A study of the potential use of membrane perturbants in enhancing the hyperthermic treatment of cancer

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A STUDY OF THE POTENTIAL USE OF MEMBRANE PERTURBANTS IN ENHANCING THE HYPERTHERMIC TREATMENT OF CANCER

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To my parents, with love and gratitude.

Many tumour cells are more sensitive to hyperthermia than non-cancerous cells. The nature of this greater thermal sensitivity is not clear. The present study indicates that a likely cause for this increased thermal sensitivity is membrane-associated. Plasma membrane enriched fractions were obtained from two solid rat tumours: D23, a hepatoma, and Mc7, a sarcoma. Lipids from these membranes were extracted, characterized, and compared to equivalent fractions from control tissue (liver). In both cases the tumour membranes had lowered cholesterol:phospholipid ratios. There was little difference in the phospholipid classes, but there was some difference in the fatty acid composition of the individual phospholipids. Fluorescence polarization studies were carried out on whole membranes and indicated that the overall 'order' of the tumour membranes was decreased with respect to the controls. In addition a plasma membrane bound enzyme, the Mg2+ ATPase, was found to be considerably more thermolabile in the tumour cells. The addition of the membrane pertubant tetracaine produced a greater degree of disorder in the tumour membranes compared to controls, and enhanced the thermolability of the Mg2+ATPase. These differences are further evidence that the plasma membrane is a likely site for the primary lesion in cell heat injury. Results from in vivo studies support the above mentioned in vitro work. D23 and Mc7 tumours, grown in the foot, were subject to hyperthermia and the simultaneous application of a membrane perturbant, tetracaine. The addition of the tetracaine significantly increased the efficacy of the treatment. When the D23 tumour was grown in ethanol-dependent rats there was no difference in the 'adaptive' response of the tumour, compared to the normal, plasma membranes. There was no difference in the heat sensitivity of foot tumours grown in ethanol-fed rats compared to tumours from pair fed controls.

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Finally I would like to thank my friends from my housegroup attached to St. Nic's church, for their love and moral support, and the following friends: Andy & Jeannette, Frank & Ali, Franc & Jan, and Marianne.

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GLOSSARY

A.N.S.	8-Anilino-1-napthalenesulphonic acid, sodium salt
B.H.T.	Butylated hydroxytoluene
B.S.A.	Bovine serum albumin
D.D.S.A	Dodecenyl succinate
D.M.P.	2,4,6-Tri(dimethylaminomethyl) phenol
D.O.C.	7-Deoxycholate
D.P.H.	1,6-Diphenyl-1,3,5-hexatriene
Na+/K+ATPase	Sodium and potassium stimulated, magnesium -dependent adenosine 5′-triphosphatase
Mg2+ATPase	Magnesium-dependent, adenosine 5′-triphosphatase
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PI	Phosphatidylinositol
PS	Phosphatidylserine
rav.	Average radius of rotation
S.D.S.	Sodium dodecyl sulphate
SM	Sphingomyelin
Tetracaine hydrochloride	4-(Butylamino)benzoic acid, 2-(dimethylamino)ethyl ester hydrochloride

Other abbreviations were the commonly used ones suggested by the Biochemical Journal (Policy of the Journal and Instructions to Authors (1984): Biochem. J. 217, 1-26.).

MATERIALS

Materials were obtained from the following sources:

Agar Aids

Araldite

D.D.S.A.

D.M.O. 30

Aldrich

D.P.H. 'puriss' grade

Alltech

Fatty methyl ester standards for G.L.C.

10% Alltech CS-5 on Chromasorb WAW 100-120 mesh support

B.O.C Ltd.

Nitrogen

J. Borrough Ltd.

Absolute alcohol

Eastman Kodak

A.N.S. (sodium salt)

Sigma chemical Co.

Adenosine monophosphate (Sigma grade) disodium salt

Adenosine triphosphate (Sigma grade)disodium salt

Bovine serum albumin - fraction V

Boron trifluoride methanol reagent

Cholesterol - Sigma grade

Cytochrome c - horse heart Type III

Deoxycholate -sodium salt

Diagnostic Kit -for the determination of ethanol
No. 332-UV

E.D.T.A. (Sigma grade) disodium salt

Glycine

Imidazole - grade 1

-Nicotinamide adenine dinucleotide phosphate, reduced form

L- -phosphatidyl choline, dipalmitoyl

S.D.S. (lauryl sulphate, sodium salt

Tetracaine -hydrochloride

Thymidine 5'-monophosphate p-Nitrophenol ester,
sodium salt

Tris (hydroxymethyl)-amino methane (tris)- Trizma base

Supelco

Phospholipid standards (for T.L.C)

All other reagents were of analytical grade and were obtained from B.D.H. Ltd. Lubrol was a generous gift from I.C.1 Dyestuffs Division.

1. GENERAL INTRODUCTION

In a study which seeks to potentiate the use of hyperthermia in the clinical treatment of cancer there are two crucial questions to be asked. First what is the nature of cellular heat injury? And secondly, what factors are involved in making some tumour cells more susceptible to heat injury?

The first question is fundamental, but very difficult to answer. Exposure to heat has been reported to damage a variety of cell structures. For example it has been reported that the following cellular components are susceptible during heating: DNA, RNA, proteins, nuclear proteins, lipids, chromosomes, nucleoli, membranes, lysosomes, microtubules, as well as a large variety of synthetic pathways and metabolic functions within the cell. This diversity of proposed sensitive sites for heat damage in cells reflects the rather intractable nature of the problem. Some differences may well arise as a result of different cell types and/or organisms that are under study. However it is more likely that difficulties in identifying lesions in heat injury arise because of the need to identify primary from secondary and tertiary damage. This problem has been discussed by Bowler, (1981). A number of these sites have been suggested to have a more significant role in cellular heat death than others. That is i) Lysomomal damage (eg.Hume &

Field, 1977; Hume et al. 1978; and Magan, 1981), this is because of the role of proteolytic enzymes in cell destruction. ii) Protein denaturation/inactivation (eg. Ushakov, 1964) and damage to the cytoskeleton (eg. Bass et al. 1982; Coss et al. 1982. iii) Damage to the nucleus in various forms (review Warters & Roti Roti, 1982).iv) Damage to the plasma membrane. evidence coming from a number of groups: Bowler and co-workers (Bowler et al. 1973; Bowler, 1981; and Bowler et al. 1982). Gerner and co-workers (Cress & Gerner, 1980 and Cress et al. 1982) Hahn and co-workers (Fajardo et al. 1980, Li et al. 1980). Lepock and co-workers (Lepock et al. 1981 and Lepock, 1982). Yatvin and co-workers (eg. Yatvin et al. 1983) and others eg. Cavaliere et al. (1967); Strom et al. (1973).

Lysosomal damage can not be a universal feature of cellular heat injury,as some workers report no increase in lysosomal enzyme activity during hyperthermia (Tamulevicius & Streffer, 1983), and others have shown that any lysosomal membrane damage is likely to be a secondary, not a primary, event (Rogers et al. 1983). Variations in the results between different groups may also reflect the techniques used to study lysosomal damage. For example, Hume and Field, (1977) use histochemical techniques, where as Tamulevicius and Streffer, (1983) used a biochemical approach. The two sites most favoured for the primary heat lesion are the plasma membrane and the nucleus.

The nucleus has been shown to be damaged at various sites after heating. Although the structure of chromatin is not denatured by heat shock, there is an increase in non-histone

chromosomal proteins tightly associated with the cell nucleus,(Roti Roti & Winward, 1978). DNA synthesis is rapidly inhibited by heat, with replicon initiation stopped and the rate of chain elongation greatly reduced (Waters & Stone, 1983; Wong & Dewey, 1982; and Warters, 1982). It is clear that cell heat sensitivity changes in the cell-cycle, and this could be a result of changes within the nucleus (Westra & Dewey, 1971).

Recent evidence from Roti Roti (Roti Roti, 1982, and Roti Roti & Wilson, 1984) has shown that increase in chromosomal protein content which is thought to lead to reproductive heat death can also be induced by membrane peturbants eg. alcohols and local anaesthetics, without any heating. Rofstad et al. (1984) has indicated that DNA damage, in the form of micronucleus formation, during hyperthermia, is a secondary effect, resulting from damage in another cellular compartment. In addition Yi et al. (1983) has suggested that the inhibition of DNA synthesis may be due to decreased levels of intracelluar K^+ , which is, in turn, a result of plasma membrane damage. Thus the plasma membrane is emerging as a critical site of primary heat lesion. Before the evidence for this is presented in more detail, it is important to understand the structure of the plasma membrane, and how thermal energy might perturb this structure.

The Fluid Mosaic model of Singer and Nicholson, (1972), is the basis of current understanding of membrane structure. They proposed a dynamic structure of a phospholipid bilayer with a hydrophobic core, in which proteins were floating. Originally

HEAT

↓ 1° LESION

Say: Damage to PLASMA MEMBRANE

↓ Causing 1° EFFECTS

Say: Loss of K^+ and metabolites from cell
Entry of Na^+ & Ca^{++} into cell
Direct inactivat$^{n.}$ of membrane proteins

↙ ↘ Causing 2° LESIONS

Eg. Intracellular rise in Ca^{++}

1. Δ compartment permeability
2. Activates proteases & protein kinases

Extracellular Δ in Na^+ & K^+

1. Disrupts biophysical properties of cells
2. Loss of ionic gradients

↓

Disrupt$^{n.}$ of metabolism

↓ Causing 3° LESIONS

Breakdown of cellular & physiological homeostasis

↓

DEATH

protein movement was thought to be unrestricted but now it is realized that some are anchored by the cytoskeleton. The other important element, particularly in mammalian plasma membranes, and missing from the original model, is cholesterol. This molecule has a 'condensing' effect on the phospholipids in the natural fluid phase of membranes and is thought to stabilize membranes structure (Oldfield & Chapman, 1972 and DeKruff et al. 1972). The structure of the plasma membrane, and hence it's physical properties, plays a vital role in maintaining a semi-permeable barrier which controls the intracellular environment, maintaining cellular homeostasis.

Thermal energy increases the fluidity (or decreases the order) of the hydrophobic acyl chain domain of the membrane. The possible consequences of this peturbation are first an increase in membrane permeability, and secondly a disruption of the functioning of plasma membrane proteins. These can be seen as essentially reversible changes and the membrane will revert to normal when the heat insult is removed. However if the perturbation is maintained the intracellular ionic balance will be erroded and membrane proteins will eventually undergo irreversible changes in tertiary structure which would lead to 'denaturation' and the loss of function. It is postulated that these primary events will have 'knock-on' secondary and tertiary effects which eventually lead to cell death (Bowler, 1981) Bowler's hypothesis is illustrated in figure 1.1 The evidence for the plasma membrane as a primary site of heat lesion, responsible for the eventual hyperthermic killing of the cell will now be presented.

Evidence that increased plasma membrane permeability, caused by heat, can lead to cell death has been produced by a number of workers. Raaphorst and Dewey, (1978), showed that the enhancement of hyperthermic killing of cultured mammalian cells, was produced by treatment with anisotonic NaCl. They suggested that this implied that, either the plasma membrane was a primary site of heat inactivation of mammalian cells, or changes in ion concentrations enhance thermal damage occurring in critical intracellular structures. Anghileri et al. (1983) showed that increased membrane permeability preceeded hyperthermic killing of cells. Schanne et al. (1979) have suggested, following studies with a variety of membrane destructive reagents, that the influx of extracellular calcium ions, and the drastic change of the intracellular environment, is a common final pathway in cell death. Certainly the cell death involved in liver necrosis is thought to be mediated by calcium ion influx, following membrane damage as a result of acute ethanol intoxication (Kane et al. 1980)

Membrane 'fluidity', or order has also been correlated with the hyperthermic killing of a number of cells. This has been done in bacterial systems by Yatvin and his co-workers, using a unsaturated fatty acid requiring E. coli auxotroph (Yatvin, 1977; Yatvin & Dennis, 1978; Dennis & Yatvin, 1981; and Yatvin et al. 1983). They describe increasing the proportion unsaturated fatty acids, and thus decreasing membrane order, in mammalian cells either by nutritional manipulation or changing growth temperature, enhanced heat killing (Yatvin et al. 1978; Hidvigi et al. 1980; and Anderson et al. 1981). However some

of the interpretation of these results is disputed by Lepock et al. (1981), who questioned the role of membrane 'fluidity' in hyperthermic cell killing. Yatvin et al. (1983) has conceded that there is not always a direct relationship, but pointed out that often that the way that 'fluidity' is measured is only crude, and is unable to detect subtle, but important changes in the physical state of the membrane. In later work Lepock and co-workers have shown a correlation between membrane protein denaturation and hyperthermic killing (Massicotte-Nolan et al. 1981 and Lepock et al. 1983). Cress & Gerner have shown that there is an inverse relationship between membrane cholesterol and hyperthermic killing in a number of mammalian cell lines, though it must be noted that, these workers did not separate out the component membranes of the cell, and so an extrapolation of these results to the plasma membrane is not strictly correct (Cress & Gerner, 1980 and Cress et al. 1982).

It has also been shown that drugs and other agents which are known to perturb membrane structure can potentiate hyperthermic killing. For example, local anaesthetics (Yatvin, 1977 and Yau, 1979). Yatvin has also shown that anaesthetic potency correlates inversely with bacterial survival when used synergistically with heat. Li et al. (1980) have shown a similar effect with an aliphatic alcohol series. Polyamines, when exogenously applied, potentiate the effects of hyperthermia, and are thought to interact with a plasma membrane component (Fuller & Gerner, 1982).

The membranes of many organisms have been shown to adapt to

changing environmental temperature. When the environmental temperature changes, the membrane order will change as a direct consequence. The cell then makes compensatory changes to bring membrane order within 'acceptable' levels. This phenomenon has been observed in bacteria, plants and poikilotherms, and termed homeoviscous adaption by Sinensky, (1974). This response indicates, indirectly, that the physical condition of the plasma membrane is important in the cell's response to temperature.(Reviews: Hazel & Prosser, 1974 and Cossins, 1983). There is also evidence of similar adaptive responses in mammalian cells, for example, Anderson et al. (1981) have reported temperature induced homeoviscous adaption in Chinese hamster ovary cells.

As other agents, besides temperature, perturb membrane structure, one might expect that they would elict a similar adaptive response if their presence was maintained in the plasma membrane for some time. This appears to be the case with ethanol. Chin and Goldstein, (1978) have reported that the long-term administration of ethanol, in mice, produced an adaptive response (an increase in membrane cholesterol) in synaptic and erythrocyte membranes. The fact that the adaptive response to heat and the membrane perturbant ethanol is similar has been confirmed by Anderson et al. (1983). These workers have shown that long-term ethanol administration protected mouse ear from heat induced necrosis if the ethanol was removed prior to treatment. Thus increasing plasma membrane stability, by elicting an adaptive response to a membrane perturbant, will protect the cell against the effects of hyperthermia.

The use of hyperthermia as a treatment for cancer is thought to go back into antiquity, but the first clinical reports date from the middle of the nineteenth century. The first two reports from Busch, (1866) and Bruns, (1887), concern the complete regression of sarcomas in patients with high fever, that is over 40^{o}C. In the second paper there was no reoccurrence of the tumour after an eight year period. A review by Rohdenburg, (1918) showed that a high proportion of spontaneous human tumour regression (carcinomas and sarcomas) was accompanied by high fever in patients. The history of the association of hyperthermia with tumour regression has been reviewed by Cavaliere et al. (1967). From this extensive survey, and many other more recent reports, it is clear that many tumours are more sensitive to hyperthermia than normal tissues. The basis of this, like the heat injury of normal cells, is not entirely clear. However, the identification of either the cause of injury by heat in normal cells, or the critical difference between normal and tumour cells which makes the the latter more sensitive to heat, would help in the design of improved methods of therapy.

The plasma membrane of tumour cells, as with normal cells, has been suggested as a site of primary heat lesion in cancer cell death, and some evidence will be presented here for this. It may indeed be that changes in the plasma membrane of tumours are the basis of the differential heat sensitivity of tumours, but there are other physiological factors which are important in tumour regression in vivo, and these will be discussed later. Mondovi et al. (1967) in their study of respiration in a Morris

hepatoma showed that this process was irreversibly damaged at temperatures of 42-43°C where as in normal proliferating tissue (regenerating liver) respiration was insensitive to more prolonged heatings at 44°C. This suggests that tumour heat sensitivity is not just a reflection that it is proliferating tissue. These workers also showed that ethanol, a membrane perturbant, enhanced this thermal sensitivity of the tumours, suggesting the site of lesion in tumour hyperthermic killing is the same as normal tissues. Other early evidence for the association of plasma membrane lesion with tumour heat killing has been presented by Strom et al. (1973), They have shown that heated tumours are more permeable to glutamate than normal tissues. Fekete (1978) has shown that Ehrlich and lymphoma cells are protected against normally lethal doses of heat by the addition of glycerine which is thought to stabilize the plasma membrane. This gives indirect evidence that the tumour membrane is crucial in the tumour cells' response to heat. Fajardo et al.(1980) produced good evidence, using electron microscopy, that there is a breakdown of the plasma membrane in tumours following heating. More recently Yi et al. (1983) and Boonstra et al. (1984) have shown that the plasma membrane is an important site of heat lesion in tumours.

Studies of the membrane biochemistry of tumours cells have revealed differences between tumour and normal plasma membranes. For example, many hepatoma plasma membranes have raised cholesterol levels compared to liver membranes (review Chen et al. (1978). Others have shown lowered plasma membrane cholesterol in leukaemic and lymphoma cancer cells, this correlated with increased membrane

fluidity (Inbar, 1976; Van Blitterswijk et al. 1977 & 1982). Since membrane cholesterol is thought to help stabilize cells to heat (Cress & Gerner, 1980), one might expect that some tumours are more sensitive to heat than normal cells, whereas others are more resistant. However Sabine (1983) has suggested that deviations in membrane cholesterol levels (either elevated or reduced) can lead to the impairment of membrane function. The fatty acyl chain pairing in tumour phosphatidyl choline is reported to be abnormal in some tumours, (Bergelson & Dyatlovitskaya, 1973). When such phospholipids are incorporated into the plasma membrane, the structure would have a greater permeability than a normal membrane and therefore is another possible source of tumour differential heat sensitivity.

As indicated previously, when considering the differential thermosensitivity there are important physiological factors which need to be taken into consideration. This is illustrated by the study of Kang et al. (1980). They pointed out that SCK tumour cells were far more sensitive to heat in vivo than they were in vitro. The most important factors are blood flow and local pH.

In normal tissue, blood flow increases at commonly used hyperthermic temperatures (42-45°C), but in tumours blood flow increases slightly when they are heated to temperatures below 41-42°C but above 42°C the flow is drastically reduced. Vascular occulsion occurs, and because heat is ineffectively dissipated the tumour temperature builds up above that of the surrounding tissues. It is suggested that the decrease in

tumour pH which occurs during hyperthermia is due to the decrease in the nutrient supply and oxygen, the cells becoming hypoxic (review Song, 1982). There is some dispute whether there is a decrease in intracellular pH as well as extracellular pH and the relative importance of these two factors in hyperthermic killing. Eg. Hofer & Mivechi, (1980) favour intracellular pH changes, whereas Dickson & Calderwood, (1979) have shown that intracellular pH does not mediate the effect of hyperthermia or that hyperglycaemia leads to decreased pH which sensitizes tumour to heat damage.

Another important physiological factor in the effect of hyperthermia on tumours in vivo is the involvement of the immune system. The way the immune system participates depends on the type of heating regime being used. There is some circumstantial evidence that an anti-tumour immune response occurs after curative local hyperthermia in animals and perhaps in man. This is a non-specific response which includes a raised macrophage content. Conversely whole body hyperthermia tends to suppress the immune response (review Dickson & Shah, 1980). Thus the differential sensitivity of tumours to hyperthermia has a physiological as well as a cellular basis.

The work undertaken in this thesis has three main aims: i) To clarify further the role of the plasma membrane in tumour hyperthermic killing. In order to do this the plasma membranes from two tumours D23 a hepatoma, and Mc7 a sarcoma were isolated and their lipid composition determined particularly with respect to the fatty acid content of their phospholipids and the level

of cholesterol. Physical measurements using steady state fluorescence polarization will establish whether there is any change in the order of these membranes, and will be correlated with the lipid composition and compared to similar determinations carried out on liver plasma membranes from the same animals. ii) To assess the role of membrane fluidizers in enhancing the hyperthermic killing. This will comprise of an initial biochemical study of the effects of membrane perturbants on the integrity of the plasma membrane of tumour as compared to that from liver and the synergistic effect of heat on these different plasma membranes.

The long term effect of one membrane fluidizer, ethanol, on the adaptive response of tumour plasma membranes, as compared to similar membranes from normal tissue will also be studied. The aim of this part of the work is to see whether the tumour cells are able to respond to the same extent as normal cells, to the presence of a membrane perturbant. If not, this scenario offers the possibility of increasing the difference in thermosensitivity between normal and tumour cells, which could be exploited in the clinical treatment of the disease. Again the tumour plasma membranes from tumour and normal tissue will be isolated and characterized to determine any differences in their lipid composition and physical state after ethanol administration. iii) The final aim of the work is to try and correlate any differences between normal and cancer plasma membranes, and the effect of heat and membrane perturbants on these membranes, with the *in vivo* situation. Obviously if any differential thermosensitivity exists *in vitro* it must also

reflect the situation *in vivo* if it has any application in enhancing the hyperthermic treatment of cancer clinically. In order to do this the two tumours, D23 and Mc7, whose plasma membranes have been characterized earlier in this study, will be passaged into the feet of rats and the effect of the membrane perturbant, tetracaine on the the hyperthermic cure of the tumours will be assessed. A further series of experiments will be carried out to see if the long-term exposure of the tumour to the membrane perturbant, ethanol, produces any differences in the thermosensitivity between the tumour from ethanol-fed and control rats. Again the results will be examined to see whether there is any correlation between the biochemical changes in the plasma membrane, found in the earlier study, and the tumour's sensitivity to hyperthermia.

SECTION 1 : CHARACTERIZATION OF TUMOUR PLASMA MEMBRANES

2. PURIFICATION OF A PLASMA MEMBRANE FRACTION FROM D23 AND MC7 TUMOURS

INTRODUCTION

In order to assertain the effects of hyperthermia and membrane fluidizers on tumour plasma membranes it is, of course, first necessary to isolate those membranes from the tumour. Plasma membranes from erythrocytes ('ghosts') are relatively easy to isolate, and so have been much studied. The simple 'bag-like' structure of the erythocytes makes the preparation of pure membranes relatively simple. Plasma membranes from cells with a more complex ultrastructure were not isolated with any degree of success until 1960 (Neville, 1960). Since then plasma membranes have been isolated by many workers from a great variety of tissues (for review see Evans, 1982). Isolation of plasma membranes from tissue and cells other than erythrocytes requires complex fractionation procedures in order to separate out contaminating intracellular membranes, and structures such as the nucleus.

Generally speaking, different methods of purification are

used for cells in culture, and whole tissue, particularly in the initial stages of separation; plasma membranes from tumour tissue often require a slightly different isolation procedure than that used for the tissue from which it has originally been derived (Emmelot et al. 1974). Isolation follows three main steps.

i) Cellular disruption, usually by homogenization.

ii) Preliminary low speed centrifugation to remove gross contamination, for example, cytosolic proteins, undistrupted cellular organelles, and extraneous extracellular material.

iii) Preparative density gradient centrifugation, often using sucrose, to resolve individual fractions ie. mitochondrial, microsomal (golgi & endoplasmic recticulum) and the plasma membrane fraction.

Although a large literature exists on the methodology and use of membrane fractions from tumour material, direct reference will be made here only to those studies directly pertinent to the present Chapter. A large amount of work on the isolation of liver and hepatoma membranes has been carried out by Emmelot, Bos and co-workers, (review Emmelot et al. 1974) usually on rat or mouse tissues. Other workers have also used solid rat hepatomas eg., Upreti et al. (1983), and Raftell & Blomberg (1973), who isolated plasma membranes from the D23 tumour that was used in this current study. Plasma membranes from cultured hepatoma cells have been isolated too, Koizumi et al. (1976)

and Sauvage et al. (1981). Leukaemic and lymphoma plasma membranes have been studied by van Bitterswijk et al. (1975) and Koizumi et al. (1981), respectively. Plasma membranes from many other cancerous cell types have been studied eg. Chicken sarcomas (Perdue et al. 1973).

Normal tissue, and tumour tissue which is minimally deviated from it, can be isolated by similar methods. However, in many passaged tumours, including those used in this study (see Appendix 2), the nuclei are large in relation to the cytoplasm and so are easily disrupted during homogenization, forming a gel which handicaps further separation of the plasma membranes. The plasma membranes themselves are often more fragile. In order to separate such plasma membranes gentle homogenization is carried out, using a loose-fitting pestle, in the presence of Ca2+. This prevents the rupture of the nuclear envelope, and binds the plasma membrane into large sheets which can be sedimented with the undisrupted nuclear pellet. These problems, and their solutions are dicussed by Emmelot et al. (1974) in their review. The plasma membranes of one of the tumours used in this study has been previously isolated, Raftell & Blomberg, (1973); but the reported recovery of marker enzyme activity was poor. Ray (1970), reported a method for the isolation of liver plasma membranes which yielded a high recovery of marker enzymes. This method was a modification of the original Neville isolation (Neville, 1960), and included several of the important steps required for the successful isolation of the deviated tumour type described above; for example: gentle homogenization in the presence of Ca2+.

Therefore,this method was used to isolate the plasma membranes from the two tumour types, D23 hepatoma and Mc7 sarcoma, as well as liver from the same animals.

The purification, during fractionation, of the plasma membranes was assessed by monitoring the enrichment of 'marker' enzyme activity. Enzyme markers are proteins which are known to associate exclusively with a particular sub-cellular fraction. In the case of the plasma membrane the most commonly used markers are the Na+/K+-ATPase and 5'-Nucleotidase. It is also important to know to what degree the plasma membrane enriched fraction is contaminated with other membrane types, and again specific enzyme markers can be used to assess the degree of purity of the plasma membranes fraction obtained.

MATERIALS AND METHODS

1. Isolation of Plasma membrane enriched fractions from D23 and Mc7 tumours

The isolation of tumour plasma membranes was carried out by the method of Ray(1970), with some modification. Liver plasma membranes were isolated in a bicarbonate buffer containing 0.5mM Ca2+, whereas tumour membranes, as suggested by Emmelot(1974), had an identical buffer which was fortified with 2.0mM Ca2+.

Tumours were excised from the flanks of rats. The healthy tissue was dissected out, leaving any neocrotic

material found in the centre. All subsequent operations were carried out at 0-4°C. Approximately 1g, wet weight, of tissue was homogenized with 20ml of either 0.5 or 2.0mM $CaCl_2$, as appropriate in 1mM $NaHCO_3$, pH 7.5, using 25 gentle strokes of a loose fitting 'Douce' homogenizer. Up to 8g of starting tissue was treated in this way in any given isolation. The homogenate was then diluted in more of the buffer so that the total volume was 100fold the weight of the starting material. This was then allowed to stand for 5 minutes at 4°C, with occassional stirring.The suspension was then filtered through 2 layers of pre-washed muslin, and the filtrate spun at 2000g(rav=228mm) in a MSE Coolspin for 30 minutes. The supernatant, (S1), was discarded, and the pellet, (P1), resuspended in half the original volume by 3-4 gentle strokes of a hand-held homogenizer. The resuspended pellet was respun at 1700g(rav=228mm) for 15 minutes. The supernatant, (S2),was again discarded, the pellet resuspended in half the previous volume of buffer and then respun in the same way as the last step. The resulting pellet, (P3), was resuspended in 2mM EDTA, 20mM imidazole buffer, pH7.2 and sufficient 70% sucrose was added to give a final sucrose concentration of 48% (w/v).

Sucrose solutions: 45, 41, and 37%(w/v); containing 1mM EDTA, 20mM imidazole, pH7.2; were carefully layered in 8ml bands on top of the membrane layer already placed at the bottom of a 38ml centrifuge tube. This produced a discontinous density gradient. Great care was taking in

ensuring that the sucrose solutions were of the correct strength, all were checked, at 25°C, using an Abbe Refractometer with a sucrose scale. The density gradients were centrifuged in a MSE Prepspin 50 at 86,000g(rav=123mm), in a 6x38ml capacity, swing-out rotor for 2 hours. Discrete bands of membrane formed at the interface of the different sucrose layers; the plasma membrane fraction, (B1), was formed at the interface of the 37 and 41% layers (see Fig. 2.1). All the bands, B1-B3, and the pellet, (P4), were removed using a pasteur pipette and washed by resuspending in 1mM EDTA, 20mM imidazole buffer, pH7.2 and respun at 100,000g(rav=77.52mm), in a 8x50ml capacity rotor, for 1 hour. The pellets were resuspended in a minimum volume of the same buffer, and stored on ice until required for enzyme assay. (When lipid or other non-enzymatic determinations were to be performed on the fractions, the membranes were stored under nitrogen at -20°C until required.)

2. Marker Enzyme characterization of isolated fractions

a) Alkaline Phosphodiesterase I

The reaction media contained a final concentration of 100mM $MgCl_2$, and 1mM thymidine p-nitrophenyl phosphate in 50mM glycine buffer, pH 9.7. The reaction was started, after 5 minutes pre-incubation at 37°C, by the addition of 0.3ml of diluted membrane suspension, to give a final volume of 1.5ml. The reaction was stopped after 30 minutes

by the addition of 1.5ml of 0.2M NaOH. The tubes were spun at 900g for 10 minutes to remove any cloudy suspension. The yellow colour was read at 400nm on a Pye Unicam SP8-100 using water as a blank, and the concentration of the nitrophenol produced was calculated from its extinction coefficient at 400nm.

b) Na+/K+ATPase(EC 3.6.1.3)

The reaction conditions were as follows: 10mM K+, 3mM Mg2+, and 100mM Na+ in 20mM imidazole buffer, pH 7.2, in the presence or absence of 1mM ouabain. ATP (sodium salt) was added to give a final concentration of 3mM. Incubations were carried out at 37°C. The reaction was started by the addition of 0.5ml of enzyme suspension to give a final volume of 2ml. Na+/K+ ATPase activity was determined as that activity which was sensitive to the presence of ouabain.The reaction was stopped after 30 minutes, by the addition of 4ml of an acid molybdate/lubrol reagent. This consisted of 1%Lubrol added to an equal volume of 1% ammonium molybdate in 0.9M sulphuric acid. The reagent reacts quantitatively with the released inorganic phosphate, forming a yellow colour which was read at 390nm (Atkinson et al. 1973). A standard curve was constructed using Na_2HPO_3 which was linear in the range 0-1.2 umoles of phosphate.

c) 5' Nucleotidase(EC 3.1.3.5)

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5′ Nucleotidase activity was determined using the method of Emmelot et al. (1964). The reaction media contained 100mM KCl, 5mM $MgCl_2$ in 50mM Tris, pH7.2 (at 37°C). 1.8ml of this media was added to 0.1ml of 0.1M AMP, disodium salt. The tubes were pre-incubated at 37°C and the reaction started by adding 0.1ml of membrane suspension. After an appropriate time the reaction was stopped and inorganic phosphate released was assayed as described for the Na+/K+ATPase.

d) Glucose-6-phosphatase(EC 3.1.3.9)

This enzyme was determined by the method of Tsai et al. (1975). A 0.1ml aliquot of membrane suspension was added to 0.3ml of maleic buffer (0.1M, pH6.5), and 0.1ml of a 0.1M glucose-6-phosphate solution which had been pre-incubated at 37°C for 5minutes. The tubes were then incubated. After an appropriate time the reaction was stopped, and inorganic phosphate (Pi) was determined using the methods described for the Na+/K+ ATPase.

e) NADPH cytochrome c reductase(EC 1.6.2.3)

This assay was based on the method of Tsaiet al. (1975). The assay was performed at 20°C as suggested by Emmelot et al., (1964). 50uL of the membrane suspension was added to each of duplicate cuvettes containing 0.9ml of phosphate buffer (0.1ml, pH7.4) and 0.1ml of cytochrome c (1% w/v). The reaction was initiated by the addition of

0.1ml of NADPH in one cuvette, in the other the same volume of distilled water was added and this acted as a blank. The cuvettes were read immediately, against each other, and the rate of decrease of absorbance of the cytochrome c was measured in a Pye Unicam SP8-100 dual-beam spectrophotometer at 550nm for 2-3 minutes.

f) Succinate dehydrogenase(EC 1.3.99.1)

Succinate dehydogenase activity was determined using the method of Tsai et al. (1975). To duplicate cuvettes containing 1.2ml of 20mM phosphate buffer/1% B.S.A., pH7.0 was added 0.1ml of 1%(w/v) cytochrome c and 50uL of membrane suspension. The cuvettes were throughly mixed, and read against each other to form a zero value. When a stable base line was achieved, 0.4ml of 10mM KCN was added to the reference cuvette, and 0.4ml of 10mM KCN containing 50mM succinate, was added to the other. The increase in absorbance at 550nm was measured immediately and then for several minutes on a dual-beam SP8-100 spectrophotometer at either i)20°C or ii)30°C.

3. Electron Microscopy

The final membrane suspension was pelleted, and prepared for electron microscopy by the methods outlined in Appendix 2.

4. Protein determinations

Protein estimations were carried out using a modified Lowry method, (Peterson, 1977), which is particularly relevant for determining membrane protein, as it contains a deoxycholate solublisation step. A standard curve was constructed using B.S.A. in the range 0-30ug.

: Reagents

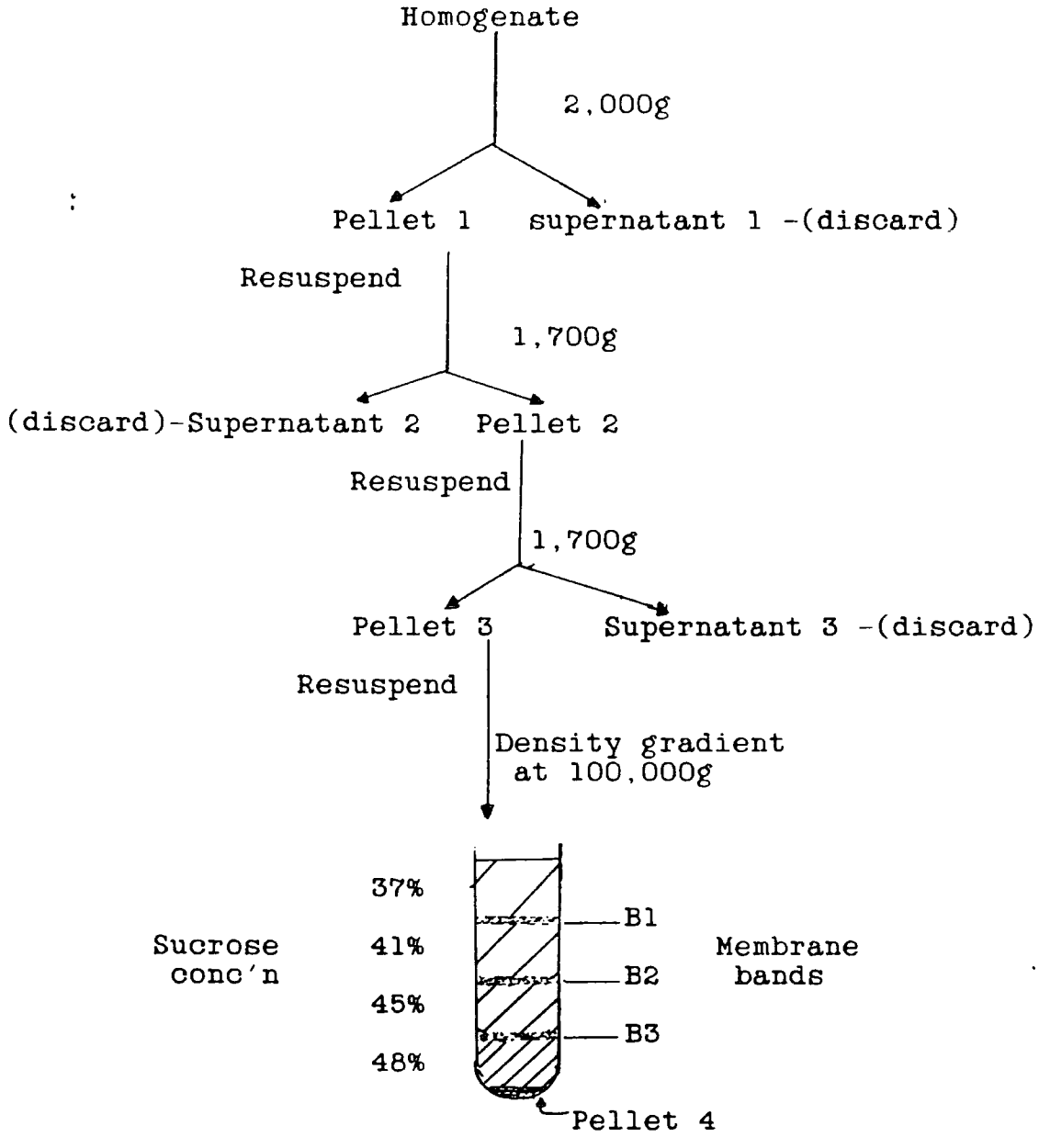
Reagent A: This was made up freshly, by mixing equal volumes of the following stock solutions together. i)C.T.C. (0.1 %copper sulphate, 0.2% potassium tartrate and 10% sodium carbonate). ii)0.8M NaOH. iii) 10% S.D.S. and iv) distilled water.

Reagent B: This was a six-fold dilution (in water), of the Folin-Ciocalteau phenol reagent.

A small volume of membrane/protein suspension was added to a plastic Eppendorf micro-tube (vol. 1.7ml) and made up to a total volume of 1ml with distilled water. To this was added 0.1ml of 0.15% deoxycholate, the samples were gently mixed and allowed to stand for 10 minutes. After this time 0.1ml of 72% T.C.A. was added and mixed using a vortex mixer. The tubes were then spun for 2 minutes in an Eppendorf minifuge, the supernatant decanted, leaving a very small white pellet of precipitated protein. To this pellet was added 0.5 ml of distilled water,

FIGURE 2.1

Plasma membrane Purification Scheme
for Liver, and tumours
(from Ray, 1970)



followed by 0.5ml of reagent A. The tube was vigorously mixed on a vortex mixer, and allowed to stand for 10 minutes, before the addition of 0.25ml of reagent B. After the addition of this last reagent, the tubes were rapidly mixed and allowed to stand for 30 minutes before reading the blue colour formed at 750nm in an SP8-100 dual beam spectrophotometer using water as a blank.

