cell. In contrast, the expression of certain cell surface antigens, thought to be restricted to cells of the haemopoietic cell lineage, by SCLC has implicated a haemopoietic origin. The probability of a separate histogenesis for SCLC has become less secure with the recent data demonstrating that neuroendocrine features can be found in nonSCLC as well as SCLC. Moreover, the findings that SCLC and nonSCLC can co-exist within the same tumour deposit and that transitions between SCLC and nonSCLC may occur, in vivo and in vitro, has suggested that a closer link may exist between the different histological subtypes of lung cancer.

Mixed cell types of SCLC with large cell or other histological types of lung cancer are frequently recognised at autopsy in patients who initially presented with "pure" SCLC in their biopsy specimen. Moreover, in those patients in whom a mixture of SC and large cell carcinoma are detected at diagnosis the prognosis for these patients is significantly worse than for those patients with "pure" SCLC. It is unclear, however, whether this observation represents the evolution of two separate lung cancers or the transformation of the initial "pure" SCLC to another cell type. However, since nonSCLC tumours generally harbour a relative resistance to radiation and chemotherapy a change in SCLC morphology and/or neuroendocrine properties towards the nonSCLC non-neuroendocrine types could be a factor in the inevitable resistance to therapy that emerges with time in patients with SCLC. The aim of this study was to investigate whether the changes in pathological and biochemical characteristics of SCLC and the associated changes in sensitivity to radiotherapy and/or chemotherapy, could be due to an alteration in the SCLC phenotype. SCLC cells were treated with phenotypic inducer agents to see whether the SCLC phenotype could be altered. Following alteration of the SCLC phenotype the chemosensitivity of the cells was determined to see whether SCLC phenotypic change had any effect on the sensitivity of the cells to cytotoxic agents.

SCLC cell lines were treated with drugs known to induce phenotypic changes in other cell lines. Following treatment, selected SCLC phenotypic markers were measured; DDC, CKBB and BLI. Changes in the levels of these markers were taken as representing an alteration in the SCLC phenotype. The SCLC growth characteristics and cellular morphology were also examined following phenotypic inducer treatment. Three cell lines were included in this study, NCI-H187, NCI-H69 and NCI-H128d. These cell lines differed in a number of characteristics, namely cellular morphology, DDC and BLI expression.

When the SCLC cell lines were treated with various phenotypic inducer agents changes in the levels of the selected SCLC phenotypic markers were found. Taking the SCLC cell lines as a whole, there was no consistent pattern of phenotypic change following drug treatment. However, within a cell line, the phenotypic shift induced by the various drugs always appeared to be in the same direction.

Changes in the levels of the selected SCLC phenotypic markers was taken as representing an alteration in the SCLC phenotype. In particular, HMBA induced a phenotypic shift in the H69 cells, represented by decreased expression of DDC and BLI and an increased expression of CKBB. Furthermore, HMBA induced a cytostatic effect and a change in the H69 cellular morphology. Also, a phenotypic alteration was noted when H187 cells were treated with dbcAMP. DbcAMP induced a significant increase in the expression of DDC, BLI and CKBB. It also induced a cytostatic effect and altered the H187 cellular morphology. These SCLC phenotypic changes induced by either HMBA or dbcAMP were found to require de novo protein synthesis, since incubation with cycloheximide inhibited the drug induced effects.

Having established that the SCLC phenotype could be altered by phenotypic inducer agents the effect of this change on the chemosensitivity to cytotoxic drugs was examined. It was found that in general the SCLC phenotypic change, as represented by changes in the expression of specific markers, did not alter the cells' chemosensitivity. However, dbcAMP treated H187 cells were found to be significantly more resistant to VP16 than the untreated controls.

CHAPTER 1.

INTRODUCTION.

Early in this century, lung cancer was regarded as a rarity but during the last 50 years there has been such an alarming increase in the incidence of the disease that it is now the leading form of cancer affecting man. There appears to be no single aetiological agent responsible for lung cancer. However, the increase in mortality that has been demonstrated in various parts of the world has been associated with the introduction of carcinogenic agents in the respiratory environment. A careful study of the environment has revealed three major groups of factors in connection with the aetiology of lung cancer; in order of importance these are: cigarette smoking, atmospheric pollution and industrial hazards.

Most primary tumours of the lung arise from bronchial epithelium, and of these the overwhelming majority are bronchogenic carcinomas. Bronchogenic carcinoma can be classified into four main histological types: squamous lung cancer, adenocarcinoma, large cell carcinoma and small cell lung cancer (SCLC). SCLC is further classified into lymphocyte-like (oat cell type) and intermediate cell type.

Small Cell Lung Cancer.

SCLC has been viewed as a unique clinical and pathological entity from the other types of lung cancer, collectively referred to as non small cell lung cancer (nonSCLC). The distinction between SCLC and the other types of lung cancer has been based upon: (1) a relatively high growth fraction, (2) an extremely early potential for metastatic dissemination, (3) sensitivity to both radiation therapy and/or chemotherapy as opposed to most other types of lung cancer and (4) expression of neuroendocrine properties and frequent ectopic hormone secretion.

SCLC accounts for approximately 25% of lung cancers. It is a particularly aggressive neoplasm. Metastatic spread to extrapulmonary organs usually starts at an early stage of the disease, and by the time pulmonary SCLC is diagnosed metastatic spread is often extensive. Consequently, surgical removal of the tumour is, in most cases, unlikely to result in a cure from the disease. Chemotherapy is the most important treatment modality for SCLC. A number of single agents or classes of drugs are active against this tumour including alkylating agents (especially cyclophosphamide), adriamycin, periwinkle alkaloids (especially vincristine), VP16, cisplatinum, methotrexate, nitrosoureas, procarbazine, hexamethylmelamine and bleomycin. Single agents alone were initially shown to impact slightly upon survival, but the major progress including potential cure was first appreciated when combination chemotherapy regimens, with or without radiotherapy, were employed. The most widely used drug combinations in the treatment of SCLC include, traditionally, three of the following drugs: vincristine, adriamycin, cyclophosphamide or VP16 (Klastersky, 1988). However, subsequent relapse is common with a 2 year survival of only 10%. The high rate of relapse and failure of chemotherapy is believed to be due to a large degree to drug resistant cells either existing prior to or arising during treatment.

Prior to any therapy a given tumour can be composed of a number of cell populations of variable size and heterogeneous in terms of drug sensitivity phenotype. Within a tumour, drug sensitive cells and drug resistant cells can co-exist. Tumour cells that are drug resistant may be resistant to one, or more than one, drug or class of drugs which can be structurally and biologically unrelated.

Although drug resistance is unquestionably the single most important factor impairing the curative outcome of chemotherapy a number of other factors may also limit the overall success of this modality. Solid tumours often have a limiting blood supply that impairs direct exposure of some tumour cells to adequate cytotoxic drug concentrations. The use of radiotherapy and/or surgery has been employed as an additional approach to eradicate this type of disease. A major problem in the administration of anticancer agents is that they may not always be distributed throughout the body and sanctuary sites may exist which protect the tumour cells from the cytotoxic therapy. For SCLC sanctuary sites are often found in the CNS where the blood brain barrier prevents adequate diffusion of the most effective agents to the tumour. Chemotherapeutic agents demonstrate a dose response effect and so potentially a greater cell kill might be appreciated by utilising regimens in which one or more of the drugs are given in higher doses. However, the cost of higher cytotoxic drug concentrations is considerably greater toxicity including more treatment related deaths. Toxicity is therefore a primary limiting factor to the administered concentration of chemotherapeutic agents.

The eventual goal in the treatment of SCLC is to develop effective systemic therapy that will produce high response rates and a high percentage of long term disease-free survivors (cures). Currently, the results that can be obtained with maximally delivered combination chemotherapy (with or without additional radiotherapy) are limited by the available agents. In the past decade studies on SCLC have accumulated a vast amount of data although no therapeutic progress has been made during this time. Information derived from biological studies as well as the development of new active anticancer agents look to be the most promising avenues to a more effective control of SCLC.

Biology of Small Cell Lung Cancer.

The development of in vitro systems for supporting the continual growth of cell lines of SCLC obtained from patients with this disease has greatly advanced our knowledge of the origin and biology of SCLC. Approaches that have been utilised to develop these cell lines include (1) direct culture in liquid medium (serum-supplemented medium or selective chemically defined medium) of specimens obtained directly from patients, and (2) establishment in culture of nude mouse SCLC heterotransplants developed by the inoculation of clinical material into these animals. The nude athymic mouse also serves as a useful means of evaluating the tumourigenicity of both fresh specimens and cell lines and for studying the effects of chemotherapeutic agents on tumours established in nude mice. Cell lines, as well as providing a tool to aid the further understanding of the biology and origin of SCLC, provide a tool for the development of monoclonal antibodies with specificity for this tumour and also provide an in vitro model for the screening of new cytotoxic agents with potential clinical activity in SCLC. Moreover, the use of SCLC cell lines have permitted the identification of a specific cytogenetic abnormality associated with SCLC. All of these have direct application to patient management.

Since the late 1970's, several laboratories have been successful in the establishment of continuous cell lines from SCLC (Gazdar et al 1980; Carney et al 1985; Baillie-Johnson et al 1985). While few have been cultured from primary tumours, cell lines have been cultured from a variety of metastatic sites. Although earlier attempts to establish cell lines of this tumour met with limited success, with improved techniques and better culture conditions cell lines can now be established from 50-70% of all adequate specimens. The use of conditioned media from established SCLC cell lines (Gazdar et al 1980) and the use of a chemically defined serum-free medium (Carney et al 1981) has greatly enhanced the ability to establish new cell lines.

Once established the tumour cells grow either as floating cell aggregates or attached to the substrate. In the latter cases, as cell density increases large clumps of cells detach from the surface of the dish and float. Based on their appearance the lines can be subgrouped into four major categories: types 1,2,3, & 4 (Carney et al 1985). Type 1 grow as tightly packed spherical aggregates of floating cells, which frequently demonstrate areas of central necrosis in the larger spheroids. Type 2 grow as relatively densely packed floating aggregates, amorphous and irregular in outline, and lacking central necrosis. Type 3 lines grow as very loosely adherent floating aggregates growing in small clumps and intertwined cords. Central necrosis is absent in these lines. Type 4 grow attached to substrate. These cells consist of large overlapping polygonal cells lacking the epithelioid appearance of adherent nonSCLC cultures.

In continuous culture, cell lines of SCLC have a relatively prolonged doubling time, express human isoenzymes, form colonies in soft agarose (1-5%) and form tumours in athymic nude mice with the typical morphological characteristics of SCLC (Gazdar et al 1980). The cells in culture have the typical cytological characteristics of SCLC and also express many APUD properties associated with SCLC, including neurosecretory granules, which may be present either singly or in clusters, formaldehyde-induced fluorescence and the presence of the key APUD enzyme, L-DOPA decarboxylase (DDC) (Gazdar et al 1980). DDC activity has been demonstrated in clinical specimens, cell lines and nude mouse tumours of SCLC (Baylin et al 1980). At one time the presence of DDC activity was believed to distinguish SCLC from nonSCLC. However, DDC activity has been found in surgical and autopsy tissues from both SCLC and nonSCLC, although the highest DDC activity was found in SCLC. Therefore the presence of DDC in surgical and autopsy specimens does not necessarily distinguish SCLC from nonSCLC. The average SCLC DDC activity in tissue culture and in athymic nude mouse heterotransplants was found to be much higher than

in the _in vivo_ specimens (Baylin et al 1980). The presence of high levels of DDC activity in culture and in nude mouse heteroplants clearly distinguished SCLC from nonSCLC.

Recent studies _in vitro_ have clearly demonstrated that certain markers are very useful in separating tumour cell lines of SCLC from cell lines of other histologic types of lung cancer. These markers include the BB isoenzyme of creatine kinase (CKBB), bombesin and neurone specific enolase (NSE). CKBB is normally found in large amounts only in striated muscle, brain, bladder and gastrointestinal tract (Shatton et al 1979). Clinical tumour specimens and cultures (classic and variant) of SCLC were found to produce 10- to 100- fold higher quantities of CKBB than the other types of lung cancer (Gazdar et al 1981). Therefore, CKBB expression clearly differentiates cell lines of SCLC origin from those of other forms of lung cancer. Moreover, serum CKBB levels correlated with extent of disease and sequential measurements of CKBB demonstrated an excellent correlation with observed clinical response to therapy. CKBB may be of value in estimating the extent of tumour dissemination, assigning prognosis and monitoring response to therapy in patients with SCLC.

NSE is a neuronal form of the glycolytic enzyme enolase which is most frequently found in brain tissue although it is also present in a variety of APUD cells and neurones of the diffuse neuroendocrine system,but not in other peripheral cells (Schmechel, Marangos & Brightman 1978). It was also found to be produced in considerable quantities by neuroendocrine tumours including SCLC (Tapia et al 1981). Serum NSE was found to be raised in 69% of all patients diagnosed SCLC and the levels of NSE in continuous cultures of SCLC were much higher than those observed in nonSCLC cells (Carney et al 1982). Although NSE has been proposed as a specific marker of the SCLC phenotype it has been reported that a specific monoclonal antibody for NSE reacted

extensively with all SCLC cell lines and biopsy specimens and also displayed considerable reactivity with nonSCLC cell lines and biopsy specimens (Reeve et al 1986). Therefore, the presence of NSE does not necessarily distinguish SCLC from nonSCLC.

Bombesin is a tetradecapeptide found in high amounts in brain, stomach, intestine and fetal lung (Moody et al 1981). It was first isolated from frog skin (Anastasi, Erspamer & Bucci 1971). A number of biological activities have been ascribed to the effects of bombesin including hyperglycaemia, anorexia, hypothermia and brain-site dependent analgesia. Human SCLC tumours and cell lines produce and secrete bombesin or its mammalian counterpart gastrin-releasing peptide (GRP) (Moody et al, 1981; Moody et al 1983) and can express high affinity receptors for bombesin/GRP (Moody et al 1985). Measurement of intracellular bombesin in cell lines of lung cancer has revealed that only SCLC cell lines express high levels of bombesin, nonSCLC cell lines did not contain detectable levels of bombesin-like immunoreactivity (BLI) (Moody et al, 1981). Furthermore, exogenously added bombesin and GRP stimulated colony formation in SCLC in vitro but had no stimulatory effect on nonSCLC cell lines (Carney et al 1987). These findings have led to the suggestion that bombesin may function as a growth promoting factor for SCLC (autocrine growth factor).

Recently, Carney et al (1985) and Gazdar et al (1985) proposed a new subclassification of SCLC cells based on extensive morphological and biochemical analysis of more than 50 cultured SCLC cell lines. In the new classification, SCLC cells were subdivided into two major classes, classic and variant SCLC cells. Classic SCLC cells express elevated levels of all four markers, DDC, BLI, NSE and CKBB, exhibit either type 1 or 2 morphology in culture and exhibit features typical of the intermediate cell type of SCLC in nude mice xenografts. Variant SCLC cells fail to express DDC and BLI but still continue to express

elevated levels of CKBB and/or NSE. The majority of variant cell lines have either type 3 or 4 morphology. SCLC variant lines are further divided into (1) biochemical variant lines having variant biochemical profile but retaining typical SCLC morphology and growth characteristics and exhibiting intermediate cell type of SCLC in nude mice xenografts; and (2) morphological variant lines having variant biochemical profile, altered morphology, growth characteristics and have a histological appearance in nude mice xenografts of large cell undifferentiated carcinoma.

The use of established cell lines has allowed the identification of a specific cytogenetic abnormality associated with SCLC, a deletion of all or a portion of the short arm of chromosome 3 (Whang-Peng et al 1982). The abnormality was found in cell lines from males and females from treated and untreated patients and in lines established from a variety of metastatic sites. The 3p abnormality was identified in all fresh specimens examined confirming that its presence was not an artifact of culture but rather a specific defect associated with SCLC.

In the past several years a remarkable advance in cancer research has been achieved by the study of oncogenes. Amplification of three myc family oncogenes, c-myc, N-myc and L-myc, has been found in SCLC (Little et al 1983; Nau et al 1985; Nau et al 1986). SCLC morphological variant cell lines have a higher degree of amplification of the c-myc oncogene and express high amounts of c-myc m-RNA compared to the biochemical variant and classic cell lines (Little et al, 1983; Gazdar et al 1985). The high degree of c-myc oncogene amplification and expression in cell lines with a more aggressive phenotype suggests that this amplification may be associated with, or responsible for, the malignant behaviour of these variant tumours. This hypothesis was examined by transfecting a normal human c-myc gene into a cloned classic SCLC cell line not amplified for or expressing detectable c-myc mRNA (Johnson et al 1986). It was found that c-myc expression in

the transfected clones was associated with altered large cell morphology, a shorter doubling time, and increased cloning efficiency but no difference in DDC levels or BLI. Therefore, increased c-myc expression correlates with some of the phenotypic properties distinguishing c-myc amplified variants from unamplified classic SCLC lines. Amplifications of L-myc and N-myc oncogenes have no constant association with both cell types of SCLC. In all SCLC cell lines examined, if at all, only one gene out of the three myc-related oncogenes were amplified (Nau et al, 1985; Nau et al, 1986).

SCLC is frequently associated with the presence of paraneoplastic syndromes, thus many investigators have studied the expression of a wide range of biomarkers and hormones in these tumours. A wide variety of hormones have been recognised in SCLC, both in vivo and in vitro, these include calcitonin, ACTH, ADH and neurophysins (Sorenson, et al 1981). The presence of many peptide hormones in SCLC suggests that these peptides may have an important physiological role in this tumour.

Although there are four major histological types of lung cancer only SCLC remains curable with combination cytotoxic therapy. Considerable advances in the understanding of the biology of this tumour have occurred through the ability to establish continuous cell lines of SCLC. The cell lines have allowed confirmation of the APUD properties of SCLC including the presence of neurosecretory granules' and high levels of the key APUD enzyme DDC. The characterisation of these lines has identified markers not previously associated with SCLC, including CKBB, NSE and bombesin. Although the functional role of these markers has not yet been determined data suggest that serum measurements of these markers may prove useful in both the staging and management of patients with SCLC.

The Origin of SCLC.

The cell of origin for SCLC has not been identified. It has been suggested that different forms of lung cancer originate from specialised normal cells. In this concept, SCLC is thought to stem from the normal APUD cells of the lung, the K cells, which were originally supposed to be of ectodermal neural crest origin. In contrast, another theory of lung carcinoma histogenesis is that all lung cells arise from a "common stem cell" of endodermal origin and that the different histologically discernible tumour types represent different lines of differentiation. A haemopoietic stem cell origin has also been proposed for SCLC based on the expression of cell surface antigens thought to be restricted to cells of the haemopoietic cell lineage. It has been suggested that cancerous cells may arise from macrophage precursors in bone marrow and these precursors migrate to the lung to participate in the repair of damaged tissue a consequence of continual heavy smoking, often associated with SCLC.

The K Cell Hypothesis:-

The neuroendocrine properties of SCLC, which includes the presence of dense core granules in the cytoplasm and the production of amine and peptide hormones, have suggested that this neoplasm may have a separate histogenesis from the other major types of human lung tumours. This tendency of SCLC to produce amine and peptide hormones, such as vasopressin and adrenocorticotrophin, has led some workers to include SCLC among the group of peptide hormone and amine synthesising cells termed by Pearse as the amine precusor uptake and decarboxylation (APUD) system (Pearse 1969). According to this hypothesis, the proposed SCLC cell of origin is the K cell in the lung.

The role of the K cell is poorly understood, but even more basic aspects about this cell such as its prevalence, life span, physiologic factors affecting its turnover and response to various stimuli, including carcinogens are unknown. K cells are present in human bronchial and bronchiolar lining of adults as well as newborn infants and fetuses. In the mucosa of the adult human respiratory tract these cells are usually located singly close to the basement membrane. Interestingly, in contrast to the adult, such a cell, or sometimes even groups of them, appear to be present more frequently on the bronchial lumen in the lungs of human fetuses and neonates (Bonikos & Bensch, 1977). The precise function of these cells either singly or in groups, and the interplay between these two components, under physiological and pathological conditions is not entirely clear. It has been suggested that the groups of K cells may function mostly as intrapulmonary chemoreceptors, whereas, the solitary K cells may act locally in a regulatory or paracrine manner. The comparative frequency of the groups of K cells in foetal lungs and their relative scarcity in adult lungs may also indicate a role related to pulmonary growth and development. The feature most characteristic of this cell is its large complement of spherical, electron dense cytoplasmic granules which closely resemble neurosecretory granules, the contents of which are yet to be identified. Immunohistochemical studies have shown that both solitary K cells and the groups of K cells possess immunoreactive serotonin, bombesin, neurone specific enolase and calcitonin whereas only solitary K cells display immunoreactive leu-enkephalin (Gould et al 1983).

The K cell groups and solitary K cells are regarded as the bronchopulmonary components of the dispersed neuroendocrine APUD system. The APUD system is based on the common property of some cells to accumulate and to store fluorogenic amines (dopamine and serotonin), which, however, may also be synthesised within these cells by decarboxylation of the appropriate amino acid precusors (L-DOPA and

5-hydroxtryptophan). This system is supposed to consist of a variety of solitary or aggregates of polypeptide producing endocrine cells, including those of the anterior pituitary, the parafollicular cells of the thyroid, the pancreatic islets, the gastrointestinal endocrine cells and the lung endocrine cell (K cell) (Pearse 1969). Pearse originally postulated that cells belonging to the APUD system may have a common embryologic origin in the neural crest. This view however did not go unchallenged and subsequently embryological and histological evidence have indicated that the neuroendocrine cell populations of the gastrointestinal tract are probably endodermally derived (Sidhu, 1979), and given that the bronchopulmonary tree arises from the primitive oesophagus it has been tentatively suggested that the respiratory tract APUD cells are also endodermally derived (Gould et al, 1983). Moreover, careful analysis of neuroendocrine-related biochemical properties in all the major histological forms of lung cancer reveal the presence of immunoreactive peptide hormones (Yamaguchi et al 1983) and amine synthesis capacity (Baylin et al 1980) although concentrations are usually less than those detected in SCLC. The demonstration that neuroendocrine features can be found in non-SCLC as well as SCLC favours the "common stem cell" hypothesis of lung cancer histogenesis.

The "Common Stem Cell" Origin of SCLC.

The probability of a separate histogenesis for SCLC has become less secure with the recent data demonstrating that neuroendocrine features can be found in nonSCLC as well as SCLC. One of the key APUD cell properties, the presence of the enyzme DDC, is not restricted to SCLC but is present in surgical and autopsy tissues from all forms of lung cancer, albeit at lower concentrations (Baylin et al 1980). Furthermore, DDC activity has been found in 12% of nonSCLC cell lines, but limited to adenocarcinomas and large cell carcinomas and not observed in squamous cell carcinomas (Gazdar et al 1988). Moreover, immunoreactive gastrin-releasing peptide, also believed to be restricted to SCLC, was detected in about 17% of nonSCLC surgical and autopsy tissues (Yamaguchi et al 1983).

A closer link between SCLC and nonSCLC has also been suggested by the findings that SCLC and nonSCLC can co-exist within the same patient and even within the same tumour deposit. Moreover, it has been proposed that transitions may occur between SCLC and nonSCLC in vivo (Abeloff et al 1979). In a group of patients with biopsy diagnosed SCLC it was found that at autopsy 12% had completely nonSCLC histology, whereas 15% of the autopsies showed mixed SC/nonSCLC histology. The remaining 73% showed SCLC histology at autopsy. Furthermore, it was found that where no SCLC was present at autopsy the level of DDC activity was markedly lower than the levels found in SCLC. These changes in the pathological and biochemical characteristics of these lung cancers could reflect the emergence of another tumour which was present from the outset, the development of a second tumour, or an effect of cytotoxic therapy or differentiation of the initial tumour.

The apparent change from SCLC towards nonSCLC has been found to occur in vitro as well as in vivo. A cell line, OH-1, established from a patient with SCLC underwent a subtle morphological change which was

associated with a complete loss of neuroendocrine differentiation (as judged by electron microscopy studies and a 12 fold loss of DDC activity) after 16 months in culture (Goodwin & Baylin, 1982). Moreover, this morphological change and loss of neuroendocrine differentiation was accompanied by the emergence of radiation resistance.

Also, a cell line, OH-2, established from a patient diagnosed as having SCLC displayed characteristic features of nonSCLC in culture and as xenografts in nude mice (Goodwin et al 1983). The cells grew as anchorage-dependent monolayer, lacked high levels of DDC activity and grew typical large cell undifferentiated lung carcinomas in nude mice. Interestingly, analysis of the cell surface phenotype, previously shown to distinguish between SCLC and nonSCLC in culture (Baylin et al 1982), showed that both the parent OH-2 cells and OH-2 clones expressed the complete cell surface protein phenotype characteristic of SCLC. Importantly, each clone and the parent line simultaneously expressed some of the surface proteins characteristic of cultured nonSCLC. This suggests that SCLC and a form of large cell carcinoma may be linked through a change in cellular differentiation.

SCLC growing in cell culture possesses biological properties that allow classification into two categories: classic and variant (Carney et al 1985). Recent in vitro studies have introduced a new subclass of SCLC called transitional cell type (Bepler et al 1987). Two SCLC cell lines were established from different metastatic sites within 15 days from the same patient. These two lines (designated SCLC-22H and SCLC-21H) had distinct biochemical, morphological and kinetic features. At diagnosis the patient's tumour revealed pure small cell morphology. When the tumour specimen resulting in the SCLC-22H cell line was taken, the patient's tumour showed morphological features of mixed SC/LC. The SCLC-22H line had morphological features of the classic and variant cell types, biochemical and kinetic features of

the classic cell type and molecular biological features of the variant cell type. The tumour specimen taken 15 days later showed more obvious SC/LC features, and the resulting cell line SCLC-21H exhibited all properties of the variant cell type. At autopsy the patient's tumour had converted into a mixed SC/LC tumour with a dominance of large cell-like elements in all metastases examined. It was proposed that the patient's tumour may have slowly altered its whole biological behaviour from one characteristic for the classic cell type to one characteristic for the variant cell type. Cell line SCLC-22H was established during the transitional phase, which would account for its mixed classic/variant properties, and cell line SCLC-21H was established after the transition and thus expresses all variant properties. If, as Goodwin proposed, SCLC is linked to large cell carcinoma through a change in cellular differentiation then SCLC-22H may prove to be a valuable tool in understanding this link.

The transitional SCLC cell type has been described recently again in reference to the proposed link between SCLC and a form of large cell carcinoma. A cell line, SCLC-MO, was established from a patient with SCLC (Watanabe et al 1988). Following 9 months continuous culture of the parent cells in suspension, some cells attached to the flask. These adherent cells were routinely cultured as a monolayer for an additional 8 months and then cloned. Two clones that differed in the antigenic phenotype from the parent cell line were selected and established; MOA1 and MOA2. When the parent cell line and MOA1 were transplanted into nude mice, an intermediate cell type histology was found. In contrast, MOA2 showed a picture of large cell carcinoma. SCLC-MO exhibited biological features of a transitional phase between classic and variant type SCLC: expressed low levels of DDC, high levels of NSE and CKBB and had classic type morphology. The MOA1 clone was characteristic of variant SCLC in that it lacked DDC but still expressed NSE and CKBB and grew as a monolayer. MOA2 however was more characteristic of nonSCLC in that it expressed low

DDC activity, lacked NSE and CKBB and grew as a monolayer, the cells were larger than MOA1. Analysis of the cell surface phenotype with a series of monoclonal antibodies, which have been used previously to distinguish between classic SCLC, variant SCLC and nonSCLC supports this classification of SCLC-MO, MOA1 and MOA2. The presence of several marker chromosomes shared by these three lines supports the belief that MOA1 and MOA2 sublines originated from the parent SCLC-MO line and not as a result of cell selection from a heterogeneous cell population, which cannot however be ruled out. Therefore, these results suggest that a there may be a transition from SCLC to nonSCLC via a transitional SCLC cell type/variant cell type.

These in vitro findings could be explained by a change in cellular differentiation which may underly the progression of the early-passage neuroendocrine cells to the late passage non-neuroendocrine cells. Alternatively, a process of cell selection could account for the time-related morphological and biochemical changes, small numbers of the non-neuroendocrine type cells could be present in the early passages and with time come to predominate in the cultures. However, in many cases the parent SCLC cell line has been maintained for many months without the appearance of the non-neuroendocrine cells. Moreover, the change from neuroendocrine type cells to non-neuroendocrine cells has often occurred quite suddenly which might suggest a mechanism other than simple cell selection with time. Furthermore, as discussed above, OH-1 cells established from human SCLC, underwent a subtle morphological change which was associated with a virtually complete loss of neuroendocrine differentiation which was accompanied by the emergence of radiation resistance. Following irradiation of the early passage cells the cells that regrew retained their neuroendocrine phenotype, suggesting that radiation-resistant non-neuroendocrine cells were not selected for. The evidence seems to suggest therefore that, SCLC and a form of large cell carcinoma appear linked through a continuum of differentiation events that (1) may explain clinically

important transitions that occur between the major types of human lung cancer and (2) provide evidence for a common cellular origin of endocrine and nonendocrine cells in the bronchial mucosa.

The use of monoclonal antibodies have been used extensively to try and determine the origin of SCLC and the relation to nonSCLC. MOC-1, a monoclonal antibody raised against a human SCLC cell line, was found to react with a subset of cells in the normal lung with a histological appearance consistent with that of the K cell (de Leij et al 1985). Outside the lung the MOC-1 antibody reacted with a subset of normal endocrine cells (in the adrenal, thyroid, ovary and pancreas) as well as neural cells (brain and peripheral Schwann cells). Since some of these positively reacting cells are ectodermally derived whereas others are of proven endodermal origin the MOC-1 related antigen is not a cell lineage specific antigen. Instead, the common expression of the antigen by cells with a neural, endocrine or neuroendocrine function suggests that the antigen relates to a neuroendocrine differentiation state of these cells. The presence of the MOC-1 antigen on neoplastic tissue paralleled its normal tissue distribution, indicating that the antigen is generally retained upon malignant transformation. In lung cancer, the MOC-1 antigen was found in nearly all SCLC tissue examined. In addition adenocarcinoma and mixed adenosquamous carcinoma could also express the antigen, whereas pure squamous cell carcinoma generally did not. Since the antigen detected by the MOC-1 antibody is not related to a specific embryological lineage the question about the cell lineage of SCLC remains unanswered. However, the apparent reactivity by both endodermal and neural crest derived cells to the MOC-1 antibody suggests that both cell lineages may have similar differentiation possibilities. Moreover, since all major histologically different lung cancers can express an antigen, which in the normal lung is confined to APUD cells, suggests that these different normal and

malignant lung cells are interrelated, which is in favour of a "common stem cell" theory of lung cancer histogenesis.

The concept of a "common stem cell" origin of lung cancer was further supported by the findings of Waibel, O'Hara & Stabel, (1987). They described two antibodies which react with SCLC cell lines and tissues; LAM2 and LAM8. LAM2 was found to be an epithelial type membrane antigen reacting with lung carcinomas of every histology and adenocarcinomas of the breast, colon and ovary. The uniform expression of the LAM2 antigen in SCLC as well as in nonSCLC suggests that this phenomenon is common to lung tumours independent of their differentiation. In contrast, LAM8 was found to be a tumour associated antigen reacting with SCLC tissue only.

The use of monoclonal antibodies in the study of SCLC has highlighted four major groups of antigens: (1) antigens expressed by other epithelial structures such as normal bronchial epithelium, nonSCLC and nonpulmonary carcinomas e.g. LAM-2, (2) antigens associated with neuroendocrine differentiation e.g. MOC-1, (3) antigens commonly expressed in cells of macrophage differentiation of natural killer cells e.g. Leu-7 (see below), (4) antigens which are tumour associated, based on the absence of expression in normal bronchial epithelial tissues, other carcinomas and tissues of neuroendocrine differentiation e.g. LAM-8.

In the past few years, it has been recognised that in most cases carcinoma cells contain similar patterns of keratin subtypes as their normal cellular counterparts. This suggests that analysis of keratin content may help delineate the relationship between the lung carcinomas. SCLC cells contain a characteristic set of keratins (Elias, Cohen & Bernal 1988). This keratin pattern has been detected in SCLC cells and tissues and in classic and variant SCLC histologies, but not in nonSCLC or non-lung tumours although some individual

keratins are shared. Normal bronchial epithelial cells contain all the keratins expressed by SCLC, but in addition exhibit keratins that are observed in nonSCLC histologies. This finding is consistent with the concept of a "common stem cell" origin for the lung cancer histologies. Moreover, the presence of keratin, a fundamental marker of epithelial differentiation, casts serious doubt on the interpretation that SCLC has a monocytic origin simply due to the presence of surface antigens shared with monocytes (Ruff & Pert, 1984).

The Haemopoietic Origin of SCLC.

Several groups have examined SCLC cell lines for the presence of certain cell surface antigens, using monoclonal antibodies, in an attempt to determine the origin of SCLC. Ruff and Pert, 1984, found that several myeloid cell-associated monoclonal antibodies including OKM1, which binds to the receptor for a complement fragment C3bi on granuloyctes and monocytes, were present on human SCLC cell lines but not on representatives of nonSCLC cell lines nor normal foetal lung. They suggested that these cell surface similarities indicated that SCLC was not of lung origin but actually derived from a haemopoietic stem cell. However, examination of a large panel of well characterised cell lines from SCLC tumours for the expression of several myeloid cell-associated antigens (including Leu-7, associated with natural killer cells, C3bi defined by OKM1, and two myeloid cell specific antigens, My23 antigen and AML-1-99 defined antigen) failed to confirm the findings of Ruff and Pert (Ball, Sorenson & Pettengill, 1986). Furthermore, they found that the overall expression of myeloid cell-associated and histocompatibility antigens on the SCLC cell lines examined was variable. It was suggested that although two apparently diverse cell types expressed the same antigens this did not necessarily place them in the same lineage, as proposed

by Ruff & Pert. Rather, an alternative explanation may be that the function served by the antigens is shared by different tissues. Since the functions of the Leu-7 and My23 antigens are unknown, studies that seek to determine the functional role of these antigens may play a part in explaining why lung cancer, natural killer cells and phagocytic cells should share such cell surface molecules.

Phenotypic Modulator Agents.

Dibutyryl Cyclic Adenosine 3':5'-Monophosphate.

Dibutyryl cyclic adenosine 3':5'-monophosphate (dbcAMP) has been shown to modulate the growth, differentiation and cAMP-dependent protein kinase activity in various tumour cells (Haddox, Magun & Russell, 1980; Boonstra et al 1987; Prashad, Lotan & Lotan 1987; Tagliaferri et al 1988). The mechanism by which dbcAMP suppresses growth and promotes differentiation of these tumour cells is unknown but it has been suggested that these changes were due to increased levels of cellular cAMP. Cyclic AMP has been implicated in the regulation of the growth and differentiation of various normal and malignant cells. The mechanism by which cAMP modulates these fundamental cellular processes is not fully understood. However, it has been proposed that most, if not all, of the effects of cAMP are mediated by protein kinase A of which there are two forms, type 1 and type 2, in many mammalian tissues. The enzymes are complexes containing each two catalytic subunits and two regulatory subunits. The catalytic subunits of type 1 and type 2 protein kinase A are identical, but the regulatory subunits are different. The activation of protein kinase A by cAMP is initiated by the binding of cAMP to the regulatory subunits of the holoenzyme which leads to the release of the active catalytic subunits from the complex. Recent studies however indicate that an increase in cellular cAMP levels does not necessarily determine cell transformation and it is more likely that it is cAMP-dependent protein kinase which plays an important role in these processes. Prashad et al, (1987) showed that exogenously added dbcAMP increased the intracellular cAMP level in neuroblastoma cells and also induced the synthesis of type 1 regulatory cAMP-binding protein. Since growth of these cells was inhibited by dbcAMP and their differentiation was stimulated it has been proposed that the free type 1 regulatory cAMP-binding protein may play a role in the regulation of these cellular

processes. More recently dbcAMP was found to inhibit the growth and alter the morphology characteristic of untransformed fibroblasts in transformed NIH/3T3 cells. In this case, these changes were found to correlate with an increase of the type 2 regulatory cAMP-binding protein and a decrease in the type 1 regulatory cAMP-binding protein. It has been shown that a mixture of type 1 and type 2 kinases are present in most mammalian cells and perhaps a selective modulation of these enzymes in the different cell types may be a crucial function of cAMP.

Hexamethylene Bisacetamide.

Hexamethylene bisacetamide (HMBA) is one of the most effective polar-planar compounds that trigger cell differentiation. It is a low molecular weight (i.e. < 300 daltons) compound with a polar hydrophilic moiety and a planar hydrophobic portion. HMBA has been widely studied, it has activity in both solid tumour derived and leukemic cultured cell lines including neuroblastoma (Palfrey, Kimbi & Littauer 1977), glioblastoma multiforme (Rabson et al 1977), Friend murine erythroleukemia (MEL) (Reuben et al 1976) and the human promyelocytic leukemia, HL-60 (Collins et al 1980). HMBA has been selected for phase 1 clinical trials since it had the maximum differentiation potency in a series of bisacetamides, and also because it is possible to achieve concentrations of HMBA in patient plasma equal to the concentrations required for induction of differentiation in vitro.

The precise mechanism of action of HMBA is unknown. Bisacetamides are taken up by the cell, an intracellular concentration equal to the extracellular concentration is achieved by 6-8 hours (Reuben, 1979). Cellular uptake of HMBA appears to be necessary for action since HMBA linked to glutathione (which cannot penetrate the cell membrane) is inactive (Gabrilove, 1986). Commitment to

differentiation is both concentration and time dependent. It takes at least 10 hours after equilibration before differentiation is detected. The lag time may indicate that there is a need for continuous HMBA presence during a sequence of biochemical changes and/or that induction is cell-cycle dependent.

It has been recently shown that changes in intracellular polyamines are an intimate part of the differentiation programme induced in MEL cells by HMBA, and they appear to be associated with a regulatory event in this process (Meilhoc, Moutin & Osborne 1986). The first polyamine-biosynthetic enzyme, ornithine decarboxylase, is negatively controlled by synthetic $\alpha-\omega$ diamines as well as by the natural polyamines. Since HMBA is a diacetylated diamine, its deacetylation would produce molecules that could potentially regulate polyamine biosynthesis.

N-Acetyl-1,6-diaminohexane and 1,6-diaminohexane formed by deacetylation of HMBA are shown to accumulate rapidly inside MEL cells (Meilhoc et al 1986) and HL-60 cells (Egorin et al 1988). In the absence of HMBA, exogenous N-acetyl-1,6-diaminohexane was able to induce the complete differentiation process in both MEL and HL-60 cells. Moreover, N-acetyl-1,6-diaminohexane was able to cause changes in polyamine biosynthesis in MEL cells (Meilhoc et al 1986). This therefore implies that the catabolites of HMBA may be directly responsible for the changes in polyamine biosynthesis and maybe for initiating other events regulatory for the differentiation of these cells.

A complete characterisation of HMBA catabolism has recently been carried out both in vivo and in vitro. After intravenous administration of HMBA to humans the urine contained the parent compound and at least five metabolites formed by deacetylation and oxidation pathways. Metabolites identified included the major

metabolite 6-acetamidohexanoic acid, its monodeacetylated product N-acetyl-1.6-diaminohexane, its bis-acetylated product 1,6-diaminohexane and 6-aminohexanoic acid and its lactam, caprolactam (Callery et al 1986). A number of leukemic cell lines have also been shown to take up HMBA and to convert it to the same metabolites identified in the plasma and urine of patients treated with HMBA (Egorin et al 1988). One enzyme likely to carry out the sequential deacetylation of HMBA to N-acetyl-1,6-diaminohexane and 1,6-diaminohexane is N^8-acetylspermidine deacetylase. This particular enzyme metabolises a substrate produced by nuclear acetylases which are also involved in acetylation of histones. Therefore HMBA may inhibit histone deacetylation and thereby maintain the histones in an hyperacetylated state, which in turn might facilitate transcription of chromatin. However this possible mechanism of action for HMBA induced changes in gene expression and differentiation requires further studies.

Sodium Butyrate.

Sodium butyrate, (NaBut), a four carbon fatty acid, is a natural occurring fatty acid and is formed by the hydrolysis of ethylbutyrate. Recent studies on mammalian cells in culture show that this fatty acid affects morphology, growth rate and gene expression. Sodium butyrate is a potent differentiation agent in some cancer cells such as colorectal tumours (Tsao et al 1983), erythroleukaemia (Leder & Leder 1975), human hepatoma (Nakagawa et al 1985) and human medullary thyroid carcinoma (Nakagawa et al 1988). The mechanism by which NaBut acts on cells is not completely understood, but in some systems the effects of this agent have been related to its inhibitory action on histone deacetylase, leading to hyperacetylation of chromatin-associated histones and thereby to changes in gene expression (Riggs et al 1977). However a causal relationship between the accumulation of polyacetylated histones in the presence of NaBut and the inhibition

of DNA synthesis and the switching of malignant cells to non-malignant differentiating cells requires further work.

Retinoic acid.

The retinoids comprise a group of compounds including retinoic acid, retinol (vitamin A) and a series of natural and synthetic derivatives that together exert profound effects on development and differentiation in a wide variety of systems (Sporn & Roberts 1983). Although early studies focussed on the effects of retinoids on epithelial growth and differentiation, their actions have been shown to be more widespread than previously suspected. Many recent studies have examined the effects of these molecules on a variety of cultured cell lines including neuroblastomas, melanomas and fibroblasts. The mechanism of action of the retinoids is unknown but the recent identification of a human retinoic acid receptor belonging to the family of nuclear receptors suggests that the hormone/receptor complex may control differentiation by inducing regulatory events as a result of activation of specific sets of genes (Petkovich et al 1987).

Dexamethasone.

Steroid hormones act via specific and seemingly mobile receptor proteins. Steroids enter all cells from the blood by passive diffusion. If specific receptors are present, a high affinity hormone receptor complex is formed which, after a conformational change or activation, is translocated to the nucleus to occupy large numbers of so called acceptor sites, composed of DNA and non-histone nuclear proteins. Dexamethasone is a synthetic analogue of the naturally occurring corticosteroids. Dexamethasone has been shown to induce growth inhibition in a number of cell lines, including human lung adenocarcinoma (Jones, Anderson & Addison 1978), human glioma (Freshney et al 1980) and breast cancer cells (Darbre & King 1987).

Furthermore, dexamethasone has been shown to have a differentiation inductive effect and a repressive effect on malignancy associated properties in human gliomas and in lung carcinoma (Freshney, 1985).

Chemosensitivity of SCLC.

This study involved examining the effect of phenotypic inducer treatment of some SCLC cell lines on the cells' sensitivity to various cytotoxic agents. As discussed earlier, adriamycin, vincristine and VP16 are among the chemotherapeutic agents used in the treatment of SCLC.

Adriamycin belongs to a group of antibiotics, the antracyclines, which are isolated from Streptomyces peucetius. Adriamycin is the 14-hydroxy derivative of daunorubicin. Daunorubicin is produced by S. peucetius but after treatment with the mutagen, n-nitroso-N-methyl urethane, a modified strain of the organism develops which secretes adriamycin. Daunorubicin appears to owe its cytotoxicity to daunorubicinol, a metabolite formed by enzymatic reduction of the keto group at C13. This compound intercalates itself between adjacent base pairs of the DNA molecule which becomes partly untwisted so that further synthesis of DNA, and hence RNA, is interfered with. Adriamycin is more potent than daunorubicin in inhibiting the synthesis of nucleic acids. It also owes its cytotoxic activity to a reduction product (doxorubicinol) that intercalates itself into the DNA molecule. Adriamycin has numerous biological activities, including targeting to specific enzymes like topoisomerase II and the generation of free radicals, and because of this diversity of effects

it is difficult to assign a single activity as being the cytotoxic action.

Drugs that block the replication of DNA stop mitosis in the S stage of interphase whereas drugs that impair protein synthesis inhibit the cell growth that occurs in the G1 or G2 stages of interphase. One group of drugs that inhibit the metaphase of mitotic division is the vinca alkaloids (vincristine and vinblastine from the periwinkle plant). The vinca alkaloids bind to microtubules and cause them to disrupt into subunits. These drugs therefore inhibit those cellular processes that depend on microtubules including mitosis. Since microtubules are involved in a number of cellular functions, inhibition of spindle formation is only one aspect of the action of these drugs.

The double helical structure of DNA poses several mechanical problems for the functioning of the molecule. The principal difficulty is that the helix cannot replicate itself unless reversible swivelling takes place around the replication fork, thereby preventing entanglement of daughter DNA molecules during mitotic segregation. Similarly, a swivel mechanism is probably required during gene transcription to prevent the nascent RNA transcript being serially wrapped and dragged around the helix. Two types of such molecular swivels have been characterised. Type I topoisomerases are ubiquitous intranuclear enzymes that effect the transient breaking and rejoining of single DNA strands in living cells, allowing the helix to change its twist. These enzymes have been heavily implicated in the mediation of gene transcription, although they do not appear essential for cell survival. Type II topoisomerases induce transient double-stranded breaks in DNA, with subsequent relaxation of DNA twist via strand-passage of duplex DNA. DNA replication, recombination, transcription and repair have all been linked with topoisomerase II activity, and

this enzyme is probably required for cell survival (Epstein, 1988).

Topoisomerases have now been recognised as targets of several commonly used antitumour drugs. For topoisomerase II, these agents include the epipodophyllotoxin VP-16 and the intercalating anthracycline derivative adriamycin. Although the cytotoxicity of such agents may not prove to be mediated exclusively via topoisomerases, it is the formation of a stabilised enzyme:DNA intermediate (and not inhibition of enzyme per se) that appears to be responsible for topoisomerase-mediated component of toxicity. This enzyme:DNA intermediate, or cleavable complex, can be revealed as DNA strand-breaks in DNA damage assays even though such breaks seem likely to be physiologically concealed in vivo. Pronounced increases in cellular topoisomerase II activity have been associated with cell proliferation both in vivo and in vitro, and this proliferative activity has in turn been associated with increased tumour cell sensitivity to topoisomerase-II-interactive drugs (Sullivan et al 1986; Markovits et al 1987).

Project Aim.

Histological variants of SCLC have long been recognised including the oat cell type and the intermediate types. However, no significant difference has been observed in the clinical presentation, extent of disease, sites of metastases nor response to therapy among these histological subtypes of SCLC (Carney et al 1980). In contrast, in those patients in whom a mixture of SC and large cell carcinoma are detected at diagnosis the prognosis for these patients is significantly worse than for those patients with "pure" SCLC (Radice et al 1982). These mixed-histology patients have a significantly lower complete response rate to cytotoxic therapy and poorer survival.

