COMPOSITION AND FUNCTION OF CELL MEMBRANES

Application to the Pathophysiology of Muscle Diseases

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COMPOSITION AND FUNCTION OF CELL MEMBRANES

Application to the Pathophysiology of Muscle Diseases

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PREFACE

The present volume contains the edited transcript of a Totts Gap Colloquium held May 19-21, 1980 sponsored by the Muscular Dystrophy Association. The aim of the colloquium was to bring into focus data relating to cell membranes that might contribute to understanding the pathogenic mechanism of Duchenne muscular dystrophy.

A major impediment to progress in understanding the pathogenesis of muscular dystrophy has been the failure, so far, to identify the basic genetic defect. Pending the identification of the genetic lesion in Duchenne dystrophy and, in view of scattered but persistent indications of a basic membrane disturbance, it seemed worthwhile to explore in open dialogue the current state of knowledge of membrane morphology and chemistry with an eye to possible leads for further investigation.

The participants, drawn from a variety of interested disciplines, attempted to synthesize and reconcile their findings and to identify crucial areas of ignorance in need of exploration. For the most part they avoided specialized jargon and spoke in a language that could be understood by the rest of the group. Apart from providing a review of widely varying approaches to the study of the composition and behavior of cell membranes, the discussions brought together current thinking on strategies and approaches to the study of the pathogenesis of muscular dystrophy. Already the personal contacts made at the colloquium have led to new inter-institutional collaborative investigations.

The discussions were recorded, transcribed, edited and to some extent, rearranged to fit into a sequence of chapters. Valuable assistance was provided by Helen Goodell who helped with the editing and Joy Colarusso Lowe whose patience and expertise made possible the manuscript itself.

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CHAPTER 1

PHYLOGENY, STRUCTURE AND COMPOSITION OF CELL MEMBRANES

DR. BLOBEL: In an average eukaryotic cell there are as many as 1 billion protein molecules, 10,000 to 50,000 of which are separate species. These various species of protein fall into groups which have similar topology such as lysosomal proteins, peroxisomal proteins and those which are found exclusively in the mitochondrial matrix or the intermembrane space of the chloroplast stroma, etc.

The information for the protein localization resides in the protein in discrete sequences that are shared by proteins which have a similar typology. The information in these discrete sequences has to be decoded by specific machinery very much like the mail zip code system. The sequences in membrane associated proteins affect their translocation across the membrane and further affect their subsequent sorting into other compartments.

While the general principle is relatively clear and easy to understand, as are many other biological phenomena, the details are lacking. We do not know much about the details of this process.

In order to help those of you who are not cell biologists, I would like to give you a little evolutionary crutch which will help you to sort out what kind of membranes we have.

A primordial cell (Figure 1-1) has one membrane, a plasma membrane, and it has one compartment which we can conveniently call the endoplasmic compartment. This plasma membrane is endowed with a couple of specific functions. For instance, it can bind ribosomes and it can bind chromatin. Apparently what has happened in evolution is that the plasma membrane of the primordial cell began to invaginate at some point so that intracellular membranes started

to appear. Some of the functions originally present in the prokaryotic plasma membrane have been removed so that in a eukarytic cell the ribosome binding function, for example, is exclusively localized in the rough endoplasmic reticulum. The chromatin binding function, originally present in the pluri-potential prokaryotic cell membrane has been entirely delegated to the inner nuclear membrane. Therefore in the invagination process two functions have been removed from the plasma membrane and delegated to intracellular membranes. Two separate compartments have thereby been generated, an ectoplasmic and an endoplasmic compartment. In addition, there is a nucleoplasmic compartment and a cytoplasmic compartment that are both members of the endoplasmic compartment. The membrane between the nuceloplasmic and the cytoplasmic compartment contains pores on the order of 1000 Å in diameter which permit the travel of large molecules such as ribonucleic acid or ribosomes out of the nucleus and they permit the entry of proteins such as RNA polymerase and histones from the cytoplasm into the nucleus without having to cross a membrane barrier. I haven't indicated many of the other intracellular membranes you are familiar with such as peroxisomes, lysosomes and the Golgi apparatus. They could have developed a similar invagintion process or they could represent outgrowths of these endoplasmic reticulum membranes.

Evolutionary Development of Cell Membranes

Many people who think about evolution of cellular membranes believe that mitochondria and chloroplasts have a quite different history. The suggestion is that at one point in evolution a cell engulfed another prokaryotic progenitor cell. The ribosomes and the plasma membrane of this cell and the chromatin binding sites are indicated in Figure 1-2. When this endocytotic process is concluded you end up with a double membrane structure. The outer mitochondrial membrane is derived from the plasma membrane of the host cell, very much like the other intracellular membranes. It is derived phylogenetically from the plasma membrane of the same cell, whereas according to the theory just mentioned, the inner mitochondrial membrane is really a membrane of the foreign cell. Therefore, if you like Greek you can call this compartment the xenoplasm if you wish, where the ribosomes and chromatin binding sites are still present. The chloroplast has a similar history in that the invagination process of the inner membrane has proceeded further so that you get pinched off vesicle structures which are called thylakoid membranes. This description gives on an idea of how these membranes may have developed. This is not just a game which one can play. It brings a certain order into thinking about cellular membranes and it may help us to understand the way proteins are sorted on and within a cell. Some of the protein traffic may have to do with the way these membranes evolve, reflecting their evolutionary history. Proteins, as you know, are synthesized

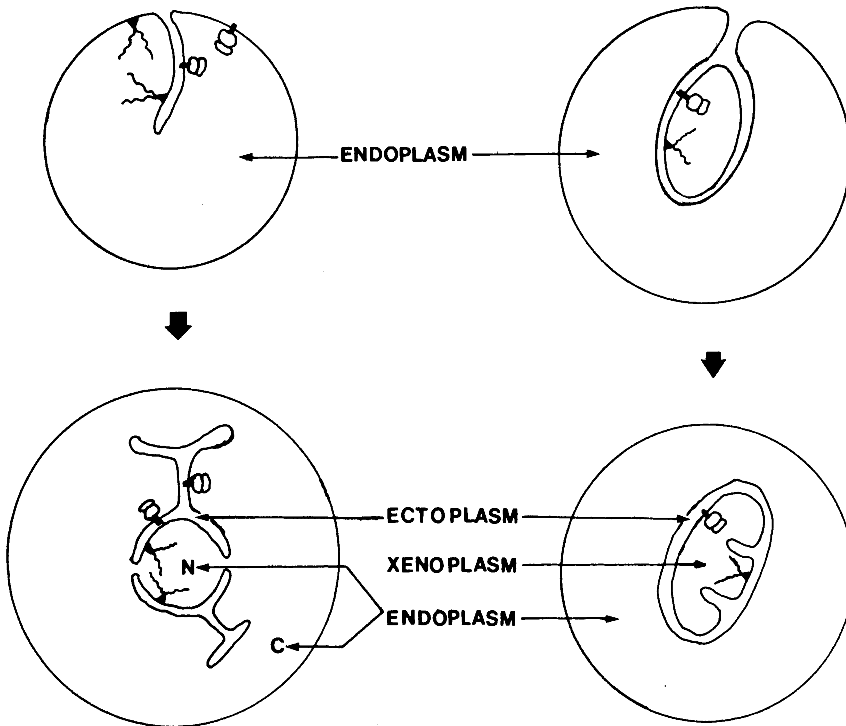


Figure 1-1: Primordial cell with single endoplasmic compartment showing beginning invagination and binding of ribosomes and chromatin. N - nucleoplasm, C - cytoplasm. (Reproduced again as Figure 4-24a).

Figure 1-2: Primordial cell having engulfed another prokaryotic progenitor cell. (Reproduced again as Figure 4-24b).

only in the endoplasmic compartment because this is where one finds the ribosomes either bound to the membrane or free in the cytoplasm and in the cytoplasmic compartment. In the course of evolution much of the DNA of a prokaryotic progenitor cell was probably transfered into the genome of the host cell in a transformation-like process. The subcellular compartment must import its proteins from the cytoplasm. Most proteins in a subcellular compartment are not synthesized by the ribosomes but in the endoplasmic reticulum from which they must be translocated. In some cases the proteins must cross two membranes, and in some cases they must only cross one membrane because there are also many proteins in the intermembrane space, the outer compartment of the mitochondria.

Non-Bilayer Lipids and the Inner Mitochondrial Membrane

DR. de Kruijff:* In the last decade the fluid mosaic model (Singer and Nicholson, 1972) of biological membranes has become generally accepted as it provided a rationale for many structural and functional features of membranes. More recently, however it has become increasingly clear that this model is incomplete for reasons relating to lipid composition as well as functional abilities of biological membranes. First, although the chemical variation in membrane lipids is enormous, it is surprising that most of them can be divided into only two groups on structural grounds: The lipids of the first group, including phosphatidylcholine and sphingomyelin, will organize themselves in bilayers when they are in the fully hydrated state (bilayer lipids). It is obvious that this property has greatly contributed to the bilayer concept of biological membranes. In contrast, the lipids in the second group do not form bilayers when they are dispersed in excess buffer (non-bilayer lipids). This group includes major lipids such as phosphatidylethanolamine, monoglucosyl and monogalactosyl diglyceride and cardiolipin (in the presence of Ca^{2+}) (Cullis and de Kruijff, 1979). These lipids prefer the hexagonal H_{II} phase (Figure 1-3). This phase consists of cylinders of lipids surrounding long aqueous channels. The unique feature of the H_{II} phase both from a structural and functional point of view is that it allows polar lipids to be organized in a low energy configuration inside a hydrophobic environment. The reason for the abundant presence of these non-bilayer lipids in membranes is difficult to understand in terms of membrane models in which the bilayer is suggested to be the only organization available to the lipids.

* Work done in collaboration with A.J. Verkleij, C.J.A. van Echteld, W.J. Gerritsen, P.C. Noordam, C. Mombers, A. Rietveld and J. de Gier, Utrecht, The Netherlands, and P.R. Cullis, M.J. Hope and R. Nayer, Vancouver, B.C. Canada. Also in press in <u>Cell Biology</u>.

Figure 1-3: Schematic representation of the bilayer and the hexagonal H_{II} phase.

The second property of biological membranes, not explained by present membrane models, is of a functional nature. In a large number of membrane mediated processes, part of the lipids would appear to temporarily have to leave the bilayer configuration. Clear examples are all forms of membrane fusion and transbilayer movements of lipids (flip-flop). These processes have as a common characteristic that in some intermediate state part of the lipids must reside within the hydrophobic environment of the membrane. These two considerations have led us to suggest an alternative model of biological membranes (Cullis and de Kruijff, 1979) in which non-bilayer lipids and the non-bilayer structures they allow play active functional roles.

I would like to illustrate the various aspects of non-bilayer structures employing the lipids of the inner membrane of the mammalian mitochondrion. It will be shown that this membrane contains a large fraction of lipids which when hydrated (in isolated form) will adopt various non-bilayer phases under a variety of different physiological conditons. The implications of these findings for the structure of the inner mitochondrial membrane will be discussed especially in relation to Ca^{2+} transport, protein insertion and cytochrome oxidase activity.

Structural properties of inner mitochondrial lipids

Single Lipid Systems

Phosphatidylcholine, phosphatidylethanolamine and cardiolipin are the main lipids found in the inner membrane of the mammalian mitochondrion in beef heart mitochondria, for example, amounting to 39, 33 and 25 mol % respectively of the total lipids (Krebs et al., 1979). When these lipids in isolated form are dispersed in excess buffer they will undergo a process of self-association, thereby forming large aggregates, the structure of which will depend on the type of lipid and the conditions. This polymorphic phase behavior of phospholipids can be conveniently monitored by ^{31}P NMR (Cullis and de Kruijff, 1979). In the case of lipids organized in extended bilayers, the proton decoupled 31P NMR spectrum has a characteristic asymmetrical lineshape with a low field shoulder and a high field peak. Such a spectrum is observed for an aqueous dispersion of inner mitochondrial phosphatidylcholine at 37°C. Figure 1-4 demonstrates that this lipid is organized in extended bilayers as has been found for all other long chain PC's tested so far. Phospholipids organized in the hexagonal H_{II} phase have a ^{31}P NMR spectrum of a reduced width and a reversed asymmetry. Furthermore, the dominant spectral feature has a characteristic chemical shift. Inner mitochondrial phosphatidylethanolamine in excess buffer at 37°C shows such a spectrum (Figure 1-4) demonstrates that this lipid is organized in the hexagonal H_{II} phase at 37°C. In agreement with data on other natural phosphatidylethanolamines (Cullis and de Kruijff, 1978) at lower, non-physiological temperatures the bilayer phase is preferred (Figure 1-4).

Cardiolipin, the third main inner mitochondrial lipid dispersed in a salt solution at neutral pH, forms extended bilayers (Rand and Sengupta, 1972; Cullis et al., 1978) as is illustrated in Figure 1-5. In addition to a preferential localization in the inner mitochondrial membrane this lipid has another very distinctive property in that it is the only major negatively charged membrane phospholipid which will adopt the hexagonal H_{II} phase in the presence of various divalent cations (Rand and Sengupta, 1972; Cullis et al., 1978). The preference of cardiolipin for the H_{II} phase in the presence of equimolar Ca^{2+} is illustrated in Figure 1-5. Since the mitochondrion can actively accumulate large amounts of Ca^{2+} into the matrix space (Nicholls and Crompton, 1980; Carafoli, 1979) and cardiolipin is preferentially located on the inner monolayer of the inner mitochondrial membrane (Krebs et al., 1979) (thus facing the matrix space) we have the remarkable possibility that for this biomembrane the majority (60) of its lipids will <u>not</u> prefer the bilayer but rather the H_{II} phase configuration under physiological conditions. Furthermore, this phase behavior can be expected to be strongly Ca^{2+} dependent.

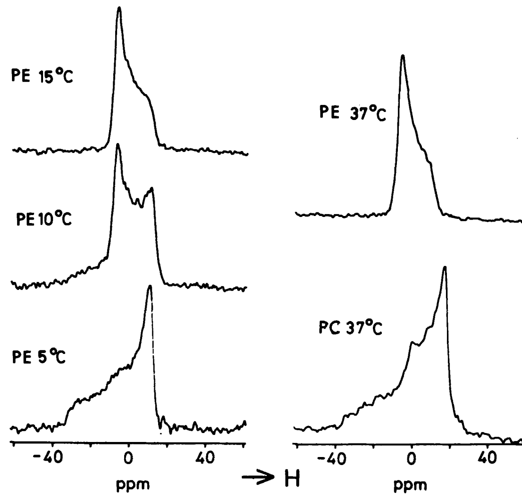


Figure 1-4: 81 MHz ^{31}P NMR spectra of aqueous dispersions of rat liver inner mitochondrial PE and PC. 30 μmoles of phospholipid was hydrated with 1.0 ml 100 mM NaCl, 10 mM Tris-acetic acid (pH 7.2) and 1 mM EDTA. Reproduced by permission (Cullis et al., 1980).

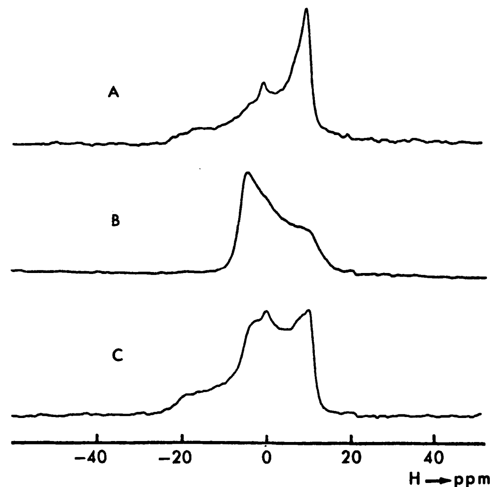


Figure 1-5: 81 MHz ^{31}P NMR spectra at 30°C of an aqueous dispersion of cardiolipin (A) in the presence of Ca^{2+} (B) and cytochrome c (C). 50 μmoles beef heart cardiolipin was dispersed in 1.0 ml 100 mM NaCl, 10 mM Tris/HCl, 0.2 mM EDTA pH 7.0. In (B) 0.1 ml 1 M $CaCl_2$ and in (C) 0.2 ml buffer containing 36 mg oxidized cytochrome c was added.

At this point of our structural analysis two important and related questions should be considered. In the first place, does the H_{II} phase occur in mixtures of the inner mitochondrial lipids. Secondly, is the presence of H_{II} phase in a membrane compatible with the functioning of that membrane. Intuitively, it seems difficult for instance to reconcile extended areas of H_{II} phase with the barrier function of the membrane. Therefore, we have to consider the possibility that the H_{II} forming tendency of phosphatidylethanolamine and cardiolipin can be expressed in alternative, possibly functionally, more relevant, non-bilayer structures.

Mixed Lipid Systems

The major binary mixtures of the inner mitochondrial membrane lipids are phosphatidylcholine-phosphatidylethanolamine, phosphatidylcholine-cardiolipin and phosphatidylethanolamine and cardiolipin mixtures. The results obtained with the one lipid systems indicate that these lipid mixtures will have different polymorphic phase properties, therefore, we will discuss them in turn. It should be noted that as the ^{31}P NMR measurements require substantial amounts of lipids (the isolation of which from the inner mitochondrial membrane is rather tedious), these experiments employ more readily available natural phosphatidylethanolamines and phosphatidylcholines which have a very similar phase behavior to inner mitochondrial lipids.

In mixtures of unsaturated phosphatidylcholine and phosphatidylethanolamine the phosphatidycholine component will stabilize a bilayer structure for the phosphatidylethanolamine component (Cullis and de Kruijff, 1979; Cullis and de Kruijff, 1978). When this bilayer stabilization by phosphatidylcholine is monitored by ^{31}P NMR unexpected intermediate situation is encountered. Instead of gradually going from a "hexagonal H_{II}" to a bilayer spectrum with increasing phosphatidylcholine concentration, a narrow symmetrical resonance at the chemical shift position of phospholipids undergoing rapid isotropic motion is observed as an intermediate (Cullis and de Kruijff, 1979; Cullis and de Kruijff, 1978). This "isotropic" signal can become the dominant spectral feature in the ^{31}P NMR spectrum (de Kruijff et al., 1979). In mixtures of phosphatidylcholine and mitochondrial cardiolipin in the absence of divalent cations, as expected, only the lamellar phase is observed (de Kruijff et al., 1979). Addition of Ca^{2+} to these mixtures results again in an unexpected intermediate situation. Instead of a mixture of bilayer and H_{II} phase an isotropic ^{31}P NMR signal is observed for a fraction of the phospholipids (de Kruijff et al., 1979).

Cardiolipin-phosphatidylethanolamine are of particular interest for two reasons. In the first place both lipids are preferentially localized in the inner monolayer of the inner mitochondrial membrane (Krebs et al., 1979). Secondly it can be expected that cardiolipin will stabilize the bilayer configuration of phosphatidylethanolamine but that this stabilization will be extremely Ca^{2+} sensitive. This

is illustrated in Figure 1-6 for a 2:1 mixture of soya phosphatidylethanolamine (which adopts the H_{II} phase above 10°C (de Kruijff and Cullis, 1980)) and cardiolipin where, in the absence of Ca^{2+} predominantly a bilayer structure is observed at 30°C. Addition of a small amount of Ca^{2+} (Ca^{2+}/cardiolipin = 0.05) has a dramatic effect on the spectrum in that a large isotropic peak is formed at the expense of the bilayer signals (Figure 1-6). With higher amounts of Ca^{2+} as expected only the H_{II} phase is formed. The picture which emerges from these experiments is that in a mixed system of a bilayer and a non-bilayer lipid, the non-bilayer preference of the latter lipid is not expressed in formation of the H_{II} phase but in an intermediate structure in which the phospholipids can undergo rapid (on the NMR time scale) isotropic motion. This situation is also observed for the total inner mitochondrial lipids. A large isotropic ^{31}P NMR signal is observed superimposed on a bilayer lineshape (Cullis et al., 1980). Addition of Ca^{2+} increases the isotropic component and also induces the formation of some H_{II} phase (Cullis et al., 1980).

^{31}P NMR cannot give precise information on the structure of this "isotropic" intermediate structure. Since these systems are macroscopically large the isotropic signal cannot originate from small vesicles in which vesicle tumbling or lateral diffusion of

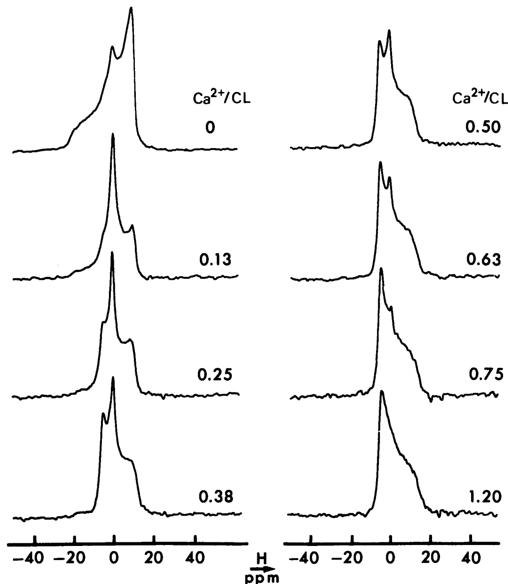


Figure 1-6: Effect of Ca^{2+} addition on the 81 MHz ^{31}P NMR spectra of soya (PE-CL (2:1) mixed lipsomes. To 50 µmoles phospholipid dispersed in 1.0 ml of 100 mM NaCl, 10 mM Tris/HCl, 0.2 mM EDTA pH 7.0 aliquots of a 100 mM $CaCl_2$ solution were added to give the Ca^{2+}/cardiolipin ratios indicated in the figure.

the lipids would provide an isotropic averaging mechanism. Non-bilayer structures such as inverted micelles or lipids, undergoing rapid lateral diffusion around curved bilayer surfaces in a large structure are most likely candidates for this "isotropic" signal.

Freeze-fracturing provides a less ambiguous interpretation of the structure of the "isotropic" phase. In these systems large numbers of small (60-120 Å, depending on the system) uniformly sized particles and pits are often present (de Kruijff et al.,1979; Verkleij et al., 1979 and de Kruijff et al., 1980) on the fracture faces. The observation of these intramembranous "lipid particles" (these systems contain no protein), an example of which is shown in Figure 1-7 for phosphatidylcholine-cardiolipin (1:1) bilayers in the presence of Ca^{2+}, together with the ^{31}P NMR data suggest that the "isotropic" intermediate structures are intra bilayer inverted micelles (Figure 1-8a). Freeze-fracturing indicates that the inverted micelles can be both randomly dispersed or linearly arranged in the lipid bilayer. The linearly arranged particles appear to be localized on the nexus of intersecting bilayers. This situation might be encountered in multi-layered liposomes in which, upon fusion of bilayers inside the liposomes, a honeycomb network of bilayers can be formed (Figure 1-8b). This latter structure, in which two bilayers are joined via inverted micelles might be relevant for the possible sites of contact between outer and inner mitochondrial membrane (Block et al., 1971).

Inner Mitochdondrial Lipids and Transbilayer Transport

Since the lipidic particle appears to be the favored non-bilayer structure in mixtures of inner mitochondrial lipids, it is useful to consider the way this structure can be formed in a bilayer. In addition to its occurrence as an intermediate in bilayer fusion (Verkleijj et al., 1979 and 1980) it can be envisaged that within a bilayer inverted micelles can be formed by local high concentrations of the H_{II} lipid (Cullis and de Kruijff, 1979). This could result in an invagination of the bilayer resulting in the formation of the inverted micelle which can be dissociated in the opposite or the original monolayer. This model predicts two properties of the inverted micelle containing bilayer. In the first place phospholipid flip-flop which in pure lipid bilayers under equilibrium conditions is extremely slow (Rothman and Lenard, 1977) should be increased (Cullis and de Kruijff, 1979). Secondly, when the inverted micelle is formed as a result of divalent cation - cardiolipin interactions (Figure 1-9) the divalent cation is translocated across the membrane. In this case the inverted micelle would act as an ionophore for the divalent cation. In Figure 1-10 it is shown that for mixed phosphatidylcholine-cardiolipin bilayers both the phosphatidylcholine flip-flop and the Mn^{2+} permeability are greatly increased when lipidic particles are present in the

bilayer, supporting the above model (Gerritsen et al., 1980).

The increased divalent cation permeability in the cardiolipin containing bilayer is particularly interesting as the mitochondrion is capable of a very rapid Ca^{2+} uptake (Nicholls and Crompton, 1980; Carafoli, 1979). To elaborate further on this suggestion we studied the effect of Ruthenium red, a potent inhibitor of the Ca^{2+} transport in mitochondria, on the polymorphic phase behavior of cardiolipin (Cullis et al., 1980). Addition of equimolar Ruthenium red to cardiolipin bilayers had no effect on the ^{31}P NMR lineshape. However, its presence completely blocked the Ca^{2+}-induced bilayer to H_{II} transition of cardiolipin (Cullis et al., 1980).

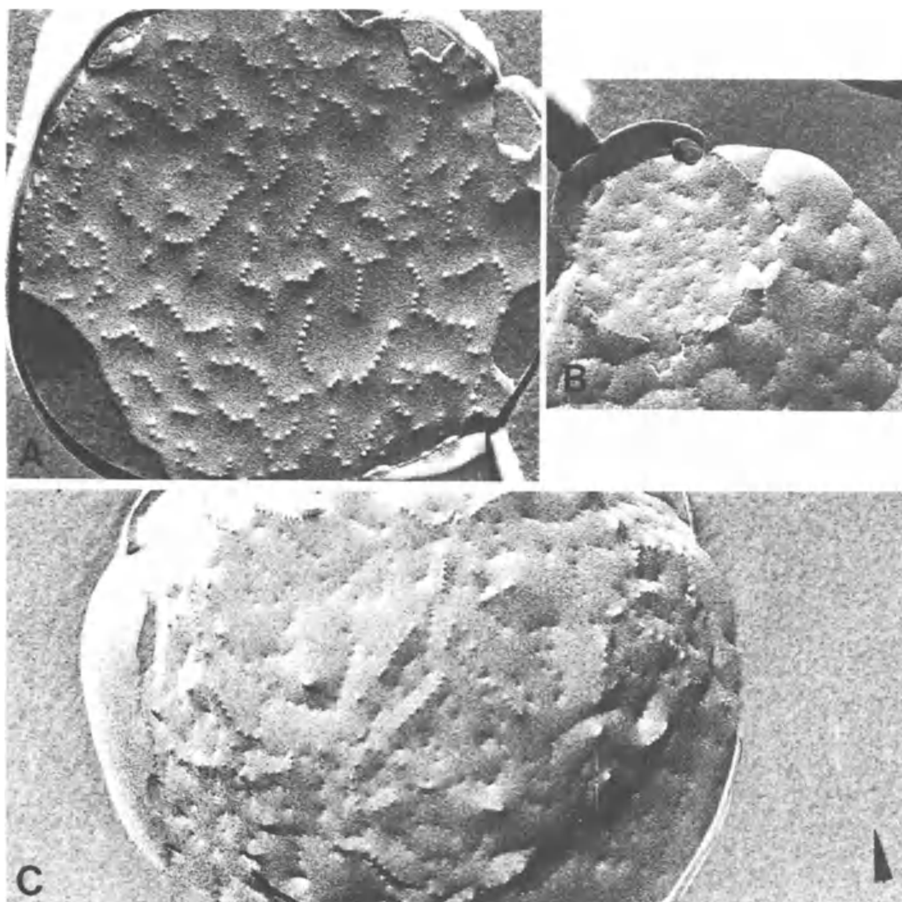


Figure 1-7: Freeze-fracture pictures of Ca^{2+} containing egg PC/CL (1:1) large unilamellar vesicles. Magnification about 100,000 x (Verkleij et al., 1979).

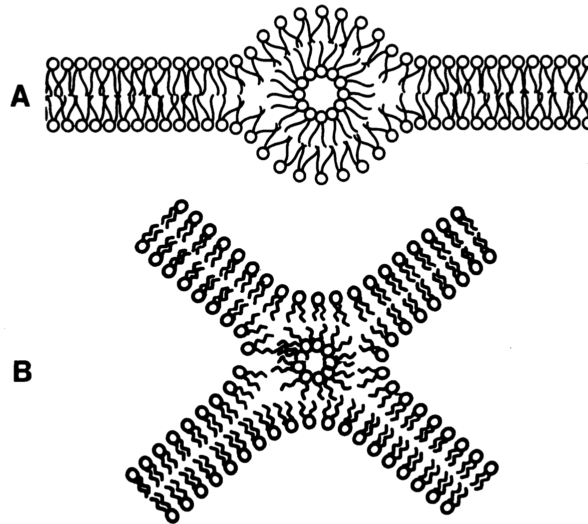


Figure 1-8: Intrabilayer inverted micelles as models for the lipidic particle. In (A) the inverted micelle is located inside a single bilayer in (B) it is shown at the nexus of two intersecting bilayers.

If divalent cation transport across the membranes could proceed via inverted micelles as ionophores it can be expected that lipids forming these structures also would be able to translocate the divalent cation into a bulk phase of low dielectric contrast such as chloroform (Cullis et al., 1980). Using the techniques developed by Green and co-workers (1976) we studied the uptake of $^{45}Ca^{2+}$ from an aqueous solution into chloroform for various membrane lipids. Table 1 shows both cardiolipin and phosphatidic acid (which can adopt under some cases the H_{II} phase in the presence of divalent cations can partition $^{45}Ca^{2+}$ into the organic phase (Cullis et al., 1980). Also in full agreement with a proposed role of cardiolipin in Ca^{2+} transport in mitochondria Ruthenium red blocks the Ca^{2+} uptake into chloroform by cardiolipin as was reported before (Tyson et al., 1976).