RESULTS

Figure 2.1 shows the purification procedure from Ray, (1970). The notation used to indicate the fractions in this figure will be used in all the following text. Initial studies were carried out using 0.5mM Ca^{2+} in the bicarbonate isolation media, but following the suggestion of Emmelot et al, (1975) 2.0mM Ca^{2+} was included in the latter procedures, and this improved the yield without effecting the purity of the membranes. In the first studies involving 2.0mM Ca^{2+}/bicarbonate buffer the Na^{+}/K^{+}ATPase was used as a plasma membrane marker, to determine the degree of purity at each stage in the isolation, and the final purity of the plasma membrane enriched fraction. These membranes were checked for the presence of contaminating membranes using other enzyme markers: Glucose-6-phosphatase for endoplasmic reticulum and cytochrome oxidase for mitochondrial contamination. All markers were low in activity in the tumour fractions as compared with those from liver. The Cytochrome oxidase was only detected in the original

homogenate and P4 fraction, and not recorded here. However during the course of the work other markers were examined which conflicted with the earlier results. The enrichment of a plasma membrane fraction will now be described in more detail.

1. Plasma membrane enrichment

Three markers for the plasma membrane were used in this study i) The Na+/K+ ATPase (the most commonly used marker) ii) 5′ Nucleotidase and Alkaline phosphodiesterase I . The Na+/K+ATPase will be considered first.

Tables 2.1 and 2.2 show the purification of the Na+/K+ATPase from D23 and Mc7 tumours respectively. They show the specific activity (umoles Pi liberated/mg protein/hour) of each of the 9 fractions examined, the relative purity of each compared to the original homogenate, and the %age recovery of initial enzyme activity. Ray, (1970) reported a plasma membrane enriched fraction concentrating between the 37 and 41% sucrose bands of the gradient (ie. band B1). The two tumours, D23 and Mc7 produce a similarly enriched fraction at this junction, with specific activities of: 7.79+2.62 and 6.05+1.47 umoles Pi/mg protein/hour; giving a purification factor over the original homogenate of 8.3 and 6.1 fold, respectively. This represents about 2% of the original activity, and is a poor yield. Examination of the two lower bands on the gradient, B2 and B3, reveals that some ATPase of a lower specific activity than the putative plasma membrane

TABLE 2.1 Na^+/K^+-ATPase in D23 fractions

Fraction	Specific Activity umolesPi liberated/ mg protein/ hour	Purification Factor	Yield of Activity %
H	1.08±0.38 (3)	1.0 (3)	100 (3)
S1	0.110±0.110 (3)	0.34±0.34 (3)	16.7±11.1 (3)
P1	2.22±0.38 (3)	3.19±1.68 (3)	74.6±40.7 (3)
S2	1.18±0.78 (3)	2.93±2.64 (3)	14.6±10.3 (3)
P3	1.97±1.36 (3)	2.04±0.15 (3)	14.8±8.34 (3)
B1	7.79±2.62 (4)	8.27±3.50 (4)	1.67±0.76 (4)
B2	0.636±0.331 (4)	0.544±0.37 (4)	0.162±0.042 (4)
B3	0.912±0.460 (4)	0.590±0.457 (3)	0.131±0.125 (3)
P4	0.162±0.058 (4)	0.307±0.157 (4)	1.93±1.56 (4)

No.s in brackets are no. of experiments
Values ± S.E.M.

TABLE 2.2 Na+/K+-ATPase in Mc7 fractions

Fraction	Specific Activity umolesPi liberated/ mg protein/ hour	Purification Factor	Yield of Activity %
H	1.33±0.43 (4)	1.0 (4)	100 (4)
S1	0.505±0.107 (4)	0.455±0.074 (4)	25.8±4.00 (4)
P1	2.60±0.22 (4)	2.81±0.90 (4)	65.3±14.6 (4)
S2	1.52±0.088 (4)	1.68±0.56 (4)	8.27±2.34 (4)
P3	1.48±0.36 (4)	1.32±0.26 (4)	15.3±3.20 (4)
B1	6.05±1.47 (4)	6.10±2.65 (4)	2.47±0.53 (4)
B2	3.46±0.69 (4)	3.22±0.81 (4)	0.948±0.134 (4)
B3	1.85±0.57 (4)	1.74±0.75 (4)	0.678±0.175 (4)
P4	0.308±0.260 (4)	0.203±0.126 (4)	1.32±1.05 (4)

No.s in brackets are no. of experiments
Values ± S.E.M.

TABLE 2.3 5′ Nucleotidase in D23 fractions

Fraction	Specific Activity umolesPi liberated/ mg protein/ hour	Purification Factor	Yield of Activity %
H	0.450±0.099 (4)	1.0 (4)	100 (3)
S1	0.044±0.044 (4)	0.083±0.083 (4)	2.47±2.47 (3)
P1	1.05±0.52 (4)	2.53±1.16 (4)	46.5±33.9 (3)
S2	1.01±0.049 (4)	1.90±0.116 (4)	6.81±0.42 (3)
P3	2.11±1.66 (4)	1.29±0.010 (4)	21.1±4.6 (3)
B1	2.18±0.41 (4)	6.24±2.52 (4)	1.52±0.72 (3)
B2	0.290±0.167 (4)	3.35±2.47 (4)	0.626±0.477 (3)
B3	0.800±0.255 (4)	2.46±1.34 (4)	0.667+0.476 (3)
P4	0.166±0.066 (4)	0.63±0.38 (4)	4.01±2.02 (3)

No.s in brackets are no. of experiments
Values ± S.E.M.

TABLE 2.4 5′-Nucleotidase in Mc7 fractions

Fraction	Specific Activity umolesPi liberated/ mg protein/ hour	Purification Factor	Yield of Activity %
H	0.497±0.105 (3)	1.0 (3)	100 (3)
S1	0.34±0.34 (2)	0.35±0.35 (2)	-
P1	0.65±0.11 (2)	1.09±0.07 (2)	-
S2	1.60±0.51 (2)	2.75±1.05 (2)	-
P3	0.60±0.37 (2)	1.08±0.72 (2)	-
B1	2.45±0.49 (3)	5.54±1.8 (3)	2.56
B2	1.38±0.79 (3)	3.95±2.78 (3)	2.11
B3	1.53±0.41 (3)	4.17±2.51 (3)	-
P4	0.215±0.156 (3)	0.38±0.29 (3)	-

No.s in brackets are no. of experiments
Values ± S.E.M.

TABLE 2.5 Alkaline phosphodiesterase I in D23 fractions

Fraction	Specific Activity umoles nitrophenol liberated/mg protein /hour	Purification Factor	Yield of Activity %
H	0.088±0.051 (3)	1.0 (3)	100 (3)
S1	0.0303±0.0152 (3)	0.395±0.228 (3)	16.6±11.3 (3)
P1	0.244±0.123 (3)	3.30±0.70 (3)	59.0±27.4 (3)
S2	0.253±0.082 (3)	2.94±0.10 (3)	9.95±0.55 (3)
P3	0.355±0.169 (3)	5.44±3.87 (3)	19.7±0.25 (3)
B1	0.200±0.088 (3)	3.98±1.78 (3)	0.429±0.188 (3)
B2	0.717±0.480 (3)	8.32±5.13 (3)	0.452±0.407 (3)
B3	0.0455±0.0335 (3)	0.737±0.380 (3)	0.082±0.066 (3)
P4	0.017±0.007 (3)	0.268±0.004 (3)	2.42±1.31 (3)

No.s in brackets are no. of experiments
Values ± S.E.M.

TABLE 2.6 Alkaline phosphodiesterase I in Mc7 fractions

Fraction	Specific Activity umoles nitrophenol liberated/mg protein /hour	Purification Factor	Yield of Activity %
H	0.135±0.060 (2)	1.0 (2)	100 (2)
S1	0.0618±0.0258 (2)	0.465±0.015 (2)	30.4±5.25 (2)
P1	0.345±0.174 (2)	2.47±0.405 (2)	91.0±42.9 (2)
S2	0.317±0.097 (2)	2.53±0.41 (2)	24.2±4.45 (2)
P3	0.069±0.025 (2)	0.536±0.054 (2)	8.64±1.66 (2)
B1	0.143±0.050 (2)	1.52±1.05 (2)	0.542±0.198 (2)
B2	0.127±0.014 (2)	1.20±0.588 (2)	0.335±0.060 (2)
B3	0.159±0.038 (2)	1.56±1.36 (2)	0.299±0.003 (2)
P4	0.005±0.005 (2)	0.007±0.007 (2)	0.001±0.001 (2)

No.s in brackets are no. of experiments
Values + S.E.M.

fraction. The pellet, P4, contains a relative large %age of the gradient ATPase activity, however this of a low specific activity, and results from the incomplete resolution of the plasma membrane from the nuclear fraction.

An inspection of fractions 1-9 reveals that a considerable amount of material of high specific activity is lost in supernatants S1 and S2; this is greatest in the Mc7 isolation. The other main source of loss of activity occurs during the discontinuous sucrose gradient spin. Of the activity placed on the gradient only 26%(D23) and 35%(Mc7) is recovered. This is not due to a loss of protein, and therefore the purification factor of the membranes is considerably reduced, as a result of protein inactivation, not loss of membrane material.

Tables 2.3 and 2.4 show similar purification patterns using the 5′ Nucleotidase as a plasma membrane marker. Again the putative plasma membrane fraction reported by Ray, (1970), for liver was the same in both tumours. The nucleotidase was present at a lower specific activity in the tumour fractions than the Na+/K+ ATPase , that is : 2.18 (D23) and 2.45 (Mc7); the purification factors were lower as well, 6.24 and 5.54 for the D23 and Mc7, respectively. As with the previous enzyme, distribution of this marker is also found in bands B2 and B3. Again, there was the loss of relatively high specific activity material from supernatants S1 and S2.

The pattern of purification appears to be similar from a consideration of both plasma membrane marker enzymes examined so far. However, the final marker enzyme , Alkaline phosphodiesterase I (Tables 2.5 and 2.6)showed slightly different patterns, and little or no increase in specific activity, in the Mc7 plasma membrane fraction. The specific activity, umoles of substrate utilized/mg protein/hour, of the enzyme in B1 (the putative plasma membrane fraction) was low in both tumours: 0.200, D23 and 0.143, Mc7. These values were similar, but there appeared to be a greater purification of the D23 hepatoma fraction, ie. about 5 fold to the lower 1.5 fold enrichment for the Mc7. The distribution of this marker enzyme in the remaining bands of the sucrose gradient are different in the two tumours. In the D23 there appears to be a greater, though variable, enrichment (8.32+5.13) in band B2, than the supposed plasma membrane fraction, B1. Band B3 is of lower specific activity, as in the other plasma membrane markers. In the Mc7 the purification is similar in all three bands; B1-B3. Examination of the yield of phosphodiesterase activity during the fractionation procedure, reveals a considerable loss at all stages, which means the resultant activity in the membrane fractions is low. There was the same kind of loss of activity in the S1 and S2 supernatants, and during density gradient centrifugation, as reported for the other two plasma membrane markers. The substantial loss of activity makes this an unsuitable marker for these two tumour types.

2. Microsomal contamination

Glucose-6-phosphatase and NADPH cytochrome-c reductase are microsomal membrane markers, indicating the degree of contamination of the purified plasma membrane fraction.

Tables 2.7 and 2.8 show glucose-6-phosphatase(G6Pase) activities and relative purity for the D23 and Mc7 isolation procedures. In the putative plasma membrane fraction, B1, the specific activity was 2.08 and 1.49 umoles of Pi liberated/mg protein/hour, for the D23 and Mc7 isolations; this represents a slight purification over the original homogenate, of about 2-fold, in both tumour types. This indicates that the G6Pase has to some extent co-purified with the plasma membrane marker enzymes. However, in fraction B2 the purification factor of this enzyme is even greater than that of the B1; D23 2.6-fold, Mc7 4.2-fold. This represents higher specific activities in the B2 than the B1 fraction.(3.5, D23 and 3.7 umolesPi/mg protein/hour, Mc7. Fraction B3 is also enriched with respect to this marker in both tumour types. In both Mc7 and D23 most of the activity placed on the gradient goes to the nuclear pellet, P4. This represents a greater proportion of the total activity than in the case of say the Na+/K+ATPase. In both tumours the recovery of activity after sucrose density gradient oentrifugation is greater than in the plasma membrane marker enzymes, and is greatest in the Mc7. This distorts the relative purity indicated by marker enzyme oonsideration alone. In the

TABLE 2.7 Glucose-6-phosphatase in D23 fractions

Fraction	Specific Activity umolesPi liberated/ mg protein/ hour	Purification Factor	Yield of Activity %
H	1.14±0.36 (3)	1.0 (3)	100 (3)
S1	ND (3)	ND (3)	ND (3)
P1	1.78±1.04 (3)	1.78±0.80 (3)	38.2±14.9 (3)
S2	1.75±0.64 (2)	2.14±0.26 (2)	7.18±0.75 (2)
P3	3.81±2.10 (2)	3.85±1.26 (2)	22.4±11.7 (2)
B1	2.08±0.35 (3)	2.07±0.42 (3)	0.497±0.122 (3)
B2	3.56±2.28 (3)	2.57±1.09 (3)	0.196±0.015 (3)
B3	1.94±0.89 (3)	1.81±0.55 (3)	0.475±.077 (3)
P4	0.458±0.017 (3)	1.18±0.55 (3)	5.56±2.53 (3)

No.s in brackets are no. of experiments
Values ± S.E.M.

TABLE 2.8 Glucose-6-phosphatase in Mc7 fractions

Fraction	Specific Activity umolesPi liberated/ mg protein/ hour	Purification Factor	Yield of Activity %
H	1.18±0.34 (4)	1.0 (4)	100 (4)
S1	0.012±0.012 (4)	0.037±0.037 (4)	3.00±2.60 (4)
P1	0.673±0.203 (4)	1.90±0.89 (4)	50.4±25.0 (4)
S2	1.00±0.77 (4)	5.04±4.60 (4)	10.0±6.40 (4)
P3	0.700±0.289 (4)	1.68±0.93 (4)	7.47±2.57 (4)
B1	1.49±0.53 (4)	2.12±0.91 (4)	1.13±0.53 (4)
B2	3.69±0.89 (4)	4.19±1.29 (4)	4.05±2.46 (4)
B3	1.71±1.08 (4)	1.62±1.13 (4)	0.604±0.310 (4)
P4	0.519±0.236 (4)	0.596±0.300 (4)	11.8±4.93 (4)

No.s in brackets are no. of experiments
Values ± S.E.M

TABLE 2.9 NADPH cytochrome c reductase in D23 fractions

Fraction	Specific Activity umoles cyt.c reduced/mg protein /hour	Purification Factor	Yield of Activity %
H	0.146±0.029 (3)	1.0 (3)	100 (3)
S1	0.167±0.043 (3)	1.23±0.349 (3)	43.7±6.83 (3)
P1	0.276±0.116 (3)	1.78±0.469 (3)	41.0±10.0 (3)
S2	0.553±0.388 (3)	4.11±2.69 (3)	21.6±2.90 (3)
P3	1.00±0.86 (3)	5.14±3.28 (3)	16.9±2.0 (3)
B1	0.362±0.091 (3)	2.43±0.19 (3)	0.585±0.062 (3)
B2	0.918±0.292 (3)	7.31±3.45 (3)	0.679±0.126 (3)
B3	0.593±0.037 (3)	4.46±1.02 (3)	1.28±0.18 (3)
P4	0.064±0.027 (3)	0.410±0.107 (3)	4.36±2.18 (3)

No.s in brackets are no. of experiments
Values ± S.E.M.

TABLE 2.10 NADPH cytochrome c reductase in Mc7 fractions

Fraction	Specific Activity umoles cyt.c reduced/mg protein /hour	Purification Factor	Yield of Activity %
H	0.243±0.132 (3)	1.0 (3)	100 (3)
S1	0.144±0.024 (3)	0.869±0.238 (3)	49.0±8.8 (3)
P1	0.287±0.032 (3)	1.89±0.70 (3)	43.9±7.5 (3)
S2	0.497±0.020 (3)	3.55±1.53 (3)	24.3±5.6 (3)
P3	0.388±0.135 (3)	1.75±0.84 (3)	21.9±8.9 (3)
B1	0.190±0.032 (3)	2.37±1.43 (3)	0.944±0.543 (3)
B2	0.454±0.159 (3)	3.09±0.80 (3)	0.883±0.136 (3)
B3	0.788±0.429 (3)	5.49±2.43 (3)	2.19±0.94 (3)
P4	0.112±0.094 (3)	0.518±0.441 (3)	4.93±3.48 (3)

No.s in brackets are no. of experiments
Values ± S.E.M.

initial stages of purification, the G6Pase follows the pattern of the plasma membrane markers although proportionally less is lost in the supernatants S1 and S2.

Tables 2.9 and 2.10 show the distribution of the microsomal marker NADPH cytochrome c reductase in the plasma membrane isolation. Inspection of the membrane fractions from the sucrose gradient indicates differences in the distribution of this marker between the two tumour types. However in both cases the putative plasma membrane fraction has the lowest specific activities and purification value. In the D23 band B2 contained the highest specific activity of the gradient fractions giving a purification factor of 7.3-fold; where as the Mc7 had the highest reductase activity in the B3 band, giving a purification factor of 5-fold. In both tumour types the greatest %age yield of reductase activity in the bands was in band B3. The pellet, P4, contained the greatest proportion of activity, but there is a loss, in both tumours, of reductase activity during density gradient centrifugation. The loss of activity in the NADPH cytochrome c reductase in supernatants S1 and S2 is greater than that for the glucose-6-phosphatase marker. The distribution pattern of the reductase in the final membrane fractions B1-B3 is different from that of the plasma membrane markers N+/K+ATPase and 5' Nucleotidase, but it differs also from the other microsomal marker, G6Pase.

3. Mitochondrial contamination

Preliminary results with cytochrome oxidase at 25°C revealed little or no activity in any of the fractions; only in fractions H and P4 was there any significant activity detected. The same was true of succinate dehydrogenase, tested under similar conditions. These conditions were those indicated by Emmelot et al. (1964). Other workers have used higher temperatures, and when the succinate dehydrogenase was assayed at 30°C there was a considerable difference in the activity and pattern of contamination. Unfortunately this was only discovered later on in the study.

Tables 2.11 and 2.12 show the distribution of the succinate dehydrogenase(S.D.H.) in the various fractions of the isolation procedure. In both the D23 and Mc7 tumours the mitochondrial fraction co-purified with the plasma membrane fraction, although a substantial amount of activity is lost particularly in supernatants S1 and S2. This is not just owing to a loss in protein, but also a loss in the activity of the protein remaining. There is however very little activity lost on the density gradient, contrasting with many of the other marker enzymes so far described, including the plasma membrane markers. About 75% and 80% of the total activity of the P2 fraction for D23 and Mc7, respectively, is recovered. This of course affects the purification and specific activity of this marker enzyme. The highest specific activity of S.D.H. is

TABLE 2.11 Succinate dehydrogenase in D23 fractions

Fraction	Specific Activity umoles cyt.c reduced/mg protein /hour	Purification Factor	Yield of Activity %
H	0.231±0.213 (3)	1.0 (3)	100 (3)
S1	0.027±0.019 (3)	0.121±0.107 (3)	7.63±6.99 (3)
P1	0.568±0.142 (3)	2.09±0.576 (3)	51.5±14.9 (3)
S2	0.430±0.430 (3)	0.63±0.63 (3)	2.17±2.17 (3)
P3	3.46±1.68 (3)	9.45±1.65 (3)	8.51±3.93 (3)
B1	1.80±1.10 (3)	5.11±4.41 (3)	0.654±0.406 (3)
B2	2.53±1.03 (3)	10.54±6.19 (3)	0.919±0.399 (3)
B3	0.776±0.186 (3)	2.85±0.76 (3)	0.808±0.149 (3)
P4	0.120±0.031 (3)	0.535±0.276 (3)	3.96±1.17 (3)

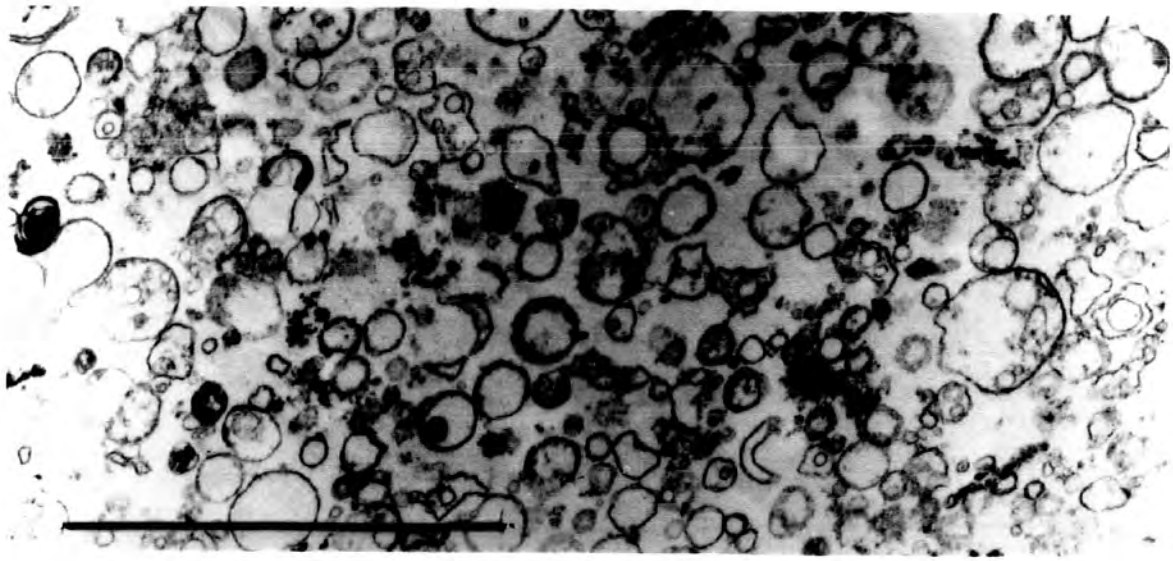
No.s in brackets are no. of experiments
Values + S.E.M.

TABLE 2.12 Succinate dehydrogenase in Mc7 fractions

Fraction	Specific Activity umoles cyt.c reduced/mg protein /hour	Purification Factor	Yield of Activity %
H	0.216±0.084 (2)	1.0 (2)	100 (2)
S1	0.059±0.034 (2)	(2)	17.3± (2)
P1	0.225±0.176 (2)		53.6±14.1 (2)
S2	0.091±0.091 (2)		
P3	1.10±0.85 (2)		15.8±4.87 (2)
B1	1.45±0.78 (2)	7.23±3.77 (2)	5.20±1.5 (2)
B2	1.25±0.71 (2)	6.10±0.77 (2)	1.96±0.41 (2)
B3	0.215±0.115 (2)	1.42±1.09 (2)	1.93±0.43 (2)
P4	0.194±0.07 (2)	0.406±0.263 (2)	3.50±1.41 (2)

No.s in brackets are no. of experiments
Values + S.E.M.

found in different fraction in the two tumours. In D23 the greatest specific activity and purification factor is found in the B2 band, however there is considerable contamination due to this marker found in band B1, the plasma membrane fraction, with a specific activity of 1.8umole of cytochrome c reduced/mg protein/hour representing a 5-fold purification over the homogenate. Most of the S.D.H. activity resides in the nuclear pellet, P4. This was the same in the MC7 purification,but the marker was more evenly distributed between the B1 and B2 fraction. Clearly this co-purification of the S.D.H. represents significant contamination of the plasma membrane fraction, however, it is important to note at this stage, that this contamination was erratic. This contamination was almost certainly due to the presence of intact mitochondria in the final preparation, and Ray, (1970), in his original method suggested an additional spin on a continuous sucrose gradient, to remove this type of contamination.. This additional purification step was tried, but failed to change the nature of the plasma membrane fraction, and produced no further resolution of the B1 fraction. Appendix 2 shows electron micrographs of the two tumour types, and few mitochondria could be seen particulary compared to liver cells. Electron micrographs were also taken of the plasma membrane fractions (Plate 2.1). The micrographs show mainly smooth membrane vesicles with little evidence of heavy mitochondrial contamination. Since such contamination could have a serious effect on the interpretation of the results, the level of mitochondrial



contamination in the plasma membranes of liver, the control tissue was investigated. Contamination was just as extensive in the liver plasma membrane fraction with specific activities of succinate dehydrogenase of 1.2Umoles cyt.c reduced/mg protein/hour. This level of contamination, present to the same extent in liver, Mc7 and D23 plasma membrane fractions, allows other comparative studies to be made.

DISCUSSION

An overall examination of the results reveals that in both tumour types, the fraction with the greatest plasma membrane enrichment is band B1; the same fraction as reported by Ray, (1970), and therefore this method of isolating the plasma membranes from liver is applicable to the two tumours, D23 and Mc7. It must be noted that the plasma membrane markers were also found in bands B2 & B3, and in the nuclear pellet (P4), this latter observation indicates that at least some of the plasma membranes cannot be separated from the nuclear fraction. The enrichment is clearly demonstrated by the plasma marker enzymes, Na+/K+ATPase and the 5′ Nucleotidase with purification factors of 8-fold (D23) and 6-fold (Mc7): and 6, 5.5-fold(D23,Mc7), for the two enzymes respectively. The substantial loss of activity of the Alkaline phosphodiesterase, particularly during the sucrose density gradient stage, makes it an unsuitable marker in these two tumours. The rather unusual distribution of this enzyme in

bands B1-B3 may indicate the existence of domains within the tumour membranes, as indicated in other tumours (eg. Haeffner et al. 1980, and Hoessli & Rungger-Brandle, 1983) and this latter enzyme does not share the same domain as the other two.

The purification factor quoted for other tumour and liver plasma membranes is usually higher than shown in Tables 2.1-2.6, in the range of 10-20 fold eg Ray, (1970), Raftell and Blomberg, (1973), although Upreti et al. (1983) reported 8-fold purification factor in the Na+/K+ATPase, and one of 0.6-fold for 5′ Nucleotidase in the membranes of a hepatoma. The relatively poor enrichment may be partly due to the low specific activity of the marker enzymes in the plasma membrane. Raftell and Blomberg, (1973), for example, isolated D23 tumour plasma membranes and reported a specific activity, for the 5′ Nucleotidase of 30 umoles of Pi released/mg protein/hour for the final fraction where as in the D23 isolation presented here was 6.2-fold. It is important to note that the specific activity for the original homogenate, reported by these workers was 4-fold higher than for this current purification procedure, and it must be remembered that this earlier work was carried out over 14 years ago, and Emmelot and Bos, (1969) have reported differences in enzyme activities of the same tumour maintained in passage over several generations. Raftell and Blomberg, (1973) noted, in their study of the D23 hepatoma, that the marker enzymes had decreased activity compared to the original tissue,

liver. For example, 5′ Nucleotidase in liver membranes has been reported with various specific activities: 82.0, 58.6 umoles Pi lib./mg protein/hour, for Ray, (1970) and Coleman et al. (1967) respectively, both of which are higher than for the D23 tumour membranes reported here and by Raftell and Blomberg, (1973). Emmelot et al. (1964), have reported an activity of 11.6 umoles/Pi liberated/hr. for the Na+/K+ ATPase in liver. From several reports on hepatoma membrane enzymes, it is clear that variation from the original tissue can either be in the form of an increase, or decrease. Emmelot & Bos, (1969) have reported an increase in the 5′ Nucleotidase activity in some hepatomas, but a decrease in others. Other workers have reported specific activities in 5 hepatomas which are less than those reported here, (Koizumi et al, 1976). The Mc7 sarcoma is not comparable with the liver and so it is difficult to assess the purification and recovery of marker enzymes. Low specific activities in the homogenate obviously may mean a proportionally greater loss of activity during purification, as a certain amount of activity will aways be lost anyway. Loss of fairly high specific activity material in supernatants S1 and S2 is due to vesicle formation; the presence of Ca2+, used to bind the membranes together in sheets is not fully effective. Loss of specific membrane marker activity during differential density gradient centrifugation is due to loss of enzyme activity, rather than loss of membranes per se. It must be remembered that one is purifying plasma membranes and not necessarily their component proteins, no

matter how good that purification might seem! In both tumour types, 70% of the activity of the plasma membrane markers has been lost in the density gradient centrifugation step, this indicates that the purification factors could be up to 3-fold higher, well within the most successful literature purifications, if the recovery of activity had been better. This loss may be due to the formation of tight vesicles during centrifugation. The calcium, which might have prevented the formation of vesicles, had been removed by E.D.T.A., because of its property to act like 'glue', binding the membranes together. The entrapment of markers in vesicles is well reported, Evans, (1982). Forbush, (1983), has shown that S.D.S pre-incubation, which breaks up vesicles, can increase the specific activity of the Na+/K+ATPase considerably. This latent activity has been demonstrated in the D23 plasma membrane fraction during some preliminary work in this laboratory. The poor enrichment may be purely due to this process in both of the important plasma membrane markers. It is pertinent to remember that the entrapment of the markers of contaminating membranes, in vesicles, may also be possible. For example there appears to be a loss of activity during density gradient centrifugation for the GDPase of D23 but not for Mc7, where recovery is 100%.

Both microsomal marker enzymes, NADPH cytochrome c reductase and GDPase, concentrate in different bands to that of the plasma membrane, although both are present in

the putative plasma membrane fraction, B1. Other workers have reported similar specific activities in purified plasma membrane eg. Koizumi et al. (1976), reported GDPase activities of 0.47-1.72 umolesPi/mg protein/hour in various rat hepatomas; and Emmelot and Bos, (1969) reported specific activities of 2.7 umolesPiliberated/mg/protein/hr. in mouse hepatomas The results obtained for the D23 and Mc7 presented here are: 2.08, and 1.49 respectively. One would expect higher levels of this enzyme in the D23 because it is derived from a gluconeogenic tissue. Values for liver are even higher still eg. 2.4, Koizumi et al. (1976) and 3.05, Coleman et al. (1967). The 2-fold purification factor in both tumour plasma membranes indicates that the contamination is not great from this membrane source.

The NADPH cyt.c reductase is another important microsomal marker and is studied as an alternative to GDPase, because of the latter's limitation to gluconeogenic tissue. In the D23 isolation the highest specific activity of this enzyme is found in B2 whereas it is more concentrated in the band B3 of the Mc7 gradient fraction. This indicates a difference in the sub-cellular distribution of this enzyme in these two tumours. In both tumours the specific activity of this microsomal marker is considerably lower in the B1 fractions than in any other gradient fraction, ie. 0.36 (D23) and 0.19 (Mc7). Reports of the specific activity of this enzyme in liver vary eg. 0.144 (Tsai et al. 1975) and 7.68 umole cyt.c/mg

protein/hour (Emmelot et al. 1970). Sauvage et al. (1981), in their study of HTC hepatoma cells, report that this enzyme might be a plasma membrane enzyme in tumours. As other workers have postulated (eg Upreti et al. 1983) marker enzymes in liver will not necessarily have the same distributions in tumour sub-cellular fractions when the metabolism of the cell is disrupted. The results presented here show that there may be even variation between different tumours.

The greatest contamination in the putative plasma membrane fraction, as indicated by S.D.H. enzyme activities and distribution, come from a mitochondrial source. The first point to be made is that the recovery of this marker during density gradient centrifugation is 100% (that is, all the activity of this enzyme in the P3 pellet placed on the gradient, is recovered). This contrasts starkly with the recovery of other markers, notably those of the plasma membrane. This difference clearly distorts the index of purity due to markers alone. If the recovery of plasma membrane markers was the same as for the S.D.H., then the proportion of contamination would be greatly reduced. Although most workers report low or no mitochondrial contamination in the plasma membrane fractions, Ray, (1970), in his orginal method did not report the assay of a mitochondrial marker, relying on their absence from Electron micrographs of the final pellet. The same is true of Koizumi et al. (1976), in their study of the plasma membranes of 5 rat hepatoma cell

lines. The Electron micrographs presented in this current study also reveal very little mitochondrial contamination, and in fact there is a low frequency of this organelle in the original cells (see Appendix 2). This anomaly might best be explained by the apparent greater stability of the mitochondrial marker during plasma membrane isolation, when compared to the normal plasma membrane markers. It is also noted, that, under the conditions for the assay of respiratory enzymes recommended by Emmelot, et al. (1964) of 20°C, little S.D.H. or cytochrome oxidase activity was detected, and none was found in the plasma membrane fractions. The difference is so great, that the rise in activity in the S.D.H. between the two sets of conditions, ie 20-30°C, cannot be explained by direct temperature effects alone, and may be due to opening of closed vesicles. Additional evidence is presented in the next chapter which supports the idea that mitochondrial contamination is not excessive. The phospholipid classes presented in Table 3.1, do not show changes which would implicate contamination from a mitochondrial source (eg. cardiolipin, a mitochondrial lipid, was not detected in the T.L.C separation of the tumour phospholipids).

Further separation, using a discontinuous sucrose gradient, as recommended by Ray, (1970) to remove mitochondrial contamination, did not further resolve these two membrane types. Since Ca2+ has been effectively removed by the addition of E.D.T.A., the binding of different membrane types together, by this cation, seems

unlikely. Any separation by buoyant density relies on reasonable differences between the membranes. The plasma membrane, due to its high cholesterol content, is usually easy to resolve; however if these tumour membranes have a lowered cholesterol content, the component membranes may be very difficult to resolve. Rapidly dividing cells, eg. regenerating liver, have lowered cholesterol levels in the plasma membrane, Koizumi et al. (1976). The same may be true of these tumour cells. This problem of resolution is not uncommon, Tulkens et al. (1974) and Lewis et al. (1975) have used digitonin, a complex polymer, to increase differentially the buoyant density of the plasma membrane above that of the contaminating membranes. The principal is that digitonin binds to the cholesterol in the membranes, increasing the buoyant density. Obviously the amount of binding depends on the the levels of cholesterol, and small differences in the buoyant density, due to this lipid, can be enhanced using this polymer. The disadvantages of using this molecule are i)It can inhibit plasma membrane bound enzymes (Lewis et al. (1975). ii) It also disrupts the physical structure of the membrane, making it unsuitable if any physical measurements are to be carried out later. Despite these disadvantages some workers have used digitonin to resolve hepatoma plasma membranes which were normally inseparable from contaminating fractions, (Suavage et al. 1981). An important part of the work reported in this thesis involves measurement of the physioal state of the tumour membrane and so use of digitonin to further purify the plasma

membrane was undesirable. Clearly the contamination reported here could interfer with the interpretation of the results obtained in further studies of these tumour membranes. However, since this was a comparative study between normal and tumour membranes, if the contamination of liver membranes is similar to that of the tumours, then this, to some extent, allows one to infer that any changes taking place between the two membrane types are a result of neoplasia, not contamination. The results here indicate that this is the case, as the liver plasma membrane used in these studies has the same level of mitochondrial contamination as the D23 and Mc7 tumour membranes.

3. LIPID CHARACTERISATION OF D23 & MC7 TUMOUR PLASMA MEMBRANES

INTRODUCTION

The fluid mosiac model of the cell membrane structure (Singer & Nicholson, 1972) forms the basis of the current understanding of plasma membrane structure. They proposed a bilayer of phospholipids with a hydrophobic core, in which proteins floated. This model has since been expanded to include cholesterol, which, in the normal liquid phase has a condensing effect on the fatty acid chains of the phospholipid molecules. (Oldfield & Chapman, 1972, and DeKruff *et al*. 1972). A further modification specifies that all proteins cannot float freely when at least some are anchored by the cytoskeleton. The lipids play a crucial role in determining the physical nature of the membrane, and are responsible for maintaining a semi-permeable barrier, without which cell function would breakdown.

Thermal energy, as postlulated in the General Introduction, decreases the stability of the membrane by increasing the motion of the lipid constituents, and therefore decreasing membrane order. This could have two deleterious consequences for membrane function, if the thermal perturbation became excessive. First, the increased 'fluidity' would effect the semi-permeable nature of the membrane and solute leakage would occur (Gladwell

et al. 1973). Secondly, the less ordered lipid environment would allow heat perturbation of protein structure, and could lead to 'denaturation' (Bowler et al. 1973; Bowler, 1981). Clearly the nature of the lipids in any given membrane will determine its responsiveness to heat. If the lipid composition is such that a disordered membrane is produced, less heat will be required to upset these membrane functions. The lipids which are most important for determining the physical state or order of the membrane are the fatty acyl chains of the phospholipid molecule, and in mammalian plasma membranes, cholesterol. Unsaturated fatty acids by virtue of their greater molecular area tend to disorder the structure (Stubbs, 1983). That these fatty acids play a role in the cells response to heat has been shown by indirect evidence. It has been known for some time that many organisms eg. bacteria, plants and poikilotherms change the degree of saturation of the fatty acyl chains of their membranelipids in response to changes in environmental temperature (Hazel & Prosser, 1974). Homeoviscous adaptationwas the term used by Sinensky, (1974) to describe this process, and it has been recently reviewed by Cossins, (1983). In mammalian systems increases in membrane cholesterol in response to other 'fluidizing' agents such as ethanol (Chin & Goldstein, 1978) have been reported and Sinensky (1978), showed, in cholesterol deficient mutants, that there was a correlation between heat sensitivity, membrane cholesterol levels and membrane 'microviscosity'. More importantly the relationship between heat sensitivity and cellular cholesterol content was shown in a variety of cell lines.(Cress & Gerner, 1980, and Cress et al. 1982).

There is a large literature on the lipid composition of tumours which has been extensively reviewed elsewhere (eg. Wood, 1973)

Although the lipids of many tumours have been characterised, usually these studies have been on whole tumours, rather than sub-cellular fractions like the plasma membrane, as noted by Upreti et al. (1983). Largely these studies have been attempts to understand the nature of the cancer phenotype (review Weber, 1983) rather than how such changes can be related to the stability of these membranes to hyperthermia. In their studies of plasma membranes from leukaemias and lymphomas some workers have reported decreased plasma membrane cholesterol which would make these cells more unstable to heat (Inbar, 1976; Koizumi et al. 1981; and van Blitterswijk 1982). But there are many tumour cells which show an opposite pattern with increased plasma membrane cholesterol, particularly hepatomas (for review see Chen et al. 1978).

The aim of this chapter is to characterise the lipids of isolated plasma membranes from D23 and Mc7 tumours, and to compare these with those of liver from the same animal, to see if there are any changes in the tumour lipids which might explain the heat sensitivity of many tumours. Plasma membrane enriched fractions of the tumours will be isolated using the purification procedure described in Chapter 2. The lipid characterization of the liver and two tumour plasma membranes will be examined by phospholipid classes; by the fatty acid composition of these phospholipid classes; and by

cholesterol/phospholipid molar ratios. The tumour lipids will be compared to the lipids from the equivalent plasma membrane fraction of liver, derived from the same animal, and to literature values for liver. Only the D23, being a hepatoma, can be directly compared to liver; no equivalent normal tissue comparison was available for the Mc7 sarcoma. consequently the main emphasis will be to underline the features common to both tumours, but distinct from liver, as these are more likely to result from a common property of tumour cells, rather than variation due to a different tissue source.

MATERIALS AND METHODS

1. Isolation of plasma membranes

Plasma membrane enriched fractions were isolated from liver, D23 and Mc7 tumours using the methods in the preceding section.