As discussed previously, mixed cell types of SCLC with large cell or other histological types of lung cancer are frequently recognised at autopsy in patients who initially presented with pure SCLC in their biopsy specimen (Abeloff et al, 1979). It is unclear whether this observation represents the evolution of two separate lung cancers or the transformation of the initial "pure" SCLC to another cell type. Similar to patients who initially present with a mixed cell type, patients with SCLC who relapse from initial cytotoxic therapy are frequently resistant to the effects of further cytotoxic therapy. SCLC has been shown to undergo a similar transition in vitro (Goodwin & Baylin 1982) and in nude mice heteroplants (Goodwin et al, 1983). In particular, an established line of human SCLC (OH-1) underwent a subtle morphological change which was accompanied by a profound loss of neuroendocrine differentiation and the emergence of radiation resistance (Goodwin & Baylin 1982). Furthermore, comparison of five classic SCLC cell lines, which included two large cell variant SCLC cell lines, revealed that the large cell variants, which had lost APUD properties, were more resistant to radiation compared to the classic SCLC cell lines. Therefore a change in SCLC morphology and/or neuroendocrine properties towards the large cell nonneuroendocrine

types could thus be a factor in the inevitable resistance to therapy that emerges with time in patients with SCLC.

The changes in pathological and biochemical characteristics of SCLC with the associated changes in sensitivity to radiotherapy and/or chemotherapy, could reflect the emergence of another tumour which was present from the outset, the development of a second tumour, differentiation of the initial tumour or an effect of cytotoxic therapy on the morphology of SCLC. The aim of this work was to investigate whether these changes observed in SCLC could be the result of an alteration in the phenotype of the SCLC cells. The major question addressed in this study is: Can the SCLC phenotype be altered and if so does this affect chemosensitivity ?

Various markers of the SCLC phenotype were selected. SCLC cell lines were treated with drugs known to induce phenotypic alterations in other cell types. Following treatment, the levels of the SCLC phenotypic markers were measured. Changes in the levels of these markers were taken as representing an alteration in the SCLC phenotype. The chemosensitivity of the SCLC cell lines was measured following phenotypic inducer treatment, to determine whether phenotypic change altered chemosensitivity.

CHAPTER 2.
MATERIALS AND METHODS.

Tissue Culture.

Materials:-

RPMI 1640 10x		--- Northumbria Biologicals Ltd.
L-Glutamine 200mM (100x)		--- Gibco, Europe Ltd.
HEPES Buffer Solution 1M		--- Gibco, Europe Ltd.
Foetal Calf Serum		--- Biocon, UK Ltd.
Sodium Bicarbonate 7.5%		--- Gibco, Europe Ltd.

Phosphate Buffered Saline:-

	g/l	
NaCl	8.0	
KH_2PO_4	0.2	
$Na_2HPO_42H_2O$	1.45	
KCl	0.2	--- BDH
Hoechst 33258		--- Sigma

Cell Maintenance:-

The human small cell lung cancer (SCLC) cell lines used in this study are characterised in Table 1. The cell lines are all derived from pleural effusions and have been categorised as classic SCLC by their in vitro characteristics (Carney et al, 1985). The SCLC cell lines were maintained as non-adherent cultures in Roswell Park Memorial Institute medium 1640 (RPMI 1640) supplemented with 10% foetal calf serum (FCS), in gas phase of 2% CO_2 98% air, at 37°C. The selection of 2% CO_2 to buffer with the bicarbonate in the medium, instead of the commonly used 5% CO_2, provided pH stability during microtitration plate manipulations particularly with HEPES. The cells

were passaged once per week and the medium was changed between subcultures.

Table 1 : <u>SCLC Cell Lines Used in This Study</u>.

Cell Lines	Pathology	Origin	Reference
NCI-H187	SCLC (classic)	PE	Carney et al,(1985) (C.R. 45, pp 2913).
NCI-H69	SCLC (classic)	PE	Carney et al,(1985) (C.R. 45, pp 2913-2923).
NCI-H128d	SCLC (classic)	PE	Carney et al, (1985) (C.R. 45, pp 2913-2923).

Mycoplasma Staining:-

The cells were tested for mycoplasma contamination once a month. The medium from exponentially growing cultures of SCLC cells was added to 35mm petri dishes containing an indicator cell line (A549 adenocarcinoma cell line) known to be free of mycoplasma. After 2-3 days the indicator cells were fixed with 1 part acetic acid to 3 parts methanol and stained using the Hoechst 33258 fluorescent dye. The Hoechst dye binds specifically to DNA. Mycoplasma contamination could be detected by the presence of extranuclear fluorescence (Chen, 1977).

Cultures were mycoplasma free at the time of all experiments.

Cell Freezing:-

A using stock of each cell line was stored in liquid nitrogen, $-196^{\circ}C$. Every three months the culture stock was replaced by cells thawed from the freezer.

Cell Counting:-

For day to day estimation of cell number cultures were repeatedly pipetted to disaggregate the cell clumps and the cell number was measured using an electronic particle counter (Coulter Counter Z_{B1}).

Determination of the growth cycle was carried out either by electronic cell counting, total protein determination or by use of the MTT assay:-

   a.   Cell Growth by Electronic Cell Counting.

Cell suspensions were seeded into 24 well plates at $5x10^4$ cells/ml in RPMI 1640 supplemented with 10% FCS and incubated at $37^{\circ}C$ in 2% CO_2. At regular intervals throughout the growth curve 0.4ml samples were removed from the appropriate wells, following repeated pipetting, and 19.6mls of PBS were then added. The cell number was determined by electronic particle counting.

Where the effect of various drugs on the growth of SCLC cell lines was examined, the cells were plated out in medium containing the drug and the drug was present throughout the growth curve.

In order to measure cell growth accurately by electronic cell counting a single cell suspension is required. This requirement was found to be the main drawback of electronic cell counting for the SCLC cell lines. Since the cell lines grew as floating aggregates a single

cell suspension was not easily obtained. Photographs 1,2,3 & 4 show that H187 and H69 can be disaggregated fairly well, although a complete single cell suspension was still not achieved. For these cell lines electronic cell counting was used as an estimation of cell growth. This was supported by the finding that measurement of the growth curve by electronic cell counting showed good correlation with the increase in total cellular protein (Figs 1 & 2). However, electronic cell counting was found to be unreliable for estimating cell growth of H128d which grew as tight aggregates. Photographs 5 & 6 show that even after repeated pipetting of H128d large aggregates remained.

b. <u>Cell Growth by Total Protein.</u>

Cell suspensions were seeded into 25cm^2 tissue culture flasks. On appropriate days thereafter the cells were harvested and washed twice with PBS. The cell pellet was dissolved in 0.5M NaOH and acid soluble protein was determined using the Biorad Protein Assay (see later).

c. <u>Cell Growth by MTT Assay.</u>

Cell suspensions were plated out at 5x10^4 cells/ml into 96 round bottomed microtitration plates in RPMI 1640 with 10% FCS, incubated at 37°C in 2% CO_2. At regular intervals throughout the growth curve one plate was harvested and an MTT assay (see below) was carried out to determine cell number. Where the effect of drugs on the growth of SCLC cell lines was examined, the cells were plated out at twice final concentration and the drug was added to the plate at twice final concentration. Following the appropriate incubation period, the drug was removed by spinning the plates at 200g for 5mins, aspirating the supernatant and resuspending the cells in fresh medium. The plates were fed on alternate days thereafter.
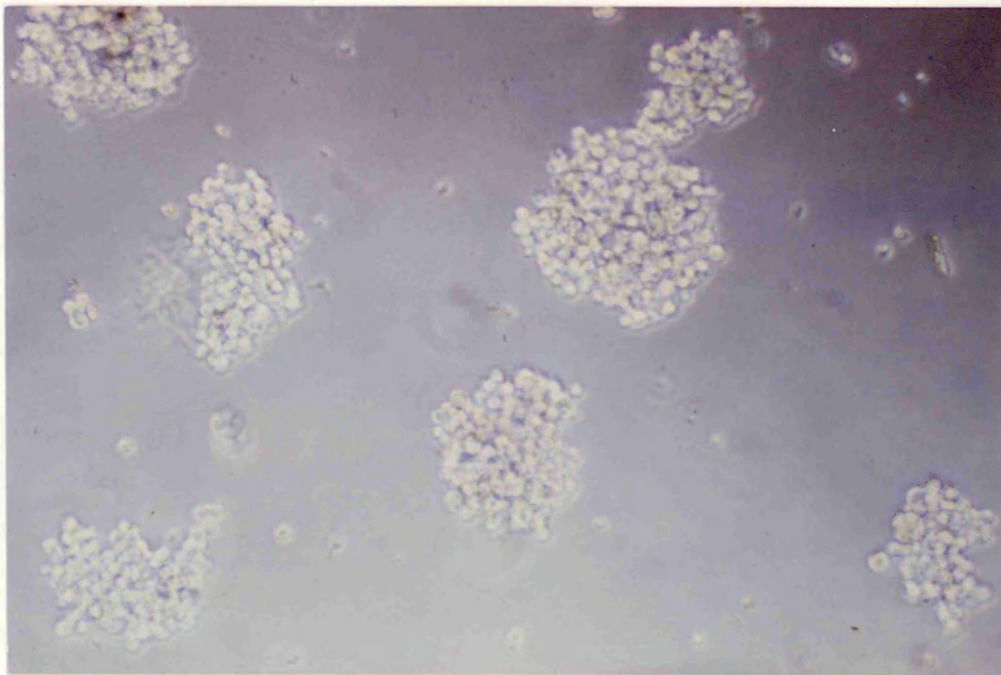
Photograph 1:- H187 Cells (10x objective).

H187 cells were grown in RPMI 1640 medium supplemented with 10% FCS.
The cells were photographed in late log phase of growth.
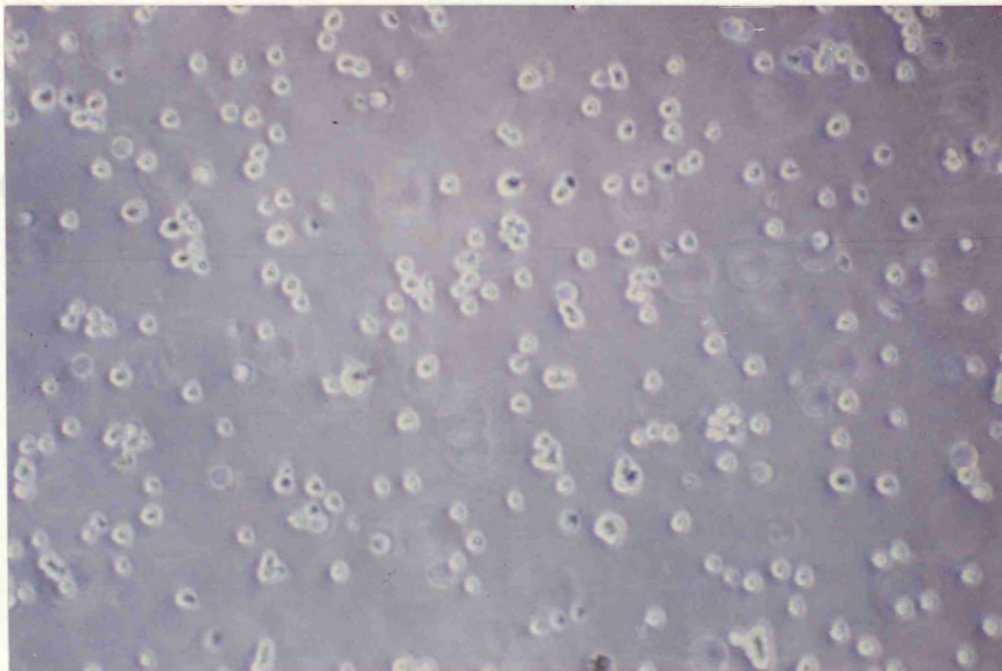
Photograph 2:- H187 Cells Disaggregated by Repeated Pipetting (10x objective).

H187 cells were grown to late log phase, and then pipetted up and down
using a 10ml glass pipette. A cell suspension consisting of single
cells and aggregates of two or three cells was produced.
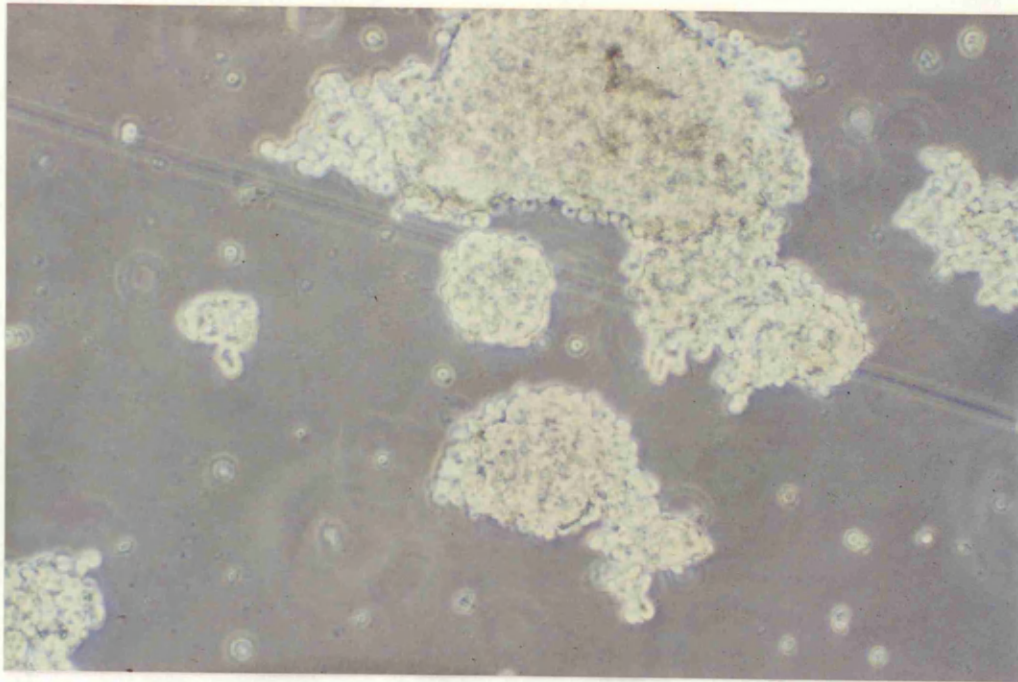
Photograph 3:- H69 Cells (10x objective).

H69 cells were grown in RPMI 1640 medium supplemented with 10% FCS.
The cells are in late log phase of growth.
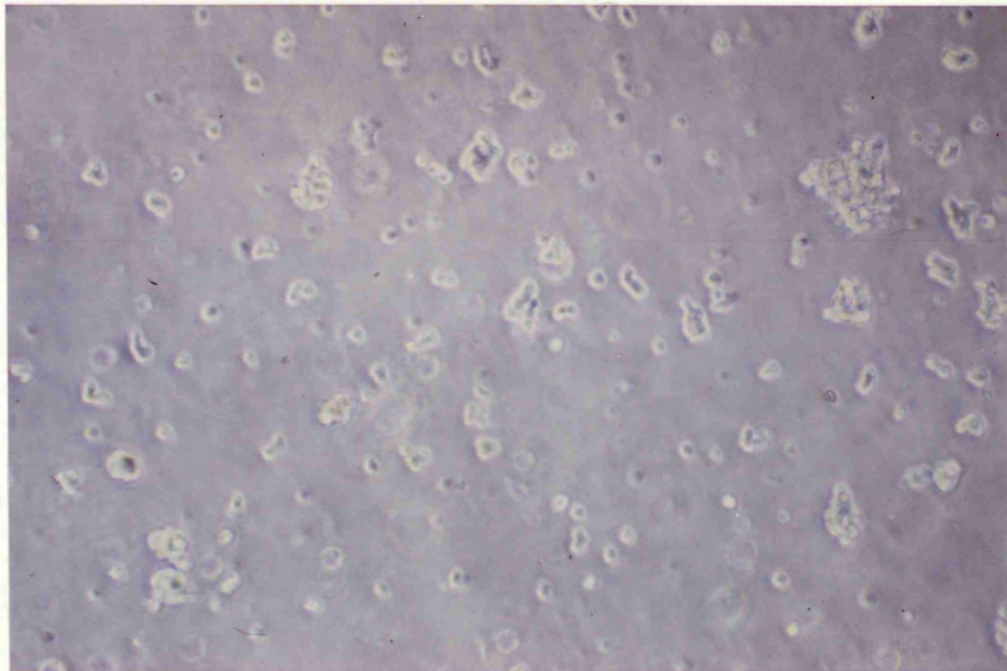
Photograph 4:- H69 Cells Diaggregated by Repeated Pipetting (10x objective).

H69 cells were grown to late log phase of growth. The cells were then
pipetted up and down using a 10ml pipette to disaggregate the cell
clumps. The H69 cell suspension consisted of single cells and
aggregates of varying cell number.
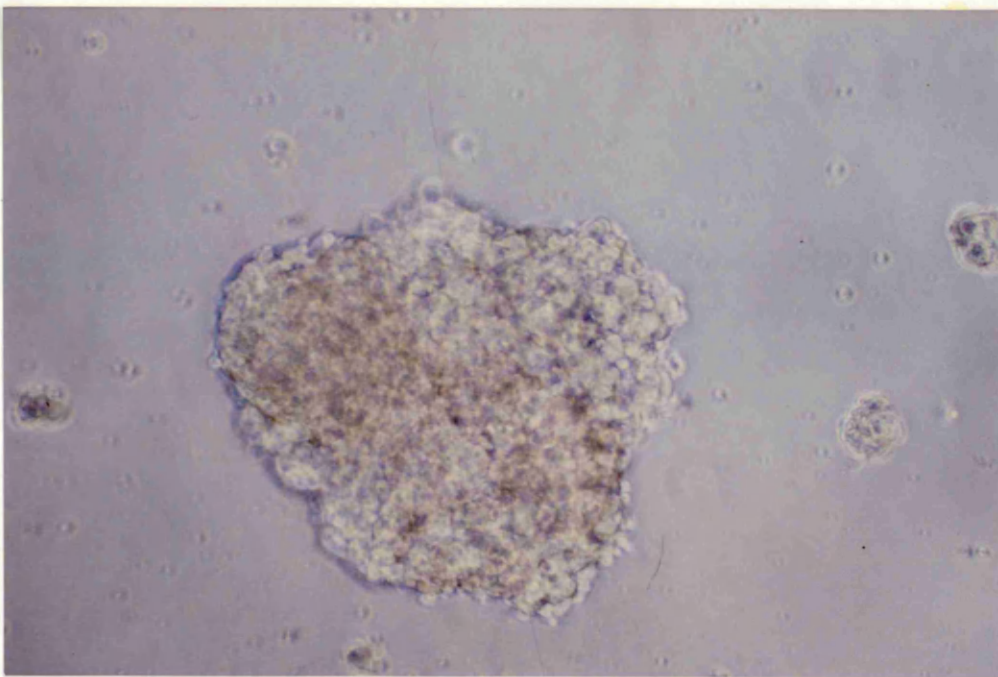
100 μm

Photograph 5:- H128d Cells (10x objective).

H128d cells were grown in RPMI 1640 supplemented with 10% FCS. The
cells were grown to late   log phase of growth.
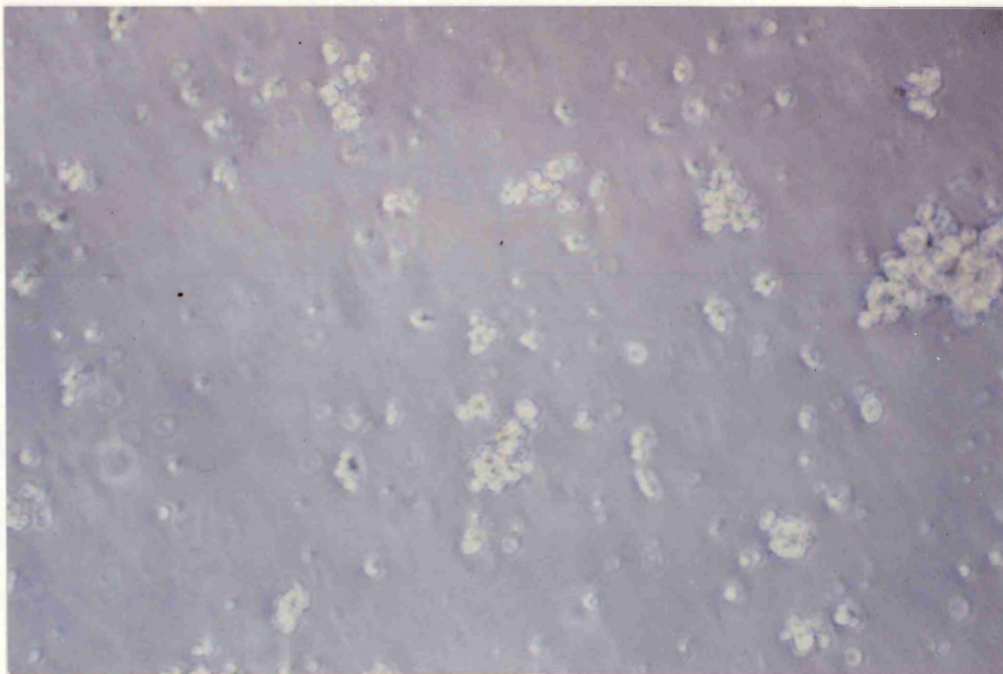
100 μm

**Photograph 6:- H128d Cells Disaggregated by Repeated Pipetting (10x
         objective).**

H128d cells were grown to late log phase. The cells were then
pipetted up and down using a 10ml pipette. The cell suspension
consisted of aggregates of various sizes.

A microtitration assay has a number of advantages which makes it an attractive means of determining cell growth. It is less time consuming, a large number of replicates can be handled easily, it enables the determination of the effect of a number of drugs simultaneously and it can be semi-automated.

Measurement of cell growth by microtitration based on the MTT assay was found to correlate well with electronic cell counting (Figs 3 & 4, and Table 2). Therefore, the MTT assay could be used to determine the growth cycle of the SCLC cell lines. Furthermore, since the MTT assay only measures viable cells one could distinguish between dead and live cells, which is not possible by electronic cell counting.

Table 2: Doubling Times (td) of SCLC Cell Lines.

Cell Line.	td by electronic cell counting (days)	td by MTT assay (days)
H187	2.33 ± 0.15, (n=6)	2.57 ± 0.32, (n=4)
H69	3.02 ± 0.22, (n=5)	3.80 ± 0.22, (n=4)

(results represent the mean of n determinations ± standard error).
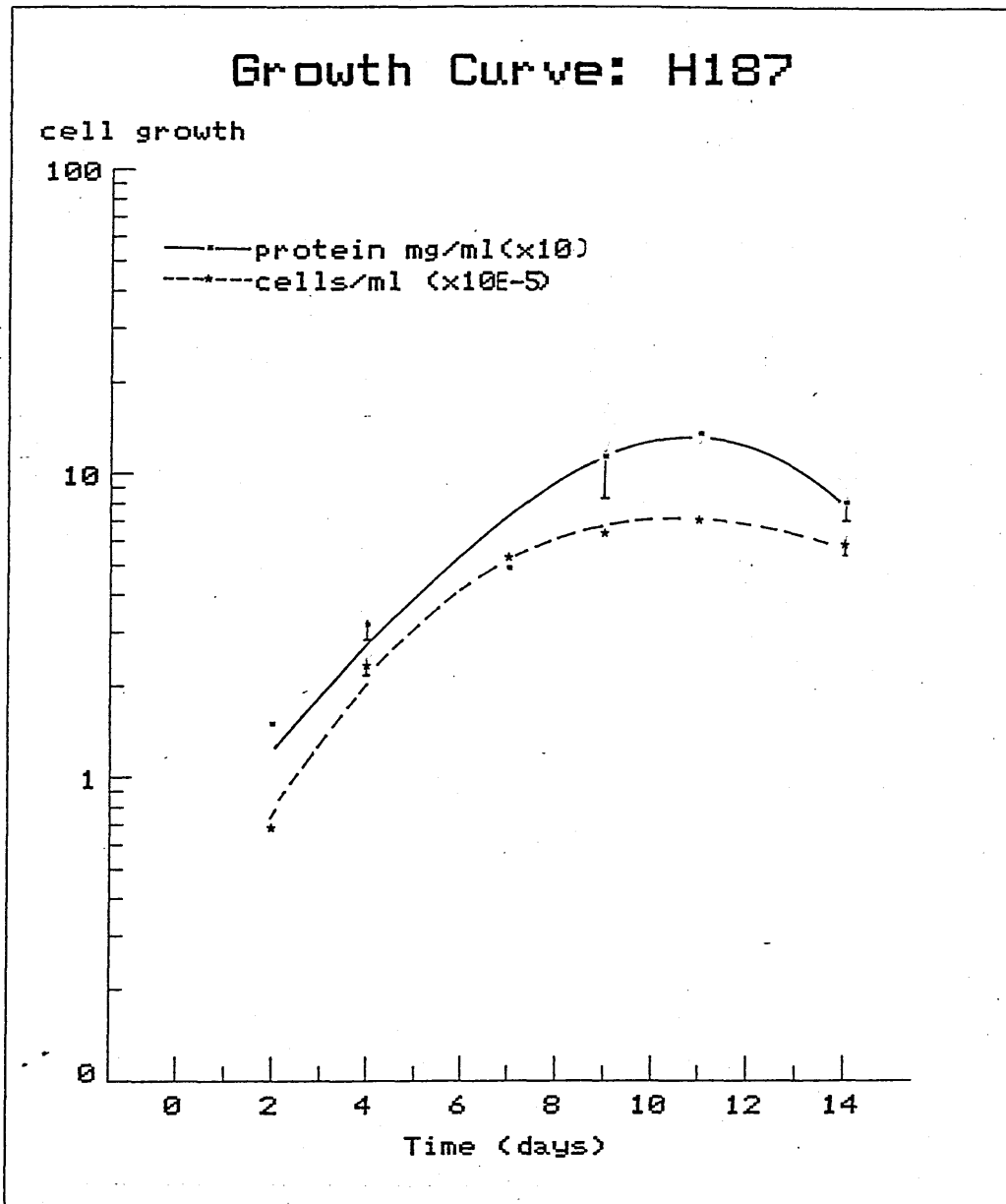
Figure 1:- Growth Curves of H187 Cells.

The cells were seeded into either 24 well plates or 25cm^2 flasks at 3x10^4 cells/ml. On the appropriate days the cell growth was measured by cell counting (24 well plate) using an electronic counter or by measuring protein content (25cm^2 flask).
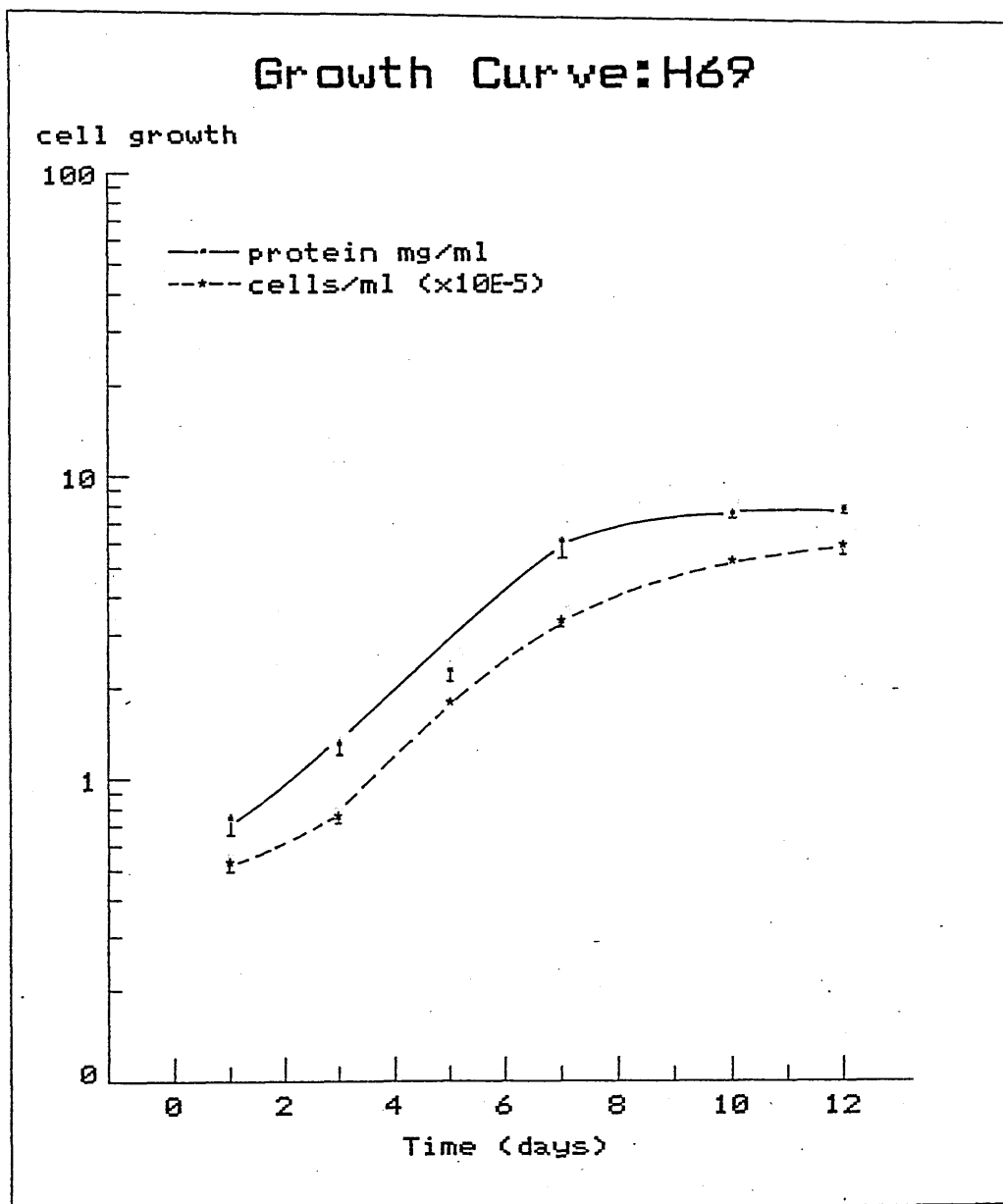
Figure 2:- Growth Curves of H69 Cells.

The cells were seeded into either 24 well plates or 25cm^2 flasks at 3x10^4 cells/ml. On the appropriate days the cell growth was measured by cell counting (24 well plate) using an electronic counter or by measuring protein content (25cm^2 flask).
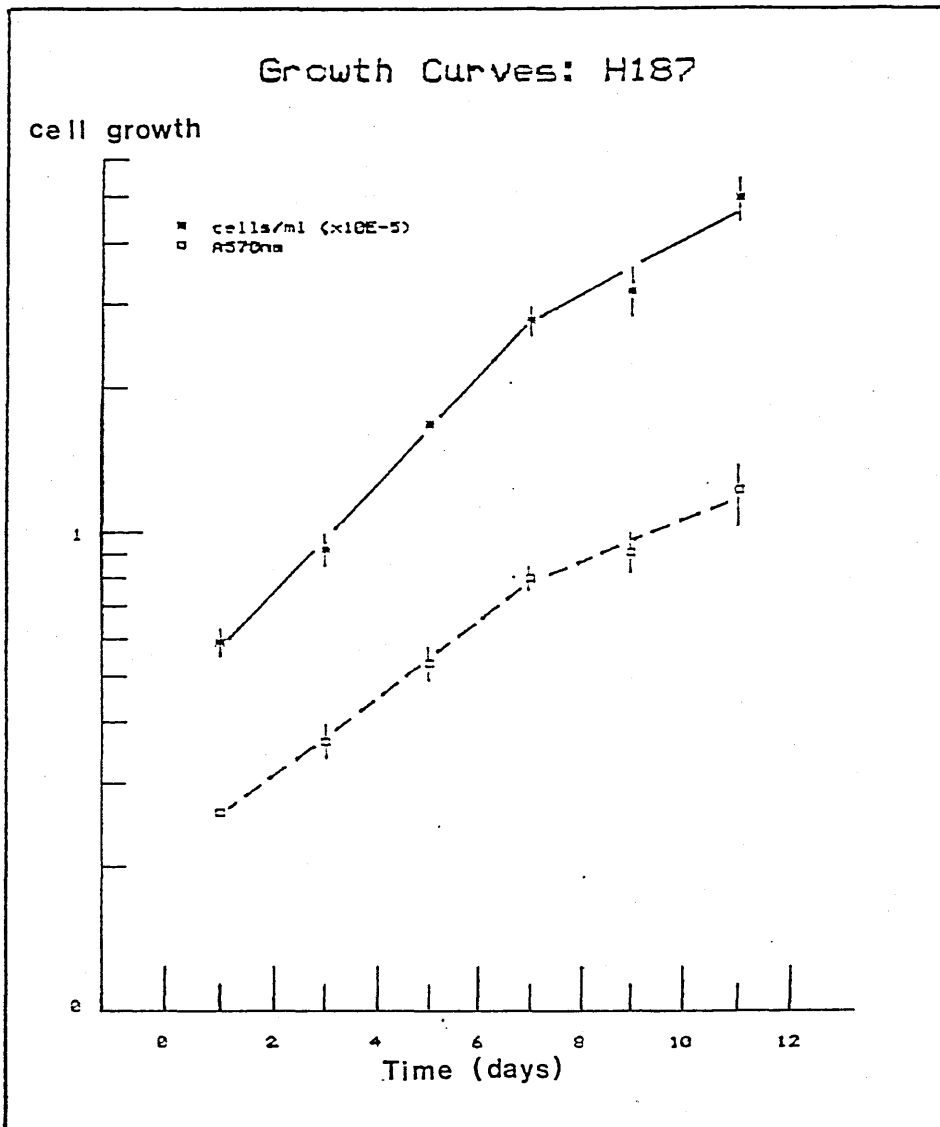
Figure 3:- Growth Curves of H187 Cells.

The cells were seeded into either 24 well plates or into 96 round
bottomed well plates. On the appropriate days the cell growth was
measured by cell counting (24 well plate) or by carrying out an MTT
assay (96 well Plate).
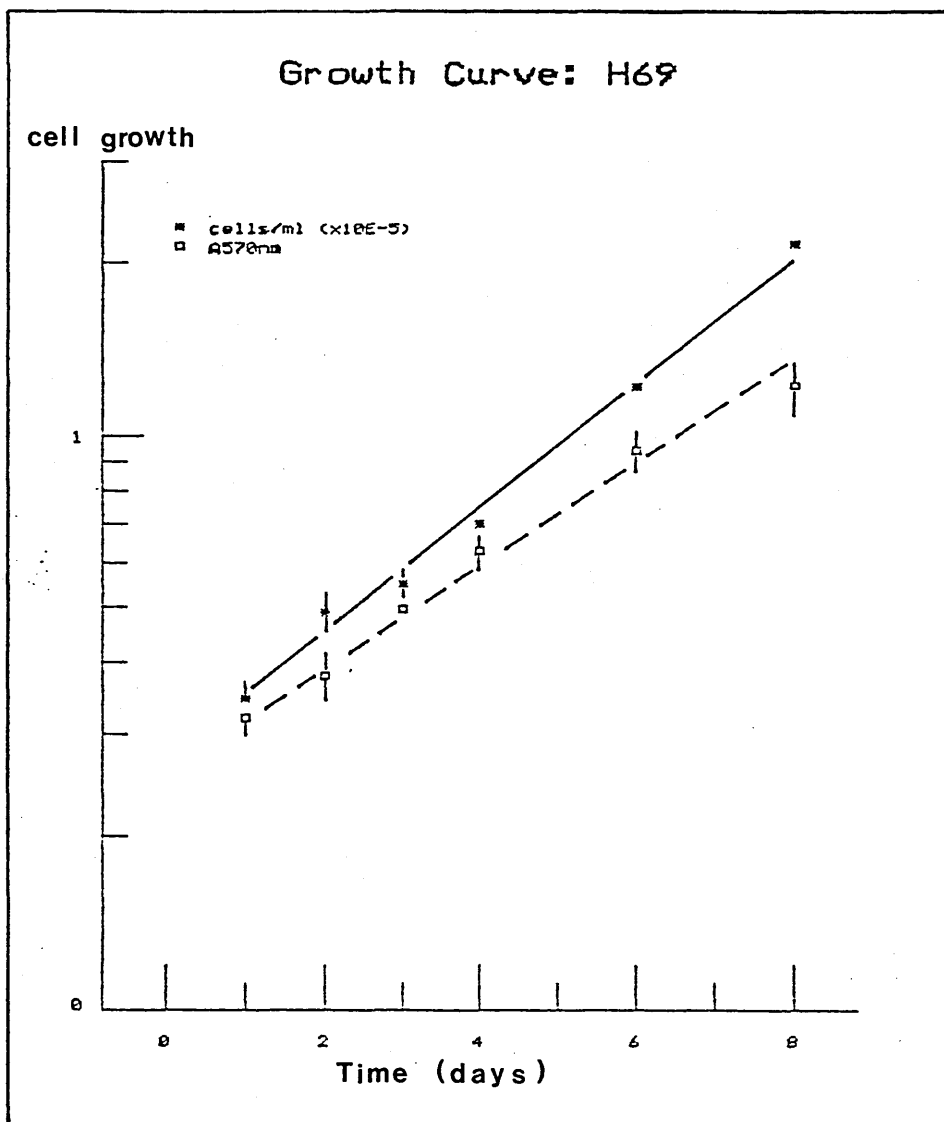
Figure 4:- Growth Curves of H69 Cells.

The cells were seeded into either 24 well plates or into 96 round bottomed well plates. On the appropriate days the cell growth was measured by cell counting (24 well plate) or by carrying out an MTT assay (96 well Plate).

Bombesin Assay.

Assay of Bombesin Levels by the Degree of Mitogenicity in Swiss 3T3 Cells.

Rozengurt and Sinnet-Smith (1983) showed that bombesin was a potent mitogen for Swiss 3T3 cells. Bombesin was found to induce DNA synthesis in quiescent cultures of these cells in a concentration dependent manner, with the maximal effect being achieved at 3nM. This effect has been shown to be receptor mediated, antagonised by [D Arg^1, D Pro^2, D Trp^{7,9}, Leu^{11}] substance P (Zachary & Rozengurt 1985).Therefore, since bombesin can stimulate Swiss 3T3 cell mitogenesis, could this be used as a means of determining unknown levels of bombesin?

SCLC cell lines are known to produce and secrete bombesin (Moody et al, 1981). Therefore, if conditioned medium from exponentially growing cultures of SCLC cells was added to quiescent cultures of 3T3 cells would it be possible to quantify the amount of bombesin present by the degree of mitogenesis which may occur?

Swiss 3T3 cells were rendered quiescent by removal of serum. 24hrs later known amounts of bombesin were added to the cells and the cells were incubated for 24hrs at 37°C in 2%CO_2. The cell number was measured at regular intervals thereafter using the MTT assay.

Protocol:-

1. Swiss 3T3 cells were trypsinised in 0.25% trypsin in PBS and resuspended in RPMI 1640 10% FBS at a final concentration of 5x10^4 cells/ml.

2. The cells were aliquotted into 96 well plates, 200μl/well.
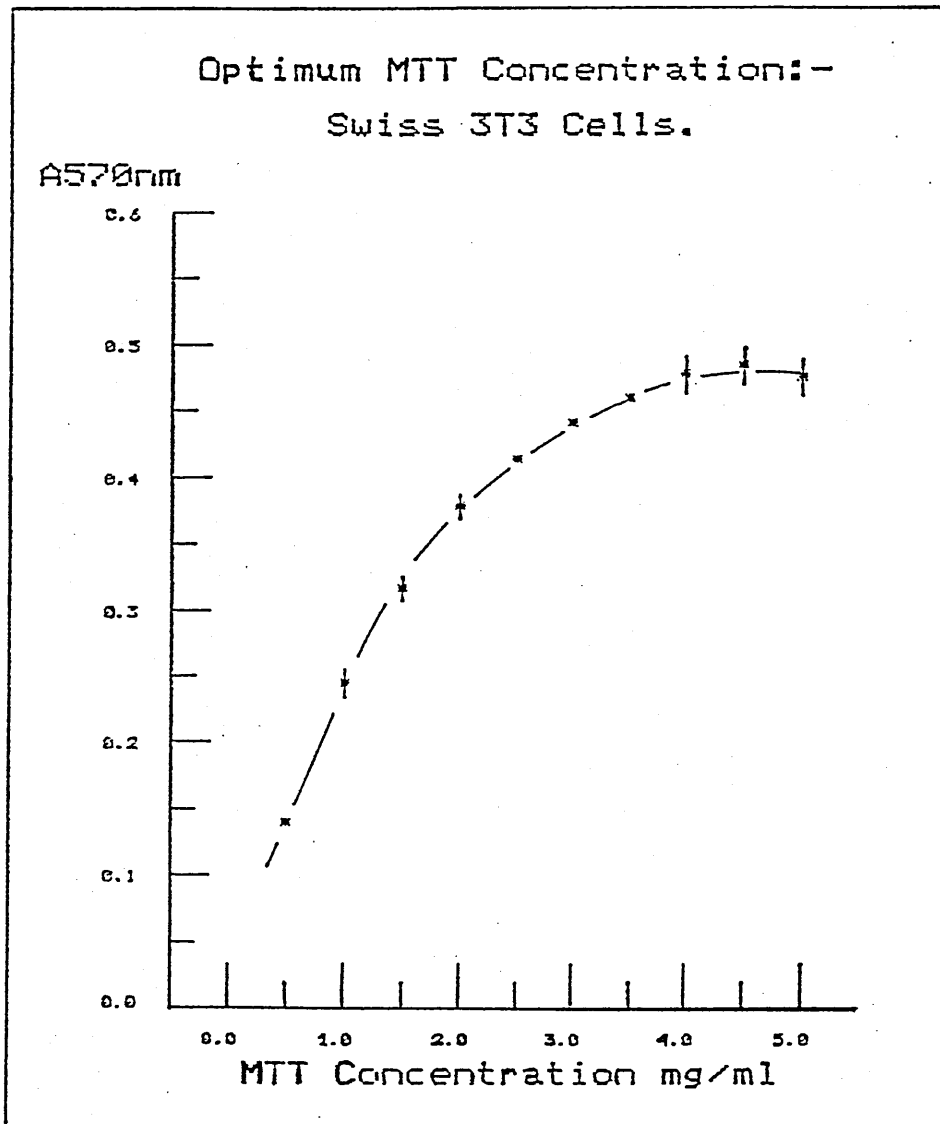
Figure 5:- Optimum MTT Concentration for Maximum Formazan Production in Swiss 3T3 Cells.

Swiss 3T3 cells were seeded into 96 well plate at 5x10^4 cells/ml (1x10^4 cells/well) and incubated for 24 hours at 37°C in 2% CO_2. After the incubation period MTT was added to the plate at various concentrations. Following a 4hour incubation period the absorbance at 570nm was determined, as a measure of the amount of formazan produced.

Each point represents a mean of 8 determinations ± standard error.

3. The plates were incubated at 37°C in 2%CO$_2$ for 24hrs to allow cell adhesion and the initiation of cell proliferation.

4. After 24hrs the medium was replaced with serum free medium to render the cells quiescent.

5. Bombesin was added to the cells at a concentration range 10^{-6}M - 10^{-13}M, incubated for 24hrs.

6. The cell number was determined using the MTT assay (see below).

Note:- The optimum concentration of MTT for maximum formazan production in 3T3 cells was taken as 4.5mg/ml, fig 5.

Results and Conclusions:-

Following a 24hr exposure of 3T3 cells to bombesin no stimulation of cell proliferation was found, even although a marked increase in cell number was found by Rozengurt and Sinnet-Smith (1983) after a 24hr exposure. Perhaps DNA synthesis had been stimulated in these cells but the effect was not significant enough to be manifested into an increase in cell number after 24hrs. However even longer exposure times to bombesin did not result in a increase in Swiss 3T3 cell mitogenesis.

Since our Swiss 3T3 cells did not respond to bombesin they could not be used to determine unknown levels of bombesin in SCLC.

Radioimmunoassay of Bombesin.

Materials:-

Bombesin RIA Kit   ---   INCstar Ltd.
Acetic Acid   ---   BDH

Radioimmunoassay (RIA) depends on the precise antigen specificity of antibodies. A known amount of radioactive antigen ^{125}I-Bombesin, is added with a fixed amount of antibody, rabbit antibombesin, to a sample containing an unknown quantity of unlabelled antigen, bombesin. The unlabelled bombesin competes with the labelled bombesin for antibody binding sites so that the greater the amount of bombesin in the unknown sample the smaller the amount of radioactive bombesin bound to the antibody. The antibody with bound bombesin is precipitated using a second antibody, goat anti-rabbit immunoglobulin. The radioactivity in the precipitate is counted using a gamma counter. The amount of bombesin in the sample is calculated from a standard curve, where known amounts of unlabelled bombesin are added to the ^{125}I-bombesin/rabbit antibombesin incubation mixture.

This assay has a number of advantages. It is specific and reproducible and it can be used to measure both cellular and secreted bombesin levels.

Cells were harvested, washed and cellular bombesin acid extracted. The amount of bombesin in the cells was measured by RIA.

Protocol:- Adapted from method of Bepler et al, 1987.

1. The cells were harvested by centrifugation 200g for 5mins, and the cell pellet washed twice with PBS.

2. The pellet was resuspended in 1ml 2M Acetic Acid, and boiled for 10mins.

3. The samples were clarified by centrifugation at 10,000g. Supernatant lyophilised and stored at -70°C.

4. The freeze dried extract was dissolved in 1ml PBS, and the amount of bombesin was assayed by RIA following the manufacturers instructions.
   Briefly, a sample of cell extract for analysis was added simultaneously to rabbit antibombesin and ^{125}I-bombesin and incubated at 4°C overnight. The second antibody, goat antirabbit, was added and following a 20min incubation at 25°C the samples were centrifuged, decanted and the precipitate counted.

5. The acid soluble protein was measured using the Biorad Protein Assay (see later).

The results are expressed as pg bombesin/ μg acid soluble protein.

Creatine Kinase.

Materials:-

Creatine Kinase   --- Sigma

Creatine Kinase Electrophoresis :

   Cardiotrac-CK (CK isoenzyme substrate set)

   3 in 1 Electrophoresis Control Serum ("level II")

   MOPSO Buffer Set   --- Corning Diagnostics Ltd.

Creatine phosphate acts as a storage form of high energy phosphate groups in vertebrate muscle and other tissues. When energy is required it is creatine kinase (CK) which is responsible for transferring a high energy phosphate group from creatine phosphate to adenosine diphosphate (ADP) to form adenosine triphosphate (ATP) and creatine.

$$\text{Creatine Phosphate + ADP} \xrightarrow{\text{CK}} \text{ATP + Creatine}$$

The soluble cytoplasmic forms of CK occur as dimers consisting of three possible combinations of two antigenically distinct chains, M (muscle form) and B (brain form).

MM, MB and BB.

In order to establish the amount of CK isoenzyme activity initially one has to measure total CK activity. Total CK activity is measured indirectly by means of a NAD linked reaction. CK catalyses the reaction between creatine phosphate and ADP, forming creatine and ATP, in the presence of hexokinase (HK). The ATP formed phosphorylates glucose to glucose-6-phosphate (G-6-P). Glucose-6-phosphate dehydrogenase (G-6-PDH) then catalyses the oxidation of G-6-P in the

presence of nicotinamide adenosine diphosphate (NAD). During this oxidation an equimolar amount of NAD is reduced to NADH.

