ROLE OF PLASMA MEMBRANE IN CYCLIC ADENOSINE 3',5'-MONOPHOSPHATE PRODUCTION AND REGULATION OF GROWTH IN NORMAL, MALIGNANT AND HYBRID CELLS IN CULTURE

A thesis presented in part fulfilment
of the requirement for the
degree of

DOCTOR OF PHILOSOPHY

in the

FACULTY OF SCIENCE

VICTORIA UNIVERSITY OF MANCHESTER

by

Anne White

Manchester

September, 1978

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 - 1346

To Stuart and my family

"The universe is full of magical things,
patiently waiting for our wits to grow sharper"

Eden Phillpots.

Following graduation from the University of Manchester in June 1975, the author has undertaken research in the Department of Biochemistry at the University of Manchester under the supervision of Dr. S.R. Ayad.

The results presented in this thesis have not been submitted for any other degree and to the best of the author's knowledge are her own work, except where due acknowledgement is made.

The author wishes to express her gratitude to Professor G.R. Barker for the provision of facilities to undertake this work.

Thanks are due to my supervisor Dr. S.R. Ayad for his enthusiasm and encouraging advice; to Miss M.R. Schofield for her assistance in the presentation of this thesis and to Miss M. Barber for typing the manuscript.

I would also like to thank colleagues and friends of the Department of Biochemistry for many interesting discussions.

The receipt of financial support from the Cancer Research Campaign is gratefully acknowledged.

CONTENTS

SUMMARY

Comparative studies of cAMP synthesis in a human embryonic lung cell line (AP-9) and two malignant cell lines (SKL-2; lymphoblastic leukaemia cells and EB_2; Burkitt lymphoma cells) showed that both basal and hormone-stimulated cAMP levels were reduced in the two malignant cell lines. It would appear that this occurs by multiple aberrations in the components involved in cAMP metabolism.

The local anaesthetic, tetracaine, which is known to alter the mobility of plasma membrane components, was used as a probe to study the effects of these changes on cAMP responsiveness to hormones in the three cell lines. The cAMP levels in normal AP-9 fibroblasts displayed a biphasic response to tetracaine in the presence of either prostaglandin E_1 (PGE_1) or isoprenaline, with a peak at 0.5mM tetracaine, followed by inhibition at higher concentrations. In contrast, tetracaine enhanced hormone-stimulated cAMP levels in SKL-2 cells and produced a combined response in EB_2 cells. Since a similar effect was observed with different hormones, it is proposed that the local anaesthetic acts on a component distal to the hormone receptor. These results are considered with regard to the effects of tetracaine on (1) the translational mobility of membrane components and (2) the disruption of cytoskeletal elements.

Hybrids (PCM) between a Chinese hamster fibroblast cell line (CH23) and a mouse lymphoma suspension cell line (P338F-36) were originally isolated in this laboratory by non-selective pressure (Ayad and Delinassios, 1974). One of these hybrids, designated PCM3, has been investigated because of its unusual growth. It exists as a monolayer (PCM3-M) with a considerable proportion (5-15%) of its cells in suspension (PCM3-S) and either form of the hybrid can perpetuate the cell line indefinitely. The clonal origin and the karyotype analysis indicate that the hybrid is not a heterogeneous population but different forms of the same cells and further studies have shown that the suspension form is not just the result of overcrowding on the monolayer.

Experiments were carried out to determine the mechanisms regulating the growth of this hybrid, to examine the interrelationship with cAMP and to investigate the role of the plasma membrane.

The growth of the PCM3 hybrid was inhibited when the cells were incubated in the presence of agents which increase cAMP concentrations. Furthermore, the percentage of cells in suspension was specifically reduced and this appeared to result from increased adhesion of the PCM3-M cells.

PGE_1 stimulation of cAMP was observed to be markedly enhanced in both the monolayer and suspension forms of the PCM3 hybrid, when compared with the parental cell lines and this confirms earlier observations that the PGE_1 response is inherited as a positively dominant character in the PCM series of hybrids. Comparison of the basal levels of cAMP in the two forms of PCM3 indicated that these were lower in PCM3-S cells. However, this difference in cAMP levels was caused by determination of cAMP with respect to protein content, which was greater in the suspension cells. Estimation of intracellular cAMP on a cellular basis proved that the cAMP levels were slightly higher in PCM3-S cells. Examples are given of reports where this observation might affect the interpretation of results.

It was postulated that the two forms of the hybrid existed at different parts of the cell cycle and evidence was obtained from time lapse photography and examination of the mitotic index which suggested that the mitotic phase of the cell cycle occurred in the suspension form of the hybrid. However, further investigations proved that the PCM3-S were capable of synthesising DNA, which suggested that the PCM3-M cells become detached during the S-phase and remain in suspension until after mitosis. This was substantiated by inducing synchronisation with serum deprivation. This collected cells in the G_2 phase and significantly increased the proportion of suspension cells. After serum addition the PCM3-S cells were observed to divide and form monolayer cells.

A cell surface glycoprotein of molecular weight 220,000 has been identified on PCM3-M cells and found to be trypsin sensitive. The properties of this protein correlate well with the protein designated CSP or LETS, characterised by Hynes (1973) and found to be absent from the surface of transformed cells. Using lactoperoxidase-catalysed iodination, it was possible to identify this protein on the membrane of PCM3-M and CH23 cells. However, it was found to be absent from the membranes of PCM3-S and P388F-36 cells. Incubation of PCM3-S cells with partially purified CSP isolated from CH23 cells produced a culture consisting almost completely of PCM3-M cells and it was postulated that the CSP caused increased adhesion of the hybrid cells which suppressed the formation of the suspension cells.

In conclusion, it is thought that the unusual growth of the hybrid results from the loss of cell surface glycoportein from the surface of PCM3-M cells during the S phase of the cell cycle. This causes decreased adhesion and movement into suspension where the cells undergo mitosis. With the organisation of the new plasma membranes after mitosis, it could be envisaged that the CSP is once more exposed, so the cells are able to attach to the substratum.

I N T R O D U C T I O N

The discovery of cyclic adenosine 3',5'-monophosphate (cAMP) in 1958 made a powerful contribution to our understanding of cellular regulation. It was isolated and identified by Dr. Earl Sutherland and co-workers in the course of investigations into the epinephrine stimulation of glycogenolysis in the liver and as a result the Second Messenger hypothesis was formulated to describe how hormone action is mediated by increased cAMP levels within the cell. It is now known that hormone receptors activate adenylate cyclase in the plasma membrane producing increased levels of cAMP, which are able to activate various protein kinases in the cell and thereby regulate many diverse intracellular processes.

The development of cell culture techniques has led to significant advances in several fields of research. It has become possible to study cAMP metabolism in vitro and to undertake a direct comparison of normal and malignant cells. These investigations have been complimented by research into somatic cell hybridisation which studies the inheritance of genetic material after cell fusion.

Thus the expansion in the field of cell culture resulted in recognition of the correlation between altered cAMP levels and abnormal growth characteristics in malignant cells. However, despite the exponential

growth in research effort the mechanisms underlying these phenomena have not been resolved.

With this in mind, the research presented in this thesis has attempted to study the variations in cAMP levels in relation to alterations in growth in normal, malignant and hybrid cells in culture. Initially the role of the plasma membrane in the hormonal stimulation of cAMP was compared in normal and malignant cells, because it is known that the membrane is intimately involved with alterations in growth associated with malignancy. Subsequently the isolation of a somatic cell hybrid, between a Chinese hamster fibroblastic cell line and a mouse lymphoma suspension, provided a unique opportunity to study a hybrid cell line with altered growth characteristics and to examine the associated changes in cAMP levels. The approach taken has been to examine the mechanisms responsible for growth aberrations in an attempt to determine if cAMP is involved.

A. CELL CULTURE AND SOMATIC CELL HYBRIDISATION

I. Cell Culture

Since the beginning of this century research workers have attempted to grow cells and tissues *in vitro*. This technique has proved to be of great advantage for the analysis of many diverse phenomena. The maintenance of cells in culture requires careful isolation from fragments of dissociated animal tissues and growth in specially defined media. These techniques are now well established and have been described by Paul (1970).

Because of the necessity to grow these cells on plastic and glass surfaces and to passage them by cell dilution, there are a limited number of cell types which can be cultured. The following normal cell types are most often found in culture:

a. Fibroblastic cells.
   These are usually of mesenchymal origin from connective tissue and grow as long spindle-shaped cells with cytoplasmic processes. They are often capable of migration on their supporting surfaces.

b. Epithelial-like cells.
   These consist of rounded or polygonal cells of ectodermal and endodermal origin. They grow to form sheets resembling epithelium and while single cells are relatively static, whole patches may move

across the surfaces.

c. Other cell types.

This category includes macrophages, lymphocytes, monocytes and some nerve cells, all of which are known to divide at a slower rate, which suggests that they are less likely to be maintained in culture. Lymphocytes are often stimulated to grow in culture by the use of agents called mitogens.

It is possible to maintain transformed cells *in vitro* and this allows a direct comparison with normal cells, without the complications observed *in vivo*. Transformation is most commonly defined as the stable acquisition of those characteristics which increase the cell's potential to form a tumour i.e. neoplastic transformation and this can be brought about spontaneously or by the action of viruses, chemical carcinogens or x-rays. Transformed cells can be isolated from malignant tissue *in vivo* or produced by one of the techniques above.

Most of the research effort in the study of transformed cells has been concerned with viral transformation and this has described in detail by Studzinski (1977). The oncogenic viruses which cause transformation may be divided into those containing DNA and those containing RNA. The groups of DNA viruses include papovaviruses, adenoviruses, poxviruses

and herpesviruses, while the RNA containing viruses are avian and murine sarcoma groups and the mouse mammary cancer virus.

The characteristics of growth of normal and transformed cells *in vitro* have been reviewed by Macpherson (1970) and will be described in detail in a subsequent section, where the comparison will be discussed in relation to cAMP levels. However, it is important to realise that transformation produces a neoplastic cell which will be recognisable by the alteration of certain characteristics but which may not be oncogenic i.e. it may not have developed the ability to produce tumours. Therefore it is often necessary to test for tumourigenicity.

The absolute test for tumourigenicity is to prove that the cell is capable of producing tumours in isogeneic hosts. Foley *et al.* (1962) have developed an assay to test for tumourigenicity whereby the cells are injected into the cheek pouch of the Syrian hamster, which by virtue of it's structure provides protection from immunological rejection. Oncogenic cells are capable of forming tumours under these conditions. It is also possible to identify oncogenic cells by the presence of virus-induced antigens on their cell surface (Vogt *et al.*, 1965).

The decision to use cell culture depends on the nature of the research. Obviously some investigations require experimentation in whole animals or organs, while other studies might be more suited to experimentation with extracts from specific tissues. However, there are distinct advantages in using cultured cells as outlined below:

1. One of the most important advantages is that cloned cells are identical and therefore eliminate variations observed with tissue preparations, which often consist of a variety of cells.

2. Cloned cell lines also eliminate variations observed between different preparations of the same tissue.

3. The use of cultured cells also provides a direct comparison of different cell types as exemplified by studies of normal and malignant cells.

4. Another advantage is that individual cells are in contact with incubation media in experiments, thus eliminating diffusion problems observed with tissue preparations.

5. The ability to examine the behaviour and metabolism of whole cells often provides valuable information which can complement the studies with extracts.

The disadvantages of using cells grown *in vitro* can often be overcome by using appropriate controls and by the correct monitoring of the genetic material of these cells. Thus spontaneous alterations in the cell lines will be eliminated and gradual changes occurring with increasing time in culture will be corrected. An obvious disadvantage is that the quantity of material obtained is small in comparison with that obtained from tissue extracts and this may limit certain investigations.

II. Somatic Cell Hybridisation

Production of a somatic cell hybrid is brought about by the fusion of two different cell types to produce a heterokaryon, containing genetic material from both parental cell lines. Cells from different species may be fused to produce interspecific hybrids. In this way it is possible to fuse cells from such diverse species as mouse and man (Harris and Watkins, 1965).

The observation of multinucleate cells nearly a century ago suggested that cell fusion might occur, but it was not until 1960 that hybrid cells were first isolated after spontaneous fusion in culture (Barski *et al.*, 1960). This type of fusion occurs at a very low rate under natural conditions and therefore the observation that viruses can induce fusion, greatly facilitated research (Okada, 1962).

Since these early experiments, hybridisation has been used extensively and has provided the *in vitro* system for the analysis of eukaryotic genetics. Reviews by Ringertz and Savage (1976) and Croce (1977) provide an excellent insight into this topic and describe its importance in many areas of research.

Induction of Fusion

Experimentally, fusion can be induced by a number

of agents, including various viruses (Poste, 1972) lysolecithin (Croce et al., 1971) and polyethylene glycol (Pontecorvo, 1975). Initially hybrids were produced by the action of a paramyxovirus called Sendai or haemagglutinating virus of Japan (H.V.J.) (Harris and Watkins, 1965; Okada and Murayama, 1965) and this is still used extensively. The fusion process is a property of the viral envelope (Hosaka, 1970) and so the virus is inactivated by UV irradiation (Okada, 1962) or β-propriolactone (Neff and Enders, 1968) to inhibit infectivity. More recently polyethylene glycol has been used to induce fusion (Pontecorvo, 1975; Pontecorvo et al., 1977) and the results suggest that this agent may be more useful than viruses, because it eliminates problems associated with incomplete inactivation of viral infectivity.

Mechanism of Fusion

Fusion is a property of the cell membrane and it has been suggested that fusogenic agents cause phase changes in the structure of the cell membranes which make them more susceptible to fusion (Cullis and Hope, 1978). Papahadjopoulos et al. (1977) have studied the fusion of phosphatidylserine vesicles and have shown that these phase changes occur and can be induced by calcium. This has been substantiated by several reports which suggest that calcium is necessary for fusion (Okada and Murayama, 1966). However, Hart et al. (1976) have recently produced evidence to suggest that calcium inhibits fusion.

The mechanism underlying virally-induced fusion involves virus particles binding to cells and causing agglutination at 4°C. This is followed by a brief incubation at 37°C when membrane fusion occurs. Evidence presented by Knutton (1977) suggests that the cell fusion is a result of simultaneous binding of a virus particle with two cells, and Scheid and Choppin (1974) have shown that a glycoprotein of 53,000 daltons is responsible for the virus's ability to fuse cells.

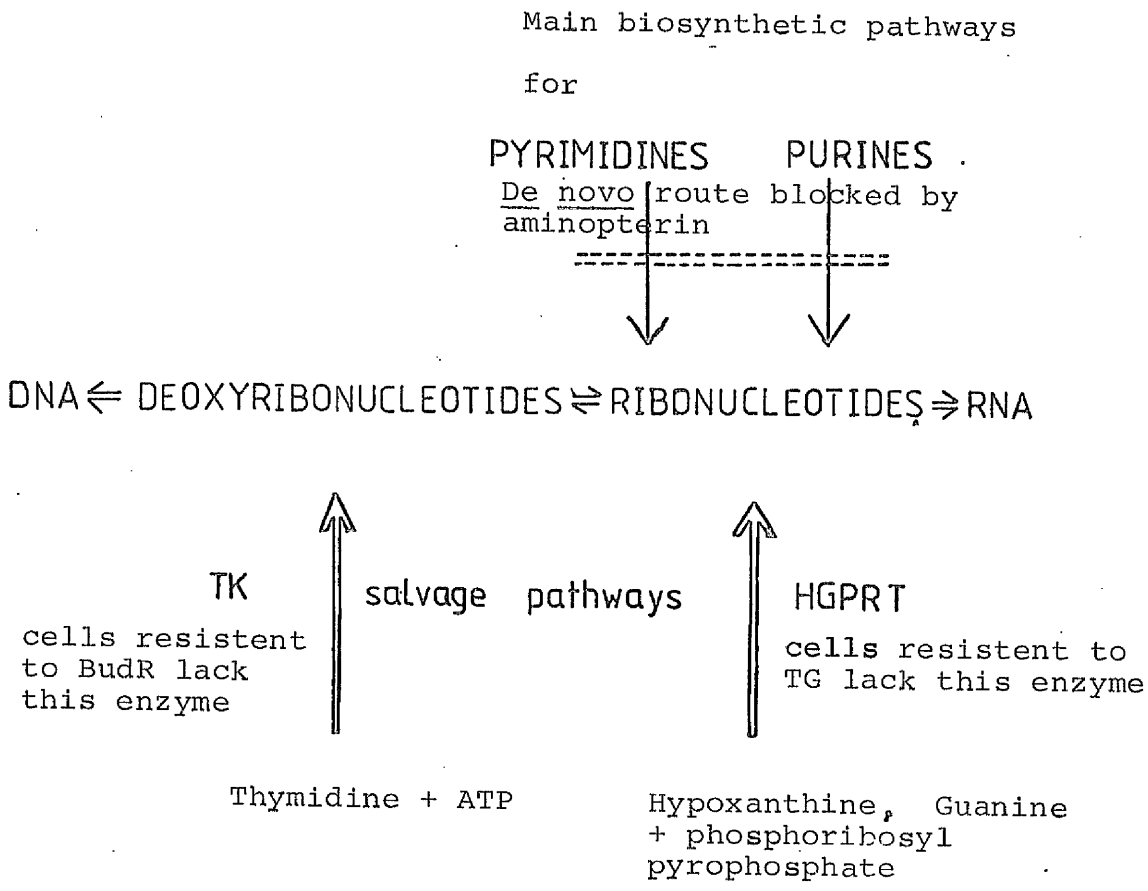
Chromosome Loss

Membrane fusion is followed by nuclear fusion when chromosomes from the two cells are brought together at mitosis. If the chromosomes are from different cell types then the resulting hybrid is described as a heterokaryon and a proportion of these are often capable of indefinite multiplication *in vitro*. Intraspecific hybrids usually retain most of the chromosomes from both parental cell lines, but interspecific hybrids tend to exhibit a slow rate of chromosome loss. (Yoshida and Ephrussi, 1967). One set of parental genes is usually lost preferentially as shown by hybrids between human and rodent cells, where the human chromosomes are not retained. (Weiss and Green, 1967). It has been observed by Kao and Puck (1970) that hybrids from cells with different growth rates tend to lose chromosomes from the slower growing parent.

Isolation of Hybrids

Successful isolation of hybrid cells in early experiments was attributed to their high growth rate, which allowed separation from parental cell lines. However, development of selective methods of isolation (Littlefield, 1966) provided more frequent isolation of different hybrids. The principles of the technique are outlined below in Figure 1:-

FIGURE I. SELECTIVE ISOLATION OF HYBRID CELLS



Fusion is carried out between cells selected in 5-bromo-2'-deoxyuridine (BUdR) which lack thymidine kinase (TK) and cells resistent to thioguanine (TG) which have lost hypoxanthine guanine phosphoribosyl transferase (HGPRT). Both these enzymes are required to synthesise nucleotides by salvage pathways, when the main biosynthetic pathways are blocked by aminopterin. Therefore after fusion, cells are cultured in hypoxanthine, aminopterin, thymidine (H.A.T.) medium (Szybalski et al., 1962) and only hybrid cells which have inherited both enzymes for the salvage pathways will grow. There are some limitations to the use of mutants and these are described by Siminovitz in a review of the nature of heritable variation (1976).

Another method of hybrid isolation uses semi-selective pressure, by isolating a rapidly proliferating hybrid from fusion between a slow growing parent and an actively dividing cell line which is eliminated by selection. (Davidson and Ephrussi, 1965). Nutritional mutants have also been used to select for hybrids (Kao et al., 1969), as have techniques involving temperature-sensitive mutants (Basilico, 1974).

Finally a process of non-selective pressure has been developed in this laboratory (Ayad and Delinassios, 1974). This exploits the differences in cellular adhesiveness between parental and hybrid cells. In this way, hybrids have been isolated from fusion between a

fibroblastic Chinese hamster cell line (CH23) and a mouse lymphoma cell line (P388F-36) by a series of cell dilution operations. The hybrids grown as monolayers (PCM) were identified and cloned.

Identification of Hybrids

Following the isolation of hybrid cells it is necessary to prove that they do contain chromosomes from both parental cell lines. This is achieved directly by karyotype analysis and by differential banding techniques (Caspersson et al., 1971). It is also possible to distinguish between the isozyme patterns of the parental and hybrid cells on the basis of their electrophoretic mobilities (Ayad and Delinassios, 1974)
An alternative approach is to examine the surface antigens which are co-dominantly expressed in hybrid cells (Gershon and Sachs, 1963).

Applications of Hybridisation

Hybridisation has developed as a very powerful technique in the study of gene regulation in mammalian cells. It is possible to examine the expression (dominance) or extinction (recessiveness) of phenotypic traits in hybrid cells, although in most experiments

it is not possible to show whether this occurs at the level of structural genes or regulatory processes. In this way, experiments have shown that membrane ATPase activity was retained in hybrids between mouse macrophages and L cells (Gordon et al., 1971) while melanin production was selectively extinguished in hybrids between pigmented Syrian hamster melanoma cells and mouse L cells (Davidson et al., 1966). Phenotypic traits may also be coexpressed as exemplified by esterase patterns (Ayad and Delinassios, 1974).

Perhaps the most important use of hybridisation has been in the location of genes on specific chromosomes in mammalian cells. With the observations by Weiss and Green (1967) that human chromosomes were preferentially lost from intraspecific hybrids, it was realised that comparisons of phenotypes and chromosome patterns would allow the assignment of genes to specific chromosomes. This also provided the possibility of establishing gene linkage groups and as a result of these studies over 100 different markers have been assigned to specific human chromosomes (Baltimore Conference, 1975).

Analysis of the expression of malignancy has been undertaken using hybridisation techniques (Harris, 1975) and this has allowed the assignment of the chromosomal integration site of oncogenic

viruses to chromosome 7, (Croce and Koprowski, 1975). Unfortunately at present there appears to be conflicting evidence concerning the expression of malignancy. Work by Croce and Koprowski (1975) suggested that the transformed phenotype was expressed in hybrids between normal mouse cells and SV40-transformed human cells. However, experiments by Harris and co-workers (Harris et al., 1969; Jonasson et al., 1977) suggested that malignancy was suppressed when tumour cells were fused with normal diploid fibroblasts.

Somatic cell hybrids between differentiated and undifferentiated cells have been used to investigate gene regulation of the expression of differentiated functions. The extensive range of characteristics studied has been reviewed by Davis and Adelburg (1973) and Ringertz and Savage (1976).

Interest in the use of hybridisation to study hormone action was stimulated by observations that the cAMP responsiveness to catecholamines was recessive in hybrid cells (Gilman and Minna, 1973) while prostaglandin stimulation of cAMP was expressed by hybrids (Minna and Gilman, 1973; Ayad and Foster, 1974). This has led to the use of hybrids to study various aspects of cAMP metabolism and this will be described in a subsequent section.

B. CYCLIC ADENOSINE 3',5'-MONOPHOSPHATE METABOLISM

I. Introduction

Since it's discovery in 1958 cyclic-AMP has been recognised as the mediator of the actions of a wide range of hormones, including glucagon (Sutherland and Rall, 1960) catecholamines (Robison et al., 1971) and prostaglandins (Hinman, 1972). Thus it has been shown to be involved in many diverse cellular functions ranging from increasing the rate of glycogenolysis in the rat liver (Sutherland and Rall, 1960) to acting on cellular growth in vitro (Pastan et al., 1975). Over the last few years cAMP has been detected universally in tissues of widely different animal species and in bacteria.

The discovery of this cyclic nucleotide has therefore proved very important to the studies of physiology and pharmacology at the cellular level. However, despite the vast catalogue of actions of cAMP there is still much confusion about its ability to regulate so many processes in vivo.

The effect of hormonal stimulation of a cell's metabolism is controlled by a balance of all the components involved in cAMP production and degradation and these are presented in a simplified model of

cAMP metabolism, shown in Figure 2. Most of the reactions mediated by cAMP involve hormones, which are released from specific tissues and which travel via the blood to act on target cells in order to elicit a response. These hormones do not enter the cells, but instead bind to specific receptors on the cell surface, which then activate adenylate cyclase (ATP:pyrophosphate lyase (cyclising) E.C. 4.6.1.1), situated on the inner surface of the cell membrane. This catalyses the cyclisation of ATP to produce cAMP and there is considerable evidence to suggest that there is a Mg^{2+} binding site and a GTP dependent regulatory subunit on this enzyme.

Thus the hormone signal is translated into increased cAMP levels, within the cell and this is thought to bring about the cellular response by activating various specific protein kinases. These may phosphorylate enzymes to initiate metabolic changes in the cytoplasm or they may regulate gene expression by phosphorylating proteins in the DNA-histone complexes.

Cyclic-AMP degradation is brought about by cyclic 3',5'-nucleotide phosphodiesterase (E.C. 3.1.4.16) and activation of this enzyme provides another level of control of cAMP metabolism.

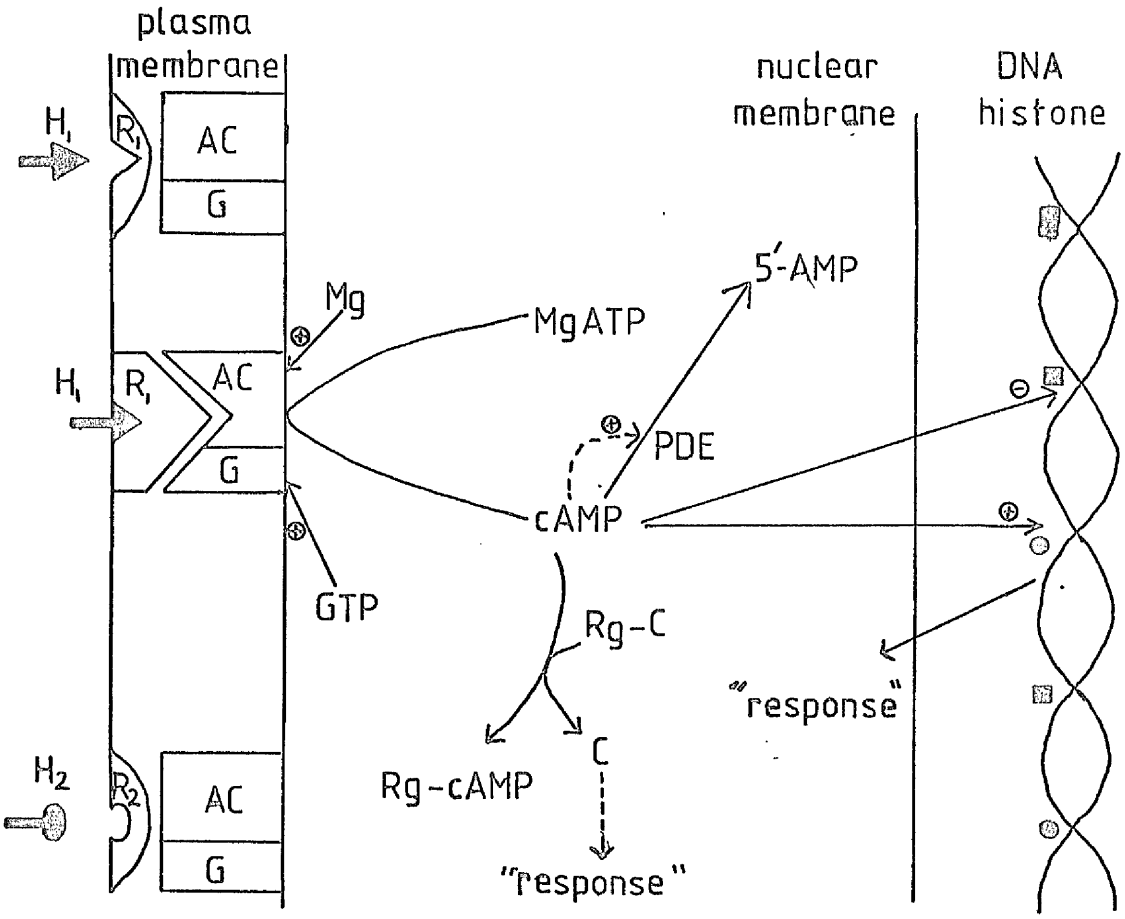


FIGURE 2. A SIMPLIFIED MODEL OF CYCLIC-AMP METABOLISM

H_1 H_2	different hormones
R_1 R_2	different hormone receptors specific for H_1 and H_2 respectively.
AC	adenylate cyclase
G	guanyl nucleotide-dependent regulatory site
PDE	cyclic 3',5'-nucleotide phosphodiesterase
Rg	regulatory subunit of protein kinase
C	catalytic subunit of protein kinase (active form of enzyme.
Rg-C	inactive form of protein kinase (holoenzyme)
+	stimulatory effect
-	inhibitory effect

It can be seen from this brief outline, that although cAMP influences numerous processes in various cells, it is capable of producing a precise response to a particular signal as a result of the specificity inherent in the components of the cAMP system. Thus the type of hormone receptor present in the cell membrane, the regulation of adenylate cyclase and the degree of cyclic 3' 5'-nucleotide phosphodiesterase activity will all act to control the cAMP activity. A further level of specificity is achieved by the presence of specific kinases and all these factors contribute to the determination of the cellular response.

II. Methods used to Study Cyclic-AMP Metabolism

In an attempt to understand the mechanisms underlying the role of cAMP in the whole cell, a variety of experimental approaches have been taken. The use of cultured cells to study cyclic nucleotide metabolism has been reviewed by Chlapowski *et al*. (1975) and Makman *et al*. (1977), while an alternative approach has been to investigate isolated components from tissue extracts. The relative merits of these two approaches have been discussed previously and whichever is taken it is necessary to bear in mind that the experimental procedure may have affected the response.

However, using cultured cells it enables the examination of genetic variants of the system and thereby provides a better understanding of the normal mechanisms. This has been achieved by the investigations described below:-

1. Viral Transformation

Numerous studies have compared normal cell lines with their transformed counterparts in an effort to understand the aberrations associated with cyclic nucleotides and malignancy. Many reports suggest that low levels of cAMP can be correlated with certain transformed characteristics (Pastan and Johnson, 1974)

and this has been examined in a series of eloquent experiments by Pastan and colleagues. They have used cells transformed by temperature sensitive mutant viruses, which exhibit transformed phenotypes at permissive temperatures (32°C) and more normal characteristics at non-permissive temperatures (39°C). Using this technique they have shown that normal rat kidney cells infected with temperature sensitive Kirsten sarcoma virus exhibit increased cAMP levels with increasing cell density, when incubated at the non-permissive temperature, but fail to show increased cAMP levels at the permissive temperature (Carchman et al., 1974). More recently Yoshida et al. (1977) have used this technique and suggested that the change in activity of adenylate cyclase is secondary to transformation.

2. Isolation of Mutants

The classical method for studying genetic variation is the isolation of mutant cell clones which grow in selective conditions such as altered temperature, or the presence of certain drugs. Using this approach, Tomkins and his colleagues have contributed greatly to the understanding of cAMP metabolism (see review by Bourne et al., 1975).

It was known that growing cells in dibutyryl cAMP or agents which increase endogenous cAMP caused cytolysis and this was used to isolate S49 mouse lymphoma cells resistent to increased cAMP. Examination of these mutant clones showed that they were defective in certain components of the cAMP system. Thus Daniel et al. (1973) obtained variants deficient in protein kinase activity and it has subsequently been shown that the inactivity is caused by an alteration in the regulatory subunit of the protein kinase (Coffino et al., 1975; Hochman et al., 1977). Studies on kinase deficient mutants have confirmed that protein kinases mediate cAMP-induced growth arrest (Insel et al., 1975) and cAMP activation of phosphodiesterase (Bourne et al., 1973).

Using this technique, a 'deathless mutant' cell line has been isolated which appears resistant to cytolysis but remains responsive to growth arrest and phosphodiesterase activation and it has been suggested that the altered gene product is distal to the protein kinase (Lemaire and Coffino, 1977). Bourne et al. (1975) have also isolated a variant of S49 lymphoma cells which is deficient in hormone-responsive adenylate cyclase and more recently Haga et al. (1977) have obtained an 'uncoupled' mutant which possesses hormone receptors and adenylate cyclase but fails to

respond to hormones, suggesting that the interaction between hormone receptor and catalytic unit is uncoupled. It can be seen that these variant S49 lymphoma cells have provided a valuable approach to the study of cAMP metabolism and a great deal of information concerning the interactions of the various components has been deduced from these experiments.

3. Somatic Cell Hybridisation

Fusion of cells differing in phenotype with respect to cAMP metabolism allows genetic analysis of the mechanisms governing the expression of functions related to cAMP and the isolation of novel phenotypes provides new systems for the biochemical examination of the relationships between the components involved in cAMP metabolism. This approach, using cell hybrids, has been reviewed briefly by Makman et al. (1977).

Investigations in this field were initiated by Gilman and Minna (1973) who studied the inheritance of cAMP responsiveness to hormones in various hybrid cell clones. The fusion of cells responsive to catecholamines with unresponsive clones produced hybrids with greatly diminished activity. This suggested that the catecholamine receptor is under negative control. However, fusion of cells responsive to prostaglandin E_1 (PGE_1) with unresponsive clones produced hybrids which responded to PGE_1 with similar or enhanced levels

of cAMP (Minna and Gilman, 1973). These results were obtained from the same hybrid clones and provided evidence for the independent regulation of different hormone receptors. Hormone binding studies have subsequently shown that catecholamine receptors are decreased in the hybrids, while the number of PGE_1 binding sites correlate with the degree of activation of cAMP (Brunton et al., 1976).

Using the hybridisation of cell lines exhibiting either hormone receptor binding or adenylate cyclase activity it has been possible to reconstitute hormonally stimulated cAMP production and prove that the components are independent and compatible in heterologous systems. (Orly and Schramm, 1976; Schramm et al., 1977; Schwarzmeier and Gilman, 1977).

In this laboratory, investigations of cAMP metabolism in a hybrid cell line designated PCM_1 confirmed the studies of Minna and Gilman (1973) by showing an enhanced responsiveness to PGE_1 (Ayad and Foster, 1974). Examination of the adenylate cyclase in PCM_1 has established that the enzyme has properties in common with both parental cell lines (Ayad and Foster, 1977). In conjunction, Ayad and Burns (1977) have analysed PGE_1 binding and propose that receptors from both parents are expressed in the hybrid.

The preliminary report by Ayad and Foster showed that cyclic 3',5'-nucleotide phosphodiesterase (PDE) activity was reduced in PCM_1 and this has since been extensively studied in PCM2 and PCM3 hybrid cell lines (Ayad and Wright, 1977). These investigations also suggest that certain characteristics of this enzyme have been inherited from each parent. In contrast to these observations, Tisdale and Phillips (1976) propose that increased cAMP levels correlate with increased PDE activity in hybrids examined at different cell densities.

III. Hormonal Stimulation of Cyclic-AMP Synthesis

Hormones are produced by specific organs and travel in the blood to act on target cells, where they bind to specific receptors, and modify cellular processes. The group of hormones classified as prostaglandins are an exception to this, in that they are produced by the target tissues. The hormone receptors function to discriminate between hormones and to generate a signal which will trigger the cellular response. Interaction between hormone and receptor is highly specific, readily reversible and must be sustained to produce a response. Studies have shown that the receptor-adenylate cyclase complex, composed of protein and phospholipid, spans the plasma membrane with the receptor exposed on the outer surface and the enzyme situated on the inner surface.

It is obvious that different hormones may act on the same cell and therefore different types of receptors must exist on the surface of that cell. It was originally thought that each receptor was bound to a distinct adenylate cyclase but it is now understood that different receptors can activate a common enzyme (Bär and Hechter, 1969; Birnbaumer and Rodbell, 1969). This may be explained by the mobile receptor hypothesis which will be described later, in relation to activation of adenylate cyclase.

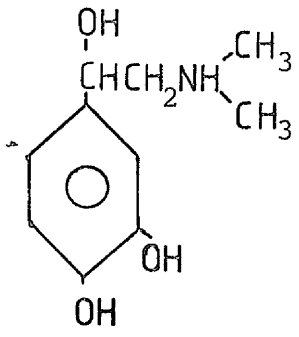
Various hormones are mediated by cAMP and detailed studies of the glucagon and insulin receptors have been discussed by Helmeich et al. (1976). However, this thesis is concerned with catecholamines and prostaglandins, so it is pertinent that they are described in more detail.

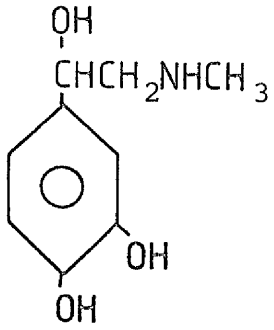
1. Catecholamines

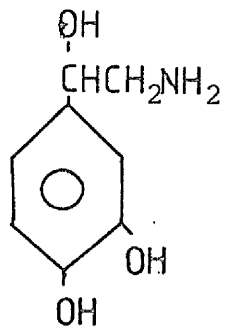
The problem associated with summarising the information about these hormones is the vast amount of literature which has been published. This may be judged by the fact that four reviews have been produced recently (Lefkowitz et al., 1976; Helmeich et al., 1976; Levitzki, 1976; Maguire et al., 1977).

The main catecholamines found in the body are adrenaline (epinephrine) and noradrenaline (norepinephrine) and their widespread action both as hormones and neurotransmitters led to numerous studies which began as early as 1906. Their receptors were designated adrenoreceptors and Ahlquist (1948) classified α and β receptors according to the relative potency of these agonists on different physiological actions. (Furchgott, 1955). Because of their enormous potential in pharmacology synthetic catecholamines such as l-isoprenaline (isoproterenol) were developed (see Table 1).

TABLE 1. LIGAND SPECIFICITY OF α-RECEPTORS AND β-RECEPTORS







(-) Isoprenaline		(-) Adrenaline		(-) Noradrenaline
α	1	<	2	< 3
β	1	>	2	⩾ 3

Since then the β-receptors have been further classified into either β_1 or β_2 categories as a result of the production of synthetic antagonists such as propranolol, which is specific for β_1 and β_2 receptors and practolol, which is specific for β_1-receptors only. Using these agents it has been possible to show that β_1-receptors mediate actions such as cardiac stimulation and β_2-receptors mediate increased glycogenolysis, while α-receptors act to cause increased contraction of smooth muscles, as in the bladder.

(Levitski, 1976). With the discovery of cAMP, it was soon realised that the β-receptors were responsible for activating adenylate cyclase. However, the primary event following α-adrenergic stimulation is less clear. It has been proposed that an influx of Ca^{2+} ions occurs following hormone binding and this suggests that Ca^{2+} may act as the 'second messenger'. (Schramm and Selinger, 1975).

A direct examination of the β-adrenergic receptors has been hampered by the small number of receptors, low affinity, high levels of non-specific binding and lack of highly specific radio-ligands. From the activation studies it was apparent that the receptors were stereoselective for l-isomers and this was used as a necessary criterion for the identification of specific binding. The different approaches to binding studies have been excellently reviewed by Maguire et al., (1977).

A variety of ligands have been employed in attempts to identify binding sites with the properties expected. The early studies relied on [^{3}H]-catecholamines and these did not fulfil the conditions for specific binding. In particular (+) and (-) stereoisomers were shown to possess equal binding capacity (Bilezikian and Aurbach, 1973) and it is now

suggested that the very high numbers of receptors reported, were probably the result of non-specific binding to a heterogeneous group of sites (Lefkowitz et al., 1976). With the advent of highly specific radio-labelled antagonists, the true β-receptors have been identified. Thus 600-1000 β-receptors per cell have been estimated in turkey erythrocytes using [^{3}H]-propranolol, (Levitzki et al., 1974) and a similar number of binding sites have been observed using [^{125}I]-iodohydroxybenzylpindolol in a different cell preparation (Brown et al., 1976). However, it has recently been shown that rat skeletal myoblasts (L6 cells) are a rich source of β-receptors, since they appear to contain 80,000 per cell (Atlas et al., 1977). By synthesising [^{3}H]-hydroxybenzylisoproterenol, Lefkowitz and Williams (1977) have provided a more potent agonist, with the advantage over antagonists of allowing a direct comparison of binding with adenylate cyclase activation. Recently direct binding studies have been employed to assay for solubilised β-receptors and using digitonin it has been possible to isolate β-receptors with a molecular weight of 1.3 - 1.5 x 10^{5} (Caron and Lefkowitz, 1976).

With the wide variation in methods used to detect binding sites, there has been some confusion concerning the relationship between hormone binding and activation. It has been shown that the highest physiological level of hormone only saturates 0.01% to 1.0% of the total

receptors, although maximum stimulation is expressed (Levitzki, 1976). This had led to the conclusion that 'spare receptors' exist, and analysis of Scatchard plots for hormone binding reveals that negative cooperativity exists, not only for catecholamine receptors (Limbird and Lefkowitz, 1976), but also for insulin binding, (De Meyts et al., 1976). It is proposed that the function of cooperativity is to provide exquisite sensitivity to low concentrations of hormone, while buffering against acutely elevated levels. (De Meyts, 1976). However, controversy surrounds the ubiquitous nature of cooperativity among catecholamines, since evidence of interactions is lacking in certain cell lines (Brown et al., 1976).

2. Prostaglandins

In comparison with catecholamines this group of hormones have been discovered relatively recently, and for a good review the reader is referred to Samuelson et al., 1975). They are derived from prostanoic acid and are classified according to the substituents attached to the cyclopentane. Prostaglandins are synthesised by the prostaglandin synthetase complex, in the tissues which they act on. (Horton, 1969).

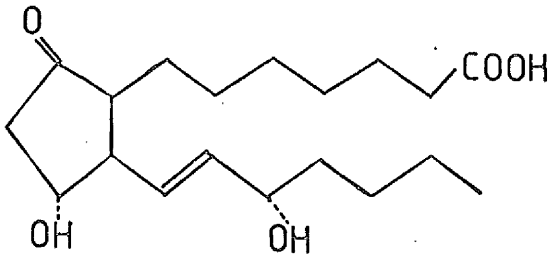


FIGURE 3. PROSTAGLANDIN E_1 (PGE_1)

The importance of this group of hormones lies in their involvement in inflammation, fever analgesia and gynaecological events (Horton, 1976). It has also been revealed that the prostaglandins are associated with platelet aggregation (Gorman et al., 1977). Evidence has shown that some of their effects are mediated by cAMP and it is known that PGE_1 acts in this way (Hinman, 1972; Kuehl, 1974).

Evaluation of the characteristics of prostaglandin receptors has proved difficult because of the problems associated with binding studies. However, Lin and Rao (1977) have reported discrete binding sites for PGE_1 and $PGF_{2\alpha}$ in luteal cells and they have shown that these hormones bind to their own receptors with high affinity, but to each other's receptors with low affinity. Ayad and Burns (1977) have used [^{3}H]-prostaglandin E_1 in binding studies and observed that binding closely parallels activation of adenylate cyclase.

Examination of cells in culture has recently revealed that these cells produce PGE_2 and $PGF_{2\alpha}$ during their growth (Hammarstrom, 1977). It has been suggested by Taylor and Polgar (1977) that these endogenously produced prostaglandins may function as regulators of cell division.

IV. Adenylate Cyclase

Adenylate cyclase is something of an enigma, in that despite intensive research, the mechanisms governing cAMP synthesis have remained elusive. The enzyme is situated on the inner surface of the plasma membrane and catalyses the cyclisation of ATP to produce cAMP. It is optimally active at concentrations of 1-2mM ATP with a pH range 7.5-8.5 and temperatures between 30-40^{o}C. The molecular weight of the solubilised enzyme is estimated as 1.6 x 10^{5} (Housley et al., 1977; Neer, 1976).

A coherent review is provided by Perkins (1973).

1. The Catalytic Unit

It is now agreed that the true substrate for adenylate cyclase is an MgATP complex (Drummond and Duncan, 1970) and various reports suggest the presence (Garbers and Johnson, 1975; Londos and Preston, 1977) or absence (de Haën, 1974; Londos and Rodbell, 1975) of a separate allosteric site for magnesium ions (Mg^{2+}). An alternative proposal to explain the excess requirement for Mg^{2+} is that the enzyme is inhibited by an unchelated form of ATP which is reduced by conversion to the MgATP complex (Lin et al., 1975). It has been suggested that the stimulation of adenylate cyclase by hormones and other activators involves a decrease in the requirement for Mg^{2+} as an activator

(Alvarez and Bruno, 1977). Other metal ions are known to interact with the catalytic unit and several reports suggest that Mn^{2+} can replace Mg^{2+} (Birnbaumer, 1973) while ions such as Cu^{2+} and Zn^{2+} have been found to inhibit the enzyme (Birnbaumer et al., 1969).

It is interesting to speculate about the true role of calcium in cAMP metabolism, since there are brief reports of it's involvement in several different aspects. The problem lies in the difficulty of determining Ca^{2+} levels within the cytoplasm and in understanding the compartmentalisation involved. It has been postulated that Ca^{2+} acts as a negative allosteric regulator binding to a separate site on adenylate cyclase (Steer and Levitzki, 1975), while other investigations have shown that a calcium dependent activator of cyclic 3',5'-nucleotide phosphodiesterase is capable of stimulating the enzyme (Brostrom et al., 1976; Cheung et al., 1975). Separately calcium acts as an additional second messenger (Schramm and Selinger, 1975) and is known to interact with cAMP in several regulatory processes. (Durham, 1974; Berridge, 1975; Rasmussen et al., 1975).

During the early investigations into cAMP synthesis, it was observed that fluoride ions were capable of eliciting a maximum response in most mammalian tissues studied (Tao and Lipman, 1969). NaF acts optimally at 5mM by increasing the V_{max} and lowering $K_a(Mg^{2+})$

without altering the K_m (MgATP). (Birnbaumer et al., 1969). Activation of adenylate cyclase by NaF requires Mg^{2+} (Schramm and Naim, 1970) and is ineffective on intact tissues. It has previously been suggested that fluoride ions act directly on the catalytic unit, but recently a requirement for an endogenous protein factor has been revealed. (Bradham, 1977). There is also a report suggesting that NaF and prostaglandin E_1 may activate adenylate cyclase by dephosphorylating an inhibited form of the enzyme (Najjar and Constantopoulos, 1973). Whatever the mechanism of fluoride activation, the use of this anion has been valuable as a true indication of enzyme activity in experiments where reconstitution of hormonally stimulated adenylate cyclase has been studied.

2. Coupling between Receptor and Enzyme

The most important aspect of cAMP synthesis is the activation of adenylate cyclase by the hormone receptor. It has generally been assumed that the receptor is part of a complex in the membrane, such that ligand binding induces an altered conformation in the receptor, which changes the catalytic properties of the complex. This implies a fixed arrangement of the subunits in a single macromolecular entity and does not allow for coupling of different types of receptors, to a common adenylate cyclase. An alternative possibility proposes that receptors and enzyme units can diffuse laterally within the plane of the membrane in an

independent manner. This is described as the mobile receptor hypothesis. (Cuatrecasas, 1974; de Haën, 1976) and owes it's existence to increasing evidence for the fluid nature of the plasma membrane (Singer and Nicolson, 1972). The receptors trigger the cellular response by reversibly associating with adenylate cyclase and this occurs with greater affinity in the presence of hormone (Jacobs and Cuatrecasas, 1977). It is thought that binding of ligand may alter the amphipathic nature of the receptor, thus facilitating coupling to enzyme.

Several investigations postulating that the receptor and enzyme are separate entities, have subsequently provided convincing evidence for the 'floating' receptor. An impressive approach has been employed by Orly and Schramm (1976) with the reconstitution of hormone responsive adenylate cyclase from fusion between turkey erythrocytes with inactivated enzyme and Friend erythroleukaemia cells which do not contain β-receptors. Alternative evidence using x-ray inactivation and target theory reveals that receptor and enzyme only couple in the presence of hormone (Housley et al., 1977). Finally, resolution of ligand binding sites from adenylate cyclase activity, by gel filtration chromatography substantiates the suggestion that these components act as separate

entities (Limbird and Lefkowitz, 1977). Unfortunately, in most of these experiments, recombination of the solubilised components has not restored hormonally responsive catalytic activity. At present reconstitution has been observed only after triton X305 solubilisation (Ryan and Storm, 1974; Storm et al., 1976).

3. The GTP Site

Rodbell and his colleagues were responsible for recognition of the fundamental importance of guanine nucleotides, following their discovery that these nucleotides enhance glucagon stimulation of adenylate cyclase in plasma membranes from rat liver (Rodbell et al., 1971). It has subsequently been observed that enhancement of adenylate cyclase sensitivity is a widespread phenomenon (Rodbell et al., 1975) and recently it has been suggested that GTP is essential for hormonal activation, although it may already be present as a contaminant of ATP in some experimental conditions (Kimura and Nagata, 1977). Thus confusion surrounds the effect of GTP on basal adenylate cyclase activity. (Hanoune et al., 1975; Cuatrecasas et al., 1975).

The non-hydrolysable nucleotide analogue 5'-guanylylimidodiphosphate (Gpp(NH)p) has been used extensively in studies on the GTP site. Unlike GTP, it readily activates adenylate cyclase (Bennett and

Cuatrecasas, 1975b) and when incubated with hormone, Gpp(NH)p acts synergistically to produce cAMP levels which are often greater than those observed after fluoride stimulation (Schramm and Rodbell, 1975). The activation by analogue is irreversible in the presence or absence of hormone and it is still uncertain whether this is due to a change in enzyme stability after nucleotide removal (Cuatrecasas et al., 1975) or to persistent binding of Gpp(NH)p (Cassel and Selinger, 1977a).

Activation of Gpp(NH)p is competitively inhibited by GTP, which proves that these nucleotides act at the same site (Lefkowitz, 1975) and since Gpp(NH)p is more active than GTP it was suggested that the analogue stabilises an active form of the enzyme because it is resistent to phosphotransferase action. These proposals have been used in schemes to explain the role of hormones in adenylate cyclase activation. Thus Levitzki (1977) described the following model:-

$$R{\cdot}E{\cdot}GTP \underset{k_2}{\overset{H\ k_1}{\rightleftharpoons}} H{\cdot}R{\cdot}E{\cdot}GTP \xrightarrow[k_3]{} H{\cdot}R{\cdot}E'{\cdot}GTP \xrightarrow[k_4]{} H{\cdot}R{\cdot}E + GDP + P_i$$

Interaction of hormone (H) and receptor (R) facilitates the conversion of enzyme (E) to an active form (E') which is inactivated by hydrolysis of GTP. Similar schemes have been proposed by Rodbell (1975) and Cuatrecasas et al., (1975). Recently evidence for a specific GTPase has accumulated and Cassel and Selinger (1977b) propose that the guanine nucleotide regulatory site of adenylate cyclase is identical to the GTP binding site of the catecholamine stimulated GTPase.

