A THESIS

ENTITLED

PHOSPHOLIPIDS AND THE BETA-ADRENERGIC RESPONSE

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by

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PAGE NUMBERS ARE CUT OFF IN THE ORIGINAL

DEDICATION

To Belinda,

who put up with so little,

for so long.

ABBREVIATIONS USED IN THIS THESIS

ATP	Adenosine 5'-triphosphate
cAMP	Adenosine 3':5'-cyclic monophosphate
CPT-cAMP	Chlorophenylthio-adenosine 3':5'-cyclic monophosphate
CDP-choline	Cytidine 5'-diphosphocholine
Dibutyryl cAMP	$N^6,O^{2'}$-Dibutyryl adenosine 3':5'-cyclic monophosphate
DHA	Dihydroalprenolol
DME	N,N'-Dimethylethanolamine
DPH	1,6-Diphenyl-1,3,5-hexatriene
ESR	Electron spin resonance
HE	Haematoxylin-eosin
LPC	Lysophosphatidylcholine
NAD^+	Nicotinamide adenine dinucleotide
NME	N -Monomethylethanolamine
PA	Phosphatidic acid
PBS	Phosphate buffered saline
PC	Phosphatidylcholine
PDME	Phosphatidyl-N,N'-dimethylethanolamine
PE	Phosphatidylethanolamine
PI	Phosphatidylinositol
PMT	Phospholipid methyltransferase
PNME	Phosphatidyl-N,-monomethylethanolamine
PS	Phosphatidylserine
SAM	S-Adenosyl-L-methionine
SDS	Sodium dodecyl sulphate
SPM	Sphingomyelin
DMPC	Dimyristoylphosphatidylcholine
GTP	Guanosine 5'-triphosphate
ADP	Adenosine 5'-diphosphate
Gpp(NH)p	Guanyl-5'-yl imidophosphate
PG	Phosphatidylglycerol
CoA	Coenzyme -A

SUMMARY

The phospholipid polar headgroup composition of membranes of C6 cells was modified in vivo by growth for 24h in media supplemented with the polar headgroup precursors, N, N'-dimethylethanolamine, N-monomethylethanolamine or ethanolamine. These modifications were achieved without alteration of the cholesterol and phospholipid content of the membranes, and without changes in the fatty acyl or protein composition of these membranes. No changes were found in the physical properties of membranes isolated from these cells.

Enriching the cell membranes with PDME, PNME or PE elevated the basal intracellular cAMP content, but decreased the degree of stimulation of intracellular cAMP content in response to β-adrenergic stimulation. This reduction of the β-adrenergic response appeared to be due to enhanced phosphodiesterase activity.

β-adrenergic stimulation did not affect bulk physical properties of the membrane. Exposure to isoproterenol increased the amount of [^{3}H] methyl label recovered from TLC plates but the different distribution of label indicated that such analysis is insufficient to demonstrate specific phospholipid methylation.

This system is a useful means of studying the relationship between phospholipid composition, phospholipid metabolism and the β-adrenergic response in the C6 cell line.

CONTENTS

Publications arising from this work.

INTRODUCTION

"It is easier to collect anecdotes,
the tall tales
That travellers, some centuries ago,
brought home
Of wisecracks and the drolleries of fools,
Than to travel in the mind to that place
Where the map becomes reality, where
cracks are gullies"

Ruthven Todd

1.1 THE C6 GLIOMA CELL LINE

The C6 glioma (AT CCL107) is a stable cell line derived from a nitroso-methylurea-induced tumour isolated from rat brain (for a review, see Pfeiffer et al., 1978). This cell is believed to be of astroblast character, but the exact glial affinities of the cell line have been questioned (Benda et al., 1971). C6 cells express the glial markers, S-100 protein and glial fibrillary acidic protein, which suggests that they are of astrocytic character. However, they also express the oligodendroglial marker enzyme 2':3'-cyclic nucleotide phosphohydrolase, as well as the astrocyctic marker glutamine synthetase. Parker et al. (1980) have described markedly high cyclic nucleotide phosphohydrolase activity in early passage (21 to 26) cells whereas glutamine synthetase activity was low in these early passage cells. This relationship was reversed in high passage (82 to 88) cells. On the basis of these differences in marker enzyme activity and in morphological appearance, they suggested that in early passages, C6 show oligodendrocytic character which transdifferentiates to astrocytic character in late passages.

C6 cells possess adrenergic receptors which are coupled to adenylate cyclase, and which respond to stimulation with β-adrenergic agonists by increases in their cyclic-3':5'-adenosine monophosphate (cAMP) content of "heroic magnitude" (Gilman and Nirenberg, 1971). Much is known about the β-adrenergic-adenylate cyclase system of C6 cells (reviewed in Pfeiffer et al., 1978). Mechanisms of cAMP accumulation and desensitisation of the response to prolonged stimulation have been recorded; these

mechanisms will be considered in greater detail later.

The C6 cell possesses other receptors, some of which are coupled to adenylate cyclase. Benzodiazepine receptors are present (Syapin and Skolnick, 1979). Dopamine receptors and prostaglandin receptors have been identified (reviewed in Van Calker & Hamprecht, 1980) which, like benzodiazepine receptors, are coupled to adenylate cyclase. Histamine and adenosine receptors are also present but they are not coupled to adenylate cyclase.

The lipid composition of C6 cells has also been extensively studied (Robert et al., 1976 ; Eichberg, 1976). In general terms, much is known about the C6 cell system and several reviews are available (Benda, 1978; Pfeiffer et al., 1978; Van Calker and Hamprecht, 1980).

1.2 PHOSPHOLIPID BIOSYNTHESIS

1.2.1 Introduction

Thudichum emphasised the importance of phospholipids in his book "A Treatise on the Chemical Constitution of the Brain" (1884). He made extensive studies of the lipid composition of many tissues, which demonstrated that phospholipids comprised approximately 47% of the total lipid content of the human brain.

The difficulties in preparing pure individual lipid classes precluded detailed study of their physiological role and prevented studies to challenge the prevalent notion that phospholipids had only a structural role, and were metabolically inert. However, Hevesey (1947) observed that ^{32}P, as inorganic orthophosphate could be rapidly incorporated into tissue phosphatides in vivo and in vitro. McMurray et al. (1957)

demonstrated that ^{32}P, as labelled ATP, was incorporated into rat brain phosphatides faster than ^{32}P from labelled inorganic orthophosphate. This suggested that phosphate incorporation proceeded by an energy-dependent process. These studies illustrated that dynamic turnover of phospholipid occurs, and stimulated further studies on phospholipid metabolism.

It is now clear that membrane phospholipids are continually turned over; both the polar head group and the fatty acyl chains are subject to rapid modification. These modifications are the means by which the physical and chemical properties of the membrane are changed in response to environmental or physiological changes. Thus, phospholipid metabolism is also a homeostatic mechanism. In many tissues, phospholipids play an important role in the transmission of biological signals (Crews et al., 1982; Berridge, 1983) and regulation of cell-surface phenomena.

The biosynthetic pathways of phosphatidylcholine (PC) formation are of most importance to the studies in this thesis. In the following pages, the principal pathways are discussed.

1.2.2 The CDP-choline pathway

The most important means of PC biosynthesis in mammals is by the CDP-choline pathway (Fig.1). Experiments by Kornberg and Pricer (1952) showed that phosphorylcholine is incorporated as a unit into the PC of rat liver preparations. The phosphorylcholine is derived from the transfer of a phosphoryl group from ATP catalysed by the enzyme choline kinase (ATP:choline phosphotransferase, EC 2:7.1.37).

Kennedy and Weiss (1956) made the important discovery

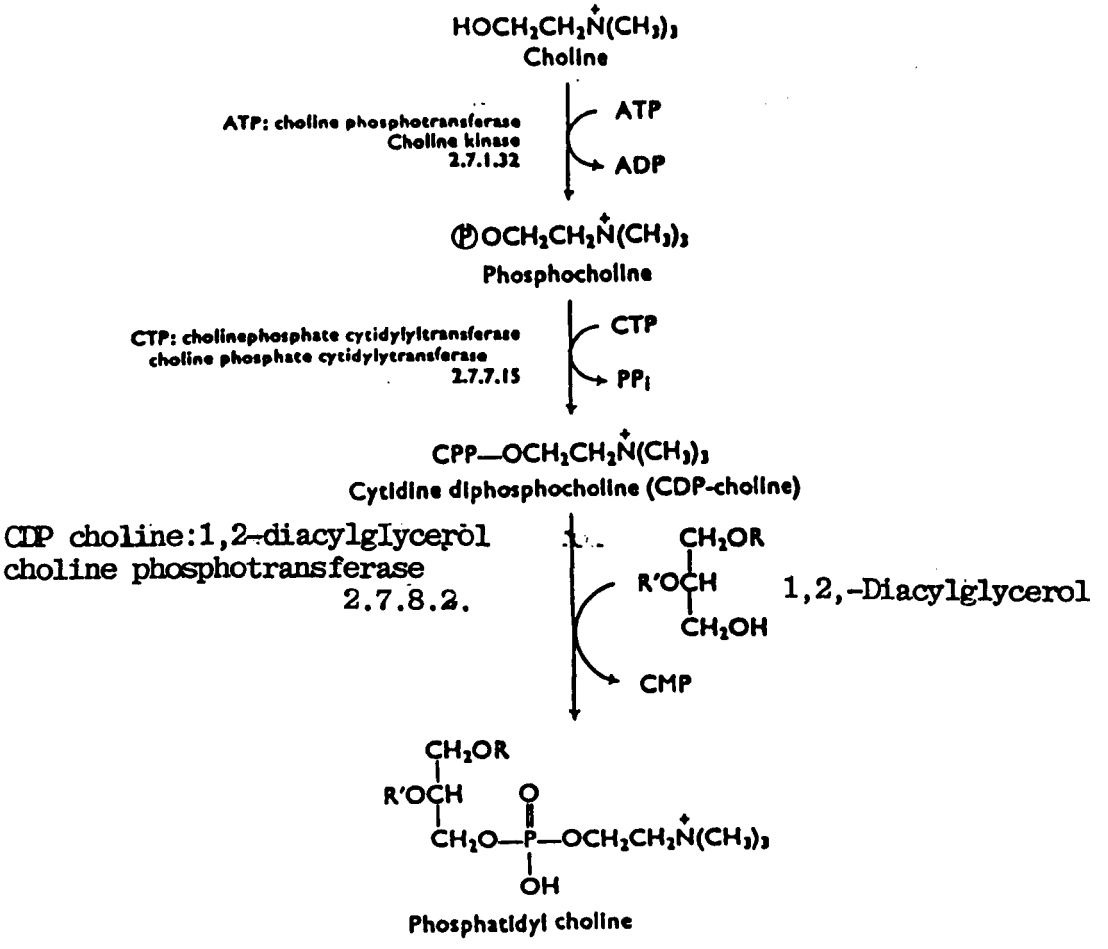


Fig.1: BIOSYNTHESIS OF PC BY THE CDP-CHOLINE PATHWAY

that cytidine triphosphate (CTP), but none of the other nucleoside 5'-triphosphates, is essential for the incorporation of phosphorylcholine into PC in chicken liver preparations. The transfer of a cytidyl group from CTP to phosphorylcholine is catalysed by cytidyltransferase (CTP:cholinephosphate cytidylyl transferase EC2.7.7.15) with the release of pyrophosphate (Borkenhagen and Kennedy, 1957).

The transfer of the phosphorylcholine from CDP-choline to 1,2-diacylglycerol is carried out by choline phosphotransferase (CDP-choline:1,2-diglyceride cholinephosphotransferase EC2.7.8.2), first described by Kennedy and Weiss (1956) in chicken liver. Cytosine monophosphate is released, and CTP regenerated from it using ATP as a phosphate donor. It is now known that phosphotidylethanolamine (PE) is formed by a similar pathway.

The ethanolamine and choline kinase activities appear to be due to different enzymes in the cytosol from rat liver preparations (Brophy et al., 1977). Cytidylyltransferase is found in both the microsomal and cytosolic fractions in rat liver preparations (Choy et al., 1977) and it shows high substrate specificity (Carter and Kennedy, 1966).

Choline and ethanolamine phosphotransferases are exclusively microsomal in liver (Ostrow and Getz, 1973). Freysz et al. (1982) have presented evidence that suggests that in brain microsomes, the choline phosphotransferase is embedded in the outer leaflet of the bilayer, and that the ethanolamine phosphotransferase is on the inner leaflet. This may be a contributory factor to the maintenance of an asymmetric distribution of PE and PC. Studies in vivo (Akesson et al., 1970)

suggest that choline phosphotransferase shows a preference for dienoic and tetraenoic diglyceride species, whereas ethanolamine phosphotransferase is more active with tetraenoic and hexaenoic species, although differences in substrate solubilities make these type of experiments difficult to interpret. However, Kahno and Ohno (1975) avoided these problems, and were able to show that ethanolamine phosphotransferase preferentially utilised hexaenoic species, whereas the choline phosphotransferase showed no substrate preference between diglycerides. This may account for the relatively high level (more than 20%) of hexaenoic fatty acids normally found in rat liver PE (White, 1973).

The substrate specificities of the enzymes of phospholipid biosynthesis may be one of the means of tailoring phospholipid molecules for their roles in the maintenance of membrane fluidity. However, the constant amounts of phospholipids in a given membrane, despite turnover, suggests that both catabolic and anabolic processes regulate membrane lipid composition.

1.2.3 Regulation of PC Biosynthesis

Although the CDP-choline pathway had been elucidated by the mid-1950's, it was the mid-1970's before it was clear how the pathway was regulated (for review, see Vance and Choy, 1979).

The regulatory step is now known to be the conversion of phosphocholine to CDP-choline, which is catalysed by cytidylyltransferase. One of the first definitive studies of this reaction was performed by Paddon et al. (1979). HeLa cells were pulse-chased with [^{3}H]choline. The [^{3}H]choline was very rapidly converted to phosphocholine. When the disappearance of label from phosphocholine was monitored, it was found that it

was transiently associated with CDP-choline which was immediately converted to PC.

Cytidylyltransferase from rat liver is associated with both the microsomal and soluble fractions. In the cytosol, the enzyme exists in two different forms. The major form (L-form) has a molecular weight of 2×10^5 and requires a lipid fraction for full activity. The presence of lysophosphatidylethanolamine (LPE), phosphatidylserine (PS), or phosphatidylglycerol (PG) stimulates the enzyme activity seven-fold. Diacylglycerol appears to cause aggregation of the L-form to high molecular weight aggregates, which show increased enzymic activity. LPC appears to inhibit the L-form. CTP has been proposed as an additional regulator of the enzyme (Vance and Choy, 1979).

Sundler and Akesson (1975) showed that the rate of PG synthesis is also influenced by the availability of diacylglycerol, and by its fatty acid composition. Both PE and PC are synthesised in mammals principally by the CDP-base pathway, however there are three other pathways of minor importance to consider for PC production.

1.2.4 Base Exchange

A number of major phospholipid types, including phosphatidylserine (PS), phosphatidylinositol (PI), PC and PE can have their polar headgroup (base) exchanged with a different base:-

$$\text{PE} + \text{choline} \xrightarrow{Ca^{2+}} \text{PC} + \text{ethanolamine}$$

The base exchange enzyme is stimulated by Ca^{2+}, and is usually found in greatest concentration in the microsomes (Porcellati

et al., 1971). The reaction is not dependent on high-energy compounds, and operates best in the brain at alkaline pH s. It is similar to the reverse reaction of phospholipase D.

Although the specificity of the enzyme(s) involved is questionable, Miura and Kanfer (1976) claim to have successfully separated base exchange enzymes specific for ethanolamine, serine and choline. PE appears to be the best donor in brain preparations, and ethanolamine and serine are the free bases most frequently utilized in several systems (Saito and Kanfer, 1973), so the pathway is of negligible importance for PC synthesis. In fact, Plagemann (1971) has shown that base exchange is of little importance for PC biosynthesis in Hepatoma cells in culture.

Base exchange is of interest, since it seems that PE derived from PS by base exchange can be N-methylated to PC. Mozzi et al. (1982) have described such a system in microsomes derived from rat brain cortex.

1.2.5 Acylation of Lysophosphatidylcholine

Merkle and Lands (1963) showed that liver preparations contain selective acyl CoA:phospholipid acyltransferases that are able to bring about acylation of LPC:

$$\text{LPC} + \text{Acyl CoA} \rightarrow \text{PC} + \text{CoA}.$$

Similar enzymes are present in other tissues. Lands and Hart (1965) showed that enzymes acting on 1-acylglycerol-3-phosphorylcholine (1-acyl-GPC) could be distinguished from 2-acyl-GPC. The pattern of acyltransferase activity varies considerably, both with different fatty acids and with different acyl acceptors. Presumably the well known preferential esterification of

unsaturated fatty acids at position 2 of 1-acyl-GPC, and of saturated fatty acids at position 1 of 2-acyl-GPC, is the result of the selective specificity of these acyltransferases. LPC is only present in minor amounts in biological membranes, due to its disruptive properties, and so acylation of LPC is unlikely to contribute significantly to the bulk PC content. The enzyme responsible seems to be microsomal in location (Fisher et al., 1982).

1.2.6 The N-Methylation Pathway of Phosphatidylcholine Synthesis

PC can also be formed by a successive methylation of the primary amino group of PE, via mono and dimethylated derivatives. These reactions are catalysed by two methyl-transferases which use S-adenosyl-methione as a methyl donor (SAM). The pathway was first demonstrated in rat liver cells by Bremer and Greenberg (1961) and has been recorded in many different systems (Mato and Alemany, 1983). Mozzi and Porcellati (1979) and Blusztajn and Wurtman (1981) have demonstrated the presence of the pathway in rat brain.

In 1976 Katyal and Lombardi (1976) showed that the methylated intermediates, PDME and PNME, were present in lipid extracts from rat liver. Since then a vast amount of work has been done by Axelrod's group (see Hirata and Axelrod, 1980, for review). It has been suggested that two methyltransferases exist (Hirata and Axelrod, 1980; Mozzi and Porcellati, 1979; Crews et al., 1980).

In adrenal medulla homogenates, the addition of Mg^{2+} stimulated the incorporation of radioactive methyl groups from SAM. Thin-layer chromatography of lipid extracts showed that

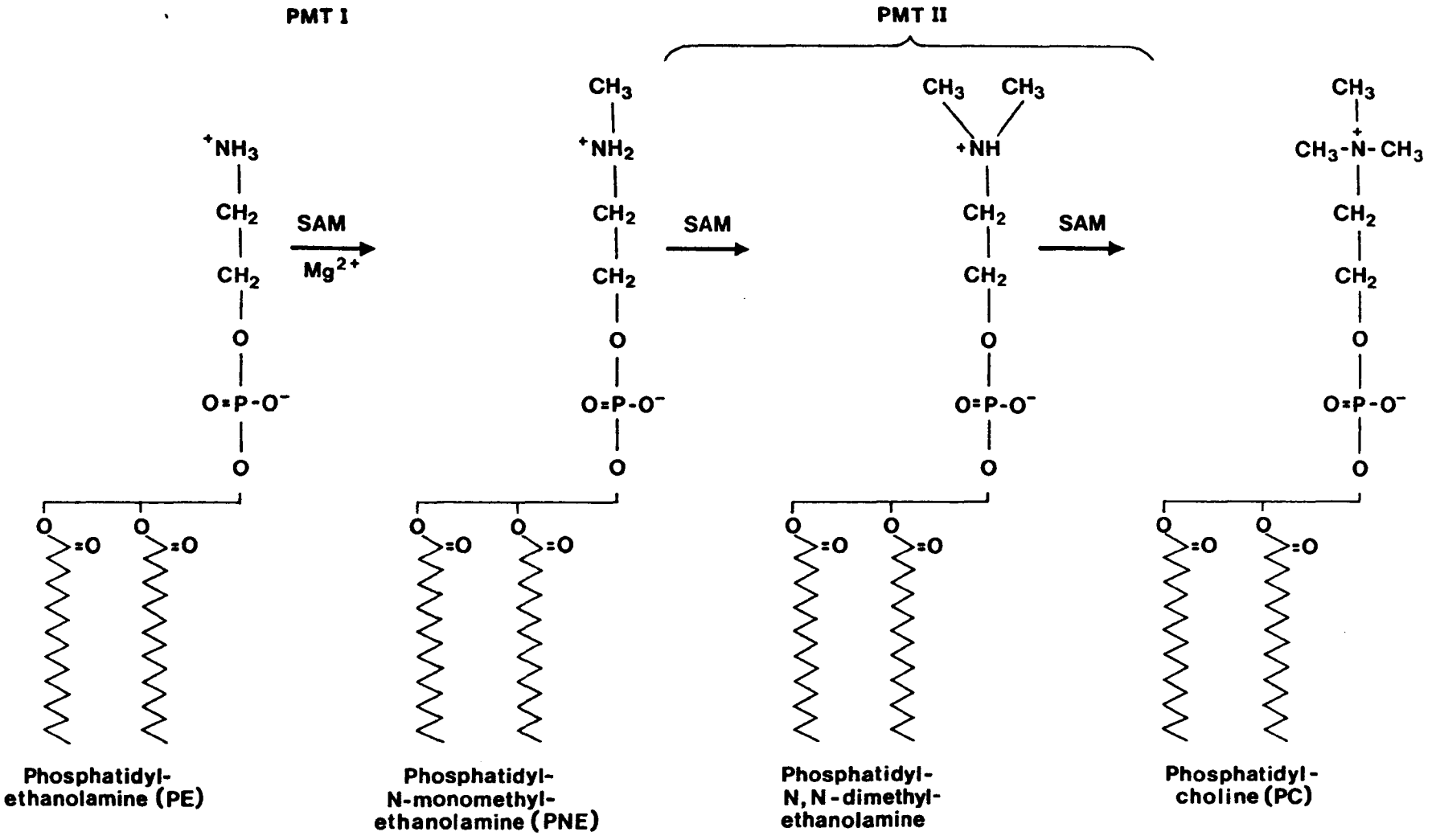


Fig.2: **CONVERSION OF PE TO PC BY N-METHYLATION**

the methyl groups were incorporated into PNME, PDME and PC. It was suggested that two methyltransferases with different properties were responsible for the incorporation of label into these intermediates. Methyltransferase I converts PE to PNME, requires Mg^{2+}, has a low Km for SAM (about 2 μM) and an optimal pH of 7.0. Methyltransferase II catalyses the stepwise methylation of PNME to PDME to PC. This enzyme has a high Km for SAM (100 μM), a pH optimum of about 10.0, and no Mg^{2+} requirement. These enzymes are located in the microsomes and mitochondria of the adrenal medulla. Similar activities have been found in a variety of systems (Hirata and Axelrod, 1980).

Experiments with phospholipase C, trypsin and erythrocyte ghosts indicated that PE and phospholipid methyltransferase I (PMT I) are distributed on the inner leaflet of the membrane, whilst PMT II and PC are localised in the outer leaflet. It was suggested that methylation causes a rapid translocation of phospholipid across the membrane. This enzymatically-stimulated flip-flop occurred rapidly (within 2 min.), and was suggested to be responsible for such a diverse range of events as the maintenance of membrane lipid asymmetry, calcium ion fluxes, membrane fluidity changes, and cell surface receptor function (Hirata and Axelrod, 1980). These events will be considered in detail later.

Audubert and Vance (1983) have argued that erroneous conclusions have been drawn from these studies on Km's, and that a single methyltransferase enzyme with a pH optimum of about 10.0 is responsible for all three methylations.

1.2.7 The Relationship between the N-Methylation and CDP-Choline Pathways of PC Biosynthesis

Phospholipid methylation is another means by which PC can be produced, and has been suggested as an alternative source of PC production when choline deficiency reduces the output of the CDP-choline pathway in the liver (Mato and Alemany, 1983).

In the liver, where the pathway was first discovered by Bremer and Greenberg (1961), it is estimated that as much as 20% of liver PC is synthesised by this pathway (Sundlerand Akesson , 1975). The liver shows the highest methylation pathway activity of all the body tissues, and much useful work has been done with liver membrane preparations and hepatocytes in order to understand the function of the pathway, and its relationship to the CDP-choline pathway (reviewed by Mato and Alemany, 1983).

It seems that the methylation and CDP-choline pathways are regulated by similar agents, but in an antagonistic fashion, Pelech and Vance (1982) showed that a cAMP-dependent protein kinase is involved in the regulation of cytidylyltransferase.

Incubation of hepatocytes with cAMP analogues for 5h reduced the activity of cytidylyltransferase, however by 15h, the activity of the cytidylyltransferase was stimulated (Pelech and Vance, 1982). Similarly, (Pelech et al., 1981) CPT-cAMP, a cAMP analog, decreased PC synthesis from [^{3}H]choline by 40% and these effects were potentiated by phosphodiesterase inhibitors.

The transmethylation pathway is stimulated by some cAMP analogues. Mato et al. (1982) found that cAMP stimulated methylation when added in the presence of micromolar ATP, which suggests the involvement of a cAMP-dependent protein kinase.

Hormones and Ca^{2+} ionophores stimulate methylation but inhibit the CDP-choline pathway (Alemany et al., 1982). Methylation inhibitors such as deaza adenosine stimulate the CDP-pathway (Pritchard et al., 1982), and choline deficiency stimulates methylation, concomitant with a decrease in CDP-pathway activity (Schneider and Vance, 1978). Maziere et al. (1982) have also demonstrated a coordinated regulation of the two pathways. In virus-transformed hamster fibroblasts, they were able to show that CDP-pathway activity is depressed, while there is an increase in methylation pathway activity.

So it seems that the two pathways are regulated in a coordinated manner, and that the methylation pathway provides an auxiliary system for PC production in times of dietary choline deficiency. PC turns over within 10 hours in the liver, and it may be necessary to have an auxiliary synthetic system to maintain the PC content of the cells.

1.2.8 Other Functions of N-Methylation of PE

Because of the importance of choline for acetylcholine production, interest has been focussed on the pathways of free choline production in the brain. The methylation pathway has been studied, as a possible additional means of choline production. Experiments by Ansell and Spanner (1982) showed that the methylation pathway in brain tissue in vitro and in vivo, is of negligible importance as a source of choline. In experiments in which [1,2-^{14}C]DME was injected intracerebrally into rats this was shown to be phosphorylated and incorporated into PDME, which was formed at a rate of about 5 nmol/g brain/h. However, only a small amount of this was methylated to PC in 7h. The

amount of PC formed by methylation in synaptosomes is 2.6-10 pmol/mg protein/h, compared with around 2700 pmol/mg protein/h in the liver, so quantitatively, the pathway is unlikely to be of much significance for choline production.

The enzymes responsible for phospholipid methylation do not appear to display rigid substrate specificity. Mogelson and Sobel (1981) have demonstrated that ethanolamine plasmalogens in rabbit myocardial membranes can be N-methylated, although at a slower rate than that of phosphoglycerides. Phospholipid methylation is stimulated by a variety of membrane and cell surface events. Some of the cases documented are described in Table 1, together with their presumptive function. Phospholipid methylation associated with the β-adrenergic response is dealt with in section 1.7.0.

1.2.9 Non-polar Lipid Methylation

It is clear that a large percentage of the methyl groups of L-[methyl-^{3}H]methionine are incorporated into the neutral lipid fraction (Zatz et al., 1982).

Zatz examined the fate of label incorporated into human red blood cells. It was found that over 50% of the radioactivity was incorporated into the non-polar lipid fraction, and in particular, into fatty acyl methyl esters. Zatz et al. (1981) found 90% of the non-polar-located label in ubiquinone in rat basophilic leukemia cells, retinal cells, and parotid cells. Ubiquinone appears to be methylated by catechol-o-methyltransferase, whilst fatty acid methyl esters are formed by fatty acid carboxymethyltransferase. It is clear that lipid methylation is not limited to phospholipids, but rather, that events which increase the rates of

TABLE 1 : EFFECTS OF DIFFERENT ADDITIVES ON PHOSPHOLIPID N-METHYLATION

System	Stimulus	Effect on Rate of Methylation	Presumptive Function	Reference
Pancreatic Islets	Glucose	Stimulation	Insulin release	Mato & Alemany (1983)
Glioma Cells	Isoproterenol	Stimulation	cAMP production	Munzel & Koschel (1982)
Red Blood Cells	Immunoglobulin E	Stimulation		Crews et al. (1980 b)
Mast Cells	Immunoglobulin E	Stimulation	Histamine release	Hirata & Axelrod (1980)
Dictyostelium discoideum	cAMP	Stimulation	Chemotaxis	Alemany et al. (1980)
Neurites	Nerve growth factor	Stimulation		Pfenninger & Johnson (1982)
Pituitary	Corticotropin release factor	Stimulation		Hook et al. (1982)
Chicken embryos	Differentiation	Stimulation		Zelenka et al. (1982)
Rat brain explants	Carbamylcholine	Stimulation	Increase in GABA receptor affinity	Giesinget & Gerken (1982)
Rat cerebellum	S-Adenosyl methionine	Stimulation	Increase in affinity of β-adrenergic, benzodiazepine & GABA receptors	Perri et al. (1983)
Rat brain synaptosomes	Ageing	Stimulation	Maintenance of membrane fluidity	Crews et al. (1981)
Rat brain neurons	Dopamine	Stimulation	cAMP production?	Leprohon et al. (1983)
Lymphocytes	Mitogens	Stimulation	Mitogenesis	Hirata & Axelrod (1980)
Glioma cells	Benzodiazepine	Stimulation	cAMP production	Strittmatter et al. (1979)
Dictostyleum discordeum	Calmodulin	Stimulation	Chemotaxis?	Gil et al. (1980)
Erythrocyte	S-Adenosyl methionine	Stimulation	Increase in Ca^{2+}/Mg^{2+} ATPase activity	Strittmatter et al. (1979b)

Table 1 (continued)

TABLE 1 (continued)

System	Stimulus	Effect on Rate of Methylation	Presumptive Function	Reference
Neutrophils	Attractant	Inhibition	Chemotaxis	Hirata et al. (1979)
Neutrophils	Phagocytosis	Inhibition		Gil et al. (1981)
Macrophages	Attractant	Inhibition	Chemotaxis	Pike et al. (1979)
Platelets	Aggregation	Inhibition		Shattil et al. (1981)
Neuroblastoma Cells	Deaza-adenosine	Inhibition	Neurite extension	Murato & Monard (1982)
Liver microsomes	Glucagon	Stimulation	No effect on calcium flux	Kraus-Friedmann & Zimniak (1983)
Lymphocytes	Mitogens	No effect	Mitogenesis	Moore et al. (1982)
Kidney cortex	S-Adenosyl methionine	Stimulation	No effect on fluidity or membrane transport function	Chauhan et al. (1982)

methylation cause a general increase in lipid methyltransferase activity.

1.3 THE β-ADRENERGIC SYSTEM

1.3.1 Components of the System

It is now accepted that the β-adrenergic system consists of three components. These are listed in Tables 2 and 3. The hormone receptor (R) is exposed at the cell surface and binds ligands with specificity and high affinity. The R protein has a hydrophobic portion inserted into the membrane which probably interacts with the G-protein. The G-protein mediates the R protein stimulation of the catalytic (C) protein, and is subject to the influence of mono- and divalent cations. The C protein is exposed on the cytoplasmic side of the membrane. Hormonal stimulation, mediated by R and G proteins, or stimulation of the G protein-C protein complex by NaF results in an increase in the rate of cAMP synthesis. For this to occur in intact cells, the three components, which are mobile in the plane of the bilayer, must couple together (Cuetrecases, 1974). There is a continuous low basal activity of adenylate cyclase in the unstimulated cell, but hormonal stimulation elicits up to thousand-fold increases in cAMP production.

Investigations on the structure and function of the β-adrenergic-adenylate cyclase system have employed a variety of genetic, physical and biochemical approaches, such as cell fusion, solubilisation, target site analysis, kinetic and ligand binding studies (for review see Stadel et al., 1982).

At least three proteins are involved in the β-

adrenergic system. This has been shown by genetic and cell fusion studies. The C protein can be inactivated by N-ethyl maleimide, without effect on hormone binding in avian erythrocytes. (Schramm and Naim, 1970).

More definitive evidence that separate R and C proteins couple in the plane of the bilayer to yield an active adenylate cyclase was provided by Orly and Schramm(1975). In these elegant experiments, Friend cells which lack the R protein, but have G and C proteins, were fused with turkey erythrocytes containing C units inactivated by N-ethylmaleimide.

Hormonal stimulation was reconstituted by cell fusion (reviewed, Ross and Gilman, 1980). This is evidence which supports the "mobile receptor" hypothesis

1.3.2 The β-Adrenergic Receptor

β-Adrenergic receptors are divided into two classes, on account of their ability to bind pharmacological agents that show different degrees of stimulation of cAMP production.

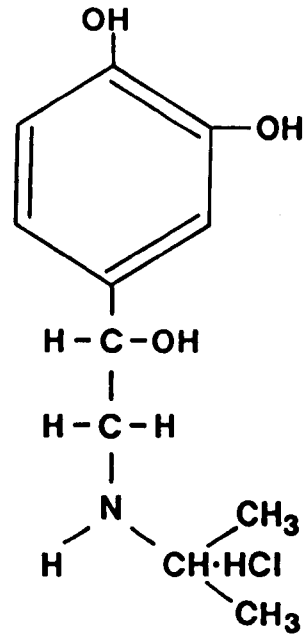
The β_1-receptor - stimulation of adenylate cyclase activity by β-antagonists follows the order of potency:

isoproterenol > noradrenalin > adrenalin

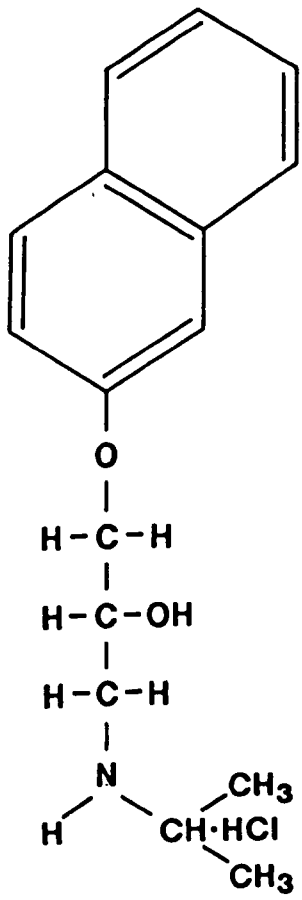
and is blocked by the antagonist practolol. These β_1-receptors are present in cardiac and adipose tissue. The β_2-receptor-stimulation of cAMP accumulation order of potency is:

isoproterenol > adrenalin > noradrenalin

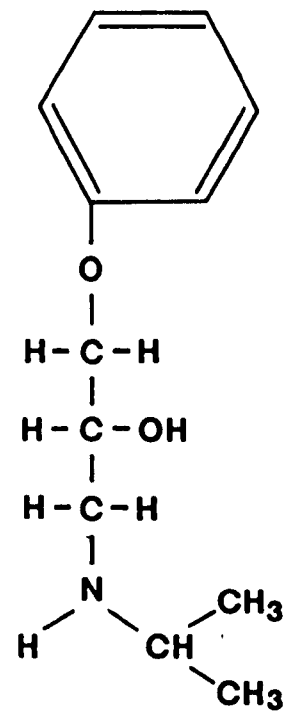
and is blocked by propranolol (Fig. 3). These receptors are located in the bronchi and striated muscle. In both types of receptors, the (-)-isomers of the antagonists are more potent than the (+)-isomers.



ISOPROTERENOL



PROPRANOLOL



DIHYDROALPRENALOL

Fig.3: β-ADRENERGIC AGONIST AND ANTAGONISTS

Recently, the mammalian β_2-adrenergic receptor has been purified 50,000-fold, after solubilisation by digitonin from canine lung tissue. A major band, due to a protein of 52,000 daltons, on SDS polyacrylamide gel electrophoresis gels was shown to be the β-receptor binding site on account of its binding of [^{3}H]-labelled photo-affinity probes for the β-adrenergic receptor (Kirolovsky and Schramm, 1983).

Cherksey et al. (1981) presented evidence which suggests that the β-adrenergic receptor of frog erythrocytes is bound to the cytoskeleton, and that agonist, but not antagonist, binding releases the receptor and allows it to interact with the G and C units.

1.3.3 The Guanine Nucleotide-Binding Regulatory Protein

Much is known about the molecular characteristics of the G-protein, principally since it is relatively easy to purify from membrane preparations. The protein binds guanine nucleotides, a property which was utilised by Pfeuffer (1977) to identify the protein as a 42,000 dalton protein in pigeon erythrocyte membranes labelled with [^{32}P]GTP-γ-azidoanilide. Similarly, various studies using cholera toxin in order to ADP-ribosylate membrane proteins using [^{32}P]NAD, identified the G protein as a 42,000-45,000 dalton protein in a variety of systems (reviewed in Ross and Gilman, 1980).

Cassel and Selinger (1976) discovered that turkey erythrocyte membranes contain a GTPase activity, stimulated by catecholamines. Braun et al. (1982) have shown that the G-protein contains two GTP-binding sites, one of which has a GTPase activity. This may be of importance to the model

proposed by Cassel and Selinger (1977), and Ross and Gilman (1980), who proposed that a "regulatory GTPase cycle" controls hormone-stimulated adenylate cyclase activity.

On the basis of results obtained by target-size analysis, Rodbell (1980) has proposed that the G unit exists in two different forms. In the G stimulatory form, coupling of R with the G unit results in a complex which will activate adenylate cyclase. On the other hand, stimulation of certain receptors, e.g. α-adrenergic receptor, leads to an R-G complex which inhibits adenylate cyclase. A separate population of G inhibitory proteins have been proposed. This forms the basis for a dual regulation of the C protein by inhibitory and stimulatory hormones mediated by RG complexes (the disaggregation-coupling model).

The cytoskeleton of the cell appears to be involved in regulation of adenylate cyclase on account of its linkage to the G protein (Rasenick, 1981). Agents such as cis unsaturated fatty acids, and colchicine which disrupts microtubules enhance mobility of the G protein, and increase its binding to the catalytic unit. This suggests that the G unit is bound to microtubules in the absence of hormonal stimulation.

Recent work by Gilman's group (Northup et al., 1983) has demonstrated that the G protein can be resolved by gel filtration into two subunits (Tables 2 and 3).

The resolved α subunit was able, when activated with guanidine nucleotide analogues, to activate purified preparations of C protein. The β subunit binding to the α.C complex promotes a slow deactivation of the activated C unit.

TABLE 2: PROTEIN COMPONENTS OF HORMONE-SENSITIVE ADENYLATE CYCLASE

Hormone receptor (R)

Contains hormone binding site on extracellular face. One or more different receptors per target cell.

Catalytic protein (C)

Activity with Mg^{2+}.ATP less than 10% of activity with Mn^{2+}.ATP. Not stimulated by hormones, fluoride or guanine nucleotides. Molecular weight = 190,000. Sensitive to mild heating, low concentrations of sulphydryl reagents.

Guanine nucleotide-binding regulatory protein (G)

Confers upon C ability to use Mg^{2+}.ATP as substrate.
Mediates regulation of C's activity by fluoride and guanine nucleotides.
Binds guanine nucleotides and fluoride.
Probable GTPase.
Contains 41,000-45,000 dalton cholera toxin substrate.
More stable to heat or sulphydryl reagents than is C.
The G protein consists of two subunits (α and β) on binding of R-H, the β subunit dissociates.
The α subunit activates the C unit. A slow re-association of the $\alpha.\beta$ complex terminates activation of C.