Cardiolipin Specific Formation of Non-bilayer Structures by Cytochrome c

Cytochrome c is a small (m.w. 12,000), nearly spherical (radius of gyration 12-14 Å) highly basic (8 net positive charges at neutral pH) inner mitochondrial protein which is involved in electron transport between cytochrome b,c_1 and cytochrome oxidase. Since it experiences strong electrostatic interactions with negatively charged phospholipids (de Kruijff and Cullis, 1980) and

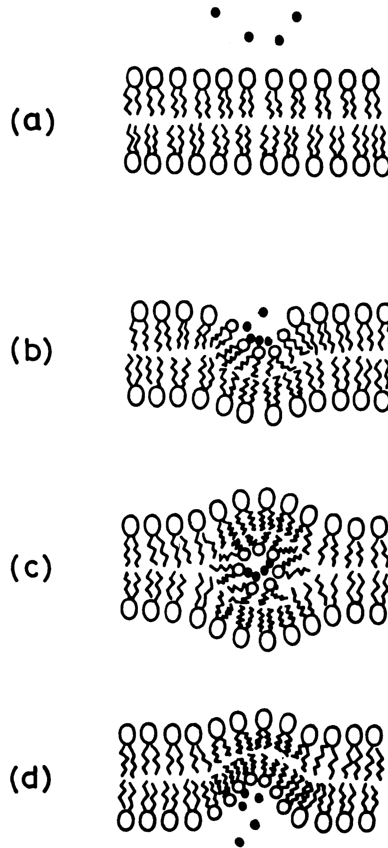


Figure 1-9: A model of facilitated transport of Ca^{2+} (or other divalent cations) via formation of an intermediate intrabilayer inverted micellar cation-CL complex (Fig. 1-2c). The headgroups of CL interacting with the cation are depicted as being smaller in order to indicate a reduction in the area per phospholipid molecule in the headgroup region arising from the Ca^{2+}-CL interaction (Cullis et al., 1980).

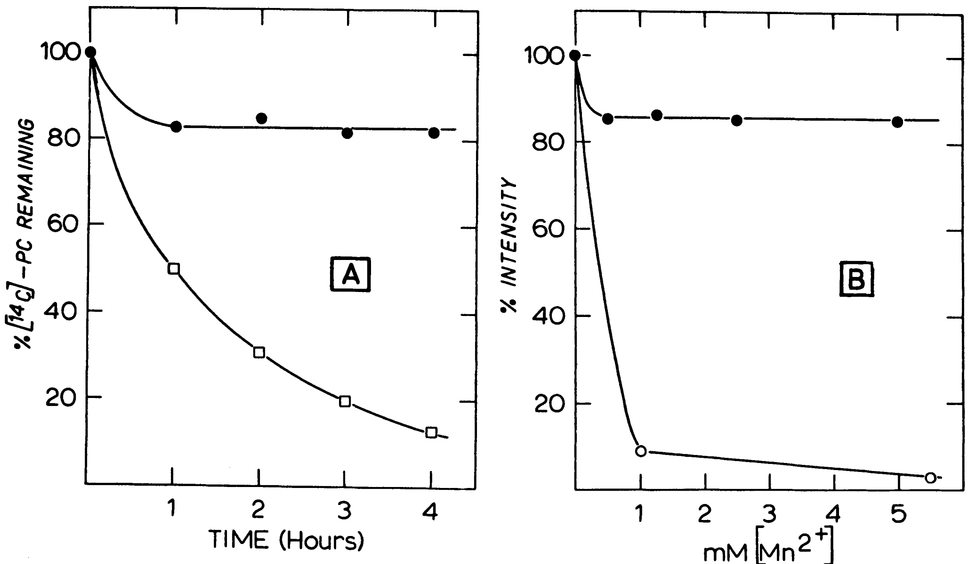


Figure 1-10: PC flip-flop (A) and Mn^{2+} permeation (B) through PC/CL (1:1) liposomes (0---0) containing no lipidic particles and PC/CL (1:1)-Ca^{2+} vesicles prepared by an ether injection method which have bilayers containing large numbers of lipidic particles (0---0). The PC flip-flop is measured by determining the fraction of ^{14}C-PC exchangeable with PC-exchange protein. The Mn^{2+} permeability is measured as a reduction in the ^{31}P NMR signal intensity of the phospholipids after the addition of aliquots of the paramagnetic cation. In the multilayered liposomes approximately 15% of the molecules are located with the outer monolayer. Reproduced by permission (Gerritsen et al., 1980).

TABLE I

Phospholipid Mediated Ca^{2+} Uptake into Chloroform

	Amount of Ca^{2+} taken up (μmoles) *
Cardiolipin	4.7
Cardiolipin + Ruthenium red	0.5
Phosphatidic acid	10.7
Phosphatidylserine	1.1
Phosphatidylcholine	0.4
No Lipid	0.0

* Phospholipid corresponding to 6 μmoles of phosphorus was dissolved in chloroform. Subsequently 4 ml 100 mM NaCl, 10 $CaCl_2$ (containing 1 μCi 45 Ca^{2+}), 10 mM Tris/HCl pH 7.4 was added. In some cases 6 μmoles of Ruthenium red was added to the aqueous buffer. The resulting two phase system was shaken for 3 h at 20°C when after the amount of 45 Ca^{2+} in the chloroform phase was determined (Cullis et al., 1980).

cardiolipin is the only main negatively charged phospholipid in the membrane,we became interested in the question as to whether cytochrome c would show a specific interaction with cardiolipin and in particular whether this interaction would result in the formation of non-bilayer structures.

Interacting cytochrome c (oxidized or reduced) with a variety of different negatively charged membrane phospholipids revealed a specific change in the ^{31}P NMR spectrum indicative of the formation of the H_{II} phase and an isotropic phase (de Kruijff and Cullis, 1980) (Figure 1-5c) only when cardiolipin was present in the bilayer. This result was confirmed by freeze-fracture electron microscopy where the H_{II} phase structure and numerous small (< 60 Å diameter) particles are observed on the fracture face of the bilayer (Figure 1-11). These latter structures are highly unusual for a protein which is believed to be extrinsic. These data, together with the notion that cytochrome c upon inter-action with negatively charged phospholipids can form isooctane soluble complexes (Das and Crane, 1964) which upon rehydration give rise to H_{II} phase (Borovjagin and Moshkov, 1974), can be rationalized as shown in Figure 1-12. Upon electrostatic

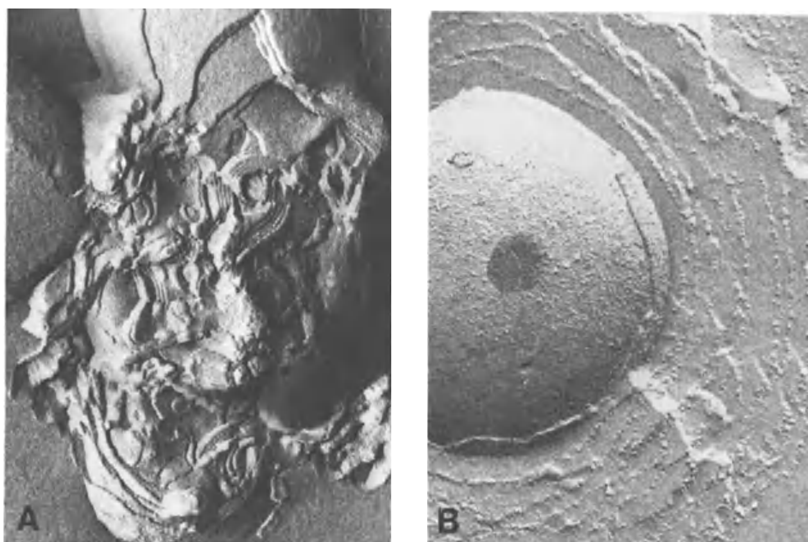


Figure 1-11: Freeze fracturing of CL liposomes after the addition of cytochrome c. Details as in the legend of Fig. 1-5c. Next to the areas of H_{II} phase (A) multilayered bilayer vesicles are observed on the bilayers of which (including the innermost) numerous small paritcles are visible (B). This is also evident in cross-fractures of the bilayers. Magnification about 100,000 x.

interaction with cardiolipin the lipid molecule will adopt a shape permitting the bilayer to invaginate thereby allowing cytochrome c partly to "penetrate" the bilayer (without interacting to a great extent with the acyl chains of the lipids!). This process can finally result in the intrabilayer inverted micellar or short H_{II} configuration in which the cytochrome c is present. This complex can dissociate in a way analogous to that described in Figure 1-9 on either side of the bilayer permitting the cytochrome c to move across the bilayer. This latter process has been observed in both model membranes (de Kruijff and Cullis, 1980) and in the membrane of the sub-mitochondrial particle (Nicholls, 1974). The possibility of the cardiolipin specific formation of a low energy intrabilayer configuration of cytochrome c offers new possibilities for the involvement of lipids in cytochrome oxidase activity which will be discussed in more detail.

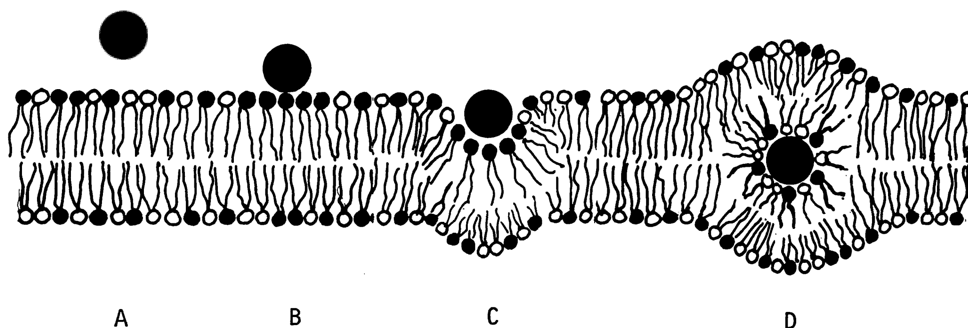


Figure 1-12: Model for the formation of intra-bilayer non-bilayer structures of cytochrome c in CL (dark lipids) containing bilayers. Soluble cytochrome c (A) binds to the bilayer surface, thereby clustering the Cl (B) which results in bilayer invagination (C) and subsequent formation of cytochrome c containing inverted micelles or H_{II} phases (D).

Despite the fact that the mitochondrion has been an extremely popular system for many membrane scientists it is surprising to discover how little is actually known about the precise structure of the inner mitochondrial membrane, in particular with respect to its membrane lipids. The question of the structure of the lipid component of this membrane becomes even more pertinent in view of our findings that the lipids can adopt non-bilayer structures and that both Ca^{2+} and cytochrome c will promote the formation of these structures.

Phospholipid Structure in the Inner Mitochrondrial Membrane

Although thin sectioning electron microscopy indicates a trilamellar structure typical of a bilayer structured membrane, considerable controversy exists about the interpretation of these pictures (Sjostrand, 1978). Freeze-fracturing studies have given firm evidence that extended areas of bilayer exist in this membrane (Hackenbrock, 1977). However, quantitative information on the extent of this structure is not available. Classical structure probing techniques such as x-ray have, to our knowledge, not been successfully applied to the mitochondrion. This most likely is related to the extreme biochemical instability of this metabolically very active membrane. This will become clear in the following sections in which we will summarize our own structural ^{31}P (Cullis et al., 1980) NMR studies on the isolated inner membrane ghost and the intact mitochondrion.

The isolated inner mitochondrial membrane was first studied in order to avoid complications caused by the presence of small phosphorous containing molecules present in the matrix.

Inner Membrane Ghost

Inner mitochondrial membrane ghosts prepared by osmotic lysis of mitoplasts show ^{31}P NMR spectra which do not change when the membranes are incubated for up to 30 minutes at temperatures ranging from 4-37°C (Cullis et al., 1980). Since the data accumulations take on the order of 10 min we feel confident that we obtain information on the "native" membrane. The ^{31}P NMR spectrum of these membranes at 4°C has the characteristic line shape of phospholipids organized in extended bilayers (Figure 1-13). At the 0 ppm position a small (± 10% of the total intensity) signal characteristic of phospholipids undergoing isotropic motion is observable. Increasing the temperature to 37°C results in a spectral change where the isotropic component has grown at the expense of the bilayer component. Due to the fact that both resonances overlap, no exact quantification on the amount of isotropic signal can be given. Incubating the membranes at 37°C with increasing amounts of Ca^{2+} results in a marked increase in the amount of isotropic signal (Figure 1-13). The experiments demonstrate that the majority of the phospholipids in the isolated inner mitochondrial membrane are localized in extended

bilayers but that a small fraction of the phospholipids undergoes rapid isotropic motion at 37°C. This fraction is increased in the presence of Ca^{2+}. The exact nature of the structures giving rise to this motion is unknown.

Since the inner mitochondrial membrane is quantitatively by far the most abundant membrane in the mitochondrion we attempted to study its phospholipid organization in the intact mitochondrion by ^{31}P NMR. The ^{31}P NMR spectrum of rat liver mitochondria is composed of a bilayer component on top of which narrow resonances of various phosphorous containing molecules such as Pi, ATP and ADP are present (Cullis et al., 1980). Incubating these mitochondria for times as short as 5 min at 37°C results in large spectral changes, mainly because of ATP hydrolysis, resulting in a growth of the phosphate peak which makes it virtually impossible to say anything of the structure of the membrane phospholipids. These problems can be partially circumvented by using more stable beef-heart mitochondria, 20 mm sample tubes containing 8 ml of mitochondrial suspension (50 mg protein/ml) thereby cutting down the accumulation time to approximately 1 min, and by keeping

Intact Mitochondrion

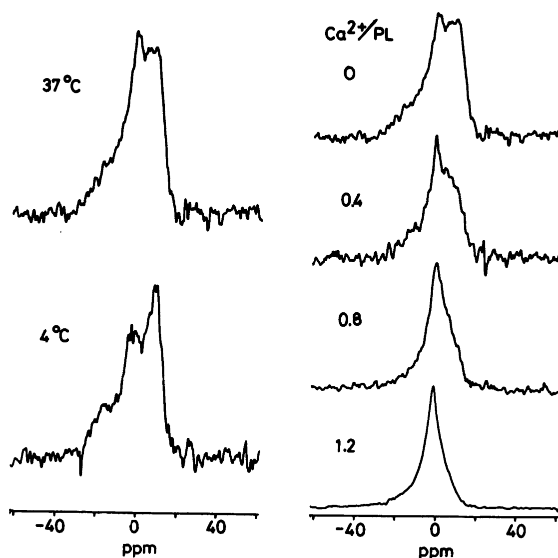


Figure 1-13: 81.0 Mhz ^{31}P NMR spectra of isolated rat liver inner mitochondrial membranes. The Ca^{2+} titration was done at 37°C. The amount of Ca^{2+} is expressed as a molar ratio to total inner mitochondrial phospholipid (PL). Reproduced by permission (Cullis et al., 1980).

the mitochondria (in the presence of succinate) well oxygenated by continuously injecting H_2O_2 to the catalase containing stirred sample. Under these conditions we were able to obtain 31P NMR spectra at 37°C and 0°C which remained stable for up until 10 minutes (Figure 1-12). At 0°C, except for two small resonances originating most likely from inorganic phosphate and a phosphate, the spectrum consists entirely of the typical bilayer spectrum and demonstrates that at this temperature all phospholipids are organized in extended bilayers. At 37°C a similar spectrum is observed with the additional feature of a small but signficant broad isotropic peak centered at 0 ppm. At the same time the definition of the low field shoulder became less clear. Both of these observations demonstrate increased isotropic motion of a small part of the membrane phospholipids. Finally, incubating the intact beef-heart mitochondria with 100 mM Ca^{2+} results in a large increase in the isotropic component.

Functional Implications

The occurrence of non-bilayer structures in the hydrated preparation of inner mitochondrial lipids and the observation of isotropic motion for part of the inner mitochondrial phospholipids at 37°C in the intact system suggests that non-bilayer structures might occur in this membrane *in vivo*. It is therefore useful to consider how the mitochondrion may benefit functionally from these structures. In this discussion we will speculate on the involvement of non-bilayer lipids in three areas of mitochondrial functioning, e.g. (1) Ca^{2+} uptake, (2) protein insertion and (3) cytochrome oxidase activity.

Ca^{2+} Transport

Many cellular processes and enzymes are sensitive to and often regulated by Ca^{2+} present in the cytosol. It has been postulated that the mitochondrion can act as a regulator of this Ca^{2+} pool (Nicholls and Crompton, 1980) since it is capable of a very fast energy-dependent Ca^{2+} uptake and release. The molecular details and biochemical nature of this process are not very well understood. However, our results show that Ca^{2+} induces the formation of lipidic particles in cardiolipin containing bilayers, that these particles facilitate flip-flop and divalent cation transport, that Ruthenium red blocks the formation of non-bilayer phases by Ca^{2+} in cardiolipin containing bilayers and that this inhibitor of Ca^{2+} transport in mitochondrion also blocks the uptake of Ca^{2+} in an organic phase by cardiolipin. We therefore propose that non-bilayer structures (most likely inverted micelles) formed as a result of the Ca^{2+} -cardiolipin interaction are involved during some stage of the Ca^{2+} transport process across the inner mitochondrial membrane.

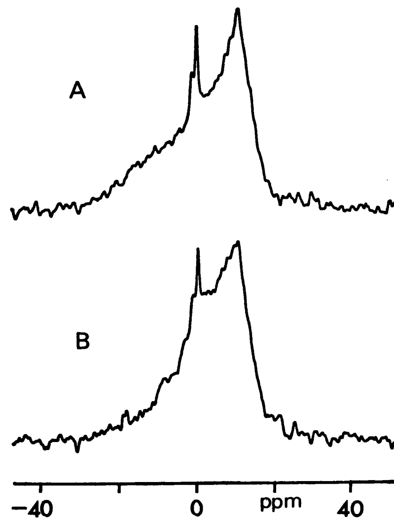


Figure 1-14: 81.0 MHz ^{31}P NMR spectra of intact beef heart mitochondria at (A) 0°C and B (37°C). The sample consisted of 8 ml mitochondria (50 mg protein/ml) in 0.25 M sucrose pH 7.4 suspension containing 50 mM Na-succinate and 1 mg catalase which was placed in a 20 mm NMR tube and was kept continuously oxygenated by injecting at a rate of 100 µl/min a 3% H_2O_2 solution into the stirred suspension.

Many different proteins of the inner mitochondrial membrane such as cytochrome c and several subunits of the cytochrome oxidase are synthesized on endoplasmic reticulum bound ribosomes and are subsequently transported to and inserted in the inner mitochondrial membrane. Furthermore it is known that cytochrome c can move across the inner mitochondrial membrane (Nicholls, 1974) which process might be related to the turnover of this protein. Although it is clear that protein factors will be involved in these processes, we suggest that inverted non-bilayer structures could provide a low energy pathway for the insertion and translocation of these proteins. Our own data on the cardiolipin specific cytochrome c induced non-bilayer phases support this hypothesis. Furthermore, it is a remarkable observation that many if not all of the "integral" inner mitochondrial membrane proteins which are synthesized on endoplasmic reticulum bound ribosomes are, like cytochrome c, basic proteins with a relatively low hydrophobicity (Iyengar and Iyengar, 1980). Examples of such proteins include: creatine kinase, the ADP-ATP porter, subunit V of the ATPase and subunits VI and VII of cytochrome oxidase (Iyengar and Iyengar, 1980). Furthermore, in model experiments it has been demonstrated that for incorporation of isolated cytochrome c oxidase in a bilayer cardiolipin (in the presence of phosphatidylcholine) or a mixture of another (bilayer type) negatively charged lipid with unsaturated phosphatidylethanolamine is required (Eyton et al., 1977). Saturated phosphatidylethanolamine's (which cannot adopt the H_{II} phase (Cullis and de Kruijff, 1978)) inhibit the proper incorporation of cytochrome oxidase (Eyton et al., 1977). In addition, it has been shown that the mitochondrial $^{31}P_i$-ATP exchange reaction only can be properly reconstituted in mixed phosphatidylcholine/phosphatidylethanolamine systems (Racker et al., 1975) in which, as we have demonstrated, non-bilayer structures can occur.

Protein Insertion

Cytochrome oxidase has been recently demonstrated to have an absolute requirement for cardiolipin for activity (Fry and Green. 1980; Vik and Capaldi, 1980). We demonstrated that cytochrome c, the substrate for cytochrome oxidase, shows a very specific and unique interaction with cardiolipin resulting in the formation of an intra membrane cytochrome c-cardiolipin complex. We, therefore, propose that the formation of this complex is important for the electron transport between cytochrome c and cytochrome oxidase. Possible modes of interaction between cytochrome c in this complex and cytochrome oxidase are shown in Figure 1-13. By extrapolation to the intact membrane we suggest that cytochrome c could be involved in lateral intramembrane electron transport between cytochromes b, c_1 and cytochrome oxidase. Although recent data (Schneider et al., 1980) indicate that the cytochromes b, c_1 and c and cytochrome oxidase form one free diffusable complex, others (Roberts and Hess, 1977) have reported that cyto-

Cytochrome Oxidase Activity

chrome c is a highly diffusible membrane component in the process of the electron transfer.

The abundant occurrence of non-bilayer lipids in the inner mitochondrial membrane offers exciting new dimensions to our understanding of the functioning of the mitochondrion. Further research will be directed towards answering two fundamental questions; (1) what is the role of non-bilayer lipids in the coupling between electron transport and ATP synthesis and (2) what will be the structural organization of the non-bilayer lipids under conditions of active oxidative phosphorylation?

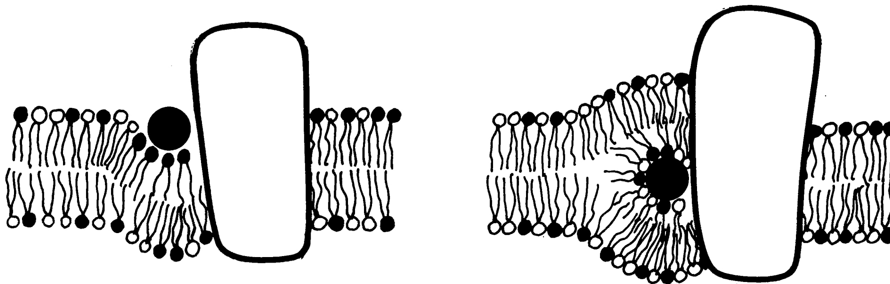


Figure 1-15: Two possible ways in which cytochrome c-CL non-bilayer complexes could interact with cytochrome oxidase

DR. SANDRA: What do you think is the role of cholesterol in your system?

DR. de KRUIJFF: It is a remarkable fact that the bilayers from membranes that have a high PE content hardly ever have cholesterol. In bacterial systems, if you have a high PE content, there is no cholesterol. Microsomal membranes have a high PE content but no cholesterol. If you come across a membrane with a high cholesterol content, with a low PE content, in effect what we have demonstrated is a system where PE is present and it is all in the non-bilayer phase. For example, if you have inverted micelles, cholesterol has a tendency to make its own bilayer. So we think it might be one reason why it is favorable for those systems not to be too close together at all times.

DR. SANDRA: I would like to comment on the exchange protein experiment. If you incubate phospholipid exchange protein (PLEP) together with inverted micelle, is the exchange at an acceptable level? Would you expect that it would not work or would you expect the full outer leaflet of the membrane to be acquired accessible for the exchange protein?

DR. de KRUIJFF: The outer monolayer lipids will be exchanged to the outside of the vesicle and we believe that to fit this mechanism of the inverted micelles, we have flip-flop from the inside to the outside and thereby exposing the inner monolayer lipids to the external environment.

DR. SANDRA: I guess what I am saying is that I do not consider that proof of the inverted micelle. Is there convincing evidence for the flip-flop in inverted micelles?

DR. de KRUIJFF: The proof of the inverted micelle stems from the fact that we do see a freeze fracturing inside the lipid bilayer structure. It is obvious it was formed from what you would expect from these lipid micelles. We see either a particle or a depression shown in the fracture plane either underneath or above the structure. We see the phosphorylation between external phases as we know it in an inverted phase and those inverted micelles.

DR. SANDRA: Could the structures you see at freeze fracturing be microlenses of trapped organic solvents retained during the preparation of the inverted micelle?

DR. de KRUIJFF: No. For instance, in the case of PC, we, of course, have all the controls in our hands. If you don't add calcium you don't see them.

DR. BUTTERFIELD: Let's go back to the red cells as it relates to the manganese story. Of course, manganese does not penetrate red cells within the period of about three hours. If measured from when using it first you broaden the proton resonance in water and you measure water transfer.

DR. de KRUIJFF: There is no cardiolipin in the erythrocyte. The unique thing about cardiolipin is that it is the only negatively charged lipid you find in a membrane. It can go into a new bilayer phase counteracting the calcium. Phosphoserine upon infusion of calcium stays in the lamellar phase. So since it is both positively and negatively charged and in effect both cardiolipin and calcium, active calcium, do occur in the mitochondrion. But in the erythrocyte you don't have cardiolipin and phosphoserine cannot form a non-bilayer phase on its own.

DR. BRANTON: I am extremely disturbed by the size you attribute to the particle in the freeze fracturing. I think the published results indicate that the particles are clearly larger than the size you give them. Even if they were the size you give them, 80 Å, it is hard to reconcile that with a micelle made up of lipids in which, in a bilayer configuration, knowing the length of the hydrophobic domain, should be on the order of 40 or 50 Å in diameter. The diameter I measured for the particles is more frequently in excess of 100 Å. On the basis of what I have seen so far, I am totally convinced that the existence of these micelles must rest entirely on the NMR results, mostly because I understand them less well. So I would like to ask how firm the evidence is that your peak can be attributed exclusively to a micelle. Are there other interpretations? How do you reconcile the presence of that peak with a very small amount of lipids?

DR. de KRUIJFF: It is certainly true that in the case of monoglucosyl triglyceride lecithin, the particles you see are 60 Å. In the case of the PC cardiolipin, they are about 90 Å, in the case of the PE cholesterol PC about 110. So it appears that the size is determined by the lipid and it makes sense.

DR. BRANTON: We are talking about lipids with a known chain length. And those chain lengths do not vary 50 Å going from one lipid to another. How do we get to a size of 120 Å.

DR. de KRUIJFF: Because they are inverted micelles and there is an internal space which is not made up by the lipid bilayer so the minimum diameter of the vesicle will be greater than two times the chain length of the lipid chain.

DR. BRANTON: I see.

DR. de KRUIJFF: I think I have to make it totally clear that in an inverted micelle you have water inside your lipids. Of course, those lipids will always be about 50 Å, but they are dependent for the quantity of water they include. I think you can take the case of cholesterol, in the case of the PCP cholesterol system you have large structures. So it would mean that the inverted micelle would be relatively large and it could have to do with the flexibility of a lipid molecule to fit into a curved surface. It is amazing to see that if you are going to make a conicated vesicle for the same lipid you see a large size inverted micelle. Even without going into those arguments I think the freeze fracturing does certainly show a good deal of evidence for those inverted micelles.

DR. BRANTON: The difference in size is attributable to the amount of water?

DR. de KRUIJFF: Yes. The other point is how strong is the NMR. NMR, of course, is not a structure determining technique.

PROTEIN INTERACTIONS IN THE CYTOSKELETON

The Cytoskeleton of the Erythrocyte

DR. BRANTON: The red cell membrane, is one of the few membranes where it has been possible to investigate in considerable detail the molecular interactions between its constitutent proteins. Other cell membranes are not as easy to obtain in large quantities. Moreover it is not easy to obtain many membranes in a desired orientation. A detailed molecular analysis requires quantitation and a knowledge of what fraction of the membrane one has and also in what state that particular membrane is. For example, aspects of a membrane may or may not be accessible depending on their direction. The human red cell has a defined shape that is attributable to a series of proteins which make up something called the red cell cytoskeleton. Cytoskeletons in general, in a variety of different eukaryotic cells are defined in terms of what there is after you have extracted the cell with a detergent. What is left after one extracts the variety of eukaryotic cells with a detergent such as Triton X-100 is usually made up of actin filaments containing the protein actin or intermediate filaments containing a protein such as vimentin and of microtubules containing the protein tubulin. In the case of the red cell, as we will see, the number of components in the cytoskeleton makes it look somewhat impoverished. It contains actin but neither tubulin nor vimentin. Now, if one extracts the cell to make what are called membrane ghosts of the erythrocyte the lipids and many of the other molecules are removed so that what one

has left is the so-called cytoskeleton or shell. It is the fibrillar anastomosing meshwork that underlies the cell membrane, which as I indicated, is somehow involved in giving the cell its shape. It is also involved in determining the distribution of some of the membrane contours. In a freeze-fracture rendition of the cell membrane one sees a typical convex face of the membrane composed of smooth areas with intervening particles attributable to some of the transmembrane proteins. They appear to be located in a rather random or uniformly distributed fashion in the plane of the membrane of a fresh cell or freshly prepared ghost. Under certain conditions, for example, certain pH's, the intramembrane particles become aggregated. This is a very striking change in the organization of the membrane which again can be attributed to the underlying meshwork of protein in the cytoskeleton. I shant go into the evidence indicating how we know that the cytoskeleton is involved in changing the distribution of particles but rather I will address more specifically the question of how the cytoskeleton is related to these transmembrane elements and how the membrane is put together.

The Binding of Structural Proteins in the Red Cell Membrane

The Coomassie blue staining shows about 8 or 9 major proteins starting with bands 1 and 2 which together make up a protein called spectrin, one of the major proteins of the red cell membrane. Underlying it is the protein formerly known as 2.1 which is now called ankyrin because it provides the attachment site for spectrin to the membrane. Then we have another major membrane protein, band 3 which is the transmembrane protein responsible for anion transport and possibly also for sugar transport in the red cell membrane. Underlying that we have two proteins, band 4.1 and 4.2 which have no further name and 4.5 which also has no further name. One of the remarkable things about red cell membranes is that some of the relatively few polypeptides still remain nameless. Band 5 has been characterized as being erythrocyte actin, a very standard actin kind of molecule such as found in any number of prokaryotic cells. Underlying that are band 6 and 7 which again have no name, although it is known that band 6 represents glyceraldehyde-3-phosphate dehydrogenase. With periodic acid-Schiff staining where we reveal the position of the glycosylated proteins, we identify three major proteins names here, PAS 1,2 and 3. PAS 1 is also called glycophorin and it has been extensively studied and characterized by Vincent Marcessi. The cytoskeleton is made up predominantly of three proteins, polypeptides 1 and 2 together which make up spectrin; polypeptides, band 5, which is actin and band 4.1. When one extracts an erythrocyte ghost with Triton-100, spectrin, actin and band 4.1 are left and the question we have been trying to ask is how are these proteins related to each other and how are they related to the cell membrane. The predominant protein clearly is spectrin, from the fact that it stains quite heavily.