These observations have been augmented by studies using cholera toxin. The mechanism by which this protein elevates cAMP levels has been discussed by Gill (1977). Briefly, cholera toxin consists of two components; the B component made up of five subunits which bind to G_{M1} gangliosides on the cell surface and the A subunit which is more hydrophobic and fragments to produce a large component A_1 capable of activating adenylate cyclase (Matuo et al., 1976). Cholera toxin was shown to elevate cAMP levels in whole cells after a significant lag period (Bennett and Cuatrecasas, 1975a) and subsequently stimulation of cAMP in purified plasma membranes was observed in the presence of NAD^+ and ATP (Martin et al., 1977). Several reports suggested that cholera toxin converted the enzyme to a purine nucleotide sensitive state (Bennett et al., 1976) but it has recently been proposed that cholera toxin acts

on the GTPase to inhibit GTP hydrolysis thus stabilising the active form of adenylate cyclase (Levinson and Blume, 1977; Johnson and Bourne, 1977; Cassel and Selinger, 1977c).

Investigations into the role of GTP in cAMP production have provided a great deal of conflicting evidence concerning the reciprocal effect of purine nucleotides on the kinetics of hormonal activation of cAMP. Several reports suggest that GTP acts by decreasing the affinity of hormone receptors for agonists, but not antagonists (Ross et al., 1977; Lefkowitz et al., 1976b). This effect is not ubiquitous (Hanski and Levitzki, 1978) and is not easily reconciled with the other schemes proposed to describe GTP action.

In order to explain these observations, Rodbell and his colleagues have now postulated the existence of two GTP binding sites which act separately on receptor conformation and enzyme activity (Lad et al., 1977). Solubilisation experiments suggest that these two nucleotide sites are structurally as well as functionally distinct (Welton et al., 1977) and the following scheme has been proposed to explain the overall mechanism (Lad et al., 1977).

$$GTP + R_d \rightleftharpoons R_s \cdot GTP \qquad I$$

$$GTP + E_1 \rightleftharpoons E_2 \cdot GTP \qquad II$$

$$R_s \cdot GTP + E_2 \cdot GTP \rightleftharpoons (R_c \cdot GTP)(E_2 \cdot GTP) \overset{H}{\rightleftharpoons} (H \cdot R_c \cdot GTP)(E_2 \cdot GTP) \qquad III$$

In reaction I GTP binds with hormone receptor (R_d) to decrease the affinity for hormone (R_s). Similarly, in reaction II the nucleotide converts the enzyme from an inactive (E_1) to an active (E_2) form. This allows coupling of receptor and catalytic unit (Reaction III) which produces an increased affinity of the receptor (R_c) for ligand (H). It has subsequently been proposed that turkey erythrocytes which do not exhibit reduced affinity towards agonists, do not possess a GTP regulatory site associated with the hormone receptor. (Hanski and Levitzki, 1978).

The different approaches taken in the relevant reviews (Maguire et al., 1977; Helmreich et al., 1976) reflect the confusion surrounding the role of GTP in cAMP synthesis. The discovery of a specific GTPase and the evidence that two GTP regulatory sites exist may clarify the situation, although at present it is not known whether these observations will explain other reports. Evidence for a cytosolic protein-

nucleotide complex (Pecker and Hanoune, 1977) and a partially purified GTP-binding protein (Pfeuffer, 1977) may help in the characterisation of the guanine nucleotide interaction.

A schematic diagram of the relationship between the components involved in hormonal stimulation of cAMP synthesis is depicted in Figure 4. It is not possible, at present, to provide an exact diagram of the precise interactions between the various components, but it is perhaps helpful to visualise how they may act in an integrated manner, on the basis of recent experimental evidence.

4. Desensitization

Hormone receptors appear to be capable of protecting the cell from chronic exposure to hormones and this is described as tachyphylaxis *in vivo* and desensitization *in vitro*. The process of receptor regulation has been discussed in reviews by Maguire *et al*. (1977) and Kolata (1977).

In several cell types, it has been observed that stimulation by hormone produces increased cAMP levels which subsequently decrease in a time dependent manner, despite continual potency of hormone (Makman, 1971; Franklin and Foster, 1973). This is followed by a refractory period when despite the

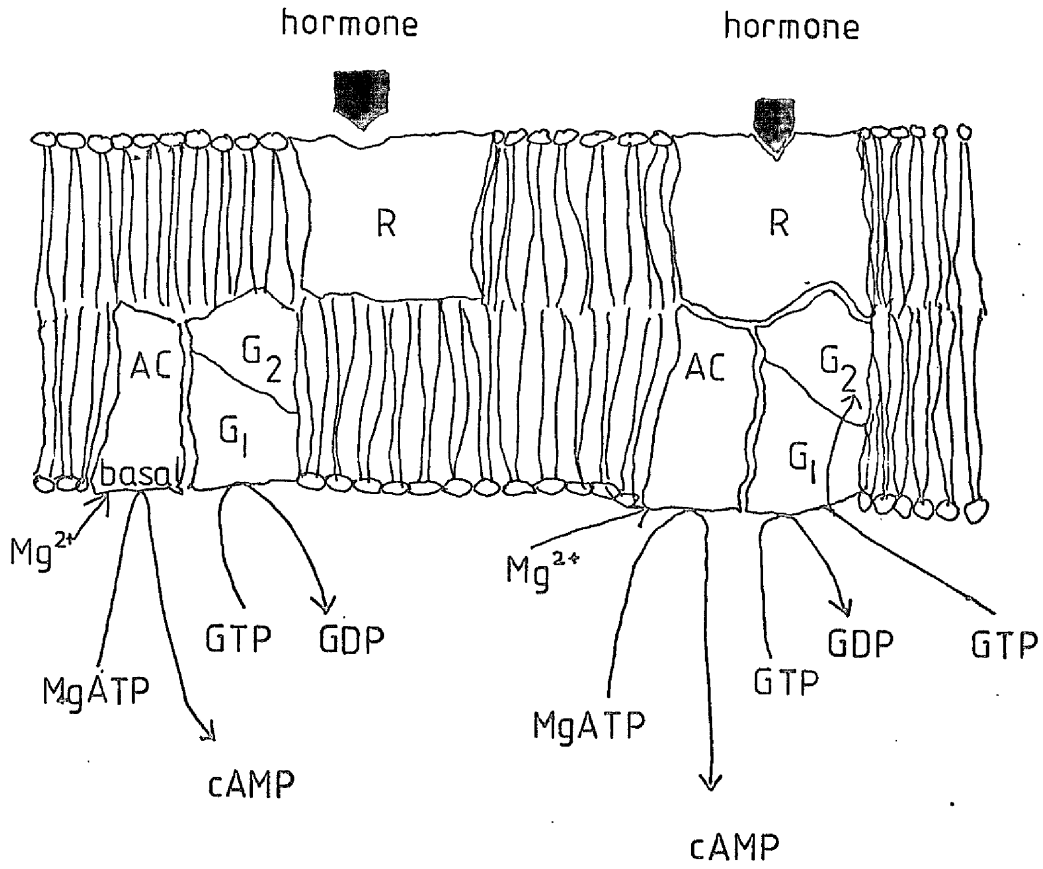


FIGURE 4. THE COMPONENTS INVOLVED IN CYCLIC-AMP SYNTHESIS

The diagram shows possible interactions between the different components involved in cAMP synthesis. It illustrates the importance of their situation in the plasma membrane, which allows mobility of the components and regulation by both extracellular (hormones) and intracellular (ATP, Mg^{2+}, GTP) factors in an integrated manner.

(AC = adenylate cyclase, G_1 and G_2 = guanyl nucleotide binding sites, R = hormone receptor).

removal of hormone, cells fail to respond to further hormone challenge (Ayad and Foster, 1974). Chronic exposure to a particular hormone produces desensitization specific for that hormone, thus *in vivo* or *in vitro* incubation with isoprenaline causes reduced sensitivity to further stimulation by that hormone but does not affect the rates of NaF or PGE_1 activation (Mukherjee et *et al.*, 1975; Mickey *et al.*, 1976). Concurrent incubation with β-receptor antagonists prevents the loss of responsiveness to isoprenaline.

Several reports suggest that desensitization is the result of increased cyclic 3',5'-phosphodiesterase activity (Manganiello and Vaughan, 1972) but this has been disputed (Franklin and Twose, 1976) and although it may account for desensitization shared by different classes of agonists (heterologous desensitization) it would not explain agonist specific changes (Su *et al.*, 1976). It would appear that this homologous desensitization is a characteristic of the receptor and this is confirmed by investigations which show that decreased cAMP responsiveness correlates with reduced hormone binding (approximately 60% loss) while the affinity and Hill coefficient do not alter (Mukherjee *et al.*, 1976.)

The mechanism of agonist induced loss of binding sites remains unclear, but it is unlikely that the receptors are lost from the membrane (Mukherjee *et al.*, 1976) and more credible that persistent binding of

hormone masks the receptor (Brunton et al., 1976). An interesting theory is that guanine nucleotides, which are known to increase the dissociation of agonist from receptors (Maguire et al., 1976) may relieve desensitization (Mukherjee and Lefkowitz, 1977; Hanski and Levitzki, 1978). However, this does not fully account for the refractory period in the whole cell as guanine nucleotide action is relatively rapid.

This mechanism of agonist specific desensitization is complicated by the observation that an S49 mutant clone lacking hormone responsive adenylate cyclase but retaining β-receptors, does not appear to show desensitization by loss of receptors, thus suggesting that refractoriness requires an intact adenylate cyclase (Shear et al., 1976).

A 'Locking Receptor' model for hormonal activation of adenylate cyclase which accounts for desensitization has been proposed by Swillens and Dumont (1977) and is represented in Figure 5. This is an adaptation of the general model used to describe the mobile receptor hypothesis (Cuatrecasas, 1974) and the scheme assumes that activation is immediate, whereas inhibition (reaction 2) is slower.

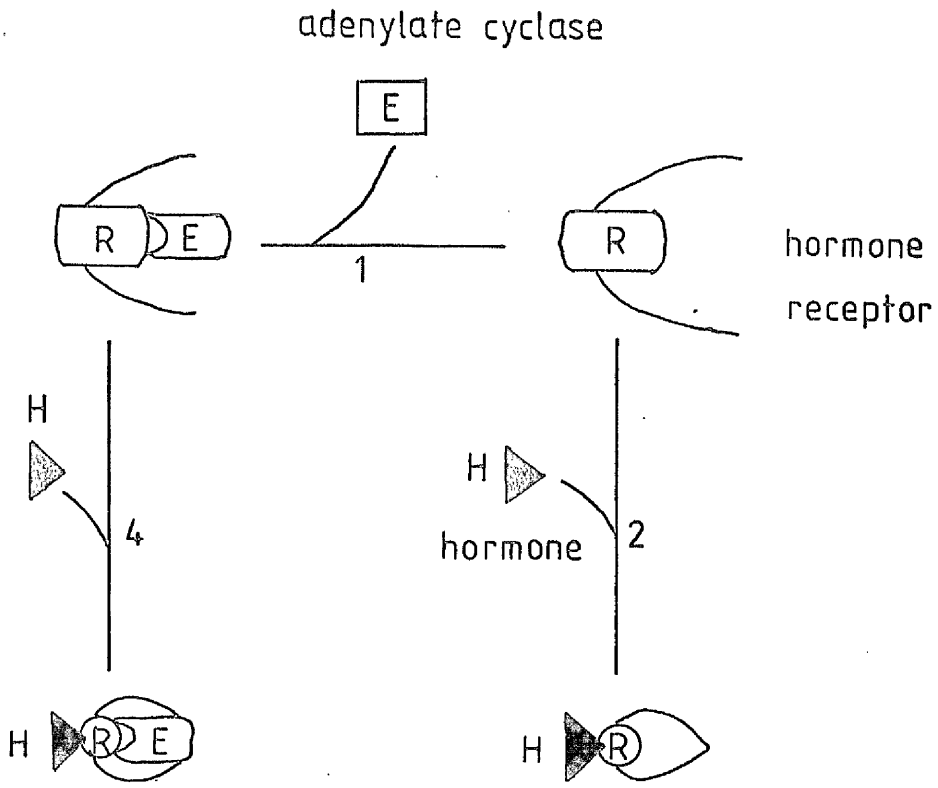


FIGURE 5. THE 'LOCKING RECEPTOR' MODEL

This model can accommodate the involvement of two guanine nucleotide regulatory sites as described in the previous section, where GTP binding lowers the affinity of the desensitized form of the receptor.

V. The Role of the Plasma Membrane

The hormone receptors and the catalytic units involved in cAMP production are found embedded in the plasma membrane, as an integral part of it's structure. Thus it is not surprising that the membrane environment of these components affects the response observed.

The importance of certain phospholipids is revealed, when phospholipases are used to digest the plasma memrbane. This severely disrupts cAMP production and addition of phosphotidylinositol is required for partial reactivation of adenylate cyclase, while phosphotidylserine appears to restore catecholamine sensitivity of the enzyme (Rethy et al., 1972). Thus phospholipids are thought to be involved in the linkage between hormone receptor and adenylate cyclase (Levey and Klein, 1972). Evidence for the involvement of cholesterol in this coupling is provided by the interaction of the antibiotic, filipin with cholesterol in the membrane. This abolishes hormonal activation of adenylate cyclase, without affecting hormone binding or enzyme activity (Puchwein et al., 1974). Finally the evaluation of temperature dependence of adenylate cyclase suggests that membrane lipids are capable of altering hormone, fluoride and GTP stimulation of adenylate cyclase activity (Counis and Jutisz, 1977; Orly and Schramm, 1975).

The Fluid Mosaic Model of plasma membrane structure proposes that components of the membrane are free to move laterally within the plane of the membrane (Singer and Nicolson, 1972). This provides the basis for the 'floating receptor' hypothesis which suggests that hormonal stimulation of cAMP production occurs by the coupling of hormone receptors and adenylate cyclase units which diffuse independently within the plane of the membrane. Thus alterations in the membrane associated with fluidity changes would profoundly affect the coupling of receptor and catalytic unit.

Tetracaine

The local anaesthetic tetracaine has been used as a probe in investigations into the effect of changes in membrane fluidity on hormonally stimulated cAMP production, (Ayad and White, 1977; Ayad and Morgan, 1977). Local anaesthetics such as tetracaine have been shown to enhance the fluidity of phospholipid bilayers (Papahadjopoulos et al., 1975) and plasma membranes from mouse cells (Poste et al., 1975).

Tetracaine appears to act by penetrating the plasma membrane (Koblin et al., 1975) and interacting with the polar groups of acidic phospholipids (Papahadjopoulos et al., 1975), thus displacing calcium from the membrane (Papahadjopoulos, 1972).

Investigations into cell agglutination by plant lectins have provided evidence to suggest that local anaesthetics also disrupt microtubules and microfilaments which form the membrane-associated cytoskeletal elements found beneath the plasma membrane (Poste et al., 1975(b)). The microfilaments, composed of actin-like molecules tend to lie parallel to the membrane, while the microtubules, which are formed from the polymerisation of tubulin subunits appear to radiate into the cytoplasm. Microtubule polymerisation requires GTP hydrolysis and is inhibited by calcium ions (Soifer, 1975).

The cytoskeletal elements are involved in the transmembrane control of receptor mobility and distribution (Nicolson, 1976). This regulation occurs by microtubules limiting receptor mobility while microfilaments act in opposition via a contractile mechanism (Poste et al., 1975(b)). The mechanism by which local anaesthetics alter receptor mobility appears to be by displacement of calcium which disengages microfilaments from the plasma membrane and disrupts microtubule organisation (Nicolson and Poste, 1976). The cytoskeletal elements are also involved in the maintenance of cell shape (Borman et al., 1975; Nicolson et al., 1976) and the anchorage of membrane components. It seems possible that the cytoskeletal elements could be involved in the control

of hormone receptor mobility and this has already been proposed for insulin (Van Obberghen et al., 1976).

A schematic diagram of the plasma membrane is presented in Figure 6. It illustrates the various mechanisms for regulation of receptor mobility (Nicolson, 1976). Since the hormone receptors and catalytic units responsible for cAMP synthesis are embedded in the membrane they may well be controlled by the same general principles.

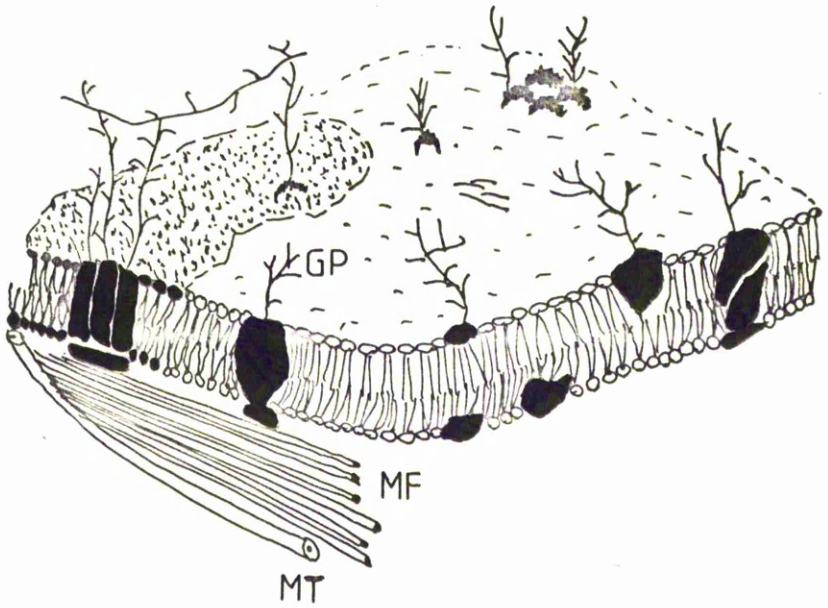


FIGURE 6. CONTROL OF CELL SURFACE RECEPTOR MOBILITY

This schematic diagram represents the various mechanisms thought to control the mobility of cell surface receptors. Thus some glycoprotein complexes (GP) are free of restraints while others are sequestered in lipid domains (shaded area) or anchored by outer surface components. The lateral movement of integral glycoprotein complexes can also be controlled by transmembrane linkages to membrane-associated cytoskeletal elements, which are composed of microtubules (MT) and microfilaments (MF).

VI. Degradation of Cyclic AMP

Cyclic 3',5'-nucleotide phosphodiesterase (PDE), the enzyme uniquely responsible for degradation of cAMP, was first discovered by Butcher and Sutherland (1962). The numerous reports concerning this enzyme have been reviewed extensively (Appleman et al., 1973; Wells and Hardman, 1977) and in discussing these it is necessary to emphasise the trends without cataloging the exceptions, thus tending to provide a slight oversimplification of the current research.

This enzyme has been isolated from both particulate and soluble preparations and is known to exist in several forms (Brooker et al., 1968; Cheung, 1970). The low K_m form of the enzyme displays anomalous kinetics and is relatively specific for cAMP as substrate, while the high K_m form catalyses the hydrolysis of both cAMP and cGMP. A third form of PDE which may not be as widely distributed as the other two, is characterised by the ability of cGMP to act as an allosteric activator of cAMP hydrolysis (Terasaki and Appleman, 1975).

These forms appear to be interconvertible and it has been suggested that aggregation of enzyme subunits may be responsible for the changes in kinetic behaviour.

This could provide an important regulatory mechanism, since cAMP enhances the conversion of the associated (low K_m) form of the enzyme to the dissociated (high K_m) form. (Pichard and Cheung, 1976).

The cyclic nucleotide phosphodiesterase requires Mg^{2+} ions for activity, although Mn^{2+} and Ca^{2+} can be substituted (Lin et al., 1974). The enzyme is inhibited by methyl xanthines and both theophylline (Schroder and Plagueman, 1972) and isobutyl methyl xanthine (Schultz and Hamprecht, 1973) have been used extensively to potentiate cAMP production.

The existence of a cyclic nucleotide phosphodiesterase activator was first demonstrated independently by Cheung (1967) and Kakiuchi et al. (1970) and has since been detected in every vertebrate tissue studied. The activator is a non-dialysable Ca^{2+} binding protein, which interacts specifically with the high K_m form of PDE and is stable to extremes of temperature and pH. It has been purified to homogeneity and has a molecular weight of 18,000 (Lin et al., 1974). The activator lacks tissue and enzyme specificity and comparisons with other Ca^{2+} binding proteins reveal that it is homologous to troponin C (Stevens et al., 1976).

The exact stoichiometry of the interaction between Ca^{2+}, protein activator and PDE has not been established,

but it is known that the activator possesses two types of Ca^{2+} binding sites (Lin et al., 1974). A mechanism for the interaction of the three components has been proposed by Gnegy et al. (1976; 1977) following the observation that a cAMP dependent protein kinase is involved in the release of phosphodiesterase activator from membranes. (Figure 7.)

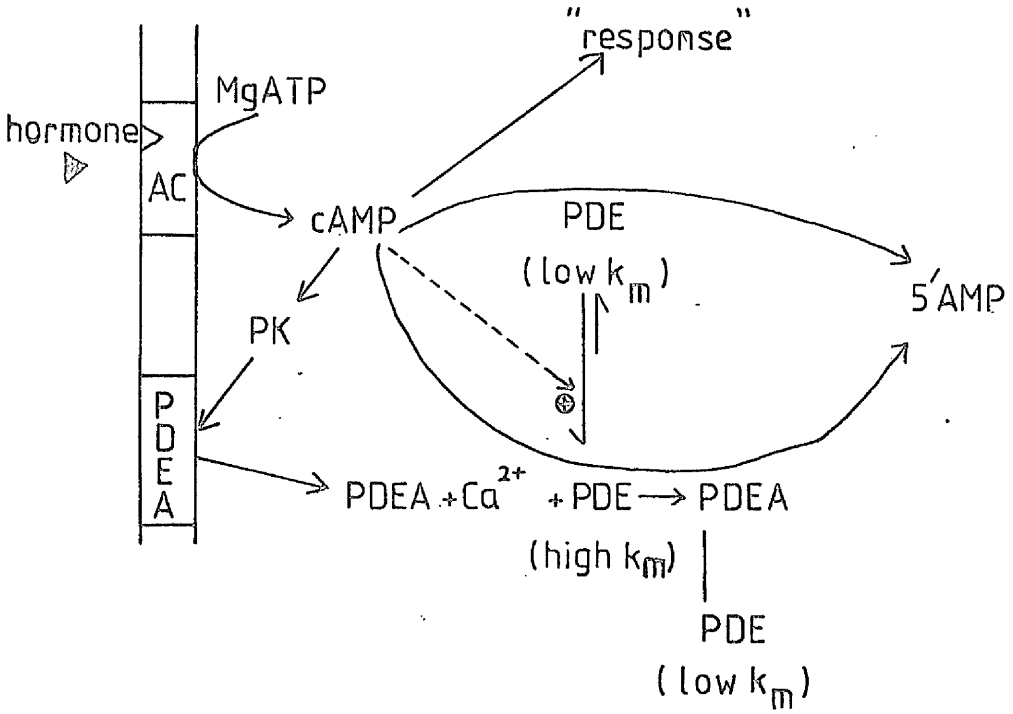


FIGURE 7. THE PARTICIPATION OF ACTIVATOR (PDEA) IN THE REGULATION OF cAMP

PDE = phosphodiesterase. AC = adenylate cyclase. PK = protein kinase.

Increases in cAMP levels are accompanied by an increase in phosphodiesterase activity which can apparently occur via cAMP induction of a rapid mechanism and a slower process involving protein synthesis. (Pawlson et al., 1974; Bourne et al., 1973). The importance of cAMP-dependent protein kinase is emphasised both in the scheme above and in the report by Bourne et al. (1973) which establishes that this kinase is necessary for induction of phosphodiesterase.

VII. Action of Cyclic AMP on Protein Phosphorylation

Most if not all hormonally stimulated cAMP actions are mediated by protein phosphorylation involving cAMP dependent protein kinases. These kinases are very specific *in vivo* for relatively few proteins, which they recognise by the presence of some common features, including basic amino acids close to the target serine. However, the ultimate action of cAMP depends on the types of substrate for kinases which are present in the cell.

Cyclic AMP dependent kinase consists of two subunits; a regulatory subunit (R) which binds cAMP and a catalytic subunit (C) which contains the active site. The intact enzyme is described as a holoenzyme and interacts with cAMP according to the following mechanisms (Hofman *et al.*, 1975):-

$$\underset{\text{(inactive)}}{R_2C_2} + 2\,cAMP \rightleftharpoons R_2(cAMP)_2 + \underset{\text{(active)}}{2\,C}$$

The holoenzyme has been purified to homogeneity (Beavo *et al.*, 1974) and two forms (Peak I and Peak II) have been extensively characterised. These differ with respect to their R subunits (Corbin and Keely, 1977;

Lemaire and Coffino, 1977b) and may exist to provide different physiological responses. A non-competitive protein inhibitor has also been isolated and purified (Walsh et al., 1971). This binds to the free catalytic subunits and may function to increase the sensitivity of the cellular response.

The action of cAMP dependent kinases in triggering cytoplasmic response mechanisms has been described in a comprehensive review by Nimmo and Cohen (1977). These kinases also mediate the actions of cAMP by phosphorylating histones and non-histone proteins present in the nucleus and this has been reviewed by Johnson (1977).

XIII. Cyclic AMP Metabolism and Malignancy

Numerous reports suggest that altered cyclic AMP metabolism is in part responsible for some of the abnormal properties associated with malignant cells especially growth regulation and this interrelationship between cAMP, growth control and malignancy will be discussed in detail in the following section. Although it is now well established that many malignant cell lines exhibit reduced cAMP levels (Pastan et al., 1975) there does not appear to be a clearly defined aberration at a single site and indeed defects in various components involved in both cAMP synthesis and degradation have been observed.

Thus adenylate cyclase activity is decreased in both malignant and transformed cells (as catalogued in a review by Ryan and Heidrick, 1974) although this is contradicted in reports on hepatocarcinomas (Christoffersen et al., 1972) and SVT2 transformed 3T3 cells (Peery et al., 1971). Viral mutants that are temperature sensitive in their transformed function have been used in chick embryo fibroblasts to show that decreased adenylate cyclase activity is due to transformation (Anderson et al., 1973) but paradoxically it has recently been suggested that the reduced enzyme activity appears after the changes in morphology and therefore cannot be responsible for them (Yoshida et al., 1977).

Hormonal stimulation of cAMP synthesis is reduced in leukaemic leukocytes (Polgar et al., 1973) and more recently a reduction in the number of catecholamine receptors has been detected in similar cells (Sheppard et al., 1977). In contrast several reports suggest that increased cAMP phosphodiesterase activity may be responsible for altered cAMP metabolism in malignant cells (Kedan et al., 1976; Hait and Weiss, 1977) while a lesion in a cAMP dependent protein kinase has been correlated with increased tumourigenicity in neuroblastoma cells resistent to the cytotoxic effects of dibutyryl cAMP (Simantov and Sachs, 1975).

Since malignancy is associated with many alterations in the plasma membrane (Nicolson, 1976(b)) it could be envisaged that changes in the membrane itself are responsible for aberrations in the membrane-bound component of cAMP metabolism or in the coupling between them. Comparisons of normal and transformed 3T3 cells suggest that transformation is accompanied by increased membrane viscosity associated with reduced fluidity (Fuchs et al., 1975) although the opposite situation exists in leukaemic lymphocytes (Ben-Bassat et al., 1977) and this might be explained by the variation in the cholesterol:phospholipid ratio in these cells (Shinitzky and Inbar, 1976). Thus uptake of cholesterol from liposomes causes decreased adenylate cyclase activity in fibroblasts and this may arise from a reduction in membrane fluidity brought about by incorporation of increased cholesterol into the

membrane (Klein et al., 1976).

The membrane-associated cytoskeletal elements may also be coupled to adenylate cyclase (Helmreich, 1976) so that a reduction in the array of microtubules and microfilaments in malignant cells (Brinkley et al., 1975) would be likely to disrupt cAMP synthesis.

IX. Cyclic GMP

In comparison with cAMP, relatively little is known about the role of cyclic GMP in the cell. This could be because cGMP is present at concentrations 1 to 2 orders of magnitude lower than cAMP and hence is more difficult to detect, or because the actions of cGMP are relatively limited and not easily observed (Goldberg et al., 1973).

Cyclic GMP was first discovered in urine (Ashman et al., 1963) and is synthesised by both particulate and soluble guanylate cyclase (Kimura and Murad, 1975) which requires manganese ions for activity (Hardman and Sutherland, 1969). Degradation of this cyclic nucleotide is catalysed by a specific cGMP dependent phosphodiesterase (Davis and Kuo, 1977) and by a phosphodiesterase which degrades both cAMP and cGMP (Appleman and Terasaki, 1975) while the actions of cGMP appear to be mediated by protein kinases which are homologous to cAMP dependent protein kinases (Lincoln and Corbin, 1977). The metabolism of cGMP has been described by Goldberg et al. (1973).

Many of the investigations into cGMP action have been concerned with the inverse relationship between cGMP and cAMP, in the mediation of cell proliferation

(Goldberg et al., 1974) and this will be discussed in a subsequent section. Alternatively cAMP and cGMP may mediate the action of different hormones in the same tissue, as exemplified by the pancreas where cAMP causes increased fluid and ion secretion in response to secretin, while acetylcholine stimulates cGMP synthesis which increases enzyme secretion (Albano et al., 1976).

C. CYCLIC AMP AND GROWTH CONTROL IN NORMAL AND MALIGNANT CELLS

The characteristics of growth of normal and transformed cells have been reviewed by Macpherson (1970) and the membrane changes associated with transformation and malignancy are described by Nicolson (1976b). With the observation that cAMP inhibited growth in normal and transformed cells in culture (Burk, 1968; Ryan and Heidrick, 1968) intensive research was initiated to determine the role of cAMP in growth control (Pastan et al., 1975; Friedman, 1976; Makman et al., 1977; Abell and Monohan, 1973) and the possible implications in malignancy (Ryan and Heidrick, 1974; Pastan and Johnson, 1974).

I. Morphology and Adhesion

Normal cells in culture require support from plastic or glass surfaces for growth. They attach to these surfaces and assume a flattened appearance. Virally transformed cells tend to show an altered morphology (Medina and Sachs, 1963) and early investigations revealed that addition of N^6,2'-O-dibutyryl cAMP (dbcAMP) produced a reversion in the cells from a random arrangement of polygonally shaped cells to a parallel array of elongated cells

characteristic of normal fibroblasts (Hsie and Puck, 1971). This restoration of normal morphology appeared to involve assembly and alignment of microtubules (Porter et al., 1974; Willingham and Pastan, 1975) which was substantiated by the observation that virally transformed cells contained diminished numbers of microtubules (Brinkley et al., 1975).

A loss in the adhesive properties of transformed cells and a temperature sensitive mutant from 3T3 cells was also attributed to decreased cAMP levels. (Schields and Pollock, 1974; Willingham et al., 1973). This may be connected with the loss of anchorage dependence in transformed cells, which is characterised by growth in soft agar and the ability to form suspension cultures of round cells. (Macpherson, 1970). Anchorage independence correlates well with tumourigenicity in vivo (Shin et al., 1975; Spandidos and Siminovitch, 1977) and when considered with increased agglutinability of malignant cells may be important in metastasis.

The ability of dbcAMP to induce morphological differentiation in neuroblastoma cells has been studied extensively, by Prasad and his colleagues (Prasad and Kumar, 1974) and the effects of cAMP on various forms of differentiation, adhesion and morphology have been discussed in detail by Friedman (1976).

II. Cell Proliferation

cAMP levels are inversely related to growth in both normal and malignant fibroblasts (Otten et al., 1971). Thus malignant cells characterised by a higher growth rate possess decreased levels of cAMP, which suggests that growth control could be restored by increasing cAMP levels. Numerous reports now substantiate the early observations that dbcAMP inhibits cell proliferation (as tabulated in Makman et al., 1977) and normal fibroblasts such as WI-38 cells exhibit elevated cAMP levels at quiescence (D'Armiento et al., 1973).

Agents such as serum and insulin stimulate DNA synthesis and proliferation (Leffert and Koch, 1977). It has now been established that the three factors from serum which are required together to stimulate growth are epidermal growth factor, insulin and vitamin B_{12}, (Mierzejewski and Rozengurt, 1976). Addition of serum to quiescent cells is followed by a rapid fall in cAMP levels (Seifert and Rudland, 1974) and co-incubation with dbcAMP or agents known to increase cAMP, antagonises the action of these mitogenic factors (Froehlich and Rachmeler, 1972). This led to the conclusion that a reduction in cAMP concentration was the trigger for DNA synthesis.

However, this has now been disputed since exogenous cAMP did not inhibit serum stimulation of DNA synthesis until 8 hours after serum addition. This is consistent with the presence of a cAMP sensitive inhibitory locus in mid G_1 of the cell cycle (Rechler et al., 1977).

Regulation of growth in vivo may also be controlled by the synthesis of prostaglandins since self-regulation in vitro is achieved by PGE_2 which inhibits division and $PGF_{2\alpha}$ which stimulates proliferation (Taylor and Polgar, 1977).

Several reports indicate that cGMP is the positive modulator of growth stimulation although it has been suggested that the inverse relationship between cAMP and cGMP described by the yin-yang hypothesis may be the true mediator of cell proliferation (Goldberg et al., 1974).

An alternative approach to examing cell proliferation in fibroblasts has been the study of peripheral lymphocytes which exist in a state of quiescence unless stimulated to grow by antigens or plant lectins. There are two conflicting schools of thought; one proposes that cAMP mediates the stimulation by mitogens (Parker et al., 1974) while the other suggests that cAMP acts

in concert with cGMP as described above for fibroblasts. (Hadden et al., 1972). The considerable evidence for both proposals has been comprehensively reviewed by Friedman (1976).

Calcium ions may also be important as intracellular mediators of growth, and Berridge (1975) has proposed a model for cell division in which calcium acts as the primary mitogenic signal and the cyclic nucleotides interact to modulate regulation (Figure 8).

The role of calcium is substantiated by the mitogenic effects of the Ca^{2+} ionophore (A23187) in human peripheral lymphocytes (Jensen et al., 1977) and by the lack of responsiveness of transformed WI-38 cells to changes in calcium (Boynton et al., 1977)

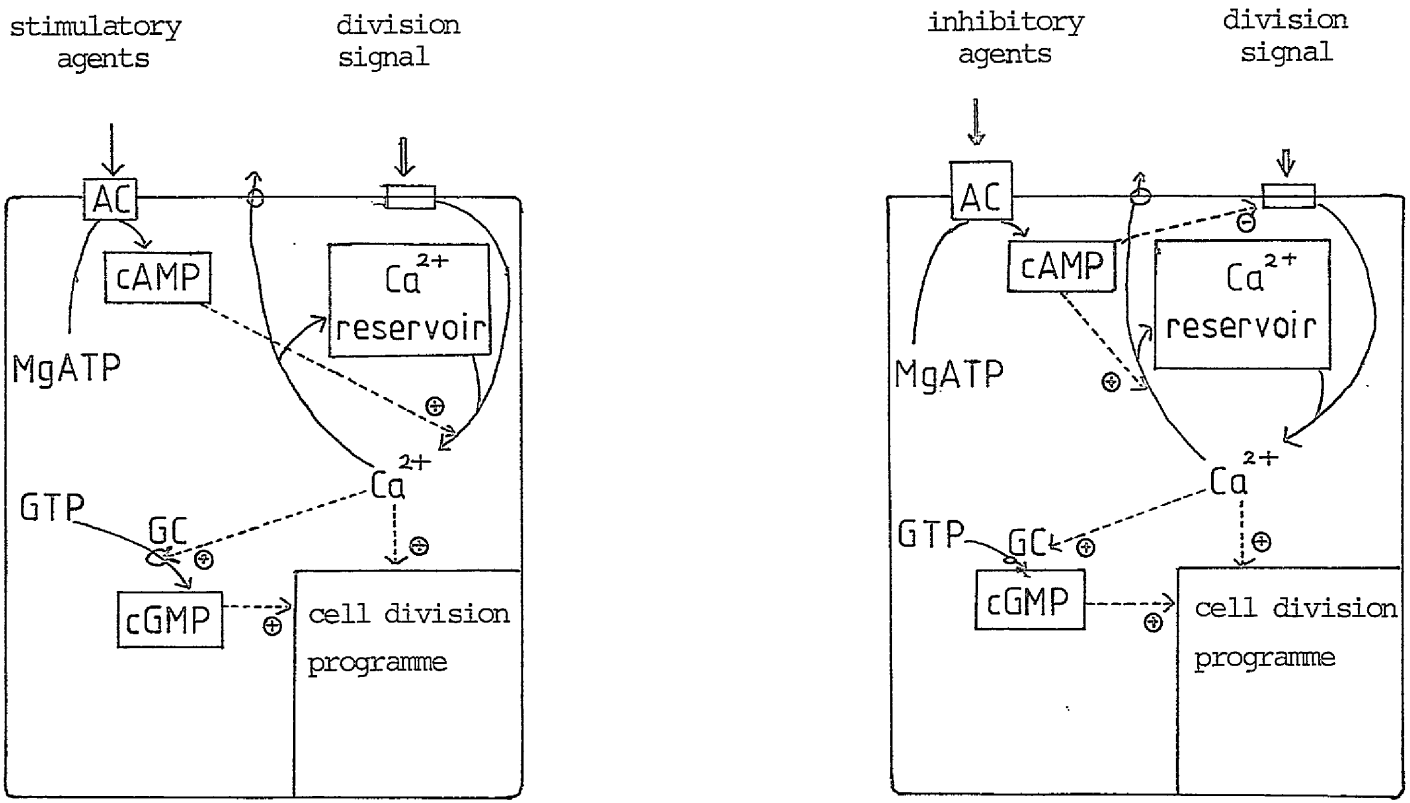


FIGURE 8. The Control of Cell Division

In (A) cAMP augments the Ca^{2+} signal while in (B) cAMP opposes the Ca^{2+} signal (GC = guanylate cyclase. AC = adenylate cyclase).

III. Inhibition of Growth

With the observation that cell contact induced a quiescent state in cells (Abercrombie and Heaysman, 1954) it was proposed that as cells became crowded at confluency contact inhibition of growth occurred. Transformed cells did not show contact inhibition and tended to pile up in random arrays (Temin and Rubin, 1958).

It has subsequently been suggested that growth inhibition is due to serum restriction which can be relieved by humoral factors (Mierzejewski and Rozengurt, 1977; Shields, 1976) and this agrees with the decreased serum requirement observed in transformed cells. Thus density dependent inhibition of growth is now used to describe quiescence.

Several reports have shown that as normal fibroblasts reach confluency their cAMP levels rise (D'Armiento et al., 1973) and in comparison transformed cells do not appear to exhibit this increase (Otten et al., 1971). However, induction of quiescence by serum restriction and density dependent inhibition may occur by independent processes and while the former is known to produce increased cAMP levels, conflicting reports suggest that cAMP levels increase (Otten et al., 1971) or remain the same

(Oey et al., 1974) as a result of density dependent inhibition. These mechanisms may be resolved by the application of more rigorous experimental conditions.

It is interesting to note that the response of cAMP to hormones and fluoride appears to change with increasing cell density in WI-38 fibroblasts (O'Neill and Hsie, 1975) and C_6 astrocytoma cells (Morris and Makman, 1976).

IV. The Cell Cycle

The foregoing sections suggest an involvement of cAMP in growth control which can only be fully understood when the role of cAMP in the regulation of the cell cycle is resolved. The cell cycle has been divided into 4 main phases, for convenience, and these are depicted in Figure 9, which shows the cell undergoing DNA replication (S) or mitosis (M) or preparing for these events (G_1 and G_2). G_o is the resting or quiescent state and it is not known whether this is a distinct phase or just an extension of G_1 (Friedman, 1976).

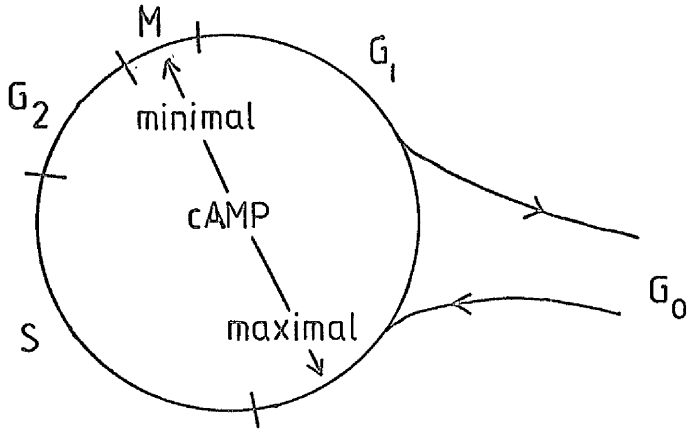


FIGURE 9. THE CELL CYCLE

The cell cycle of transformed cells appears to be similar except that the ability to enter G_o is lost.

Cyclic AMP levels are generally considered to be minimal in mitosis and maximal in late G_1 with fluctuation throughout the other stages (Zeilig et al., 1976). In human lymphoid cells, adenylate cyclase activity appears to follow this trend while cAMP phosphodiesterase increases to maximal in mitosis (Millis et al., 1974). Much of the information on the modulation by cAMP has been obtained from the addition of cAMP analogues and the subsequent determination of the inhibition point in the cell cycle. Thus cells incubated with cAMP appear to be arrested in both G_1 (Coffino et al., 1975b) and G_2 (Kurz and Friedman, 1976) depending on the cell type and the stage in the cycle when cAMP is added. The interpretation of cAMP levels in synchronised cells is limited by the difficulties inherent in the synchronisation protocols.

In comparison with cell cycle traverse, quiescence appears to arrest cells in G_1 (G_o) and the interrelationship between cAMP, cGMP and calcium in regulating entrance to and exit from the G_o stage of the cell cycle has been discussed previously.

The role of cyclic nucleotides in the cell cycle has been reviewed by Friedman *et al.*, (1976).

V. Cell Surface Glycoprotein and Adhesion

A major cell surface glycoprotein (CSP or LETS protein) is apparently decreased or absent from the surface of transformed cells (Hynes, 1973). This cell surface glycoprotein has been identified in several fibroblastic cell lines and isolated by urea extraction (Yamada and Weston, 1974). It represents 3% of the total cell protein in chick embryo fibroblasts and has a characteristic subunit molecular weight of 200-250,000 daltons, by SDS-polyacrylamide gel electrophoresis.

CSP is sensitive to proteolysis by trypsin and is present on the cell surface as disulphide-bonded complexes (Hynes and Destree, 1977), the distribution of which varies among cell strains in a tissue specific manner (Mosher et al., 1978). CSP is secreted into the medium suggesting that it is similar to collagen, although collagenase treatment reveals that it is distinct. (Yamada and Weston, 1974).

The loss of CSP from transformed cells can be compensated by incubation with CSP, isolated from normal fibroblasts. This produces a reversion in their morphology in that they become elongated and flattened showing increased adhesion to the substratum (Yamada

et al., 1976). Several transformed cell lines also showed increasing alignment upon incubation with CSP, suggesting a role in restoration of contact inhibition of movement. Examination of the levels of CSP in normal fibroblasts at different densities showed that the CSP concentration increased at confluency and this was also observed when cells were inhibited in G_1 of the cell cycle by serum starvation. Readdition of serum produced a decrease in CSP which reached a minimum at mitosis when the cells had rounded-up (Hynes and Bye, 1974).

The primary action of CSP appears to be on cell adhesion, since it failed to slow the growth rate of transformed cells (Yamada et al., 1976). This is corroborated by the fact that this cell surface glycoprotein acts as an agglutinin, causing aggregation of formalised sheep erythrocytes. (Yamada et al., 1975). However, the effects of cAMP and CSP appear to be distinct since incubation with isolated CSP does not elevate cAMP levels (Yamada et al., 1976).

The effects of this cell surface glycoprotein on morphology and adhesion have been confirmed in normal and HSV-transformed NIL hamster fibroblasts. (Ali et al., 1977). Furthermore, CSP appears to restore the arrangement of actin cables, suggesting an interaction with cytoskeletal organisation. This is in agreement

with observations that cytochalasin B, which disrupts microfilaments, increases the quantities of CSP in the medium. (Ali and Hynes, 1977). However, Schlessinger et al., (1977) have shown that agents which disrupt microtubules and microfilaments do not alter the relative immobility of CSP, suggesting that the glycoprotein is not attached to the cytoskeletal elements.

The absence of CSP from transformed cells is in part due to a reduced rate of synthesis, although increased turnover also appears to contribute to the loss of CSP (Olden and Yamada, 1977). It has not been ascertained whether the route of processing of the glycoprotein is internal → surface → medium or internal → medium → surface and it may be that an equilibrium exists between the two routes (Hynes et al., 1977). Clearly the loss of CSP from transformed cells cannot be understood fully until the mechanism of incorporation into the membrane has been established.

The alterations in cell surface proteins associated with transformation have been comprehensively reviewed by Hynes (1976) and Yamada and Pastan (1976).

METHODS

CELL LINES

HUMAN EMBRYONIC LUNG CELLS: AP-9

These cells were obtained from an aborted embryo and kindly donated by I.C.I. Alderley Park. They are primary diploid fibroblasts and as such undergo 22 passages before their growth is exhausted. Being fibroblasts they grow adhering to clean glass surfaces and appear as long spindle-shaped cells. At high cell density AP-9 cells align to produce a monolayer of whorl-like threads.

This cell line has been maintained in Eagle's minimal essential medium (MEM) supplemented with 10% foetal bovine serum (FBS) as described below, and is normally subcultured every 4-5 days with a split ratio of 1:3. The generation time of these cells is about 28 hours.

HUMAN BURKITT LYMPHOMA CELLS: EB_2

The Burkitt lymphoma cells were isolated from the jaw tumour of a nine year old Ugandan boy and established *in vitro* as a continuous cell line. They grow in suspension and at high cell density tend to form clumps which are just visible as a cloudy suspension of particles.

In this laboratory, EB_2 cells have been cultured in MEM supplemented with 10% FBS and cells are usually passaged to give an initial density of $2.5 - 7.0 \times 10^5$ cells ml^{-1}. This cell line has a generation time of 16-20 hours.

HUMAN LYMPHOBLASTIC LEUKAEMIA CELLS: SKL-2

SKL-2 cells were isolated by Dr. Baynard Clarkson at Sloane Kettering and obtained from Flow Laboratories in 1974. The interesting morphological characteristic of these cells is that they do not readily adhere to glass surfaces but are generally found adhering to each other so that they can be observed in suspension as white balls 1-2mm in diameter, especially at limiting cell density.

This is a continuous cell line maintained in MEM supplemented with 10% FBS as described below and usually subcultured to give an initial cell density of 4.0×10^5 cells ml^{-1}.

CHINESE HAMSTER CELLS: CH23

This cell line was isolated after a biopsy of an inbred Chinese hamster (*Gricetulus griseus*) in 1958 (Tjio and Puck, 1958) and has since been maintained *in vitro* as a continuous cell line. It was cloned in this laboratory (Ayad and Delinassios, 1974) and the clone used in these studies (CH23) was characterised by the presence of 23 chromosomes. The chromosomal composition fluctuates between 21-26 in diploid cells and 8% polyploidy exists. Karyotype analysis has shown the presence of X and Y chromosomes which indicate that the cells were isolated from a male hamster (Delinassios, 1974).

CH23 cells grow as a monolayer, adhering to glass surfaces and exhibit characteristics typical of a fibroblast in that they grow as long narrow cells which form tightly packed parallel arrays as the cell density increases. They have a generation time of 20-21 hours and are passaged to give an initial cell density of 5.0×10^4 cells ml^{-1}. Throughout these studies CH23 cells have been grown in MEM supplemented with 10% newborn calf serum (NCS) as described below.

MOUSE LYMPHOMA CELLS: P388F-36

Isolation of P388F cells has been described by Fox and Gilbert (1966) and the clone used (P388F-36) was obtained by Delinassios (1974). This clone is characterised by a dominant karyotype of 36 from a range in chromosome number of 34-46. Polyploid cells account for 27% of the population and the diploid cells contain mainly acrocentric chromosomes, with only 3-4 that are metacentric.

This is a continuous cell line growing to high cell density in suspension, with a generation time of 12-13 hours. They are normally passaged to give a cell density of approximately 2.0×10^5 cells ml^{-1} but they can be seeded at much lower densities. These cells have been routinely grown in MEM supplemented with 10% NCS for direct comparison with CH23 cells.

HYBRID CELLS: PCM3

Fusion of Chinese hamster cells CH23 and P388F-36 mouse lymphoma cells using inactivated Sendai virus produced a series of hybrids that were isolated by a method of non-selective pressure. (Ayad and Delinassios, 1974). The hybrids growing as monolayers were designated PCM and karyotype analysis showed selective loss of mouse chromosomes resulting in a relatively stable chromosomal composition of no fewer than 19 hamster chromosomes coexisting with several mouse chromosomes. Investigation of the hybrids isolated as suspension cells PCS showed that they selectively lost hamster chromosomes retaining mainly mouse chromosomes. Cloning of the PCM hybrid produced several clones characterised by distinct isozyme patterns for esterase activity which were clearly of a hybrid nature, (Ayad and Delinassios, 1974) and by cyclic nucleotide dependent phosphodiesterase activity which shows hybrid characteristics (Ayad and Wright, 1977).

One of the clones, designated PCM3, grows as a monolayer with 10-20% of the population either loosely attached or in suspension. The cells which adhere are much rounder than CH23 cells and are characterised by several small processes which extend from the cell body. Either monolayer (PCM3-M) or suspension (PCM3-S) can be used to seed a culture at a cell density of approximately 4.0×10^4 cells ml^{-1}. The medium used for these cells throughout the study has been MEM supplemented with 10% NCS which is described below.

MEDIA

FBS Medium

The human cell lines (AP-9, EB-2, SKL-2) were routinely cultured in this medium which was made by mixing sterile solutions of the following:-

654ml	double distilled water
100ml	Eagles Minimal Essential Medium (Gibco Bio-cult)
100ml	foetal bovine serum (Gibco Bio-cult)
20ml	200mM glutamine (Flow Labs)
25ml	4.4% sodium bicarbonate solution (Wellcome Labs.)
100ml	non-essential amino acids
0.2ml	0.5g streptomycin sulphate BP (Glaxo Labs.)
0.4ml	250,000 units/ml benzyl penicillin (Na) BP (Glaxo Labs.)

The antibiotics were alternated at intervals with:

1.0ml	2.5mg/ml amphotericin B (Squibb Ltd.)
5.0ml	40mg/ml gentomycin (Flow Labs.)