2. Protein estimates

Proteins were assayed as described in Chapter 2, section 4.

3. Lipid extraction

Total lipid extraction was carried out on the isolated plasma membranes using the method of Bligh and Dyer(1959), modified by Hajra *et al*. (1968). All solvents contained

0.05% B.H.T..

To a 2.4ml aliquot of the plasma membrane suspension (1.7-3.0mg protein), was added the following: 9ml of methanol:chloroform mixture(2:1) and 0.192ml of concentrated hydrochloric acid. The mixture was homogenized in a Potter homogenizer, using ten manual strokes. The one-phase mixture was poured into a glass centrifuge tube and allowed to stand for 5 minutes. A further 3ml of chloroform was used to rinse out the homogenizing tubes, the resulting liquid was then added to the centrifuge tube and mixed for 30 seconds on a MSE vortex mixer. To this was added with further mixing, 3ml of a solution of 0.2M potassium chloride in 2M Orthophosphoric acid. To assist the separation of the two resulting phases, a 5 minute low speed centrifugation(500g) was carried out. The lower (chloroform layer) was decanted(12ml); then a further 12ml of dried chloroform was added to the remaining mixture, vortex mixed, and recentrifuged. The combined lower layers were dried down under dry white spot nitrogen and resuspended in an appropriate volume of dry chloroform/methanol (2:1). This extract was stored under nitrogen, in a sealed glass tube, at -20°C, in the dark, until required.

4. Assay of Total Cholesterol.

Total cholesterol was assayed enzymatically using a B.D.H. Kit (BDH Choloxidate No.1), and based on

cholesterol oxidase. The assay was designed for use with blood serum, and so was modified as described by Johnson,(1979).

The assay was linear in the range 0-0.4 µmoles of cholesterol. An appropriate volume of lipid extract, together with cholesterol standards were placed in clean, thick-walled glass tubes and evaporated to dryness in an oven set at 100°C. The lipids were then redissolved in 0.1ml of isopropanol. To this solution was added 2.5ml of the reconstituted buffered enzyme solutions 10 minutes at 37°C.

The pink colour which formed was read at 500nm on a Pye Unicam SP8-100 spectrophotometer, in a glass cuvette with a 1cm path length. (Any cloudy suspension was removed before reading by using low speed centrifugation.)

5. Assay of Total Phospholipid.

Phospholipid estimations were carried out by the method of Raheja et al. (1973). This method had the advantage of assaying phospholipid directly, and was insensitive to any contaminating inorganic phosphate.

Preparation of Chromogenic Solution.

Ammonium molybdate solution (A)

Ammonium molybdate(8g) was dissolved in 60ml of distilled water. A few drops of concentrated hydrochloric acid were added to complete the dissolution. This primary solution (A) was used to make solutions 1 and 2.

b)Solution 1

Redistilled mercury(5g) was added to a mixture of 20ml of concentrated hydrochloric acid, and 40ml of the ammonium molybdate solution (A) described above. The mixture was stirred on a magnetic stirrer for 45minutes and filtered; a red-brown filtrate was formed.

Solution 2

The subsequent operations were carried out by stirring in an ice bath to prevent denaturation of the reagents by boiling. This was done in a fume cupboard. An acid molybdate solution was prepared by very carefully adding 100ml of concentrated sulphuric acid to 20ml of the prepared molybdate solution (A)

The final chromogenic reagent was prepared by very carefully adding solution 2, with stirring, to solution 1. A dark green solution was formed -if dark blue, the reagent had been denatured during preparation. The dark green solution was mixed with other solvents in the following ratios: chromogenic reagent(25vol.); methanol(45vol.); chloroform(5vol.); and distilled water(20vol.). The dark

green mixture was then stored at 0-4°C, and had a shelf-life of in excess of 6 months.

Assay procedure

Standards and samples were dried down in thick walled glass test tubes in an oven at 100-110°C. The range of the standard curve was 0-0.3umoles of phospholipid phosphorus. To these dried down samples was added 0.4ml of chloroform, followed by 0.2ml of the chromogenic reagent. These were mixed then heated in a boiling water bath for exactly 3 minutes. The tubes were then cooled , and 3ml of chloroform was added. The solution was vortexed and spun at low speed, in a centrifuge(500g), for 5 minutes to separate the two layers. The absorbance of the blue colour in the lower, chloroform, layer was measured at 716nm in a glass cell with a 1cm path length, using a Pye Unicam SP8-100 spectrophotometer. The assay was linear in the range 0-0.3umoles of lipid phosphorous.

6. Two dimensional Thin Layer Chromatographic separation of phospholipid classes.

Thin layer plates, 20cm square, were coated with a slurry of slica gel H (Kieselghur H), 0.5mm thick. These were air-dried and then activated in an oven at 100-110°C for at least 2 hours.

An aliquot of the lipid extract, up to 1mg, was dried

down to a small volume under nitrogen and placed, using a microsyringe, in the corner of the plate. The plate was first developed in a solvent mixture which contained the following: chloroform(65vol.), methanol(30vol.), and 28%ammonia(4vol.). The plate was removed and air-dried, then either i) Heated in an oven at 100^{o}C for 5minutes (for the determination of phospholipid class)or ii)Dried in a flow of dry nitrogen, for at least 2 hours, (for fractions used to determine the fatty-acid content). After drying the plate was turned through 90 degrees to the first running position, and then run in a second solvent mixture containing : chloroform(135vol.), methanol(65vol.), glacial acetic acid(18vol.),and distilled water(3vol.). The plate was then air-dried and the phospholipid fractions located and identified.

7. Location and identification of separated phospholipids

Initially the phospholipid classes were identified in two ways: i)Comparison with standards.

Comparable chromatography using known phospholipid standards was carried out.

ii)Use of specific, destructive chemical tests.

Free amino group: PE.

Ninhydrin spray reagent, obtained from Sigma Chemical Co., was applied and the plate heated for 5 minutes at

105°C. Lipids containing the amino group showed up as a distinct red-violet on a pink background.

Choline containing lipids: PC,LysoPC and Sph.

Solution(i) 1.7g of bismuth subnitrate III in 100ml of 20% glacial acetic acid

Solution(ii) 40g of KI in 100ml of water.

The spray reagent was formed by mixing 20ml of solution(i) and 5ml of (ii) with 70ml of distilled water, to form the so called 'Dragendorff's reagent. Choline containing lipids appeared as orange-red spots immediately after spraying , or on mild warming (Wagner et al. 1961).

After this initial identification procedure, the two following locating methods were used for routine purposes.

a)For quantitative assessment of individual phospholipid classes iodine was used, then removed by evaporation in a moving air stream.

b)For fractions used to characterize their fatty-acids, the plates were lightly sprayed with 0.1%solution of A.N.S.reagent, and identified with a U.V. lamp, using a 350nm filter. The phospholipids appeared as bright fluorescent spots against a dark background.

8. Assay of phospholipid fractions from T.L.C.

Identified phospholipid fractions were removed from the plate using a vacuum line, attached at one end to a pasteur pipette which was tightly packed with a glass wool plug. The lipids were eluted using a chloroform:methanol mixture(1:9). The eluant was dried down under nitrogen, and the phospholipid was determined by the method described earlier.

9. G.L.C. of fatty-acids derived from separated phospholipid classes

a)Sample preparation

Spots were scraped off the thin-layer plates and placed in a small bijou with a Tuf-Bond teflon seal in the the screw cap. The methyl esters of the fatty -acids were formed by adding sufficient boron triflouridmethanol reagent to just wet the sample; the bottle was sealed tightly, and heated to 100°C for 15 minutes. The bottle was then cooled, the volatile contaminates allowed to evaporate in a stream of air, and the fatty-methyl esters extracted by washing with hexane, and filtering the resulting suspension through a glass wool-plugged pasteur pipette -which had been previously washed in the solvent. The sample was then dried down, under nitrogen, to a minimum volume, and injected onto the column.

b)Chromatography

Gas-liquid chromatography was carried out on a Pye Unicam 104series chromatogram. The columns used were 2.1m long, with a 2mm internal diameter, and were also obtained from Pye Unicam. They were packed with a cyano-silicone stationary phase, 10% Alltech CS-5, on a chromasorb WAW 100-120mesh support. The columns were run isothermally at 210^{o}C, with nitrogen as the carrier gas. The carrier gas flow rate was 20ml/minute. Peaks were identified by comparison of their retention times with known standards. When standards were unavailable, peaks were identified using the semi-log extrapolation procedure of Ackman, (1963).

Quantitative determination of the fatty-acid methyl esters was obtained using a Trivector Scientific Ltd. Trilab 2(with graphics) computer integrator, which was linked to the chromatogram. The integrator included a sophisticated programme which was used to calculate peak area. The results were expressed as the relative percentage weight contribution of individual fatty-acids as compared to total fatty acid weight.

RESULTS

1. Phospholipid Classes

Table 31 shows the distribution of plasma membrane phospholipid classes in the two tumours and liver, expressed as percentage composition. The pattern in D23 tumour plasma membranes and those from its source tissue, liver, are similar ie. Phosphatidylcholine (PC) is the most abundant phospholipid present and all the classes found in liver membranes are also present in the tumour membranes. However the levels of Phosphatidylethanolamine (PE), Phosphatidylserine/-inositol (PS/PI), and sphingomyelin (SM) are lower in tumour membranes, whereas the levels of PC and lysoPC are higher. The Mc7 tumour membranes display a different pattern of phospholipid classes. The biggest difference between liver/D23 and the Mc7 membranes is the balance between PC and PE, each being present in roughly equal proportions in the Mc7 membranes. No lysoPC was detected, and the level of SM was the same as the liver, greater than the D23.

2. Phospholipid fatty acid composition

Tables 3.2-3.4 show the fatty acid composition, class by class, of the plasma membrane phospholipids from liver, D23 and Mc7 tumours, respectively. Each phospholipid class will be examined separately, and any differences in fatty acid composition between the liver and two tumour membranes noted. At the end of this description the fatty acid composition of the total phospholipids will be examined in order to pick out the overall changes in fatty acid composition. (The notation used to describe the

TABLE 3.1

Phospholipid classes of liver and tumour plasma membranes

	% wt.				
Class	PC	PE	SPH	PS/PI	Lyso-PC
Tissue					
LIVER	43.1±2.0	25.6±0.3	13.8±1.8	15.4±2.4	2.7±2.0
Lit. Values B	46.1	24.7	16.8	10.5	0.5
Lit. Values R	34.9	18.5	17.7	16.3	3.3
D23	51.9±8.7	17.2±3.1	8.3±1.3	11.7±2.8	9.6±1.9
Mc7	34.6±7.2	35.8±3.8	13.5±1.3	11.4±0.7	nd.

n=3 values ±S.E.M.

PC = Phosphatidylcholine

PE = Phosphatidylethanolamine

SM = Sphingomyelin

PS/PI = Phosphatidylserine/
Phosphatidylinositol

Literature References

B= Bergelson et al. (1970)

R= Ray, (1970)

TABLE 3.2

Fatty acid composition of main phospholipid classes from liver plasma membranes

relative % weight

Fatty acid	Phosphatidyl ethanolamine	Phosphatidyl choline	Sphingomyelin	Phosphatidyl serine/inositol
16:0	25.3±2.8	18.4±1.9	15.8±7.35	26.2±13.8
16:1	7.2±3.3	7.7±0.9	9.4±5.5	8.8±3.9
18:0	18.1±2.8	20.3±0.8	14.8±0.4	27.4±12.5
18:1	6.2±0.8	11.4±0.4	9.8±0.8	6.2±1.3
18:2	12.1±0.7	14.1±1.3	12.2±0.7	5.9±1.2
18:3	2.0±1.5	1.6±1.0	3.9±0.5	0.4±0.4
20:0	0.9±0.7	0.2±0.1	0.45±0.05	-
20:1	-	-	-	-
20:2	-	-	-	-
20:3	-	-	-	-
20:4	18.6±5.1	19.3±0.00	13.4±2.8	15.7±8.3
20:5	-	-	-	-
22:0	-	-	1.6±0.1	-
22:1	-	-	-	-
22:2	-	-	-	-
22:3	-	-	-	-
22:4	-	-	-	-
22:5	-	-	-	-
22:6	4.5±4.5	4.5±0.2	-	2.4±2.4
24:0	-	-	18.9+13.2	-
24:1	-	-	-	-

n=2 values ± S.E.M.

TABLE 3.3

Fatty acid composition of main phospholipid classes from from D23 tumour plasma membranes

relative % weight

Fatty acid	Phosphatidyl ethanolamine	Phosphatidyl choline	Sphingomyelin	Phosphatidyl serine/inositol
16:0	12.2±4.7	19.8±3.8	7.6±3.2	11.9±4.8
16:1	12.1±2.1	0.6±0.6	8.0±1.3	4.3±1.9
18:0	16.0±6.2	10.1±0.2	8.1±3.2	11.3±2.8
18:1	13.1±3.9	15.4±1.5	17.5±2.4	11.5±3.2
18:2	13.2±2.7	13.4±2.2	11.5±0.05	9.4±3.2
18:3	0.1±0.1	-	-	0.2±0.2
20:0	11.0±1.1	8.8±2.9	12.0±0.5	8.5±0.9
20:1	trace	-	-	-
20:2	0.6±0.5	0.8±0.6	-	-
20:3	7.2±1.5	5.4±2.7	5.8±0.6	5.3±1.5
20:4	9.3±5.3	5.9±1.5	7.2±0.9	7.0±1.1
20:5	-	-	trace	trace
22:0	2.1±2.1	4.3±2.0	5.0±0.8	7.9±2.1
22:1	-	trace	-	trace
22:2	-	trace	trace	-
22:3	1.9±1.9	2.9±1.5	-	10.6±6.3
22:4	-	-	-	1.2±1.2
22:5	-	-	-	-
22:6	2.7±2.7	4.7±4.7	-	2.1±1.4
24:0	-	-	2.5±2.5	4.4±0.8
24:1	-	-	-	-

n=3 values ± S.E.M.

TABLE 3.4

Fatty acid composition of main phospholipid classes from Mc7 tumour plasma membranes

relative % weight

Fatty acid	Phosphatidyl ethanolamine	Phosphatidyl choline	Sphingomyelin	Phosphatidyl serine/inositol
16:0	17.2±2.7	22.2±5.8	19.0±3.3	13.7±2.1
16:1	10.4±4.6	5.8±2.6	14.0±7.1	8.3±4.2
18:0	19.5±4.2	9.5±1.8	8.8±4.9	14.7±7.1
18:1	17.7±2.7	18.5±6.3	7.8±3.0	8.8±1.9
18:2	8.2±2.2	8.0±3.4	7.9±2.7	5.7±2.6
18:3	1.2±0.6	0.7±0.3	1.5±0.8	1.6±1.1
20:0	2.5±0.9	0.5±0.5	1.8±1.8	6.2±1.9
20:1	0.5±0.5	-	-	0.1±0.1
20:2	-	-	-	-
20:3	1.2±0.3	-	-	0.9±0.9
20:4	4.7±2.0	8.4±5.9	4.5±1.6	5.4±2.3
20:5	-	-	-	8.3±4.1
22:0	3.3±2.5	-	8.8±4.4	0.7±0.7
22:1	-	3.6±3.6	-	trace
22:2	-	-	-	-
22:3	-	-	1.8±1.8	-
22:4	-	0.6±0.3	-	-
22:5	-	-	-	-
22:6	0.9±0.5	11.1±10.3	-	16.6±13.8
24:0	0.4±0.3	-	15.6±14.7	-
24:1	-	-	-	-

n=3 values ± S.E.M.

individual fatty acids refers to the carbon chain length and the number of unsaturated bonds, ie. 18:1 refers to a fatty acid with a carbon chain length of 18 having one unsaturated bond.)

In the PE of liver plasma membranes the predominant fatty acids are 16:0, 18:0, 18:2, and 20:4. These fatty acids are also important in the two tumour plasma membrane phospholipids, though their relative contributions are different. The level of 16:0 is lower in the PE of both tumour membranes, but not significantly so. Conversely the level of 16:1 was higher in the phospholipids from the tumour membranes than it was in that of the liver. In the C-18 fatty acids there was no significant difference in the levels of 18:0 and 18:2, but the 18:1 fatty acid was elevated in the PE from the tumour plasma membranes, as compared to the same phospholipid from the liver plasma membranes. This elevation was statistically significant in the case of the Mc7 fatty acid ($p<0.05$). In the C-20 fatty acids the level of 20:4 was lower in both the Mc7 and D23 PE when compared to the phospholipid from the liver, but 20:3, which was undetected in the liver appeared in both the tumour phospholipids. Another fatty acid which was undetected in the liver but appeared in both the tumour membrane phospholipids was 22:0. The level of 22:6 was similar in all three membrane types. In addition a small amount of 22:3 was detected in the D23, but not the Mc7 and liver PE.

The liver plasma membrane PC has roughly equal proportions of 16:0, 18:0, and 20:4. These three fatty acids make up 60% of the total fatty acids. A further 25% of the total fatty acids are 18:1 and 18:2, these are present in equal amounts. The proportion of the remaining fatty acids (eg. 16:1 and 18:2) is small. There is no difference in the level of 16:0 in the PC of the liver and the two tumour plasma membranes, but the proportion of 16:1 in the D23, is significantly lower ($p<0.01$), than either the liver or Mc7 phospholipid. In the C-18 fatty acids the level of 18:0 is significantly lower in the PC fraction of both tumour plasma membranes ($p<0.002$, for the D23 and $p<0.02$, for the Mc7), compared to the liver plasma membrane phospholipid. Conversely, the level of 18:1 is higher (though not significantly so) in the PC from the two tumour membranes, as compared to liver membrane PC. Thus there is no overall change in the proportion of C-18 fatty acids between the liver and tumour membrane PC, but there is an elevation of the mono-unstaturated fatty acid at the expense of the fully saturated fatty acid, when comparing the tumour membrane phospholipid to the same lipid from liver membranes. There is no difference in the proportion of C-20 fatty acids between the liver plasma membrane PC and the D23 tumour plasma membrane PC, but there is less of this fatty acid chain length (compared to liver PC) in the PC from the Mc7 tumour plasma membrane. Although the overall level of 20 carbon atom fatty acids remains unchanged in the D23 tumour membrane phospholipid, there is an elevation in the level of 20:0 and 20:3 fatty acids with

a corresponding lowering of the level of 20:4 type. The Mc7 PC has no 20:3 unlike the D23 phospholipid, and the level of 20:0 is similar to the liver PC. There is, however, less 20:4 in the Mc7 membrane phospholipid than in liver membrane phospholipid. In this respect the two tumour plasma membranes display similar patterns. The level of 22:6 in the PC is similar in the liver, D23 and Mc7 plasma membranes, though the level of this fatty acid is very variable in the Mc7 phospholipid.

The predominant fatty acids in SM from liver plasma membranes are 16:0, 18:0, 18:2, 20:4 and 24:0, though the level of the last fatty acid listed is very variable. The level of 16:0 in the D23 plasma membrane phospholipid is lower than in the liver plasma membrane phospholipid. The level of the Mc7 membrane fatty acid is the same as it is in the liver membrane. The level of the 16:1, 18:0 and 18:2 fatty acids is also similar in the SM of all three plasma membrane types, but there is considerable variation in these values. The level of 18:1 in the D23 membrane phospholipid is significantly higher ($p<0.05$) than the level of this fatty acid in the liver membrane phospholipid. The level of this fatty acid in the SM from Mc7 tumour membranes was, however, the same as the SM from liver membranes. The differences in the C-20 fatty acids between the liver plasma membrane SM and those for the D23 and Mc7 tumour plasma membrane SM were similar to those already reported in the case of the PE and PC phospholipids. That is there was a lowering of the level

of 20:4 in the SM of both tumour membrane types and the appearance of 20:3 in the D23, but not the Mc7 phospholipid when compared to the SM of liver membranes. The level of 20:0 was significantly higher ($p<0.001$) in the SM of the D23 tumour plasma membranes than the level in the SM of liver and Mc7 tumour plasma membranes. The only other difference in fatty acid composition between liver and tumour plasma membrane SM was the lowered level of 24:0 in the D23 phospholipid, though, because of the variability of the data, this was not statistically significant.

The predominant fatty acids in the PS/PI liver plasma membrane phospholipids were 16:0 and 18:0, which were present in roughly equal proportions, and accounted for 50% of the total phospholipid fatty acid. The 20:4 was also fairly abundant and there was a significant level of 16:1, 18:1 and 18:2 present. In the PS/PI from the plasma membranes of the two tumours there were two major differences in the fatty acid profile of these membranes compared to those of the liver. Firstly the level of 16:0 and 18:0 fatty acids from the tumour phospholipids were lower than those from the liver, and secondly the level of 20:4 was lower in the tumour phospholipids than it was in those from the liver. As has been reported in the other three phospholipid classes, 20:3 was present in the D23 PS/PI phospholipids, but not in the same lipid from the liver . It was present in the Mc7 phospholipids, but only in trace amounts. The only other difference of note was that the proportion of 22:6 was higher in the Mc7 PS/PI

phospholipids than in either the D23 or liver PS/PI phospholipids.

Most of the differences between tumour and liver fatty acid profiles of the phospholipid classes presented in Tables 3.2-3.4 were not statistically significant, any which were have been noted in the text. In order to resolve any broad differences between normal and tumour plasma membranes in their fatty acid composition, and whether both tumours show similar differences, the data from the total phospholipid fatty acid composition is presented in barchart form in Figs. 3.1 & 3.2. The total phospholipid composition was not determined separately, and so these figures represent weighted averages produced from the product of the contribution of any given fatty acid, weighted according to the abundance of the particular phospholipid it was derived from. Obviously this is not an ideal method, but it does give a better idea of the type of differences in fatty acid composition and summarizes the results described in more detail in the preceding section. The figures correspond to the relative %age weight plotted against i) carbon chain length and ii) degree of fatty acid unsaturation.

Figure 3.1 indicates that the proportion of C-16 and C-18 chain lengths is about the same in all three plasma membranes types, although C-18 is 10-15% lower in the Mc7 phospholipids. There is also a decrease in C-20 in this tumour compared with the two other types. There is an

Fig.3.1 Fatty acid chain length of total phospholipids

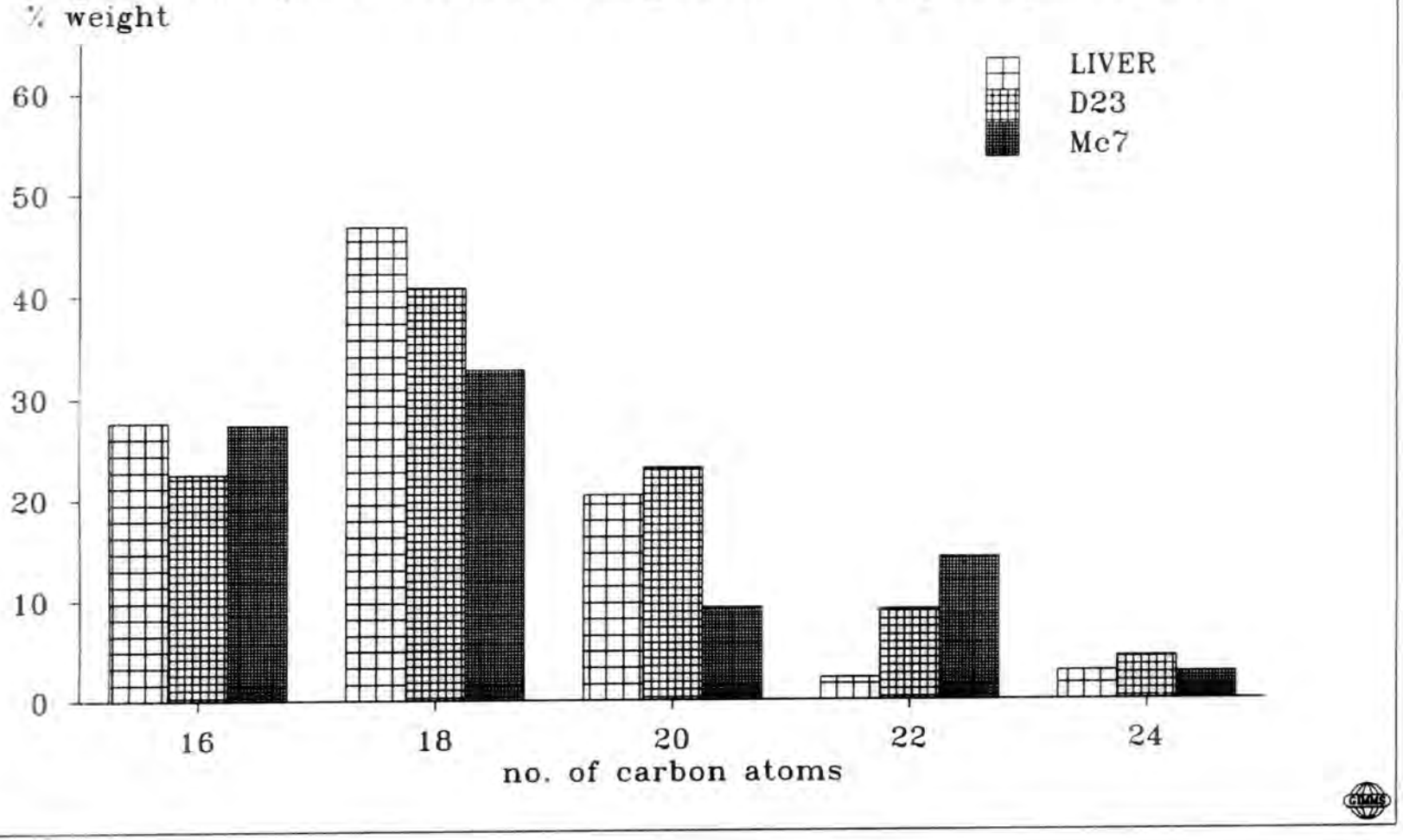
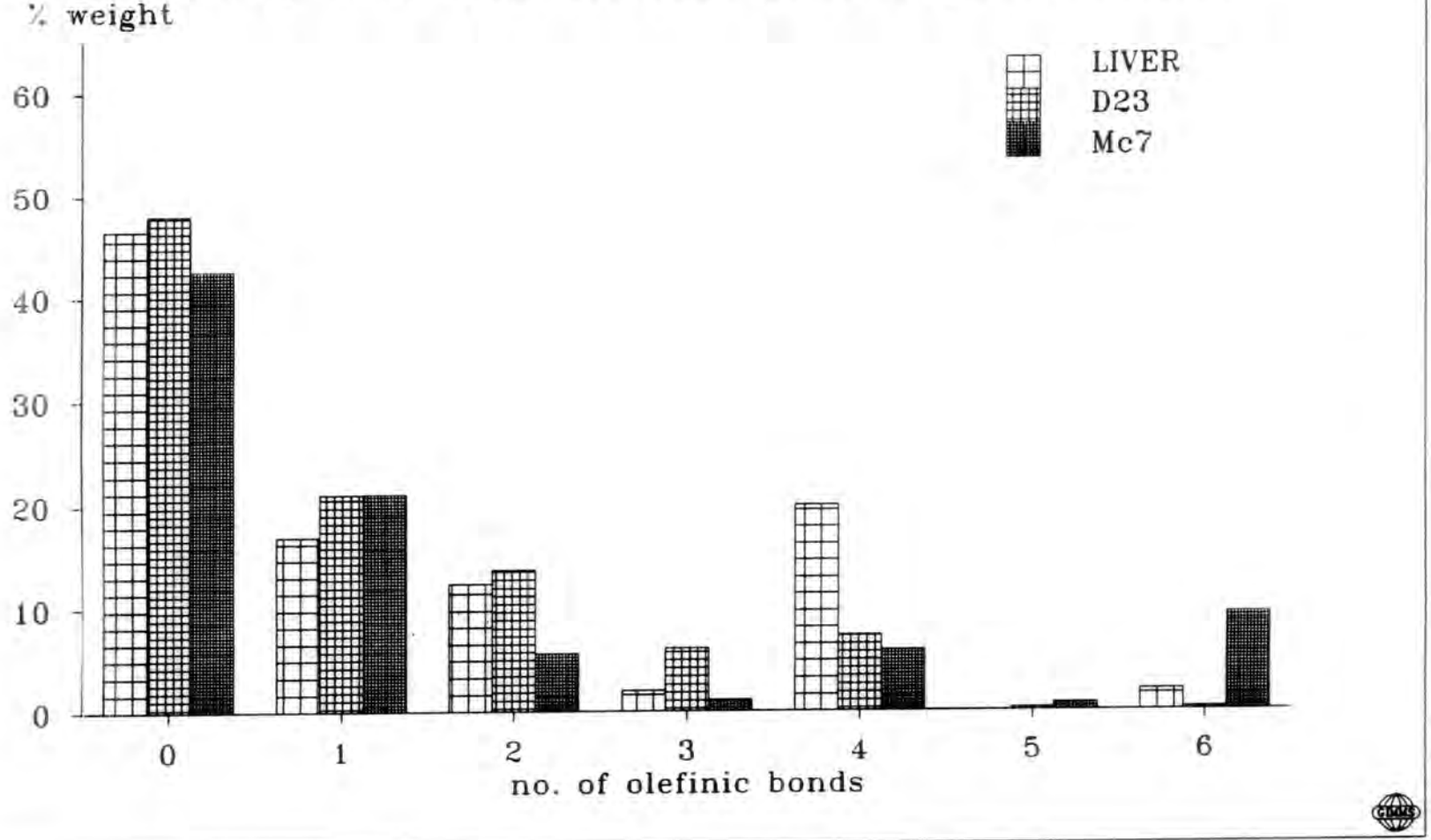


Fig.3.2 Fatty acid unsaturation of total phospholipids



increase in the level of C-22 chain length fatty acids in both tumours compared to liver.

Figure 3.2 shows the degree of unsaturation of the fatty acids. The percentage of unsaturated fatty acids in the phospholipids of all three membrane types is similar, and lies between 43%(Mc7) and 48%(D23). The main difference is the type of unsaturation. Both tumours have greater levels of mono-unsaturated fatty acids than liver, this difference is small, but it is the result of quite a large change in the level of 18:1. The other difference change common to both tumours is a decrease in the levels of arachadonic acid (20:4). Other differences are not reflected in both tumours, for example: the level of di-unsaturated fatty acid in the Mc7 phospholipids is lower than in the liver phospholipids, but the level of this fatty acid type is the same in liver and D23 phospholipids. The percentage of tri-unsaturated fatty acids is higher in the D23 phospholipids, compared to those from the liver, but this is not so for the other tumour (Mc7) phospholipids, as the tri-unstaturated fatty acid levels are similar to those from the liver.

3. Cholesterol/phospholipid ratios

Table 3.5 shows the plasma membrane cholesterol content of liver (including literature values) and the two tumours, both in terms of cholesterol/mg of protein and cholesterol/phospholipid molar ratios. The phospholipid

TABLE 3.5

Cholesterol/Phospholipid molar ratios for liver and tumour plasma membranes

Membrane type	Cholesterol/ protein umoles/mg	Phospholipid/ protein umoles lipid P /mg	Cholesterol/ phospholipid Molar ratio
LIVER n=3	0.24±0.03	0.46±0.012	0.51±0.055
Lit. values	0.13-0.56	0.50-0.61	0.74-0.91
D23 n=7	0.13±0.03	0.39±0.035	0.30±0.038
Mc7 n=7	0.09+0.013	0.41+0.088	0.28+0.049

values ± S.E.M.

content/mg of protein is also displayed.

It should be first noted that the values for liver cholesterol levels presented here are slightly lower than the range of cholesterol levels in the literature, both in terms of cholesterol/mg of protein, and cholesterol/ phospholipid ratios. The values for the D23 and Mc7 tumours are not significantly different from each other, though it must be pointed out that the levels did change during the 2 year course of the study. At the beginning of the study the Mc7 had slightly higher cholesterol levels than the D23, at the end this was reversed. These changes however, in no way detract from the most important trend in the table; that is the large and significant decrease in the levels of plasma membrane cholesterol in both tumours compared to liver ($p<0.05$ for D23 and Mc7). The levels of phospholipid do not change compared to the liver, and illustrate the fact that the lower molar ratios are due to a lowered level of cholesterol alone.

DISCUSSION

A detailed lipid characterization has not previously been carried out on the plasma membranes of either of the two tumours studied, so it is difficult to compare the results presented here. However the results obtained for liver membranes can be compared with literature values. If good agreement between these data is obtained, this is considered to allow any differences demonstrated in tumour

membrane lipid profiles, when compared to the normal tissue (liver), to be related to the nature of the tumour alone.

The phospholipid classes, shown in table 3.1, demonstrate that there are variations between different literature values, and that the results for the liver plasma membranes presented here are within the range of these values. The variation in these results may reflect procedural or strain differences between laboratories. Another possible source of variation could be that regional differences in function and compostion have been noted in plasma membranes (eg. Schroeder, 1983). These could be an important source of inter laboratory variation in data if differences in technique isolate different regions of the plasma membrane. It is possible to compare directly hepatoma cell membranes with liver cell membranes, owing to the liver origin of the D23 tumour. The study of the Mc7 sarcoma membranes provides an opportunity to try and identify features of membrane lipid composition that might be a common feature of neoplasia. D23 plasma membranes have a slightly higher PC content but a lower PE content than liver but, owing to the variability between separate isolations, the observed differences may not be significant. Other workers have noted higher levels of PE and lower levels of PC in hepatoma plasma membranes, as compared to the same type of membrane from liver, eg. Upreti et al. (1983) and Kiozumi et al. (1976). An increase in the PE/PC ratio was also noted by van Hoeven & Emmelot, (1973) in both rat and mouse hepatoma plasma

membranes ,as compared with liver plasma membranes from the same animals. Such changes were observed in the Mc7 sarcoma, but the comparison with liver is not really valid.

The SM content has been reported to be elevated in some tumours (Koizumi et al. (1976); Upreti et al. (1983); and van Hoeven & Emmelot, (1973) but lower in leukaemic cells (Van Blitterswijk et al. 1982) and lymphomas (Koizumi et al. 1981). These latter reports agree with the present data for D23 membranes showing a reduced level over the liver membranes. Changes in sphingomyelin levels in the membranes of other organisms have tended to parallel changes in cholesterol levels (Patton, 1970). This may be due to the stable complex formed by the association of these two molecules (Vandenheuvel, 1963) and also the high affinity the sphingomyelin has for cholesterol (Demel et al. 1977) The Mc7 membranes do have similar sphingomyelin levels to the liver, although levels of PE and PC are very different from this tissue, and the hepatoma derived from it.

The high level of lysoPC observed in the D23 membranes could be due to phospholipase action, as it is reported that isolated membranes are far more vulnerable to this type of degradation.(Ibrahim & Thompson, 1965, and Zwaal et al. 1970) Some lysoPC was observed in liver membranes but was not detected in Mc7 membranes. The high level of this phospholipid in D23 membranes and its presence in liver membranes may reflect the common tissue source. However in

solid tumours some necrotic tissue is often present, and although care was taken to exclude it in samples it is possible some contamination may have occurred. It may be significant that Mc7 was less necrotic throughout than D23, and this may have contributed to the difference in LysoPC levels in the two tumour membranes.

What role do these changes in phospholipid class have in membrane structure? This is a difficult question to answer as the physical properties of the phospholipid are not just dependent on the type of head group, but also the fatty acyl chain composition. For example the enhanced rigidity conferred by sphingomyelin molecule is largely the result of its relatively more saturated acyl chain compostion, which results in a decreased molecular area (Jain & Wagner, 1980). In considering differences in phospholipid class with relation to structure, the asymmetric distribution of phospholipids in the two leaflets of the bilayer must be taken into account (Bretscher, 1972). PC and SM are principally concentrated in the outer leaflet, where as PE , PS and PI are in the inner one. Emmelot, 1977) has suggested that increased levels of PE help to stabilize the inner leaflet of the bilayer, and Boyle & Dean, (1982) have shown that increased microviscosity correlates with decreases in the PC/PE ratio, although it must be noted that they did not look to see if there were any changes in the fatty acid composition of these phospholipids. The role of sphingomyelin in membrane stabilization is better established, (Shinitzky &

Barenholz, 1974 and Borochov et al. 1977), and with the difference in the level of this lipid between the D23(8.3%) and Mc7(13.5%) tumour membranes, one might expect that the Mc7 membrane was a more stable structure. However one must also take into account the changes in other lipids like cholesterol, and the levels of unsaturation in the fatty acyl chains of the phospholipids, before such speculation has any credance.

The fatty acid composition of the plasma membrane phospholipids is a most important factor in determining membrane stability, but, as Stubbs & Smith, (1984), recently reported in their review, the relative proportion of fatty acid types do not always give predictable effects in terms of membrane physical properties.

It first must be noted that there are some differences in the level of 16:1 fatty acid between the liver plasma membrane phospholipids presented here compared to the literature values of say, Ray, (1970). (That is 8% compared to 4.5%, respectively). It is also noted that some groups, eg. Upreti et al. (1983), did not find any 16:1 at all. This may be due to dietary variations, since these can considerably affect the fatty acid profile of any given cell membrane. Another possibility is that the G.L.C. peak is not a fatty acid, but some contamination. This could only be resolved by undertaking a mass-spectroscopic analysis of the G.L.C. separated peaks, and was not attempted in this study.

Another anomaly is the liver membrane SM fatty acid composition in the present analysis, as compared to literature values (eg. Keenan & Morre, 1970). They differ particularly with reference to 16:1, 18:1, 18:2, and 24:0. The 16:1 does not occur in the data presented in the literature, and 18:1 and 18:2 are only present in trace amounts in the literature, whereas in the current data they each contribute about 10% of the total fatty acid composition. Conversely, the level of 24:0 in the current data is lower than that quoted in the literature, though there is considerable variation in the percentage contribution of this fatty acid. This may be a result of the methylation step used in this study, which was obtained from Lewis, (1978). This procedure involved the methylation of all phospholipid classes at 100°C for 15 minutes. However it is reported in the review Stein and Smith, (1982), that sphingomyelin requires a much longer reaction time than other phospholipids (ie. 90 minutes) This difference in methodology may account for the discrepancies sphingomyelin, both in liver, and presumably in the tumour phospholipids as well.