TABLE 3 : MOLECULAR WEIGHT OF COMPONENTS OF THE ADENYLATE CYCLASE SYSTEM (IN DALTONS)

Source	Hormone receptor	G-protein	C-protein	References
Rat renal medulla	-	-	1.59×10^5	Neer (1974)
Bovine cerebral cortex	-	-	2.2×10^5	Neer (1978a
S49 lymphoma	7.5×10^4	4.5×10^4	2.5×10^5	Haga et al. (1977)
S49 lymphoma (cyc^- clone)	-	α subunit $5.15 \times 10^4 \pm 3,300$ β subunit $5.22 \times 10^4 \pm 2,500$		Northup et al. (1983)
Rat liver	-	4.5×10^4	1.8×10^5	Stengel and Hanoune (1980)
Rat testis	-	-	1.9×10^5	Neer (1978b)

Accordingly, they have proposed a model in which the hormone-promoted dissociation from the α subunit provides a mechanism for inhibitory regulation of the C unit:

$$\begin{array}{ccccc} & & \overset{L}{\rightleftharpoons} & & \\ & \alpha.\beta & & L.\alpha.\beta & \\ Mg^{2+} & \uparrow\downarrow & & \uparrow\downarrow & Mg^{2+} \\ & a+\beta & \rightleftharpoons & \beta + L.\alpha & \rightleftharpoons L.a.C^* \\ & & L & & C \end{array}$$

where L is an activating ligand (GTPγS)

and C* represents the active C unit.

Thus the rate of activation of G is inversely related to α.β concentration, and is reduced by β which has a low affinity for activating ligands. The dissociation of α.β is the slow step in the activation of G.

1.3.4 Adenylate Cyclase [ATP pyrophosphate-lyase (cyclising), EC 4.6.1.1]. Much less is known about the physical properties of the C protein, because of the difficulty in obtaining pure preparations of the protein. Details of the size of C proteins from different sources are given in Table 3. Nothing is known about the subunit composition of the C protein, although partial specific volume determinations make it seem likely that C has a large hydrophobic surface area. This is supported by the ability of cholate-solubilised C to be reincorporated into monolamellar phospholipid vesicles upon the removal of detergent (Ross, E.M., unpublished).

The MnATP-dependent adenylate cyclase activity of C is sensitive to several proteases and sulphydryl reagents (Ross and Gilman, 1978). Cysteine residues appear to be involved in both catalysis and G-C interaction, since both activities can

by destroyed by N-ethylmaleimide (Ross et al., 1978).

Other Components

Mg^{2+} and Mn^{2+} have stimulatory effects on adenylate cyclase, as does a diterpenoid compound called forskolin. The exact site to which these agents bind is not known, but evidence collated by Rodbell (Rodbell, 1983) suggests that a distinct unit proximal to the catalytic unit contains all these sites. Brooker et al. (1983) have demonstrated the presence of a component which is essential for forskolin-stimulated cAMP accumulation in C6-2B cells. This component has a shorter half-life than the other three components, and is turned over rapidly. Blocking protein synthesis causes a progressive loss of forskolin stimulation, presumably due to loss of the labile component. In certain cells, in particular brain cells, adenylate cyclase is present in calmodulin-dependent and calmodulin-independent forms (Bradham and Cheung, 1980). The dependent forms are activated by calmodulin or calcium independently of the G protein.

Another influence on adenylate cyclase activity is Ca^{2+}, whether acting via calmodulin, or by itself it stimulates adenylate cyclase at low concentrations, but inhibits at higher concentrations, possibly due to competition for Mg^{2+} and Mn^{2+} sites. (Bradham and Cheung, 1980). Ca^{2+} also stimulates phosphodiesterase resulting in an increase in the rate of cAMP hydrolysis. (Lin and Cheung, 1980).

Studies by Rodbell and Martin's groups using target size analysis of the glucagon-activated adenylate cyclase system, indicate that R-G complexes exist in large molecular weight oligomers (Rodbell, 1980; Martin, 1983), whether

similar oligomers exist in the β-adrenergic adenylate cyclase system, remains to be seen.

Although not usually classed as a component of the β-adrenergic adenylate cyclase system, 3':5'-cyclicAMP phosphodiesterase [EC3.1.4.17] is worthy of mention. This enzyme hydrolyses cAMP, so terminating the hormonal response. It is mainly located in the cytosol, and catalyses the reaction:

$$3':5'\text{-cyclicAMP} \xrightarrow{H_2O} 5'\text{-AMP}$$

1.3.5 Mechanism of β-adrenergic Activation of Adenylate Cyclase

The theory which fits in best with the majority of experimental data to explain adenylate cyclase activation is the "collision coupling" theory of Tolkovsky and Levitski (1978). This theory was derived from an algebraic treatment of kinetic data from experiments in which different components of the β-adrenergic system were investigated.

Hormone binding to R and GTP binding to G induce activation of C to its cAMP producing state C' thus

$$ATP \xrightarrow[GTP,Mg^{2+}]{Hormone} cAMP + PPi$$

The hormonal signal (H) is terminated upon GTP hydrolysis to GDP and P_i at the GTPase site. Reactivation requires removal of GDP, binding of a new GTP molecule, and the continued presence of hormone. Thus the steady state level of activated adenylate cyclase is determined by the relative rate of conversion of inactive enzyme (C) to active enzyme (C') which

is termed the 'on reaction':-

$$HR + G_{GTP}\cdot C \rightleftharpoons [HR\cdot G_{GTP}\cdot C] \rightarrow HR + G_{GTP}\cdot C'$$

transient complex

$$HR + G_{GDP}\cdot C$$

The rate of GTPase activity determines the rate of deactivation of the enzyme (the 'off reaction').

The steady state level of activated enzyme is described by the amount of enzyme in the activated state or 'on reaction', and that in the 'off reaction' state:-

$$C \underset{K_{OFF}}{\overset{K_{ON}}{\rightleftharpoons}} C'$$

$$[C_{TOTAL}] = [C] + [C'].$$

Therefore at steady state: $K_{ON}[C] = K_{OFF}[C']$

$$\therefore [C'] = [C_{TOTAL}] \Big/ 1 + \frac{K_{OFF}}{K_{ON}}$$

The model accounts for a number of properties of the system, and is a useful means of predicting what the order of coupling of the components is.

The 'on reaction' kinetics in the presence of p[NH]ppG are always first order, and K_{ON} is always a saturating function of the hormone concentration. These findings support the view that G is complexed with C at all times. The results obtained by target size analysis (Martin, 1983) do appear to be in agreement with the collision coupling model. In these studies the existence of a transitory HRGC complex was found, its existence having been predicted by the collision coupling model.

Other models for adenylate cyclase activation have been produced (Ross and Gilman, 1980; Stadel et al., 1982) but do not appear to have gained the popularity of the collision coupling version.

Apart from the termination of response by GTP hydrolysis, when intact cells are continuously exposed to β-agonist, there is a progressive decrease in the ability of the β-adrenergic system to respond. This densensitisation, as it is called is due to subtle changes in the responsiveness of the components of the system, and is dealt with in section 1.6.1. It probably represents a method of preventing cellular damage by over-exposure to β-agonists. Comprehensive reviews have been written on this subject by Leftkowitz and co-workers (Lefkowitz et al., 1980).

1.4 ROLE OF PHOSPHOLIPIDS IN MEMBRANES

Phospholipids contribute to membrane structure, as well as regulating the function of proteins contained within, or loosely attached to the membrane.

There is a considerable variety of phospholipid species, each with its own distinct polar headgroup and fatty acyl composition. If phospholipids had merely a structural role to play, then one would not expect to find such a diverse variety present. Consequently, these different phospholipids must have a role in modulating membrane protein function.

Phospholipids could control membrane proteins by two possible means. Specific interaction between given phospholipids and membrane proteins could occur. Alternatively, phospholipids could exert effects by virtue of their physical and chemical properties, which influence the environment in which membrane proteins are located.

The first possibility, that phospholipids specifically interact with membrane proteins found favour a decade ago when studies on solubilised membrane-bound enzymes began to have some degree of success. It was found that, in some cases, enzymic activity could be restored by adding back a given lipid, or mixture of lipids. (Fourcans and Jain, 1974). The problem about this type of approach is that one cannot unequivocally demonstrate that the required lipids act on the protein directly so causing specific alterations in tertiary structure rather than general effects on the hydrophobic packaging of the protein molecule. Differences in the physical state of the lipid species

themselves at a given temperature make it difficult to compare the efficacy of different lipids in restoring membrane protein function.

In an attempt to demonstrate the effect of specific phospholipid headgroups on enzyme function, different strategies have been devised. These methodologies include: detergent-mediated lipid substitution; the insertion of defined lipids by supplementing the growth medium with polar headgroups, or precursors (discussed in section 1.5.2); phospholipase treatment; and dietary manipulations. However all these methods are capable of modifying other parameters other than headgroup composition, and so results obtained by such tactics have to be appraised with caution. For example, changes in headgroup composition may also alter membrane physical properties, or fatty acyl chain and cholesterol content (reviewed in Houslay and Gordon, 1983).

These drawbacks have been considered in greater detail in reviews by Sandermann (1978) and Houslay and Gordon (1983). These approaches are not invalidated by these criticisms, but it is essential that experiment design takes these other effects into account.

To date the only membrane-bound enzyme which shows an unambiguous preference for specific phospholipid polar headgroups is the mitochondrial β-hydroxybutyrate dehydrogenase which requires the choline group of PC in order to bind its co-enzyme, NAD^+ (Isaccson et al., 1979).

It has been observed that this enzyme, cytochrome C

oxidase and the $Ca^{2+}Mg^{2+}$ ATPase can all be activated more effectively by a mixture of lipids than by a single phospholipid

This may be of significance in vivo, since biological membranes are almost exclusively heterogenous in lipid composition. When presented with mixtures of phospholipids, only 2.5-4 molecules of PC were required per polypeptide chain for activation of β-hydroxybutyrate (Gazzotti et al., 1975). Possibly the enzyme is activated by a combination of non-specific lipid:protein interactions and specific binding to high affinity sites.

Another membrane protein, for which there now appears to be good evidence to suggest specific phospholipid:protein interactions is the proteolipid protein, the major integral protein from myelin. Negatively charged phospholipids such as PA and PS bind with highest affinity to the delipidated protein. (Brophy et al., 1983).

Before discussing approaches to the study of phospholipid:protein interactions, I shall briefly list some concepts of membrane structure, protein and lipid mobility which are relevant to this thesis.

1.4.1 Lipid:Protein Interactions

The fluid mosaic model of Singer and Nicolson (1972) is now generally accepted. Proteins and lipids both display varying degrees of mobility in the lateral plane of the bilayer; in addition proteins show a rotational diffusion, and to a lesser degree lipids show a slow, transverse motion across the bilayer (flip-flop). This area has been reviewed by Edidin (1981).

The lateral mobility of proteins in the membrane is of particular importance for the β-adrenergic adenylate cyclase system, since it requires the coupling of receptor, G protein and adenylate cyclase units for activation of cAMP production. The freedom of motion of membrane proteins is influenced, in turn, by the freedom of motion or fluidity of the membrane lipid molecules.

Fluidity comprises two distinct parameters: lipid order which is a structural property, and lipid motion which is a dynamic property. The fluidity of a lipid membrane is influenced by the polar headgroup and fatty acyl chain composition of the phospholipids and the cholesterol, and protein content of the membrane.

At the top of the acyl chain, near to the polar headgroup, motion is highly anisotropic, whereas the section of the acyl chain nearest to the core of the bilayer undergoes relatively isotropic motion.

Electron spin resonance (ESR) is a useful technique with which the physical properties of the membrane can be studied because nitroxide spin labels can be located near the polar headgroup (as with 5-doxyl stearic acid), or close to the bilayer core (using 16-doxyl stearic acid). Using 5-doxyl stearic acid spin labels, one can study the extent of anisotropic motion, from which structural (order) parameters can be derived. The 16-doxyl stearic acid spin label on the other hand, allows measurements of the rates of motion within the bilayer; consequently a more complete assessment of the physical properties of the membrane can be derived (Schreier et al., 1978).

The problem with fluorescent probe molecules such as diphenylhexatriene, is that they are sensitive to both the structural and dynamic properties of the bilayer, and cannot differentiate between changes in these two individual components of fluidity.

The degree of methylation of the phospholipid polar headgroup, from PE to PC in particular, has been shown to cause a significant decrease in the phase transition temperature of model membranes, due to steric effects of the headgroup disrupting packing. Thus, changes in the membrane polar headgroup composition might be expected to cause significant changes in membrane fluidity (Vaughan and Keough, 1974; Jain and Wagner, 1980).

1.4.2 Effect of Lipid Physical Properties on Membrane Protein Function

Since membrane proteins are contained within a lipid matrix, it follows that changes in the physical properties of the lipid, either by composition changes, or by the effect of external factors such as temperature, or cations (Kimelberg, 1977) would be likely to alter membrane protein activity.

First, it might be wise to consider what types of lipid:protein interactions there are. Much of this work has been done on membrane-bound enzymes, since their enzymic activity is a convenient index by which to measure the effects of alteration in the nature of these lipid:protein interactions. This topic has been extensively covered in good reviews (Freedman, 1981; Lenaz, 1977).

Apart from specific phospholipid:protein interactions, covered in later sections, the physical properties of lipids play several roles in membrane protein function. Some of the

more important roles include:

1. Anchorage of the protein to the membrane;
2. Maintaining the protein in a conformation appropriate to the function of the protein - particularly important for membrane bound enzymes;
3. Orientating the protein in the membrane (e.g. Na^+/K^+ATPase);
4. Providing an appropriate environment for processes requiring non-polar media.

Thus, changes in lipid physical properties could modulate membrane protein function.

1.4.3 Boundary Lipid

Apart from the influence of the bulk lipid on membrane proteins, the proteins may also be influenced by a small population of lipid molecules which interact directly with the protein, the boundary lipid.

Jost et al. (1973) demonstrated, using electron spin resonance techniques, that the cytochrome C oxidase interacts with a small population of lipid molecules. The boundary lipid was calculated to be comprised of 30-35 mol. of phospholipid per mol of protein.

It was suggested that these phospholipids, rather than the bulk lipid modulated the properties of the enzyme.

The existence of boundary lipid is controversial (see Quinn, 1981). Watts (1981) has offered a plausible explanation for the differing conclusions drawn from ESR and NMR studies. He has suggested that the detection of boundary lipid by ESR, and the failure to observe this lipid population

by NMR is due to the different time regimes sampled by the two spectroscopic techniques. One role of boundary lipid was suggested to be the maintenance of the protein in a conformation compatible with its activity (Watts, 1981). It is interesting to speculate that phospholipid methylation could modify membrane protein function, despite its limited capacity for PC production, if it were to change the composition of boundary lipid.

Much evidence for a physical effect of lipids on membrane proteins has come from the study of Arrhenius plots (Kimelberg, 1977). These studies indicate that phase changes in the lipid can cause changes in the activation energy of enzyme-catalysed reactions manifested by changes in the temperature at which breakpoints in the plot occur. These results have to be interpreted with caution, since some alterations in activity appear to be due to thermal effects on the protein itself. (Jain and Wagner, 1980).

1.4.4 Modification of Lipid Composition in vivo as a means of Studying Specific Lipid-Protein Interactions

The problems of solubilisation of membrane proteins have been discussed. Because of the difficulties encountered with this approach, the modification in vivo of membrane lipid composition has offered an alternative approach which would not appear to cause damage to the proteins, since they are not subjected to harsh chemical treatments. Membrane-bound proteins, as the name suggests, are intended to function within the hydrophobic environment of a biological membrane.

For complexes such as the β-adrenergic adenylate cyclase system, the membrane provides the correct orientation

for the function of the complex. Modification of membrane lipid in vivo allows the complex to be studied in an intact membrane, rather than each element having to be taken in isolation.

By growing cells in the presence of fatty acids, polar headgroup precursors or by fusing cells with liposomes containing defined phospholipid or cholesterol mixtures, the lipid composition can be readily manipulated. The principal problem with this strategy is that often variables other than the one under study are also altered. Apart from unintended changes in membrane composition, or cell growth, changes in the physical properties of the cell membranes can occur. Changes in the physical properties are often compensated for by cellular processes which lead to a 'homoviscous adaptation' (Schroeder, 1978) which maintain the normal physical state of the membrane in spite of the perturbation.

This technique is also a useful tool for studying the lipid dependence of a variety of cellular processes. Table 4 lists some effects of phospholipid polar headgroup manipulation on a variety of different cell functions. Possible mechanisms for the incorporation of polar headgroup precursors have been suggested. (Sundler and Akesson, 1975; Yavin, 1977).

1.5 EFFECTS OF LIPID ON ADENYLATE CYCLASE ACTIVITY AND THE β-ADRENERGIC RESPONSE

As discussed in section 1.4, lipid composition can affect membrane bound enzyme activities through either specific effects of given lipids, or by general effects on membrane physical properties.

The main approaches have been:

(i) solubilisation of the protein, and adding back defined lipid mixtures to the protein preparation to study their effects on the restoration of protein function

(ii) the removal of specific lipids by phospholipase treatment, and determining the effect on activity in membrane preparations

(iii) modification in vivo of lipid composition and observing the effects on protein function.

The results of modifications in vivo on the β-adrenergic response will be described in section 1.5.5. Bearing in mind the drawbacks of solubilisation and phospholipase treatments, I would like to discuss the conclusions drawn from such studies in this section. Before describing the effects of specific lipids on the β-adrenergic response, I shall describe some studies in which the physical state of the membrane was modified by the application of exogenous agents such as anaesthetics, since these agents exert their effects by changing the physical state of the lipid components of the membrane. These studies have been summarised by Houslay and Gordon (1983).

1.5.1 Effects of Modified Membrane Fluidity on β-Adrenergic Function

Many studies have come from the laboratory of Houslay describing the effect of benzyl alcohol on adenylate cyclase activity in preparations of rat liver membranes (Houslay and Gordon, 1983). Benzyl alcohol is a small, water soluble molecule which readily partitions into bilayers, with its aromatic nucleus directed into the apolar core of the bilayer, and its hydroxyl group orientated in the polar headgroup region of the phospholipids. When inserted into the bilayer, it increases

the fluidity and decreases the lipid phase transition temperature. Since benzyl alcohol is a neutral molecule, charge effects on the phospholipid polar headgroups on protein molecules can be ruled out.

When 40 mM benzyl alcohol is added to liver plasma membranes, the hormone response of adenylate cyclase to glucagon was activated twofold, although the effect on basal activity is small. Considerable effects on NaF and guanine nucleotide stimulation of activity were observed. NaF-stimulation was inhibited at low benzyl alcohol concentrations (10 mM), and was believed to be due to a disruption of the polar headgroup packing of the bilayer. A reduced stimulation by guanine nucleotides implied a change in the conformation of the enzyme, or altered interaction with bilayer lipids, perhaps analogous to the effects described by Bakardjieva (1982) of phospholipid substitutions on receptor function. The increased hormonal response was probably due to increased fluidity of the bilayer relieving physical constraints on the enzyme imposed by lipids, thus increasing the conformational flexibility of the enzyme, and so increasing its activity.

However, at high benzyl alcohol concentrations all activities are inhibited, although fluidity was increasing. This may be due to displacement of boundary lipids by benzyl alcohol. The results of these studies imply that enzyme activity is constrained by its lipid environment, but that increased fluidity can disrupt the organisation of lipid-protein domains essential for normal function, and eventually, disrupt boundary lipid conformations with a resultant loss of enzyme activity.

Increased lipid fluidity has also been caused by increases in temperature, and presumptive effects of lipid fluidity on protein function have been claimed on account of changes in the position of breakpoints in Arrhenius plots. However, there is much disagreement about the meaning of such data since some of the differences in the position of the breakpoints are undoubtedly due to thermal effects on the protein itself. (Houslay and Gordon, 1983).

1.5.2 Effect of Specific Lipids on the β-Adrenergic System

I: Manipulation of Membrane Lipid Composition in vivo

In this discussion I shall concentrate on the effect of membrane lipid modification on β-adrenergic function, and I shall describe, in turn, the effects of fatty acyl chain modification, cholesterol content and phospholipid polar headgroup manipulation.

1.5.3 Effects of Fatty Acyl Chain Composition on the β-Adrenergic Response

Orly and Schramm (1975) investigated the effects of fatty acids of different chain length and degree of unsaturation on adenylate cyclase activity in turkey erythrocytes. Certain of these fatty acids stimulated a 3-fold activation of catecholanine-stimulated adenylate cyclase activity at 37°C, and as much as 16-fold activation at 20°C. The most potent activator, cis-vaccenic acid, caused an increase in membrane fluidity as detected by fluorescent probes. However, there was no close correlation between the increase in fluidity and activation of adenylate cyclase (Houslay and Gordon, 1983) suggesting that they do act directly on adenylate cyclase as well. This approach does not, however, seem particularly useful, since membrane physical properties are so drastically altered.

1.5.4 Effects of Cholesterol on Adenylate Cyclase

Various studies have been made on the role of cholesterol on adenylate cyclase (for review, see Houslay and Gordon, 1983). All these studies have involved treating intact cells with cholesterol-rich liposomes, or by using cell mutants defective in cholesterol biosynthesis. The problem with this approach is that it is likely that the cells will adapt to these alterations by altering their phospholipids composition.

Techniques have been developed using liposomes to increase or decrease the cholesterol content of rat liver plasma membranes. (Houslay and Gordon, 1983). Some interesting results have been obtained by this approach.

Basal activity seems to be relatively insensitive to increased cholesterol concentrations. On the other hand, glucagon, glucagon + GTP and NaF stimulated activities are decreased dramatically if the cholesterol-phospholipid ratio is increased or decreased from the range 0.65-0.72. This implies that cholesterol plays an important role in the regulation of adenylate cyclase.

Increased cholesterol levels seem to increase the order parameter of the 5-doxyl stearic acid spin probe. Therefore it may be that cholesterol inhibits the enzyme partly by rigidifying the membrane, or by decreasing the number of cholesterol-poor domains available to the enzyme. This could result in increased protein-protein interaction by restricting the availability of phospholipids to the enzyme.

The decreased activity in cholesterol-poor membranes could be due to lack of interaction of cholesterol with the

enzyme itself, or due to the release of phospholipids inhibitory to the enzyme from complexes with cholesterol.

1.5.5 Phospholipid Polar Headgroup Modification and Effects on Adenylate Cyclase

Phospholipid polar headgroup manipulation is carried out by supplementing the growth medium of cells in tissue culture with polar headgroups. These are rapidly incorporated in the corresponding phospholipids, and this technique has proved successful in a number of different cell types (Table 4).

Apart from my own work, it seems that this technique has not been extensively utilised in the study of β-adrenergic function. Only two papers have appeared which have described the effects of polar headgroup manipulation on adenylate cyclase activity. These are discussed below.

Engelhard et al. (1976, 1978) grew LM fibroblasts in the presence of the polar headgroups choline and ethanolamine (Engelhard et al., 1976) or in the presence of ethanolamine, NME, DME, 3-aminopropanol or 2-amino-1-butanol. (Engelhard et al., 1978).

In all cases the fatty acyl chain composition remained unchanged. No changes were found in the protein composition of membranes from the treated cells, as assessed by SDS-polyacrylamide gel electrophoresis, although supplemented membranes showed an increase in viscosity, as measured by fluorescent depolarisation measurements of the probe diphenyl-hexatriene. (Engelhard et al., 1976).

These modifications caused significant changes in basal, NaF- and prostaglandin E_1 (PGE_1)-stimulated adenylate cyclase activity. Basal activities were increased in all the

Table 4: Effect of Polar Headgroup Manipulations on Cellular Functions

System	Phospholipid(s) Enriched	Effect	Reference
Neuroblastoma cells	PNME, PDME or PE	No change in cholesterol or fatty acid composition. Increase in phospholipid content	Robert et al., (1978)
Neuroblastoma cells	PNME or PDME	Increase in total cellular phospholipids	Yavin (1977)
LM fibroblasts	PNME, PDME or PE	No effect on membrane-bound enzymes. Sterol content decreased. LPC content increased.	Schroeder et al. (1976a)
LM fibroblasts	PNME, PDME or PE	No change in physical properties measured by fluorescence depolarisation	Schroeder et al., (1976b)
Plasmacytoma cells	PNME, PDME or PE	Increase in rate of basal phospholipid methylation	Maeda et al., (1980a)
LM fibroblasts infected with virus	PNME, PDME or PE	Increased release of viral glycoprotein	Maeda et al. (1980b)
LM fibroblasts	PNME or PDME	Reduced endocytosis	Schroeder (1981)
LM fibroblasts	PNME, PDME or PE	No change in physical properties of membranes (fluorescence depolarisation). Fatty acid composition of incorporated phospholipid intermediate in saturation between PE and PC.	Schroeder (1978)
Yeast cells	PE	Reduction in rate of amino acid uptake	Trivedi et al., (1982)
Plasmacytoma cells	PNME, PDME or PE	Changes in golgi structure and reduced IgG secretion	Nakano et al. (1982)
Mouse liver cells	PE:PC ratio increased	Reduction in membrane fluidity measured by fluorescence depolarisation.	Boyle and Dean (1982)

treated cells, but this could be abolished by solubilisation, indicating that the stimulation was due to specific phospholipid protein interactions. The magnitude of NaF-stimulation was decreased in all the base-treated membranes relative to choline-treated cells. A similar pattern was observed for the PGE_1 + GTP stimulation, although when stimulated with PGE_1, the cAMP response was unchanged, except for NME-treated membranes which showed an increase in the degree of stimulation. (Engelhard et al., 1978). No correlation could be found between the viscosity changes and the basal and PGE_1-stimulated activities. A good correlation was found, however, between these activities and the PE:PC ratio in the treated membranes (Engelhard et al., 1978).

Solubilisation abolished the basal activity increases due to the bases. No correlation could be found between the degree of NaF- and PGE_1 + GTP stimulation of activity and the viscosities of the treated cells. Taken together, these results suggest that the differences in activity were due to specific phospholipid:protein interaction, and not merely a result of physical changes in membrane properties.

1.5.6 Effects of Specific Phospholipids on the Activity of the β-Adrenergic System II:Solubilisation Studies

Since these studies have been carried out on detergent-solubilised enzyme preparations, it is argued, that the effects observed are due to the preference of the enzyme for individual phospholipids or phospholipid mixtures of defined composition, and not subject to the effects of changes in membrane fluidity. Two studies are of particular interest since, unlike earlier studies, they employed nonionic detergents. It has been demonstrated

by Johnson and Sutherland (1973) that nonionic detergent itself can activate brain adenylate cyclase.

In a study by Hebdon et al. (1981), rat brain adenylate cyclase was solubilised using sodium deoxycholate. In this form, enzymic activity was undetectable with either Mg^{2+} or Mn^{2+} in the absence of nonionic detergent or specific phospholipids. Maximal restoration of activity to both cations was found with PC, SM, PNME, or LPC liposomes. Nonionic detergents such as Triton X-100 and Nonidet P40 were also able to restore full activity. PE and PDME were capable of partially restoring full activity (40-60% and 10-20% respectively). All other lipids were without effect. When a second phospholipid was mixed with PC in the ratio 75% PC:25% of the other phospholipid, maximum activity was observed. This decreased, as the amount of PC was reduced.

Ross (1982) presented results which are in agreement with those of Hebdon's group, insofar as the importance of PC is concerned. In a preparation of rabbit hepatic adenylate cyclase which was free of endogenous lipids PC was found to be important for the interaction of C with the G protein.

The lipid-free enzyme was stimulated by Mn^{2+}, and by forskolin. The addition of purified G protein to the preparation, and stimulation with the activator GTPγS or NaF resulted in slight increases in activity .

However, when dimyristoylphosphatidylcholine (DMPC) was added or several other species of PC were added, an eight-fold stimulation by the activator-liganded G protein was observed. Since the basal and forskolin activities were unaffected by PC,

it was suggested that PC is important primarily for the regulatory interaction of C with G, rather than the intrinsic activities of either protein.

The detergents Lubrol 12A9 and Triton X-100 could not substitute for PC, and there seemed to be a preference for DMPC, Dipalmitoyl-, 1-Palmitoyl, 2-oleoyl- and Dielaidoyl PC species were much less effective. The presence of the choline moiety alone, in trials with LPC, was not sufficient for activity. This suggests that PC is specifically required by the enzyme. In contrast to previous studies, (Rubalcava and Rodbell, 1973), acidic and negatively charged phospholipids had no effect on the restoration of activity.

It seems that one can tentatively conclude from these two studies that adenylate cyclase does show a specific requirement for PC.

Houslay and Palmer (1979) have reported that low concentrations (0.001-0.01 mM) of Lysomyristoyl PC stimulated the activity of adenylate cyclase in rat liver plasma membrane preparations, although at higher concentrations (0.5-1 mM) the enzyme was inhibited. Glucagon stimulation of the enzyme was more susceptible to inhibition than the NaF stimulation. It seems that the coupling of the R-G complex was perturbed, since no effect on glucagon-binding was observed.

1.5.7 Effect of Specific Phospholipids on the β-Receptor

Kirolovsky and Schramm (1983) prepared a delipidated β-adrenergic receptor and reconstituted it with defined phospholipids to investigate whether specific interactions occurred. It was found that PE restored maximum agonist binding. In contrast to earlier studies by Rubalcava and Rodbell (1973), acidic phospholipids proved ineffective in restoring binding ability.

1.5.8 Phospholipase Studies

Some of the earliest studies to investigate the question of phospholipid specificity were performed using phospholipases. (Rubalcava and Rodbell, 1973). Treatment of liver plasma membranes with phospholipase C from Bacillus cereus causes a preferential hydrolysis of acidic phospholipids (PS). The polar headgroup is removed, leaving diacylglycerol in the membrane. The enzyme from Clostridium perfringens hydrolyses neutral phospholipids preferentially. Treatment of the membranes with the Bacillus enzyme caused a loss of the glucagon-stimulation of adenylate cyclase, however, even after substantial hydrolysis, the Clostridium enzyme had little effect on the hormonal response.

This was interpreted by these workers to imply that certain acidic phospholipids were necessary for the hormonal response. However, these type of experiments cause perturbation of the structure and properties of the membrane, and have proved difficult to reproduce (Ross and Gilman, 1980).

1.5.9 Conclusion

The results of these studies indicate that activity of the hormone-stimulated adenylate cyclase is influenced by both the physical properties of the membrane, and possibly by specific phospholipid requirements. The coupling between the receptor, G protein and C protein seems to be the process most sensitive to these factors.

Phospholipid specificity itself, and any resultant changes in membrane physical properties could be a subtle means of regulating the response of the C protein to hormonal stimulation. A means of modulation could be the protein-catalysed exchange of

one phospholipid for another or, for example, if PC was hydrolysed to yield LPC, an increase in activity of the C protein might be expected. In view of the importance of specific lipid classes, possibly in close association with the protein, rapid interconversion of PE to PC by phospholipid methylation could ostensibly be a method of regulating the hormone response of the C protein. The role of phospholipid methylation on the β-adrenergic response is considered in section 1.7.

1.6 THE C6 β-ADRENERGIC SYSTEM

The β-adrenergic system of C6 cells has been thoroughly studied and excellent reviews have been written. (Pfeiffer et al., 1978; Van Calker and Hamprecht, 1980). The latter review is particularly extensive.

Stimulation of cAMP accumulation in C6 cells was first described by Gilman and Nirenberg (1971). A significant increase in cAMP accumulation was observed when (-)-isoproterenol (0.01-100 μM) was administered. Noradrenalin and adrenalin, but not α-adrenergic agonists, also resulted in a stimulation of cAMP accumulation. This accumulation was enhanced by 1 mM theophylline, an inhibitor of phosphodiesterase.

Extremely high concentrations of isoproterenol ($> 10^{-3}$M) slightly depressed the response, and stimulation was maximal in the concentration range 0.1-1 μM. The stimulation of cAMP content rose 40-fold within 30 seconds, and was maximal (200-fold) 5-15 min. after addition of the drug. The cAMP content decreased thereafter, but was still 40-fold greater than the basal level after one hour of exposure to the drug. All these effects were blocked by β-adrenergic , but not α-adrenergic, antagonists.

The β-adrenergic receptors of C6 cells were studied using [^{3}H]-dihydroalprenalol (DHA) (Van Calker and Hamprecht, 1980). The number of β-sites was found to be 10,000 per cell, and they exhibited the same specificity profile as the β-receptors which mediate cAMP accumulation. Later work by this group (Homburger et al., 1981) demonstrated that C6 contains both β_1 and β_2 receptors, but that only the β_2 receptor effectively stimulated adenylate cyclase. Occupation of all the receptor sites is necessary for a maximum cAMP response to be achieved (Van Calker and Hamprecht, 1980).

The number of receptors expressed by cells in culture is influenced by various factors. Dibner and Insel (1981) showed that the presence of serum in the growth medium reduces the number of receptors measured by DHA binding studies. In a sub-clone of C6, the C6 T3VA line, transformation by virus caused a three-fold decrease in the number of DHA binding sites, and a significant decrease in the maximum stimulation of adenylate cyclase by isoproterenol, which appeared to be due to an incomplete coupling of the receptors to adenylate cyclase (Higashida et al., 1982).

The adenylate cyclase phosphodiesterase and cAMP-dependent protein kinase activities of C6 cells have been studied by various groups, and extensively characterised (Van Calker and Hamprecht, 1980). The mechanism of adenylate cyclase control and coupling with the β-adrenergic receptor was studied, and found to conform to the collision coupling model of Tolkovsky and Levitski (1978).

The adenylate cyclase of C6 exists in two forms, one

of which shows an absolute calcium dependence, the effects of which are mediated by calmodulin. Brostrom et al. (1976) observed a biphasic response of C6 adenylate cyclase to calcium. Both the basal and noradrenalin-stimulated activities were increased by < 1 μM Ca^{2+}, but higher concentrations of Ca^{2+} (100 μM) inhibited the enzyme. When calmodulin was added to the membrane preparations, the calcium concentration required for activation was lowered, and in solubilised enzyme preparations calmodulin was shown to be tightly bound to the enzyme. These findings indicate that calcium probably plays a significant role in the regulation of the β-adrenergic response in C6 cells. A large percentage of the C6 adenylate cyclase is calcium dependent. β-Adrenergic stimulation of C6 cells in Ca^{2+}-free medium reduced the magnitude of the response by 60-70%. (Brostrom et al., 1982). The number of β-receptors, as measured by DHA binding, did not change, confirming that Ca^{2+} effects occur at the level of the adenylate cyclase itself.

Phosphodiesterase exists in two forms with different K_mS and V_{max}S for cAMP. (Van Calker and Hamprecht, 1980). Schultz et al. (1972) showed that noradrenalin stimulation of cAMP content was followed by an increased rate of cAMP degradation, due to the induction of both the high and low K_m forms of phosphodiesterase. Induction of the phosphodiesterase activity appeared to be due to the presence of cAMP itself, since the effect could be mimicked by adding dibutyrl cAMP. De novo RNA and protein synthesis was required for the increase in activity, since cycloheximide and actinomycin D blocked the effect. (Van Calker and Hamprecht, 1980).

β-adrenergic stimulation also causes a rapid activation of other enzymic activities: noradrenalin stimulation changes the pattern of protein phosphorylation and induces a subcellular redistribution of protein kinase activity. (Van Calker and Hamprecht, 1980). Previous studies showed that the increase in cAMP content due to β-receptor stimulation caused a 2- to 4-fold increase in protein kinase activity. (Van Calker and Hamprecht, 1980).

Other effects, apparently caused by elevated intracellular cAMP levels have been described. Doore et al. (1975) noted that β-adrenergic stimulation is followed by a release of cAMP into the medium. Introducing the cAMP analogue dibutyrl cAMP into cells in culture, caused a progressive increase in the release of prostaglandins PGE_1 and small amounts of PGA and $PGF_{2\alpha}$. (Hamprecht et al., 1973). Although PGE_1 was shown to stimulate cAMP accumulation in the glioma clone C6-BU-1, (Van Calker and Hamprecht, 1980) it is not clear what the physiological significance of this prostaglandin release is. A consequence of elevated intracellular cAMP is an increase in phosphodiesterase activity, which is one of the mechanisms by which desensitisation occurs.

1.6.1 Desensitisation

Noradrenalin stimulation causes increases in intracellular cAMP content which is followed by an increased rate of cAMP degradation, due to the induction of both the high and low K_m forms of phosphodiesterase (Van Calker and Hamprecht, 1980). This appeared to be a direct result of cAMP itself as the effect could be mimicked by dibutyrl cAMP. The refractoriness to receptor-mediated increases in cAMP content, also extends to

stimulation by prostaglandin E_1, which also fails to elicit maximal cAMP accumulation, thus the desensitisation observed in C6 cells appears to be of the heterologous type.

Desensitisation consists of three distinct stages; a loss of coupling between the receptors and the G protein:C protein complex, followed by a loss of hormone receptors, and an increased rate of cAMP degradation (Perkins, 1983).

The role of cAMP itself, in bringing about desensitization has been well described by Koschel (1980). Elevation of the intracellular cAMP content using phosphodiesterase inhibitors caused a subsequent refractoriness of adenylate cyclase to challenge by β-agonists. The number of β-receptor sites was unchanged by this treatment, nor was the stimulation of the adenylate cyclase complex by either NaF or guanine nucleotides affected. This suggests that the desensitisation is caused by an inhibition, or defect in coupling of the receptors to the G protein. It was suggested that loss of hormone receptors occurred as a secondary event, and not by a cAMP-mediated mechanism.

Some evidence to suggest that cAMP-independent desensitisation occurs has been presented by Franklin and Twose (1979). Cells were pre-incubated with β-agonists that caused cAMP accumulations of only 1% of the maximum caused by isoproterenol. The cells were then challenged with isoproterenol and found to be desensitised. These results suggest that occupation of the receptor by an agonist can cause desensitisation in the absence of significant rises in intracellular cAMP.

The increase in phosphodiesterase activity, appears to

occur as a later event. Several hours pre-incubation with noradrenalin were necessary before a 53% increase in phosphodiesterase activity was observed. (Franklin and Twose, 1979).

Studies by Fishman et al. (1981) have confirmed the view that desensitisation to β-agonists in C6 cells is caused initially by a defect in the coupling between the receptor and the C/G complex. The mechanism by which cAMP causes this decoupling is unknown. Kinetic studies by Homburger et al. (1982) show that the K_{off} constant of adenylate cyclase from desensitised membranes is increased, concomitant with a decrease in the coupling constant. Increased GTPase activity was suggested as a possible cause.

The action of phospholipase A_2 has also been implicated in the mechanisn of desensitisation. Cells exposed to isoproterenol for 2h showed an 80% decrease in cAMP production in response to a subsequent challenge. The number of β-receptors decreased by 20-30%, and an increase in the release of arachidonic acid into the medium was noted. The arachidonic acid release is modulated by phospholipase A_2. Phospholipase A_2 inhibitors could prevent the desensitisation and loss of receptors, but addition of activators of the enzyme to cell cultures, caused receptor loss and refractoriness even in the absence of pre-incubation with agonist. These results suggest that changes in the phospholipid environment of the β-receptor could be responsible for the receptor loss (Mallorga et al., 1980).

1.6.2 Effects of Cell Culture Conditions on the β-adrenergic System

Differences in the activity of various components of the β-adrenergic system have been observed due to the effects of

age, and of cell density.

Phosphodiesterase activity is influenced by the stage of growth of C6 cultures (Van Calker and Hamprecht, 1980). During the rapid growth phase, phosphodiesterase activity was only 50% of that observed in stationary phase cultures, when corrections were made for protein content.

Similar results were found by Morris and Makman (1975). These workers also demonstrated, that despite increased phosphodiesterase activity, the magnitude of cAMP accumulation in response to either adrenalin- or NaF-stimulation was highest in confluent cell cultures. No change was found in the receptor affinity for hormones, so it was concluded that either synthesis de novo or activation of nascent receptor-cyclase units occurs with increased cell density.