The non-essential amino acids were dissolved in double distilled water and sterilised by filtration (0.22μ Millipore filters):-

g per litre	amino acids
0.089	L-alanine
0.150	L-asparagine H_2O
0.133	L-aspartate
0.075	glycine
0.147	L-glutamine
0.115	L-proline
0.105	L-serine

NCS Medium

All other cell lines (CH23, P388F-36, PCM3) were cultured in medium containing 10% newborn calf serum, and the following sterile solutions were combined:

764ml	double distilled water
100ml	Eagles minimal essential medium (Flow Labs.)
100ml	newborn calf serum (Flow Labs.)
10ml	200mM glutamine (Flow Labs.)
25ml	4.4% sodium bicarbonate solution (Wellcome Labs.)
0.2ml	0.5g/l streptomycin sulphate BP (Glaxo Labs.)
0.4ml	250,000 units/ml benzyl penicillin (Na) BP (Glaxo Labs.)

The antibiotics were alternated at intervals with:

1.0ml	2.5mg/ml amphotericin B (Fungizone)(Squibb Ltd.)
5.0ml	4.0mg/ml gentomycin (Flow Labs.)

The pH of the medium was maintained at 7.4 by the presence of $NaHCO_3$ and CO_2 which was introduced by gassing the cultures with 5% CO_2 in air. The medium was stored at $4^{\circ}C$ for up to two months without detrimental effect on cell growth.

Several experiments used cells grown on the bottom of scintillation vials and as this involved large numbers of vials it was impractical to gas each vial. The correct pH was therefore maintained with the organic amine buffer

HEPES (N-2-hydroxyethyl piperazine-N'-2-ethanesulphonic acid) at a concentration of 20mM. The cells do not show any altered characteristics in HEPES buffered medium and other cell lines have also been shown to grow favourably in these conditions (Itzgaki and Kimura, 1974; Franklin and Twose, 1976).

CELL CULTURE METHODS

The cells were routinely grown at 37°C in 100ml glass medical bottles and cultured in bottles of differing capacity for experimental purposes:

Flat glass bottles	10ml medium
Flat glass bottles	20ml medium
Roux bottles	100ml medium
Carrel flasks	200ml medium.

The volume of medium in each type of bottle should not be excessive as too much medium resulted in limited growth as a result of restricted gaseous exchange.

These bottles were washed at regular intervals with detergent (RBS-25), acid (dilute HCl) and finally double distilled water. After each incubation the bottles were soaked and then rinsed with double distilled water. Sterilization of bottles and salt solutions was achieved by autoclaving for 20 minutes at 15 lbs. per square inch. All pipettes were soaked in lysol after use, rinsed in double distilled water and sterilised by dry heat in an oven at 180°C for 90 minutes.

Passage of Cells

Suspension cells: These were subcultured by placing a small aliquot of cells from a stationary culture into a sterilised bottle with the required amount of fresh medium

to dilute the cell number by the appropriate factor.

AP-9 cells. The cell sheet of a stationary culture was washed with a solution of Hank's balanced salt solution (HBSS) and then trypsinized using 2-4ml of 0.025% trypsin (Worthington) for 5 minutes at 37°C. The trypsin stock solution had been sterilised by millipore filtration and diluted with HBSS before storage at -20°C. After trypsinization, fresh medium was added to inhibit the activity of the enzyme and the cells dispersed by gentle pipetting. The appropriate volume of fresh medium was then added and the cell suspension divided into sterile bottles.

The Hank's balanced salt solution (HBSS) (Paul, 1970) was used without Ca^{2+} and Mg^{2+} ions which caused cell aggregation. The following salts were dissolved in double distilled water and the solution autoclaved, as described above:

g/l	
8.34	NaCl
0.4	KCl
0.06	$Na_2HPO_4 2H_2O$
0.06	KH_2PO_4
1.00	D-glucose
0.35	$NaHCO_3$
0.002	phenol red

The pH of the solution was corrected to 7.6 at 37°C using 1M HCl.

PCM3-M and CH23 Cells: These cells were subcultured by washing the cell sheet with trypsin salt solution and by trypsinizing the cells for 5 minutes at 37°C with 2-4mls of 0.05% trypsin (Difco) dissolved in trypsin salt solution. When the cell sheet was visible as an opaque layer and the cells had started to detach fresh medium was added to inactivate the trypsin. The cells were then gently dispersed with a pipette and diluted with the appropriate amount of fresh medium, before being transferred to sterile bottles.

The trypsin salt solution was obtained by dissolving the salts in double distilled water and correcting the pH to 7.7, which is optimal for trypsin action, without causing cell damage. Sterilisation was achieved by autoclaving, as described above, and the solutions were stored at 4°C. The following concentrations were used:

g/l	
6.85	NaCl
0.54	KCl
8.11	D-glucose
6.04	tris-HCl
0.01	phenol red

Storage of Cell Lines

Cells may be stored indefinitely in liquid nitrogen in a biological freezer without any morphological change or viability loss. This is most important for primary cell lines such as AP-9 which do not grow in culture after 22 passages. It is therefore necessary to freeze large quantities of these cells at an early generation to provide enough cells for later studies. Continuous cell lines are often subjected to spontaneous alterations in the distribution of chromosome number and in chromosome morphology (Barski, 1964). Therefore, frequent karyotype analysis is required to monitor for any such alterations and regular replacement of cell lines, from stock cultures in liquid nitrogen, is advisable.

Suspension cells were prepared for freezing by centrifugation at 750g for 5 minutes under sterile conditions. The cells were then resuspended to give 6ml of approximately 10^6 cells per ml. Cells attached to glass were first trypsinized as described above and then resuspended in 6ml fresh medium to give 10^6 cells per ml. Both cell types were then treated similarly in that the solutions used were:-

6ml	cell suspension in fresh medium
3ml	foetal bovine serum
1ml	DMSO (dimethyl sulphoxide)

After careful mixing the resulting suspension was pipetted into sterile 1ml ampoules which were sealed and placed in the biological freezer within 10 minutes of DMSO addition. The cells were cooled at 1°C per minute to -70°C in a Union Carbide BF-6 biological freezer, and once at -70°C the ampoules were stored in liquid nitrogen (-196°C). The DMSO is used to prevent cell damage which might occur due to ice crystal formation and also to reduce osmotic changes within the cell.

When necessary the cells were removed from liquid nitrogen and thawed rapidly, by placing the ampoule in warm water, so that the cells could be quickly diluted in 10ml medium under sterile conditions.

MORPHOLOGICAL OBSERVATION AND VIABILITY ESTIMATION

The morphology of monolayer cells was examined by subculturing the cells into sterile petri dishes containing coverslips and allowing the cells to grow to the necessary density. The coverslips containing cells were then washed with physiological saline (0.9%), fixed in methanol (2 changes of 3 minutes each) and then air dried. Staining was accomplished by putting the coverslips in 1% methyl violet (dissolved in distilled water) for 2-3 minutes, and then rinsing with distilled water before air-drying Methyl violet stains the nuclei dark purple and the cytoplasm light blue.

Suspension cells were collected by centrifugation at 750g for 5 minutes and then treated in a similar manner to monolayer cells. The cells were fixed on the coverslips as a result of briefly flaming the drop of cell suspension after it had been spread over the glass surface.

Microscopic examination of these cells was carried out using a Zeiss microscope with a Zeiss camera attachment and the black and white films used were Kodak Panatonic X.

It is particularly important to be able to ascertain the number of viable cells in stationary cultures, as decreased viability can seriously affect the results of growth experiments with suspension cells and significantly alter enzyme activities, measured as a function of protein concentration.

Several techniques were used to study cell viability including:-

1. The dye exclusion technique using 1% trypan blue in 0.1% HBSS. The method involves mixing cells in suspension with stain (1:1) and leaving them 10 minutes before counting on a Hawksley-Neubauer counting chamber. Viable cells exclude the dye and remain unstained while non-viable ones stain dark blue (Paul, 1970).

2. The opaque nature of cells under phase contrast may also be used to determine viability.

3. Janus green can be used in a viability test and in this instance the viable cells take up the dye.

Monolayer cells can only be absolutely tested in this way if they are grown on coverslips. However, in experiments where the cell number is determined using an electronic counter, then a small aliquot may be removed after trypsinization and resuspension and this aliquot can then be tested for relative viability after trypsin treatment.

KARYOTYPE ANALYSIS

Karyotype analysis is necessary to characterise new cultures and to maintain continuous cultures with stable genetic composition. Replenishment of continuous cultures from cells stored in liquid nitrogen should also be followed by a comparative study of the chromosomal patterns to ascertain whether any changes have occurred. This method for isolating the cells in metaphase has also been used to examine the mitotic phase of the cell cycle by estimating the percentage of cells undergoing mitosis.

This technique is an adaption of the method used by Tjio and Puck (1958) and depends on the fact that colchicine distrupts microtubules and hence causes cells to accumulate in metaphase. It also relies on the fact that mitotic cells swell in hypotonic solutions causing the chromosomes to separate so that after flattening they are easily visible under the light microscope (Hsu and Pomerat, 1953).

The method involved growing monolayer cells in 100ml medical bottles until the cells reached mid-log and then incubating them for 4-6 hours at 37^{o}C with 20µg/ml colchicine dissolved in HBSS. Concentrations of colchicine in the range 0.5 - 25.0µg/ml were used initially but it was decided that a final concentration of 20µg/ml was

optimal for accumulation of mitotic cells. It was also observed that addition of 10% fresh medium was advantageous to isolation. After incubation with colchicine the cells were scraped from the glass surface, using a rubber policeman. They were then combined with the medium and centrifuged at 750g for 10 minutes. Cells in suspension were incubated with colchicine in the same way and collected by centrifugation as described above.

Both types of cells were then resuspended in 5mls hypotonic HBSS (1ml HBSS: 9ml double distilled water) at 37^{o}C for 15-30 minutes depending on the type of cell. The centrifugation was then repeated to collect the cells and 0.2-0.5ml of acetic acid:methanol (1:3) added to fix the cells. After 10 minutes at 37^{o}C the cells were dispensed on to clean microscope slides, air dried, and flattened by the application of even pressure over the coverslip. Staining was then achieved using acetic-orcein and semi-permanent mounts made using Canada balsam.

DETECTION AND PREVENTION OF CONTAMINATION

It is relatively simple to detect contamination of cell cultures by bacteria and fungi because they usually grow faster than the mammalian cells producing a dense culture with a characteristic smell. They can easily be characterised under the microscope and once this contamination has been detected it is necessary to autoclave all the bottles and then discard all the solutions. The best method of prevention is good aseptic technique and the presence of antibiotics can help to suppress their growth, especially if the antibiotics are varied as this protects against resistance.

Intracellular contamination can also occur due to the presence of viruses and mycoplasma (pleuroneumonia-like organisms PPLO). These organisms are more difficult to detect and unlike the other types of contamination they do not harm the cells but they do seriously alter experimental results. If cells become infected it is better to dispose of them and replenish the cell line from stocks which have been frozen and are known to be free of contamination.

The method used for detection of viruses and mycoplasma is that of Nardonne *et al* (1965) and the general technique of autoradiography is described by Prescott (1964). Monolayer cells in 100ml medical bottles were grown for 6 generations in the presence of 0.1-0.2μCi/ml ^{3}H-thymidine and then trypsinized and resuspended in fresh medium without radioactive label. They were then placed in sterile petri dishes containing coverslips, to which the cells adhered

and overnight they were able to metabolise free ^{3}H-thymidine. The pH of the medium in the petri dishes was maintained using either HEPES buffered medium or a CO_2-saturated atmosphere, obtained by storing the dishes in sealed plastic boxes which have been gassed. After overnight incubation the coverslips were washed in HBSS (Hank's balanced salt solution) and fixed in methanol. After drying they were mounted cell side up onto slides using Canada balsam.

Suspension cells were also incubated in 100ml medical flats for 6 generations, in the presence of 0.1-0.2μCi/ml ^{3}H-thymidine and were collected by centrifugation at 750g for 10 minutes. The cells were then resuspended in fresh medium without radioactive label and incubated overnight to allow them to metabolise any free ^{3}H-thymidine. Washing of the cells was achieved by centrifugation, as described previously and resuspension in HBSS. They were then collected by centrifugation and fixed in methanol. Drops of this suspension were placed on clean slides and allowed to dry.

Autoradiographs were prepared in the dark room, with only the red filter safety lamp on. Pieces of film approximately 3 x 2cm were cut from Kodak fine grain autoradiographic plate AR-10 and floated, emulsion side down for 3 minutes on distilled water at 21^{o}C. The slides containing the cells were then placed under the film at an angle of 30^{o} to the horizontal and gradually lifted, so that one edge came into contact with the film first and then the rest of the film clung round the slide. These were then

placed in racks, dried with a fan and stored in boxes containing silica gel for 1 to 4 weeks at 4°C to allow labelling to occur. Slides were removed at intervals during this time to examine the degree of labelling.

The film coated slides were developed as described below:-

1. The slides were equilibrated at 21°C and then dipped into Kodak D-19 developer (at 21°C) for 4 minutes.

2. They were then rinsed for not less than 30 seconds in double distilled water at 21°C.

3. The slides were then fixed in Kodak Metaphix (21°C) for 10-15 minutes.

4. Finally they were rinsed in running water or 2% acetic acid for 10 minutes and air dried.

High background can be decreased by keeping the slides away from the safety lamp, while maintenance of temperature helps to prevent film slippage. This can also be eliminated by subbing the slides and this is carried out by dipping the slides into a filtered solution made from:

5g Gelatin
0.5g Chrome alum
1L distilled water

The slides were then dried at 60°C before being used.

The cells were stained either before film application or after development and the slides were dipped into a filtered solution of Giemsa for 5-30 minutes and then rinsed in distilled water. They were examined using a Zeiss microscope to determine the location of the silver grains. Grains found over the whole surface of the cell and in intercellular spaces suggest contamination is present, while uninfected cells would be expected to exhibit labelling only over their nuclei, as a result of incorporation of ^{3}H-thymidine into their DNA.

DETERMINATION OF CELL NUMBER

Two techniques can be used to estimate cell number. It is possible to count cells viewed under a microscope using a Hawksley-Neubauer haemocytometer chamber, while it is often more convenient to count cells electronically with a Coulter counter.

To estimate cell number using a haemocytometer, monolayer cells were grown on coverlsips in sterile petri dishes and after the specified time they were removed and placed on the haemocytometer, cell side down. The coverslips were pressed down firmly until Newton's rings were observed and then the cells were examined under the microscope. Trypsin treated monolayer cells and cells grown in suspension were counted by placing a drop of culture on the haemocytometer and pressing a coverslip on them as described above. Care had to be taken to reduce the number of cell clumps in these suspensions and this was achieved by keeping the cells at $4^{o}C$ and by gently pipetting the suspension before counting.

The concentration was obtained by counting the number of cells in the 25 squares. This covers an area of $1mm^2$ and has a depth of fluid of 0.1mm, so that the volume is 1×10^{-4}ml and hence the number of cells per ml can be ascertained. The advantage of this method is that cell clumps and non-cellular material can be recognised and cell viability estimated.

However, when large numbers of samples are involved it is impractical to use a haemocytometer and therefore cells have to be counted electronically. They are prepared by obtaining cell suspensions, as described above, and then washing these at $4^{o}C$ with filtered isotonic saline (0.9% NaCl) followed by centrifugation at 750g for 10 minutes. The cells are suitably diluted in isotonic saline, which has previously been filtered using a 0.22μ Millipore filter to remove dust particles. The Coulter counter (Model B Coulter Electronics, St. Albans) operates by sucking 0.5ml cell suspension through a 100μm aperture between two electrodes so that the cells trigger an electric signal. The threshold settings are 9-100 to eliminate very small particles. The setting for 1/aperture current = 1,while the 1/amplification varied depending on the cell type.

The cell number recorded on the counter was corrected using a coincidence factor of 2.5 to give a value for the number of cells in 0.5ml, thus allowing the concentration of cells to be obtained. This method does not differentiate between viable and non-viable cells and therefore it is necessary to determine viability microscopically. Another disadvantage of this method is that cell clumps can reduce the actual cell number so clumping must be kept minimal during preparation.

PROTEIN DETERMINATION

The protein concentration was determined by the method of Lowry *et al.* (1951). The following reagents were used:-

A. 2% Na_2CO_3 in 0.1M NaOH

B. 0.5% $CuSO_4$ $5H_2O$

C. 1.0% NaK Tartrate

D. 50ml A + 0.5ml B + 0.5ml C (freshly made)

E. Diluted Folin-Ciocalteau phenol reagent (1:2 in double distilled water)

F. Standard protein (bovine serum albumin) 0-1.0mg/ml in double distilled water.

2.5ml of solution D was added to 200µl each of water blank, buffer blank, standards (F) and samples. These solutions were mixed and left for 10 minutes before the addition, with rapid mixing of 0.25ml of solution E. After 30 minutes incubation to allow colour development the optical density of the solutions at 750nm was determined.

Where possible the samples were dissolved in solution A which was then included as a blank. If any other buffers were used they were accounted for by the appropriate controls. A standard curve was prepared from the results and the appropriate dilutions of samples made so that values could be obtained from the linear portion of the standard curve.

INVESTIGATION OF THE CYCLIC-AMP LEVELS IN INTACT CELLS

Preparation of Cells for Determination of Intracellular cAMP

The levels of cAMP in cells grown in vitro were determined as a function of both protein concentration and cell number. Careful isolation of cells as well as the use of a sensitive cAMP assay were required to measure the low concentration in the cells.

The cells were cultured to the required cell density in 200ml medical flat bottles and care was taken that the cells received identical culture conditions, because cell number was determined using a representative bottle. Monolayer cells in the remaining bottles were washed twice with H-HBSS (HBSS buffered with 20mM HEPES) at $4^{o}C$, scraped from the glass surface with a rubber policeman and collected in H-HBSS. This suspension was then centrifuged at 750g for 10 minutes to collect the cells which were then resuspended in a known volume of H-HBSS at $4^{o}C$.

Suspension cells were collected at $4^{o}C$ by centrifugation as described above, and the cells were then washed twice in H-HBSS followed each time by recentrifugation. Finally the cell pellet was resuspended in H-HBSS to give a suspension of 1/100 of the original volume.

Both cell types were then treated similarly in that 20% of each solution was used to estimate protein concentration while the remaining 80% was prepared for cAMP determination. Samples were obtained for protein estimation by washing the cell suspension in isotonic saline (0.9% NaCl), centrifuging to collect the pellet and boiling the resuspended pellet in 1.0ml 2% Na_2CO_3 in 0.1M NaOH (see protein determination).

The cell suspensions were prepared for cAMP estimation by boiling for 5 minutes in covered vials and by sonication for 10 seconds to disrupt the cells. They were then stored at -20^{o}C until assayed. cAMP determination could be deferred for several months without notable decay of the samples.

Hormonal Incubation with Intact Cells

(a) Monolayer Cells

In order to examine the hormonal stimulation of cAMP in cells which adhere to glass, experiments were carried out on monolayer cultures grown on the bottom of scintillation vials which had been specially washed, as described previously for cultures bottles (Franklin and Foster, 1973). The cells were grown in 1-2ml of the appropriate medium, which was buffered with HEPES to maintain pH.

At the appropriate cell density the medium was removed, the cell sheet washed twice with 1ml H-HBSS and drained. H-HBSS (0.5ml) was then added and pre-incubation achieved at 37°C for 15-30 minutes. At the end of this time the incubation was initiated by the addition of 0.5ml H-HBSS containing twice the final concentration of hormone and phosphodiesterase inhibitor (1-methyl-3-isobutyl xanthine; IBMX, 100µg/ml). The various hormone concentrations and incubation times are given in the text, but usually the reaction was stopped after 10 minutes by boiling the vials, with the caps in place, for 5 minutes. After cooling, the suspensions were sonicated for 10 seconds and therefore the values obtained were the sums of intracellular and extracellular concentrations. All samples were stored at -20°C and before assay they were thawed, transferred to 1ml eppendorf tubes and centrifuged using a microcentrifuge to remove cell debris from the supernatent.

The phosphodiesterase inhibitor (IBMX) was dissolved in 0.5ml absolute ethanol and then diluted in H-HBSS, which was then used to dilute the hormone solutions. The catecholamine, isoprenaline, was dissolved directly in this buffer but Prostaglandin E_1 (PGE_1) was stored as a concentrated solution (10mg/ml) in absolute ethanol at -20°C and diluted as required. PGE_1 was only stable for a few days at 4°C (Roseman et al., 1973) and whilst remaining stable at pH 5-7 it was degraded at pH 8 and above (Karim et al, 1968). All hormone solutions were diluted just before incubation and kept at 4°C until addition. The final concentration of ethanol was less than 5% and usually below 1%, but blanks

containing the respective ethanol concentrations were always included.

The protein concentration obtained for monolayers was the average of determinations from 6 vials. These were emptied of medium, washed twice with isotonic saline, boiled for 5 minutes with 1ml of 2% Na_2CO_3 in 0.1M NaOH, and assayed for protein as described previously.

(b) Suspension Cells

Measurement of hormonal stimulation of cAMP required that large numbers of suspension cells were grown to the required density and collected by centrifugation at 750g for 10 minutes. These cells were washed twice by resuspension in H-HBSS at 1/20 of their original volume. A small aliquot was removed for protein determination and washed as described in the previous section. The remaining suspension was dispensed into the specially washed scintillation vials in 0.5ml aliquots and the procedure above, for monolayer cells, used for the incubation.

Cyclic AMP Assay

The assay used to measure cAMP in these experiments must be sensitive to levels of less than 1 pmole; capable of determining cAMP in over 100 tubes per assay and relatively inexpensive. It is also important that the technique is not subject to interference from contaminating nucleotides. The method used is a slightly modified form of the cAMP saturation

binding assay of Brown et al. (1971). The modifications have been based on details from several reports (Albano et al., 1974; Tovey et al., 1974; Wunderwald et al., 1974).

Reagents

1. Cyclic AMP Standards

These range from 0.1 to 20pmoles per 20µl in double distilled water. They were stored at -20°C for several months without degradation of cAMP.

2. Tritiated cAMP

[8-^{3}H]cAMP (30Ci/mmol) was diluted 1500 times for the assay in double distilled water, and stored in 1ml aliquots in eppendorf tubes at -20°C.

3. Tris-EDTA buffer

50mM Tris-HCl pH 7.4 at 20°C

4mM EDTA

6mM β-mercaptoethanol.

4. cAMP-Binding Protein

Bovine adrenals, collected as soon after slaughter as possible, were kept on ice and dissected at 4°C. All fatty tissue was removed, along with medullae and the cortices were chopped, and homogenised with 1.5 volumes of an ice-cold medium of:

0.25M sucrose

50mM Tris-HCl pH 7.4 at 20°C

25mM KCl

5mM $MgCl_2$

The homogenate was centrifuged at 2000g for 5 minutes at 4°C and the resultant supernatent stored in 1ml aliquots at -20°C. Negligible loss of activity was found after 9 months storage. Prior to assay, the preparation was thawed and centrifuged for 2 minutes in the microcentrifuge before the supernatent was diluted 1/40 with tris-EDTA buffer (3).

5. Albumin-Charcoal

1g bovine serum albumin

2.0g charcoal norit SX-1

50ml tris-EDTA buffer (3)

This was stirred to give a thoroughly mixed solution at 0°C.

6. Scintillator

either

8g/l butyl PBD

100.4g/l napthalene

1L scintillation grade, peroxide free, dioxan

or

4g/l PPO

0.2g/l POPOP

667ml scintillation grade, sulphur free, toluene

333ml scintillation grade triton X-100

60ml double distilled water.

Procedure

The assay was performed in plastic eppendorf centrifuge tubes, arranged in rows of 12 in a grid over a tray of ice. The solutions were added according to the following scheme:

	20µl	100µl		20µl		200µl	
	[8-^{3}H]cAMP	sample	buffer	standard cAMP	distilled water	buffer	binding protein
Blanks	+		+		+	+	
Standard S_o	+		+		+		+
Standards S	+		+	+			+
Samples	+	+			+		+

+ denotes an addition to tube

The assay was started by addition of binding protein to successive rows at 5 minute intervals. The solutions were incubated for 90 minutes at 4^{o}C and the reaction stopped by the addition of 500µl of the ice-cold charcoal albumin mixture to the tubes in each row in the same sequence. Each tube was immediately shaken and groups of 12 centrifuged for 2 minutes in the microcentrifuge. The row of tubes was then carefully placed in a rack and left until the following row was being centrifuged, when 500µl of supernatent was removed from them and placed in vials with 9.5ml scintillator. Alternatively, the samples were placed in 7ml plastic vial inserts with 3.3ml scintillator (ii) prepared without water.

The vials were counted (usually 5 minutes each) in a Packard model 3385 Liquid Scintillation Spectrometer using the factory-optimised settings for ^{3}H with an efficiency of 55%. The results were calculated using a computer programme described in the appendix.

THEORY AND CALCULATIONS

Brown et al. (1972) gives a good description of the theoretical aspects of saturation assays, such as described here. The binding protein isolated from adrenal cortices contains soluble protein kinases with a high affinity for cAMP, which binds subsaturating concentrations of [8-^{3}H]cAMP. The charcoal-albumin mixture functions to remove all unbound nucleotides by sedimentation, so that in the S_o standard all the [8-^{3}H]cAMP exists bound in the supernatent and gives maximal counts of about 3000 c.p.m. When unlabelled cAMP is added, in the form of standards or samples, radioactive cAMP is displaced from the soluble binding protein and the counts decrease. A blank is obtained when no binding protein is present.

A plot of c.p.m. against cAMP concentration is not linear at low and high values of cAMP, so the results are expressed as the ratio:-

$$\frac{S_o}{S} = \frac{S_o \text{ standard c.p.m. - blank c.p.m.}}{\text{standard c.p.m. - blank c.p.m.}}$$

and a plot of S_o/S against standard cAMP concentrations is used. When saturating concentrations of [8-^{3}H] cAMP are added the response is linear up to 10-15 pmoles cAMP, although some sensitivity is lost (see Figure 10).

Very low basal levels of cAMP are detected by diluting the [8-^{3}H]cAMP a further 1/3 before assay and by diluting the binding protein 1/100 after thawing, instead of 1/40.

Having obtained values of cAMP for the samples, volume corrections can be applied and specific activity expressed as pmoles cAMP per mg protein per incubation time.

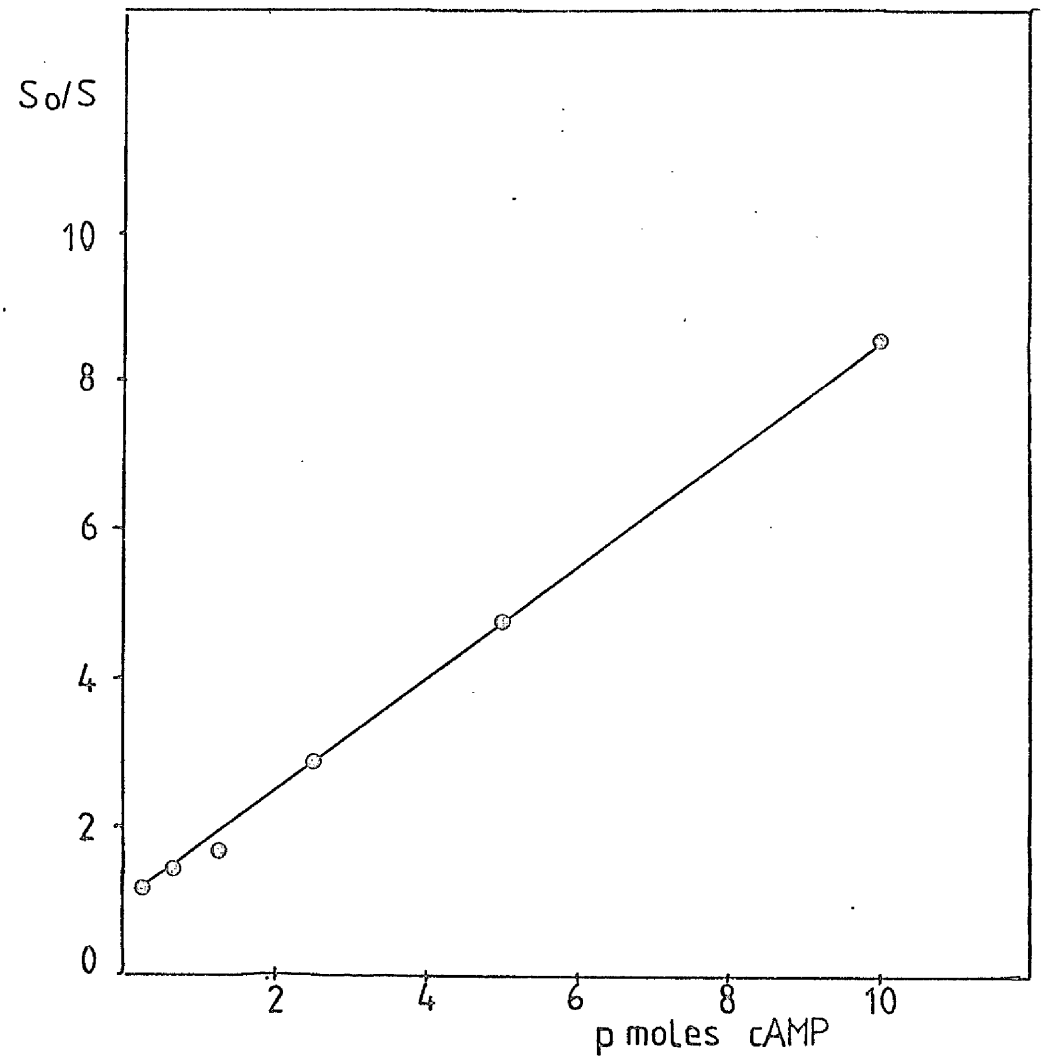


FIGURE 10. STANDARD CURVE FOR CYCLIC AMP ESTIMATION

A representative plot of S_o/S vs. cAMP. The gradient obtained from this graph = 0.75. Estimations of cAMP levels below 2.5 pmoles cAMP were obtained by the dilution of [8-^{3}H]cAMP and binding protein which increased the sensitivity of the assay.

TIME LAPSE PHOTOGRAPHY

Examination of cells over 48 hours was achieved by time lapse photography using a Bolex X16 cine camera attached to a Zeiss microscope with a 10X objective lens and an 8X eye piece lens in position. The film used was a kodak tri-X reversible cinematography film with 1 frame exposed for 1 second every 40 seconds.

The cells were subcultured, 12 hours before photography, into a bottle specially designed in this laboratory by Dr. S.R. Ayad. The bottle has a medium capacity of 3-5ml which covers the lower surface leaving an air pocket near the neck of the bottle to allow gaseous exchange. The photography was carried out at $37^{o}C$, with light supplied during exposures only, and the films were examined using a single frame analyser.

STUDY OF DNA SYNTHESIS

Radioactive Labelling of DNA

In order to determine whether certain cells were capable of synthesising DNA, a study was made of the rate of incorporation of tritium-labelled thymidine into DNA. The method used was based on the experiments of Yang et al., (1976). Non specific incorporation was observed by the inhibition of DNA synthesis using hydroxyurea, (Ayad et al., 1969) which acts by blocking ribonucleotide reductase (Nicolini, 1976).

Incorporation of [^{3}H]-thymidine and separation of the labelled DNA are described below:-

1. Cell cultures were grown in Roux bottles under identical conditions for 48 hours before assay. Inhibition of DNA synthesis, in a control culture, was achieved by pre-incubation for 5 minutes with hydroxyurea, at a final concentration of 1×10^{-2}M.
2. All cultures were then incubated at 37^{o}C with 2μCi/ml [^{3}H] thymidine (5Ci/mmole) at 37^{o}C for 2 hours.
3. The incubation was stopped by placing the bottles on ice and the cells were collected by centrifugation of the suspension at 750g for 10 minutes or by trypsinization of the monolayer. They were then washed and resuspended in 4ml of a solution of HBSS containing 1% BSA.

4. 3ml aliquots were then removed from each preparation and used to estimate cell number (described in a previous section) or DNA content (described below).
5. The remaining 1ml portions were treated with 5ml ice-cold trichloroacetic acid (TCA) and left to flocculate for 15 minutes at $4^{o}C$.
6. Millipore filtration units were prepared by presoaking glass fibre filter papers (GF/C 2.1cm) in 5% TCA containing 100µg/ml unlabelled thymidine. After assembling, the discs were washed with ice-cold 5% TCA, and the flocculated material poured through.
7. Each disc was then washed with:-

   3 x 5ml 5% TCA (ice-cold)

   3 x 5ml 95% ethanol (ice-cold)

   3 x 5ml ether (ice cold)

   This was necessary to remove TCA as it is known to fluoresce.
8. The discs were then dried using an infra-red lamp and placed in vials with 10ml toluene scintillator:-

   1 litre Toluene

   0.1g POPOP

   4g PPO

   Radioactivity was counted using a Packard liquid scintillation spectrometer, model 3385 with factory-optimised settings for ^{3}H and an efficiency of 55%. Each vial was counted for 10 minutes.

Estimation of DNA Content

Incorporation of [^{3}H] thymidine may be examined as a function of DNA content or cell number. The procedure for measurement of DNA involves fluorimetric analysis of ethidium bromide intercalation, and the method described below is an adaption of the technique used by Karsten and Wollenburger (1972).

Reagents

1. Buffer A.

   1M sodium acetate

   1M sodium chloride adjusted to pH 5.6 at 20^{o}C.

2. Ethidium bromide

   20µg/ml dissolved in buffer A and prepared fresh.

3. Standard DNA

   Calf thymus DNA at approximately 200µg/ml was weighed accurately on a Cahn electric balance and dissolved over 48 hours in buffer A at 4^{o}C. This stock solution was diluted with buffer A to obtain a series of concentrations in the range 20-200µg/ml.

4. Pronase

   Nuclease free pronase was dissolved in buffer A to give 80µg/ml. Fresh solutions were prepared for each assay.

5. Ribonuclease

DNase free enzyme was used at a concentration of 20µg/ml buffer A and heated to 80°C for 10 minutes, before incubation, to remove any contaminating DNase.

6. Bentonite

Bentonite is a hydrated aluminium silicate clay, which is used as an inhibitor of DNase and RNase activity and absorbs protein particles such as ruptured cell membranes.

Bentonite was prepared by the method of Brownhill et al., (1959) in that 10g crude bentonite was homogenised for 5 minutes in 200ml distilled water using a Waring blender. The solution was then centrifuged at 1000g for 20 minutes to remove large particles, followed by centrifugation at 9000g for 20 minutes. The resulting pellet was re-homogenised for 2 minutes in 200ml buffer A and collected by centrifugation at 9000g for 20 minutes. The resuspension was repeated a further 3 times, and the pellet finally diluted to give a 5% w/v suspension in buffer A. Aliquots were stored at -20°C and thawed just before use.

Procedures

Samples were stored deep frozen, prior to assay when they were thawed, homogenised with 5 strokes of the glass homogeniser and diluted to 4ml for incubation with 0.04ml of 5% w/v bentonite at 4°C for 30 minutes,

accompanied by frequent shaking. Duplicate 4ml aliquots of the series of standards were also incubated in this way and a series of standards was incubated without bentonite, to estimate the sedimentation of DNA. At the end of the incubation the solutions were centrifuged at 20,000g for 20 minutes at 4°C and the clear supernatents mixed with the reagents according to the following protocol.

Solution	Buffer A	Pronase	DNA Standards	Ribonuclease	Homogenates	Ethidium bromide (added last)
A	1ml	1ml	1ml			1ml
B	2ml	1ml				1ml
C	4ml					
D*		1ml		1ml	1ml	1ml
E	3ml				1ml	

*Solutions D were equilibrated at 37°C for 20 minutes in a water bath before addition of ethidium bromide.

After mixing the solutions, values for fluorescence were obtained using a Perkin Elmer fluorescence spectrofluorimeter, model 204. The excitation and analyser wavelengths used were 360nm and 580nm respectively. The selector knob was set at x10 and the sensitivity at x8. The fluorimeter was set at 0% using a water blank and at 100% using the stock solution of standard DNA.

Calculation

Values of fluorescence intensity (f) were read directly from the fluorimeter and substituted in the following equation:-

$$[DNA]_D = \frac{[std]\ (f_D - f_B - f_E + f_C}{f_A - f_B}$$

$[DNA]_D$ = amount DNA per solution D (µg/ml)

[std] = concentration DNA per solution A (µg/ml)

A standard curve of fluorescence intensity vs. DNA concentration was plotted to prove that a linear relationship exists over the concentration range studied (see Figure 11).

In determining the concentration of DNA in the initial solutions, allowances were made for the dilution after homogenisation.

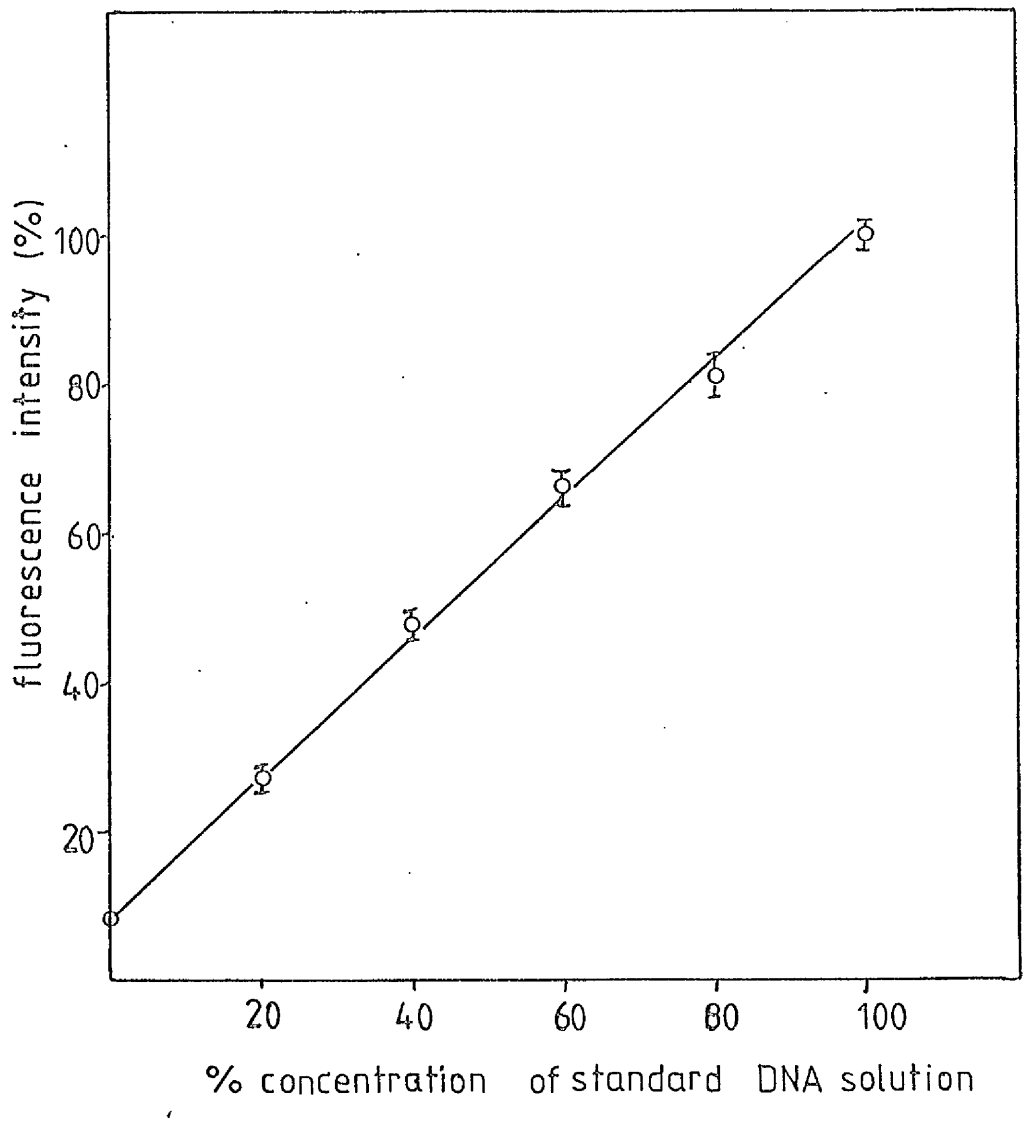


FIGURE 11. STANDARD CURVE FOR DNA ESTIMATION

The fluorescence intensity of a known concentration of DNA is adjusted to 100%. This is a representative plot to show that the relationship between fluorescence intensity and DNA concentration is linear over the dilution range studied.

DETERMINATION OF MITOTIC INDEX

The purpose of this experiment was to examine the percentage of cells undergoing mitosis in a population in which cells grow attached and in suspension. The microtubule inhibitor, colchicine was made use of to increase the number of mitotic cells. Rapidly growing monolayer and suspension cells were incubated together or separately, as described in the text, with fresh medium containing 20µg/ml colchicine. After 4 hours the cells were prepared for examination by the method described in the section on karyotype analysis.

Mitotic cells were scored only if the chromosomes were clearly visible and several slides were counted for each preparation to eliminate error in spreading.

SYNCHRONISATION TECHNIQUES

A critical review of synchronisation techniques has been written by Nias and Fox (1971) and since this publication very little progress has been made. However, the use of synchronisation in these studies was for analysis of the variation in cell number attached and in suspension throughout the cell cycle. Therefore the choice of technique was limited since it was not possible to synchronise cells by mitotic shake-off (Terasima and Tolmach, 1963) nor was it feasable to use agents such as N^6,O^2-dibutyryl cAMP (Froehlich and Rachmeler, 1972) because of the associated effect of growth and morphology. Because of these limitations, synchronisation was attempted using the block and release method with excess thymidine.

Synchronisation by Double Thymidine Block

Excess concentrations of the specific DNA precursor, thymidine, are thought to act by feedback inhibition, causing cells to accumulate at the G_1/S boundary in the cell cycle (Bootsma et al., 1964). Preliminary experiments using the single thymidine block described by Millis et al (1974) were not productive, so it was decided to follow the protocol described by Zeilig et al (1976) for the double thymidine block. Several papers have been published (Thomas and Lingwood, 1975; Tobey et al., 1972) which describe adverse effects observed with thymidine-block experiments and therefore objective examination of the results is necessary.

The purpose of obtaining a synchronised culture was to allow cell number analysis throughout the cell cycle, in a hybrid cell line which grows with cells attached to the substratum and in suspension. Thus it was necessary to subculture these cells into a series of 200ml medical flat bottles until they attained exponential growth. The bottles were then separated into control (-T) and thymidine treated (+T) cultures, which were incubated in medium containing 2mM thymidine (dissolved in HBSS and sterilised by Millipore filtration).

After 17 hours the suspension cells from the +T cultures were centrifuged at 750g for 10 minutes, washed with HBSS, resuspended in thymidine-free medium and replaced in the same +T cultures containing monolayer cells. The control bottles were treated similarly and given the same medium. The incubation in thymidine-free medium was interrupted after 9 hours by the readdition of thymidine (final concentration 2mM) to the +T cultures, which were then left 15 hours before removal of thymidine and replacement with thymidine-free medium, as described above. The cells were thus released from the thymidine block and the cultures in -T and +T bottles could be studied for changes in cell number at different time intervals. Cell number determinations were made using a Coulter counter and the method has been described previously.

Degree of Synchrony

The effectiveness of thymidine as a tool for producing synchronised cell cultures can be examined using the mitotic index or the rate of DNA synthesis. This latter method has been studied by pulse labelling the cells with [^{3}H]-thymidine and analysing the incorporation into DNA by autoradiography. The technique has been adapted in this laboratory (Winstanley, 1971) from the method of Terasima and Tolmach (1963) and is described as follows:-

1. The cells were incubated with [^{3}H]-thymidine 24Ci/mmole (dissolved in HBSS) at a concentration of 1μCi/ml for 15 minutes at 37^{o}C.

2. They were then collected by centrifugation of the suspension at 750g for 10 minutes or by trypsinization of the monolayer and incubated in 5ml warm HBSS at 37^{o}C for 15 minutes.

3. The suspension was recentrifuged and the pellet dispersed in 5ml warm hypotonic HBSS (0.5ml HBSS + 4.5ml double distilled water) for an incubation of 15 minutes at 37^{o}C.

4. Removal of the cells from hypotonic solution was achieved by centrifugation, so that they could be fixed in 3ml of methanol:glacial acetic acid (1:3) for 10 minutes.

5. The fixative was also removed by centrifugation and the cells resuspended in a small aliquot of fresh fixative, dispensed onto slides and stained for 30 minutes in a filtered Giemsa solution. The excess was washed away with distilled water.

6. Slides were stored in silica gel and prepared for autoradiography as described in a previous section (page 97). The preparations were exposed simultaneously for periods of 4-7 days and after developing, any unstained slides were treated with methyl violet for 3 minutes.

7. Silver grains were detected over the nuclei of labelled cells and background grains were negligible, so that determination of the percentage of labelled cells was easily acheived.

Incubation in Serum-Free Medium

Several reports suggest that serum-deprived cells become quiescent (Lawrence and Jullien, 1975) and are arrested at the G_1/G_o phase of the cell cycle (Mierzejewski and Rozengurt, 1976). Readdition of growth factors of serum induces 80-90% of the population to undertake DNA synthesis, which is usually preceded by a decrease in the cAMP levels within the cells. (Pledger et al 1975). Therefore incubation in serum-free medium has been

used to examine the growth characteristics of a hybrid cell line.

Cells were grown in standard medium in 200ml medical flat bottles until they attained exponential growth, when they were 'stepped down' into medium containing 1.0% newborn calf serum. After 40 hours the serum was replaced and the cell number determined at regular time intervals, using the Coulter counter method described previously.

IDENTIFICATION OF CELL SURFACE PROTEIN

A study has been made of a major cell surface glycoprotein (CSP), which when incubated with transformed cells caused increased adherence to the substratum and altered morphological appearance (Yamada et al., 1976). Identification of CSP was achieved by homogenisation using the method of Hynes (1973) and separation, with sodium dodecyl sulphate (SDS) gel electrophoresis as described by Yamada and Weston (1974).

Homogenisation of cells

Cells were grown to confluency in Roux bottles and prepared for homogenisation by washing in 10mM sodium phosphate buffer pH 7.0 containing 2mM phenyl methyl sulphonyl fluoride (PMSF) which was used to inhibit proteolysis. (PMSF was dissolved in 95% ethanol at 9mg/ml and added to the buffer with rapid stirring). The monolayer cells were scraped from the glass into the buffer using a rubber policeman and centrifuged at 750g for 10 minutes. Suspension cells were also collected by centrifugation as described above, and then the pellets were resuspended in 0.5ml aliquots of homogenisation buffer containing:-

10mM	sodium phosphate pH 7.0
2%	sodium dodecyl sulphate (SDS)
2mM	PMSF
10%	glycerol
0.001%	bromophenol blue

Before electrophoresis the solutions were reduced by the addition of dithiothreitol to give a concentration of 0.1M,

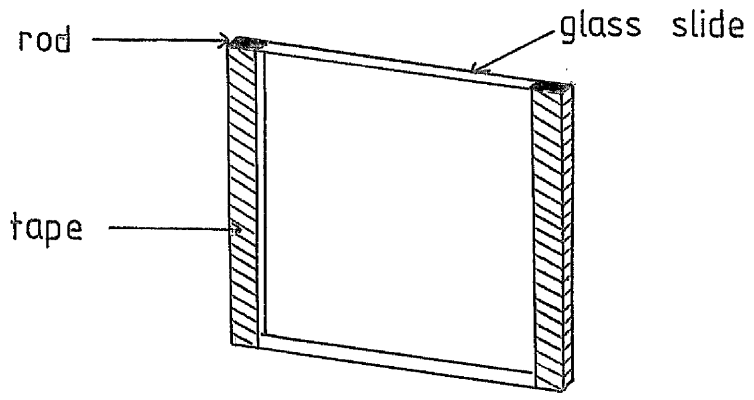
and boiled for 3-6 minutes. The amount of homogenate applied to the gels was equivalent to 7.5×10^5 cells.

Sodium Dodecyl Sulphate Gel Electrophoresis

Proteins can be separated by SDS gel electrophoresis, because their relative mobilities are linear functions of the logarithms of their molecular weights. This can be explained by the fact that SDS binds hydrophobically with a constant weight per weight of protein and swamps the charge of the protein. This gives a complex which is more soluble and which has a uniform shape; with length dependent on the number of residues. The theoretical and practical aspects of electrophoresis are adequately described by Morris and Morris (1976) and Gordon (1969).

1. Preparation of Gels

Slab gels were cast in glass cassettes made by separating two glass slides (cleaned in chromic acid) with glass rods. The rods were stuck in place with Bostik (number 1) and covered with radioactive tape:-



The slabs were held vertically in a gel slab casting apparatus, with the aid of a wedge, and 10ml ethanol (20% v/v) poured into the bottom of the tank to provide a flat top for the gels.

Gels were made with 5% acrylamide, according to the method of Laemmli (1970). The following solutions were mixed and deaerated after each addition:

22.5ml per gel	Buffer 0.75M Tris-HCl pH 8.8 at 20°C 0.2% SDS
12.75ml per gel	Double distilled water
7.5ml per gel	Acrylamide stock solution 30g acrylamide 0.8g N,N'-bis-methylene acrylamide dissolved to give 100ml in double distilled water and stored at 4°C in dark
2.25ml per gel	Ammonium persulphate 15mg/ml double distilled water Made immediately before use.
0.05ml per gel	TEMED final concentration of 0.1% in the gel.

After addition of the catalysts the gel solution was immediately poured into the inlet tubing of the casting apparatus and polymerisation aided by the use of a fluorescent light. Gels were stored at 4°C in a humidified atmosphere.

2. Preparation of Standard Proteins

All proteins used as standards were prepared by boiling for 3 minutes in sample buffer (Yamada and Weston, 1974) as described below:-

10mM	Sodium phosphate pH 7.0 at 20°C
1%	SDS
10%	glycerol
0.1M	dithiothreitol
0.001%	bromophenol blue

The following proteins were used to prepare standard curves:-

Ovalbumin (m.w. 43,000):
Dissolved directly in sample buffer to give a concentration of approximately 0.1mg/ml

Bovine serum albumin (m.w. 68,000):
Dissolved directly in sample buffer to give a concentration of approximately 0.1mg/ml

β-Galactosidase (m.w. 130,000):

This enzyme exists as an oligomer under native conditions.

20µl of the suspension is mixed with 20µl sample buffer.