It has become clear during the past year or so that spectrin is an actin binding protein, similar to many actin binding proteins in being able to cross-link actin filaments and also in having an elongate floppy shape. The general distribution of the polypeptides of the erythrocyte membrane was reviewed by Ted Steck in 1975. Band 3 and PAS 1 are two major transmembrane proteins but most of the polypeptides I spoke of are on the inside surface.

Spectrin itself is an elongate floppy molecule which can be visualized in the electron microscope by drying out of glycerol and low angle rotary shadowing. It is approximately 1000 Å long and is made up of 2 physical strands which we now know represent the two polypeptides which we see in polyacrylamide gels. These two polypeptides appear to be bound to each other at their termini but interact weakly and in a variety of different ways along their lengths. Spectrin can also exist as a tetramer made up of two polypeptides of band 1 and 2 of band 2. This tetramer is an end to end association of the heterodimer. It is an end to end association because the tetramer is twice as long as the dimer. To determine how this basic molecule of the cytoskeleton is bound to the red cell membrane we have focused our attention in part on the structure of the molecules and in part on the biochemistry of their interaction. To study how spectrin was associated with the red cell membrane, we devised an assay in which we reduce the red cell membranes to smaller vesicles. These vesicles were either right side out or inside out. If one measures the amount of spectrin that is bound to these vesicles as a function of the amount of spectrin that is added, one obtains a saturable plot. The spectrin association with inside out vesicles is slow, it is pH dependent, it is abolished by heating spectrin, it requires salt, is saturable at about 200 μg spectrin per mg of membrane protein. This is the saturation one obtains with spectrin tetramer. Using the spectrin dimer it is closer to about 100-120 μg spectrin per /mg membrane protein. In the native cell membrane there is approximately 200 μg spectrin per /mg membrane protein. So the saturation level here is the same as the proportion of spectrin to total membrane protein one finds in the native cell. Finally, one can obtain from this kind of analysis the dissociation constant which, at pH 7.6 is about 10^{-7} indicating a relatively high affinity binding of spectrin to the inside out vesicle. The obvious question is, what is the nature of the interaction, to what is spectrin binding?

Further work indicated that spectrin binds to band 2.1, which has been named ankyrin and that ankyrin in turn binds to the major transmembrane protein, band 3. Having purified the ankyrin molecule, we sought to learn about the structure of its linkages to spectrin. Ankyrin has a different structure from spectrin, being a more globular polypeptide with an elongated axis of about 70 Å and a short axis of about 50 or 60 Å. If one incubates together, the purified ankyrin molecule with the purified spectrin molecule,

one sees that the binding site on the spectrin molecule is approximately 200 Å from one end of the polypeptide, in other words about 800 Å from the other end. The next question was which end? We can define the ends in terms of an end that participates in tetramer formation. Doing the same kind of experiments not with dimers but with tetramers. One finds that the ankyrin molecule binds about 200 Å from the center of the tetramer suggesting that the binding site is located at the end closest to the end that participates in tetramer formation. This view of the molecule also confirms that the end to end association between the spectrin heterodimers is a head to head association rather than a head to tail association.

Using similar experimental maneuvers, we have now explored the interaction of band 4.1 and of actin with spectrin. The picture which emerges is that of a complicated network in which ankyrin binds to band 3, spectrin tetramers bind to ankyrin and band 4.1 and actin bind to spectrin tetramers. Thus, nearly all of the major erythrocyte membrane proteins appear to bind together, via band 3, into a protein complex which stabilizes the red cell membrane and provides it with the elasticity and flexibility required to pass through the circulatory system.

DR. ENGEL: I wonder if you might comment on how the cytoskeleton might regulate the aggregation and distribution of membrane particles.

DR. BRANTON: Spectrin might, in some cases at least, be regulating the distribution of intramembrane particles, that is to say causing them to aggregate. In addition, there appear to be many other factors that can induce intramembrane particle aggregation. Even in the total absence of spectrin, under certain conditions, one can get intramembrane particle aggregation.

DR. STRITTMATTER: Can you tell us more about band 3 in terms of its membrane function?

DR. BRANTON: It has been studied most extensively by Ted Steck, who is right now in the process of trying to determine its amino acid sequence. It exists in the membrane as a dimer and is known to traverse the membrane and probably does so more than once.

DR. KENT: Is there any indication that the ankyrin binding to band 3 is random?

DR. BRANTON: In the native cell there is not enough ankyrin to bind to every band 3 and so it is readily understandable that only one out of eight band 3's are bound. However, there is a considerable puzzle right now in that if we do these experiments with inside-out ankyrin depleted vesicles, and bind to them ankyrin

in saturating amounts, we can add back as much ankyrin as we wish and still we find that only one out of eight band 3 molecules appear to have the potential for binding ankyrin at high affinity. In other words, seven of them seem to be masked or else it has been suggested that they may be different molecules. However, I don't think they are different molecules. There is biochemical evidence that we are dealing with a homogeneous polypeptide even though the sugar molecules may differ from one copy to another of band 3, the polypeptide region is homogeneous. We also have independent evidence that all of the band 3 molecules including those that are incapable of binding ankyrin, would be extracted out of the red cell membranes with Triton and put back into liposomes. Again we find that one out of eight of those are capable of binding. So there is something about the way those molecules are coming together in the membrane that make the site so that only some of them are accessible. We don't understand what that is, but I am speculating or believe right now that there may be stearic hindrance. We know for example that they come together at least as a dimer. There has recently been evidence that they may exist in fact as a tetramer in the membrane.

DR. PARK: Is there any competition between ankyrin and band 6?

DR. BRANTON: No there isn't. We have done experiments in which band 6 is bound and yet ankyrin binds independently. Because only one out of eight or so of the band 3's are binding ankyrin, it may be that band 6 is binding to one copy and ankyrin is binding to another copy.

DR. GONZALEZ-ROS: Has anyone checked any of these interactions previously mentioned in pathological cases when studying the cytoskeleton?

DR. BRANTON: As I indicated, some of these studies are now beginning. Up until fairly recently, the main thing known about the cytoskeleton was that it contained a lot of spectrin and everybody was looking for defects in spectrin or lack of spectrin or differences in spectrin phosphorylation. Now that it's become clear that spectrin is bound to 2.1 and 4.1 people are looking for alterations in those bindings in diseases states, but I don't know of any correlations or results to date.

DR. STROHMAN: Two questions, first do the 2.1 and 4.1 bind to the same strand or do they bind to different strands in the dimer?

DR. BRANTON: We don't know where 4.1 binds. From the electronmicroscopy we know that it only binds at the end or very close to the end. We haven't yet isolated a segment or piece of spectrin that binds. Those experiments hopefully will be done in the near future.

DR. STROHMAN: The second question is, where is the myosin that is active there?

DR. BRANTON: As far as I know, there is no myosin, there is actin but no myosin. Let me take that question a step further, and that is something perhaps I should emphasize. In non-muscle cells where one frequently doesn't have much myosin, the cytoskeleton is made up of actin, vimentin and tubulin. The shape and disposition of these molecules in the cell has been the focus of considerable attention, because using flourescent antibodies one can gain a fairly good understanding of the distribution of these proteins. Much less attention has been paid, I think, to the linking proteins, of which I would say, myosin is certainly one. In a sense I think the linking proteins are perhaps the more interesting because they provide the dynamics which are necessary to explain movement. In so far as these linking proteins are concerned, we are dealing with rather elongated flexible molecules with covalent interaction. Actin binding protein from macrophages or other proteins that bind actin filaments, appear in the electronmicroscope as wiggly little strands. Both actin binding protein and filament are dimeric molecules, homodimers not heterodimers as in the case of the spectrin and they elongate in one case a little bit longer and in another case shorter than spectrin. The thing that has brought these actin binding proteins, these flexible entities to my attention, has been the availability now of a technique to examine them in the electronmicroscope so one can get a better feeling of what they look like. Up until very recently, understanding of these actin binding proteins has been at a state where they are viewed as something biochemical, as capable of binding actin but there hasn't been any understanding whatsoever of their structure.

DR. WILLNER: Does the removal of single proteins or the insertion of them have any influence on the activity of enzymes in the red cell membrane?

DR. BRANTON: To my knowledge no one has really asked those questions. Some of those ATPases have been correlated with some of the molecules directly, for example, it has been suggested that spectrin itself has an ATPase activity and so if one removes it then one removes the aspect of the enzymatic activity. But other than that I don't know of any studies of firmly bound enzymes whose function is altered as a consequence of removing these proteins.

DR. HYDE: Is the actin in red cells identical to that in muscle as far as you know?

DR. BRANTON; No, it is not identical. Muscle actin is called alpha actin. Red cell actin is more like beta actin. It differs from muscle actin but appears to be similar to the kind of actin found in the large number of eukaryotic non-muscle cells.

DR. ENGEL: Do antibodies to one actin bind the other actin?

DR. BRANTON: Yes.

DR. CHARNOCK: Do you see any role for Mg^{++} in any of these processes? Does the phosphorylation of spectrin have any pertinence?

DR. BRANTON: All studies directed at elucidating a specific role for the phosphorylation of spectrin have failed to find it. For example, if you phosphorylate or dephosphorylate spectrin, it binds equally well to the red cell membrane and in fact, more careful recent studies by Jonathan Tyler and Jim Anderson in my lab have shown that the correlations between phosphorylation and shape change are such that the phosphorylation or dephosphorylation occurred after, rather than before the shape changes. It follows rather than preceeds the shape of things. Spectrin has yet to have shown any effect on actin binding. But that doesn't mean that it doesn't have an effect; no one has found it as yet.

DR. ENGEL: Is it not possible that as you purify the system you remove important factors which are absolutely essential to energy?

DR. BRANTON: Yes, you are quite right. It's very possible that you remove important factors. For example, we don't understand how this band 4.1 mediates the interaction between spectrin and actin. It is an essential form of interaction. One can get a direct interaction between spectrin and actin but that interaction appears to be different from the one in the presence of 4.1. And among other things, the interaction among spectrin and actin and the presence of 4.1 is very calcium sensitive. At calcium concentrations greater than about 80 micromolar, the viscosity changes are inhibited. This inhibition by calcium to the interaction is a property of the interaction that is conveyed upon the interacting species by band 4.1. In the absence of 4.1 you can also get interaction, but those interactions are not calcium sensitive. That may be the kind of interaction which we may be purifying away.

Strength and Flexibility of the Cytoskeleton

DR. STROHMAN: What is known about the tension varying capacity, again I'm looking for analogies to muscle. What's known about the effect of distortions in the cell if you stretch it or press on it mechanically? What do we know about the tension variation? If you break things can you see it all clearly with respect to the actin-spectrin interaction.

DR. BRANTON: Well, it's known that in the absence of spectrin the membrane is much more fragile, in fact, characteristically, as you remove spectrin the membrane fragments although it need not do so if you treat it very gently. But usually if you are stirring even

mildly as you are removing the spectrin by low ionic strength treatment as the spectrin elutes, the membrane vesicles frequently turn inside out and break into little pieces. So clearly the spectrin together with the actin which is being released helps to hold the membrane together and has been cited as being responsible for giving the red cell membrane the strength and plasticity to exist in our circulatory system and go through small passages and get squeezed and pushed around as it does for so many days without breaking down. It's also possible if one stretches the membrane, as has been done for electronmicroscopic purposes, to actually visualize the spectrin underneath the tear in the lipid bilayer. These are experiments in which membranes are placed on form bar and then the form bar sheeting is stretched so that one gets a tear in the lipid bilayer and can visualize it directly in the electron-microscope. It's acting there as some kind of covalent sheet of threads holding things together. Then there have been a very large number of studies that explore the correlation of the binding of spectrin to the membrane or the interaction of spectrin with the various components that have been shown to alter the fragility of cells. For example, effects of calcium: calcium is known to aggregate spectrin and precipitate it. If you introduce calcium into the cell you grossly change its fragility and its form and shape.

DR. M. GLASER: Let me ask you about how the spectrin network maintains its soft shape under normal conditions. Band 3 is, of course, mobile, moving very slowly, but will move. Is spectrin a rigid molecule itself?

DR. BRANTON: No, spectrin is not a rigid molecule. As far as we could tell it is a very floppy molecule, and in that respect it is a rather unusually elongated molecule. We're not really talking here about the tertiary or quaternary structure of the protein but about the long range folding which occurs in really a huge molecule which, unlike myosin which is fairly stiff because of a coil configuration, appears to be a very flexible multi-domain protein. It could keep the transmembrane proteins apart by binding them due to its charge or for other reasons.

DR. M. GLASER: I was just thinking that if the spectrin was pulling, everything would pull together rather than stay apart.

DR. BRANTON: Yes, but if you remove all of the spectrin, the transmembrane proteins still appear to remain apart.

DR. de KRUIJFF: With respect to the functional aspects of spectrin, it is not clear whether the lateral distribution of the proteins has any functional significance to the membrane. Nor is it clear what the shape of the erythrocyte is to the carrying of oxygen and carbon dioxide. Just from a medical point of view is this spectrin a phylogenetically early protein that may be

present in early development of the erythrocyte, serving an especially important function?

DR. BRANTON: I think that one can visualize the functions of spectrin in terms of the elasticity and plasticity of the cell membranes and I think that most cells will not tolerate the kind of high pressure squeezing that the red cell has to put up with in going through small capillaries. The general view is that the very function of the spectrin together with its associated protein is to provide a kind of meshwork underlying an otherwise delicate bilayer, allowing the membrane to hold together under very strong forces without being disrupted. It's like pulling an ordinary cell through a French press, so to speak, where the cell does hold up and maintains its ability to carry oxygen.

DR. MOSS: Aren't these descriptions of post-mortem properties? Is this what is going on in the red cell when it is intact or is there room for post-mortem artefacts?

DR. BRANTON: There is a lot of room for post-mortem artefacts. A really crucial question is what are all these proteins like in the intact cell? We frankly don't know the answer to many of those questions. We assume, I think frequently in biology in general and in the red cell specifically, that if we find high-affinity binding sites between two proteins, that it is as likely as not that these sites are representative of interactions that occur in vivo as well as in the test tube. That isn't necessarily true but otherwise it is difficult to understand why such specific amino acid sequences and hence specific folding of the proteins would have evolved unless they serve some function in the cell. We still know very little of what the shape of the spectrin molecule is inside the cell. I illustrate the spectrin molecule as it is seen in the electronmicroscope after it is laid down on a piece of mica and shadowed with carbon and platinum and you see an elongated strand. That doesn't mean it exists in the cell that way. We know it is floppy and it might be folded into a little ball or a fluffy ball. These happen to be useful images for analyses but they really shouldn't be construed to convey an image of the molecule in the membrane.

DR. WOLF: Is anything known about the metabolic requirements of these cytoskeleton proteins, their turnover or how long they last?

DR. BRANTON: I don't know of any studies of the turnover of any of these proteins. In the mature red cell, of course, there cannot be any turnover because there is no machinery there for turning it over. I don't know that nucleated cells have been studied from this standpoint.

DR. PARK: It wasn't clear to me whether you had found any disease state or abnormal condition in which spectrin or actin or ankyrin or band 3 were altered.

DR. BRANTON: No, we haven't looked. I think time spent trying to elucidate the basic interactions would be more revealing because then you would know which function to look at in a disease state.

DR. PARK: I agree with you completely. I was just thinking if there were, for instance, an abnormality which would be reflected in say hemolytic anemias, one might speculate that the fragility of the cells associated with spherocytes could reflect a defect in spectrin, for example.

DR. BRANTON: The only clear correlation is with a very rare genetic disorder in certain mice due to a lack of spectrin because the spectrin is broken down by proteolysis very rapidly. The mice do hemolyse and they do not survive.

DR. ENGEL: To what extent can what you have learned of the red cell cytoskeleton be applied to other cell types such as cultured muscle cells.

DR. BRANTON: There have been studies with Hela cells and I think also with lymphocytes in which a gentle Triton extraction reveals some kind of a lamellar meshwork underlying the cell membrane and clearly an extensive cytoskeleton internally. This cytoskeleton consists of the proteins which I spoke of which retain something of the shape of the cell after one has removed the membrane. The photographs of what underlies the membrane are particularly striking. It's even clear that some of these proteins may be attached to a cytoskeleton and they would be transmembrane proteins because they can be labeled in the native state and after Triton extraction still exist in this shell. The labeling demonstrates that they must be transmembrane proteins. However, in most cells, it seems as though there is much less material of this nature underlying the cell membrane allowing for a mobility and more fragility. I doubt that we can conclude that the same proteins are present. I view spectrin as one of a class of actin binding proteins which in the case of the erythrocyte serves to interlink filaments or short protofilaments of actin and to bind them to the membrane. We do not know if this is true for other cell types.

DR. MURRAY: Is it certain that the band 3 proteins that are not linked to ankyrin are totally equivalent to those that are linked? In other words, can you extract the ones that are not linked to the ankyrin and then show by _in vitro_ binding experiments that there is not some slight modification?

DR. BRANTON: We extracted with Triton X-100 those band 3 molecules that are presumeably not associated with spectrin. We do that by extracting in the presence of salt where spectrin is associated with 2.1 and 2.1 is associated with band 3. We get out only a limited fraction of band 3, about 60-70%. That band 3 has been reincorporated into vesicles and it has been shown that those with the vesicles combined with 2.1 just as the main cell does with the same affinity. Again, a limited number of binding sites are available as though the band 3 had bolted itself back into the membrane so as to expose only a limited number of sites. Another kind of experiment, which I think in a sense is really more significant has been to dissolve the band 3 in Triton and clip off the 45,000 dalton fragment, either from the total band 3 contained in the membrane or the fraction of band 3 extractable in the presence of salt (i.e. the unbound band 3). Then the 45,000 dalton fragments from both of those fractions are used in competition experiments to see if they compete with the inside out vesicles for the binding of 2.1. Those fragments are equally effective in competing, in other words, both total band 3 separation and the limited fraction which in a given cell at a given time is not bound to band 3 are equally effective in competing for binding with inside out vesicles. So all indications are that all of the 2.1 protein molecules are potentially capable of binding but for some reason they are not all bound at a given time.

DR. MURRAY: Is there some agent that causes or some disease state in which there is an alteration of the ratio of bound to free 2.1 proteins?

DR. BRANTON: In the case of 2.1 binding through the inside vesicles, we really don't have any good evidence that the rebinding is reconstituting the native binding. It maybe that what we are measuring is something totally irrelevant and hence the 1 out of 8 may also be totally irrelevant. For some reason we don't have the correct conditions which effect the correct *in vivo* binding.

DR. SCHOTLAND: How do changes in the cell surface affect the cytoskeleton of the red cell?

DR. BRANTON: The picture that I have in mind is one in which there is an interconnection. It may be an indirect connection via several proteins or polypeptides between the inside surface and the outside surface. If you perturb the inside surface by pulling all the spectrin molecules together in some way and hence pull all of the intra membrane particles together because they are bound to spectrin, you would also alter the distribution of charges and receptors on the cell surface. Conversely if you came in at the cell surface and pulled things together or redistributed them in the plane of the membrane, you would presumably induce alterations on the inside of the membrane and on the cytoskeleton -- if they remain connected.

DR. SCHOTLAND: It is such a complex situation, but if, for example, there were alterations in binding sites on the surface, which we've observed in Duchenne muscle, how would that effect the cytoskeleton?

DR. BRANTON: I really have no idea.

DR. WOLF: The peculiarity of the red cell, as you brought out, is that it gets squeezed and twisted but it doesn't do anything on its own in terms of motility. Do we have evidence relating to the cytoskeleton in cultured cells, lets say fibroblasts, muscle cells and others that are responsible for a more active life than the red cell. I wonder does anyone in the group have any evidence in this regard. I think it is important to know what differences are involved with structure in cells that are less passive than the red cell.

DR. BRANTON: Perhaps I should mention one very obvious difference. If spectrin were binding actin in the form of long filaments in the cell membrane, one would expect that we would see a lateral association between the actin filaments and spectrin. In other words one can visualize a filament bound at various places along its length to the tail end of the spectrin. In fact when one does look at other eukaryotic cells which are more mobile, actin appears to be bound in a terminal fashion to the cell membrane and in a fashion quite different from that of the red cell.

ANATOMICAL AND FUNCTIONAL CHARACTERISTICS OF MUSCLE CELL MEMBRANES

DR. FLEISCHER: Muscle is an enormously complex tissue. A detailed understanding of how a tissue works requires an understanding of cells, organelles, membranes and molecular components. The study of successively simpler levels of complexity makes possible the understanding of more and more about less and less. In the limit, molecules can be described in ultimate detail. The primary sequence and the three dimensional structure are available for a number of proteins. However, with molecules we can no longer observe complex processes which are essential for life, i.e., integration and coordination of metabolic processes in time and space, regulation and homeostasis. Therefore, the information obtained on the simplest levels must be related back into the framework of the entire system. I will try to illustrate this multilevel approach to the study of muscle physiology as studied in our laboratory.

Contraction and relaxation of muscle is a membrane mediated phenomenon. Control is exercised by regulating the intracellular Ca^{2+} concentration. That is, when muscle is stimulated by nerve, (excitation-contraction coupling), calcium is released from the sarcoplasmic reticulum (SR) compartment, the calcium concentration in the sarcoplasm rises to $\sim 1\mu M$ and muscle contraction is triggered. For muscle relaxation to occur, calcium must be pumped back into the SR compartment, reducing the Ca^{2+} concentration to approximately $0.1\mu M$. Membranes account for a very minor fraction of the muscle. Most of the mass consists of contractile filaments.

Sarcoplasmic reticulum (SR) is one of the most extensively studied membrane systems since it serves as a prototype of a membrane system capable of active transport, and because of its central role in muscle physiology. The Ca2+ uptake process can readily be studied using isolated SR vesicles and has been extensively studied in many laboratories (Tada et al., 1978).

Skeletal Muscle Sarcoplasmic Reticulum

More than 10 years ago, we initiated a molecular biology approach to the study of SR from rabbit skeletal (Meissner et al., 1971) muscle. The aims were to: (1) Isolate and characterize highly purified SR. (2) Disassemble SR into its components and relate the properties of these constituents to SR function. (3) Reconstruct membrane vesicles, which are similar to normal SR and capable of transport function. (4) Correlate composition with structure, and structure with function. Such studies required development of methodology to reconstitute membranes of defined lipid-to-protein ratio which could be varied in the range of high protein content similar to that of natural membranes. (5) Carry out biophysical studies to obtain detailed membrane structure including motional parameters which are relevant to understanding the nature of lipid-protein interaction and the exercise of function. We will review our progress in the sequence as outlined.

Isolation of highly purified normal SR (N-SR) in sizeable quantity (hundreds of mg protein) was achieved by a procedure making use of zonal rotors for large scale centrifugation (Meissner et al., 1973). In our preparation of SR, three proteins predominated as judged by polyacrylamide gel electrophoresis using dissociating conditions (Figure 1-14, gel 2). The calcium pump protein of 119,000 daltons (Rizzolo et al., 1976) (sometimes referred to as calcium ATPase), the calcium binding protein and a polypeptide of approximately 55,000 daltons (designated M_{55}), comprise about 75, 10 and 10 per cent respectively of the protein of the purified SR fraction.

The morphology of normal SR can be viewed by electron microscopy using three different methods of sample preparation (Figure

1-17). In thin sections, membrane vesicles can be visualized with trilaminar appearance of the membrane. (Figure 1-17a). Negative staining (Figure 1-17b) reveals small particles (40 Å) at the outer surface of the membrane (Ikemoto et al., 1968). Freeze-fracture electron microscopy (Figure 1-17c) shows an asymmetric distribution of particles at the hydrophobic fracture faces with most of the intramembranous particles observable in the outer leaflet (concave face) of the membrane (Deamer and Basin, 1969).

Highly purified SR can be further subfractionated into heavy and light SR vesicles based on their isopycnic densities (Figure 1-18). Heavy SR contains electron-dense matter within its compartment (Figure 1-18b), whereas light SR (Figure 1-18a) is practically devoid of such contents (Meissner, 1975; Saito et al., 1978).

Polyacrylamide gel patterns of light SR (gel 1) and heavy SR (gel 3) were compared with the normal SR (gel 2) from which they were derived (Figure 1-14). The electron opaque contents of heavy SR are preferable mainly to calcium-binding protein with the SR compartment. Light SR is essentially all membrane vesicles, of which the calcium pump protein comprises 90 per cent or more of the membrane protein. The SR membrane from heavy SR likewise consists mainly of calcium pump protein. Indeed, the SR membrane

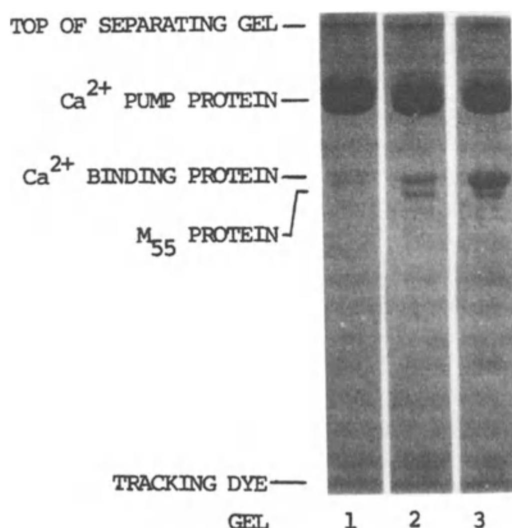


Figure 1-16: Polyacrylamide gel electrophoresis (PAGE) of SR. The samples of the gels are of: (1) light SR; (2) normal SR; (3) heavy SR.

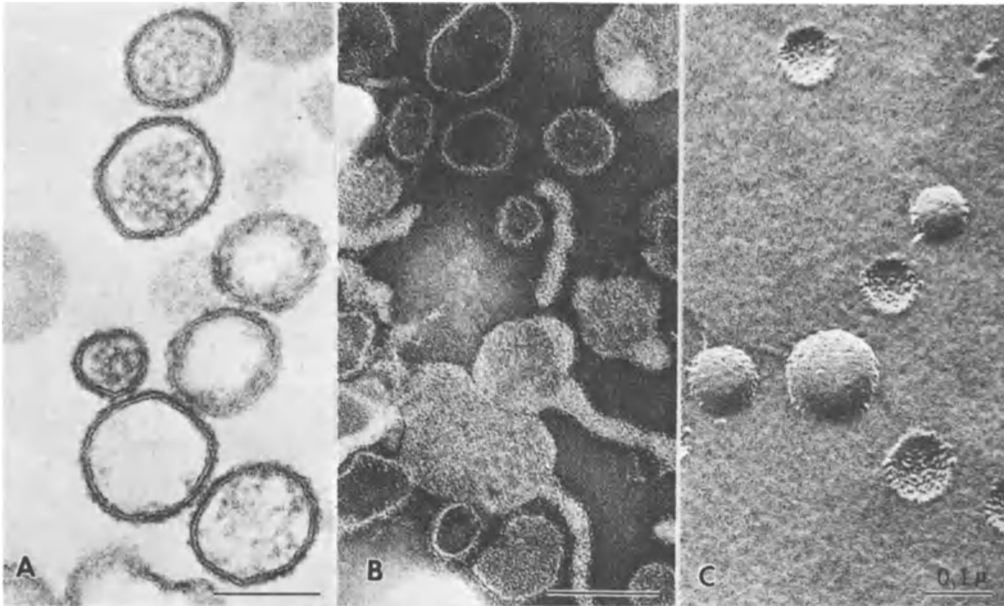


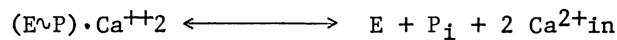
Figure 1-17: Normal SR as visualized by electron microscopy (A) in thin sections, (B) using negative staining, and (C) by freeze fracture.

is highly enriched with regard to the calcium pump protein (Meissner, 1975; Saito et al., 1978).

Isolated SR is capable of energized Ca2+ ion uptake. In muscle, the uptake of Ca2+ results in the lowering of the intracellular concentration to approximately 0.1 mm so that relaxation of muscle can then occur.

The energized uptake of Ca^{2+} by SR can be formulated as follows:

$$2Ca^{2+}out + ATP\ (Mg^{++}) + E \longleftrightarrow (E{\sim}P\ Ca^{2+} + ADP + Mg^{++}$$



$$(E{\sim}P){\cdot}Ca^{++}2 \longleftrightarrow E + P_i + 2\ Ca^{2+}in$$

$$2\ Ca^{2+}out + 1\ ATP \longleftrightarrow ADP + P_i + 2\ Ca^{2+}in$$

In equations (1) and (2), E refers to the enzyme(s) responsible for catalyzing the Ca^{2+} translocation catalyzing process. This simplified scheme points out that a phosphoenzyme intermediate (Yamamoto and Tonomura, 1967) is involved in the Ca^{2+} pumping cycle. The equations also define some functional characteristics which can be measured to characterize SR function. Both Ca^{2+} and ATP are substrates for the reaction and so binding sites should be

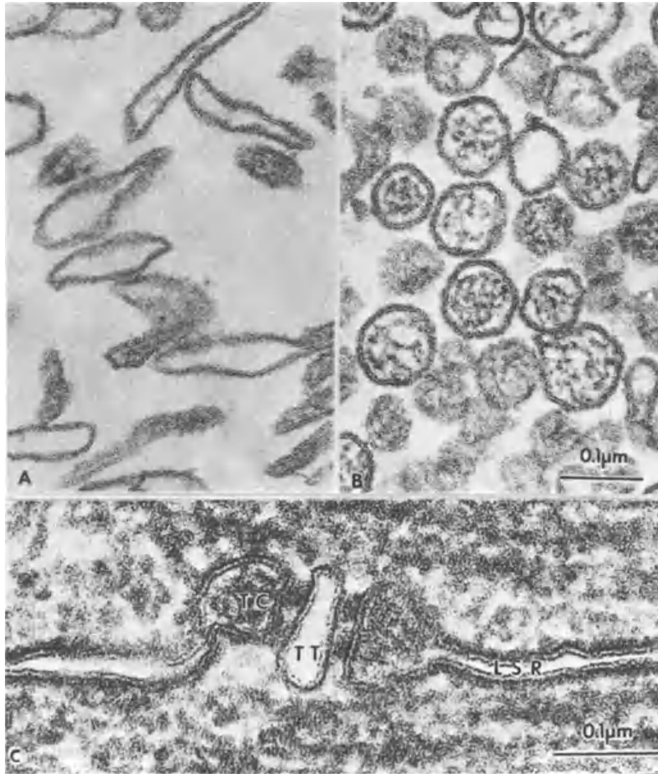


Figure 1-18: Electron micrographs of isolated (A) light and (B) heavy SR and (C) the triad region in situ. (A) and (B), light and heavy SR were prepared from normal SR by isopycnic centrifugation. (C) The triad region is emphasized in the thin section of the rabbit skeletal muscle. The sample was fixed using tannic acid enhancement. The asymmetry of the SR membrane can readily be visualized. TT - T-tubule; TC - terminal cisternae of SR; LSR - lateral cisternae of SR.

measurable. The membrane component which forms the phosphoenzyme intermediate should be identified. The rates of Ca^{2+} uptake as well as ATP hydrolysis by SR can be measured. The efficiency of Ca^{2+} transport, i.e., the mole ratio of Ca^{2+} transported per ATP hydrolyzed is 2 in isolated SR. The catalytic cycle is more complex than shown, consisting of at least 8 steps.