Mallorga et al. (1981) have investigated the effect of continuous subculturing on the β-adrenergic system. Cells of high passage number (115-175) showed an inability to effect large increases in cAMP content in response to isoproterenol stimulation compared with low passage (39-80) cells. The defect was not due to any changes in the G and C proteins, as NaF and cholera toxin stimulation of cAMP accumulation was similar in both cell types. The receptor number of the low passage cells was 2-4 times greater than the high passage cells, and in the high passage cells phosphodiesterase activity was 2-3 times higher than in low passage cells.

These studies illustrate the need to take cell density and passage number into account when assessing experimental results.

1.7 PHOSPHOLIPID METHYLATION AND THE β-ADRENERGIC RESPONSE

Hirata and Axelrod (1980) have proposed that phospholipid methylation is a general mechanism for facilitating the transduction of biological signals across cell membranes. The purpose of this section is to review the evidence supporting a role for phospholipid methylation in the β-adrenergic response, principally from the laboratory of Hirata and Axelrod. Many of the experiments which will be described are contained in a comprehensive review by Hirata and Axelrod (1980), and many of the studies described are reviewed in that work.

Contrasting evidence will be discussed at the end of this section. It is clear that β-adrenergic stimulation causes an increase in the activity of lipid methyltransferases, what is not clear is whether the methylation is an obligatory event for adenylate cyclase activation, or merely a simultaneous, but unrelated phenomenon.

1.7.1 Stimulation of Phospholipid Methylation by β-Adrenergic Agonists

When rat reticulocytes, previously loaded with S-adenosyl methionine (SAM) were stimulated by β-adrenergic agonists, phospholipid methylation was increased. The increase in labelling was dose-dependent and stereospecific for the (-)-isomer of β-agonists (Hirata and Axelrod, 1980). The value for half-maximal activation of methylation by isoproterenol was close to that required for half-maximal activation of adenylate cyclase in C6 cells and rat reticulocyte ghosts (Hirata and Axelrod, 1980).

In the intact C6 cell, using [^{3}H]methionine as a methyl donor the order of potency for β-agonist stimulation of

methylation was consistent with the effect being mediated by a β_2 receptor, (Hirata and Axelrod, 1980), which is the receptor that mediates cAMP accumulation in the C6 cell.

GTP, which plays an important role in the coupling of β-receptors to adenylate cyclase, shifted the dose response to the left by two orders of magnitude, suggesting a possible involvement of coupling factors in enhancing phospholipid methylation (Hirata and Axelrod, 1980). Hashizume et al. (1983) have claimed that GTP is bound to, and inhibits, PMT_I. When a β-agonist is bound to the receptor, GTP is released from PMT_I activating it. PMT_{II}, on the other hand, is activated by the GTP released from PMT_I. Thus, constraints imposed on the PMT s by the β-adrenergic receptor, are relieved by the binding of agonist to the receptor, causing activation of both of the PMT s.

Isoproterenol-stimulated cAMP formation was markedly increased in reticulocyte ghosts, which had previously had SAM sealed inside them. No change in the number of β-receptors was observed under these conditions (Hirata and Axelrod, 1980). This concentration of SAM was previously found to stimulate PMT_I, producing PNME. From this they inferred that PNME production by phospholipid methylation promoted the coupling of β-receptors to adenylate cyclase (See Fig. 4.).

The replacement of PE by PC, and vectorial translocation of the methylated phospholipid was found to increase the fluidity of the membrane of erythrocyte ghosts, as measured by the polarisation of fluorescence of 1,6-diphenyl-1,3,5-hexatriene. The formation of PNME was claimed to be responsible for the observed increase in membrane fluidity, and this could be abolished by

Fig.4: Phospholipid Methyltransferases and their Relationship to the β-Adrenergic Receptor

Key

PMT I)
PMT II) Phospholipid methyltransferases

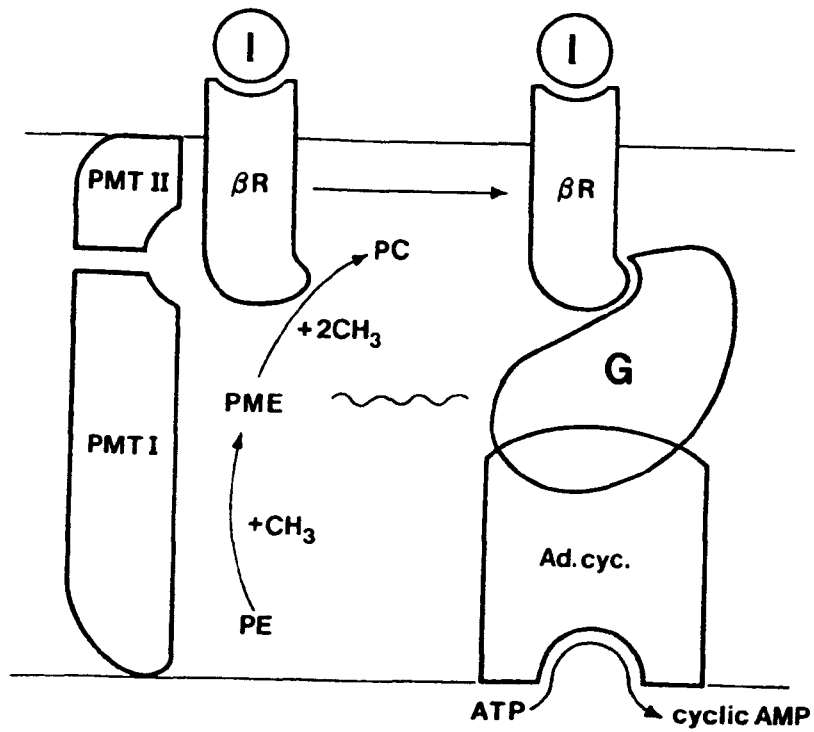
I = Isoproterenol

βR = β-Adrenergic receptor

G = Guanylnucleotide binding protein

Ad.Cyc. = Adenylate cyclase

Binding of I to βR activates PMT s catalysing methylation of PE. Formation of PME causes fluidity changes in the membrane which promote coupling of βR to G./Ad.Cyc.



the addition of S-adenosylhomocysteine (SAH) which blocks methylation (Hirata and Axelrod, 1980).

1.7.2 Masking and Unmasking of β-adrenergic Receptors

When reticulocyte ghosts loaded with SAM were incubated for 1h, the number of β-adrenergic receptors increased by 40%, as measured by the binding of DHA. By varying the concentration of SAM, it was shown that activation of PMT_{II} and subsequent PC production was responsible for the increase in binding sites. The increase in sites could be blocked by SAH, since protein synthesis de novo is unlikely in the membrane preparation, the results suggest that PC synthesis unmasked cryptic receptor sites (Hirata and Axelrod, 1980).

Similarly, when HeLa cells were treated with the methylation inhibitor 3-deazaadenosine, the number of β-adrenergic receptors, as well as isoproterenol-stimulated cAMP formation were decreased. Upon removal of the inhibitors by washing the cells, the β-adrenergic receptor number returned to normal (Hirata and Axelrod, 1980). The conclusion drawn from these studies was that PC produced by phospholipid methylation, can rapidly modulate the β-adrenergic response, by alteration in receptor number.

Benzodiazepine receptors which are coupled to adenylate cyclase are present in C6 cells (Syapin and Skolnick, 1979). When C6 cells were incubated with [^{3}H]methionine, benzodiazepines stimulated [^{3}H]methyl group incorporation in a dose-dependent manner and this paralleled the increase in cAMP production. When benzodiazepines and β-agonists were added together, an additive stimulation of methylation was observed (Hirata and Axelrod, 1980). This suggests that the two different receptors both have

their own cluster of PMT s which are stimulated specifically by the ligand whose receptor they surround.

There is some evidence that PMT s regulate receptor number in intact animals. Repeated injections of 3-deazaadenosine into rats resulted in a decrease in the β-adrenergic receptor number in the heart and the brain (Hirata et al., 1980).

To summarise, the essence of Axelrod's theory is that β-adrenergic receptors are associated with a small cluster of PMT s. Binding of agonist releases and activates the PMT s which cause a rapid translocation across the membrane of methylated phospholipids. The formation of PNME causes local fluidity increases which enhance coupling of the receptor and adenylate cyclase, facilitating transduction of the hormonally-transmitted signal. Methylation of PNME to PC uncovers receptor sites which are normally inactive, so amplifying the signal.

1.7.3 Evidence Against a Functional Role for Phospholipid Methylation

In the last three years considerable evidence has accumulated which argues against the proposals of Axelrod and his co-workers. The two aspects of phospholipid methylation which have proved most contentious are the PMT-induced changes in membrane fluidity, and the claim that phospholipid methylation is an essential step in hormone-stimulated cAMP formation. I shall deal with the fluidity question first.

Vance and de Kruijff have raised several objections to there being any functional significance of PE methylation (Vance and de Kruijff, 1980). They have calculated that only 0.00033% of the total PC in rat erythrocyte experiments was derived by PMT action, and that such small amounts of PC would

be unlikely to have any effect on the fluidity of the bulk lipid. They insist that some other explanation must be sought for the recorded fluidity changes.

In a reply to these criticisms, Axelrod and Hirata (1980) argue that the small amounts of methylated lipid need not be important, if they are located in the boundary lipids around membrane proteins. The probe molecule that was employed, preferentially localises in the boundary of lipid domains, and is not necessarily reporting bulk lipid fluidity. Axelrod and Hirata maintain that the methylation reactions satisfy all biochemical criteria of enzymic reactions, and therefore are unlikely (as claimed by Vance and de Kruijff) to be the product of experimental artefacts.

However, since regulatory mechanisms exist to maintain an appropriate homoviscous membrane environment it seems unlikely that small amounts of PNME could affect fluidity when, as Schroeder (1978) has shown, 50% of the membrane phospholipid can be substituted for by PNME, without alteration in physical properties as measured by fluorescent probes.

Several workers have shown that the hormone-stimulated cAMP formation can function normally in the absence of phospholipid methylation.

Koch et al. (1983) investigated the β-adrenergic response of three lines of myogenic cells. In all cell lines, isoproterenol stimulated cAMP synthesis, but no increases in phospholipid methylation were observed. Colard and Breton (1981) increased PMT activity in preparations of rat liver membranes by adding SAM to the incubation mixture. No increase in NaF-

stimulation or of GTP + glucagon stimulation of adenylate cyclase could be found. Schanche et al. (1982) blocked methylation using 3-deazaadenosine in rat liver membrane preparations, without any effect on glucagon-stimulated cAMP accumulation. Similarly Padel et al. (1982) blocked 88% of the methylation reaction in guinea pig and rat parotid gland preparations, with no effect on hormonal stimulation of cAMP accumulation.

Doubts have also been raised as to the role of methylation on receptor modulation. Munzel and Koschel (1982) examined methylation in C6 cells persistently infected with subacute sclerosing panecephalitus virus (SSPE). In these cells the accumulation of cAMP in response to isoproterenol was impaired, and the stimulation of β-adrenergic methylation was completely abolished. However, the number of β-adrenergic receptors was comparable to that of normal C6 cells.

In addition, in infected cells, the basal methylation was actually depressed by isoproterenol stimulation. This loss of methylated lipid could not be blocked by propranolol. Analysis of the location of label by t.l.c. revealed that the isoproterenol-stimulated demethylation in C6/SSPE cells was not due to a degradation of methylated PE, but rather an inhibition of PC synthesis by N-methylation. The results of these studies do not support any involvement of PC production by N-methylation in the control of β-receptor number.

The sequence of events after hormone receptor stimulation has also been questioned. Hirata and Axelrod (1980) maintain that phospholipid methylation is a pre-requisite for coupling of the receptor to the adenylate cyclase. Mato et al. (1982) have shown that although glucagon and vasopressin stimulate phospho-

lipid methylation in hepatocytes, the methyltransferases are located on membranes distal to the plasma membrane, and that stimulation of methylation is dependent on transmission of the hormonal signal by the secondary messengers Ca^{2+} and cAMP. Hormonal responses in methylation rate are abolished by maintaining the cells in calcium-free media. The cAMP messengers appear to activate a cAMP-dependent protein kinase, which in turn activates the methyltransferases.

Similarly Pritchard et al. (1981) showed that analogues of cAMP stimulate methylation in hepatocytes. If, as Hirata and Axelrod suggest, methylation is instrumental in controlling cAMP production, it seems strange that cAMP does not cause feedback-inhibition of methylation, but rather stimulates it.

1.7.4 Summary

It seems clear that phospholipid methylation accompanies β-adrenergic stimulation. Whether methylation is a prerequisite for, or a consequence of, cAMP accumulation is still unclear, as is any unquestionable physiological role for methylation. Possibly, much of the confusion has arisen from attempts to compare results from a diverse variety of systems and unite them into a common hypothesis. As Mato and Alemany (1983) have pointed out, the phospholipid, cholesterol and protein content of cells varies considerably. In particular, PC content varies from cell to cell type. PC comprises 30% of the phospholipids of rat erythrocytes, but constitutes only 5% in sheep erythrocytes. Thus it is not wise to make sweeping generalisations. The stimulation of phospholipid methylation that accompanies β-adrenergic stimulation could be explained by

two possible schemes.

1. Methylation is an essential event in the transduction of β-adrenergically-mediated signals, and that methylation causes physico-chemical changes in membrane properties.

2. Methylation is not directly involved in biological signal transduction, but by-products of the methylation pathway affect membrane-bound enzyme activities which result in a termination of the β-adrenergic response and in the desensitisation of the receptors.

One aim of this project is to test if either of these possibilities are plausible.

1.8 AIMS OF THIS STUDY

The goal of this work was to manipulate the membrane lipid composition of intact C6 cells in tissue culture in order to study the effects of modifying the membrane lipid environment on the β-adrenergic response. The intention was to grow C6 cells in media containing the bases ethanolamine, N, N,'-dimethylethanolamine (DME) and N -monomethylethanolamine (NME). I could reasonably expect, from previous reports, that rapid and extensive modification of the phospholipid composition of the cell membranes might occur, without toxic effects on the cells.

Since the β-adrenergic system is membrane-bound, it was hoped that I might infer how the phospholipid composition of the membranes affects the β-adrenergic function in an intact organism. The changes in polar headgroup composition might also be accompanied by changes in acyl chain composition and sterol content, if membrane fluidity did not change. Therefore, it was important to investigate the physical properties of the modified membranes.

The second aspect of this project was to study the role of phospholipid methylation in modulating β-adrenergic responses. For this reason the choice of bases was such that when they were incorporated into the membrane, they would enrich the membrane in intermediates of the phospholipid methylation pathway. This would amplify the magnitude of the response of the pathway. The effect of an amplified phospholipid methylation response on cAMP accumulation in response to β-adrenergic stimulation could then be studied. This would allow

me to test some of the postulates of Hirata and Axelrod (1980) concerning the effect of these intermediates on the physical properties of the membrane lipid.

METHODS

Hexagram 43: Renewed Advance

He who resolves to proceed must first exhibit the culprit's guilt in the royal court, appealing for sympathy in an earnest and sincere manner. At the same time he must be conscious of the peril his actions place him in. Then let him proceed and he will be accompanied by good fortune.

The Book of Changes

2.1 CELL CULTURE

The cells used in this study were from the C6 rat glioma (Benda 1978), American Tissue Culture Collection No. CCL107, obtained from Flow Laboratories. All cells used in these experiments were between passage numbers 44 to 54, unless stated otherwise.

Cells were maintained as monolayers in petri dishes of tissue culture grade plasticware (90 x 15mm), containing 20mls of growth medium. The growth medium was Dulbecco's Modification of Eagle's Medium (Dulbecco and Freeman, 1959) containing penicillin (100 IU/ml), streptomycin (100µg/ml) and L-glutamine (4mM). Growth medium was supplemented with bovine foetal calf serum (5% v/v). All growth media and sterile plasticware were purchased from Flow Laboratories, Irvine, Scotland.

All cell culture manipulations were carried out under stringent aseptic conditions in a Laminar flow hood (Microflow) which was cleaned with "Lysol" (Gallenkamp) before and after manipulations. Cells were grown in an atmosphere of 5% CO_2/95% air (v/v) at 37°C. Pipettes and glassware were sterilised by autoclaving.

(a) Cell storage

Cells were stored in plastic ampoules immersed in liquid nitrogen. An auxiliary supply was kept in a -70°C freezer as a safety measure.

Petri dishes of confluent cells were prepared for storage as follows. The monolayers were removed by trypsinisation, transferred into culture tubes and collected by centrifugation.

The cell pellets were resuspended in 40% Foetal Calf serum (v/v) in Dulbecco's modified Eagle's medium (0.5ml of serum for each plate of cells in the pellet). After trituration, the cell suspensions were transferred to storage ampoules kept on ice (0.5ml/ampoule). A solution of 20% dimethylsulphoxide (v/v)(0.5ml) in Dulbecco's modified Eagle's medium was carefully layered onto the serum in the ampoules. After sealing the ampoules, the contents were gently mixed, and transferred to a polystyrene box with sides 4cm thick. This was closed, and placed in a -70°C freezer for 2h. After that, the ampoules were placed in the liquid nitrogen.

(b) Transfer of cells into culture

An ampoule containing the cells was removed from the liquid nitrogen and warmed between gloved hands, before being thawed in a waterbath at 37°C. These operations were carried out wearing gloves and a face visor for protection in case of explosion of the ampoule on removal from the liquid nitrogen.

When the contents were thawed, the ampoule was removed from the waterbath and washed with 70% ethanol (v/v) and allowed to dry in the aseptic conditions within the flow hood. The ampoule was opened, and the contents transferred to a plastic culture tube. Slowly, and with gentle agitation, 5mls of growth medium, prewarmed to 37°C, were added to the tube. The tube was capped and gently inverted. Another 5mls were added in the same manner, the tube gently inverted, and the contents poured into a petri dish (90 x 15mm). The dish was swirled to disperse the contents. Another 10mls of growth medium were added, before the dish was placed in the incubator, and the cells were grown to confluency.

(c) Cell passage

Trypsin (2.5% v/v) was thawed and mixed well. The medium was removed from petri dishes containing confluent cells by aspiration. Trypsin (2ml) was added to each dish, and the plate swirled so that the liquid covered all the cells. After one minute the liquid was removed, and the plate allowed to incubate at room temperature for 5 minutes, or until the cell monolayers began to detach from the plastic. Growth medium (5mls) was added to inhibit the trypsin.

The cell suspension was then transferred into culture tubes, which were sealed and centrifuged for 5 minutes at room temperature in an MSE minor, speed, position 1. The supernatant was discarded, and the cell pellet resuspended in complete growth medium. The cell suspension was mixed with an appropriate amount of growth medium such that the contents of a single petri dish were split between five new petri dishes, each containing 20mls of cell suspension. This split ratio of 1:5 was used for all subsequent experiments. The petri dishes were replaced in the growth incubator and allowed to reach confluency before use in experiments. Growth medium was changed every three days.

(d) Polar head group manipulation

The bases ethanolamine , N ,N',-dimethylethanolamine (DME) (both Analar grade, B.D.H.) and N-monomethylethanolamine (NME),(Fluka A.G.)were made up in water as stock solutions (50mM). The pH was adjusted to 7.0 using 13M hydrochloric acid, and the solutions sterilised by autoclaving.

The sterile base solutions were added to complete growth medium to a final concentration of 5mM. Cells were grown in this medium for 24 hours, unless stated otherwise.

(e) Measurements of cell growth

Cells were grown in Linbro trays and the original plating density was noted using the haemocytometer. A low initial density (1 x 10^7 cells/well) was found to be best for accuracy in plotting growth curves. Duplicate wells were used for each timepoint in the study.

After removal of the medium, the cells were washed twice with saline 0.9% (v/v), and trypsin (0.5ml) was added to each well. After 2 minutes incubation at 37°C, cells were washed from the wells using complete growth medium until no cells were found remaining when the well was examined under the microscope.

The cell suspension was transferred to culture tubes and spun on the bench centrifuge for 5 minutes to pellet the cells. The cell pellet was suspended in an appropriate volume (1-5mls) of saline containing 50% (v/v) Trypan blue to identify non-viable cells, and an aliquot was counted by haemocytometer. The cell numbers recorded were of intact, unstained cells only. Care was taken to keep Trypan blue off the skin, as it is considered to be carcinogenic.

(f) Photomicrography

Examination of the cell cultures was necessary to ensure that manipulations were made at the correct time and stage of growth. An "Olympus" CK inverted stage microscope with phase contrast optics was used to examine the cultures.

Morphological changes in treated cells were recorded by monochrome micrography, using an "Olympus" C-35 camera, PM-10M adaptor and "Olympus" light meter. Pan F50 ASA film (Kodak) was used to record the observations.

Colour photographs were taken using the same camera system, but it was attached to an "Olympus" BHB microscope, since a higher magnification was required to see the fine nuclear structure. Tungsten 160 ASA film (Kodak) was used for these studies.

(g) Haematoxylin-Eosin staining

Haematoxylin-Eosin staining (HE staining) was used to determine whether base treatment affected cell ultrastructure. Nuclei and nucleoli are shown clearly against a counter-stained cytoplasm.

Cells were grown on coverslips. The coverslips were cleaned by boiling in 0.1M sodium hydroxide, rinsed overnight in tapwater, rinsed 3 times in distilled water, and sterilised by heat in glass petri dishes.

After growth in the presence of bases, the coverslips containing cells were removed, rinsed twice with Dulbecco's phosphate buffered saline (PBS) pH 7.4, and the cells fixed in a 2.5% (v/v) solution of glutaraldehyde (EM scope) in PBS overnight at 4°C.

The monolayers were stained with HE (Drury and Wallington, (1980) and then mounted on glass microscope slides for photography using D.P.X.(B.D.H.). The preparation of reagents for HE staining is detailed in the Appendix.

2.2 MEMBRANE PREPARATION

Membranes for gas liquid chromatographic analysis of fatty acid, electron spin resonance, or any other determinations were prepared as follows.

(a) Cell harvesting

Petri dishes of confluent cells were harvested by washing twice with Dulbecco's phosphate buffered saline pH 7.4 (PBS) after removal of the growth medium by aspiration. The cells were scraped from the dish using a blunt spatula, and washed into centrifuge tubes with PBS. The cell pellet was collected after centrifugation (38,000 x g. for 20 mins. at 4°C). Cell pellets were stored at -20°C until required.

(b) Membrane preparation

A crude membrane preparation was made from the cell pellet by resuspending the pellet in PBS and sonicating the suspension for 15 seconds, on ice, with an MSE sonicator. The suspension was centrifuged (38,000 x g. for 20 mins. at 4°C). The membrane pellet was retained and stored at -20°C until required.

2.3 LIPID ANALYSIS

(a) Lipid Extraction

Cell or membrane pellets were collected in 15ml glass centrifuge tubes for lipid extraction. After decanting the supernatant, chloroform/methanol (2:1, v/v), (3mls) was added to each tube, which was vortexed to break up the pellet. After 10 minutes at room temperature, 0.1M potassium chloride (1.2mls) was added to each tube, and the tube vortexed. The tube was spun at full speed in an MSE bench centrifuge for 2 minutes to accelerate phase separation.

The upper aqueous phase was removed by aspirating along with cell debris and 0.1M potassium chloride/50% (v/v) methanol

(1.2mls) was added to each tube. The tubes were vortexed, centrifuged, and the aqueous phase removed. This was repeated. The chloroform phase was transferred to a glass screw cap tube and methanol was added dropwise until one phase was obtained. This was evaporated to dryness using oxygen-free nitrogen. The residue was dissolved in chloroform/methanol (1:1 v/v) and stored under nitrogen at -70°C for analysis. All solvents were Analar grade.

(b) Thin layer chromatography

Before an attempt could be made to modify the lipid composition of the C6 cells, a thin layer chromatography (t.l.c.) system had to be found which would resolve the transmethylation pathway phospholipids. An unsatisfactory result from 1-dimensional systems (Hirata et al. 1979b) prompted me to try 2-dimensional t.l.c.

(c) Preparation of t.l.c. plates

Glass plates (20 x 20cm) were washed, and all trace of grease removed by wiping with acetone. Silica G. (Merck) (60g) was mixed with "Florisil" (Merck) (1g) in a conical flask. "Florisil" aids in the resolution of acidic phospholipids. Distilled water (120mls) was added, and the flask was shaken well for 30 seconds. The slurry was applied to the clean glass plates, clamped in a Shandon casting stand, as a layer 0.4mm thick. The plates were allowed to dry for 1 hour before activation by heating in an oven at 100°C for 1 hour.

(d) T.L.C.

The activated plates were marked with 2 margins scratched

into the silica at right angles to each other, and 2cm from the edge of the plate. A lipid extract from control cells was spiked with PNME and PDME (Sigma) and applied to the plates. PDME and PNME alone were applied to other plates run in the same solvent systems, for comparison. The plates were then developed in tanks equilibrated with different solvent systems.

The plates were developed in the first dimension solvent system until the solvent front reached the margin. The plates were then removed and solvent was removed with a hot air stream. When the first solvent was removed, the plate was allowed to cool, turned at right angles to the direction of the first development, and developed in the second dimension system. When the solvent reached the margin, the plate was removed, blown dry, and retained for further analysis, or placed in a tank containing Iodine vapour to visualise the lipids. All lipids appeared as yellow spots. The plates were compared to determine whether a given 2-dimensional solvent system was capable of resolving PNME and PDME from endogenous C6 lipids.

(e) Solvent systems

1. (Brophy et al. 1978).

1st Dimension	Chloroform/methanol/water/Ammonia 35% (v/v) 58:35:5:2.5 (v/v)
2nd Dimension	Chloroform/acetone/methanol/glacial acetic acid/ water 40:15:13:12:18 (v/v)

This system resolved the endogenous lipids well, but failed to resolve PDME or PNME from PE.

2. (Maeda et al., 1980a).

1st Dimension	Chloroform/methanol/water 65:25:4 (v/v)
2nd Dimension	Butanol/acetic acid/water 6:2:2 (v/v)

PNME and PDME were resolved from the endogenous lipids, but PI was poorly resolved from PS.

3. A system I devised.

1st Dimension	Chloroform/methanol/water/ammonia 58:35:5:2.5 (v/v)
2nd Dimension	Propionic acid/n-propylalcohol/chloroform/water 2:2:1:1 (v/v)

This system resolved all the endogenous lipids well, as well as resolving PNME and PDME (Plate 1). It was used for all subsequent t.l.c.

(f) Characterisation of the t.l.c. system

To ensure that the resolved lipids were correctly identified, the identity of all the spots was confirmed by co-chromatography with authentic standards, and by the use of specific chemical tests.

The following chemical tests were employed:

(g) Zinzadze reagent

This identifies the spots due to phospholipid, and distinguishes them from neutral lipid (Plate 2).

(h) Ninhydrin stain

Lipids containing free amino groups (PS and PE) were identified using this reagent (Plate 3).

(i) Dragendorff reagent

A test specific for lipids containing choline groups. (PC, LPC and SM) (Plate 4).

(j) Periodate-Schiff's reagent

Lipids containing vicinal diol groups (PI, PG and glycolipids) were identified using this reagent. PG stained very weakly. (Plate 5).

(k) Ferric chloride stain

The red-violet spot in the top left-hand corner of

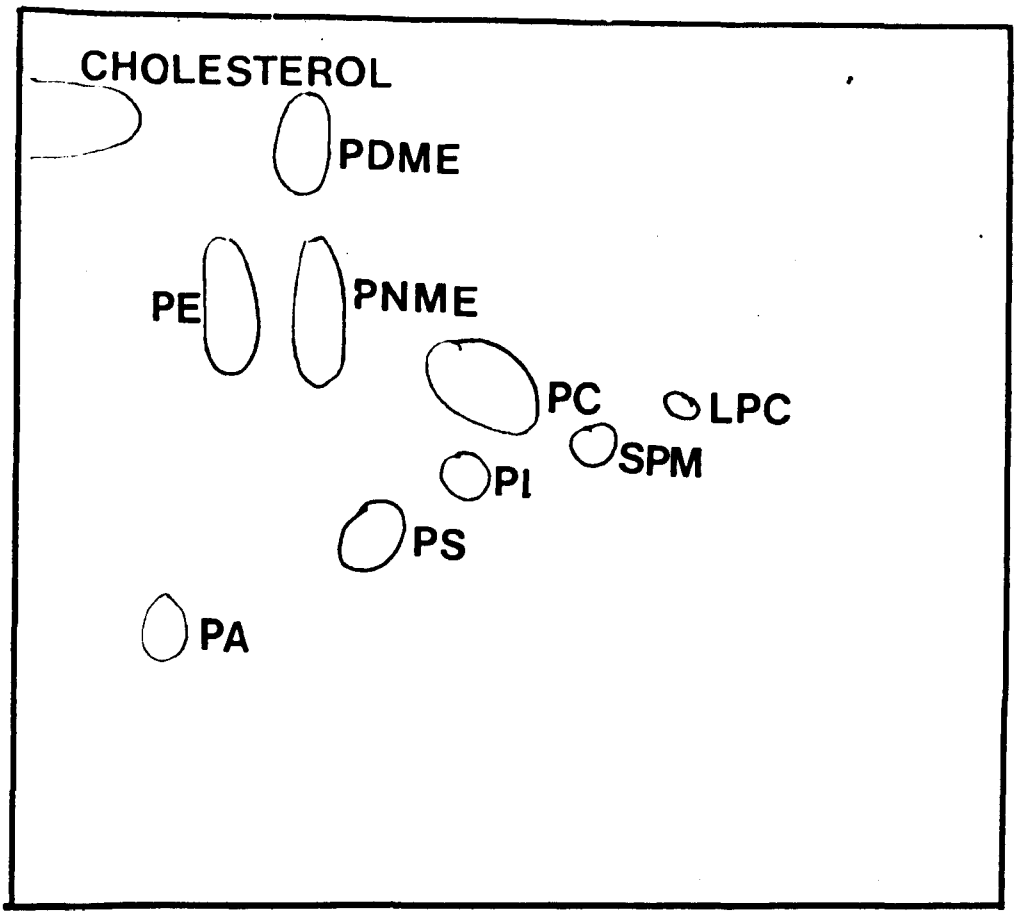


Plate 1: Lipids stained with iodine

Plate 2: Phospholipids stained with Zinzadze reagent

Plate 3: Phospholipids containing primary amino groups (Ninhydrin Stain)

Plate 4: Phospholipids containing choline (Dragendorff reagent)

Plate 5: Phospholipids containing vicinal diol groups (Schiff's reagent)

Plate 6: Cholesterol (Ferric chloride stain)

Plate 1:

Plate 2:

Plate 3:

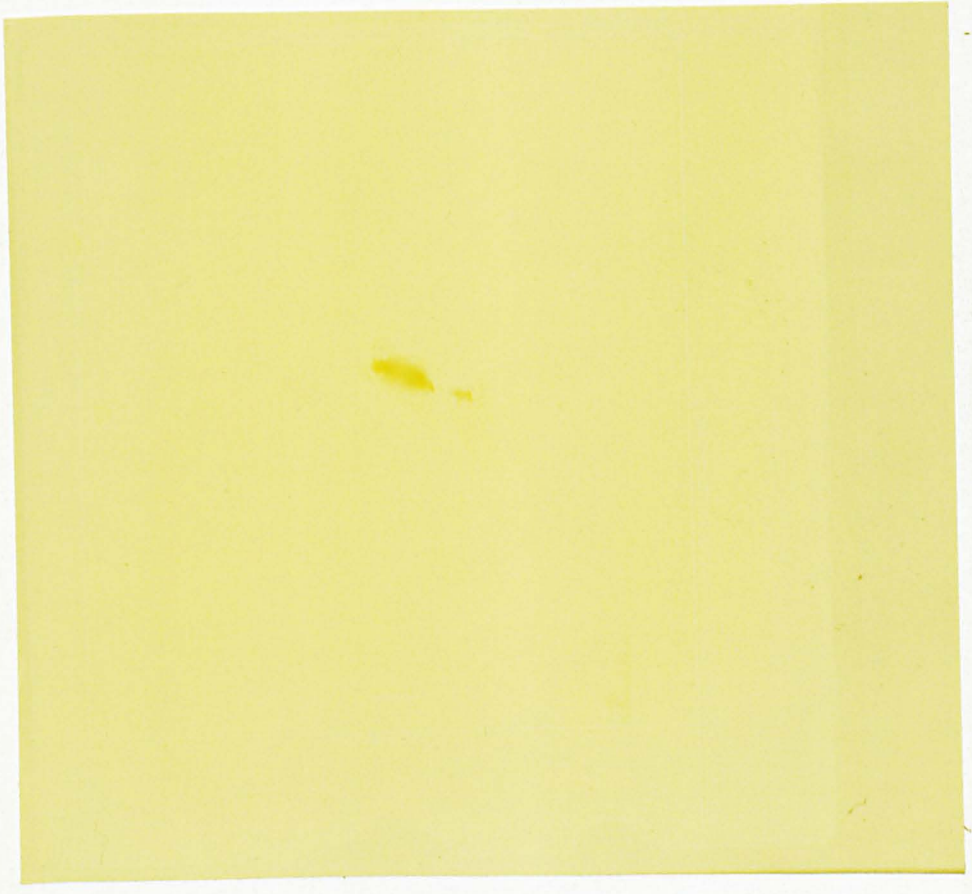


Plate 4:

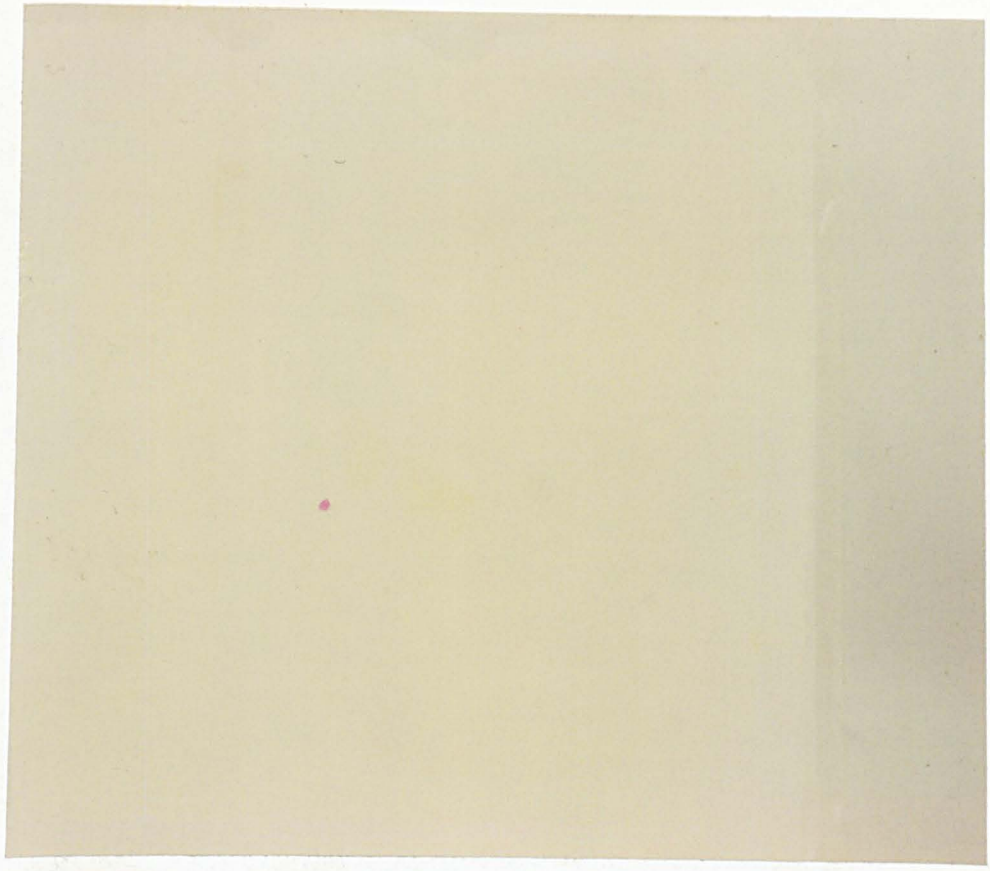


PLATE 5:

PLATE 6:

Plate 6 indicates the location of cholesterol and cholesterol esters on the t.l.c. plate.

Full instructions on the recipes for, and use of these reagents is given in the appendix.

(1) Phosphorus determination (Ref. Rouser et al. 1972)

Phospholipid content was determined by measuring the amount of phospholipid phosphorus present. This method was used for both lipid extracts and phospholipid spots on t.l.c. plates.

Thick wall glass test tubes were soaked for 2 hours in chromic acid, rinsed well with distilled water and dried. 50-100µl of lipid extract was added to the tubes and washed to the bottom with methanol, which was evaporated at 100°C. 70% (v/v) perchloric acid (650µl) was added to each tube. Acid was also added to sufficient tubes for the blank and standard curve. It is vitally important that all the solvent is evaporated otherwise an explosion may occur on heating with perchloric acid.

All tubes were then heated with marbles on top of the tubes, in a heating block (Tecam Dri-block DB-3H) for 1 hour at 180°C. Standards were not added until after the acid digestion.

Standards (30-400nmol) were added from a standard of 1mM KH_2PO_4. The final volume of all the tubes was brought to 3.95mls with distilled water. 0.5ml of 2% (w/v) aqueous ammonium molybdate was added to each tube and mixed well. Ascorbic acid (10%, w/v) was freshly prepared, and 0.5ml added to each tube. After mixing, the tubes were placed in a boiling waterbath for exactly 5 minutes each tube. No marbles were placed on top of the tubes.

The tubes were allowed to cool and the absorbances read, within 1 hour, at 800nm. The spectrophotometer was zeroed using the reagent blank.

The phosphorus content of spots from t.l.c. plates was measured by scraping the spots revealed by iodine staining into tubes and digested as before. A small amount of silica gel was added to the zero standard, since silica increases the absorbance. The protocol was followed as before. Before noting the absorbances, tubes containing silica gel were centrifuged for 5 minutes in a bench centrifuge. Aliquots were removed by pipette for absorbance measurements.

(m) Cholesterol Determinations

Two different methods were used to measure the cholesterol content of membrane lipid extracts. These methods detect free cholesterol only, but since cholesterol esters are present in only very minor amounts, I felt that for the purposes of these studies they could be disregarded.

I began by using a chemical method (Huang et al. 1961), but then switched to an enzymic method (Ott et al. 1982), since it was more sensitive. In either analysis, samples were assayed in triplicate.

(n) Free Cholesterol Assay (Ref. Huang et al. 1961).

This is essentially a modification of the standard Liebermann-Burchard method.

Reagents

Concentrated sulphuric acid (10mls) was added dropwise to acetic anhydride (60mls) and stirred, ensuring that the

temperature did not rise more than 10°C. Glacial acetic acid (30mls) was added and anhydrous Na_2SO_4 was dissolved in the mixture. Care must be taken in the addition of sulphuric acid, rapid addition will result in the glass vessel shattering. The reagent was found to be stable for two weeks.

A standard was made by dissolving cholesterol (Sigma) in 99% (v/v) ethanol to give a 1mg/ml solution.

Procedure

Lipid extracts and standards (20-200µg cholesterol) were evaporated to dryness using nitrogen, and the residue dissolved in 200µl of glacial acetic acid. The reagent (5mls) was added, and the tubes incubated for 20 minutes at 25°C. The absorbances were measured at 610nm, and a standard curve constructed.

Colour stability of the assay was poor, and colour begins to fade after 30 minutes. A spectral scan (550-620nm) indicated the λ_{max} to be 610nm. The assay was found to be linear up to 500µg cholesterol, but as can be seen in fig.5 the sensitivity is low, which is why the enzymic method was adopted. The possibility of phospholipid interference was tested for by spiking unknown samples with cholesterol and checking that the increment in cholesterol content was correct compared with unspiked controls. No trace of interference by phospholipid was noted for either the chemical or enzymic methods.