<div align="center">

CK

Creatine Phosphate + ADP -------> Creatine + ATP

HK

ATP + Glucose -------> ADP + G-6-P

G-6-PDH

G-6-P + NAD -------> 6PG + NADH

</div>
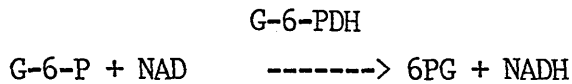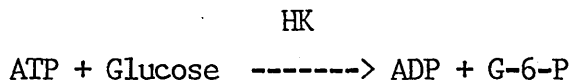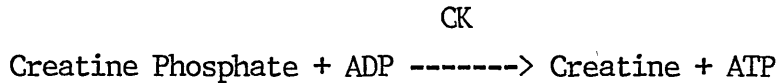
The increase in amount of NADH is measured as a change of absorbance at 340nm. The rate of change in absorbance is directly proportional to CK activity.

Having established the total CK activity the percentage of each CK isoenzyme is measured. The CK isoenzymes are separated by gel electrophoresis. The distinctly separate zones can be detected by overlaying the agarose gel with creatine kinase substrate. The identification of the CK isoenzymes by this method also involves a NAD linked reaction, as above. In this case the zones with CK activity fluoresce under U.V. light and can be quantified using a scanning fluorimeter.

Protocol:-

Extraction of Cellular Creatine Kinase.

CK was extracted from the cells by the same method used to extract DOPA decarboxylase (see below). Extraction by freeze thawing 3x was found not to affect the enzyme activity.
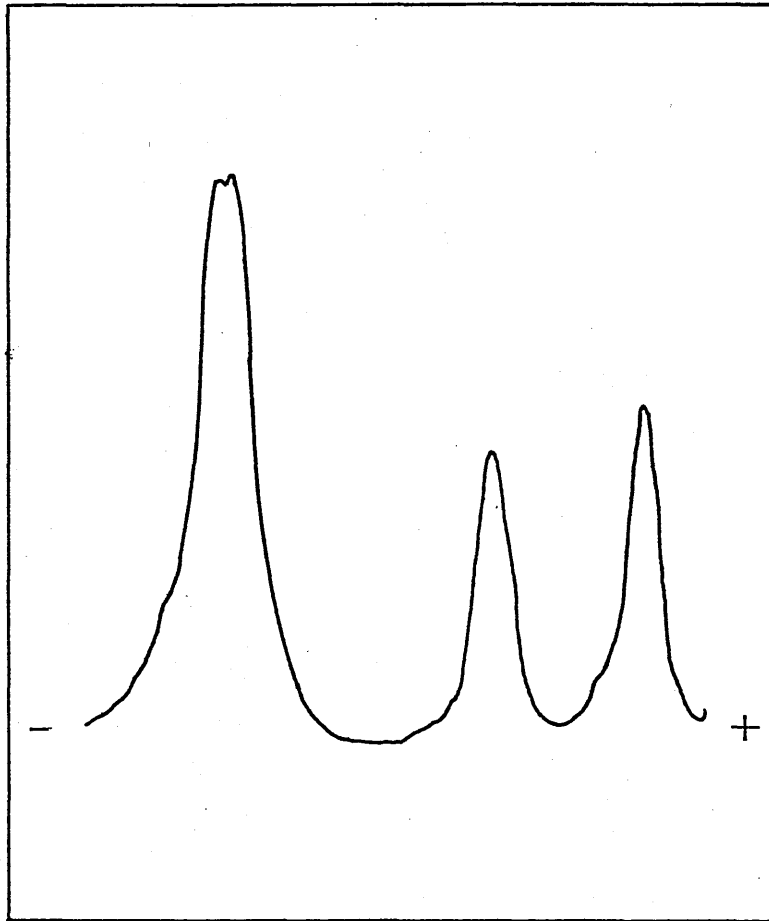
Figure 6:- Electrophoretic Scan of the Creatine Kinase Isoenzymes.

The scan shows the result of electrophoretic separation of the three CK isoenzymes in control human serum. The first peak represents the MM isoenzyme, followed by the MB isoenzyme and lastly the BB isoenzyme.

Assay of Creatine Kinase Activity.

CK activity was quantified from a standard U.V. kinetic assay supplied by Sigma. The assay was performed following the manufacturers instructions. Briefly, a sample of the cell extract for analysis was added to the creatine kinase reagent (Sigma) and incubated at $30^{\circ}C$ for 5mins in a spectrophotometer (Gilford 250). During this time, absorbance readings at 340nm were taken every 30 seconds. An increase in absorbance at 340nm represented an increase in the formation of NADH. One unit of CK activity was defined as the amount of enzyme which produced one μmole of NADH per minute under the conditions of the assay procedure.

Isoenzyme distribution of enzymatic CK was determined by electrophoresis using reagents and equipment supplied by Corning Diagnostics Ltd. The assay was performed following the manufacturers instructions. The isoenzymes were separated by electrophoresis on preformed 1% w/v agarose sheets (Corning Diagnositics) at 9V/cm for 23 mins in a 0.05M MOPSO [3-(N-Morpholino)-2-hydroxypropane sulfonic acid] buffer, pH 7.8. The CK isoenzymes were identified by overlaying the gel with creatine kinase substrate (Corning) and measuring the NADH produced using a scanning fluorimeter (Helena Densitometer). Figure 6 is an example of an fluorimetric scan of the CK isoenzymes.

DOPA Decarboxylase Assay.

Materials:-

Pyridoxal 5'phosphate	---	Sigma
L-β-3,4- dihydroxyphenylalanine (L-DOPA)	---	Sigma
DL-β-3,4- dihydroxyphenylalanine (DL-DOPA)	---	Sigma
D-β-3,4- dihydroxyphenylalanine (D-DOPA)	---	Sigma
3-Hydroxytyramine (dopamine)	---	Sigma
Nialamide	---	Sigma
EDTA	---	BDH
Sodium tetraphenylboron	---	BDH
Heptan-3-one	---	BDH
Borax	---	BDH
KH_2PO_4	---	BDH
Na_2HPO_4	---	BDH
NaH_2PO_4	---	BDH
Dihydroxyphenylalanine L-3,4-[ring -2,5,6-^{3}H] ([^{3}H] L-DOPA)	---	Du Pont
(2,5,6-^{3}H) dopamine	---	Amersham UK
Ecoscint	---	National Diagnostics

DOPA decarboxylase (DDC) was first discovered in the kidney; it also occurs in large amounts in the liver as well as in adrenergic neurones and chromaffin cells. The enzyme appears to be free in the cytoplasm and not associated with any subcellular structure. It requires pyridoxal 5'phosphate for activity, and is specific for the L-forms of amino acids.

DDC decarboxylates L-DOPA to dopamine with the loss of carbon dioxide.

DDC
L-DOPA ----------------> Dopamine
CO_2

$$HO-\text{[benzene ring]}-CH_2-CH-NH_2 \\ | \\ COOH \\ OH$$

$$HO-\text{[benzene ring]}-CH_2-CH_2-NH_2 \\ OH$$
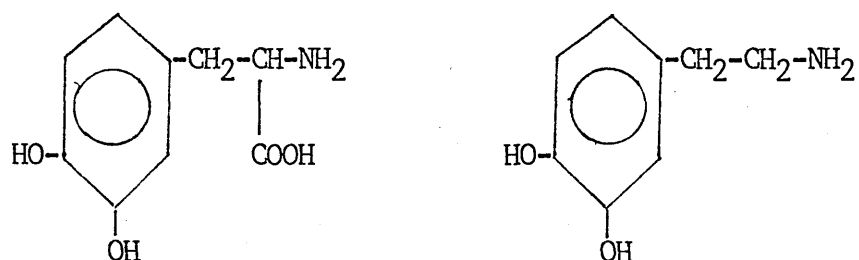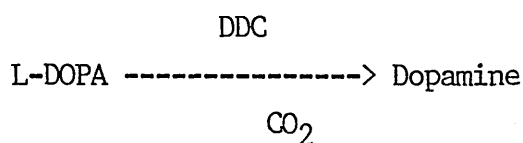
DDC activity can be determined by the rate of formation of dopamine from L-DOPA.

DDC was extracted from the cells. The activity of the enzyme was determined using radioactively labelled L-DOPA and separating the radioactive dopamine formed from the unchanged amino acid. The amount of dopamine formed was directly related to the activity of the enzyme.

1. Extraction of Cellular DDC.

Protocol:-

1. Cells were harvested by centrifugation at 200g for 5mins.

2. The cell pellet was washed twice with ice cold PBS.

3. Finally the cell pellet was resuspended in 1ml borate buffer pH7.6 (minimum), in 15ml plastic test tubes, and an aliquot removed and stored for assay of protein and DNA content.

4. The remaining cell/buffer suspension was frozen thawed 3x using solid CO_2 (Drikold)/methanol and a 37°C water bath. Freeze thawing 3x was found to cause 100% lysis of the cells, examined by trypan blue exclusion. It did not have any adverse effect on the enzyme activity.

5. The sample was then transferred to Eppendorf tubes and centrifuged at 8000g, for 5mins.

6. The supernatant was assayed for DDC activity.

2. Enzyme Substrate Reaction.

Incubation Mixture (final concentrations)

Borate Buffer	--- 0.025M Borax + 0.05M KH_2PO_4 pH 7.6
Pyridoxal 5'Phosphate	--- 100µM
Nialamide	--- 125µM
EDTA	--- 1.5mM
L-DOPA	--- 1200µM
^{3}H-L-DOPA	--- 30µCi/ml

Protocol:-

1. The reaction mixture (above) was prepared, omitting the pyridoxal 5'phosphate.

2. 45µl samples were aliquoted out into 15ml glass stoppered centrifuge tubes.

3. The samples were then incubated at 37°C for 5mins.

4. 200μl of the DDC extract was added to 100μl of pyridoxal 5'phosphate (1200μM) and mixed thoroughly. (Adding pyridoxal 5'phosphate to the enzyme minimises any reaction with the substrate).

5. 15μl of the enzyme/pyridoxal 5'phosphate mixture was then added to the appropriate tubes to start the reaction.

6. The samples were incubated at 37°C for 1 hour.

7. The reaction was stopped by adding 5mls ice cold sodium phosphate buffer 10mM pH 6.5. The tubes were then transferred to ice.

Blanks:-

a. Reagent Blank - Incubation mixture without enzyme incubated for 1 hour.

b. Sample Blank - Incubation mixture incubated for 1 hour, add enzyme extract after incubation period.
Similar results were obtained in both cases.

The quantity of DOPA carried over into the dopamine extracted samples has been found to be equivalent to that extracted in the blanks (Laduron & Belpaire 1968). Therefore it is necessary to subtract the value of the blank from the samples in order to obtain an accurate value of the dopamine formed. This also accounts for any nonenzymatic decarboxylation of DOPA which may occur.

3.Extraction of Dopamine.

The amine, dopamine, is extracted from the unchanged amino acid, DOPA, by liquid cation exchange with sodium tetraphenyl boron (Fonnum 1969). Sodium tetraphenyl boron forms a complex with cations that is insoluble in water but soluble in organic solvents such as higher ketones.   In a weak acid buffer dopamine becomes protonated whereas DOPA undergoes internal exchange and forms a Zwitterion.   Sodium tetraphenylboron complexes with dopamine to form an insoluble salt. DOPA does not combine with the sodium tetraphenylboron as the Zwitterion has no overall charge.   Addition of an organic solvent separates DOPA and dopamine : the dopamine/tetraphenylboron complex dissolves in the organic phase whereas the DOPA remains in the aqueous phase. Dopamine is not extracted by ketone in the absence of sodium tetraphenylboron.

Sodium tetraphenylboron in heptan-3-one (ketone) was added to the samples. The organic layer was extracted, washed and the ^{3}H-dopamine present counted.

Protocol:-

1.   1ml of heptan-3-one containing 25mg sodium tetraphenylboron was added to each sample tube.

2.   The samples were mixed gently by inversion for approximately 1min.

3.   The two phases were separated by centrifugation at 1500g for 5mins at 4°C.

4. The aqueous phase was removed using a Pasteur pipette and discarded.

5. The organic phase was washed with 2mls of 10mM sodium phosphate buffer pH 7.4, containing 1mg sodium tetraphenylboron, (any DOPA remaining is back extracted into the aqueous phase).

6. The samples are centrifuged at 1500g for 5 mins at 4°C.

7. An 0.5ml aliquot was removed from the organic layer and 10mls Ecoscint was added. The radioactivity present was counted for 10mins using a scintillation counter (Packard Liquid Scintillation Spectrometer).

Note:- Heptan-3-one quenches by 50%

Extraction Efficiency:-

% recovery of dopamine 76.3%

% recovery of DOPA 0.33%

MTT Cytotoxicity Assay.

Materials:-

MTT ([3-(4,5-Dimethylthiazol-2-yl)-2,5-
         diphenyltetrazolium bromide)   --- Sigma

Glycine   --- BDH

NaCl   --- BDH

Dimethyl sulphoxide (DMSO), Analar grade --- BDH

96 round bottomed well microtitration plates
        (Cat.N° 16332 0A)   --- Nunclon

Cytotoxic Drugs.

Adriamycin   --- Farmitalia, Carlo Erba Ltd.

Vincristine Sulphate   --- David Bull Laboratories

Vepesid (Etoposide VP16-213)   --- Bristol-Myers Pharmaceuticals

Clonogenic and biochemical assays have been widely used to measure the chemosensitivity of tumour cell lines.

Clonogenic assay measures cell survival by proliferative capacity. It is a widely accepted method but is time-consuming and it is difficult to automate. Another problem with this assay is that some cells have a low plating efficiency, so that there is low survival in the untreated controls. A clonogenic assay as a measure of chemosensitivity was not suitable for SCLC cell lines. The cells grew as tight aggregates and it was difficult to obtain a single cell suspension, a prerequisite of the clonogenic assay. Repeated pipetting reduced the cell viability.

Biochemical assays based on the principle of microtitration have been used to measure radiolabelled precursor incorporation into nucleic acid or protein as a measure of cell survival. A microtitration assay provides a means for quick assessment of cytotoxicity. It allows measurement of a number of variables easily e.g. drug concentration, drug exposure time and cell density. Replicate samples can be handled easily and it can be semiautomated. However in many cases these assays involve a number of manipulations and can therefore introduce variability into the system. The recent introduction of a microtitration colorimetric assay based on the ability of living metabolically active tumour cells to reduce a tetrazolium based compound (MTT) to a purple formazan product (Mosman 1983; Carmichael et al, 1987), has provided a real alternative to the clonogenic assay for assaying chemosensitivity of tumour cells, including SCLC cell lines.

The MTT assay has been shown to correlate well with the clonogenic assay (Plumb et al, 1987). There is a linear relationship between cell number and the amount of formazan produced for both adherent (Fig 7) and non-adherent cell lines (Fig 8, Milroy personal communication), i.e. the amount of formazan produced is directly proportional to cell number. This allows the MTT assay to be used not only as a chemosensitivity assay but also as an estimation of cell number, especially where cell counting by conventional means is inappropriate (non-adherent cell lines which grow as tight aggregates).

The assay involved the use of 96 well microtitration plates. The cells were seeded into the plates and treated with the appropriate drug at various concentrations, for the required time. At the end of the assay cell survival was measured by adding MTT directly to the medium in the wells and incubating for 4 hours. Formazan crystals were formed in the viable cells. The medium was removed and the
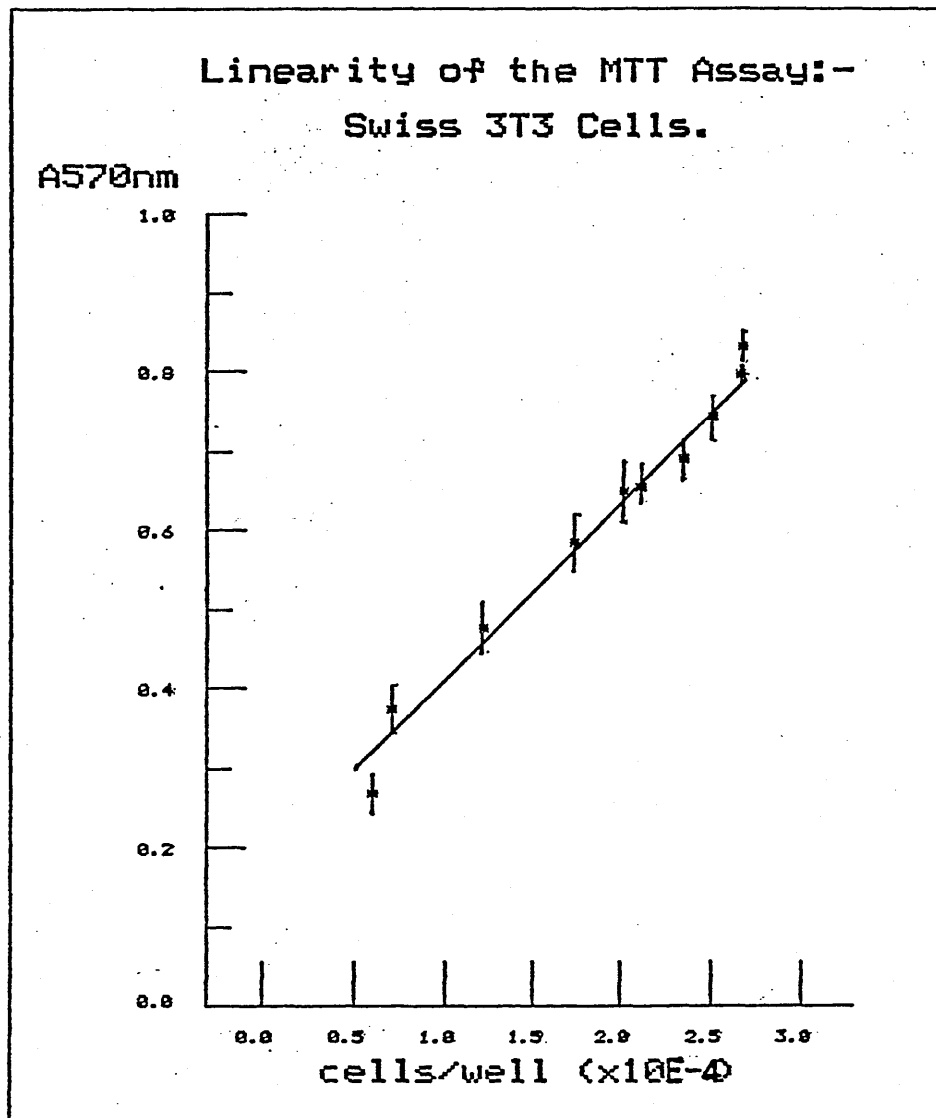
Figure 7:- Linear Relationship Between the Cell Number of a Monolayer Culture and the Amount of Formazan Produced from MTT.

Swiss 3T3 cells were seeded into two 96 well plates in RPMI 1640 + 10% FCS, using a multichannel pipette. The cells were diluted serially across the plate by altering the delivery volume of the multichannel pipette. Medium was added to the plates to give a final volume of 200μl. After 48hrs, an MTT assay was carried out on one plate, the other plate was used to determine cell number. The amount of formazan formed, as measured by absorbance at 570nm, was plotted against the cell number.

Linearity of the MTT Assay:-
H187 Cells.

A570nm
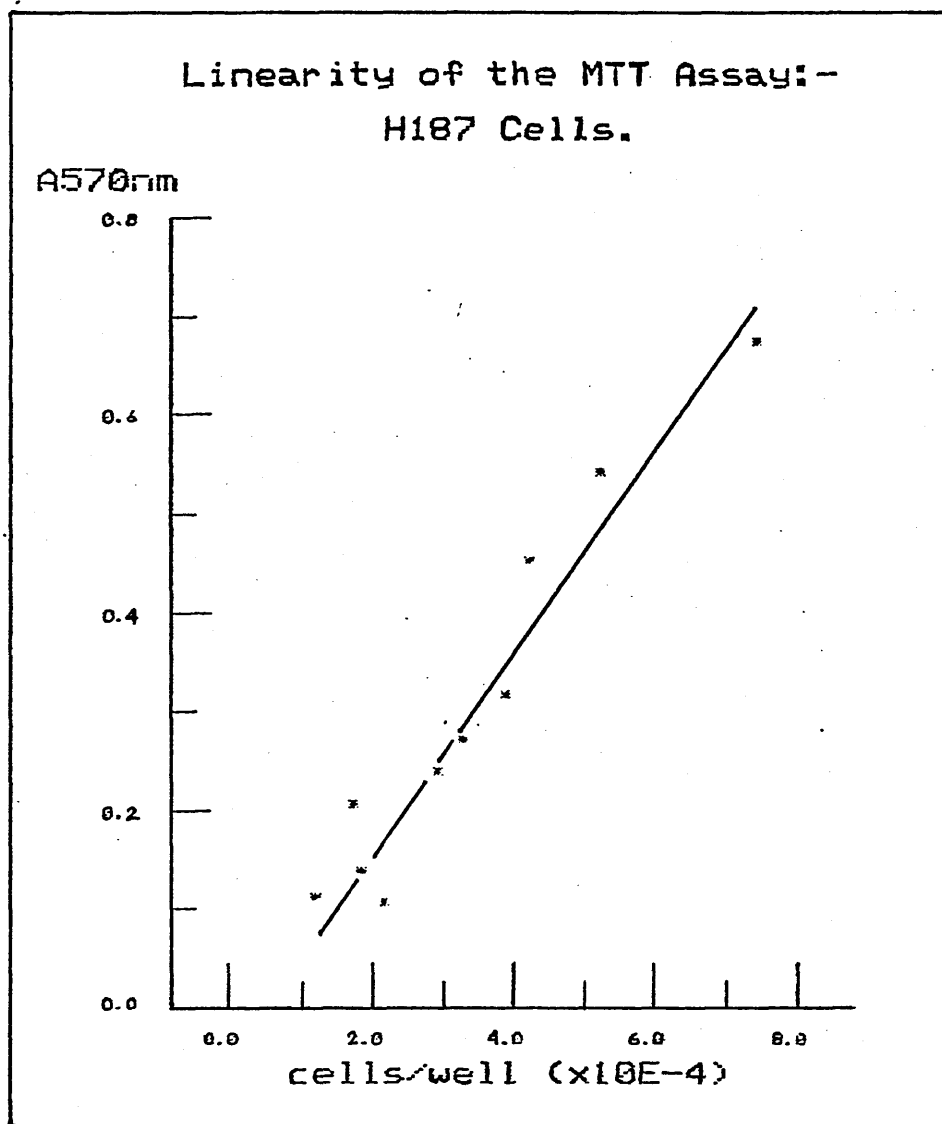
Figure 8:- Linear Relationship Between the Cell Number of a
Suspension Culture and the Amount of Formazan Produced
from MTT.

H187 cells were seeded into two 96 well plates in RPMI 1640 + 10%
FCS, using a multichannel pipette. The cells were diluted serially
across the plate by altering the delivery volume of the multichannel
pipette. Medium was added to the plates to give a final volume of
200µl. After 48hrs, an MTT assay was carried out on one plate, the
other plate was used to determine cell number. The amount of
formazan formed, as measured by absorbance at 570nm, was plotted
against the cell number. Data was kindly provided by R.M. Milroy.

formazan crystals were solubilised with DMSO. The formazan production
was analysed using a microtitration plate reader (Biorad).

Protocol:-

1. <u>Cell Plating:-</u>
   a. Monolayer Cultures: A cell suspension of appropriate cell
   concentration was prepared.
   200µl aliquots were dispensed into a 96
   well plate using a multi-channel dispenser.
   The cells were left for 2 days to allow
   cell adhesion and proliferation.

   b. Suspension Cultures: The cells were seeded into 96 round
   bottomed well plates, 100µM, at 2x final
   concentration.

2. <u>Drug Addition:-</u>
   (cells were in exponential growth at the time of drug
   addition).

   a. Monolayer Cultures: Medium was removed from the wells &
   replaced with fresh medium containing drug.
   The cells were incubated for the
   appropriate period at $37^{o}C$ in 2% CO_2.

   b. Suspension Cultures: Drug was added at the time of cell seeding.
   100µl of the drug, 2x final concentration,
   was added to the appropriate wells.
   The plates were then incubated for the
   appropriate period at $37^{o}C$ in 2% CO_2.

For drug exposure times longer than 24hrs, fresh drug was added every 24hrs at the final concentration.

3. Recovery Period:-

A recovery period is an essential part of the chemosensitivity assay. It allows the distinction between proliferating cells and cells that may appear viable but have lost their proliferative capacity, following a toxic insult. Also, after the drug is removed lethal damage may still accumulate, the effect of which would be lost if a recovery period was not included. It is important to allow time for cell death and the loss of metabolic activity. It is also possible that the effect of a drug could be reversed following removal. This effect would be lost if no recovery period was allowed.

The recovery period for each cell line was chosen to allow approximately 2-3 population doubling times. The cells were still in exponential growth at the end of the experiment.

Monolayer cultures were fed every day.
Suspension cultures were fed on alternate days:-
> the plates were centrifuged at 200g for 5mins, 150µl of the medium was removed, and replaced with 150µl of fresh medium.

4. MTT Assay:-

1. On the final day medium was replaced by medium containing 10mM HEPES.

> (addition of HEPES to the medium was found to reduce the scatter between replicate samples (Plumb, personal communication)).
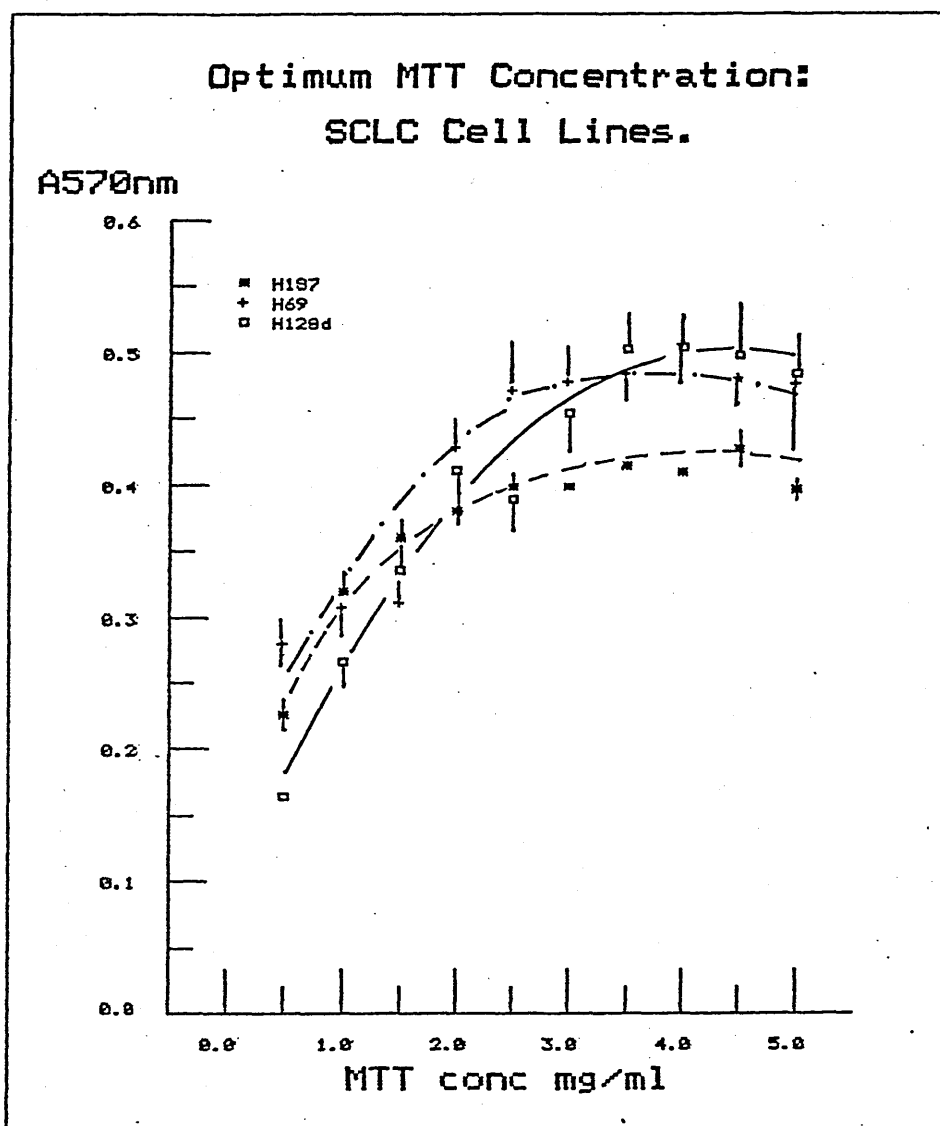
**Figure 9:- Effect of MTT Concentration on the Formazan Produced in
           SCLC Cell Lines.**

SCLC cell lines were incubated with various concentrations of MTT,
4hrs, 37°C, 2% CO_2. The amount of formazan produced by the cells in
the presence of varying MTT concentrations was measured by the
absorbance at 570nm.
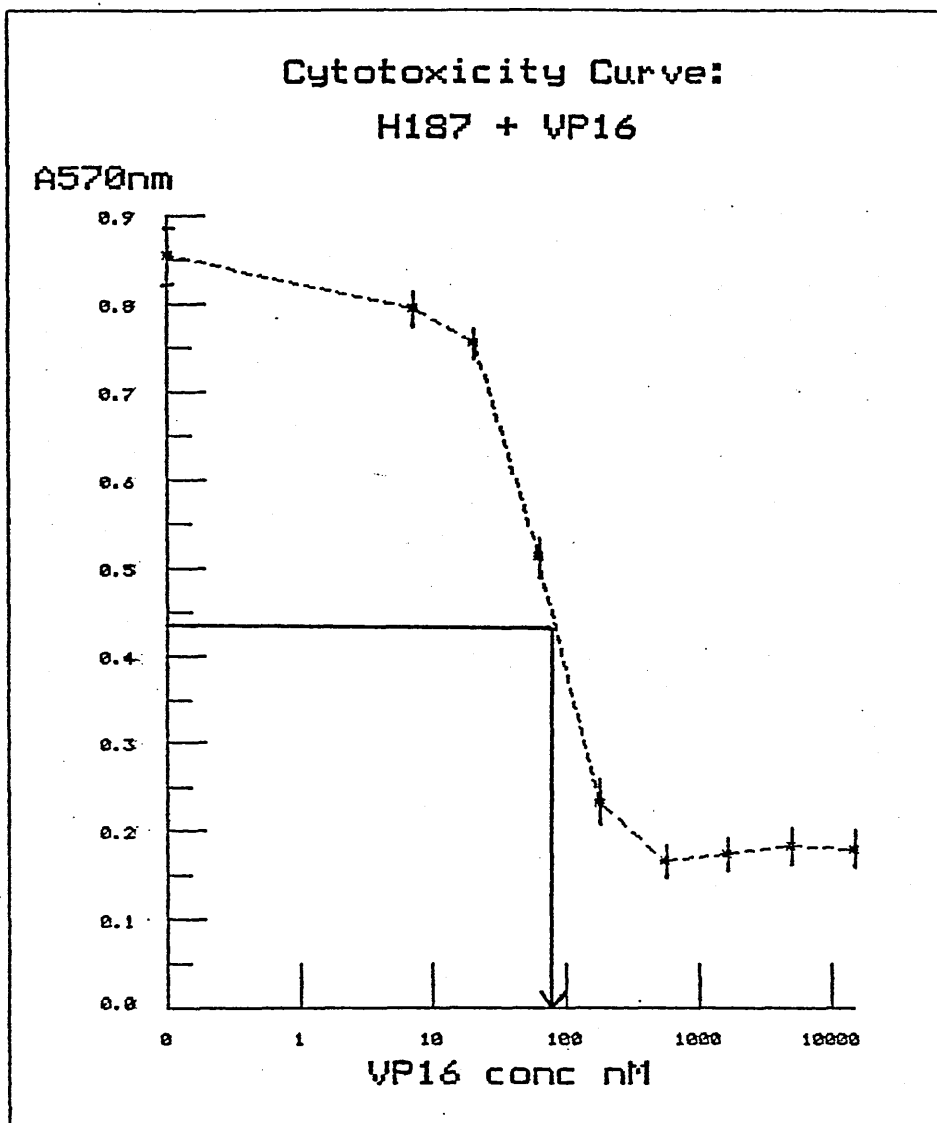
Figure 10:- Cytotoxicity Curve of H187 Cells after a 24 hour Exposure to VP16.

This is a cytotoxicity curve obtained from the MTT assay shown in photographs 7&8. The ID_{50} is taken as the concentration of drug which reduced the control absorbance at 570nm by 50%. In this instance the ID_{50} was found to be 90nM.

Each point represents the mean of 8 replicate samples $\pm$ standard error.

2. 50µl of MTT was added to each well of the plate at a concentration of MTT which allows maximum formazan production (Fig 9):-

NCI-H187 3.0mg/ml
NCI-H69 3.0mg/ml
NCI-H128d 4.0mg/ml.

3. The plates were incubated for 4 hours in the dark. Photograph 7.

4. Medium containing unreduced MTT was removed (suspension cultures were centrifuged at 200g for 5mins).

5. 200µl of DMSO was added to solubilise the formazan crystals followed by 25µl glycine buffer - 0.1M glycine + 0.1M NaCl pH 10.5 (addition of buffer is required to ensure the formazan, which can exist in two forms, is present in one form. (Plumb, personal communication)). Photograph 8.

6. The formazan production was measured using a microtitration plate reader (Biorad Model 2550 EIA Reader).

7. The ID_{50} for a cytotoxic drug was determined by plotting the absorbance at 570nm against the concentration of drug (Fig 10).
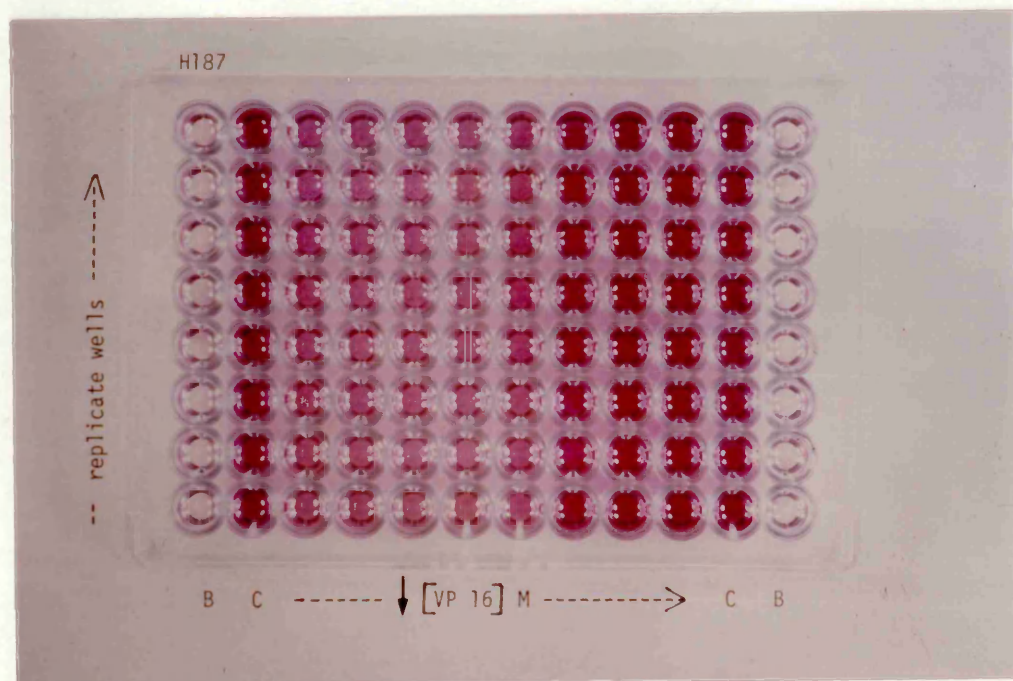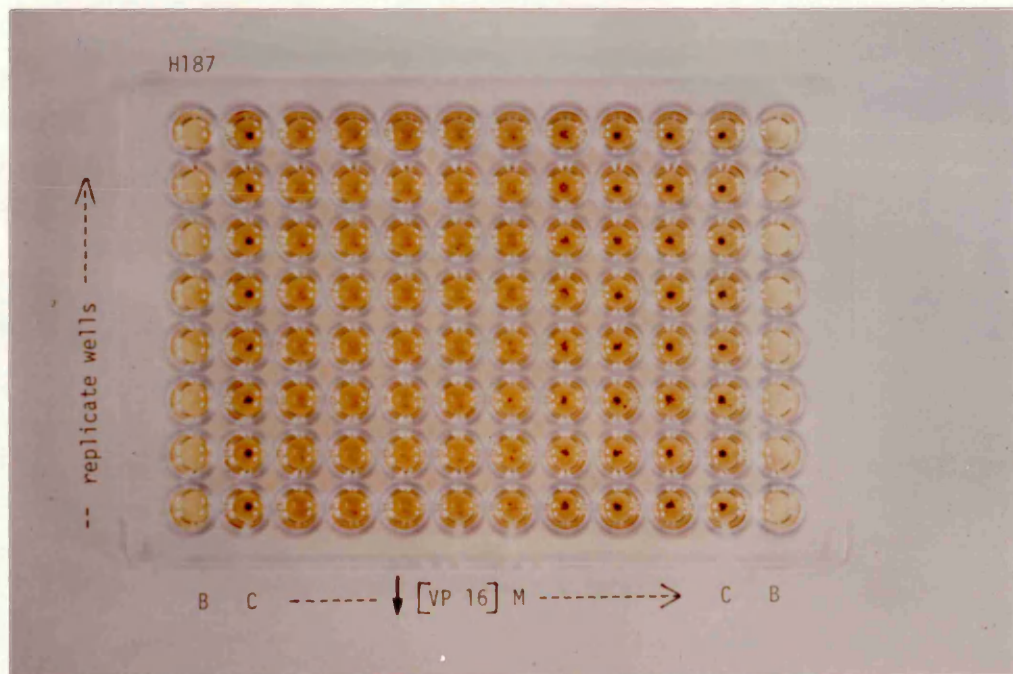
Photograph 7:- MTT Microtitration Plate Before DMSO Addition.

H187 cells were plated into a 96 well microtitration plate and exposed
tp VP16 for 24 hours. The cells were allowed a 5 day recovery period
at which time 3.0 mg/ml of MTT was added to each well. The photograph
shows the viable H187 cells as purple dots in the centre of the
appropriate wells.

   B - blank   C - control wells (no VP16).

Photograph 8:- MTT Microtitration Plate After DMSO Addition.

DMSO has been added to the microtitration plate shown in Photograph 7.
DMSO has solubilised the purple formazan crystals which are present in
viable cells. The intensity of purple colour in each well gives a
measure of the viable cells present.

   B - blank   C - control wells (no VP16).

Biorad Protein Assay.

Materials:-

Protein Dye Concentrate   --- Biorad

Bovine Serum Albumin   --- Sigma

NaOH   --- BDH

The Biorad Protein Assay is based on the observation by Bradford (1976), that the binding of Coomassie Brilliant Blue G-250 to protein causes a shift in the absorption maximum of the dye from 465nm to 595nm. The increase in absorption at 595nm is a measure of the amount of protein present.

Since the assay only measures soluble protein the protein was first solubilised in 0.5M NaOH. A standard curve was performed for every assay where the standard, Bovine Serum Albumin (BSA), was also dissolved in 0.5M NaOH.

The assay was performed following the manufacturers instructions. Briefly, the protein content was measured by mixing an aliquotted volume of the unknown protein solution with the Dye Reagent Concentrate and the absorbance at 595nm was measured. At high protein concentrations diluted Dye Reagent Concentrate (1:4 with distilled water) was used and the absorbance was read on a spectrophotometer (Gilford 250). Where the concentration of protein was low, undiluted Dye Reagent Concentrate was used and the absorbance was measured using a microtitration plate reader (Biorad Model 2550 EIA Reader).

DNA Assay.

As of the method of Le Pecq & Paoletti (1966), modified by Karsten & Wollenburger (1972) and then Gardner & Plumb (1979).

Materials:-

Ethidium Bromide	---	Sigma
Deoxyribonucleic Acid (Calf Thymus Type 1)	---	Sigma
Ribonuclease A (Bovine Pancreas Type 111-A)	---	Sigma
Protease (Streptomyces Griseus Type X1V)	---	Sigma
PBS reagents	---	BDH
NaCl	---	BDH

The amount of nucleic acid can be determined using a dye that becomes fluorescent only when it binds to nucleic acid. The intensity with which a sample fluoresces is directly proportional to the amount of nucleic acid present.

Ethidium bromide (2,7-diamino-9-phenylphenanthridine 10-ethyl bromide) was added to the cell sample containing DNA. The resulting fluorescence was due to both RNA and DNA. The fluorescence due to DNA alone was measured following treatment of the cell sample with RNAase.

Protocol:-

1. The following solutions were prepared:-

      a. Phosphate Buffered Saline:

	g/l
$CaCl_2$ $2H_2O$	0.13
KCl	0.2

KH_2PO_4	0.2
$MgCl_2 6H_2O$	0.1
NaCl	8.0
Na_2HPO_4	1.15

b. Ethidium Bromide - 2.5mg in 200ml PBS

c. DNA - 5mg in 100ml 0.9% NaCl (overnight 4°C).

d. Protease - 6mg in 100ml PBS (reduces protein nucleic acid
                                interactions).

e. RNAase - 20mg in 1ml H_2O.

2. To 0.2ml of - DNA standard,
             - PBS blank,
             - homogenised cell sample - 1.0ml PBS
                                           0.8ml Protease
                                           0.04ml RNAase

   were added and incubated for 1 hour.

3. 2ml of ethidium bromide was then added to each tube, and mixed.

4. The fluorescence was read at - excitation wavelength 360nm
                                  emission wavelength 587nm
   using fluorophotometer (Shimadzu RF-540).

Other Materials:-

Dexamethasone sodium phosphate (Decadron) --- Merck, Sharp & Dohme Ltd

N,N'-Hexamethylene bisacetamide   --- Sigma

N-Butyric acid, sodium salt   --- Sigma

Retinoic acid, all trans Type XX   --- Sigma

Verapamil HCl   --- Abbott Laboratories Ltd

N^6-2'-O-Dibutyryl adenosine 3':5'-cyclic

        monophosphate, sodium salt   --- Sigma

Millex - GS 0.22μm Filter Unit   --- Millipore

Statistical Analysis.

All statistical analysis was carried out using a two tailed Students t-test. Where the results are expressed as a percentage of the control the two tailed Students t-test was carried out by comparing the experimental data to a known value i.e. 100.

CHAPTER 3.

DEVELOPMENT OF THE DOPA DECARBOXYLASE ASSAY.

The DDC assay was carried out following the method of Emson, Burrows & Fonnum (1974). This method described the assay of DDC activity in motor neurons of the locust, it was therefore important to confirm that the method was suitable for the assay of DDC activity in human SCLC cell lines, and if not make the necessary modifications.

1. Selection of Radioactive Substrate.

Emson et al assayed DDC activity using $(2-^{14}C)$ DOPA as a substrate in an incubation medium containing (final concentrations):-

Sodium phosphate buffer (NaPi) pH7.6	---	60mM
Pyridoxal 5'phosphate	---	100μM
Nialamide	---	125μM
EDTA	---	1.5mM
D,L-$(2-^{14}C)$ DOPA (52mCi/mM)	---	400μM

Due to the lack of availability of DL-$(2-^{14}C)$ DOPA it was necessary to select another radioactively labelled substrate. Two labelled forms were found:-

(i) L-3,4-[ring-2,5,6-^{3}H]-DOPA   (ii) 1-[^{14}C]-DOPA
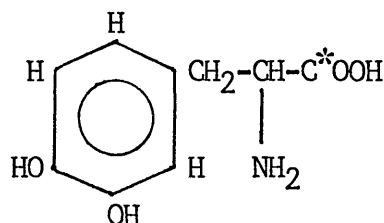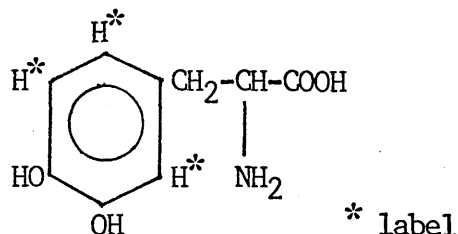
* label

If one used the radiolabelled substrate (i) the extraction of [^{3}H]-dopamine was required as a measure of the DDC activity, whereas (ii) required the extraction of [^{14}C]-carbon dioxide. One main disadvantage of using [1-^{14}C]-DOPA in a radioassay is that one cannot distinguish between enzymatic and nonenzymatic decarboxylation of DOPA. Nonenzymatic decarboxylation of DOPA has been reported (Okuno & Fujisawa 1983), although this can be minimised if the assay is preformed in the presence of EDTA. Another disadvantage in using [1-^{14}C]-DOPA was the hazard of possible escape of radioactive CO_2. Therefore form (i) was selected as the labelled substrate for the DDC assay.

2. Specificity of DOPA Decarboxylase.

As DDC is reported to act only on the L-isomer of aromatic amino acids, it was useful to determine if our enzyme reaction was specific for the L-isomer of DOPA. If this was the case it would support the initial assumption that is was in fact DDC that was being assayed. Therefore does the presence of D-DOPA or DL-DOPA have any effect on the formation of dopamine ?

DDC was prepared from NCI-H128d cells, a SCLC cell line with high DDC activity. DDC was added to a reaction mixture (final concentrations):-

NaPi buffer pH7.6	--- 60mM
Pyridoxal 5'Phosphate	--- 100μM
Nialamide	--- 125μM
EDTA	--- 1.5mM
^{3}H-L-DOPA (^{3}H-D-DOPA not available)	--- 25μCi/ml
L-DOPA, DL-DOPA or D-DOPA	--- 400μM

The reaction was allowed to continue for 30mins, at which time the reaction was stopped and the dopamine formed extracted.

Results.

Isomer	mean cpm $\pm$ S.E.
DL-DOPA	3545.7 ± 107.5
L-DOPA	4397.5 ± 120.8
D-DOPA	48414.3 ± 6168.4

The presence of D-DOPA resulted in higher levels of ^{3}H-dopamine formed compared to L-DOPA or DL-DOPA. At first glance this was quite alarming considering the putative specificity of DDC for L-isomers. The increase in the amount of product formed could be due to either an increased rate of reaction in the presence of D-DOPA or the result of an increase in the specific activity of ^{3}H-L-DOPA. The former explanation is unlikely since an increased rate of reaction was not seen in the presence of the racemic mixture. If the increase in product formed is due to an increase in the specific activity of the radioactively labelled substrate and not to an increase in the reaction rate then the concentration of D-DOPA in the reaction mixture should be unimportant. That is, as long as the concentration of L-DOPA remains constant then varying the concentration of D-DOPA should not alter the amount of dopamine formed.