We have purified and characterized the major components of SR (Meissner et al., 1973).

The calcium pump protein contains two specific calcium-binding sites, and one ATP binding site, and is the membrane component which forms the phosphoenzyme intermediate (Meissner and Fleischer, 1971; Meissner et al., 1973; Meissner, 1973).

Thus the calcium pump protein contains some key requisites for the Ca^{2+} pumping molecule. But is it capable of catalyzing energized pumping of Ca^{2+}? To answer this question we made use of the reconstitution approach. Achievement of function in the reconstituted membrane means that the components involved can be identified, characterized and the pumping machinery can be studied in detail.

Normal SR can be dissociated using deoxycholate and then reconstituted to form functional membrane vesicles by removing the detergent (Meissner and Fleischer, 1973; Meissner and Fleischer, 1974). Conditions are stringent for reassembly of the membrane to form functionally reconstituted SR (R-SR). Reconstitution must be carried out at 15-20°C in order to achieve good energized accumulation of calcium. The protein of R-SR, like normal SR membranes, consist mainly of calcium pump protein. Using the procedure described previously, most of M_{55}, but little of the calcium-binding protein is retained in R-SR. Reconstitution can also be achieved with purified calcium pump protein to yield functional membrane vesicles (Meissner and Fleischer, 1973). Thus, the calcium pump protein has all the known characteristics required for calcium pumping and appears to be the calcium pumping molecule (E in equations 1 and 2). We have, therefore, termed E the "calcium pump protein." "Calcium pump protein" is a more appropriate designation than Ca^{2+} ATPase, since it is possible to have ATPase activity without the Ca^{2+} pumping capability; Ca^{2+} pumping is the more inclusive function. The procedure developed in our laboratory achieves reconstitution of membrane vesicles with lipid content comparable to that of N-SR membrane (Meissner and Fleischer, 1974; Fleischer et al., 1979). Functionally, the energized calcium pumping rate and capacity of reconstituted SR is somewhat less than one-half that of the original SR (Fleischer et al., 1979; Fleischer et al., 1979).

The reconstitution procedure which we have described has three key characteristics (Fleischer et al., 1979; Fleischer et al., 1979; Inesi et al., 1980) which differ from that of others (Racker, 1972): (a) R-SR membrane vesicles like N-SR consist mainly of protein (60 per cent protein and 40 per cent lipid) and are not liposomes mildly doped with trace amounts of calcium pump protein; (b) R-SR does not require trapping agents, such as oxalate, within its compartment during vesicle formation in order to achieve subsequent energized calcium pumping; and (c) R-SR is capable of energized calcium pumping in the absence of a trapping agent in the assay medium.

Recently, we developed a procedure to visualize the asymmetry of the SR membrane in thin sections using tannic acid to enhance contrast (Saito et al., 1978). In normal SR, the outer layer is 70 Å wide compared with 20 Å each for the middle and inner layers (Figure 1-19). The asymmetry of the normal SR membrane can thus be visualized in thin sections as well as using negative staining (Figure 1-17b), and by freeze fracture electron microscopy (Figure 1-17c).

R-SR differs from N-SR in one key aspect, i.e., the asymmetry of the membrane has not been retained in the membranes formed in the test tube. This difference can be seen using each of the three methods of sample preparation for electron microscopy. In thin sections, the trilaminar appearance of the R-SR membrane with high protein content is symmetrical, i.e., the trilayer is 70 Å, 20 Å, and 70 Å wide (Figure 1-19c). When the lipid-to-protein ratio of the R-SR membrane is increased, there is less material on the membrane surfaces (Figure 1-19b). With negative staining, the surface particles can be visualized on both inner and outer membrane faces of R-SR (Figures 1-19d and 1-19e). With freeze-fracture, R-SR has a more symmetrical distribution of particles between convex and concave fracture faces (Figures 1-20b through 1-20f) whereas in normal SR, the particles are observed mainly in the outer (concave) fracture face (Figures 1-17c and 1-20a).

The reconstitution procedure has been modified to prepare R-SR membranes of defined phospholipid composition and methodology has been developed to vary the lipid content of the reconstituted SR (Fleischer et al., 1979; Wang et al., 1979; Wang et al., 1981). The lipid content which we found most useful for correlative studies ranges from approximately one-half to twice the lipid content of N-SR. Such a series of preparations has permitted correlation of composition with structure. The study is illustrated using freeze-fracture electron microscopy. A series of R-SR membrane vesicles of increasing lipid content is shown in Figure 1-20. The lipid content varies from approximately less than one-half (Figure 1-20b), to the same (Figure 1-20d) to more than twice the lipid content (Figure 1-20f) of normal SR (Figure 1-20a). The concentration of particles observed at the hydrophobic center in the R-SR membrane vesicles of both convex and concave faces was found to decrease proportionately with the protein content of the membrane. Concommitantly, the particle-free area increased with the lipid content. Since the protein content of these membranes consist mainly of one type of protein (>90 per cent), the concentration of intramembranous particles is directly proportional to the concentration of the calcium pump protein molecules in the membrane. The number of pump molecules calculated to be in the membrane is greater by at least a factor of 2 than the number of particles observed. Thus, it would appear that the particles, on the average, consist of two or more calcium pump protein molecules (Wang et al., 1979).

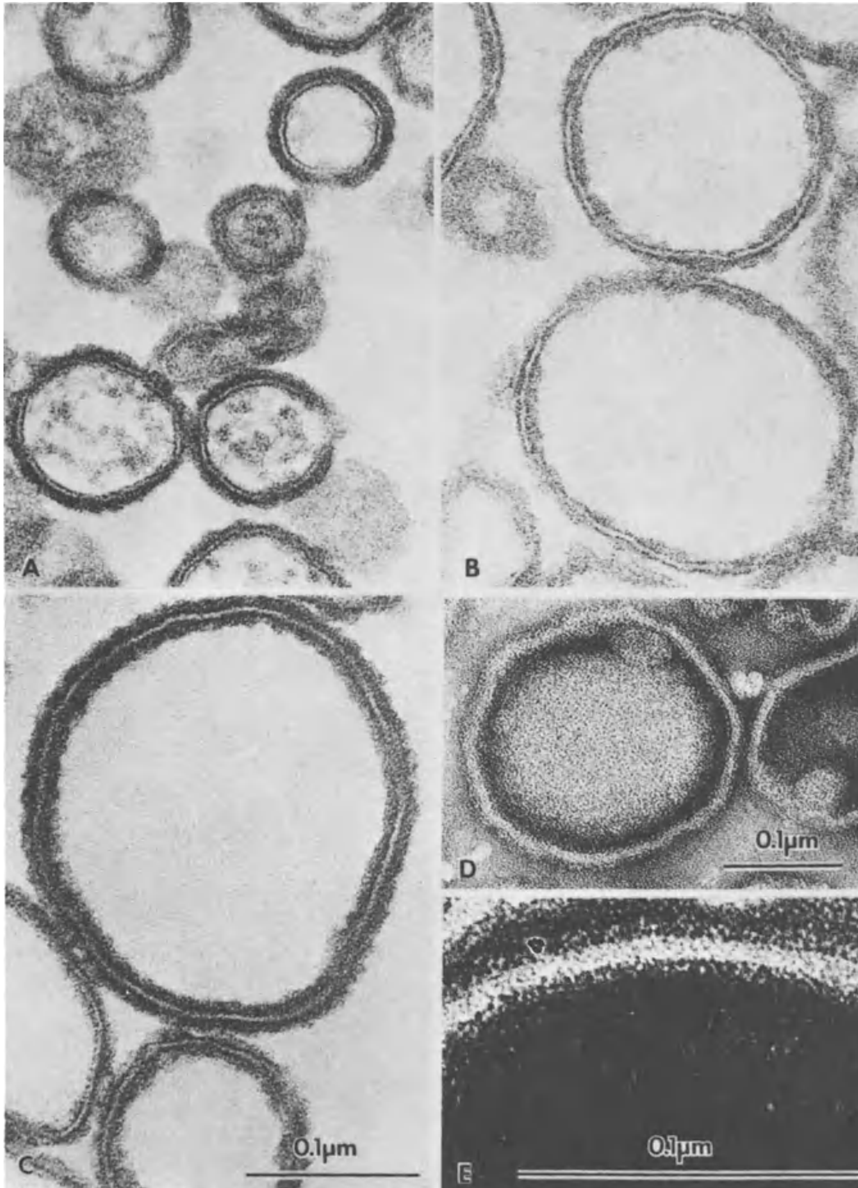


Figure 1-19: SR as visualized in thin sections (A-C) and by negative staining (D-E). (A-C) Tannic acid was used to achieve enhanced contrast of the SR membranes. A is normal SR; B and C are reconstituted SR of lower and higher protein content, respectively. The 0.1μ bar in C gives the enlargement for A-C. Figures D and E are negative staining of reconstituted SR.

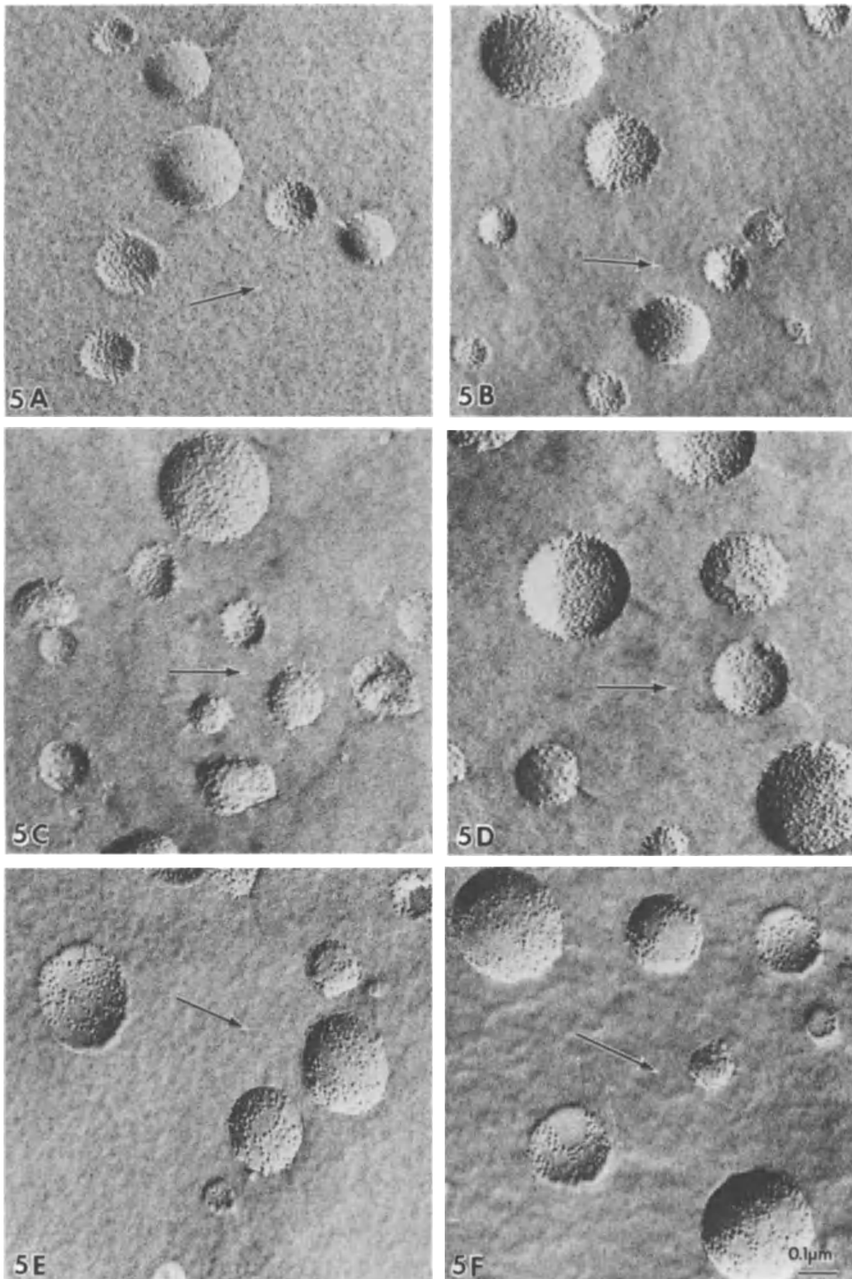


Figure 1-20: Freeze-fracture electron microscopy of normal SR (A) and reconstituted SR of varying phospholipid content (B) 0.38; (C) 0.57; (D) 0.78; (E) 1.03; and (F) 1.29 µmoles phospholipid per mg protein; the value for normal SR is 0.78.

The studies described above bear on the orientation of the calcium pump protein in the SR membrane (Figure 1-21). The main protein component of the SR membrane is the calcium pump protein. Phospholipid vesicles alone have a symmetrical appearance of the trilayer; the width of each layer is approximately 20 A° (Figure 1-21a frame E). Therefore, the asymmetry of the SR membrane which is observable in thin sections (frame B) and by negative staining (frame A) must be referable to the unidirectional alignment of the calcium pump protein in the membrane, i.e., a portion of the calcium pump protein extends beyond the phospholipid bilayer from the outer surface of the membrane. The calcium pump protein must be transmembrane (Figure 1-21b) since calcium is pumped across the membrane from the outside to the inside of the vesicle. This interpretation is supported by x-ray and neutron diffraction studies (Blasie et al., 1981). In R-SR, the pumps are more bidirectionally aligned giving rise to the symmetrical appearance in both thin sections (Figure 1-21a frame C) as well as by negative staining (Figure 1-21a, frame D). The calcium pump is depicted as transmembrane and as an oligomer for the reasons cited above (Figure 1-21b).

The ability to prepare functional membrane vesicles of defined composition and with varying phospholipid content, within the range of high protein content of the type described here, makes possible biophysical studies to characterize membrane structure including motional parameters (Fleischer et al., 1979). Such studies using x-ray and neutron diffraction have been carried out in collaboration with Leo Herbette, Antonio Scarpa and Kent Blasie of the University of Pennsylvania (Blasie et al., 1981). Nuclear magnetic resonance studies have been carried out: [P-NMR] with A.C. McLaughlin of the Brookhaven National Laboratory, Upton, New York (McLaughlin et al., 1981; [D-NMR and P-NMR] with Joachim Seelig and his colleagues at the University of Basel, Switzerland (Seelig et al., 1981); [H-NMR] with Edward Dratz and Alan Deese, University of California, Santa Cruz (Deese et al., 1980); and EPR studies with Larry Dalton SUNY, Stony Brook and J. Oliver McIntyre at Vanderbilt University (McIntyre et al., 1981).

X-ray and neutron diffraction studies were carried out on normal and reconstituted SR membrane vesicles containing protonated and specifically deuterated phospholipids. The total electron scattering profile obtained by x-ray diffraction on oriented multilayers of membrane vesicles was resolved into separate phospholipid and protein electron densities. These studies provide an electron density profile of the Ca^{2+} pump protein in the SR membrane. The Ca^{2+} pump was found to be transmembrane with a major portion of the molecule extending out from the surface of the membrane (Blasie et al., 1981). Thus, similar conclusions have been reached independently using electron microscopy and diffraction approaches (Saito et al., 1978; Fleischer et al., 1979; Blasie et al., 1981).

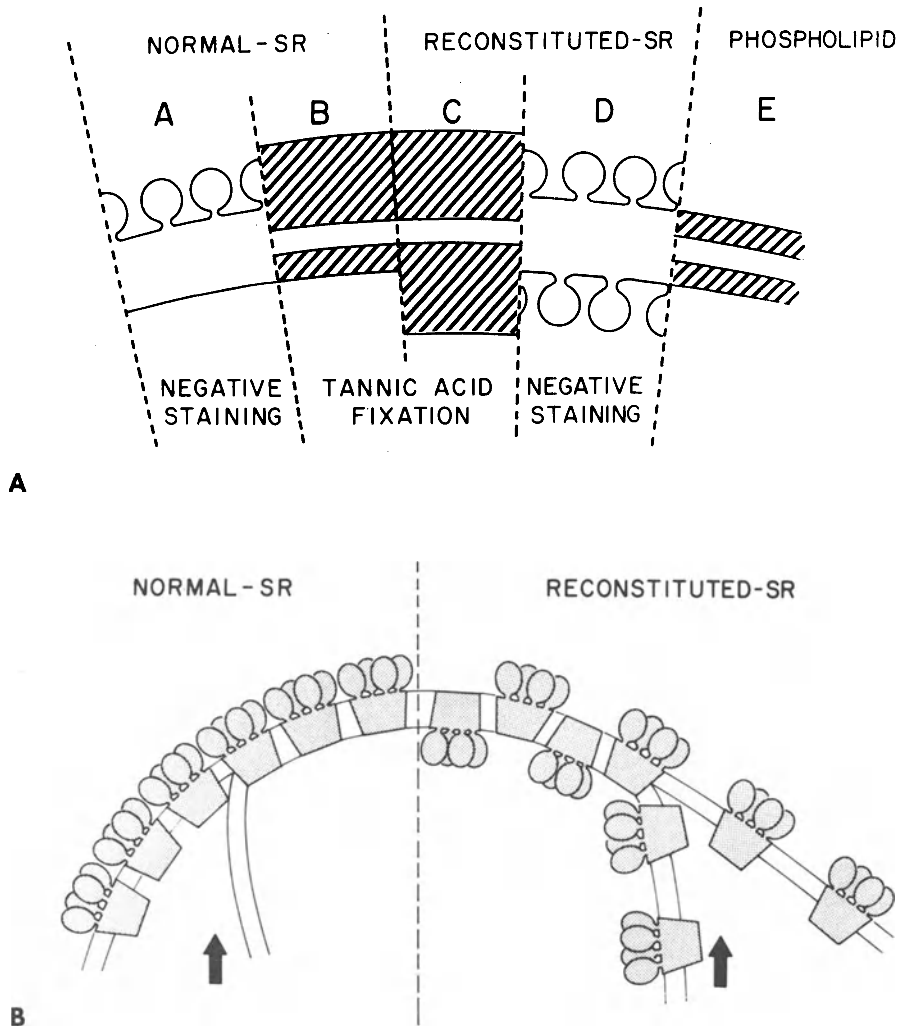


Figure 1-21: Diagrammatic representation of normal and reconstituted SR membranes: A. Normal and reconstituted SR as visualized by negative staining and in thin section electron microscopy using tannic acid. The 40-Å particles observed with negative staining (Frames A and D) and the broadened outer band in thin section using tannic acid extend 50 Å from the surface (Frames B and C), compared with phospholipid (Frame E). The membrane is asymmetric in normal SR (Frames A and B) and symmetric in reconstituted membranes (Frame C and D); B. Diagrammatic representation of normal SR and reconstituted SR vesicles illustrating the effect of freeze-fracture on these membranes.

Deuterium and phosphorus nuclear magnetic resonance were used to characterize the motion of the hydrophobic and polar portions of the phospholipid in the membrane, respectively. The quadrupole echo technique makes possible quantitation of signal. The deuterium and phosphorus spectra of the phospholipid in the membrane were characteristic of a homogeneous liquid crystalline bilayer. Thus, to a first approximation, within the time resolution of NMR (10^{-5} sec or less), we do not detect phospholipid which appears to be immobilized by the calcium pump protein, i.e., we find no evidence to support the notion of immobilized "boundary layer" phospholipid. The structure related order parameters are obtained from the deuterium quadrupole splitting constant and the phosphorus chemical shielding constants and indicate a small but uniform disordering of the lipid in the membrane as compared with the phospholipid alone. The dynamic property is obtained from spin lattice relaxation time measurements (T_1) and shows that the reorientation rate of motion of the hydrophobic and polar moieties of the phospholipid is slowed by 10-20 per cent as compared with bilayer phospholipid (liposomes) (Seelig et al., 1981; McLaughlin et al., 1981).

The concept of boundary lipid was originally suggested from EPR studies (Jost et al., 1973). Such measurements are sensitive to motion in a faster time domain than NMR, i.e., 10^{-7} to 10^{-11} seconds. We therefore looked for immobilized phospholipid (spin labeled lecithin, sn-2 stearoyl doxyl-16) in reconstituted SR. The motion of the lipid in the reconstituted membrane is motionally similar to that of bilayer phospholipid. However, a spectral component referable to immobilized phospholipid in the presence of CPP was detected, equivalent to approximately 8 moles of phospholipid per mole CPP. This small amount of phospholipid appears to be insufficient to constitute a boundary of immobilized phospholipid surrounding the CPP (McIntyre, 1981).

Both NMR and EPR studies lead to similar conclusions: 1) To a first approximation the lipid environment in the membrane is homogeneous and typical of a PL bilayer; 2) the CPP in the membrane increases the disorder of the PL:3) there is insufficient PL immobilized to account for a lipid "boundary" or "annulus" around the CPP. The immobilized PL seems to be referable to PL which is entrapped intramolecularly within the oligomeric structure of the pump unit or due to intermolecular entrapment of PL (Seelig et al., 1981; Deese et al., 1980; McLaughlin et al., 1981; McIntyre et al., 1981).

Thusfar, we have discussed mainly the Ca^{2+} pumping process which is mediated by SR. A substantial amount of information is known about the Ca^{2+} uptake process because this capability is retained by isolated SR and can be recovered in reconstituted SR.

The Calcium Release Process

By contrast, Ca^{2+} release is not readily studied in isolated SR and little is known about this process (Endo, 1977). The Ca^{2+} release process involves two types of membranes, the sarcolemma and the SR. Both membranes are associated in the form of the triadic junction which is composed of two terminal cisternae of SR in junctional linkage with the transverse tubule. The transverse tubule is the specialized portion of the sarcolemma which invaginates and extends transversely into the interior of the muscle fiber. Thus, in excitation-contraction coupling, the pathway of excitation proceeds first a) from nerve to muscle via the neuromuscular junction, the junction between nerve and muscle plasma membranes. The action potential continues along b) the sarcolemma and then intracellularly via c) the transverse tubule and across d) the triadic junction triggering calcium ion release from e) the terminal cisternae of sarcoplasmic reticulum . Ca2+ release cannot readily be studied in vitro and hence little is known about this process. Bob Mitchell in our laboratory has recently isolated a preparation enriched with respect to triads (Mitchell et al., 1981), that is, transverse tubule in junctional association with the terminal cisternae. The signal to release Ca^{2+} from the SR is transmitted across this junction (Figure 1-22). Robert Mitchell and Phil Palade are approaching the study of Ca^{2+} release using the triads in an attempt to simulate physiological conditions for Ca^{2+} release. If an in vitro assay to study Ca^{2+} release can be worked out, then it may be possible to study the molecular details involved in the Ca^{2+} release process.

The asymmetry of the SR membrane can now be visualized, in situ, in thin sections of muscle using tannic acid enhancement (Figure 1-20c).

In Vivo Correlations

The broad band (70 A°) is on the outer face of the SR membrane so that calcium ions are pumped from the broad outer face **across** the membrane to within the SR compartment. This asymmetry is retained in the isolated SR vesicles (Figure 1-20a). It may also be noted in the structure of the triad (Figure 1-18c) the compartment of the terminal cisternae of SR contains opaque material, whereas the lateral cisternae of SR is devoid of such contents. These studies suggest that heavy and light SR derive from the terminal and lateral cisternae, respectively (Meissner, 1975; Saito et al., 1978; Fleischer et al., 1979) and that the contents of the terminal cisternae contain calcium binding protein. The asymmetry of the SR membrane both isolated and in situ, is clearly referable to the unidirectional orientation of the Ca^{2+} pump protein.

Muscle contraction and relaxation is regulated by membranes, of which sarcoplasmic reticulum (SR) serves a central role. A molecular biology approach has been directed at the study of SR structure and function and is reviewed here.

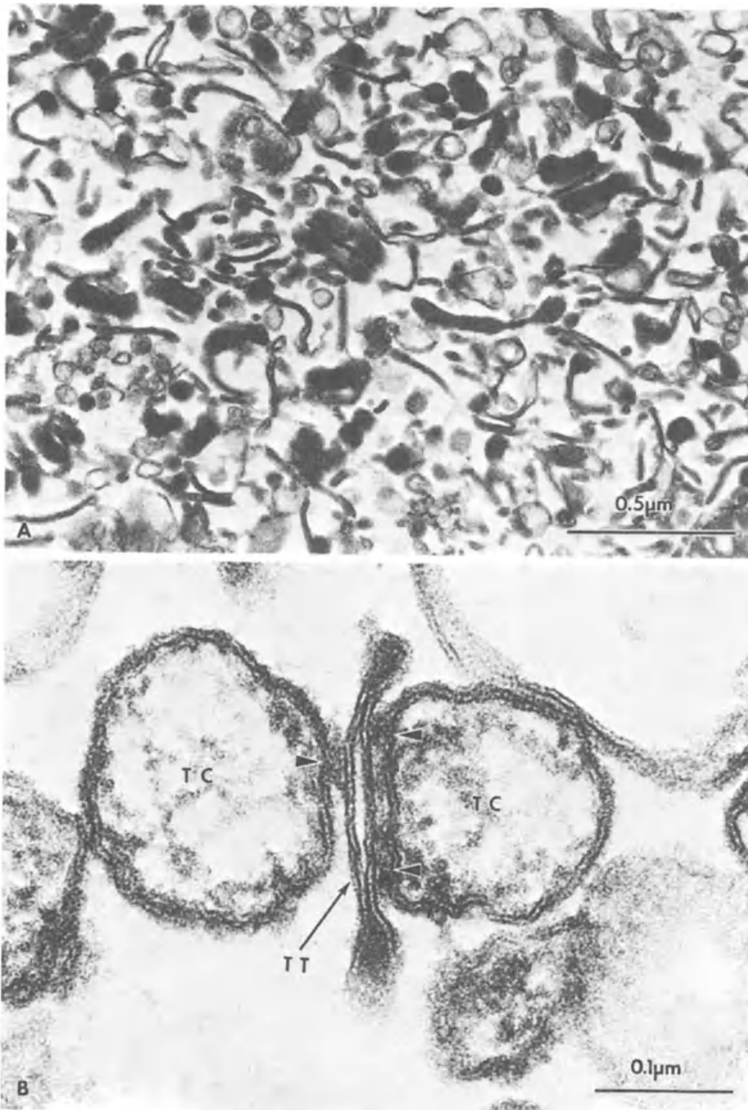


Figure 1-22: An enriched preparation of triads (A). The triads are composed of transverse tubules in junctional association with the terminal cisternae of sarcoplasmic reticulum. The sample was fixed using tannic acid enhancement. The isolated triads can be compared with the triadic structure in situ (cf. Fig. 1-1c) A higher power section is shown in B. The transverse tubule (TT) and terminal cisternae (TC) of SR are indicated. Bridge protein structures are clearly discernable (triangles).

Highly purified SR has been isolated and subfractionated into heavy and light SR which appear to be referable to terminal and lateral cisternae of SR respectively. Isolated SR is capable of energized uptake of Ca^{2+}, in vitro, and so the Ca^{2+} uptake process can readily be studied. The dissociation and reconstitution approach of sarcoplasmic reticulum makes possible study of components at the molecular level. Information obtained can then be related back to the level of the membrane. The Ca^{2+} pump protein has two high affinity Ca^{2+} binding sites and one ATP binding site. It forms the phosphoenzyme intermediate and has been shown by reconstitution studies to be the pump molecule. The Ca^{2+} pump protein is oriented transmembrane with a major portion extending out from the bilayer from the cytoplasmic face. By reconstituting membranes of different lipid to protein ratios and defined lipid environment, we can carry out experiments to correlate membrane composition with structure and structure with function. The motional characteristics of the lipid and protein components in the SR membrane are being studied by NMR and EPR techniques. To a first approximation, we detect a homogeneous lipid environment typical of bilayer phospholipid. There is no evidence for a "boundary" of immobilized lipid surrounding the Ca^{2+} pump protein. The Ca^{2+} release process cannot readily be studied in isolated SR. Toward this end, we have recently isolated triads, i.e., the triadic junction, consisting of transverse tubule in junctional association with the terminal cisternae of SR. The aim of this approach is to try to develop an *in vitro* system to study the Ca^{2+} release process.

I think the essence of the problem is that a human being is an extremely complex machine. If one cog in the machine is defective, progressive degeneration of the system can occur. The defect would have to be relatively minor in order for a baby to even be born, let alone reach adolescence. This complex machine needs to be understood, therefore the argument for *basic research*. Is muscular dystrophy referable to a membrane defect? *It may be.* We need to learn more about membranes, how they work, and how they are regulated. Our chairman, and Dr. Schotland have pointed out the importance of being able to study the plasma membrane. The isolation of skeletal muscle plasma membrane is particularly difficult because it constitutes only a very small portion of the membranes of the muscle cell. It is encased in the basement membrane and not readily released. Further, it is heterogeneous in form and function consisting of four discrete portions: 1) the portion associated with the neuromuscular junction; 2) the surface membrane; 3) non-junctional transverse tubule; and 4) transverse tubule in junctional association with the sarcoplasmic reticulum (terminal cisternae). In addition to such heterogeneity, membrane vesicles can be isolated which are sealed right-side-out, sealed inside-out, or may be leaky.