Molecular weight markers:

1ml of sample buffer was added to the contents of a vial, mixed and boiled for 3 minutes. This stock solution was kept at -20°C and 100µl aliquots were taken for electrophoresis, mixed with 100µl sample buffer and boiled for 3 minutes.

Myosin (m.w. 220,000)

This protein exists as an oligomer under native conditions. It was isolated by the method of Perry (1955), modified by Weeds and Hartley (1968). Briefly the dorsal and leg muscles were dissected from a freshly killed rabbit and minced in a meat grinder at 4°C, before being stirred for 15 minutes in 3 volumes of:-

0.3M	KCl
0.1M	KH_2PO_4
0.05M	K_2HPO_4
1mM	EDTA

dissolved in double distilled water at pH 6.5 (4°C)

After centrifugation at 1000g for 15 minutes at 4°C the supernatent was filtered through a 1 inch pad of paper pulp, previously washed with buffer. Precipitation was achieved by slow addition with stirring of 10 volumes

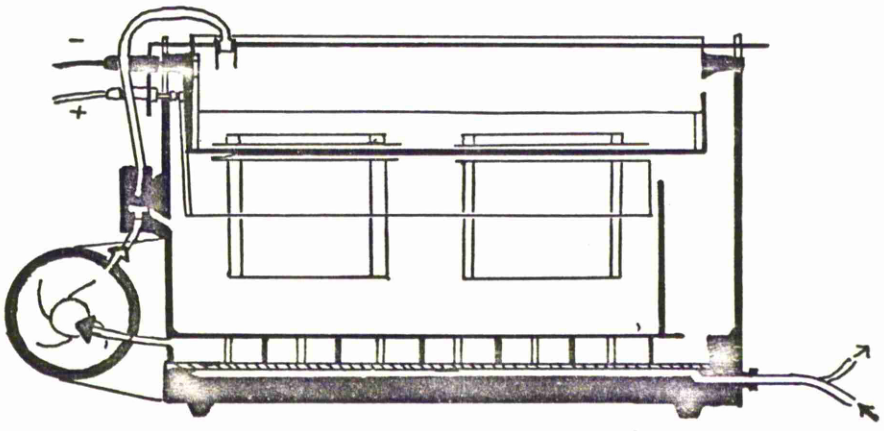
of ice-cold double distilled water containing 1mM EDTA and the myosin settled out overnight at 4°C. The resultant crystals were collected by centrifugation at 1000g for 30 minutes. The volume was noted and the myosin brought into solution by addition of sufficient KCl to bring the ionic strength to 0.5.

Actomyosin was precipitated out by adjusting the pH to 6.6 with solid $NaHCO_3$ and the ionic strength slowly lowered to 0.3M by the addition of double distilled water containing 1mM EDTA. The precipitate, collected by centrifugation at 10,000g for 15 minutes was resuspended in a small volume of supernatent and recentrifuged. The resulting precipitate was lyophilised for storage purposes.

Myosin was brought into solution by the method of Weber and Osborn (1969) in which 5mg protein was dissolved in 10ml sample buffer containing 8M urea. This was incubated for 2 hours at 37°C and then dialysed against buffer without urea for several hours at room temperature. The solution was stored for several weeks at room temperature without adverse effects. Before electrophoresis a small aliquot was boiled for 3 minutes and bromophenol blue added to give a 0.001% solution.

3. Electrophoresis

The electrophoresis apparatus (GE-4) was used with a power supply model number EPS 500/400, and was assembled as described in the instruction leaflet.



The gel slabs were placed in position in the gaskets and pre-equilibrated at 50mA for 20 minutes in running buffer:-

25mM	Tris-HCl pH 8.3 at 20^{o}C
192mM	glycine
1mM	EDTA
0.1%	SDS

The samples were applied by addition of 20µl portions and electrophoresis carried out for 3-4 hours at 25mA per gel until the bromophenol blue reached the bottom of the gel.

4. Staining

Gels were stained for 2-10 hours at room temperature in a solution made by mixing the following substances and filtering any insoluble material through Whatman No. 1 filter paper:

1.25g	Coomassie brilliant blue G250
227ml	methanol
46ml	glacial acetic acid
227ml	double distilled water

Destaining was achieved by rinsing the gels in double distilled water and repeatedly incubating in the following solution at 37°C:-

75ml	acetic acid
50ml	methanol
875ml	double distilled water

Gels were stored in the destaining solution in covered dishes.

5. Calculation of Molecular Weight

Movement of proteins was determined by direct measurement and by scanning using a gel scanner attached to an Acta Spectrophotometer. Absorbance was measured at 600nm on 1cm wide strips cut from the gel.

The gels swell some 5% in acid solution and therefore mobility measurements of standards and samples were calculated as:

$$\text{mobility} = \frac{\text{length gel before staining}}{\text{length gel after staining}} \times \frac{\text{distance protein migration}}{\text{distance dye migration}}$$

The molecular weights expressed on a semi-logarithmic scale were plotted against mobilities of the standard proteins to obtain a calibration curve, from which molecular weights of unknown proteins could be obtained. A typical standard curve is shown in Figure 12.

Trypsin Treatment of CSP

One of the characteristics of the major cell surface glycoprotein is that it is remarkedly sensitive to trypsin and therefore identification of CSP on gels can be achieved by comparing the pattern of bands from homogenates of control and trypsin-treated cells.

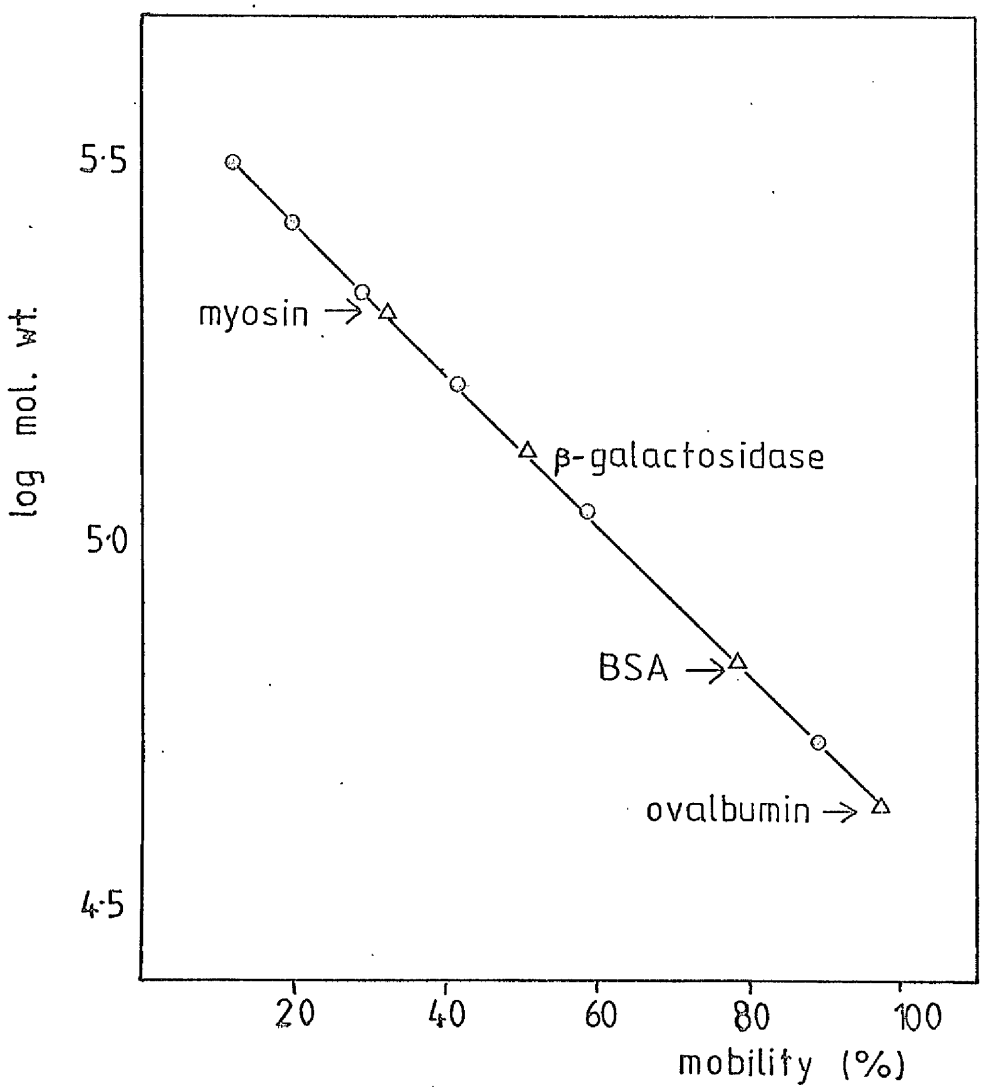


FIGURE 12. CALIBRATION LINE FOR STANDARD PROTEINS ON SDS-POLYACRYLAMIDE GELS.

A representative calibration line for 5% gels. Calculation of mobility referred to that of Bromophenol blue is described in the text. The reference proteins (Δ) used were ovalbumin (M.W. 43,000), bovine serum albumin (M.W. 68,000) β-galactosidase (M.W. 130,000) and myosin (M.W. 200,000). The molecular weight markers (●) are oligomers made by cross-linking a homogeneous monomer such that the molecular weights are 53,000 (monomer) 106,000 (dimer) 159,000 (trimer) 212,000 (tetramer) 265,000 (pentamer) 318,000 (hexamer)

The experiment was carried out by incubating confluent monolayer cells with 1μg/ml trypsin at 37°C for 3 minutes, inhibiting further proteolysis by washing with phosphate buffer containing PMSF, and then homogenising control and trypsin-treated cells, as described previously. Some cell lines may require longer incubation with trypsin, but this should be inhibited before the cells detach.

^{125}I-LABELLING OF CELL SURFACE GLYCOPROTEIN

Iodination of CSP was achieved by a lactoperoxidase-mediated reaction which labels only external membrane proteins. (Phillips, 1972). The technique is based on the method of Hynes (1973) and is described below:-

Incubation with Radioactive Label

1. Cells were grown to confluency in 200ml medical flat bottles. Suspension cells were collected by centrifugation and monolayer cells by scraping from the glass, with a rubber policeman. The cell number of each culture was determined and adjusted to give 4×10^6 cells in total.
2. The following reactions were carried out in conical centrifuge tubes at room temperature. Each cell type was washed 3 times with phosphate buffered saline (PBS) pH 7.2:-

8g/l	NaCl
0.2g/l	KCl
1.15g/l	Na_2HPO_4
0.2g/l	KH_2PO_4

3. Cells were then resuspended in PBS containing 5mM glucose, followed by the addition of carrier free $Na^{125}I$ to a final concentration of 400μCi/ml.

4. The reaction was initiated by the addition of lactoperoxidase and glucose oxidase to final concentrations of 20µg/ml and 0.1 units/ml respectively.
5. The reaction was allowed to continue for 10 minutes at room temperature with occasional swirling and labelling was stopped by the addition of phosphate-buffered iodide PBI (NaCl replaced by NaI in PBS) containing 2mM PMSF.
6. Free ^{125}I was removed by centrifugation and the cells washed twice more with PBI + PMSF.

Separation by Electrophoresis

The cell pellets were resuspended for electrophoresis in 200µl homogenisation buffer containing:-

10mM	sodium phosphate pH 7.0
2%	sodium dodecyl sulphate
2mM	PMSF
10%	glycerol
0.001%	bromophenol blue
0·1M	dithiothreitol

After boiling for 3 minutes the extracts were separated by gel electrophoresis, as described in the previous section, with the equivalent of 4.0×10^5 cells being applied to the gels.

Autoradiography

After staining the gels were dried by setting overnight in 2% agar and then drying very slowly using an infra-red lamp. (Reid and Bieleski, 1968). When dry the gels were able to form very close contact with Kodirex X-ray film. Exposure times varied but 4-6 hours was usually considered sufficient. The negatives were then compared directly with photographs of stained gels.

ISOLATION OF CELL SURFACE PROTEIN

Yamada et al. (1975) have isolated CSP from the membranes of cells in culture using urea extraction. This technique has been modified to include reducing agents in the buffers, as a result of work by Hynes and Destree (1977), who observed that cell surface proteins are disulphide bonded to the cell membrane.

Urea Extraction

This extraction was carried out with cells at saturating density, as it has been observed that recovery of CSP is density dependent (Yamada and Weston, 1974). The procedure is described below:-

1. Monolayer cells grown to confluency in Roux bottles, were washed 4 times with 50ml aliquots of HBSS containing 10mM HEPES (H-HBSS) pH 7.4 at 37°C.
2. The cultures were then rinsed for 60 minutes at 37°C with 25ml serum-free medium containing 2mM PMSF (medium A).
3. After washing with H-HBSS, the cells were extracted for 2 hours at 37°C with 25ml medium A containing 2mM dithiothreitol plus 1.0M urea (Analar) added immediately before use.
4. The medium was then poured off the cell sheet and centrifuged at 25,000g for 15 minutes at 4°C.

Concentration of Extract

Several techniques were tested in an attempt to develop a protocol for collection of the extract, for gel electrophoresis and for incubation with cells:-

Lyophilization

The supernatent, collected after centrifugation, was dialysed against 3 x litre changes of double distilled water for 48 hours and then freeze-dried in siliconised tubes. Siliconisation was used to prevent the extract adhering to the glass and was achieved using the method of Airhart et al. (1973). The samples stored at $4^{o}C$ were resuspended in sample buffer for gel electrophoresis.

Amicon Filtration

After centrifugation the supernatent was dialysed for 20 hours against 3 changes of 40 volumes each of calcium, magnesium free Dulbecco's phosphate buffered saline (PBS) at $4^{o}C$ with vigorous stirring. The resulting solution was filtered in Amicon CF 50A cones at $4^{o}C$ and 750g until 2ml remained. This was then stored at $4^{o}C$ and aliquots used for gel electrophoresis.

Ammonium Sulphate Precipitation

The supernatent was dialysed for 20 hours against 3 changes of 40 volumes each of PBS at 4°C with vigorous stirring. This was then mixed with a solution of PBS containing:-

2mM	PMSF
10mM	N ethylmaleimide
25mM	EDTA

which was used to rinse the dialysis bag. Ammonium sulphate was slowly added with stirring to give 75% saturation and the pH adjusted to 7.4 with NH_4OH. This solution was left overnight and then centrifuged at 49,000g for 1½ hours at 4°C to collect the precipitate. This was dissolved in 0.5ml of sample buffer containing PMSF (2mM) and dialysed at room temperature with 4 changes of 20ml of the above buffer. The extracts were then ready for electrophoresis.

Incubation of Cells with Isolated CSP

The cell surface glycoprotein was extracted with urea from 3 Roux bottles containing CH23 cells, collected by precipitation with ammonium sulphate and dissolved in 1/20 volume of buffer A:-

0.15M	NaCl
1mM	$CaCl_2$
10mM	cyclohexylaminopropane sulphonic acid pH 11.0

The extracts were dialysed overnight against two changes of 400 volumes of buffer A and then stored at -70°C. A small aliquot was removed before this final dialysis and dialysed against double distilled water for protein determination.

PCM3-S cells were obtained at saturating density, centrifuged at 750g for 10 minutes and resuspended in fresh medium to give 1.0×10^4 cells/ml. Isolated CSP was added to 100ml of medium containing PCM3-S cells to give a concentration of 20µg/ml and the number of attached and suspended cells compared with that of a control culture after overnight incubation. Cell number determinations have been described previously.

RESULTS

SECTION A. CYCLIC AMP SYNTHESIS IN NORMAL AND MALIGNANT CELLS

In these studies, the hormonal stimulation of cAMP synthesis was characterised and the local anaesthetic, tetracaine used as a probe to examine how alterations in the plasma membrane might affect this synthesis.

I. CHARACTERISTICS OF THE HUMAN CELL LINES

Human embryonic lung fibroblasts (AP-9), lymphoblastic leukaemia cells (SKL-2) and Burkitt lymphoma cells (EB_2) were used to compare the accumulation of cAMP in normal and malignant cells. These three cell lines have been described in the previous section and their morphology is presented in Figure 13.

AP-9 cells are typically fibroblastic and exhibit normal growth characteristics, while the two malignant cell lines are characterised by growth in suspension to high cell density. All three cell lines have maintained a stable chromosomal complement throughout the period of culture, as shown by karyotype analysis. Examination of autoradiographs after [^{3}H]-thymidine labelling has established that these cells are free from contamination by viruses and mycoplasma.

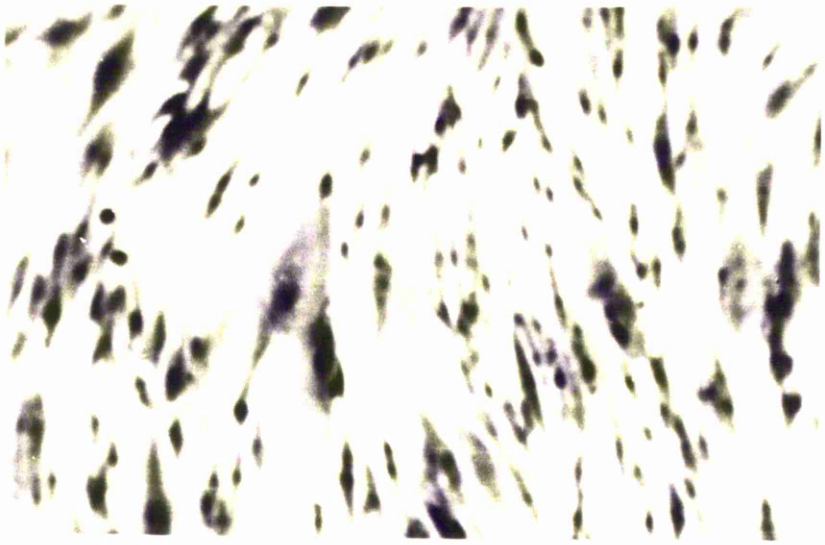
FIGURE 13. MORPHOLOGY OF (A) AP-9, (B) SKL-2 AND EB_2 CELLS

(A) AP-9 cells were grown on coverslips, in plastic petri dishes. Prior to examination the cells were fixed and stained with 1% methyl violet. The cells were photographed using a Zeiss camera fitted to a Zeiss microscope with a magnification of x190.

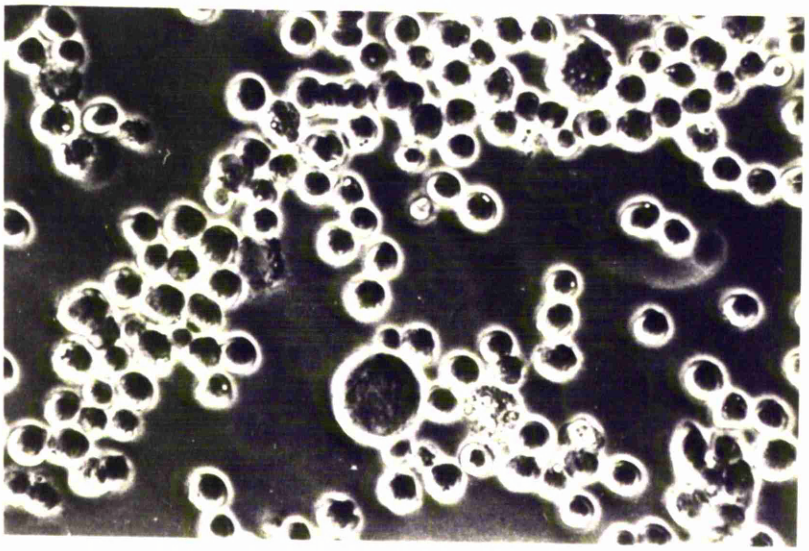
(B) & (C) SKL-2 and EB_2 cells were grown in medical flat bottles and collected by centrifugation at 750g for 5 minutes. After resuspension in a small volume of medium the cells were examined directly under phase contrast using a Zeiss microscope. The magnification for SKL-2 cells was x 480 and for EB_2 cells x 1200.

The Zeiss camera was fitted with Kodak Panatonic-X (black and white) film ASA/BS/16 DIN.

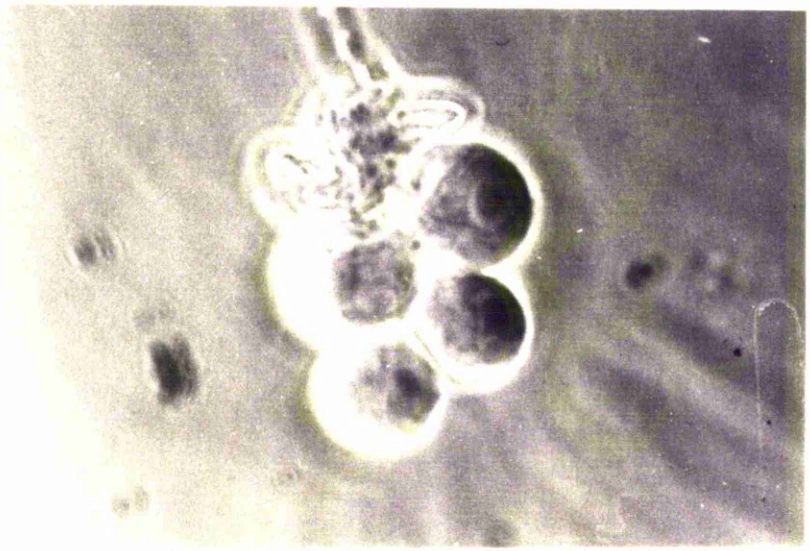
A



B



C



II. HORMONAL STIMULATION OF CYCLIC AMP SYNTHESIS

The ability of these human cell lines to synthesise cAMP in response to the hormones, isoprenaline and prostaglandin E_1 (PGE_1) is shown in figures 14 and 15. In order to compare the responses in different cell lines, all incubations were carried out at 37°C for 10 minutes, with monolayer cells which had reached confluency and suspension cells which had ceased to divide. The cyclic nucleotide phosphodiesterase inhibitor, 3-isobutyl-1-methyl xanthine (IBMX) was co-incubated with the various hormone concentrations to potentiate the cAMP levels.

AP-9 fibroblasts responded to an optimal isoprenaline concentration of 10μM by producing cAMP levels of 5.0 nmoles/mg protein. The concentration required to produce a half-maximal response was estimated to be approximately 0.1μM. In comparison, PGE_1 at optimal concentrations of 2.8μM elevated cAMP levels to 3.0 nmoles/mg protein, with a half-maximal response at concentrations of approximately 0.28μM.

In contrast the malignant SKL-2 and EB_2 cells were much less responsive to hormonal stimulation, producing cAMP levels 2 orders of magnitude lower than AP-9 cells, although the concentration of

FIGURE 14. A COMPARISON OF ISOPRENALINE-STIMULATED CYCLIC-AMP LEVELS OF INTACT CELLS

Cells were equilibrated for 15 minutes at 37°C before prewarmed solutions containing the respective hormone concentrations were added to start the reaction. The phosphodiesterase inhibitor 3-isobutyl-1-methyl xanthine (IBMX) (0.23mM final concentration) was present in all hormone incubations.

The incubations were continued for 10 minutes at 37°C and results represent the levels of cAMP accumulated over the 10 minutes.

They are expressed as means of duplicate cAMP determinations from duplicate incubations. Error bars indicate one standard deviation. Basal levels of cAMP are determined from incubations in the absence (B) or presence (I) of IBMX. Dose response curves were obtained for AP-9 cells (■—■); SKL-2 cells (▲—▲) and EB_2 cells (●—●).

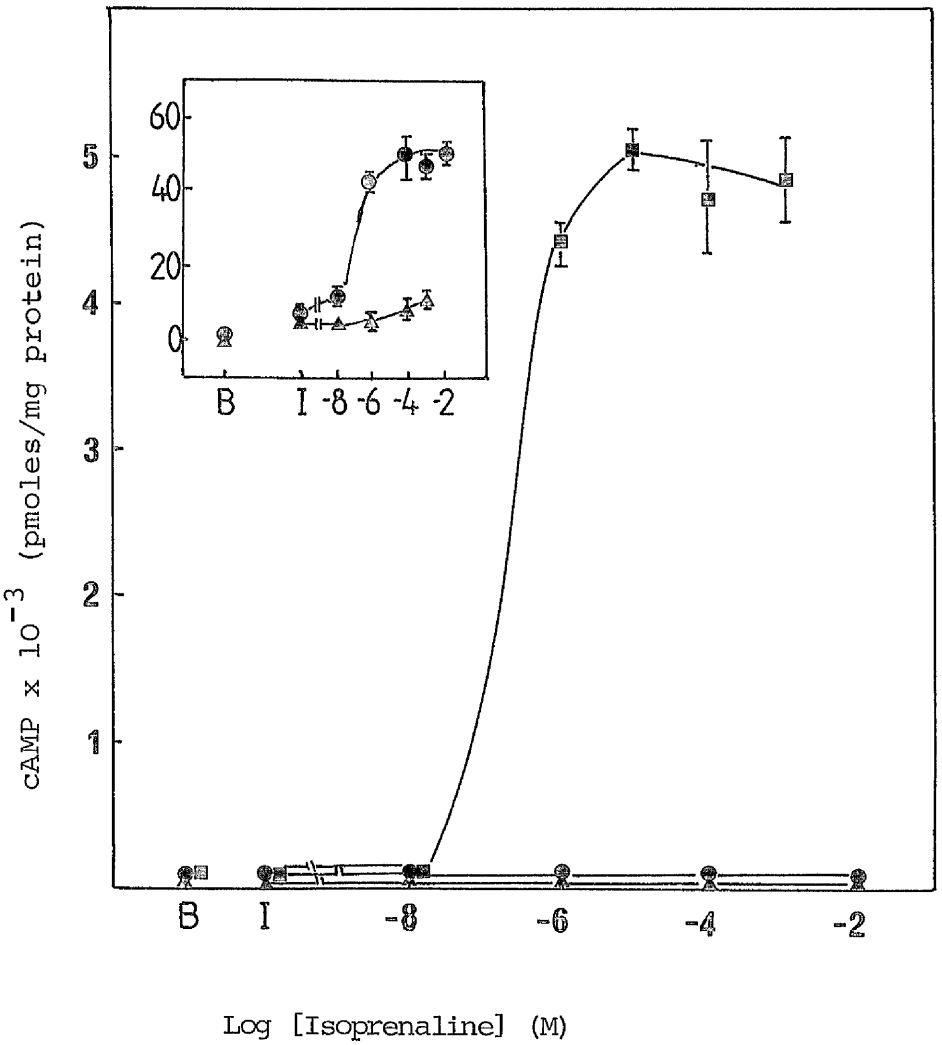

cAMP x 10^{-3} (pmoles/mg protein)
5
4
3
2
1
B
I
-8
-6
-4
-2
60
40
20
0
B
I
-8
-6
-4
-2
Log [Isoprenaline] (M)

FIGURE 14

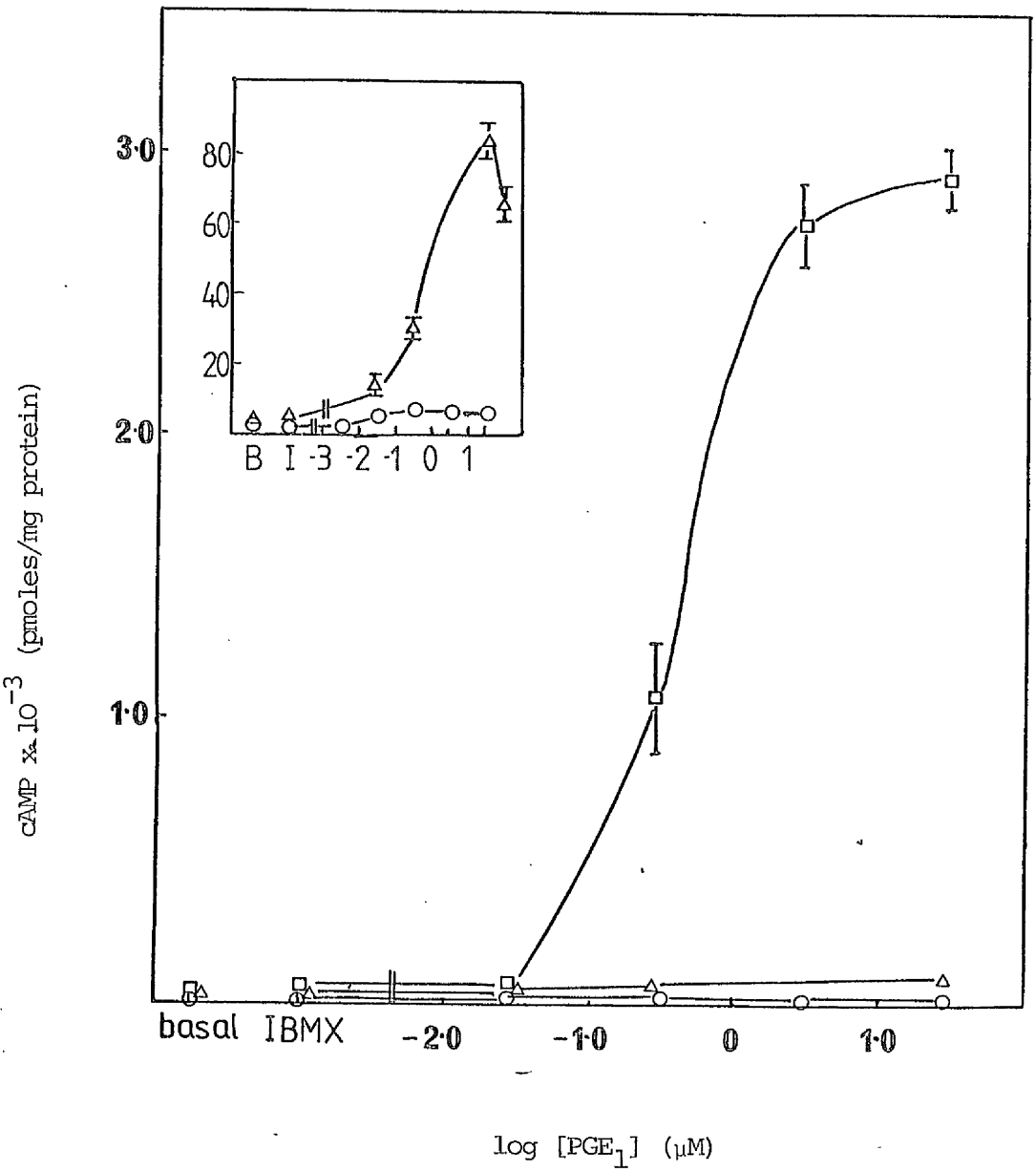


FIGURE 15. A COMPARISON OF PROSTAGLANDIN E_1 STIMULATED CYCLIC-AMP LEVELS IN INTACT CELLS

The experimental details are described in the legend to Figure 14. Dose response curves were obtained for AP-9 cells (□ — □); SKL-2 cells (△ — △) and EB_2 cells (o — o).

hormone required to produce half-maximal stimulation did not appear to be significantly altered. In order to detect the low levels of cAMP it was necessary to use large numbers of cells (10^7 per ml) so that consistently reliable concentrations of cAMP could be obtained. This reduced ability of malignant cells to respond to hormonal stimulation is in agreement with the general observation that cAMP levels are altered in malignant cells.

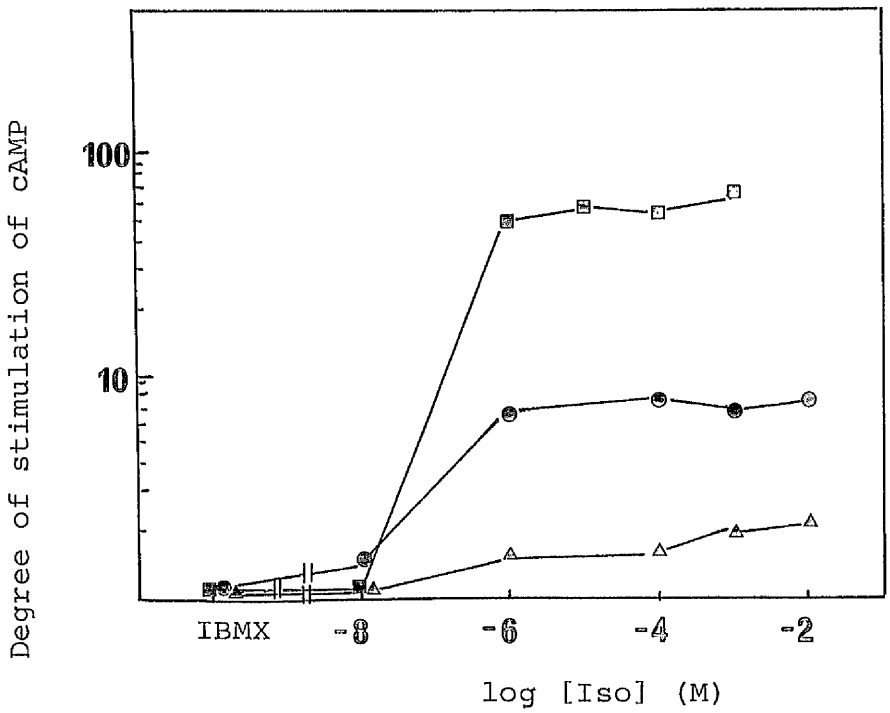
The degree of stimulation of cAMP synthesis can be seen in Figure 16, and is presented relative to control values of cAMP concentrations determined from incubations in the presence of IBMX alone. Thus it appears that in terms of adenylate cyclase activation; PGE_1 has a greater effect than isoprenaline in SKL-2 cells, while EB_2 cells respond to isoprenaline but not PGE_1.

Table 2 shows that the trends observed after hormone incubation were also seen when basal cAMP concentrations were compared. Throughout this series of experiments the absolute levels of cAMP in the presence or absence of hormone were observed to vary slightly between experiments, but in all cases the ratios between cell types were consistent in parallel experiments.

FIGURE 16. THE DEGREE OF STIMULATION OF CYCLIC-AMP

These figures are constructed from the data shown in Figures 14 and 15. The degree of stimulation of cAMP is expressed relative to control values of cAMP concentrations in the presence of IBMX alone.

The points represent the effect of isoprenaline in AP-9 cells (■—■), SKL-2 cells (▲—▲) and EB_2 cells (●—●) and the effect of PGE_1 in AP-9 cells (□—□), SKL-2 cells (△—△) and EB_2 cells (O—O).


Degree of stimulation of cAMP
100
10
IBMX
-8
-6
-4
-2
log [Iso] (M)

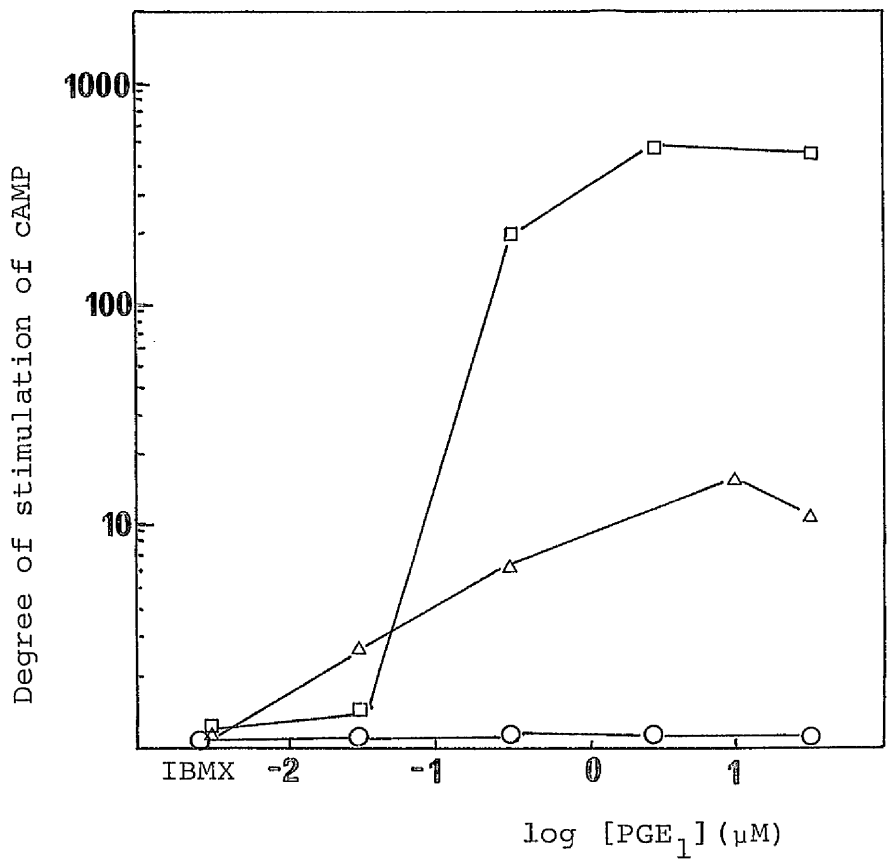

Degree of stimulation of cAMP
1000
100
10
IBMX
-2
-1
0
1
log [PGE1] (μM)

FIGURE 16.

TABLE 2. CONTROL LEVELS OF CYCLIC-AMP

Treatment	Cyclic-AMP (pmoles/mg protein)		
	AP-9	EB_2	SKL-2
No addition	8.6	0.9	2.6
3-Isobutyl-1-methyl-xanthine (IBMX) (50µg/ml = 0.23mM)	11.4	5.4	3.6

The experimental details are given in the legend to Figure 14. The data represent the average of at least 4 independent experiments.

The β-adrenergic antagonist, (±) propranolol, binds specifically to β-receptors without activating adenylate cyclase. Preincubating this antagonist for 10 minutes before isoprenaline incubation, at 1μM; a concentration known to completely abolish β-adrenergic agonist action in C6 glioma cells (Gilman and Minna, 1973), it was possible to show that the isoprenaline-stimulation of cAMP synthesis was reduced by 92%, 83% and 86% in AP-9, SKL-2 and EB_2 cells respectively. Addition of (+) propranolol had no effect on the elevation of cAMP, while incubation with the β_1-adrenergic antagonist, practolol produced 94% blockade in AP-9 cells. This suggested that isoprenaline activated adenylate cyclase by binding in a stereoselective manner to β-adrenergic receptors and at least in AP-9 cells the receptors should be classed as β_1.

III. TETRACAINE ACTION

Striking differences were observed in the effects of various concentrations of tetracaine on isoprenaline and PGE_1 stimulation of cAMP synthesis in AP-9 cells (figures 17 and 18), SKL-2 cells (figures 19 and 20), and EB_2 cells (figures 21 and 22). The experimental conditions were analogous to those described for characterisation of cAMP responsiveness to hormones. Tetracaine was added to the various concentrations of hormone before the assay and then co-incubated with the hormone for 10 minutes. The range of tetracaine concentrations examined was chosen

after preliminary experiments showed that other concentrations were less effective. Data presented in the graphs are representative results of at least four independent experiments.

AP-9 Cells

Figures 17 and 18 show the effects of various concentrations of tetracaine on cAMP responsiveness to isoprenaline and PGE_1 respectively. The most interesting observation is the ability of low concentrations of tetracaine to potentiate cAMP accumulation, while higher concentrations act to virtually abolish the effect of hormone. This potentiation is particularly noticeable with cells incubated in the presence of isoprenaline at 0.5mM tetracaine. Cells incubated with IBMX in the absence of hormone do not exhibit significantly altered cAMP levels, with tetracaine. Both 1.0μM isoprenaline and 0.28μM PGE_1; hormone concentrations which are close to those which produce half maximal cAMP stimulation can be seen to be optimally potentiated by lower tetracaine concentrations.

With the limitations imposed by the large number of samples assayed, it is difficult to determine the exact concentration of tetracaine which produces the maximal response. Therefore it is more accurate to point out that with optimal isoprenaline concentrations a peak of cAMP occurs between concentrations of

FIGURE 17. EFFECT OF TETRACAINE ON ISOPRENALINE-STIMULATED CYCLIC-AMP LEVELS IN AP-9 CELLS

Cells were equilibrated at 37°C for 15 minutes and tetracaine, dissolved in salt solution, containing the hormone, was warmed to 37°C before use. Tetracaine and Isoprenaline were co-incubated for 10 minutes at 37°C in the presence of the phosphodiesterase inhibitor, 3-isobutyl-1-methyl xanthine (0.23mM final concentration).

The results represent the levels of cAMP accumulated over the 10 minute incubation period and are expressed as the means of duplicate cAMP determinations from duplicate incubations. The standard deviation was less than 15%. Incubations occurred in the presence of no hormone (△—△) and isoprenaline concentrations of 10^{-8}M (○—○); 10^{-6}M (▲—▲); 10^{-5}M (□—□); 10^{-4}M (●—●); 10^{-3}M (■—■).

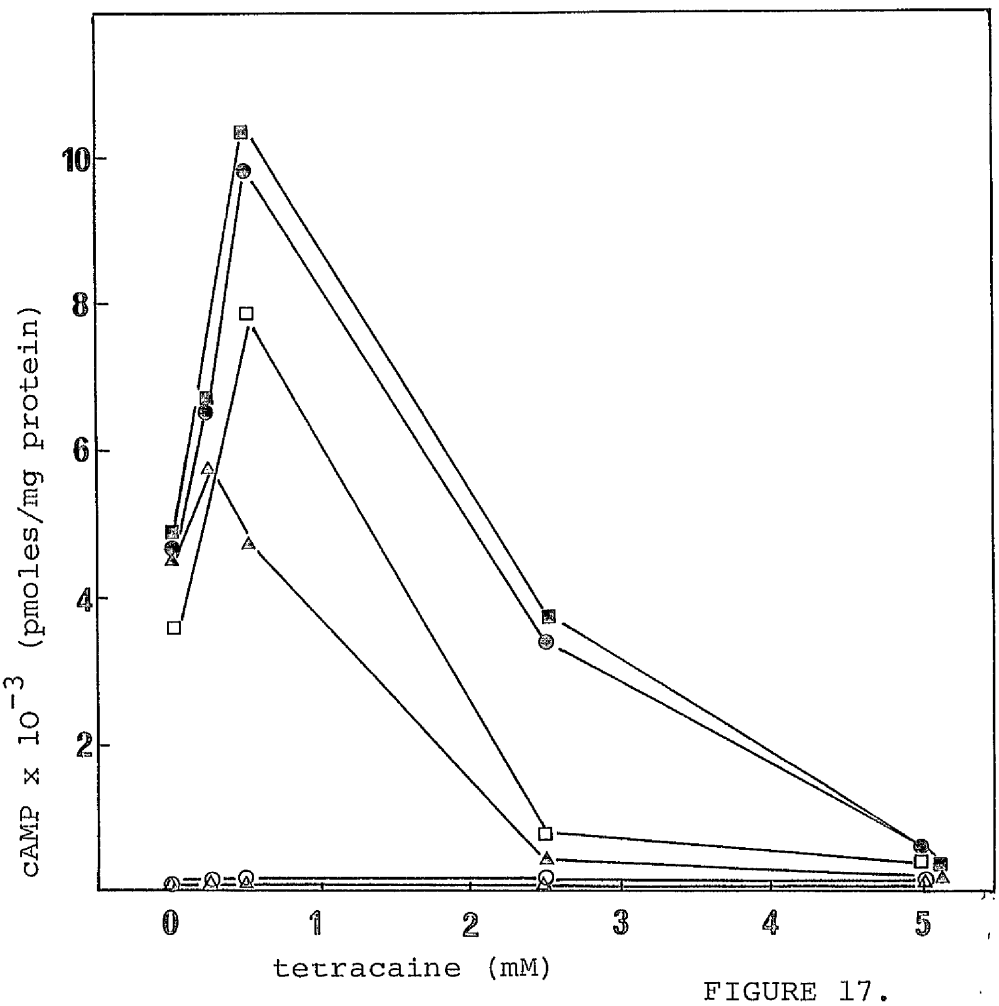


FIGURE 17.

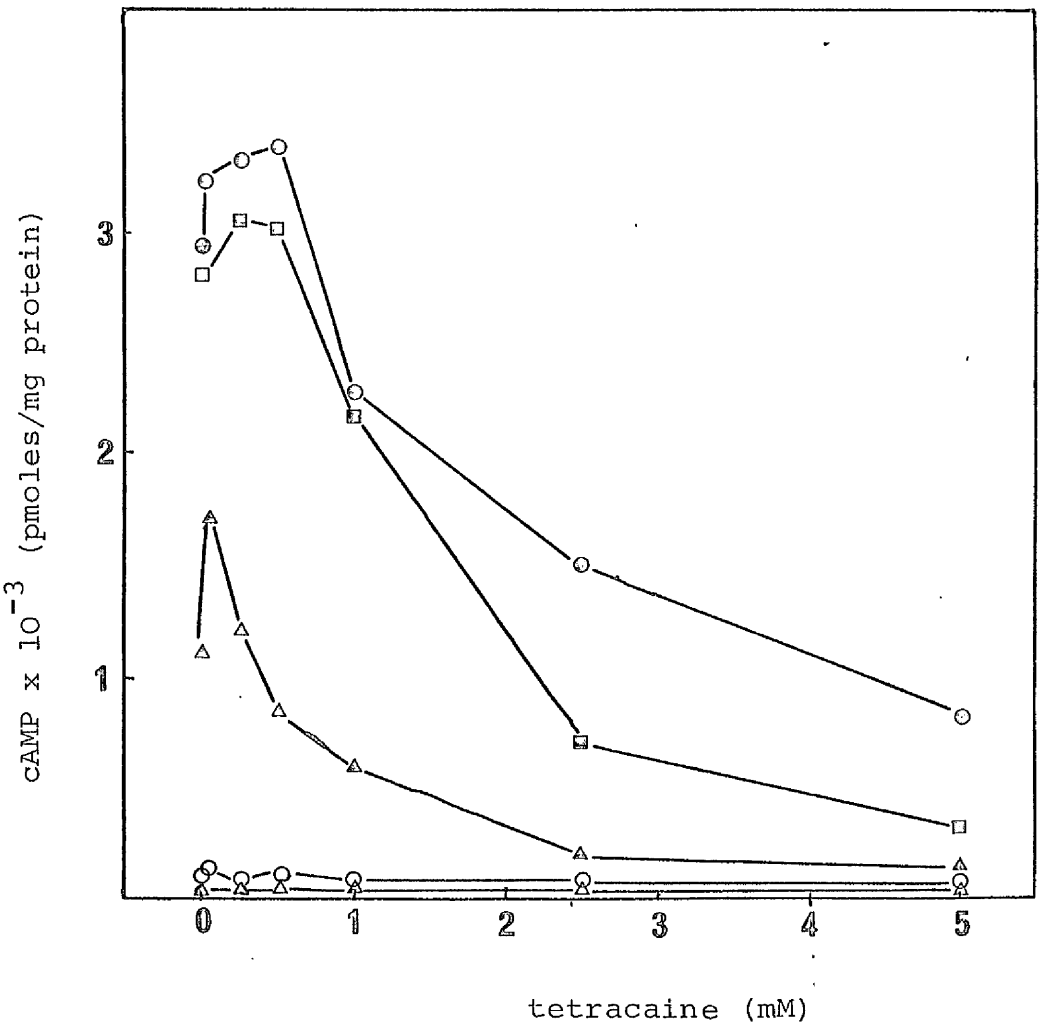


FIGURE 18. EFFECT OF TETRACAINE ON PROSTAGLANDIN-E_1 STIMULATED CYCLIC-AMP LEVELS IN AP-9 CELLS

The experimental details are described in the legend to Figure 17. Incubations occurred in the absence (△—△) and presence of prostaglandin E_1 concentrations of 0.028μM (o—o); 0.28μM (△—△); 2.8μM (□—□); 28.0μM (o—o).

0.25 and 2.5mM, while optimal PGE_1 concentrations produce a peak within the tetracaine range of 0.25 to 1.0mM. With sub-optimal hormone concentrations the peak occurs at tetracaine concentrations of less than 0.5 and 0.25mM respectively.

SKL-2 Cells

In marked contrast to AP-9 cells, both basal and hormonally stimulated cAMP levels are increased by tetracaine in SKL-2 cells (figures 19 and 20). Thus 5mM tetracaine produced 2-3 fold elevation of cAMP with cells incubated in the absence or presence of hormone. However, similar trends were not observed when tetracaine was incubated with 10^{-3}M isoprenaline. This may be the result of an interaction between the local anaesthetic and the high isoprenaline concentrations, which tends to produce an acidic solution, (pH 5.0 after 10 minute incubation).

It should be noted that the potentiation observed with tetracaine does not constitute a restoration of hormone responsiveness to these malignant cells.

EB_2 Cells

The response of EB_2 cells was more complicated than that exhibited by the other two cell lines and could be described as being a combination of the effects previously observed (figures 21 and 22). The

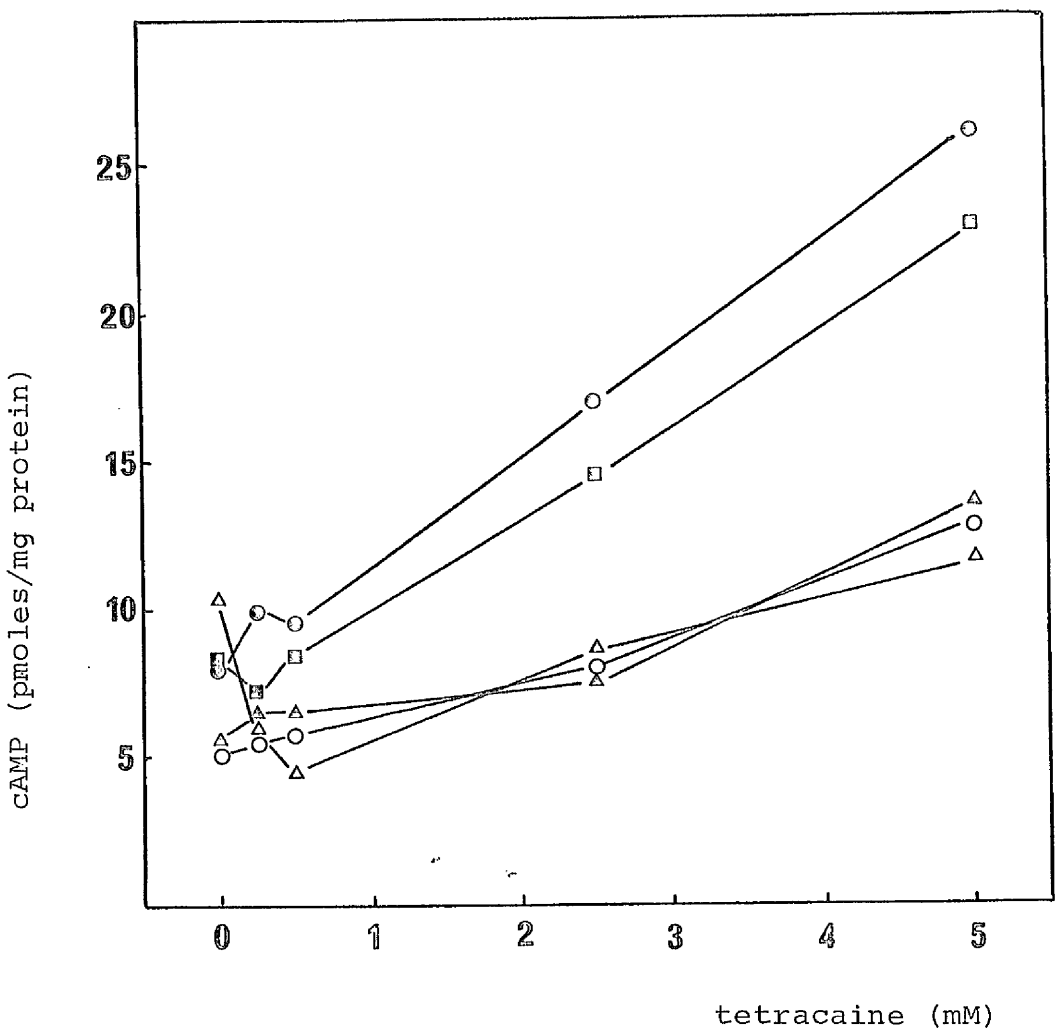


FIGURE 19. EFFECT OF TETRACAINE ON ISOPRENALINE STIMULATED CYCLIC-AMP LEVELS IN SKL-2 CELLS

The experimental details are described in the legend to Figure 17. Incubations occurred in the absence (o—o) and presence of isoprenaline concentrations of 10^{-8}M (△—△); 10^{-6}M (□—□); 10^{-4}M (o—o); 10^{-3}M (△—△).