DDC was prepared as before. Two reaction mixtures were prepared, one with a ^{3}H-L-DOPA specific activity of 25Ci/mol and the other with a specific activity of 75Ci/mol. A DDC assay was performed with increasing concentrations of D-DOPA. After 30mins the reaction was stopped and the dopamine formed extracted.
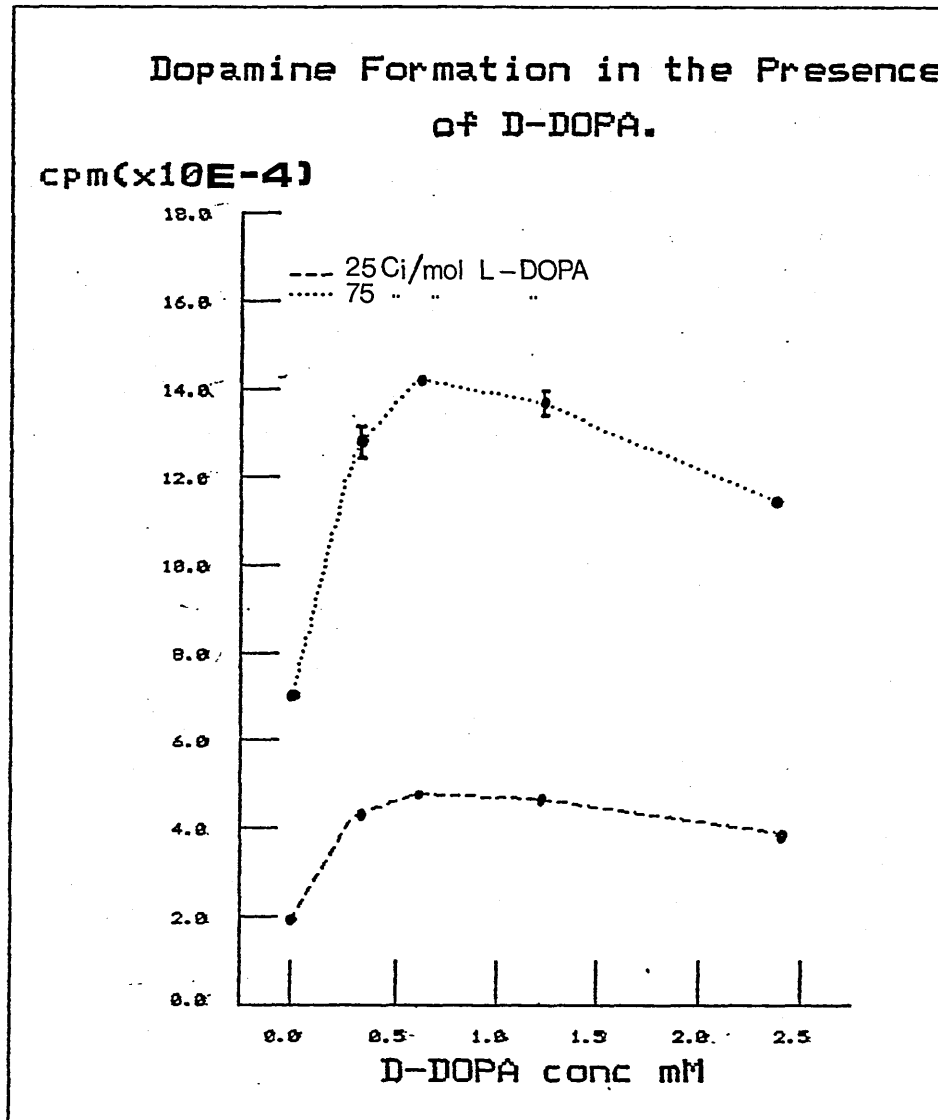
Figure 11:- Effect of Alteration in D-DOPA Concentration on the Formation of Dopamine from a Constant Amount of L-DOPA.

The activity of DDC extracted from H128d cell line was examined in the presence of increasing concentrations of D-DOPA. The dopamine formed is expressed as counts per minute.

Each point represents the mean of 3 determinations ± standard error.

Figure 11 shows that increasing concentrations of D-DOPA, up to 0.6mM, increased the formation of dopamine. If our assumption was true that DDC is specific for L-isomers then why does D-DOPA affect the amount of ^{3}H-dopamine formed? Perhaps DOPA is being metabolised by other enzymes resulting in the depletion of the substrate for DDC; D-DOPA may be competing with L-DOPA for these enzymes with the result that more L-DOPA is available for decarboxylation by DDC.

Alternatively, it has been reported that DOPA can react with DDCs' coenzyme pyridoxal 5'phosphate with the formation of a tetrahydroisoquinoline derivative (Schott & Clark 1952), this could have two effects both resulting in a fall in product formed. Firstly, if the concentration of DOPA is low then reaction with the coenzyme could sufficiently reduce the substrate concentration to cause a decreased rate of reaction and subsequently a decrease in dopamine formed. Secondly, this reaction could essentially be removing pyridoxal 5'phosphate from the reaction mixture which could indirectly reduce the amount of product formed by reducing enzyme activity. Therefore by increasing the concentration of DOPA in the form of the D-isomer, less L-DOPA will react with the coenzyme and subsequently more L-DOPA will be available for decarboxylation by DDC.

Somerville, (1964), showed that if borate was used to complex the active m hydroxy on DOPA it was possible to suppress the reaction of DOPA with its coenzyme. When this experiment was repeated but the reaction mixture was prepared using a borate buffer instead of the NaPi buffer addition of D-DOPA had no effect on the amount of dopamine formed. Moreover, when the borate buffer was directly compared to the NaPi buffer it was found that under the same assay conditions the dopamine formed in the presence of borate buffer was 4 fold greater than that found using the NaPi buffer. Therefore since the D-DOPA effect can be inhibited by addition of borate buffer it can be concluded that the L-DOPA is being lost from the system via conversion to a

tetrahydroquinoline derivative and not by enzymatic metabolism by other enyzmes.

In order to optimise the conditions for the DDC assay borate buffer was selected for use in the assay.

3. Selection of the Substrate Concentration.

In an enzyme-substrate reaction the rate of reaction varies with substrate concentration.   If the concentration of L-DOPA was too low the enzyme DDC would not be fully saturated and the reaction rate would be submaximal.   However, at high L-DOPA concentrations the DDC would be fully saturated and the rate of reaction would be at its maximum and the amount of product formed per unit time constant. Therefore it was necessary to determine a suitable L-DOPA concentration that would not limit the rate of reaction.

DDC was prepared from H128d cells.   The enzyme was incubated with the reaction mixture (final concentrations):-

$$
\begin{array}{ll}
\text{Borate Buffer pH7.6} & \text{--- } 0.025\text{M Borax + } 0.05\text{M } KH_2PO_4 \\
\text{Pyridoxal 5'phosphate} & \text{--- } 100\mu M \\
\text{Nialamide} & \text{--- } 125\mu M \\
\text{EDTA} & \text{--- } 1.5\text{mM} \\
^3\text{H-L-DOPA} & \text{--- } 30\mu Ci/ml
\end{array}
$$

+ various concentrations of L-DOPA.

The reaction was allowed to continue for 30mins, at which time it was stopped and the dopamine formed extracted.

Figure 12 shows the effect of increasing substrate concentration on the rate of catalysis of L-DOPA.   In order to ensure near maximum rate
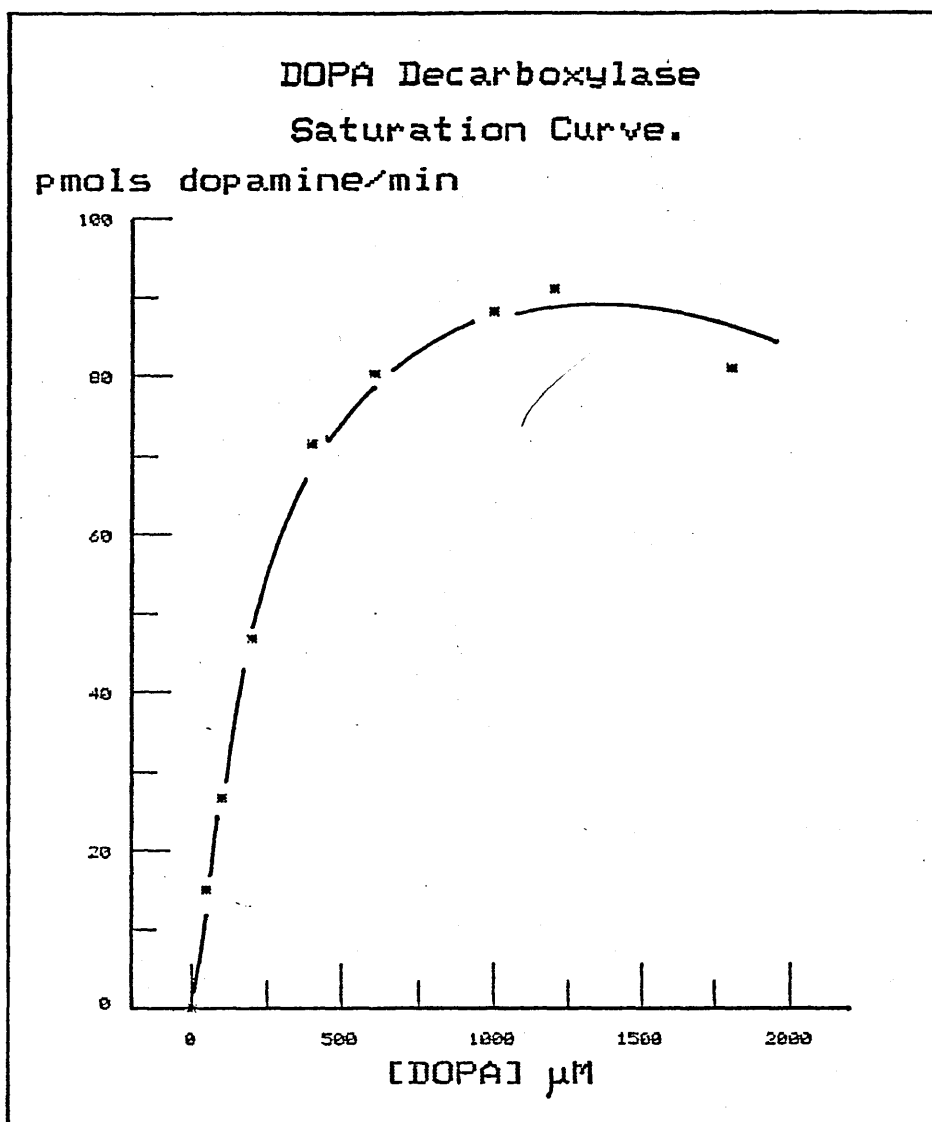
Figure 12:- Effect of Substrate Concentration on the Rate of Catalysis of L-DOPA.

A DDC aasay was preformed with various concentrations of L-DOPA. The rate of reaction, V, was determined by measuring the dopamine formed per minute.

Each point represents the mean of 3 determinations ± standard error.

of reaction in the DDC assay, 1200µM L-DOPA was selected as a suitable substrate concentration since it did not limit the reaction rate. The results from Figure 12 were further analysed using a Lineweaver-Burke Plot (Figure 13). Analysis of the data in the Lineweaver-Burke Plot, using a computer programme which draws the best-fit line, found that:-

Maximum rate of reaction V_{max} = 118.6 pmol dopamine/min
Dissociation Constant K_m = 3.03 x 10^{-4}M

Note:-

The dissociation constant for DDC was found to be similar to that found for the enzyme by other workers:-

K_m for DDC

Bovine adrenal medulla
        (Laduron & Belpaire 1968).   5.7 x 10^{-4}M

Hog kidney (Christenson, Dairman &
                Udenfriend 1970).   1.9 x 10^{-4}M

Pig kidney cortex (Okuno & Fujisawa 1983).   0.4 x 10^{-4}M

4. Selection of the Coenzyme Concentration.

DDC requires the presence of pyridoxal 5'phosphate for full activity. The concentration of pyridoxal 5'phosphate in the reaction mixture was therefore an important factor in ensuring full enzyme activity. Therefore, what pyridoxal 5'phosphate concentration is sufficient to allow maximum enzyme activity ?
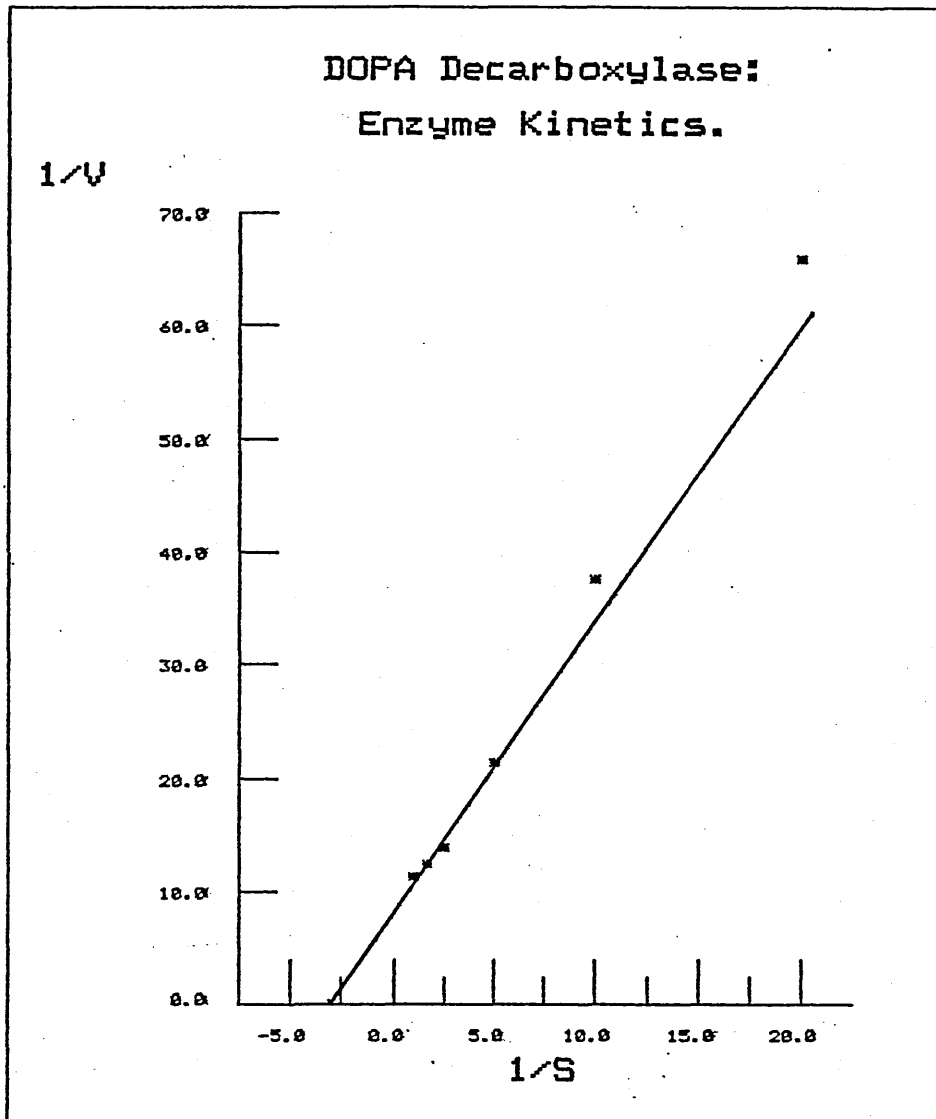
Figure 13:- Effect of Substrate Concentration on the Rate of Catalysis of L-DOPA: Lineweaver-Burke Plot.

By plotting 1/V versus 1/[S] (reciprocal of the substrate concentration) the dissociation constant K_m and the maximal reaction rate V_{max} can be derived. Rate of reaction, V, is expressed as pmols of dopamine formed per minute and the substrate concentration (S) is in μM.

Each point represents the mean of 3 determinations $\pm$ standard error.

DDC was prepared from H128d cells. The enzyme was incubated with the reaction mixture (final concentrations):-

Borate Buffer pH7.6	--- 0.025M Borax + 0.05M KH_2PO_4
Nialamide	--- 125μM
EDTA	--- 1.5mM
L-DOPA	--- 1200μM
^{3}H-L-DOPA	--- 30μCi/ml

+ various concentrations of Pyridoxal 5'Phosphate.

The reaction was stopped after 30mins and the dopamine formed extracted.

Figure 14 shows that in the absence of pyridoxal 5'phosphate the amount of dopamine formed was significantly reduced, illustrating the necessity of the coenzyme for full DDC activity. The level of dopamine formed reached a plateau in the presence of 50μM pyridoxal 5'phosphate. Therefore 100μM pyridoxal 5'phosphate was selected to ensure optimal enzyme activity.

5. Selection of Nialamide and EDTA Concentration.

It was important that once the dopamine was formed it was not metabolised by other enzymes which may have been present. There are two principal enzymes concerned with the catabolism of catecholamines catechol-O-methyl transferase (COMT) and monoamine oxidase (MAO). COMT is a cytoplasmic enzyme which requires adenosyl methionine and magnesium or other divalent cations for activity. The presence of EDTA not only minimises the nonenzymatic decarboxylation of DOPA (discussed earlier), but also inhibits the activity of COMT by chelating divalent cations. MAO is a mitochondrial enzyme which is inhibited by nialamide.
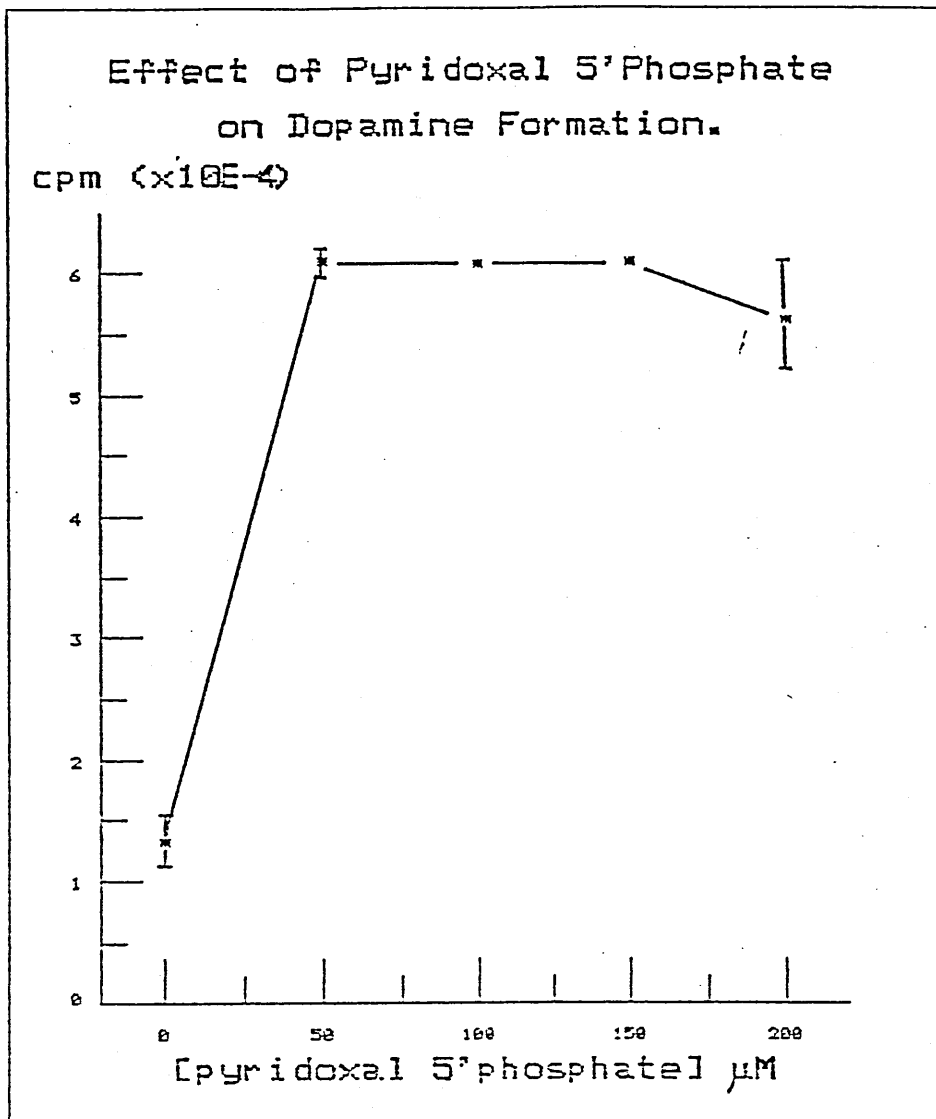
Figure 14:- Effect of the Coenzyme Concentration on the Formation of Dopamine from L-DOPA by DDC.

A DDC assay was carried out in the presence of increasing concentrations of pyridoxal 5'phosphate. The dopamine formed was extracted and counted.
The dopamine formed is expressed as counts per minute.

Each point represents a mean of 3 determinations $\pm$ standard error.

Linearity of DDC Assay: H187
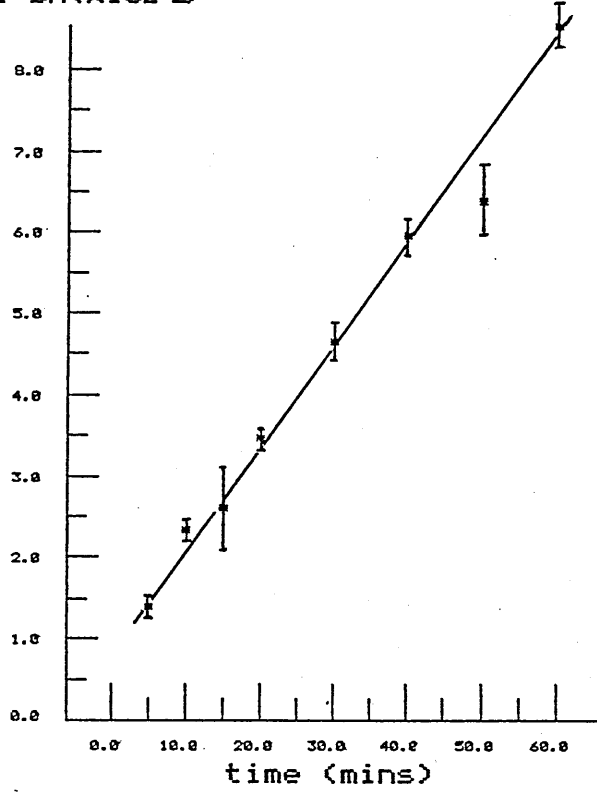
Linearity of DDC Assay: H69
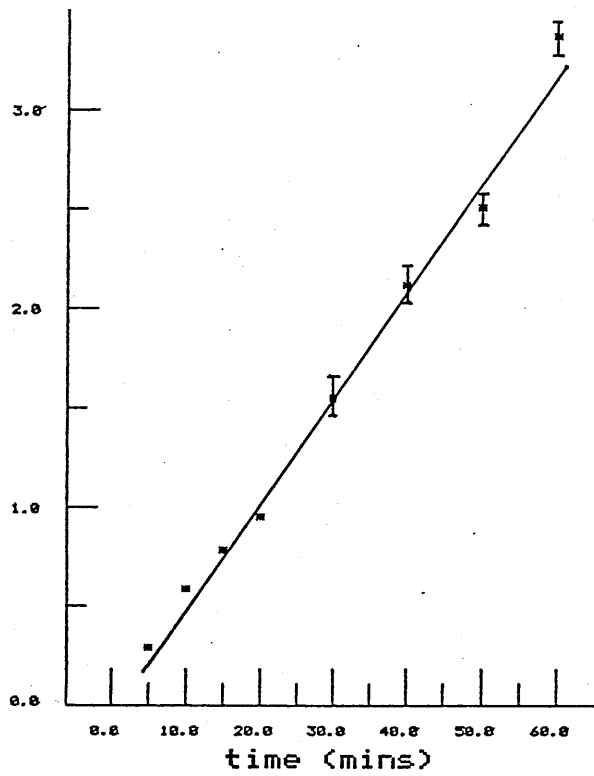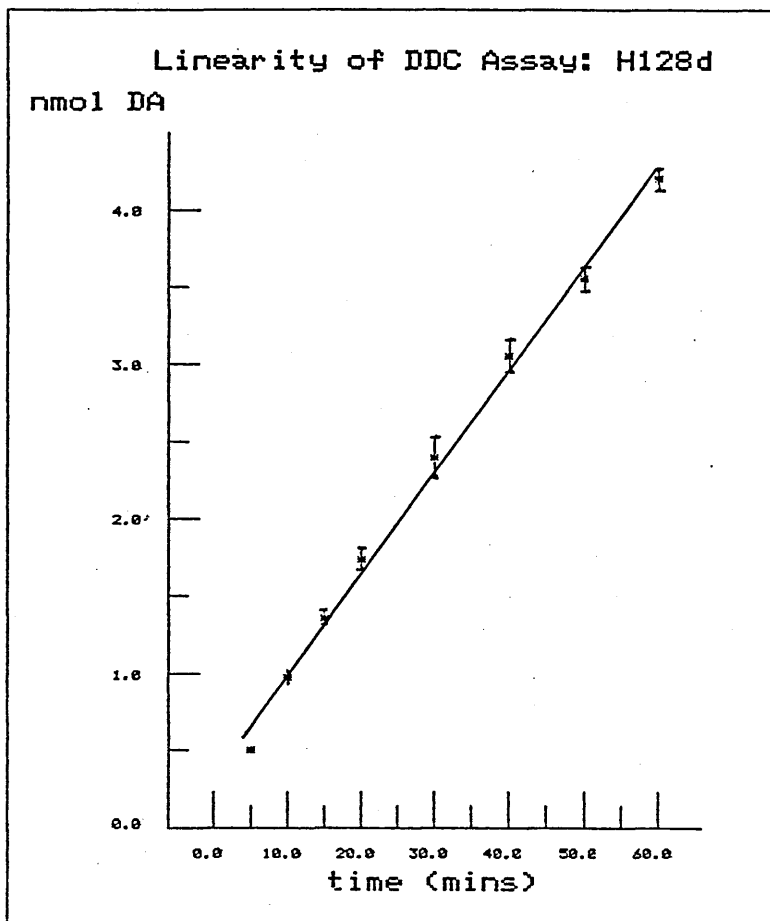
Figure 15, 16 & 17:- Measurement of the Reaction Rate for SCLC Cell Lines over the Incubation Period.

DDC was extracted from SCLC cell lines: H187, H69 and H128d. The amount of dopamine formed at various intervals throughout the incubation period was measured. The rate of formation of dopamine from L-DOPA is represented by the amount of dopamine formed (nmols) versus time (minutes).

Each point represents the mean of 3 determinations $\pm$ standard error.

DDC was extracted from H128d. A DDC assay was carried out with or without EDTA or nialamide. The assay was allowed to continue for 30mins, at which time the dopamine formed was extracted and measured.

In the absence of either nialamide or EDTA there was no change in the amount of dopamine formed. It would therefore appear that during the incubation period dopamine is not metabolised. Furthermore, the amount of dopamine formed was not affected by the presence of 125μM nialamide and 1.5mM EDTA. EDTA and nialamide were retained in the reaction mixture at the concentrations specified by Emson et al 1974, to eliminate the possibility of dopamine catabolism and also to prevent the nonenzymatic decarboxylation of DOPA (EDTA).

6. Linearity of the DDC Assay.

In order to use the DDC assay to measure enzyme levels in SCLC cell lines it was essential that the rate of reaction was linear with time.

A DDC assay was carried out with DDC extracted from H187, H69 and H128d cells. The reaction was stopped at regular intervals throughout the incubation period, and the amount of dopamine formed was extracted and measured.

The DDC reaction rate was found to be linear over the incubation period for the enzyme extracted from the three SCLC cell lines (Fig 15, 16 & 17).

CHAPTER 4.
THE EFFECT OF PHENOTYPIC MODULATORS ON THE GROWTH
OF SCLC CELL LINES

The differentiation of most adult stem cells is associated with the loss of proliferative capacity (Wier & Scott 1986, Till 1982). Furthermore, a growth suppressive effect has often accompanied the induction of differentiation in many cell systems following treatment with various differentiating agents. For example, induction of differentiation in mouse neuroblastoma cell lines following treatment with either HMBA (Palfrey et al, 1977) or dbcAMP (Prashad et al, 1987) was accompanied by inhibition of cell proliferation. Also, NaBut was shown to induce a more differentiated phenotype in human medullary thyroid carcinoma cells and also decrease cellular proliferation (Nakagawa et al, 1985). Dexamethasone, another phenotypic inducer, has been shown to induce a more differentiated phenotype in some non small cell lung cancer cell lines with an accompanied cytostatic effect (J.McLean personal communication, 1987). How then does treatment of the SCLC cell lines with the various putative phenotypic inducers affect cellular proliferation?

The effect of a number of phenotypic inducers, HMBA, dbcAMP, NaBut and dexamethasone, on the proliferation of the SCLC cell lines was examined. Cell growth was measured using the MTT assay or by electronic cell counting, in the presence of drug for 72hrs, and for a further 7 days (approx.) in the absence of drug. A 72hr exposure time was selected as this was the exposure time used to examine the effect of the phenotypic inducers on the SCLC phenotype. When electronic cell counting was used to determine cell growth, the drug was present throughout the growth curve.

Both H187 and H69 cell lines exhibited growth suppression in the presence of 5mM HMBA (Figs 18 & 19). During 5mM HMBA exposure H187 cells experienced a growth delay, but following drug removal on day 3
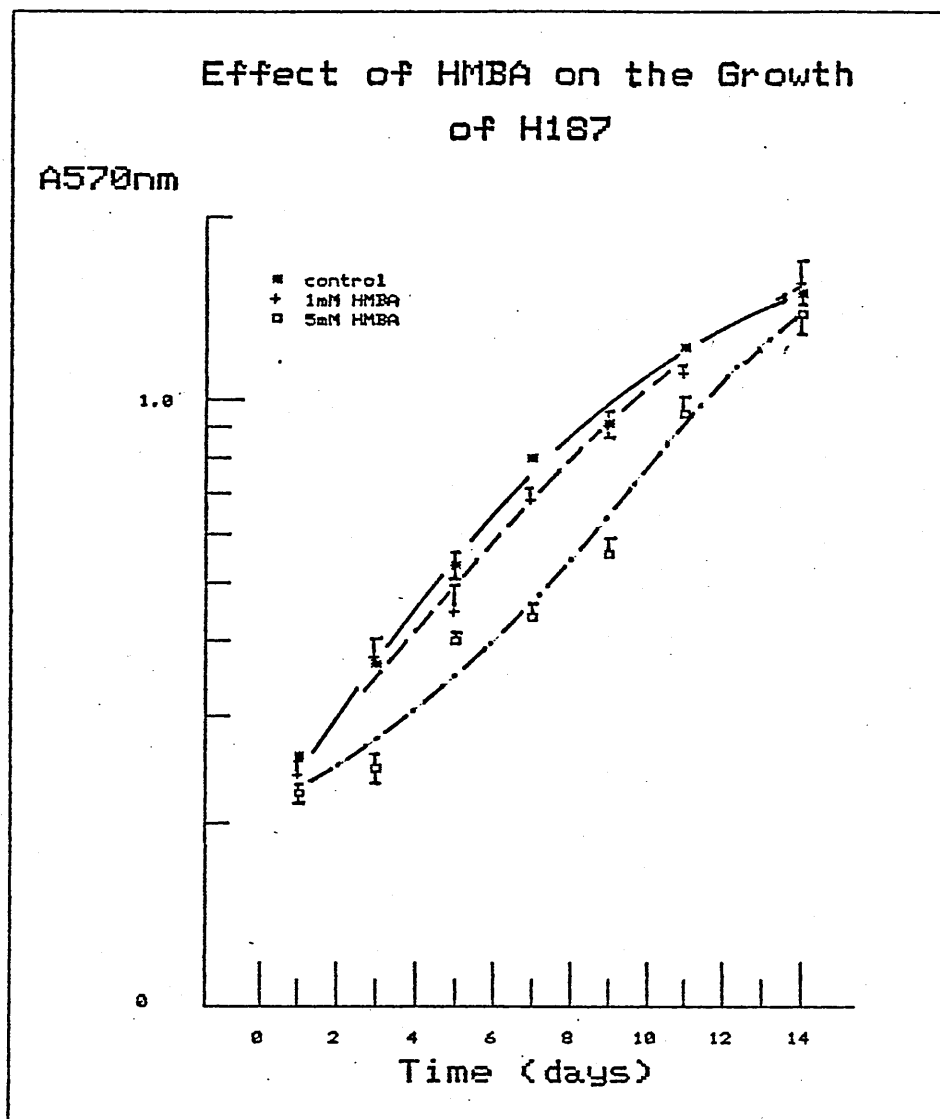
Figure 18:- The Effect of HMBA on the Growth of H187 Cells.

H187 cells were seeded into 96 well plates in the presence or absence of either 1mM HMBA or 5mM HMBA at a final cell concentration of $5x10^4$ cells/ml. The cells were incubated at $37^{\circ}C$ in 2% CO_2 throughout the experiment. On day 3 the HMBA was removed from the cells by spinning the microtitration plates at 200g for 5 minutes, aspirating the supernatant and replacing with fresh medium. The cells were fed on alternate days thereafter. The growth of the cells was measured at regular intervals throughout the growth curve using the MTT assay.

The results represent mean of 8 replicate samples ± standard error.

The figure is a representative from 3 experiments.

Figure 19:- The Effect of HMBA on the Growth of H69 Cells.

H69 cells were seeded into 96 well plates in the presence or absence of either 1mM HMBA or 5mM HMBA at a final cell concentration of 5×10^4 cells/ml. The cells were incubated at 37°C in 2% CO_2 throughout the experiment. On day 3 the HMBA was removed from the cells by spinning the microtitration plates at 200g for 5 minutes, aspirating the supernatant and replacing with fresh medium. The cells were fed on alternate days thereafter. The growth of the cells was measured at regular intervals throughout the growth curve using the MTT assay.

The results represent mean of 8 replicate samples $\pm$ standard error.

The figure is a representative from 3 experiments.

Table 3: Doubling Times (t_D) of SCLC Cell Lines Following Treatment with Phenotypic Inducers.

Cell Line	Control t_D (days)	Phenotypic Inducer P.I.	t_D (days) following P.I. Treatment
H187	2.57 ± 0.32	DbcAMP 0.5mM	4.0 ± 0.7 **
		1.0mM	5.3 ± 0.8
		NaBut 0.5mM	3.5 ± 0.6 *
		1.0mM	4.5 ± 0.66
		HMBA 1.0mM	2.7 ± 0.43
		5.0mM	3.6 ± 0.7
H69	3.80 ± 0.22	DbcAMP 0.5mM	4.35 ± 0.4 *
		1.0mM	6.0 ± 0.7
		NaBut 0.5mM	4.12 ± 0.15 ***
		1.0mM	6.47 ± 0.4
		HMBA 1.0mM	3.60 ± 0.15
		5.0mM	4.70 ± 0.43

SCLC cell lines were seeded into 96 round bottomed well plates in the presence and absence of the appropriate drug. The cells were exposed to the phenotypic drugs for 72 hours. The growth of the cells was measured using the MTT assay during drug exposure and for approximately one week after drug exposure. The doubling times were calculated from the exponential part of the growth curve following drug removal.

Results represent the mean of t_D ± standard error from 4 separate experiments.

Statistical analysis was carried out using the Students t-test.

* $P<0.05$ ** $P<0.02$ *** $P<0.002$

the cells entered an exponential growth phase with a doubling time
similar to the untreated cells (Table 3). The effect of 1mM HMBA
on the growth of H187 cells was also examined but it was found that
at this concentration HMBA did not have a growth suppressive effect.
A slightly different pattern was found following treatment of H69
cells with HMBA. During 5mM HMBA exposure H69 cells had a decreased
proliferation rate. However when HMBA was removed, unlike HMBA
treated H187 cells, H69 cells did not immediately enter an
exponential growth phase. The H69 growth rate gradually increased
and exhibited a control growth rate approximately 4 days after HMBA
withdrawal. It may be that HMBA induces a growth suppressive effect
only on a certain population of H69 cells, and the pattern of H69
cell growth following HMBA treatment is the net result of a
heterogenous population of cells growing at different rates. The
uninduced H69 cells continue to proliferate at the control level and
eventually outgrow the HMBA growth suppressed population and hence
the return to the control proliferative rate. The effect of 1mM HMBA
on the growth of H69 cells was also examined and it was found that at
this concentration HMBA had no effect on the H69 cell growth.

Following exposure of the SCLC cell lines to dbcAMP two different
patterns of growth suppression were found. When H187 cells were
exposed to dbcAMP the growth rate of the cells was reduced and this
was maintained even after drug removal (Fig. 20). This growth
suppressive effect of dbcAMP was found to be dose dependent where 1mM
dbcAMP induced a greater cytostatic effect than 0.5mM dbcAMP. Table
3 shows that the doubling times of the 1mM dbcAMP treated H187 cells
was increased significantly. Therefore, dbcAMP treated H187 cells
exhibited a suppressed proliferation rate which was maintained for at
least 11 days after drug removal. In the presence of either 0.5mM or
1.0mM dbcAMP H69 cells exhibited a growth delay (Fig. 21). On removal
of the drug on day 3, the cells entered an exponential growth phase.
However, even after 7 days in drug free medium the cells did not
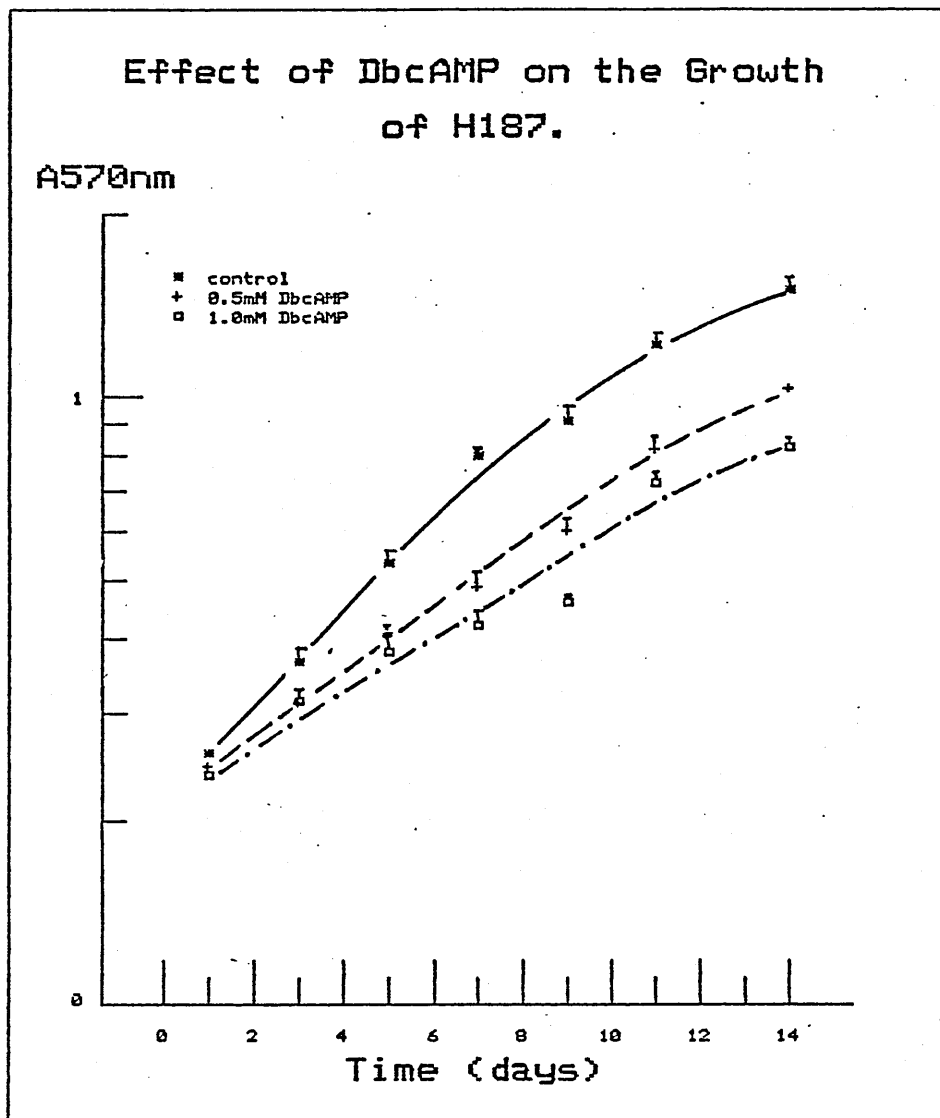
Figure 20:- The Effect of DbcAMP on the Growth of H187 Cells.

H187 cells were seeded into 96 well plates in the presence or absence
of either 0.5mM or 1.0mM dbcAMP at a final cell concentration of
$5x10^4$ cells/ml. DbcAMP was removed on day 3 and the cells were fed
on alternate days thereafter. The growth of the cells was measured
using the MTT assay.

The results represent the mean of 8 replicate samples $\pm$ standard
error.

The figure is a representative from 3 experiments.

Effect of DbcAMP on the Growth
of H69

A570nm

■ control
+ 0.5mM dbcAMP
□ 1.0mM dbcAMP
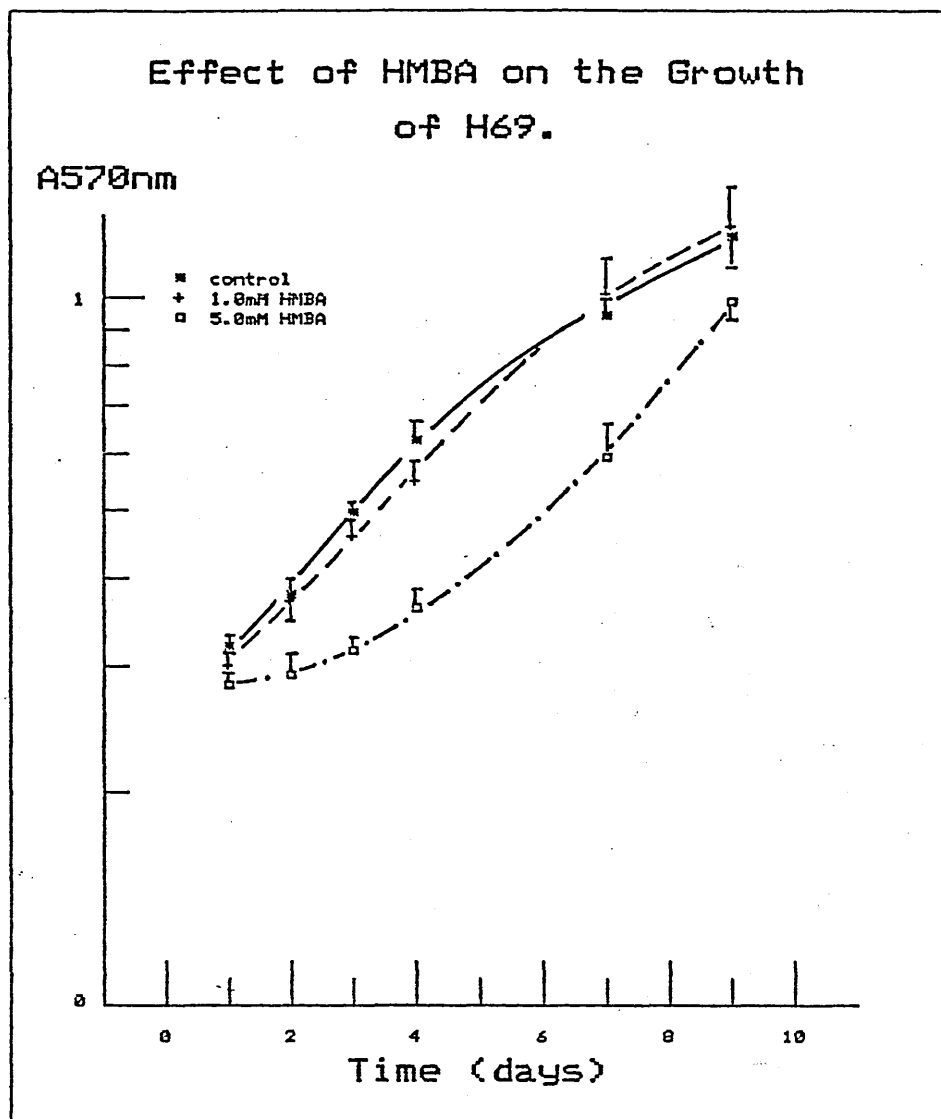
1.0

0

0   2   4   6   8   10

Time (days)
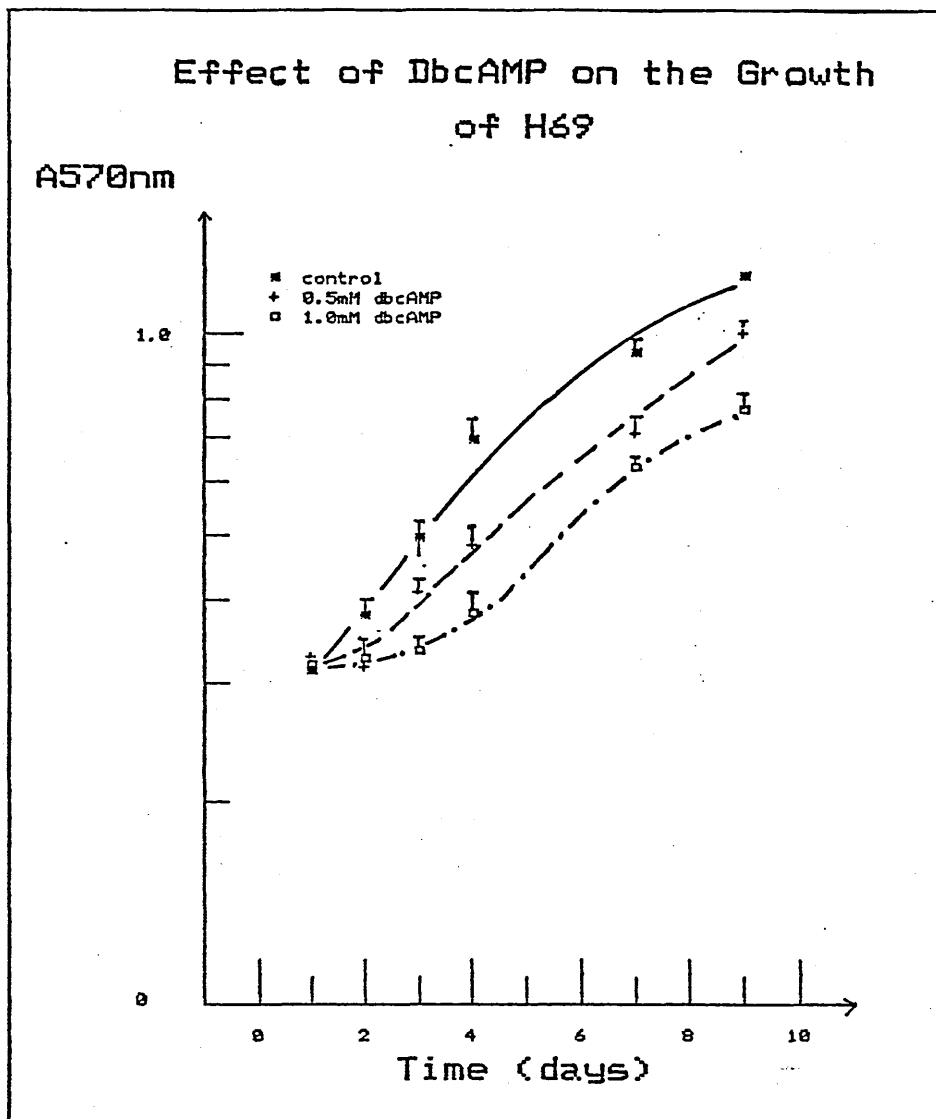
Figure 21:- The Effect of DbcAMP on the Growth of H69 Cells.

H69 cells were seeded into 96 well plates in the presence or absence of either 0.5mM or 1.0mM dbcAMP at a final cell concentration of 5×10^4 cells/ml. DbcAMP was removed on day 3 and the cells were fed on alternate days thereafter. The growth of the cells was measured using the MTT assay.