Notwithstanding the elevation of 16:1 fatty acyl chains in the liver phospholipids as compared with the literature, there is a small elevation, in all the tumour phospholipid fractions, of this fatty acid, a greater elevation of 18:1, and some elevation of 18:2 when comparing tumour membrane phospholipid fatty acid profiles with those from liver membrane phospholipids. The

percentage of polyunsaturated fatty acids, particularly 20:4, is lower in the tumour membranes as compared with liver membranes. This elevation of mono- and di-unsaturated fatty acids, together with the lower level of polyunsaturated fatty acids, in tumour membrane phospholipids compared to normal membrane phospholipids has been widely reported in the plasma membranes of hepatomas and other tumours. Eg. Upreti et al. (1983) and Van Hoeven & Emmelot, (1973) -hepatomas; Schroeder & Gardiner, (1984), in a melanoma; Van Blitterswijk et al. (1982), in leukaemic G.R.S.L. cells. Upreti et al. (1983), in their study of liver and hepatoma lipids reported that this was true in nearly all subcellular fractions, and Hartz et al. (1982) again reported similar changes in the mitochondrial and microsomal fractions of another hepatoma. Lipid extracts of whole tumours have also revealed elevations of 16:1, 18:1 and 18:2 and decreases in polyunsaturated fatty acids like 20:4. For example Araki et al. (1974) in human hepatomas, Bergelson et al. (1973) in rat hepatomas, Daniel et al. (1980) in cultured transformed cells, derived from fibroblasts, Ruggieri et al. (1979) in revertant cells, and Yau & Weber, (1972) in transformed chicken embryonic fibroblasts. It is important to note that this change in the proportion of mono- and di-unsaturated to polyunstaturated fatty acids has also been reported in some proliferating normal cells. eg, regenerating rat liver, Gershbein & Singh, (1970) and Glende & Morgan (1968), and in foetal liver Jahn et al. (1967), and Luit et al. (1975). Thus this may not be a

phenomenon of neoplasia, but of rapidly dividing cells. Hartz et al. (1982), has suggested several explanations for these fatty acid changes:

i)The dominance of de novo synthesis, with decreased reacylation could explain increases in 16:1, 18:1, 18:2, and the decreased level of 20:4.

ii)Changes in the phosphatide fatty acyl pool, available for of incorporation fatty acids into phospholipids, due to an increased or decreased fatty acyl CoA precurser observed in some tumours.

iii)Mobilization of depot fat. Depot fat and hepatoma phosphatides show many similarities eg. increased 16:1, 18:1, and decreased 20:4, and therefore in its rapidly dividing state the tumour uses this fat. (This could correlate with the high density of lipid deposits found in the tumours cells used in this current study)

Hartz et al, (1982) related these changes to the increased growth rates of the hepatomas in their study. The transplanted tumours used in this present study are rapidly growing ones, and would agree with this explanation.

Consequently in view of the differences in fatty acid composition reported to exist between these membranes, the question arises as to how such differences might affect

membrane function? A primary aim of this study is in the correlation of membrane structure (chemical and physical) with the sensitivity of many tumour cells to hyperthermia and membrane perturbants. Unfortunately, as Stubbs and Smith , (1984), pointed out in their recent review, changes in fatty acid composition do not always affect the membrane structure in a predictable fashion. It was thought, for instance, that the increased unsaturation of the fatty acids, due to the greater space their molecular structure occupied, would increase the fluidity of the membrane. Therefore the increase in 18:1, would not compensate for the equivalent decrease in 20:4. However, as indicated in model systems the first double bond introduced into a fatty acid causes the greatest effect on the bilayer, the second, not so great an effect, and further unsaturation probably very little change in fluidity takes place, (Van Deenen et al. 1971). In biological systems the first double bond is normally introduced towards the centre of the acyl chain, and the second close to it, then the third and fourth etc. progressively to the opposite end of the chain, Stubbs & Smith, (1984). Obviously changes in configuration will have a greater effect at the middle, than at the 'tail' end of the chain, due to the molecular dynamics involved. It is also interesting to note, from the point of view of tumour susceptibility to hyperthermia, that Boonstra et al. (1982), showed that the supplementation of oleic(18:1) or linoleic(18:2) into the media of neuroblastoma cells increased permeablility, whilst increases in stearic acid(18:0) had no effect. Increased permeability is one of

the postulated primary events in the heat death of cells by a membrane related mechanism (Bowler, 1981). Another unexpected perculiarity, reported by Bergelson & Dyatlovitskaya, (1973), is that archidonyl phospholipids (20:4) produce more stable membranes than saturated species, due to their ability to bind to proteins more strongly than the latter.

One important consideration, which has received comparatively little attention, is the positioning of different fatty acids in the 1 and 2 positions on the phospholipid molecule. This, in fact, has a very important role to play in the stabilty of membrane structure. For example: 18:2/18:0 gives a more expanded monolayer than 18:0/20:4 does.(Demel et al, 1972). Bergelson & Dyatlovitskaya, (1973) reported in their examination of tumour phosphatidyl choline, that there was an increase in the less common fatty acid pairing, ie. 2 unsaturated molecules on the same phospholipid molecule. These structures would produce very leaky membranes which might explain the nature of tumour heat sensitivity. Therefore an increase in mono-unsaturated fatty acids might be expected to be compensated by a decrease in say 20:4 fatty acids. In terms of change to membrane physical structure, there are more subtle changes in phospholipid fatty acid structure, not considered in this present work, which might be more significant in tumour membrane thermal stablility. It must be remembered that other lipids also play an important role in determining the stability of the plasma

membrane structure, particularly cholesterol. Thus the differences observed between membranes in their fatty acid profiles, in chain length and degree of unsaturation cannot be easily equated with specific membrane properties.

Before considering the relative levels of cholesterol between liver and tumour membranes it is important to ask why the results presented here for the liver membrane cholesterol are lower than those in the literature. A number of possible explanations exist: i)The contamination noted in Chapter 2 might significantly affect the lipid composition of the plasma membrane fraction, particularly reducing cholesterol level. This is because the membranes from other sub-cellular organelles have significantly less cholesterol in them than the plasma membrane does. This is a possibility, but the phospholipid class analysis (Table 3.1) did not indicate obvious contamination by mitochondria for example. The more likely explanation was given by Johnson, (1979) ii) She showed that many of the older chemical methods of assaying for cholesterol were subject to interferences from the oxidation of other lipids, notably phospholipids giving cholesterol to phospholipid ratios up to 30% higher than those obtained by the more sensitive enzymatic method used in this present study. This could then easily account for the differences between literature values and the ones given here. An additional complexity is that the lipids of liver plasma membranes have been shown to exist in domains (eg. Schroeder, 1983), and different isolation procedures could favour the

enrichment of domains of different cholesterol content. This would explain the great variation in the literature values of plasma membrane cholesterol for liver.

In Table 3.1 both tumour membranes have considerably less cholesterol in their plasma membranes than those of liver. For the D23 hepatoma this is unexpected, as generally speaking the literature reports elevated cholesterol levels in hepatoma plasma membranes. For example: Van Hoeven & Emmelot, (1973) reported raised plasma membrane cholesterol levels in 5 hepatomas from mice and rats, though the magnitude of the elevation was far less in the former. Kiozumi et al. (1976), examined 4 hepatomas and found two with raised cholesterol levels over liver (one considerably higher), one unchanged, but one with decreased levels of this lipid. Therefore the result presented here for the D23 hepatoma do not form a precedent for this tumour type, though it must be noted it is exceptional. In fact elevation in hepatoma cholesterol is so widespread that a number of workers have correlated these changes with the nature of liver malignancy (Chen et al. 1978 and Coleman & Peterson, 1981)

Other tumour types have been reported to have had lowered plasma membrane cholesterol compared to normal tissue. eg. Kiozumi et al. (1982) in lymphoma cells and Van Blitterswijk et al. (1982) in leukaemic cells. Indeed lowered cholesterol levels together with the changed phospholipid and fatty acid pattern observed in the D23 and

Mc7 tumours have been interpreted by some as a measure of metastatic potency, Schroeder & Gardiner, (1984). However the two tumours in this study were not known to metastase.

The lowered cholesterol levels may well be a function of rapidly dividing cells (as noted previously by Hartz et al.1982). Foetal, newborn and regenerating liver are all known to have considerably lowered cholesterol/ phospholipid ratios than the adult rat liver (Kiozumi et al. 1976). Thus the differences in lipid composition need not be attributable to a direct expression of the cancerous state, but rather the rapidity of cell division in these tumour cells.

One other important observation made recently by Fisher et al. (1984) has an important bearing on this work. They noted that decreased levels of membrane cholesterol make PC considerably more susceptible to phospholipase A2. This may explain the increased levels of lysoPC found in the D23 tumour membranes.

Two important questions remain. i) Does the summation of all the postulated effects of these changes in tumour plasma membrane lipid composition have an expression in the physical stability of the membranes? and ii) Do these changes relate to the differential heat sensitivity of many tumours? The first question will be dealt with in the next chapter when a physical study, based on the fluorescence polarization of the fluorescence probe D.P.H. in normal

cells. If this is the case then one might expect that the tumour cells, particularly hepatomas, which have been reported to have elevated plasma membrane cholesterol (Chen et al. 1978), to be less heat sensitive than normal cells. No work has been attempted which would answer this question, but as Sabine, (1983), has recently suggested, it may be the absolute level of membrane cholesterol which is important for normal membrane functioning. Thus deviations from the norm (either elevation or lowering) might impair membrane function.

4. STEADY STATE FLUORESCENCE POLARIZATION OF D.P.H. IN TUMOUR AND LIVER PLASMA MEMBRANES

INTRODUCTION

The physical properties of plasma, and other membranes, is largely determined by the properties of the fatty acyl chains of the phospholipid bilayer. These acyl chains form the hydrophobic core of the membrane structure (Singer & Nicholson, 1972). There are now a number of different techniques available to investigate the physical state of this hydrophobic core, for example: E.S.R., N.M.R., and various fluorescence techniques, steady state fluorescence polarization being the most popular. These techniques have been reviewed recently and their relative merits in determining membrane 'fluidity' dicussed in some detail (Stubbs, 1983). Although all these approaches are said to measure membrane 'fluidity', this is at best an ill-defined term, as the techniques mentioned above often measure very different physical interactions. Fluidity, in terms of the motion of acyl chains, could describe either the orientation (angular range of rotational motion), or the rate of rotational motion (Stubbs, 1983); and the techniques mentioned above may consist of either a rate or range component, or indeed both. Steady state fluorescence polarization, the technique selected in this current study, is now thought to produce information

concerning the 'range' of lipid motion, and is therefore principally monitoring lipid order or packing (eg. Pottel et al. 1983).

There are a number of reasons for the popularity of this technique, and hence its use in this study. The instrumentation is cheap and easy to operate, when compared to techniques such as E.S.R. and N.M.R.. Measurements can be performed easily on relatively small amounts of membrane samples, and are very reproducible. (for reviews of the technique see Shinitzky & Barenholz, 1978, and Lee, 1982)

The principal of steady state fluorescence polarization is the insertion of a hydrophobic fluorescent dye deep into the core of the membrane bilayer. The dye molecules are immobilized in the rigid matrix of the membrane, and when excited by polarized light, they emit fluorescence that is polarized parallel to the excitation light. The extent of movement during the nanoseconds of the excited state determines the proportion of light which is depolarized on fluorescence. In the most extreme case (which would not occur in the constraining structure of a membrane) all the light will be depolarized.

The most commonly used probe molecule is 1,6-Diphenyl-1,3,5-hexatriene (D.P.H.). This has a number of advantages over other fluorescent probes, for example: it only fluoresces when it is in the hydrophobic core of the membrane, and not in the surrounding aqueous environment; it has a high extinction coefficient and an absorption maximium at 355nm, well

removed from tryptophan fluorescence. One disadvantage is that it undergoes photo-isomerization, and so exposure to excitation light should only occur just before a measurement is to be taken (Lee, 1982). It has now been shown that lipid domains of varying order exist within many plasma membranes.(eg Gordon et al. 1983; Lentz et al. 1983 and Schroeder, 1983) The polarization value obtained from such membranes will be a weighted average of these different regions, and will not reflect the state of any given region in the membrane. Therefore the main use of this technique is in determining any bulk changes in the hydrophobic core of membranes. In addition it has been reported that D.P.H. partitions equally well in hydrophobic regions which are either rich in lipids or proteins (Stubbs et al. 1976)

Shinitzky and Barenholz, (1978), converted the polarization values, obtained using steady state fluorescence polarization, into 'microviscosity' by comparing the values obtained from biological membranes with the values of the same probe in oil of known viscosity. This, however, has been shown to be inappropriate, as the others have shown that the standard viscosities varied with different hydrocarbons, and the motion of D.P.H. in membranes is anisotropic, whereas it is isotropic in the oil (Hare & Lussan, 1977). More recently, Pottel et al. 1983) have shown that there is an empirical relationship between order parameter and steady state fluorescence anisotropy of D.P.H., thus the need to convert polarization values into relative 'microviscosities' is unnecessary.

Relatively few studies on tumour plasma membranes have been carried out using this technique, (eg. Inbar, 1976; Inbar & Shinitzky, 1974; Van Blitterswijk et al. 1977, and Johnson, 1981), but all these workers found decreased D.P.H. polarization values (lower order) in tumour cells. Van Blitterswijk et al. (1982) correlated the changes in plasma membrane order found in their earlier paper (Van Blitterswijk et al. 1977) with changes in lipid composition (particularly cholesterol levels) in the plasma membranes from the same cell type. It may be that the decreased level of membrane cholesterol found in the D23 and Mc7 tumour plasma membranes described in Chapter 3 (Table 3.5) can also be correlated with a decreased membrane order as determined by D.P.H. fluorescence polarization measurements. It is important to determine the level of membrane order in this study because Lepock, (1982) has indicated that decreased plasma membrane order may be important in determining the susceptibility of tumour cells to hyperthermia.

MATERIALS AND METHODS

1. Membrane preparation and introduction of probe

Tumour and liver membranes were isolated using the methods described in Chapter 2, section 1; and resuspended in 0.1M phosphate buffer, pH7.7. This membrane suspension was added to 2.4ml of the same phosphate buffer in a quartz 10mm fluorescence cuvette, to give an optical density of 0.1 at 500nm, on a dual beam Cecil spectrophotometer. The

probe, D.P.H., was introduced into the membranes as follows: 2μL of a 2mM solution in glass-distilled tetrahydrofuran, was added into the cuvette with rapid mixing. The cuvette was then incubated for 15 minutes at 37°C. Cuvettes were then cooled to about 4°C before the start of a temperature run between 4-50°C. Several cuvettes could be taken through any given run so that the three membrane types had their fluorescence polarization measurements determined in exactly the same conditions. Cuvettes, when read, were placed in a thermostatically controlled chamber; the remaining cuvettes were maintained at about the same temperature, using a separate chamber. This system was maintained within ±0.1°C of the required temperature with a Jubabo thermostated circulator; cuvette temperature was measured with a calibrated thermistor. The thermostated cuvette chamber was constructed to include provision for the gassing of cuvette surfaces with dry nitrogen gas at sub-ambient temperatures, to prevent condensation in a humid atmosphere.

2. Instrumentation and Steady State Fluorescence Polarization measurement

Steady state polarization of D.P.H. fluorescence was measured with an analogue T-format fluorimeter (Applied Photophysic Ltd.), similar to that described by Jameson et al, (1980), but with modified excitation optics, analogue photomultipliers, and radiometric amplifier. The fluorimeter was mounted on a 1/4 inch aluminium optical

bench. The excitation wavelength was 360nm; the excitation path was filtered with a Corning 7-54 broadband pass filter and the emission path with a Corning 3-73 sharp out filter. The photomultiplier voltage was smoothed by continuously averaging the polarization ratio using a BBC microcomputor (model B, Acorn Computers Ltd., Cambridge), connected to an Acorn 12-bit analogue-to-digital converter. The computer was programmed to provide an average of 256 individual conversions which were themselves continuously averaged until a stable value to 3 decimal places was obtained.

Polarization of fluorescence (p) was calculated from the following equation:

$$P = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}}$$

where $I_{\parallel}$ and $I_{\perp}$ are the intensities of fluorescent light detected through polarizers orientated parallel and perpendicular, respectively, to the excitation light.

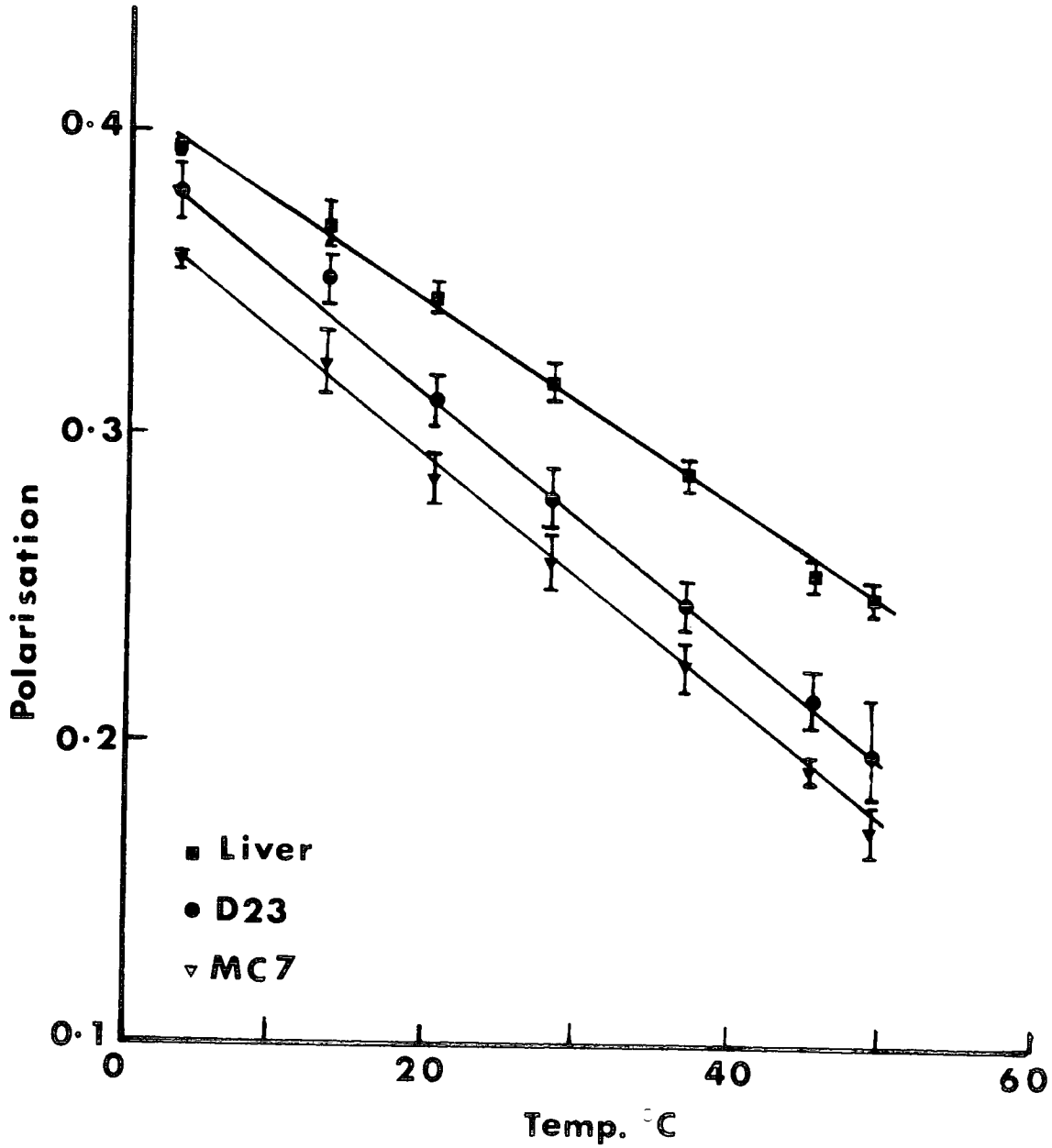
In practice the ratio of $I_{\parallel}$ to $I_{\perp}$ was determined and this was inserted into the the following equation:

$$p = \frac{(I_{\parallel}/I_{\perp})-1}{(I_{\parallel}/I_{\perp})+1}$$

RESULTS & DISCUSSION

The D.P.H. fluorescence polarization of the plasma membranes (Fig. 4.1) indicate that both the D23 and Mc7 tumour plasma membranes were less ordered than those from the liver at all temperatures from 4-50°C, and that this difference increases with increasing temperature. The intercepts of the graphs for the D23 and Mc7 tumour plasma membranes are significantly different from the intercept of the graph for liver membranes, ($p<0.01$ & $p<0.001$, respectively). There is, however, no satistical difference between the slopes of all three membrane types, and no difference between the two tumour plots in terms of either slope or intercept. Therefore it appears that at hyperthermic temperatures, there is a difference in order between the normal and tumour membranes, which could contribute to the differential sensitivity of some tumour cells to hyperthermia.

In a more detailed analysis of the results, the first thing to consider is the greater variation within the polarization values of the tumour membranes compared to those from liver. There are a number of possible explanations. Within a given tissue membrane variation has several components. Experimental and measuring errors ought to be common and so the greater variability in the tumour membranes, as compared to those from the liver, must reflect variability in the tissue source material. This is emphasised by the reported larger variability in tumour


0·4
0·3
0·2
0·1
Polarisation
0
20
40
60
Temp. °C
Liver
D23
MC7

lipid composition (Chapter 3). Solid tumours are heterogenous and contain, for example, host macrophages (Evans, 1972). Serious macrophage contamination was avoided by taking 'healthy' tissue, but clearly a differential macrophage composition of starting tissue could also contribute to the variation observed in Fig. 4.1.

As stated in the introduction little work has been done on the D.P.H. fluorescence polarization of tumour plasma membranes. In the main leukaemic and lymphoma cell types have been studied, and in these cell types the plasma membranes have been found to be less ordered, or more 'fluid' than the normal, non-cancerous ones, Inbar, (1976), Inbar & Shinitzky, (1974), Van Blitterswijk et al. (1977). Johnson, (1981), also found decreases in D.P.H. fluorescence polarization in mouse leukaemic cell lines, but did not observe any decreases in the fluidity of human leukaemias and lymphomas, as suggested by Inbar and Shinitzky, (1974). This difference Johnson attributed to the degree of cell purity. Patients with untreated lymphocytic leukaemias have a large number of lymphocytes in their blood, so there is little contamination from cell types (eg. leukocytes and platelets) and it is possible to obtain a virtually pure lymphocyte preparation. Normal healthy people have a much lower proportion of lymphocytes in their blood so there is proportionally more contamination from other cell types. Thus the membrane preparation obtained from 'normal' lymphocytes by Shinitzky

and coworkers were mixtures and not suitable controls for the membranes obtained from the cancerous cell type.

The question is how to relate this lower order of tumour membranes to the composition of the plasma membrane, particularly the lipids studied in chapter 3.

There is a problem in relating phospholipid head group to membrane order. The problem is that although head group can effect the acyl chains of the bilayer, it is difficult to understand their relative importance, due to the variable fatty acid composition of the chains (Stubbs, 1983). Nonetheless, sphingomyelin is clearly important in membrane physical structure, and is know to stabilize membranes (Shinitzky & Barenholz, 1974 and Borochov et al. 1977). The Mc7 plasma membranes had far more SM than those of the D23, so from the known nature of SM it might be predicted that the D23 plasma membranes should be far more fluid than the those of the Mc7; this is not the case, there is no significant difference between the fluidity of the two tumour plasma membranes. The other changes in phospholipid class were a slight elevation in the PC/PE ratio in the D23 plasma membranes, and a lowering in this ratio in the Mc7 plasma membranes when comparing the tumour membranes with the liver. Boyle and Dean, (1982), reported that lower PC/PE ratios correlated with an increase in microviscosity (or a increase in order) as indicated by in vitro fluorescence polarization. Again it is possible to suggest that the D23 plasma membrane is less ordered than

those of liver because of an increased PC/PE ratio, but one might expect, using similar extrapolation, the Mc7 plasma membranes to have an increased order due to their lower PC/PE ratios, and this is not the case. Again it is difficult to predict changes in membrane order from a consideration of plasma membrane phospholipid class alone. A clearer relationship between lipid content and plasma membrane order is apparent when considering the fatty acid unsaturation of the phospholipids and the levels of membrane cholesterol.

Fatty acid unsaturation is an important means of modifying cell membrane physical properties in many organisms eg., bacteria, plants and fish (Hazel & Prosser, 1974), but is not as important in mammalian plasma membranes. A great deal of work has been done on modifying the fatty acid saturation of plasma membranes enzymatically or by diet. Workers have often been able to produce decreased membrane order when the unsaturation of the membrane phospholipid fatty acids has been increased, particularly when 18:1 and 18:2 levels have been raised.(Review Stubbs & Smith, 1984). These workers have suggested that the changes in fatty acid saturation required to elicit observable changes in membrane order are often extreme, and therefore may not reflect a situation which could ever occur naturally. However, Cossins and Prosser, (1978), in their study of synaptic membranes from a variety of animal species, have shown that membrane fluidity is principally influenced by the fatty acid

composition of the phosphoglycerides. They found a very good correlation between fluorescence polarization and the ratio of saturated/unsaturated fatty acids. It has been suggested by Johnson, (1981) that free fatty acids could be important in determining membrane order, but this was not investigated in the current study.

The relative importance of fatty acid unsaturation has been brought in question by a number of other studies. For example, Owen et al. (1982), showed that the erythocyte plasma membrane in patients with a liver disease had increased cholesterol content and increased fatty acid saturation. When cholesterol levels were normalised by incubation with normal plasma, the order of the membranes, as indicated by D.P.H. fluorescence polarization, returned to normal levels, despite the raised fatty acid saturation. Also in the study of Herring et al. (1980), on the microrganism Dictostelium discoideum, no change in order was seen by D.P.H. fluorescence polarization or the E.S.R. of 5' deoxystearate when fatty acid saturation changed. The relative importance of cholesterol over other membrane lipids in determining the physical state of the plasma membrane in tumour cells has been shown by Van Blitterswijk et al. (1977, 1983) in their studies of leukaemic plasma membranes showed that there was a lowered membrane order (as detected by D.P.H. fluorescence polarization) in cancerous plasma membranes compared to normal membranes. Unfortunately, it was difficult to ascribe this change in order to a single change in membrane structure because

there were several differences in the lipid composition between normal and cancer cells (eg. lower SM , lower cholesterol/phospholipid ratios and elevated levels of phospholipid fatty acid unsaturation). However these workers in the same study looked at the differences in order between plasma membranes and extracellular vesicles (ECVs) shed from them. There was a decrease in order when going from the plasma membranes to the ECVs, but the only change in lipid composition was a decreased phospholipid/cholesterol ratio. Thus the level of membrane cholesterol is again seen as the the most important factor in detemining changes in plasma membrane order in mammalian cells.

The plasma membranes of both tumours had lowered cholesterol levels compared to the equivalent membranes from liver, and this is likely, as argued above, to be the major cause of their lowered order compared to liver. As discussed in Chapter 3, the cellular level of cholesterol is related to the degree of sensitivity of many cell lines to hyperthermia (Cress & Gerner, (1980). But until this study little work has been done to try and correlate the level of plasma membrane cholesterol, the order of this membrane and the sensitivity of the tumour to hyperthermia.

Lepock et al (1983) have shown that there is a lack of correlation between plasma membrane lipid phase transitions and hyperthermia, but as Lepock himself has suggested, (Lepook, 1982) some if not all membranes undergo

irreversible structural transitions after mild hyperthermia (41-44°C), and these are probably due to the denaturation of membrane proteins, leading to the impairment of cell function. Thus the importance of the changes in the lipid composition and order of the D23 and Mc7 tumour plasma membranes in the hyperthermic sensitivity of these tumours to heat may be only fully realized when considering how these factors affect membrane protein thermostability. Therefore it seems crucial to investigate the effect of heat, and other membrane perturbants, on the stability of membrane proteins. This is the aim of the work presented in the next chapter.

It is important to note, as Bowler et al. (1982) has suggested, that such gross structural changes might not been necessary for the onset of hyperthermic cell death as the decreased order of the tumour plasma membranes makes them more fluid, and therefore more permeable at any given temperature, as compared with normal plasma membranes.

SECTION 2. THE EFFECT OF MEMBRANE PERTURBANTS ON TUMOUR PLASMA MEMBRANES

5. THE EFFECT OF TETRACAINE ON TUMOUR AND LIVER PLASMA MEMBRANES AND THE MEMBRANE BOUND Mg2+-ATPase

INTRODUCTION

The first section of this thesis has estabished the characteristics of the plasma membranes from two tumours, the D23 hepatoma and the Mc7 sarcoma; the membrane purity has been assessed, the major lipids characterized in some detail, and the physical state of these membranes determined. The data has confirmed the suggestion by some (eg. Yatvin, 1977, and Dennis & Yatvin, 1981) that the increased susceptibility of many tumour cells to hyperthermia could lie in the differences in lipid composition and decreased membrane order of their plasma membranes. Hyperthermia is known to have a disordering effect on the plasma membranes which may eventually lead to increased membrane permeability and loss of function as indicated by Bowler, (1981), eventually leading to cell death. However there are other membrane perturbants besides heat, including a wide

range of drugs and other chemicals (for recent review see Goldstein, 1984). These membrane perturbants include alcohols, eg. ethanol; and local anaesthetics, eg. phenobarbitol, procaine and tetracaine. The effectiveness of such drugs in enhancing the hyperthermic killing of cells has already been investigated , principally by Yatvin and co-workers (Yatvin, 1977, Yatvin & Dennis, 1978, and Yatvin et al 1982). They have established that tetracaine had the greatest effect in potentiating the hyperthermic killing of bacteria. The interaction of tetracaine with membranes has been extensively studied, both in model systems (eg. Sikaris & Sawyer, 1982, Boulanger et al 1981, and Schreier et al 1984), and also in biological membranes (eg. Ondrias et al 1983, Coakley et al 1983, and Mahler & Singer, 1984). For these reasons this cationic local anaesthetic was selected in the current study to determine the combined effect of hyperthermia and membrane fluidizers on the structure of the plasma membranes from liver and the two tumours, D23 and Mc7.

This study involved two separate sections: a) The effect of tetracaine on the physical properties of the three plasma membranes, and b) A study of the thermo-lability of an integral membrane protein in the three membrane types and how that lability is modified by the introduction of tetracaine, a membrane perturbant.

The first technique, using D.P.H. fluorescence polarization has been described in detail in Chapter 4. Unfortunately, although tetracaine is the best candidate in

terms of the potentiation of hyperthermia, it, in common with other amine based local anaesthetics, is a fluorescence quencher. Fortunately at the pH used in these studies the amount of quenching is small, (Sikaris & Sawyer, 1982).

The second approach, examining the thermo-stability of an intergral membrane protein, is one which has been used by Bowler, Cossins and co-workers to monitor homeoviscous adaption and the consequent changes in membrane lipid composition and membrane order, (Cossins & Bowler, 1976, Cossins et al 1981). In other words the integral membrane protein serves as a natural probe of membrane function and complements the approach using artifical probes which themselves may disrupt membrane structure. It also has the advantage of monitoring a defined environment, that is the adjacent region of the lipid bilayer. It does not give the same kind of 'picture' as D.P.H. fluorescence polarization, which represents a weighted average of all the different membrane domains. This kind of approach in studying tumour plasma membranes is rare in the literature, however there is one study by Emmelot and Bos, (1968), which looked at the thermo-lability of the Na+/K+-ATPase and the Mg2+-ATPase in hepatoma plasma membranes.

In this current study the Mg2+-ATPase was selected. This was because of its relative abundance, compared to say the Na+/K+-ATPase, in the tumour membranes, not because of its functional significance in the plasma membrane. In fact its function is obscure, though Forgac & Cantley, (1984), have recently shown that it is not an ionic pump in human erythrocyte

membranes.

MATERIALS AND METHODS

1. Isolation of plasma membranes

Plasma membrane enriched fractions were obtained from liver, and the two tumours, D23 and Mc7, using the methods described in Chapter 1, section 1.

2. D.P.H. Steady state Fluorescence Polarization

The plasma membranes from liver and the D23 and Mc7 tumours were prepared for fluorescence polarization measurement using the methodology described in Chapter 4, Materials and methods section 1. A concentrated solution of tetracaine was added to three cuvettes to give final concentrations of either 0, 1.0 or 5mM tetracaine. On addition of the anaesthetic the cuvettes were allowed to stand for 15 minutes in order to allow for the equilibration of the reagent with the membranes. After this time the change in polarization against temperature 4-50^{o}C was carried out as previously described in Chapter 4. A statistical treatment of the results was carried out using the analysis of covariance option from the MIDAS statistical package.

3. Thermal inactivation of the Mg2+-ATPase

Plasma membranes were diluted using a medium containing 1mM E.D.T.A., 20mM imidazole buffer, pH7.2. 0.3ml of the membrane suspension was added to pre-equilibrated glass tubes held in a 'Forbes Bar'. This equipment maintained a temperature gradient between 37-63°C. At each temperature there were 4 different conditions, 3 tubes contained tetracaine at several concentrations (for details see Figs. 5.6-5.8), and the fourth tube acted as a control. The tubes were incubated for exactly 10 minutes, and then they were placed on ice, to prevent further inactivation. The residual Mg2+ATPase activity was then assayed.

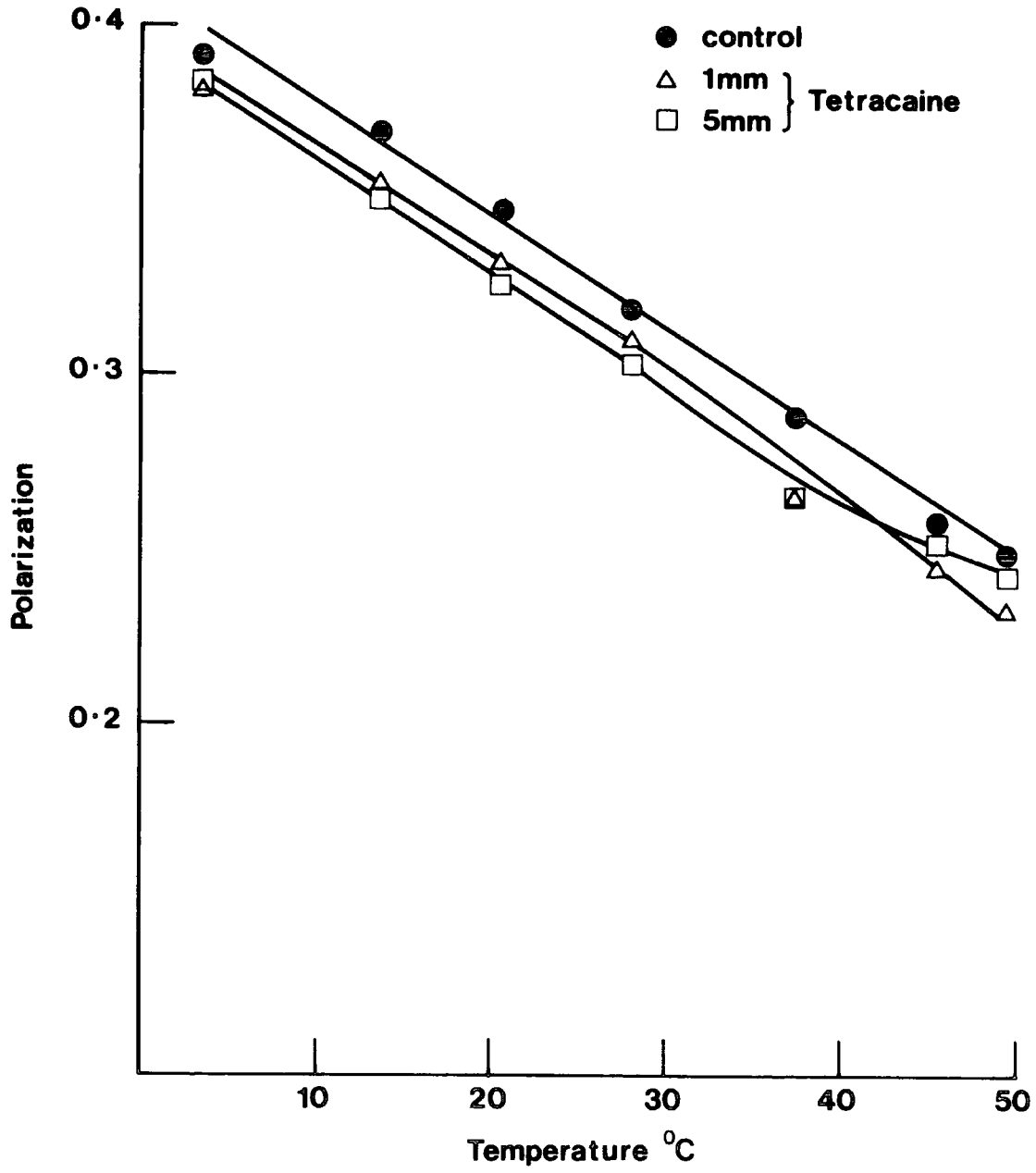
4. Assay of residual Mg2+ATPase activity

The enzyme was assayed in a similar way to the Na+/K+ATPase described in Chapter 1. The reaction conditions were as follows: 10mM K+, 3mM Mg2+, and 100mM Na+ in 20mM imidazole buffer, pH 7.2, in the presence of 1mM ouabain. ATP (sodium salt) was added to give a final concentration of 3mM. Incubations were carried out at 37°C. The reaction was started by the addition of 0.3ml of enzyme suspension to give a final volume of 2ml. The reaction was stopped and the inorganic phosphate liberated was determined in the same way as for the Na+/K+-ATPase described in Chapter 2, section 2b.

RESULTS

Figures 5.1-5.3 show the effect of tetracaine on the liver, D23 and Mc7 plasma membranes respectively. The plots are averages of 3 separate membrane isolations taken over a period of 18 months. The standard errors about each point are omitted for clarity, but a statistical treatment of these results is presented in Table 5.1. The equations of the lines for control and 1mM tetracaine runs are presented in Table 5.1. Lines generated by the addition of 5mM tetracaine were not linear and therefore could not be treated in this manner. No statistical difference in the slope of the lines is produced by the addition of 1mM tetracaine to either liver, D23 or Mc7 plasma membranes. There is, however, a significant change in the intercept on the addition of 1mM tetracaine to the D23 and Mc7 tumour membranes ($p<0.002$). Although there is a similar change in intercept produced by the addition of 1mM tetracaine to the liver plasma membranes, it is not statistically significant.