The isolation of plasma membrane from skeletal muscle is more complicated than the isolation of SR.

It would be instructive to consider the many mutations which are known in human hemoglobin. More than 100 abnormal hemoglobins have been identified in patients, one of which is responsible for Sickle Cell Anemia. The defect is understood at a molecular level. The defective hemoglobin, has a single amino acid substituted in the b-chain, i.e., valine for glutamic acid. This substitution results in reduced solubility of the deoxygenated hemoglobin and sickling occurs when there is a high concentration of the deoxygenated form. Therefore, the patient is most vulnerable when the oxygen tension is decreased such as at high altitudes or as a consequence of physical exertion. There is clearly a basic defect(s) in Muscular Dystrophy(s). We must identify the component which is modified to understand this lesion(s).

Dr. Robert Mrak in our laboratory has been studying sarcoplasmic reticulum from normal and genetically dystrophic mice to see whether he can find a defect in the SR membrane. He has previously reported differences between sarcoplasmic reticulum isolated from normal and genetically dystrophic mice. For the past two years, he has refined the fractionation procedures for skeletal muscle SR from mice. It should be pointed out that special care needs to be exercised in the isolation and characterization of subcellular organelles from dystrophic tissue. Dystrophic tissue has a different consistency and is more difficult to homogenize. It is not a trivial task to adapt an isolation procedure for normal tissue to dystrophic tissue. Dr. Mrak now does not find any major functional differences in isolated, well characterized SR from dystrophic muscle. He studied the protein and lipid composition of the purified SR from normal and dystrophic mice and finds them to be qualitatively similar with small quantitative differences. Therefore, for the genetically dystrophic mouse model, we cannot find any fundamental difference in these well defined SR fractions. It should be kept in mind that muscular dystrophy is a family of different diseases. A defect in one disease does not necessarily have to be the same as that in another. So that if there were no defect in SR of skeletal muscle from dystrophic mice, it would not preclude a defect in SR in Duchenne muscular dystrophy or muscular dystrophy in the chicken. That is to say, different molecular defects could give rise to similar symptoms. For example, lysosomal defects which give rise to a variety of storage disease, some with neurological symptoms, were found to result from defects in different hydrolytic enzymes. This understanding came after the lysosome was discovered. It could then be recognized that the common etiology was referable to defects in different enzymes of the lysosome.

EDITORIAL SUMMARY

The aim in this chapter has been to provide an anatomical basis for the study of cell membrane function and to identify molecular arrangements and interactions as they may affect the behavior of cells. Considerable information is available about the cytoskeleton of the red cell and its relationship to the plasma membrane. There is relatively little information of this type available on nucleated cells, including muscle cells. An extensive study of the organization of membrane lipids has been included to lay the ground work for an understanding of the regulation of transmembrane traffic. Finally some of the membrane bound enzymes responsible for cellular function have been described. With respect to the problem of identifying a basic membrane defect in Duchenne dystrophy the impediment is a lack of knowledge of the primary genetic abnormality and the gene product associated with it. Until such information is at hand it will be necessary to work with what alterations are identifiable, including doubtless many changes that are secondary to the degenerative process in muscle.

CHAPTER 2

CHARACTERISTICS AND BEHAVIOR OF PLASMA MEMBRANES

Diffusive Processes in Membranes

DR. HYDE: I want to review briefly three topics that in my judgment are relevant to muscular dystrophy studies and persons involved in these studies using ESR spectroscopy. These topics are: (1) the positions of rotational diffusion of membrane constituents in lipid bilayers; (2) the behavior of oxygen in lipid bilayers and the ways in which it can interfere with ESR spin-label measurements; and (3) a review of some recent ESR instrumental approaches that might be useful in investigations of membranes.

Rotational Diffusion

This section is abstracted from a recent review by Hyde and Thomas, 1980. The most general rigid body rotational diffusion problem in ESR spectroscopy involves an anisotropic inertial tensor of arbitrary orientation with respect to an anisotropic restoring potential, and a molecular probe located in the rigid body characterized by a tensor describing the magnetic interactions that is also of arbitrary orientation. In a membrane, a great simplification occurs. The restoring potential is axially symmetrical about the normal to the bilayer and the inertial tensor of most membrane constituents is axially symmetric, with the principal axis also perpendicular to the bilayer surface. As a consequence, lipid or protein components can reasonably be assumed to have an axially symmetric diffusion tensor characterized by $D_{\parallel}$ and $D_{\perp}$. Here $D_{\parallel}$ is the rotational diffusion constant parallel to the normal of the bilayer. $D_{\perp}$ is the diffusion constant perpendicular to the normal of the bilayer.

If the membrane constituent is reasonably rigid, as for example cholesterol (or its equivalent spin label -- cholestane) or a protein, $D_\perp$ may be close to 0, and rotational diffusion can be characterized by a single diffusion constant, $D_\parallel$. The orientation of the spin-label probe with respect to the bilayer normal remains uncertain. To a good approximation the tensor describing the magnetic interactions is axially symmetric and the magnitudes of the interactions are always about the same for all spin labels. Under this approximation, there is just one unknown characterizing the relative orientations of the diffusion tensor and the magnetic tensor -- namely θ, the angle between the principal axes of the two tensors. The problem, which initially seemed hopelessly complex, then reduces to a determination of just two unknowns, D_{II} and θ. This is a particularly good approximation for proteins undergoing rotational diffusion in lipid bilayers. An appropriate methodology to obtain θ is, where possible, to work with oriented membranes. In our laboratory we are currently investigating several approaches to determination of θ when one has an isotropic spatial distribution (i.e., all orientations in our sample cell are equally probable).

DR. de KRUIJFF: Could you discuss $D_\parallel$ and $D_\perp$ in more detail.

DR. HYDE: Consider again the inertial tensor and the restoring potential.

A cigar-shaped object has an axial inertial tensor. It would be expected to undergo much more rapid diffusion about an axis along the cigar than perpendicular to the cigar.

But how it actually diffuses depends on the restoring potential. In a fluid with an isotropic potential, such as water, the principal coordinate system of the rotational diffusion tensor coincides with that of the inertial tensor. There are just two components of the diffusion tensor of a cigar, $D_\parallel$ and $D_\perp$.

The potential in a bilayer, however, is extremely anisotropic. The bilayer itself places constraints that essentially prohibit a protein from rotating about an axis parallel to the bilayer surface. The anisotropic potential arises from hydrophobic interactions and stearic effects.

The notation $D_\parallel$ and $D_\perp$ is also used to describe ^{31}P NMR experiments in lipid bilayers. On the NMR time scale, $D_\parallel$ is large, effectively averaging out some components of the magnetic interaction tensors, while $D_\perp$ is very small and does not affect other components. The notation can in addition be used to describe experiments using fluorescence probes.

Returning to the general discussion of diffusional processes, I will consider next the subject of "wobbles."

For lipid labels, such as those with the doxyl moiety (Spin labeling: Theory and Applications, 1976), θ is about 0°, which is an important simplification. But the lipids are so flexible that a new concept, "wobble," must be introduced.

Hubbell and McConnell, 1971, introduced the concept of "effective Hamiltonian" in studying lipid fluidity in membranes, and Griffith and Jost, 1976, have developed the concept further. The model involves the assumption that restricted angular fluctuations or "wobbles" of the lipid occur with magnitude and frequency that increase from the surface of the membrane to the center of the bilayer. These motions are assumed to be fast enough that they result in new time-independent average magnetic interactions. These motions give rise to the so-called "fluidity gradient," and the "order parameter" concepts described in detail by Hubbell and McConnell, 1971 and Griffith and Jost, 1976. Here I wish only to emphasize that these models are extremely stylized. There is an assumption that the restoring potential has infinitely sharp walls. The assumption leads to simplification of computer generated spectral simulations. I suggest that it is now time to consider use of more complicated theoretical models that better resemble the real situation. I sugggest furthermore that the word "fluidity" has little specific science connotation and that a more precise vocabulary should be employed. It is at this point that $D_{\perp}$ can be introduced, since the wobble motion represents in fact rotational diffusion about an axis perpendicular to the bilayer normal.

Another cautionary note: sometimes workers in the field report correlation times rather than diffusion constants. In systems undergoing isotropic rotational diffusion, the correlation time and the diffusion constant are inversely proportional to each other, and there is no particular advantage in using one or the other. In an anisotropic system, one can speak of loss of correlation along x, y or z in a molecular coordinate system, but these three correlation times do not have tensorial properties. Relationships between correlation times and diffusion constants are given in Tao, 1969. Whether one measures a loss of correlation or a diffusion constant depends on the particular technique of measurement.

In summary, what I am saying is that in our studies of diffusion in membranes we ought to be better physicists and more precise in reporting the results of our experiments.

DR. ENGEL: Could you consider a three dimensional macroscopic object and simulate the motions you are talking about in a membrane?

DR. HYDE: Consider this stick or pointer. As I spin it around its own axis, twisting it in my hands, I caused the D_{II} type motion. But of course it is not a diffusive motion, which is a random walk.

Molecular collisions cause many small changes in angular motion. These are random but result on balance in rotational diffusion.

Similarly, as I turn the stick end over end, I think about $D_{\perp}$. But it is a much slower diffusive process. Many more collisions are required in order to cause noticeable rotational diffusion.

If I grasp the stick somewhere near one end and rotate my wrist rapidly back and forth by about 15°, I simulate the wobble motion.

This wobble simulation is a particularly difficult problem. Consider the stick lying always within a solid cone, which is equivalent to the assumption of infinite potential walls: the stick is prohibited from going outside the cone. Nevertheless, within the cone many motions are possible. The tip of the stick might move around in circles, which is called perimeter diffusion. It might move back and forth from one side of the cone to the other. It is reasonable to guess that there is some long-range order -- neighboring lipids interact with each other. This interaction, one would guess, must affect the nature of the wobble motion. We can make many such models. I consider it a personal challenge, which I invite you to accept also, to devise experimental methods to distinguish these models of wobble motion and to describe this motion with increased precision. This program must include rather sophisticated computer modeling.

DR. SCHOTLAND: Once again, can you give us a physical understanding of $D_{\perp}$?

DR. HYDE: Consider this stick that I grasp now at the middle and twist all about. The ends of the stick always lie on the surface of a sphere. Let the floor and the ceiling be the two sides of the bilayer. (Figure 2-1). Then the normal to the bilayer goes from floor to ceiling. Visualize this normal passing through the center of the sphere. Let θ be the angle between the normal and the stick. Then $D_{\perp}$ describes the average change of θ because of thermal diffusion. With a complicated anisotropic restoring potential as in a membrane, $D_{\perp}$ is a function both of θ and the distance from the polar headgroups (floor or ceiling).

DR. SCHOTLAND: And the correlation time?

DR. HYDE: Assume each of us in the room has a pointer and that at time $t = 0$ all point straight up. We say that the pointers are perfectly correlated. Then as rotational diffusion proceeds each of our pointers will, after some length of time, be oriented in a different direction. Correlation is lost, and the correlation time is a measure of how long this took.

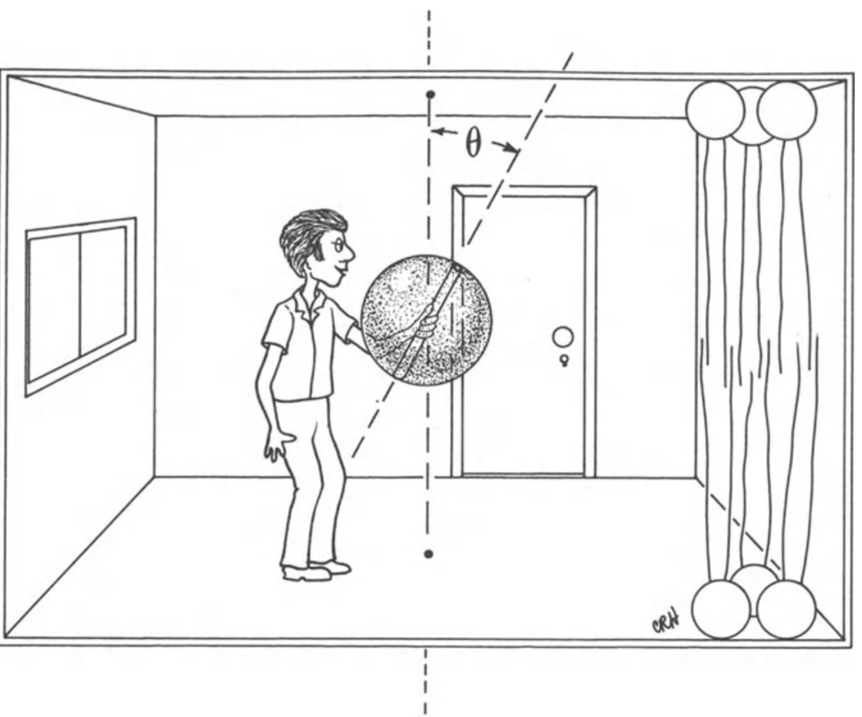


Figure 2-1: Let the floor and ceiling correspond to the surfaces of a lipid bilayer as suggested by the modern art in the corner of the room. The pointer pivots randomly about the wrist of the man such that the ends lie always on the surface of a sphere. The normal to the bilayer passes through the wrist. In this analogy the pointer is in the coordinate system of a segment of a fatty acid chain. Then $D_{\perp}$ can be related to the average change of θ per unit time.

The technique of fluorescence depolarization directly measures loss of correlation. In fixing one's ideas on correlation times, this is a good technique to study.

Oxygen

Molecular oxygen is paramagnetic. It interacts with many molecular probes, including not only nitroxide radical spin labels but also nuclei detected by NMR and fluorescence probes (quenching the latter). Then quite obviously: (a) oxygen should be eliminated when using molecular probes to study membranes and (b) if interaction between oxygen and probe is observed, it can permit the study of transport properties of oxygen.

I consider this two-sided role of oxygen in the context of spin-label experiments in membranes.

The oxygen concentration in water at 40°C is given in standard tables as 1.06×10^{18} molecules/cm^3. Usual textbooks give the concentration in membranes as 4.4 times that in water on a single experiment on olive oil. (Battino et al., 1968). The biomolecular collision rate of oxygen with a molecular probe is given by the Smoluchowski equation:

$$\omega_{ex} = 4\pi \, R(D_O + D_P) \, [O_2]$$

where D_O and D_P are the translational diffusion constants of oxygen and probe, and R is the interaction distance.

The classical magnetic dipole-dipole interaction between spin label and oxygen is now established as being less significant than the quantum mechanical Heisenberg exchange interaction in membranes. (Windrem and Plachy, 1980; Subczynski and Hyde, in press). Heisenberg exchange broadens the spin-label lines (Windrem and Plachy, 1980) and also changes the thermal contact with the environment. (Subczynski and Hyde, in press). (That is, because of oxygen, any non-equilibrium Boltzmann populations that might exist between two levels for whatever reason are brought back to equilibrium more rapidly.)

Almost without exception, investigators using spin labels in membranes have not bothered to remove oxygen. Popp and Hyde (in press) have now shown that in some cases this is likely to have led to error in the reported data. Problems are particularly serious in those forms of ESR spectroscopy that are dependent on non-equilibrium populations of energy levels. They, therefore, urge all workers routinely to remove oxygen from their samples.

Subczynski and Hyde have emphasized the other side of the oxygen--spin-label interaction. The question they ask is whether or not oxygen transport properties in membranes are mediated in biologically relevant ways by membrane constituents. It occurs to me that this might be a good question to ask in the context of muscular dystrophy. They report a discontinuity in the bimolecular collision rate at the main transition temperature of dimyristolyl-phosphatidylcholine (DMPC) bilayers, and differences in the rate for different phospholipids. In unpublished work they have observed further differences upon addition of cholesterol.

DR. PARK: You ought to tell us how to remove oxygen.

DR. HYDE: We use for sample tubes a methylpentene polymer known as TPX produced by Mitsui Petrochemical Industries Limited, Tokyo, and obtained by us from Westlake Plastics Company, Lenni, Pennsylvania. This material is very permeable to gases and quite impermeable to water. By flowing nitrogen over the sample tube, "gas phase dialysis" occurs and oxygen is removed from the sample. For control purposes, it can be readily put back by flowing oxygen over the sample. We use capillaries of about 1 mm i.d., 0.2 mm wall thickness. They can be produced on a lathe rather quickly by a skillful machinist. More details are given by Popp and Hyde. A similar approach has also been used by Placy and Windrem.

Some Modern ESR Techniques

If one wants to use changes in spectral features of spin-label spectra to make statements about transport in membranes, the width of the spectral feature determines the time scale for transport that is amenable to study. The relevant equation is:

$$T_x = \frac{1}{\Delta H \cdot 1.76 \cdot 10^7} ,$$

where T_x is a typical characteristic time for a transport process, ΔH is the spectral width in gauss, and 1.76×10^7 is a physical constant (the magnetogyric ratio). Since spin-label widths are typically a few gauss, we have severe constraints placed on the range of dynamic events that can be studied. This range may or may not be biologically relevant. The best experiments are those that have been designed to ask relevant questions in this time scale.

This T_x is a spectrally determined "clock." In my own research I have developed spin-label methods that use a different clock -- namely the time to reach equilibrium Boltzmann population of levels, which is called T_1. In spin labels, T_1 is 100 to 1,000 times longer than T_x.

I argue that these methods make it possible to ask a substantially wider range of biologically relevant questions concerning transport in membranes.

It is not possible to go into all of these methods here, but a brief list and relevant citations are appropriate.

A. Measurement of oxygen transport by observing the decrease of T_1 because of collisions with oxygen (Subczynski & Hyde, in press). T_1 is measured directly using the technique of saturation recovery (Hyde, 1979) or indirectly using the method of continuous wave (CW) saturation.

B. Saturation Transfer Spectroscopy. The technique permits the determination of rotational correlation times in the range of 10^{-3} to 10^{-7} sec, compared with the usual range of T_x using the equation above of 10^{-7} to 10^{-11} sec. Recent reviews are by Hyde and Thomas, 1980 and by Hyde and Dalton, 1979. The role of anisotropic rotational diffusion is considered in these reviews. The technique is particularly suitable for studying rotational diffusion of proteins in membranes.

C. Electron-Electron Double Resonance (ELDOR). C.A. Popp and I (unpublished) have recently completed an ELDOR experiment to measure translational (i.e., lateral) diffusion of lipid components in membranes. While several competing technqiues have been developed previously, we believe our methods are about one order of magnitude more precise. This permits asking many new biophysical questions concerning lateral diffusion. Among such questions are the effects on lateral diffusion of viscosity of the medium, radius of curvature of the membrane, cholesterol, lipid composition, and metal ions.

These techniques that use T_1 as the "clock" require specific knowledge of T_1 on the systems under investigation. Only in recent years has the capability of measuring T_1 of spin labels existed, and it remains a difficult measurement. However, techniques are improving. T_1 data are given in: Hyde and Sarna, 1978; Husijen and Hyde, 1974; Percival and Hyde, 1976; Schartz et al., 1979 and Forrer et al., in press).

It is my hope that these new techniques have been developed to a sufficient degree that they now can be applied to biomedical problems and that they can make contributions in the search for defects that result in muscular dystrophy.

DR. M. GLASER: There are differences in the lipid composition and the activity of membrane-bound enzymes in muscle and in some other tissues of patients with muscular dystrophies compared with the corresponding tissues from normal patients. There are also changes in the lipid composition when cells undergo normal differentiation processes and when they are malignantly transformed. One of the things we would like to know is whether these changes in lipid composition are primary or secondary events of the state of the cell and whether the changes in lipid composition that are observed can be responsible for the changes that are seen in the membrane.

Significance of Lipid Composition

My first impulse was to present a table by way of introduction which would give a list of membrane-bound enzymes and how they depend on lipids for activity. Although it is relatively easy to start making up a list of enzymes that depend on lipids for activity, there are only a very limited number of enzymes where the details of how they depend on lipids for activity have been well worked out. In many cases the literature is quite ambiguous and there are conflicting reports on how an enzyme depends on lipids for activity. In many cases the problems are with the methods that are used. For example, one result can be obtained if an enzyme activity is reconstituted using one technique and another result is obtained using another technique. One approach towards understanding the physiological significance of how lipids affect membrane structure and function in animal cells involves manipulating the lipid composition of cells as they are growing in tissue culture. The idea is to manipulate one component of the bilayer at a time in a defined way. The effect of this component on a functional parameter of interest, say on an enzyme activity, can be examined and correlated with how the component changed the structure and fluidity of the bilayer. It is especially important to carry out the modification while the cells are growing and healthy in order to be able to interpret the biological significance of the results. I would like to present some of the results you can get using this approach. I also would like to use it as a vehicle to present some current thinking about how lipids affect membrane structure and function and to point out some areas that I think are important for future research.

The system of choice for many of these studies was LM cells for two main reasons. (1) They can be grown in suspension culture, so it is relatively easy to get a large quantity of these cells and (2) they can be grown in a chemically defined medium. That is, the tissue medium contains vitamins, salts, amino acids, glucose and a few other things, but not protein, no lipids and

Manipulation of fatty acid Composition in Cultured LM Cells

no serum. It is completely chemically defined and that makes manipulations much easier. One of the things that has to be put in the medium in order to get growth of LM cells is choline, the structure of which is given in Table 1. The major phospholipid of these cells happens to be phosphatidylcholine. The manipulation of the phospholipid polar head groups is conceptually quite simple. Tissue culture medium was made that did not contain choline but instead contained an analogue. The cells were very obedient and they incorporated the analogue and put it into membrane phospholipids in place of choline. If dimethylethanolamine was added, for example, the cells incorporated this analogue and made the corresponding phospholipid, phosphatidyldimethylethanolamine. Initially when the cells are grown on choline, there was no detectable amount of phosphatidyldimethylethanolamine present in the cells. When dimethylethanolamine was added it was incorporated and over 50% of the phospholipids became phosphatidyldimethylethanolamine while phosphatidylcholine and phosphatidylethanolamine decreased. When analogues were added that the cells never see, such as aminobutanol or aminopropanol, these were incorporated to quite substantial extents, 35% or 45%, respectively. Besides manipulating the phospholipid polar head groups, the fatty acids can be manipulated. When cells were grown on normal medium containing choline, there were four major species of fatty acids: 16:0, 16:1, 18:0 and 18:1. Interestingly enough, in these cells there there were no essential fatty acids or polyunsaturated fatty acids. When a fatty acid was added to the medium, the cells incorporated the fatty acid, provided it was in a form where it was non-toxic such as a BSA complex. When linoleate was added under the conditions of Table 1 it was incorporated to about 35%. There was very little change in the polar head group composition. Thus, the phospholipid polar head group and the fatty acid composition can be manipulated individually or they can be manipulated at the same time. If both linoleate and a polar head group analogue were added at the same time, linoleate was still incorporated to about 35 percent and the head group was incorporated to the same extent whether or not linoleate was present.

Figure 2-2 illustrates a couple of points regarding the incorporation of fatty acids. Initially there was no linoleate in the medium and there was no linoleate in the cells. When increasing amounts of linoleate were added, linoleate was incorporated by the cells until over 50% of the fatty acids became linoleate. As the amount of fatty acid was increased, the biosynthetic capacity to make phospholipids or to use the fatty acids as energy was eventually exceeded. Consequently, the fatty acid was stored as triglycerides. The cells became crammed full of the triglyceride droplets and eventually the cells burst.

Table 1

Phospholipid composition of LM cells grown in medium containing different analogues of choline

Supplement	Structure	Phospholipid composition (%)						
		PE	PC	Other	PMEA	PDMEA	PBA	PPA
Choline	$HOCH_2CH_2N^+(CH_3)_3$	22.4	51.8	25.8	-	-	-	-
Methylethanolamine	$HOCH_2CH_2NHCH_3$	9.8	15.2	26.7	45.1	3.1	-	-
Dimethylethanolamine	$HOCH_2CH_2N(CH_3)_2$	17.3	8.9	22.4	0.7	50.6	-	-
	NH_2							
ℓ-2-Aminobutanol	$HOCH_2CHCH_2CH_3$	13.9	11.1	39.6	-	-	35.4	-
3-Aminopropanol	$HOCH_2CH_2CH_2NH_2$	15.0	17.8	22.4	-	-	-	45.0
Choline + linoleate		24.4	50.6	25.0	-	-	-	-
Methylethanolamine + linoleate		10.2	14.9	24.0	47.4	3.6	-	-
Dimethylethanolamine + linoleate		13.9	9.4	23.0	1.4	52.3	-	-
ℓ-2-Aminobutanol + linoleate		13.6	22.0	25.4	-	-	39.1	-

Cells were incubated for 3 days in medium containing choline or different analogues (40 μg/ml). In some samples, linoleate (20 μg/ml) was added as the bovine serum albumin complex 16 hr before cells were harvested. From Glaser et al., 1974.

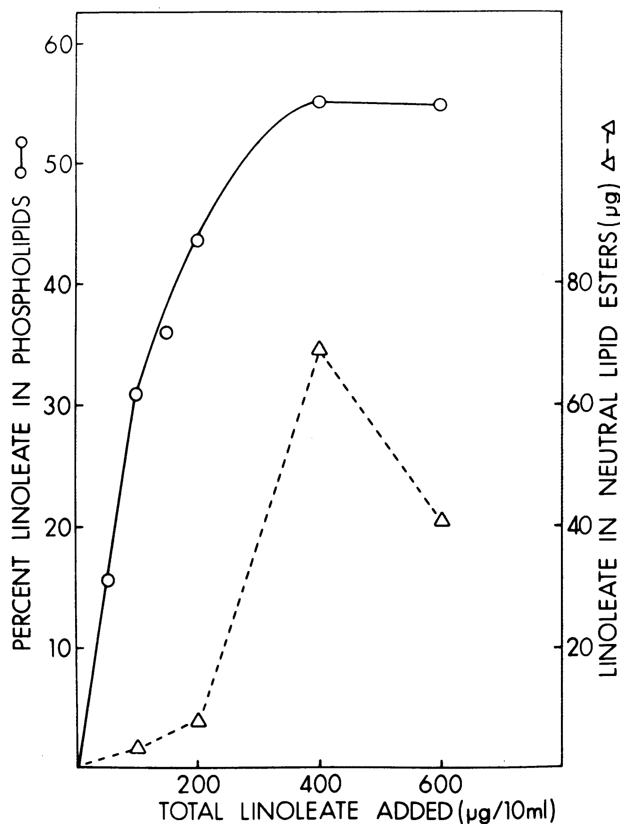


Figure 2-2: Concentration dependence of linoleate incorporation. Identical aliquots of LM cells were grown for 24 hours with increasing concentrations of linoleate in the medium. (0-0) per cent linoleate in phospholipids (Δ---Δ) total amount of linoleate in neutral lipid (μg/flask) (Ferguson et al., 1975).

Figure 2-3 illustrates the effect of changing phospholipid polar head group composition on the morphology of normal and Rous sarcoma virus-transformed chicken embryo fibroblasts. The lipid composition of these cells can be manipulated in a similar manner to LM cells, although more work is involved. The normal cells were flat and they had very few surface features. When they were transformed, they rounded up and displayed microvilli blebs and so forth. When normal cells were in methylethanolamine-containing medium, the cells instead of being elongated became broader and more epitheliod in shape. With butanolamine about 50% of the cells rounded up. In fact, they looked and behaved a little like the transformed cells. For example, they did not adhere as tightly to the surface of the tissue culture dish. There also must have been alterations in cytoskeleton.

Lipid Manipulations in Transformed Cells

Altering the lipid composition produced large changes in the properties of membrane-bound enzymes. For example, if cells were supplemented with ethanolamine there was an increase in the amount of PE that was present, a three-fold change in Km of adenylate cyclase, three-fold change in the specific activity, and so forth. (Engelhard et al, 1978). But in order to really understand what was going on, it was important to also understand how these modificaions changed the structure and fluidity of the membrane. There are a variety of techniques that can be used to study membrane structure: ESR, NMR, fluorescence, calorimetry, etc. Each technique has its own advantages and disadvantages that need to be appreciated. I would like to discuss two, one is fluorescence polarization and the other is deuterium NMR. One way to look at the motion of molecules in a membrane is to use fluorescence polarization. In order to do that it is necessary to have a fluorophore. One fluorescent probe that has been fairly popular is DPH, diphenylhexatriene, which was introduced by Shinitzky and his colleages. It is a hydrophobic molecule, so if it is put in a cuvette with membranes it will partition right into the interior of the membrane. In a polarization experiment, plane polarized light at the appropriate wavelength is used to excite the molecules. If DPH is aligned with the incoming light, it will be absorbed. If the medium has a high viscosity, DPH will not have a chance to rotate during its fluorescent lifetime, and consequently, it will absorb and emit the light with the same orientation. Thus, the light coming out will be highly polarized. Conversely, if DPH is in a medium of low viscosity, it will have a chance to move. Some molecules are likely to move a little, some a bit further, and some will move in the opposite direction before emitting the light. Thus, the light coming out will be depolarized or have a low polarization. The other thing that is important to measure beside the polarization is the lifetime. If two membrane samples are compared that have the same viscosity but cause DPH to have different lifetimes, DPH will have more or less time to move before emitting the light.