The results represent the mean of 8 replicate samples ± standard error.

The figure is a representative from 3 experiments.

resume the control growth rate and in fact the doubling time of 1.0mM dbcAMP treated H69 cells was significantly different from the untreated controls.

NaBut had a marked effect on the growth of the SCLC cell lines. Treatment of H187 cells with either 0.5mM or 1.0mM NaBut resulted in growth suppression which was maintained for 7 days after drug removal on day 3 (Fig. 22). This cytostatic effect of NaBut on the growth of H187 cells was dose dependent since 1.0mM NaBut induced a greater growth suppressive effect than 0.5mM. Following 1.0mM NaBut treatment the H187 cell doubling time was significantly increased (Table 3). A growth suppressive effect was also noted in NaBut treated H69 cells. NaBut 0.5mM did not significantly alter the growth rate of H69 cells (Table 3). However in the presence of 1.0mM NaBut the H69 cell growth was completely suppressed (Fig. 23). When the drug was removed on day 3 the cells exhibited a reduced growth rate for a further 24 hours after which time the cells entered an exponential growth phase. Table 3 shows that 1.0mM NaBut treated H69 cells have a significantly longer doubling time than the untreated controls. This pattern of NaBut induced growth suppression in H69 cells was not unlike that found following HMBA exposure. Therefore it may be that NaBut induced a cytostatic effect in only a certain proportion of H69 cells and eventually the remaining uninduced cells outgrew the slower growing population. It was also possible that the observed growth delay was due to a cytotoxic effect of NaBut. This was found not to be the case. The cell viability was examined by trypan blue exclusion on day 3 and found to be greater than 90%.

Finally, the effect of dexamethasone on the growth of the SCLC cell lines was examined. The growth of the cells was measured by electronic cell counting and the drug was present throughout the experiment. Dexamethasone, 25μM, reduced the H187 cell growth rate and the saturation density (Fig. 24). The cell concentration at

Figure 22:-- The Effect of NaBut on the Growth of H187 Cells.

H187 cells were seeded into 96 well plates at a final cell concentration of 5×10^4 cells/ml. The cells were grown in the presence or absence of either 0.5mM or 1.0mM NaBut for 3 days. The cells were fed on alternate days following drug removal. The growth of the cells was determined using the MTT assay.

The results represent the mean of 8 replicate samples $\pm$ standard error.

The figure is a representative from 3 experiments.
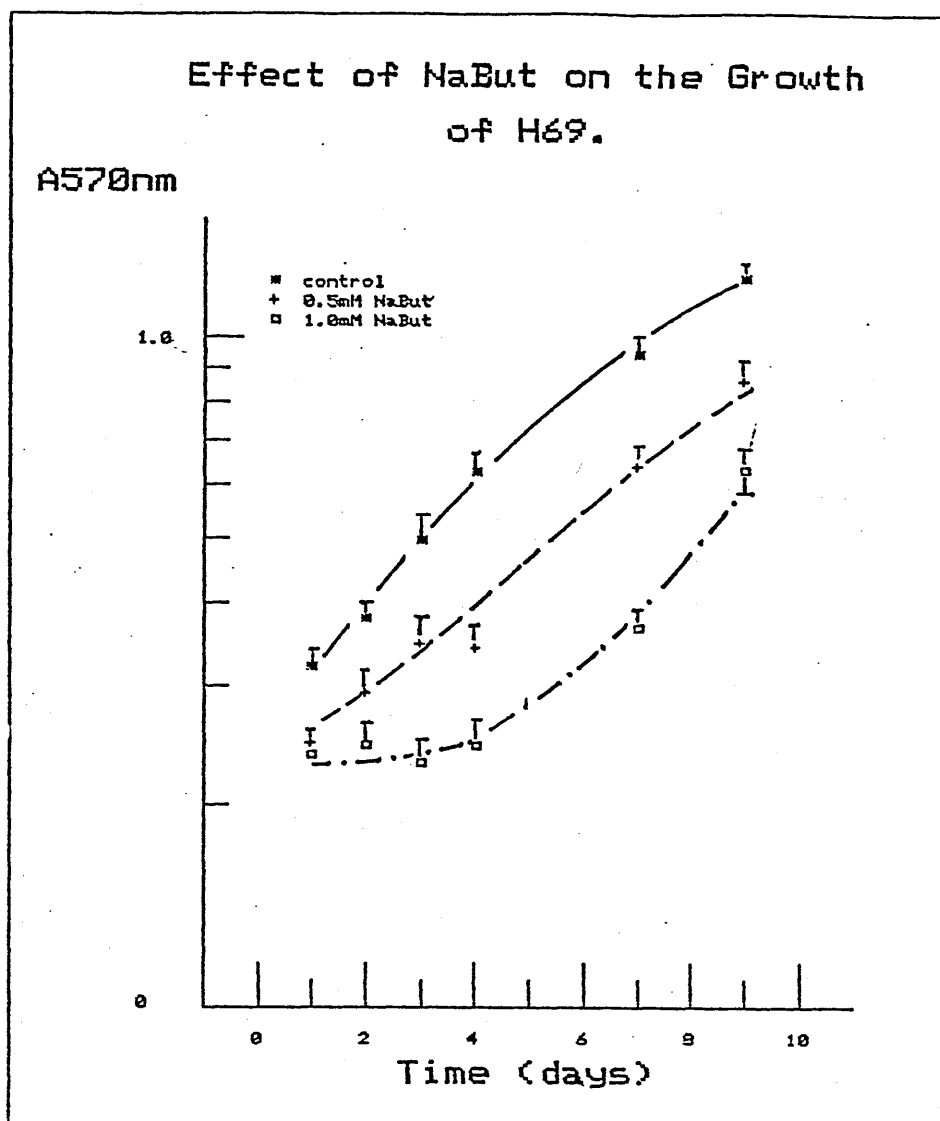
Figure 23:- The Effect of NaBut on the Growth of H69 Cells.

H69 cells were seeded into 96 well plates at a final cell concentration of $5x10^4$ cells/ml. The cells were grown in the presence or absence of either 0.5mM or 1.0mM NaBut for 3 days. The cells were fed on alternate days following drug removal. The growth of the cells was determined using the MTT assay.

The results represent the mean of 8 replicate samples ± standard error.

The figure is a representative from 3 experiments.

plateau phase of growth in the drug-treated cells was found to be significantly different from the untreated controls. Dexamethasone, however, had no effect on the growth of H69 cells (Fig. 25). It was found that glucocorticoid receptors were present in H187 cells, in low levels, but were absent from H69 cells (S. Cowan, personal communication). It may be that the selective effect of dexamethasone on the growth of H187 cells compared to H69 cells is due to the glucocorticoid receptor concentration.

The effect of the various phenotypic inducers on the growth of H128d cells was also examined, however the aggregate nature of this cell line proved to be a major obstacle in measuring cell growth. As discussed in the methods section H128d cells grow as large tight aggregates. Attempts to disaggregate H128d cells resulted in a suspension consisting of smaller aggregates of various sizes. Rapid seeding of this disaggregated H182d cell suspesion into a MTT microtitration plate gives an even distribution of cells across the plate. However for a growth experiment at least six microtitraton plates were required and it was found that even dispensing of the H128d cell suspension into replicate microtitration plates was not obtained. This error between plates became more noticeable as the growth curve proceeded and unfortunately satisfactory growth curves of H128d cells in the presence and absence of phenotypic inducers were not obtained.

The Effect of Dexamethasone on the Growth of H187.

cells/ml (x10E-5)

control
25μM DX

Time (days)
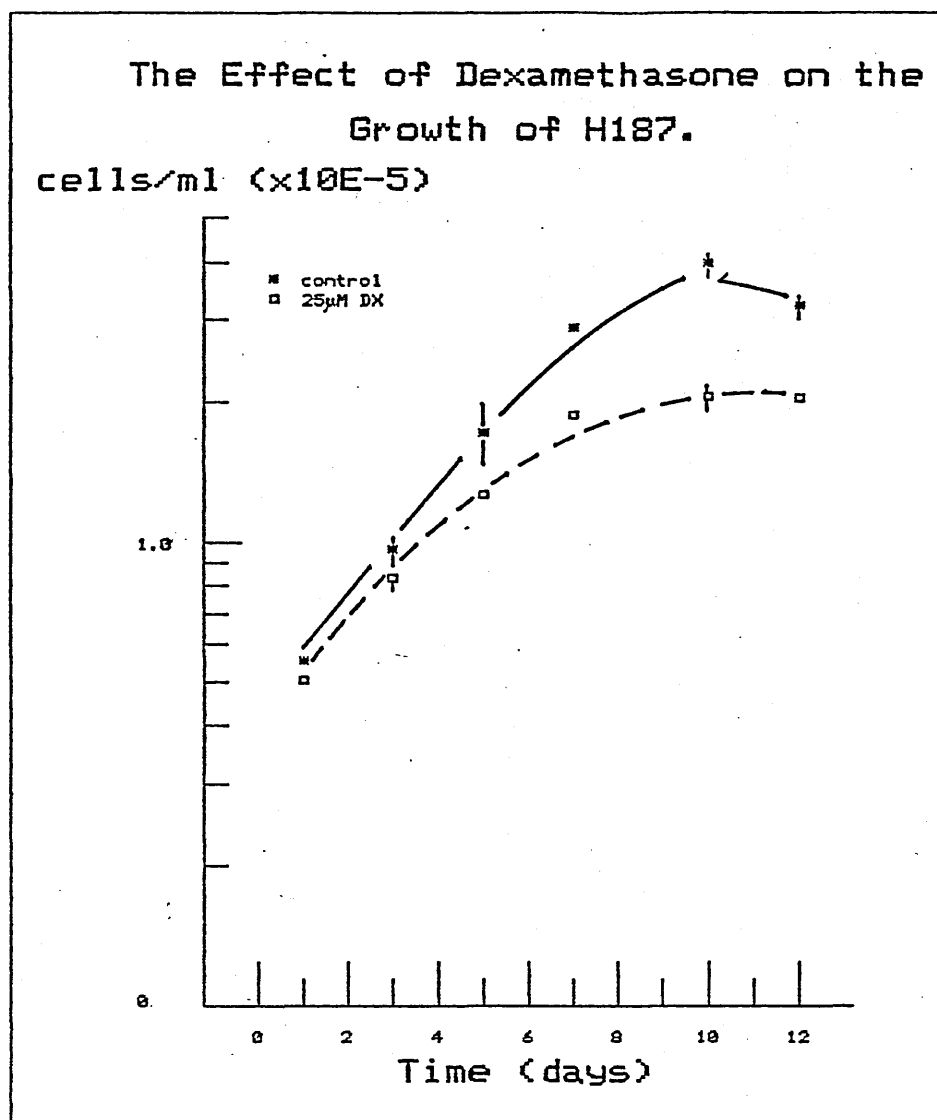
Figure 24:- The Effect of Dexamethasone on the Growth of H187 Cells.

H187 cells were seeded into 24 well plates at a final cell concentration of $5x10^4$ cells/ml. The cells were grown in the presence of 25μM dexamethasone for approximately 12 days. The cell growth was determined by electronic particle counting.

The results represent the mean of 3 replicate samples ± standard error.

The figure is a representative from 3 experiments.
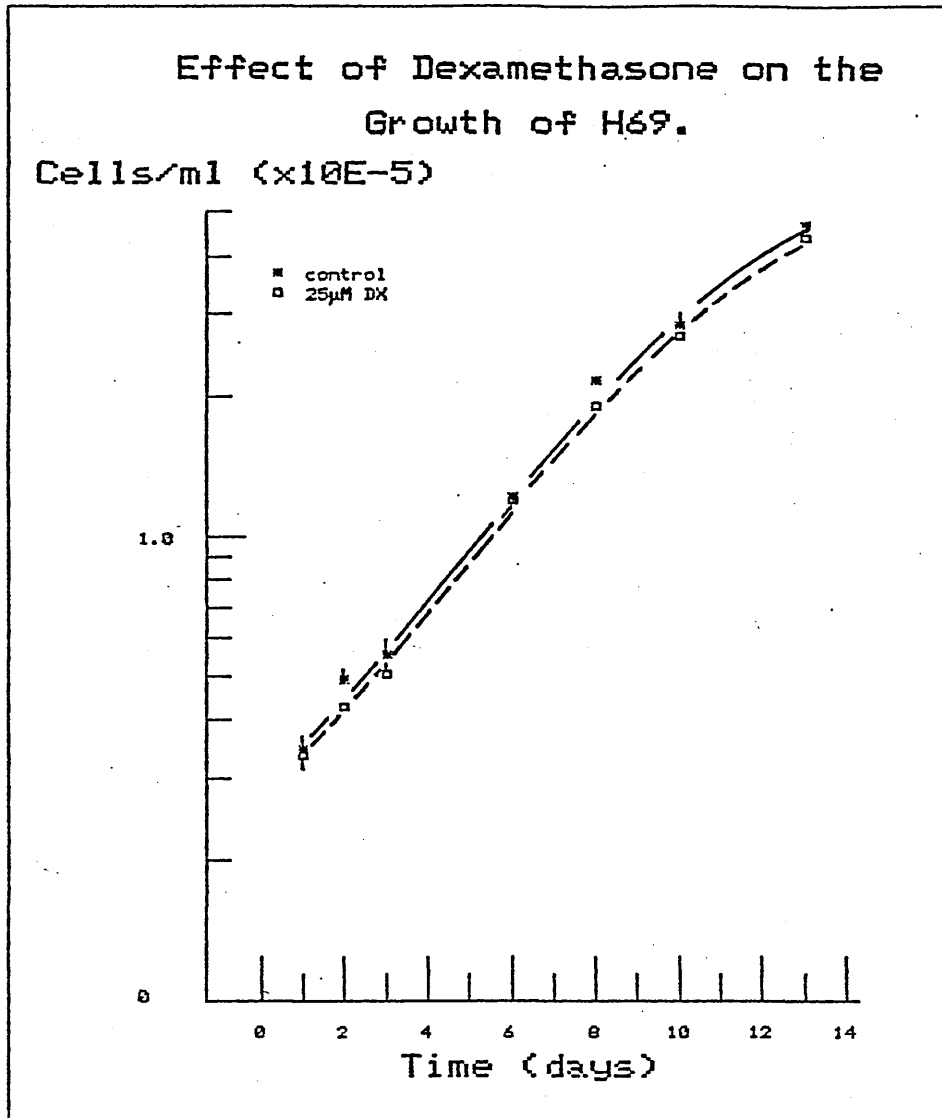
Figure 25:- The Effect of Dexamethasone on the Growth of H69 Cells.

H69 cells were seeded into 24 well plates at a final cell concentration of 5×10^4 cells/ml. The cells were grown in the presence of 25µM dexamethasone for approximately 12 days. The cell growth was determined by electronic particle counting.
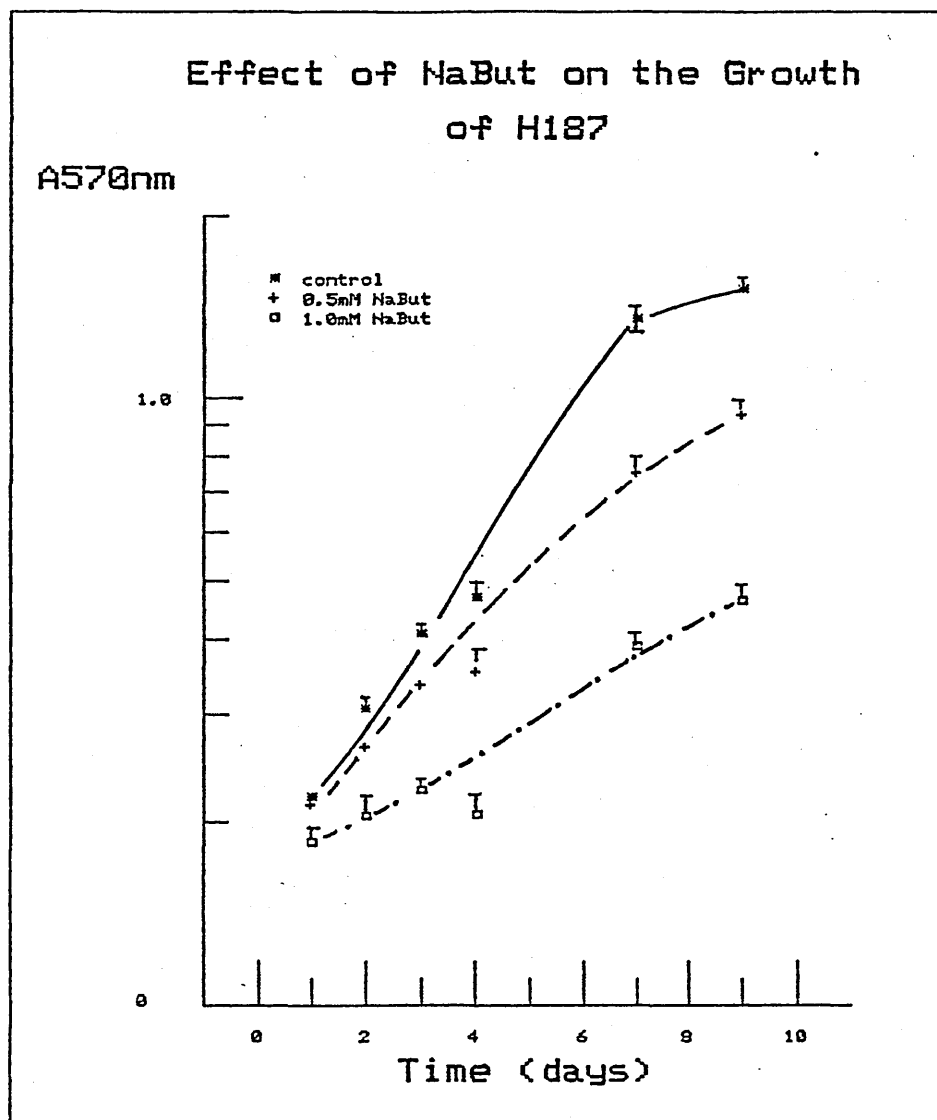
The results represent the mean of 3 replicate samples ± standard error.

The figure is a representative from 3 experiments.

Table 4: Control Levels of L-DOPA Decarboxylase Activity in the SCLC
Cell Lines.

Cell Line.	L-DOPA Decarboxylase Activity	
	$\mu IU/mg$ protein	$\mu IU/\mu g$ DNA
H187 (n=9)	200.9 ± 31.8	3.2 ± 0.53
H69 (n=11)	3977.6 ± 349.0	65.7 ± 12.3
H128d (n=11)	8085.2 ± 808.1	101.4 ± 15.2

The DDC activity in the three SCLC cell lines is expressed as
international units (IU)/mg protein or IU/μg DNA, where one IU is
equivalent to one μmole of product formed per minute.

The results represent the mean DDC activity ± standard error from n
independent experiments.

CHAPTER 5.
EFFECT OF PHENOTYPIC MODULATORS ON THE EXPRESSION
OF SCLC PHENOTYPIC MARKERS.

Part 1:- The Effect of Phenotypic Inducers on the DDC Activity in some
        SCLC Cell Lines.

The aim of this project was to investigate whether the SCLC phenotype
could be altered by phenotypic inducers and whether an alteration in
the SCLC phenotype had any effect on chemosensitivity. In order to
examine the .ffect of phenotypic inducers on the SCLC phenotype it was
necessary to select suitable phenotypic markers.   It has been
proposed that SCLC cells belong to the group of peptide hormone- and
amine-synthesising cells which Pearse called the amine precusor uptake
and decarboxylation (APUD) system (Pearse,1969). L-DOPA decarboxylase
(DDC) is a key enzyme step in the synthesis of biogenic amines and as
such plays an integral role in the APUD concept.   Furthermore, DDC
activity has been reported to be present in SCLC cell lines at
relatively higher activity than in other lung tumours (Baylin et al,
1980).   DDC activity was therefore selected as a marker to represent
the SCLC phenotype.

Table 4 shows the control DDC activities of the SCLC cell lines used
in this study. The DDC activity is expressed either as µIU/mg protein
or µIU/µg DNA. The three cell lines expressed marked differences in
their control levels of DDC activity. The highest expressor of DDC
activity was found to be H128d, which had a DDC activity level 40 fold
greater than H187, the lowest expressor.   H69 was found to have an
intermediate level of DDC activity, approximately 2 fold lower than
H128d.

The DDC activity of the three SCLC cell lines was measured during the
growth cycle to determine whether the enzyme activity was altered by
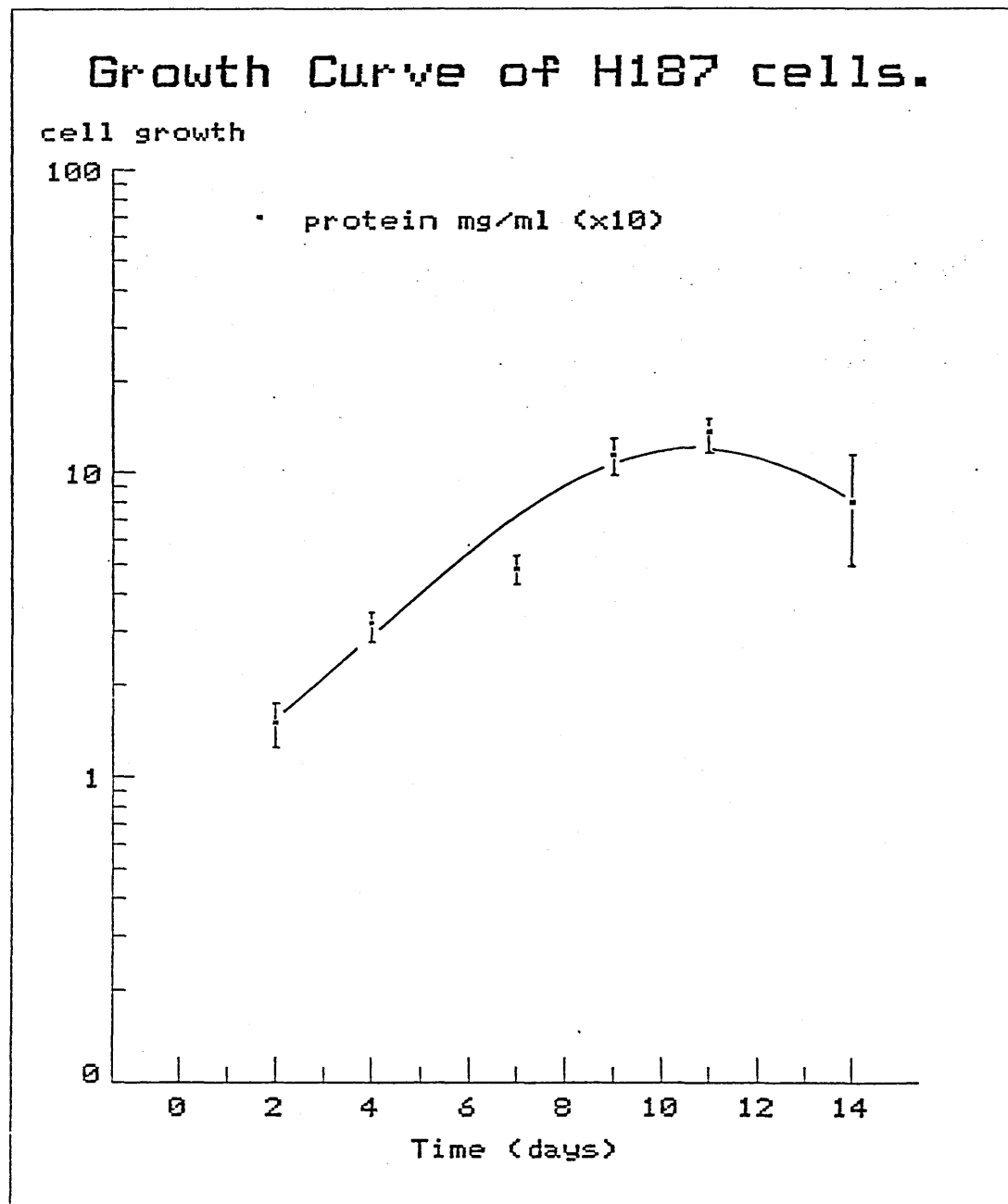
Figure 26:- Growth Curve of H187 Cells.

H187 cells were seeded into 25cm^2 flasks at low density. On the appropriate days the cells were harvested and the cell growth was determined by measuring protein content using the Bradford Protein Assay.
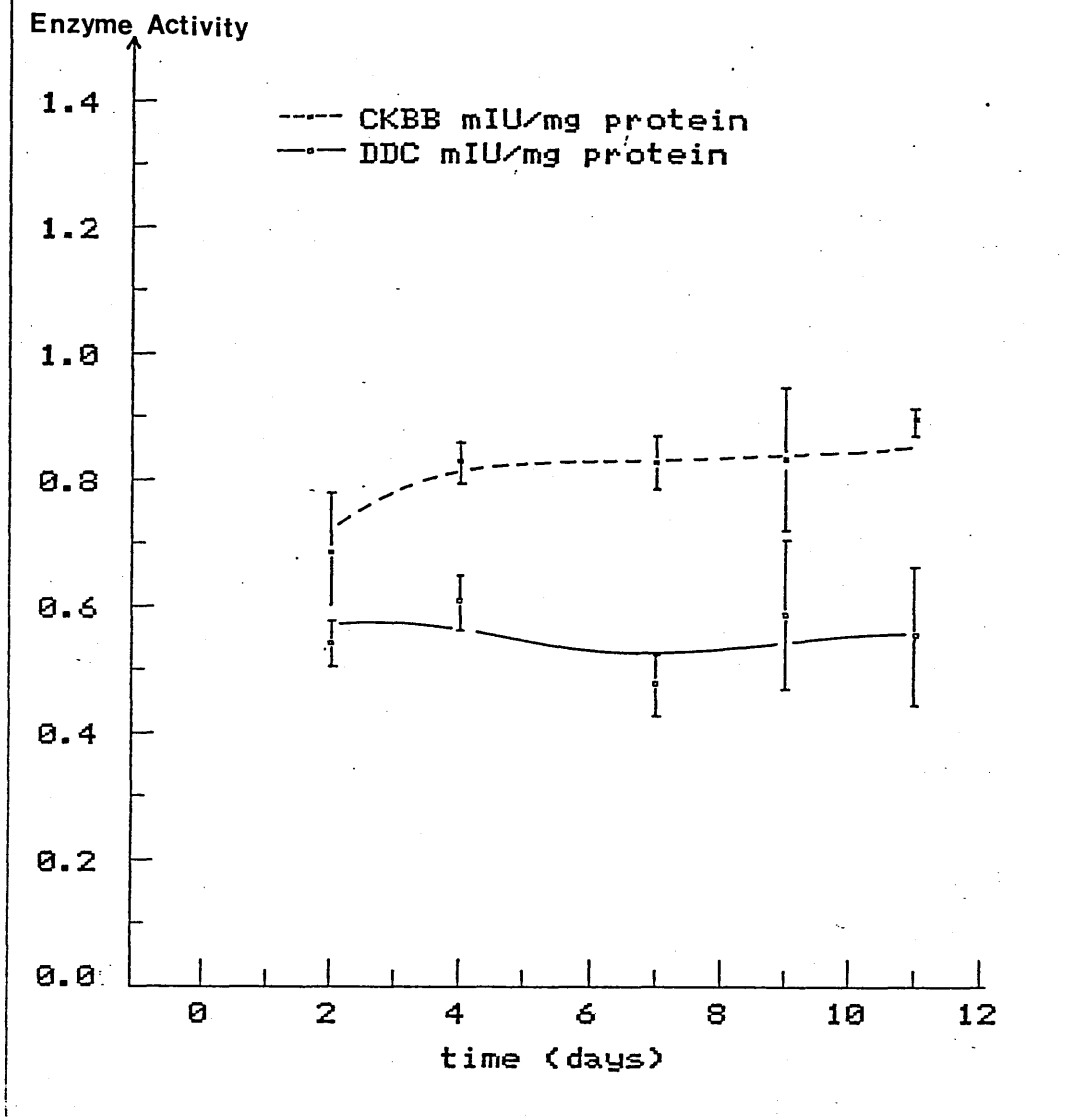
Figure 27:- Growth Related Expression of DDC and CKBB Activities in
H187 Cells.

H187 cells were grown from low density in 25cm^2 flasks (see Fig. 24). At regular intervals throughout the growth curve H187 cells were harvested and their DDC and CKBB activities determined. The results represent the mean enzyme activity $\pm$ standard error of 3 replicate samples.

Figure 28:- Growth Curve of H69 Cells.

H69 cells were seeded into $25cm^2$ flasks at low density. On the appropriate days the cell growth was determined by measuring protein content using the Bradford Protein Assay.
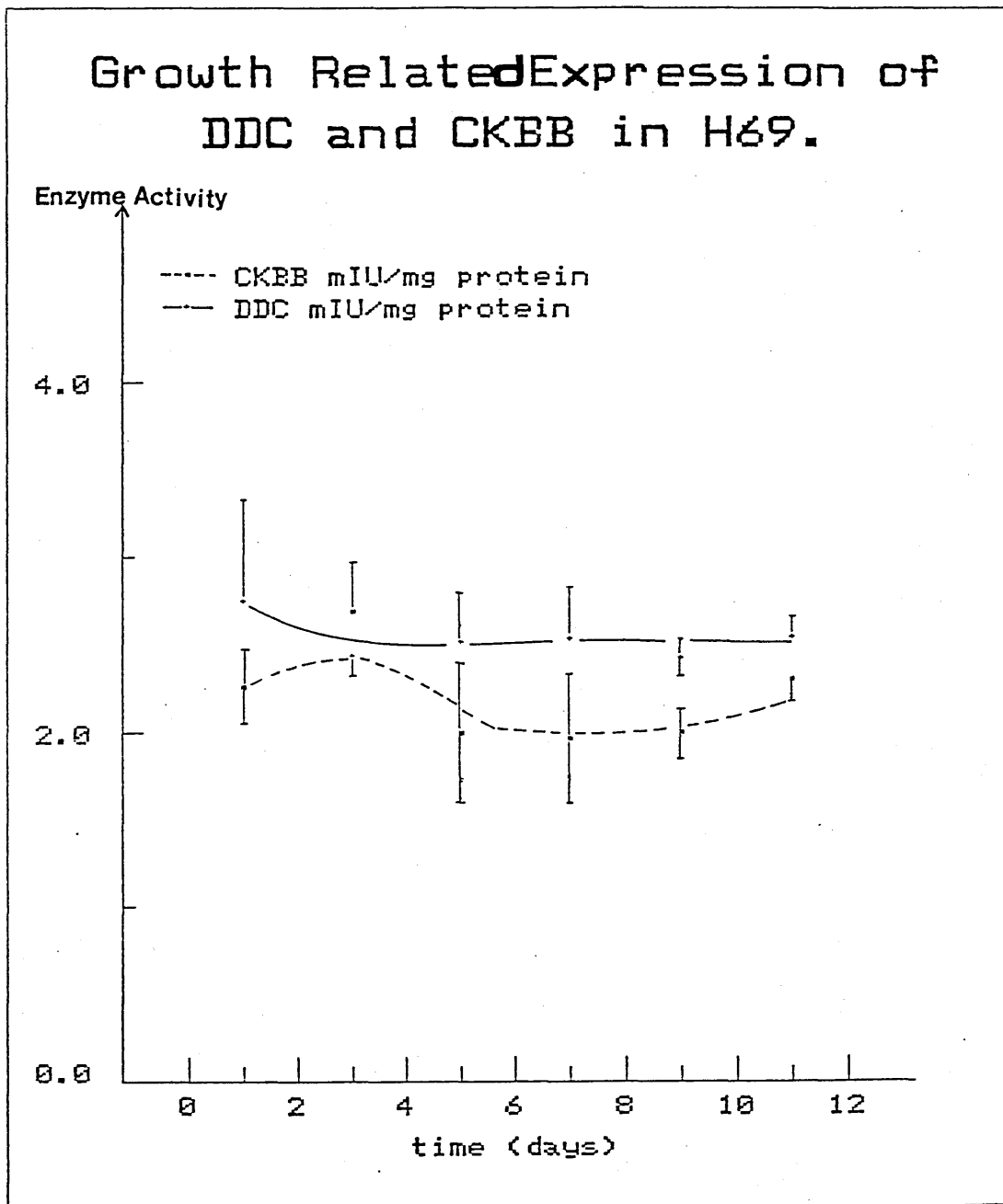
Figure 29:- Growth Related Expression of DDC and CKBB Activities in H69 Cells.

H69 cells were grown from low density in $25cm^2$ flasks (see Fig. 25). At regular intervals throughout the growth cycle H69 cells were harvested and their DDC and CKBB activities determined. The results represent the mean enzyme activity ± standard error of 3 replicate samples.

Figure 30:- Growth Curve of H128d Cells.

H128d cells were seeded into 25cm^2 flasks at low density. On the appropriate days the cell growth was determined by measuring protein content using the Bradford Protein Assay.
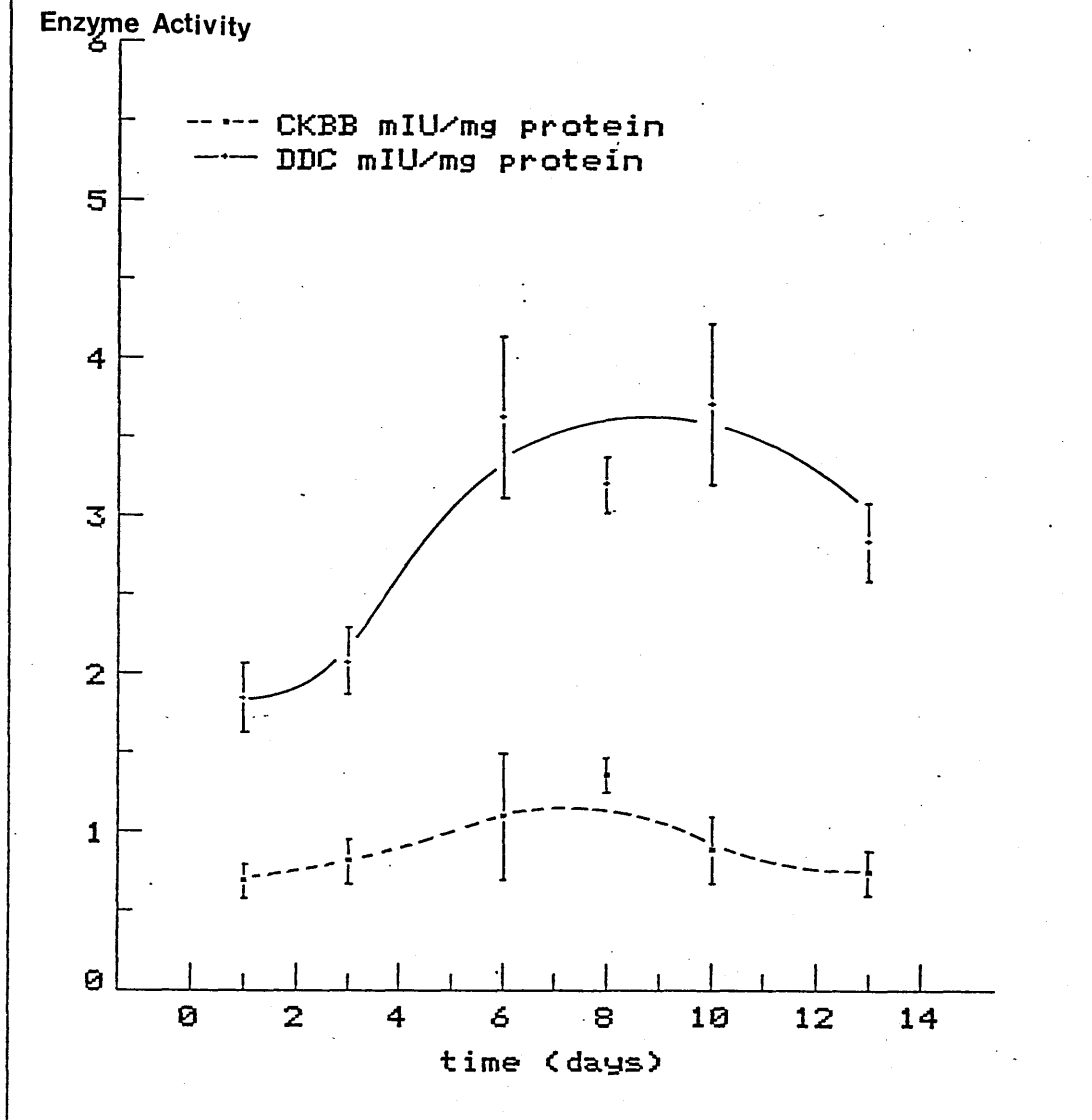
Figure 31:- Growth Related Expression of DDC and CKBB Activities in H128d Cells.

H128d cells were grown from low density in $25cm^2$ flasks (see Fig. 27). At regular intervals throughout the growth cycle H128d cells were harvested and their DDC and CKBB activities determined. The results represent the mean enyzme activity $\pm$ standard error of 3 replicate samples.

increasing cell density. The cells were seeded into 25cm^2 flasks at low density and grown for approximately 11 days. The cell growth was determined by measuring the protein content at intervals throughout the growth curve (Figs. 26, 28 & 30), and concurrent measurement of the cellular DDC and CKBB activity was carried out (Figs. 27, 29 & 31).

Figures 27 & 29 show that during the H187 and H69 growth cycle the cellular DDC activity did not change significantly. Some fluctuation in H187 DDC activity was noticed at low cell densities, however this may be due in part to the preparation procedure. Since the H187 DDC activity was found to be low, several flasks of cells had to be pooled together in order to have enough assayable material at low cell densities. This introduced a number of steps which lengthened the preparation procedure which may have indirectly affected the enzyme's activity. Figure 31 shows that the DDC activity in H128d increased as the cell density increased. However, after about 6 days the DDC activity did not significantly change. The cellular DDC activity was therefore measured during the late log phase of the growth cycle, where the enzyme activity showed minimum fluctuation. The DDC activity has previously been shown not to fluctuate during the cell cycle (Baylin et al 1980).

The effect of a number of phenotypic inducers on the DDC activity in some SCLC cell lines was examined. The SCLC cell lines were treated with the appropriate drug in late log phase for 72hrs, approximately 72hrs after cell seeding. A 72hr exposure time was selected because in other systems at least a 3 day drug exposure time was required before phenotypic change was observed (Lever 1979, Fibach et al 1977 and Nakagawa et al 1985). Following treatment of the cell lines with or without the appropriate phenotypic inducer the DDC activity was calculated as μIU/mg protein and IU/μg DNA. It is possible that expression of enzyme activities as IU/mg protein may give a false impression of changes in enzyme activity, since the

Table 5: Effect of HMBA Treatment on the L-DOPA Decarboxylase Activities in SCLC Cell Lines.

[HMBA] mM.

Cell Line.	0.1	0.5	1.0	5.0
H187	119.6	81.1 $\pm$ 7.5	94.5 $\pm$ 15	95.3 $\pm$ 14.5
H69	93.4 $\pm$ 9.7	87.5 $\pm$ 4.0*	77.3 $\pm$ 5.7*	49.3 $\pm$ 9.8*
H128d	87.0 $\pm$ 1.8	89.1 $\pm$ 5.1	94.5 $\pm$ 8.0	71.2 $\pm$ 7.1

SCLC cell lines were grown to late log phase and exposed to HMBA for 72hrs. The cells were then washed 2x with PBS and their DDC activities determined.

The results are expressed as the % control DDC activity, where the control values are taken as 100. The results represent the mean of 3 replicate experiments $\pm$ standard error.

Statistical analysis was carried out using the Students t-test.

* $P < 0.05$

Effect of HMBA on the DDC Activity
in H69.

% control DDC activity

HMBA conc. mM
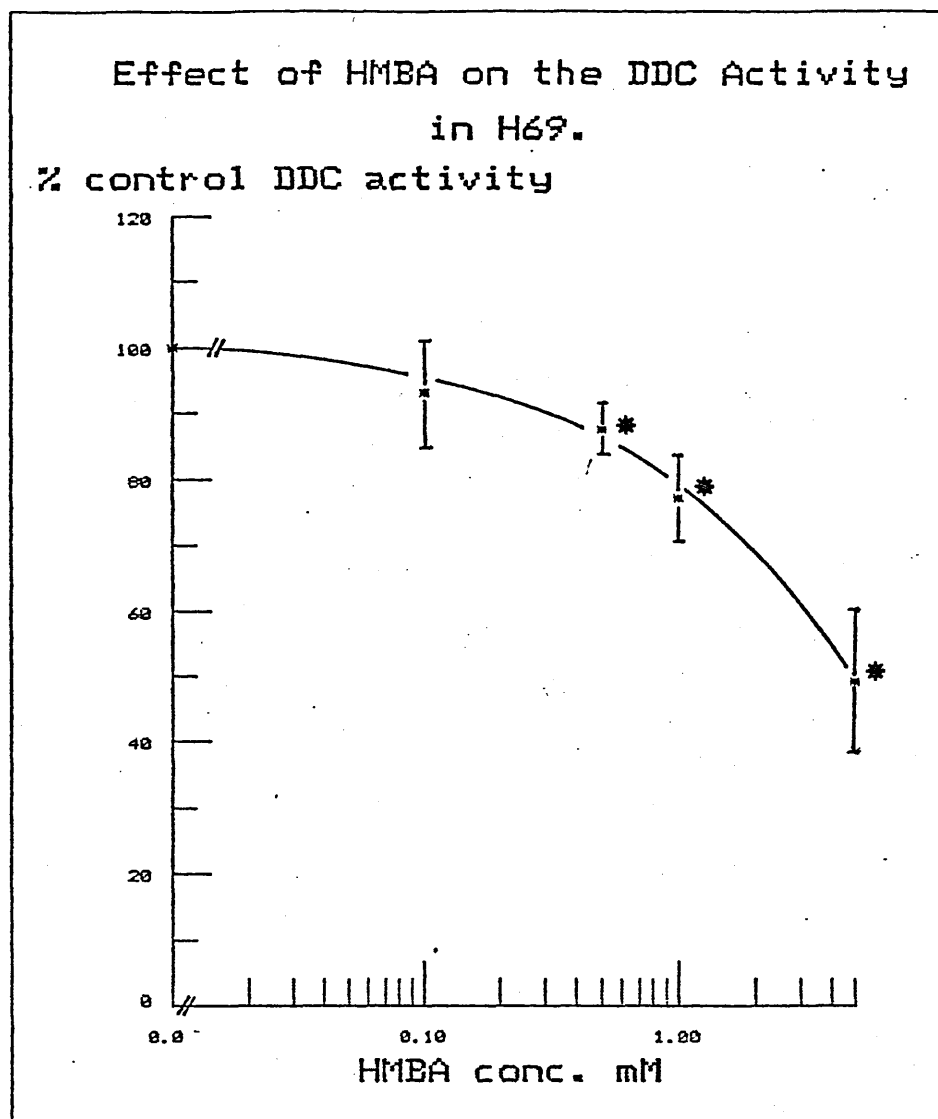
Figure 32:- The Effect of HMBA on the DDC Activity of H69 Cells.

H69 cells were exposed to HMBA at various concentrations for 72hrs.
Following drug exposure the cells were washed 2x with PBS and their
DDC activity determined.

The results are expressed as the % control DDC activity where the
control value equals 100. The results represent the mean of 3
experiments ± standard error.

Statistical analysis was carried out using the Students t-test.

* P<0.05

Table 6: Effect of DbcAMP Treatment on the L-DOPA Decarboxylase Activities in some SCLC Cell Lines.

[dbcAMP] mM.

Cell Line	0.05	0.1	0.5	1.0
H187	111.3 ± 8.4	133.7 ± 4.4	162.4 ± 4.2***	194.5 ± 22.3*
H69	101.5 ± 11.0	95.0 ± 0.7	79.9 ± 5.4*	69.7 ± 2.6***
H128d	89.2	83.7	93.4 ± 16.0	149.6 ± 7.7*

SCLC cell lines in late log phase of growth were incubated with dbcAMP for 72hrs. Following the incubation period the cells were washed 2x with PBS and their DDC activities determined.

The results are expressed as the % of control DDC activity, where the control values are taken as 100. The results represent the mean of 3 replicate experiments ± standard error.

Statistical analysis was carried out using the Students t-test.

* P<0.05   ** P<0.02   *** P<0.01

observed changes may have been due to an alteration in the amount of
protein per cell. If the drugs were to either increase or decrease
the protein content per cell this would obviously alter the specific
activity of the enzyme without actually altering the amount of enzyme
protein. In order to ensure that this was not the case the enzyme
activity was also expressed per µg DNA. Irrespective of whether the
enzyme activity was calculated as µIU/mg protein or IU/µg DNA any
change found in enzyme activity compared to the controls was found to
be the same.

The DDC activity of the drug treated cells is expressed as a
percentage of the control, where the control value is taken as 100%.

The SCLC cell lines were incubated with a range of HMBA concentrations
for 72hrs. Table 5 shows that HMBA treatment did not significantly
affect the DDC activity in either H187 or H128d. However, when H69
cells were exposed to HMBA a dose dependent decrease in the DDC
activity was found (Fig. 32). Moreover, this dose dependent decrease
in the DDC activity in H69 cells following HMBA treatment was found to
be significant. The maximum effect was found following 5mM HMBA
treatment, where a 2 fold decrease was noted.