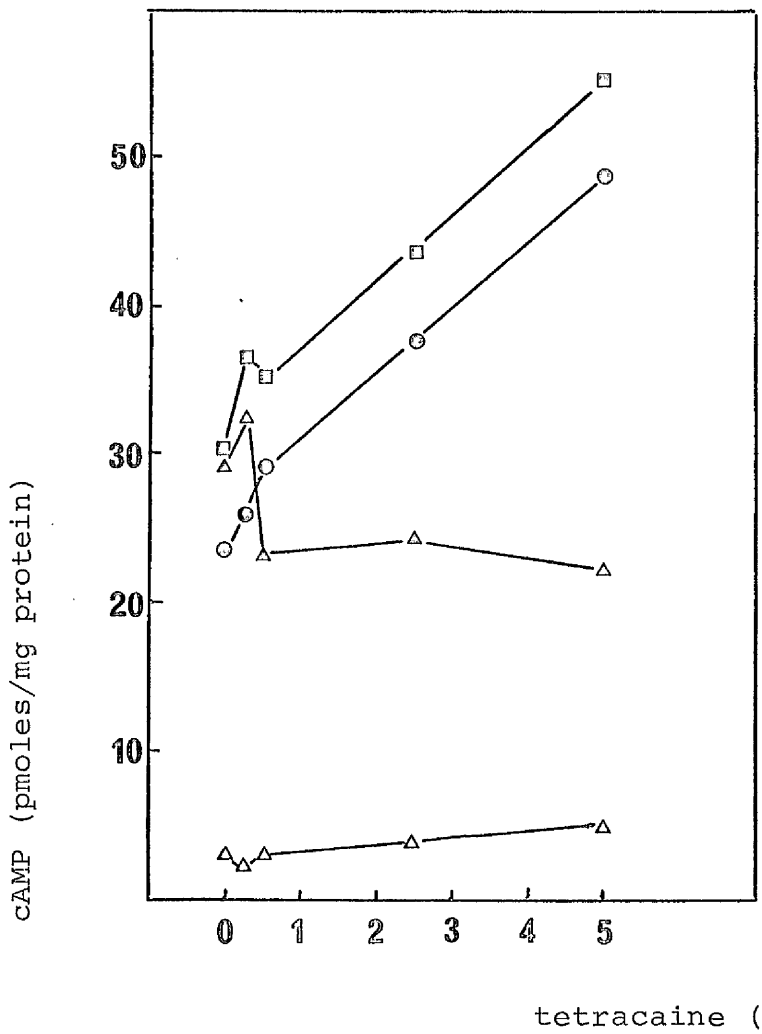


FIGURE 20. EFFECT OF TETRACAINE ON PROSTAGLANDIN E_1 STIMULATED CYCLIC AMP LEVELS IN SKL-2 CELLS

The experimental details are described in the legend to Figure 17. Incubations occurred in the absence (△ — △) and the presence of prostaglandin E_1 concentrations of 0.028μM (△ — △); 2.8μM (⊙ — ○); 28μM (▣ — □).

enhanced hormonal stimulation at low tetracaine concentrations and the inhibition at higher concentrations was similar to the biphasic response of cAMP in AP-9 cells. However, this inhibition produced a minimum in cAMP levels at approximately 2.5mM tetracaine and at 5.0mM the cAMP levels were generally equal to or greater than those observed in the absence of tetracaine. This complex response was observed in five independent experiments with two different hormones and although not easily explained, it could not be attributed to any recognisable experimental error.

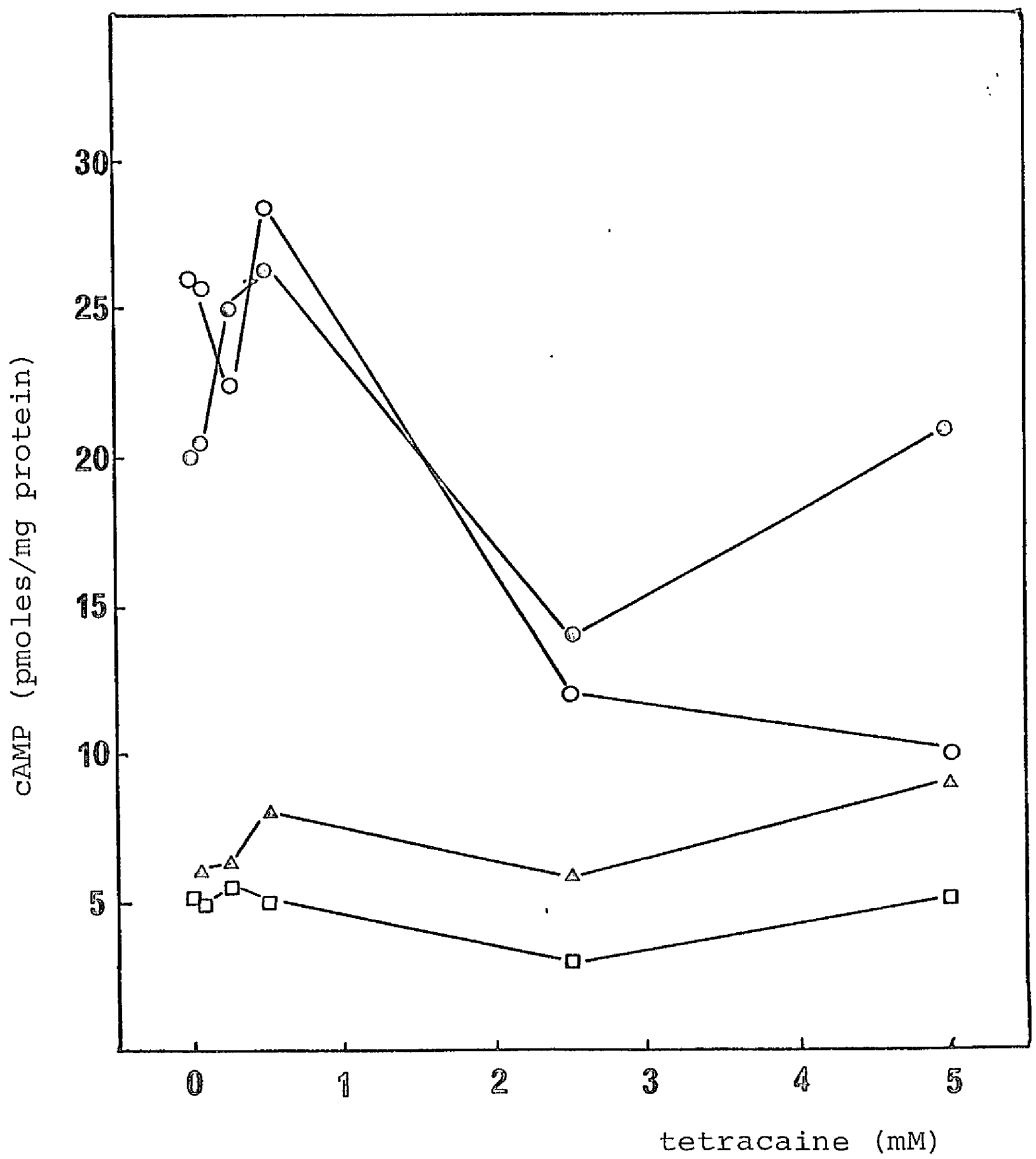


FIGURE 21. EFFECT OF TETRACAINE ON ISOPRENALINE STIMULATED CYCLIC-AMP LEVELS IN EB_2 CELLS

The experimental details are described in the legend to Figure 17. Incubations occurred in the absence (△ — △) and presence of Isoprenaline concentrations of 10^{-8}M (□ — □); 10^{-6}M (○ — ○); 10^{-4}M (○ — ○).

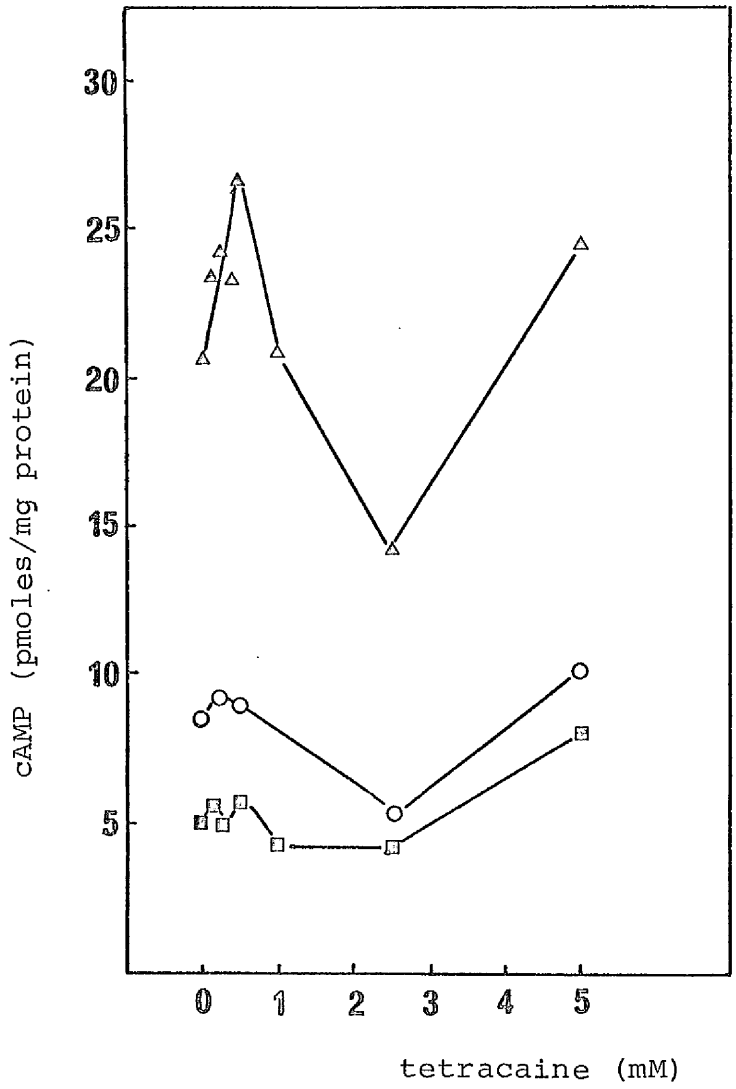


FIGURE 22. A COMPARISON OF THE EFFECT OF ISOPRENALINE AND PROSTAGLANDIN E_1 STIMULATED CYCLIC-AMP LEVELS SUBJECTED TO VARYING CONCENTRATIONS OF TETRACAINE IN EB_2 CELLS.

The experimental details are described in the legend to Figure 17. Incubations occurred in the absence (□—□) or presence of prostaglandin E_1 28μM (o—o) or Isoprenaline 10^{-4}M (△—△)

SECTION B. A STUDY OF GROWTH REGULATION IN A SOMATIC CELL HYBRID

The ability of a hybrid (PCM3) to grow as a monolayer with an unusually large proportion of it's population in suspension has provided an ideal opportunity to examine changes in cell growth and to investigate the interrelationship with cAMP, within a single cell line. By studying changes in adhesion it has been possible to examine another plasma membrane defect,associated with malignancy.

I. CHARACTERISTICS OF THE PARENTAL AND HYBRID CELLS

As previously mentioned Ayad and Delinassios (1974) obtained a series of hybrids (PCM) from fusion between Chinese hamster fibroblasts (CH23) and mouse lymphoma cells (P388F-36). The resulting hybrids were isolated by non-selective pressure, and after cloning their hybrid nature was established by esterase isozyme expression and cyclic nucleotide phosphodiesterase inheritance (Ayad and Wright, 1977).

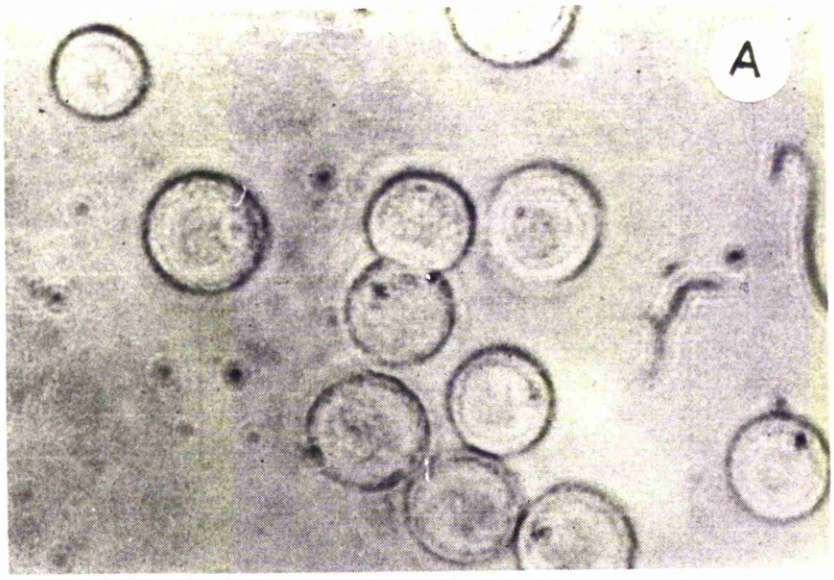
The parental cell lines have been described in the Materials and Methods section and from figure 23, it can be seen that the morphology of the two cell lines is quite different. CH23 cells are spindle - shaped monolayer cells which align in parallel arrays

FIGURE 23. MORPHOLOGY OF (A) P388F-36 (B) CH23 AND (C) PCM3-M CELLS

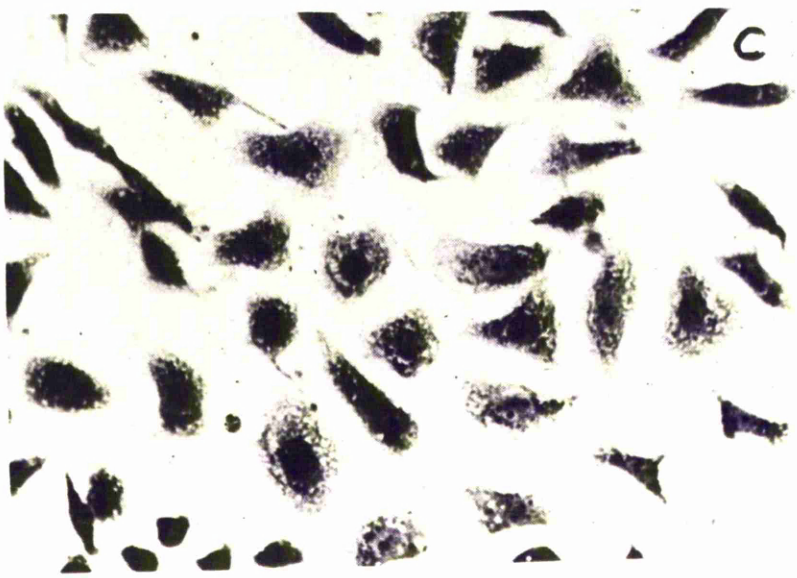
(A) P388F-36 cells were grown in medical flat bottles and collected by centrifugation at 750g for 5 minutes. The cells were then resuspended in a small volume of medium and examined directly under phase contrast using a Zeiss microscope, with a magnification of x480.

(B) & (C) CH23 and PCM3-M cells were grown on coverslips in plastic petri dishes. The cells were fixed and stained with 1% methyl violet, and then examined using a Zeiss microscope. The magnification for CH23 cells was x190 and for PCM3-M was x 480.

A Zeiss camera fitted with a Kodak Panatonic-X (black and white) film was used to photograph the cells.


A


B


C

of tightly packed whorls as they reach confluency. In comparison P388F-36 cells are spherical with large nuclei and grow in suspension to high density.

In contrast, one of the hybrids designated PCM3 grows as a monolayer with 10-20% of the cells either loosely attached or freely floating in suspension. The cells which adhere to the substratum (PCM3-M) are quite distinct from the parental CH23 cells in that they are more polygonal in shape with shorter processes (figure 23) and at confluency they are easily distinguishable because they do form tightly packed whorls. Thus, they cease to grow at lower densities than CH23 cells and tend to overlap, instead of aligning in parallel arrays.

The suspension form of the hybrid (PCM3-S) is visualised under the microscope as spherical cells situated just above the monolayer and characterised by the haloes surrounding them (figure 24). These cells are also observed in the medium. Examination of viability by dye-exclusion techniques has proved that these cells are viable and this has been substantiated by their ability to divide after passage. PCM3-S cells are present at all densities throughout the growth of the hybrid and are therefore not a consequence of over-crowding of the monolayer.

FIGURE 24. MORPHOLOGY OF PCM3 CELLS

The PCM3 cells were cultured on coverslips in plastic petri dishes and then examined directly with minimal disturbance, which allowed the observation of loosely-attached PCM3-S cells (arrows). The cells were observed under phase contrast using a Zeiss microscope with a magnification of x480.

A Zeiss camera fitted with a Kodak Panatonic-X (black and white) was used to photograph the cells.

Examination of the chromosomal complement of the PCM series of hybrids was facilitated by the fact that the mouse lymphoma cells contained 36, mainly acrocentric chromosomes, while the CH23 cells, with 23 chromosomes, had a large proportion which were metacentric (figure 25). The PCM hybrid cells stabilised with no fewer than 19 hamster chromosomes coexisting with 2-5 mouse chromosomes. The PCM3 hybrid contained 4-5 mouse chromosomes and the karyotype has remained stable and identical in both PCM3-M and PCM3-S during these investigations. This has been confirmed by detailed studies in this laboratory (Tobia, 1976; unpublished data).

The most obvious explanation for the coexistence of monolayer and suspension cells in PCM3 was that the population was heterogeneous. However, the clonal origin and the karyotype analysis would tend to discount this proposal. Further evidence against this suggestion was obtained from the examination of growth curves.

PCM3 cells were subcultured from either monolayer or suspension cells of equivalent density and the newly passaged cells grow in an identical manner, in that both monolayer and suspension cells were observed regardless of the origin of the seeded cells. Continual routine passage of the PCM3-S cells over several months showed that they were capable of perpetuating the cell line in the same way as the monolayer.

FIGURE 25. KARYOTYPES OF (A) P388F-36, (B) CH23 AND (C) PCM3 CELLS

(A) P388F-36 metaphase preparation containing 36 mainly acrocentric chromosomes.

(B) CH23 metaphase preparation containing 23 mainly metacentric chromosomes.

(C) PCM3 metaphase preparation containing 19 hamster chromosomes and 4 mouse chromosomes (arrows)

The metaphases were prepared as described in the Materials and Methods section and the chromosomes stained with acetic-orcein. The preparations were photographed using a Zeiss camera fitted to a Zeiss microscope with an oil-immersion lens and a magnification of x1200.


A
B
C

It could be envisaged that the PCM3-S cells used for subculturing were contaminated with some rounded monolayer cells or that the PCM3-M cells were contaminated by loosely attached suspension cells. However, this would be detected by altered growth curves and this was not observed (figure 26). In the experiments described subsequently both monolayer and suspension forms of the hybrid were used together for the subculture of cells.

II. CYCLIC AMP AND CELL GROWTH IN THE PARENTAL AND HYBRID CELLS

1. Cell Growth in the Presence of Elevated Cyclic AMP Levels

Since the now classical studies by Bürk (1968) who showed that exogenous cAMP inhibited cell division and by Johnson *et al.* (1971) who demonstrated that $N^6,2'$-O-dibutyryl cAMP (dbcAMP) produced a restoration of 'normal' morphology to transformed cells, numerous reports have confirmed and extended these observations to provide evidence for the role of cAMP in the regulation of proliferation, (review by Ryan and Heidrick, 1974). It was therefore of interest to study the effects of increased cAMP levels on the growth and morphology of the monolayer and suspension forms of PCM3.

FIGURE 26. GROWTH CURVES FOR PCM3

PCM3-M cells from a confluent culture were trypsinized and passaged to give 4.9×10^5 cells per culture bottle. The resultant PCM3-M (●—●) and PCM3-S (▲—▲) were counted until they reached stationary phase.

PCM3-S cells from the same confluent culture were carefully removed and seeded in parallel bottles at equivalent densities. The resultant PCM3-M (o—o) and PCM3-S (Δ—Δ) were counted in an identical manner.

The cell number was determined using a Coulter counter and expressed as total cells per culture bottle. Viability was not less than 98%.

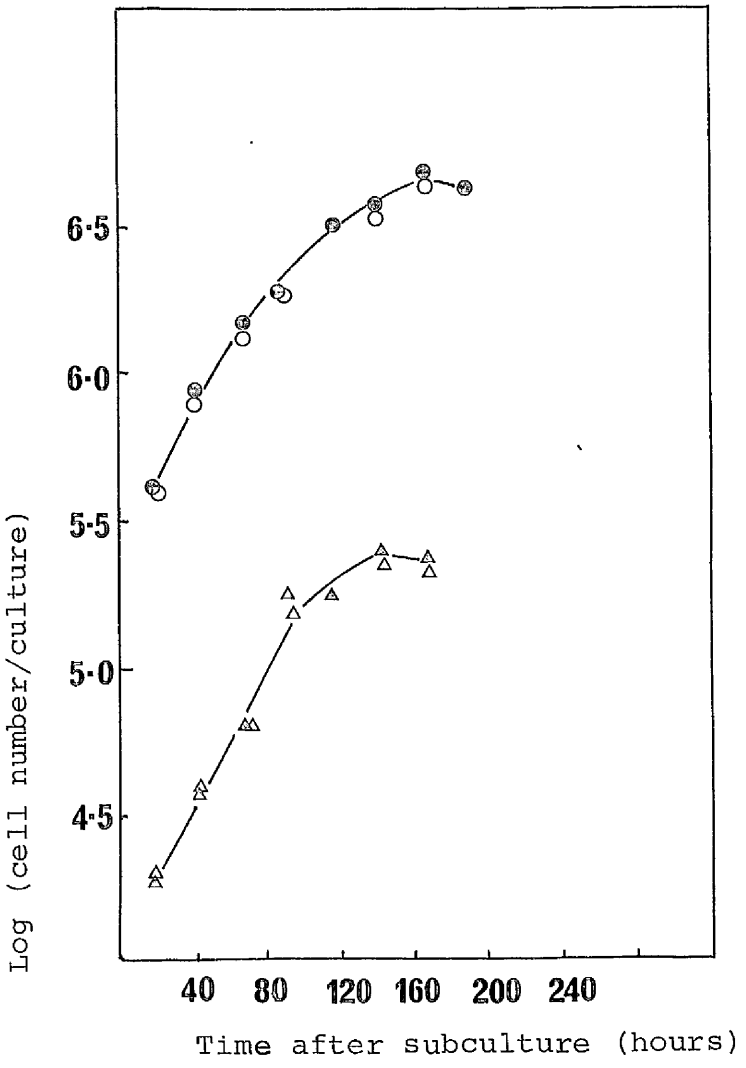

6·5
6·0
5·5
5·0
4·5
Log (cell number/culture)
40
80
120
160
200
240
Time after subculture (hours)

FIGURE 26

This was achieved by comparing the growth of hybrid cells, incubated in the presence and absence of the hormone PGE_1 at a concentration of 2.8μM which significantly stimulates cAMP synthesis. Parallel cultures were incubated in medium containing 0.1mM IBMX, which is known to inhibit the cyclic nucleotide phosphodiesterase and thereby increase intracellular cAMP. Cell growth was also monitored in cultures which contained both PGE_1 and IBMX.

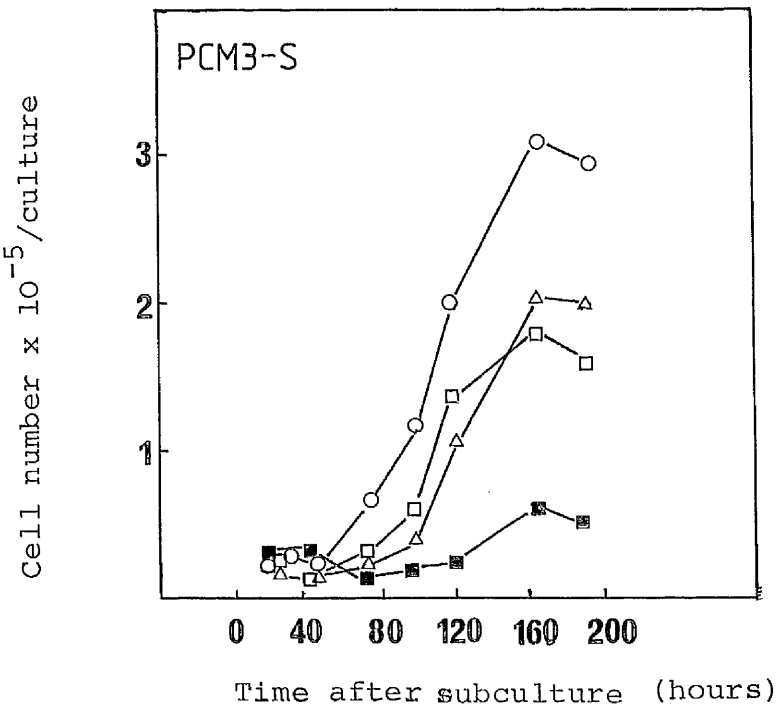
These agents inhibited proliferation by differing degrees in both PCM3-M and PCM3-S (figure 27). PGE_1 and IBMX incubated together produced the most striking effect by inducing a premature quiescence which virtually abolished the suspension form of the hybrid. When incubated separately IBMX was as effective as PGE_1 at slowing growth rate and reducing cell number in the stationary phase. However, co-incubating these agents resulted in a synergistic inhibition, which suggested that they were acting specifically via an increase in intracellular cAMP concentrations. This test for synergism was a useful control which precluded non-specific inhibition.

Examination of the ratio of PCM3-M to PCM3-S cells revealed that incubation with PGE_1 + IBMX resulted in a decrease in the percentage of cells in suspension from 5% at 24 hours to 2% from 71 hours onwards. This

FIGURE 27. GROWTH OF PCM3 IN THE PRESENCE OF AGENTS WHICH INCREASE cAMP

A single mixed culture of PCM3-M and PCM3-S was used to seed 4 sets of bottles containing no additions (o—o), 0.1mM IBMX (□—□), 2.8μM PGE_1 (△—△) and a combination of PGE_1 + IBMX at the same concentrations (■—■). PGE_1 concentrations were maintained by daily replenishments. 200ml bottles containing 20ml medium were used so that PCM3-M and PCM3-S could be counted from the same bottle.

Cell number was determined using a Coulter counter and expressed as total cells per bottle. Viability was not less than 95% and graphs show results representative of three independent experiments.


PCM3-S
Cell number x 10^{-5}/culture
3
2
1
0
40
80
120
160
200
Time after subculture (hours)

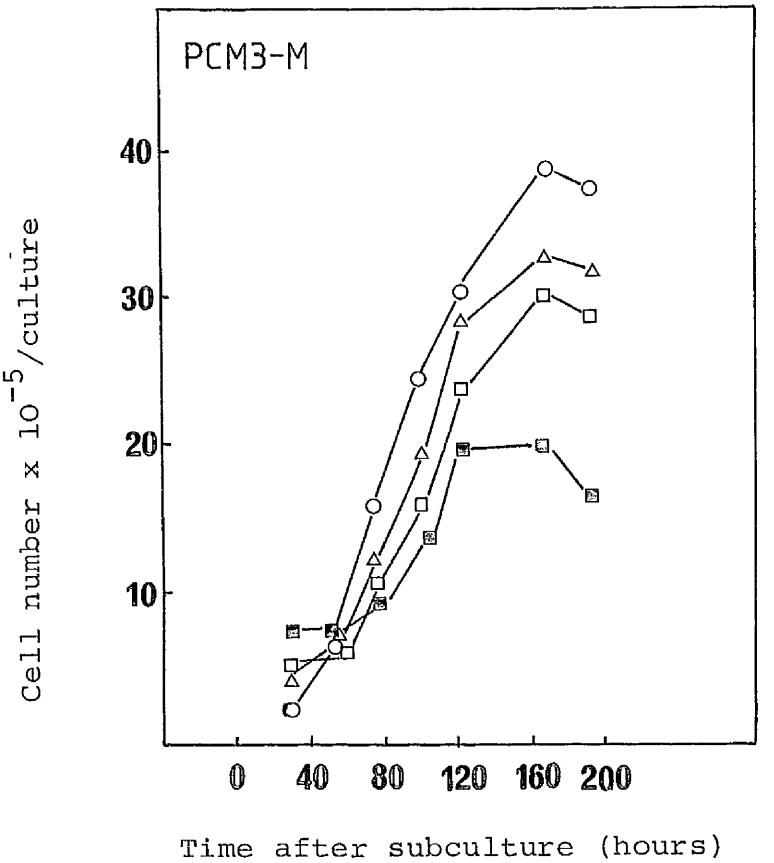

PCM3-M
Cell number x 10^{-5}/culture
40
30
20
10
0
40
80
120
160
200
Time after subculture (hours)

FIGURE 27

trend was not observed when the agents were incubated separately, since the percentage of suspension cells remained constant at approximately 5%.

During this experiment it was noted that the PCM3-M cells, incubated in the presence of these agents exhibited an altered morphology. This was examined in more detail by time-lapse photography. Cells incubated in PGE_1 + IBMX under identical conditions appeared to increase in size during the first 24 hours in culture. However, these monolayer cells may have developed a more flattened morphology, as a result of increased adhesion and this would have given the appearance of an increase in size. This was suggested by the fact that movement over the substratum was severely restricted during the incubation, and was substantiated by the lack of difference in cell size after trypsinization of control and treated monolayer cells (Table 3).

Removal of PGE_1 + IBMX-containing medium at 95 hours, when cultures had begun to show growth inhibition and replacement by conditioned medium from control cultures allowed partial but not complete growth restoration of PCM3-M (figure 28). In comparison, an increase in cell number was not observed with PCM3-S cells, although this may have been due to cell loss during the replacement of medium. Thus the effect of

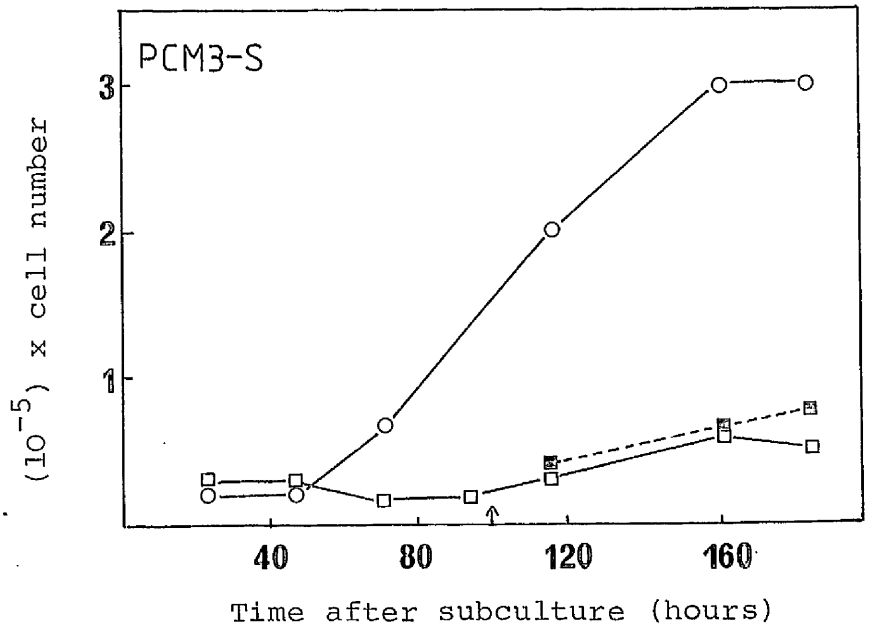
TABLE 3. CELL SIZE ANALYSIS OF PCM3

Treatment	PCM3-M		PCM3-S	
	volume (μm^3)	radius (μm)	volume (μm^3)	radius (μm)
Control	25	7.8	24	7.7
PGE_1 (2.8μM) + IBMX (0.1mM)	24	7.7	23	7.6

The experimental details are described in the legend to Figure 27. Cell size was determined 24 hours after subculture. A size distribution graph was plotted using a Coulter counter with an amplification of 8 and an aperture current of 1.0.

FIGURE 28. REMOVAL OF AGENTS WHICH INCREASE CYCLIC-AMP

The experimental details are described in the legend to Figure 27. At 95 hours (arrows) medium containing PGE_1 + IBMX was removed by centrifugation of PCM3-S and replaced by conditioned medium from control cultures incubated in an identical manner. Graph shows control cultures without additions (o—o), cells incubated with PGE_1 + IBMX (□—□) and parallel cultures with conditioned medium (■—■).


PCM3-S
3
2
1
(10^{-5}) x cell number
40
80
120
160
Time after subculture (hours)

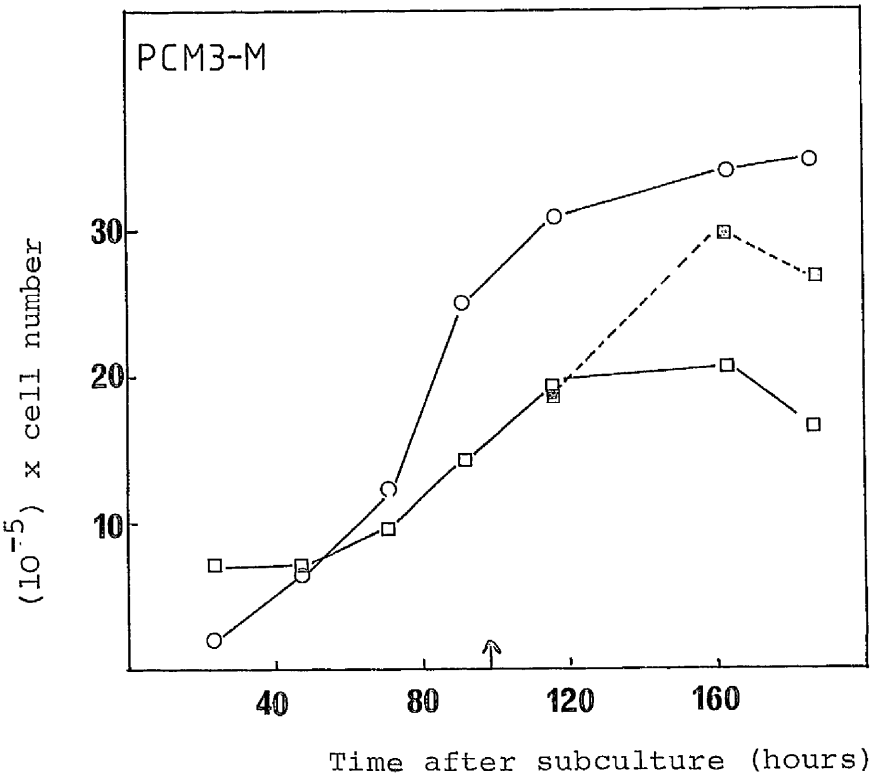

PCM3-M
30
20
10
(10^{-5}) x cell number
40
80
120
160
Time after subculture (hours)

FIGURE 28

cAMP on cell growth may be partially reversed, but the reinitiation of cell division is not observed until at least 20 hours after removal of the agents which elevate cAMP.

As a comparison, the effect of PGE_1 and IBMX incubated together and separately was examined in the parental cell lines (figures 29 & 30). CH23 cell growth was inhibited more effectively by IBMX, although co-incubation of hormone and phosphodiesterase inhibitor still produced the greatest response. The trends observed in P388F-36 cells were remarkably similar to those in PCM3-S cells and this tends to suggest some common denominator in suspension growth control. Thus the inhibition of growth in PCM3-S may be comparable with that in P388F-36 or may reflect an alteration in the ratio of monolayer and suspension cells.

2. Cyclic AMP Accumulation in Response to PGE_1

Prostaglandin E_1 stimulation of cAMP synthesis was characterised in the monolayer and suspension forms of PCM3 and compared with the cAMP responsiveness in the parental cell lines. As seen with other PCM hybrids (Ayad and Foster, 1974) the basal and hormonally elevated cAMP levels were enhanced in both PCM3-M and PCM3-S cells in comparison with CH23 and P388F-36

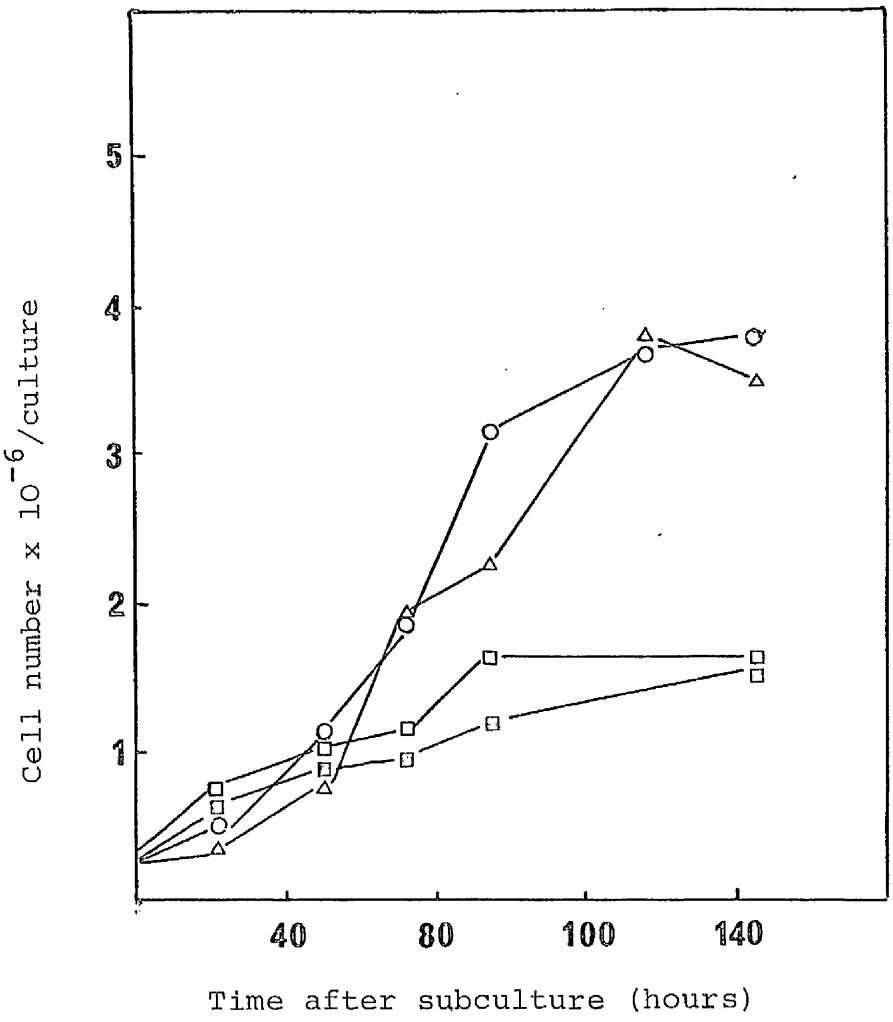


FIGURE 29. EFFECT OF CYCLIC-AMP ELEVATION ON GROWTH OF CH23 CELLS

A trypsinized suspension of confluent CH23 cells was used to seed 4 sets of bottles containing no additions (o—o) 0.1mM IBMX (□—□) 2.8μM PGE_1, (△—△) and a combination of PGE_1 + IBMX at the same concentrations (▣—▣). Experimental details are the same as those described in the legend to Figure 27, except that 100ml bottles containing 10ml medium were used.

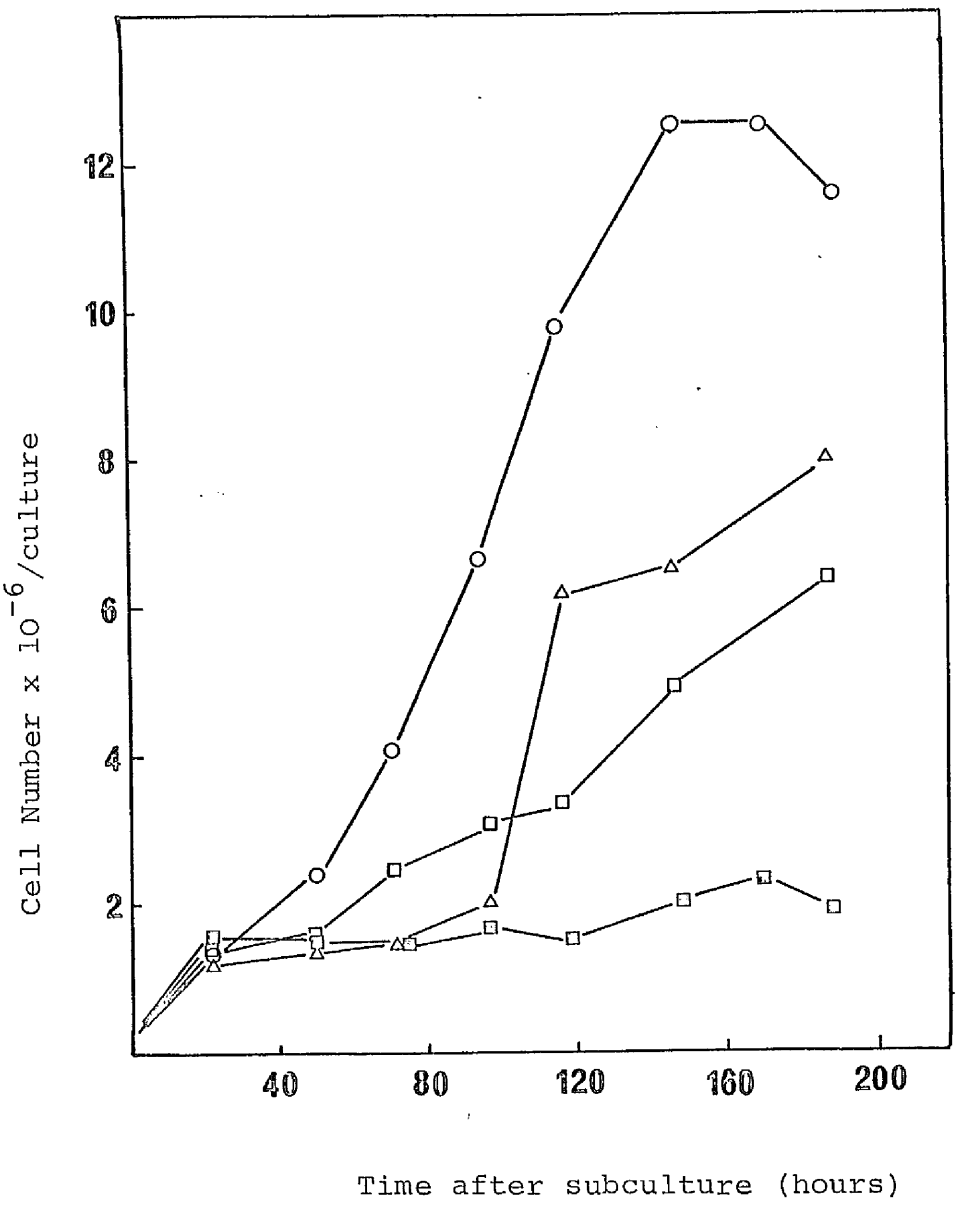


FIGURE 30. EFFECT OF CYCLIC-AMP ELEVATION ON GROWTH OF P388F-36 CELLS

A single suspension of confluent P388F-36 cells was used to seed 4 sets of bottles containing no additions (o—o) 0.1mM IBMX (□—□) 2.8μM PGE_1 (△—△) and a combination of PGE_1 + IBMX at the same concentrations (▣—▣). Experimental details are the same as those described in the legend to Figure 29.

cell lines (figure 31). Thus the parental cells exhibited a three-fold increase in cAMP levels after incubation with PGE_1 while PCM3-M and PCM3-S cells showed a 30-fold and 50-fold stimulation respectively. All the cell lines appeared to be maximally stimulated by 2.8μM PGE_1, but the hormone concentration which produced a half-maximal stimulation was observed to vary slightly from 0.028μM in CH23 cells to 0.14μM in P388 and PCM3-M cells and to 0.35μM in PCM3-S cells.

The effect of PGE_1 was examined in both actively dividing cells at the logarithmic phase of growth and in cells which had reached quiescence. The cAMP responsiveness to PGE_1 in PCM3-M cells was significantly reduced in mid-logarithmic growth while that of PCM3-S cells was slightly elevated (figure 32). In comparison the response of both CH23 and P388F-36 cells tended to be greater in actively dividing cells, with P388F-36 exhibiting a 2-fold elevation (figure 33). However, this did not provide an explanation for the difference observed between parental and hybrid cells.

An optimal concentration of PGE_1 (2.8μM) was used in a study of the time course of cAMP accumulation (figure 34). It was apparant that the enhanced stimulation in the hybrid occurred at all the incubation times studied. In both hybrid and parental cell lines, the greatest increase in cAMP was elicited after a

FIGURE 31. CYCLIC AMP RESPONSE TO PGE_1 IN PARENTAL AND HYBRID CELLS

Dose response curves were obtained for PCM3-M (o—o), PCM3-S (●—●) CH23 (□—□) and P388F-36 (△—△).

Monolayer cells were grown to confluency in vials and before the assay PCM3-S cells were removed from PCM3-M by rinsing the cell sheet carefully. Suspension cells were collected at confluency by centrifugation and resuspended to give a protein concentration for P388F-36 of 1mg/ml and to make PCM3-M and PCM3-S equivalent. The cells were pre-equilibrated for 15 minutes at 37°C and then incubated with hormone for 10 minutes in the presence of IBMX (0.23mM final concentration). The basal levels of cAMP were determined from incubations in the absence (B) or presence (I) of IBMX. Results represent levels of cAMP accumulated over 10 minutes and are the average of at least 2 independent experiments. Each experiment is also the mean of duplicate cAMP determinations from duplicate incubations.