The results show that in all three membrane types tetracaine decreases the polarization value and therefore decreases membrane order. This effect does not appear to follow a classic dose response, that is 5mM tetracaine has little more effect than 1mM tetracaine. The overall effect of 1mM tetracaine is more pronounced on the tumour plasma membranes than the same type of membranes from liver at all temperatures. The effect of tetracaine is to shift the curve along the temperature axis, so that for 1mM tetracaine the shift is 3, 7, and 6°C for the liver, D23,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Polarization
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Tetracaine
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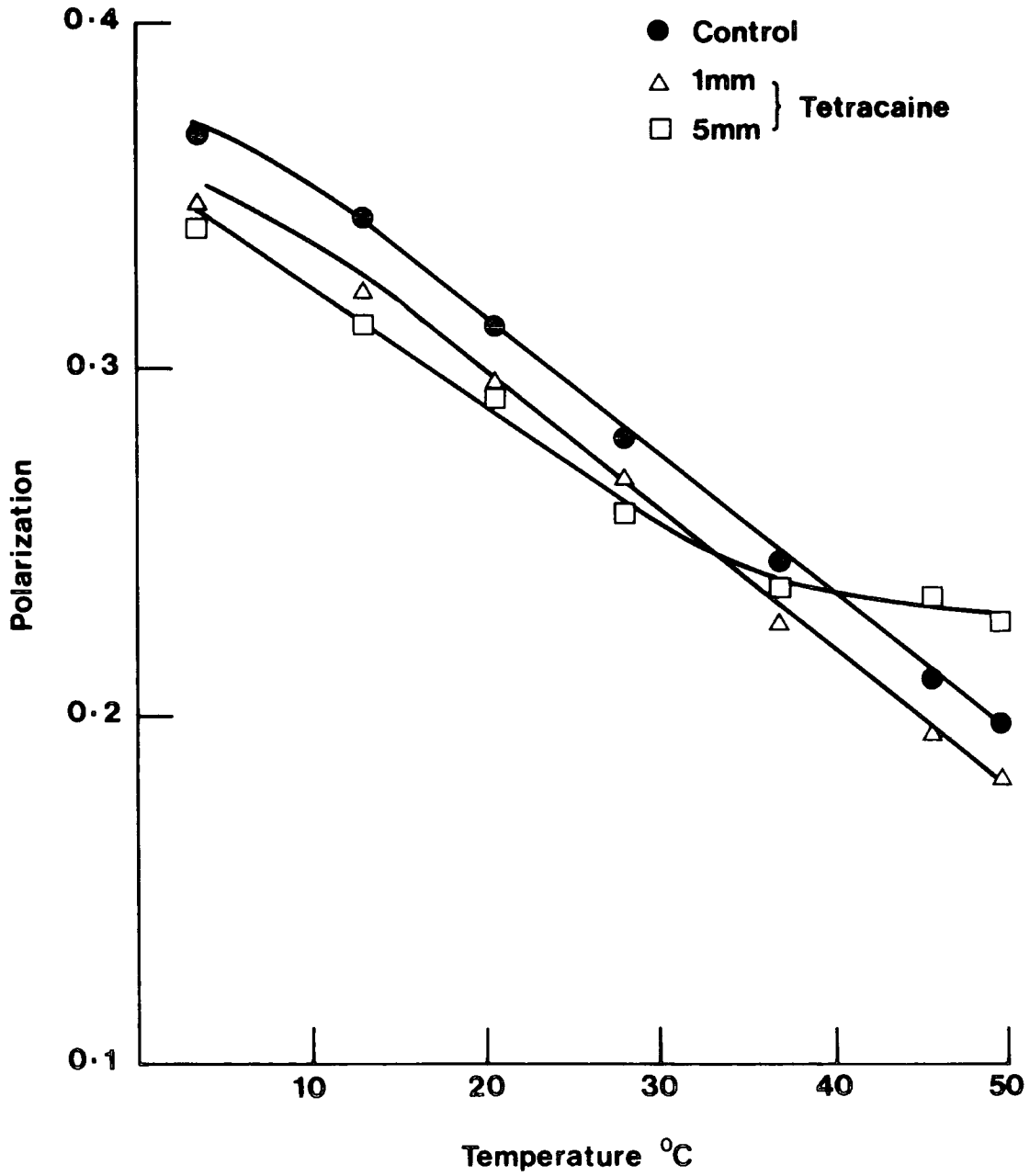
Temperature °C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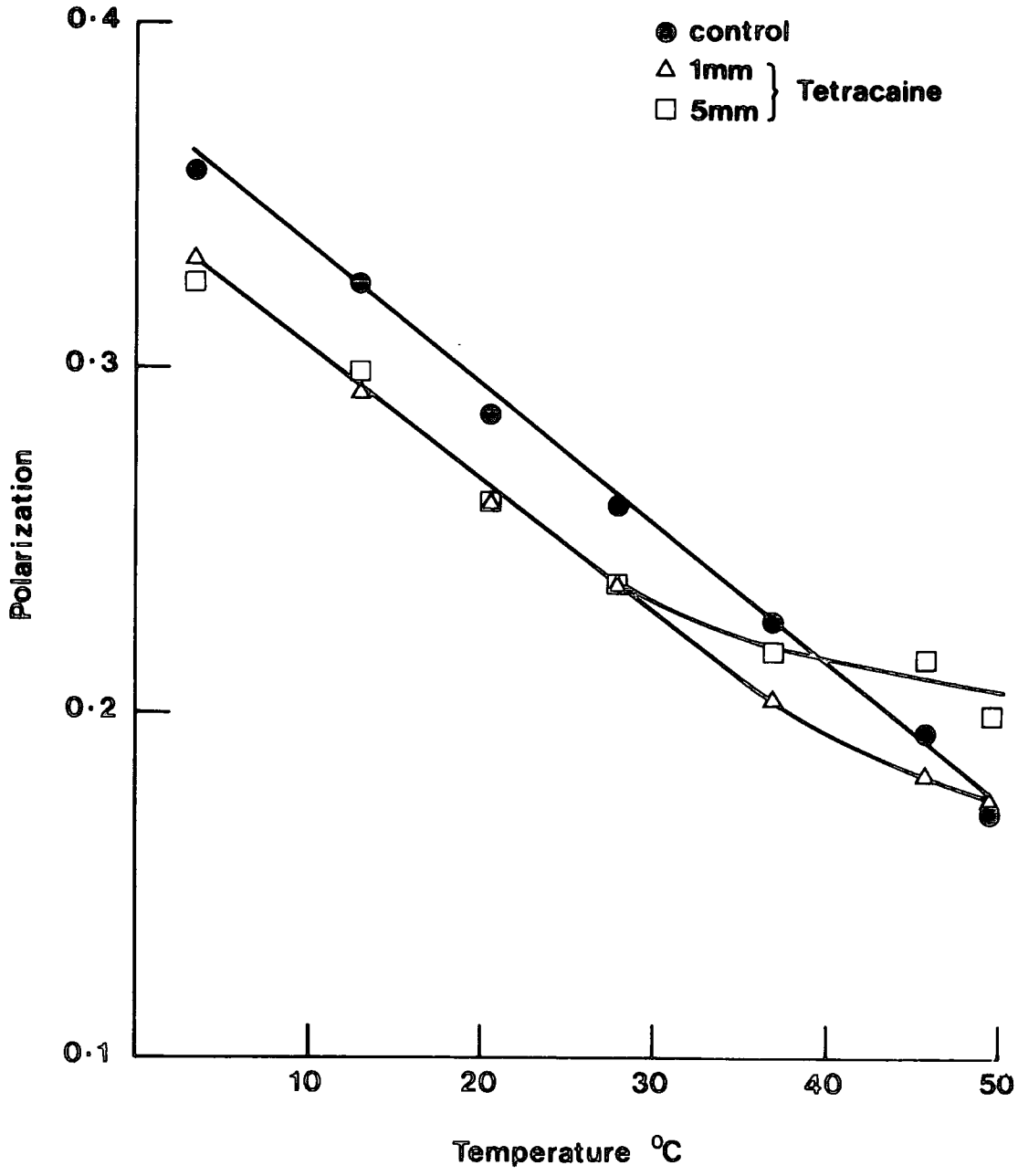
0·4
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Polarization
control
1mm
5mm
Tetracaine
10
20
30
40
50
Temperature °C

TABLE 5.1

Effect of tetracaine on the steady state fluorescence polarization of D.P.H. in liver and tumour plasma membranes. Equations of the lines generated using the MIDAS statistical package.

Membrane type	Tetracaine conc'n/mM	M x 10^2	C
LIVER	0	-0.325	0.409
	1	-0.327	0.397
D23	0	-0.381	0.387
	1	-0.386	0.370
Mc7	0	-0.411	0.376
	1	-0.345	0.339

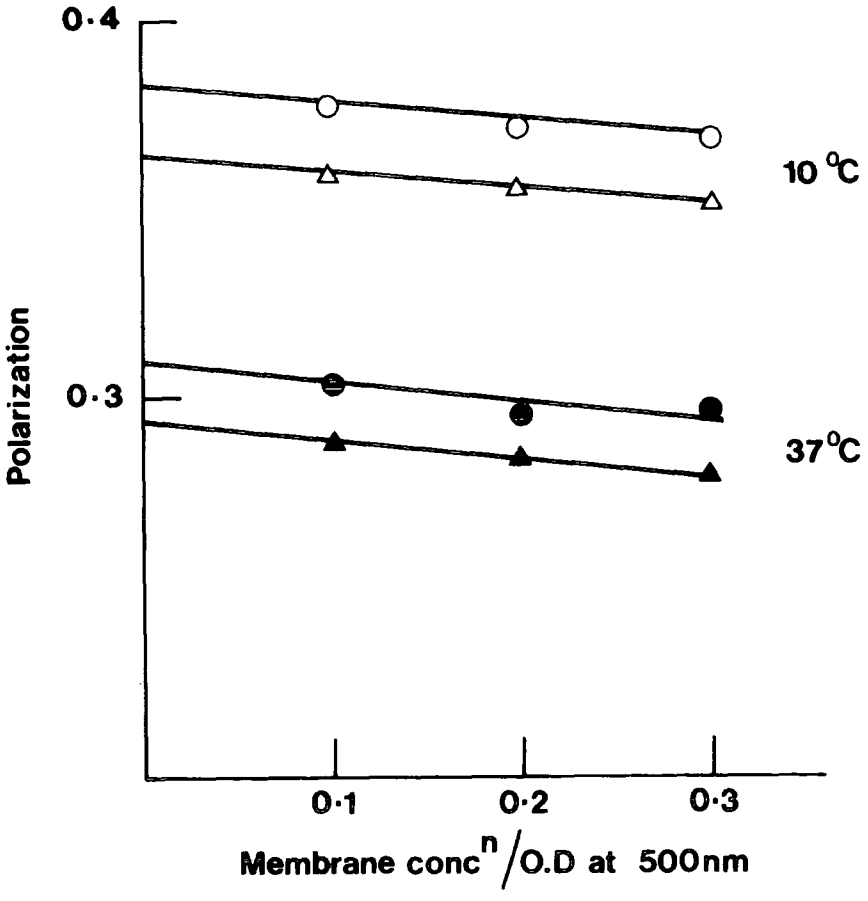
Values fit the form of equation:

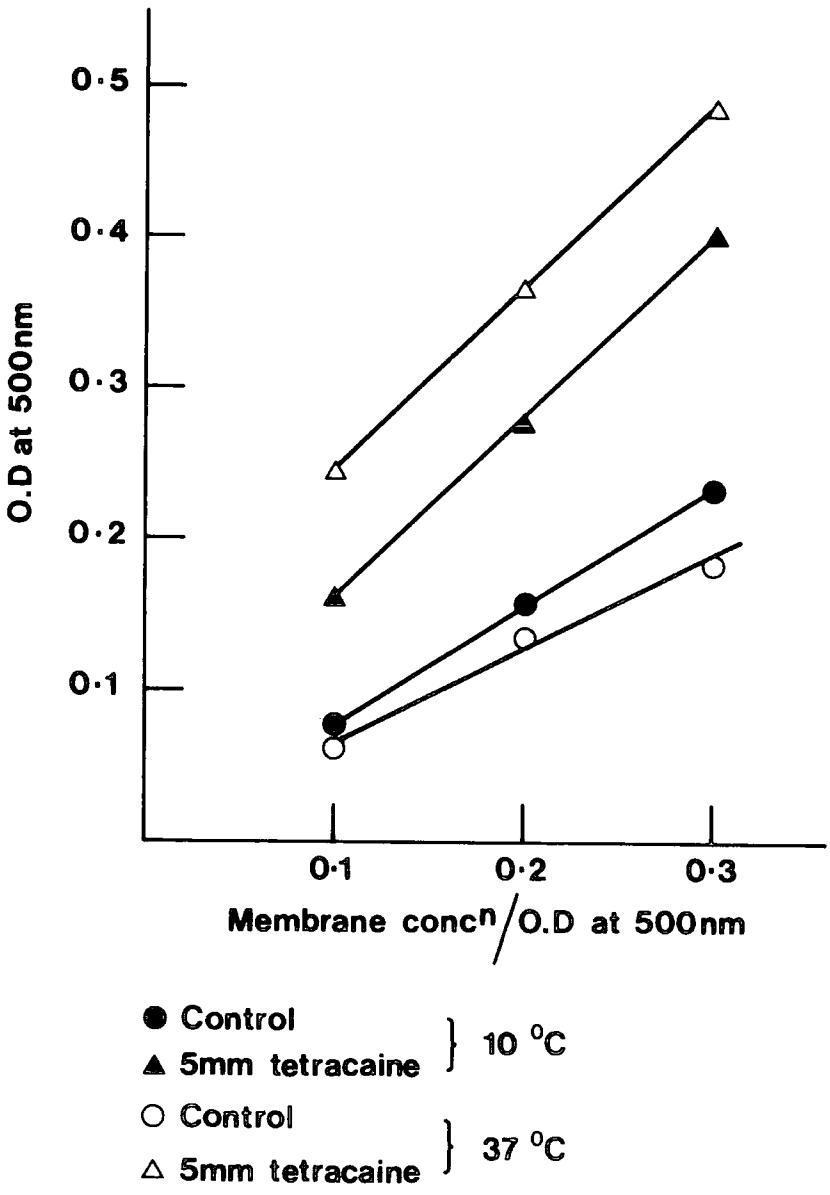
$$Y = MX + C$$

The data was from 3 separate experiments

and Mc7 membranes, respectively. Because the slopes of the curves are not significantly different, the effect of tetracaine on membrane order does not appear to vary much with temperature. For 5mM tetracaine at lower temperatures, there is a greater decrease in polarization, and therefore order, in the two tumour plasma membranes than for the liver plasma membrane. However at higher temperatures in all three plasma membrane types there is a deviation from the linear plots so that at the highest temperatures the plasma membrane appears to be more ordered in the presence of 5mM tetracaine than in its absence! This effect is considerably more pronounced in the tumour plasma membranes, and certainly would not be predicted from an understanding of the action of tetracaine; therefore the phenomenon was further investigated.

It was observed, for example, that the cuvettes which gave these peculiar values were cloudy and there was evidence of membrane floculation. To eliminate from consideration any effect which was independent of the membranes, the following combinations of components was tried to see if any of them produced the cloudiness and floculation observed: i)D.P.H. probe + phosphate buffer + increasing temperature, to see if any temperature dependent pH change was taking place which might precipitate the probe or buffer. There was no effect. ii) Buffer + 5mM tetracaine + increasing temperature, this had no effect. And lastly iii) D.P.H. + buffer + 5mM tetracaine + increasing temperature, again there was no effect.


0·4
0·3
Polarization
10 °C
37°C
0·1
0·2
0·3
Membrane conc^{n}/O.D at 500nm
○ Control
△ 5mm tetracaine
⊖ Control
▲ 5mm tetracaine


0·5
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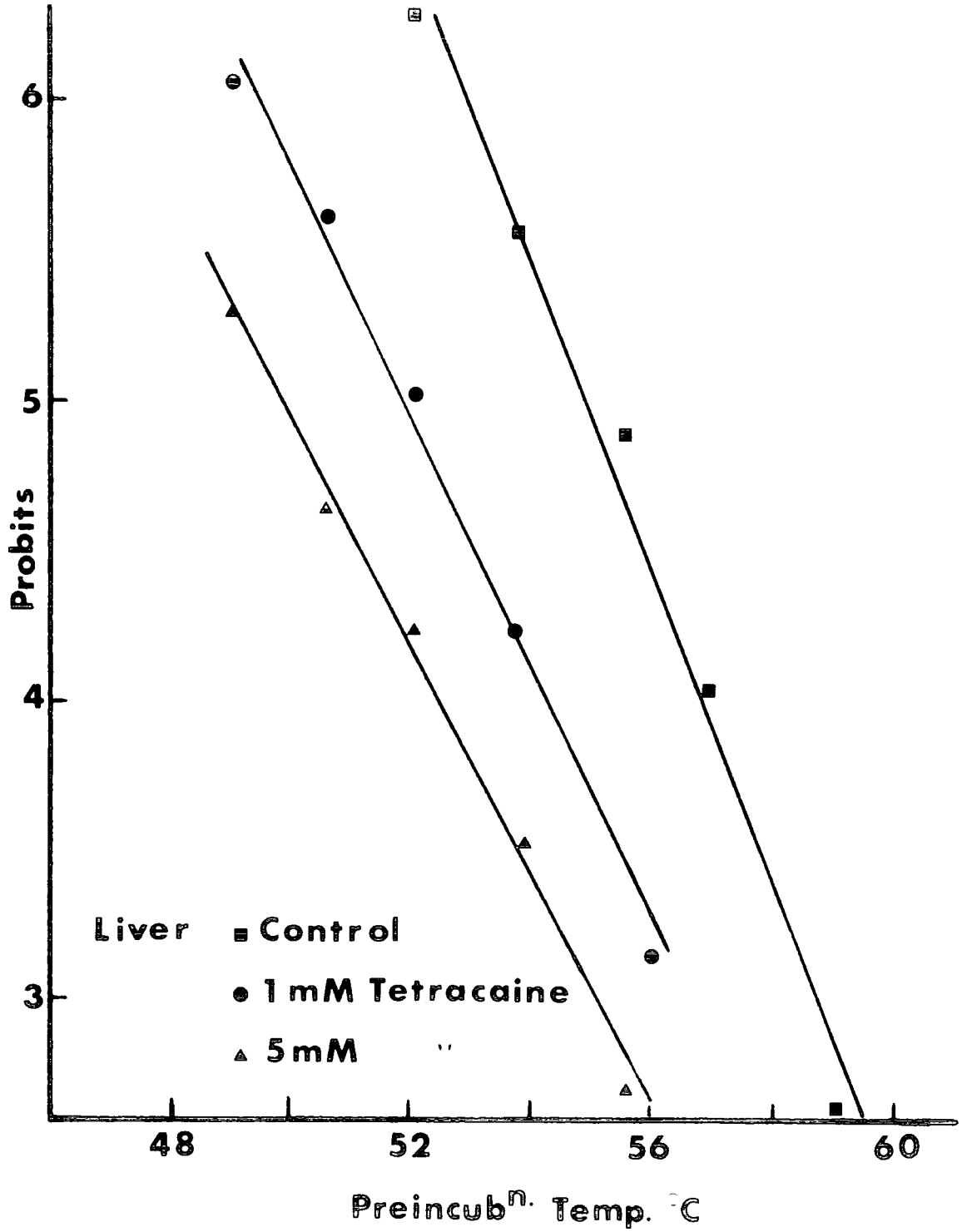
0·1
O.D at 500nm
0·1
0·2
0·3
Membrane concⁿ/O.D at 500nm
● Control
▲ 5mm tetracaine
10 °C
○ Control
△ 5mm tetracaine
37 °C

Therefore the cloudiness and floculation was the result of a direct effect of high tetracaine concentration on the plasma membranes themselves. To attempt to quantify the effect the following further experiment was carried out. Liver membranes were taken at various concentrations (to give ODs at 500nm of 0.1, 0.2, and 0.3), and then the effect of 5mM tetracaine at two different temperatures (10 and 37°C was assessed in terms of i) polarization ii)optical density 500nm. After the polarization value for a given sample had been obtained the cuvette was immediately removed and placed in a spectrophotometer which was equilibrated to the same temperature, and the OD at 500nm was recorded. The results are presented in figs. 5.4 & 5.5. Fig. 5.4 shows the effects on polarization of temperature and tetracaine confirms first, that there is very little effect on polarization value when membrane concentration is changed, and second, that increased temperature leads to decreased polarization values and hence 'order'. Fig. 5.5 records the effect on the OD of 5mM tetracaine and shows some interesting trends. The figure indicates that when liver plasma membranes without tetracaine are taken from 10 to 37°C there is an overall decrease in OD. The effect with the membranes plus 5mM tetracaine is the reverse, ie. the OD is greater at 10 than 37°C. But more importantly, at both 10 and 37°C there is a large increase in OD when compared to the liver plasma membranes, at the same temperatures, without tetracaine.

Figures 5.6-5.8 show the effect of increasing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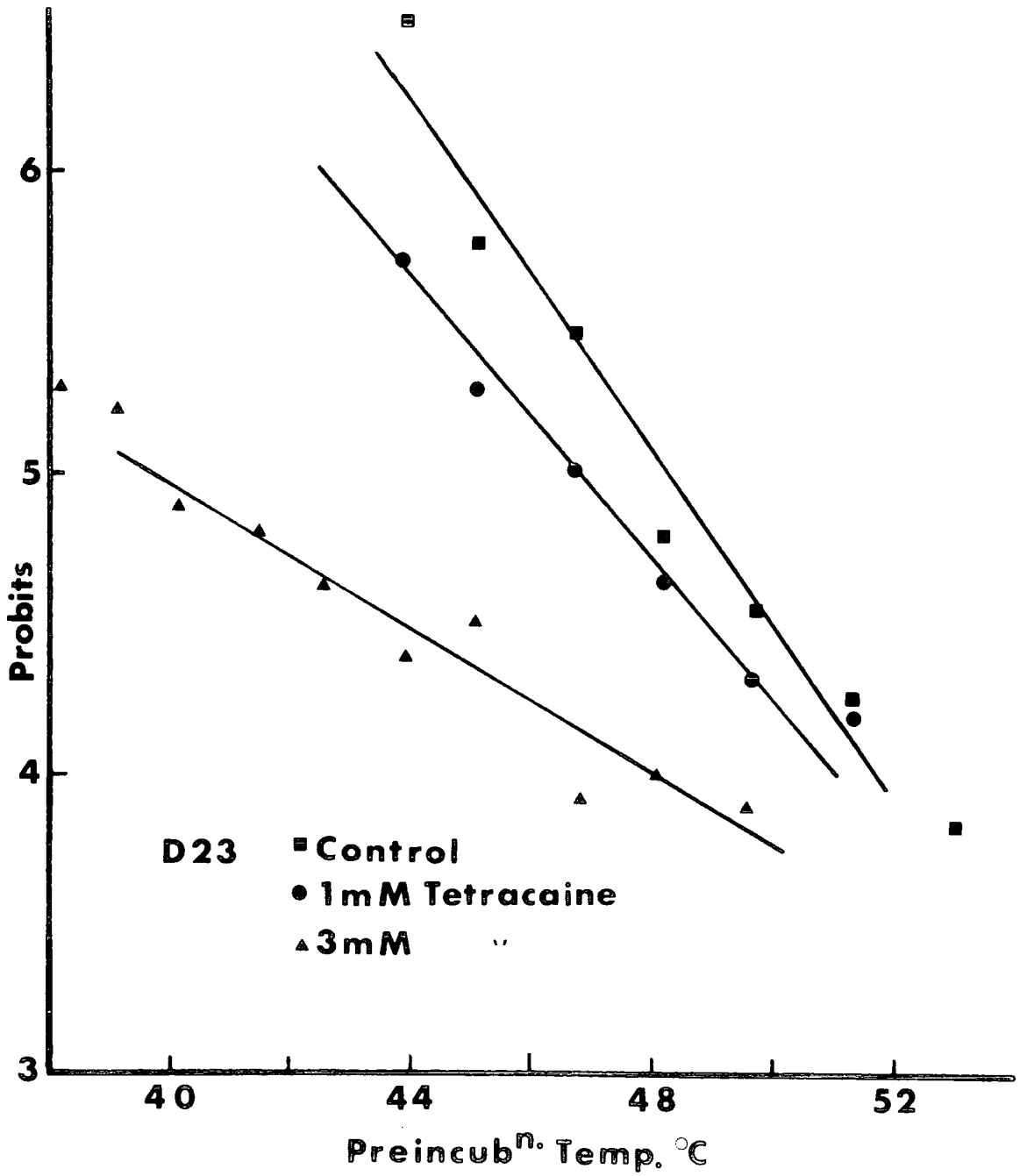
3
Probits
Liver
Control
1 mM Tetracaine
5 mM
48
52
56
60
Preincubⁿ. Temp. °C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Probits
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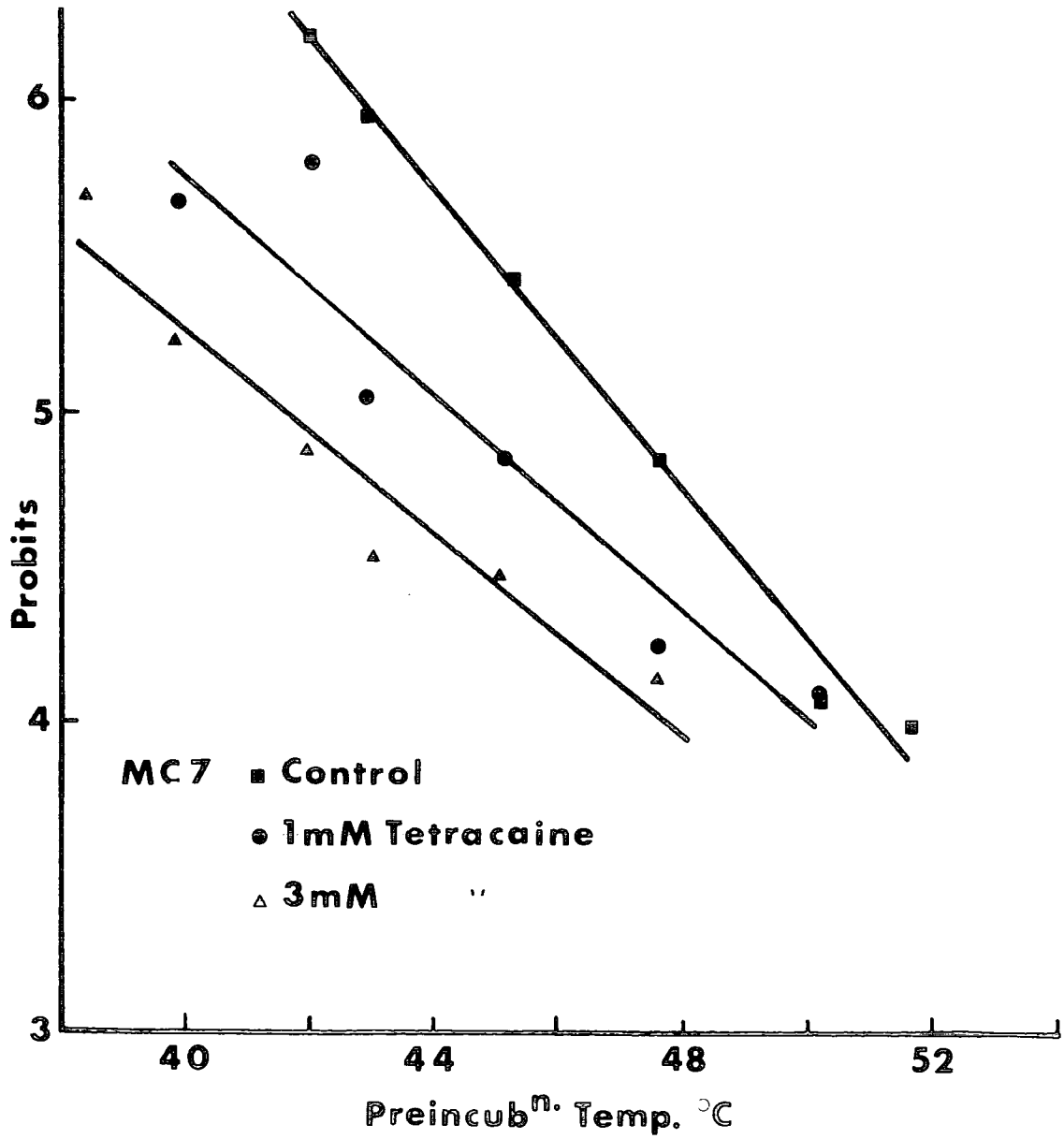
52
Preincubⁿ. Temp. °C
D23
Control
1mM Tetracaine
3mM


6
5
4
3
Probits
MC7 ■ Control
● 1mM Tetracaine
△ 3mM
40
44
48
52
Preincubⁿ. Temp. °C

tetracaine concentration on the thermostability of the membrane bound Mg2+-ATPase from liver, D23 and Mc7 plasma membranes, respectively. Probits were used to convert the sigmoidal dose response curve into a linear form, and then plotted against temperature. A probit of 5.0 represents 50% inactivation of the enzyme. They show typical results, not means, and were fitted to a straight line using an unweighted least squares method. The slope of the liver enzyme is steeper than the slope of the two tumour enzymes which are similar in gradient. The temperatures at which 50% of the Mg2+-ATPase is inactivated, at each tetracaine concentration, for each type of plasma membrane, are presented in the legends of Figures 5.6-8. These are the averages from three separate experiments, and were again produced using an unweighted least squares method. The 95% confidence limits of the temperature which produces 50% inactivation (probit of 5) of the enzyme were calculated using Fieller's Theorum using the methods described by Finney, (1978). It is very clear from figures 5.6-5.8, that the tumour enzymes are considerably more thermo-labile than the same enzyme from liver. The D23 Mg2+-ATPase is 50% inactivated at $47.51\pm0.185^{o}C$, and the Mc7 enzyme at $46.34\pm0.25^{o}C$, where as the Mg2+-ATPase from liver plasma membranes is not 50% inactivated until the temperature reaches $53.87\pm0.317^{o}C$. This represents a difference between the tumour and normal enzyme of about $7^{o}C$.

The effect of tetracaine is to potentiate further the

inactivation, so that with increasing tetracaine concentration the 50% inactivation temperature of the enzyme is progressively lower. The effect of tetracaine produces a similar drop in the 50% inactivation temperature of the Mg2+-ATPase in all three types of plasma membranes. For example 1mM tetracaine produces a drop in the 50% inactivation temperature of 3.4, 2.0, and 1.8°C for the liver, D23, and Mc7 enzymes, respectively. 5mM tetracaine reduces the temperature required to inactivite 50% of the liver enzyme activity by 6.6°C, and 3mM tetracaine reduces the temperature required to inactivate the D23 and Mc7 tumour enzyme by 50% by 7.3 and 4.4°C, respectively. All these difference are statistically significant, and the statistics are presented in the legends of Figs. 5.6-8.

DISCUSSION

The relationship between local anaesthetics, membrane order, protein stability and temperature is a complex one. In the literature controversy abounds, and it would be easy to lose the significance of the results presented here in speculation of the detailed interactions that take place. Therefore, it seems sensible to highlight the central conclusions which can be drawn from the results presented here.

The results clearly indicate that:

i)Tetracaine disorders the plasma membranes of the two

tumours to a greater extent than those from liver.

ii)The Mg2+ATPase is far more thermally sensitive in the D23 and Mc7 tumour plasma membranes than in those from the liver; and,

iii)This sensitivity can be enhanced by the addition of tetracaine to the plasma membranes of all three tissues.

Goldstein, (1984) has considered the action of drugs on membranes from the standpoint of fluidity changes. There is no clear consensus over the site of action of anaesthetics, which may be lipid, protein, or both. However, she suggested that of the drug-induced changes in the hydrophobic phase of the membrane, 'fluidity may be the most significant. It is probably the change in membrane order or fluidity which is most important when considering the exploitation of local anaesthetic action in the enhancing of tumour sensitivity to hyperthermia.

The results presented here clearly show that tetracaine has a disordering effect on tumour plasma membranes which is apparently greater than its effect on liver plasma membranes. There is however a bizzare effect on membrane order in all the plasma membranes (though this is far greater in magnitude in the tumour membranes), whereby, at high tetracaine concentrations (above 3mM) and at temperatures above 30°C membrane order is apparently increased rather than decreased. Observation and Fig. 5.5

indicate that these apparent increases in order are paralleled by an increase in light scattering, which is almost certainly due to protein denaturation. Since D.P.H. only fluoresces when in a hydrophobic environment, this gain in order cannot be the result of displacement of the probe from the membrane. It may be due to the fact that a significant proportion of the probe molecules are trapped in a hydrophobic region of the denatured protein which is more ordered than the intact lipid bilayer. Alternatively, it could be that the probe is trapped in micelles formed from the tetracaine (which is amphipathic in nature) and this environment is again more ordered than the normal undisrupted membrane. It is unlikely that this effect is related to fluorescence quenching as it only occurs at a high temperature. It is interesting to note that the effect becomes apparent at about the same polarization value (0.25) for all three plasma membrane types. Thus it may mark a value of membrane disordering which causes irreversible changes to the membrane structure. Although the peculiar nature of this effect is difficult to understand, its physical manifestation is that tetracaine has a destructive effect on membranes which is induced at temperatures similar to those used in clinical hyperthermia. At high concentrations tetracaine and other amphiphilic local anaesthetics have a 'detergent' effect on membranes (eg. Mahler & Singer, 1984). These workers also observed that this effect promoted gross redistributions of components in the plane of the bilayer and it is almost certainly this detergent action which leads to the protein

denaturation observed in the present study.

The effect of tetracaine does not follow the classic 'dose response' action often seen with drugs. This is due to the fact that it has more than one possible site of action. Although many studies on the effect of tetracaine on biological membranes and model systems have been carried out, there is uncertainty about its orientation in the bilayer. Sikaris and Sawyer, (1982), using fluorescence quenching techniques showed that the aromatic amine of tetracaine resided deep in the phospholipid bilayer. However this study was not carried out at physiological pH, but at pH 9.5. Boulanger et al (1981) showed, in a deuterium/ P31 N.M.R. study, that tetracaine exerted different effects on phospholipid dispersions depending on whether it was positively charged (at pH 5) or uncharged (at pH 9.5). Clearly the former form will tend to interact ionically at the plasma membrane surface, whereas the latter will have mainly a hydrophobic interaction deeper within the lipid bilayer, as suggested by Sikaris and Sawyer, (1982). These two workers have also suggested that at physiological pH the positive form is unable to penetrate the bilayer, and Mahler and Singer, (1984), suggest this is the only form at this pH(7.4). This would mean that the tetracaine would not penetrate the bilayer at all at normal physiological pH. Other workers, however, argue that tetracaine has a hydrophobic action (eg. Ondrias et al 1983 and Grof & Belagyi, 1983). These current studies were carried out at pH 7.7 which is not

significantly different from physiological pH, in terms of the form in which tetracaine will adopt, and therefore its effect could be predominantly hydrostatic in nature. Certainly Chang and Wang, (1984), in a spin label/ fluorescence probe study, suggest that local anaesthetics interact hydrostatically with proteins. The variation in literature is to some extent a function of different pHs at which the studies have been carried out, and the different physical techniques used. A further complication, suggested recently by Schreier et al (1984), is that in model membrane systems the apparent pK of tetracaine decreases in the presence of membranes as a function of membrane concentration. All of the above discussion concerns the effect of tetracaine at fairly high concentrations; it is, however worth noting that at lower concentrations tetracaine in common with other membrane perturbants has an ordering effect on membranes. Pang and Miller, (1978), have proposed that at low concentrations the perturbant stabilizes the acyl chain, however with increasing concentration of perturbant (or cholesterol) there are an increasing number of interactions of the perturbant with stable cholesterol/phospholipid complexes which disrupt them and lead to an overall loss in order.

There are several other properties of tetracaine which relate to membrane order which must be considered. It has been suggested that there is differential fluidity between the monolayers in liver plasma membranes (Whetton et al 1982), and this might well occur in the tumour plasma

membranes as well. Cationic local anaesthetics, such as tetracaine, have been shown to bind preferentially to the inner leaflet of the bilayer, (Houslay et al 1981, Ogiso et al 1981, & Dipple et al 1983). If the inner leaflet from the tumour plasma membranes was already more fluid than normal this could have important implication in the interaction of anaesthetics and heat with membrane proteins, and will be discussed later. Another important cation which affects membrane order and is reported to interact with cationic local anaesthetics is Ca2+.

The fact that Ca2+ reduces the fluidity of rat liver plasma membranes has been established by studies using at least two physical techniques: a fluorescence probe study, Livingstone and Schachter, (1980), and a spin label study, Gordon et al (1983). The latter workers have also observed that cationic local anaesthetics compete with Ca2+ binding sites on the inner leaflet of the membrane. This was first noted by Seeman, (1972). Other workers have suggested that on the axonal membrane surface, there is no functionally essential cation binding site which the Ca2+ and local anaesthetics compete, (Deschenes et al 1981). Calcium ions have also been shown to modulate free fatty acids in liver plasma membranes, increasing the rigidity of the membrane structure, particularly in the solid domains (Schroeder & Soler-argilaga, 1983). The fact that cationic local anaesthetics can compete with Ca2+ binding sites offers another route whereby these agents can disorder or fluidize plasma membranes.

The thermal inactivation studies of the Mg2+ATPase reveal that the enzyme is a great deal more thermally labile in the plasma membranes of the two tumours than in those of the liver. The only similar study in the literature confirms that this is true in other tumours as well. Emmelot and Bos, (1968), found that the Mg2+ATPase from a hepatoma was far more thermally labile at 40 and 50°C than the equivalent liver enzyme. However they also found the complete opposite with another plasma membrane enzyme the Na+/K+-ATPase, ie. the liver enzyme was more thermally sensitive than that of the hepatoma. A preliminary investigation in this laboratory using the Na+/K+-ATPase in the plasma membranes of the Mc7 tumour showed that there was little difference between the thermolability of this enzyme from liver. Emmelot and Bos attributed the differences they observed to temperature dependent changes in membrane structure in the tight junctions of liver plasma membranes which were not seen in hepatoma plasma membranes. This may well be the case in the present study as the tumours have an undifferentiated morphology. It has also been reported that the activity of the Mg2+-ATPase from liver plasma membranes decreases markedly with increasing temperature above 30°C. Therefore at hyperthermic temperatures the protein could be more unstable as its ability to function at these temperatures is impaired.

What are the possible relationships between the observed thermo-lability of the tumour enzyme and the lipid

composition and order of the tumour plasma membranes reported in Chapters 3 and 4? Cossins et al (1981), looked at the temperature inactivation of the Na+/K+-ATPase in goldfish synaptosomes, and related decreases in membrane order with the thermoliability of this enzyme. Cossins and Bowler, (1976) have also reported that integral membrane proteins were far more thermo-labile than aqueous enzymes in the same organism. This they related to the need for greater flexibility in the tertiary structure of the membrane proteins in order to maintain conformational flexibility in the distinctly ordered and anisotropic environment of the plasma membrane. The thermal stability of the tumour membrane enzyme presented here, as in the case reported by Cossins et al (1981), may be related back to the decreased membrane order and therefore the changes in lipid composition, particularly the lowered cholesterol levels.

As indicated in the introduction, the function of the Mg2+-ATPase is unclear. Forgac and Cantley, (1984), have shown it is not an ion pump in human erythrocytes. Two earlier studies on this type of ATPase activity in the same cell type have been carried out by Singer and co-workers (Sheetz & Singer, 1977 and Birchmeier & Singer, 1977). These latter workers found that the activity was associated with the conversion of a component of spectrin (part of the cytoskeleton) in a phosphorylated form which in turn was responsible for shape changes in erythrocyte ghosts. In a completely different system, a membrane fraction of rat

skeletal muscle, it was proposed that the Mg2+-ATPase which was found in abundance in these membranes required the mobility of proteins within the membrane to be regulated by ATP. This is again suggestive of a similar role to the one above. There are two important consequences of the observation that the Mg2+-ATPase may be involved in the cytoskeleton.