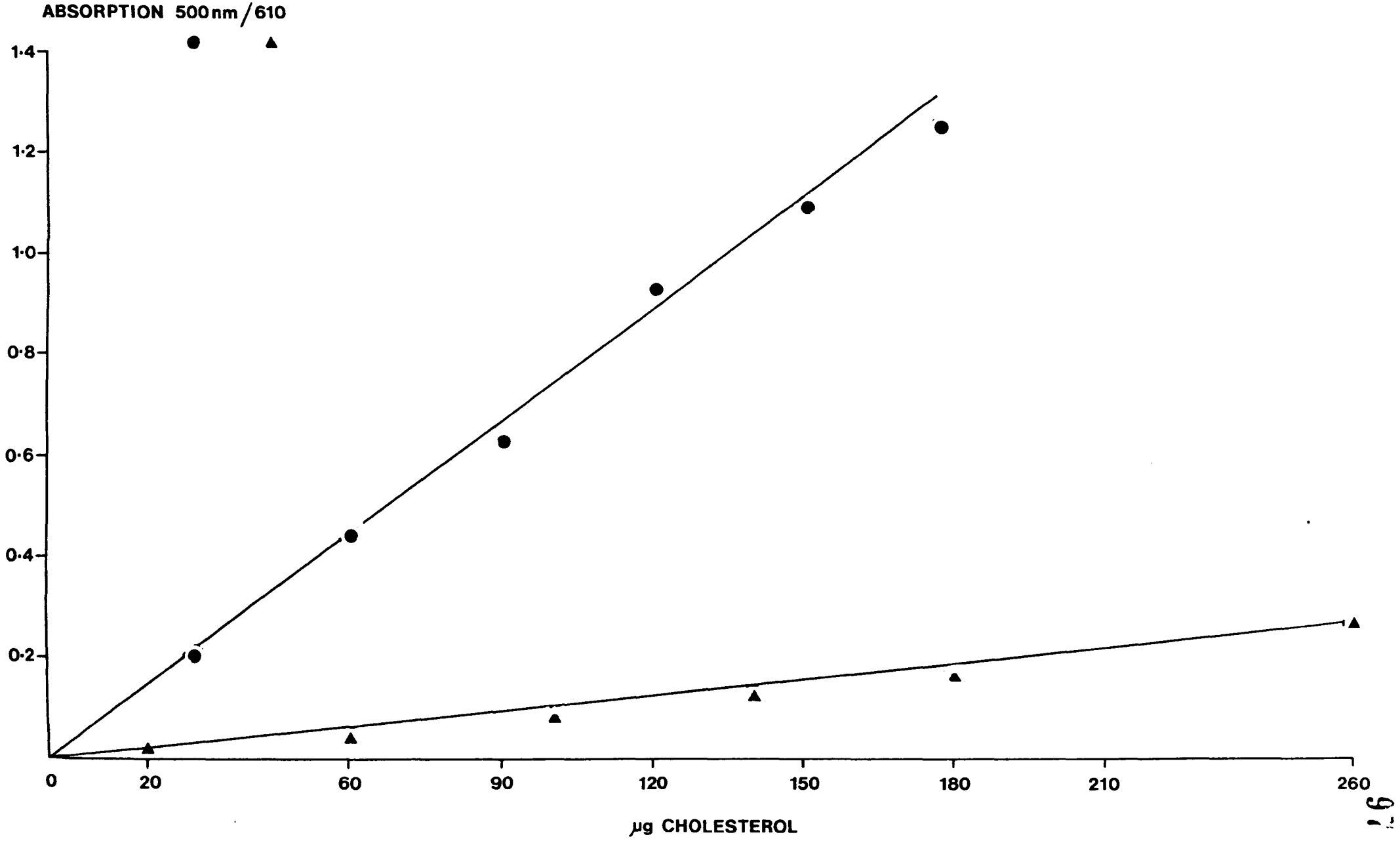
(O) Enzymic Assay (Ref. Ott et al., 1982).

A cholesterol oxidase kit (Cholesterol C-system, CHOP-PAP method) was purchased from Boehringer Mannheim Gmbh Diagnostica, and employed as in the method of Ott et al. (1982).

Fig.5: Cholesterol assay calibration curves

● = Enzymatic method (Ott et al., 1982)

▲ = Chemical method (Huang et al., 1961)


ABSORPTION 500nm/610
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0
20
60
90
120
150
180
210
260
µg CHOLESTEROL

The reagent was made up according to the manufacturer's instructions, but Triton X-100 was added to a concentration of 0.5% (v/v).

Aliquots of membrane lipid extracts and cholesterol standards in ethanol were dried down under nitrogen in the assay tubes. Reagent (2mls) was added to each tube, the tubes mixed, and incubated at 20°C for 30 mins. Absorbances were read at 500nm, and a standard curve constructed. The useful range of the assay was 20µg-150µg of cholesterol. Colour remained stable for at least one hour.

Calculation of cholesterol content

Cholesterol content was converted from µg to µmols to allow cholesterol:phospholipid ratios to be derived. The molecular weight of cholesterol was taken to be 386.

(p) Fatty acid analysis (Nordoy and Lund, 1968).

Fatty acid analysis on lyophilized membranes was done by a modification of the method of Nordoy and Lund (1968). Fatty acid methyl esters were prepared by the addition of methanol (4ml) containing H_2SO_4 (1.5% v/v) and butylated hydroxy toluene (0.01%, w/v) to a dry membrane sample (1.5-2mg protein). After incubation at 80°C for 1 hour under argon, the fatty acid methyl esters were extracted three times with 3ml of light petroleum (b.p. 35°-60°C) containing butylated hydroxy toluene (0.01%, w/v). The pooled extracts were washed twice with 2ml of $KHCO_3$ solution (2%, w/v). The extract was then dried with anhydrous Na_2SO_4 and evaporated under argon.

Gas liquid chromatography

The methyl esters were dissolved in diethyl ether and

identified by chromatography on a Pye Model 104 gas liquid chromatograph equipped with a column containing 15% EGSS-Y on Diatomite CQ (Field Instruments Co. Ltd., Middx) at 194°C or one packed with 15% EGSS-X on Diatomite CQ (Field Instruments Co. Ltd., Middx.) at 178°C. The fatty acid methyl esters were identified by comparison of their retention times with those of standards (Chromatography Services, Merseyside).

Quantification was done by triangulation. The peaks were triangulated, and the peak area calculated by multiplying the height by the width at half the peak height. Fatty acid composition was expressed as the % of the total area that each individual peak occupied.

2.4 PROTEIN CONTENT AND COMPOSITION

(a) Protein Assay (Ref. Lowry et al., 1957).

This method is a modified version that includes sodium dodecyl sulphate (SDS) in the incubation mixture to solubilise membrane proteins.

Protein samples were sonicated to ensure homogeneity, and pipetted into assay tubes in duplicate. The sample volume was made up to 200µl. A standard was prepared by making a 1mg/ml solution of bovine serum albumin (Fraction V, Sigma) in the same buffer as the samples were in. Standards (30-200µg) were added to the assay 250µl of 1M NaOH/0.25% (w/v) SDS was added to each tube and the tubes were incubated for 15 minutes at 60°C. Reagent B (2.5mls) was added to each tube, mixed and incubated for 15 minutes at room temperature. The composition of reagent B is given in the appendix.

Finally 250µl of Folin-Ciocalteu reagent (B.D.H.) freshly diluted (1:1) v/v with distilled water, was added and the tubes mixed. The tubes were left to stand for 45 minutes at room temperature, and the absorbances read at 750nm.

(b) SDS Polyacrylamide gel electrophoresis (Ref. Laemmli, 1970).

Analysis of membrane protein composition was performed by S.D.S. gel electrophoresis in 12% acrylamide slab gels. For recipes for gel preparation, see appendix.

Membrane preparations from C6 cells (0.7mg protein) in 250µl of PBS were pelletted by centrifugation at 40,000 x g for 20 minutes at 4°C. To the resulting pellet was added 160µl of 4% (w/v) SDS, 20µl of 2-mercaptoethanol, and 80µl of sample buffer. The mixture was heated at 100°C for 5 minutes in a boiling waterbath. 40µl of the mixtures containing the

samples were added to the sample wells in the gel. The running conditions are detailed in the appendix.

2.5 PHYSICAL STUDIES

(a) Polarisation of fluorescence of 1,6,-diphenyl-1,3,5,-hexatriene (Ref. Shinitsky and Barenholz, 1974).

Reagents and procedure

A crude membrane preparation made as described earlier. 1,6-Diphenyl-1,3,5,-hexatriene (DPH, Sigma) was made up as a 200µM stock solution in tetrahydrofuran.

The membrane preparation was resuspended in buffer, containing 150mM NaCl, 5mM $MgCl_2$ and 100mM Tris-HCl pH 8.0, to a protein concentration of 2mg/ml. DPH stock solution (25µl) was added, the tube mixed and incubated in the dark for 1 hour at 37°C. Duplicates were run for each sample.

The membrane suspensions were removed at the end of the incubation period, washed once with incubation buffer, and pelletted by centrifugation at 40,000 x g for 15 minutes at 4°C. The membrane pellets were resuspended in incubation buffer to a final protein concentration of approximately 0.1mg/ml, and used for polarisation of fluorescence measurements.

Spectrofluorimetry

The measurements were made on a Perkin Elmer MPF-3 fluorescence spectrophotometer fitted with a Hitachi polarisation accessory. The sample cell was maintained at 27°C for these measurements.

The optimum settings for slit widths, excitation and emission wavelengths were obtained by following the instructions in the instrument manual. Excitation and emission wavelengths used were 360 and 470nm respectively. The excitation slit width used was 5nm, and the emission slit width was 10nm.

The excitation and emission spectra obtained are

illustrated in figs. 19 and 20 . In the excitation spectrum, a peak was observed at 700nm. This was suspected to be artefactual. A scan of the absorbance spectrum of DPH (fig.21) revealed no absorbance anywhere near that wavelength.

Polarisation values

The steady state polarisation values (P) were calculated using the following equation:-

$$P = \frac{(0,0) - t(0,90)}{(0,0) + t(0,90)}$$

The notation refers to the orientation in degrees of the two polarising filters with respect to each other. The first figure in the brackets refers to the orientation of the filter over the emission slit. Thus, one can measure the fluorescence parallel to the electric vector of the plane polarised light emission (0,0) and the fluorescence perpendicular to that vector (0,90).

The term t is a constant to take into account light scattering within the machine, and is calculated from:-

$$t = \frac{(90,0)}{(90,90)} .$$

Electron spin resonance studies

(b) Studies on membranes

Stearic acid with the 4,4-dimethyl-3-oxazolidynyloxy (doxyl) group attached to either carbon 5 (5-doxylstearic acid) or 16 (16-doxylstearic acid) was purchased from Syva Assoc., California. Stock 0.86mM solutions were prepared in ethanol. Spin-labelled stearic acid was incorporated into membrane

preparations by first drying the spin label (4.4nmol) at the bottom of small glass test-tube to which was added 100µl of membrane suspension (1µmol phospholipid). After incubation for 10 minutes at room temperature the membranes were removed and centrifuged at 35000xg for 30 minutes. The membrane pellet was resuspended in 50µl of 100mM NaCl/1mM EDTA/2mM Hepes (pH 7.4) and transferred to a capillary for electron spin resonance (ESR) measurements.

ESR spectra were recorded with a JEOL JES-PE-1X or a Varian E3 spectrometer equipped with a nitrogen flow temperature control system. The temperature of the sample was monitored with a thermocouple placed close to the sample. The order parameter (S) can be calculated for 5-doxylstearic acid undergoing rapid motion from the equation:-

$$S = \frac{(A_{//} - A_{\perp})}{A_{zz} - A_{xx}}$$

where $A_{//}$ and $A_{\perp}$ are half the separation of the outer and inner hyperfine splittings (fig. 6) respectively and A_{zz} and A_{xx} are the hyperfine splitting tensors in the z and the x direction respectively. A value of 26.3G was taken for $A_{zz}-A_{xx}$ (Schreier et al., 1978).

The correlation time (τ) was calculated for 16-doxylstearic acid undergoing rapid isotropic motion from the equation:-

$$\tau = 6.5 \times 10^{10} W_o \left[\sqrt{\frac{h_o}{h_{-1}}} - 1\right] \quad \text{(Kivelson, 1960)}$$

where W_o is the peak to peak width and h_o is the height of the centre line and h_{-1} is the height of the high field line (fig. 7).

Fig.6: Electron spin resonance spectrum of 5-doxylstearic acid incorporated into C6 cell membranes

$2A_{\parallel}$ = Outer hyperfine splitting

$2A_{\perp}$ = Inner hyperfine splitting

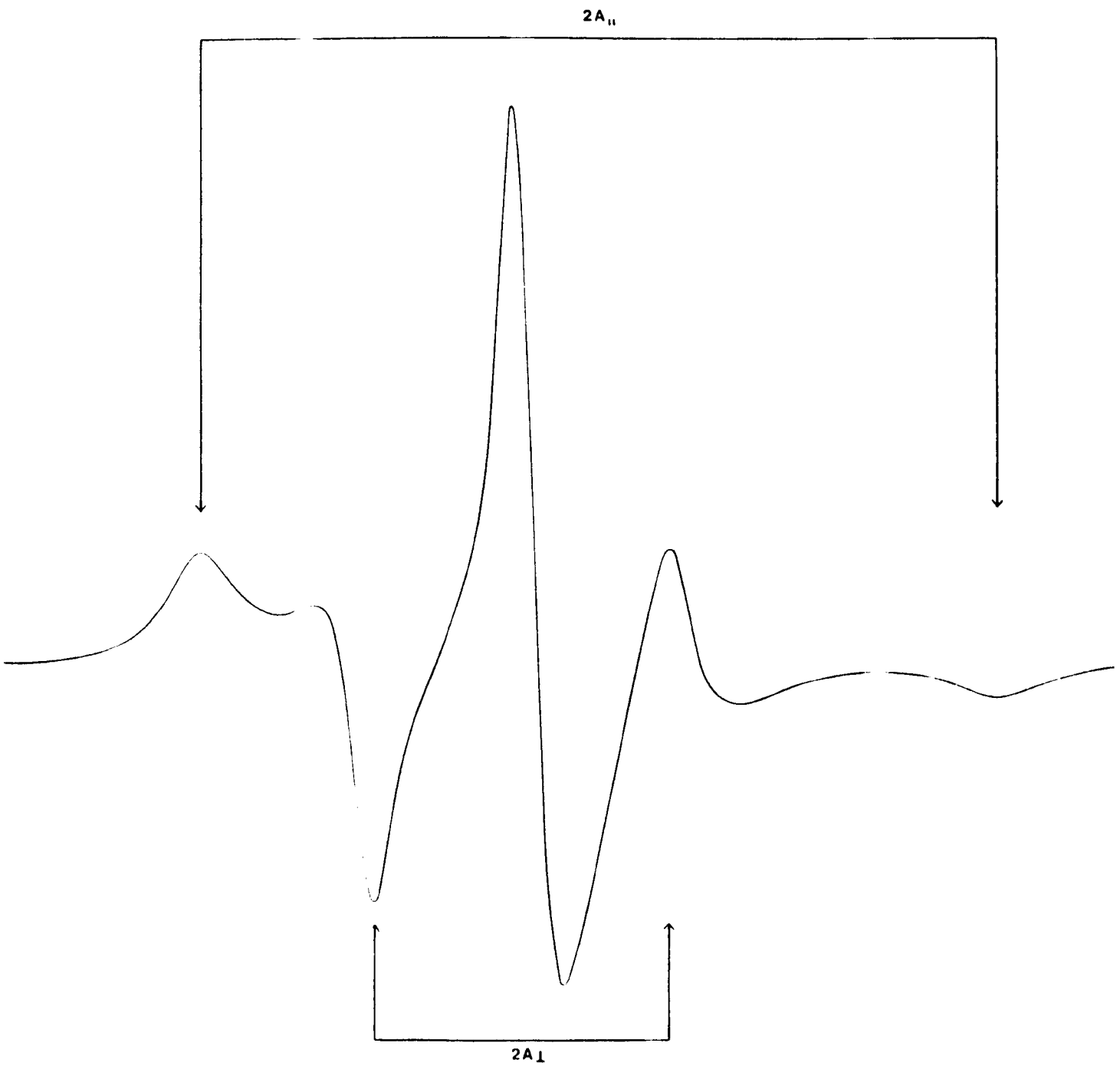
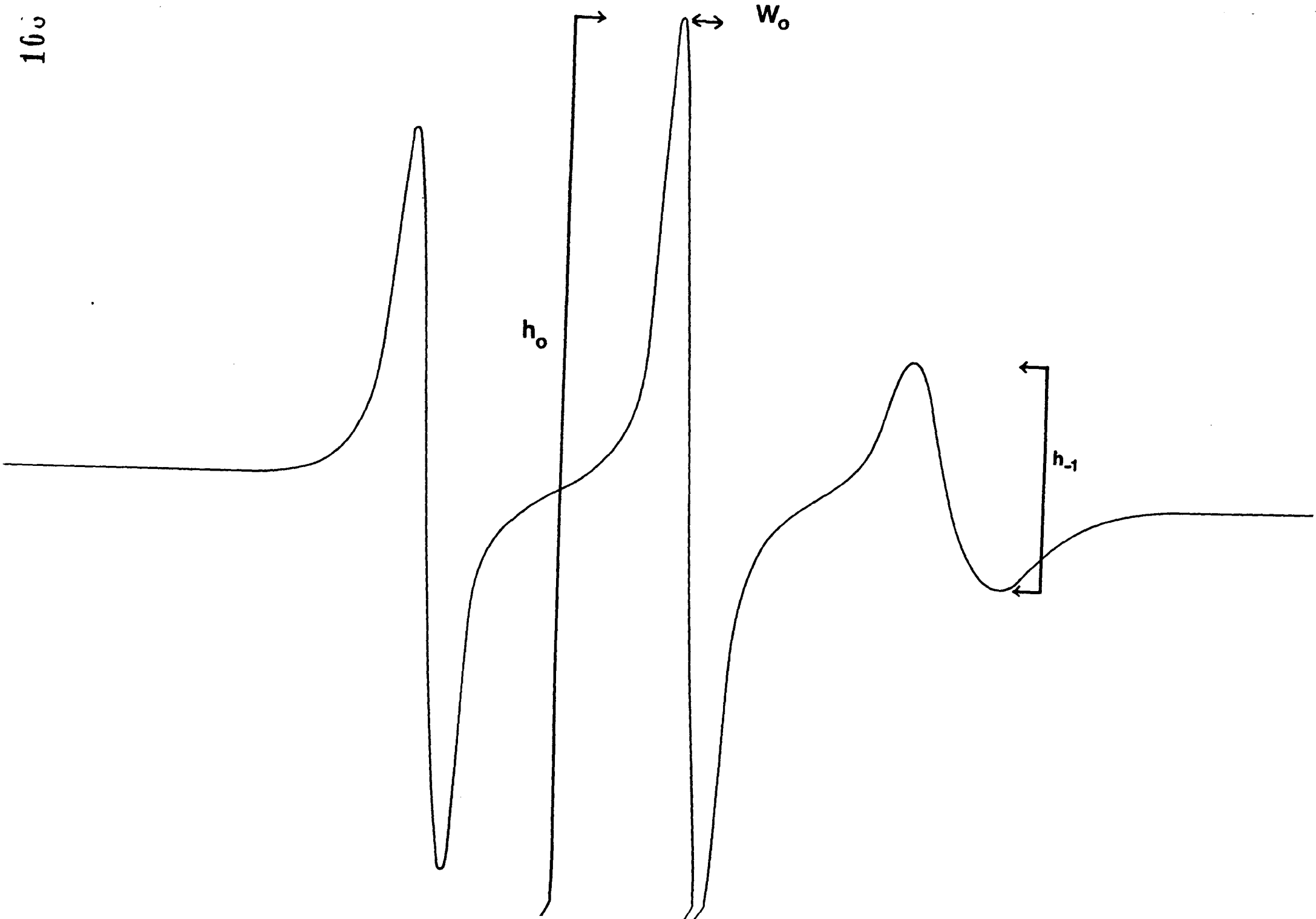

2A||
2A⊥

Fig.7: Electron spin resonance spectrum of 16-doxylstearic acid incorporated into C6 cell membranes

W_0 = peak to peak width

h_0 = height of the centre line

h_{-1} = height of the high field line


W_o
h_o
h_-1

(b) Live cells

The cells were grown in petri dishes (9cm diameter, 1.6-2.0mg protein). Cells were incubated in Medium 199 (10ml) for 25 minutes and incubated for a further 30 minutes in the presence of the drugs. 10 minutes before the end of the incubation in the presence of the drugs, 3µl of an ethanolic solution of 5-doxyl stearic acid (5-(4',4'-dimethyl-oxazolidine-N-oxyl) stearic acid) (50µg, Syva Research Chemicals) were added. Cells were harvested by scraping with a rubber policeman, centrifuged at 1000 rpm for 5 minutes and then transferred to a capillary for the electron spin resonance measurements. Spectra were recorded with a JEOL JES-PE-1X spectrometer equipped with a temperature control system. In order to minimize internalization and destruction of the spin-labelled fatty acid, spectra were recorded at 30°C. There was no significant contribution to the spectra from unincorporated label and repeat scans on the same sample showed little diminution in signal intensity over a 15 minute period. The molecular order of the spin-labelled fatty acid was assessed by measuring the hyperfine splitting of the outer extrema of the electron spin resonance spectra (Hubbell and McConnell, 1971; Pang, 1980).

2.6 CYCLIC AMP ASSAYS (Ref. Brown et al. 1971)

A competitive protein binding assay was used to determine cAMP. The binding protein was prepared from fresh specimens obtained from the slaughterhouse. The procedure for the assay is essentially that of Brown et al. (1971) with some modification.

(a) Cell culture

C6 cells were subcultured from confluent 90mm dishes. For basal cAMP determinations the contents of one plate were split between five other plates and grown for 2 days, after which they were incubated in the presence of various bases for 24 hours. Final protein content was 1.40-2.0mgs/dish.

Cell cultures for isoproterenol stimulation were established by passaging the contents of one 90mm dish between 30mm diameter dishes. The initial cell density was 0.8-1.2 x 10^6 cells/well. These cultures were allowed to grow for 2-3 days, before a further 24h growth in medium containing the bases. The final protein content was 0.8-1.1mg protein per cell. Triplicate dishes were established for each condition.

The cells were washed twice with pre-warmed (37°C) medium 199, and 3mls of 199 were added per 30mm dish, or 10mls to a 90mm dish.

Isoproterenol HCl (200μM) was freshly prepared in distilled water and added to the cultures to a final concentration of 10μM. The cultures were replaced in the incubator for 30 minutes, unless specified otherwise.

Incubations were stopped by washing the cells with ice-cold saline (0.9%) w/v, and the cAMP extracted with 0.1M HCl (1ml). After 30 minutes the acid extract was removed and lyophilized. The cAMP content was measured by the protein binding method. The protein content of the cellular residue was measured by the method of Lowry et al. (1957), after precipitation with 10% (w/v) trichloroacetic acid.

(b) Preparation of binding protein

Bovine adrenal glands were obtained from a local slaughterhouse. The glands were removed from the carcase within 20-30 min of death, and brought to the laboratory on ice.

The adrenal cortices were dissected and scraped free of fat and connective tissue. The cortices were chopped and homogenized in 1.5 volumes of ice-cold Littlefield's medium (0.25M sucrose, 25mM KCl, and 5mM $MgCl_2$ in 50mM Tris-HCl, pH 7.4). The homogenate was centrifuged at 1250xg for 15 min at 4°C. The pellet was discarded, and the supernatant was centrifuged at 5000xg for 15 mins at 4°C. The supernatant was decanted and strained through muslin to remove fat droplets. The filtrate was stored in aliquots (1ml) at -20°C. It was found that binding capacity of the preparation increased over a period of months upon storage at -20°C.

Binding protein was serially diluted and assayed for its ability to bind cAMP in the absence of added cold cAMP. A binding protein dilution was chosen such that the zero standard would bind 50% of the added tracer (Chard, 1978) to optimise the sensitivity of the assay. Each new batch of binding protein was tested in this way before use in the assay.

(c) Assay procedure

To each tube 625 nCi of [8-^{3}H]-3': 5' cyclic AMP (79 mCi/mg), Amersham, was added in 50µl of assay buffer (50mM Tris-HCl pH 7.4 containing 8mM theophylline, and 6mM 2-mercaptoethanol).

Standards (0-15pmol of cAMP) were added from a stock solution (100pM) in assay buffer containing 1mM sodium azide.

The lyophilized acid extracts to be assayed were

dissolved in assay buffer and an aliquot taken (30-150µl) for assay. The volume in all tubes was brought to 200µl with assay buffer. Samples and standards were run in triplicate.

The total radioactivity added to the assay tubes was ascertained by determining the radioactivity in an aliquot (100µl) from tubes containing 50µl of tracer and 150µl of buffer. No binding protein or charcoal was added to these tubes. An appropriate dilution of the binding protein was made, and binding protein (100µl) added to each tube, and vortexed. The tubes were covered, and incubated at 4°C for 1½ hours.

A charcoal suspension was made containing BDH charcoal (0.5g) in assay buffer (5mls) containing 2% (w/v) bovine serum albumin (Fraction V, Sigma). The suspension was stirred constantly for at least 30min before use. At the end of the incubation, 100µl of the suspension was added to each tube, the tubes were mixed and centrifuged (7000xg for 20 min at 4°C) within 3 min of addition of charcoal.

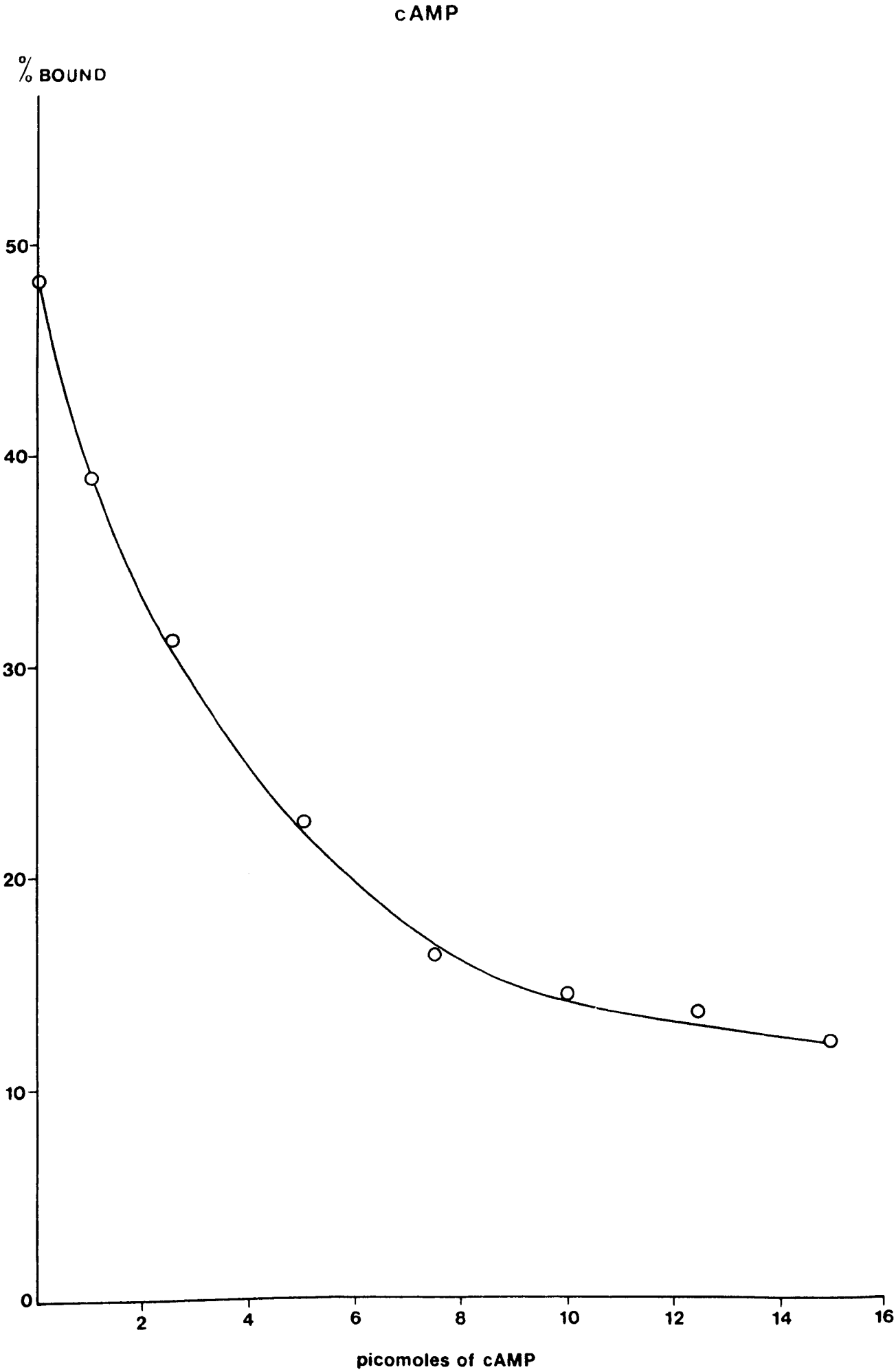
An aliquot (200µl) of the supernatant was taken and added to 'Fisofluor' cocktail (5ml) and the radioactivity was determined. The radioactivity in the total counts tubes was determined from a 100µl aliquot.

The bound fraction (% bound) was calculated as follows:-

$$\frac{\text{total radioactivity in standard or sample}}{\text{total radioactivity added}} \times 100$$

A calibration curve (fig. 8) was obtained by plotting the percentage of radioactivity bound in the standards against pmols cAMP. Values for cAMP content of the samples were derived from this curve. In practice the steepest, and therefore most

Fig.8: Calibration curve for cAMP assay (Brown et al., 1971)


cAMP
% BOUND
50
40
30
20
10
0
2
4
6
8
10
12
14
16
picomoles of cAMP

sensitive part of the curve was found to be between 1-10 pmols.

(d) Theophylline experiments

Cell culture was as described before, but during the incubation the medium 199 contained 1mM theophylline (Sigma), which had previously been made up fresh as a 20mM solution, the pH of which had been adjusted to 7.0 with NaOH. All other procedures were unchanged.

2.7 LIPID METHYLATION STUDIES

(a) Cell culture

Cultures were established in six-well Linbro trays, which have 6 x 35mm diameter wells. It was important for the methylation experiments to have a comparable cell density. To this end standard plating densities were used. The cells from one confluent (90mm) dish were passaged into four Linbro trays by adding 3mls of cell suspension (0.2-0.5 x 10^6 cells/ml) to each well. The cultures were grown for 2 days or until the gaps between the cell bodies had just begun to close, before a 24h incubation with base-containing medium.

(b) Lipid methylation assay

The cells were washed twice with medium 199 pre-warmed to 37°C. Medium 199 (1ml) was added to each well with 20µl of [methyl-^{3}H] methionine (85Ci/mmol).

One Linbro tray was used for each treatment. 4 wells were used for methylation measurements, and 2 wells were used for protein determinations.

All trays were replaced in the incubator for 25min to equilibrate the label with intracellular methionine. The unstimulated cultures were left in the incubator. Cells for drug treatments were briefly removed, the drugs were added, and the cells replaced in the incubator for a further 30min.

At the end of the incubation, the medium was removed, and the cells were washed twice with ice-cold 0.9% (w/v) saline. 1ml of 10% trichloroacetic acid (w/v) was added to each well to terminate the reaction.

The cells were either stored frozen on the trays at -70°C,

or scraped from the wells, using a spatula, into glass centrifuge tubes. After centrifugation, 35000xg for 20 minutes at 4°C, the radioactively-labelled lipids were extracted from the cell pellet. Protein determinations were carried out on the remaining two wells by the method of Lowry et al. (1957).

The lipid extract was either dried at 100°C prior to addition of liquid scintillation fluid (10ml), or subjected to analysis by t.l.c.

The location of radioactive methyl groups was determined by scraping lipid-containing spots, visualized by iodine staining, into scintillation vials and adding a suitable cocktail. Radioactivity in either case, was determined by liquid scintillation spectrometry.

(c) Methionine uptake

Cultures in Linbro trays were established, some were treated with medium containing base analogues for 24h. Three wells of the six well tray were used for methionine uptake determinations, and 2 wells were used for protein determinations. All wells were washed twice with medium 199, and medium 199 (1ml) was added to each well. To each of the 3 wells was added 20μCi of [methyl-^{3}H] methionine (85 Ci/mmol, Amersham), and the cultures were returned to the incubator. At the end of the different incubation periods, the wells were washed twice with ice-cold 0.9% (w/v) saline, after removal of the medium 199. The contents of methionine-treated wells were scraped into test tubes in a small volume (2 x 0.5ml aliquots) of saline. NCS tissue solubilizer (3ml, Amersham) was added to each tube and the tubes were

heated, with marbles closing their mouths at 45°C for 30min.

The contents of each tube were tipped into a scintillation vial, and the incorporation of radioactivity was determined.

RESULTS

"There are spaces
where infringements are possible.
There are notices that say:
Trespassers will be welcome."

Norman MacCaig.

3.0 CELL CULTURE

Initial studies were conducted with the aim of obtaining satisfactory cell growth on the minimum amounts of foetal calf serum. The purpose of this was twofold: first, serum is extremely expensive, and it was hoped that less could be used, secondly, it has been shown that the number of β-adrenergic receptors in C6 cells is reduced by the presence of serum (Dibner and Insel, 1981).

The effect of different concentrations of serum on cell growth is displayed in Fig.9. 5% (v/v) Foetal calf serum appeared to give the best cell growth. I decided that the benefits of reduced serum usage would be outweighed by decreased growth rates, and consequent increases in medium consumption. An attempt to substitute for serum was made by using Iscove's modified Dulbecco's medium, obtained from Flow Labs (Iscove, 1978). This medium was supplemented with: 100mg/litre Soybean lecithin, 1mg/litre transferrin, and 100mg/litre bovine serum albumin. This supported C6 growth in the absence of serum, but at slower rates, so I decided to continue using 5% (v/v) foetal calf serum in Dulbecco's modified Eagle's medium.

3.1.0 GROWTH OF CELLS IN MEDIA CONTAINING POLAR HEAD GROUP ANALOGUES

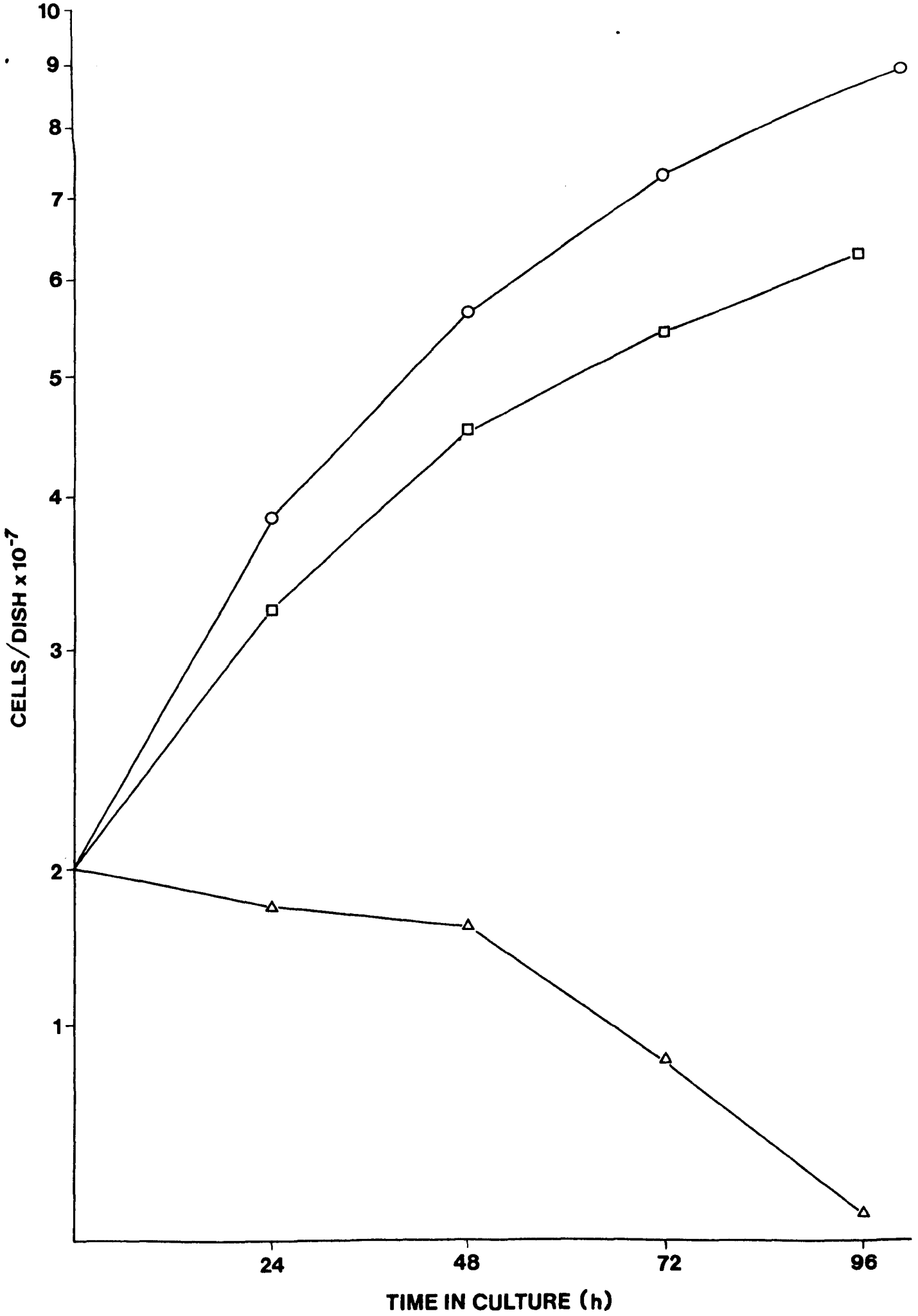
In order to modify the phospholipid polar headgroup composition, cells were grown in media supplemented with bases as described in section 2.1. I found that bases added to a final concentration of 5mM in the growth medium resulted in rapid and extensive changes in the phospholipid polar headgroup composition without any evidence of toxic effects on the cells. After 24h

Fig.9: Dependence of rates of C6 cell multiplication on the concentration of foetal calf serum in the growth medium.

O = Cells grown in 5% (v/v) serum

□ = Cells grown in 1% (v/v) serum

Δ = Cells grown in media without serum


10
9
8
7
6
5
4
3
2
1
CELLS/DISH x 10⁻⁷
24
48
72
96
TIME IN CULTURE (h)

growth in the presence of 5mM DME approximately 30% of the total phospholipid phosphorus consisted of PDME (see Table 5).

These results were similar to those presented by Finkel and Volpe (1979) for the incorporation of DME into PDME. The rate of cell growth is illustrated in Fig.10. Initially the polar headgroup analogues caused a slight decrease in growth rate relative to the control. This lower growth rate was only maintained in the cells grown in the presence of ethanolamine.

Cells grown in the presence of DME and NME reached stationary phase at a cell density comparable to that of the controls, although after 4 days the growth rate of the treated cells declined rapidly. Most experiments never continued longer than 2 days in the base-containing medium, and most involved exposure of the cells to bases for only 24h.

3.1.1 Effect on Cell Morphology

Plate 8 shows cells grown in DME-supplemented medium. After 24h, the cells became rounded and lost their extended processes; gaps between the cell bodies were observed in what were previously continuous monolayers. No such effect was found with either NME or ethanolamine, but photomicrographs of single cells indicated that a loss of processes had occurred, although not to the same extent as with DME treatment (Plates 7-10).