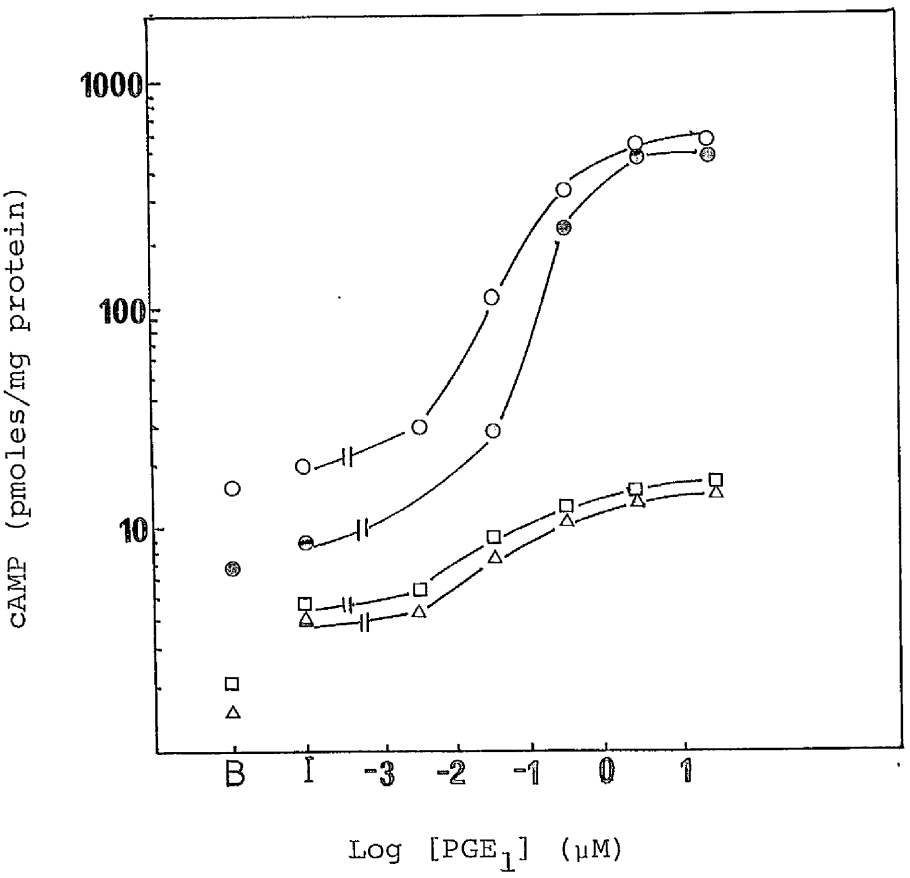


FIGURE 31

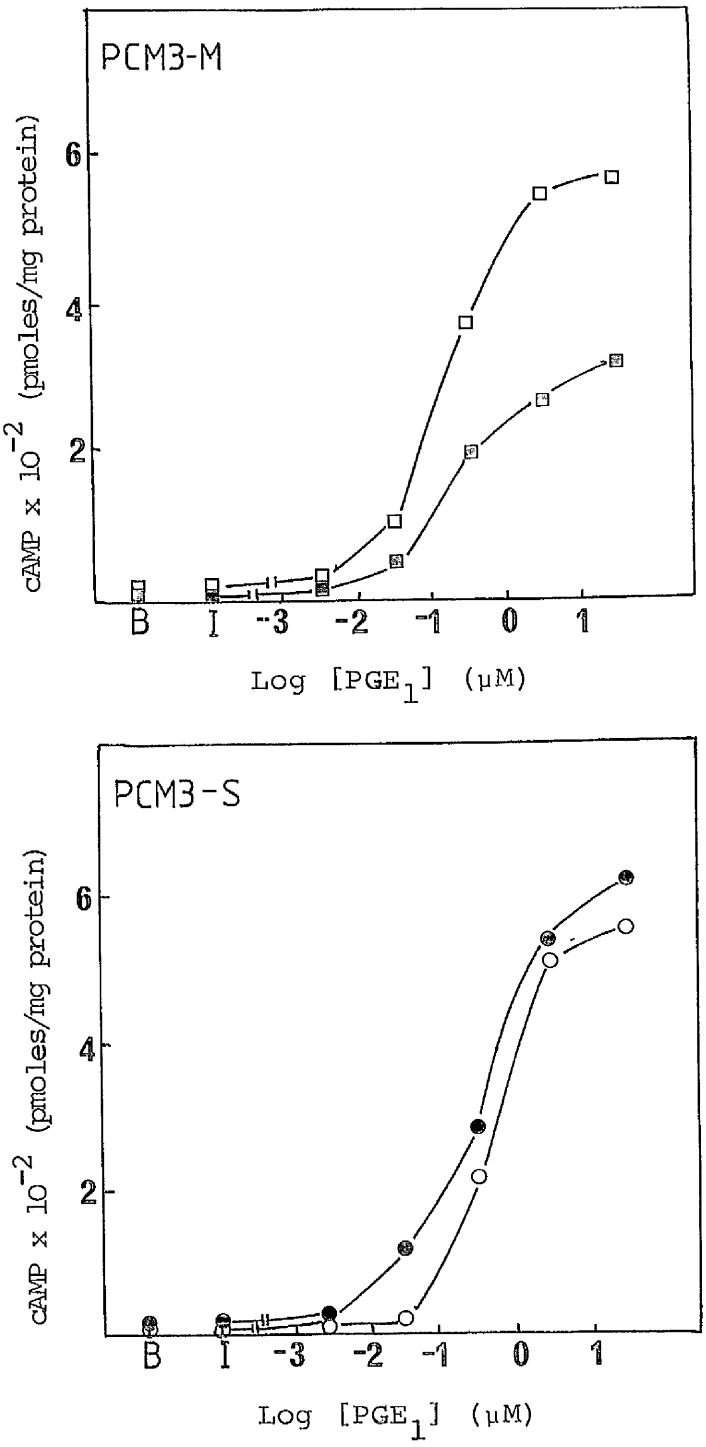


FIGURE 32. CYCLIC AMP RESPONSE TO PGE_1 IN PCM3 AT DIFFERENT STAGES OF GROWTH

The experimental details are described in the legend to Figure 31. PGE_1 stimulation of cAMP synthesis was measured after incubation for 10 min. at 37°C in PCM3-M at mid-log (□—□) and quiescence (□—□) and in PCM3-S at mid-log (●—○) and at quiescence (○—○).

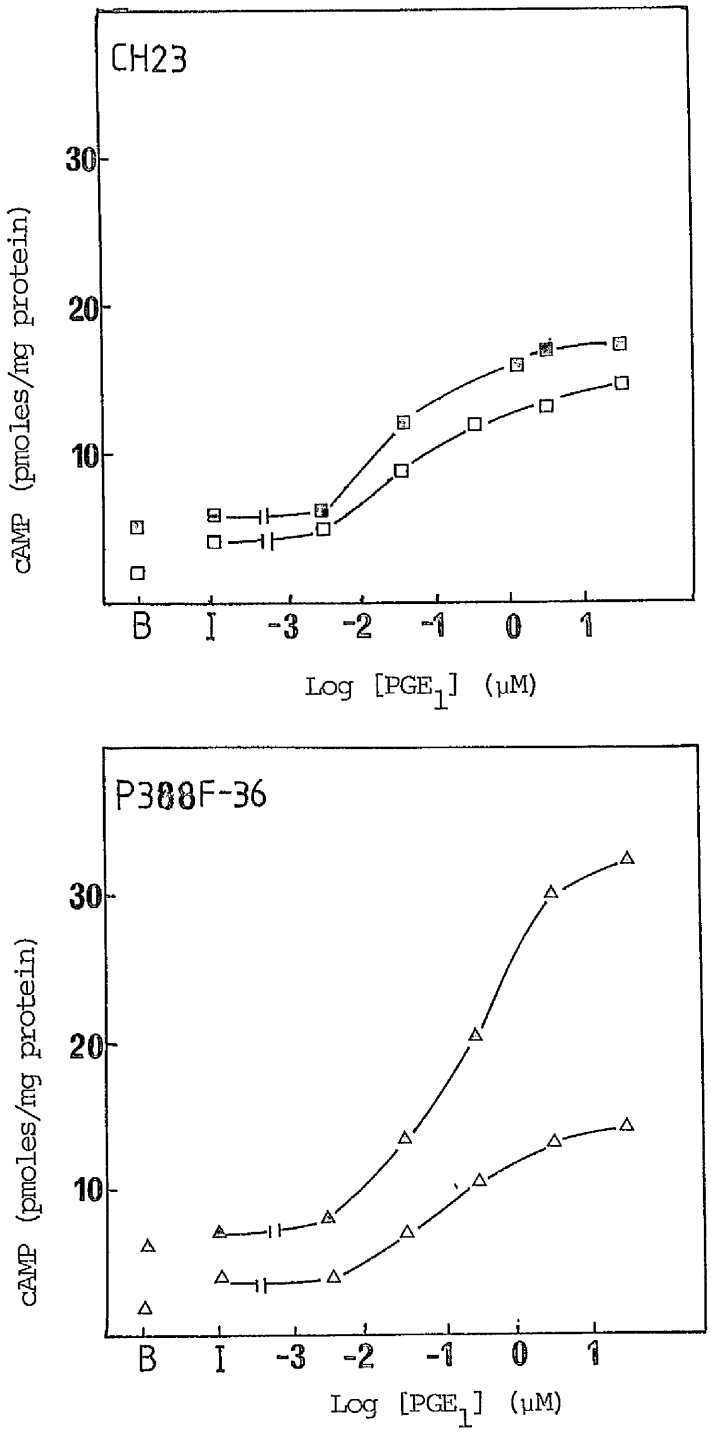


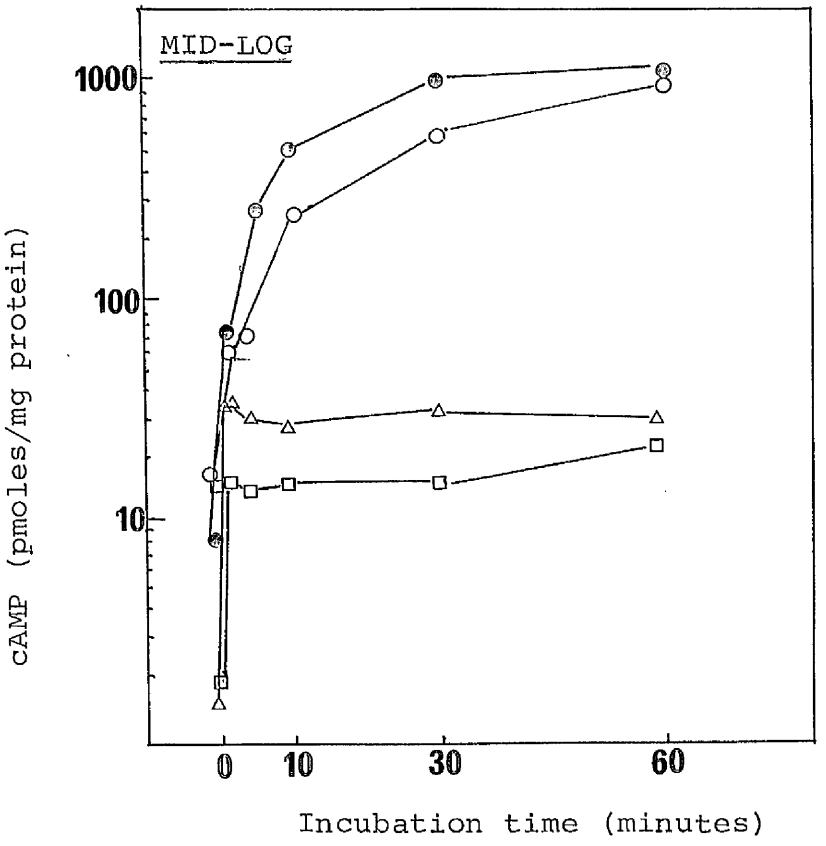
FIGURE 33. CYCLIC AMP RESPONSE TO PGE_1 AT DIFFERENT STAGES OF GROWTH

Experimental details are described in the legend to Figure 31. PGE_1 stimulation of cAMP synthesis was measured after incubation for 10 minutes at 37°C in CH23 cells at mid-log (■—■) and at quiescence (□—□) and in P388F-36 cells at mid-log (△—△) and at quiescence (△—△).

FIGURE 34. TIME COURSE OF CYCLIC AMP RESPONSE TO PGE_1 AT DIFFERENT STAGES OF GROWTH

The experimental details were the same as those described in the legend to Figure 31 except that the incubation time was varied while a constant PGE_1 concentration of 2.8μM was incubated.

The cAMP levels in PCM3-M (o—o), PCM3-S (●—●), CH23 (□—□) and P388F-36 (△—△) were assayed in cells at mid log (top graph) and quiescence (bottom graph).


MID-LOG
1000
100
10
cAMP (pmoles/mg protein)
0
10
30
60
Incubation time (minutes)

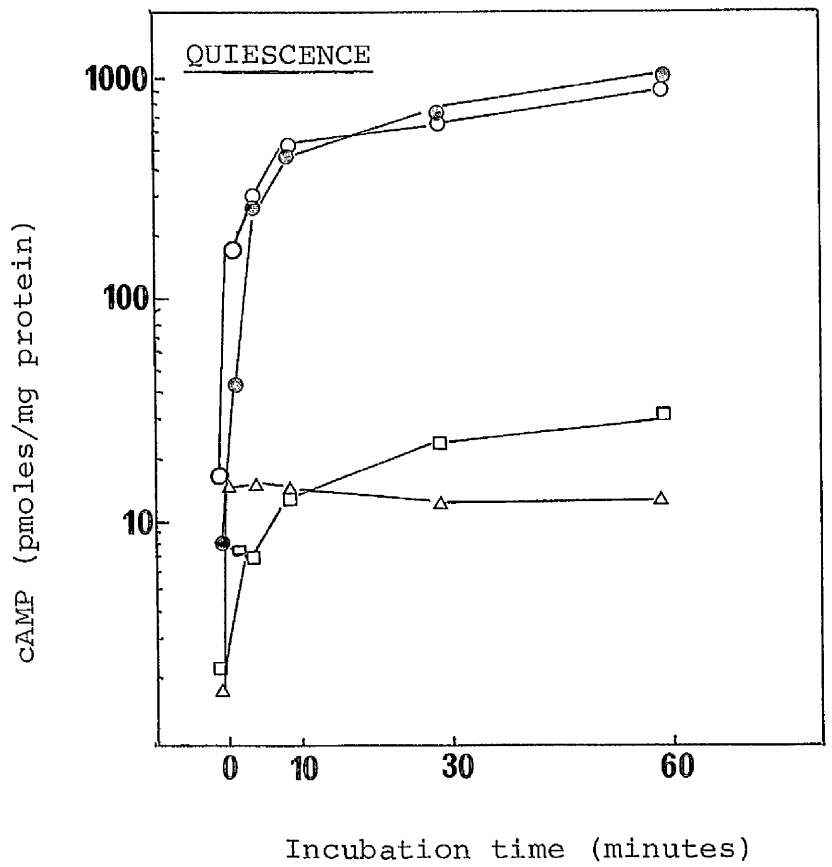

QUIESCENCE
1000
100
10
cAMP (pmoles/mg protein)
0
10
30
60
Incubation time (minutes)

FIGURE 34

10 minute incubation of hormone, although the PCM3 cells continued to accumulate cAMP upto 60 minutes after hormone addition.

Thus the enhanced hormonal stimulation of cAMP in PCM3 cells was observed at all the PGE_1 concentrations studied, with cells in both logarithmic and stationary phases of growth and after widely different incubation times, at different states of cell growth. The monolayer and suspension forms of PCM3 appeared to respond similarly under most conditions and the only notable difference was the higher basal cAMP levels in PCM3-M cells (figure 31).

3. Intracellular Cyclic AMP Concentrations

If this difference in basal cAMP levels was responsible for the difference between the monolayer and suspension growth, it would explain the decrease in the percentage of cells in suspension after incubation in agents which increase cAMP, since PCM3-S was capable of responding to PGE_1 (2.8μM) + IBMX (0.23mM) in a similar manner to PCM3-M, (as shown by figure 31).

However, comparison of the protein concentration and cell density in the experiments on hormonal stimulation of cAMP led to the determination of protein content/cell (Table 4), which showed that the suspension form of the hybrid contained twice as much protein as the monolayer form. Thus the basal levels of cAMP would be greater in PCM3-S cells than in PCM3-M cells when measured as a function of cell number. The cAMP responsiveness to PGE_1 would also be greater in PCM3-S cells when measured in this way, although the difference between parental and hybrid cells would not be significantly altered.

It was therefore necessary to examine the intracellular cAMP levels in PCM3 cells both as a function of cell number and protein content, (Table 5). This confirmed the calculations of protein concentration per cell and proved that on a cellular basis the cAMP levels were greater in PCM3-S than PCM3-M.

This suggests that the differences in the two forms of PCM3 are not related to decreased cAMP levels in suspension cells, although it does not detract from the fact that elevation of cAMP alters the growth of the hybrid.

TABLE 4. COMPARISON OF PROTEIN CONTENT IN PARENTAL AND HYBRID CELLS

	CH23	P388	PCM3-M	PCM3-S
Protein/cell(μg)	0.7×10^{-4}	1.1×10^{-4}	1.9×10^{-4}	4.1×10^{-4}
	(0.37X)	(0.58X)	(1.0)	(2.16X)

Protein concentration and cell number were determined in confluent and limiting-density cultures which were used for the study of hormonal stimulation of cAMP. Figures in parentheses show the ratio of protein/cell using PCM3-M cells as reference.

TABLE 5. INTRACELLULAR CYCLIC-AMP LEVELS IN PCM3 CELLS

Cyclic AMP Levels	PCM3-M	PCM3-S
Total cAMP (pmoles/ culture)	9.0 ± 1.5	1.0 ± 0.3
cAMP (pmoles/mg protein)	10.7	7.7
cAMP (pmoles/cell)	2.4×10^{-6}	3.3×10^{-6}

Intracellular cAMP levels were determined in confluent cells, and results show average of duplicate cAMP assays from duplicate incubations. PCM3-M and PCM3-S cells were separated and samples removed for estimation of cell number and protein content before being treated for cAMP determination. Protein/cell for PCM3-M = 2×10^{-7}mg and for PCM3-S = 4.0×10^{-7}mg.

III. A STUDY OF THE CELL CYCLE IN PCM3

The growth characteristics and the clonal origin of PCM3 suggest that the monolayer and suspension are different forms of the same cells. This would be explained if the hybrid cells existed in suspension for part of the growth cycle. The appearance of rounded cells at division has been observed in many fibroblast cultures (Terasima and Tolmach, 1963) and it could be envisaged that this characteristic has become pronounced in PCM3 cells as a manifestation of P388F-36 gene expression.

1. Time-Lapse Photography

Examination of hybrid cultures by time-lapse photography was used to explore the visual relationship between the monolayer and suspension forms of PCM3. Cells were subcultured at both high and low densities and films taken over 48 hours of growth. This enabled one frame to be exposed for one second every 40 seconds and by using a phase analyser it was possible to study the cell growth frame by frame.

In the films, rounded cells were distinctly visible because of the haloes of light surrounding them (as observed in figure 24) and while some were free to float in the medium, others appeared loosely attached above the plane of the monolayer. At

random intervals monolayer cells retracted their processes, lost contact with the substratum and formed suspension cells which subsequently divided before reforming contacts with the glass.

The time interval between cells rounding and division varied from 15-60 minutes, while the average time for the whole process from retraction of the parental cell to spreading of the daughter cell ranged from 30 minutes to 2 hours, although the majority of cells accomplished it in 1 hour. The average length of the complete cell cycle was determined to be 20 hours.

In the five films studied no monolayer cell divided while attached to the substratum and equally no newly divided suspension cell was observed to undergo mitosis without first adhering to the substratum, becoming indistinguishable from other monolayer cells and returning to the suspension.

2. DNA Synthesis

To provide evidence for the occurrence of different forms of PCM3 at different parts of the cell cycle, the rate of incorporation of [^{3}H]-thymidine into DNA was investigated. Hybrid cells

were incubated with [^{3}H]-thymidine for 2 hours and the incorporation determined with respect to DNA content or cell number (Table 6). When cells were incubated together, PCM3-S incorporated more radioactive label than PCM3-M. This could have been the result of interchange between the two forms of the hybrid or simply incorporation by the monolayer followed by movement into suspension.

To investigate this possibility, PCM3-M and PCM3-S were incubated separately with [^{3}H]-thymidine and this proved that the suspension cells were capable of synthesising DNA. It also established that PCM3-S cells were capable of synthesising more DNA than PCM3-M cells. As these results could have been biased by the need for new medium, a comparison was made of cells incubated in fresh and conditioned medium. This showed that the medium did not influence the previous results and confirmed that PCM3-S cells were able to incorporate [^{3}H]-thymidine into DNA.

These results are difficult to interpret in view of the observations made by time-lapse photography. They could be explained if the cells become rounded during the S-phase of the cell cycle and remain in suspension until after mitosis. This would require that the G_2 phase be very short in these cells.

TABLE 6. DNA SYNTHESIS IN PCM3

Protocol	Incorporation of [^{3}H]-thymidine					
	cpm/μg DNA			cpm/10^5 cells		
	PCM3-M	PCM3-S	(S/M)	PCM3-M	PCM3-S	(S/M)
Cells incubated together	2800	3060	(1.1)	3950	6550	(1.7)
Cells incubated separately	2030	3250	(1.6)	7150	12450	(1.7)

Cells were incubated for 2hr with 2μCi/ml [^{3}H]-thymidine at 37^{o}C. Non-specific incorporation was determined by pre-incubating cells in the presence of 2mM hydroxyurea and this was subsequently deducted from the total incorporation. At the end of the incubation the cells were collected and aliquots removed for determination of DNA content or cell number before estimation of incorporation. Results are the average of triplicate determinations in 2 (cpm/μg DNA) or 3 (cpm/10^5 cells) experiments. Figures in parentheses represent the ratio of incorporation of PCM3-S to PCM3-M cells.

3. Mitotic Index

If the suspension form of the hybrid represents cells in the mitotic phase of the cell cycle then they should be easily distinguishable by examination of the mitotic index. In order to detect a reasonable number of dividing cells, the microtubule inhibitor, colchicine was used to collect cells at mitosis. Two procedures were followed, in the first method a logarithmically growing culture of both monolayer and suspension cells was incubated with colchicine and then separated for analysis of the mitotic index, while in the second method PCM3-M and PCM3-S cells were incubated and analysed separately.

The results of the different approaches are presented in Table 7. In both protocols the PCM3-S cells had a higher mitotic index than the PCM3-M cells, although when the cells were incubated together the result was remarkably greater. It was envisaged that if the monolayer cells were separately capable of undergoing mitosis they would be less firmly attached during division and therefore in a mixed culture they might be collected with the suspension, thus biasing the results. Therefore when the cells were incubated separately, the medium from the PCM3-M cells was collected and was observed to contain cells in suspension which had a mitotic index of 14%.

TABLE 7. MITOTIC INDEX OF PCM3 CELLS

PROTOCOL	MITOTIC INDEX		
	PCM3-M		PCM3-S
PCM3-M and PCM3-S incubated together	4% (224)		31% (108)
PCM3-M and PCM3-S incubated separately	2.5% (171)	a.	12.5% (93)
		b.	14% (163)

Cells were collected at mitosis by incubation with colchicine (20μg/ml) for 4 hours and then examined microscopically after staining with acetic orcein. The results are presented as the means of 5 separate experiments and the figures in parentheses represent the average number of cells counted in each experiment. (a. isolated before colchicine; b. isolated after colchicine.)

From these results it was still not possible to decide whether the PCM3-M cells had moved into suspension before division or whether the gentle removal of medium had still dislodged some dividing monolayer cells. However, in a control experiment it was observed that CH23 cells collected in mitosis by colchicine were still associated with the monolayer and no cells were observed in suspension. This tended to suggest that the suspension was the major source of mitotic cells in the hybrid.

It was interesting to note that the cumulative mitotic index of the two suspension cultures analysed separately was approximately equal to the mitotic index of the PCM3-S cells incubated in the presence of PCM3-M. This showed that during the 4 hour incubation, half the mitotic cells originated from the suspension and half from monolayer cells which had moved into suspension. This is in agreement with the proposed time interval between cells moving into suspension and mitosis.

4. Synchronisation

PCM3 hybrid cells were synchronised in an attempt to obtain exclusive formation of monolayer or suspension at different parts of the cell cycle. Excess thymidine, which halts cells at the beginning of the S phase was chosen as the most suitable synchronisation protocol and preliminary experiments showed that the double thymidine block, as used by

Zeilig *et al.*, (1976) produced better synchrony.

Figure 35 shows the effect of synchronisation on the cell density of PCM3-M and PCM3-S cells and figure 36 presents the percentage of labelled PCM3-M cells, which was monitored to determine the degree of synchronisation. This labelling index showed that the majority of cells synthesised DNA immediately after release from the block, but the degree of synchronisation diminished rapidly.

Estimation of cell density over the 12 hours after thymidine removal showed a burst of cell division between 8 and 12 hours. However, both PCM3-M and PCM3-S cell number increased at the same time and the degree of synchronisation was not sufficient to allow observation of a preferential increase in cell number. Control cultures which had not received thymidine increased constantly over the same 24 hour time interval. This experiment was adapted to examine more closely the increase in cell number between 8 and 12 hours but it was still not possible to detect a difference between the increase in PCM3-M and PCM3-S cells.

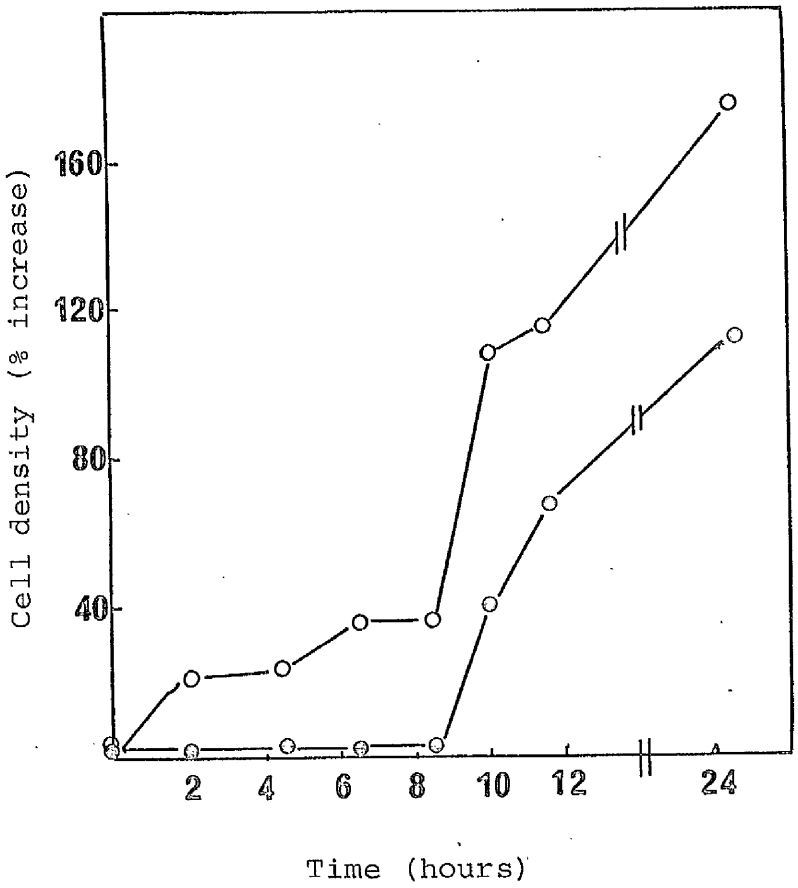


FIGURE 35. CELL DIVISION IN SYNCHRONISED PCM3 CELLS

PCM3 cells were synchronised by a double thymidine block and progress through the cell cycle after removal of thymidine at 0 hours was monitored by measuring the % increase in cell density in PCM3-M (o—o) and PCM3-S (o—o) with initial cell densities of 6.6×10^5 and 0.9×10^5 respectively.

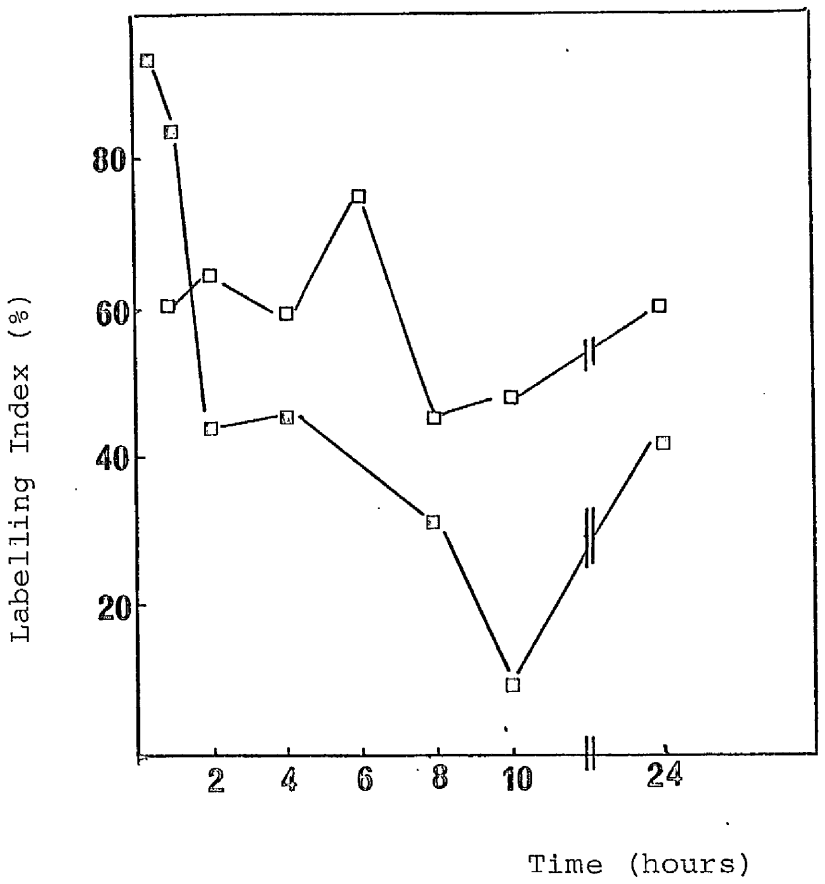


FIGURE 36. SYNCHRONISATION IN PCM3 CELLS

PCM3 cells were released from a double thymidine block and progress through the cell cycle was determined in PCM3-M cells from measurement of the labelling index in treated (□—□) and untreated cells (□—□). The percentage of labelled cells was obtained after pulse labelling with [^{3}H]-thymidine (1μCi/ml) for 15 minutes at the times indicated followed by autoradiography.

The percentage of cells in suspension was calculated in both control and thymidine-treated cells and observed to remain constant at 15 and 10% respectively. Thus the S phase of the cell cycle was not characterised by an increase in the percentage of suspension cells, as expected.

5. Serum Deprivation

It has long been known that serum deprivation induces quiescence and that readdition of fresh serum stimulates a partially synchronised initiation of cell growth. This knowledge was utilised to study the relationship between PCM3-M and PCM3-S cells by reducing the serum concentration to 1.0% in logarithmically growing cultures for 24 hours and then monitoring cell density after replenishment of serum.

The most striking observation was the increase in the percentage of cells in suspension after serum deprivation which was followed by a further increase two hours after restoration of normal serum concentrations (figure 37). The percentage of cells in suspension then diminished during the next 11 hours to the levels observed in control cultures. This represented a decrease in PCM3-S cells which correlated with an increase in PCM3-M cells suggesting a shift from suspension to monolayer.

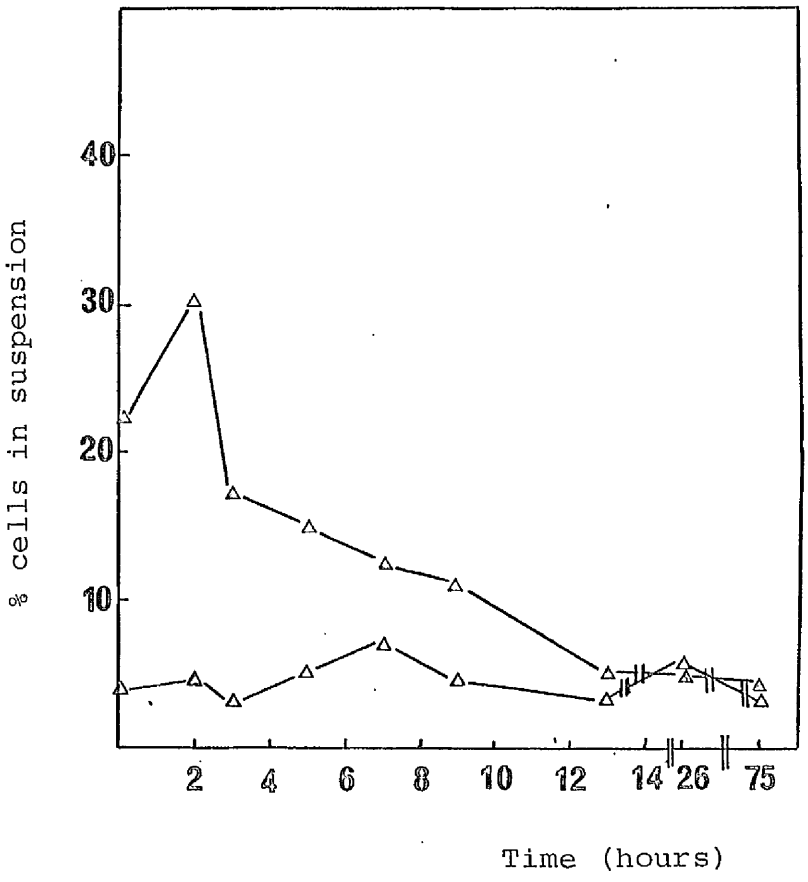


FIGURE 37. SERUM SYNCHRONISATION OF PCM3

PCM3 cells were incubated in 1% serum for 24 hours and then in 10% serum from time 0 hours onwards. The PCM3-M and PCM3-S cell numbers were then determined at the times shown in control (△—△) and serum treated cultures (△—△).

The increase in the percentage of cells in suspension by 8% after 2 hours occurred reproducably in three independent experiments and incubation of PCM3-M and PCM3-S cells separately from 0-2 hours proved that it occurred by division of suspension cells rather than movement from monolayer to suspension. This could be explained if reducing the concentration of serum had inhibited cells in the G_2 phase of the growth cycle.

In a parallel experiment, examination of cell number at intervals from 10 to 20 hours after serum replenishment showed no wave of division at about 12 hours which would have been consistent with a block in the cell cycle at G_1. However, a small peak in the percentage of suspension cells was observed at approximately 19 hours which provided evidence for inhibition in the G_2 phase.

Although this tended to substantiate other evidence concerning the relationship between the two forms of the hybrid it did not provide a complete synchronisation of the cell culture.

IV. INVESTIGATION OF A CELL SURFACE GLYCOPROTEIN

During the studies of the PCM3 hybrid, the question repeatedly raised was concerned with the basic difference in the adhesive properties of the monolayer and suspension. It became apparent that some alteration, probably in the plasma membrane, was responsible for the interchange between PCM3-M and PCM3-S cells. Reports of a cell surface glycoprotein (CSP) which could be added to transformed cells to produce an alteration in morphology and an increase in adhesion (Yamada et al., 1976) provided a likely candidate for the plasma membrane changes in PCM3.

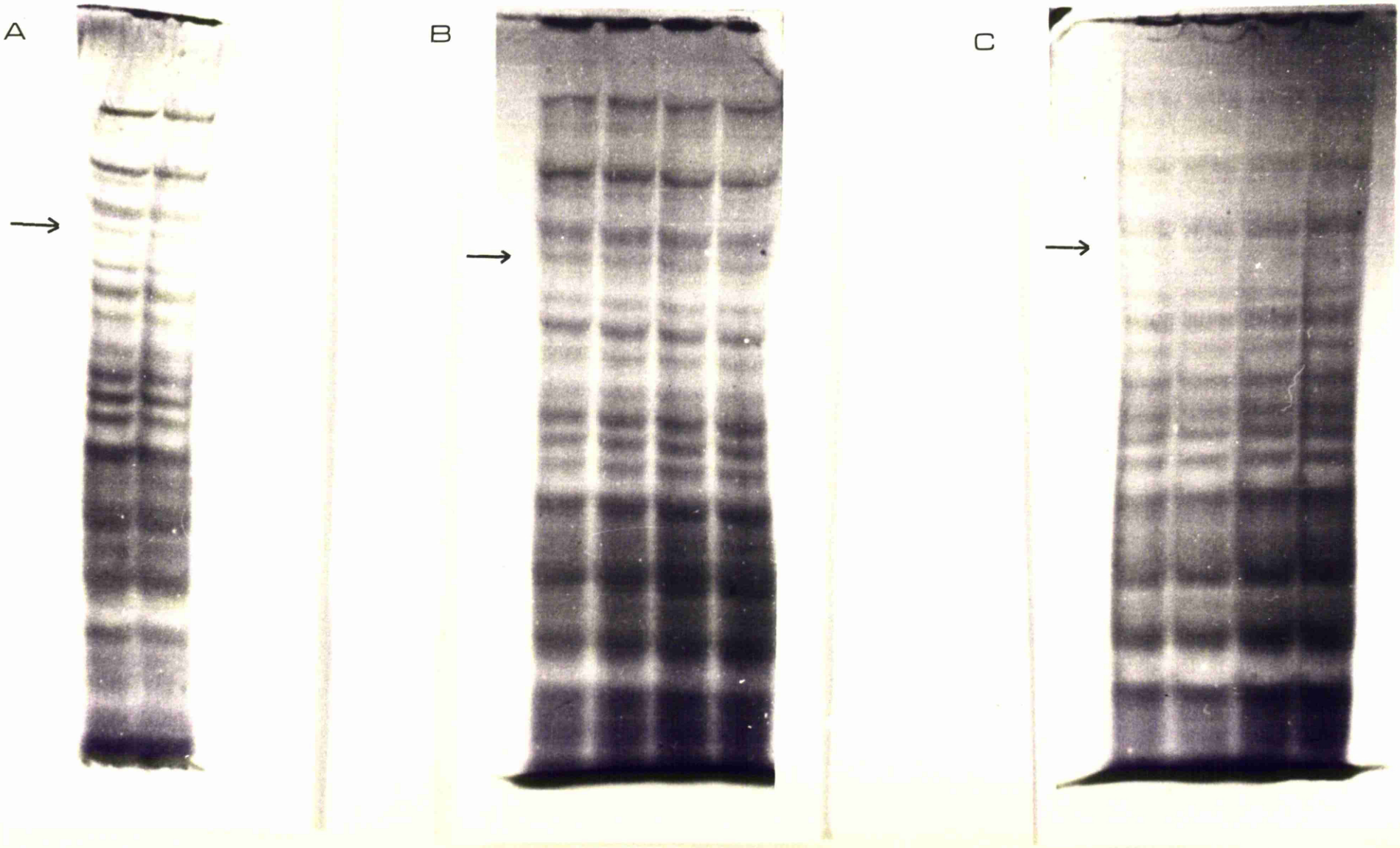
1. Identification of CSP

SDS-polyacrylamide gel electrophoresis of PCM3-M homogenates identified a protein band of molecular weight 219,000 ± 5,000 (figure 38) which corresponded to the cell surface glycoprotein of chick embryo fibroblasts, identified by Yamada and Weston (1974). It was sensitive to mild proteolytic digestion (figure 38c) which suggested that it was exposed on the external surface of the plasma membrane. It should be noted that some other proteins of higher molecular weight were also modified by trypsin treatment. This cell surface protein was not observed in homogenates of PCM3-S cells.

FIGURE 38. CHARACTERISATION OF CSP ON PCM3-M CELLS

(A) PCM3-M cells from a confluent culture were homogenised in 2% SDS and then subjected to electrophoresis on 5% SDS-polyacrylamide slab gels. After electrophoresis the gels were stained with Coomassie blue and the molecular weights of the protein bands calculated from standard curves obtained by running proteins of known molecular weight on the same gel. The band indicated (⟶) has a molecular weight of 219,000.

(B) & (C) The proteolytic sensitivity of CSP was examined by comparing control cells (B) and cells treated with trypsin (1µg/ml) at 37°C for 3 minutes. Both cultures were then homogenised and the proteins identified as described above. The band indicated (⟶) has a molecular weight of 224,000 and is significantly reduced after trypsin treatment.


A
B
C

2. ^{125}I-Labelling of CSP

The cell surface glycoprotein was further characterised by lactoperoxidase catalysed iodination, which labels only external membrane proteins. A preliminary experiment was carried out with CH23 fibroblasts, since it was thought that they might contain more CSP than the hybrid cells.

After iodination the cells were homogenised and subjected to SDS-polyacrylamide gel electrophoresis. The gel was then stained with Coomassie blue and after identification of the protein bands the gel was dried and processed for autoradiography. A comparison of the stained gel and the autoradiograph is presented in figure 39. Of the seven labelled bands, the one most heavily labelled coincides with the CSP band of molecular weight 224,000.

A comparison of the levels of the cell surface glycoprotein in parental and hybrid cultures was achieved by simultaneous iodination of the different cell lines at equivalent cell densities followed by identification using gel electrophoresis and autoradiography (figure 40). The autoradiographs showed that the most heavily labelled band which corresponds to CSP was severely reduced or absent from

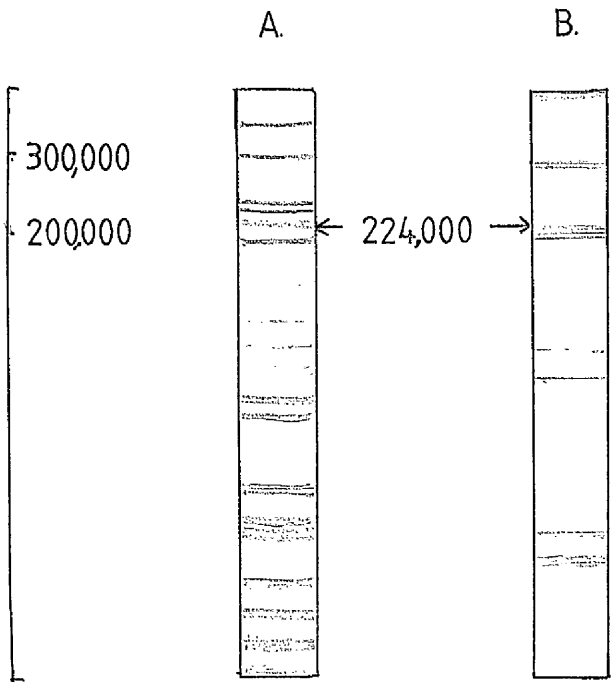


FIGURE 39. ^{125}I LABELLING OF CSP ON CH23 CELLS

Schematic representation of (A) stained gel and (B) autoradiograph of homogenate from CH23 fibroblasts after lactoperoxidase-catalysed ^{125}I labelling of external membrane proteins. The molecular weight and position of CSP are indicated (arrow). SDS-polyacrylamide gel electrophoresis was carried out in an identical manner to that described in the legend to Figure 38. 4.6×10^6 cells were incubated and homogenate equivalent to 0.9×10^5 cells added to the gel.

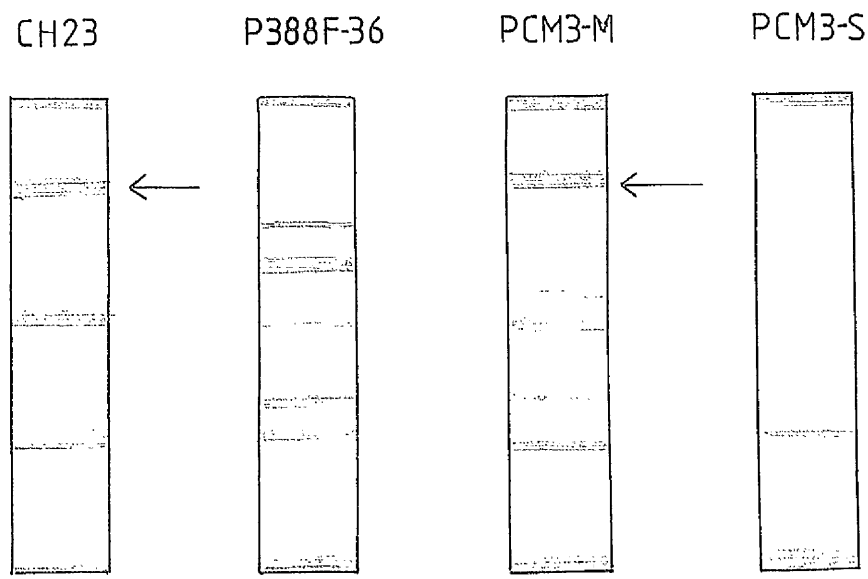


FIGURE 40. ^{125}I LABELLING OF CSP ON PARENTAL AND HYBRID CELLS

Schematic representation of autoradiographs of lactoperoxidase-catalysed ^{125}I labelling of external membrane proteins. Suspension cells were counted and their cell density adjusted to be equivalent to PCM3-M (4.0×10^6) before iodination. CH23 cell density determined in a parallel culture was adjusted to that of PCM3-M after iodination. All cell homogenates were then treated identically as described in the legend to Figure 38. Homogenates equivalent to 40×10^5 cells were added to the gels.

both PCM3-S and P388F-36 cells, although other bands representing proteins of different molecular weight were still present. This indicated that the suspension cells specifically lacked the cell surface glycoprotein.

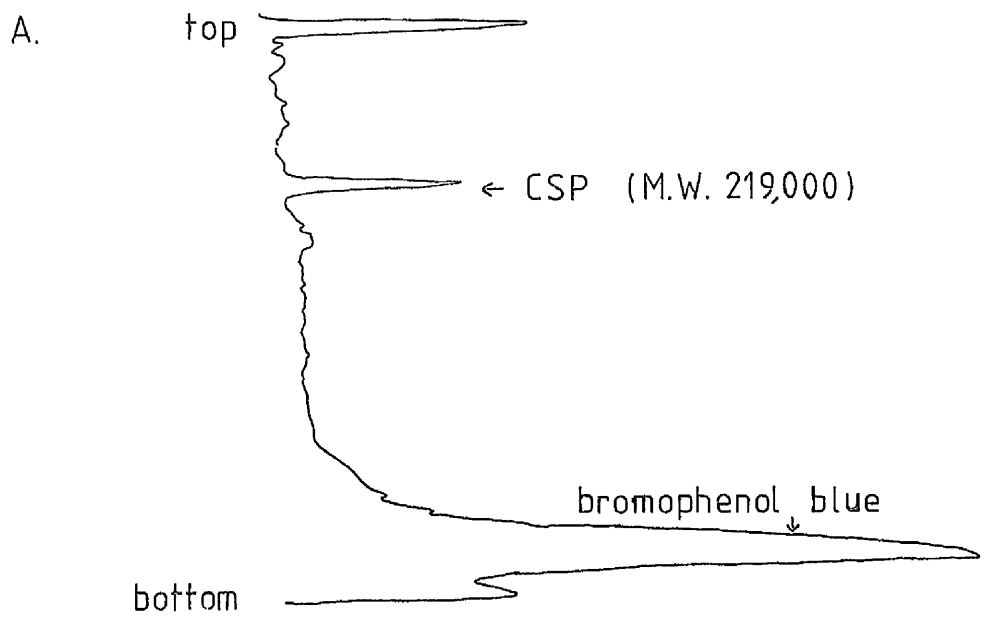
3. Isolation of CSP

Isolation of the cell surface glycoprotein from intact cells was achieved by incubation with 1.0M urea in serum-free medium. The yield was increased by the inclusion of the reducing agent, dithiothreitol (2mM) which was added because it had been observed that CSP was disulphide-bonded at the cell surface (Hynes and Destree, 1977).

Attempts to concentrate the extract using dialysis and freeze-drying or filtration in Amicon cones proved unsuccessful, and it was thought that the protein had remained on the glass surfaces, although siliconisation of glassware did not increase the yield. Ammonium sulphate precipitation circumvented these problems and the resultant extract from CH23 and PCM3-M cells was observed to be fairly homogeneous after SDS-polyacrylamide gel electrophoresis (figure 41). The observed band corresponded to the trypsin-sensitive protein band of the cell homogenate.

FIGURE 41 . ABSORBANCE TRACE OF ISOLATED CSP

CSP isolated from (A) PCM3-M cells and (B) CH23 cells. The CSP was isolated from confluent cultures of PCM3 (1.5×10^7 cells) and CH23 (9.0×10^5 cells) by urea extraction and then purified with ammonium sulphate precipitation. The extract was dialysed with sample buffer and then subjected to electrophoresis as described previously. The absorbance trace was obtained at 580nm from stained gels. The shoulder of the CSP peak from CH23 cells is probably a CSP degradation product.


A.
top
← CSP (M.W. 219,000)
bromophenol blue
bottom

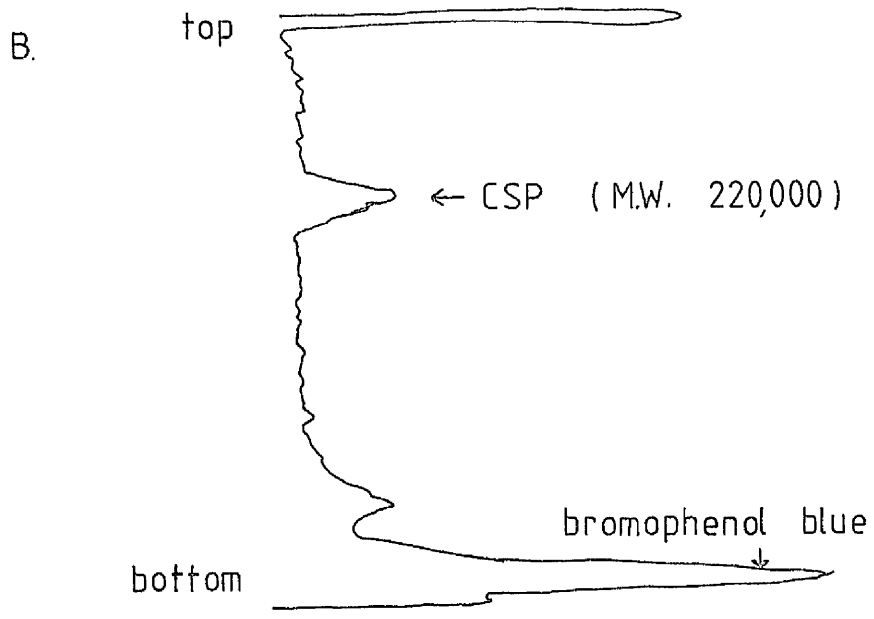

B.
top
← CSP (M.W. 220,000)
bromophenol blue
bottom

FIGURE 41

4. Addition of CSP to PCM3-S Cells

Three Roux of CH23 cells were used to obtain CSP as described above and after determination of protein content the extract was stored at -70°C to maintain active CSP. This protein was added to PCM3-S cells (1×10^6) at the time of subculture to give a final concentration of 20µg/ml and the addition repeated at 24 hour intervals. PCM3-S cells at equivalent density were also passaged to provide control cultures.

The cell density of the monolayer and suspension forms of PCM3 was then determined at 16 and 72 hours after subculture. After 16 hours the cells had just begun to divide, but nevertheless the most striking observation was the 50% decrease in the number of suspension cells in the treated cultures in comparison with control cultures. After incubation with CSP for 72 hours the suspension cells were reduced to negligible levels and the monolayer cells had ceased to divide.

The morphological appearance of the PCM3-M cells had also altered with incubation in the cell surface glycoprotein. After 72 hours the cells were larger and flatter which tended to indicate that CSP had increased the adhesion of these PCM3 cells.

D I S C U S S I O N

A. CYCLIC AMP SYNTHESIS IN NORMAL AND MALIGNANT CELL LINES

I. HORMONAL STIMULATION OF CYCLIC AMP

A comparison of the increased cAMP accumulation after incubation with hormone in AP-9, SKL-2 and EB_2 cells provides striking evidence for the decreased responsiveness of the malignant cell lines. Thus although SKL-2 cells produce a slightly better response to PGE_1 than to isoprenaline, the normal fibroblasts (AP-9) still show a 30-fold greater degree of stimulation and while EB_2 cells produce a slightly better response to isoprenaline than to PGE_1, the AP-9 fibroblasts still have a 10-fold greater degree of stimulation with isoprenaline (section A.II). The difference observed is reflected in the relative basal levels of cAMP in the three cell lines.

These results are consistent with alterations detected in the hormonal stimulation of cAMP in a mouse lymphoma cell line (Ayad and Foster, 1974), with alterations in the intracellular level of cAMP in a transplantable mouse lymphoma (Tisdale and Phillips, 1976) and with the numerous reports of aberrations in cAMP metabolism in both malignant and transformed cell lines (review by Pastan et al., 1975) where the predominant finding has been a decrease in cAMP levels although particularly in tumour cells, increases have been observed.

The reduction in cAMP levels in the malignant cells could have been caused by an increase in cyclic-nucleotide phosphodiesterase, but this is unlikely in view of the inclusion of the phosphodiesterase inhibitor, IBMX in all incubations. A more likely explanation is the reduction in the number of PGE_1 receptors in EB_2 cells, as evidenced by prostaglandin binding studies (Ayad and Burns, 1977) and the implication of decreased hormone receptors as the cause of reduced cAMP responsiveness in lymphocytic leukaemic lymphocytes (Sheppard et al., 1977). However, the basal levels of cAMP and the reduced response to isoprenaline indicate that the PGE_1 receptor is not the only site of aberration in EB_2 cells and the decreased adenylate cyclase activity observed in human lymphocytic leukaemia cells (Polgar et al., 1973) may be significant.