When the DDC activities in the SCLC cell lines were examined following
treatment with dbcAMP a different pattern of phenotypic change was
observed. Table 6 shows that dbcAMP altered significantly the DDC
activity in H187 and significantly reduced the DDC activity in H69 in
a dose dependent manner (Fig. 33). Incubation of H187 with 1mM dbcAMP
resulted in an 100% increase in the DDC activity. In contrast, 1mM
dbcAMP treatment reduced the DDC activity in H69 by 30%. The DDC
activity in H128d was found to be unaffected by dbcAMP at lower
concentrations, however at the highest concentration examined, 1mM,
the DDC activity increased by approximately 50% (Tab. 6).
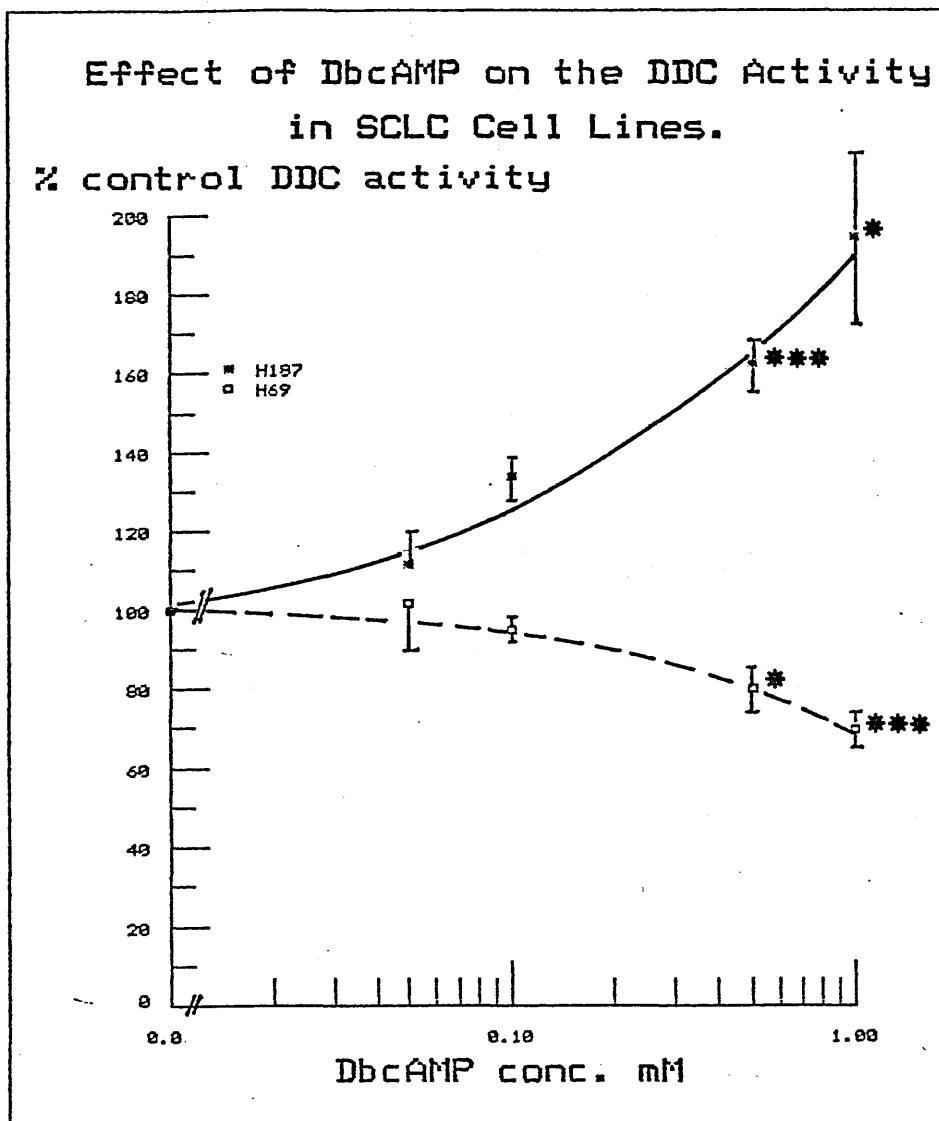
Figure 33:- The Effect of DbcAMP on the DDC Activity in SCLC Cell Lines.

SCLC cell lines were incubated with various concentrations of dbcAMP for 72hrs. The cells were then harvested and their DDC activities determined.

The results are expressed as the % control DDC activity where the control value is taken as 100. The results represent the mean of 3 replicate experiments ± standard error.

Statistical analysis was carried out using the Students t-test.

* P<0.05 ** P<0.02 *** P<0.01

Table 7: Effect of Sodium Butyrate Treatment on the L-DOPA Decarboxylase Activities in SCLC Cell Lines.

[NaBut] mM.

Cell Line	0.1	0.5	1.0
H187	96.8 ± 13.6	115.4 ± 9.9	129.9 ± 11.1
H69	74.8 ± 23.0	48.7 ± 12.2*	51.0 ± 4.2***
H128d	101.2 ± 11.7	76.0 ± 8.3	72.8 ± 2.3***

SCLC cell lines in late log phase were treated with NaBut for 72hrs. The cells were harvested, washed 2x with PBS and their DDC activities determined.

The results are expressed as the % control DDC activity, where the control value is taken as 100. The results represent the mean of 3 replicate experiments ± standard error.

Statistical analysis was performed using the Students t-test.

* P<0.05   ** P<0.02   *** P<0.01

Table 8: The Effect of Dexamethasone on the L-DOPA Decarboxylase Activity in the SCLC Cell Lines.

	[Dexamethasone] μM		
Cell Line	0.25	2.5	25
H187	107.6 ± 0.8	113.5 ± 12.6	95.4 ± 4.0
H69	88.3 ± 15.1	95.3 ± 11.6	90.5 ± 10
H128d	97.1 ± 1.6	92.3 ± 5.3	83.3 ± 0.8

SCLC cell lines were grown to late log phase and exposed to dexamethasone for 72hrs. The cells were then washed 2x with PBS and their DDC activities determined.

The results are expressed as the % control DDC activity, where the control values are taken as 100. The results represent the mean of 3 replicate experiments ± standard error.

Statistical analysis was carried out using the Students t-test.

The SCLC cell lines were also exposed to NaBut, another phenotypic inducer.   It was found that NaBut significantly reduced the DDC activity in both H69 and H128d but had no effect in H187 (Tab. 7). The maximum effect of NaBut on the DDC activity in H69 was at 0.5mM where a greater than 50% reduction was found.   H128d cells were not as sensitive to NaBut as H69 cells, following 1.0mM NaBut exposure only a 27% fall in the DDC activity was observed.

When the cells were incubated with dexamethasone for 72hrs their DDC activity did not change significantly (Tab. 8).

Part 2:- The Effect of Phenotypic Inducers on the Creatine Kinase BB Activity in some SCLC Cell Lines.

As discussed earlier, SCLC can be distinguished from most other lung cancers by the presence of high levels of the key amine-handling cell enzyme L-DOPA decarboxylase. SCLC can also be characterised by the presence of high levels of creatine kinase in the form of its BB isoenzyme (CKBB) (Gazdar et al, 1981).

CK catalyses the transfer of a high energy phosphate bond from creatine phosphate to ADP. It has been suggested that CK is present in all body tissues and organs, and its levels and the relative proportion of its isoenzymes, CKMM CKMB and CKBB alter during embryonic development and differentiation and show considerable organ-to-organ variation. Apart from striated muscle, brain, bladder and gastrointestinal tract most organs have low levels of enyzme predominantly in the form of CKBB (Shatton, Morns & Weinhouse 1979).

As mentioned above, high levels of CKBB have been found in tumour specimens and cultures of SCLC. Electrophoretic analysis of the CK in normal and neoplastic lung samples showed that CK is predominantly in the CKBB form (Gazdar et al 1981). Therefore the CK activity found in SCLC tumours and cultures is quantitatively but not qualitatively different from those in normal and other lung cancers. CKBB was selected as a biochemical marker of the SCLC phenotype for a number of reasons. In contrast to DDC, high levels of CKBB have not been identified in lung cancers and cultures other than those of the SCLC lineage. Furthermore, some SCLC tumours have been reported to undergo an alteration in their morphology both in vivo and in vitro (Abeloff et al, 1979) with an accompaning loss of many distinctive SCLC features including DDC, however high levels of CKBB are maintained. Therefore CKBB was selected as a biochemical marker to represent the SCLC phenotype.

Table 9: Control Levels of Creatine Kinase BB Activity in the SCLC Cell Lines.

Cell Line	Creatine Kinase BB Activity	
	IU/mg protein	mIU/µg DNA
H187 (n=10)	1.13 ± 0.09	19.2 ± 2.2
H69 (n=12)	2.42 ± 0.21	41.4 ± 6.7
H128d (n=12)	1.39 ± 0.11	18.5 ± 2.6

The CK BB activity in the three cell lines is expressed as international units (IU)/mg protein or IU/µg DNA, where one IU is equivalent to one µmole of NADH produced per minute.

The results represent the mean CK BB activity ± standard error from n independent experiments.

The control CKBB activities in the SCLC cell lines used in this study
are given in Table 9. CKBB activity is expressed as international
units IU/mg protein and as mIU/µg DNA. In either case H69 cells were
found to have the highest CKBB activity, approximately twice that of
the other two lines, H187 and H128d. The percentage of the CKBB
isoenzyme in H187, H69 and H128d was found to be 81.3 $\pm$ 2.8, 85.4 $\pm$
5.1 and 88.8 $\pm$ 2.8 respectively (mean CKBB activity $\pm$ standard error
of 8 replicate experiments).

In order to determine the effect of phenotypic inducers on the CKBB
levels in the SCLC cell lines it was important to determine whether
the CKBB activity fluctuated at all during the growth cycle of the
cells. If the CKBB activity did change over the growth cycle of the
cells it would be important to ensure that the control cells and the
drug treated cells were at the same point in the growth cycle so that
any change in the CKBB activity was due to phenotypic change and not
to cell density. Figures 27, 29 & 31 show that the CKBB activity of
the SCLC cell lines did not change significantly during the growth
cycle. Some fluctuation was found at low cell densities but this may
have been due to low sample volumes. Therefore in order to ensure
constant control levels of CKBB activity the enzyme was assayed
following phenotypic inducer treatment in late log phase (day 6
onwards). The cells were incubated with the appropriate phenotypic
inducer for 72hrs, following which time the cells were harvested and
their CKBB activity determined. As in the case of the DDC results,
CKBB activity was also calculated both as IU/mg protein and mIU/µg
DNA. In either case any change in the CKBB activity following
phenotypic inducer treatment was found to be the same. The results
are presented as a percentage of the control CKBB activity where the
control activity is taken as 100%.

The effect of HMBA on the CKBB activities in the SCLC cell lines was
examined. HMBA was found to change the CKBB activity significantly in

Table 10: Effect of HMBA Treatment on the Creatine Kinase BB Activities in SCLC Cell Lines.

[HMBA] mM.

Cell Line.	0.1	0.5	1.0	5.0
H187	79.0 ± 8.8	110.7 ± 4.4	102.7 ± 14	$156.2 \pm 11.8^{*}$
H69	$115.7 \pm 1.6^{**}$	$127.0 \pm 5.7^{**}$	$120.5 \pm 4.9^{*}$	100.3 ± 14.6
H128d	90.4 ± 3.8	$88.0 \pm 1.8^{*}$	$83.8 \pm 0.9^{*}$	$69.7 \pm 2.0^{***}$

SCLC cell lines were grown to late log phase and then incubated with HMBA for 72hrs. The cells were harvested, washed 2x with PBS and their CK BB activities determined.

The results are expressed as the % control CK BB activity where the control value is taken as 100. The results represent the mean of 3 replicate experiments ± standard error.

Statistical analysis was carried out using the Students t-test.

* P<0.05 **P<0.02 *** P<0.01
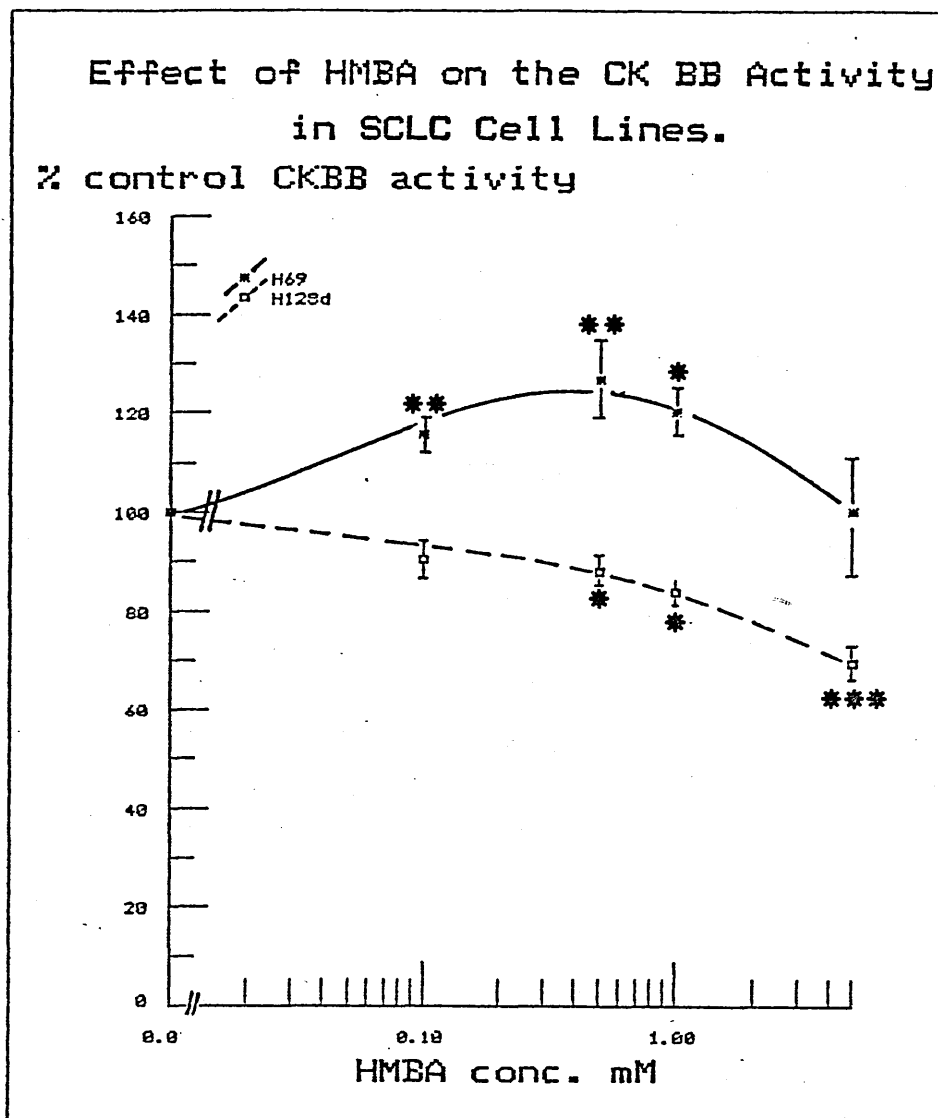
Figure 34:- The Effect of HMBA on the CKBB Activity in some SCLC Cell Lines.

SCLC cell lines were exposed to various concentrations of HMBA for 72hrs. Following drug exposure the cells were wahed 2x with PBS and their CKBB activities determined.

The results are expressed as the % control CK BB activity where the control value is taken to be 100. The results represent the mean of 3 experiments ± standard error.

Statistical analysis was carried out using the Students t-test.

$*$ P<0.05 $**$ P<0.02 $***$ P<0.01

all three cell lines examined, although the pattern of HMBA-induced change in each case is different (Tab. 10). HMBA, 5mM, significantly increased the CKBB activity in H187. However, HMBA induced a change in the CKBB levels of H69 at lower concentrations. A significant increase in the CKBB levels in H69 was found, with a peak effect at 0.5mM (Fig. 34). This effect was lost when the HMBA concentration was increased above 1.0mM, and, in fact, 5mM HMBA had no effect on the CKBB activity in H69. The pattern of HMBA induced CKBB change in H128d was different. The CKBB levels in H128d were signficantly reduced in a dose dependent manner by HMBA (Fig. 34).

Following treatment of the SCLC cell lines with dbcAMP a selective change in the CKBB activity was found. DbcAMP significantly increased the CKBB activity in H187 in a dose dependent manner (Fig. 35). The maximum effect was found following treatment with 1mM dbcAMP, where a 62% increase in activity was found. DbcAMP did not significantly effect the CKBB activity in the other two cell lines examined (Tab. 11).

With NaBut a selective change in the CKBB activity was again found. NaBut increased significantly the CKBB activity in H187 in a dose dependent manner (Fig. 36). The greatest effect was seen following exposure of H187 cells to 1.0mM NaBut, where a 46% increase in CKBB acitivity was found. NaBut treatment did not significantly affect the CKBB activity in the other two lines examined (Tab. 12).

Finally, the effect of dexamethasone on the CKBB activities in the SCLC cell lines was examined. However, dexamethasone up to 25μM was found to have no effect on the CKBB activity in the SCLC cell lines examined (Tab. 13).

The effect of retinoic acid on the DDC and CKBB activities in H69 cells was also examined. However, it was found that the drug treated cells had the same enzyme activities as the untreated controls.
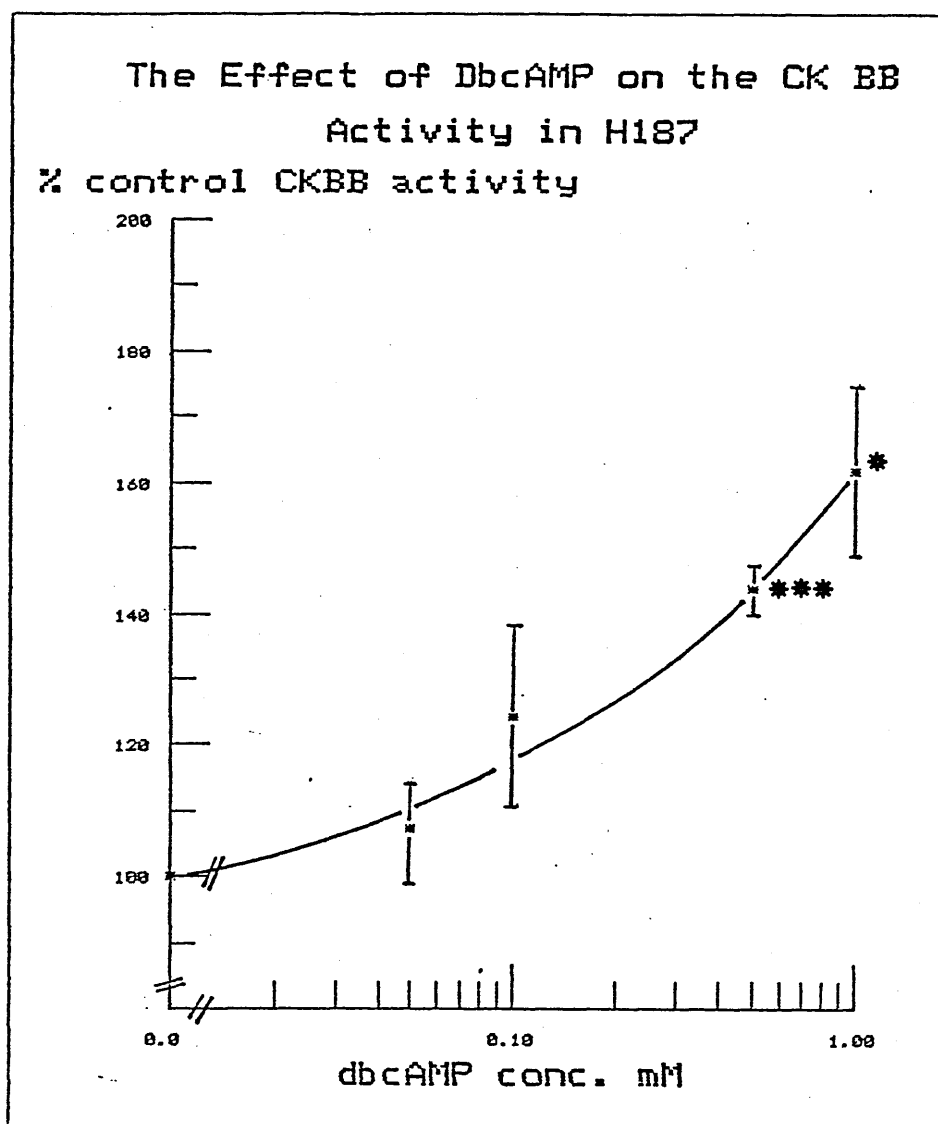
Figure 35:‐ **The Effect of DbcAMP on the CKBB Activity of H187 Cells.**

H187 cells were incubated with dbcAMP for 72hrs. Following drug exposure the cells were washed and their CKBB activity determined.

The results are expressed as the % control CKBB activity where the control value is taken to be 100. The results represent the mean of 3 experiments $\pm$ standard error.

Statistical analysis was performed using the Students t-test.

       * P<0.05   ** P<0.02   *** P<0.01

Table 11: **Effect of DbcAMP Treatment on the Creatine Kinase BB Activities in SCLC Cell Lines.**

[dbcAMP] mM.

Cell Line	0.05	0.1	0.5	1.0
H187	107.2 ± 7.5	124.2 ± 13.0	$143.8 \pm 3.2^{***}$	$161.9 \pm 15.1^{*}$
H69	153.4	146.6 ± 9.2	137.1 ± 19.1	108.1 ± 17.0
H128d	66.2	73.4	84.1 ± 10.5	124.4 ± 9.9

SCLC cell lines were treated in late log phase with dbcAMP for 72hrs. The cells were harvested, washed and their CK BB activities determined.

The results are expressed as the % control CK BB activity, where the control value is 100. The results represent the mean of 3 replicate experiments $\pm$ standard error.

Statistical analysis was performed using the Students t-test.

* P<0.05 ** P<0.02 *** P<0.01
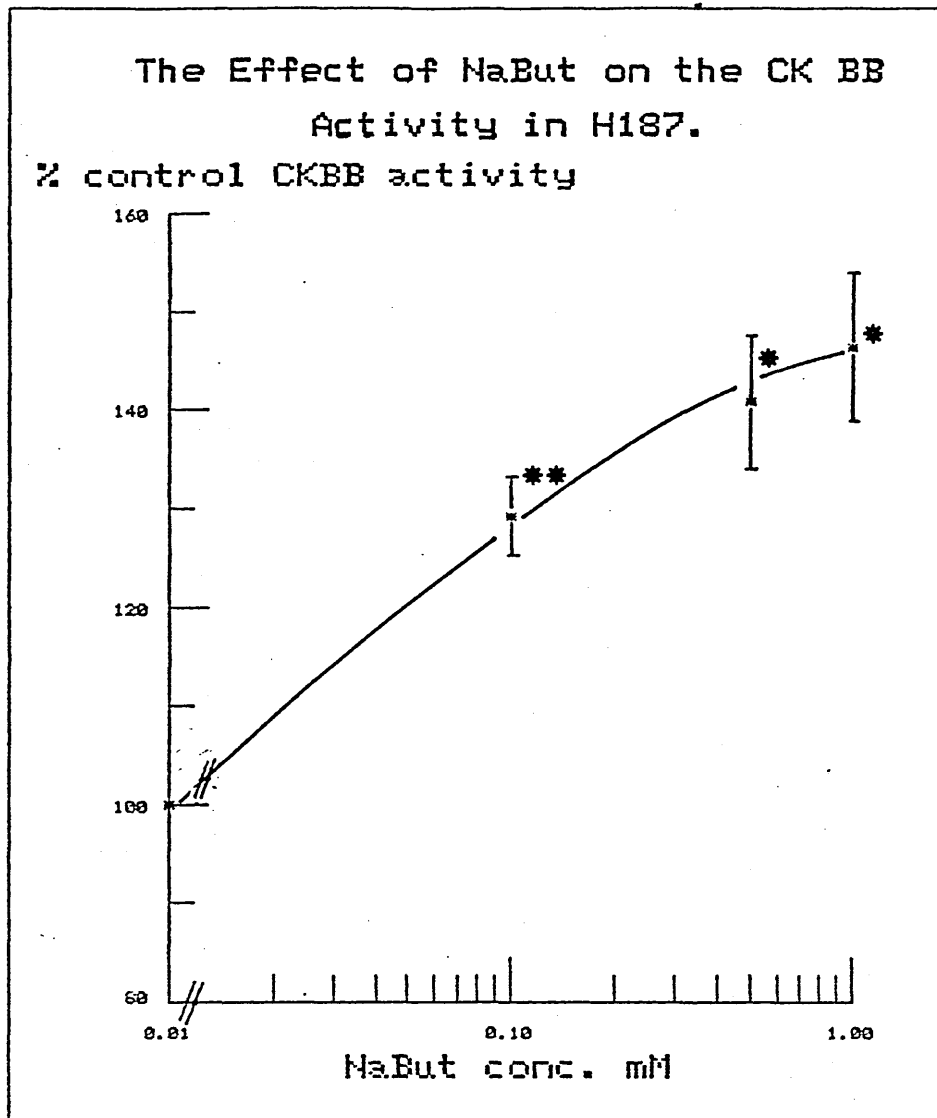
Figure 36:- The Effect of NaBut on the CKBB Activity in H187.

H187 cells were incubated with NaBut for 72hrs. Following the drug incubation period, the cells were harvested and their CKBB activity determined.

The results are expressed as the % control CKBB activity where the control value is taken to be 100. The results represent the mean of 3 experiments ± standard error.

Statistical analysis was carried out using the Students t-test.

* P<0.05   ** P<0.02

Table 12: Effect of Sodium Butyrate Treatment on the Creatine Kinase BB Activities in SCLC Cell Lines.

[NaBut] mM.

Cell Line	0.1	0.5	1.0
H187	$129.1 \pm 3.9^{**}$	$140.9 \pm 7.1^{*}$	$146.4 \pm 8.3^{*}$
H69	110.3 ± 9.2	93.6 ± 2.4	96.2 ± 13.6
H128d	105.7 ± 9.1	92.4 ± 22.2	102.3 ± 12.3

SCLC cell lines were treated with NaBut for 72hrs in late log phase of growth. The cells were then harvested, washed 2x with PBS and their CK BB activities determined.

The results are expressed as the % control CK BB activity, where the control value is taken as 100. The results represent the mean of 3 replicate experiments $\pm$ standard error.

Statistical analysis was performed by the Students t-test.

* P<0.05 ** P<0.02

Table 13: The Effect of Dexamethasone on the CKBB Activities in the SCLC Cell Lines.

Cell Line	[Dexamethasone] μM		
	0.25	2.5	25
H187	117.3 ± 5.0	121.0 ± 10.0	117.4 ± 9.9
H69	100.0 ± 5.4	103.6 ± 11.3	100.0 ± 5.4
H128d	100.7 ± 9.0	103.3 ± 17.0	98.9 ± 16.8

SCLC cell lines were grown to log phase and exposed to dexamethasone for 72hrs. The cells were then washed 2x with PBS and their CKBB activities determined.

The results are expressed as the % control DDC activity, where the control values are taken as 100. The results represent the mean of 3 replicate experiments ± standard error.

Statistical analysis was carried out using the Students t-test.

Part 3:- The Effect of Phenotypic Inducers on the Bombesin-Like Immunoreactivity in Some SCLC Cell Lines.

Bombesin is a 14-amino acid peptide which was initially isolated from the skin of a frog (Anastasi et al 1971). In mammals, bombesin-like immunoreactivity has been found predominantly in the brain (Moody et al, 1979) lung (Wharton et al 1978) and intestine (McDonald et al 1979). In the human lung, bombesin-like immunoreactivity (BLI) has been found in the bronchial and bronchiolar epithelium both as single cells and as groups (Wharton et al, 1978). It is readily found in full term foetal and neonatal lung, but is greatly reduced or even absent in the adult lung. Moreover, in neonates with the acute respiratory distress syndrome, there is a significantly lower bombesin content in all regions of the respiratory tract compared to either normal full-term infants or 24- to 28-week-old fetuses and it has been proposed that bombesin-like peptides may have a role in the normal development of the human lung (Ghatei et al 1983). The mammalian equivalent to bombesin is believed to be gastrin-releasing peptide (GRP). GRP is a 27-amino acid peptide with a carboxy-terminal heptapeptide sequence similar to bombesin.

Human SCLC cell lines have been shown to contain significant levels of BLI, while the non-SCLC cell lines examined had undetectable BLI (Moody et al, 1981). It has been suggested that bombesin is an autocrine growth factor in SCLC cell lines since some cell lines produce bombesin (Moody et al, 1981)and for the following reasons: (1) some SCLC cell lines have high affinity bombesin receptors which bind detectable bombesin (Moody et al, 1985), (2) some SCLC cell lines have been reported to respond to bombesin in vitro (Weber et al 1985; Carney et al 1987), and (3) a monoclonal antibody to bombesin which binds to the C-terminal region inhibits the clonal growth of SCLC in vitro and the growth of SCLC xenografts in vivo. Since the presence

Table 14: Control Levels of Bombesin-like Immunoreactivity in the SCLC Cell Lines.

Cell Line	Bombesin-like Immunoreactivity pg/µg acid soluble protein
H187 (n=3)	undetectable
H69 (n=3)	8.4 $\pm$ 1.18
H128d (n=3)	57.2 $\pm$ 8.6

The bombesin-like immunoreactivity (BLI) of the three SCLC cell lines used in this study were examined by radioimmunoassay.

The results represent the mean BLI of n independent experiments.

of BLI distinguished SCLC from other lung cancer types, BLI was selected as another marker to represent the SCLC phenotype.

Table 14 shows the control levels of BLI in the SCLC cell lines used in this study. The BLI is expressed as pg/μg soluble protein. BLI was undetectable in H187 but was found in both H69 and H128d. H128d was found to have the highest level of BLI which was approximately 7 fold higher than the BLI found in H69.

The effect of a number of phenotypic inducers on the BLI in the SCLC cell lines was examined. However due to the cost of the RIA a limited investigation was carried out. Based on the results of phenotypic inducer treatment on the DDC and CKBB activities in the SCLC cell lines, only the drugs which produced the most significant change in the enzyme levels were examined for their effect on the BLI. Table 15 summarises the effect of phenotypic inducer treatment on the BLI in the three SCLC cell lines examined.

DbcAMP 1mM was found to significantly increase both the DDC and CKBB activities in H187. When H187 cells were treated with dbcAMP 1mM for 72hrs detectable levels of BLI were found compared to an undetectable BLI level in the untreated control cells. Therefore dbcAMP 1mM treatment increased the BLI in H187 cells.

The effect of phenotypic inducer treatment on the BLI in H69 cells was examined. Although both dbcAMP and NaBut reduced the DDC activity significantly in H69 cells, treatment for 72hrs with these drugs had no effect on the BLI. HMBA has previously been shown to increase the CKBB activity significantly and reduce the DDC activity significantly in H69 cells (see above) and when H69 cells were treated with 5mM HMBA for 72hrs a significant decrease in the BLI was found.

Table 15: The Effect of Phenotypic Inducer Treatment on the Bombesin Like Immunoreactivity (B.L.I.) in some SCLC Cell Lines.

Cell Line	Control Levels of B.L.I.	Phenotypic Inducer P.I. Treatment	B.L.I. following P.I. treatment.
H187	Undetectable	dbcAMP 1.0mM	0.064 $\pm$ 0.04
H69	8.4 $\pm$ 1.18	dbcAMP 1.0mM	8.8 $\pm$ 1.7
		NaBut 1.0mM	7.6 $\pm$ 2.4
		HMBA 5.0mM	2.3 $\pm$ 0.7*
H128d	57.2 $\pm$ 8.6	dbcAMP 1.0mM	43.9 $\pm$ 11.8
		NaBut 1.0mM	51.6 $\pm$ 16.4
		HMBA 5.0mM	92.4 $\pm$ 26.7

SCLC cell lines were incubated with the appropriate phenotypic inducer for 72hrs. The cells were then harvested and their BLI determined.

The results represent the mean BLI pg/µg acid soluble protein $\pm$ standard error from 3 independent experiments.

Statistical analysis was performed using the Students t-test.

* $P < 0.01$

When the effect of phenotypic inducer treatment on the BLI in H128d was examined it was found that the BLI in H128d did not change in response to treatment of any of the phenotypic inducers selected.

Bombesin-like peptides have been shown to be secreted by SCLC cells in culture (Moody et al, 1983). These studies however investigated the effects of the various phenotypic drugs on the cellular BLI levels and did not examine the effect of drug treatment on the secretion of BLI. It is possible that these drugs may have altered the secretion rate of BLI from the SCLC cells which may account for the differences found. Interestingly, dbcAMP has been shown to increase the secretion of bombesin-like peptides from a SCLC cell line and a role for cAMP in the mechanism of bombesin-like peptide secretion has been implicated (Korman et al 1986). DbcAMP treatment of the SCLC cell lines did not significantly reduce the levels of BLI and in fact increased the BLI in H187 cells. Therefore if dbcAMP increased the secretion of BLI from the SCLC cell lines this must have been matched with an increased synthesis of BLI. If the reduction in BLI in H69 cells following exposure to HMBA was due to an increased secretion of BLI then the synthesis rate of BLI did not match the secretion rate. It will be important to determine the effects of these drugs on the secretion of BLI from these cell lines. Moreover, how will the presence of cycloheximide affect the BLI levels following exposure to these drugs ?

Table 16 summarises the phenotypic changes found following incubation of the SCLC cell lines with either HMBA, dbcAMP or NaBut for 72hrs. Taking DDC, CKBB and BLI as representing the SCLC phenotype, phenotypic inducer treatment caused an alteration in the phenotype of the SCLC cell lines examined. There does not however appear to be any consistent pattern of phenotypic change induced by these drugs taking the SCLC cell lines as a whole this may reflect differences in the position of the SCLC cell lines within the lung cancer lineage.

Table 16: Summary of the Significant Phenotypic Changes.

	Phenotypic Inducer.								
	HMBA			dbcAMP			NaBut		
Marker	DDC	CKBB	BLI	DDC	CKBB	BLI	DDC	CKBB	BLI
Cell Line									
H187	NE	↑	ND	↑	↑	↑	NE	↑	ND
H69	↓	↑	↓	↓	NE	NE	↓	NE	NE
H128d	NE	↓	NE	↑	NE	NE	↓	NE	NE

Table 16 summarises the significant phenotypic changes observed following treatment of some SCLC cell lines with either HMBA, dbcAMP or NaBut.

Key:-   NE - no effect, ND - not done.

However, within a cell line the change in the phenotypic markers in response to the phenotypic inducers does appear to be consistent. For example, the CKBB activity in H187 cells always increases in response to drug treatment, and the DDC activity in H69 cells always decreases following exposure to the phenotypic inducers. The exception to the rule is H128d DDC activity, where both an increase and decrease have been found following exposure to phenotypic drugs.

Table 17: Effect of Cycloheximide on the Phenotypic Drug Induced Changes in the SCLC Phenotype.

Cell Line	Drug(s) Added	DDC Activity µIU/mg protein	µIU/µgDNA	CKBB Activity IU/mg protein	mIU/µgDNA
H187	control	119.8 ± 4.8 ,	1.96 ± 0.08	1.29 ± 0.07 ,	21.0 ± 1.1
	cyclohex. 0.5µg/ml	104.8 ± 6.3 ,	1.59 ± 0.2	1.32 ± 0.03 ,	19.6 ± 2.9
	dbcAMP 1mM	317.1 ± 19.6 ,	5.28 ± 0.5	1.89 ± 0.05 ,	31.5 ± 2.7
	dbcAMP cyclohex.	121.7 ± 17.6 ,	1.85 ± 0.2	1.38 ± 0.10 ,	23.8 ± 1.9
H69	control	2535.9 ± 224 ,	45.7 ± 1.98	2.95 ± 0.37 ,	52.9 ± 2.9
	cyclohex. 0.5µg/ml	2655.6 ± 142 ,	37.2 ± 5.5	3.12 ± 0.22 ,	47.5 ± 6.9
	HMBA 5mM	1105.8 ± 59 ,	17.5 ± 2.8	2.92 ± 0.37 ,	46.0 ± 6.4
	HMBA + cyclohex.	2321.4 ± 243 ,	38.6 ± 1.6	3.49 ± 0.48 ,	42.4 ± 3.1

H187 and H69 cells were treated with 1mM dbcAMP and 5mM HMBA, respectively, in the presence and absence of 0.5µg/ml cycloheximide for 72 hours. The DDC and CKBB activity of the cells was then determined.

The results represent the mean enzyme activity ± standard error from 3 determinations.

Part 4:- The Effect of Cycloheximide on the Phenotypic Drug Induced Changes in the SCLC Phenotype.

In order to investigate whether de novo protein synthesis was required for the changes in the DDC and CKBB activities found in some SCLC cell lines following phenotypic inducer treatment, the SCLC cell lines were exposed to the appropriate phenotypic inducer with or without cycloheximide. Cycloheximide blocks the peptidyl transferase reaction on ribosomes and so inhibits the elongation phase of translation in eukaryotes.

Following phenotypic inducer treatment of the SCLC cell lines it was found that the most significant changes in the SCLC DDC and CKBB activities were noted after 1mM dbcAMP treatment of H187 cells and 5mM HMBA treatment of H69 cells. Therefore it was examined whether inhibition of protein synthesis had any effect on the changes in the enzyme activities seen.

H187 cells were treated with and without 1mM dbcAMP in the presence and absence of 0.5μg/ml cycloheximide (Tab. 17). As noted earlier, 1mM dbcAMP significantly increased the DDC and CKBB activities in H187 cells. In the presence of cycloheximide the dbcAMP induced enzyme changes were inhibited. This therefore suggests that the changes in DDC and CKBB activities in H187 cells following treatment with 1mM dbcAMP require de novo protein synthesis.

H69 cells were treated with and without 5mM HMBA in the presence and absence of 0.5μg/ml cycloheximide (Tab. 17). As before HMBA significantly reduced the DDC activity in H69 cells. This effect was found to be completely inhibited by the presence of cycloheximide. Therefore, HMBA induced alteration in DDC activity in H69 cells appears to require de novo protein synthesis.

CHAPTER 6.
THE EFFECT OF PHENOTYPIC INDUCERS ON THE MORPHOLOGY OF THE SCLC CELL LINES.

Considerable heterogeneity is observed in the gross appearances of SCLC cell lines. In general, most cell lines grow as floating aggregates of tightly to loosely packed cells that may frequently demonstrate areas of central necrosis. There are however a few SCLC cells lines which grow attached to the substrate. Carney et al in 1985 described four distinct SCLC morphological classes, type 1,2,3 and 4. Type 1 cell lines grow as tightly packed spherical aggregates of floating cells. In the larger type 1 aggregates areas of central necrosis are frequently seen. Type 2 cell lines grow as relatively densely packed floating aggregates with an indefinite and irregular outline. These cell lines do not display areas of central necrosis. Type 3 cell lines grow as loosely adherent floating aggregates growing in small clumps and intertwined cords, central necrosis is absent in these cell lines. Type 4 cell lines grow attached to the substrate.

The three cell lines used in this study could be distinguished from each other by their morphological characteristics. H128d cells grew as tight aggregates with a spherical shape where individual cells could not be seen (Photograph 5). In the larger H128d aggregate an area of central necrosis was not uncommon. These cells are classed as type 1 cells. H69 cells, however, grew in suspension as irregular shaped aggregates where the individual cells within an aggregate could not be easily identified (Photograph 13). These cells are classed as type 2 cells. Finally, H187 cells grew as irregular floating aggregates with a morphology that resembled a "bunch of grapes" (Photograph 9). Individual H187 cells could be identified within an aggregate. H187 cells are classed as type 3 cells.

100 μm
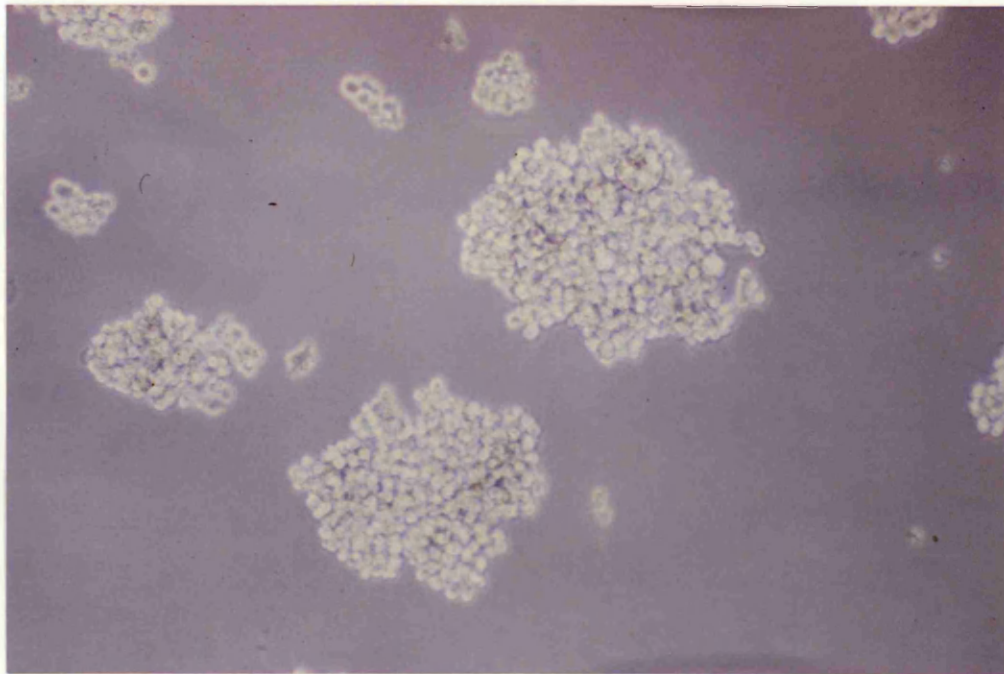
Photograph 9:- Control H187 Cells (10x objective).

H187 cells in late log phase of growth.

100 μm
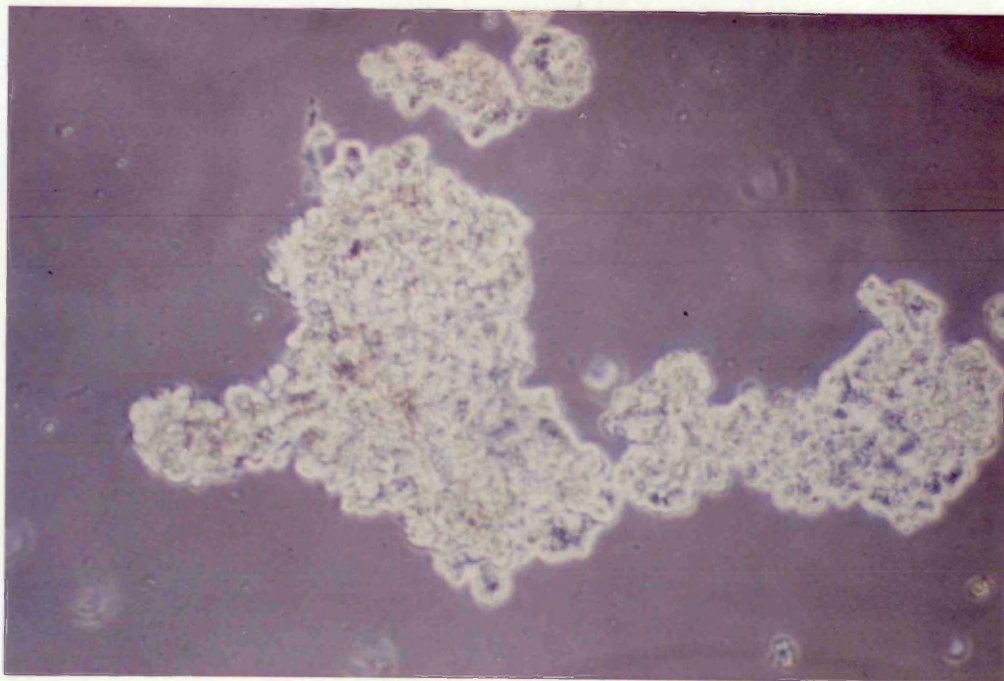
Photograph 10:- H187 Cells After 72Hr Exposure to 5mM HMBA (10x objective).

H187 cells in late log phase were exposed to 5mM HMBA for 72hrs. Cells were photographed on day 3.
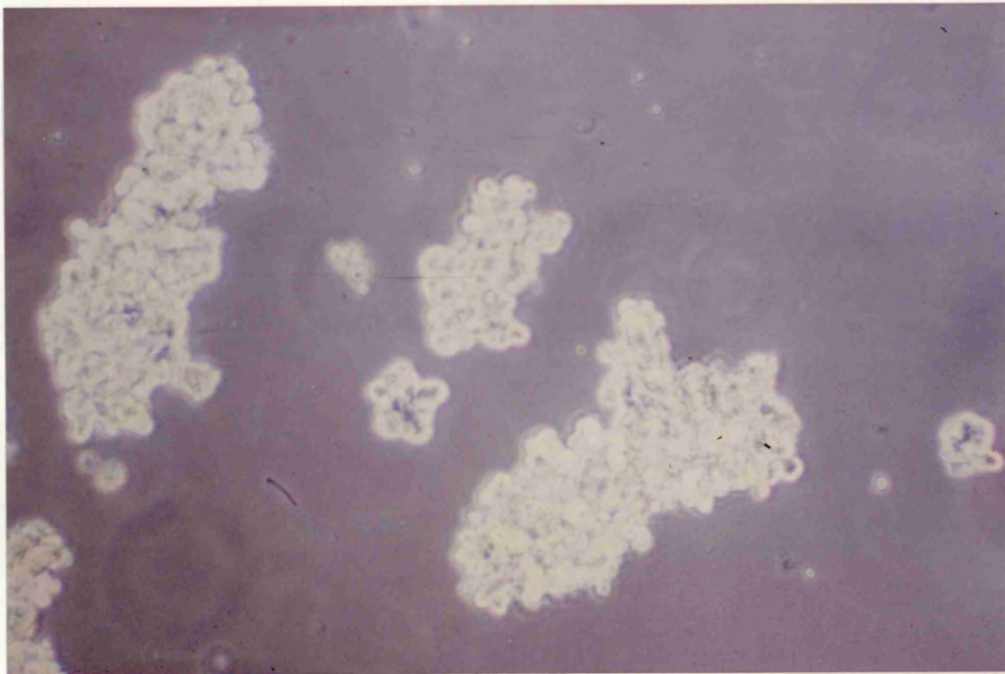
Photograph 11:- H187 Cells After 72hr Exposure to 1mM DbcAMP (10x objective).

H187 cells in late log phase were exposed to 1mM dbcAMP for 72hrs. Cells were photographed on day 3.
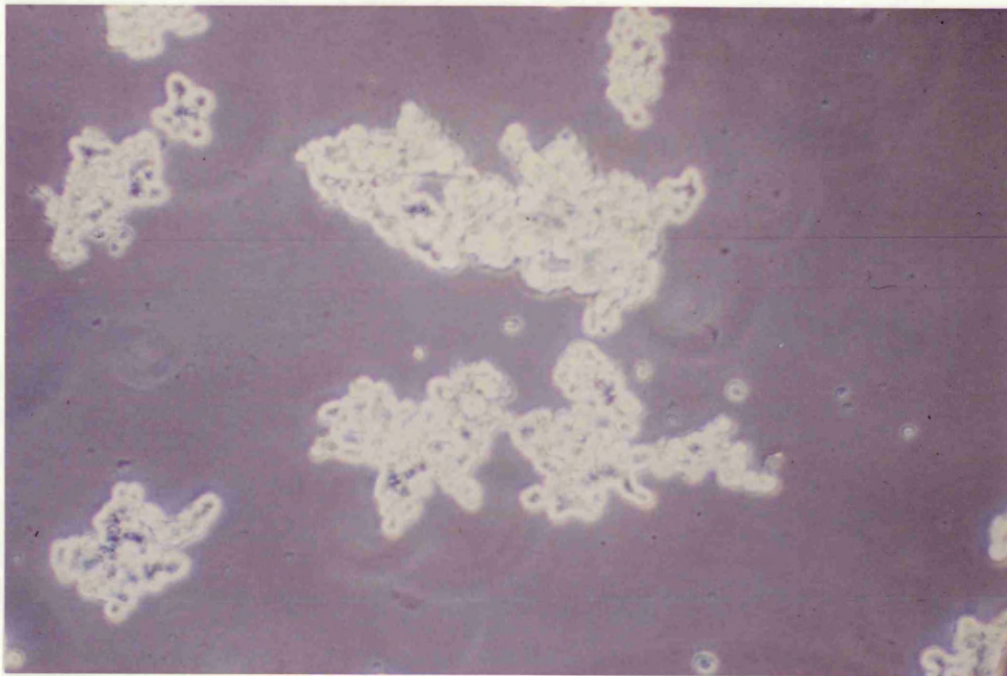
Photograph 12:- H187 Cells After 72hr Exposrue to 1mM NaBut (10x objective).

H187 cells in late log phase were exposed to 1mM NaBut for 72hrs. Cells were photographed on day 3.