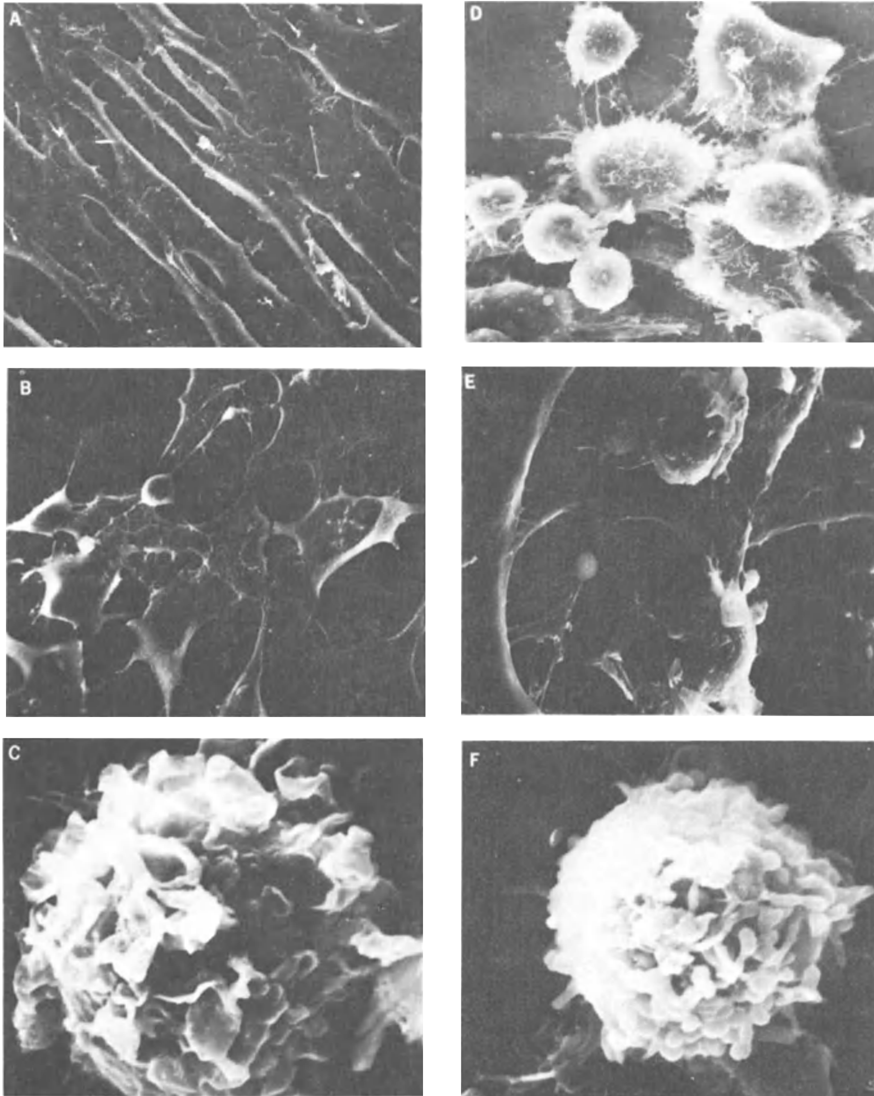


Figure 2-3: Scanning electron micrographs of normal and Rous sarcoma virus-transformed chicken embryo fibroblasts supplemented with choline analogues. Normal cells were grown in: A, delipidated medium plus choline for 50 hr (x 1200); B, delipidated medium plus N-methylethanolame for 50 hr (x 1000); C, E, and F, delipidated medium plus ℓ-2-amino-1-butanol for 50 hr (x 7800 x 3000 and x 8000, respectively); D, transformed cells were grown in delipidated medium plus choline for 50 hr (x 2000) (Hale et al., 1979).

Consequently it is necessary to make measurements of both the polarization and the lifetime. Then the Perrin equation can be used to calculate the viscosity or rotational relaxation time. The rotational relaxation time is the time it takes for the molecule to move through a given angle. If DPH is in a medium of high viscosity, it will take a long time to move through the angle and it will have a long rotational relaxation time.

Viscosity or microviscosity can be calculated directly from polarization and lifetime measurements. As has been shown by a number of investigators, this is valid if DPH is in homogenous solvent, but a bilayer is not homogeneous. The motion of DPH and other molecules in a bilayer is not isotropic, it is hindered (Andrich and Vanderkooi, 1976; Dale et al., 1977; Kawato et al., 1977; Hare and Lussan, 1977; Lakowicz and Prendergast, 1978; Veatch and Stryer, 1977). It is significantly hindered below the phase transition of a phospholipid or at low temperatures. Thus, it is not strictly valid to calculate a microviscosity and it appears more appropriate to express the data in terms of the polarization or the rotational relaxation time. There is one other problem that I want to point out and that concerns the fact that cells have triglycerides. Most tissue culture cells are grown in the presence of serum, and serum has lots of fatty acids either in the form of serum lipoproteins or as fatty acids bound to albumin. When fresh medium is added to cells, the fatty acids will rapidly be taken up by the cells. When the biosynthetic capacity of the cells to make phospholipids or use the fatty acids as energy is exceeded the fatty acids will be stored as triglycerides as I pointed out previously.

Figure 2-4 shows normal and Rous sarcoma virus-infected cells plated at different densities in fresh serum. Triglycerides increased, reached a maximum at about 20 hours, and then decreased. When measurements were made on these cells using DPH or other hydrophobic molecules, the molecules not only partitioned into the membranes of the cell, they partitioned into all hydrophobic compartments including triglycerides. Depending on the conditions, there were more triglycerides in the cells than phospholipids. It turns out that the triglycerides had a very low viscosity and strongly biased the measurements. What was measured under these conditions was the amount of triglycerides and changes in triglyceride rather than reflecting the properties of the membranes. Polarization started out high and it reached a minimum at the same time triglycerides reached their maximum. Then as triglycerides were used up, polarization increased. When the cells were plated at a little higher density, the triglyceride maximum was reached sooner and it was not quite as high. There was a good inverse correlation with the polarization. The reason for pointing this out is because this problem is still not widely recognized in the

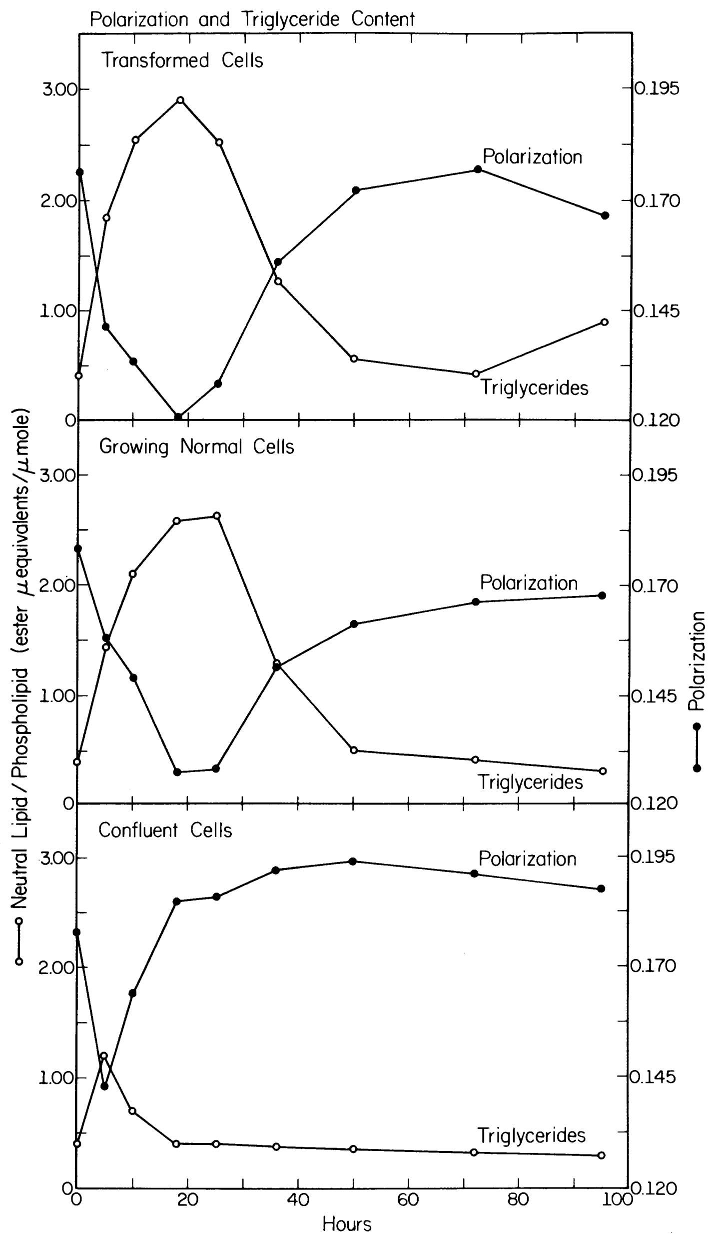


Figure 2-4: Whole cell fluorescence polarization of DPH at 37°C and accumulation of triacylglycerols and alkyldiacylglycerols (0) in transformed, normal growing, and confluent chicken embryo fibroblasts (Pessin et al., 1978).

literature, and it is not only a problem with the DPH but it is also a problem that needs to be considered whenever a hydrophobic probe is used. Care has to be taken in membrane preparations since vesicles are formed depending on the homogenization conditions and it is very easy to trap triglycerides in the vesicles. So there can be variable amounts of triglycerides present that will bias the measurements.

Figure 2-5 shows the temperature dependence of the rotational relaxation time of DPH in a pure phospholipid, dimyristoyl, phosphatidylcholine, and for the plasma membrane of LM cells. The pure phospholipid showed the typical phase transition from the gel to the liquid-crystalline state while the plasma membrane did not give an indication of a distinct transition or a discrete region of lateral phase separation.

Each type of membrane in a cell has a different lipid composition and this gives rise to different motions of DPH. The curves in Figure 2-6 show the rotational relaxation time of DPH for the plasma membranes, ER, and mitochondrial membranes of cells supplemented with either choline, ethanolamine or linoleate. The values for the plasma membrane were about two to three fold higher than for the mitochondrial membrane. When ethanolamine was added, the curves were shifted up, and when linoleate was added the curves were shifted down. The extent that they went up or down depended on the extent of supplementation. Interestingly enough though, the ratio between the plasma membranes and mitochondrial membranes stayed roughly constant. The same was true for the ratio between the plasma membrane and ER. Thus, there seems to be some feature the cell wants to control. A question that remains to be answered is what gives rise to these differences in lipid composition and what are the factors that regulate it so as to maintain the differences between the various membranes.

Figure 2-7 shows an analysis of the different phospholipid classes that were found in these different membranes. The major point is that there were characteristic motions of DPH in the different phospholipid classes. The only one that showed a clear gel to liquid crystalline phase transition above 0°C was sphingomyelin. It could be that PE was undergoing a hexagonal to bilayer transition but this would not be observed with DPH. The sphingomyelin transition was very similar to the type of transition for dimristoyl phosphatidylcholine except in this case it was a little bit broader because sphingomyelin contained more than one species of fatty acid. The rotational relaxation time of DPH in phosphatidylcholine, for example, was in the range characteristic of lipids well above the phase transition. The slight upward curvature might indicate it was reaching a transition below zero degrees. Most of the phospholipids in the cell were well above

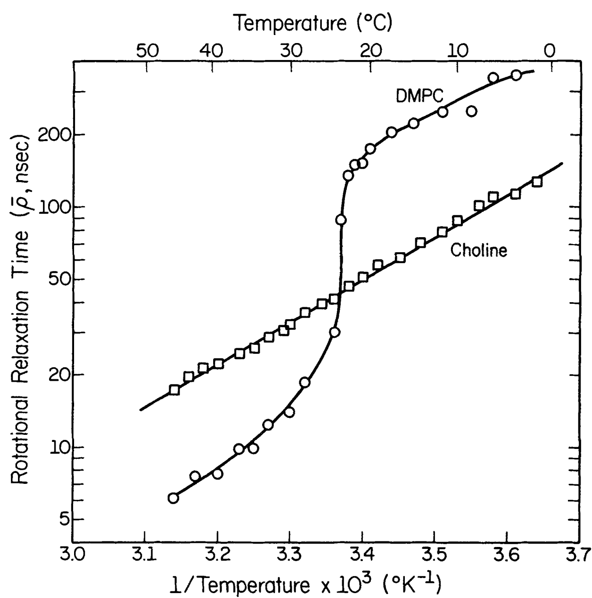


Figure 2-5: The temperature dependence of the rotational relaxation time of DPH in plasma membranes isolated from LM cells supplemented with choline and in DMPC vesicles (Esko et al., 1977).

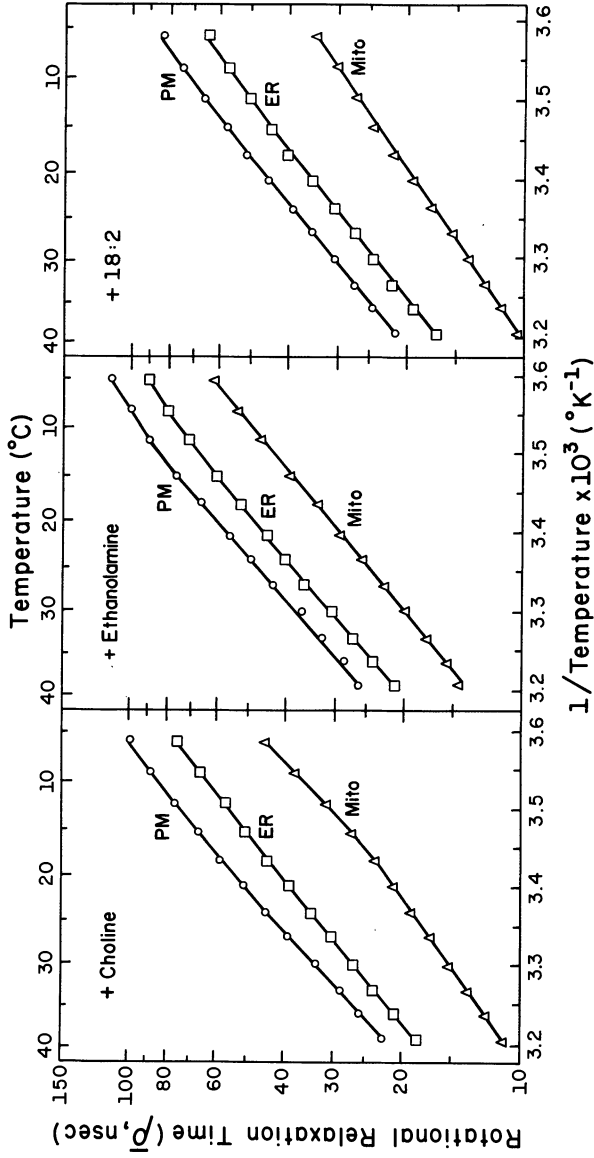


Figure 2-6: The temperature dependence of the rotational relaxation time of DPH in (O-O) plasma membranes, (□ - □) microsomes and (Δ-Δ) mitochondria from choline-supplemented, ethanolamine-supplemented, and linoleate-supplemented LM cells (Gilmore et al., 1979).

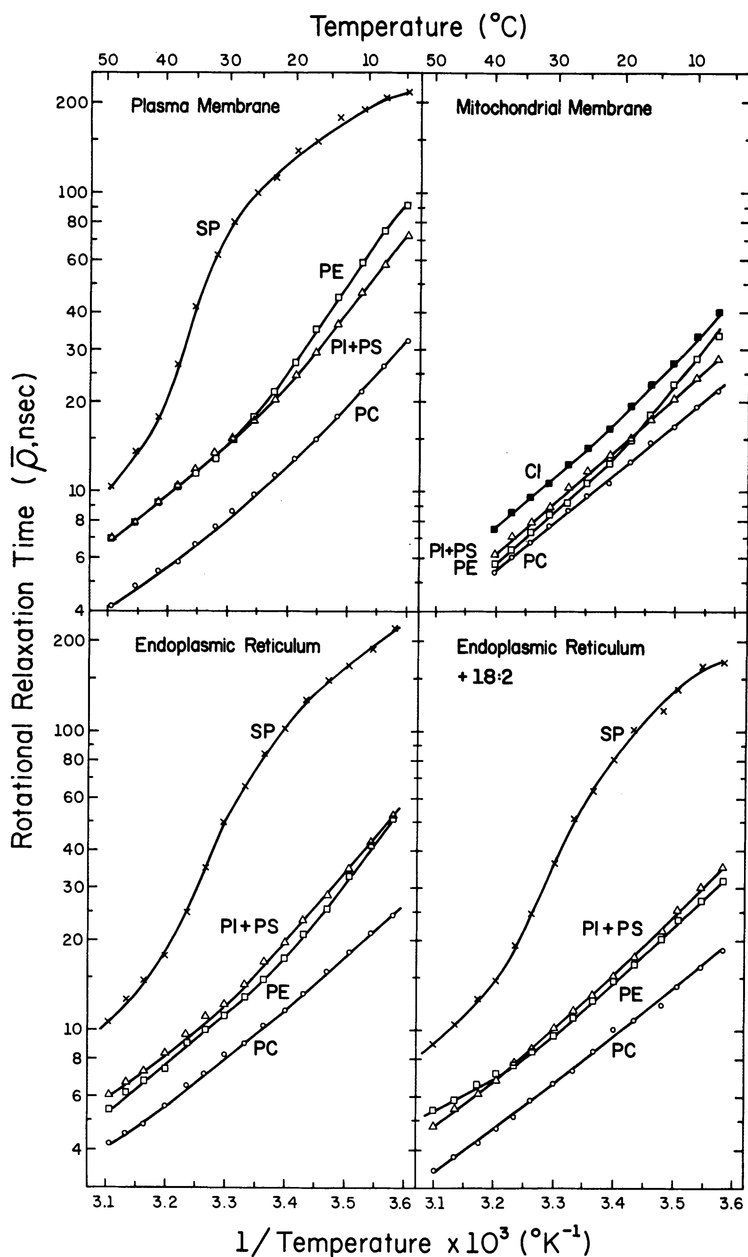


Figure 2-7: The temperature dependence of the rotational relaxation time of DPH in phospholipid fractions isolated from plasma membranes, mitochondrial membranes, microsomal membranes from choline-supplemented LM cells, and microsomal membranes from linoleate-supplemented LM cells (Gilmore et al., 1979).

their phase transition at zero degrees. At 37 degrees, where the cells were growing, sphingomyelin was also above its phase transition below zero degrees. Most of the phospholipids in the cell were well above their phase transition at zero degrees. At 37 degrees where the cells were growing, sphinogomyelin was also above its phase transition.

When the concentration of sphingomyelin was reduced to the concentration found in a membrane, the transition was damped out as shown in Figure 2-8. When the sphingomyelin concentration was first reduced to 75 percent, the transition shifted to the right and became broader. Then as it was reduced to around 10 percent, which was approximately the concentration found in the plasma membrane, there was little sign of the phase transition. Consequently, it is misleading to talk in terms of gel to liquid crystalline phase transitions above zero degrees when the membrane phospholipids in these cells are in a fluid state at the growth temperature.

Effects on Rotational Relaxation Time

One of the things that is implied when talking about the rotational relaxation time of DPH in the plasma membrane is that the membrane is homogeneous. In the fluid mozaic model, protein and lipids are free to diffuse freely in the membrane and to assume a random distribution. As we talked about earlier this morning, the protein components of the membrane can be organized into discrete arrays. But there is no firm evidence that lipids are restricted to localized lipid regions or domains. Since lipids are fluid and free to move, they should be randomly dispersed in the membrane. I would like to present data which suggest that, in fact, the lipids of the membrane can be organized into fairly large localized regions on the order of a tenth of a micron or larger. The evidence for this comes from looking at the composition of enveloped viruses. Vesicular stomatitis virus and Rous sarcoma virus are surrounded by a lipid bilayer. The bilayer is derived during the budding process from the plasma membrane of the cells (Patzer et al., 1979; Lenard, 1978). The virally coded envelope proteins migrate to the plasma membrane where they form a patch. The RNA core comes up to that patch and then buds from the plasma membrane during which the host proteins are excluded and the lipid is picked up from the plasma membrane. The general idea that has arisen in the literature is that the lipid composition of the virus is the same as the lipid composition of the plasma membrane (Lenard, 1978; Patzer et al., 1979). That idea has arisen because a given virus can be grown on different types of cells, cells that have different lipid compositions, and the lipid composition of the virus always reflects the lipid composition of the host cell.

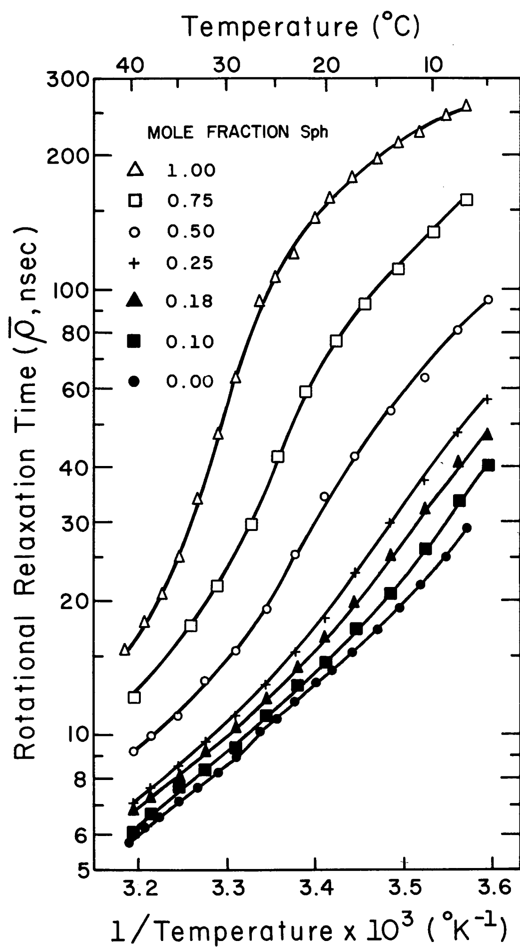


Figure 2-8: The temperature dependence of the rotational relaxation time of DPH in mixtures of sphingomyelin and phosphatidylcholine. Sphingomyelin and phosphatidylcholine were isolated from whole LM cells (Gilmore et al., 1979).

Table 2 shows a comparison of the phospholipid composition of vesicular stomatitis virus with the ER and the plasma membrane from the host cells which were chicken embryo fibroblasts. There were some differences in choline-containing medium. For example, phosphatidylcholine in the virus was 31.6 percent and in the plasma membrane it was 43.4 percent. There was also a small change in phosphatidylethanolamine and in some other phospholipids. Similar differences have been observed by a number of investigators (in particular McSharry and Wagner, 1971; and Quigley, et al. 1971, 1972. One argument that can be made is that the plasma membrane is really the same as the virus, but the reason that the plasma membrane value was higher was because of contamination with other types of cellular membranes. Even using membrane marker enzymes or electron microsocpy, it is hard to say whether it is 50% or 100% pure. The argument could be made that the values of phosphatidylcholine were really the same in the plasma membrane and in the virus, namely 31.6%, but the plasma membrane was contaminated by ER. ER had lots of phosphatidylcholine and if it was contaminating the plasma membrane preparation, it would raise the true value for the plasma membrane and give the value that was observed. The same argument can be made for phosphatidylethanolamine and the other phospholipids. In order to determine whether the virus did reflect the lipid composition of the plasma membrane, the lipid composition of the cells and viruses were altered. In some cases there were large differences between the plasma membrane and the virus. For example, when cells were supplemented with dimethylethanolamine or aminobutanol, there was almost a 20% difference in phosphatidyldimethylethanolamine or phosphatidylbutanolamine. But it is still possible to make arguments about contamination. In order to definitely resolve this problem the kinetics of incorporation were examined.

Figure 2-9 shows data for Rous sarcoma virus grown on chicken embryo fibroblasts. The differences between the virus and the plasma membrane were quite large. When cells were grown on choline the amount of phosphatidylcholine never changed. When the cells were grown on dimethylethanolamine, the amount of phosphatidyldimethylethanolamine increased fairly linearly in the virus, while it went up and leveled off in the plasma membrane. For aminobutanol, the incorporation was similar for both the virus and the plasma membrane.

As the analogues were being incorporated, something had to go down. Figures 2-10 and 2-11 were taken from the same experiment and show what happened to phosphatidylcholine and sphingomyelin. The amount of phosphatidylcholine stayed the same (Figure 9) when the cells were supplemented with choline. When the cells were supplemented with dimethlethanolamine, the amount of phosphatidylcholine decreased dramatically in the plasma membrane and in the ER. For the virus, on the other hand, phosphatidylcholine dropped only a little bit for about 30 hours and then it leveled off. For

Table 2

Phospholipid composition of the plasma membrane, endoplasmic reticulum, and vesicular stomatitis virus isolated from chicken embryo fibroblasts

Phospholipid Composition (% phosphate)

	PC	PE	PI+PS	SPH	PDMEA	PBA	Other
			Choline				
ER	59.2	23.9	10.1	5.4	-	-	1.4
PM	43.4	26.2	14.1	15.2	-	-	1.1
VSV	31.6	32.7	16.8	17.8	-	-	1.1
			Dimethylethanolamine				
ER	21.3	9.1	10.7	3.9	53.4	-	1.6
PM	17.8	16.1	13.4	11.5	39.9	-	1.3
VSV	18.2	27.5	16.5	17.6	19.2	-	1.0
			ℓ-2-Amino-1-butanol				
ER	43.5	9.8	12.9	5.2	-	26.5	2.1
PM	32.0	13.5	14.0	15.1	-	22.8	2.6
VSV	24.2	32.0	16.3	22.2	-	4.7	0.6

Cells were grown for 36 hr in the presence of choline or choline analogues (Pessin and Glaser, unpublished data).

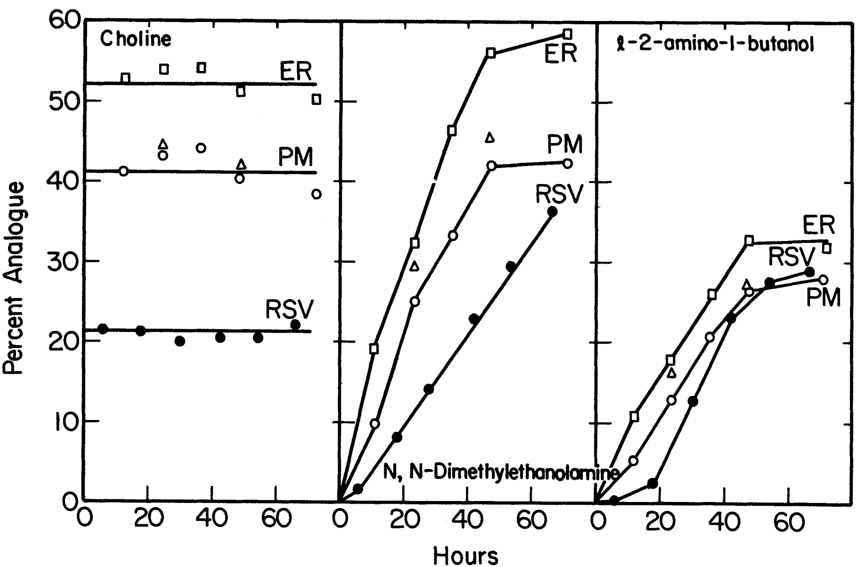


Figure 2-9: Rate of incorporation of choline, N,N-dimethylethanolamine, or ℓ-2-amino-1-butanol into phospholipids of the endoplasmic reticulum, plasma membrane and Rous sarcoma virus isolated from chicken embryo fibroblasts. Open triangles (Δ) represent phagosome preparations (Pessin and Glaser, 1980).

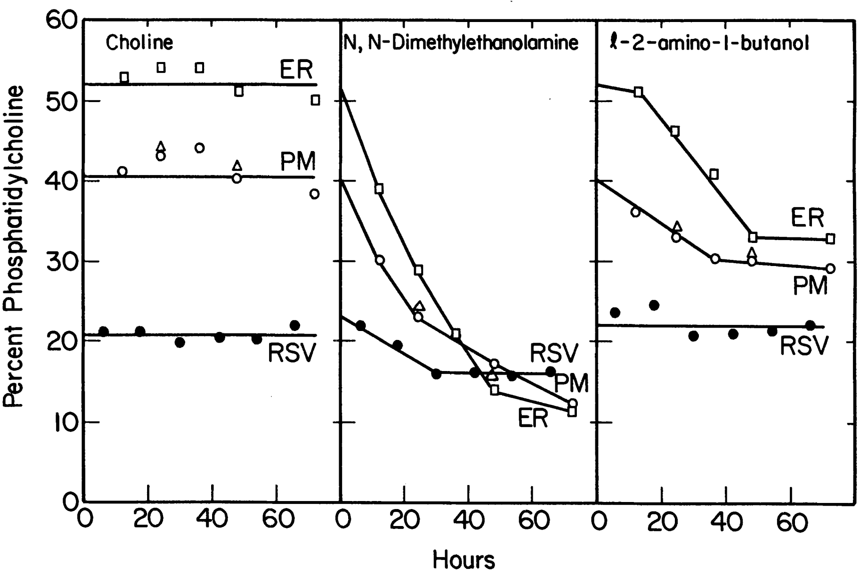


Figure 2-10: Percent of phosphatidylcholine in the endoplasmic reticulum, plasma membrane, and Rous sarcoma virus during growth of chicken embryo fibroblasts on choline, N,N-dimethylethanolamine, or ℓ-2-amino-1-butanol. Open triangles (Δ) represent phagosome preparations. The data are from the same experiment as shown in Figure 2-9 (Pessin and Glaser, 1980).