Notwithstanding our uncertainty of the functional role of the Mg2+-ATPase, it can be used as a 'probe' of membrane responsiveness to temperature change. If it has an important role in the structure of the plasma membrane ie. modulation of the cytoskeleton, the marked thermoliabilty of the tumour enzyme compared to that of normal tissues may be a possible reason for the differential thermosensitivity of tumour cells in general.

The second point that can be made is that if it is involved with the cytoskeleton, it will almost certainly be located in the inner leaflet of the plasma membrane, and therefore is assymetrically distributed (like for example the 5'-Nucleotidase). In at least one plasma membrane (that of the hamster liver cell) the two lipid bilayers have been shown to be independently controlled (Houslay & Palmer, 1978). It may be that tumour cells have lost the ability to regulate which might explain why the Na+/K+-ATPase, a bilayer transversing protein, is not affected as much as the Mg2+-ATPase, which rests in the inner leaflet.

The denaturing property of heat on the Mg2+-ATPase is potentiated by tetracaine in all three plasma membrane types. The synergistic effect of heat and the anaesthetic produces 50% deactivation of the tumour enzymes at 45.54 and 44.49°C for the D23 and Mc7 respectively, a similar temperature has been used in hyperthermic treatment regimes. This is very significant as it indicates that at relatively low temperatures (compared to liver) the combination of heat and membrane perturbant had a devastating effect on the plasma membrane. It is also worth noting that even at 37°C there was some inactivation due to the presence of 5mM tetracaine alone without the input of further heat. This is almost certainly the result of the 'detergent' like action of tetracaine when it is present in fairly high concentrations. This effect has been dicussed earlier. These results considerably re-inforce the view that the adminstration of membrane fluidizers should have a major role in improving the hyperthermic treatment of cancer, a point dealt with more fully in Chapter 7.

6. THE EFFECT OF ETHANOL ADMINSTRATION ON THE COMPOSITION AND PHYSICAL PROPERTIES OF Mc7 PLASMA MEMBRANES

INTRODUCTION

The first section of this thesis was concerned with the characterization of plasma membranes from two solid tumours; the Mc7 sarcoma, and the D23 hepatoma. The last chapter dealt with the immediate effects of tetracaine, a potent membrane fluidizer, on plasma membrane structure. This next section of the work is concerned with the long-term effects of a membrane disordering agent (ethanol) on the lipid composition and physical state of the Mc7 tumour plasma membrane. The reason for this interest in the long term effects of membrane perturbants is that it has been shown with a least one perturbant, ethanol, that long-term administration will induce tolerance; and recently Littleton *et al*. (1983) has shown that ethanol produces cross-tolerance with hyperthermia. Thus there may exist a method to manipulate the resistance of tumours and normal tissues to heat which could be very important in hyperthermia therapy. Curran and Seeman (1977), have suggested that this is the result of changes in the membrane lipid compostion, which is, in turn, an attempt to compensate for the fluidizing effects of ethanol on the membranes. Chin *et al.* (1978), for example, have shown that erythrocyte and

synaptic membranes had a significant increase in membrane cholesterol after ethanol administration in mice. Cholesterol is known to stabilize membranes(Oldfield & Chapman, 1972 and DeKruff et al. 1972). Also Littleton and John, (1977). in a similar ethanol dependency experiment, have reported changes in plasma membrane phospholipid fatty acids which could be seen as compensatory. These kind of adaptive changes have been observed to take place in the presence of other types of membrane perturbing drugs, for example morphine has been shown to increase cholesterol/phospholipid ratios and decrease membrane fluidity or order, (Heron et al. 1982).

This kind of adaptation has been termed 'homeoviscous' by Sinensky (1974) and has been shown to be part of many organisims response to changes in environmental conditions, such as temperature, which alter the physical state of the plasma, and other membranes. The organism response is to change the lipid composition of the membranes in a compensatory manner, (Hazel & Prosser, 1974, and Cossins, 1983). The cross-tolerance of heat and membrane perturbants such as drugs, suggests the mechanism of compensation may be similar in both cases, and that the plasma membrane is the key site of heat action.

Normal cells will tend to change their plasma membrane structure to compensate for the presence of a fluidizer, but many tumour cells are reported to have more fluid plasma membranes than normal cells(eg.Inbar, 1976; Van Blitterswijk et al. 1977 ; and the D23 & Mc7 plasma membranes from this current study, Chapter 4). It is questionable whether such cells could

respond as well to the presence of a membrane perturbant when their plasma membranes are already considerably more disordered than those of normal cells. If this is indeed the case then a differential heat sensitivity between normal and tumour cells could be established using the adaptive response induced by membrane fluidizers. The object of the current series of experiments is to see whether this suggestion is indeed a practical possiblility. Ethanol was used as the membrane perturbant because its long-term effects are well documented in normal tissues.

The method of ethanol administration selected for this study was that of Chin and Goldstein, (1978). They used a liquid diet based on chocolate flavoured Carnation Slender. Ethanol was added to the experimental animal's slender diet and its pair-fed control received a similar slender diet which had been balanced iso-calorifically by using sucrose to substitute for the ethanol. The liquid feeding of ethanol has been well established (review, Lieber & DeCarli, 1982), but there are other methods of ethanol administration reported. For example IP injection (La Droitte et al. 1984a); by ethanol vapour, (eg. La Droitte et al. 1984b & Dunbar et al. 1981); and by intubation (eg. Crews et al. 1983). The disadvantages of these methods are that they induce stress (IP injection, and intubation), which itself can induce changes in lipid metabolism; or adequate iso-calorifically fed controls are difficult to establish (vapour).

Plasma membranes from the Mo7 tumour grown in either

ethanol tolerant rats or pair-fed controls were isolated, along with the synaptic membranes from the same animals, and their lipid composition determined. The physical properties of the membranes were also studied. Thus any differences in the response of normal and tumour plasma membranes to the presence of a membrane perturbant were determined.

MATERIALS AND METHODS

1. Ethanol administration

Ethanol-dependent rats were produced using the methods described by Chin et al. (1978). This method involved the addition of ethanol to a value of 33% of the total calorific intake by using Carnation chocolate flavoured 'Slender', a liquid diet which had all the essential mineral salts and nutrients. Rats of the inbred Nottingham strain used for the first part of this study were pair-fed. The intake of alcoholic Slender of an individual rat was measured daily, and its pair-fed partner was given an equivalent volume of slender, made iso-calorific by the addition of an appropriate amount of sucrose. Each experiment involved 20 rats, usually female, but in one instance male, and the animals were maintained on this diet for 10 weeks.

After 10 weeks, a suspension of tumour cells was passaged into the animals flank in the manner described in Appendix 1, section ; the Mc7 into the females, the D23

into the males; and allowed to grow for 10 days. After this time the animals were sacrificed, blood ethanol determined, and membranes from the tumour and brain isolated.

2. Blood ethanol measurement

The rats were sacrificed by cervical dislocation and immediately a sample of blood 2ml was obtained by cardiac puncture. Ethanol was determined using a Sigma kit (332-UV Ethanol), based on alcohol dehydrogenase, linked to NADH. The whole blood was spun down in an Eppendorf minifuge for 2 minutes at 11,400g (rav.48mm), the serum was removed, and deproteinized by precipitation with 6.25% (w/v) T.C.A. A 0.1ml aliquot was removed, diluted, and assayed using the kit. Samples were kept cold, in sealed tubes to prevent the evaporation of the ethanol.

3. Isolation of tumour membranes

Tumour plasma membranes were isolated using the methods outlined in Chapter 2, section 1.

4. Isolation of synaptic membranes

Synaptic membranes were isolated by the method of Rodriguez de Lorez Arnaiz et al. (1967). All operations were carried out at 0-4°C. The brain from each rat was removed, the grey matter dissected out, and then the pooled

tissue (5g) was homogenized in 20ml of 0.32M sucrose, 1mM E.D.T.A., in 20mM imidazole, pH7.2 (Isolation medium 1). The homogenate was then spun for 10minutes at 900g(rav. 155mm) in an MSE Coolspin The supernatant was then decanted. the pellet resuspended in isolation medium 1 and respun as before. The combined supernatants were then diluted in isolation medium 1 and spun for 30 minutes at 20,000g(rav.76.2mm)in a 8X50ml rotor using an MSE High speed 18 centrifuge. The supernatant was discarded, and the pellet resuspended in 1mM E.D.T.A., 20mM imidazole, pH7.2 (Isolation medium 2). This suspension was then respun at 20,000g for 30minutes in the same rotor. The pellet was then resuspended in 5ml of isolation medium 1 and layered carefully on top of a discontinuous sucrose gradient in each of 3x25ml centrifuge tubes. The gradient was comprised of 1.2, 1.0, 0.9, and 0.8 M sucrose solutions each in 1mM E.D.T.A., 20mM imidazole pH7.2. The sucrose solutions were carefully pipetted on top of each other, in order of decreasing density; and allowed to stand for 1 hour at room temperature to allow a merging of the layers, before the brain fraction was added. The tubes were spun in a 3X25ml swing-out head at 93,000g(rav.73.77mm) for 2.5 hours using an MSE Superspeed 40. The 2 middle bands were aspirated, diluted in isolation medium 2, and spun at 100,000g(rav. 77.52mm). The final pellet was resuspended in the minimum volume of isolation medium 1 using gentle homogenization. An aliquot was removed for protein assays and another for fluorescence polarization measurements. These, together with the bulk of the synaptic membranes,

were stored frozen at -20°C. Storage time was not more than 2 weeks.

5. Steady state fluorescence polarization

Isolated tumour and synaptic membranes were resuspended in 0.1M phosphate buffer, pH7.7. Steady state fluorescence polarization of the probe D.P.H. in the membranes was carried out using the methods described in Chapter 4, Materials and Methods, section 1.

6. Protein determinations

Protein assays were carried out using the methods in Chapter 2, section 3.

7. Lipid extraction and characterization

See materials and methods, Chapter 3.

RESULTS

1. Blood alcohol levels

Table 6.1 shows the blood ethanol levels at the time of sacrifice. It should be noted that there is considerable variation between individuals within any given experiment as well as variation in the means between the three separate experiments. The mean blood alcohol level

TABLE 6.1

Blood Ethanol levels in rats on sacrifice

EXP'T	Ethanol concentration	
	RANGE %(w/v)	AVERAGE %(w/v)
1	0.00126-0.0697	0.0246±0.0099
2	0.00397-0.144	0.0910±0.0223
3	0.006-0.192	0.0971±0.0166

n=10 values ± S.E.M.

is lower in the first experiment than the last two (ie 0.0246 of 0.0910 &0.0971% (w/v) respectively).

The effect of diet on plasma membrane lipid composition

During the course of these experiments it was noted that the lipid composition of the Mc7 plasma membranes from the animals fed on slender and sucrose (ie the controls) was different from the same plasma membranes taken from animals on the normal laboratory diet. In making this comparison it must be remembered that the calorific content of the two diets was different, and this too might affect membrane lipid composition. It is therefore important to deal with the differences in lipid composition which occur when the diet is changed from normal to slender, before considering the effect on lipid composition of adding ethanol to this changed diet.

A comparison of Table 6.4 from this chapter and table 3.1 from Chapter 3 shows that there were no significant differences between the phospholipid classes of the Mc7 plasma membranes from animals fed on slender diet, as compared to those membranes obtained from animals fed on the normal diet.

A comparison of Figure 3.5 from chapter 3 and Figure 6.5 from this chapter reveal changes in fatty acid profile of the Mc7 tumour plasma membrane phospholipids when the

diet is changed from normal to slender. There appears to be an elevation in the level of 18:1 in the PE and PC from the Mc7 plasma membranes when the animals are put on the slender diet and a small elevation of 18:1 in the SM of the tumour plasma membranes after this diet change. There is no such change in the level this fatty acid in the PS/PI classes of the Mc7 membranes when the diet is changed. Only the elevation of the 18:1 in PC is statisically different, ($p<0.02$). There also appears to be a lower level of 16:1 in the PE, SM, and PS/PI from the tumour membranes from slender fed rats compared to the level of this fatty acid in the same phospholipids from tumour membranes which had come from animals on the normal diet. None of these apparent differences were statistically significant. The overall effect however is seen in Figure 6.2 which shows a net elevation in mono-unsaturated fatty acids of the tumour plasma membrane phospholipids from animals fed on the slender diet compared to the equivalent membrane phospholipids from animals fed on the normal diet. In Table 6.2 the fatty acid composition of the two diets is examined. There are two main differences in the fatty acid profiles of the two diets. First there is an increase in the proportion of 18:0 and 18:1 in the slender diet when compared to the normal one, and second, there is less 18:2 in the slender diet than the normal one.

A comparison of Table 3.5 from Chapter 3 and Table 6.8 from this current chapter shows that there is a decrease in the levels of Mc7 plasma membrane cholesterol when the

TABLE 6.2

Fatty acid composition of rat diets

Fatty acid	relative % wt.	
	FOOD PELLET	SLENDER
16:0	28.1	28.9
16:1	0.3	2.1
18:0	2.5	21.0
18:1	15.1	26.9
18:2	47.1	15.3
18:3	4.6	1.7
20:0	-	0.3
20:1	-	-
20:2	-	-
20:3	0.3	-
20:4	-	1.0
24:0	-	0.2

Values are duplicates of two experiments

tumours are grown in animals fed on the slender diet rather than the normal one. This difference is statistically significant, $p<0.05$. Although the tumour plasma membranes from animals on the normal diet appear slightly more ordered than those from rats fed on the slender diet, these differences are not statistically different, either by considering the difference in polarization at a given temperature or by considering the lines each as a single entity.

2. Phospholipid classes

Synaptic membranes

Table 6.3 shows the phospholipid classes of the synaptic membranes taken from rats on the alcohol diet and their pair-fed controls. The predominant phospholipid classes are PC and PE, accounting for 70% of the total phospholipid, the percentage contribution of the PE is however slightly higher than the PC. The remaining phospholipids SM and PS/PI were present in roughly equal proportions. There is no difference in proportion of phospholipid class when one compares the synaptic membranes from the animals which have been administered ethanol with the synaptic membranes of their pair-fed controls. It is noted, however, that due to the loss of sample from one experiment the number of replicates is only two and futher experiments would have to be performed in order to be certain that no changes were taking place.

TABLE 6.3
Phospholipid classes of Synaptic membranes from rats fed on either sucrose or ethanol liquid diets

	% wt.			
Class	PC	PE	SM	PS PI
synaptic membrane sucrose diet	32.0±6.0	39.1±11.9	14.9±7.2	13.5±13.5
alcohol diet	30.0±4.0	49.8±4.3	11.1±0.1	7.6±1.3

n=2 values ±S.E.M.

PC = Phosphatidylcholine

PE = Phosphatidylethanolamine

Sph= Sphingomyelin

PS/PI = Phosphatidylserine & inositol

Mc7 tumour plasma membranes

Table 6.4 shows the phospholipid classes of Mc7 plasma membranes taken from rats on the alcohol diet and their pair-fed controls. PC and PE are the predominant types accounting for 70% of the total phospholipid and are present in roughly equal proportions in the two membrane types. The rest of the phospholipid moiety is made up of similar proportions of SM and PS/PI. There is also a very small amount of LysoPC present. There appears to be an increase in the ratio of PC/PE in the tumour plasma membranes of the alcohol-fed animals compared to the same membranes from the sucrose-fed controls, and also a decrease in the proportion of SM; these differences however are not statistically significant ($p > 0.05$).

3. Phospholipid fatty acid compositions of membranes from ethanol-fed rats

Synaptic membranes

Table 6.5 shows the total phospholipid fatty acid composition from the synaptic membranes of ethanol-fed and control rats. The predominant fatty acid in both cases is 16:0, and the other major ones are 18:0 and 18:1. The polyunsaturated fatty acids, 20:4 and 22:6 each account for about 5% of the total, there is also some 24:0 present and trace amounts of 20:3 and 20:0. There is no statistically significant difference between the fatty acid composition

TABLE 6.4
Phospholipid classes of Mc7 tumour plasma membranes from rats fed on either sucrose or ethanol liquid diets

		% wt.			
Class	PC	PE	SM	PS/PI	Lyso-PC
Tissue Mc7 sucrose diet	35.0±5.6	35.0±5.6	17.6±3.9	14.0±1.3	1.7±1.7
alcohol diet	45.5±7.6	26.7±7.9	13.7±4.0	12.7±3.1	1.0±1.0
Mc7 from normal diet	34.6±7.2	35.8±3.8	13.5±1.3	11.4±0.7	nd.

n=3 values ±S.E.M.

PC = Phosphatidylcholine

PE = Phosphatidylethanolamine

SM = Sphingomyelin

PS/PI = Phosphatidyl serine & inositol

TABLE 6.5

Fatty acid composition of total phospholipid fraction from synaptosomes

	relative % weight	
Fatty acid	CONTROL	ALCOHOL
16:0	35.3±2.7	37.9±2.5
16:1	-	-
18:0	27.4±2.9	25.7±4.2
18:1	21.0±1.5	21.3±1.1
18:2	-	-
18:3	-	-
20:0	0.2±0.2	0.4±0.3
20:1	0.4±0.4	-
20:2	-	-
20:3	0.2±0.1	0.1±0.1
20:4	5.5±2.8	4.8±0.5
20:5	-	-
22:0	-	-
22:1	-	-
22:2	-	-
22:3	-	-
22:4	-	-
22:5	-	-
22:6	3.9±2.9	4.1±0.4
24:0	2.0±1.7	1.7±0.3
24:1	-	-

n=3 values ± S.E.M.

of the synaptic membrane phospholipids of ethanol-fed and control rats.

Mc7 tumour plasma membranes

Tables 6.6 and 6.7 show the phospholipid fatty acid composition of Mc7 tumour plasma membranes from ethanol-fed and control, sucrose-fed, rats. These will be described class by class. The fatty acid profile from PE is not significantly different when comparing the two experimental groups. The dominant fatty acid is 18:0 and then 16:0 and 18:1 in roughly equal proportions. The other important fatty acid which is present in any amount is 22:6 (10%). The fatty acid profile for PC is similar with 16:0, 18:0 and 18:1 forming the bulk of the total % by weight; there is considerablely less 22:6 in this phospholipid class than in the PE, however. There is an elevation of 18:1 in the tumour phospholipid from the alcohol fed rats, when compared to the control. These differences are not statistically significantly different. The only significant differences between the plasma membrane fatty acid profile of ethanol-fed and control rats, lie in the SM class of phospholipid. The fatty acid 20:0 is lower in the tumour membranes from ethanol-fed rats, compared to control membranes, and 18:2 is higher. The elevation of the 18:2 is statistically different ($p>0.05$). There are insignificant differences between the fatty acids from the PS/PI fraction between ethanol and sucrose-fed rats. In total composition there is a decrease in 16:0 and an

TABLE 6.6
Fatty acid composition of main phospholipid types from Mc7 tumour plasma membranes after slender diet + sucrose CONTROL

	relative % weight			
Fatty acid	Phosphatidyl ethanolamine	Phosphatidyl choline	Sphingomyelin	Phosphatidyl serine/inositol
16:0	15.6±4.3	26.3±5.3	22.1±8.7	24.9±6.7
16:1	0.7±0.7	6.0±1.7	1.9±0.9	0.6±0.6
18:0	13.5±1.7	9.4±1.6	3.8±0.6	24.2±7.7
18:1	30.0±5.8	30.5±3.5	12.3±3.0	11.4±5.1
18:2	2.1±1.4	3.7±0.9	10.5±0.3	4.1±2.3
18:3	-	0.2+0.2	-	-
20:0	3.0±1.3	1.9±1.1	12.5±3.6	5.9±2.3
20:1	0.5±0.5	0.3±0.1	-	-
20:2	-	-	-	5.3±4.4
20:3	0.3±0.3	1.7±0.6	6.9±1.1	2.8±2.2
20:4	4.7±0.9	3.2±1.5	5.1±2.3	4.7±0.8
20:5	-	-	-	-
22:0	3.0±1.9	1.9±1.5	7.3±2.8	1.9±1.9
22:1	-	-	-	-
22:2	-	0.3±0.3	0.5±0.5	0.4±0.4
22:3	4.2±4.2	1.8±1.0	2.5±2.5	2.4±2.4
22:4	0.8±0.8	=	0.6±0.6	-
22:5	3.0±3.0	2.8±1.4	-	-
22:6	15.1±4.1	4.6±3.73	-	1.4±1.4
24:0	2.1±0.9	-	7.9±1.6	3.7±1.8
24:1	-	-	1.3±0.8	-

n=3 values ± S.E.M.

TABLE 6.7
Fatty acid composition of main phospholipid types from Mc7 tumour plasma membranes after slender diet + ethanol EXPERIMENTAL

	relative % weight			
Fatty acid	Phosphatidyl ethanolamine	Phosphatidyl choline	Sphingomyelin	Phosphatidyl serine/inositol
16:0	20.3±6.7	26.2±1.1	33.7±6.6	10.5±4.9
16:1	1.7±0.5	4.4±2.1	3.3±2.7	0.1±0.1
18:0	16.8±4.6	8.7±1.0	6.2±2.1	13.3±3.2
18:1	32.3±7.8	38.7±1.8	15.0±4.5	14.8±5.3
18:2	3.1±1.2	5.4±1.6	3.6±1.4	5.2±2.4
18:3	-	-	-	-
20:0	1.2±0.6	1.8±0.6	5.2±1.8	3.4±3.2
20:1	0.5±0.5	1.2+0.6	-	0.2±0.2
20:2	-	-	-	0.7±0.4
20:3	0.4±0.2	1.7±0.2	3.0±0.8	4.1±2.2
20:4	4.6±0.6	2.5±0.3	4.4±0.6	4.4±1.0
20:5	-	-	-	=
22:0	1.2±1.2	1.4±0.5	3.3±2.5	6.5±2.0
22:1	0.5±0.5	-	-	-
22:2	-	-	0.5±0.5	3.0±2.5
22:3	0.1±0.1	4.7±4.3	1.7±1.7	-
22:4	0.3±0.3	-	-	-
22:5	-	-	-	0.8±0.8
22:6	9.9±9.1	-	-	18.4+9.9
24:0	3.0±1.8	-	11.4±6.7	7.4±3.8
24:1	-	-	2.3+1.3	-

n=3 values ± S.E.M.

increase in 20:0 in the phospholipid fatty acid profiles from ethanol-fed rats, when compared to the same profile from control rats.

Figures 6.1 and 6.2 show the overall trends in the the fatty acid profiles of the total phospholipid fractions of the Mc7 tumour plasma membranes from rats on sucrose (control) and alcohol (experimental) diets. Figure 6.1 indicates that there is an overall increase in C-18 chain length, in the alcohol group, compared to the control group and figure 6.2 shows that this is due to an increase in mono-unsaturated fatty acids. This increase in mono-unsaturates in the tumour membranes from the alcohol bearing rats is almost certainly a product of the elevation of 18:1 in the PC fraction and the elevation of this phospholipid compared to other phospholipid classes.

4. Cholesterol/phospholipid ratios

Table 6.7 shows the relative level of cholesterol, phospholipid, and protein in both synaptic membranes and Mc7 tumour plasma membranes, with or without ethanol administration. In both membrane types dietary administration of alcohol causes an apparent increase in the level of cholesterol as compared to the control group. The level phospholipid is not different between experimentals and control, and therefore the cholesterol/phospholipid molar ratios are also elevated in the experimental membranes compared to the membranes from

Fig.6.1 Fatty acid chain length of total P.M. phospholipids

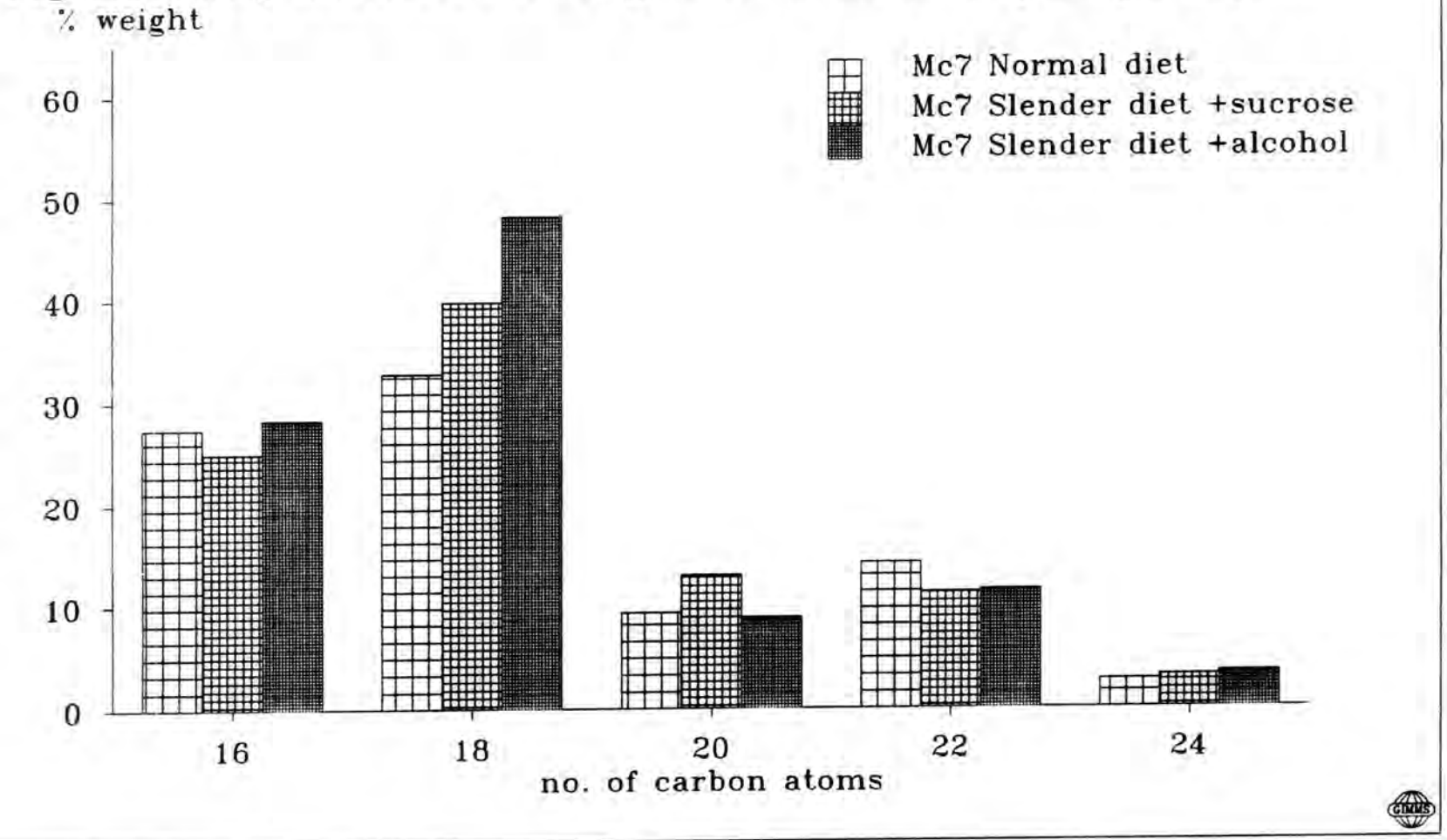


Fig.6.2 Fatty acid unsaturation of total P.M. phospholipids

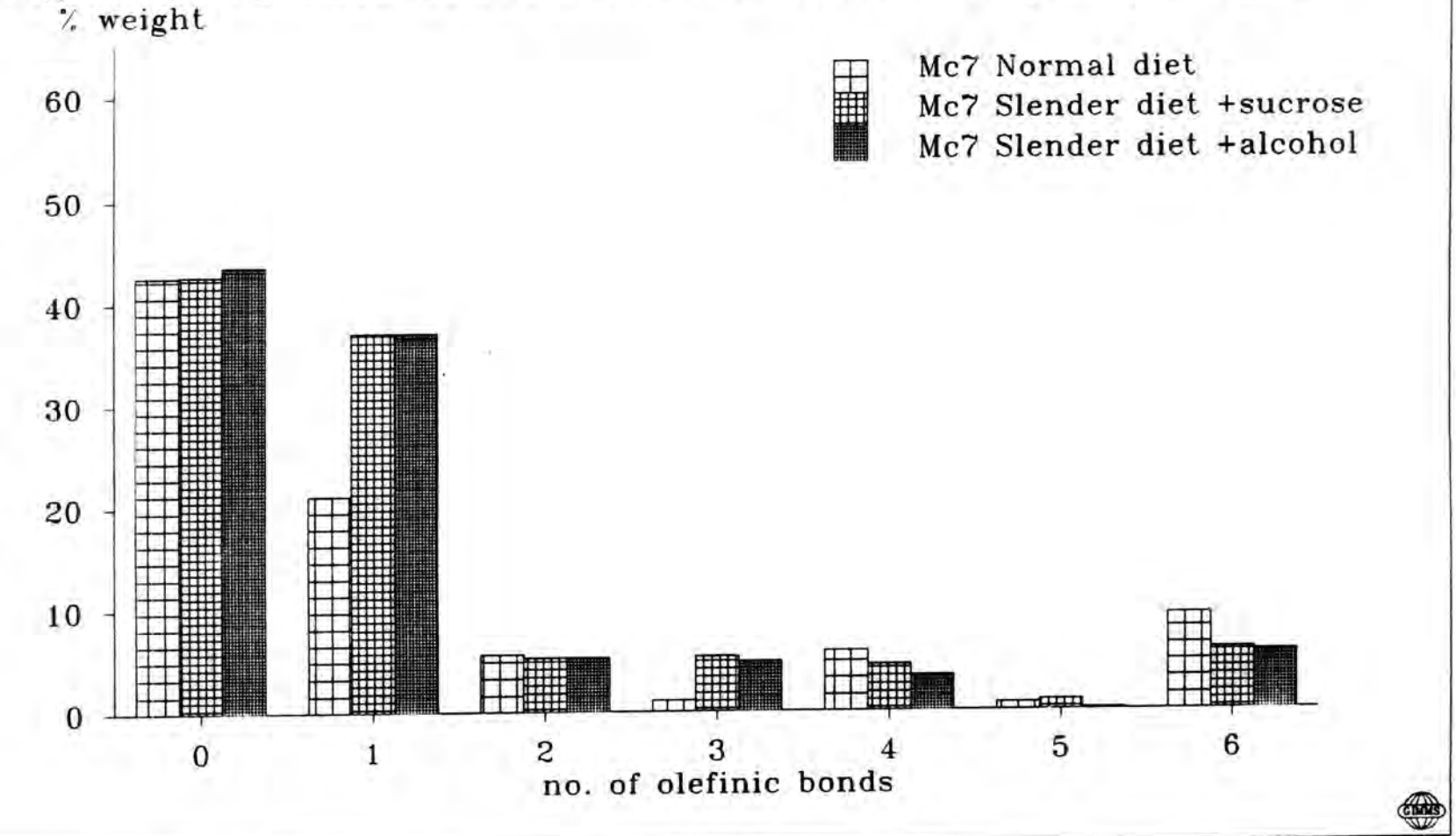


TABLE 6.8

Cholesterol/Phospholipid molar ratios of synaptosomes and tumour membranes from rats fed on ethanol or sucrose liquid diets

Membrane type	Cholesterol/ protein umoles/mg	Phospholipid/ protein umoles lipid P /mg	Cholesterol/ phospholipid Molar ratio
synapt'			
sucrose n=3	0.0303±0.0042	0.185±0.022	0.175±0.045
ethanol	0.0429±0.008	0.171±0.027	0.277±0.081
Mc7			
sucrose n=3	0.0125±0.0034	0.106±0.035	0.120±0.0006
ethanol n=3	0.0147±0.024	0.0812±0.004	0.178±0.029

the controls. This difference is not statistically significant, ($p<0.1$).

5. D.P.H. Steady state fluorescence polarization measurements

Synaptic membranes

Figure 6.3 and Table 6.9 show the change of the steady state fluorescence polarization of D.P.H. in the synaptic membranes from rats fed on either ethanol or sucrose, over the temperature range from 4-50^{o}C. No significant differences in polarization were found between membranes obtained from rats on an alcohol diet and those from sucrose-fed rats.

Figure 6.4 and Table 6.10 show a similar polarization vs. temperature plot for the Mc7 tumour membranes from control and ethanol-fed animals. A small consistent, but insignificant difference in polarization between membranes from ethanol-fed and control rats can be seen.

DISCUSSION

The aim of this aspect of the work was to study the long-term effects of ethanol administration on tumour cell plasma membranes. The purpose was to establish whether adaption to long term ethanol administration occurred, and whether a

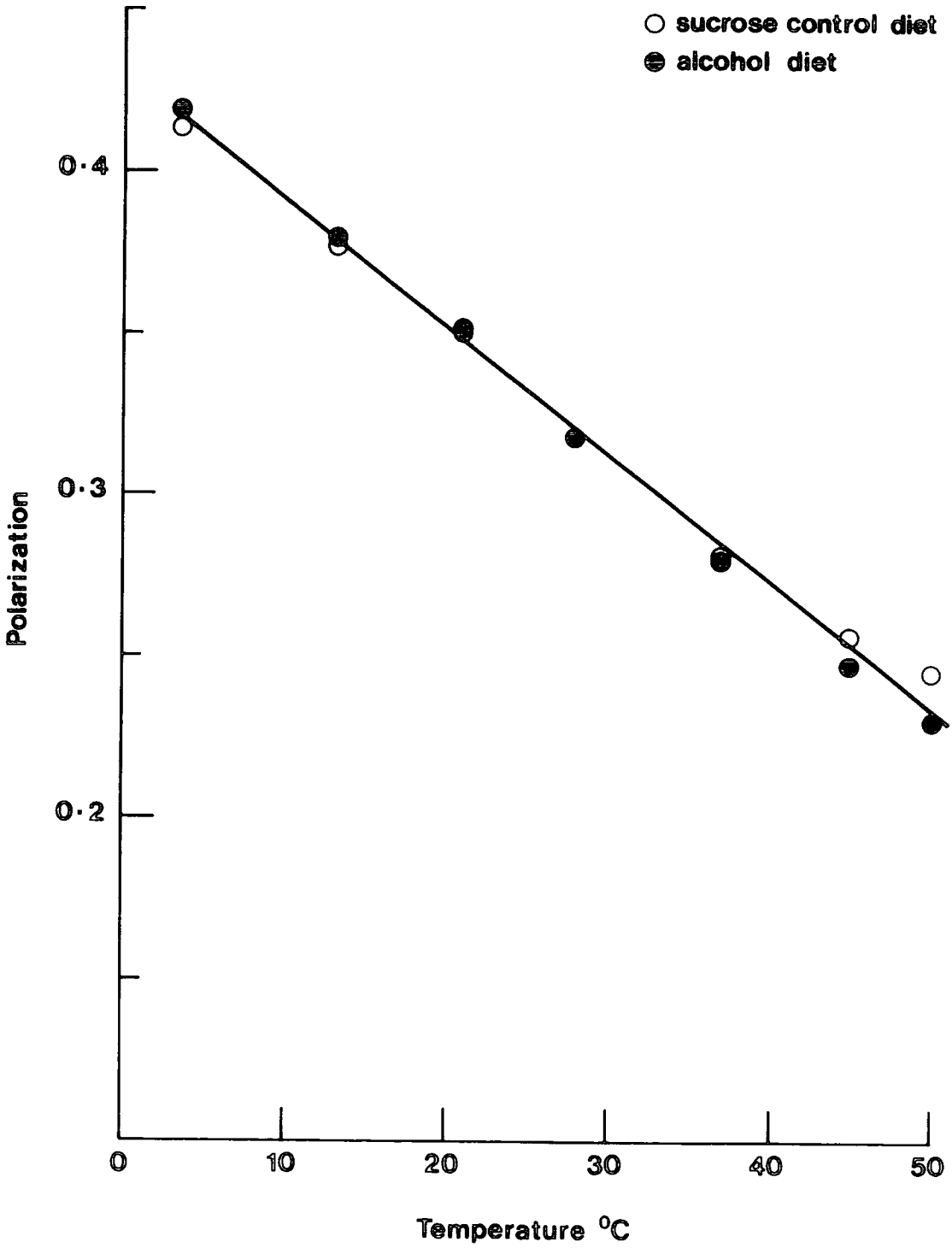

○ sucrose control diet
● alcohol diet
0·4
0·3
0·2
Polarization
0
10
20
30
40
50
Temperature °C

TABLE 6.9

Steady state fluorescence polarization of D.P.H. in the synaptic membranes from ethanol- and sucrose-fed rats

	Polarization	
Temp. °C	CONTROL	ETHANOL
3.5	0.414±0.0157	0.418±0.016
13.6	0.378±0.0042	0.379±0.0072
20.6	0.350±0.0028	0.351±0.002
28.0	0.319±0.0038	0.319±0.0043
36.8	0.282±0.0032	0.281±0.0057
45.0	0.257±0.01	0.247±0.007
50.4	0.246±0.016	0.230±0.009

n=3 values ± S.E.M.