3.1.2 Haematoxylin-Eosin Staining of Base Treated Cells

Since morphological changes appeared to occur, I was interested to see if the base treatments were causing any effects on the microscopic structure of the cells. Haematoxylin-Eosin staining was employed to determine whether any changes in the relative size of the nuclear components and cytoplasm had

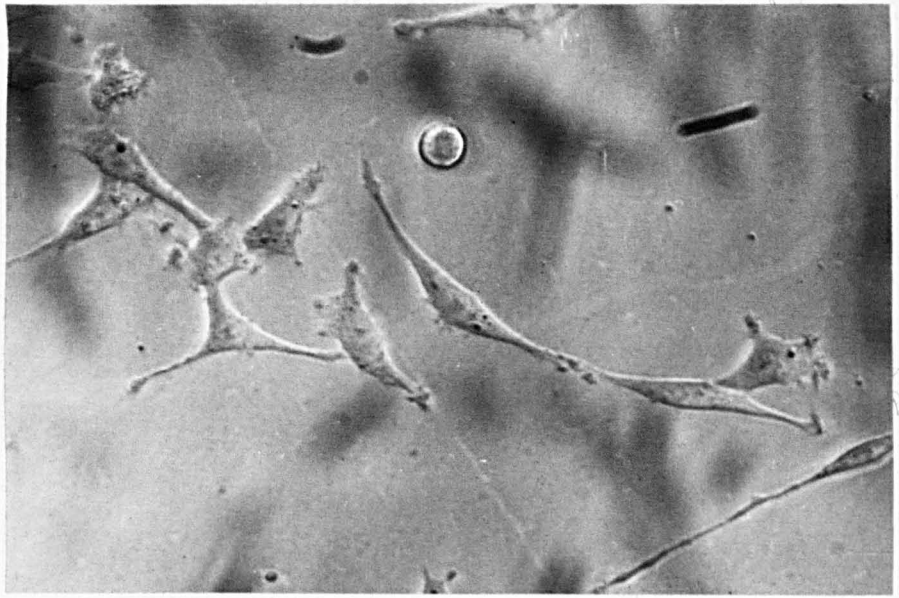


PLATE 7

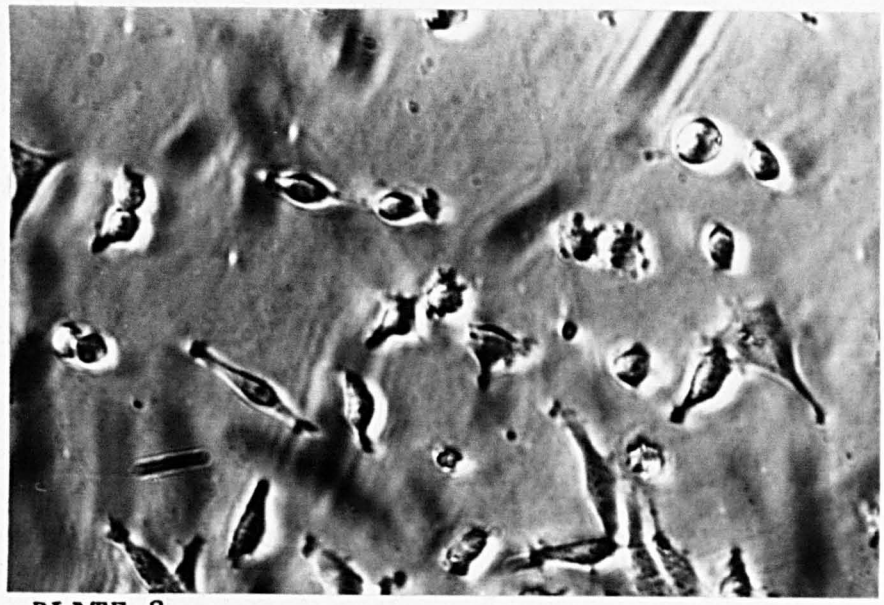


PLATE 8

PLATE 9

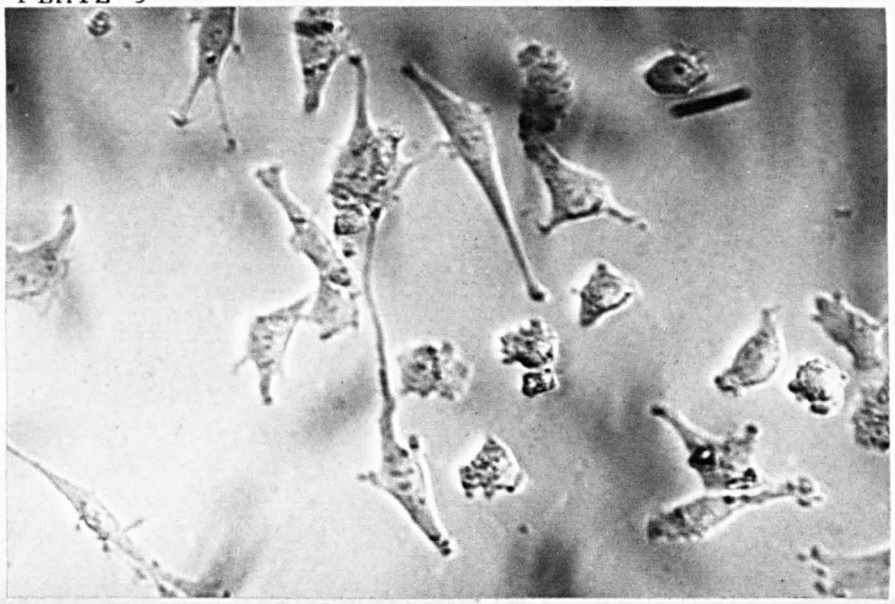
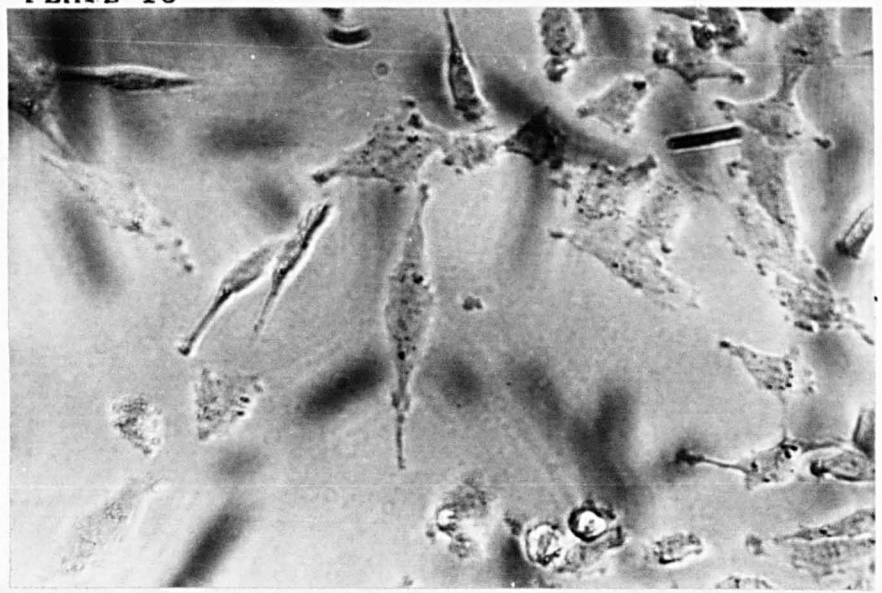


PLATE 10



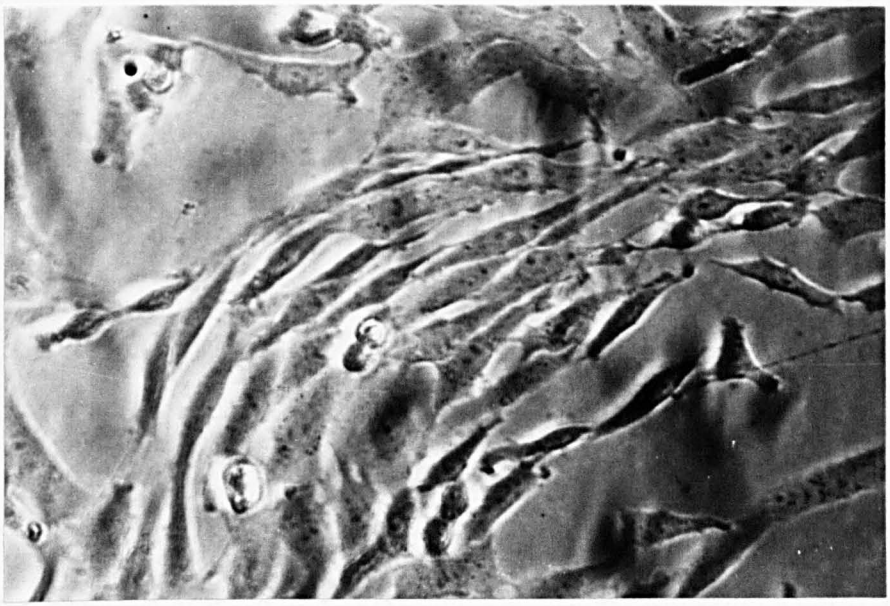


PLATE 11

PLATES 7-11: C6 CELLS VIEWED BY PHASE-CONTRAST MICROSCOPY X200

PLATE 7: Control Cells

PLATE 8: Cells exposed to 5mM DME for 24h

PLATE 9: Cells exposed to 5mM NME for 24h

PLATE 10: Cells exposed to 5mM Ethanolamine for 24h

PLATE 11: Cells exposed to 250mM Dimethysulphoxide for 72h

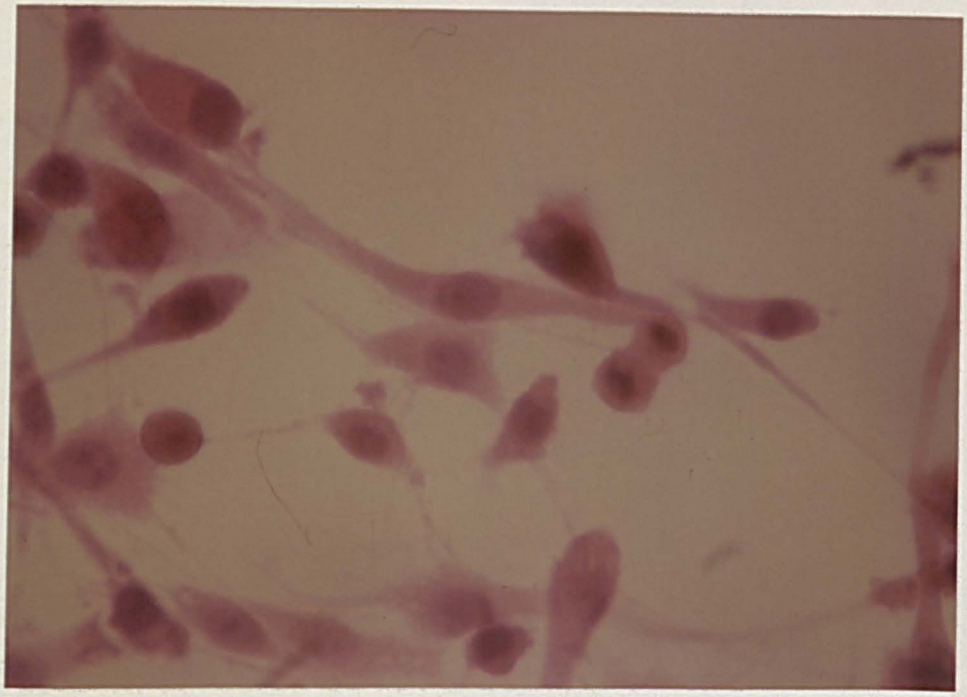


PLATE 12: C6 cells stained with Haematoxylin-Eosin X500.

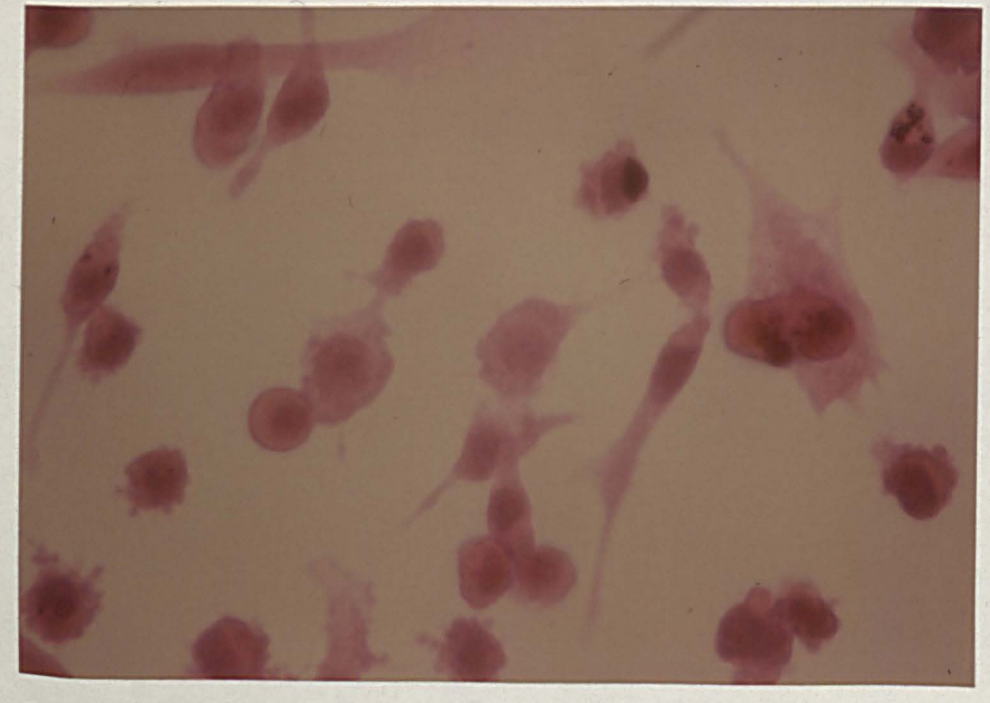


PLATE 13: C6 cells in media containing 5mM DME, grown for 24h stained with Haematoxylin-Eosin X500.

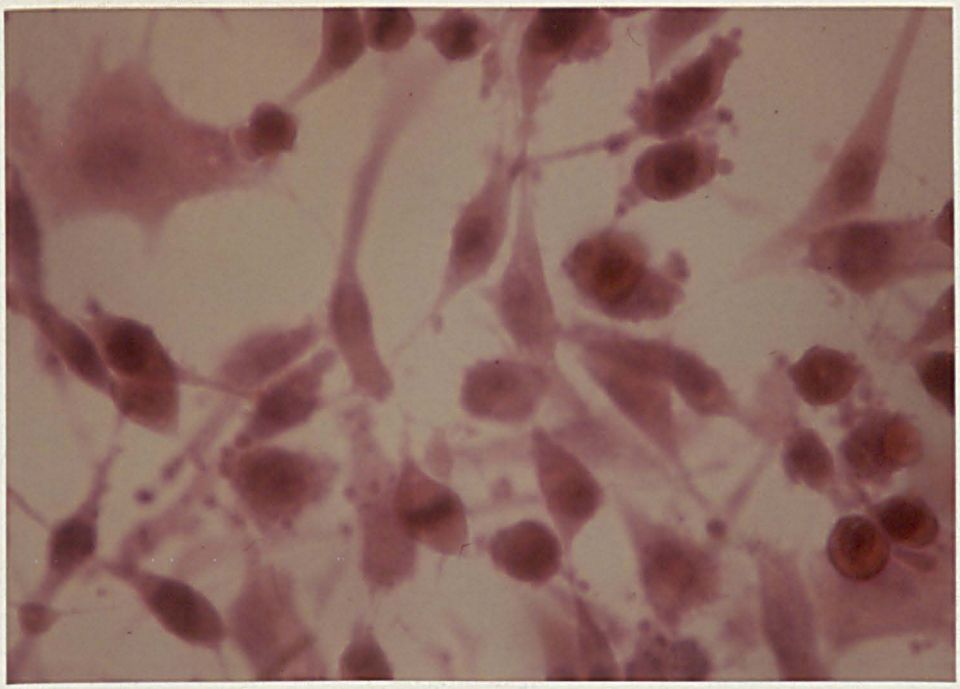


PLATE 14: C6 cells grown in media containing 5mM NME for 24h and stained with Haematoxylin-Eosin X500.

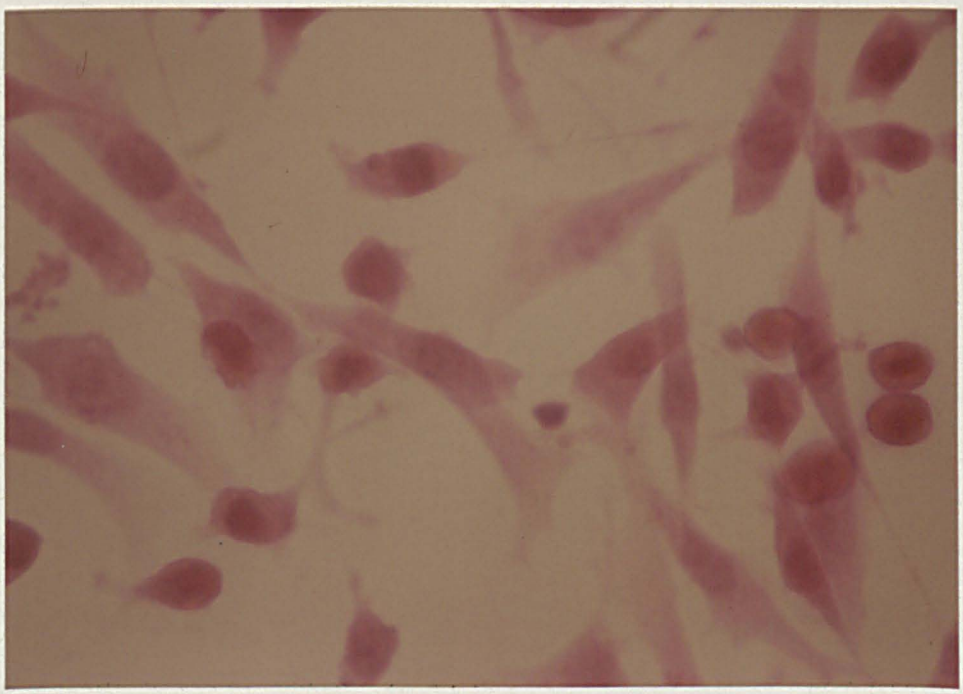


PLATE 15: C6 cells grown in media containing 5mM Ethanolamine for 24h and stained with Haematoxylin-Eosin X500

Growth of C_6 Cells in the presence of polar head group precursors

(○–○, control; ●–●, dimethylethanolamine; □–□, monomethylethanolamine; ■–■, ethanolamine)

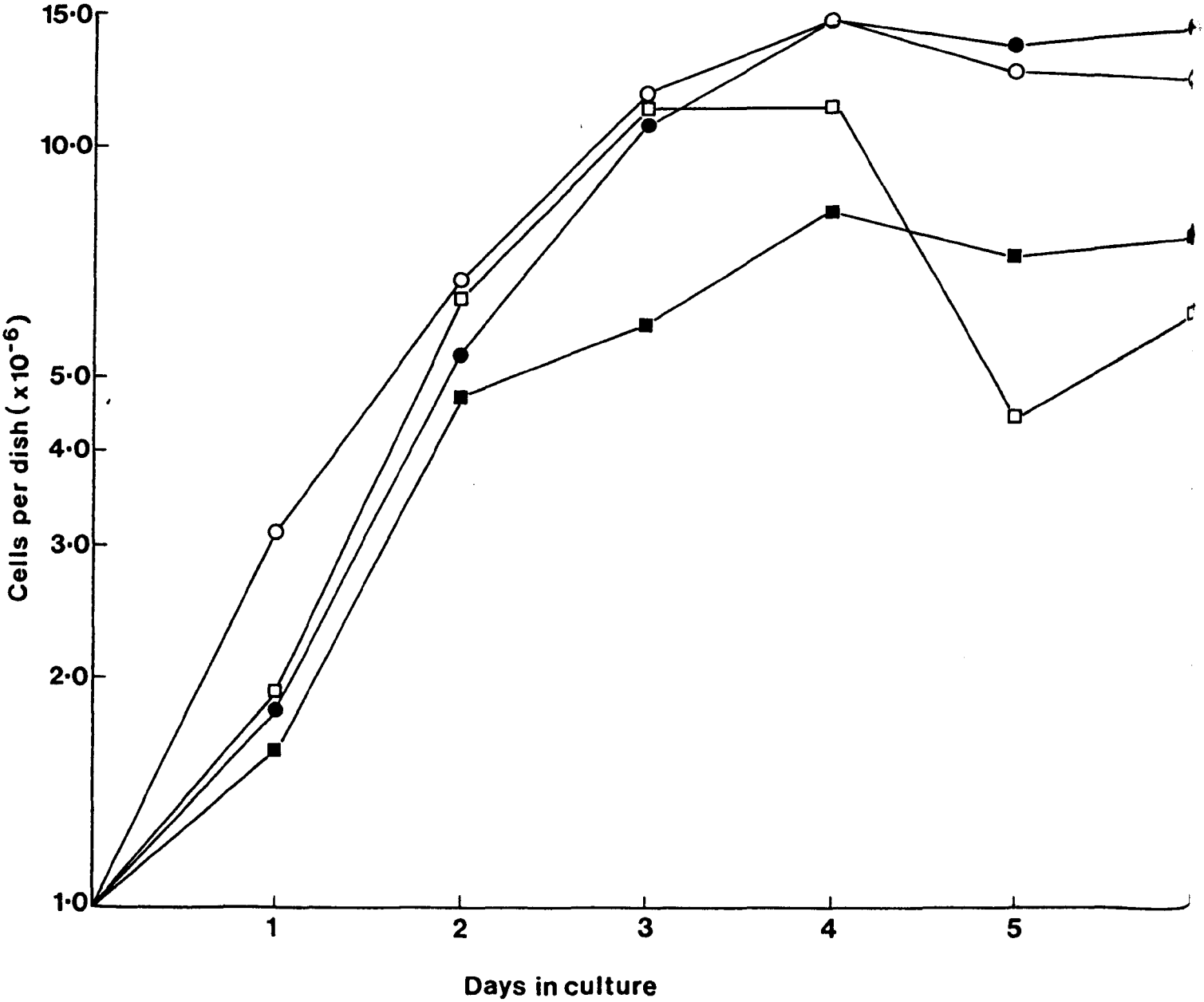


Fig.10. Growth rates of C6 cells in the presence of 5mM polar headgroup analogues; control, (O); N,N'-dimethylethanolamine, (●); N-monomethyl-ethanolamine, (□); ethanolamine, (■). Cells were grown in 20 cm^2 dishes and harvested after trypsinization. Viable cells were counted using a haemocytometer.

occurred. Plates 12-15 illustrate the results of those studies.

The "rounding-up" of the DME-treated cells is apparent, with little change in the shape of the NME- and ethanolamine-treated cells. No change in either the cell size, the shape or distribution of the nucleoli were observed. This is of importance, since any disturbances in nuclear function could have far-reaching effects that could extend further than just modification of the function of membrane-bound receptors or membrane-bound enzymes. The nuclei of the ethanolamine treated cells did not stain as intensely as the other cells, including the controls. This correlates with the reduced growth rate observed with ethanolamine treatment.

3.2.0 EFFECT OF BASE TREATMENT ON CELL MEMBRANE COMPOSITION

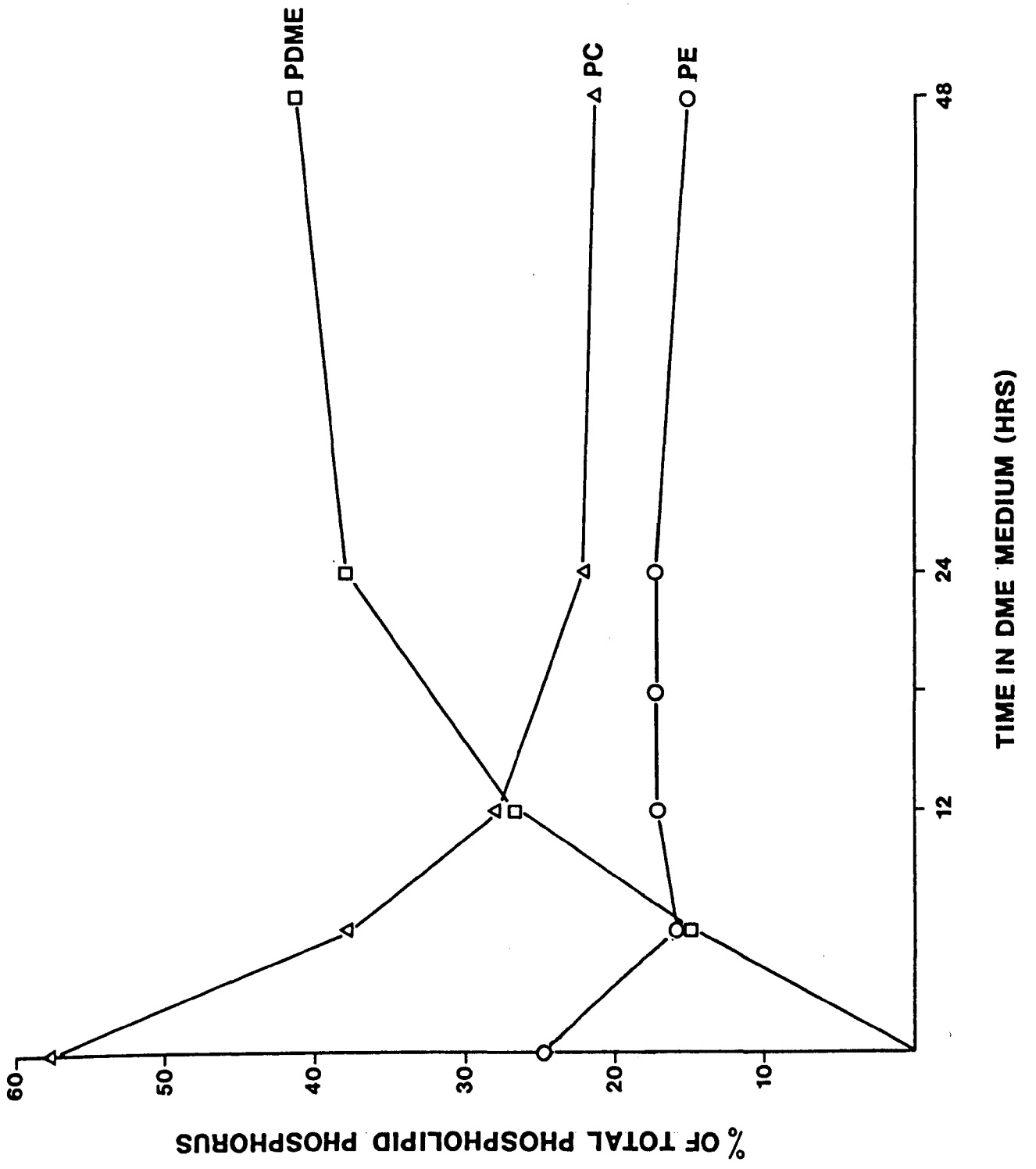
3.2.1 Incorporation of Bases into Cell Membranes

A study of the rate of headgroup incorporation was made for growth in the presence of DME. Cells were harvested and the lipid extract subjected to analysis by t.l.c. as described in Section 2.3. Changes in the major components of the phospholipid composition were measured, and are presented in Fig.11. No change was observed in the amounts of the minor components (Sphingomyelin, LPC, PI, PS and PA).

After 24hrs, the changes in phospholipid composition had almost reached a maximum, and this length of incubation was used in all other experiments.

From Fig.11 it is clear that the increase in PDME was at the expense of PC and PE. PC decreased from 57% to 27%, and PE content declined from 27% to 17% of total phospholipid phosphorus.

Fig.11: Growth of C6 Cells in medium containing 5mM DME: Effect on major components of the phospholipid composition.


PDME
PC
PE
60
50
40
30
20
10
12
24
48
% OF TOTAL PHOSPHOLIPID PHOSPHORUS
TIME IN DME MEDIUM (HRS)

Plates 16-19 show the gradual change in cell shape caused by DME treatment. It is clear from Plate 16 that within 12 hours of treatment, changes in the shape of the cells had occurred. Rounding of the cells continued beyond 24 hours of treatment (Plate 19), although no significant changes were observed after 48 hours.

The morphological changes were found to be reversible if the base-containing medium was replaced with normal growth medium. After 72 hours in normal medium, the cells had almost completely reverted to normal shape.

3.2.2 Effects of Base Treatment on Phospholipid Polar Headgroup Composition

Cells were grown for 24 hours in medium containing DME, NME or ethanolamine and the resulting changes in phospholipid composition were measured. The results of these experiments are presented in Table 5. NME and DME treatments caused the corresponding phospholipids PNME and PDME to accumulate to $\sim$ 32% of the total phospholipid phosphorus, without any change in the PI, PS and LPC content. An inverse relationship was found between the sphingomyelin content and the number of methyl groups on the polar headgroup nitrogen of the base supplement with which the cells were treated. The increase in sphingomyelin content in the base treated cells may reflect an attempt by the cells to maintain the percentage of choline phospholipids in the cells.

Ethanolamine treatment caused an increase in the PE content from 27% to 37%, whilst there was only a small decrease in the PC content of these cells (from 49% to 46%).

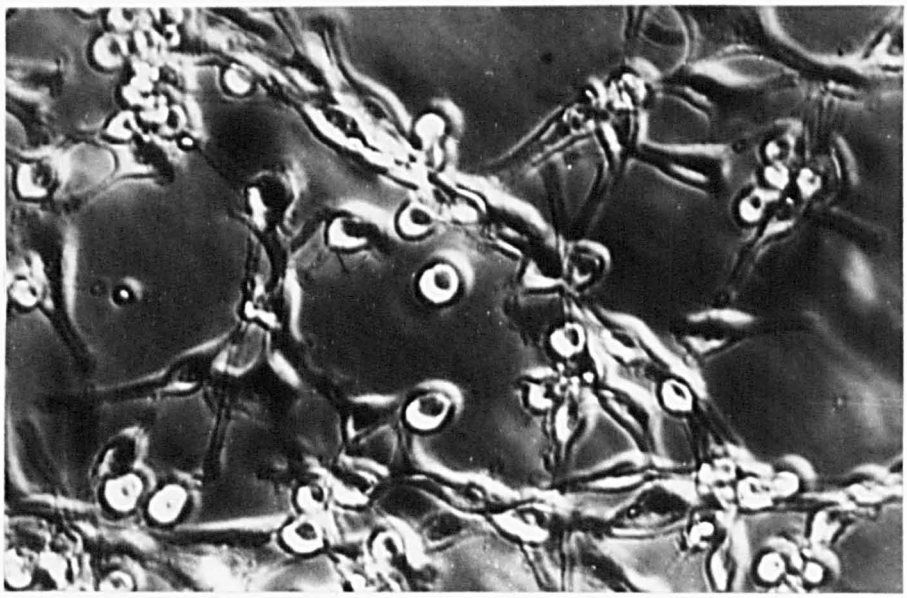


PLATE 18

PLATE 17

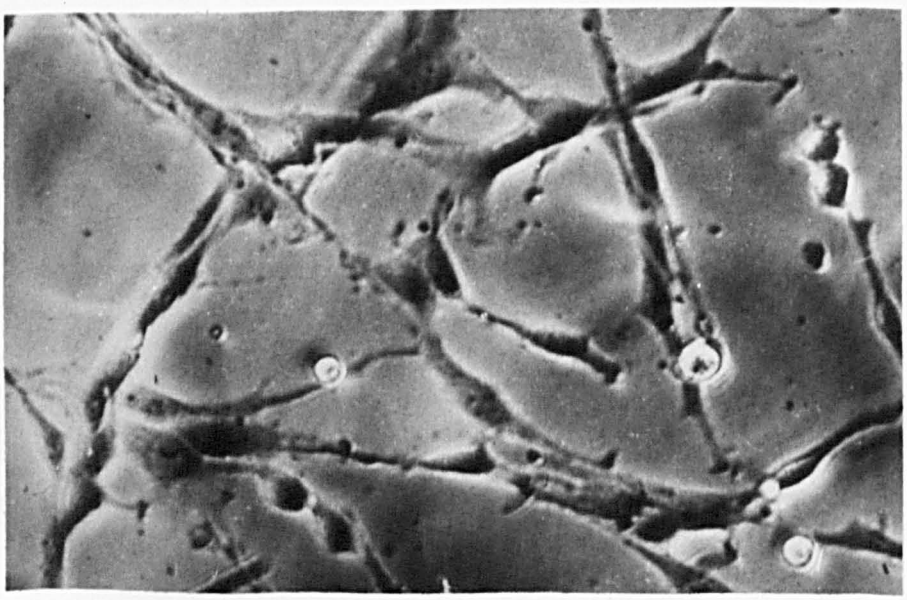
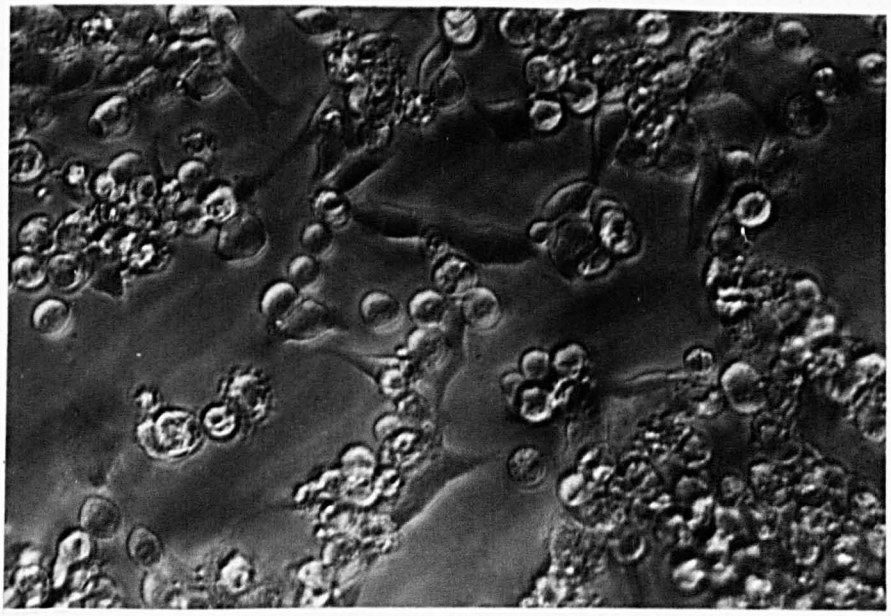


PLATE 16

PLATE 19



PLATES 16-19: TIME COURSE OF THE MORPHOLOGICAL CHANGES IN C6 CELLS GROWN IN MEDIA CONTAINING 5mM DME VIEWED BY PHASE-CONTRAST MICROSCOPY X200

PLATE 16: 0h growth in medium containing DME

PLATE 17: 12h growth in medium containing DME

PLATE 18: 24h growth in medium containing DME

PLATE 19: 48h growth in medium containing DME

TABLE 5

Phospholipid polar headgroup composition of C6 cells grown in medium containing bases or DMSO

Molar % of Phospholipid Phosphorus ± SEM

Supplement	PE	PNME	PDME	PC	PS	PI	Sphingo-myelin	PA
None (3)	27.4 ± 0.4	-	-	49.4 ± 0.5	6.6 ± 0.4	9.5 ± 0.4	5.6 ± 0.6	-
DME (3)	17.8 ± 0.2	-	32.6 ± 1.5	27.5 ± 7.7	6.8 ± 0.5	8.9 ± 1.2	6.4 ± 0.4	-
NME (3)	16.8 ± 0.8	31.0 ± 1.2	-	27.7 ± 0.8	5.6 ± 1.1	7.0 ± 0.5	10.5 ± 0.5	-
Ethanol-amine (3)	37.1 ± 0.3	-	-	46.5 ± 1.4	5.2 ± 0.6	7.0 ± 0.5	10.0 ± 0.7	1.8 ± 0.6
DMSO (4)	20.3 ± 1.1	-	-	54.5 ± 4.0	5.8 ± 0.6	7.6 ± 1.3	7.3 ± 1.7	3.67 ± 0.6

LPC was found to be $\lesssim$ 2% of the total phospholipid phosphorus in each case.

Cells were grown for 24h in medium containing 5mM bases (DME, NME and ethanolamine) or for 3 days in medium supplemented with 250mM DMSO.

Lipids were extracted and analysed by TLC.

Numbers in brackets indicate the number of measurements.

However, there was a small but significant decrease in the amounts of PS and PI, concomitant with an increase in the sphingomyelin content of these cells.

An attempt was made to increase the amount of PC in the membrane. Zwingelstein et al. (1982) showed that a large increase in the amount of PC could be obtained by growing Friend cells in 250mM dimethylsulphoxide(DMSO) for 3 days. However with C6 cells, this treatment resulted in very little increase in the PC content (Table 5). Interestingly, DMSO caused changes in the shape and packing of the C6 cells (Plate 11).

3.2.3 Cholesterol:Phospholipid Ratios in Base-treated Cells

Since Finkel and Volpe (1979) found that DME treatment caused a reduction in the activity of hydroxymethylglutaryl-CoA reductase, a key regulatory enzyme of cholesterol biosynthesis, it was thought that the amount of cholesterol in the membranes of these cells might decrease. This could have important consequences for both the physical properties of the membranes and the activity of membrane-bound enzymes.

This possibility was investigated using two different methods of cholesterol determination. As the important parameter for the maintenance of membrane fluidity is not the cholesterol content per se, but rather its value relative to phospholipid, the cholesterol:phospholipid ratios in the various cells were compared.

First, the cholesterol:phospholipid ratio was measured during the time course of DME-incorporation. This was done by measuring the phospholipid phosphorus content of harvested cells as described (Section 2.3). Cholesterol content was determined

using a modified Liebermann-Burchard method. The results of this study are presented in Table 6.

TABLE 6

Cholesterol Content of Membranes Prepared from C6 Cells Grown in Medium containing 5mM DME

Supplement	Cholesterol:Phospholipid Ratio ± SEM (mol:mol)
None	0.33 ± 0.02 (5)
24h with DME	0.37 ± 0.09 (5)
48h with DME	0.33 ± 0.07 (5)

Lipid extracts were prepared from cell membranes.

Statistical analysis (Students t-test $p < 0.05$) of these results indicated that no change had occurred in the cholesterol:phospholipid ratio after 48h exposure to DME.

This study was extended to check the effects of NME, DME and ethanolamine treatment on the cholesterol-phospholipid molar ratio (Table 7).

TABLE 7

Effect of Modified Phospholipid Polar Headgroup Composition on Cholesterol content in Membranes prepared from C6 Cells grown in the Presence of 5mM bases for 24h

Supplement	Cholesterol:Phospholipid Ratio (mol:mol)
None	0.40
DME	0.38
NME	0.37
Ethanolamine	0.40

The values are the means of determinations on two separate

samples. Lipid extracts were prepared from cell membranes. Cholesterol was determined by an enzymic method (Section 2.3). Phospholipid phosphorus was determined as described in the methods (Section 2.3).

It would appear from the results in Table 7 that exposure to the bases, NME, DME or ethanolamine does not change the cholesterol:phospholipid ratio of C6 cells.

The values obtained in Table 7 were obtained by using an enzymatic method to determine cholesterol. This was adopted since it was found to be more sensitive than the Liebermann-Burchard method (Fig.5).

However, an unusual effect was noted with the enzymatic method. The cholesterol:phospholipid ratio of samples which had been stored for several months at -70°C increased. This was quite reproducible, and inexplicable. However, even under these conditions no change was observed in the cholesterol: phospholipid ratios of the treated cells compared with controls (Table 8).

TABLE 8

Effect of Modified Phospholipid Polar Headgroup Composition on Cholesterol content in Membranes prepared from C6 Cells grown in the presence of 5mM Bases for 24h. Membranes were stored for several months at -70°C.