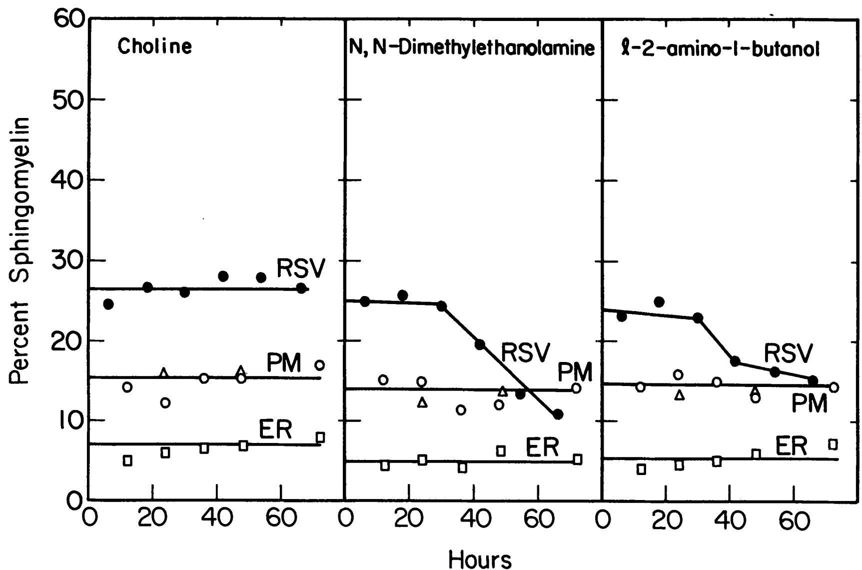


Figure 2-11: Percent of sphingomyelin in the endoplasmic reticulum, plasma membrane and Rous sarcoma virus during growth of chicken embryo fibroblasts on choline, N,N-dimethylethanolamine, or ℓ-2-amino-1-butanol. Open triangles (Δ) represent phagosome preparations. The data are from the same experiment as shown in Figure 2-9 (Pessin and Glaser, 1980).

aminobutanol, phosphatidylcholine decreased also in the plasma membrane and in the ER, but it stays relatively constant in the virus. The behavior of the plasma membrane and the virus was very different. In the virus, it was sphingomyelin that decreased while it stayed constant in the plasma membrane and ER (Figure 10). This was true for supplementation with either dimethylethanolamine or aminobutanol. There is no way that the data can be explained by contamination; the virus was quite different from the plasma membrane.

Figure 2-12 shows a comparison of the rate of analogue incorporation into Rous sarcoma virus and into vesicular stomatitis virus. The two viruses were different from each other and both of them were different from the plasma membrane. This suggests that the viruses bud from distinct regions in the plasma membrane. The regions must be quite large since the virus is on the order of a tenth of a micron. This has numerous implications for membrane structure and function. For example, in considering how an enzyme depends on lipids for activity, it is important to know what lipids surround the enzyme. Determining the composition of the whole plasma membrane could be misleading.

Lipid-Protein Interdependence

An important question is what gives rise to these differences in lipid composition between the viruses and the plasma membrane. Two main mechanisms can be envisioned. (1) The virus picks a pre-existing patch of the plasma membrane. For example, the virus may need to bud from a region of constant fluidity. (2) Alternatively, the viral proteins could sequester specific lipids, and form a type of "boundary lipid." This type of an argument goes against some of the recent deuterium NMR data which suggests that ideas on boundary lipids have to be modified.

Figure 2-13 shows deuterium NMR spectra of dimyristoyl phosphatidylcholine which had been deuterated in the methyl group of the fatty acid chain. The resonance was split into two and the magnitude of the splitting reflected the rate and type of motion the deuterium nuclei were undergoing. At very low temperatures the splitting was very large, and it decreased progressively as the sample was warmed.

Figure 2-14 shows the effect of adding cholesterol at 30°C which was above the gel to liquid crystalline phase transition temperature. The bilayer became more rigid and the splitting increased. In order to understand how the membrane regulates the activity of a membrane-bound enzyme or the motion of an enzyme moving through the bilayer, it is important to consider what lipids are bound to it. Since we

Effect of Cholesterol on Rigidity

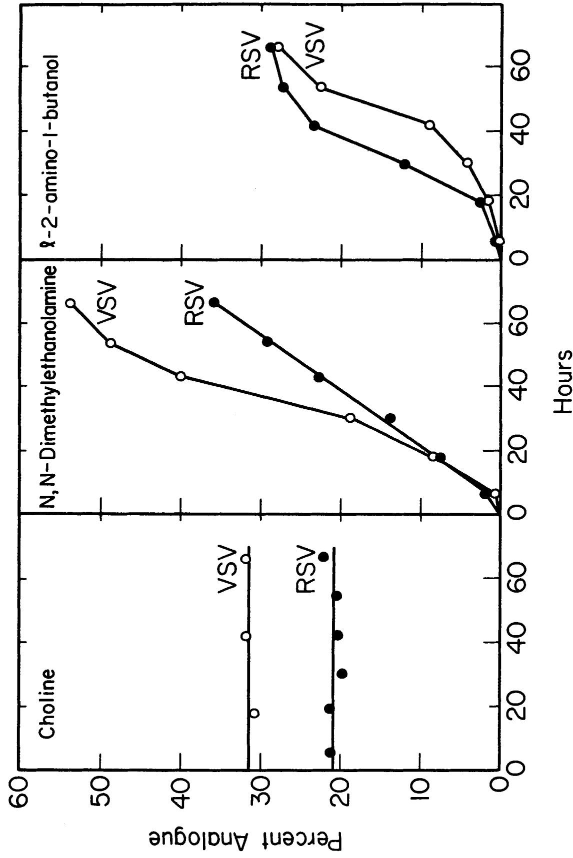


Figure 2-12: Rate of incorporation of choline, N,N-dimethylethanolamine or ℓ-2-amino-1-butanol into Rous sarcoma virus and vesicular stomatitis virus (Pessin and Glaser, unpublished data).

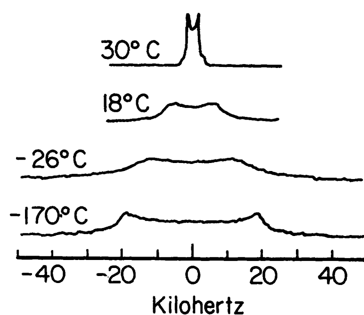


Figure 2-13: Deuterium Fourier-transform NMR spectra of DMPC-d3 bilayers in excess deuterium-depleted water obtained at 34.1 MHz by using the quadrupole echo pulse technique (Oldfield et al., 1978).

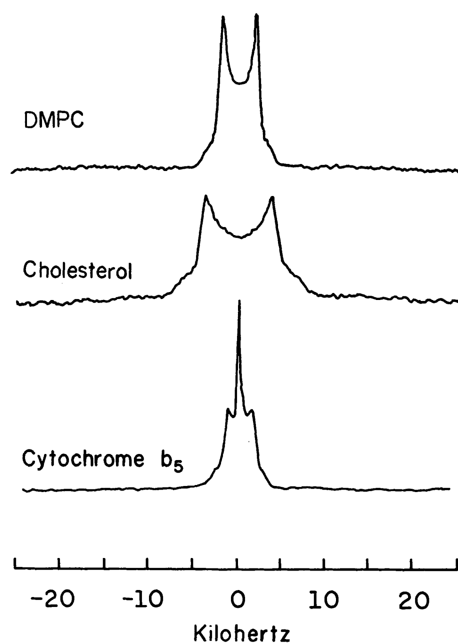


Figure 2-14: Deuterium Fourier-transform NMR spectra of DMPC-d_3 bilayers, and DMPC-d3 bilayers containing cholesterol or cytochrome b5, obtained at 34.1 MHz by using that quadrupole echo pulse technique. The sharp central component in the cytochrome b_5 spectrum was due to the natural concentration of deuterium in water that was not completely removed in this experiment (Oldfield et al., 1978).

were interested in the activity of cytochrome b_5, we wanted to know how much boundary lipid was really associated with this protein. Cytochrome b_5 is an example of a protein that has been shown to have boundary lipid associated with it by techniques such as ESR (Dehlinger et al. 1974). But it was not clear exactly how much lipid was associated with it. The splitting in the NMR spectrum should have increased when cytochrome b5 was added to DMPC, similar to what happened when cholesterol was added. The result was exactly the opposite. Instead of the splitting getting larger, it actually got a little bit smaller. When deuterium was attached to different parts of the phospholipid molecule, it also gave results consistent with the absence of bound lipid. This type of experiment has now been done by a number of workers with different proteins and the deuterium NMR results were similar (Seelig and Seelig, 1978; Oldfield et al., 1978). Namely, when a protein was added to the bilayer there was no change or a narrower component appeared in the spectrum instead of a rigid component. One explanation for this type of discrepancy with other methods is that ESR and fluorescence look at motion on a different time scale then deuterium NMR. ESR, for example, averages motion on a much slower time scale, 10^{-4} to 10^{-5} seconds. If a protein is added to a bilayer, some of the fatty acids do associate with the protèin, but they stay associated for only a very short period of time. On the ESR time scale, there is a component in the spectrum due to this boundary lipid. But over longer time periods, the fatty acid is bound, then comes off a little bit, and then goes into the bulk bilayer. In deuterium NMR the different conformations are averaged. Thus, the net effect is a disordering and that is what gives rise to the narrowing in the NMR spectra. This would indicate that the boundary lipid is exchanging at a fairly rapid rate instead of what was once considered to be a very rigid and non-exchanging boundary lipid. Further studies are necessary to determine if this view is correct and to determine how localized lipid regions can be formed in the membrane. Perhaps the viral proteins are different or have a higher affinity for one phospholipid and that is all that is necessary.

DR. BUTTERFIELD: Just refresh my memory on the structure of fatty acid in this case.

DR. M. GLASER: It is a saturated fatty acid, 14:0, myristic acid. The experiment has been done with some others but most of the studies that we have done have been with myristic acid. Other workers have used other fatty acids.

DR. BUTTERFIELD: I am just interested in the middle part here for a second. Again, maybe the similarity will prevail here but Ian Smith says, "add cholesterol to unsaturated lipids indeed you get a rigidity. However, add cholesterol to a saturated lipid you get a more fluid type of ESR pattern."

DR. M. GLASER: Yes, if you are below the phase transition and you were to add cholesterol you would get a disordering effect. These NMR experiments were done above the phase transition and under these conditions you do not see the disordering effect with cholesterol, you see a rigidifying effect which is the opposite case from cytochrome b_5. If you dropped below the phase transition, you would see a disordering with both cholesterol and cytochrome b_5.

DR. BUTTERFIELD: In an unpublished work that Betty Gafney and I have done on cholesterol below the phase transition in the same system, we would say that cholesterol decreases d-parallel, makes these easy motions faster and makes the entire system more rigid and therefore changes d-perpendicular. It is more complicated than just saying it makes it more fluid or less fluid. If one moves the d-parallel in one direction making that faster motion and changes d-perpendicular in the other way, it makes the entire structure more rigid with less fluidity, if you will, in the middle of the bilayer, but an increase in d-parallel.

Effect of Lipid Composition on Activity of Membrane Bound Enzymes

DR. CHARNOCK: The membrane-associated enzyme ($Na^+ + K^+$)-ATPase is the biochemical expression of the coupled transport of Na^+ and K^+ ions across the membranes. When isolated from a wide variety of mammalian sources, it is found to be a lipid-dependent-system which displays marked temperature dependence.

The temperature-activity relationship of ($Na^+ + K^+$)-ATPase is frequently illustrated by Arrhenius plots of the logarithm of the rate constant for ATP hydrolysis vs. the reciprocal of the absolute temperature. After some years of debate it is now accepted that the Arrhenius plots of ($Na^+ + K^+$)-ATPase are non-linear and that there is an inflection point at about 18-20°C. Above this temperature the apparent activation energy of the process (Ea_1) is about 15 kcals/mole, whereas below this temperature the value is about 35 kcals/mole.

While investigating this aspect of the enzyme system some years ago we found that although the Arrhenius plots of ($Na^+ + K^+$)-ATPase were not significantly altered by treatment with the detergents DOC or SDS, treatment of the isolated membrane fragments with phospholipase-A produced a marked change in the Arrhenius plot of this enzyme activity. This is shown in Figure 2-15.

After treatment with PPL-A the inflection point is no longer evident and only a single activation energy can be calculated ($\simeq$ 24 kcals/mole) over the entire physiological temperature range.

However, when this phospholipase-A treated material was reincubated with phosphatidylserine, the original temperature activity relationship was restored: i.e. the inflection point at 18-20°C was recovered and values for Ea_I and Ea_{II} were again 15 and 35 kcals/mole respectively.

After incubation with PPL-A and PS the activity of the preparation was about 86% of the original control value. (Charnock, Cook, Almeda and To, 1973).

Subsequently we were able to show that these temperature: activity relationships were much more apparent when the enzyme activity was determined as a function if its INHIBITION by OUABAIN, than was the case when enzyme activity was measured as a function of activation by CATIONS. (Charnock, Almeda and To, 1975).

Ouabain Binding

We deduced from these results that the ouabain-binding sites of the $(Na^+ + K^+)$-ATPase were modulated by the presence of lipids within the membrane matrix in a manner more apparent than was the case for either the Na^+ or K^+ activation sites.

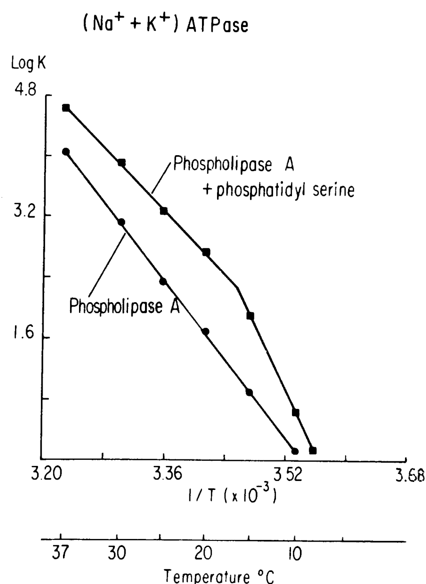


Figure 2-15: The effect of phospholipase-A on the temperature: activity relationship of NaK-ATPase. (■) after reconstruction with PS. (●) after treatment with PPL-A.

Direct examination of the effects of both TEMPERATURE and LIPOLYSIS upon the binding of $[^3H]$-oubain to $(Na^+ + K^+)$-ATPase considerably strengthens this view. We found that under certain experimental conditions (i.e. Polytron disintegration of brain preparations) BOTH DOC and SDS treatment of ox-brain membranes consistently results in membranes which displayed LINEAR Arrhenius plots of ^3H-oubain binding, while continuing to display NONLINEAR Arrhenius plots of ENZYME ACTIVITY (Charnock, Simonson and Almedia, 1977). Figure 2-16.

We can conclude from these studies that the ouabain-binding (i.e. drug-receptor) characteristic of $(Na^+ + K^+)$-ATPase is a lipid dependent process which is more susceptible to change BY DETERGENT EXTRACTION than is the hydrolysis of substrate by the enzyme!

It should therefore be possible to find situations in nature, where different drug-binding properties of $(Na^+ + K^+)$-ATPase can exist, through different lipid environments of the same enzyme protein. I was reminded of the original observations of Skou (1960) where in the enzyme preparation from the shore crab (Carcinus maenus) he was only able to demonstrate about 60-70% inhibition of the enzyme at 10^{-4} M ouabain, whereas this drug concentration was capable of 100% inhibition of $(Na^+ + K^+)$-ATPase preparations obtained from mammalian tissues.

It is also well known that the lipid composition of marine cells is quite different from that of mammalian tissues.

I therefore determined to compare the dose-response curve for ouabain inhibition of the enzyme from mammalian sources (rabbit, kidney, beef brain) with that from the cold-water crab *Cancer magister*. The results are shown in Figure 2-17.

Clearly the sensitivity of the CRAB $(Na^+ + K^+)$-ATPase system to inhibition by ouabain is 2 log units LESS than that of the inhibition of $(Na^+ + K^+)$-ATPase from beef brain (or many other enzyme preparations from mammalian sources).

In this context, it is also of great interest that the fatty acid composition of the two membrane associated ENZYME preparations is also widely different in some important respects. This is shown in Figure 4. Table 1 gives only the major fatty acid composition of our preparations.

Note that the beef system contains about 10% more saturated fatty acid than the crab (made up almost entirely of 16° and 18°).

The crab contains some 15% more total unsaturated fatty acid than beef (about 5% more 20' and an entirely "new" fatty acid

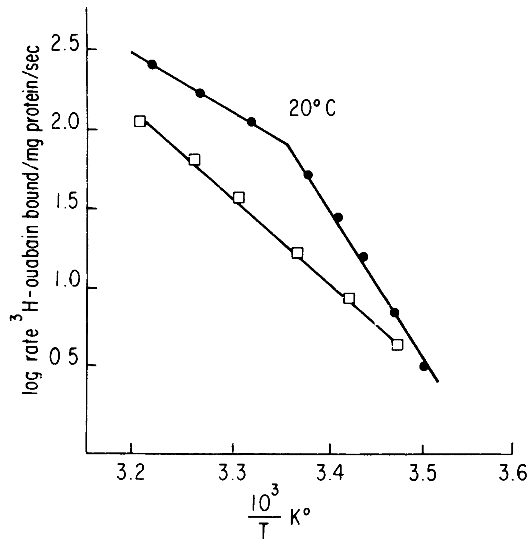


Figure 2-16: The effect of temperature on the binding of $[^3H]$-ouabain to NaK-ATPase. (●) minimal disruption during preparation. (□) vigorous disintegration with polytron homogenizer.

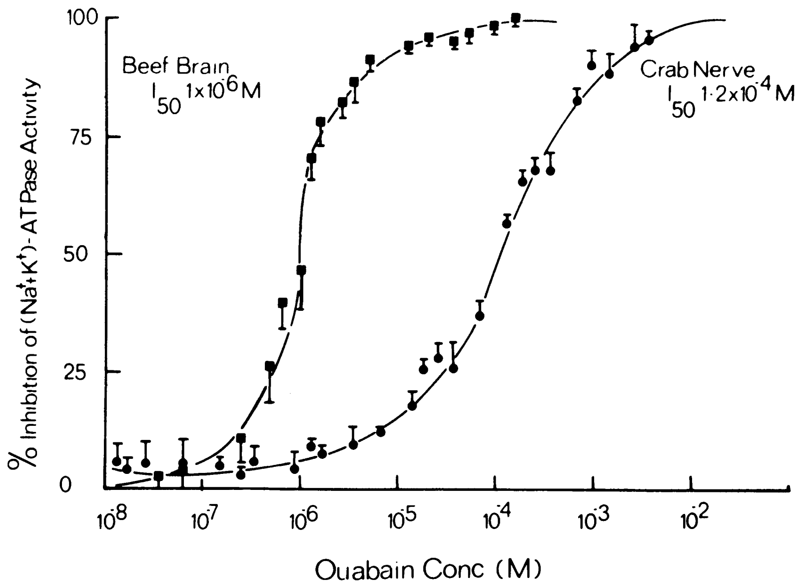


Figure 2-17 Dose response curves for ouabain inhibition of NaK-ATPase (■) beef brain enzyme. (●) crab nerve enzyme.

20^5 ω3 which was just a trace amount in beef but now is >20% of the total fatty acid content of the system in the crab).

There is also considerably more CHOLESTEROL in the crab membrane than there is in the beef, about double on a % (wt/wt) basis.

These changes in fatty acid and cholesterol composition are compatible with a change in the FLUIDITY of the membrane MATRIX in which the $(Na^+ + K^+)$-ATPase protein is embedded. The possibility was examined by using the fluorescent probe 12-(9-anthroyl)-stearic acid (12-AS) and measuring the effect of temperature upon the mobility of this probe by the fluorescence polarization procedure (Charnock and Simonson, 1977). The results are shown in Figure 2-18.

There is a marked change in the fluidity of beef membranes at 26°C, thus one could reasonably expect two phases of ENZYME FUNCTION within the physiological temperature range.

Conversely, the crab enzyme does not show any change in its fluidity (below 32°C) and therefore one would only anticipate a single state of ENZYME FUNCTION through the normal physiological temperature range of this animal. Perhaps of even more significance, at any given temperature the "fluidity" of the crab enzyme is considerably LESS than that of the beef preparation.

This is born out by the comparison of the activation energies for crab $(Na^+ + K^+)$-ATPase and beef $(Na^+ + K^+)$-ATPase where the values for the crab system are less than those for the beef enzyme at any temperature which we examined. The results are shown in Table II.

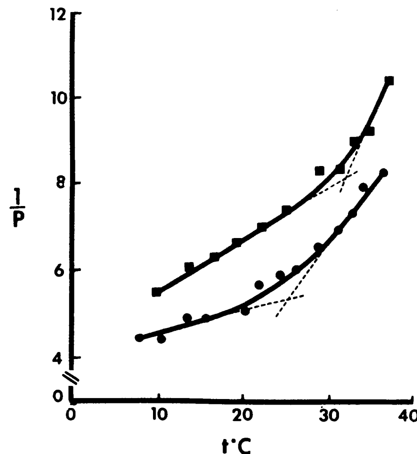


Figure 2-18: Fluorescence polarization of 12-AS labeled membrane preparations of NaK-ATPase. (■) crab nerve. (●) beef brain.

Table I: Fatty acid composition of beef and crab (Na^{+} + K^{+})-ATPase containing membrane preparations.

SATURATED FATTY ACIDS	% TOTAL LIPIDS	
	BEEF BRAIN	CRAB AXON
14^{0}	1.8	trace
15^{0}	trace	trace
16^{0}	21.7	10.4
17^{0}	0.3	0.4
18^{0}	19.6	11.0
20^{0}	0.2	3.3
22^{0}	0.2	6.0
24^{0}	1.1	1.5
total saturates	44.9	32.6
UNSATURATED FATTY ACIDS		
14^{1}	0.2	trace
15^{1}	trace	trace
16^{1}	1.8	1.5
17^{1}	0.1	0.2
18^{1}	19.6	15.8
$18^{2}\omega6$	0.8	0.9
$18^{3}\omega3$	0.1	0.9
20^{1}	trace	5.2
$20^{2}\omega6$	0.4	1.4
$20^{3}\omega3$	0.5	0.9
$20^{4}\omega6$	6.2	3.0
$20^{5}\omega3$	trace	21.2
21^{1}	1.1	trace
22^{1}	1.2	trace
$22^{4}\omega6$	3.1	1.6
$22^{5}\omega3$	1.1	0.2
$22^{6}\omega3$	12.7	13.4
24^{1}	2.2	trace
total unsaturates	51.1	66.2

However, one point that needs commenting upon is that in NEITHER of these systems is there direct correspondence between the transition temperature of the enzyme activity and the temperature at which the mobility of the fluorescent probe 12-AS was shown to undergo a major change. While there is some similarity to the beef system (Tc° 20° and 26°) there is none to the crab. Apparently we are not detecting coincident events within the membranes - clearly the fluorescent probe 12-AS is not measuring changes in lipid ordering which are reflected in changes in enzyme activity.

From this comparative study of beef brain and crab axon $(Na^+ + K^+)$-ATPase it is possible to believe that the pharmacological properties - that is the binding of drug to an enzyme receptor molecule - may be influenced by the lipids of the membrane matrix surrounding the enzyme receptor.

Of course, this does NOT exclude the possible more direct role of the enzyme receptor protein displaying different properties as well.

We have examined another ATPase system in detail, where the biological model provides for VARIABLE lipid composition of certain membranes throughout the year.

This is the situation encountered in hibernating mammals where summer feeding on grass and seeds coupled to a body temperature of 37°C, leads to a very different lipid composition of the tissues than that found during the deep torpor of hibernation at less than 5°C, where neither food nor water is consumed for periods of many months.

Hibernating Animals

The Richardson ground squirrel *Spirmophilus richardsonii* is a good example of this type of mammal, and is fortunately abundant on the plains of central Alberta. Figures 2-19 and 2-20.

Please remember that during torpor all the organs have greatly reduced function as the organ temperature itself is only a 1° - 2° above freezing point.

For example in this animal both respiration and heart beat slow enormously. Heart beat drops from 150-200 beats/min. in the summer to 1 beat every 2 or 3 minutes during torpor!

We removed the hearts from these animals and dissected out atrial and papillary muscle for pharmacological study, while using the much larger ventricles (>97% total wet weight) for biochemical examination of myocardial $(Na^+ + K^+)$-ATPase.

Table II: Arrhenius activation energies (Ea, in Kcal/mole) for beef and crab $(Na^+ + K^+)$-ATPase above and below the critical temperatures.

	Ea_I	Ea_{II}	T°C
BEEF BRAIN	18.1	37.0	20.0
CRAB AXON	12.5	28.5	16.2

Figure 2-19: Awake summer Richardson ground squirrel.

Figure 2-20: Winter hibernating Richardson ground squirrel in deep torpor.

Our first series of experiments were with the cardiac glycoside ouabain which as you know is the most water soluble of all the naturally occurring cardiac glycosides. However, its extremely slow 'binding constant' in whole heart preparations and its pseudo-irreversible action on isolated heart muscle preparations make these experiments both difficult and tedious.

It takes all day to elicit a maximal pharmacological response with low concentrations of the drug, and then one is unable to wash the drug out of the reaction system. Hence you can only determine the effect of one concentration of drug on one cardiac muscle preparation from each animal and the experimental design we were obliged to work with was quite unsatisfactory.

However, we did get some results which have recently been published (Charnock, Dryden, Lauzon and Skoog, 1980).

Fortunately, at about that time we were able to obtain a sample of a relatively new semi-synthetic cardiac glycoside manufactured by the Ayerst Pharmaceutical Co. in Montreal. This compound (Actodigin) was said to have all the qualitative pharmacological properties of the naturally occurring cardiac glycosides, but has a much shorter binding time in both man and experimental animals. It can be readily washed out of cardiac muscle preparations in vitro and is thus an excellent tool for the sort of studies we had attempted with ouabain. It's structure is shown in Figure 2-21: It is derived from digitoxigenin.

However, Actodigin is a very new compound so before we used it to study its possible inhibitory action on cardiac muscle function we thought it would be a good idea to check its inhibitory properties against myocardial NaK-ATPase.

We prepared the enzyme from the myocardium of the awake ground squirrel, the guinea pig and the rat as the literature describes these latter two species as cardiac glycoside sensitive and cardiac insensitive species respectively. The results are shown in Figures, 2-22, 2-23, 2-24 (Charnock, Dryden, Lauzon, 1980).

Clearly, Actodigin inhibits the myocardial NaK -ATPase of the ground squirrel, the guinea pig and the rat in the same order of potency that it affects the isometric twitch tension of the isolated atrial and papillary muscle from these same species.

We therefore felt that we had justified the use of Actodigin as both an inotropic agent and an inhibitor of cardiac NaK -ATPase.

With this agent I determined to reexamine my biological model of the hibernating ground squirrel where we had already shown:

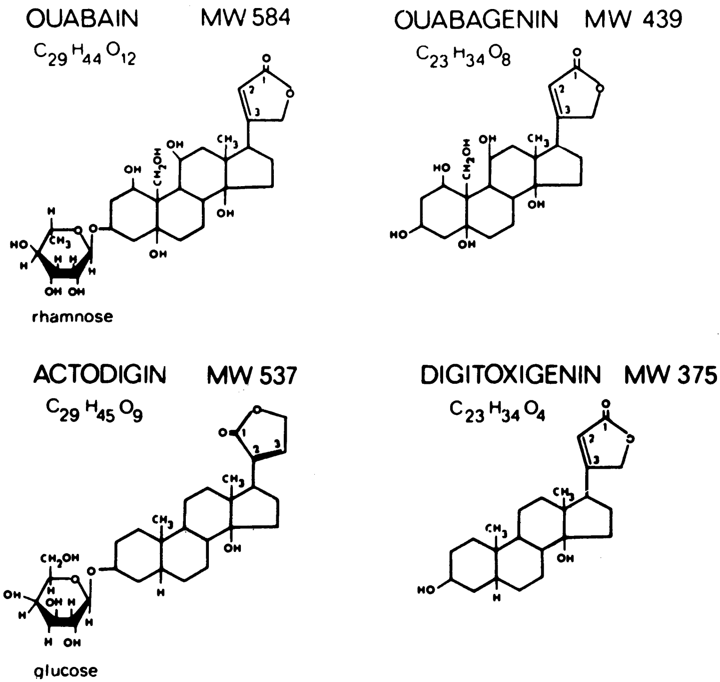


Figure 2-21: Chemical structures of actodigen and ouabain and the genins from which they were derived.

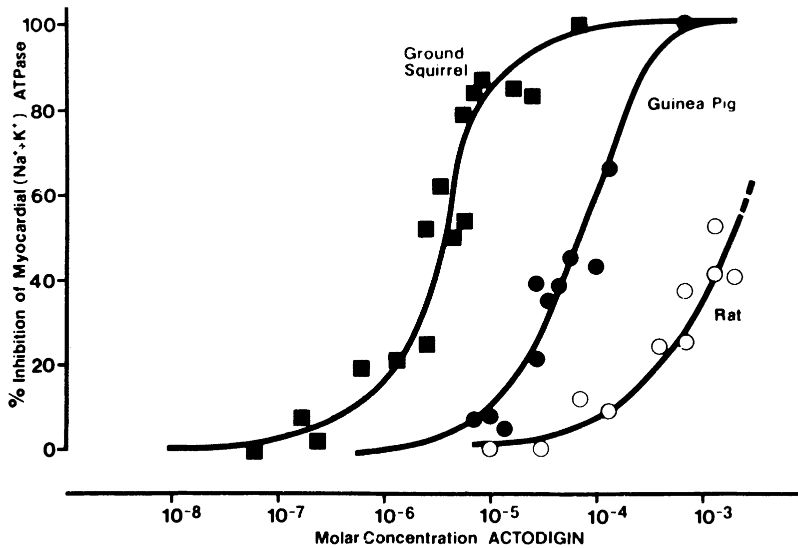


Figure 2-22: Species difference in sensitivity of myocardial (Na^+ + K^+)-ATPase to inhibition by actodigen.

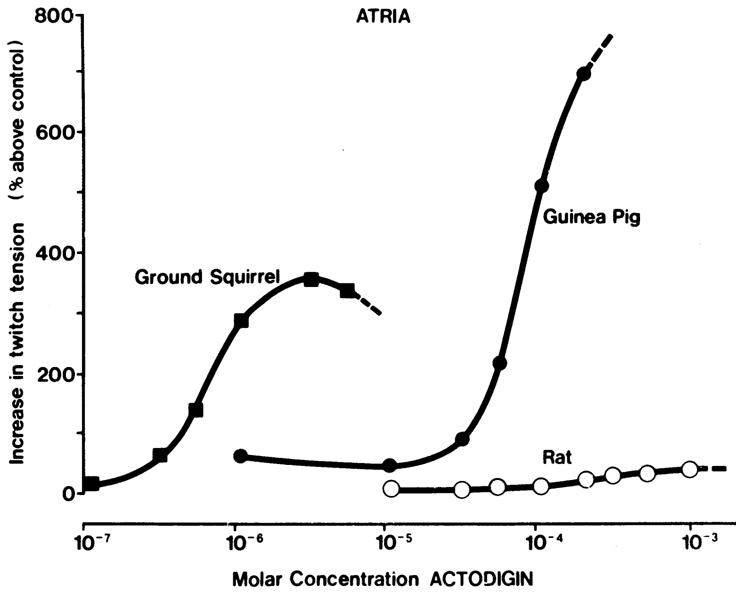


Figure 2-23: Species difference in positive inotropic action of actodigen on isolated atrial muscle.