Since the three cell lines used in this study had distinctive morphological features it was of interest to investigate whether the cellular morphology changed following phenotypic inducer treatment. The SCLC cell lines were exposed to the appropriate phenotypic inducer for 72hrs after which time the cellular morphology was viewed using a phase contrast microscope, 10x objective.

Following treatment with 5mM HMBA the H187 cellular morphology was found to change (Photograph 10). The cells grew as irregular floating aggregates more densely packed than the untreated controls. In the untreated H187 cells individual cells within an aggregate could be easily identified (Photograph 9); this was not the case in the HMBA treated cells. Moreover, when H187 cells were exposed to either 1mM dbcAMP or 1mM NaBut a similar morphological change was observed; the cells grew as more densely packed irregular shaped floating aggregates. Therefore, when H187 cells were exposed to either HMBA, dbcAMP or NaBut the cellular morphology changed from type 3 to type 2.

When H69 cells were treated with phenotypic inducers for 72hrs morphological changes were also observed. 5mM HMBA treated H69 cells (Photograph 14) adopted a morphology more typical of type 1 SCLC cell lines. The H69 aggregates lost their irregular shape and became more spherical. Incubation with 1mM dbcAMP for 72hrs did not appear to have any effect on the H69 cellular morphology (Photograph 15). The dbcAMP treated H69 cells retained the type 2 SCLC cell line morphology. When H69 cell were treated with 1mM NaBut the aggregates became more spherical in shape compared to the untreated controls (Photograph 16), however this effect was not as marked as following exposure to HMBA. Therefore when H69 cells were exposed to some phenotypic inducers the morphological changes seen implied a shift from a type 2 SCLC cell line towards a type 1 SCLC cell line.
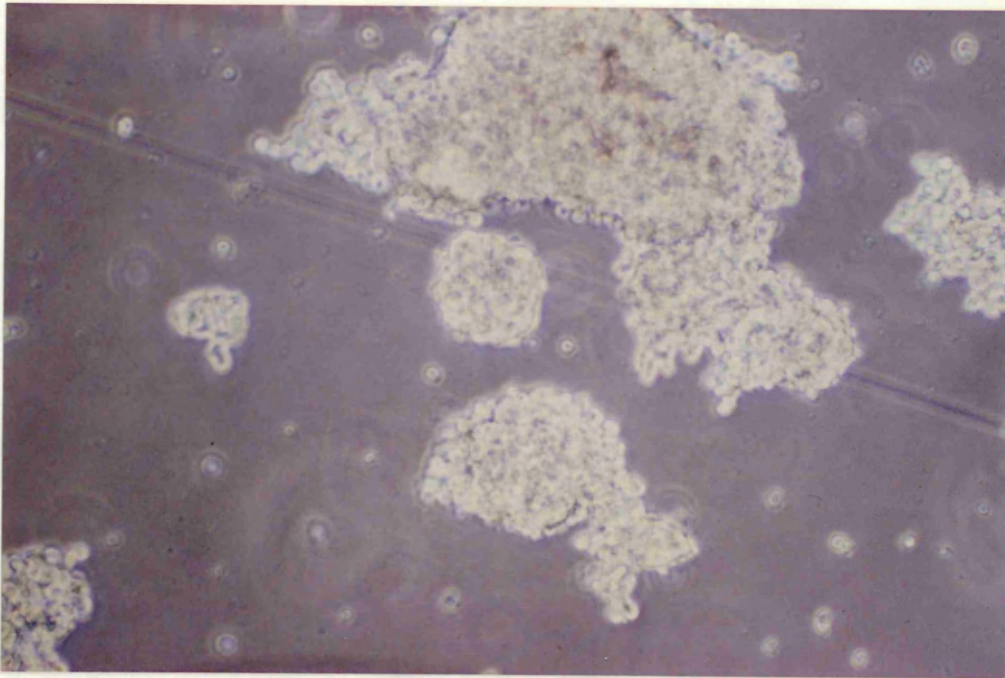
100 µm

Photograph 13:- Control H69 Cells (10x objective).

H69 cells in late log phase of growth.
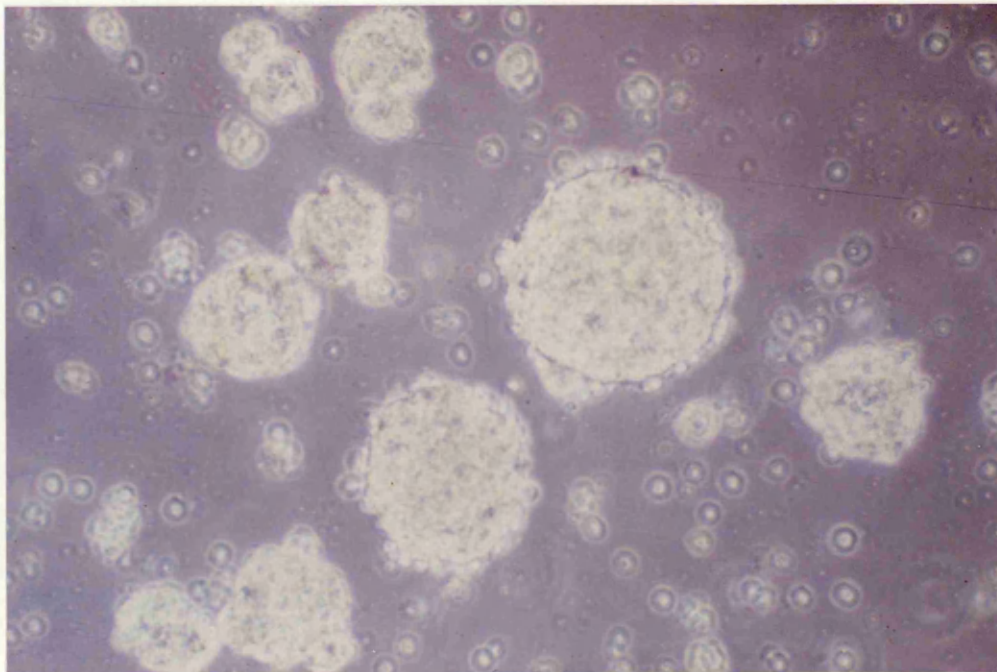
100 µm

Photograph 14:- H69 Cells After 72hr Exposure to 5mM HMBA (10x objective).

H69 cells in late log phase were exposed to HMBA for 72hrs. The cells were photographed on day 3.
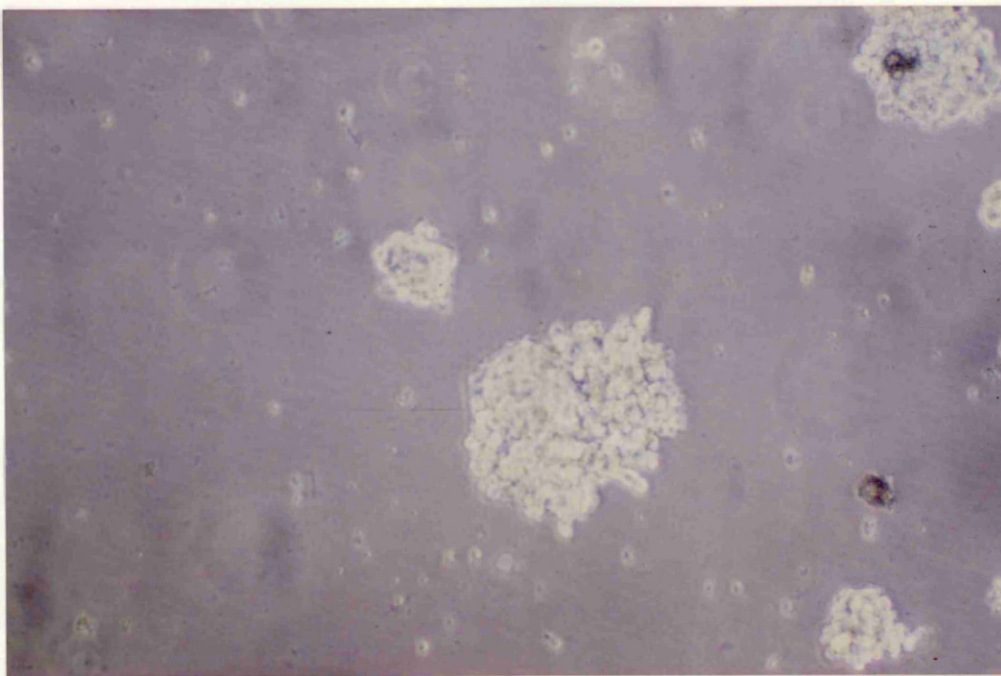
Photograph 15:- H69 Cells After 72hr Exposure to 1mM DbcAMP (10x objective).

H69 cells in late log phase were exposed to 1mM dbcAMP for 72hrs. The cells were photographed on day 3.
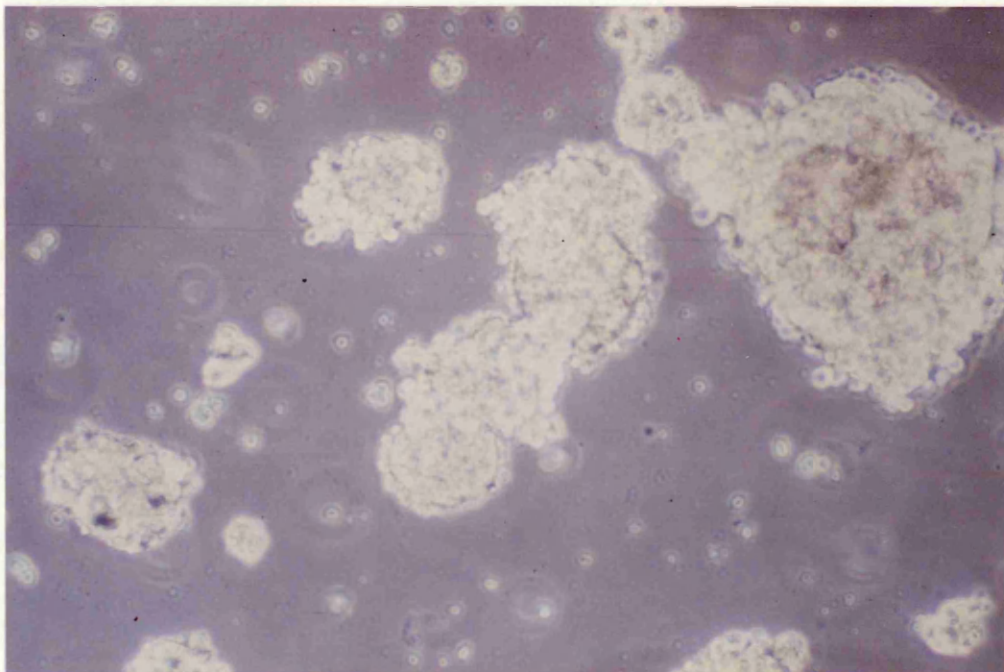
Photograph 16:- H69 Cells After 72hr Exposure to 1mM NaBut (10x objective).

H69 cells in late log phase were exposed to 1mM NaBut for 72hrs. The cells were photographed on day 3.

Finally, H128d cells were exposed to 5mM HMBA, 1mM dbcAMP and 1mM NaBut for 72hrs and the cellular morphology examined. However, in this cell line no morphological changes were noticed following phenotypic inducer treatment, the cells remained growing as tight spherical aggregates typical of type 1 SCLC cell lines.

CHAPTER 7.
THE EFFECT OF PHENOTYPIC CHANGE ON THE CHEMOSENSITIVITY
OF THE SCLC CELL LINES.

The aim of this project was to investigate whether the SCLC phenotype could be altered by phenotypic inducer agents, and if so whether this change in the SCLC phenotype had any effect on chemosensitivity. Having determined that the SCLC phenotype could be altered following treatment with various inducers the effect of this phenotypic change on chemosensitivity was investigated.

SCLC is one of the few solid tumours which shows an initial exquisite sensitivity to radiation and/or chemotherapy. This characteristic separates SCLC from the other three major forms of human lung carcinomas: squamous cell, adeno- and large cell undifferentiated. The eventual goal in the treatment of SCLC is to develop effective systemic therapy that will produce high response rates and a high percentage of long term disease free survivors. Currently, the results that can be obtained with maximally delivered combination chemotherapy (with or without additional radiotherapy) are limited by the available agents. The most widely used drug combinations include traditionally three of the following four drugs: vincristine, adriamycin, cyclophosphamide or etoposide (VP16).

HMBA, dbcAMP and NaBut have been shown to alter the growth rate, DDC CKBB activities, levels of BLI and the cellular morphology of some SCLC cell lines. The effect of these phenotypic changes on the adriamycin, vincristine and VP16 chemosensitivity of the SCLC cell lines was examined to determine whether phenotypic change had any effect on the SCLC chemosensitivity.

The cells were exposed to the appropriate phenotypic inducer for 72hrs. The cells were then washed and resuspended in drug free
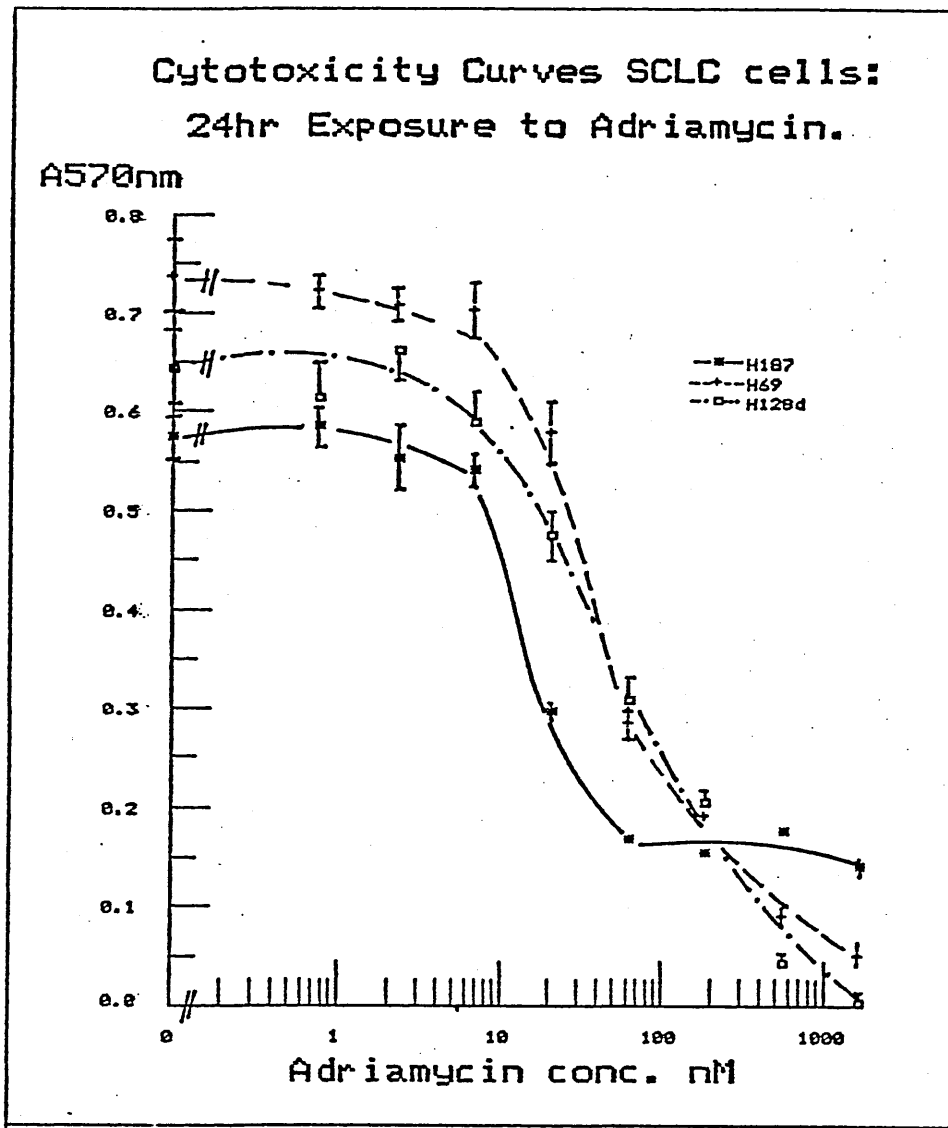
Figure 37:- Cytotoxicity Curves for SCLC Cell Lines Exposed to Adriamycin for 24hrs.

SCLC cell lines were exposed to adriamycin for 24hrs and the cells were allowed to recover for the appropriate period (H187 4 days, H69 and H128d 7 days). An MTT assay was carried out to determine the adriamycin chemosensitivity of the cell lines.

The results represent mean of 8 replicate samples $\pm$ standard error.

Table 18 : Effect of Phenotypic Change on the Adriamycin Chemosensitivity of the SCLC Cell Lines - 24hr exposure.

Cell Line.	Control ID_{50} Adriamycin nM.	Phenotypic Inducer P.I.	ID_{50} Adr nM following pretreatment of P.I.	
			1.0mM	5.0mM
H187	36.0 ± 3.6	HMBA	50.6 ± 2.0	26.6 ± 9.0
H69	45.0 ± 7.3	HMBA	43.2 ± 5.0	49.0 ± 6.6
H128d	70.0 ± 2.5	HMBA	95.0 ± 18.0	72.0 ± 16.1
			0.5mM	1.0mM
H187		dbcAMP	45.0 ± 11.4	53.0 ± 14.0
H69		dbcAMP	43.6 ± 5.0	39.3 ± 2.9
H128d		dbcAMP	101.1 ± 19.6	115.0 ± 25.0
			0.5mM	1.0mM
H187		NaBut	78.0 ± 26.0	29.0 ± 10.0
H69		NaBut	71.2 ± 16.0	44.0 ± 1.15
H128d		NaBut	83.0 ± 2.5	67.0 ± 23.0

The SCLC cell lines were treated with the appropriate phenotypic inducer for 72hr. The cells were then washed 2x with PBS and resuspended in drug free medium. The cells were plated out at 5×10^4 cells/ml and exposed to adriamycin for 24hrs. After the appropriate recovery period, cell survival was measured using the MTT assay.

The results represent the mean ID_{50} ± standard error from 3 replicate experiments.

Statistical analysis was carried out using the Students t-test.
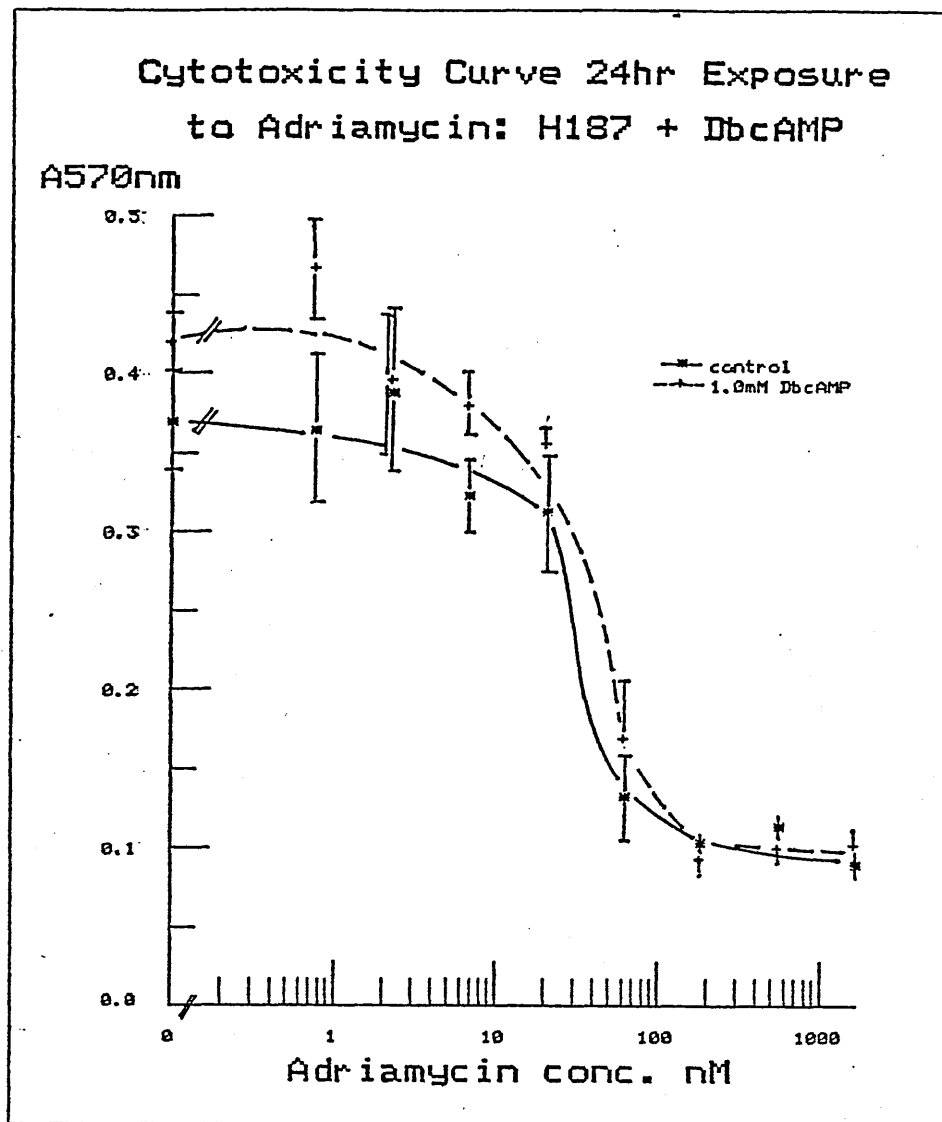
Figure 38:- The Effect of DbcAMP Pretreatment on the Adriamycin Chemosensitivity of H187 cells.

H187 cells were pretreated with 1mM dbcAMP for 72hrs. The cells were then washed 2x with PBS and resuspended in drug free medium. The cells were plated out into 96 well plates at a final cell concentration of $5x10^4$ cells/ml. The cells were exposed to adriamycin for 24hrs and following drug removal, allowed to recover for 4 days. An MTT assay was performed to determine the adriamycin chemosensitivity of dbcAMP treated and untreated H187 cells.

The results represent the mean of 8 replicate samples ± standard error.
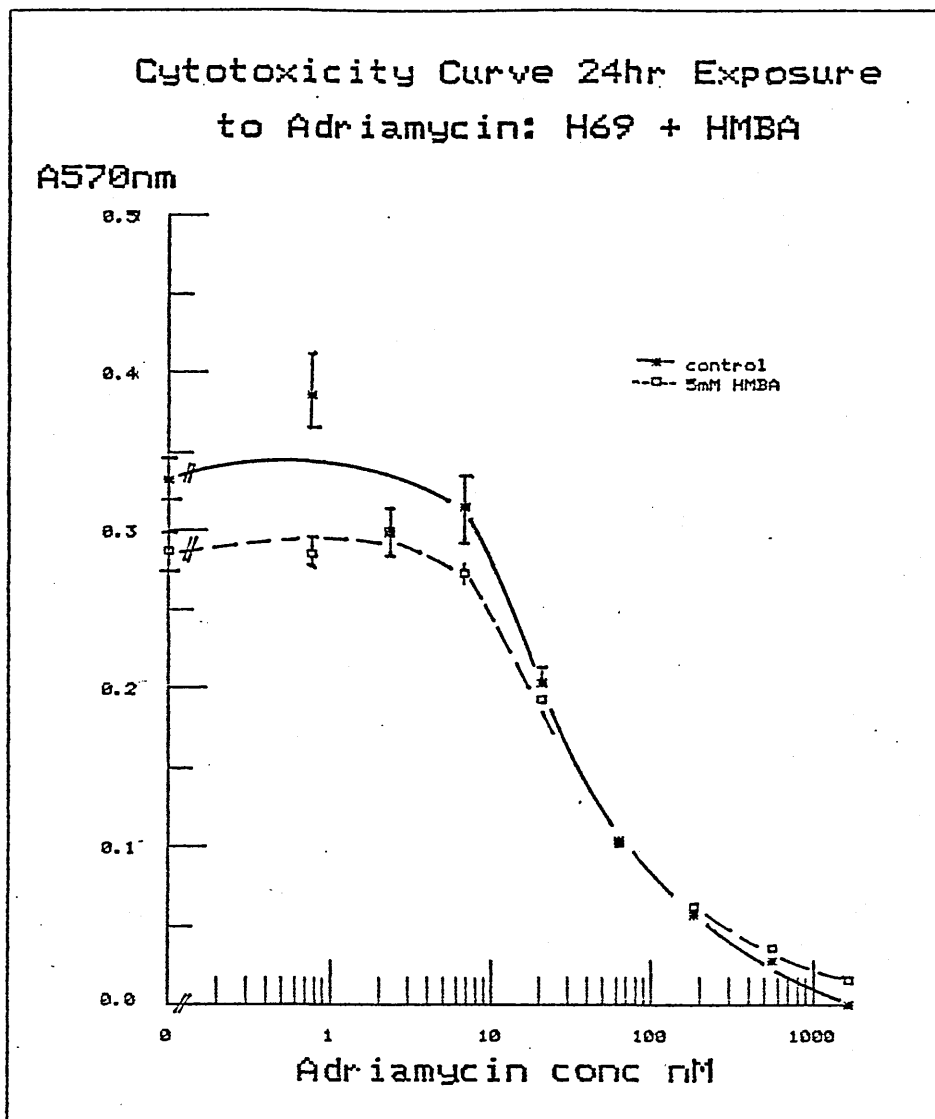
Figure 39:- The Effect of HMBA Pretreatment on the Adriamycin
Chemosensitivity of H69 Cells.

H69 cells were pretreated with or without 5mM HMBA for 72hrs. The
cells were washed 2x with PBS and resuspended in drug free medium.
The cells were then plated out into 96 well plates at a final cell
concentration of $5x10^4$ cells/ml, and exposed to adriamycin for 24hrs.
After a 7 day recovery period an MTT assay was performed to determine
the affect of HMBA pretreatment on the H69 adriamycin
chemosensitivity.

The results represent the mean of 8 replicate samples $\pm$ standard
error.

medium, and plated out into microtitration plates. The cells were then exposed to the appropriate cytotoxic agent (adriamycin, vincristine or VP16) and their chemosensitivity was determined using the MTT assay.

Typical cytotoxicity curves for the three SCLC cell lines following a 24hr exposure to adriamycin as shown in Figure 37. The three curves are similar except at high adriamycin concentrations, where the H187 cytotoxicity curve levels off. This implies that there are still viable H187 cells remaining at high concentrations of adriamycin following a 24 hour exposure. The mean adriamycin ID_{50}'s following a 24hr exposure of the three SCLC cell lines were found to be 36nM, 45nM and 70nM for H187, H69 and H128d respectively (Table 18). Statistical analysis of the SCLC cell lines ID_{50}'s using the Students t-test showed that the H187 and H69 ID_{50}'s were not significantly different. However, comparison of the H187 ID_{50} with the H128d ID_{50} s showed that the chemosensitivity of these two lines were significantly different, $p < 0.001$. Moreover, the H69 adriamycin ID_{50} was found to be significantly different from the H128d ID_{50}, $p < 0.01$.

When the SCLC cell lines were pretreated for 72hrs with the appropriate phenotypic inducer the adriamycin ID_{50} was not significantly different from the untreated controls (Tab.18). More specifically, dbcAMP pretreatment of H187, previously shown to cause the most significant change in the H187 cellular phenotype, did not affect the chemosensitivity of H187 to adriamycin. It is possible however that phenotypic inducer treatment may have caused a shift in the chemosensitivity of a selective H187 cell population without an associated shift in the adriamycin ID_{50}, perhaps with the emergence of an resistant fraction. This however is unlikely since the cytotoxicity curve obtained for dbcAMP treated H187 cells mimicked that of the untreated control cells (Fig 38). If a resistant fraction was present following phenotypic inducer treatment this may have been
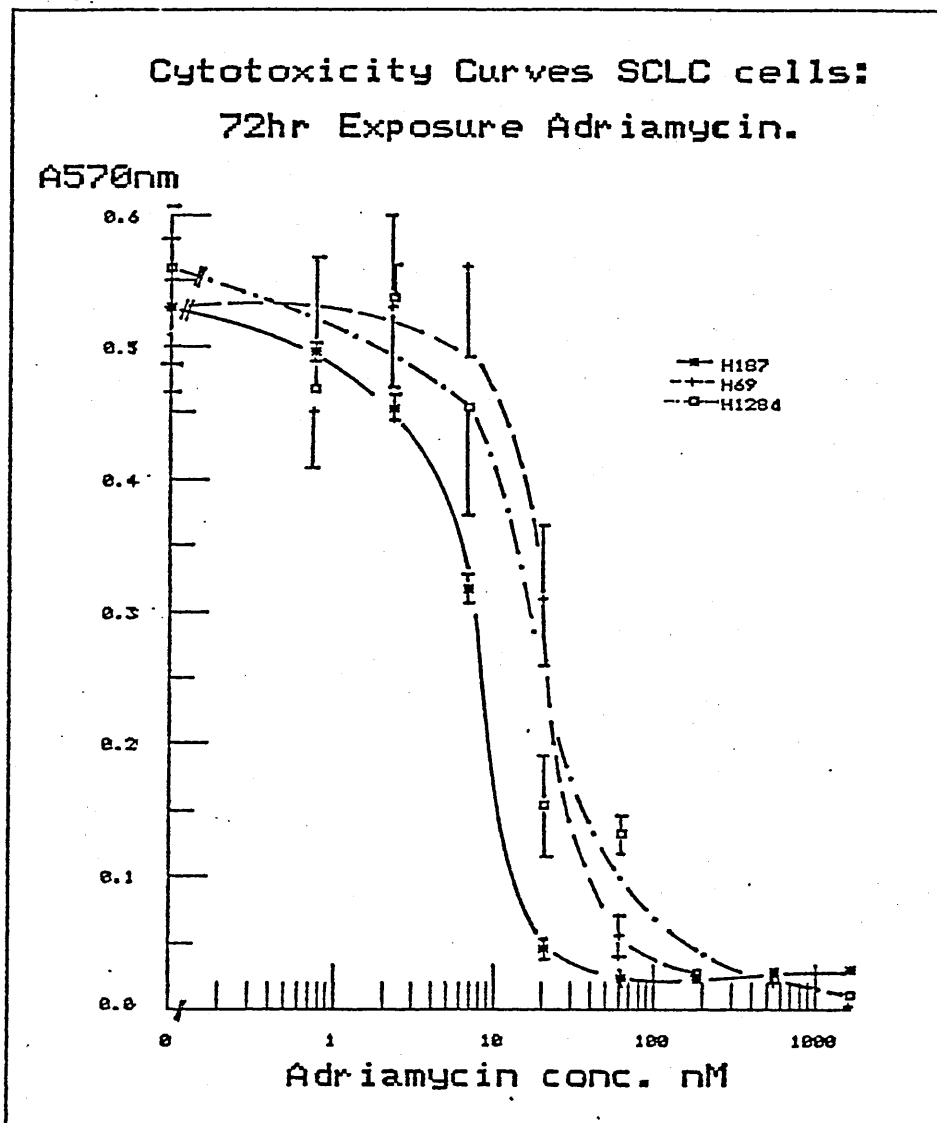
Figure 40:- Cytotoxicity Curves for SCLC Cell Lines Exposed to Adriamycin for 72hrs.

SCLC cell lines were exposed to adriamycin for 72hrs and then allowed to recover. An MTT assay was carried out to determine the adriamycin chemosensitivity of the cell lines.

The results represent mean of 8 replicate samples ± standard error.

Table 19 : **Effect of Phenotypic Change on the Adriamycin Chemosensitivity of the SCLC Cell Lines - 72hr exposure.**

Cell Line	Control ID_{50} Adriamycin nM.	Phenotypic Inducer P.I.	ID_{50} Adr nM following pretreatment of P.I.
H187	9.06 ± 0.29	dbcAMP 1.0mM	9.5 ± 2.5
H69	22.6 ± 1.73	dbcAMP 1.0mM	26.0 ± 4.6
		NaBut 1.0mM	28.3 ± 5.2
		HMBA 5.0mM	24.0 ± 6.4
H128d	25.3 ± 8.2	dbcAMP 1.0mM	29.6 ± 15.1
		NaBut 1.0mM	39.3 ± 16.0

SCLC cell lines were incubated with the appropriate phenotypic inducer for 72hrs. The cells were then washed 2x with PBS and resuspended in drug free medium and plated into 96 well plates at a final cell concentration of $5x10^4$ cells/ml. The cells were exposed to adriamycin for 72hrs. After the appropriate recovery period an MTT assay was performed to determine cell survival.

The results represent the mean ID_{50} ± standard error from 3 replicate experiments.

Statistical analysis was performed using Students t-test.

Table 20 : The Effect of Phenotypic Change on the Vincristine Chemosensitivity of the SCLC Cell Lines.

Cell Line	Control ID_{50} Vincristine nM	Phenotypic Inducer P.I.	ID_{50} Vinc nM following pretreatment with P.I.
72hr Exposure:-			
H187	0.56 ± 0.14	dbcAMP 1.0mM	0.27 ± 0.08
24hr Exposure:-			
H69	1.17 ± 0.19	NaBut 1.0mM	2.03 ± 0.3
		HMBA 5.0mM	0.89 ± 0.11

SCLC cell lines were pretreated for 72hrs with the appropriate phenotypic inducer. The cells were then harvested, washed 2x with PBS and resuspended in drug free medium. The cells were plated out into 96 well plates at a final cell concentration of 5×10^4 cells/ml and exposed to vincristine for either 24hrs (H69) or 72hrs (H187). After the appropriate recovery period the vincristine chemosensitivity was determined using the MTT assay.

The results represent the mean $ID_{50} \pm$ standard error of 3 replicate experiments.

Statistical analysis was carried out using the Students t-test.
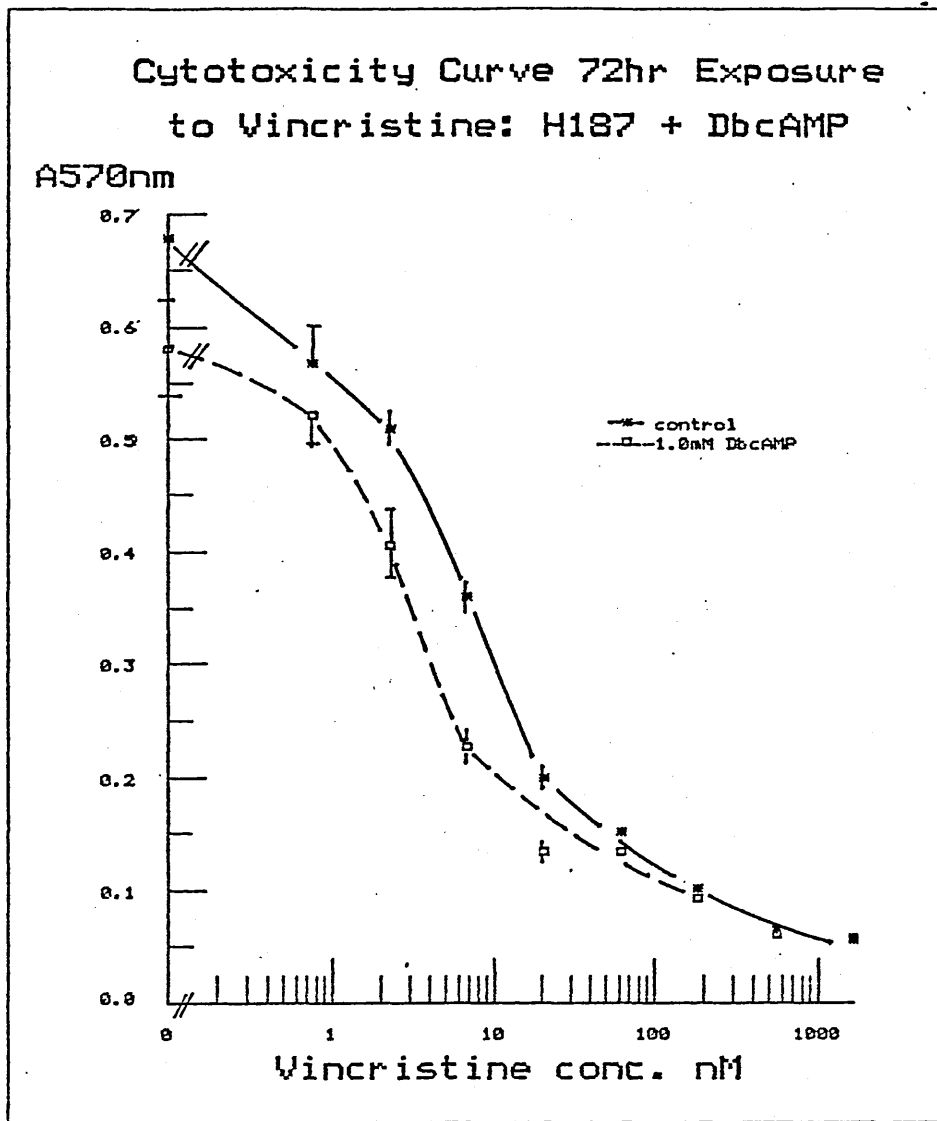
**Figure 41:- The Effect of DbcAMP Pretreatment on the H187 Vincristine
Chemosensitivity.**

H187 cells were pretreated with dbcAMP 1mM for 72hrs. The cells were
harvested, washed 2x with PBS and resuspended in drug free medium.
The cells were plated out into 96 well plates at a final cell
concentration of $5x10^4$ cells/ml. The cells were exposed to
vincristine for 72hrs and after a 4 day recovery period an MTT assay
was carried out to examine the effect of dbcAMP pretreatment on the
vincristine chemosensitivity of H187 cells.

The results represent mean of 8 replicate samples ± standard error.
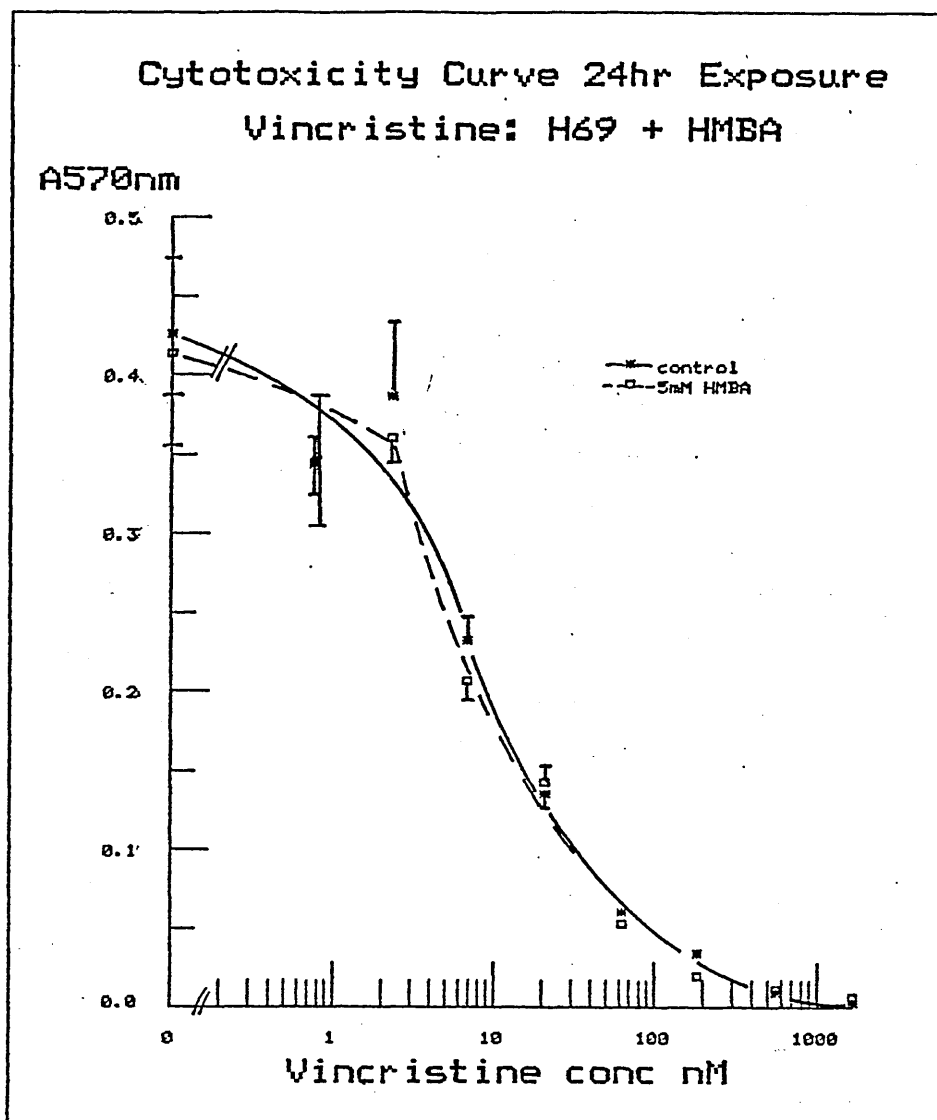
**Figure 42:- The Effect of HMBA Pretreatment on the H69 Vincristine Chemosensitivity.**

H69 cells were exposed to 5mM HMBA for 72hrs. The cells were then washed, and resuspended in drug free medium. HMBA treated and untreated H69 cells were seeded into 96 well plates at a final cell concentration of 5×10^4 cells/ml. The cells were exposed to vincristine for 24hrs. After drug removal the cells were allowed to recover for 7 days. An MTT assay was carried out to determine the vincristine chemosensitivity of the HMBA treated and untreated H69 cells.

The results represent mean of 8 replicate samples ± standard error.

apparent by a tailing off of the cytotoxicity curve at high adriamycin concentrations compared to the untreated controls. Therefore dbcAMP induced phenotypic change in H187 has no effect on the chemosensitivity of H187 to adriamycin. Furthermore, pretreatment of H187 cells with either HMBA or NaBut had no effect on the H187 adriamycin chemosensitivity. These findings were further supported by examination of the effect of phenotypic change on the chemosensitivity in H69 and H128d cell lines. In spite of the fact that HMBA caused a significant change in the phenotype of H69 cells, pretreatment of H69 with either 1.0mM or 5.0mM HMBA failed to alter H69 adriamycin chemosensitivity (Table 18). Moreover the cytotoxicity curves of H69 with or without HMBA pretreatment did not differ (Fig 39). DbcAMP or NaBut pretreatment of H69 cells had no effect on the cells' chemosensitivity to adriamycin (Table 18). The chemosensitivity of H128d to adriamycin was not significantly altered by pretreatment with the phenotypic inducers (Table 18).

It may be however that phenotypic inducer treatment does alter the SCLC chemosensitivity but a 24hr exposure to adriamycin is insufficient to allow the effect to be seen. Therefore the effect of phenotypic change on the chemosensitivity of the SCLC cell lines was examined following a 72hr exposure to adriamycin. Figure 40 shows the typical cytotoxicity curves obtained for the three SCLC cell lines following a 72hr exposure to adriamycin. The control cytotoxicity curves were found to be similar even at high adriamycin concentrations. It was found that H187, H69 and H128d had a mean adriamycin ID_{50} of 9nM, 22.6nM and 25.3nM respectively (Tab.19). Statistical analysis of the SCLC cell lines ID_{50}'s showed that the H187 ID_{50} was significantly different to both the H69 ID_{50} ($p<0.002$) and the H128d ID_{50} ($P<0.05$). However the H69 ID_{50} was not significantly different from the H128d ID_{50} unlike that found following a 24hr exposure to adriamycin.

Table 21 : The Effect of Phenotypic Change on the VP16
Chemosensitivity of the SCLC Cell Lines - 24hr Exposure.

Cell Line	Control ID_{50} VP16 nM	Phenotypic Inducer P.I.	ID_{50} VP16 nM following pretreatment with P.I.
H187	102.0 ± 9.0	dbcAMP 1.0mM	166.0 ± 12.0 *
H69	860.0 ± 140	HMBA 5.0mM	925.0 ± 130

SCLC cell lines were pretreated with the appropriate phenotypic inducer for 72hrs. The cells were then harvested, washed 2x with PBS, resuspended in drug free medium and plated out into 96 well plates at a final cell concentration of 5×10^4 cells/ml. The cells were exposed to VP16 for 24hrs. The VP16 chemosensitivity was determined following the appropriate recovery period using the MTT assay.

The results represent the mean $ID_{50} \pm$ standard error from 3 replicate experiments.

Statistical analysis was performed using the Students t-test.

* $P < 0.02$

The cells were pretreated with the phenotypic inducers seen previously to cause the greatest shift in the cellular phenotype and then exposed to adriamycin for 72hrs. It was found however that even after a 72hr exposure to adriamycin no change in the cells' chemosensitivity to adriamycin was seen (Table 19).

Although phenotypic change did not appear to affect the adriamycin chemosensitivity of the cell lines it may alter the response of the SCLC cell lines to other cytotoxic drugs. Therefore, the effect of phenotypic inducer treatment on the vincristine and VP16 chemosensitivity of the SCLC cell lines was examined.

As most pronounced changes in cellular phenotype were found following treatment of H187 with dbcAMP and treatment of H69 with either HMBA or NaBut, these combinations were selected to investigate the effect of phenotypic change on the vincristine chemosensitivity.

In order to determine the effect of phenotypic change on the vincristine chemosensitivity of H187 cells a 72hr exposure time was required. When dbcAMP treated H187 cells were exposed to vincristine for 24hrs the cytotoxicity curve was very shallow and the ID_{50} could not be accurately derived. Since vincristine is a cell cycle specific drug, acting at the M phase of the cell cycle, an alteration in the cycle time or position of the cells within the cell cycle could affect the cytotoxicity exerted. DbcAMP has been shown to significantly affect the growth of H187 cells and this may affect the vincristine cytotoxicity found following a 24hr exposure. When a 72hr exposure to vincristine was adopted a cytotoxicity curve for the dbcAMP treated and untreated H187 cells was obtained where the ID_{50} could be determined (Fig 41).