Since both the hormone receptors and the adenylate cyclase catalytic units are found embedded in the plasma membrane and since it is widely acknowledged that the cell surface is the site of many aberrations associated with malignancy (Nicolson et al., 1977) it is envisaged that the membrane defects could alter either the receptor or the enzyme or the coupling between the two, so that cAMP synthesis appears to be altered by different mechanisms in different cells.

II. TETRACAINE ACTION

It is therefore of interest to examine the changes in membrane fluidity, because this could alter coupling between receptor and enzyme, which occurs by lateral movement in the plane of the membrane (Jacobs and Cuatrecasas, 1977). It has been shown that local anaesthetics increase the fluidity of phospholipids in both artificial bilayers (Papahadjopoulos et al., 1975) and plasma membranes (Poste et al., 1975) and therefore tetracaine was used as a probe to study the effects of fluidity changes on cAMP responsiveness to hormones.

The results presented are complex to interpret, but the biphasic response of cAMP levels in the normal AP-9 fibroblasts (Figures 17 and 18) could result from an enhanced translational mobility of components in the membrane, which initially aids coupling but eventually has adverse effects on the interaction between receptor and enzyme.

It has been observed that malignant cells tend to show alterations in the mobility of membrane components (Nicolson, 1976(b)) and this could explain the different tetracaine action in SKL-2 cells (Figures 19 and 20) such that the local anaesthetic may compensate for the altered mobility and therefore produce a 2-3 fold enhancement of cAMP accumulation. It does not however, restore cAMP responsiveness to normal. In

comparison, the response in EB_2 cells (Figures 21 and 22) is similar to that in AP-9 cells at low tetracaine concentrations and to that in SKL-2 cells at higher tetracaine concentrations which tends to suggest that the aberration in the membrane is different in the two malignant cell lines.

Since the results of tetracaine action are similar for completely different hormones, it would appear that the local anaesthetic acts on a component of the cAMP system which is common to both PGE_1 and isoprenaline activation and is therefore distal to the hormone receptors.

Further research is required before the exact nature of fluidity changes in malignant cells can be characterised. The present evidence is of variability in different types of cells (Burger, 1977) thus fluorescence polarisation techniques have shown that malignant fibroblasts possess a lower lipid fluidity and a higher receptor mobility than normal fibroblasts, whereas malignant lymphocytes have a higher lipid fluidity and a lower receptor mobility than the normal lymphocytes (Shinitzky and Inbar, 1976).

In view of evidence implicating the cytoskeletal elements as the site of tetracaine action it is necessary to consider how the cAMP response in the three cell lines could result from disruption of microtubules and microfilaments. At the concentrations

used, electron microscopy studies have shown that the cytoskeletal elements disappear, the cells become rounded and finally detach (Nicolson et al., 1976).

In AP-9 cells, it could be envisaged that disruption of the membrane-associated cytoskeletal elements, might release a component held in restraint by them, thus allowing enhanced cAMP synthesis, while further disruption at increased tetracaine concentrations might alter cAMP accumulation adversely. A similar situation is found where microtubules and microfilaments are known to anchor receptors involved in plant agglutination and disruption by tetracaine leads to receptor redistribution (Poste et al., 1975(b)). It has also been observed that disruption of microfilaments by Cytochalasin B reduces the number of insulin and human growth hormone receptor sites thus providing evidence of a role for microfilaments in the distribution of polypeptide hormone receptors (Van Obberghen et al., 1976).

Evidence has been presented for a reduction in the cytoskeletal array of transformed cells (Brinkley et al., 1975) and therefore it is likely that the malignant SKL-2 and EB_2 cells would respond differently to tetracaine. The results suggest that the inhibitory action of tetracaine in normal cells is alleviated by the decrease in microtubules and microfilaments, while disruption of the remaining cytoskeletal elements can enhance hormonal stimulation of cAMP.

It must be emphasised that tetracaine could be producing the cAMP response by a general chaotropic effect on the membrane or by a combined action on the fluidity of membrane components and the disruption of cytoskeletal elements. Finally the inhibitory phases may occur as a result of calcium acting on adenylate cyclase, since tetracaine is known to displace calcium from the membrane (Papahadjopoulos et al., 1975).

This work has subsequently been substantiated by a more detailed comparison of tetracaine action at different temperatures in AP-9 and EB_2 cells, (Ayad and Morgan, 1977). Thus a reduction in the incubation temperature, which is known to reduce the mobility of membrane components, causes an enhancement of the PGE_1-stimulated cAMP levels in EB_2 cells and an inhibition in AP-9 cells. At the lower temperatures, tetracaine potentiates the increase in EB_2 cells and produces a further inhibition in AP-9 cells. This would suggest that tetracaine is acting in concert with temperature on membrane mobility.

Since a reproducible change in hormone-stimulated cAMP levels was not observed after treatment of AP-9 and EB_2 cells with the anti-microtubule agent, colchicine or the microfilament modifier, Cytochalasin B, (S. Morgan, personal communication) it is unlikely that tetracaine was exerting it's effect via an action on the cytoskeletal elements in these cells. However, colchicine

does enhance PGE_1 and isoprenaline stimulation of cAMP accumulation in human leukocytes, suggesting that disruption of microtubules relieves a constraint which is restricting hormone activation of adenylate cyclase, (Rudolph et al., 1977). A similar enhancement by colchicine in S49 lymphoma cells provided evidence for a site of action subsequent to the hormone receptor because hormone binding studies showed that interaction between receptor and hormone was not altered by colchicine, (Insel and Kennedy, 1978).

B. A STUDY OF GROWTH REGULATION IN A SOMATIC CELL HYBRID

I. CHARACTERISTICS OF THE PCM3 HYBRID

As indicated in the introduction, malignancy is characterised *in vitro* by properties such as growth in soft agar, loss of density dependent inhibition and alterations in morphology and adhesion. Many of these properties have been associated with changes at the cell surface. However, exceptions to almost all these correlations have been observed and the ability to relate some of these growth properties to tumourigenicity *in vivo* has recently been questioned (Strauss *et al.*, 1977). Thus it is necessary to be cautious in interpreting the properties of malignant cells which have not been directly correlated with tumourigenicity.

The observation that a hybrid (PCM3) isolated after fusion between a monolayer and a suspension is capable of growing simultaneously as both monolayer and suspension is therefore of interest in view of the correlation between loss of anchorage dependence and tumourigenicity *in vivo* (Shin *et al.*, 1975).

It has already been postulated that the series of hybrids isolated after fusion between Chinese hamster fibroblasts and mouse lymphoma suspension cells grow as monolayer (PCM) or suspension (PCS) depending on the relative number of hamster and mouse chromosomes

in the hybrid (Ayad and Delinassios, 1974). In this thesis, the characteristics of PCM3 cells tend to suggest that suspension growth is the result of the expression of a gene or series of genes derived from the P388F-36 parental cell line, which has not been included in the chromosomal complement of other PCM hybrid clones.

It is of interest to note that a similar situation to PCM3 with floating cells in a monolayer culture has been observed after HSV-transformation of NIL fibroblasts (Ali et al., 1977) since this tends to indicate that the appearance of suspension cells in the hybrid is closely correlated with the inheritance of malignant traits from the P388F-36 lymphoma.

The clonal origin and the karyotype analysis of the hybrid indicate that it is not a heterogeneous population of monolayer and suspension cells, but a culture of identical cells existing in different forms. The growth characteristics substantiate this and show that the suspension consists of viable cells, which are present throughout the growth of the culture. Thus these cells are not the result of overcrowding as has been observed in some transformed cultures.

An understanding of the mechanism of monolayer and suspension growth in PCM3 is therefore relevant in this context.

II. CYCLIC AMP AND CELL GROWTH

1. Cell Growth in the Presence of Elevated Cyclic AMP Levels

Incubation of parental and hybrid cells in agents which have been shown to increase cAMP in these cells produced varying degrees of growth inhibition. PGE_1 and IBMX acted synergistically to induce premature quiescence which indicated that the quiescence was brought about specifically by elevation of cAMP. This has also been observed after incubation with cAMP analogues and cyclic nucleotide-phosphodiesterase inhibitors (Ryan and Heidrick, 1974; Friedman, 1976).

Consistent with these observations are reports that PGE_1 inhibits growth of leukaemia cells in culture (Yang et al., 1976) and tumour growth in vivo (Santoro et al., 1976), although the role of cAMP in this inhibition was not investigated. It has been suggested that synthesis of endogenous prostaglandins may regulate cell growth, with PGE_2 inhibiting and $PGF_{2\alpha}$ stimulating proliferation, (Taylor and Polgar, 1977). The inverse relationship between PGE production and growth rate in HeLa cells is contradictory (Hammarström, 1977).

Since the early studies on the effects of hormone incubation on cell growth, it has become clear that the

cells are able to protect themselves from prolonged hormonal stimulation by desensitization of the hormone receptors, (Ayad and Foster, 1974). Therefore, after several hours the cAMP levels decline despite the continued presence of hormone and readdition of fresh hormone does not restore the elevated cAMP until after a refractory period in the absence of hormone. In C_6 glioma cells morphological changes observed after incubation with noradrenaline were seen to undergo reversion at a time when the cells had become desensitized to hormone (Oey, 1975).

The results presented in Section B.II.1 show that growth was inhibited when the cells were incubated with hormone and the inhibition continued even though the cells were probably desensitized after only a few hours. This suggested that the hormonal elevation of cAMP before desensitization determined the subsequent quiescence. However, in PCM3 cells incubated in the presence of PGE_1 and IBMX, removal of these agents after 95 hours incubation resulted in a re-initiation of growth which indicated that the inhibition of proliferation by cAMP was reversible. Therefore at least under these conditions it would appear that inhibition of cAMP degradation by IBMX maintained sufficiently high cAMP levels to inhibit cell division.

When the PCM3 cells were incubated with both PGE_1 and IBMX the growth of cells in suspension was virtually non-existent and the percentage of PCM3 cells in suspension was specifically reduced. The most likely explanation

for this phenomena would be an increase in the adhesion of PCM3-S cells to the substratum or a decrease in the detachment of PCM3-M cells. In a similar situation human lymphocytes grown in semi-suspension culture have been observed to attach to a substratum when incubated with dbcAMP, but growth then proceeded at a rate comparable to the control suspension (Smith et al., 1974).

Further evidence for an increase in adhesion in the presence of these agents was provided by the flattened appearance and the restricted movement of the PCM3-M cells. The morphological alteration of PCM3-M cells was not due to an increase in cell size and was probably the result of increased cell spreading, and therefore increased adhesion.

Pastan et al., (1977) and Pastan and Willingham (1978) have proposed that morphological transformation is characterised by rounded cell shape, surface microvilli, high agglutinability by plant lectins and low adhesion; the latter being primarily responsible for the other responses. They also propose that dbcAMP causes a reversion of all four properties and that these characteristics can be distinguished from loss of growth control. It would appear that in PCM3 cells, increasing cell adehsion by elevating cAMP does exert an effect on growth control. This is consistent with the action of dbcAMP on the growth of CHO cells (Puck, 1977). In these studies it was postulated that a cAMP-dependent network of

microtubules and microfilaments which connects the cell surface to the genome, carries information which regulates cell growth. In transformed cells disruption of this network causes uncontrolled growth while dbcAMP restores this network and therefore limits growth. This is consistent with a recent report which suggests that the cell shape as controlled by substratum adhesiveness is critical for DNA synthesis in normal cells (Folkman and Moscona, 1978).

2. PGE_1 Stimulation of Cyclic AMP

The PCM3 hybrid responded to PGE_1 stimulation by producing much greater cAMP levels than either of the parental cell lines and the enhanced accumulation of cAMP occurred in both monolayer and suspension forms of PCM3. This indicates that the PGE_1 responsiveness is inherited dominantly in the hybrid, which is consistent with results obtained from other PCM hybrids, (Ayad and Foster, 1974) and from hybrids obtained after fusion between mouse fibroblasts and rat glioma cells (Brunton et al., 1977).

In all the hybrids studied, the cAMP levels obtained after PGE incubation were not additive, but significantly greater than the sum of the parental responses. In the PCM3 hybrid cells the enhanced stimulation was dose dependent and detected in both actively growing and stationary cultures. Time course studies also proved that the difference between the parental and hybrid cells was not the result of a variation in the optimal incubation time.

Further investigation of a PCM hybrid has shown that the lower cyclic nucleotide phosphodiesterase activity in the hybrid contributes to, but does not fully account for the enhanced responsiveness to PGE_1 (Ayad and Foster, 1974). It is now known that cells possess PGE_1 receptors inherited from both parental cell lines, but only express a high affinity receptor in the activation of adenylate cyclase (Ayad and Burns, 1977). Surprisingly this high affinity receptor has been inherited from the P388F-36 cells where it appears to be masked in intact cells, but available for hormone activation of adenylate cyclase in broken cell preparations.

However, another contributing factor to the enhancement of cAMP levels is the increased adenylate cyclase activity and it is now known that the adenylate cyclase enzyme in PCM cells exhibits characteristics inherited from both parental cell lines (Ayad and Foster, 1977). Thus the hybrid nature of the enzyme, the high affinity PGE_1 receptors and the low phosphodiesterase activity may all contribute to determine the enhanced accumulation of cAMP in intact cells, while changes in the plasma membrane may also affect the response.

Experiments are at present being carried out in this laboratory to determine the 'ancestry' of the components of cAMP synthesis in PCM3 cells. It has already been observed that the adenylate cyclase displays characteristics in common with the P388F-36 enzyme,

while the guanyl-nucleotide regulatory subunit appears to have been inherited from CH23. (R. Hughes, personal communication). This hybrid also displays lower phosphodiesterase activity and the enzyme exhibits properties of both parental systems, thus expressing it's hybrid nature (Ayad and Wright, 1977).

3. Intracellular Cyclic AMP Levels

During the studies of cAMP responsiveness to PGE_1 in the PCM3 hybrid, it was noted that the basal levels of cAMP were higher in the monolayer than the suspension. This indicated that the intracellular levels were different in the two forms of the hybrid and it was thought that this might explain the differences in the adhesion of these cells, especially as it had already been observed that elevating cAMP levels favoured the monolayer form of the hybrid.

However, these levels were determined as a function of protein content and results showed that PCM3-S cells contained twice as much protein as PCM3-M cells. Therefore the actual cAMP content per cell was slightly higher in the suspension cells.

If the PGE_1-stimulated cAMP levels were to be expressed as a function of cell number rather than as a function of protein content, the response of the PCM3-S cells would have been slightly greater than that of PCM3-M

cells. However, since protein content does not vary sufficiently between the parental and hybrid cell lines, expression of cAMP levels as a function of cell number would still show an enhanced response in the hybrid compared to the parental cell lines.

Although the relationship between cell density and protein content was not measured in these studies, it is known that in mouse L cells the protein content per cell in a sparse culture is always greater than that in a dense culture (Tsuboi et al., 1976). Thus it is important to determine the state of growth of the cells studied and to use cells at the same state of growth when comparing cAMP measured as a function of protein content.

From these results it appears that when comparing different cells or different conditions of growth it is preferable to express cyclic nucleotide levels on a cellular basis. It could be envisaged that anomalies may also arise if cAMP levels measured as a function of protein content are examined in cells which have ceased growth at different stages of the cell cycle, since cells in the G_2 phase have approximately twice as much protein as cells in the G_1 phase and this could influence the results. In view of the cell cycle studies discussed later it is thought that this may indeed be the case with PCM3 cells.

Most reports of cAMP changes have used protein concentration as the reference, even when comparing the relationship between cAMP levels and growth rate at different cell densities (Tisdale and Philips, 1976; Morris and Makman, 1976). It is therefore difficult to compare these reports with others which have measured cAMP levels per cell at different cell densities (O'Neill and Hsie, 1975) and this may explain some of the contradictory evidence for the role of cAMP in growth control. In a similar situation, it has recently been shown that neoplastic mammary tissue exhibits increased specific levels but decreased cellular levels of cAMP and this difference may help to explain anomalies observed in other malignant tissues (Kung et al., 1977).

III. A STUDY OF THE CELL CYCLE IN PCM3

From examination of the characteristics of these cells it was concluded that the monolayer and suspension of PCM3 were different forms of the same cells. The most likely explanation for this phenomenon is that the cells exist in the two forms at different parts of the cell cycle and it is now well known that cells tend to round up at mitosis. In fact this property is utilised to separate mitotic cells from a heterogeneous culture and thereby obtain synchronised cells, (Terasima and Tolmach, 1963).

However, in a normal culture, the mitotic cells do not detach completely without perturbation and the percentage of cells undergoing mitosis is not usually sufficient to distinguish them. Therefore, it could be envisaged that in PCM3 the formation of rounded cells at mitosis has become more pronounced, such that the cells round up at an earlier point in the cell cycle or the daughter cells formed after mitosis do not attach immediately after division.

This explanation for the formation of suspension cells in the PCM3 culture was substantiated by the time-lapse photography since all the PCM3-M cells were observed to round up before dividing and all the PCM3-S cells attached to the substratum, becoming indistinguishable from the monolayer, before moving back into suspension to divide. A higher proportion of the suspension cells were pre-mitotic rather than post-mitotic but the considerable variation between individual cells meant that it was not possible to prove conclusively that passage into the G_1 phase coincided with increased adhesion of the cells.

A surprising feature of the cell cycle studies was the increased incorporation of [^{3}H]-thymidine into the DNA of PCM3-S cells, which signified that the suspension form of the hybrid was capable of DNA synthesis and indeed that the rate of incorporation was greater in these cells. It was at first thought that PCM3-M cells with radioactively labelled DNA

had moved into suspension during the two hour incubation, which had resulted in PCM3-S cells with labelled DNA. However, incubating the two forms of the hybrid separately proved that the PCM3-S cells were themselves able to synthesise DNA.

It is conceivable that the hybrid cells become detached from the substratum at some point during the S phase of the cell cycle and remain loosely attached above the monolayer or in suspension until after mitosis. This would require that the G_2 phase be very short in these cells and this would agree with observations in lymphoid cells which tend to have a pronounced S phase (Friedman et al., 1976).

In conjunction with studies of DNA synthesis the mitotic index was investigated in the hybrid cells and this provided further evidence for the mitotic phase of the cell cycle occuring in the suspension. The results indicated that the percentage of cells undergoing mitosis was far greater in the suspension form of PCM3, although once again it was necessary to question how much the co-incubation of PCM3-M and PCM3-S had influenced the results.

Separating the two forms of the hybrid before the experiment showed that very few monolayer cells divided, but during the incubation some cells had become detached and the mitotic index of these was equivalent to that of the PCM3-S cells incubated separately. For comparison

the mitotic index of CH23 fibroblasts was examined and the results indicated that mitotic cells in suspension were not a general feature of monolayer cells. Thus it was concluded that the PCM3 hybrid was distinguishable because the cells moved into suspension before undergoing mitosis.

It is of interest that the whole of the suspension did not undergo mitosis during the incubation, since this signifies that the suspension also represented cells in other phases of the growth cycle. It was noted that the mitotic population was made up equally of cells which had been in suspension before the incubation and cells which had moved into suspension during the incubation. This is consistent with the proposals made earlier concerning cells moving into suspension at a fixed time before mitosis.

The block and release method of synchronisation using excess thymidine was utilised as the most appropriate technique to examine the concerted movement of PCM3 cells through the cell cycle. Furthermore, the double thymidine block procedure was chosen as it is known that this helps to reduce the unbalanced growth resulting from the necessity for cells to be held at the S phase boundary for different lengths of time (Friedman et al., 1976).

This synchronisation protocol was effective in producing a wave of division beginning 8 hours after release of the block. The labelling index indicated that this division was preceeded by a period of DNA synthesis which declined to a minimum prior to mitosis. It was not however possible to differentiate between the increase in PCM3-M and PCM3-S cells and it was thought that this was due to the decay in the degree of synchrony, which has been shown to decrease from 100% at S phase to 33% at M phase in HeLa cells, (Zeilig et al., 1976).

Although attempts to observe a synchronous movement from monolayer to suspension, after excess thymidine treatment were unsuccessful, greater success was achieved by inducing quiescence with serum deprivation. Division of PCM3-S cells 2 hours after serum re-addition and a smaller wave of division at 19 hours indicated that removal of serum had collected the cells in the G_2 phase of the cell cycle. This was further substantiated by the absence of an increase in cell number at approximately 10 hours, which would have been consistent with inhibition in the G_1 phase.

Thus these results provide further evidence for the proposed regulation of monolayer and suspension growth in PCM3, since removal of serum which induced quiescence in the G_2 phase of the cell cycle caused a specific and substantial increase in the number of suspension cells. Furthermore, when the conditions for

growth were re-established cell division was observed only in the PCM3-S cells and this was followed by settling of the suspension onto the monolayer, with the result that the PCM3-M cell number increased. The time taken for the percentage of cells in suspension to reach that of control cultures probably reflects incomplete synchronisation such that some cells are still moving into suspension and dividing when the synchronised cells are attaching to the monolayer.

Lawrence and Jullien (1975) used serum deprivation to obtain quiescent chick embryo fibroblasts for the examination of growth stimulation by cyclic-nucleotide phosphodiesterase. In these studies they observed that serum removal caused a change in morphology to rounder cells, although they did not explain their finding. The results obtained with PCM3 cells would suggest that the cells were suspended at the G_2-M boundary of the growth cycle.

A great deal of evidence has accumulated to show that increased cAMP levels mediate serum restriction (e.g. Oey et al., 1974) and incubation with agents that elevate cAMP levels inhibits growth of normal 3T3 cells in both G_1 and G_2 phases of the cell cycle (Willingham et al., 1972) while growth of transformed 3T3 cells is inhibited only in G_2 (Paul, 1973). This is consistent with the observations of endogenous cyclic nucleotide levels, where malignant cells which appear to have lost the ability to enter G_o from G_1 do

not exhibit the coincident peak in cAMP levels observed in normal cells (Friedman, 1976). Therefore it is quite conceivable that serum deprivation has inhibited the growth of PCM3 cells at the restriction point in the G_2 phase. However, it would appear that incubation with agents which elevate cAMP inhibits growth at the G_1 restriction point since the number of suspension cells is strikingly reduced.

The results presented for the study of the cell cycle in PCM3 are consistent with the proposal that these cells are attached to the substratum throughout the G_1 phase, but round-up and detach during the S phase. The cells then remain in suspension until after mitosis when the progeny attach and form the monolayer. Further evidence is provided by the higher levels of protein detected in PCM3-S cells, during the determination of cAMP concentration, since it is known that cells in G_2 contain more protein (Kimball et al., 1971).

IV. INVESTIGATION OF A CELL SURFACE GLYCOPROTEIN

Although the molecular basis of cell adhesion is poorly understood it is clearly a complex process involving a variety of cell surface glycoproteins and several observations suggest that the cell surface glycoprotein characterised by Hynes (1973) is important

in the cell-substratum adhesion of normal cells. These observations include a decrease in CSP after mild proteolytic digestion, (Yamada and Weston, 1974), absence of CSP from virally-transformed cells which show decreased adhesion and an increase in adhesion to the substratum, following the incubation of these cells with CSP isolated from normal fibroblasts (Yamada et al., 1976; Ali et al., 1977).

Therefore, in view of the known differences in the adhesion of PCM3-M and PCM3-S cells it is interesting to observe that the CSP in PCM3M cells, identified and characterised by sensitivity to trypsin treatment, was absent from the outer surface of PCM3-S cells. In comparison, the CSP was observed to be present on the membrane of the parental CH23 fibroblasts, but lost from the parental P388F-36 lymphoma cells, which grow in suspension.

PCM3-S cells incubated with CSP isolated from CH23 fibroblasts showed enhanced attachment to the substratum, which resulted in the formation of a culture consisting almost completely of monolayer cells. These cells subsequently acquired a more flattened morphology, which along with the enhanced attachment signified an increase in their adhesion, as observed with transformed NIL fibroblasts, (Ali et al., 1977).

This increased adhesion was accompanied by the inhibition of growth of the monolayer, which tends to suggest that the PCM3 cells are unable to accomplish division without first rounding-up. This feature appears to be peculiar to the hybrid, since addition of CSP to transformed chick embryo fibroblasts causes a reversion in the morphological properties but does not restore growth control (Yamada et al., 1976). The distinction between morphology and growth is consistent with the observation that a mutant of 3T3 cells possesses many of the morphological properties of transformed cells but retains normal growth control, which has led Pouyssegur et al. (1977) to propose that these morphological properties are not linked to the mechanism which controls cell division.

It would appear that CSP is not the only membrane glycoprotein involved in adhesion (Pastan et al., 1977) and does not increase adhesion universally (Juliano and Gagalang, 1977). Other glycoporteins are known to be altered in transformed cells, as exemplified by the restoration of contact inhibition of growth to malignant hamster melanocytes by a melanocyte contact inhibitory factor, which is a glycoprotein of 160,000 molecular weight, (Knecht and Lipkin, 1977). In fact it has been suggested that there are changes in the carbohydrate moiety of most, if not all membrane glycoproteins in malignant cells (Warren et al., 1977).

Recently the aberration in a glycoprotein of molecular weight 100,000 has been correlated with the tumourigenic potential *in vivo* of a variety of malignant cells (Bramwell and Harris, 1978). At present the abnormality in this glycoprotein appears to be the marker for malignancy which best fulfils the criteria required to establish an unequivocal marker (Yamada, 1978).

V. GENERAL CONCLUSIONS

The unusual growth of the PCM3 hybrid is the result of decreased adhesion of the suspension cells which appears to be caused by a loss of the cell surface glycoprotein of molecular weight 220,000. Since the cell cycle studies indicate that these suspension cells are formed during the S phase of the growth cycle and remain in suspension until after mitosis, it is thought that the cell surface glycoprotein, becomes detached from the PCM3-M cells during the S phase, thus causing the transition from monolayer to suspension.

It would be envisaged that this process occurs in normal cells immediately prior to mitosis and this is substantiated by the high levels of CSP in G_1 which decrease to a minimum at mitosis (Hynes and Bye, 1974). After mitosis it is likely that the plasma membranes of the progeny contain cell surface glycoprotein which has been newly synthesised or made available at the cell surface and is therefore responsible for attachment to the substratum.

The effects of incubating PCM3 cells in agents which elevate cAMP closely parallel the effects of CSP, in that the decrease in the percentage of cells in suspension and the increase in the adhesion of monolayer cells is observed in both situations. Also, cAMP and CSP both affect the growth of PCM3 cells, while neither alters growth control in transformed chick embryo fibroblasts. It has been shown that addition of CSP to cells does not affect the cAMP levels (Yamada et al., 1976) but the corollary of the effect of cAMP on CSP has not been fully investigated.

The CH23 fibroblasts remain attached to the substratum and exhibit the anchorage dependence of normal cells, whereas P388F-36 cells, in common with other malignant cell lines have lost this anchorage dependence and also appear to have lost the cell surface glycoprotein involved in cell adhesion. Each of these parental characteristics can be recognised in PCM3 cells. This indicates that the PCM3 hybrid has inherited anchorage dependence from CH23 fibroblasts, but that removal of CSP from the cell surface results from alterations caused by a mechanism inherited from the P388F-36 parent.

C. FUTURE WORK

As with most research, examination of the results presented, raises questions which can only be answered by further experimentation. A brief résumé of the problems which require elucidation, is given below:

1. Perhaps the most pertinent area for further research is in development of the synchronisation protocol. Provision of a more highly synchronised culture would enable further study of the transition from monolayer to suspension and would allow determination of the levels of the cell surface glycoprotein (CSP) at different stages of the cell cycle. In this way, the loss of CSP accompanying the movement from monolayer to suspension could be investigated by analysis of the levels of CSP in the medium, following the increase in the percentage of cells in suspension.

2. Differences in the levels of CSP in PCM3-M and PCM3-S cells may also be studied by examination of the synthesis, degradation and exposure of this glycoprotein. Furthermore, it would be of interest to determine the effect of partially purified CSP on P388F-36 cells, which also lack this glycoprotein.

3. The effect of cAMP on the growth characteristics of PCM3 and in particular on cell adhesion could be explained by a direct action on CSP or a more general action on cell shape and adhesion. This

could be substantiated by determination of the levels of endogenous cAMP throughout the cell cycle in a synchronous culture. Resolution of this problem may clarify the role of cAMP in the proliferation of these cells.

4. It would also be informative to determine whether endogenous prostaglandins were involved in the regualtion of cell growth and whether this action was mediated by cAMP.

5. Finally, investigation of the 'ancestry' of the components involved in cAMP metabolism in the PCM3 hybrid may explain the enhanced hormonal stimulation of cAMP levels and the subunit nature of the hormone receptors and catalytic units concerned with cAMP synthesis. This work is being studied, at present, in this laboratory.

APPENDIX I. ABBREVIATIONS

Adenylate cyclase	ATP: pyrophosphate lyase(cyclising) E.C. 4.6.1.1
5'AMP	Adenosine 5'-monophosphate
ATP	Adenosine 5'-triphospahte
ATPase	ATP phosphohydrolase
Bentonite	Hydrated aluminium silicate clay
BSA	Bovine serum albumin
Butyl-PBD	2-(4'-tert-butylphenyl)-5-(4"-biphenylyl)-1,3,4-oxodiazole
Cyclic AMP (cAMP)	Cyclic adenosine 3',5'-monophosphate
cAMP-dependent protein kinase	ATP: protein phosphotransferase E.C. 2.7.1.37
Cyclic GMP (cGMP)	Cyclic guanosine 3',5'-monophosphate
c.p.m.	Counts per minute
CSP	Cell surface glycoprotein of M.W. 220,000 (Hynes, 1973)
d.d.w.	Double distilled water
d.b.cAMP	N^6,2'-O-dibutyryl cAMP
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease E.C.3.1.4.5
EDTA	Ethylenediaminetetra-acetic acid
EGTA	Ethyleneglycol-bis-(aminoethyl ether) tetra-acetate
Ethidium bromide	2,7-diamino-9-phenyl phenanthridine 10-ethyl bromide
f	Fluorescence intensity
FBS	Foetal bovine serum
β-galactosidase	E.C.3.2.1.23
Glucose oxidase	E.C. 1.1.3.4
Gpp(NH)p	Guanosine 5'-(β,γ-imino)triphosphate
GTP	Guanosine 5'-triphosphate
^3H	Tritium
H.A.T.	Hypoxanthine, aminopterin, thymidine
H.A.U.	Haemagglutinating units
HBSS	Hank's balanced salt solution
HEPES	N-2-hydroxyethyl piperazine-N'-2-ethane sulphonic acid
H-HBSS	Hank's balanced salt solution containing HEPES
IBMX	3-Isobutyl-1-methylxanthine
Lactoperoxidase	E.C.1.11.1.7
M.E.M.	Minimal essential medium
NAD^+	Nicotinamide-adenine dinucleotide, oxidised
NCS	Newborn calf serum
PBI	Phosphate buffered iodide
PBS	Phosphate buffered saline
PDE	3',5'-cyclic nucleotide 5'-nucleotidohydrolase E.C.3.1.4.16
PDEA	Phosphodiesterase activator
PGE_1	Prostaglandin E_1
PMSF	Phenyl methylsulphonylfluoride
POPOP	1,4-bis-(4-methyl-5-phenyloxazolyl)-benzene

PPO	2,5-diphenyl oxazole
PPLO	Pleuroneumonia-like organisms
Pronase	E.C.3.4.24.4
RNA	Ribonucleic acid
RNase	Ribonuclease E.C. 3.1.4.22
SDS	Sodium dodecyl sulphate
SV40	Simian virus 40
TCA	Trichloroacetic acid
TEMED	N,N,N'N'-tetra methyl ethylenediamine
Tris	2-amino-2-hydroxymethyl-1,3-propanediol
Trypsin	E.C.3.4.4.4
V_{max}	Maximum velocity

Cells

AP-9	Human embryonic lung cell line
CH23	A clone of Chinese Hamster fibroblasts
CHO	Chinese hamster ovary cell line
EB_2	Burkitt lymphoma cell line
HeLa	Human cervix carcinoma cells
NIL	Hamster fibroblast cell line
NIL-HSV	NIL fibroblasts transformed by hamster sarcoma virus
P388F-36	A clone of P388F mouse lymphoma cells
PCM3-M	Monolayer form of hybrid isolated from fusion of CH23 and P388F-36
PCM3-S	Suspension form of PCM3
3T3	Swiss mouse embryo cell line
WI-38	A human fibroblastic cell line

APPENDIX 2. MATERIALS

Acetic orcein	Gurr chemicals
Acrylamide	BDH Chemicals
NN'-bis-methylene acrylamide	Hopkin and Williams
Bentonite	BDH Chemicals
Bovine serum albumin	Sigma
Butyl PBD	Koch Light Labs.
Canada balsam	Hopkin and Williams
cAMP	Sigma
[8-^{3}H] cAMP	Radiochemical Centre, Amersham
Charcoal Norit SX-1	Hopkins and Williams
Colchicine	Sigma
Coomassie brilliant blue G	Sigma
Cyclohexyl amino-propane sulphonic acid	Sigma
Dithiothreitol	International Enzymes Ltd.
DMSO	Hopkins and Williams
DNA (calf thymus)	Sigma
Ethidium bromide	BDH Chemicals
N-ethylmaleimide	Sigma
Folin-Ciocalteau phenol reagent	BDH Chemicals
β-Galactosidase	Sigma
Giemsa	Gurr Chemicals
Glass fibre filter papers GFC (2.1cm)	Whatman
Glucose oxidase	Worthington
Glycerol	Hopkin and Williams (Analar)
HEPES	Sigma
IBMX	Aldrich Chemical Company Inc.
Kodirex X-ray film	Kodak Ltd.
Lactoperoxidase	Calbiochem Ltd.
Molecular weight markers (no. 44230 2R)	BDH Chemicals
Na^{125}I (carrier free)	Radiochemical Centre, Amersham.
Naphthalene	Koch Light Labs.
Nutrient agar	Oxoid
Ovalbumin	Sigma
PMSF	Sigma
POPOP	Koch Light Labs.
PPO	Koch Light Labs.
Pronase (nuclease free)	Calbiochem Ltd.
Ribonuclease	Sigma
SDS	BDH Chemicals
TEMED	Kodak Ltd.
[^{3}H]-thymidine	Radiochemical Centre, Amersham
Triton	Koch Light Labs.
Trypsin (for AP-9 cells)	Worthington Chemical Inc., Ohio
Trypsin	Difco

PGE_1 was a kind gift from I.C.I. Pharmaceuticals, Alderley Park, Cheshire.

Acta Spectrophotometer	Beckman Ltd.
Gel Scanner II attachment (0.2mm Slit)	Beckman Ltd.
Gel Electrophoresis apparatus (GE4)	Pharmacia Fine Chemicals, Uppsalla
Gel slab casting apparatus (GSC-8)	Pharmacia Fine Chemicals, Uppsalla
Power supply (model IP17)	Heathkit
microcentrifuge	Netheler and Hinz GmbH.

APPENDIX 3. COMPUTER PROGRAMME

```
      PRØGRAM ANNEY(INPUT,ØUTPUT,TAPE1=INPUT,TAPE2=ØUTPUT)
      INTFGER I,NUM
      REAL S,BLANK,SLØPE,PC,DF,X,A,B,C
C---READ IN: NUMBER ØF SAMPLES,S0-BLANK,BLANK,GRADIENT
      READ (1,100) NUM,S,BLANK,SLØPE,PC,DF
  100 FØRMAT (I10,5F10.C)
      WRITE (2,200)
  200 FØRMAT (1H1,17HCØUNTS PER 10 MIN,9X,4HS/CS,9X,7HPM CAMP,7X,
     117HSPECIFIC ACTIVITY)
      DØ 2 I=1,NUM
C---READ IN SAMPLE CPM,
      READ (1,107)X
  107 FØRMAT (F10.C)
      A=S/(X-BLANK)
C---CALCULATE PMØLES CAMP
      B=(A-1.0)/SLØPE
C---CØNVERT TØ CØNCENTRATIØNS ØF CAMP
      C=(B*DF)/PC
C---WRITE ØUT: SAMPLE CPM,S0/S RATIØ,PMØLES CAMP &
C---CØNCENTRATIØN ØF CAMP
    2 WRITE (2,201) X,A,B,C
  201 FØRMAT (1HO,3X,F10.2,8X,F10.2,4X,F10.2,9X,F10.2)
      STØP
      END
```

REFERENCES

Abell, C.W. and Monahan, T.M. (1973) J. Cell Biol. 59, 549

Abercrombie, M. and Heaysman, J.E.M. (1954) Exp. Cell Res. 6, 293.

Ahlquist, R.P. (1948) Am. J. Physiol. 153, 586.

Airhart, J., Sibiga, S., Sanders, H. and Khairallan, E.A. (1973) Anal. Biochem. 53, 132.

Albano, J.D.M., Barnes, G.D., Maudsley, D.V., Brown, B.L. and Etkins, R.P. (1974) Anal. Biochem. 59, 468

Albano, J., Bhoola, K.D. and Harvey, R.F. (1976) Nature, 262, 404.

Ali, I.U. and Hynes, R.O. (1977) Biochim. Biophys. Acta. 471, 16.

Ali, I.U., Mautner, V., Lanza, R. and Hynes, R.O. (1977) Cell 11, 115.

Alvarez, R. and Bruno, J.J. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 92.

Anderson, W.B., Johnson, G.S. and Pastan, I. (1973) Proc. Natl. Acad. Sci. USA. 70, 1055.

Appleman, M.M. and Terasaki, W.L. (1975) in Advances in Cyclic Nucleotide Research (Drummond, G.I., Greengard, P. and Robison, G.A. Eds.) Vol. 5, p. 153, Raven Press, New York.

Appleman, M.M., Thompson, W.J. and Russell, T.R. (1973) in Advances in Cyclic Nucleotide Research. (Greengard, P. and Robison, G.A. Eds). Vol. 3, p. 65, Raven Press, New York.

Atlas, D., Hanski, E. and Levitski, A. (1977) Nature 268, 144.

Ayad, S.R. and Burns, G.R.J. (1977) Exp. Cell Res. 108, 7.

Ayad, S.R. and Delinassios, J.G. (1974) Biochem. Genet. 12, 147.

Ayad, S.R. and Foster, S.J. (1974) Cell. 3, 135.

Ayad, S.R. and Foster, S.J. (1977) Exp. Cell Res. 109, 87.

Ayad, S.R., Fox, M. and Fox, B.W. (1969) Mutation Res. 8, 639

Ayad, S.R., Fox, M. and Winstanley, D. (1969) Biochem. Biophys. Res. Commun. 37, 551.

Ayad, S.R. and Morgan, S.L. (1977) Exp. Cell Res. 109, 9.

Ayad, S.R. and White, A. (1977) Exp. Cell Res. 107, 201.

Ayad, S.R. and Wright, J.F. (1977) Biochem. Genet. 15, 1001.

Baltimore Conference, 1975. Third International Workshop on Human Gene Mapping (1976) Cytogenetics and Cell Genetics 16, 1.

Bar, H. and Hechter, O. (1969) Proc. Natl. Acad. Sci. U.S.A. 63, 350

Barski, G. (1964) in Cytogenetics of Cells in Culture (Harris, R.J.C. Ed.) Academic Press, New York.

Barski, G., Sorieul, S. and Cornefert, F. (1960) C.R. Hebd. Seances. Acad. Sci. 251, 1825

Basilico, C. (1974) in Somatic Cell Hybridisation (Davidson, R.L. and de la Cruz, F.F. Eds) p. 235 Raven Press, New York.

Beavo, J.A., Bechtel, P.J. and Krebs, E.G. (1974) Methods Enzymol. 38, 299.

Ben-Bassat, H., Polliak, A., Rosenbaum, S.M., Naparstek, E., Shouvai, D. and Inbar, M. (1977) Cancer Res. 37, 1307

Bennett, V., Cuatrecasas, P. (1975a) J. Memb. Biol. 22, 29.

Bennett, V., Cuatrecasas, P. (1975b). J. Memb. Biol. 27, 207.

Bennett, V., Craig, S., Hollenburg, M.D., O'Keefe, E., Sahyoun, N. and Cuatrecasas, P. (1976) J. Supramol. Structure. 4, 99.

Berridge, M.J. (1975) J. Cyclic Nucleotide Research 1, 305.

Bilezikian, J.P. and Aurbach, G.D. (1973) J. Biol. Chem. 248, 5577.

Birnbaumer, L. (1973) Biochem. Biophys. Acta 300, 129.

Birnbaumer, L., Pohl, S.L. and Rodbell, M. (1969) J. Biol. Chem. 244, 3468

Birnbaumer, L.& Rodbell, M. (1969) J. Biol. Chem. 244, 3477.

Bootsma, D., Budke, L. and Vos, O. (1964) Exp. Cell Res. 33, 301.

Borman, L., Dumont, J.N. and Hsie, A.W. (1975) Exp. Cell Res. 91, 422

Bourne, H.R., Tomkins, G.M. and Dion, S. (1973) Science 181, 952.

Bourne, H.R., Coffino, P. and Tomkins, G.M. (1975) in Advances in Cyclic Nucleotide Research (Drummond, G.I., Greengard, P. and Robison, G.A. Eds). Vol. 5, p. 759, Raven Press, New York.

Bourne, H.R., Coffino, P. and Tomkins, G.M. (1975) Science 187, 750.

Boynton, A.L., Whitfield, J.F., Isaacs, R.J. and Trembley, R. (1977) J. Cell Physiol. 92, 241.

Bradham, L.S. (1977) J. Cyclic Nucleotide Research 3, 119

Bramwell, M.E. and Harris, H. (1978) Proc. Royal Soc. London. B201, 87.

Brinkley, B.R., Fuller, G.M. and Highfield, D.P. (1975) Proc. Natl. Acad. Sci. USA 72, 4981

Brooker, G.I., Thompson, W.J. and Appleman, M.M. (1968) Biochem. J. 7, 4177

Brostrom, M.A., Brostrom, C.O., Breckenridge, B.M. and Wolff, D.J. (1976) J. Biol. Chem. 251, 4744.

Brown, B.L., Albano, J.D.M., Etkins, R.P. and Sgherzi, A.M. (1971) Biochem. J. 121, 561.

Brown, B.L., Etkins, R.P. and Albano, J.D.M. (1972) in Advances in Cyclic Nucleotide Research (Greengard, P. and Robison, G.A. Eds.) Vol. 2, p. 25. Raven Press, New York.

Brown, E.M., Hauser, D., Toxler, F. and Aurbach, G.D. (1976) J. Biol. Chem. 251, 1232.

Brownhill, T.J., Jones, A.S. and Stacey, M. (1959) Biochem. J. 73, 434.

Brunton, L.L., Wiklund, R.A., van Arsdale, P.M. and Gilman, A.G. (1976) J. Biol. Chem. 251, 3037.

Brunton, L.L., Maguire, M.E., Anderson, H.J. and Gilman, A.G. (1977) J. Biol. Chem. 252, 1293.

Burger, M.M. (1977) in International Cell Biology (Brinkley, B.R. and Porter, K.R. Eds.) p. 131, Rockefeller University Press.

Burk, R.R. (1968) Nature, 219, 1272.

Butcher, R.W. and Sutherland, E.W. (1962) J. Biol. Chem.

237, 1244.

Carchman, R.A., Johnson, G.S., Pastan, I. and Scolnick, E.M. (1974) Cell, 1, 59.

Caron, M.C. and Lefkowitz, R.J. (1976) Biochem. Biophys. Res. Commun. 68, 315.

Caspersson, T., Zech, L., Harris, H., Wiener, F. and Klein, G. (1971) Exp. Cell Res. 65, 475

Cassel, D. and Selinger, Z. (1977a) J. Cyclic Nucleotide Research, 3, 11.

Cassel, D. and Selinger, Z. (1977b) Biochem. Biophys. Res. Commun. 77, 868

Cassel, D. and Selinger, Z. (1977c) Proc. Natl. Acad. Aci. USA, 74, 3307.

Cheung, W.Y. (1967) Biochem. Biophys. Res. Commun. 29, 478

Cheung, W.Y. (1970) in Advances in Psychopharmacology (Greengard, P. and Costa, E. Eds.) Vol. 3, p.

Cheung, W.Y., Bradham, L.S., Lynch, T.J., Lyn, Y.M. and Tallant, E.A. (1975) Biochem. Biophys. Res. Commun. 66, 1055.

Chlapowski, F.J., Kelly, L.A. and Butcher, R.W. (1975) in Advances in Cyclic Nucleotide Research (Greengard, P. and Robison, G.A. Eds.) Vol. 6, p. 245, Raven Press, New York.

Christofferson, T., Moerland, J., Osnes, J.B., Elgjo, K. (1972) Biochem. Biophys. Acta 279, 363

Coffino, P., Bourne, H.R. and Tomkins, G.M. (1975) J. Cell Physiol. 85, 603.

Coffino, P., Gray, J.W. and Tomkins, G.M. (1975b) Proc. Natl. Acad. Sci. USA 72, 878

Corbin, J.D. and Keely, S.L. (1977) J. Biol. Chem. 252, 910.

Counis, R. and Jutisz, M. (1977) Molecular and Cellular Endocrinology. 7, 313.

Croce, C.M. (1977) in Growth, Nutrition and Metabolism of Cells in Culture (Rothblat, G.H. and Cristofalo, V.J. Eds.) Vol. III, p. 83, Academic Press, New York.

Croce, C.M., Sawicki, W., Kritchewsky, D. and Koprowski, H. (1971) Exp. Cell Res. 67, 427

Croce, C.M. and Koprowski, H. (1975) Proc. Natl. Acad. Sci. USA 72, 1658

Cuatrecasas, P. (1974) Ann. Rev. Biochem. 43, 169.

Cuatrecasas, P., Jacobs, S. and Bennett, V. (1975) Proc. Natl. Acad. Sci. USA 72, 1739.

Cullis, P.R. and Hope, M.J. (1978) Nature, 271, 672.

Daniel, V., Litwack, G. and Tomkins, G.M. (1973) Proc. Natl. Acad. Sci. USA 70, 76.

D'Armiento, M., Johnson, G.S. and Pastan, I. (1973) Nature New Biol. 242, 78.

Davidson, R.L. and Ephrussi, B. (1965) Nature, 205, 1170.

Davidson, R.L., Ephrussi, B., Yamamoto, K. (1966) Proc. Natl. Acad. Sci. USA 56, 1437.

Davis, C.W. and Kuo, J.F. (1977) J. Biol. Chem. 252, 4078

Davis, F.M. and Adelburg, E.A. (1973) Bacteriological Reviews 37, 197

de Haën, C. (1974) J. Biol. Chem. 249, 2756

de Haën, C. (1976) J. Theor. Biol. 58, 383

De Meyts, P. (1976) J. Supramol. Structure. 4, 241.

De Meyts, P. Bianco, A.R. and Roth, J. (1976) J. Biol. Chem. 251, 1877

Delinassios, J.G. (1974) Ph.D. Thesis, University of Manchester.

Drummond, G.I. and Duncan, L. (1970) J. Biol. Chem. 245, 976

Durham, A.C.H. (1974) Cell. 2, 123.

Foley, G.E., Handler, A.H., Adams, R.A and Craig, J.M. (1962) Natl. Cancer Institute Monograph 7, 173

Folkman, J. and Moscona, A. (1978) 273, 345.

Fox, M. and Gilbert, C.W. (1966) Intern. Radiat. Biol. 11, 339.

Franklin, T.J. and Foster, S.J. (1973) Nature New Biol. 246, 146.

Franklin, T.J. and Twose, P.A. (1976) FEBS. 66, 225.

Friedman, D.L. (1976) Physiological Reviews 56, 652

Friedman, D.L., Johnson, R.A., Zeilig, C.E. (1976) in Advances in Cyclic Nucleotide Research (Greengard, P. and Robison, G.A. Eds.) Vol. 7, p. 69, Raven Press, New York.

Froehlich, J.E. and Rachmeler, M. (1972) J. Cell Biol. 55, 19.

Fuchs, P., Parola, A., Robbins, P.W. and Blout, E.R. (1975) Proc. Natl. Acad. Sci. USA 72, 3351.

Furchgott, R.F. (1975) Pharmacology Reviews 7, 183

Garbers, D.L. and Johnson, R.A. (1975) J. Biol. Chem. 250, 8449.

Gershon, D. and Sachs, L. (1963). Nature, 198, 912.

Gill, D.L. (1977) in Advances in Cyclic Nucleotide Research (Greengard, P. and Robison, G.A. Eds.) Vol. 8, p. 85, Raven Press, New York.

Gilman, A.G. and Minna, J.D. (1973) J. Biol. Chem. 248, 6610.

Goldberg, N.D., O'Dea, R.F. and Haddox, M.K. (1973) in Advances in Cyclic Nucleotide Research (Greengard, P. and Robison, G.A. Eds.) Vol. 3, p. 155.

Goldberg, N.D., Haddox, M.K., Dunham, E., Lopez, C. and Hadden, J.W. (1974) in Control of Proliferation in Animal Cells (Clarkson, B. and Baserga, R. Eds.) p. 609, N.Y. Cold Spring Harbor Lab.

Gordon, A.H. (1969) Laboratory Techniques in Biochemistry and Molecular Biology (Work, T.S. and Work, E. Eds) North Holland Press.

Gordon, S., Ripps, C. and Cohn, Z. (1971) J. Exp. Med. 134, 1187

Gorman, R.R., Bunting, S. and Miller, O.V. (1977) Prostaglandins 13, 377.

Gnegy, M.E., Costa, E. and Uzunov, P. (1976) Proc. Natl. Acad. Sci. USA 73, 352.

Gnegy, M.E., Nathanson, J.A. and Uzunov, P. (1977) Biochem. Biophys. Acta 497, 75.

Hadden, J.W., Hadden, E.M., Haddox, M.K. and Goldberg, N.S. (1972) Proc. Natl. Acad. Sci. USA 69, 3024.

Haga, T., Ross, E.M., Anderson, H.J. and Gilman, A.G. (1977) Proc. Natl. Acad. Sci. USA. 74, 2016.

Hait, W.N. and Weiss, B. (1977) Biochem. Biophys. Acta 497, 86.

Hammarstrom, S. (1977) Eur. J. Biochem. 74, 7.

Hanoune, J., Lacombe, M-L. and Pecker, F. (1975) J. Biol. Chem. 250, 4569.