Supplement	Cholesterol:Phospholipid Ratio ± SEM (mol:mol)
None	0.53 ± 0.03 (3)
DME	0.56 ± 0.02 (3)
NME	0.58 ± 0.1 (3)
Ethanolamine	0.57 ± 0.01 (3)

3.2.4 Protein:Phospholipid Ratios in Cells with Modified Phospholipid Polar Headgroup Composition

Another important component which contributes to membrane fluidity is the protein content of biological membranes. Changes in either (a) the protein content, or (b) the phospholipid content, could be part of the mechanism by which "homoviscous adaptation" occurs.

Yavin (1977) and Robert et al. (1978) found that in M-1 Neuroblastoma cells the phospholipid content of cells with altered phospholipid composition increased. This might serve to maintain the physical properties of the membrane, or might have been simply a reflection of an increase in phospholipid synthesis de novo due to the incorporation of the polar headgroups into the cell.

The protein:phospholipid ratios were investigated in both whole cells and membranes prepared from base-treated cells (Tables 9 and 10).

TABLE 9

Protein and Phospholipid content of C6 Cells grown in Medium containing 5mM DME

Supplement	Cells/ Dish x 10^6	Mg Protein/ 10^6 cells	nmol Phospholipid/10^6 cells	Phospholipid: Protein Ratio nmol/mg
None	8.66±0.58	0.221±0.031	163.0±9.5	752±57
DME (24h)	9.08±0.23	0.201±0.025	127.0±26.5	688±285
DME (48h)	10.29±0.78	0.188±0.086	129.3±7.3	670±32

Values are the means of 3 separate samples ± SEM.

From Table 9 it is clear that no significant change occurred in cell number, protein content or phospholipid content in cells exposed to DME for up to 48h. The effects of NME and ethanolamine were not studied. These results differ from the findings of Robert et al. (1978). These workers found that exposure of neuroblastoma cells to DME, NME, or ethanolamine, resulted in an increase in the total phospholipid content. An increase in the phospholipid content does not occur in C6 cells when they are exposed to these choline analogues.

Since physical studies were carried out using membranes prepared from C6 cells treated with bases, I thought it important to check that no change was occurring in the membrane phospholipid:protein ratios. The results of this study are shown in Table 10. Crude membrane preparations were made from DME-treated cells, which had been incubated with base for 24 and 48 hours, as described in the methods (Section 2.1).

TABLE 10

Protein and Phospholipid Content of Membranes from C6 Cells grown in Medium containing 5mM DME

Supplement	Phospholipid:Protein Ratio ± SEM (nmol:mg)
None	1091 ± 65 (3)
24h with DME	1067 ± 123 (3)
48h with DME	1089 ± 241 (3)

From Table 10, it is clear that the growth of cells in DME had no effect on the membrane phospholipid:protein ratios.

3.2.5 SDS Polyacrylamide Gel Electrophoresis of Membrane Proteins

To ensure that base treatment had no effects on the protein composition of the cells, the protein profile of membrane preparations was determined, as described (Section 2.4) by SDS polyacrylamide gel electrophoresis.

The results are displayed in plate 20. No change was found in any of the band patterns, suggesting that base treatment had no effect on the gross protein composition in these treated cells. These results are in agreement with studies carried out on base treated LM cells (Engelhard et al., 1978).

3.2.6 Fatty Acid Composition of Base-treated Cells

The replacement of PC by phospholipids that contain fewer methyl groups on the nitrogen of the polar headgroup might be expected to have a rigidifying effect on the bilayer (Sklar et al., 1977). In model systems, Vaughan and Keough (1974) have demonstrated that removing methyl groups from dipalmitoyl PC raises the temperature of the phase transition temperature. It was therefore quite possible that substitutions of the polar headgroup with NME, DME, or ethanolamine might cause changes in the fatty acyl composition of membrane lipids.

Fatty acid analysis on membranes was carried out using two different g.l.c. columns as described in the methods (Section 2.3). The results of these analyses are displayed in Table 11.

It is clear that no change occurs in the fatty acyl composition, apart from a decrease in the amount of arachidonic acid in NME-treated cells. This was observed when analysis was carried out on either column. There were no other significant

PLATE 20: SDS Polyacrylamide Gel. Profile of Membrane Proteins prepared from C6 cells grown in media containing 5mM bases for 24h.

Lanes 1,2 - Control Cells

Lanes 3,4 - Cells exposed to DME

Lanes 5,6 - Cells exposed to NME

Lanes 7,8 - Cells exposed to ethanolamine

changes in fatty acyl composition.

These findings are in agreement with the results of studies by Schroeder on LM cells (Schroeder et al. 1976b) except that he did not find any change in the amounts of arachidonic acid. The changes I observed were, however, statistically significant at $p < 0.05$, calculated by the Students t-test. Similarly, Robert et al. (1978) found no change in the fatty acyl composition of M-1 Neuroblastoma cells grown in the presence of NME, DME, or ethanolamine. The values which I obtained for the fatty acyl composition of control cells are similar to those described by Robert et al. (1977) for C6 cells.

In LM cells, although base substitution causes no changes in the total fatty acyl composition, further work by Schroeder (Schroeder, 1978) has shown that the fatty acyl composition of individual phospholipid classes were different. In particular, the fatty acyl composition of the phospholipids resulting from base treatments were intermediate in unsaturated fatty acyl content between that of the PE and PC in the membranes.

The presence of butylated hydroxy toluene did not make any great difference to the fatty acyl compositions recorded for the control cells on either column. However, initial studies in which oxidation of 18:1 and 16:1 occurred prompted the use of this agent, to rule out any differences in fatty acyl composition resulting from oxidation.

TABLE 11: Effect of Phospholipid Polar Headgroup Modification on Fatty Acyl Composition of C6 Cells grown in Medium containing 5mM Bases for 24h.

EGSSX Column	Supplement				EGSS-Y	Supplement			
Fatty Acyl composition % of Total ± SEM	None	DME	NME	Ethanol-amine		None	DME	NME	Ethanol-amine
14:0	3.0±0.4	1.25±0.04	1.06±0.4	3.03±0.32	14:0	1.0±0.4	1.37±0.6	1.17±0.4	1.71±0.3
16:0	25.9±2.6	22.15±0.4	23.1 ±2.4	21.7 ±1.5	16:0	20.9±1.6	19.83±0.8	23.3 ±0.4	21.0 ±2.5
16:1	8.5±1.5	11.1 ±2.5	11.1 ±0.4	7.82±0.2	16:1	8.8±1.0	8.09±0.7	11.7 ±0.9	7.8 ±0.9
17:0	-	0.34±0.05	-	-	17:0	1.06±0.3	1.0	0.88±0.2	1.01 ±0.2
18:0	15.5±2.0	18.3 ±0.2	14.94±1.8	12.0 ±2.8	18:0	16.6±0.5	16.7±1.7	16.3 ±1.7	16.18±1.2
18:1	36.4±1.1	38.25±0.4	41.6 ±4.5	43.6 ±0.6	18:1	38.16±2.2	33.2±1.1	39.1 ±1.7	37.3 ±0.1
18:2	0.3±0.1	0.46±0.1	0.45±0.3	0.56±0.1	18:2	0.83±0.2	0.24±0.2	0.17 ±0.07	0.43±0.2
20:1 + 18:3	0.45±0.2	0.38±0.05	0.65±0.2	-	20:1 + 18:3	0.71±0.2	0.26±0.07	0.15 ±0.04	0.27±0.1
20:2	0.86±0.2	0.86±0.37	1.02±0.1	0.77±0.2	20:2	1.97±0.5	1.36±0.4	1.29 ±0.3	0.79±0.4
20:3w9	0.28	0.21±0.05	0.24±0.03	0.37±0.4	20:3w9	1.73±0.4	1.34±0.4	0.46 ±0.2	0.36±0.1
20:3w6	1.34±0.6	0.32±0.07	0.39±0.1	0.51	20:3w6	1.0 ±0.6	0.73±0.1	1.05 ±0.6	0.57±0.1
20.4	3.28±0.6	2.76±0.5	1.53±0.1	2.26±0.5	20:4	5.7 ±1.7	3.78±0.4	1.45 ±0.1	2.97±0.6
20:5	0.98±0.2	0.49±0.2	0.38±0.1	0.66±0.2	20:5	0.68±0.1	0.35±0.01	0.41 ±0.1	0.41±0.1
22:4w6	0.84±0.3	0.39±0.1	0.33±0.1	1.07±0.4	22:4w6	0.67±0.1	0.73±0.7	0.34	0.39±0.05
22:5w6	1.27±0.4	0.1 ±0.3	0.96±0.1	-	22:5w6	0.61±0.1	0.93±0.2	0.71 ±0.2	1.21±0.03
22:5w3	1.27±0.3	1.35±0.5	0.91±0.1	0.88±0.1	22:5w3	1.75±0.6	0.97±0.2	0.61 ±0.2	0.88±0.3
22:6w3	1.68±0.6	1.56±0.4	1.16±0.2	1.18±0.4	22:6w3	1.58±0.3	1.16±0.3	0.87 ±0.1	1.62±0.4

Values are the mean ± SEM of 3 determinations on 2 different samples prepared for each column as described in the methods (Section 2.3).

3.3.0 PHYSICAL PROPERTIES OF CELL MEMBRANES FROM CELLS WITH MODIFIED PHOSPHOLIPID POLAR HEADGROUP COMPOSITION

3.3.1 Fluorescence Depolarisation Studies

Hirata and Axelrod (1978) have claimed that phospholipid methylation increases the fluidity of erythrocytes, as measured by the fluorescence depolarisation of 1,6,-diphenyl-1,3,5,-hexatriene (DPH). Therefore it was important to check whether the enrichment of the phospholipids with PDME, PNME or PE had any effect on the physical properties of the cell membranes.

I decided to use the same incubation conditions employed by Hirata and Axelrod (1978), to incorporate the hydrophobic probe molecule 1,6-diphenyl-1,3,5,-hexatriene into membranes prepared from C6 cells exposed for 24 and 48h to DME. Measurements of the steady-state fluorescence polarisation were made. The values calculated were expressed as polarisation values (P) since objections have been raised to extrapolating values obtained in biological membranes to microviscosity values by using the Perrin equation (Kaunto et al., 1977; Lakowicz and Prendergast, 1978; Van Hoeven et al., 1979).

The values obtained are tabulated below (Table 12).

TABLE 12

Fluorescent Depolarisation Values of DPH Incorporated into Membranes from C6 cells treated with 5mM DME

Supplement	P Value ± SEM	
None	0.328 ± 0.008	(3)
24h with DME	0.334 ± 0.046	(3)
48h with DME	0.299 ± 0.079	(3)

No significant difference ($P < 0.05$ Students t-test) was found in the P values of the treated cells compared with the control membranes.

3.3.2 Electron Spin Resonance Studies

Schreier et al. (1978) have stated that molecular order in lipid bilayers and the rates of lipid motion are distinct parameters. Since electron spin resonance spectroscopy facilities became available to me, I thought that a more comprehensive study of possible changes in membrane fluidity could be made in the base-treated cells by measuring the order parameter of 5-doxylstearic acid spin label incorporated into membranes propared from base-treated cells. The rotational correlation times of lipid motion were derived using 16-doxylstearic acid spin label.

Table 13 shows that at 37°C no change in either the order parameter (S) or the rotational correlation times (τ) of the fatty acid spin labels were found in any of the base treated cell membranes, compared with controls.

A more extensive study was carried out where the order parameters (Fig.12) and the correlation times (Fig.13) of the spin labels were measured over the temperature range 15°C-40°C. No change in either parameter could be found relative to the control membranes.

These results are therefore in agreement with the earlier study with DPH.

Using parinaric acid and 1,6-diphenyl-1,3,5,-hexatriene, Schroeder (1978) was unable to find any significant changes in the P values of LM cells treated with ethanolamine, NME, or DME. Thus, I am confident that no change in the physical properties of base-treated cell membranes had occurred.

TABLE 13

Order Parameters and Correlation Times for Spin-labelled Fatty Acids incorporated into Membranes with a modified Phospholipid Composition.

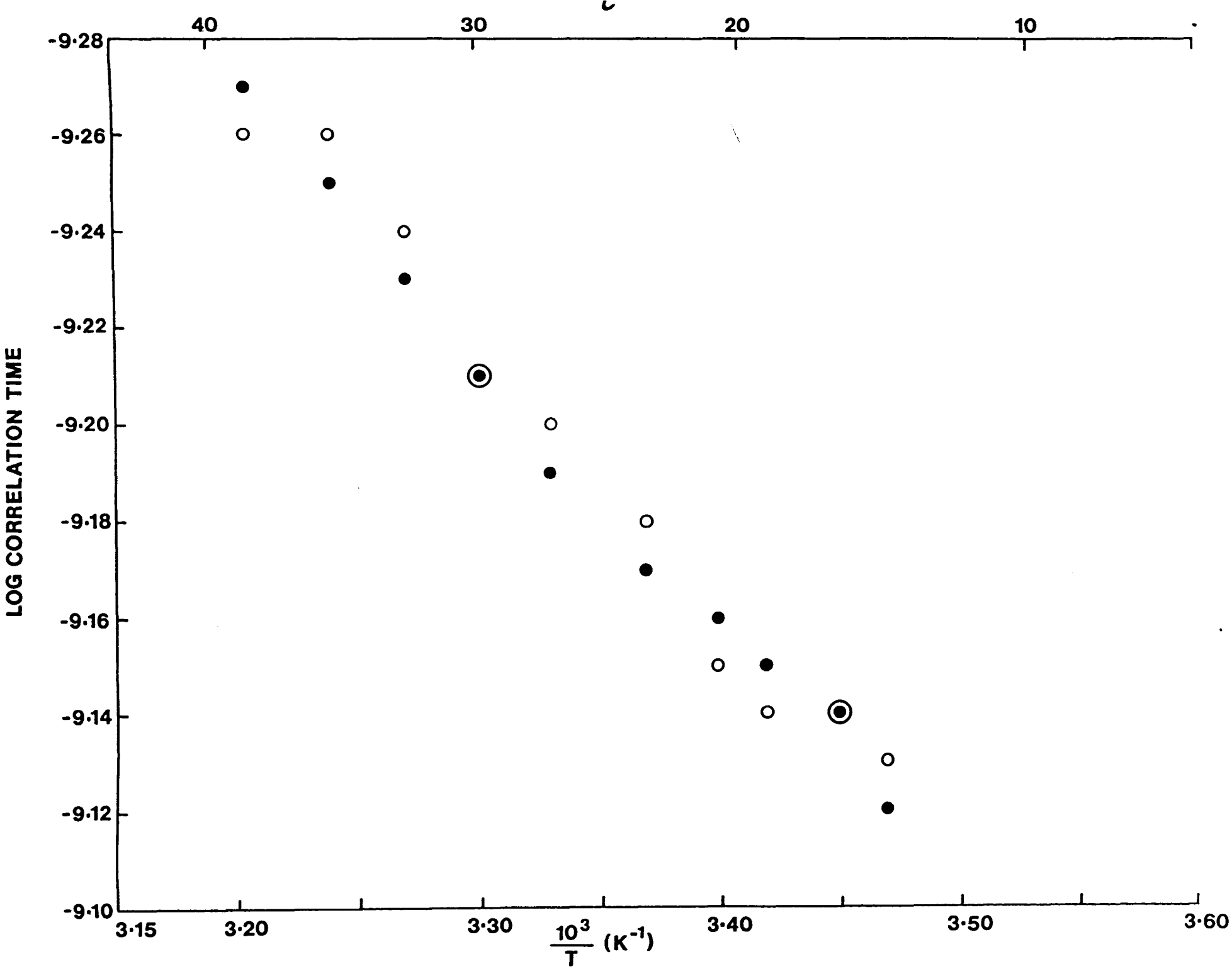
The order parameters for 5-doxylstearic acid and the correlation times for 16-doxylstearic acid in membranes from cells grown in the presence of choline analogues were measured at 37°C as described in the Materials and Methods. All values are for means of four separate experiments ± S.E.M.

Supplement	Order Parameter	Correlation time ($\times 10^{10}$s)
None	0.59 ± 0.01	5.44 ± 0.30
N,N'-Dimethyl-ethanolamine	0.59 ± 0.02	5.72 ± 0.28
N-Monomethyl-ethanolamine	0.59 ± 0.01	5.68 ± 0.17
Ethanolamine	0.58 ± 0.01	5.76 ± 0.10

Fig.12: Temperature dependence of the order parameter of 5-doxylstearic acid incorporated into the membranes of control (-O-) cells and cells grown in medium supplemented with N-monomethyl-ethanolamine (-●-). Membranes from cells grown in medium supplemented with N,N-dimethyl-ethanolamine, or with ethanolamine showed a similar profile to the controls.

ORDER PARAMETER (S)
0·9
0·8
0·7
0·6
0·5
TEMPERATURE (°C)
0
5
10
15
20
25
30
35
40
45

Fig.13: Temperature dependence of the correlation time of 16-doxylstearic acid incorporated into the membranes of control cells (-O-) and into membranes from cells grown in medium supplemented with N-monomethylethanolamine (-●-). Membranes from cells grown in medium supplemented with N',N'-dimethylethanolamine, or with ethanolamine showed a similar profile to the controls.


40
30
20
10
-9·28
-9·26
-9·24
-9·22
-9·20
-9·18
-9·16
-9·14
-9·12
-9·10
LOG CORRELATION TIME
3·15
3·20
3·30
3·40
3·50
3·60
10³/T (K⁻¹)

3.4.0 SUMMARY OF RESULTS PRESENTED IN SECTIONS 3.0-3.3.2

1. The phospholipid polar headgroup composition of cells in tissue culture can be changed radically by growing the cells in media containing polar headgroup analogues.
2. Phospholipids resulting from incorporation of the bases make up ∿ 30% of the total phospholipid phosphorus, principally at the expense of PC and PE. No change in the amounts of the minor phospholipids occurred, except after growth in the presence of ethanolamine and NME, when there was a reduction in the amounts of minor lipids, and an increase in the amounts of the other choline-containing phospholipid sphingomyelin, in addition to the decrease in amount of PC in the cells.
3. Cell growth was not significantly impaired under these conditions, with the exception of cells grown in the presence of ethanolamine, which grew more slowly and reached stationary phase at a lower density than did the other cells.
4. Cell morphology changed as a result of phospholipid modification, but no change was observed in the relative sizes of the nucleus and cytoplasmic components.
5. No major changes were observed in the fatty acyl composition of the modified cells compared to control cells.
6. No difference was found in the physical properties of the modified cell membranes compared with control membranes, when compared by measurements of the fluorescent depolarisation of DPH, or by ESR with fatty acids spin labels.

3.5.0 EFFECT OF POLAR HEADGROUP ALTERATIONS ON CYCLIC AMP ACCUMULATION

The effect of polar headgroup on basal and isoproterenol-stimulated cAMP accumulation was determined and the results of these experiments are presented in Table 14. Table 14 also shows the results of experiments in which the modified cells were treated with theophylline. These results are discussed in Section 3.5.2.

3.5.1 Effects of Phospholipid Modification on cAMP Accumulation

The basal cAMP accumulation of the cells was elevated by each of the phospholipid modifications. DME and ethanolamine treatment elevated the basal cAMP levels to a similar degree (1.5-fold) relative to the controls. NME treatment caused a greater increase in the basal cAMP levels (3.5-fold).

However, when stimulated with isoproterenol, both the degree of stimulation of cAMP accumulation, and the amount of cAMP accumulated in the modified cells were significantly reduced relative to the control cells. NME and DME treatment had the greatest effects, causing a 5-6-fold decrease in the degree of stimulation of cAMP content by isoproterenol compared to control cells. Ethanolamine treatment reduced the degree of stimulation by isoproterenol to about one half of that found in the control cells.

3.5.2 Effects of Theophylline on Cyclic AMP in Cells with Modified Phospholipid Polar Headgroup Composition

Exposure of the modified cells to theophylline, a cyclic AMP phosphodiesterase inhibitor, elevates the basal cAMP levels in all cells by 3-4-fold. The actual amount of cAMP accumulation in response to isoproterenol stimulation is

TABLE 14

Effect of Base Treatment on Cyclic AMP Accumulation

Growth Supplement	cAMP accumulation (pmol cAMP/mg protein/30min ± SEM) -theophylline basal	-theophylline + 10μM isoproterenol	Stimulation	+theophylline basal	+theophylline + 10μM isoproterenol	Stimulation
None	14.4±3.3(3)	806 ± 150 (3)	55.9X	45 (2)	685 ± 58 (3)	15.2X
DME	22.3 ± 6.6(3)	288 ± 34 (3)	12.9X	89 (2)	616 ± 179 (3)	6.9X
NME	50 ± 13 (3)	473 ± 58 (3)	9.5X	133 (2)	595 ± 50 (3)	4.5X
Ethanolamine	23 ± 3 (3)	495 ± 62 (3)	21X	92 (2)	533 ± 53 (3)	5.9X

Cells were grown for 24h in the presence of 5mM bases prior to assay for cAMP content.

Cells treated with theophylline were grown for a total of 1h in medium containing 1mM theophylline and the theophylline was present in the medium throughout the experiment.

restored in cells with a modified phospholipid composition to control levels by theophylline. However, theophylline treatment does not restore the degree of stimulation of cAMP accumulation by isoproterenol compared with the controls. This lowering of the magnitude of isoproterenol stimulation of cAMP accumulation is presumably attributable to the increased basal level in modified cells.

The degree of stimulation of the basal rates of cAMP accumulation by theophylline in all cells was similar (∿ 3-fold increase).

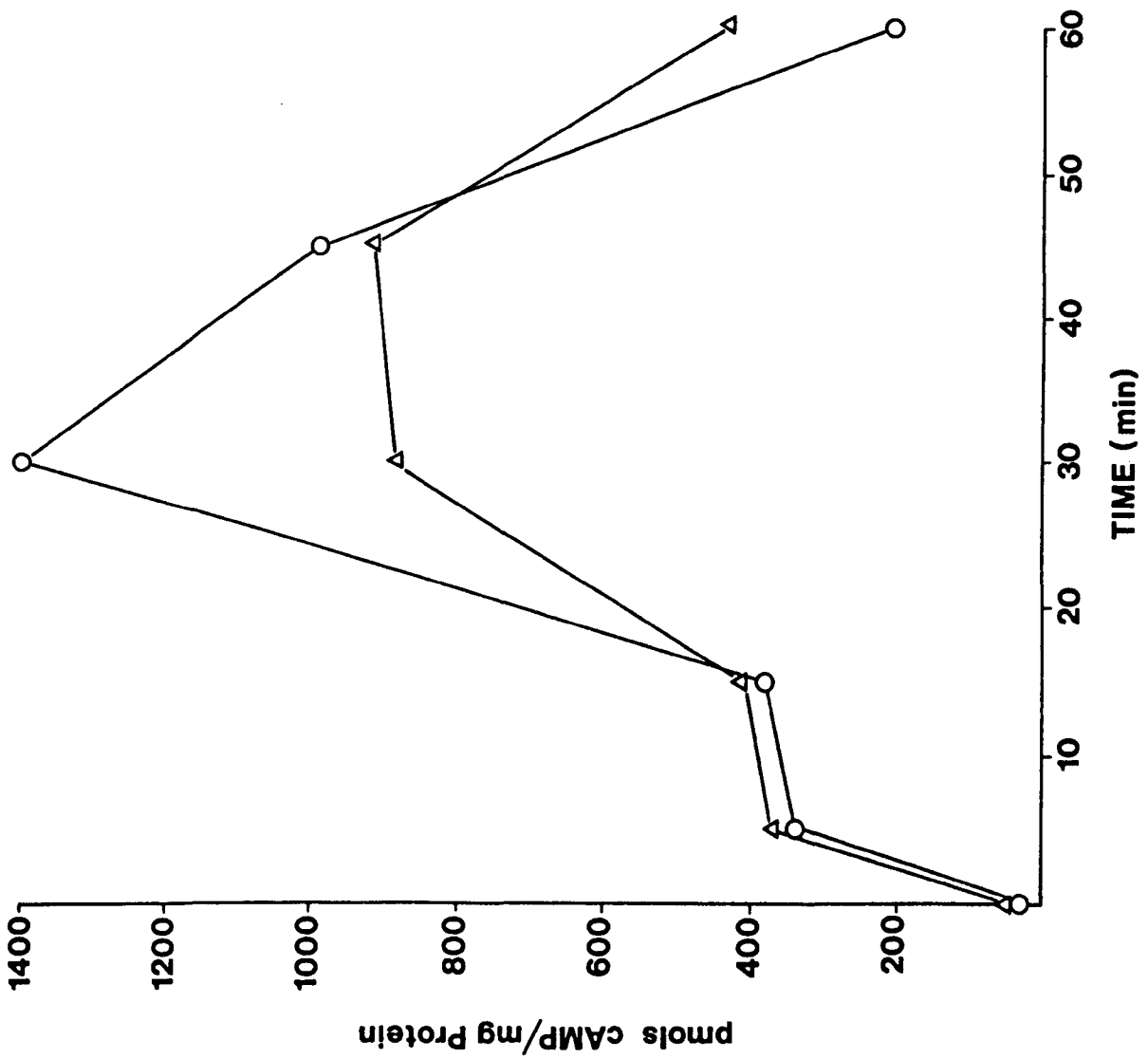
3.5.3 Time Course of cAMP accumulation in response to Isoproterenol in Cells whose Membranes are enriched in PDME

The amount of cAMP accumulated by cells treated for 24h with DME is less than the control cells (Section 3.5.1). However it was possible that the cells with altered phospholipid polar headgroup composition achieved their maximum cAMP accumulation at an earlier or later time relative to the control cells.

DME-treated cells showed the greatest decrease in cAMP accumulation relative to the control, so I decided to follow the time course of cAMP accumulation in these cells to check whether or not there was a difference in the time course of cAMP accumulation.

The results of this study are presented in Fig.14. It is clear that the maximum cAMP accumulation in the DME treated cells is less than the control cells over the 60min of the incubation with isoproterenol, although the rates of cAMP accumulation and breakdown of cAMP are similar to the controls.

Fig.14: Time course of cAMP accumulation in response to isoproterenol stimulation in C6 control cells (-O-), and in C6 cells grown in medium containing N ,N'-dimethylethanolamine (-Δ-).


1400
1200
1000
800
600
400
200
pmols cAMP/mg Protein
10
20
30
40
50
60
TIME (min)

3.5.4 Summary of Results presented in 3.5-3.5.3

1. Basal levels of cAMP accumulation in C6 cells are elevated by phospholipid polar headgroup modification.
2. The rise in cAMP content in response to β-adrenergic agonist stimulation is reduced to one-fifth of that in control cell values in cells exposed to DME and NME treatment, and to one-half of control values in cells with an elevated PE content.
3. The amount of cAMP accumulated in response to isoproterenol in cells with a modified phospholipid composition can be restored to levels similar to those of the control cells by theophylline.
4. Theophylline is unable to restore the degree of stimulation of cAMP accumulation by β-agonists in cells with a modified phospholipid composition.
5. The time course of cAMP accumulation in response to isoproterenol in DME treated cells is similar to that of the control cells.

3.6.0 LIPID METHYLATION

3.6.1 Cell Density and Lipid Methylation

Initially, difficulty was experienced in getting reproducible lipid methylation results. This was due to several factors, but mainly due to the effects of cell density and to differences between the amount of cells in the cultures used for each condition. Initially, low cell densities were used, but it was found that during the washes after the incubations with methionine, the monolayer was damaged and cells were lost which altered the total protein recovered. Better cell adhesion was obtained with denser cultures, but this led to a loss of isoproterenol-stimulation of methylation.

Morris and Makman (1976) demonstrated that the responsiveness of the β-adrenergic system of C6 cells increased with increasing cell density in culture. This prompted an investigation of the effect of cell density on the methylation response, which is presented in Table 15.

TABLE 15

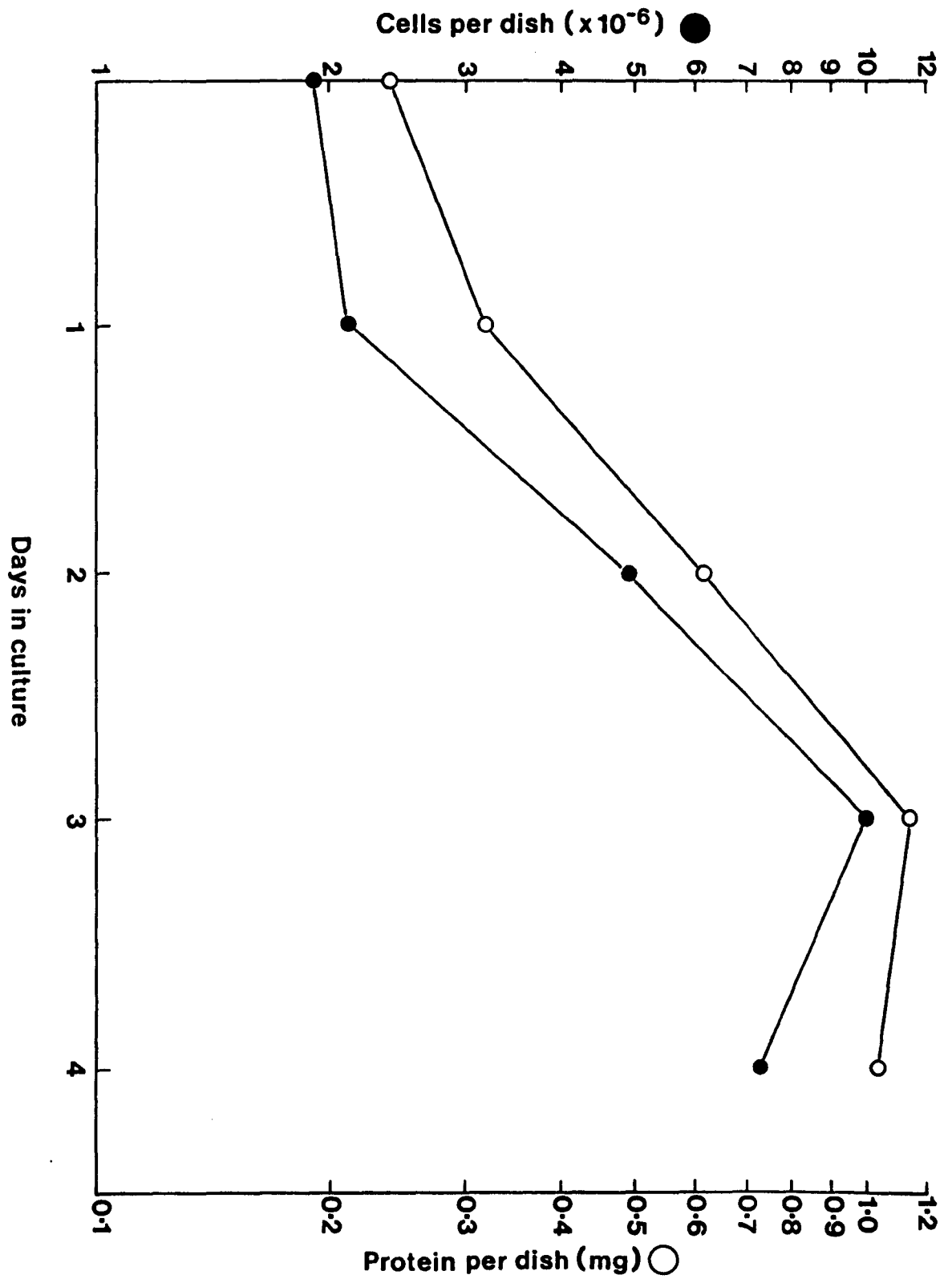
Effect of Cell Density on Lipid Methylation (methyl groups incorporated pmol/mg protein/55min ± SEM)

Condition	Low density (0.36mg/ protein/well)	High density (> 1.0mg/ protein/well)
Control	115.2 ± 7 (4)	40.6 ±10(4)
Control + 10μM isoproterenol	183 ±5 (4)	48.7 ± 8(4)

From Table 15 it seems that both the basal methylation, and the degree of stimulation by isoproterenol are reduced in high density cultures. The basal methylation was 2.8 times greater in low density cultures than in high density cultures. The stimulation of methylation by isoproterenol was 1.5-fold in low density cultures, but only 1.2-fold in high density cultures.

In Fig.15 the rate of cell growth is related to the protein content of the cultures. It appears that in the low density cultures (0.36mg protein/well) the cells are still in the logarithmic growth phase, but that the high density cultures (> 1.0mg protein/well) are in stationary phase, and that both cell number and protein begins to decrease, possibly due to nutrient starvation. It appears that the optimum isoproterenol-stimulation of methylation occurs when the cells are still undergoing rapid growth.

Fig.15. Cell growth and protein content of C6 cell cultures established in linbro wells. After growth for various periods of time, the cells were detached by trypsinisation and counted by haemocytometer. The cell suspension was then centrifuged, and protein determinations carried out on the pellet.


Cells per dish (x10⁻⁶) ●
1
2
3
4
5
6
7
8
9
10
12
Days in culture
1
2
3
4
0·1
0·2
0·3
0·4
0·5
0·6
0·7
0·8
0·9
1·0
1·2
Protein per dish (mg) ○

For all subsequent methylation experiments the cells were used whilst still in the rapid growth phase (0.4-0.6 mg protein/well).

The assay was redesigned such that four wells of the linbro tray were used for methylation studies and the remaining two wells were used for protein determinations. This greatly improved the reproducibility of the assay.

3.6.2 Lipid Methylation and the β-Adrenergic Response

The results presented in Table 16 indicate that [^{3}H]methyl group incorporation is mediated by the β-adrenergic receptor. [^{3}H]Methyl group incorporation is stimulated by the β-adrenergic agonist isoproterenol, and blocked by the β-adrenergic antagonist, propanolol.

methylation is stimulated by β-adrenergic agonists at the same concentrations which result in an isoproterenol-stimulated rise in intracellular cAMP content.

Hirata and Axelrod (1980) have claimed that stimulation of phospholipid methylation in reticulocytes leads to an increase in membrane fluidity, which is essential for the coupling of the β-receptor to adenylate cyclase. To test this claim, I incorporated 5-doxylstearic acid spin label into live cells in culture. The cells were then stimulated with isoproterenol at a concentration known, from previous experiments, to stimulate both lipid methylation and cAMP accumulation. At the end of the incubation, the cells were harvested intact and transferred to glass capillary tubes for electron spin resonance studies.

The hyperfine splitting ($2T_{\parallel}'$) of the outer extrema of the ESR spectra was measured. The larger the splitting, the more

ordered and the less fluid is the membrane. Table 16 shows that incubation of intact C6 cells with isoproterenol causes a rise in intracellular cAMP and that this is accompanied by a stimulation in the rate of [^{3}H]methyl group incorporation However, under these conditions, no change was observed in the hyperfine splitting indicating that lipid methylation is unlikely to cause changes in the physical properties of the plasma membrane. Furthermore, it seems unlikely that lipid methylation regulates the β-adrenergic response by altering the motional properties of the lipid in the plasma membrane.

On the other hand, propanolol clearly affects the physical properties of the membrane lipid in these cells, causing the membrane to become more rigid.

TABLE 16

Effects of isoproterenol and propranolol on [^{3}H]methyl group incorporation, cyclic AMP accumulation and the physical properties of plasma membranes in intact C6 cells

Drug	^{3}H-Methyl incorporated pmol per mg protein /55min)[a]		Cyclic AMP (pmol per mg protein /30min)[b]		$2T'_{\parallel}$ (Gauss)[c]
None	32±4	(4)	14±3	(3)	52.9±0.1
Isoproterenol (10μM)	75±10	(4)	806±133	(3)	52.9±0.1
Isoproterenol (10μM) + propranolol (500μM)	36±5	(4)	27±5	(3)	53.7±0.1
Propranolol (500μM)	35±11	(4)	13±1	(3)	53.9±0.1

a. [^{3}H]methyl group incorporation ± SEM
b. cAMP measurements ± SEM
c. Hyperfine splitting ± SD

3.6.3 Effect of cyclic AMP Analogues on Rate of Lipid Methylation

To determine whether cAMP could regulate lipid methylation cells were treated with either dibutyryl cAMP or with theophylline in order to raise the intracellular cAMP content. The effect on lipid methylation was then examined.

TABLE 17

Effect of dibutyryl cAMP and theophylline on the rate of lipid methylation. Values are ± SEM.

Treatment	Methyl group incorporation pmol/mg protein/55min	
None	75.5 ± 2.1	(4)
+1mM theophylline during assay incubation	72.7 ± 3.9	(4)
+1mM dibutyr cAMP for 1h prior to assay incubation	95.8 ± 6.0	(4)
+2mM sodium butyrate for 1h prior to assay incubation	71.4 ± 6.2	(4)

Theophylline treatment elevates intracellular cAMP content (Table 14), but did not have any significant effect on the rate of lipid methylation. However, dibutyryl cAMP increased the rate of methylation, and is known to elevate intracellular cAMP levels. The increased rate of lipid methylation did not appear to be an effect of butyric acid, since butyric acid on its own did not increase the rate of lipid methylation.

3.6.4 Comparison of the Effects of Stopping Lipid Methylation Incubations with Trichloroacetic acid or Phosphate buffered Saline on the Distribution of Incorporated Radioactivity in C6 Cells

Moore et al. (1982) published work which showed that terminating the lipid methylation reaction in lymphocytes with trichloroacetic acid caused a greater incorporation of

radioactivity into the cells, than when ice-cold phosphate buffered saline was used to stop the reaction.

I was concerned that the use of acid might be the cause of artefacts which might be responsible for both the incorporation of radioactive methionine, and the pattern of incorporation of radioactivity into particular areas of the TLC plate. This possibility was investigated by measuring the rate of methylation and the distribution of radioactivity in C6 cells that had been stimulated with isoproterenol. Incubations were stopped with either phosphate-buffered saline, or with trichloroacetic acid. The results of this study are presented in Table 18.

The results shown in Table 18 indicate that although trichloroacetic acid increases the amount of radioactivity incorporated into the cells, the percentage of [^{3}H] label recovered from any particular area of the TLC plate was not altered. It is also clear that although isoproterenol stimulated an increase in methyl group incorporation, the percentage of label that went into individual phospholipids was not altered. Almost a third of the total label incorporated was found in the neutral lipids. Considerable amounts of radioactivity were also found at the origin.

Stopping the reaction with trichloroacetic acid appeared to increase the percentage of label found at the origin, and decreased the amount of label associated with the neutral lipids compared to when the reaction was stopped with phosphate buffered saline.

[Methyl -^{3}H] methionine (10μCi) in medium 199 was extracted by the Folch method. The residue remaining after the solvent had been evaporated was re-dissolved in a mixture of phospholipid standards and subjected to TLC. It was found that ∿0.6% of the original radioactivity prior to extraction could be recovered from the TLC plate (data not shown).

Most of the radioactivity was located in an area above PE and PNME in the direction of the first dimension. A lesser amount of radioactivity was found in a broad band parallel to the direction of the second dimension.

This data indicates that the assignment of the recovered radioactivity in Table 18 to discrete phospholipids may not be reliable and that the majority of [^{3}H] methyl groups incorporated into cells during"lipid methylation" may be due either to free [^{3}H] methionine or to other contaminants.

Since acid conditions did not alter the distribution of radioactivity in the phospholipid fraction, I continued to use trichloroacetic acid to stop the methylation reaction.

TABLE 18

A comparison of the effects of terminating the incorporation of [^{3}H]methyl groups in C6 cells with trichloroacetic acid or with phosphate buffered saline on the distribution of [^{3}H] label recovered from TLC plates.