Pretreatment of H187 with 1.0mM dbcAMP did not have any effect on the cells' vincristine chemosensitivity. Table 20 shows that the
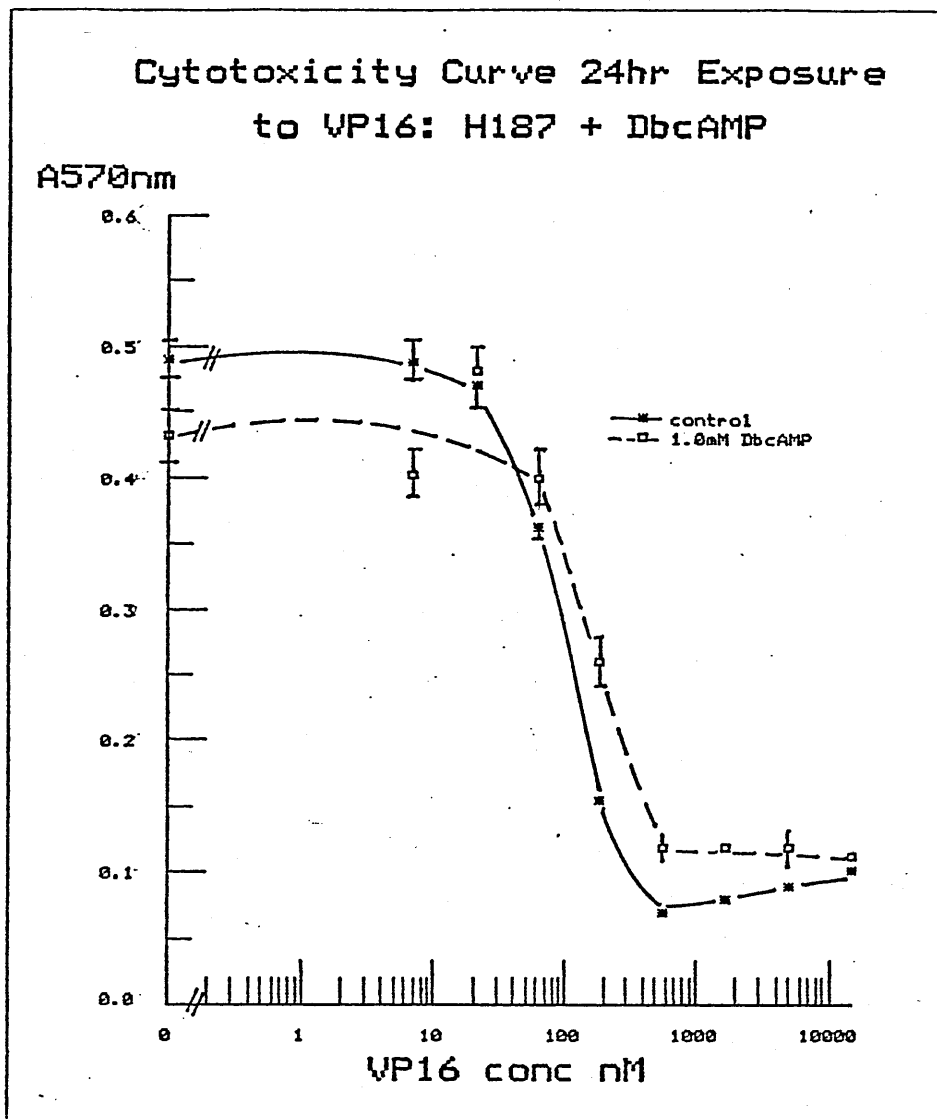
Figure 43:- Effect of DbcAMP Pretreatment on the VP16 Chemosensitivity of H187 Cells.

H187 were exposed to 1mM dbcAMP for 72hrs. The cells were washed and resuspended in drug free medium. DbcAMP treated and untreated H187 cells were seeded into 96 well plates at a final cell concentration of 5×10^4 cells/ml. Following a 24hr exposure to VP16 and a recovery period of 4 days, the cells VP16 chemosensitivity was determined using the MTT assay.

The results represent mean of 8 replicate samples ± standard error.
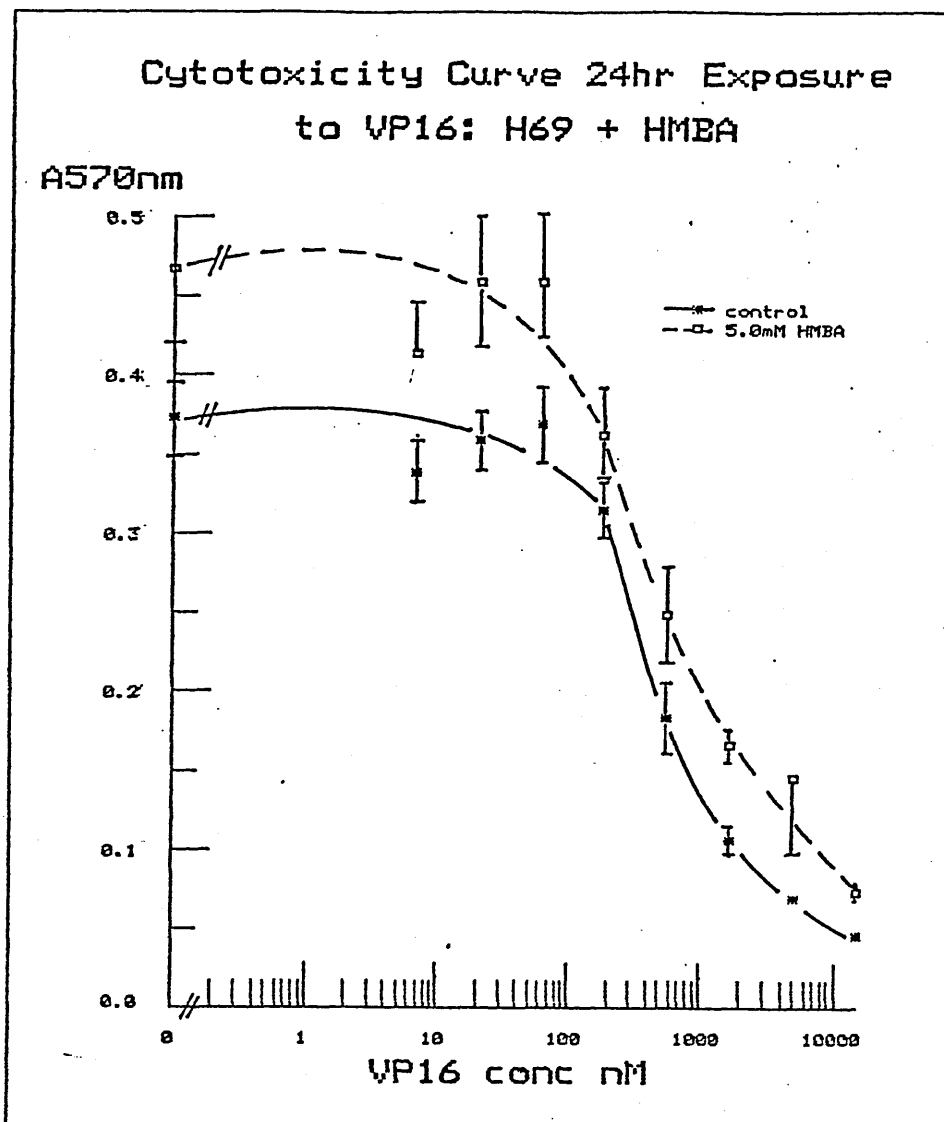
Figure 44:- Effect of HMBA Pretreatment on the VP16 Chemosensitivity of H69 Cells.

H69 cells were pretreated with 5mM HMBA for 72hrs. The cells were then washed and resuspended in drug free medium. HMBA treated and untreated cells were seeded into 96 well plates at a final cell concentration of 5×10^4 cells/ml, exposed to VP16 for 24hrs and allowed to recover for 7 days. An MTT assay was used to determine the VP16 chemosensitivity of the H69 cells.

The results represent mean of 8 replicate samples ± standard error.

vincristine ID_{50}'s of dbcAMP treated and untreated H187 cells did not differ significantly. Furthermore, the cytotoxicity curve obtained for dbcAMP treated H187 cells followed the same pattern as the cytotoxicity curve of the untreated controls (Fig 41). When the effect of pretreatment of H69 cells with either 5.0mM HMBA or 1.0mM NaBut on the vincristine chemosensitivity was examined it was found that there was no effect. Table 20 shows that the vincristine ID_{50} of the treated and untreated H69 cells did not differ significantly. Moreover, the cytotoxicity curves obtained for the HMBA treated and untreated H69 cells were superimposable (Fig 42).

Finally the effect of phenotypic change on the VP16 chemosensitivity of the SCLC cell lines was examined. Again dbcAMP treated H187 cells and HMBA treated H69 cells were selected as examples of phenotypically changed cells. Treatment of H187 with 1.0mM dbcAMP significantly reduced the VP16 chemosensitivity of these cells. Table 21 shows that following pretreatment of H187 with 1.0mM dbcAMP the VP16 ID_{50} is significantly increased. This is illustrated in Figure 43 which shows the cytotoxicity curves obtained for dbcAMP pretreated and untreated H187 cells following a 24hr exposure to VP16. However, pretreatment of H69 with 5.0mM HMBA did not significantly affect the cells VP16 chemosensitivity (Tab.21). The cytotoxicity curves obtained for HMBA pretreated and untreated H69 cells were found to be similar (Fig 44).

CHAPTER 8.

DISCUSSION.

The main objective of this study was to determine whether the SCLC phenotype could be altered, and if so, whether this alteration had any effect on chemosensitivity. The cells were exposed to various phenotypic inducers and markers of the SCLC phenotype were then measured. Changes in the levels of the phenotypic markers were taken as representing a change in the SCLC phenotype. Having established that the SCLC phenotype could be altered by certain drugs, the effect of this phenotypic change on the chemosensitivity was investigated.

A variety of phenotypic inducer agents were examined to determine whether they could alter the SCLC phenotype, these included HMBA, dbcAMP, NaBut, dexamethasone.   DDC activity, CKBB activity and bombesin-like immunoreactivity were selected as markers of the SCLC phenotype. In addition, the cellular morphology and the growth characteristics of the cell lines were also examined following phenotypic inducer treatment.

Following phenotypic inducer treatment an alteration in the SCLC phenotype was found. As a group this phenotypic change did not appear to follow a consistent pattern since in some cell lines a particular drug caused an increase in the level of a specific SCLC marker whereas in others a decrease of the same marker was found. The SCLC cell lines selected in this study were described as classic SCLC cell lines (Carney et al 1985). They nevertheless exhibited considerable diversity with respect to biochemical markers, morphology and growth characteristics. In particular differences in DDC activity were found over a 40-fold range. Similarly, the cellular bombesin-like immunoreactivity of the cell lines differed over a 50-fold range. As mentioned previously the morphology of the cell lines also differed quite considerably, being classed as type 1, 2 or 3 by

Carney's classification (Carney et al 1985). The CKBB activity of the SCLC cell lines however was found to be more consistent. Perhaps it is not surprising that a consistent pattern of SCLC phenotypic change following drug treatment was not found when one considers the differing nature of the cell lines. Within each cell line however, a distinctive pattern of phenotypic change did emerge. A similar change was found in the phenotypic markers of the cell lines, with respect to the direction (that is an increase or decrease) as opposed to the magnitude of change, irrespective of the inducer used. For example, the DDC activity in H69 cells was found to decrease following treatment with either HMBA, dbcAMP or NaBut; an increase in DDC activity was never seen. Also, an increase in the H187 cellular CKBB activity was always found following phenotypic drug treatment; a decrease was never seen. Since the cell lines were found to respond quite differently to the various inducer agents the effect of the phenotypic drug treatment on the individual cell lines will be discussed.

Alteration of the SCLC Phenotype.

(i) NCI-H187

The H187 cell line expressed the lowest DDC activity and BLI of the three lines studied. However, the CKBB activity of H187 was found to be similar to that found in the other two lines. H187 cells grew as irregular floating aggregates with a morphology that resembled a "bunch of grapes". When H187 cells were exposed to HMBA for 72 hours a number of phenotypic changes were noted. Firstly, a growth suppressive effect was found following incubation with 5mM HMBA. Following drug removal the cells reentered the exponential phase of growth with a doubling time similar to the untreated controls. Treatment with 5mM HMBA resulted in a significant increase in the CKBB activity of H187, however no effect was seen on the DDC activity.

The levels of DDC and CKBB do not appear to be concomitantly controlled. A morphological alteration was also found in H187 cells following HMBA exposure. The drug treated cells resembled type 2 cell morphology as opposed to the control cells which are classified as type 3. It is possible that the changes in the H187 cell phenotype are due to an indirect effect of the growth suppression found following exposure to HMBA. However, the cytostatic effect of HMBA on the growth of H187 cells was minimal and, as will be discussed later, there does not appear to be a direct correlation between the degree of cytostasis and the changes found in marker expression. Moreover, since the DDC activity in the H187 cells was not altered by HMBA treatment, it would appear that the control of this phenotypic marker is not directly related to the cytostatic effect induced by HMBA. In the other two SCLC cell lines the DDC activity was found to be generally repressed following treatment with the phenotypic inducers. In H187 the DDC activity is already low and it may be that the DDC cannot be further repressed.

The most active phenotypic inducer in H187 cells was found to be dbcAMP. DbcAMP exerted a growth suppressive effect in a dose dependent manner which was not reversed when the drug was removed. DbcAMP also significantly increased DDC and CKBB activities in a dose dependent manner. Furthermore, detectable levels of BLI were found in H187 cells following exposure to dbcAMP. The H187 cellular morphology was also altered. H187 cells treated with dbcAMP resembled type 2 cell morphology similar to that found following exposure to HMBA. Therefore, following exposure to dbcAMP the H187 phenotype changed in a number of repects; DDC activity increased, CKBB activity increased, BLI increased, the cellular morphology changed from type 3 to type 2 and the growth rate decreased. As discussed above, these changes in the H187 phenotype may be the result of an indirect effect of the cytostasis induced by dbcAMP. DbcAMP exerts quite a distinctive growth suppressive effect, during and following drug exposure. Since

the DDC and CKBB activities were found not to fluctuate during the growth cycle (Fig. 27) the changes in the levels of both enzymes cannot be explained by differences in the position of the cells in the growth cycle at the time the enzymes were assayed. Moreover, the degree of cytostasis exerted by dbcAMP was greater than that found following HMBA treatment but the change in CKBB activity was found to be similar.

One problem with using dbcAMP is that butyric acid is formed by the hydrolysis of dbcAMP and so the observed effects may in fact be due to the presence of butyrate rather than cAMP. When the effect of NaBut on the H187 phenotype was examined it was found than NaBut could also alter the H187 phenotype. NaBut had a cytostatic effect on the growth of H187 cells. Interestingly, H187 cells treated with 1.0mM NaBut had a similar doubling time to 0.5mM dbcAMP treated cells, so it is possible that the cytostatic effect of dbcAMP was due to the presence of butyrate. However not all the dbcAMP effects on H187 cells could be attributed to the presence of butyrate. NaBut up to 1.0mM had no effect on the DDC activity. DbcAMP at 0.5mM, however, significantly increased the DDC activity in H187 cells (Table 6). However, NaBut 1.0mM was found to significantly increase CKBB activity to a similar level found following dbcAMP exposure. NaBut treatment was found to alter the H187 cellular morphology to that found with the other inducer agents. Hence, NaBut also appears to be able to change the phenotype of H187 cells. While butyrate may also contribute to the dbcAMP induced phenotypic change of H187 cells, this does not exclude activity due to cAMP itself. With hindsight, it may have been useful to examine the effect of NaBut on the BLI in these cells to see if the change induced by dbcAMP could be attributed to the presence of butyrate.

Dexamethasone was found to induce a cytostatic effect in H187 cells at high cell density. However, dexamethasone was found to have no effect

on either the DDC or CKBB activities in H187 cells. Therefore, cytostasis does not appear to be a prerequisite or sufficient for the changes in enzyme activity found following phenotypic inducer treatment.

(ii) NCI-H69

H69 cells expressed an intermediate level of DDC activity and BLI, and a similar CKBB activity to the other two lines studied. H69 cells grew in suspension as irregular shaped aggregates where the individual cells within an aggregate could not be easily identified. The phenotypic inducer which had the greatest effect on H69 cells was found to be HMBA. HMBA induced a cytostatic effect in H69 cells during drug exposure, but following drug removal the cells entered an exponential growth phase similar to the untreated controls. HMBA was also found to significantly reduce the DDC activity in H69 cells in a dose dependent manner. This effect was noted at noncytostatic drug concentrations. HMBA was also found to increase the CKBB levels in H69 cells only at noncytostatic concentrations. HMBA significantly reduced the level of BLI in H69 cells. Furthermore, HMBA was found to have a marked effect on the H69 cellular morphology, a shift from type 2 towards type 1 cells was noted. Therefore, the H69 cellular phenotype represented by DDC activity, CKBB activity, BLI, morphology and growth rate, can undergo an alteration in response to HMBA treatment. The pattern of HMBA induced phenotypic alteration in H69 cells was quite different from that observed following exposure of H187 cells.

Although dbcAMP was found to be an effective phenotypic inducer in H187 cells, when H69 cells were treated with dbcAMP a different pattern of phenotypic change was found. In the presence of dbcAMP H69 cells exhibited a growth delay and on removal of the drug the cells entered an exponential phase but did not resume the control growth

rate even after 7 days in drug free medium. DbcAMP significantly reduced the DDC activity in H69 cells in a dose dependent manner, but had no effect on the CKBB activity, BLI and morphological characteristics. Therefore, although dbcAMP was found to be a useful phenotypic inducer agent in H187 cells its action in H69 cells was minimal. Moreover, although a significant growth suppression was found following dbcAMP exposure corresponding changes in the other phenotypic markers were not found, implying again that cytostasis is not causative in phenotypic change.

As discussed previously, the butyrate component of dbcAMP is itself a potential phenotypic inducer. When H69 cells were exposed to NaBut the cells exhibited a pattern of cytostasis similar to that found following dbcAMP treatment. On a mole to mole basis NaBut exerted a greater cytostatic effect than dbcAMP. NaBut treatment was found to significantly reduce the DDC activity in H69 cells. This effect was even greater than that found for dbcAMP treatment. H69 cells treated with NaBut had similar CKBB and BLI as the untreated controls. NaBut treatment did, however, change the H69 cell morphology, a shift towards type 1 cells was found, although this effect was not as marked as that seen following HMBA treatment. Therefore, on the basis of these results, it is possible that the effects seen following dbcAMP could be accounted for by the presence of butyrate. However, NaBut appeared to have a more potent effect than dbcAMP on a mole to mole basis and had an additional effect on the H69 cell morphology.

The effect of dexamethasone   on the phenotype of H69 cells was examined. Dexamethasone had no effect on the growth, DDC activity or CKBB activity in H69 cells.

The DDC and CKBB activties in H69 cells do not appear to be coordinately controlled. There is no direct correlation between

cytostasis and the other phenotypic changes seen. In summary therefore, the phenotype of H69 cells can be altered following treatment with selected agents.

(iii) <u>NCI-H128d</u>

Finally, the effect of phenotypic inducer treatment on the phenotype of H128d cells was examined. H128d cells expressed the highest DDC activity and the highest BLI of the three cell lines examined. The CKBB activity of H128d cells was similar to the other two cell lines. H128d cells grew as tight aggregates with a spherical shape where individual cells could not be distinguished. In the larger H128d aggregates an area of central necrosis was not uncommon. H128d cells are classed as type 1 cells.

When H128d cells were exposed to HMBA the CKBB activity was significantly reduced. Although HMBA was shown to have multiple phenotypic effects in the other two cell lines this was the only effect seen in H128d. When the effect of dbcAMP on the H128d phenotype was examined a significant increase in the DDC activity was found. Interestingly, NaBut significantly reduced the DDC activity in H128d cells. The changes seen do not follow any particular pattern and in fact this is the only instance where the expression of a phenotypic marker, DDC, can be either increased or decreased depending on the inducer treatment. The general pattern up until now has been that if the expression of a phenotypic marker changes in response to a particular inducer then that change appears to be in one direction. In conclusion therefore, H128d cells do not appear to be significantly altered by the phenotypic inducers, since changes in only one marker have been found following inducer treatment.

As mentioned earlier, the pattern of phenotypic change within the SCLC cell lines following inducer treatment was not consistent. This is

particularly evident when one considers the biochemical markers. However, when one considers morphological changes of the SCLC cell lines a consistent pattern of change was found. The SCLC cells underwent a morphological change which, if one views the morphological classes of SCLC as ranging from type 4 through to type 1, was always towards type 1. Therefore the morphological change of the SCLC cell lines does not correlate with the biochemical change.

Do These Alterations in the Various SCLC Phenotypic Markers Represent an Actual Shift in the SCLC Phenotype ?

A change in more than one marker in response to drug treatment implies a phenotypic shift of the cells. As discussed previously, a number of changes were found in the levels of the selected phenotypic markers in the SCLC cell lines following treatment with a variety of phenotypic inducer agents. However, there were some instances when only one or two changes in phenotypic marker expression were noted. For example H69 cells exposed to dbcAMP had significantly lower levels of DDC but alterations in the levels of the other phenotypic markers were not seen. It would be difficult to claim, therefore, that this represents a shift in the H69 cell phenotype. However, following treatment of H69 cells with HMBA and H187 with dbcAMP, a change in the expression of all the selected phenotypic markers was found. Therefore it would seem that treatment of these cell lines with these drugs does represent a shift in the phenotype of the cells.

Cycloheximide, a protein synthesis inhibitor, was able to inhibit the changes in DDC and CKBB expression in H69 and H187 cells following treatment with HMBA and dbcAMP, respectively. This implies that de novo protein synthesis is required for the observed changes in phenotypic marker expression in response to drug treatment. The increased expression of DDC and CKBB in H187 cells following exposure

to dbcAMP is blocked by cycloheximide. If the increased expression of DDC and CKBB in these cells was due to increased enzyme synthesis, it can be visualised how cycloheximide would block the dbcAMP effect. However, HMBA was found to reduce the expression of DDC in H69 cells, how then does cycloheximide block a reduction in enzyme activity ? It may be that DDC is under the control of an inhibitor protein and following HMBA treatment the amount of this inhibitor protein is increased with the result DDC activity is reduced. Therefore in the presence of cycloheximide the increased synthesis of this DDC inhibitor is blocked and the DDC activity remains at the control level. Such a mechanism for the regulation of an enzyme has been described for ornithine decarboxylase (Meilhoc et al, 1986). Synthesis of a protein inhibitor of ornithine decarboxylase is stimulated by putrescine, spermidine, spermine and synthetic diamines (e.g. HMBA).

The Effect of the SCLC Phenotypic Change on Chemosensitivity.

Having established that a phenotypic change in the SCLC phenotype can be achieved following exposure of the cells to the appropriate drugs the effect of this phenotypic alteration on the chemosensitivity of the cells was examined. Initially the effect of phenotypic change on the adriamycin chemosensitivity of the cells was examined and it was found that phenotypic alteration of the SCLC cell lines had no significant effect. Another important drug in the treatment of SCLC, vincristine, was also selected to determine whether the phenotypic alteration of the SCLC cell lines altered the vincristine chemosensivity. Once again no significant differences between the vincristine chemosensitivity of the control cells and the phenotypic drug treated cells was found. Finally, the effect of the phenotypic change on the VP16 chemosensitivity of the cells was examined. Only the two combinations where the maximum phenotypic changes were seen

were examined, namely H187 cells exposed to dbcAMP and H69 cells exposed to HMBA. No difference in the VP16 chemosensitivity was found in HMBA treated H69 cells, however, dbcAMP treated H187 cells were significantly more resistant to VP16 than the untreated controls.

As discussed in the Introduction, VP16 is believed to act as a cytotoxic agent via an action on topoisomerase II. Pronounced increases in cellular topoisomerase II activity have been associated with cell proliferation both in vitro and in vivo, and this proliferative activity has in turn been associated with increased tumour cell sensitivity to topoisomerase-II-interactive drugs (Markovits et al, 1987; Sullivan et al 1986). Since dbcAMP causes a marked growth suppression in H187 cells the increased resistance to VP16 may be a reflection of the decreased cell proliferation. NaBut also has a marked growth suppressive effect on H187 cells without all the other phenotypic effects of dbcAMP. It would be of interest to examine the effect of NaBut treatment of H187 cells on the VP16 chemosensitivity to determine whether a similar change in chemosensitivity was found.

From these results it appears that phenotypic change, as determined by the change in the levels of selected SCLC markers, has no effect on the chemosensitivity of the SCLC cell lines examined. It is possible however that the MTT assay was not sensitive enough to detect subtle changes in the chemosensitivity of the cells in response to phenotypic change. This possibility seems unlikely however since significant differences between the ID_{50}'s of the three cell lines could be seen using this assay and the ID_{50}'s differed by less than two fold.

As discussed in the Introduction, an established cell line of human small cell lung cancer (OH-1) underwent a subtle morphological change which was accompanied by a profound loss of neuroendocrine differentiation and the emergence of radiation resistance (Goodwin &

Baylin 1982). This change in the sensitivity of SCLC cell lines to radiation following the loss of APUD cell characteristics has been noted elsewhere. In a group of seven SCLC cell lines, two cell lines had undergone "transformation" to large cell morphological variants with loss of APUD cell characteristics. When the in vitro response of the cells lines to radiation and chemotherapeutic drugs was examined it was found that the large cell variants were 2- to 5- fold more resistant to radiation compared to the classic SCLC cell lines (Carney, Mitchell & Kinsella 1983). Therefore, it may be that an alteration in the SCLC phenotype could affect the sensitivity of the SCLC cell lines to radiation without necessarily altering the cells chemosensitivity. This avenue of investigation may well be worth pursuing.

The phenotypic change of the SCLC cells following exposure to the various drugs may have been insufficient to cause a change in the cells' chemosensitivity. It is apparent from the literature that "transformation" of classic SCLC cell lines to large cell variants is accompanied by a profound decrease in APUD properties, including DDC and the presence of neurosecretory granules, decreased levels of bombesin and a change in morphology resembling cell type 3 or 4. Although changes in the levels of the SCLC phenotypic markers were found in the present study, these changes were modest compared to the differences between classic SCLC cell lines and the "transformed" large cell variant cell lines. The cells were treated with the appropriate phenotypic drugs for 72 hours. This exposure time was selected since in other systems 72 hours was shown to be sufficient time to allow phenotypic change. It is important to extend this study to investigate the effect of longer exposure times of the SCLC cell lines to the various inducer agents on the expression of the phenotypic markers with a view to maximising the phenotypic change. Moreover, combinations of the different phenotypic agents may be more effective in inducing a change in the SCLC phenotype than using single

agents. Agents such as dexamethasone which were shown to have no effect when added alone may have a synergistic effect when added in combination with other agents.

Are the Observed SCLC Phenotypic Changes Reversible or Stable ?

The cell lines were treated with the various inducer agents at which point the levels of the various phenotypic markers were measured. It would be of interest to investigate whether the changes in the phenotypic markers following a 72 hour exposure to the various agents were still evident after a certain period in drug free medium. It is possible that a proportion of the cells underwent an irreversible phenotypic change in response to drug treatment, however a number of other cells in the population may be left unchanged. If the unchanged cells were growing at an increased rate the phenotypically altered cells would be selected against and eventually disappear from the culture and it would appear that a reversible phenotypic change had occurred. Cloning of the drug treated cells could perhaps clarify this possibility but since the cloning efficiency of SCLC cultures is extremely low it is unlikely whether this would give a conclusive answer. If indeed only a proportion of the SCLC cells had undergone a phenotypic change the levels of phenotypic marker expression found would be the net value from a heterogenous population with varying response levels. Therefore it is possible that some cells had in fact undergone a greater degree of change than the results would suggest. However, this is purely speculation and further investigation would be required to substantiate this claim. The problem of reversibility is an important one but is not easily resolved.

SCLC Phenotypic Shift: More Differentiated or Nonspecific ?

The drugs used to induce a phenotypic change in the SCLC cell lines
have been shown to be able to induce a more differentiated phenotype
in other in vitro systems. With respect to the SCLC cell lines, do
these phenotypic inducers shift the SCLC phenotype towards a more
differentiated phenotype or are the phenotypic changes a nonspecific
shift in the SCLC phenotype ?

It has been proposed that cancer is a disorder resulting from an
uncoupling of gene expression that controls cellular proliferation and
differentiation. Normal cell development is classically depicted as
proceeding from an immature stem cell, which possesses unlimited self-
renewal to commited progenitors and terminally, lineage-specific,
differentiated cells with limited, restricted or no self-renewal
capacity. The multiplication and differentiation of normal cells are
controlled by different regulatory molecules. These regulators must
interact to achieve the correct balance between cell multiplication
and differentiation during embryogenesis and during the normal
functioning of the adult individual.

Cell differentiation is the result of precise gene programming. It is
based on selective activation of only a very small part of the genome
with the vast majority of genes remaining silent in each cell type.
From one genotype many different cell phenotypes are thus generated
giving rise to the structural and functional specialisations that
constitute differentiated tissues. The origin and further progression
of malignancy result from genetic changes that uncouple the normal
balance between multiplication and differentiation so that there are
too many growing cells. In most cases neoplastic cells are often
defined by the pattern of molecular gene activation and inactivation,
so that the observed effect is the loss and/or appearance of certain

specific gene products, which are not normally found in the particular cell type at that point in its differentiation pathway.

The classification of tumours is based on the expression of morphological, biochemical and functional properties. Historically, the expression of certain specific characteristics by tumour cells has been ascribed to the origin of the tumour in question from a specific parent cell expressing the same phenotype. For example, in SCLC the expression of APUD properties has led to the proposal that SCLC originates from the endocrine cell (K cells) of the lung (Pearse, 1969). However, the expression of these differentiation characteristics do not necessarily reflect origin from a particular parent cell and there is accumulating evidence that tumours may frequently exhibit more than one type of differentiation (Mendelsohn & Maksem 1986). SCLC is a good illustration of this point; the presence of certain differentiation characteristics could imply the existence of neuroendocrine, haemopoietic and nonSCLC differentiation, but is more likely to represent the expression of nonspecific markers shared by these different cell types.

The criteria for differentiation are the expression of specific characteristics by tumour cells which are associated with the mature counterparts. In the case of SCLC where the cell of origin is unknown, differentiation associated properties cannot be determined. While there are readily identifiable markers of APUD or neuroendocrine phenotype which can be measured, the relationship of these markers to the "normal" counterpart is not understood and there is a possibility that a cell may differentiate towards an alternative phenotype showing a decrease in neuroendocrine markers in the process. Therefore, whether the phenotypic drug treatment causes a shift in the cell's phenotype towards a more differentiated phenotype cannot be determined due to the lack of suitable differentiation associated markers.

It is a premise of histopathology that those tumours which are morphologically poorly differentiated tend to be most aggressive. This raises the possibility of a modality of cancer therapy involving the shift of tumour cells towards a more differentiated phenotype more closely resembling normal cells. However, it does not necessarily follow that a cell responding to induction of differentiation becomes less malignant, although recent reports have shown that following induction of differentiation of tumour cells there is a phenotypic shift involving a reduction in some malignancy associated properties (Freshney 1985; Calabresi et al, 1979; Biedler et al 1983; Speers, 1982; MᶜLean et al, 1986; Frame, et al 1983). For this reason a marker of malignancy rather than differentiation which is not dependent on knowing the mature phenotype of the cells, were they to complete the process of differentiation, is particularly valuable in determining a phenotypic shift in response to phenotypic drug treatment.

Plasminogen activators (PA) are serine proteases which catalyse the cleavage of the inactive plasminogen to the active protease plasmin, which digests fibrin. Two main forms of PA have been described, tissue-type (t-PA) and urokinase-like (u-PA) PA. PA activity has been found to be significantly higher in a number of human neoplastic tissues compared to the normal counterparts (Nagy, Ban & Brdar 1977; Markus et al 1980; Camiolo & Greco 1986), and a role for PA in tumour growth and metastasis has been implicated. The secretion of these enzymes by neoplastic tissues and transformed cells may initiate a proteolytic cascade which reduces or eliminates the extracellular barriers for tumour growth and migration. The relationship between the level of PA produced by tumour cells and the degree of tumourigenicity and/or the metastatic potential depends greatly upon the experimental system. Data both in favour (Ossowski & Reich 1983) and against (Barratt et al 1980) a positive correlation have been

reported. Therefore, although the precise function of PA in the invasive and metastatic properties of a tumour is unclear PA does appear to be associated with malignancy. In this respect, PA was measured in lung carcinomas and anaplastic astrocytomas following exposure to various differentiating agents (Freshney 1985). It was found that stimulation of differentiation associated properties was accompanied by a dramatic decrease in the production of PA by the cells. Therefore, induction of differentiation in these tumour cells was accompanied by a reduction in this malignancy associated property.

In order to investigate the effect of the phenotypic inducer agents on the malignancy associated properties of the SCLC cell lines PA was selected as a marker of the malignant phenotype. PA activity was determined using a chromogenic substrate assay (Whur et al 1980). Using this assay the PA activity, both cellular and secreted, in the SCLC cell lines was determined. However, no PA activity could be detected in the three SCLC cell lines, H187, H69 and H128d. Therefore PA activity could not be used as a marker of malignancy in this tumour. It is possible however, that PA does not have an important role in the SCLC malignant phenotype, other proteolytic enzymes such as collagenase may be involved.

Angiogenesis has long been considered to be essential for tumour growth. Tumours can propagate to volumes of approximately $1mm^3$ by simple diffusion and exchange of nutrients and metabolites through the extracellular fluid, the critical event that converts a self-contained pocket of aberrant cells into a rapidly growing malignancy comes when the tumour becomes vascularised. The tumour must induce the host to provide it with its own network of blood vessels. Ample evidence suggests that tumours secrete various factors that promote the ingrowth of blood vessels from surrounding tissues. One such factor tumour angiogenesis factor (TAF) was recently purified using heparin-

Sepharose chromatography (Shing et al 1984). The purified factor is a cationic polypeptide with an isoelectric point of about 9.8 and a molecular weight of approximately 18,000 daltons. It stimulates capillary endothelial cell proliferation in vitro half maximally at 1ng/ml and strongly stimulates neovascularisation in the chick embryo. Since this, many other endothelial cell growth factors have been purified to homogeneity.

The ability to stimulate angiogenesis in vivo or endothelial division and motility in vitro are properties which are associated with tumours. The tendency of tumours to have a greater angiogenic effect than their normal equivalents has been used as a marker of transformation. Endothelial stimulation in vitro provides a convenient and quantifiable measure of parameters which are believed to broadly correlate with the angiogenic response in vivo. The ability of the SCLC cell lines to stimulate the growth of an endothelial cell line was examined as a measure of transformation. Preliminary data suggested that conditioned medium from the SCLC cell lines was able to induce a mitogenic effect on a quiescent culture of bovine pulmonary artery derived cells, CPAE cells (Beveridge, personal communication). The degree of mitogenesis was however difficult to reproduce on a day to day basis. Therefore this marker of malignancy could not be used convincingly to examine the phenotypic changes of the SCLC cell lines.

The growth of normal cells is largely controlled by the interplay between several polypeptide hormones and hormone-like growth factors that are present in tissue fluids. Malignant cells, however, are not subject to all the same growth controls as are normal cells. In general malignant cells require less of these exogenous growth factors than do their normal counterparts for optimal growth and multiplication and it has been suggested that transformed or malignant cells escape from normal growth controls by requiring less of such

hormones or growth factors. To explain this phenomenon, it was suggested that cells could become malignant by the endogenous production of polypeptide growth factors acting on their producer cells via functional external receptors, allowing a mitogenic response to the peptide by the same cell that produces it. This process has been termed autocrine secretion. Moreover, this autocrine hypothesis provides an explanation as to how oncogenes make cancer cells autonomous of growth factors particularly if the hypothesis is broadened to include the importance of receptors and post-receptor signal transduction as critical control elements in the normal mitogenic pathway.

Bombesin is believed to function as an autocrine growth factor in SCLC. The action of bombesin is believed to be mediated via a distinct membrane receptor (Moody et al 1985), which presumably activates a post-receptor signalling mechanism and leads eventually to a mitogenic response. If bombesin is indeed acting as an autocrine growth factor in SCLC cells then one should be able to control the growth of the cancer cells using a bombesin antagonist. This has been accomplished recently using a monoclonal antibody to bombesin (Cuttitta et al 1985). The monoclonal antibody was found to bind to the C-terminal region of bombesin-like peptides, it blocked the binding of the hormone to its cellular receptor, and inhibited clonal growth of SCLC in vitro and the growth of SCLC xenografts in vivo. More recently, [D-Arg^1, D-Phe^5, D-Trp^{7,9}, Leu^{11}] substance P, a potent bombesin antagonist in murine Swiss 3T3 cells was found to inhibit the growth of human SCLC cells in vitro (Woll & Rozengurt, 1988). Autocrine growth control appears to be associated with the transformed phenotype, therefore on the basis of this criterion bombesin expression could be viewed as a malignancy associated marker.

Following HMBA treatment of the SCLC cell line, H69, a significant reduction in the level of BLI was found. Therefore HMBA induced a

phenotypic shift in the H69 cells which involved a significant reduction in a malignancy associated property. However, dbcAMP treated H187 cells expressed an increased level of BLI. Therefore dbcAMP induced a phenotypic shift in the H187 cellular phenotype which involved an increase in the expression of a malignancy associated property. On the basis of BLI as a malignancy associated marker, SCLC cell lines can be induced to change their phenotypic status in opposite directions.

An anomaly to the hypothesis that BLI is a marker of malignancy is the apparent distribution of BLI in the three cell lines. Within the three SCLC examined BLI expression was found to be the highest in H128d cells and undectable in H187 cells. The distribution of BLI within the SCLC cell lines paralleled the distribution of DDC. That is, H128d cells expressed the highest levels of DDC activity whereas, the lowest levels were found in H187 cells. The presence of DDC activity has implied a neuroendocrine origin of SCLC and DDC activity may be associated with neuroendocrine differentiation. According to this hypothesis H128d cells exhibit elevated levels of malignancy and differentiation associated properties. Since the origin of SCLC is unclear differentiation associated markers cannot be identified unequivocally, and one cannot conclude that increased expression of DDC is associated with neuroendocrine differentiation. However, it is possible that the presence of DDC may in fact be associated with the malignancy of the cells. DDC is an enzyme involved in the synthesis of the catecholamines, dopamine, adrenaline and noradrenaline. Although SCLC may synthesise catecholamines which act as autocrine growth factors, SCLC express no clinical manifestations of over-production of catecholamines. If SCLC cells produce catecholamines then they could respond to them by dividing if the necessary receptors were present and functional. Three major questions arise from this hypothesis: (1) Do SCLC cells produce catecholamines ? (2) Do SCLC

cells have catecholamine receptors ? (3) Is mitosis induced in SCLC by catecholamines ?

In order to investigate whether DDC was involved in an autocrine growth system SCLC cells were grown in the presence of either noradrenaline, phentolamine and/or propranolol. Noradrenaline is a catecholamine which acts as an agonist on α and β receptors, phentolamine is an α receptor antagonist and propranolol is a β receptor antagonist. If catecholamines act as autocrine growth factors in SCLC via functional α and β receptors then one could hypothesise that a mitogenic response may be elicited by exogenous addition of noradrenaline or inhibited by receptor antagonists.

The effect of catecholamine agonists, antagonists and synthesis inhibitors on the growth of a SCLC cell line was investigated. However, since no change in the growth of the cells was found the data is not presented. The implications of the results will be discussed.

The growth of H187 cells, measured by either electronic particle counting or thymidine incorporation, was found to be unalterd by either noradrenaline, phentolamine and/or propranolol. SCLC cell lines have been shown to be able to take up and metabolise L-DOPA to dopamine (Pettengill, Bacopoulos & Sorenson 1982), therefore dopamine may be an autocrine growth factor in SCLC acting via dopaminergic receptors. However, exogenous addition of dopamine was also found to have no effect on the growth of H187 cells. It may be however, that endogenous production of the putative autocrine growth factor is such that the receptors are saturated and so exogenous addition of dopamine would not enhance the mitogenic effect. If the synthesis of the putative autocrine growth factor could be inhibited could this then alter the growth of the SCLC cells ? Tyrosine hydroxylase is the rate limiting enzyme in the biosynthesis of catecholamines. α-Methyl tyrosine inhibits the synthesis of catecholamines by competing with

tyrosine for the active site of tyrosine hydroxylase. When H187 cells were grown in the presence of α- methyl tyrosine it was found that the growth rate of H187 cells was unaffected. In conclusion therefore, it is unlikely that a catecholamine autocrine growth system exists in the SCLC cell line H187. With hindsight it may have been useful to extend these studies to include other SCLC cell lines which expressed higher DDC activity to examine whether these findings held true for other SCLC cell lines irrespective of the level of DDC activity.

Following phenotypic drug treatment of the SCLC cell lines (H187 and H69) various morphological alterations were noticed. H187 cells were classed as type 3 cells which are characterised as irregular shaped aggregates which resembled a "bunch of grapes" morphology. Whereas, H69 cells were classified as type 2 cells characterised as irregular shaped aggregates where individual cells can not be visualised. The pattern of morphological change was towards a more spherical aggregate shape where individual cells could not be distinguished. These morphological changes imply a change in cell-cell adhesiveness.

Development of resistance to actinomycin D, daunomycin or vincristine in Chinese hamster cells was accompanied by reduced uptake of antibiotic in proportion to the degree of resistance, a reduction in tumourigenicity and a change in morphology towards a more normal phenotype (Biedler et al 1975). The cells with acquired resistance were found to have a greater cell adhesiveness compared to their malignant counterparts. In another study, comparison of two SCLC xenografts, one sensitive to cyclophosphamide and the other a cyclophosphamide induced resistant subline of the first xenograft, indicated that induction of cyclophosphamide resistance was accompanied by expression of keratin suggesting squamous differentiation (Berman, Gusterson & Steel 1985). Moreover, the in vitro growth characteristics of the two xenografts differed; the sensitive cell line was anchorage independent whereas the induced

cyclophosphamide resistant counterpart showed flattened angular adherent culture characteristics. Therefore an increase in adhesiveness appears to be associated with an increase in drug resistance in some cell lines and also with expression of a more differentiated phenotype. The morphological changes observed with the SCLC cell lines following treatment with the appropriate drugs implies a change in the cell-cell adhesiveness of the cultures. Although it would be premature to conclude that this change in cell-cell adhesiveness represents a shift towards a more differentiated state it is nevertheless important to consider this aspect when one examines the overall SCLC phenotype change. It is perhaps also worth noting at this time that the phenotype of H128d cells, including morphological characteristics, did not undergo a significant change in response to drug treatment. H128d cells are characterised as type 1 cells (Carney et al 1985). The cells grow as tightly packed spherical aggregates of floating cells, individual H128d cells cannot be seen within an aggregate which suggests a high degree of adhesiveness between cells. If cell-cell adhesiveness is associated with the degree of differentiation of the cells then this would imply that H128d cells are the most differentiated of the three cell lines examined. If this is the case this may explain why the phenotype of H128d cells could not be significantly changed by phenotypic inducer treatment.

The entry into and exit of malignant cells from the circulation is an important requirement for the successful establishment of a secondary tumour during blood-borne metastasis. The acquisition by neoplastic cells of the capacity to invade locally and to metastasise remains the aspect of tumour progression that is of greatest clinical significance; this is still the fundamental definition of malignancy. Measurement of tumour invasiveness is difficult due to the absence of good quantifiable assays. One assay that has been used as a measure of invasiveness is the chick heart assay (Mareel, Kint & Meyvisch 1979). Chick heart fragments are co-cultured with tumour cell

aggregates in organotypic culture. This system is specific in that invasion of the chick heart fragment, its destruction and ultimately replacement is only carried out by malignant cells. The invasiveness of the SCLC cell lines, H187 H69 and H128d, was investigated using this assay, in collaboration with De Ridder. The cells were co-cultured with a fragment of chick heart for periods up to 10 days. At various time points during the co-culture the invasiveness of the cells was examined. Sections of the chick heart were taken and examined for the presence of SCLC cells. Chick heart cells were identified using a specific antibody against chick mesoderm, whereas SCLC cells were identified using an antibody against NSE. All three SCLC cell lines were shown to be invasive as defined by the ability to infiltrate the chick heart fragments. H128d cells had the greatest capacity to invade then H69 cells and finally H187 cells. Preliminary data suggests that phenotypic drug treatment of the cell lines decreases invasive ability. H69 cells treated with 5mM HMBA or 1mM NaBut did not invade the chick heart. Moreover, HMBA treated H128d cells did not invade the chick heart. Furthermore when either HMBA or NaBut were removed (following a 72 hour drug exposure) 5 days before co-culture the cells grew round the chick heart but did not invade, then some 7-9 days later the cells started to infiltrate the heart. Two things emerge from this 1) the SCLC cells remain viable after the phenotypic inducer treatment and 2) the phenotypic change is apparently reversible and decays in about 10 days.

Since the tumour cells are required to adhere to the chick heart fragment it is possible that changes in the adhesiveness of the tumour cells could account for the differences found. Both control and drug treated tumour cells were found to adhere to the chick heart fragment quite effectively.

The question originally posed was: Do these phenotypic inducers shift the SCLC phenotype towards a more differentiated phenotype or are the phenotypic changes nonspecific ? In an attempt to answer the first part of the question a number of malignancy associated markers were measured to investigate whether the cells became less malignant following phenotypic drug treatment, since an inverse relationship has been shown to exist in some cases between differentiation and malignancy. Preliminary data suggests that the SCLC phenotype becomes less malignant following phenotypic drug treatment with respect to BLI, invasiveness and cell-cell adhesiveness. However further work is required support these findings. All of these malignancy associated properties are difficult to assay and caution is required when interpreting the results. Other markers of malignancy should be measured for example tumorigenicity in nude mice or clonogenicity in soft agar. Whether the phenotypic shift observed following phenotypic inducer treatment is nonspecific cannot be answered at this time. Perhaps it would be useful to measure other cellular markers not directly associated with the SCLC phenotype for example actin or lactate dehydrogenase to see if these markers also changed following drug treatment.

Do the Changes in the SCLC Phenotype Following Phenotypic Drug Treatment Further our Understanding of the Origin of SCLC in Particular the Position of SCLC in the Histogenesis of Lung Cancer ?

In order to be able to answer these questions a more thorough investigation into phenotypic alteration in SCLC needs to be carried out. As mentioned earlier the use of other phenotypic inducer agents as well as combinations of phenotypic agents may improve the degree of

phenotypic change found. Also more phenotypic markers need to be measured to give a more detailed picture of the phenotypic change. At present the results do not give many clues to understanding the origin of SCLC. What does seem to be apparent is the considerable heterogeneity of SCLC, in particular the ability of different cell lines to undergo a phenotypic shift in opposite directions. A shift towards a more neuroendocrine-like phenotype characterised by increases in neuroendocrine associated markers has been found in some cases, whereas a loss of neuroendocrine characteristics has been found in others. In the case of an induced loss of neuroendocrine features it would be of interest to examine whether there is an associated increase in any nonSCLC phenotypic characteristics, for example keratin expression, mucin production or surfactant secretion. Moreover, analysis of cell surface proteins following phenotypic drug treatment may aid the definition of SCLC phenotypic change and may also aid the understanding of the origin of SCLC since nonSCLC cell surface proteins can also be measured. It has been proposed that all histological subtypes of lung cancer are linked via progressive differentiation, or shift in differentiation characteristics, from SCLC to large cell undifferentiated and ultimately to squamous carcinoma and adenocarcinoma (Yesner, 1978). Therefore the differences in phenotypic shifts between cell lines in response to drug treatment may reflect the position of the individual cell lines in the differentiation pathway linking the different lung cancer histologies.

Conclusions.

The aim of this study was to determine whether the SCLC phenotype could be altered and if so did this phenotypic alteration have any effect on chemosensitivity. DDC, CKBB, BLI, morphology and growth characteristics were selected as representing the SCLC phenotype. On

the basis of changes in these markers it was concluded that the SCLC phenotype could be altered by various phenotypic drugs. When the effect of this phenotypic change on the chemosensitivity was examined it was found that in general alteration of the SCLC phenotype had no effect on the chemosensitivity.

Future Considerations.

Since it has been shown that the SCLC phenotype can change following drug treatment it will be of interest to try and maximise this change with perhaps longer drug exposure times, new drugs or drug combinations. It would also be important to investigate the effect of phenotypic drug treatment on other markers associated with SCLC for example NSE, the presence of neurosecretory granules or secretion of hormones. Moreover, measurement of phenotypic markers not associated with the SCLC phenotype will also help determine whether the shift in phenotype is nonspecific or perhaps towards nonSCLC phenotype. If a greater phenotypic shift could be induced it may then be useful to determine whether there was any effect on chemosensitivity or indeed radiation sensitivity.

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