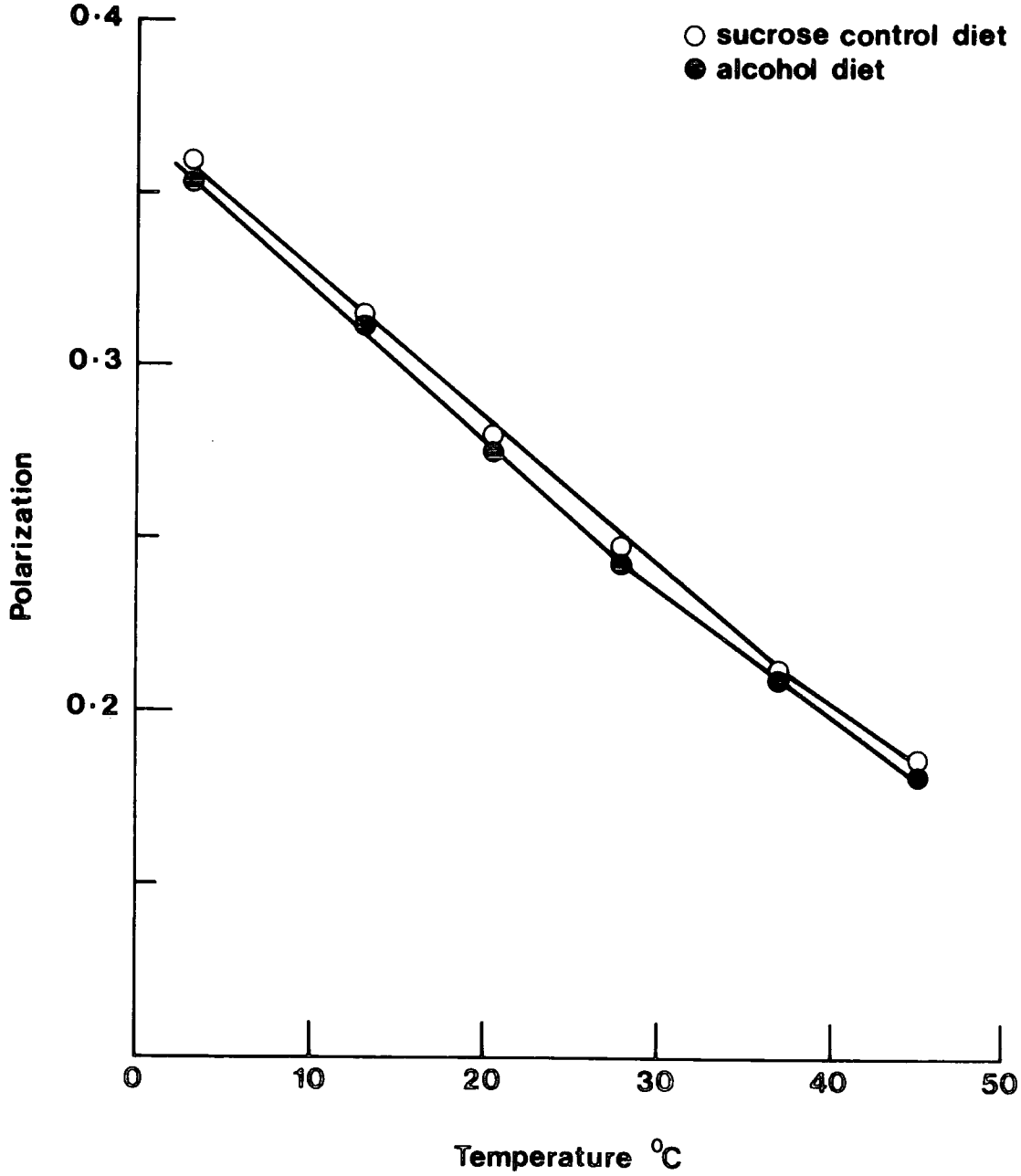

0·4
0·3
0·2
Polarization
○ sucrose control diet
● alcohol diet
0
10
20
30
40
50
Temperature °C

TABLE 6.10

Steady state fluorescence polarization of D.P.H. in the Mc7 tumour P.M.s from ethanol- and sucrose-fed rats

	Polarization	
Temp. °C	CONTROL	ETHANOL
3.4	0.358±0.0126	0.355±0.0096
13.3	0.315±0.0043	0.312±0.0078
20.6	0.281±0.0008	0.276±0.0033
28.0	0.249±0.0009	0.245±0.0023
36.8	0.213±0.0015	0.210±0.0015
45.0	0.186±0.0015	0.183±0.0012
50.4	0.164±0.0058	0.165±0.0027

n=3 values ± S.E.M.

differential pattern of adaption was achieved in tumours and control cells.

It is clearly important to appreciate the consequences of long term ethanol administration. The levels of blood ethanol, for example, may be crucial in determining the time course and magnitude of any 'adaptive' response. The measurement of blood ethanol levels revealed a wide range of values within a given set of animals, and also variation in the mean ethanol level between the three experiments. To some extent the variation within a given experiment may be due to feeding behaviour of the rats. On some occasions too some difficulties were experienced with 'Slender' blocking the food dispenser towards the end of the ration. Other workers have reported variations in ethanol levels over a 24 hour period, presumably due to behavioural variation in feeding. For example, Shorely et al. 1982 found that the ethanol level was above 0.2%(w/v) at 22:00 hours, but had dropped to 0.05%(w/v) at 7:00 hours the next day. This was on a feeding schedule which started at 5:00 hours each day. The feeding schedule in this present study started at 11:00 hours each day, and animal sacrifice was at 9:30 hours. Therefore the work of Shorely and co-workers suggests that the level of blood ethanol at sacrifice would not be maximal. and that the average levels of about 0.09%(w/v) for blood ethanol which were found in the animals from the work reported here, would be expected to produce ethanol dependent rats. Other workers who have rigorously tested the ethanol dependency of the rats in their experiments have shown that ethanol levels similar to those found in the current work produce dependency, for example

Rangaraj and Kalant, (1982), recorded blood alcohol levels of 0.104±0.068% (w/v) and LaDroitte *et al*. (1984) who reported blood alcohol levels of 0.17% (w/v).

The method by which ethanol is administered may cause metabolic effects not owing to ethanol itself. For example, IP injection or intubation can cause stress which is known to effect lipid metabolism. Inhalation, which has been used by several workers eg. Goldstein, (1972), is preferable, but it is difficult to set up the proper controls. The methodology of administration of ethanol by liquid diet has been reviewed by Lieber and DeCarli, (1982) and they suggest this is one of the best methods available. The present technique followed the method of Chin and Goldstein, (1978). Care must be taken to make sure that the control diet is balanced iso-calorifically, in our study this was done by adding sucrose. It must be appreciated, however, that it is not strictly correct to equate the calorific value of ethanol with that of sucrose, because the metabolism of the two may be very different. Shorely *et al*. (1982), have shown that fatty acid induced changes found in the phospholipids of gastrocnemius muscles after ethanol administration were not apparent if pair-fed controls were used. The discrepancy they attributed to the fact that fatty acid changes were a product of the reduced food consumption which accompanies ethanol administration. Similar changes were apparent in the current study when one considers the lipid composition of the Mc7 plasma membrane from normal diet animal fed *ad lib*. and compares it with the lipid composition of Mc7 tumour plasma membranes from the pair-fed controls, fed on

slender and sucrose (iso-calorific with the ethanol) consumed by the pair-mate. These differences in lipid composition will be discussed in more detail, before the difference between alcohol and control preparations are considered.

Changes in plasma membrane lipid composition which relate to diet

The differences between plasma membranes phospholipid fatty acid composition of the tumours in animals on normal diet and slender/sucrose-fed controls may be in part attributable to the differences in the fatty acid compositions of the two diets, shown in Figure 6.2. There was a greater proportion of 18:1 in the slender diet when compared to the normal food pellet diet: this appeared to produce an increase of this fatty acid in the plasma membrane of the Mc7 tumour. This increase occurred in all phospholipid classes, but was only statistically significant in the PC, ($p<0.02$). Although there was also a reduced proportion of 18:2 in the slender compared to normal diet, it was difficult to equate this to lowered levels of this fatty acid in the phospholipids of the tumour plasma membranes from the slender fed animals, as compared to the same membranes from animals on a normal diet. This is because the differences in the level of 18:2 in the membrane phospholipids were insignificant. There was no significant change in the phospholipid classes of the tumour plasma membranes when the diet was changed, and the only other noticeable change in lipid composition between the plasma membranes of the Mc7, when the diet was changed from normal to slender, was in the level of

cholesterol

There appears to be a lowering of plasma membrane cholesterol when the tumours are grown in animals fed on the slender diet rather than the normal one. This difference is statistically significant ($p<0.05$). The reduction in plasma membrane cholesterol of animals on the slender, as compared to the normal diet, is almost certainly due to a reduction in the fat content of the slender diet ie. it is marketed as a low-fat nutritionally balanced diet for slimmers! Since a low level of cholesterol in foods is at the moment considered virtuous, it is unsurprising to find a low level of this lipid in health food. Intake of fat is further reduced, as pointed out by Shorely *et al.* (1982), because the animals have a reduced food intake anyway. This latter point is illustrated by the fact that the growth rate of tumours from the pair-fed sucrose controls is less than it is in the same tumour fed on the normal diet *ad lib*.

In summary the differences in the tumour membrane lipid composition of rats fed on normal compared to the slender diet are: an elevation in the level of 18:1 in the membrane phospholipids of the slender-fed animals compared to the equivalent membrane phospholipids from rats on a normal diet, and a lowered level of plasma membrane cholesterol in the animals which have been on the slender compared to normal diet.

Changes in plasma membrane lipid composition due to ethanol

administration

There are no significant differences between the relative contribution of phospholipid classes in the synaptic membranes from animals fed on ethanol when compared to the same sort of membrane from animals on the control sucrose diet. However White, (1973) in his review, reports phospholipid classes which are different from the control membranes presented here, the main difference is the relative contribution of PC to PE, which is greater than in the current results. The variation between different literature reports is likely to be a reflection of i) diet and ii) the relative degree of purity of the synaptosome preparation used in the study. The synaptic membrane fraction used in this study, for example, was fairly crude, and may have contained mitochondrial contamination.

Any changes in the phospholipid classes of the Mc7 tumour membranes after ethanol administration are difficult to see as they are masked by the variation in the results obtained. For example there is a slight, but not statistically significant, increase in PC after ethanol administration. The literature appears to confirm these findings, as most workers have been unable to show changes in the phospholipid classes of plasma membranes after ethanol administration, (eg Harris *et al.* (1984) Cunningham *et al.* (1982). However, Vrbaski *et al.* (1984) report a decrease of PC, PE, and PS and an increase of PI in whole brain lipid extracts.

In the current work the synaptosome phospholipid fatty acid

compositon remains unchanged during ethanol administration. In the literature differences have been reported for example Littleton et al. (1980) have shown an elevation of 18:1 and a lowering of 22:6 in the synaptic membranes of three different strains of mice following ethanol administration; and Alling et al. (1984) report an increase in 18:1 in the PC from rat brain synaptosomes. The opposite trends have also been reported eg. Sun and Sun, (1979) have shown increases in the proportions of polyunsaturated fatty acids and decreased proportions of mono-unsaturated fatty acids in guinea pig brain synaptosomes following ethanol administration. All these reported changes are small. Other workers, in agreement with current data, have reported no changes in rat synaptosome phospholipid fatty acids after ethanol administration (Crews et al. 1983). Even those workers who have been able to show changes in the fatty acid composition are skeptical about whether such changes are the result of a homeoviscous adaptive response. Alling et al. (1982), for example, have shown that there is a correlation between changes in fatty acid composition in the blood serum, and changes in the fatty acid composition of synaptic membranes.

There are, however, noticeable changes in the fatty acid composition of the phospholipids from Mc7 tumour plasma membranes, after ethanol administration.

The elevation in 18:1 observed in the PC of Mc7 plasma membranes has also been reported in the PC of plasma membranes from other tissues besides the brain mentioned above. For example Dunbar et al. (1981) have reported an increase in this

fatty acid in phospholipids from erythrocyte membranes; and the elevation was seen in two sets of animals, one group had ethanol administered by IP injection, the other as a vapour. This increases confidence that the changes induced are not simply the result of the method of ethanol feeding. In addition, LaDroitte et al. (1984) have shown that the fatty acid changes brought about by ethanol administration are not the result of the reduction in food intake which parallel intoxification. Erythrocytes are not, however, very good models for observing any 'deliberate' adaptive process as changes in their fatty acid composition are known to reflect blood fatty acid changes which occur during ethanol administration (Alling et al. 1982). Littleton and John, (1977) as well as other workers have reported decreases in polyunsaturated fatty acid in plasma membrane phospholipids following ethanol administration. Such changes were not apparent in the Mc7 plasma membranes. Dunbar et al. (1981) have wisely pointed out that the increase in 18:1 is not proof of any adaptive reponse, as the first unsaturated bond in a phospholipid fatty acid is known to have a pronounced effect on bilayer fluidity (ie. it increases it). Sphingomyelin is known to have a stabilizing effect on membrane, and so any changes in its fatty acid composition after ethanol administration may be important. The levels of 18:2 were significantly lower ($p<0.01$) in the sphingomyelin of Mc7 tumour plasma membranes from ethanol-fed rats when compared to the sphingomyelin from tumour plasma membranes from rats on the control diet. This may be an adaptive response of the tumour cell to ethanol, although this finding is unique, there are no such changes reported in the literature for other plasma

membrane types. Finally an observation by Wing et al. (1984) has some bearing on the variation in the literature of the type of fatty acid changes elicted in membranes following ethanol administration. They noted that the same administration regime, and the same strain of mice, produced different control values, when the experiments were separated by several months. Therefore the changes seen in these two series of experiments were different because the original control levels of phospholipid fatty acids varied.

Table 6.8 shows that there is a tendency towards higher synaptic and tumour membrane cholesterol levels after ethanol administration. However neither of these differences are statistically significant. The levels of cholesterol in the synaptic membranes of animals fed on the slender diet were lower than those reported by Chin and Goldstein, (1978) using the same feeding regime. There are two crucial differences which might explain this: i)they were two different species of animal, and ii) the isolation method for obtaining the synaptosomes was different, and so the purity of the two preparations could be different. Although Chin and Goldstein have shown increases in plasma membrane cholesterol in both erythrocytes and synaptosomes, other workers have been unable to show such changes in similar systems, eg. Dunbar et al. (1981). Indeed Harris et al. (1984) have shown a slight decrease in synaptosome cholesterol levels after ethanol administration.

Although the elevations in membrane cholesterol are not statistically significant, the magnitude of the increase appears

to be greater in the Mc7 tumour plasma membranes than in the synaptosomes. This may indicate (especially since the synaptic membranes were exposed to ethanol 6X longer than the tumour plasma membranes) that the tumour membranes are able to respond as effectively, if not more so, than the equivalent membranes in normal cells, when in the presence of ethanol.

Changes in plasma membrane cholesterol after ethanol administration, like changes in phospholipid fatty acid composition, may be an indirect effect of ethanol on metabolism rather than an 'adaptive' response. Littleton et al. (1980) have remarked that such changes could have been equally caused by the following. i) nutritional deficiencies associated with ethanol intake (eg. Mansbach, 1983 has shown that ethanol administration reduced the neutral lipid absorption in the rat intestine). ii) lipid peroxidation caused by ethanol; and iii) other metabolic effects unconnected with any adaptive mechanism. For example, Baraona and Lieber, (1979) have shown that ethanol produces elevated blood cholesterol levels in humans. A very important observation has been made by Smith et al. (1982), they found, in lipid extracts from rat hepatocytes, that the nature of the change induced by ethanol was modulated by the type of diet the animals were on. They found that increases in hepatic cholesterol, during ethanol administration, were a function of the level of corn oil in the diet.

Physical studies, using fluorescent and E.S.R. probes, have shown that ethanol decreases membrane order (Chin & Goldstein, 1977; Harris & Schroeder, 1981; Harris et al. 1984;

Rottenberg et al. 1981; and Waring et al. 1981). The appropriate adaptive response to long-term ethanol administration would therefore be to increase membrane order to compensate for this fluidizing effect. This is unlikely to occur as the result of arbitrary metabolic changes due to the presence of ethanol, and therefore is more likely to indicate whether there is any true adaptive response to the ethanol.

The steady state fluorescence polarization of D.P.H. in synaptic membranes from ethanol-fed rats is not significantly different from the controls. There is, however, some difference between Mc7 tumour membranes which have been exposed to alcohol, as compared with the equivalent membranes from the pair-fed controls. Although this difference is not statistically significant, it is consistent throughout the temperature range 4-50°C. It is also in the opposite direction to what one would expect after long-term exposure to ethanol. The plasma membranes appear less ordered after ethanol administration, rather than more ordered as one might expect if there was an adaptive response (or the same order if no adaption had taken place). Chin & Goldstein, (1977), when they used E.S.R. to study changes in synaptosome order after ethanol administration, found no differences in the order between membranes exposed to ethanol and those which had not. They did note, though, that the membranes which had been exposed to long-term ethanol administration were disordered less by the presence of the alcohol than membranes which had not been so exposed. This indicates that the adaption may be a subtle one. Crews et al. (1983), however, were able to show (using D.P.H. fluorescence

polarization *in vitro*) that there was a parallel between increased synaptosome order and the elevation in the cholesterol/phospholipid ratio. They were also able to show the the *in vitro* administration of cholesterol mimicked the type of ordering seen in the membranes from ethanol dependent rats. The results of Harris *et al*. (1984) are in conflict with the above study. They showed that synaptic membranes (from ethanol dependent rats) had an increased order, but did not have an increased cholesterol/phospholipid ratio.. Furthermore, liposomes created from lipid extracts of the ethanol adapted membranes had the same order as liposomes from synaptic membranes which had not been exposed to ethanol. These latter workers have therefore suggested that changes in membrane order during ethanol administration are the result of lipid or lipid/protein complexes being rearranged. These various conflicting reports show that the problem of how membranes respond to the long-term administration of ethanol is a complex one.

It is worth noting at this stage that a single ethanol administration experiment was carried out using the male rat and D23 tumour. Several differences were seen between the tumour membranes from the ethanol fed rats and the same membranes from the pair-fed sucrose controls. There was an elevated level of cholesterol in the plasma membranes from the tumours which had been in the ethanol dependent rats. The order of the membranes from the ethanol dependent rats was less than those from the control animals. This is interesting as it supports the small insignificant trends which have been noted in the Mc7 tumour

membranes from ethanol dependent rats. It may be, therefore that these small changes would become statistically significant if enough replicates of these membrane preparations were obtained. The problem is that the variation in membranes between different isolations masks the small differences in membrane composition and structure which are due to the ethanol feeding. The most important effect which requires further investigation is that the order of the tumour plasma membranes which have been subject to ethanol administration is lower than the order of the equivalent membranes from control animals. This is the reverse of what one would expect if the membranes were adapting to the presence of a membrane perturbant such as ethanol, and is not due to the continued presence of ethanol in the membranes, as all the ethanol would have been washed out in the lengthy isolation procedure. It is also important to note that the normal synaptic membranes (after ethanol administration) did not exhibit this trend towards decreased membrane order. Therefore it appears to be specific to the tumour membranes.

The first point about this unexpected effect is that it overides the compensation one would expect from the elevation of plasma membrane cholesterol. However the level of the fatty acid 18:1 is higher in the phospholipids from tumour membranes after ethanol administration. This elevation does not occur in the synaptic membrane phospholipids which suggests it may be an important factor in determining the difference in order of the two membrane types when they respond to the presence of ethanol. This is because 18:1 is known to have a significant fluidizing

effect on the membrane when incorporated into the membrane phospholipids, (Van Deem et al. 1971). It could also be suggested that a structural change has taken place which is unrelated to the chemical composition of the membranes per se. It is important to remember that the tumour plasma membranes are already very fluid compared to those from normal cells, (See Fig. 4.1) The presence of ethanol could increase this fluidity to a level whereby the tertiary structure of the membrane proteins is disrupted, and denaturation might take place. This argument is consistent with the suggestion of Harris et al. (1984) that protein/lipid complexes are important in producing an adaptive response to ethanol. Such complexes could be damaged in the tumour plasma membranes. There are several reports of the disruptive effects of ethanol on membrane proteins in the literature. Rubin and Rottenburger, (1982) have reviewed the inhibitory effects of ethanol on amino acid transport, and Lee and Hosein, (1982) have shown that there is a decrease of glucagon binding sites in hepatic plasma membranes following ethanol administration. This persisted over 72 hours after withdrawal, and indicates some kind of irreversible damage may be taking place. Kane et al. (1980); and Schanne et al. (1980) have shown that toxic liver necrosis, following prolonged exposure to ethanol, generally involves damage to the plasma membrane, an effect that results in lethal amounts of calcium ions entering the cell. It therefore seems likely that the long-term administration of ethanol may selectively damage tumour plasma membranes because they are already more fluid than those from normal tissue.

There therefore may be some basis to the suggestion, made at the beginning of this chapter, that tumour cells may not be competent to adapt to the presence of a membrane pertubant, and therefore may be selectively sensitized to hyperthermia. Whether this damage is apparent *in vivo* will be examined in the next chapter. The main draw back of such a suggestion is that the clinical implications of the long-term administration of a membrane perturbant, such as ethanol, may not be a feasible suggestion in treatment. One further line of investigation, which has been highlighted by the current study, is that dietary manipulation of membrane composition may be a far safer method of elicting changes in plasma membrane structure in order to sensitize tumour cells to heat.

7. TUMOUR TREATMENT REGIMES USING MEMBRANE PERTURBANTS

INTRODUCTION

The results presented so far in this study have suggested that the plasma membrane is a site of primary heat lesion in cancer cell death (Chapters 2-5). They have also shown (Chapter 5) that tetracaine, a membrane perturbant, enhances the heat damage to the plasma membrane. Although the *in vitro* results suggest membrane perturbants, such as tetracaine, could serve to potentiate the hyperthermic killing of cancer, clinically, the *in vivo* situation is often different as other physiological factors eg. blood flow, pH(Song, 1982) and the immune response (Dickson & Shah, 1982) have to be taken into account.

Two studies by Yatvin's group, using the local anaesthetic, lidocaine, have shown that a membrant perturbant does enhance tumour regression when applied with hyperthermia, compared to hyperthermia treatment alone (Yatvin *et al.* 1979 & Robins *et al.* 1982). However, so far, few *in vivo* studies have been reported using this treatment regime. In this section of the work the thermal sensitivity of the D23 and Mc7 tumours, used for the *in vitro* studies earlier in this thesis, were assessed *in vivo*; and any enhancing effect of tetracaine, when applied together with heat, was determined.

A second type of *in vivo* heating experiment was carried out on tumours grown in ethanol-dependent rats. The reason for this was to see if there is any increased tolerance of the D23 tumour to heat when it is grown in the presence of a membrane fluidizer, compared to the same tumour grown in pair-fed controls. This of course means withdrawing the ethanol before heating so that any effect observed was the result of long-term adaption, and not due to the presence of the ethanol still in the plasma membranes. The basic assumption of this scenario is that the adaption due to the presence of a drug, in this case ethanol(Chin & Goldstein 1977 and Chin *et al*. 1978) is similar to the adaption produced by increased environmental temperature, (homeoviscous adaption). One study by Anderson *et al*. (1983) has shown that heat resistance is induced in mouse ear by the long-term administration of ethanol. This study indicates that there is indeed a similar adaptive response to heat and ethanol. However, Littleton has pointed out in his review (Littleton, 1983), that the type of homeoviscous adaption which is a result of changed environmental temperature is different from the type of adaptive response to drugs, particularly ethanol. This is because the changes in lipid compostion and increase in membrane order which is associated with the classic homeoviscous adaption to temperature is not a prerequisite for tolerance induced by such drugs as ethanol (Chin & Goldstein, 1977). The current experiments will indicate whether the tumour is able to adapt in a similar way to ethanol as is observed in normal tissues, or whether, in fact, it is unable to do so. If not, there is the possibility of producing a differential heat sensitivity between normal and tumour tissue *in vivo*, which could be exploited

clinically.

The convenient site for heating experiments is the dorsum of the rats foot. Although this procedure is commonly used to assess hyperthermia treatment regimes, it has been criticized as a tumour model by Hill and Denekamp, (1982), because of the difference between the extremity and core temperature. There is also some dispute about the use of anaesthetics for sedation during the treatment. The animals obviously need to be restrained in some way, and the administration of the general anaethetic, Nembutal, is the most common method (Shrivastav et al. 1983) but this anaesthetic has been shown to interfer with the vasomotor tone (Marshall & Wallman, 1980) It is also not very sensible to heat in the presence of one anaesthetic whilst trying to assess the therapeutic value of another in the treatment regime, despite the fact that control heatings, with similarly anaesthetized animals would be established. For these reasons, the animals were physically restrained, but remained unanaesthetized during the hyperthermia treatment. It must be borne in mind of course that stress can effect tumour blood flow when interpreting the results(Zanelli & Lucas, 1976)

MATERIALS AND METHODS

The D23 and Mc7 tumours were maintained in male and female Nottingham WAB/NOT inbred rats, respectively. Prior to a heating experiment 0.1ml of a chopped tumour suspension, the preparation of which is described in Appendix 1, was injected into the dorsum of the rat's left-hind foot. This was then

allowed to grow for 7-10 days. The animals were restrained in perspex tubes with the appropriate foot projecting out and taped to a spur. The foot was heated at 44°C for 1 hour in a thermostated water bath. The internal tumour temperature was measured using a thermocouple, and was found to reach ±0.1°C of the bath temperature within the first 5 minutes of heating. Prior to heating either 50uL of saline or tetracaine (0.262M), in saline was injected into the tumour mass before heating. Animals were sacrificed when the tumour volume reached 1ml.

The other type of heating experiment was carried out on tumours which had been maintained on the ethanol diet described in Chapter 7, Materials & Methods, section 1. The animals were withdrawn from the ethanol 12 hours before heating in order to ensure that no ethanol was present in the membranes during the heating. The D23 tumours, in ethanol-fed rats and sucrose controls, were heated in the same way as described in the last paragraph. Animals were again sacrificed when the tumour volume reached 1ml.

RESULTS

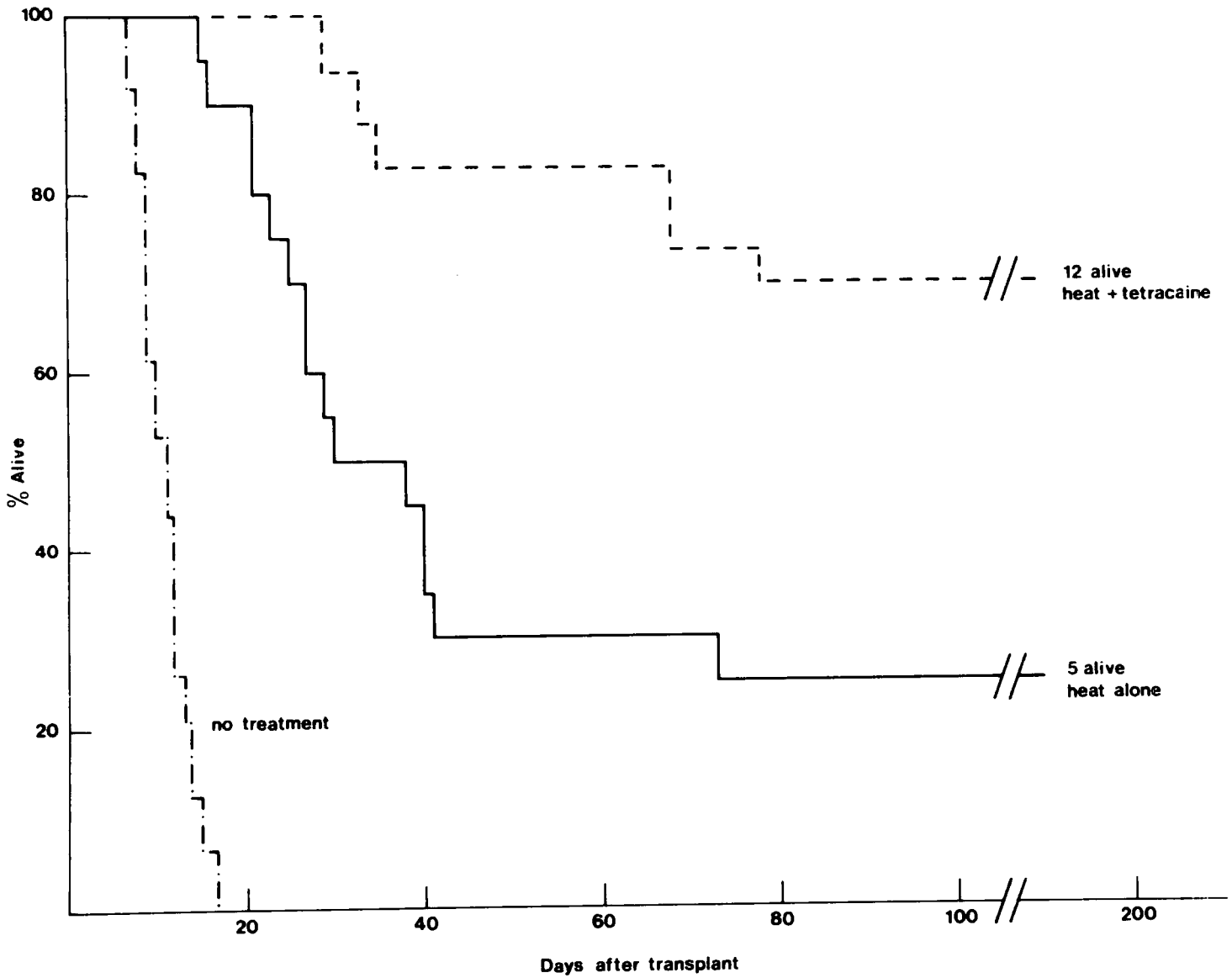
Preliminary experiments have shown that tumour growth is exponential and that tumour regression did not occur spontaneously. In consequence it was decided that the experiments would be terminated when tumour volume reached 1ml. At this size the tumour caused the animal no inconvenience nor discomfort, but it was clear that, at the observed rate of tumour growth, discomfort at least, and death at the worst was

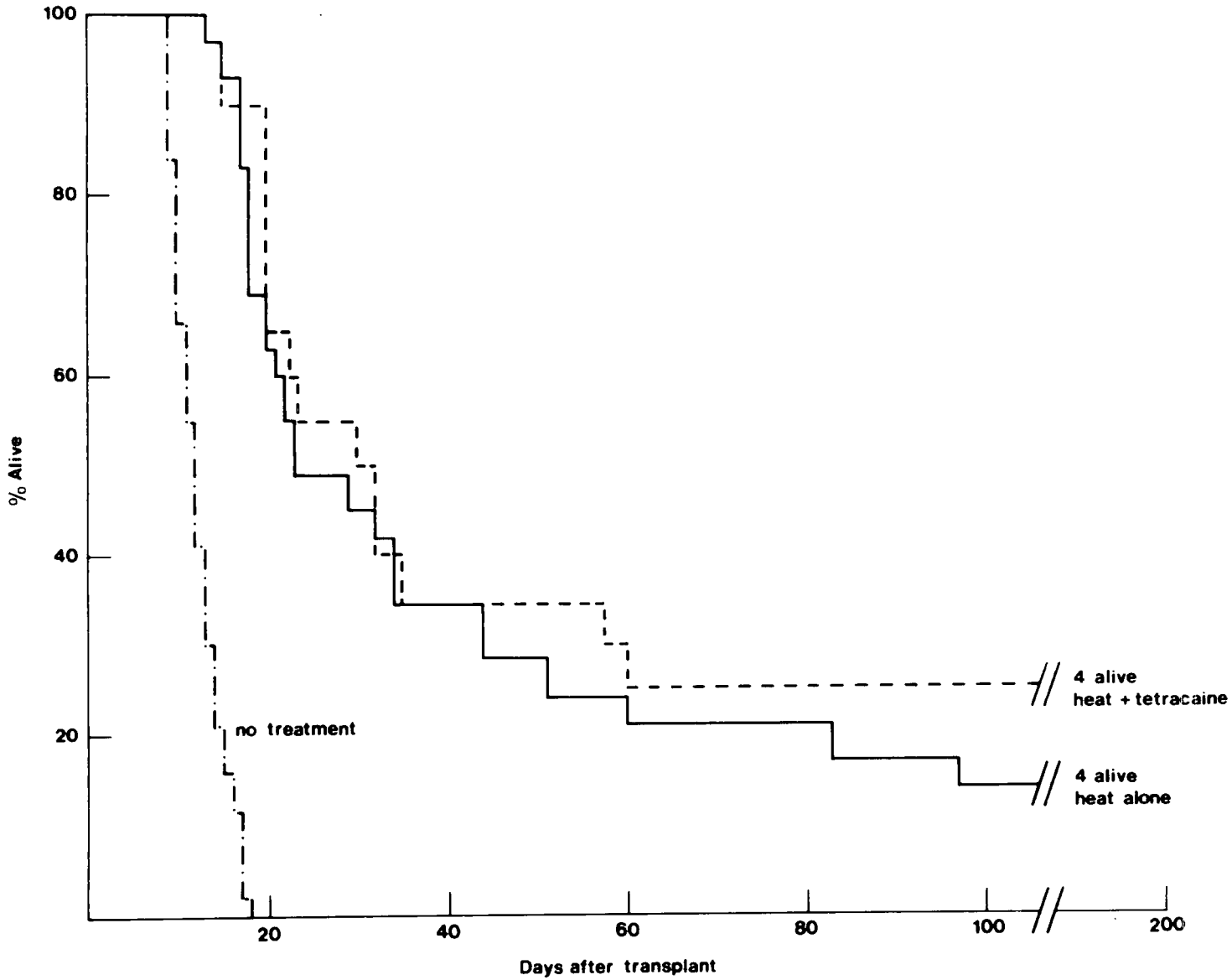
inevitable.

Figures 7.1 & 7.2 show the survival of rats with D23 and Mc7 foot tumours respectively. There are three conditions: i) no treatment, ii) heat treatment for 1 hour at 44°C with a 50uL injection of saline into the tumour, and iii) heat treatment for 1 hour at 44°C with a 50uL injection of 0.262M tetracaine in saline (dose=4mg) into the tumour. The animals were sacrificed when the tumour volume reached 1ml, thus %age Alive is not a measurement of death due to the tumours per se.

In figure 7.1, which shows the effect of the treatment regime on the D23 tumour, all the animals receiving no treatment were sacrificed within the first 16 days after the transplant, and 50% of these were sacrificed within the first 10 days. The effect of heating alone for one hour at 44°C was to delay the onset of the first death until 15 days, and considerably prolong the average lifespan of the animals, as growth of the tumour was arrested, or reduced in most of the rats. It took 30 days for 50% of the animals to die, and 33% of them were still alive, with no tumour regrowth, after 200 days. These were considered to be cured. The combination of the same 'dose' of heat and tetracaine produced even greater survival. The first death was delayed until 34 days, and 75% of the animals showed no tumour regrowth after 200 days. In the cases of heat treatment alone, or heat treatment plus tetracaine, many of the animals have now lived their natural span without any reoccurrence of the tumour.

Figure 7.2 shows the effect of the same treatment regime on


100
80
60
40
20
% Alive
no treatment
12 alive
heat + tetracaine
5 alive
heat alone
20
40
60
80
100
200
Days after transplant


100
80
60
40
20
% Alive
no treatment
4 alive
heat + tetracaine
4 alive
heat alone
20
40
60
80
100
200
Days after transplant

the Mc7 tumour, and there are considerable differences, in terms of animal survival, between this tumour, and the D23 described above. In the untreated animals the tumour reached 1ml in 50% of the animals after 12 days, and all of them had to be sacrificed by the 18th day. This is a similar growth pattern to the D23 tumour. The effect of heat treatment alone did not delay the Mc7 tumour growth to the same extent as in the D23 tumour, 50% of the animals being sacrificed after 23 days, and there was only 14% survival after 200 days. But the most important observation was that the combined effect of heat and tetracaine, which was potent in the D23, did not potentiate the effect of heat to the same degree, and after 200 days only 25% of the animals were are considered 'cured'. Therefore the response of the two tumours to the heat treatment regimes is very different.

These heatings were carried out over a 2 year period and result from some 10-12 separate heatings of tumour material from different transplants. Some differences in sensitivity were noted between experiments for both tumours, but only in the case of the Mc7 was this difference dramatic. In the early experiments (1982) Mc7 was the more sensitive of the two tumours to heat, but by 1984 it was more resistant.

In both tumours there was occasionally regrowth of the tumour higher up the leg in the thigh of the animal. This type of regrowth accounted for some of the deaths around 50, 60 and 70 days, for the D23 and Mc7.

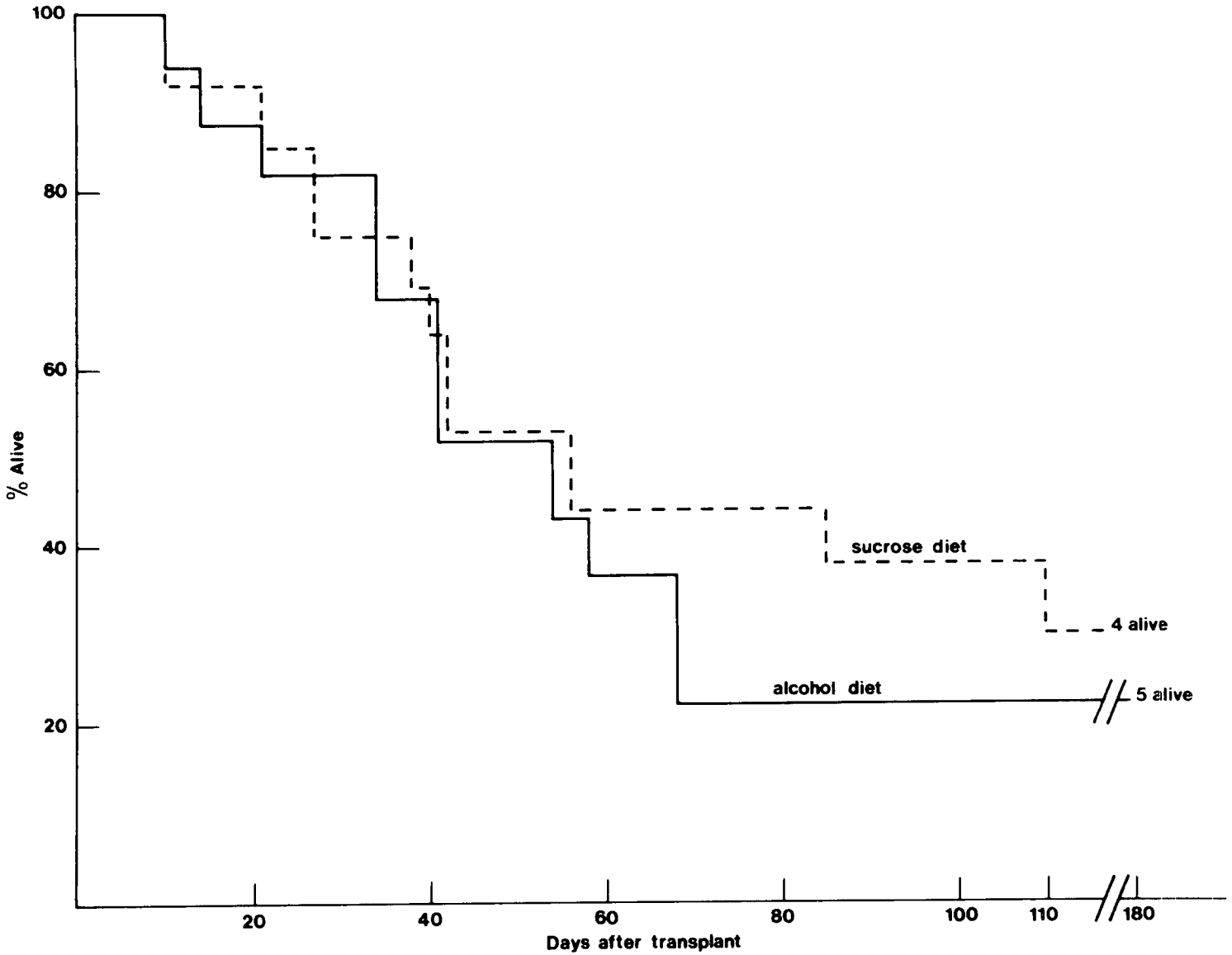

100
80
60
40
20
% Alive
sucrose diet
4 alive
alcohol diet
5 alive
20
40
60
80
100
110
180
Days after transplant

Figure 7.3 shows the effect of long-term ethanol administration on the sensitivity of the D23 tumour to heat. There is no real difference between the survival of animals on the control sucrose diet, and those which were fed ethanol. The 'cure' rate in both cases is about 30%. It must be noted, however, that these results were from a relatively small number of animals. If there was a small difference in tumour sensitivity between the two dietary regimes, they would be difficult to detect until more data had been collected.