Hanski, E. and Levitski, A. (1978) Life Sciences. 22, 53.

Hardman, J.G. and Sutherland, E.W. (1969) J. Biol. Chem. 244, 6363.

Harris, H. (1975) Cancer Research Campaign, 53rd Annual Report

Harris, H., Miller, O.J., Klein, G., Worst, P. and

Tachibana, T. (1969) Nature, 233, 363.

Harris, H. and Watkins, J.F. (1965) Nature. 205, 640.

Hart, C.A., Fisher, D., Hallinan, T. and Lucy, J.A. (1976) Biochem. J. 158, 141.

Helmreich, E.J.M. (1976) FEBS. Letts. 61, 1.

Helmreich, E.J.M., Zenner, H.P., Pfeuffer, T. and Cori, C.F. (1976) Current Topics in Cellular Regulation (Horecker, B.L. and Stadtman, E.R. Eds) Vol. 10, Academic Press.

Hinman, J.N. (1972) Annu. Rev. Biochem. 41, 161.

Hochman, J., Bourne, H.R., Coffino, P., Insel, P.A., Krasny, L. and Melmon, K.L. (1977) Proc. Natl. Acad. Sci. USA 74, 1167.

Hofmann, F., Beavo, J.A., Bechtel, P.J. and Krebs, E.G. (1975) J. Biol. Chem. 250, 7795

Horton, E.W. (1969) Physiological Reviews, 49, 122.

Horton, E.W. (1976) New Scientist 69, 9.

Hosaka, Y. (1970) in Biology of Large RNA Viruses (Barry, R.D. and Mahy, B.W.J. Eds.) Academic Press.

Housley, M.D., Ellory, J.C., Smith, G.A., Hesketh, H.T.R. Stein, J.M., Warren, G.B. and Metcalfe, J.C. (1977) Biochem. Biophys. Acta 467, 208.

Hsie, A.W. and Puck, T.T. (1971) Proc. Natl. Acad. Sci. USA 68, 358.

Hsu, T.C. and Pomerat, C.M. (1953) J. Hereditary 44, 23.

Hynes, R.O. (1973) Proc. Natl. Acad. Sci. USA 70, 3170.

Hynes, R.O. (1976) Biochem. Biophys. Acta 458, 73.

Hynes, R.O. and Bye, J.M. (1974) Cell 3, 113.

Hynes, R.O. and Destree, A. (1977) Proc. Natl. Acad. Sci. USA 74, 2855.

Hynes, R.O., Destree, A.T., Mautner, V.M. and Ali, I.U. (1977) J. Supramol. Struct. 7, 397.

Insel, P.A., Bourne, H.R., Coffino, P. and Tomkins, G.M. (1975) Science 190, 896.

Insel, P.A. and Kennedy, M.S. (1978) Nature, 273, 471.

Itagaki, A. and Kimura, G. (1974) Exp. Cell Res. 83, 351.

Jacobs, S. and Cuatrecasas, P. (1977) Trends in Biochemical Sciences 2, 280.

Jensen, P., Winger, L., Rasmussen, H. and Nowell, P. (1977) Biochem. Biophys. Acta 496, 374

Johnson, E.M. (1977) in Advances in Cyclic Nucleotide Research (Greengard, P. and Robison, G.A. Eds.) Vol. 8, p. 267, Raven Press, New York.

Johnson, G.L. and Bourne, H.R. (1977) Biochem. Biophys. Res. Commun. 78, 792.

Johnson, G.S., Friedman, R.M. and Pastan, I. (1971) Proc. Natl. Acad. Sci. USA 68, 425.

Jonasson, J., Povey, S. and Harris, H. (1977) J. Cell Sci., 24, 217.

Juliano, R.L. and Gagalang, E. (1977) J. Cell Physiol. 92, 209.

Kakiuchi, S., Yamazaki, R. and Nakajima, H. (1970) Proc. Jap. Acad. 46, 587.

Kao, F.T. and Puck, T.T. (1970) Nature, 228, 329.

Kao, F.T., Johnson, R.T. and Puck, T.T. (1969) Science 164, 312.

Karim, S.M.M., Devlin, J. and Hiller, K. (1968) Eur. J. Pharmacol. 4, 416.

Karsten, U. and Wollenburger, A. (1972) Anal. Biochem. 46, 135.

Kedan, N., Prasad, S.K., Sahu, P. and Sinha, K. (1976) J. Natl. Cancer Inst. 57, 619.

Kimball, R.F., Stella, W., Perdue, S.W., Chu, E.H.Y. and Ortz, J.R. (1971) Exp. Cell Res. 66, 17.

Kimura, H. and Murad, F. (1975) Proc. Natl. Acad. Sci. USA 72, 1965

Kimura, N. and Nagata, N. (1977) J. Biol. Chem. 252, 3829

Klein, I., Moore, L. and Pastan, I. (1978) Biochem. Biophys. Acta 506, 42.

Koblin, D.D., Pace, W.D. and Wang, H.H. (1975) Arch. of Biochem. and Biophys. 171, 176.

Kolata, G.D. (1977) Science 196, 747.

Knecht, M.E. and Lipkin, G. (1977) Exp. Cell Res. 108, 15.

Knutton, S. (1977) J. Cell Science 28, 189

Kuehl, F.A. (1974) 5, 325.

Kung, W., Bechtel, E., Geyer, E., Salokangas, A., Preisz, J., Huber, P., Torhorst, J., Jungman, R.A., Talmadge, K. and Eppenburger, U. (1977) FEBS 82, 102.

Kurz, J.B. and Friedman, D.L. (1976) J. Cyclic Nucleotide Research 2, 405.

Lad, P.M., Welton, A.F. and Rodbell, M. (1977) J. Biol. Chem. 252, 5942

Laemmli, U.K. (1970) Nature 227, 680.

Lawrence, D.A. and Jullien, P. (1975) Exp. Cell Res. 95, 54.

Leffert, H.L. and Koch, K.A. (1977) in Growth, Nutrition and Metabolism of Cells in Culture (Rothblat, G.H. and Cristofalo, V.J. Eds.) Vol. III p. 226, Academic Press, New York.

Lefkowitz, R.J. (1975) J. Biol. Chem. 250, 1006.

Lefkowitz, R.J. and Williams, L.T. (1977) Proc. Natl. Acad. Sci. USA 74, 515.

Lefkowitz, R.J., Limbird, L.E., Mukherjee, C. and Caron, M.G. (1976). Biochem. Biophys. Acta. 457, 1.

Lefkowitz, R.J., Mullikin, D. and Caron, M.G. (1976b) J. Biol. Chem. 251, 4686

Lefkowitz, R.J., Mullikin, D., Wood, C.L., Gore, T.B. and Mukherjee, C. (1977) J. Biol. Chem. 252, 5295

Lemaire, I. and Coffino, P. (1977) Cell. 11, 149.

Lemaire, I. and Coffino, P. (1977b) J. Cell Physiol. 92, 437.

Levey, G.S. and Klein, I. (1972) J. Clin. Investigation 51, 1578

Levinson, S.L. and Blume, A.J. (1977) J. Biol. Chem. 252, 3766

Levitzki, A. (1976) in Receptors and Recognition (Cuatrecasas, P. and Greaves, M.F. Eds.) Series A, vol. 2, Chapman and Hall Press, London.

Levitzki, A. (1977) Biochem. Biophys. Res. Commun. 74, 1154.

Levitzki, A., Atlas, D. and Steer, M.L. (1974) Proc. Natl. Acad. Sci. USA 71, 2773.

Limbird, L.E. and Lefkowitz, R.J. (1976) J. Biol. Chem. 251, 5007.

Limbird, L.E. and Lefkowitz, R.J. (1977) J. Biol. Chem. 252, 799.

Lin, M.C., Salomon, Y., Rendell, M. and Rodbell, M. (1975) J. Biol. Chem. 250, 4246

Lin, M.T. and Rao, Ch.V. (1977) Biochem. Biophys. Res. Commun. 78, 510.

Lin, Y.M., Liu, Y.P. and Cheung, W.Y. (1974) J. Biol. Chem. 249, 4943.

Lincoln, T.M. and Corbin, J.D. (1977) Proc. Natl. Acad. Sci. USA 74, 3239

Littlefield, J.W. (1966) Exp. Cell Res. 41, 190.

Londos, C. and Preston, M.S. (1977) J. Biol. Chem. 252, 5957

Londos, C. and Rodbell, M. (1975) J. Biol. Chem. 250, 3459

MacPherson, I. (1970) Advances in Cancer Research 13, 169

Maguire, M.E., Ross, E.M. and Gilman, A.G. (1977) in Advances in Cyclic Nucleotide Research (Greengard, P. and Robison, G.A. Eds.) Vol. 8, p. 1, Raven Press, New York.

Maguire, M.E., Van Arsdale, P.M. and Gilman, A.G. (1976) Mol. Pharmacology 12, 335.

Makman, M.H. (1971) Proc. Natl. Acad. Sci. USA 68, 2127

Makman, M.H., Morris, S.A., Ho, S.A. (1977) in Growth, Nutrition and Metabolism of Cells in Culture (Rothblat, G.H. and Cristofalo, V.J. Eds.) Vol. III, p. 295.

Manganiello, V. and Vaughan, M. (1972) Proc. Natl. Acad. Sci. USA 69, 269.

Martin, B.R., Housley, M.D. and Kennedy, E.L. (1977) Biochem. J. 161, 639.

Matuo, Y., Wheeler, M.A. and Bitensky, M.W. (1976) Proc. Natl. Acad. Sci. USA 73, 2654

Medina, D. and Sachs, L. (1963) Virology, 19, 127.

Mickey, J.V., Tate, R., Mullikin, D. and Lefkowitz, R.J. (1976) Mol. Pharmacology 12, 409.

Mierzejewski, K. and Rozengurt, E. (1976) Biochem. Biophys.

Res. Commun. 73, 271.

Millis, A.J.T., Forrest, G.A. and Pious, D.A. (1974) Exp. Cell Res. 83, 335.

Minna, J.D. and Gilman, A.G. (1973) J. Biol. Chem. 248, 6618

Morris, C.J.O.R. and Morris, P. (1976) Separation Methods in Biochemistry, Pitman Publishers.

Morris, J.A. and Makman, M.H. (1976) Mol. Pharmacology 12, 362.

Mosher, D.F., Sakselo, O., Keski-Oja, J. and Vaheri, A. (1978) J. Supramol. Struct. 6, 551.

Mukherjee, C. and Lefkowitz, R.J. (1977) Mol. Pharmacology 13, 291.

Mukherjee, C., Caron, M.G. and Lefkowitz, R.J. (1975) Proc. Natl. Acad. Sci. USA 72, 1945.

Mukherjee, C., Caron, M.G. and Lefkowitz, R.J. (1976) Endocrinology 99, 347.

Najjar, V.A. and Constantopoulos, A. (1973) Mol. and Cell Biochem. 2, 87.

Nardonne, R.M., Todd, J., Gonzales, P. and Gaffney, E.V. (1965) Science 149, 1100.

Neer, E.J. (1976) J. Supramol. Struct. 4, 51.

Neff, J.M. and Enders, J.F. (1968) Proc. Soc. Exp. Biol. Med. 127, 260.

Nesbitt, J.A., Anderson, W.B., Millar, Z., Pastan, I., Russell, T.R. and Gospodarowicz, D. (1976) J. Biol. Chem. 251, 2344.

Nias, A.H.W. and Fox, M. (1971) Cell and Tissue kinetics 4, 375.

Nicolini, C. (1976) Biochem. Biophys. Acta. 458, 243.

Nicolson, G.L. (1976) Biochem. Biophys. Acta 457, 57.

Nicolson, G.L. (1976b) Biochem. Biophys. Acta 458, 1.

Nicolson, G.L. and Poste, G. (1976) J. Supramol. Struct. 5, 65.

Nicolson, G.L., Smith, J.R. and Poste, G. (1976) J. Cell Biol. 68, 395.

Nicolson, G.L., Giotta, G., Lotan, R., Neri, A. and Poste, G. (1977) in International Cell Biology (Brinkley, B.R. and Porter, K.R. Eds.) p. 138. Rockefeller University Press.

Nimmo, H.G. and Cohen, P. (1977) in Advances in Cyclic Nucleotide Research (Greengard, P. and Robison, G.A. Eds.) Vol. 8, p. 145, Raven Press, New York.

Oey, J. (1975) Nature 257, 317.

Oey, J., Vogel, A. and Pollack, R. (1974) Proc. Natl. Acad. Sci. USA 71, 694

Okada, Y. (1962) Exp. Cell Res. 26, 98.

Okada, Y. and Murayama, F. (1965) Exp. Cell Res. 40, 154.

Okada, Y. and Murayama, F. (1966) Exp. Cell Res. 44, 527.

Olden, K. and Yamada, K.M. (1977) Cell 11, 957

O'Neill, J.P. and Hsie, A.W. (1975) Biochem. Biophys. Acta 404, 243.

Orly, J. and Schramm, M. (1975) Proc. Natl. Acad. Sci. USA 72, 3433.

Orly, J. and Schramm, M. (1976) Proc. Natl. Acad. Sci. USA 73, 4410.

Otten, J., Johnson, G.S. and Pastan, I. (1971) Biochem. Biophys. Res. Commun. 44, 1192

Papahadjopoulos, D. (1972) Biochem. Biophys. Acta 265, 169.

Papahadjopoulos, D., Jacobson, K., Poste, G. and Shephard,

G. (1975) Biochem. Biophys. Acta. 394, 504.

Papahadjopoulos, D., Vail, W.J., Newton, C., Nir, S., Jacobson, K., Poste, G. and Lazo, R. (1977) Biochem. Biophys. Acta 465, 579.

Parker, C.W., Sullivan, T.J. and Wedner, H.J. (1974) in Advances in Cyclic Nucleotide Research (Greengard, P. and Robison, G.A. Eds.) Vol. 4, p. 1, Raven Press, New York.

Pastan, I. and Johnson, G.S. (1974) Advances in Cancer Research 19, 303.

Pastan, I. and Willingham, M. (1978) Nature 274, 645.

Pastan, I.H., Johnson, G.S. and Anderson, W.B. (1975) Ann. Rev. Biochem. 44, 491.

Pastan, I., Willingham, M., Yamada, K.M., Pouyssegur, J., Davies, P. and Klein, I. (1977) in International Cell Biology (Brinkley, B.R. and Porter, K.R. Eds.) p. 161, Rockefeller University Press.

Paul, J. (1970) Cell and Tissue Culture, 4th Edn. Livingstone Press.

Paul, D. (1973) Biochem. Biophys. Res. Commun. 53, 745.

Pawlson, L., Lovell-Smith, C., Manganiello, V.C. and Vaughan, M. (1974) Proc. Natl. Acad. Sci. USA 71, 1639

Pecker, F. and Hanoune, J. (1977) FEBS 83, 93.

Peery, C.V., Johnson, G.S. and Pastan, I. (1971) J. Biol. Chem. 246, 5785

Perkins, J.P. (1973) in Advances in Cyclic Nucleotide Research (Greengard, P. and Robison, G.A. Eds.) Vol. 3, p. 1, Raven Press, New York.

Perry, S.V. (1955) Methods in Enzymology (Colowick, S.P. and Kaplan, N.O. Eds.) Vol. II, p. 582.

Pfeuffer, T. (1977) J. Biol. Chem. 252, 7224

Phillips, D.R. (1972) Biochem. 11, 4582

Pichard, A.L. and Cheung, W.Y. (1976) J. Biol. Chem. 251, 5726

Pledger, W.J., Thompson, W.J. and Strada, S.J. (1975) Nature, 256, 729

Polgar, P., Vera, J.C., Kelley, P.R. and Ruttenberg, A.M. (1973) Biochem. Biophys. Acta 297, 378

Pontecorvo, G. (1975) Somatic Cell Genetics 1, 397

Pontecorvo, G., Riddle, P.N. and Hales, A. (1977) Nature, 265, 257

Porter, K.R., Puck, T.T., Hsie, A.W. and Kelley, D. (1974) Cell 2, 145.

Poste, G. (1972) Intern. Rev. Cytology 33, 157.

Poste, G., Papahadjopoulos, D., Jacobson, K. and Vail, W.J. (1975) Biochem. Biophys. Acta 394, 520.

Poste, G., Papahadjopoulos, D. and Nicolson, G.L. (1975b) Proc. Natl. Acad. Sci. USA 72, 4430

Pouyssegur, J., Willingham, M. and Pastan, I. (1977) Proc. Natl. Acad. Sci. USA 74, 248.

Prasad, K.N. and Kumar, S. (1974) in Control of Proliferation in Animal Cells (Clarkson, B. and Baserga, R. Eds.) P. 581, Cold Spring Harbour Laboratories, New York.

Prescott, D.M. (1964) Methods in Cell Physiology Vol. 1, Academic Press, New York.

Puchwein, G., Pfeuffer, T. and Helmreich, E.J.M. (1974) J. Biol. Chem. 249, 3232.

Puck, T.T. (1977) Proc. Natl. Acad. Sci. USA 74, 4491.

Rasmussen, H., Jensen, P., Lake, W., Friedman, N. and Goodman, D.B.P. (1975) in Advances in Cyclic Nucleotide Research (Greengard, P. and Robison, G.A. Eds.) Vol. 5, p. 375, Raven Press, New York.

Rechler, M.M., Bruni, C.B., Podskalny, J.M., Warner, W. and Carchman, R.A. (1977) Exp. Cell Res. 104, 411.

Reid, M.S. and Bieleski, R.L. (1968) Anal. Biochem. 22, 374

Rethy, A., Tomasi, V., Trevisani, A. and Barnabei, O. (1972) Biochem. Biophys. Acta 290, 58.

Ringertz, N.R. and Savage, R.E. (1976) Cell Hybrids, Academic Press, London.

Robison, G.A., Butcher, R.W. and Sutherland, E.W. (1971) Cyclic AMP. Academic Press, London.

Rodbell, M. (1975) J. Biol. Chem. 250, 5826

Rodbell, M., Birnbaumer, L., Pohl, S.L. and Krans, H.M.J. (1971) J. Biol. Chem. 246, 1877

Rodbell, M., Lin, M.C., Salomon, Y., Londos, C., Harwood, J.P., Martin, B.R., Rendell, M., and Berman, M. (1975) in Advances in Cyclic Nucleotide Research (Greengard, P. and Robison, G.A. Eds.) Vol. 5, p. 3, Raven Press, New York.

Roseman, T.J., Sims, B. and Stehle, R.G. (1973) American J. Hosp. Pharm. 30, 236.

Ross, E.M., Maguire, M.E., Sturgill, T.W., Biltonen, R.L. and Gilman, A.G. (1977) J. Biol. Chem. 252, 5761

Rudland, P.S., Gospodarowicz, D. and Siefert, W. (1974) Nature, 250, 741.

Rudolph, S.A., Greengard, P. and Malawista, S.E. (1977) Proc. Natl. Acad. Sci. USA 74, 3404.

Ryan, W.L. and Heidrick, M.L. (1968) Science. 162, 1484

Ryan, W.L. and Heidrick, M.L. (1974) in Advances in Cyclic Nucleotide Research (Greengard, P. and Robison, G.A. Eds.) Vol. 4, p. 81, Raven Press, New York.

Ryan, J. and Storm, D.R. (1974) Biochem. Biophys. Res. Commun. 60, 304.

Samuelsson, B., Granstrom, E., Green, K., Hamberg, M. and Hammarström, S. (1975) Ann. Rev. Biochem. 44, 669.

Santoro, M.G., Philpott, G.W. and Jaffe, B.M. (1976) Nature 263, 777.

Scheid, A. and Choppin, P.W. (1974) Virology 57, 475

Schlessinger, J., Barak, L.S., Hammes, G.G., Yamada, K.M., Pastan, I., Webb, W.W. and Elson, E.L. (1977) Proc. Natl. Acad. Sci. USA. 74, 2909.

Schramm, M. and Naim, E. (1970) J. Biol. Chem. 245, 3225.

Schramm, M. and Rodbell, M. (1975) J. Biol. Chem. 250, 2232.

Schramm, M. and Selinger, Z. (1975) J. Cyclic Nucleotide Research 1, 181.

Schramm, M., Orly, J., Eimerl, S. and Korner, M. (1977) Nature 268, 310

Schröder, J. and Plagueman, P.G.W. (1972) Cancer Research 32, 1082.

Schultz, J. and Hamprecht, B. (1973) Naunyn Schmeideberg's Arch. Pharmacol. 278, 215.

Schwarzmeier, J.D. and Gilman, A.G. (1977) J. Cyclic

Nucleotide Research 3, 227.

Seifert, W.E. and Rudland, P.S. (1974) Nature, 248, 138

Shear, M., Insel, P.A., Melmon, K.L. and Coffino, P. (1976) J. Biol. Chem. 251, 7572

Sheppard, J.R., Gormus, R. and Moldow, C.F. (1977) Nature 269, 693.

Shields, R. (1976) New Scientist, 70, 694.

Shields, R. and Pollock, K. (1974) Cell, 3, 31.

Shin, S., Freedman, V.H., Risser, R. and Pollack, R. (1975) Proc. Natl. Acad. Sci. USA 72, 4435.

Shinitzky, M. and Inbar, M. (1976) Biochem. Biophys. Acta 433, 133.

Simantov, R. and Sachs, L. (1975) J. Biol. Chem. 250, 3236

Siminovitch, L. (1976) Cell. 7, 1.

Singer, S.J. and Nicolson, G.L. (1972) Science 175, 720

Smith, S.W., Werthamer, S. and Artman, M. (1974) In Vitro 10, 225.

Soifer, D. (1975) The Biology of Cytoplasmic Microtubules, Annal of the New York Acad. Sci. Vol. 253

Spandidos, D.A. and Siminovitch, L. (1977) Cell 12, 675

Steer, M.L. and Levitzki, A. (1975) J. Biol. Chem. 250 , 2080

Stevens, F.C., Walsh, M., Ho, H.C., Teo, T.S. and Wang, J.H. (1976) J. Biol. Chem. 251, 4495

Storm, D.R., Field, S.O. and Ryan, J. (1976) J. Supramol. Structure. 4, 221.

Strauss, D.S., Jonasson, J. and Harris, H. (1977) J. Cell Science. 25, 73.

Studzinski, G.P. (1977) in Growth, Nutrition and Metabolism of Cells in Culture (Rothblat, G.D. and Cristofalo, V.J. Eds.) Vol. III, p.356

Su, Y.F., Cubeddu, L.X. and Perkins, J.P. (1976) J. Cyclic Nucleotide Research. 2, 257

Sutherland, E.W. and Rall, T.W. (1960) Pharmacol. Rev. 12, 265

Swillens, S. and Dumont, J.E. (1977) J. Cyclic Nucleotide Research 3, 1.

Szybalski, W., Szybalski, E.H. and Ragni, G. (1962) Natl. Cancer Inst. Monog. 7, 75

Tao, M. and Lipman, F. (1969) Rec. Natl. Acad. Sci. USA 63, 86.

Taylor, L. and Polgar, P. (1977) FEBS Letts. 79, 69.

Temin, H.M. and Rubin, H. (1958) Virology 6, 669

Terasaki, W.L. and Appleman, M.M. (1975) Metabolism 24, 311.

Terasima, T. and Tolmach, L.J. (1963) Exp. Cell Res. 30, 344

Thomas, D.B. and Lingwood, C.A. (1975) Cell. 5, 37.

Thomas, D.R., Philpott, G.W. and Jaffe, B.M. (1974) Exp. Cell Res. 84, 40.

Tisdale, M.J. and Phillips, B.J. (1976) Exp. Cell Res. 99, 63.

Tjio, J.H. and Puck, T.T. (1958) J. Exptl. Med. 108, 259

Tobey, R.A., Crissman, H.A. and Kraemer, P.M. (1972) J. Cell Biol. 54, 638

Tovey, K.C., Oldham, K.G. and Whelan, J.A.M. (1974) Clin. Chim. Acta 56, 221.

Tsuboi, A., Kurotsu, T. and Terasima, T. (1976) Exp. Cell Res. 103, 257.

van Obberghen, E., De Meyts, P. and Roth, J. (1976) J. Biol. Chem. 25, 6844

Vogt, P.K., Sarma, P.S. and Huebner, R.J. (1965) Virology, 27, 233

Walsh, D.A., Ashby, C.D., Gonzalez, C., Calkins, D., Fischer, E.H. and Krebs, E.G. (1971). J. Biol. Chem. 246, 1977

Warren, L., Buck, C.A., Tuszynski, G.P. and Fuhrer, J.P. (1977) in International Cell Biology (Brinkley, B.R. and Porter, K.R. Eds.) p. 149 Rockefeller University Press.

Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406

Weeds, A.G. and Hartley, B.S. (1968) Biochem. J. 107, 531

Weiss, M.C. and Green, H. (1967) Proc. Natl. Acad. Sci. USA 58, 1104.

Wells, J.N. and Hardman, J.G. (1977) in Advances in Cyclic Nucleotide Research (Greengard, P. and Robison, G.A.) Vol. 8, p. 119 , Raven Press, New York.

Welton, A.F., Lad, P.M., Newby, A.C., Yamamura, H., Nicosia, S. and Rodbell, M. (1977) J. Biol. Chem. 252, 5947.

Willingham, M.C. and Pastan, I. (1975) J. Cell Biol. 67, 146.

Willingham, M.C., Johnson, G.S. and Pastan, I. (1972) Biochem. Biophys. Res. Commun. 48, 743.

Willingham, M.C., Carchman, R.C. and Pastan, I.H. (1973) Proc. Natl. Acad. Sci. USA, 70, 2906.

Wunderwald, P., Jurz, G. and Michal, G. (1974) Anal. Biochem. 59, 468.

Yamada, K.M. (1978) Nature, 273, 335.

Yamada, K.M. and Pastan, I. (1976) Trends in Biochem. Sci. 1, 222.

Yamada, K.M. and Weston, J.A. (1974) Proc. Natl. Acad. Sci. USA 71, 3492.

Yamada, K.M., Yamada, S.S. and Pastan, I. (1975) Proc. Natl. Acad. Sci. USA 72, 3158.

Yamada, K.M., Yamada, S.S. and Pastan, I. (1976) Proc. Natl. Acad. Sci. USA 73, 1217.

Yang, T.J., Dale, J.B. and Machanoff, R. (1976) J. Cell Sci. 20, 199.

Yoshida, M.C. and Ephrussi, B. (1967) J. Cell Physiol. 69, 33.

Yoshida, M., Ikawa, Y., Owada, M. and Toyoshima, K. (1977) Int. J. Cancer. 20, 560.

Zeilig, C.E., Johnson, R.A., Sutherland, E.W. and Friedman, D.L. (1976) J. Cell Biol. 71, 515.

have been shown to acquire increased microtubular array [33, 34]. However, our results imply a reciprocal effect suggesting that there may be a link between cytoskeletal elements and cAMP production.

Finally it must be emphasised that tetracaine may be altering hormonal stimulation of cAMP by a more general action on the components of the membrane or by a combination of the schemes proposed. It should be noted that tetracaine may be acting as a detergent having a chaotropic effect on the membrane, or it may be acting via a displacement of calcium ions from the membrane. Work is in progress to determine if the cytoskeletal elements are involved in the alteration of hormonal stimulation and to elucidate the effect of the local anaesthetic on the purified adenylate cyclase.

The authors would like to thank Professor G. R. Barker for the facilities for their research. This work was supported by a grant from the Cancer Research Campaign.

REFERENCES

1. Tisdale, M J & Phillips, B J, Exp cell res 88 (1974) 111.
2. Otten, J, Johnson, G S & Pastan, I, Biochem biophys res commun 44 (1971) 1192.
3. Monahan, T M, Marchand, N W, Fritz, R R & Abell, C W, Cancer res 35 (1975) 2540.
4. Heidrick, M L & Ryan, W L, Cancer res 30 (1970) 376.
5. Pastan, I, Johnson, G S & Anderson, W B, Ann rev biochem 44 (1974) 491.
6. Patterson, M K, Jr, J natl cancer inst 53 (1974) 1493.
7. Anderson, W B, Gallo, M & Pastan, I, J biol chem 249 (1974) 7041.
8. Polgar, P, Vera, J C, Kelley, P R & Rutenberg, A M, Biochim biophys acta 297 (1973) 378.
9. Peery, C V, Johnson, G S & Pastan, I, J biol chem 246 (1971) 5785.
10. Nicolson, G L, Biochim biophys acta 457 (1976) 57.
11. Cuatrecasas, P, Ann rev biochem 43 (1974) 169.
12. Dettaen, C, J theor biol 58 (1976) 383.
13. McGuire, R F & Barber, R, J supramol struct 4 (1976) 259.
14. Inbar, M, Shinitzky, M & Sachs, L, FEBS lett 38 (1974) 268.
15. Fuchs, P, Parola, A, Robbins, P W & Blout, E R, Proc natl acad sci US 72 (1975) 3351.
16. Papahadjopoulos, D, Jackson, K, Poste, G & Shepherd, G, Biochim biophys acta 394 (1975) 504.
17. Koblin, D D, Pace, W D & Wang, H H, Arch biochem biophys 171 (1975) 176.
18. Ritchie, J M & Greengard, P, Ann rev pharmacol 6 (1966) 405.
19. Papahadjopoulos, D, Biochim biophys acta 265 (1972) 169.
20. Poste, G, Papahadjopoulos, D & Nicolson, G L, Proc natl acad sci US 72 (1975) 4430.
21. Poste, G, Papahadjopoulos, D, Jackson, K & Vail, W J, Biochim biophys acta 394 (1975) 520.
22. Willingham, M C & Pastan, I, J cell biol 67 (1975) 146.
23. Wardone, R M, Todd, J, Gonzales, P & Gaffney, E V, Science 149 (1965) 1100.
24. Ayad, S R & Foster, S J, Cell 3 (1974) 135.
25. Lowry, O H, Rosebrough, N J, Farr, A L & Randall, R J, J biol chem 193 (1951) 265.
26. Brown, B L, Albano, J D M, Alkins, R P & Sgherzi, A M, Biochem j 121 (1971) 561.
27. Tovey, K C, Oldham, K G & Whelan, J A M, Clin chim acta 56 (1974) 221.
28. Tisdale, M J & Phillips, B J, Exp cell res 99 (1976) 63.
29. Hubbell, W L, Metcalfe, J C, Metcalfe, S M & McConnell, H M, Biochim biophys acta 219 (1970) 415.
30. Nicolson, G L, Biochim biophys acta 458 (1976) 1.
31. Nicolson, G L, Smith, J R & Poste, G, J cell biol 88 (1976) 395.
32. Brinkley, B R, Fuller, G M & Highfield, D P, Proc natl acad sci US 72 (1975) 4981.
33. Borman, L, Dumount, J N & Hsie, A W, Exp cell res 91 (1975) 422.
34. Porter, K R, Puck, T T, Hsie, A W & Kelley, D, Cell 2 (1974) 145.

Received October 15, 1976
Accepted December 16, 1976

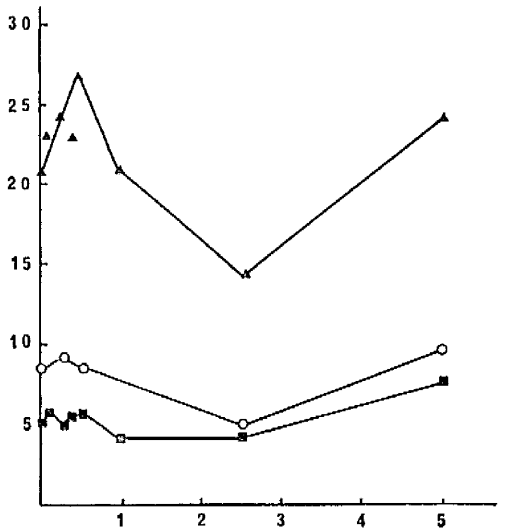


Fig. 6. Comparison of the effect of isoprenaline and prostaglandin E_1 stimulated cAMP levels subjected to varying concentrations of tetracaine. The isoprenaline concentration was 10^{-4} M (▲—▲). The prostaglandin E_1 concentration was 10 μg/ml (O—O) cells incubated without hormone (■—■).

and EB-2) follow a pattern similar to that seen with other cultured cell lines [24, 28]. We have examined the effects of the local anaesthetic tetracaine on hormonal stimulation in these cells and the response of the two malignant cell lines varied from that of the AP-9 cells.

Several reports indicate that tetracaine and other anaesthetics interact with acidic phospholipids to increase their fluidity [16, 29]. If tetracaine acts in this way on the phospholipids associated with the receptor-adenylate cyclase complex then our results indicate that it must facilitate and inhibit hormonal activation at differing anaesthetic concentration. This could easily be envisaged as it has been suggested that the hormonal receptor is capable of lateral movement in the membrane [11], and coupling with adenylate cyclase would be altered by a change in the translational mobility of components within the membrane. It has been reported that malignant cells show alterations in the mobility of membrane components [30] and this could explain the effect of tetracaine in SKL-2 cells, although it could be argued that tetracaine may be acting more generally on a membrane component, which normally restricts cAMP production. However, the malignant cell line EB-2 appears to have a more complex response to tetracaine, which suggests that there are differences in the nature of the plasma membrane in the two cell lines.

In view of the evidence implicating microtubules and microfilaments as the site of action of local anaesthetics [20], it seems probable that the action of tetracaine on the cytoskeletal elements could produce an alteration in the hormonal stimulation of cAMP. It appears that the local anaesthetics disrupt microtubules and microfilaments and over the range of concentrations used in this paper, the cytoskeletal elements disappear, cells become rounded and finally detach [31]. It may be that the action of tetracaine in our experiments initially releases a component held in restraint by the microtubules and microfilaments and this allows increased cAMP production, while further disruption and cellular rounding alters cAMP accumulation adversely. It is interesting to note that receptors involved in plant agglutination are redistributed by the action of similar concentrations of tetracaine, and this is reported to act by disrupting the microtubules anchoring these receptors [20].

If the effect of tetracaine is to alter the organisation of microtubules and microfilaments then in SKL-2 cells this disruption facilitates cAMP production. There is evidence [32] that there are fewer cytoskeletal elements in malignant cells suggesting that the response to tetracaine would be altered.

Willingham & Pastan [22] have proposed that cAMP may be involved in cytoskeletal organisation and cells grown in db-cAMP

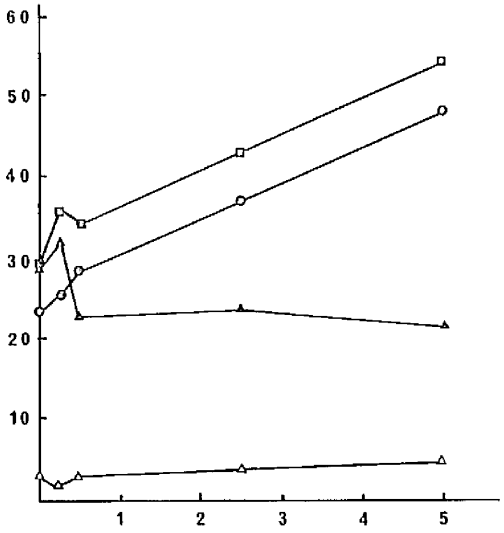


Fig. 4. Effect of tetracaine on prostaglandin E_1 stimulated cAMP levels in SKL-2 cells. Prostaglandin concentrations used were 10.0 μg/ml (■—■); 1.0 μg/ml (⊖—⊖); control without hormone (△—△).

PGE_1 has a greater effect than isoprenaline on cAMP levels in SKL-2 cells while EB-2 cells seem to respond better to isoprenaline (figs 5, 6).

The beta adrenergic receptor antagonist (±) Propranolol (1 μM) produced 92%, 83% and 86% blockade of the isoprenaline-stimulated cAMP levels in AP-9, SKL-2 and EB-2 cells, respectively.

Tetracaine action

In the studies using tetracaine as an agent to alter membrane mobility rather striking differences between normal and malignant cells were observed. Figs 1 and 2 (for AP-9 cells) show that low concentrations of tetracaine enhance hormonally-stimulated cAMP levels, while higher concentrations virtually abolish the effect of isoprenaline and PGE_1. Basal levels of cAMP are not affected in these cells.

The responses to tetracaine shown in isoprenaline-stimulated cells (fig. 3) and PGE_1-stimulated cells (fig. 4) indicate that the local anaesthetic produces an increase in both basal and hormonally stimulated cAMP levels in the malignant SKL-2 cells. However, the degree of stimulation does not justify a restoration of hormonal response.

In contrast, the effect of tetracaine on cAMP levels in EB-2 cells appears to combine characteristics of both the other cell lines. This produces three phases in the hormonal response, as the initial peak of cAMP produced by low concentrations of tetracaine is followed by a third phase of increasing cAMP levels with higher tetracaine concentrations. This response is seen both with isoprenaline (fig. 5) and PGE_1 (fig. 6) stimulation of cAMP in repeated experiments.

DISCUSSION

It has been shown that the variations in hormonal stimulation of the normal AP-9 cells and two malignant cell lines (SKL-2

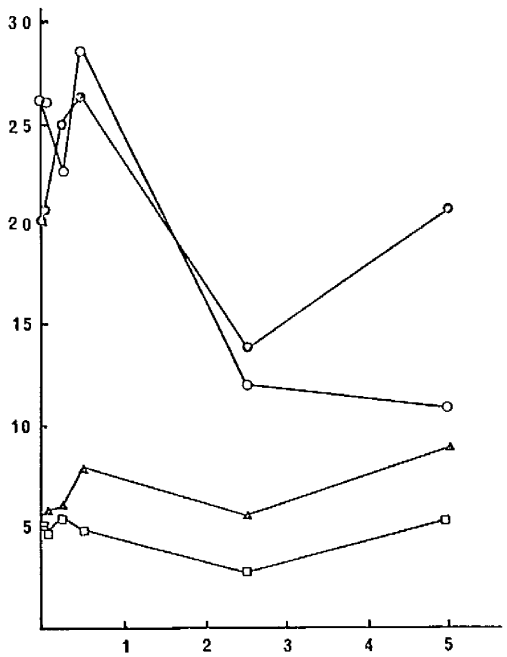


Fig. 5. Effect of tetracaine on isoprenaline stimulated cAMP levels in EB-2 cells. Isoprenaline concentration used was 10^{-4} M (⊖—⊖); 10^{-6} M (○—○); 10^{-8} M (□—□); control without hormone (△—△).

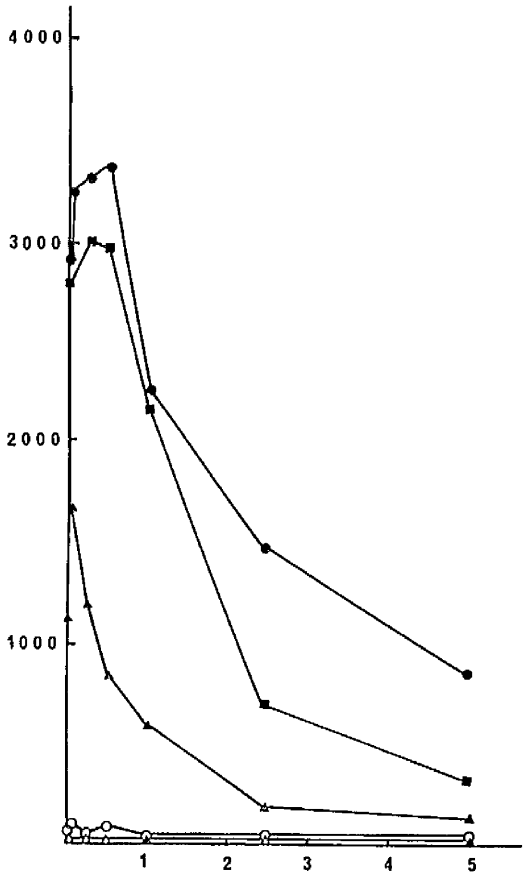


Fig. 2. Effect of tetracaine on prostaglandin E_1 stimulated cAMP levels in AP-9 cells. Prostaglandin concentrations used were 10.0 μg/ml (●—●); 1.0 μg/ml (■—■); 0.1 μg/ml (▲—▲); 0.01 μg/ml (○—○); control without hormone (△—△).

Counter (Model B. Coulter Electronics, St Albans, Herts.). The counter was equipped with 100 μm aperture. Settings of 1/amplification=1 and 1/aperture current=1/8 were used and a 0.5 ml manometer. The width of window was 9–100. All experiments were carried out on confluent fibroblasts or suspension cells at limiting cell density (2×10^6 cells/ml).

Hormonal incubation in intact cells

AP-9 fibroblasts were grown to confluency on the bottom of glass scintillation vials [24] and prior to assay washed twice with Hanks' balanced salt solution. The cells were then equilibrated at 37°C for 30 min in the salt solution. The malignant cells were grown to high cell density, centrifuged at 800 g for 5 min to remove medium, washed in Hanks' balanced salt solution and resuspended at a known concentration for preincubation in vials at 37°C for 30 min. Solutions of hormones were then added together with 3-isobutyl-1-methyl xanthine (50 μg/ml final conc.) as cAMP-phosphodiesterase inhibitor to give a final volume of 1 ml. After incubation at 37°C for 10 min the reaction was stopped by boiling the tubes for 5 min and the cell suspension sonicated for 10 sec (Kerry's Ultrasonics Ltd. Power setting 1. 0.3 mA). Thus both intracellular and extracellular cAMP was measured.

Protein concentrations were determined by the method of Lowry et al. [25] using bovine serum albumin as standard.

cAMP assay

cAMP was measured by the protein binding assay of Brown et al. [26]. The buffer was modified according to Tovey [27]. Standard curves were obtained for every assay.

RESULTS

Hormonal stimulation of cAMP

Figs 1 and 2 show that isoprenaline and prostaglandin E_1 (PGE_1) produce high levels of cAMP in AP-9 cells. In comparison these hormones stimulate cAMP to a lesser degree in the two malignant cell lines. This is in agreement with the general observation that cAMP levels are reduced in malignant cells. It can be seen from figs 3 and 4 that

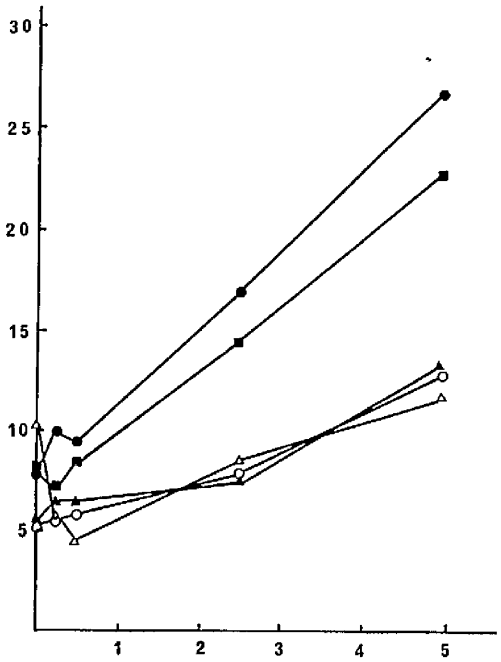
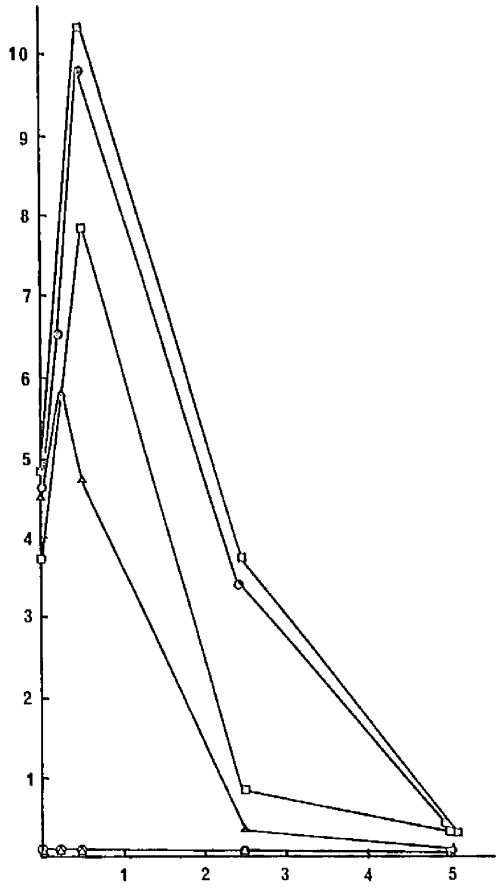


Fig. 3. Effect of tetracaine on isoprenaline stimulated cAMP levels in SKL-2 cells. Isoprenaline concentrations used were 10^{-3} M (△—△); 10^{-4} M (●—●); 10^{-6} M (■—■); 10^{-8} M (▲—▲); control without hormone (○—○).



Figs 1–6. Abscissa: tetracaine (mM); *ordinate:* spec. act. of cAMP ($\times 10^{-3}$) in pmoles/10 min/mg protein. Points shown are the means of duplicate hormone incubations and duplicate cAMP assays.
Fig. 1. Effect of tetracaine on isoprenaline stimulated cAMP levels in AP-9 cells. Isoprenaline concentrations used were 10^{-3} M (⊟—⊟); 10^{-4} M (●—●); 10^{-5} M (□—□); 10^{-6} M (▲—▲); 10^{-8} M (○—○); control without hormone (△—△).

cells [14, 15] and this may provide a partial explanation of the lower cAMP levels in neoplastic cells.

Studies by Papahadjopoulos et al. [16] using fluorescence polarisation techniques show that local anaesthetics such as tetracaine enhance membrane fluidity. Other reports indicate that tetracaine penetrates the phospholipids of the membrane [17] and exists in the membrane in the cationic form [18]. This substantiates proposals that the local anaesthetics interact with the polar groups of phospholipids [19] and that they displace calcium from these regions [16].

Recently it has been proposed that local anaesthetics such as tetracaine affect certain cellular functions by an alteration in the organisation of microtubules and microfilaments [20, 21]. The organisation of these cytoskeletal elements in relation to cell morphology is thought to involve cAMP [22].

In this paper we report the results of studies into the effect of local anaesthetic tetracaine on the hormonal regulation of adenylate cyclase. The results are discussed with regard to the various mechanisms proposed for the action of tetracaine.

MATERIALS AND METHODS

Isoprenaline HCl was purchased from Sigma, London. Prostaglandin E_1 was a generous gift from ICI pharmaceuticals, Aldeley Park, Cheshire. [^{3}H]cAMP (30 Ci/mmole) was obtained from the Radiochemical Centre, Amersham, Bucks. 3-Isobutyl-1-methyl xanthine was obtained from Aldrich Chemical Company Inc., Milwaukee, Wisc. cAMP and tetracaine were obtained from Sigma Chemicals.

Cells

Human embryonic lung fibroblasts (AP-9) were isolated at ICI Pharmaceuticals. Human lymphoblastic leukaemia cells (SKL-2) and Burkitt lymphoma cells (EB-2) were obtained from Flow Laboratories. The fibroblasts are a primary cell line and grow adhering to glass, exhibiting density-dependent inhibition of growth with a generation time of 36 h. The two malignant cell types are continuous lines and exist in suspension aggregating to various degrees at cell density, and having a generation time of 16–20 h.

Culture methods

Cells were grown in Eagle's minimal essential medium supplemented with 10% foetal bovine serum, 4 mM glutamine and 10% non-essential amino acids and maintained in an atmosphere of 5% CO_2 in air. One hundred units ml^{-1} benzyl penicillin (Na)BP and 100 $\mu g/ml^{-1}$ streptomycin sulphate (BP) were used to prevent contamination. All cells were free of PPLO contamination as shown by [^{3}H]TdR labelling and autoradiography [23].

Cells were routinely cultured in 100 ml glass bottles and passaged every 4–5 days. The fibroblasts were trypsinized using a solution of 0.025% trypsin (Worthington Biochemical Corporation, Freehold, N.J.) in Hanks' balanced salt solution.

Cell densities were determined using a Coulter

Printed in Sweden

ISSN 0014-4827

Experimental Cell Research 107 (1977) 201–206

THE EFFECT OF THE LOCAL ANAESTHETIC, TETRACAINE, ON THE ISOPRENALINE AND PROSTAGLANDIN E_1 STIMULATION OF cAMP IN NORMAL AND MALIGNANT CELL LINES

S. R. AYAD and A. WHITE

Department of Biochemistry, University of Manchester, Manchester, M13 9PL, UK

SUMMARY

A human embryonic lung cell line (AP-9) and two malignant cell lines SKL-2 (lymphoblastic leukaemia cells) and EB-2 (Burkitt lymphoma) were used to measure hormonally stimulated cyclic adenosine 3′5′-monophosphate (cAMP) levels. Isoprenaline and prostaglandin E_1 (PGE_1) produced much higher levels of cAMP in AP-9 than in either of the malignant cell lines. The local anaesthetic tetracaine was used as an agent to alter membrane mobility, and the response of hormonally stimulated cAMP varied in the three cell lines. However, the action of tetracaine on isoprenaline and prostaglandin E_1 (PGE_1) stimulation was similar in any given cell line. In AP-9 cells 0.5 mM tetracaine produced a peak in hormonally stimulated cAMP levels although higher concentrations caused inhibition. In contrast, tetracaine produced an enhancement of hormonal stimulation in SKL-2 cells and a combined response in EB-2 cells. These results are discussed with regard to recent evidence proposing that tetracaine affects cytoskeletal organisation.

Cyclic adenosine 3′5′-monophosphate (cAMP) plays an important role in regulating many properties of normal cells. Since some of these properties are altered in neoplastic and transformed cells, it is interesting to note that lower cAMP levels are also found in the cells [1, 2, 3]. This is exemplified by reports that cAMP levels are inversely related to growth rate [4], and that malignant cells are characterised by a rapid rate of growth and low intracellular cAMP levels, as reviewed by Pastan [5].

In examining the abnormally low cAMP levels in malignant cells, variations in both synthesis and degradation of the cyclic nucleotide must be considered. A great many of the characteristics of the malignant state are related to changes in the plasma membrane [6] and it seems feasible that an alteration in the membrane-bound complex involved in cAMP synthesis is responsible for the reduced level of cAMP. This alteration could involve changes in the hormone receptors or in the adenylate cyclase (ATP pyrophosphate lyase cyclizing EC4611) which catalyses production of cAMP from ATP, or both [7, 8, 9].

The fluid mosaic model of membrane structure [10] and the proposal that hormone receptors may be free to 'float' in the membrane [11, 12, 13] suggest that the translational mobility of components in the membrane may influence cAMP production in normal and malignant cells. Several reports have shown that the mobility of these components is altered in some malignant