	% of [^{3}H] label in each lipid fraction recovered from TLC plate			
	PBS		TCA	
Total d.p.m. recovered from TLC plate	Control 8907	+ isoproterenol (10μM) 12382	Control 13662	+ isoproterenol (10μM) 21007
Neutral lipid	41	42	31	26
Total phospho-lipid	29	30	28	26
PE	3	2	2	1
PNME	2	2	2	2
PDME	6	6	7	4
PC	11	11	11	12
PS	2	2	1	1
PA	1	5	2	3
PI	2	1	1	1
Sphingomyelin	2	1	2	2
Origin	30	26	41	48

Methylation was measured by incubating cells in the presence of [methyl-^{3}H]methionine for 55min, and terminating the reaction with either 10%(v/v) trichloroacetic acid or phosphate buffered saline. Cell lipids were extracted and resolved by 2-dimensional TLC. After locating the lipid spots with iodine, they were scraped off the plate and the incorporated radioactivity determined. Values are the means from two individual samples for each condition.

3.6.5 Summary of Results presented in 3.6.0-3.6.4

1. The magnitude of lipid methylation in C6 cells is dependent on the cell density of the culture. Lipid methylation is highest in low density cultures comprising cells in logarithmic growth, and lowest in cultures of cells which are in stationary phase.
2. Lipid methylation is a β-adrenergically mediated event and is stimulated by isoproterenol but plays no direct role in regulating the β-adrenergic response by promoting fluidity changes in the cell plasma membrane.
3. Cyclic AMP may regulate lipid methylation in intact C6 cells.
4. Less than a third of the [^{3}H]-methyl groups incorporated into lipid by incubating C6 cells in the presence of L-[methyl-^{3}H]methionine is localised in areas of the TLC plate to which phospholipid standards co-migrate.
5. Although isoproterenol stimulates an increase in the incorporation of L-[methyl-^{3}H]methionine, there is an increase in the amount of label incorporated into all of the lipid fractions examined. Thus, there are no differences in the percentage of label found in any particular fraction between control and stimulated cells.
6. Terminating the lipid methylation reaction with trichloroacetic acid or phosphate buffered saline makes no difference to the percentage distribution of incorporated label. However the amount of label incorporated into cells terminated by trichloroacetic acid is greater than when the reaction is stopped with phosphate buffered saline.

3.7.0 EFFECT OF PHOSPHOLIPID POLAR HEADGROUP MODIFICATION ON LIPID METHYLATION

3.7.1 Effect of Polar Headgroup Modification on the Distribution of [^{3}H]-Methyl Groups Incorporated during Lipid Methylation

Cells were allowed to incorporate L-[methyl-^{3}H] methionine for 1h, then the lipid fraction was extracted. The lipid components of the mixture were resolved by two dimensional TLC and the distribution of the incorporated radioactive methyl groups is shown in Table 19.

TABLE 19

The effect of polar headgroup manipulations on distribution of [^{3}H]label incorporated into C6 cells during basal lipid methylation.

		% of [^{3}H]label recovered from TLC plate					
		Location					
Supplement	Total d.p.m. recovered	Neutral lipid	PC	PDME	PNME	PE	Origin and other Phospholipids
None	13280 (2)	50	20	7	3	4	16
DME	26128 (2)	32	60	6	1	1	-
NME	33495 (2)	24	28	36	3	1	8
Ethanol-amine	12647 (2)	54	19	7	4	4	12

Values are the means of radioactivity recovered from two separate plates per condition.

The amount of label in the neutral lipid fraction is drastically reduced in the cells treated with DME and NME, as is the amount of radioactivity found at the origin. Ethanolamine treatment does not appear to have much effect on the distribution of label and apart from a slight increase in the neutral lipid fraction, the distribution is very similar to that in the control cells.

The percentage of label recovered in the phospholipid fraction is doubled in the NME and DME treated cells compared with the control. This increase appears to be at the expense of the percentage of label recovered from the neutral lipids and from the origin.

Base treatment causes an increase in the amount of label found in the subsequent phospholipid in the phospholipid methylation pathway. DME treatment trebles the amount of radioactivity recovered from PC in those cells. NME treatment causes a five-fold increase in the percentage of label in PDME, with only a minor increase in the amount of label in PC. Ethanolamine, on the other hand, does not show this effect.

3.7.2 Effect of Phospholipid Polar Headgroup Modification on the Rate of Lipid Methylation

Enrichment of membranes with PDME, PNME and PE will increase the amount of substrate for the phospholipid methylation pathway. Therefore I examined the effects of polar headgroup substitution on the rate of lipid methylation. The results of these experiments are presented in Table 20.

As might be expected, enrichment of the cell membranes with PE, PNME or PDME increased the basal rate of methylation. NME treatment caused the greatest stimulation of basal methylation

TABLE 20

The effect of phospholipid modification on the rate of lipid methylation in C6 cells incubated in the presence of L-[methyl - ^{3}H]-methionine.

Treatment	Methyl groups incorporated pmol/mg protein/55min ± SEM: Basal	Rate of methylation relative to control	+ 10μM isoproterenol	Rate of methylation relative to basal rate
None	70.9 ± 3.3 (4)	-	99.3 ± 4.6	130%
DME	133.2 ± 6.4 (4)	1.9 X	77.2 ± 10.3	57%
NME	210.4 ± 4.8 (4)	2.9 X	80.6 ± 6.2	38%
Ethanol-amine	95.7 ± 9.8 (4)	1.3 X	Not done	Not done

Cells were grown in linbro wells till almost confluent, then treated for 24h with medium containing 5mM bases. The rate of lipid methylation was then measured as described in Section 2.7.

by 2.9-fold. DME and ethanolamine were of similar potency and caused a 1.9 -fold and a 1.3 -fold increase in basal methylation respectively.

More interesting is the observation that exposure of the cells with modified phospholipid polar headgroup composition to isoproterenol causes a decrease in methyl group incorporation, i.e. a demethylation (Table 20).

Both NME- and DME-treated cells showed a demethylation when exposed to isoproterenol, although the effect was most marked in the NME-treated cells. In both the DME- and NME-treated cells the lipid methylation rate in the presence of isoproterenol decreased by 3-4-fold.

This prompted me to determine whether this demethylation was mediated by the β-adrenergic receptor.

3.7.3 Effect of Propranolol on Isoproterenol-induced Demethylation in DME-treated cells.

Cells were grown in the presence of DME and were then stimulated with isoproterenol in the presence of propranolol, to determine whether the presence of a β-adrenergic antagonist could block the demethylation caused by isoproterenol. The results of the experiment are contained in Table 21.

The results presented in Table 21 indicate that the isoproterenol-induced demethylation in cells whose membranes have been enriched in PDME cannot be blocked by propranolol, and is therefore unlikely to be an event mediated by the β-adrenergic receptor. Propranolol itself does not have any effect on basal methylation rates, although it blocks the isoproterenol stimulation of methylation in the control cells.

TABLE 21

The effect of propranolol on lipid methylation in control and DME-treated cells incubated in the presence of isoproterenol

Treatment	Methyl groups incorporated pmol/mg protein/ 55min ± SEM	
	Basal	+ 10μM isoproterenol
Control	63.66 ± 4.6 (4)	100.98 ± 19.2 (4)
Control + 500μM propranolol	Not done	75 ± 8 (4)
DME	90.8 ± 11.7 (4)	73 ± 5.5 (4)
DME + 500μM propranolol	84.08 ± 10.6 (4)	57.4 ± 4.5 (4)

Cells were grown for 24h in media supplemented with DME prior to measurements of lipid methylation.

3.7.4 Uptake of Methionine in Cells with Modified Polar Headgroup Composition

The rate of uptake of radioactive methionine into cells was measured in order to check whether differences in the rates of lipid methylation could be accounted for by differences in the availability of methionine.

Fig.16 shows that the initial rate of methionine uptake (0-5min) was greater under all conditions relative to the control cells. However, this enhanced rate of methionine uptake was not maintained, except by NME-treated cells, for up to 60min. Therefore in the DME and ethanolamine treated cells the enhanced methionine uptake at 60min was principally a result of the greater uptake in the first 5min of the incubation period.

Since the increase in the rates of basal methylation (Section 3.7.2) are greater than the increased amount of methionine uptake in the cells with modified phospholipid polar headgroup composition, it seems that the increased rates of basal methylation are due to the greater availability of phospholipid substrate rather than enhanced methionine uptake. This is backed up by the observation that DME and NME treatment increases the percentage of radioactivity found in the phospholipid fraction **(Table 9)**.

Figure 16

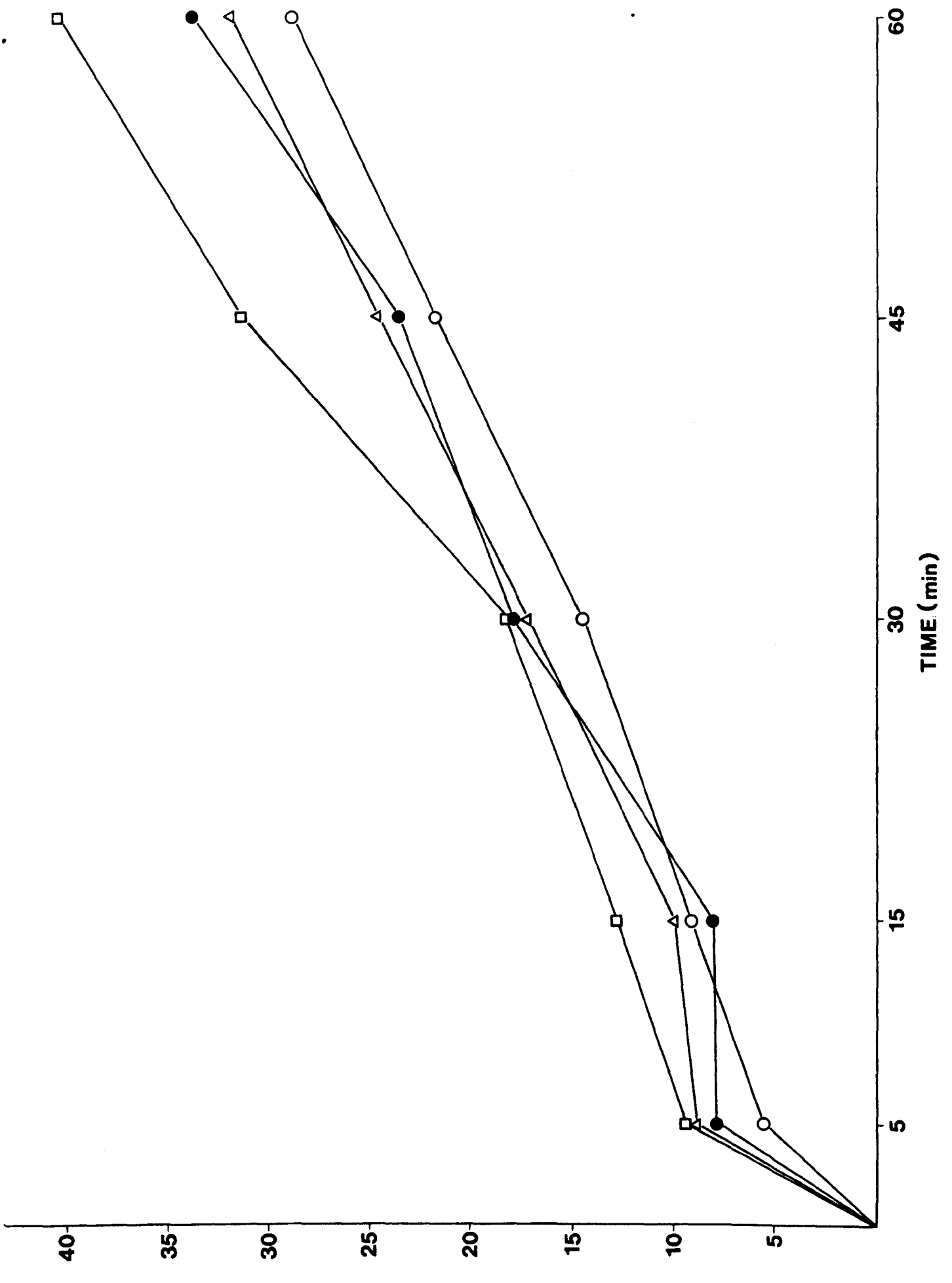
Uptake of L-[methyl-^{3}H]-methionine by C6 cells after growth in the presence of 5mM bases for 24 hours. Each point is the mean of three separate determinations.

O = control cells

△ = DME treated

□ = NME treated

● = ethanolamine treated


CPM INCORPORATED x 10^4
40
35
30
25
20
15
10
5
5
15
30
45
60
TIME (min)

3.7.5 Summary of Results Presented in Sections 3.7-3.7.4

1. Enrichment of C6 cell membranes with PDME, PNME and PE elevates the basal rate of lipid methylation. NME treatment causes the largest elevation of basal methylation. DME and ethanolamine treatments both approximately double the basal methylation rate.
2. DME- and NME- treated cells show a redistribution of the location of [methyl-^{3}H] label incorporated after incubation with L-[methyl-^{3}H] methionine. There is an increase in the amount of label found in the phospholipid fraction at the expense of the percentage of the label found in the neutral lipid fraction. Base treatment results in an increase in the percentage of label found in the subsequent phospholipid of the phospholipid methylation pathway. Cells enriched in PE show similar distribution of label in the phospholipids as in control cells.
3. Isoproterenol stimulates a demethylation in base treated cells, which is greatest in NME-treated cells. This demethylation does not appear to be mediated by the β-adrenergic receptor.
4. Base treatment leads to an increase in methionine uptake when the cells are incubated in the presence of L-[methyl-^{3}H] methionine. NME-treated cells show the highest uptake, followed by ethanolamine and DME-treated cells. The increase in methionine uptake in the supplemented cells is not sufficiently large to account for the increased basal methylation rates in the treated cells.

3.8.0 LIPID METHYLATION AND CYCLIC AMP ACCUMULATION IN HIGH PASSAGE CELLS

C6 cells are known to show marked changes in the responsiveness of their β-adrenergic system when they reach high passage number (Mallorga et al., 1981). It is also known that ageing increases the methyltransferase activity of rat brain cells (Crews et al., 1979). It is known that the β-adrenergic responsiveness of high passage cells is greatly reduced (Mallorga et al., 1981), therefore I thought that high passage cells would be a good system with which to study further the relationship between lipid methylation and the β-adrenergic response.

3.8.1 Production of High Passage Cells

High passage cells were obtained by continually passaging cells until they were approximately double the passage number of the cultures that were normally used. This ensured that the high passage cultures that I obtained were similar in age to those used by Mallorga et al. (1981), and therefore likely to display reduced β-adrenergic responsiveness to isoproterenol.

From passage number 71, the cells began to look less like the control cells. They began to look longer than the controls, more oblong in shape, and with numerous spidery processes. By passage 84, the majority of the cells were two times longer than the control cells. This enlargement of cell size was reflected in the shorter time required for the cells to reach confluency, compared with controls. Cells of passage number 102 were confluent in only 75% of the time required for cells of passage number 51. However, a decrease in the cell doubling time cannot be ruled out.

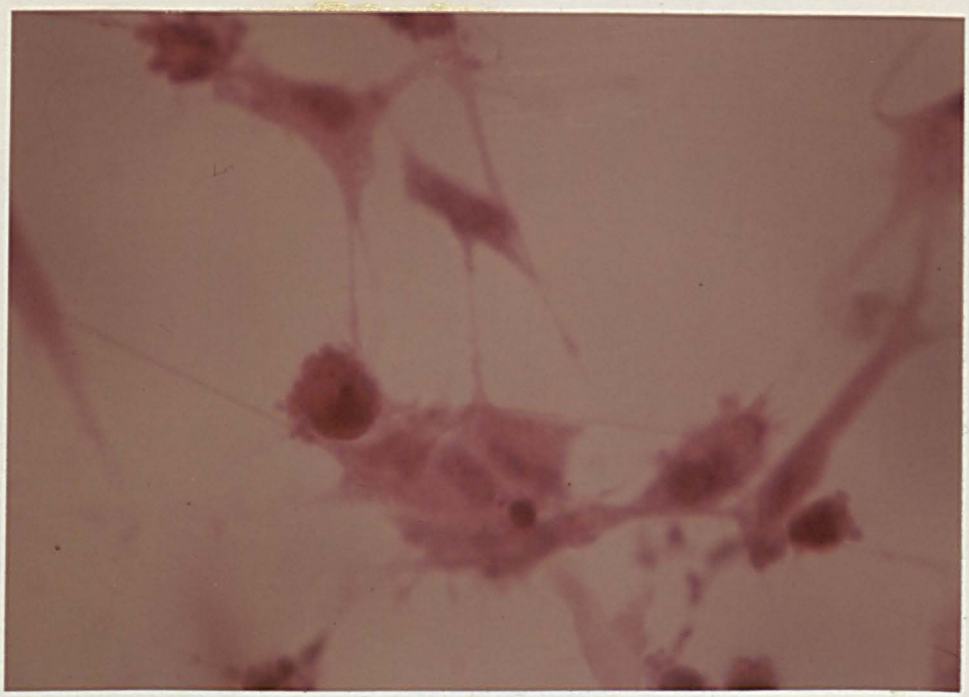


Plate 21

Plate 21: C6 cell of passage 103 stained with Haematoxylin-Eosin X500

Plate 22: Confluent monolayers of C6 cells of passage 103 X200

Plate 23: Confluent monolayers of C6 cells of passage 52 X200

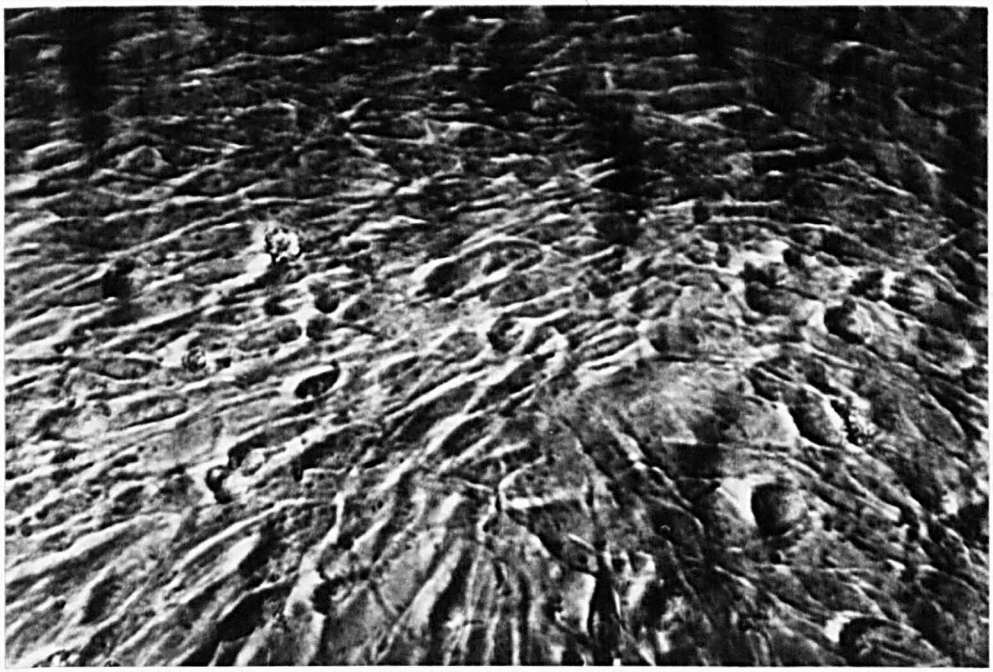


Plate 22

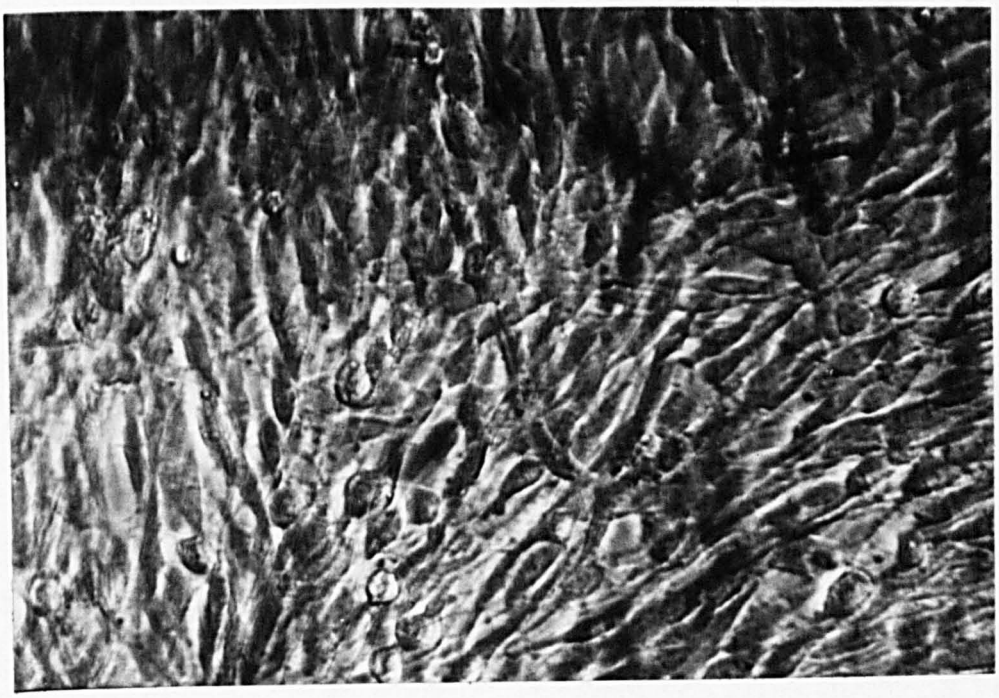


Plate 23

3.8.2 Appearance of High Passage Cells

Plate 21 shows a cell of passage number 103, stained with haematoxylin-eosin. Compared with the early passage cells (Plate 16) it is clearly larger and less astrocytic in appearance. The greater cytoplasmic-nuclear area is quite clearly visible, the nuclear contents also appeared to be more dispersed than in the early passage cells.

3.8.3 Measurements of High Passage Cell Size

Measurements of the size of the cells of passage number 103 were made using a calibrated graticule. Two measurements were made, the length of the cell and its width at the broadest part of the cell. Cells of early passage number were measured in the same way for comparison:

	Cell Size (μm) ± SEM	
Passage number 50	78.5 ± 7 x 11 ± 1	(21)
Passage number 103	179 ± 16 x 13 ± 2	(21)

The high passage cells are clearly longer than early passage cells, but the breadth of the cells is similar.

A curious effect observed in confluent cultures of high passage cells is the orientation of the cells along lines parallel to their long axis (Plate 22). This is possibly a consequence of the greater length imposing constraints on the way that the cells can pack together. This contrasts with the relatively random orientation of cells of early passage in confluent cultures (Plate 23).

3.8.4 Effect of High Passage Number on the β-Adrenergic Response

The cAMP accumulation of high passage cells in response to isoproterenol was measured. The results are presented in Table 22.

TABLE 22

Effect of Passage Number on cAMP Accumulation in C6 Cells

	cAMP accumulation (pmols/mg protein/30min ± SEM)	
Passage Number	Basal	+ 10μM isoproterenol
52	20.1 ± 2.5 (3)	973 ± 152 (3)
103	29.7 ± 6 (3)	370.9 ± 37 (3)

The basal cAMP accumulation of the early and high passage cells is similar, but the stimulation of cAMP accumulation in response to isoproterenol is clearly decreased in the high passage cells to about half that observed in the early passage cells.

3.8.5 Effect of High Passage Number on Lipid Methylation

The lipid methylation activity of the high passage cells was measured, and is recorded in Table 23.

TABLE 23

Effect of Passage Number on Lipid Methylation in C6 Cells

	Methyl groups incorporated (pmol/mg protein/55min ± SEM)	
Passage Number	Basal	+ 10μM isoproterenol
52	32 ± 3.7 (4)	75.4 ± 10.6 (4)
103	90 ± 16.1 (4)	70.3 ± 5.7 (4)

The basal methylation in the high passage cells is nearly three-fold greater than that of the early passage cells. However there is a loss of the isoproterenol-stimulated increase in methylation, and similar to the base-treated cells, there appears to be an isoproterenol-induced demethylation.

These results with the high passage cells are remarkably similar to those obtained for cells enriched in PDME.

3.8.6 Summary of Results Presented in Sections 3.8-3.8.5

1. Continuous passage causes an increase in the size of, and a change in the morphology of C6 cells in culture.
2. The stimulation of cAMP accumulation by isoproterenol is significantly reduced in these cells.
3. The rate of basal lipid methylation is three times greater than in early passage cells, but the isoproterenol-stimulation of methylation is lost, and instead isoproterenol causes a demethylation in high passage cells.

DISCUSSION

"Time may have the answers but the map is here
Now is the future that I never wished to see
I was happy dreaming and had no fear:
But now, from the map, a gun is aimed at me"

Ruthven Todd

4.0 The results of the modifications of cellular phospholipid polar headgroup composition (Sections 3.0-3.3.2) have already been discussed in those sections. This section will therefore concentrate on discussing the summarised results of Sections 3.5.4, 3.6.5, 3.7.5 and 3.8.6.

4.1 Phospholipid Polar Headgroup Modification and its Effect on the β-Adrenergic Response (Sections 3.5-3.5.3).

The elevated basal cAMP levels of cells with modified phospholipid polar headgroup recorded in Table 15 would appear to be principally due to an activation of adenylate cyclase by PDME, PNME or PE. These results are similar to those of Engelhard et al. (1978) who observed a similar pattern of activation of basal adenylate cyclase activity in LM cells. Both these results are in contrast to the work by Schroeder et al. (1976a) who found that enrichment of LM cell membranes with the same phospholipids did not alter the activity of either the Na^+/K^+ ATPase or 5'-nucleotidase, both of which are plasma membrane-bound enzymes.

Presumably the increased phosphodiesterase activity is due to an activation of the membrane-bound phosphodiesterase by the incorporated phospholipids. This is not an unreasonable assumption since Jard et al. (1972) have shown that 85% of the high Km form and 91% of the low Km form of phosphodiesterase activity in C6 cells is in the particulate fraction. It has also been shown that the decreased response of high passage C6 cells to isoproterenol is due to an increase in activity of the phosphodiesterase (Fishman et al., 1981).

Since the magnitude of stimulation of cAMP accumulation in response to isoproterenol in the cells exposed to bases is not restored to that of the controls by theophylline, it is possible that there is another reason for the reduced β-adrenergic response in these cells. There are two possible explanations. Firstly, there might be a loss of receptors in the cells enriched in PDME and PNME. Bakardjieva(1982) has shown that manipulating the phospholipid composition causes a decrease in the number of β-adrenergic receptors and a reduction in the magnitude of the β-adrenergic response in Chang liver cells. No studies of the effect of exposure of cells to bases on the β-adrenergic receptor number have been published, and it is impossible to predict if this might be the cause of the reduced response in C6 cells exposed to bases. Maximum cAMP accumulation in C6 cells in response to β-adrenergic agonists is dependent on occupation of all the available β-receptors (Bockaert et al., 1973).

The course of cAMP accumulation in cells exposed to DME (Fig.14) shows that the rate of cAMP accumulation in response to isoproterenol is similar to that of the control cells, but that the maximum intracellular cAMP content is lower than in the controls. This could point to a reduced number of β-receptors in the cells exposed to DME. Another explanation is that the coupling between the β-adrenergic receptor and adenylate cyclase might be perturbed by enrichment of the cell membranes with PNME or PDME.

Theophylline reduces the maximum cAMP response to isoproterenol in control cells. This is difficult to explain but has been recorded by other workers (Gilman and Nirenberg, 1971; Jard et al., 1972).

The low Km form of phosphodiesterase is activated by PI and LPC in rat adipocyte microsomes (Macaulay et al., 1983) and in rat brain (Wolf and Brostrom, 1976). The increased rate of phospholipid methylation in the cells with modified membrane composition (Table 20) could produce small amounts of LPC by phospholipase A_2 activity on the newly formed PC. This, although undetectable on the t.l.c. plates could be sufficient to activate the phosphodiesterase. Houslay and Palmer (1979) have demonstrated that extremely low concentrations of LPC activate adenylate cyclase. If the same were true of phosphodiesterase, it could explain the increased phosphodiesterase activity in these cells.

4.2 Lipid Methylation in C6 Cells (Sections 3.6-3.6.4)

Lipid methylation may be a misnomer since it appears from Table 18 that most of the radioactivity incorporated is not associated with phospholipids of the N-methylation pathway. Considerable radioactivity was also found in areas of the TLC plates which did not correspond to endogenous C6 lipid.

The increase in the rate of [^{3}H]methyl group incorporation caused by isoproterenol may represent a general, non-specific increase in incorporation. If this is true then lipid methylation would be unlikely to have the physiological role claimed by Axelrod's group. However if the [^{3}H] methyl group incorporation is due to incorporation into physiologically important phospholipids, some possible roles for it are discussed in the following sections and in section 5.2.

[^{3}H] Methyl group incorporation in C6 cells is a β-adrenergically mediated event and occurs under conditions in which β-adrenergic stimulation of cyclic AMP accumulation occurs. This is in agreement with the conclusions of Strittmatter et al. (1979a) and of Hirata and Axelrod (1980).

From the results presented in Table 15 it appears that both the basal and isoproterenol-stimulated rates of lipid methylation are dependent on the cell density of the cultures being examined. Morris and Makman (1976) have shown that the amount of cAMP accumulation in response to isoproterenol in C6 cells is directly proportional to the culture density. The opposite appears to be true for lipid methylation. Possibly the reduced isoproterenol stimulation of lipid methylation in high density cultures is due to a reduction in β-adrenergic receptor number in such populations.

More difficult to explain is the higher basal rate of methylation in low density cultures compared to high density cultures. Perhaps this is due to a higher rate of metabolism in cells growing at a rapid rate. Clearly, the increase in the rate of [^{3}H]methyl group incorporation on exposure of C6 cells to isoproterenol is a β-adrenergic receptor-mediated event (Table 16) and it occurs under conditions in which cAMP accumulation is stimulated. However, ESR studies indicate that lipid methylation is not responsible for fluidity changes in the membranes of cells stimulated by isoproterenol.

This observation argues against the theory of Hirata and Axelrod (1980) that PNME or any other product of N-methylation of PE promote β-adrenergic coupling by fluidising the membrane. My conclusion is backed by the results presented in Table 13 which show that cell membranes enriched in PNME, PDME or PE do not differ in physical properties from control cells.

The results presented in Table 17 indicate that [^{3}H]methyl group incorporation is stimulated by cAMP analogues. This is in agreement with the findings of Castano et al. (1980), and suggests that lipid methylation is not a primary event in the β-adrenergic response. However Pritchard et al. (1981) have shown that cAMP analogues have either little effect, or lead to a decrease in the rate of lipid methylation in hepatocytes.

The observation (Table 18) that the acid conditions used to stop the methylation reaction can increase that apparent rate of [^{3}H]methyl group incorporation in intact cells when compared to terminating the reaction with PBS is consistent with the findings of Moore et al.(1982). This does not undermine the conclusions of these experiments, since the distribution of incorporated [^{3}H] was not altered.

4.2.1 Methyltransferases and Lipid Methylation

Before speculation about the relative capacities and kinetics of the steps of the phospholipid methylation pathway are made, the question of how many phospholipid methyltransferases are present must be addressed.

Audubert and Vance (1983) argue that only one phospholipid methyltransferase, with a pH optimum of 10.0, is present in hepatocytes. In the systems examined by Hirata and Axelrod (1980) two enzymes were suggested with pH optima of 7.0 and 10.0. Similarly, Crews et al. (1980a) have provided evidence to suggest that in rat brain synaptosomes two enzymes are present with pH optima of 7.5 and 10.5.

In C6 cells an examination of the phospholipid methyl transferase activity by the method employed by Audubert and Vance (1983) indicates that there are two pH optima for phospholipid methyltransferase activity; 7.0 and 10.5 (Gillespie, McKenzie, and Brophy, unpublished results). Thus, for an explanation of the relative activity of each stage of the phospholipid methylation reaction in C6 cells it would seem appropriate to interpret the results of Tables 18 and 19 in terms of being catalysed by two phospholipid methyltransferases.

Vance and de Kruijiff (1980) have stressed that the amount of PC synthesised by N-methylation is extremely small in hepatocytes. Presumably the capacity of the pathway in C6 cells is limited. Two pieces of evidence support this conclusion. Table 5 shows that although cells could be enriched in substrate for the phospholipid methylation pathway (PE and PNME), no trace of the immediately subsequent phospholipids in the methylation pathway were found in these cells. On the other hand Table 19

shows that enrichment of the cell membranes with intermediates of the methylation pathway did stimulate phospholipid methylation.

The distribution of radioactivity associated with the incorporated methyl groups in Table 18 suggests that the isoproterenol-stimulation of the rate of methylation is due to a general increase in [^{3}H] methyl group incorporation within C6 cells, and is not specific for the phospholipids.

Careful scrutiny of the data published by Hirata et al. (1979a) shows that the same pattern was recorded. This argues against a specific role of any particular phospholipid class (such as PC) in the effects produced by the lipid methylation reaction.

The considerable amount of [^{3}H] methyl groups found in the neutral lipid fraction (Table 18) is in accord with the findings of Zatz et al. (1981) who showed that a large percentage of lipid methylation in rat lung was due to incorporation of [methyl-^{3}H] groups into neutral lipids. Kloog et al. (1982) have also demonstrated that 30% of the total radioactivity incorporated into rat parotid, retinal and basophilic cells was found associated with ubiquinone.

Table 18 shows that a considerable amount of [^{3}H] label was found at the origin. This could be due to methionine that had not been incorporated into lipid.

4.3 Phospholipid Polar Headgroup Modification and its Effects on Lipid Methylation (Sections 3.7-3.7.4)

Enriching C6 cells membranes with PE, PNME or PDME increases the basal rates of lipid methylation in intact cells. A similar pattern of stimulation was found for DME- and NME-treated MOP31C cells (Maeda et al., 1980a). However these workers did not

observe a stimulation of the basal rate of lipid methylation in ethanolamine-treated cells.

C6 cells exposed to NME and DME incorporated a greater percentage of [methyl-^{3}H] groups into the phospholipid fraction, apparently at the expense of the neutral lipid fraction, and the polar material at the origin. The decrease in the radioactivity at the origin, might be due to a greater consumption of [methyl-^{3}H]methionine by these cells. The decrease in the methylation of the neutral lipid fraction could be due to greater competition for newly synthesised S-adenosyl[methyl-^{3}H]methionine (SAM) in these cells. Although methylation of the neutral lipid fraction appears to be catalysed by distinct methyltransferases from those involved in phospholipid methylation, (Zatz et al., 1981), both classes of enzymes would presumably, compete for the same pool of SAM. Perhaps the phospholipid methyltransferases have a greater affinity for SAM than do the fatty acidmethyltransferases.

Cells enriched in PDME or PNME showed a large increase in incorporation of [^{3}H] label into those phospholipids that were next in the methylation pathway (Table 19). This is as one might expect since the enriched phospholipid will be rapidly methylated. Presumably the greater stimulation of the rate of basal methylation in cells enriched with PNME reflects two methyl groups being incorporated for each PNME molecule methylated as opposed to one per PDME molecule.

However one might expect PE molecules to incorporate three [methyl-^{3}H] groups. That the cells exposed to ethanolamine do not show a greater increase in basal methylation rate when compared to NME-treated cells, suggests some other fate for the

[methyl-^{3}H] groups incorporated into the ethanolamine-treated cells.

It could be that the cells enriched in PE have an active base-exchange pathway which causes a decrease in the availability of PE substrate for the methylation reaction. An alternative explanation is that the first step of the phospholipid methylation reaction is of limited capacity, and cannot incorporate all the [methyl-^{3}H] groups available. A decreased uptake of methionine in ethanolamine-treated cells would appear to be ruled out by Fig.16. A limited capacity of PMT I might explain why no change is seen in the percentage of label incorporated into the neutral lipid fraction in PE-rich cells. This would also explain why the radioactivity at the origin (possibly due to free methionine) does not decrease in the same fashion as in the DME- and NME-treated cells.

Methionine uptake in the cells enriched in PNME, PDME or PE is not stimulated in proportion to the increase in rate of basal lipid methylation. This indicates that the stimulation in the basal rate of lipid methylation by PDME, PNME or PE enrichment is probably due to an increase in the availability of phospholipid substrate for the PMT s, rather than increased availability of methionine for SAM biosynthesis. By contrast experiments with yeast cells which had an increased PE:PC ratio (Trivedi et al., 1982) showed that increasing the PE:PC ratio decreased methionine and other amino acid uptake .

4.3.1 Demethylation in Cells Enriched in PDME and PNME in response to Isoproterenol

The demethylation observed in Table 20 could be due to two possible causes. The first is that, since it seems that

the lipid methylation pathway is of limited capacity, it could be that in the DME- and NME-treated cells the pathway is already saturated and simply cannot be increased by β-adrenergic stimulation. This could be due to the fact that any constraints on the activity of the enzymes that are normally caused by the reception are relieved by the alteration of the membrane phospholipid composition. In addition to this point, Mato et al. (1980) have shown that the receptor-mediated stimulation of phospholipid methylation in hepatocytes is at a maximum at 15min, and declines to basal levels after 60min of exposure of the cells to glucagon.

Hashizume et al. (1983) claim that the β-receptor constrains the activity of PMT II, the enrichment of the cell membranes with PDME or PNME would stimulate PMT II, and possibly this overcomes the limitations on PMT II activity normally imposed by the β-receptor.

This however does not fully explain the decrease in lipid methylation rate that is observed. I suggest that the altered membrane phospholipid environment, or products from the methylation reaction activate a phospholipase C which is specific for the unsaturated fatty acid-rich phospholipids of the methylation pathway. Nemecz and Farkas (1980) have demonstrated that exposure to isoproterenol stimulates the activity of a phospholipase C in rat liver plasma membranes, and that this effect of isoproterenol is not mediated by the β-adrenergic receptor. Removal of the phospholipid polar headgroup by the activity of a phospholipase would explain the loss in radioactivity from the cells. If a phospholipase A_2 was activated by isoproterenol

as suggested by Mallorga et al. (1980), one would expect to see an accumulation of radioactivity in LPC relative to control cells, this was not observed in cells enriched in PDME which were stimulated with isoproterenol (data not shown). Breakdown of methylated PC to LPC would not be expected to decrease the amount of [^{3}H] recovered from the lipid fraction.

Similarly a loss of β-receptors in the cells exposed to DME, NME or ethanolamine as suggested in section 4.1 would not explain loss of [^{3}H] label from the cells on exposure to isoproterenol.

Why then does demethylation not accompany isoproterenol-stimulation of the control cells? Possibly, activation of phospholipase C is regulated by the altered phospholipid composition of the treated cells, but Castano et al. (1980) followed the time course of stimulation of lipid methylation by glucagon in hepatocytes. They found that the stimulation of lipid methylation reached a maximum after 15min exposure to a non-saturating dose of glucagon. The rate of lipid methylation decreased thereafter, reaching basal levels after 60min. This suggests that a decrease in the isoproterenol-stimulated increase in the rate of lipid methylation could eventually occur, possibly due to receptor desensitisation, or due to the activation of phospholipase C.

Perhaps the incorporation of large amounts of PDME or PNME alters the lipid domains of the membrane around phospholipase C molecules. This is unlikely, since PC synthesised by the methylation pathway is supposed to be translocated to the outer leaflet of the bilayer, presumably this is where the phospholipase C would be located.

Houslay has shown that **5-doxylstearic acid spin** label localises in the outer leaflet of the bilayer, and would be sensitive to any such domain changes suggested above. The absence of any change in the order parameter of DME- and NME-treated cells (Table 13) argues against modification of lipid domains by PDME or PNME enrichment.

4.4 β-Adrenergic Responsiveness and Lipid Methylation in High Passage C6 cells (Sections 3.8-3.8.5)

Continuous passage of C6 cells is probably a good analogy to the ageing process. On the other hand, continuous passage may produce alteration of the cell character by cumulative effects of exposure to the trypsin used in the procedure.