If one compares these last results with those for the effect of heat on the tumour in animals on a normal diet, it is noticeable that tumour regrowth takes longer in the animals fed on the slender diet ie. 50% of animals have a tumour volume of 1ml after 41 days in the 'slender' experiment, but in the same experiment with animals on a normal diet it took only 30 days for 50% of the animals to have a tumour size reaching 1ml.

DISCUSSION

The results presented in figures 7.1-7.3 confirm that hyperthermia will produce tumour regression and 'cure' either alone or in the presence of the membrane perturbant tetracaine, when rat foot tumours are heated for 1 hour at 44^{o}C. The efficacy of this cure, however, is different in the two tumours studied.

The D23 hepatoma is considerably more heat sensitive than the Mc7 sarcoma. Heat alone in the D23 will produce a 30% cure

in the rat, whereas in the Mc7 the cure is only 14%. The tumour regrowth in the Mc7 is more rapid than the D23. What is also interesting is that although the administration of tetracaine during heating produces a substantial improvement in the regression of the D23 tumour (75% are cured). the same regime only produces a slight improvement of the cure rate due to heat alone, in the Mc7.

There may be several explanations for the growth of tumours further up the leg of rats in which the primary tumour had apparently completely regressed following hyperthermia. Neither tumour is known to produce metastases, so this obvious explanation can be discarded. There are two other possibilities. For example, during the original injection of tumour cells into the foot a small population of tumour cells may have entered the blood stream and lodged further up the leg. Because they would be small in number it could take some time before the growth of a tumour was detected. Another explanation is that the heating of the tumour caused it to disaggregate, and cells were again released into the blood stream, lodging further up the leg. In the case of both explanations the late appearance of the tumour would be because only a very small population of tumour cells was involved.

The results presented for the D23 tumour are consistent with the hypothesis that the plasma membrane is a critical site of heat injury. This is because the addition of a membrane perturbant, tetracaine, considerably enhances heat therapy. Very few similar studies have been undertaken on tumours in vivo, using this kind of

heating regime, but Yatvin *et al*. (1979) and Robins *et al*. (1982), have shown a considerble increase in the regression of a transplantable adenosarcoma (grown in the hind legs of mice) when heating at 43.5°C for 1 hour, combined with lidocaine infusion, compared to heating alone. This confirms Yatvin's model study using bacteria to test the efficacy of local anaesthetics combined with heat, in cell killing (Yatvin *et al*. 1982). It would be, therefore, statisfying to say that the hypothesis of cell heat death advanced in the beginning of this thesis had been confirmed, but the Mc7 tumour shows a different response to the D23, and to some extent seems to discredit the hypothesis, as the potentiating effect of tetracaine and heat on this tumour is not marked.

The biochemical studies of the D23 and Mc7 plasma membranes, presented earlier in this thesis, Chapters 3-5, (ie. low membrane cholesterol levels, decreased plasma membrane order, and the increased thermosensitivity of the tumour plasma membrane enzyme for both tumours) would indicate that there is no real difference in the thermosensitivity of the tumours *in vivo*, *if* the plasma membrane is the critical factor in deciding the differential sensitivity of many tumours to hyperthermia. This is clearly not the case. It is therefore important to bear in mind that tumour heat sensitivity may be a combination of cellular lesions and physiological factors, which will only be applicable to tumour cells in the whole animal.

It has been shown, for example, that SPD tumour cells were more sensitive to hyperthermia *in vivo* than *in vitro*(Kang *et al*.

1980). The two most important physiological factors to bear in mind are tumour blood flow and local pH. The interaction between these two in hyperthermia has been reviewed by Song (1982). Emanmi et al. (1980) have shown, in a rat rhabadomyosarcoma, that blood flow to the tumour, at 40-41°C for 40 minutes, decreases by 50%, but returned to normal after 72 hours. However the same time of exposure, but now at 43°C, produced a more destructive effect. Capillary blood flow was eliminated and there was widespread vessel rupture and haemorrhage. Similar results have been reported by Bircher et al. (1980), in a mouse mammary adenosarcoma. They showed that below 41°C there was an increase in blood flow and tissue oxygen tension, but temperatures above 41°C caused a collapse in blood flow, a lowering of tissue oxygen tension, and a decrease in pH. They also found that the tissue oxygen tension in unheated tumours was very low in certain areas of the tumour which were also deprived of blood flow. Thus it seems clear that decreases in blood flow deprives the tumour cells of oxygen and nutrients, and they tend to go hypoxic.

Dickson and Calderwood, (1979) have shown that this condition increases the hyperthermic killing of tumours, and, using a Yoshida sarcoma, Dickson has also shown that the increase in tumour pH is due to an efflux of lactate from the tumour cells (Calderwood & Dickson, 1980). Hofer & Mivechi (1980) have proposed however, that the increased hyperthermic sensitivity of tumours is due to decreased intracellular, not extracellular, pH. It is also interesting to note that a continued low pH, after the heating has stopped, will increase

tumour regression (Freeman et al. 1980)

Tumour size has been shown to be a critical factor in determining the effect of blood flow and pH on tumour hyperthermia. Urano et al. (1980) have reported that an increase in thermosensitivity was associated with increased tumour volume, which in itself produced a decrease in pH and growth. Although these effects are desirable, in that they enhance the hyperthermic killing of tumours, Song et al. (1982) has pointed out that the hypoxic state does not favour subsequent effective radiotherapy. This is important as the two treatments are often used in combination or sequentially in clinical treatment.

The other main physiological factor to be considered in understanding the variation in heat sensitivity of tumours, is the animal's immune response. There is some circumstantial evidence that there is an anti-tumour immune response after curative local hyperthermia, in some animals. The D23 and Mc7 tumours are considered to be weakly immunogenic, the D23 more so than the Mc7 (Dickson & Simpson, (personal communication).

How might these physiological factors affect the observed difference in the thermosensitivity of the D23 and Mc7 tumours? Blood flow may be a crucial factor in the differential heat sensitivity between the D23 and Mc7. The Mc7 was observed to be better vasculated than the D23; this is almost certainly the reason why the Mc7 was rarely very necrotic, whereas the D23 often had a necrotic centre. This would mean that the D23 could

be considerably more hypoxic than the Mc7, and this is know to be an important factor in increasing tumour sensitivity to heat (Dickson & Calderwood, 1979). It is also important to remember that the hyperthermic sensitivity of the Mc7 tumour decreased markedly during the two year period of experimentation. Figure 7.2, therefore, masks the great difference between the original tumour heat sensitivity, which was similar to that of the D23, and the final heat sensitivity, which was even greater than figure 7.2 would suggest. The changing nature of the Mc7 tumour emphasises the drawback of using a passaged, rather than primary tumour system, to look at a treatment regime. It may be that there was some genetic 'drift' in the animals during the course of the experiments, but it seems unlikely that it would have affected the female and not the male when this was an inbred strain of rat. Of course the reason for using the inbred strain at all, was to eliminate, as far as possible, any genetic variation.

In the second type of heating experiment, which was performed on the D23 tumours only, there was no significant difference between the heat sensitivity of the tumour growth in ethanol-dependent rats, compared with their pair-fed controls. A report by Anderson et al. (1983), showed that ethanol administration followed by withdrawl immediately before heating, reduced heat induced necrosis in mouse ear. The lack of such a pattern of resistance in the D23 tumour may confirm the speculation in Chapter 7, when it was suggested that long-term ethanol administration may preferentially damage the tumour cells because of their high fluidity in the absence of any

membrane perturbant. It may be equally well argued that any small difference in heat sensitivity, induced by the ethanol administration, would be difficult to observe with a relative small sample size. These results do confirm the results from the biochemical study of the effects of long-term ethanol administration on tumours, that is they are also non-conclusive! It must also be remembered that the in vivo ethanol experiment, reported in this section, was undertaken on a different tumour to the biochemical study undertaken in Chapter 7. Unfortunately, at the time of the experiment, there were not enough female rats (these carry the Mc7 tumour) to attempt an experiment, and so the D23, grown in the male rats, was used instead.

Finally, there has been some recent criticism of using animal foot tumours as models for human tumours when trying to assess future clinical treatment regimes. This model is commonly used in hyperthermia and radiotherapy work, but Auerbach and Auerbach, (1982), in their review, emphasise that there are regional differences in the growth of normal and neoplastic cells, and regional differences in the immune response as well. More importantly for this current study, Hill and Denekamp, (1982), have shown that tumours grown on animal extremities may well be poor models for hyperthermia treatment, because of the low natural temperature experienced in these regions of the body. Therefore less heat might be required to kill a tumour located at the extremities than one located in the core of the animal. These latter workers also report that there are changes in the vasculation at the extremities. This may be

crucial since, as already has been shown, tumour vasculation is very important in determing tumour heat sensitivity in vivo. Despite the drawbacks of this model it represents the best available model system for the current studies.

8. GENERAL DISCUSSION

Two questions were posed at the beginning of this thesis which were the basis of the work which has now been presented. These were, first, what is the nature of cellular heat injury, and secondly, what are the factors involved in making some tumours more sensitive to heat injury? How far the work presented here has contributed towards a greater understanding of these fundamental questions will now be examined. Of course any study of this nature will raise as many questions as it will answer, and the more interesting possible future developments of the results presented here will be highlighted.

Two tumours grown in continuous passage, D23 a hepatoma and Mc7, a sarcoma, have formed the basis of these current studies, and it must be recognised that these are only a model tumour system. It is essential to have a defined system for the study of tumour membrane biochemistry undertaken here, however this system of passaged solid tumours is not ideal. Many solid tumours, for example, contain a heterogenous cell population and can contain a high proportion of macrophages, (Evans, 1972). The inbred rat strain used were difficult to breed compared to normal laboratory rats and furthermore there is some evidence, presented in Chapters 3 and 7, that the tumours changed during the course of the work (2.5 years). In Chapter 3 it was noted

that the cholesterol/phospholipid ratios of the two tumour changed gradually during the course of the work. Originally the level of cholesterol was higher in the Mc7 tumour plasma membranes than in the same membranes from the D23 tumour, but at the end of the study this pattern had reversed and the level of membrane cholesterol was similar in both the tumours. In Chapter 7 it was noted that the Mc7 tumour became progressively resistant to heat-induced regression during a 2 year period. Changes in the properties of passaged tumours has been observed by many other workers and a substantial volume of work has been published on this phenomenon, (Reviewed by Steel, 1977). Emmelot & Bos, (1969a) have shown that the properties(activities) of plasma membrane bound enzymes changed during passage. In order to reduce the chance of tumour deviation it is common practice to re-initiate the tumour line from a frozen cell suspension of the primary culture. This was done several times during the course of the work. (Despite these precautions it is inevitable that there is a certain amount of selection occurring during tumour passage.) Fresh material came from either the North of England Cancer Research Unit in the Royal Victoria Infirmary Newcastle, or the Cancer Campaign Laboratories, Nottingham. The latter Unit was responsible for the establishment of the two tumours used in this study. The number of tumour-bearing animals available for any given experiment was often limited because of problems with the breeding programme, and so the number of replicates for experiments was not as many as wished. This is particularly true of the Mc7 tumour line, because it used females for passage, and females often could not be spared from the breeding

programme.

In Chapter 2 plasma membrane enriched fractions were obtained from the liver and two tumours, D23 and Mc7. These membranes were not entirely free from contamination, but the level of the major contaminant, mitochondria, was similar in the three plasma membrane types. This allowed comparative studies to be carried out, without the contamination seriously affecting the interpretation of the results obtained. This chapter therefore highlighted the difficulty of obtaining a 'pure' plasma membrane fraction from tumours. This problem has been noted by other workers Emmelot et al. (1974) and Suavage et al. (1981) and is likely to be a result of the lack of differentiation between plasma and other subcellular membranes in this cell type. The use of liver as the control tissue allowed a direct comparison between the liver plasma membranes and the same membranes from the D23 hepatoma because this tumour was originally derived from liver. Such a control was not available for the Mc7 sarcoma and so this tumour served to indicate the kind of differences in membrane structure and function that may be common to neoplasia generally.

The results in Chapters 3 and 4, in which the lipid composition and physical state of tumour plasma membranes were compared to those membranes from liver, give the first strong indication that the plasma membrane may indeed be a basis of the differential sensitivity of tumour cells to heat.

The major differences between tumour and liver plasma

membrane lipid composition lay in the fatty acid composition of the membrane phospholipids and the amount of membrane cholesterol present. The major differences seen in the fatty acid composition of tumour plasma membrane phospholipids when compared to the equivalent membranes from liver was an elevated level of mono- and di-unsaturated fatty acids and a lowered level of polyunsaturated fatty acids particularly arachidonic acid (20:4) in the membranes from the tumours. Similar data has been obtained by a number of workers (eg. Van Blitterwijk et al. 1982 and Koizumi et al. 1981), and appears to be a fairly common feature of proliferating cells (reviewed by Hartz et al. 1982). The fatty acid composition data presented in Chapter 3 also suggested that there is an increase in the level of 22 carbon chain length fatty acids. Similar findings were not apparent in the literature. The level of membrane cholesterol in the two tumours was significantly lower than in the membranes from liver. Lower plasma membrane cholesterol levels have been reported in the literature, principally for leukaemias and lymphomas (Van Blitterwijk et al. 1982; Koizumi et al. 1981). In hepatomas other workers have reported elevated levels of plasma membrane cholesterol in tumour plasma membranes (Chen et al. 1978). Again non-cancerous proliferating tissues also have lower levels of plasma membrane cholesterol than normal 'resting' tissue, for example regenerating and foetal liver (Koizumi et al. 1976). Thus the differences in lipid composition between normal and tumour plasma membranes reported in this study may not be due to neoplasia per se. but a common feature of rapidly dividing cells.

In Chapter 4 it was shown using D.P.H. fluorescence polarization that the plasma membrane order of both tumour membranes was significantly lower than the order of the equivalent membranes from liver. In the literature others have reported that cancer cells have more fluid membranes than the normal cell type from which they are derived, (Inbar, 1976 and Van Blitterwijk et al. 1982). This lower order could be related back to the differences in plasma membrane lipid composition describe in Chapter 3. The proportion of mono-unsaturated fatty acids (particularly 18:1) in the membrane phospholipids of the two tumours was greater than in the phospholipids from the liver. As the first double bond is reported to have the most pronounced effect on membrane lipid order (Van Deenmet al. 1971) the higher level of this fatty acid type may contribute to the decreased order of the tumour plasma membranes compared to normal liver membranes. It was however the lower level of cholesterol in the tumour plasma membranes which was considered to be the most important factor in determining the reduced order of the tumours, and therefore tumour thermosensitivity. Some workers have proposed that membrane order is a key factor cell heat sensitivity (Yatvin et al. 1978 and Hidvigi et al. 1980). Others have disputed this, (Massicotte-Nolan et al. 1981 and Lepock et al. 1983), To some extent lipid order as measured by D.P.H. fluorescence polarization is not the same type of measurement as 'fluidity' defined by other physical techniques which are more concerned with motion, rather than packing. Lepock himself, in his review of the involvement of membranes in the hyperthermic killing of cells, has suggested that membrane lipid order could

be an important factor in determining cell heat sensitivity(Lepock, 1983).

The low order of the tumour plasma membranes appears to affect the temperature sensitivity of membrane proteins. In Chapter 5, the Mg2+ATPase, an integral membrane protein, was found to be considerably more temperature sensitive in the tumour membranes than it was in the membranes from liver. Emmelot and Bos, (1968) have reported similar results. It cannot be discounted that the temperature sensitivity of this enzyme is a unique property of this particular protein in tumour plasma membranes. This chapter also showed that tetracaine, a membrane perturbant, decreased lipid order to a greater extent in the tumour plasma membranes than it did in the equivalent membranes from liver. Tetracaine was shown to potentiate the thermal inactivation of the Mg2+ATPase. These results appear to confirm the idea that the perturbation of the lipid bilayer by heat and chemical agents (eg. tetracaine) will have a destabilizing effect on the proteins residing in this bilayer and that heat and chemical perturbants can act synergistically so that there is a potentiating of the denaturing effect of heat alone. Yatvin and co-workers have already shown in bacteria that tetracaine and other local anaesthetics can potentiate cell heat killing, (Yatvin et al. 1982). Thus the results presented in this chapter further substantiate the hypothesis that plasma membranes are a key site of primary heat lesion, and the 'denaturation' of membrane proteins is likely to be the irreversible membrane effect which eventually leads to heat death. Schanne et al. (1979) have suggested that the influx of

calcium ions, following plasma membrane damage, is the common basis of cell death. The data from Chapter 5 also indicates that the plasma membrane may be the key site in determining the differential sensitivity of some tumour cells to heat, that is at least one plasma membrane enzyme is considerably more thermolabile in tumour plasma membranes than it is the membranes from liver, a normal tissue.

The limitation of these studies is that they were based on a single plasma membrane enzyme, which, although abundant in most plasma membranes, has not, as yet, a clearly defined function. However, Forgac and Cantley, (1984) have recently shown in the erythrocyte that this enzyme is not an ion pump, and earlier work by Singer and co-workers has suggested that in the erythrocyte it might be involved in the cytoskeleton, (Sheetz & Singer, 1977; and Birchmeier & Singer, 1977). Further work on other membrane bound enzymes would broaden this narrow perspective. The selection of asymmetrically distributed enzymes (eg 5′ Nucleotidase & adenylate cyclase -both from the inner leaflet of the membrane bilayer), might give more information on whether there was a difference in fluidity between the inner and outer leaflets. If so these differences could be further exploited when applying heat by the introduction of anaesthetics which are known to selectively fluidize one of the two bilayer leaflets. Cationic drugs (eg. tetracaine and phenobarbitol) have been shown to partition into the inner leaflet whereas anionic drugs prefer the outer one, (Houslay et al. 1981; Ogiso et al. 1981 and Dipple et al. 1983). The ability to selectively enhance the denaturation of

protein
membrane is very appealing . This is because proteins involved in secondary messenger systems eg adenylate cyclase and the newly ellucidated system based on the release of inositol triphosphate (Berridge et al. 1983 and Berridge, 1983) are thought to be involved in cell growth regulation and it has been suggested that defects in the latter system may be responsible for the neoplastic condition itself (Michell, 1984). Therefore it may be important to determine the effects of hyperthermia on these key protein systems.

A further extension of this study might be to look at the effect of changing diet on the activity of plasma membrane enzymes, for example increasing the levels of polyunstaturated fatty acids or 18:1 which are known to disorder the membrane or decreasing membrane cholesterol. Work has already been done on the influence of membrane lipids on enzyme activity and function. Clandinin and co-workers have studied the effects of dietary lipids on certain key membrane enzymes, eg. the mitochondrial ATPase (Innis & Clandinin, 1981), synaptosomal acetylcholinesterase (Foot et al. 1983), and liver plasma membrane, glucagon stimulated adenylate cyclase activity (Needlands & Clandinin, 1983). One of the interesting observations in the ethanol administration work, presented in Chapter 6 was that a change to a low-fat diet significantly changed the lipid composition of the plasma membrane, Therefore the possibility exists for manipulating the plasma membrane lipid composition by dietary means in order to disorder the membranes so that the cells become more thermally sensitive. Mulcahy et al. (1981) have already shown that the membrane

modification of P-388 ascites tumour cells can change their heat sensitivity.

So to this point the experimental data can be interpreted to support the proposition that the plasma membrane is a site of primary heat lesion. Furthermore, the differential sensitivity of the tumour cells to heat can also be accounted for by the chemical composition and physical state of the tumour cell plasma membrane. The work presented in Chapter 6 was concerned with trying to exploit further this differential heat sensitivity. It was anticipated that normal cells might be made more resistant to heat by causing an adaptive response to the effect of long-term exposure to a membrane perturbant. This phenomenon has been demonstrated to occur when the perturbant was raised temperature, (see Hazel & Prosser, 1974 and Anderson et al. 1981)), and also such drugs as ethanol (Chin & Goldstein, 1977 and Chin et al. 1978) and morphine (Heron et al. 1982). In mammalian cells this compensation is reported to be owing to raised cholesterol content, and as tumour cells have low plasma membrane cholesterol it was considered a possibility that these cells could not make an effective adaptive response to the long term presence of a perturbant. Should this be so, adaption to ethanol may result in a widening of the thermal sensitivity of normal and tumour cells, a differential that may have value in therapy. The results of the present study were, however, inconclusive. There appeared to be an elevation in the level of membrane cholesterol both in brain synaptic membranes (normal tissue) and the plasma membranes from the tumour in alcohol-fed rats as compared to controls; these

differences however were not statistically significant. The change in fatty acid composition which occurred was small and it was difficult to eliminate the effect of ethanol on lipid metabolism which may indirectly modify membrane composition in a way that may be unconnected with the adaptive response. This also may be true of cholesterol levels in membranes as serum cholesterol levels increase during ethanol administration. One interesting observation in Chapter 6 was that the data from D.P.H. fluorescence polarization studies suggested that the tumour plasma membranes were consistently, though not significantly, lower in order after long term exposure to ethanol when compared to the equivalent membranes from controls. Two possibilities were suggested for this phenomenon, first, that it was due to the increase in 18:1 in the plasma membrane phospholipids from ethanol-fed rats as compared to the membranes from control rats. Second that it was the result of damage to the protein/protein, protein/lipid complexes which have been suggested by some (Harris et al. (1984)) to be important in the adaptive response of membranes to ethanol. Rubin and Rottenburger, (1982) and Lee and Hosein, (1982) both report that ethanol may damage protein function, others (Kane et al. 1980 & Schanne et al. 1980) have proposed that long term exposure to ethanol will damage plasma membranes. Further work would have to be done to confirm this idea as the differences were not statistically significant. However this damage was not apparent in terms of an increased cure rate when foot tumours in ethanol-fed rats were compared to controls, as was presented in Chapter 7.

Littleton (1983) has suggested that the homeoviscous adaption to temperature, which is characterised by changes in the lipid composition and order of membranes (an adaptation to maintain the fluidity of the membrane within acceptable limits for normal cell function) is different from the type of adaptation occuring in tolerance to ethanol. For example, Chin and Goldstein, (1977) were not able to show any differences in fluidity (using E.S.R. probes) in plasma membrane from ethanol-adapted mice, compared with pair-fed controls, but, the disordering effect of ethanol was considerably less in the adapted membranes than in the equivalent membranes from pair-fed controls. Therefore it would be important in future studies to establish whether such a tolerance effect was apparent in the plasma membranes of tumours which had been subjected to long-term ethanol administration. If there are only small differences occurring another approach would be to look at the thermal sensitivity of the Mg2+ATPase and other plasma membrane enzymes as these may reflect more any subtle changes occurring during ethanol-adaption, which are not detected by the D.P.H. fluorescence probe technique because it only gives an average 'picture' of the state of the membrane. Proteins are also known to be modulated by the presence of ethanol, eg. Collins et al. (1984).

The manipulation of membrane order by diet appears to be a more hopeful approach in the hyperthermic treatment of cancer, not least because it offers a less drastic method of changing the membrane composition than the long term administration of membrane fluidizers.

The real test of the efficacy of the treatment regimes using membrane perturbants locally on a short term basis is to look at the *in vivo* effect of these agents when applied together with heat. Such studies will also reveal whether the plasma membrane is the ' weak spot ' in the differential sensitivity of tumours to heat. Other workers have certainly found that membrane perturbants increased the hyperthermic sensitivity of tumours *in vivo* (Yatvin *et al*. 1979 & Robins *et al*. 1982) with the anaesthetic lidocaine. In Chapter 6 the *in vivo* effect of the membrane perturbant, tetracaine, on the hyperthermia-induced regression of the D23 and Mc7 tumours grown in the foot was ascertained. The addition of the tetracaine significantly increased the cure rate of the D23 tumour over and above normal hypertheric treatment. The cure rate (percentage alive) after 200 days was 75%. The same however was not true of the Mc7 tumour which was considerably less thermo-sensitive than the D23 tumour. Since the plasma membrane composition of the tumours were similar and they were both equally disordered, other physiological factors must come into play in deciding the thermal sensitivity of tumours to hyperthermia . The main factors are blood flow and tumour pH. The degree of vasculation will determine the ability of the tumour to maintain a normal temperature. (Review, Song 1982)

Despite the reservations expressed in the last chapter (7), which underlined the need to take into account physiological as well as cellular aspects when considering tumour heat sensitivity, the work presented in this thesis clearly indicates that the basis of the differential heat sensitivity of the D23

and Mc7 tumours studied here is related to the plasma membrane. The difference in plasma membrane lipid composition, membrane order, and the concommitant change in protein thermo-stability, are evidence for this conclusion. In addition, the use of membrane perturbants, such as tetracaine, to enhance the killing of tumour cells has been further substantiated both *in vitro* in Chapter 5, and *in vivo* in Chapter 7. Finally these studies highlight the importance of the plasma membrane in determining the sensitivity of cells to heat, and in this way underline the hypotheses of Bowler, (1981) and others which direct attention to the plasma membrane as the site of primary heat lesion in cells.

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APPENDIX 1. MAINTENENCE OF TUMOUR LINES

INTRODUCTION

The two tumours used in the work presented in this thesis were the D23 hepatocellular carcinoma and the Mc7 mammary sarcoma, both of which were originally chemically induced. These tumours were initiated at the Cancer Campaign laboratories, Nottingham, and maintained in continuous passage in inbred WAB/Nottingham wistar rats. The D23 in the male animal and the Mc7 in the female.

1. Solutions for passage

i) Antibiotic 'cocktail'

To 400ml of sterile saline was added: 20 megaunits of penicillin G, 10g of streptomycin sulphate, and 500,000 units of mycostatin. Once made up this 'cocktail was stored in small 1ml aliquots at -20 C, until required.

Anaesthetic

Commercial 'Sagatal' was diluted five-fold in sterile saline and then injected into the rat intra-peritoneally

(IP). The dose was 0.1ml per 50g of body weight.

2. Donor tumour preparation

Tumours of average size were selected (2-3ml in volume) from at least two different animals in order to reduce selection pressure which can alter the tumour characteristics over several passage generations. The area around the tumour(s) was shaved and washed with 70%(v/v) ethanol. Sterilized instruments were used to remove the tumours from the animals' flanks. The tumours, once removed were dissected in a sterile petri-dish. The tumours were enclosed in a fibrous capsule. When this was bisected the inside of the tumour could be seen to consist of a necrotic, sometimes fluid filled centre, which was often yellow, and occasionally haemorrhagic, which was surrounded by translucent, flesh-coloured tissue. The viable tumour cells were found in this flesh-coloured tissue, care was taken to avoid material from either the fibrous coat or necrotic centre.

The viable tissue, once removed, was placed in a sterile petri-dish and chopped repeatedly to a 'gel like' mass. One drop of the antibiotic 'cocktail' was added to approximately every 1ml of chopped tumour tissue. 1ml syringe barrels were then loaded with the chopped suspension and fitted with a large bore needle.

3. Implantation

Two small areas on the rat's flank were shaved and washed with 70% ethanol (the animal had been previously anaesthetized using the diluted anaesthetic describe earlier). The needle was inserted into the subcutaneous space and 0.1ml of tumour tissue (approx. 100mg) ejected. The site of injection was then wiped with 70% ethanol. This was done on both sides of the flank. The tumours reached a volume of 2-3mls in 10-12 days.

APPENDIX 2. LOW POWER ELECTRON MICROSCOPY OF TUMOUR CELLS

INTRODUCTION

The D23 hepatoma and the Mc7 sarcoma were examined using low power electron microscopy. This was in order to ascertain the general form of the tumour cells.

MATERIALS AND METHODS

The techniques used for the fixation of the tumour material was based on the methods of Karnonsky, (1965).

1. Fixation

Preparation of fixative

Solution A 2g Paraformaldehyde + 40ml of distilled water. The paraformaldehyde was heated and the precipitate formed was dissolved by slowly adding 2-6 drops of 1M NaOH.

Solution B 10ml of 25% Gluteraldehyde + 50ml of 0.2M Sodium cacodylate buffer, pH 7.3.

Solutions A and B were kept refrigerated until just

before use and then mixed together.

Small pieces of healthy tumour tissue were dissected out(or in the case of tumour membrane preparations a small pellet), placed on a piece of cardboard and immersed immediately in the Karnonsky fixative described above. The preparation was then left for 1-1.5 hours at 4 C.

2. Post Fixation

Osmium tetroxide buffer 25ml of 2% osmium tetroxide + 25ml of distilled water + 50ml of sodium cacodylate buffer, pH 7.3.

The specimens were post-fixed in 1% buffered osmium tetroxide for 0.5-1 hour at 4 C.

3. Dehydration

The material was dehydrated at room temperature through a series of 70%, 95%, and absolute ethanol, with three changes at each stage, and for a total of 15 minutes at each concentration.

4. Embedding

After dehydration the material was embedded in Araldite in the following way.

Araldite mixture: 10ml of Araldite (CY212) + 10ml of D.D.S.A. + 1ml of dibutyl phthalate + 0.5ml of D.M.P. 30. (from Glauert & Glauert, 1958)

The material was placed in a mixture of 50ml of absolute alcohol + 50ml of propylene oxide and left for 30 minutes. It was then placed in propylene oxide and the solution was changed three times. The material was then placed in a mixture which consisted of 50ml of propylene oxide + 50ml of Araldite mixture and left for 30 minutes at 45 C. The material was then placed in the Araldite mixture, which was changed after 30 minutes, at 45 C. The material was then left for 12 hours in this Araldite solution at 45 C and then transferred to 60 C for a further 2 days.

5. Sectioning and Staining

Material was sectioned on a Reichert OMU3 ultra-microtome, using glass knives. Thick (approx. 1um) sections were cut, stained with toluidine blue in 1%borax, and then scanned with a light microscope to check the fixation. Thin sections were then cut of a silver-to-gold interference colour.

Thin sections were then doubly stained with uranyl acetate followed by lead citrate as described below.

Three drops of uranyl acetate were placed on

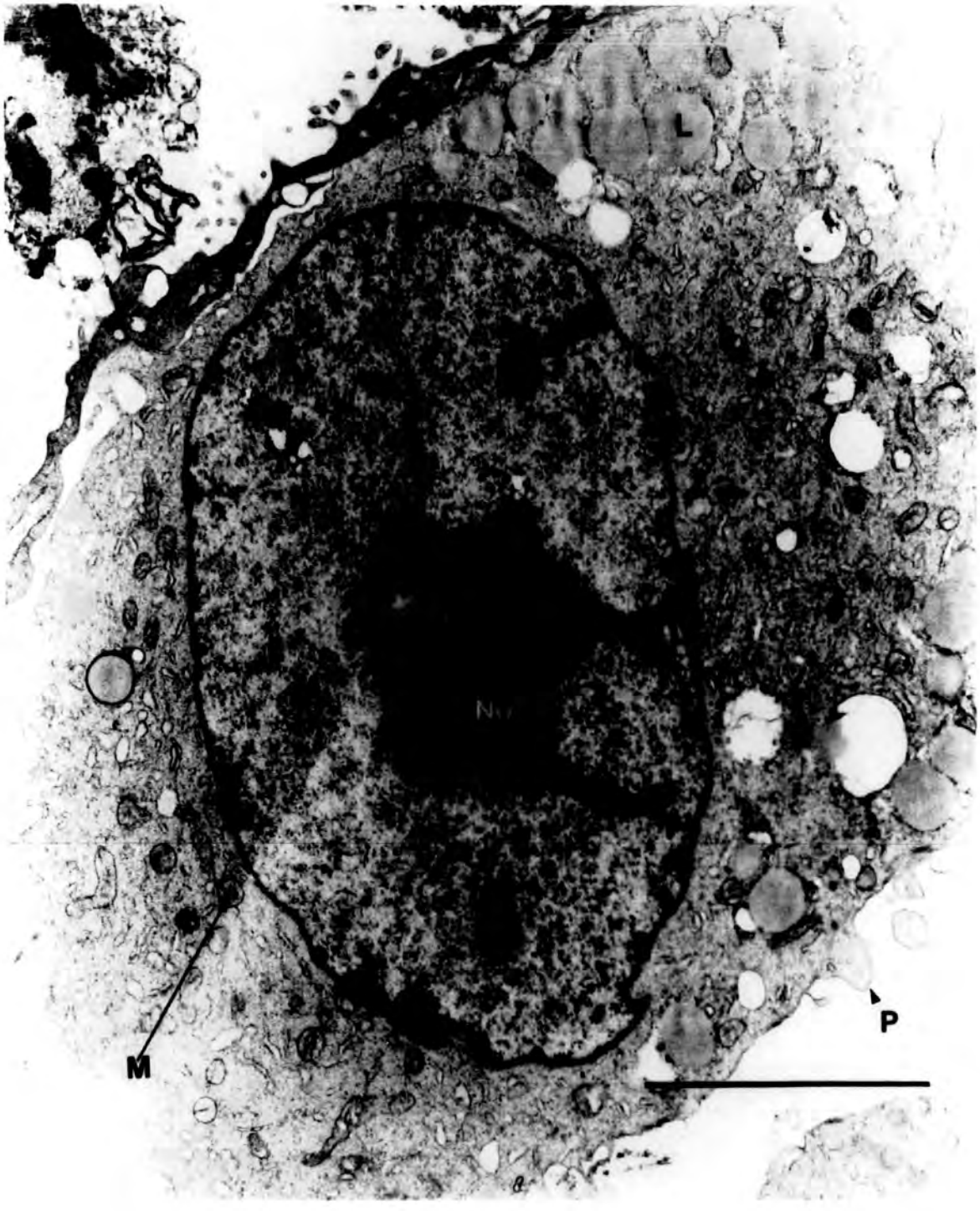
'parafilm' with the clean side up. The grid was placed Cu side down on the surface of the drop and left for 10 minutes. The grid was then washed by dripping distilled water over it for h about 40 seconds and then dried carefully. The grid was then placed in lead citrate for 10minutes using the same procedure as for the uranyl acetate. The grid was then thoroughly washed with distilled water as described previously.

Finally, the sections were examined and photographed with an AEL 801 electron microscope.

RESULTS AND DICUSSION

Plates A1.1 and A1.2 are low power electron micrographs of D23 and Mc7 tumour cells respectively.

The two cell types have several similarities. They both have large nuclei and a relatively small amount of cytoplasma. They also both appear to be fairly undifferentiated in form, which one would expect in a population of rapidly dividing cells. Because they are only low power micrographs it is only possible to pick out prominant subcellular features such as nuclei, the nuclear envelope, the plasma membrane and mitochondria. There appears to be relatively few mitochondria in the sections observed, particularly when one remembers that the D23 is a hepatoma which has originally been derived from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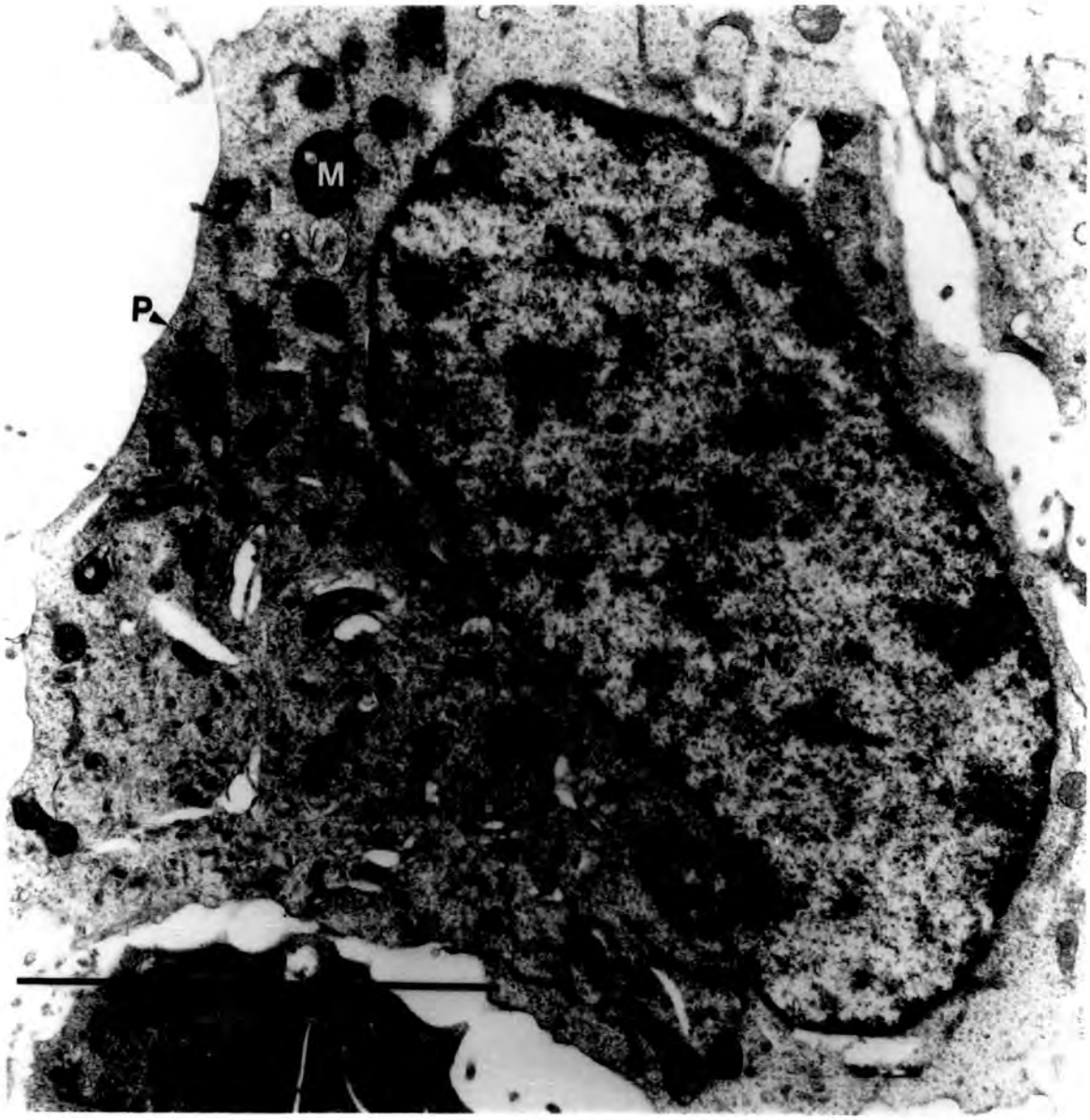
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liver cells which are rich in this organelle.

There are several differences in the appearence of the two cells, at a fairly trivial level, for example, the D23 cell is larger than the Mc7. The D23 tumour cell also appears to have more prominant nucleoli (though of course this is very much a result of the different stages in the cell cycle which the two cells are in). There is a considerable amount of lipid in the cytoplasm of the D23 cell which is not seen in the Mc7. This build up of lipids is a common feature of hepatomas presumably because they have been originally derived from liver cells, (Hartz et al. 1982). The appearence of the D23 tumour cell is similar to its appearence described by Raftell and Blomberg, (1973), therefore there has been no dramatic changes in the form of this tumour cell over a ten year period. This is an indication that the changes which occur in continuous passage are not exhibited at the level of tumour cell morphology.

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