The morphological changes, and the increase in cell size caused by continuous passage that was observed in C6 cells (Plate 21, Section 3.8.2) are similar to the relative differences observed in C6 cells of passage number 22 and 88 by Parker et al. (1980). The reduction in stimulation of cAMP content on exposure to isoproterenol (Table 22) is similar, but not as drastic as the reductions observed by Mallorga et al. (1981).

Particularly interesting is the observation (Table 23) that the high passage (HP) cells display an increase in the basal rate of lipid methylation, and a demethylation similar to DME- and NME-treated cells, on exposure to isoproterenol. Mallorga et al. (1981) showed that HP cells display an enhanced activity of the high Km form of phosphodiesterase, and a decrease in β-receptor number.

In the C6 cells enriched with PE, PNME or PDME it seems that phosphodiesterase activity is enhanced (Table 14), in this sense these cells are similar in character to the HP

cells produced by Mallorga et al. (1981). It is possible then, as hypothesised in Section 4.1, that the cells exposed to bases also have a decreased β-adrenergic receptor complement. It seems then that the HP cells and the cells grown in the presence of bases exhibit similar characteristics. It might be instructive to look for common causes of these effects.

Changes in the lipid composition of the HP cells could be responsible for the effects described; however lipid composition was not examined by Mallorga et al. (1981), or by myself, so it is impossible to comment on this possibility.

I have already mentioned that the enhanced basal methylation rate in cells exposed to bases could be due to relief of restraints on PMT activity by the β-receptor upon altering the phospholipid composition. Perhaps, since β-adrenergic receptor number in HP cells is decreased, the constraints on PMT activity are also alleviated in HP cells. If this was the case, it might explain the enhanced basal methylation in the HP cells. If that werethe case it would seem that the demethylation in response to isoproterenol in cells exposed to bases is not a result of the elevated basal intracellular cAMP levels, since in HP cells basal cAMP content was lower than in low passage cells (Mallorga et al., 1981). This would point to the altered phospholipid composition of DME- and NME-treated cells being the cause of demethylation.

The decreased β-adrenergic responsiveness of HP cells was shown to be most likely due to a defect in the coupling of the receptor to the G/C unit, since NaF- and Gpp(NH)p-stimulated adenylate cyclase activity was normal. Perhaps, a similar situation prevails in the cells exposed to DME, NME and ethanolamine.

Crews et al. (1981) have demonstrated age-dependent changes in the lipid methylation pathway in rat brain synaptosomes. They found a progressive increase in the activity of phospholipid methyltransferase I in synaptosomes prepared from rat brains of age 1-21 months. PMT II activity was not affected. The distribution of incorporated [methyl-^{3}H] groups in the HP cells was not examined, but if a similar situation prevails as in the rat synaptosomes, this might explain why the basal rate of lipid methylation was elevated in the HP cells.

CONCLUSIONS

"O Wad some Pow'r the giftie gie us
To see oursels as others see us!
It wad frae mony a blunder free us
And foolish notion"

R. Burns.

5.0 CONCLUSIONS

5.1 Phospholipid Polar Headgroups and the β-Adrenergic Response

It seems that the activity of the β-adrenergic system is modulated by the chemical properties of the phospholipid polar headgroups as well as by the physical properties of the lipid matrix. The most susceptible functions to polar headgroup influence appear to be the activity of the catalytic unit of adenylate cyclase and the coupling between it and the hormone receptor. Phosphodiesterase activity in C6 and other cell membranes is also modulated by changes in the phospholipid polar headgroup composition. It seems likely that both the rate of cAMP synthesis and its rate of breakdown can be regulated by the membrane phospholipids. Whether or not a similar regulation occurs in vivo is not clear, but clearly, the possibility exists for a sensitive control of the β-adrenergic response of the cell. Cellular phospholipid composition in a given cell line is maintained constant by a combination of catabolic and anabolic reactions. This does not exclude the possibility that rapid transient changes in phospholipid composition, such as rapid temporary changes initiated by receptor stimulation, could regulate β-adrenergic responsiveness by activation-deactivation of the production of secondary messengers. If the assumption is correct that boundary lipid regulates membrane protein function, then only small, but selective alterations in phospholipid composition would be necessary to effect changes in the activity of the membrane proteins. Phospholipid methylation could be a mechanism by which control of signal transduction is effected.

Control of membrane protein function by phospholipids may not be so simple a mechanism as suggested above. The fine

tuning necessary for rapid response of and feedback control over the system might involve other regulatory agents such as protein kinases, calmodulin and phospholipases.

The modification of phospholipid polar headgroup composition in vivo by growing cells in media containing headgroups is a useful technique with which to study phospholipid:protein interactions, since there appears to be a minimum of undesirable perturbations to the system, and of particular importance no change in the physical properties of the lipid matrix. These are the first recorded studies in which this technique has been applied to the study of the β-adrenergic system.

The conclusion that the change in the activity of adenylate cyclase in the C6 cells treated with DME, NME, or ethanolamine are due to the altered phospholipid composition is supported by the observation of Engelhard et al. (1976) that the increased basal adenylate cyclase activity in membranes prepared from cells enriched in PE could be reduced to similar values to the control cells by detergent solubilisation of the membranes which removed most of the membrane lipid from the enzyme.

Alternatively, the incorporated PDME, PNME or PE could displace endogenous boundary lipid from the enzyme and result in activation of the enzyme by changes in the composition of lipids intimately associated with the enzyme.

The reduction in the stimulation of cAMP accumulation by isoproterenol in these cells appeared to be principally due to an enhanced phosphodiesterase activity in these cells (Table 14). However the fact that theophylline cannot restore the degree of stimulation by isoproterenol to control levels indicates that the PDME-, PNME- and PE-enriched cells also have a less effective

coupling between the G/C unit and the β-adrenergic receptor, or a reduced β-adrenergic receptor number.

Defects in coupling between the β-receptor and the adenylate cyclase in virus infected C6 cells has been shown to decrease the cAMP response of these cells to isoproterenol stimulation (Munzel and Koschel, 1982; Higashida et al., 1982). A reduction in the number of β-receptors was observed by Higashida et al. (1982), but not by Munzel and Koschel (1982).

5.2 Lipid Methylation; a Role in β-adrenergic Desensitisation?

The results discussed in Section 4.2 allow some conclusions to be drawn about the nature of lipid methylation in C6 cells.

Lipid methylation results in the methylation of neutral lipids and of PE. The pathway is of very limited capacity and does not cause changes in the composition of the bulk phospholipid, nor is it likely to be a significant source of PC biosynthesis. It seems likely that two methyltransferases are responsible for catalysing the reaction, and that the first methylation of PE to PNME appears to be the rate limiting step (4.3). The rate of lipid methylation can be stimulated by β-adrenergic stimulation, and the result is a general increase in the rate of methylation of all the lipid components of the cells which is not confined to any one particular class of lipids (4.2.1). Any enhanced coupling of the β-receptor to the catalytic unit by an increased rate of lipid methylation does not come about by changes in bulk lipid order.

Calculation of the number of methylated phospholipids per β-adrenergic receptor (see appendix VIII) indicates that isoproterenol stimulation of lipid methylation in C6 cells produces 10-30 methylatedphospholipid molecules per β-adrenergic receptor. This is the same order of magnitude as the number of lipid molecules in the boundary layer (Jost et al., 1973). Therefore one cannot exclude the possibility that stimulation of lipid methylation modulates the function of β-adrenergic receptors by modifying the composition of boundary lipid.

The stimulation in the rate of lipid methylation is always accompanied by an elevation of the intracellular cAMP

content whether the elevated cAMP content is a result of increased basal adenylate cyclase activity or caused by β-adrenergic stimulation. However it is impossible to conclude whether lipid methylation expedites this increase in intracellular cAMP content or vice versa. Dibutyrl cAMP can partly mimic the β-adrenergic stimulation of the rate of lipid methylation, which implies that cAMP stimulates the lipid methyltransferases.

On the basis of these findings, and with reference to studies by other workers, I shall examine the likely role of lipid methylation in the β-adrenergic response.

The possible roles of lipid methylation in the activation of the β-adrenergic response have already been discussed. I propose that lipid methylation is unlikely to be involved in facilitating the β-adrenergic response, and that a more likely function of lipid methylation is to initiate events which terminate the β-adrenergic signal and when cells become desensitised.

Good evidence that B-adrenergic receptor desensitization in C6 cells is mediated by phospholipids comes from work by Mallorga et al. (1980). They found that Phospholipase A_2 activation was responsible for β-receptor loss after prolonged exposure of the cells to isoproterenol. Phospholipase inhibitors could prevent the loss of stimulation of cAMP content on challenge with isoproterenol. Similarly later work by this group showed that HP cells possessed an elevated phospholipase A_2 activity (Fishman et al., 1981).

It was also found that on exposure to isoproterenol there was an enhanced breakdown of methyl-^{3}H PC (Mallorga et al., 1980) which correlated

with an increased rate of release of [1-^{14}C]arachidonic acid, which had been previously incorporated into the cells. This breakdown of [methyl-^{3}H]PC and loss of [methyl-^{3}H] groups on exposure to isoproterenol supports my theory that in the cells treated with bases a phospholipase C activity is responsible for demethylation upon isoproterenol stimulation.

Nemecz et al. (1980) have shown that phospholipase C treatment of rat liver plasma membranes leads to a loss of isoproterenol-stimulated increase in intracellular cAMP content, and an elevation of basal adenylate cyclase activity. Further evidence to implicate a phospholipase C in the demethylation reaction is the observation by Leprohan et al. (1983) that the stimulation of the rate of lipid methylation by dopamine in rat brain neurons is followed by a release of [^{3}H] choline amounting to 30% of the [methyl-^{3}H] incorporated, within 30min.

As to the agents and mechanism behind the desensitisation provoked by phospholipase activity, one can only speculate. LPC and products of phospholipase A_2 or phospholipase C action would appear to be the most likely candidates. LPC itself would appear to be an unlikely agent since although Shier et al. (1976) have shown that LPC can inhibit adenylate cyclase, it seems that the primary event in C6 desensitation is an uncoupling of the receptor from the catalytic/regulatory subunits of adenylate cyclase (Fishman et al., 1981). Furthermore, if LPC was the agent involved there would be no need for a pool of unsaturated PC, since saturated acyl chain species of LPC would be as effective if the polar headgroup of the LPC molecule was the active component initiating receptor loss.

Phospholipase C activation might cause receptor loss by breaking down phospholipids in the immediate vicinity of the β-receptor. Alternatively phosphatidic acid synthesis from 1,2-diacylglycerol could act as a calcium ionophore, but this seems unlikely also since Brostrom et al. (1982) have shown that Ca^{2+} does not appear to have any direct effect on β-receptor number in C6 cells.

These objections favour a product of phospholipase A_2 activity as being the cause of desensitisation and uncoupling of the β-receptor. Some indirect evidence supports this conclusion. Shitara et al. (1982) have shown that in C6 cells an increase in the rate of uptake of [^{3}H]deoxyglucose parallels the time course of desensitisation. It is known that uptake of hexose sugars into cells is promoted by calcium ionophores (Bass et al., 1982) and is accompanied by mobilisation of intracellular Ca^{2+} pools. Bass et al. (1982) demonstrated that [^{3}H]-deoxyglucose uptake into human polymorphonuclear lymphocytes can be stimulated by products of the lipoxygenase pathway of arachidonic acid metabolism. The most potent activators of [^{3}H]-deoxyglucose uptake were found to be 5-HETE and 5,12-DiHETE. These hydroxy eicosatetraenoic acids are produced from arachidonic acid by the action of 5-lipoxygenase (see Wolfe, 1982 for a review of these pathways of arachidonic acid metabolism). Volpe et al. (1980) have demonstrated that these compounds are extremely potent Ca^{2+} ionophores.

As to the actual mechanism of desensitisation, two possibilities are likely. Since uncoupling of the β-adrenergic receptor is the first event in C6 desensitisation (Fiederich et

al., 1983; Fishman et al., 1981), it is most likely that effects of these compounds are on the receptor molecule or its immediate lipid matrix. It has been demonstrated by Stenson and Parker (1980) that these HETE compounds are incorporated into both phospholipids and membrane triacylglycerols after their release, in human neutrophils. It could be that they destabilise the binding capacity of the β-adrenergic receptor, and lead to receptor loss when they are incorporated into the plasma membrane.

The second possibility is that since these compounds are calcium ionophores, they cause a redistribution of intracellular Ca^{2+} pools. A large proportion of the phosphodiesterase and adenylate cyclase activity in C6 cells is calcium dependent (Brostrom et al., 1976; Wolf and Brostrom, 1976), the effects of the cation being mediated by calmodulin. Increasing intracellular Ca^{2+} levels to 100μM inhibits C6 adenylate cyclase, but stimulates phosphodiesterase activity.

Thus lipoxygenase metabolites could terminate the cAMP response after β-agonist stimulation. The lipoxygenase metabolites would be produced from arachidonic acid released from PC by phospholipase A_2 action. A small pool of PC enriched in arachidonic acid would be provided by the phospholipid methylation pathway. Thus, changes in calcium mobilisation by lipoxygenase products might be another explanation of the apparently increased phosphodiesterase activity in C6 cells exposed to bases. An accumulation of the desensitising agents could occur as a result of the elevated basal lipid methylation in these cells.

SUGGESTIONS FOR FURTHER WORK

"There are spaces still to be filled
before the map is complete though
these days, its only in the explored
territories that men write, sadly
here live monsters".

Norman MacCaig

6.0 SUGGESTIONS FOR FURTHER WORK

1. To verify the conclusions drawn from the data in Table 14 that phosphodiesterase activity in cells exposed to DME, NME or ethanolamine is elevated, it would be advisable to assay the phosphodiesterase activity in the modified cells.

2. To establish whether the cells exposed to the phospholipid polar headgroup analogues are indeed desensitised, it would be important to measure the β-adrenergic receptor number. Measurements of the dissociation constant (Kd) of the β-receptors in these cells would indicate whether the phospholipid polar headgroup modifications did destabilise the active conformation of the receptor units. A normal receptor complement does not necessarily imply that all the receptors present are functional. A valuable confirmatory experiment would be to determine the coupling factor in the modified cells. To do this it would be necessary to measure the Kd of a β-adrenergic ligand, e.g. dihydroalprenolol, and divide this value by the concentration of isoproterenol required to elicit an increase in cAMP content which is 50% of the maximum attainable value (Higashida et al., 1982). This would allow an index to be derived which would give an indication of how closely coupled the β-adrenergic receptors and the adenylate cyclase units are in these cells. From the data derived from such experiments one would be able to decide whether or not it was likely that enrichment of C6 cell membranes with PDME, PNME or PE produced β-adrenergic receptor-adenylate cyclase decoupling.

3. To examine the theory that the isoproterenol-induced demethylation in cells with modified phospholipid polar headgroup

composition is due to phospholipase C activity, it would be necessary to examine the phospholipase C activity in these cells.

On the other hand, if products of the phospholipid methylation reaction are responsible for the demethylation and apparent β-adrenergic desensitisation in these cells, it would be necessary to examine the phospholipase A_2 activity in these cells. If products of enhanced phospholipase A_2 activity are responsible for the desensitisation, the likely metabolites of arachidonic acid metabolism could be determined by TLC of extracts from cells exposed to bases after incorporation of [^{14}C]-arachidonic acid.

4. It seems most likely that the lesion in β-adrenergic responsiveness in the base-treated cells could be due to a defect in the β-adrenergic receptor coupling to the G/C unit. However, it would be useful to check that the adenylate cyclase activity mediated by the G-protein and directly by the catalytic unit is not compromised in these cells. This could be checked by measuring adenylate cyclase activity in response to NaF, which has its effect mediated via the G-protein, and by measuring cAMP accumulation in response to forskolin, which is believed to stimulate the catalytic unit directly.

Measurement of the adenylate cyclase activity in membrane preparations would shed light on the results shown in Table 14 . If the enhanced basal adenylate cyclase activity is indeed due to activation of the catalytic unit by the modified phospholipid composition, one would expect that detergent-solubilisation of the membranes would lower the adenylate cyclase activity to values similar to the untreated cells.

5. Finally, to determine whether the β-adrenergically-stimulated increase in cAMP content in C6 cells is dependent on phospholipid methylation, it would be interesting to measure the response to isoproterenol when lipid methylation is blocked by an inhibitor, such as cycloleucine.

APPENDICES

APPENDIX I: HAEMATOXYLIN-EOSIN STAIN
(Ref. Drury and Wallington, 1980)

(a) Preparation of Reagents

(i) Haematoxylin Stain

The following reagents were dissolved in 1 litre of distilled water overnight: haematoxylin (1g); sodium iodate (0.2g); and potassium alum (50g). To this mixture citric acid (1g) and chloral hydrate (50g) were added and the solution boiled for 5min.

(ii) Acid Alcohol

13M HCl (1 ml) was added to methanol (100ml) and mixed.

(iii) Scott's Tap Water

Sodium bicarbonate (3.5g) and magnesium sulphate (20g) and a few crystals of thymol were dissolved in 1 litre of distilled water.

(iv) Eosin Stain

Eosin 1% (w/v) was dissolved in distilled water.

Cells were grown on sterile glass cover slips and fixed in 2.5% (v/v) glutaraldehyde as described in Section 2.1.

(b) Procedure

The coverslips with the fixed cells attached were immersed in baths containing the following reagents in turn for the times indicated.

1. Xylene - 5 min.
2. Ethanol - 2 min.
3. Methylated Spirits - 1½ min.
4. Water - 1 min.
5. Haematoxylin Stain - 12 min.

6. The coverslips were washed in tap water, and dipped four times in acid alcohol.
7. Scott's tap water - 1 min.
8. The coverslips were washed with tap water.
9. Eosin stain - 5 min.
10. Methylated spirits - 30 sec.
11. Ethanol - 2 min.
12. Ethanol - 1½ min.
13. Xylene - 5 min.

Before the xylene dried out the coverslips were mounted on glass coverslips with "DPX" (British Drug Houses Ltd.) and examined by microscope. The cell nuclei are stained blue, and the cytoplasm is stained red.

APPENDIX II: PREPARATION AND USE OF CHEMICAL TESTS TO IDENTIFY LIPIDS RESOLVED ON T.L.C. SYSTEMS AFTER DEVELOPMENT

1. Ninhydrin Stain (Ref. Christie, 1973)

(a) Preparation

Ninhydrin (1g) was dissolved in acetone (100 ml).

(b) Use of the Stain

The stain was sprayed evenly over the t.l.c. plate which was then warmed for 5 min in an oven at 100°C. Lipids containing free amino groups (PE and PS) show up as bright crimson spots.

2. Zinzadze Reagent (Ref. Christie, 1973)

(a) Preparation

Two solutions were prepared. Solution I was prepared by boiling 40.1g of MoO_3 in 1 litre of 12M H_2SO_4 till dissolved. Solution II was prepared by adding powdered molybdenum (1.78g) to Solution I and boiling the solution for 15min. Solution II was allowed to cool, any residue was removed by filtration, and discarded. Equal volumes of I and II were mixed and diluted with two volumes of water, to produce the working reagent.

(b) Use of the Stain

The working reagent was sprayed evenly over the t.l.c. plate, and the stain allowed to develop at room temperature. After 5min, all phospholipids appeared as blue spots.

3. Periodate-Schiffs Reagent (Ref. Christie, 1973)

(a) Preparation of the Stain

Three reagents were required. 2% Sodium periodate and 2% sodium bisulphite solutions were made up. Schiffs reagent was used fresh from the bottle.

(b) Use of the Stain

The t.l.c. plate was sprayed with sodium periodate solution and dried at 55°C. The dried plate was then sprayed with sodium bisulphite solution and dried at 55°C. This was repeated till the iodine stain had disappeared. The cooled plate was then sprayed evenly with Schiffs reagent and allowed to develop at room temperature. Phospholipids containing vicinal diol groups (PI,PG) rapidly appear as purple spots.

4. Ferric Chloride Stain (Ref. Christie, 1973)

(a) Preparation of the Stain

$FeCl_3.6H_2O$ (50mg) was dissolved in water (90ml) with acetic acid (5ml) and conc. sulphuric acid (5ml).

(b) Use of the Stain

The t.l.c. plate was sprayed evenly with reagent and heated for 3min at 100°C. The appearance of red-violet spots indicate the presence of cholesterol or cholesterol esters.

5. Dragendorff Reagent (Ref. Christie, 1973)

This test is specific for phospholipids containing choline groups (PC, Sphingomyelin and LPC).

Two solutions were made:

I: Potassium iodide (40g) was dissolved in 100ml of water.

II: Bismuth subnitrate (1.7g) was dissolved in acetic acid (20% v/v).

Immediately before use, 5ml of I were mixed with 20ml of II and diluted with distilled water (75ml).

Use of the Stain

The t.l.c. plate was sprayed with the mixture, choline-containing lipids (PC, LPC and Sphingomyelin) appeared within a few minutes as orange spots when the plate was gently warmed.

APPENDIX III: SDS POLYACRYLAMIDE GEL ELECTROPHORESIS
(Ref. Laemmli, 1970)

(a) Preparation of Solutions

(i) Stock solutions:

acrylamide 30% (w/v) Bio-Rad Laboratories

bis-acrylamide 0.8% (w/v) Bio-Rad Laboratories

(ii) Separating Gel Buffer (dilute four-fold before use)

1.5M Tris HCl Buffer, containing sodium dodecyl sulphate (0.4%, w/v).

pH to 8.7 with concentrated HCl.

(iii) Stacking Gel Buffer (dilute four-fold before use)

0.5M Tris HCl buffer containing sodium dodecyl sulphate (0.4%, w/v)

pH to 6.8 with concentrated HCl.

(iv) Sample Buffer (dilute two-fold before use)

0.13M Tris HCl buffer containing:

20% (v/v) glycerol

2% (v/v) mercaptoethanol

4% (w/v) sodium dodecyl sulphate

0.1% (w/v) bromophenol blue

pH to 6.8 with concentrated HCl.

(v) Reservoir Buffer (dilute five-fold before use)

0.125M Tris HCl containing:

0.96M Tris glycine and 0.5% (w/v) sodium dodecyl sulphate.

After dilution prior to use, adjust pH to 8.3 with concentrated HCl.

(b) Gel Solutions

(i) Separating Gel

To make a 12% acrylamide gel (30ml/slab) the stock solutions were mixed in the proportions below in the following order:

Separating gel buffer	7.5ml
Distilled water	10.5ml
Acrylamide solution	12 ml
TEMED	20µl
10% (w/v) ammonium persulphate	100µl

The mixture was degassed prior to addition of TEMED and ammonium persulphate, after addition of the latter components the mixture was poured into the casting apparatus and allowed to set.

(ii) Stacking Gel

The stacking gel, 10mls/slab (4% acrylamide), was made by mixing the stock solutions in the proportions:

Stacking gel buffer	2.5ml
Distilled water	6.1ml
Acrylamide solution	1.34ml
TEMED	10µl
10% ammonium persulphate	50µl

The mixture was degassed as above, and the gel cast on top of the solidified separating gel.

Running Conditions

Electrophoresis was carried out using a voltage of 130V and a current of 30mA overnight. The power pack was used in the constant voltage mode.

Staining Conditions

The staining solution consisted of the following:

0.05% (v/v) Coomassie Blue
25% (v/v)isopropanolol
10% (v/v) acetic acid

The dye was dissolved in 1,320 ml of distilled water, and then the solvents were added. The stain solution was filtered, and stored till required.The SDS polyacrylamide gels were stained in this solution for 30min.

Destaining

The stained gels were left to destain overnight in 10% (v/v) acetic acid

APPENDIX IV: COCKTAILS FOR LIQUID SCINTILLATION SPECTROMETRY

Two cocktail solutions were used in this project for determining radioactivity in aqueous samples or for measuring the radioactivity in lipid samples after evaporation of organic solvent.

1. Scintillation Cocktail used with Lipid Extracts

2,5-Diphenyoxalazole (10g) and 1,4-di-2-(4-methyl-5-phenyl oxazolyl) benzene (0.6g) were dissolved in 2 litres of analar grade toluene. 10ml of this cocktail was added to lipid residue in scintillation vials. Counting efficiency was found to be 55% for tritium.

2. Scintillation Cocktail for Aqueous Samples

This cocktail was used for determining the radioactivity in silica gel samples from t.l.c. plates and for the aqueous supernatant from cyclic AMP assays.

Cocktail 1 was mixed with triton X-100 and water in the proportions:

Toluene cocktail/Triton X-100/water 10:5:1 (v/v)

counting efficiency for tritium was found to be about 25%. 15ml of cocktail were added to the sample in scintillation vials.

APPENDIX V: FATTY ACID METHYL ESTER PROFILES FROM C6 CELLS ANALYSED BY GAS LIQUID CHROMATOGRAPHY

Fig.17: Fatty acid methyl ester profile obtained after analysis of C6 cell preparations on a g.l.c. column packed with 15% EGSSX as the stationary phase.

Fig.18(overleaf): Fatty acid methyl ester profile obtained after analysis of C6 cell preparations on a g.l.c. column packed with 15% EGSSY as the stationary phase.

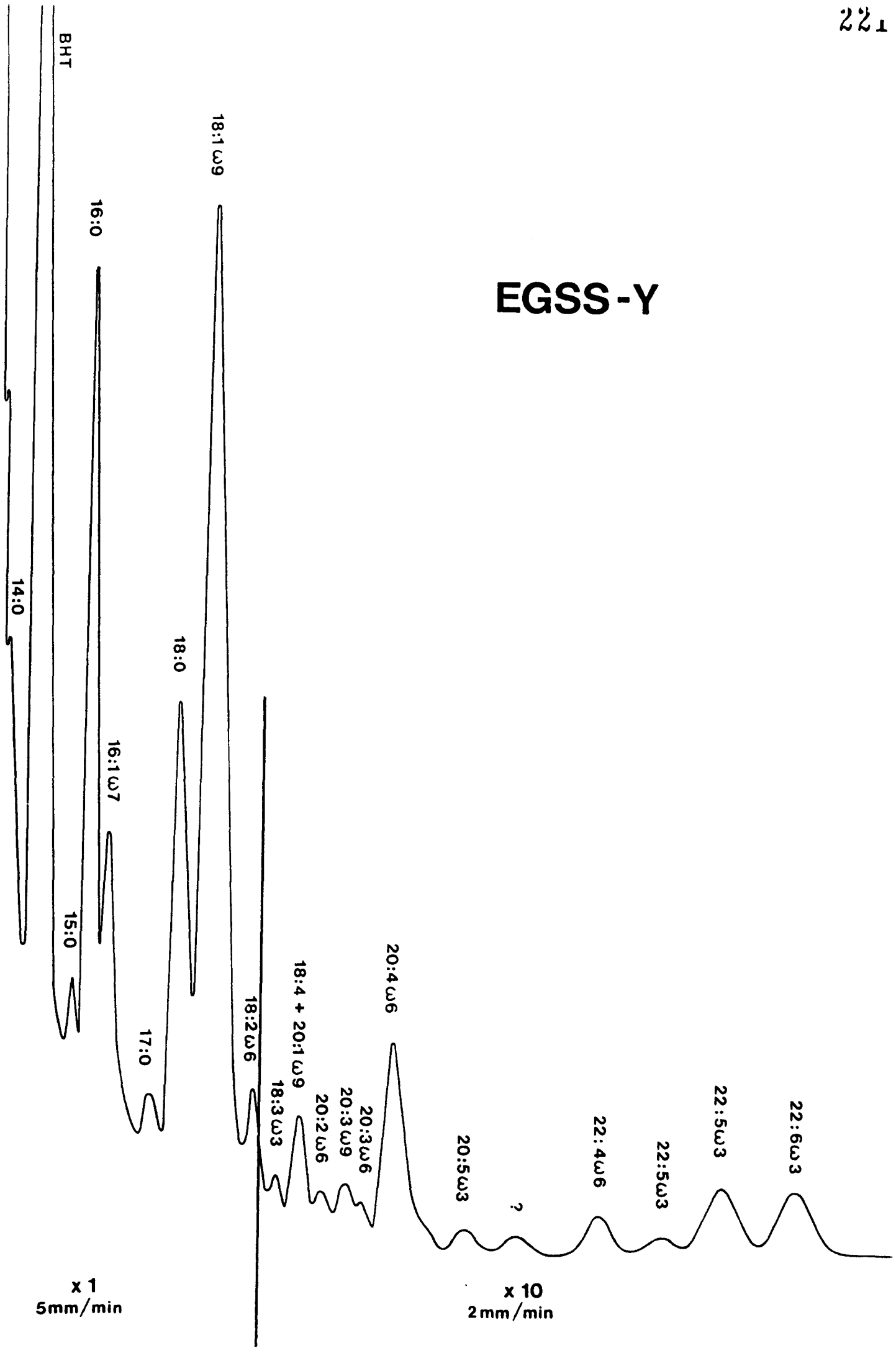
EGSS-X: A copolymer of polymeric ethylene-glycol succinate with methylsilicone

ESS-Y As above, but with a higher proportion of methyl-silicone (Christie, 1973).

22u

EGSS-X

?
22:6ω3
22:5ω3
x 20
2 mm/min
22:4ω6
20:5ω3
20:4ω6
20:3
20:2ω6
18:3ω3 + 20:1ω9
18:2ω6
18:1ω9
18:
16:1ω7
16:0
BHT
x 1
5mm/min


EGSS-Y
BHT
14:0
15:0
16:0
16:1 ω7
17:0
18:0
18:1 ω9
18:2 ω6
18:3 ω3
18:4 + 20:1 ω9
20:2 ω6
20:3 ω9
20:3 ω6
20:4 ω6
20:5 ω3
?
22:4 ω6
22:5 ω3
22:5 ω3
22:6 ω3
x 1
5mm/min
x 10
2mm/min

APPENDIX VI: DPH FLUORESCENCE SPECTRA

Fig.19: Excitation spectrum of DPH incorporated into C6 cell membranes

Fig.20: Emission spectrum of DPH incorporated into C6 cell membranes

Fig.21: Absorption spectrum of DPH in tetrahydrofuran

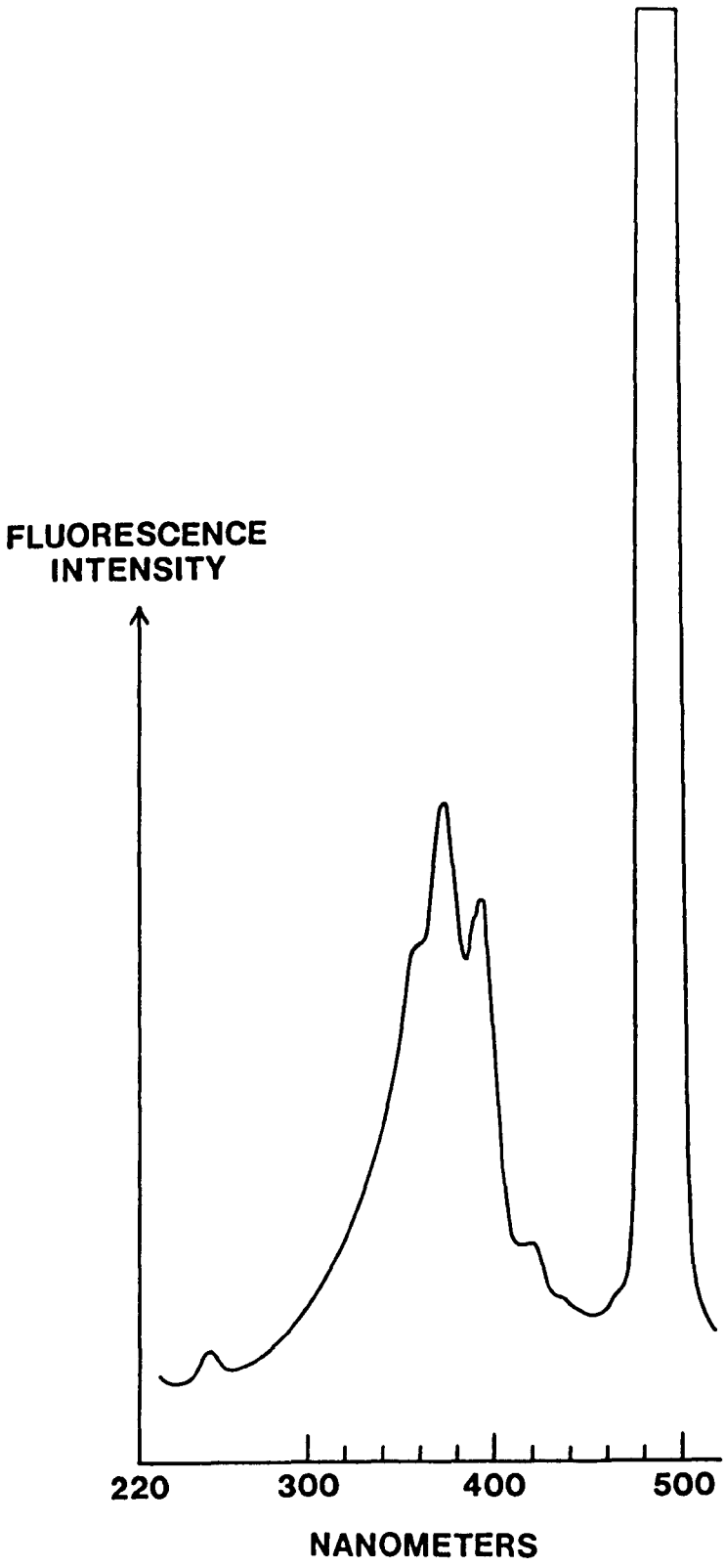


Fig.19:


FLUORESCENCE INTENSITY
340
360
380
400
420
440
460
480
500
NANOMETERS

Fig.20:

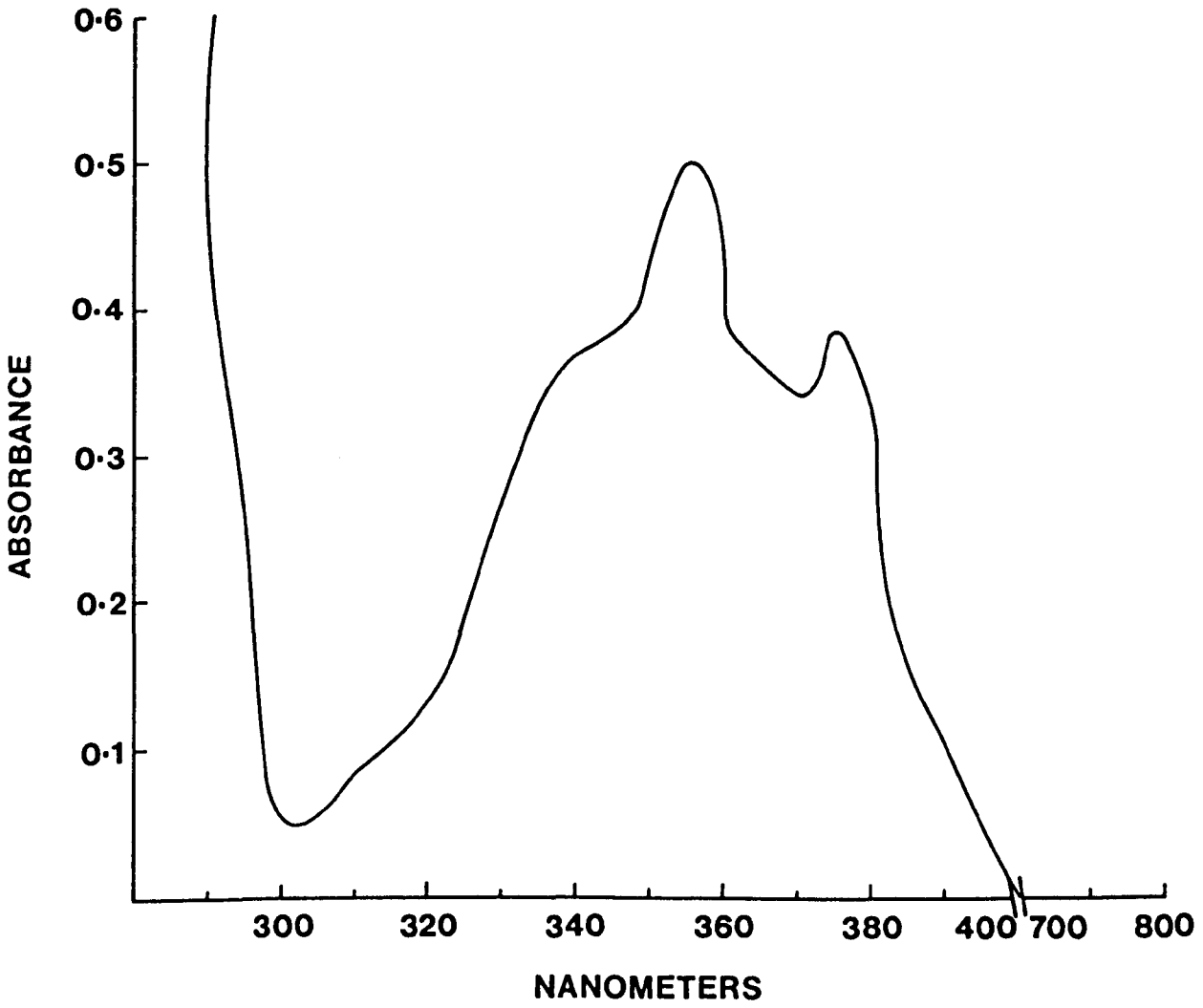

0·6
0·5
0·4
0·3
0·2
0·1
ABSORBANCE
300
320
340
360
380
400
700
800
NANOMETERS

Fig.21:

APPENDIX VII : COMPOSITION OF REAGENT B (PROTEIN ASSAY)

Reagent B was made immiedietly before use by mixing the following solutions:

2% Na K tartrate (0.5ml)
1% copper sulphate(0.5ml)
2% sodium carbonate(50ml)

APPENDIX VIII: CALCULATION OF INCREASE IN NUMBER OF N-METHYLATED PHOSPHOLIPIDS PER β-ADRENERGIC RECEPTOR AFTER ISOPROTERENOL STIMULATION.

Incubation mixture contains 1 ml of 0.1mM non radioactive methionine = 100nmol to which is added 70μCi of [^{3}H] methionine

∴ specific activity of methionine

= 70μCi/100nmol

= 0.7n Ci/pmol

Now since 1 nCi = 2220 d.p.m.

specific activity = 1554 dpm/pmol

From Table 18: PNME = 2% of total [^{3}H] recovered

(Columns 1 and 2) PDME = 6 " " " "

PC = 11 " " " "

∴ N-Methylated phospholipid = 19 " " " "

Since % does not change on isoproterenol stimulation, number of CH_3 groups incorporated = Δ 19% of 12382 - Δ 19% of 8907 = 660 dpm = 0.424 pmol

Now 0.424 pmol

$= 0.424 \times 10^{-12} \times 6 \times 10^{23}$ molecules

$= 2.54 \times 10^{11}$ molecules methylated

from Table 9, number of cells/mg protein = 10^7,

since Linbro tray contains 0.4mg protein = $\sim 2 \times 10^6$ cells

(a) Assuming 4,000 β receptors/cell = 8×10^9 receptors/0.4mg protein

(b) Assuming 10,000 β receptors/cell = 2×10^{10} receptors/0.4mg protein

(a) ∴ Ratio = $\frac{2.54 \times 10^{11} \text{ molecules}}{2 \times 10^{10} \text{ receptors}}$ = 12.8 N-methylated phospholipids per β-adrenergic receptor

(b) ∴ Ratio = $\frac{2.54 \times 10^{11} \text{ molecules}}{8 \times 10^{9} \text{ receptors}}$ = 31 N-methylated phospholipids per β-adrenergic receptor

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