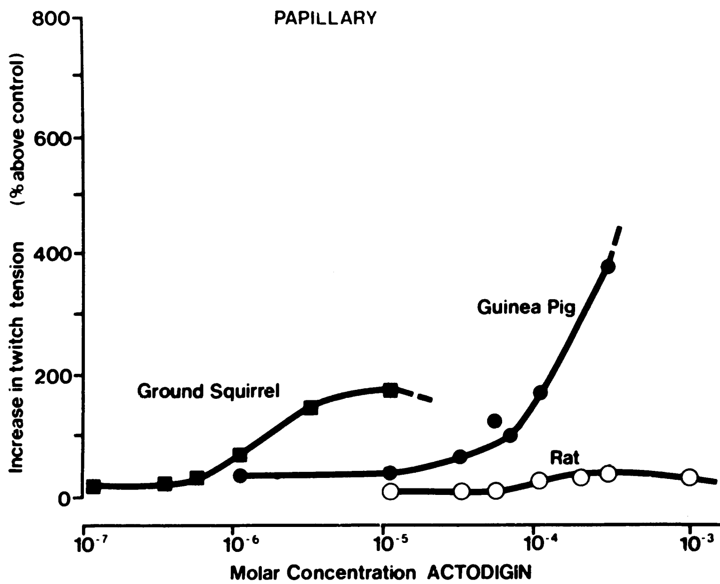


Figure 2-24: Species difference in positive inotropic action of actodigen on isolated papillary muscle.

1. Variable membrane composition with season (of Table 1)

2. Variable pharmacological response to ouabain with season (Charnock et al., 1980).

Seasonal variation in pharmacological response to Actodigen in the atrial muscle of the ground squirrel is shown in Figure 2-25.

Group I represents the response we see in muscles taken from the animal in summer when its body temperature is 37°C. The classical dose response curve is indistinguishable from that of other small laboratory homeotherms which are sensitive to cardiac glycosides.

As we go through the end of summer a most surprising thing happens. Although the body temperature remains at 37°C, and the animals are certainly not in hibernation, the magnitude of the pharmacological response (shown in Group II) begins to fall dramatically. Group III data were obtained from a group of muscles from animals that had been in hibernation for about 30 days; Group IV responses were obtained from animals in hibernation for more than 3 months.

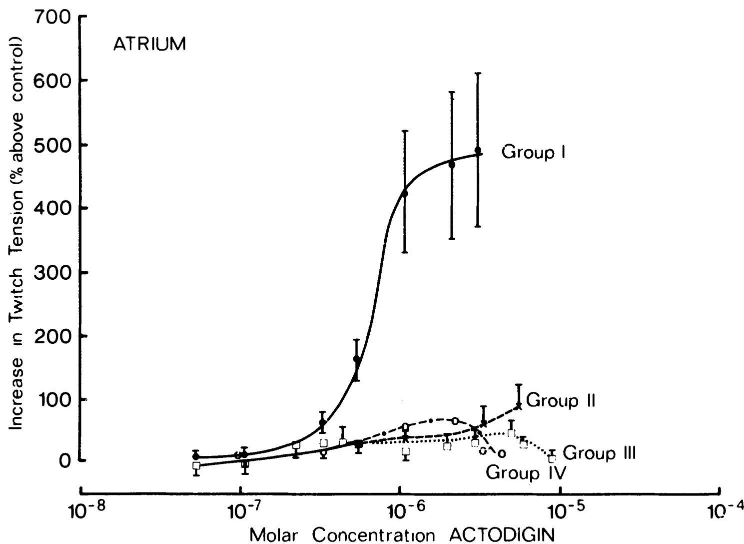


Figure 2-25: Seasonal variation in inotropic response of atrial muscle of the ground squirrel to graded concentrations of actodigen. Group I - Awake summer control animals. Group II - Immediately prior to hibernation. Group III - After one month in hibernation. Group IV - After three months in hibernation.

Clearly there is a marked loss in pharmacological response associated with hibernation.

Although not shown in Figure 2-25 we now have data showing that there is a gradual return of the pharmacological response when hibernation ceases - it is back to the summer "control" level in 5-6 weeks. Thus keeping the animals in captivity in the laboratory for 6 months which includes a period of hibernation - has not produced some irreversible change in the pharmacological or contractile properties of these cardiac muscles.

Figure 2-26 shows the complementary data of seasonal variation in the pharmacological response to actodigin in the papillary muscle.

The significant thing about this data is that the pharmacological change was found before the body temperature was lowered and persisted for a short time at least after the body temperature was raised when hibernation ceased.

So we now have a substantial seasonal variation in pharmacological properties to add to the seasonal change in membrane composition reported in Table 1. What happened to the myocardial NaK - ATPase during this time?

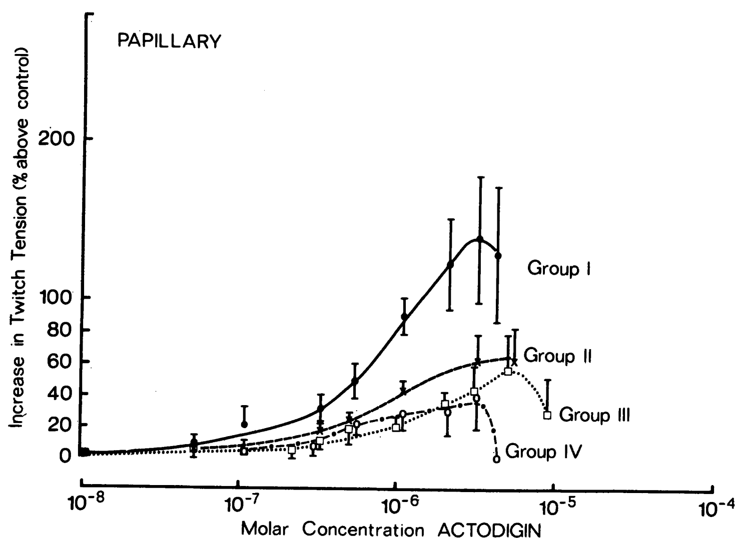


Figure 2-26: Seasonal variation in inotropic response of papillary muscle of the ground squirrel to graded doses of actodigen. Group I - Awake summer control animals. Group II - Immediately prior to hibernation. Group III - After one month in hibernation. Group IV - After three months in hibernation.

Those very features of ouabain, which make it difficult to work with in intact muscle preparations (relatively slow binding constant and pseudo irreversible reaction) make it much more suitable for studies of enzyme inhibition in vitro than is actodigen.

We therefore prepared myocardial ($Na^+ + K^+$)-ATPase from the ventricle muscle of these hearts and measured:

1. the specific activity of the preparations.
2. the amount of [^{3}H]-oubain bound to the ($Na^+ + K^+$)-ATPase preparations at equilibrium (i.e. the amount of ouabain binding sites/mg tissue).
3. the ratio of ouabain binding sites/catalytic unit of enzyme. (An empirical index first employed by Schwartz et al, 1974, 1975) to examine the "pharmacological properties" of the enzyme.

The results of our study are shown in Figure 2-27 (Charnock et al., 1980).

Here you can see that in Summer Animals (Group 1) the level of enzyme activity is relatively high as is the amount of ouabain binding, leading to a RATIO of 2.8 pmoles ouabain bound per catalytic unit of the enzyme.

This RATIO changes during both early hibernation (Group 11a) and late hibernation (Group 11b) where the enzyme activity falls as does the amount of ouabain bound, but the latter (ouabain binding) does not fall in parallel with the fall in enzyme activity (Charnock et al., 1980).

So there is some change in the relationship of the AMOUNT of [^{3}H]-oubain bound to the enzyme/catalytic unit. This is shown in Figure 2-28.

The increased amount of ouabain bound/catalytic unit of enzyme during HIBERNATION has led to a small but significant increase in SENSITIVITY of the enzyme i.e. it now takes a LOWER CONCENTRATION of ouabain to produce a 50% decrease in enzyme activity than it did in Summer, active, awake, animals.

So there has been some qualitative change in the behavior of myocardial ($Na^+ + K^+$)-ATPase during hibernation as well as the significant fall in the amount of enzyme.

Animals	Non-specific drug binding at equilibrium	ATP dependent [^{3}H]-ouabain binding Rate	Amount	Specific Activity	Ouabain bound / S.A.
Group I	0.91 ± 0.1	0.187 ± 0.029	7.45 ± 0.5	2.64	2.8
Group IIa	1.52 ± 0.2	0.095 ± 0.014	3.52 ± 0.3	0.81	4.3
Group IIb	1.34 ± 0.2	0.128 ± 0.02	5.3 ± 0.5	0.93	5.7
Group III	1.00 ± 0.2	0.204 ± 0.037	7.6 ± 0.5	2.38	3.2

Figure 2-27: Seasonal variation in specific activity and [^{3}H]-ouabain binding to myocardial membrane preparations of NaK-ATPase. Group I - Awake summer control animals. Group IIa - One month in hibernation. Group IIb - Three months in hibernation. Group III - Six weeks after hibernation ceased.

Group	Description	n	K_{I50}	P
I	Prehibernators	4	$4.7 \pm 0.5 \times 10^{-7}$M	--
IIa	'Short-term' hibernators	2	$2.3 \pm 0.3 \times 10^{-7}$M	**
IIb	'Long-term' hibernators	6	$2.2 \pm 0.3 \times 10^{-7}$M	**
III	Posthibernators	4	$3.0 \pm 0.3 \times 10^{-7}$M	n.s.

Figure 2-28: Seasonal variation in sensitivity (I_{50}) of myocardial ($Na^+ + K^+$)-ATPase to inhibition by ouabain. Group I - Awake summer control animals. Group IIa - One month in hibernation. Group IIb - Three months in hibernation. Group III - Six weeks after hibernation ceased.

Another way at looking at the function of the enzyme is by determining its activation energy. As you have seen before this is easily done by determining the effect of temperature on the initial velocity of the reaction and obtaining the Arrhenius relationship (slope) of log k_1 vs. 1/T. This is shown in Figure 2-29.

It is clear that the temperature:activity relationship of myocardial NaK^+-ATPase from awake, active, summer animals is typically non-linear.

In winter hibernation the specific activity of the myocardial enzyme falls (to 0.7 units from 212 units in this particular series) and the assays are much more difficult to perform.

However, we have no reason to believe that the plots are significantly different - and the temperature:activity relationship remains non-linear (Charnock et al. 1980).

However, the absence of any seasonal variation in the activation energy of myocardial NaK -ATPase does not exclude the possibility that some important changes occurred in the physical properties of these cardiac membranes during hibernation.

We therefore examined this possibility by subjecting these isolated membrane preparations to differential scanning calorimetry (DSC). The results are shown in Figure 2-30.

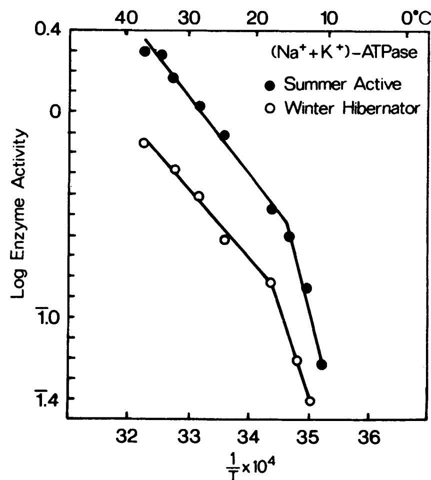


Figure 2-29: Arrhenius plots of the effect of temperature *in vitro* on myocardial $(Na^+ + K^+)$-ATPase of summer control and winter hibernating ground squirrels.

Myocardial membranes from awake active summer control animals show clear evidence of a single phase transition at 26°C. Those membranes from winter hibernating animals show a single transition commencing at 16°C i.e. 10 centigrade degrees lower.

By comparison of these exotherms with that of standard preparations of phospholipids we estimate that less than 2% of the total lipids of the membranes are involved in these phase transitions.

Only the transition at 16°C shows any correlation with myocardial NaK^+-ATPase temperature:activity relationships. This temperature also approximates 'cardiac arrest' temperature in man.

We then examined the properties of extracted lipids from these preparations by the technique of electron spin resonance. The results are shown in Figure 2-31.

Total lipid extracts of myocardial NaK -ATPase containing membranes were labeled with the nitroxyl stearic acid spin probe 16NS and the effect of temperature on probe motility determined.

Clearly plots of $\tau_o \times 10^{10}$ sec vs. 1/T° are nonlinear with points of inflection at 29°C in preparations from summer control animals and at 18°C for membranes from animals during hibernation.

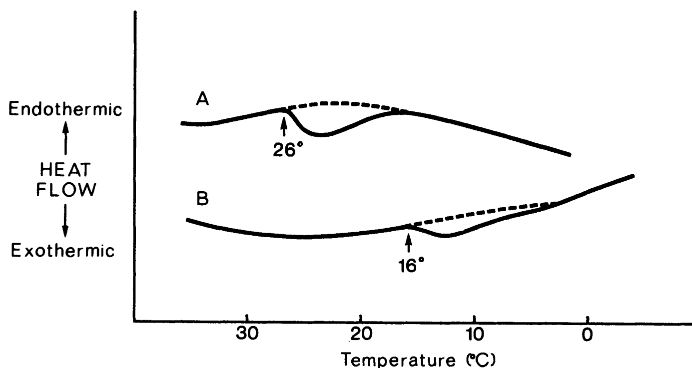


Figure 2-30: Differential scanning calorimetry traces of myocardial membranes from summer control and winter hibernating ground squirrels.

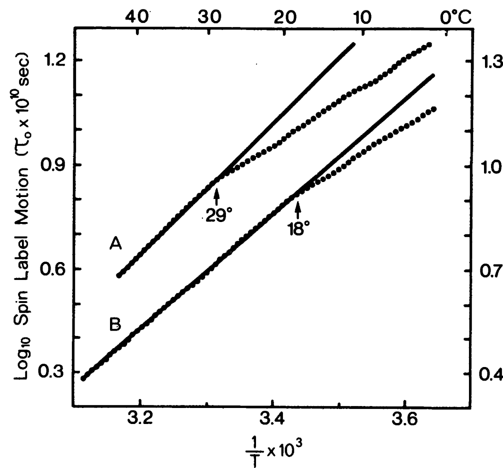


Figure 2-31: Effect of temperature on the molecular motion of 16NS intercalculated into lipid extracts of myocardial membranes from summer control (A) and winter hibernating (B) ground squirrels.

We think this is quite good agreement with the previous data we got from differential scanning calorimetry (DSC).

Thus there were changes in the molecular ordering of the spin probe in the lipid extracts, at temperatures very close to those at which exothermic transitions occurred in the whole membranes.

We know that the exotherms (phase transitions) in the whole membranes were lipid dependent phenomena as they were still present after heat denaturation of the membrane proteins.

We also know that examination of the respective $\tau_o \times 10^{10}$ sec. values at any given temperature show that the membranes from hibernating animals is in complete agreement with a lowering of the lipid phase transition T° by about 10°C.

Finally detailed lipid analysis of these preparations gives a relatively simple explanation for what has occurred. Table III shows that there is a major increase in the % unsaturation of the membrane lipids during hibernation where the total increased from 59% to 67% - the total saturates fell from 40% to 32%. Much of this increase in unsaturation could be accounted for in the 16 polyunsaturated fatty acid group which rose from 15% to 26% of the total.

Table III: Seasonal variation in the fatty acid and cholesterol composition of myocardial membranes* from summer control and winter hibernating ground squirrels.

	% Composition Total Lipids	
	Summer-awake	Winter-hibernating
Fatty Acids		
Total saturated	39.8	32.2
Total unsaturated	59.2	67.2
Total mono-unsaturated	37.5	38.6
Total $^{\omega}6$ poly-unsaturated	15.4	25.8
Total $^{\omega}3$ poly-unsaturated	6.3	2.8
Total unknowns	0.9	0.7
Cholesterol	5.3	4.6
Ratio Cholesterol: total $^{\omega}6$ poly-unsaturated fatty acids	2.9	5.6

* Results obtained from membrane preparations derived from the pooled tissues of at least three animals.

There was little change in the % cholesterol content. A more detailed examination of the % major fatty acids is given in Table IV.

There are some changes in the amount of 16° and 18° but the most important change is the large increase in 18^2 from 6% to 29% while both 22^1 and 22^5 appeared to fall.

In summary, lipid is involved in the binding of cardiac glycosides to myocardial NaK-ATPase.

In vitro - changing lipids by treatment with Phospholipase A will change enzyme characteristics.

In vivo - changing lipid composition of myocardial membranes during hibernation has only relatively small, but apparently

Table IV: Seasonal variation in major fatty acid composition of myocardial membranes from summer control and winter hibernating ground squirrels before and after activation of $(Na^+ + K^+)$-ATPase by treatment with lithium bromide.*

Fatty acid	Summer-Awake Animals		Winter-Hibernating Animals	
	Before treatment	After treatment	Before treatment	After treatment
14^0	2.0	1.1	0.4	0.3
16^0	23.6	22.1	15.0	13.9
18^0	18.0	16.2	20.4	19.5
16^1	2.0	2.5	1.1	1.0
18^1	17.5	21.6	20.1	20.0
18^2	5.7	6.0	24.8	28.7
20^4	6.2	5.9	9.3	8.9
22^1	8.7	10.9	2.2	1.2
22^5	4.1	3.4	0.4	0.5
22^6	3.1	2.1	1.3	1.7
Total	90.9	91.8	95.0	95.7

* Summer control animals sacrificed during July 1978; winter-hibernating animals sacrificed during February 1979. The membranes were prepared from the pooled tissues of at least five animals and examined before and after treatment with LiBr as described by the method of Charnock *et al.*

important effect on binding of cardiac glycosides to myocardial NaK-ATPase preparations. This is seen by changing ratios of

$$\frac{\text{Amount } ^3\text{H-ouabain bound}}{\text{Enzyme Specific Activity}}$$ which increases during hibernation.

The I_{50} is slightly decreased during hibernation i.e. the enzyme is more sensitive to drug inhibition.

There is a very significant loss of pharmacological response during hibernation which is probably due to the marked decrease in enzyme (drug-receptor) available.

There is a general increase in the fluidity of cardiac membranes during hibernation. However, only a small portion of the total membrane lipids are involved in the thermal phase transitions seen by DSC.

It now becomes possible to consider that a specialized layer of "boundary lipids" surrounding myocardial NaK-ATPase do <u>not</u>

undergo large changes in fluidity at this time but actually *insulate* the enzyme protein from the effects of changing lipid composition in the bulk phase of the membranes.

That is, their biological function may include the characteristic of maintaining a fairly constant lipid environment which is necessary for biological function of the enzyme or drug-receptor. One might think of this as "reverse modulation."

Obviously in the hibernation we are suggesting that the seasonal changes in membrane lipids are great but the boundary lipid acts to reduce the impact of this change on enzyme function.

If this were also found to be the case for several other important membrane-bound enzyme systems one might conclude that boundary lipids act by providing a constant lipid environment in the face of potential seasonal or nutritional variations.

EDITORIAL SUMMARY:

This chapter has focused on the biophysics of cell membranes including the movements of membrane lipids, the control of their dynamics and especially the interaction of membrane lipids with functions of membrane bound enzymes. Methods of measuring lipid interactions and the effects of lipid composition on enzyme function are considered in some detail, with comparisons of *in vitro* with *in vivo* data. The concept of the membrane fluidity was discussed in relation to different species of phopholipid and to the effect of varying concentrations of cholesterol. Seasonal variations in lipid composition of membranes were studied by Dr. Charnock and his associates in hibernating ground squirrels. Strikingly the changes in composition were found to occur prior to lowering of the body temperature in hibernation.

CHAPTER 3

REGULATION OF MEMBRANE FUNCTIONS

Membrane Adhesiveness and Cell Growth

DR. L. GLASER: For many years scientists have been interested in the investigation of specific cell adhesion, namely the ability of cells to specifically recognize the presence of other cells and most developmental embryologists believe that such recognition is one of the factors involved in the generation of an orderly arrangement of cells during development (Frazier and Glaser, 1979; Glaser, 1980). Cells contain on their surface a number of ligands which allow these cells to bind to molecules present on the surface of adjacent cells. For example, a number of cells have been shown to contain carbohydrate binding proteins (lectins) which will allow the cell to bind to any other cell which expresses these specific carbohydrates on the cell surface. These cells could also bind to any solid matrix synthesized in the laboratory which contains such ligands on its surface (Schnarr et al., 1978).

Many assays have been developed to try to measure the ability of cells to bind to other cells. These assays and the technical problems associated with them have been reviewed in detail recently and this presentation need not repeat this same material (Frazier and Glaser, 1979). It is clear, however, that many of these assays can measure laboratory curiosities rather than physiologically important events. That is, the presence on the cell surface of pairs of complementary molecules will allow them to bind to each other, but the developmental significance of such binding, detected under laboratory conditions is not always obvious. For example, the presence on the cell surface of a protein which can bind to carbohydrates will allow the cells to bind to any structure which contains these carbohydrates in an accessible form.

It therefore becomes of interest to study situations in which cell adhesion has physiological consequences to the cell, i.e. it alters cellular function.

The general strategy for studying such events would be to first recognize their existence and then to try to isolate the surface molecules which are involved in the recognition event. From the point of view of isolating these molecules, the most reasonable strategy is derived from the observation that cell surface membrane can be shown to retain some of the adhesive properties of the original cells (Merrell and Glaser, 1973; Santala et al., 1977). Therefore it seems reasonable to assume that plasma membranes when added to cells might mimic the physiological consequences of these adhesive events. If this can be shown to be the case, then one can assume that the relevant molecules are still present in the plasma membrane fraction in a functional form and should be isolatable from such membranes.

Two such systems have been investigated by us in detail. The first of these is contact inhibition of growth as exhibited, for example, by 3T3 cells and the second is the mitogenic effect of neurites or axonal membranes on Schwann cells. Both of these systems have been described in detail in other publications and we will briefly summarize these observations.

Contact Inhibition of of 3T3 Cells

A variety of fibroblasts when grown on tissue culture cease to grow at confluency even if the medium is changed repeatedly. Evidence from a number of sources indicates that cell contact is one, although certainly not the only component involved in this cessation of growth (Glaser, 1980; Whittenberger and Glaser, 1978; Bunge et al., 1979). We have shown that sparse and therefore logarithmically growing 3T3 cells will respond to the addition of homologous surface membranes by a cessation of growth (Whittenberger and Glaser, 1977; Whittenberger et al., 1979). The growth arrest observed upon addition of membrane to 3T3 cells is concentration dependent, is not due to a toxic effect of the membranes to the cells and is fully reversible, either by trypsinization and replating of the cells, or by the addition of higher concentrations of mitogenic factors. Addition of membranes arrests the cells early in the G1 phase of growth. By all of these criteria the arrest of the cells by the addition of membranes is identical to that observed at high cell density. In addition, the decrease in transport of a number of small molecules such as gamma-amino isobutyric acid and uridine which is observed at high cell density is also observed upon addition of membranes to cells (Lieberman et al., 1979).

While membranes represent relatively large structures which when binding to the cell surface may prevent the access of mitogenic component present in the growth medium to the cell, a series of experiments using epidermal growth factor have clearly established that at least for one such factor the membranes can block the mitogenic response without preventing access of the mitogen to the cell surface (Lieberman et al., 1980).

The next step in the identification of surface molecules involved in contact inhibition of growth, would be the extraction of these molecules from the membranes. This has been accomplished by the use of the detergent octylglucoside, a non-ionic detergent which can readily be removed from the preparations by dialysis thus avoiding toxicity to the cells (Whittenberger et al., 1978). A 10-20 fold purification of the proteins involved in contact inhibition of growth has been obtained. The proteins have been shown to be devoid of lipid and to be sensitive to denaturation either at high temperature or at low pH (Raben et al., 1980).

Work is currently in progress to further purify and characterize these proteins. One of the more interesting observations made with this partially purified material is a suggestion that the inhibition of transport of small molecules as γ-amino isobutyric acid and the arrest of the cells growth may be dissociable events and therefore be the consequence of binding of different ligands to the cell surface.

The second system that we wish to discuss is a system which is involved in regulation of the growth of Schwann cells. Work by Drs. P.M. Wood and R.P. Bunge has shown that dorsal root ganglia can be freed of fibroblasts and that proliferation of the Schwann cells in such cultures is dependent on the presence of neurons and neuronal processes (Wood and Bunge, 1975; Wood, 1976). In a detailed series of investigations by J.L. Salzer (Salzer and Bunge, 1980; Salzer et al., 1980; Salzer et al., 1980; Salzer et al., 1980), it has been possible to show that contact between Schwann cells and the axonal surface is required for the division of the Schwann cells. In addition, it has been possible to show that surface membrane fractions prepared from the axons derived from dorsal root ganglia neurons are mitogenic for Schwann cells and this mitogenic effect is highly specific. Of a variety of membranes tested only axolemma membranes prepared by the method of DeVries et al., 1979 seem to show similar mitogenic effects.

Regulation of the Growth of Schwann Cells

The biological significance of these observations is clear. The growth of the Schwann cell is restricted to the immediate vicinity of the axons with which they must interact, conversely their growth away from the axonal surface is prevented. The molecules on the surface of the axon that must interact with the Schwann cell appear to be proteins on the basis of their sensitivity to proteases as well as to heat denaturation.

The two systems which we have briefly summarized are complementary. In the one case the interaction between homologous cells results in the generation of a negative intracellular signal which brings about the cessation of cell growth. In the second case the interaction between heterologous cell surfaces, the surface of the Schwann cell and the axonal surface, generates a positive signal within the cell which allows cellular growth. At the present time our knowledge of the factors which regulate the growth of cells is very poor (Pardee et al., 1978; Scher et al., 1979) it is therefore not possible at the present time to present a better description at the molecular level of what we mean by positive and negative signals for cell growth. It is our hope that by purifying the cell surface components involved in these recognition events that we will be able to more closely define the events involved in the regulation of cell growth at least in the two systems which we have under intensive investigation in the laboratory at this time.

DR. KENT: Do you have any indication whether your 3T3 factor is a protein, and whether carbohydrate is important for its activity?

DR. L. GLASER: Dan Raben in my laboratory has succeeded in removing most, if not all, the lipid from the partially purified factor and it remains fully active. The factor is susceptible to denaturation by heat or extreme pH and thus behaves like a protein. Dr. M.A. Lieberman has shown that the factor is sensitive to inactivation by periodate, suggesting that carbohydrate may be important for its activity. Periodate, however, is a drastic reagent that may also modify amino acids.

DR. KENT: What is the experimental basis for assuming that the mitogenic signal on intact neurites is trypsin sensitive?

DR. L. GLASER: Dr. Salzer could prepare ganglia devoid of Schwann cells, the neurons in these ganglia extend neurites. Replating Schwann cells onto such preparations results in a mitogenic response, but if the ganglia have been pretreated with low doses of trypsin, then the mitogenic response is delayed by at least one day, during which one presumes the mitogenic proteins are regenerated on the neurite surface. The morphology of the ganglia during brief trypsin treatment is not altered.

DR. KENT: We have been pursuing studies along some lines that would be of interest to this group. The main area of research in our laboratory is directed toward answering the question, how is the synthesis of membranes, particularly membrane lipids, regulated? How do enzymes involved in lipid synthesis know how much of a particular lipid such as phosphatidylserine or phosphatidylcholine to put into a membrane? If the membrane is perturbed in some way which removes one of those lipids, can the enzymes respond to the deficiency in the membrane and direct the synthesis of more lipid? The model system in which we are studying this question is cultured embryonic muscle treated with phospholipase C.

Lipid Synthesis in Membranes

It was reported several years ago by Nameroff and co-workers (Nameroff et al., 1973), that if embryonic muscle cultures are treated with phospholipase C from _Clostridium perfringens_, myoblast fusion is prevented. We approached this system asking what effect, if any, does phospholipase C have on myoblast lipid composition and lipid metabolism. Initially we were a bit skeptical about whether the enzyme was active in the cultures and was degrading cellular lipids. After all, this enzyme is a toxin and is known to destroy membranes of other cells. How could the myoblasts survive such a drastic treatment? We, therefore, began an analysis of the effect of this enzyme on cellular lipid metabolism.

Figure 3-1 shows the reaction catalyzed by phospholipase C. The enzyme from _Clostridium perfringens_ prefers phosphatidylcholine as its substrate but it will also work fairly well with sphingomyelin. Phosphatidylethanolamine is a rather poor substrate and phosphatidylinositol and phosphatidylserine are not hydrolyzed by the enzyme from _C. perfringens_. Phospholipases C from other organisms have different substrate specificities and these other enzymes have not yet been found to inhibit myoblast fusion.

Phospholipase C from _C. perfringens_ prevents myoblast fusion but it allows the cells to line up as if they are recognizing and adhering to each other (Nameroff and Munar, 1976). The treatment does not prevent other aspects of myogenesis such as production of creatine kinase and myofibrils (Trotter and Nameroff, 1976).

The first question we asked was, is the phospholipase able to degrade any of the phospholipids of the cultured cells? (Kent, 1979). The muscle cells were cultured for a couple of days in the presence of ^{32}Pi to label the phospholipids, then chased in the presence of non-radioactive phosphate. Figure 3-2 shows the loss of the ^{32}P-labeled phospholipids from the cells.

Figure 3-1: Reaction catalyzed by phospholipase C from Clostridium perfringens.

The only lipid for which we observed a considerably enhanced rate of degradation is phosphatidylcholine. In 15 hours about half of the total ^{32}P-labeled phosphatidylcholine was degraded in the presence of phospholipase C. Did the phospholipase C-treated cells contain considerably less phosphatidylcholine than control cells? Not really. There was only a 5-10% reduction in total cellular phosphatidylcholine content after two days of phospholipase C treatment. We haven't done a statistical analysis to see if this reduction is significant, but presumably when plasma membranes from these cells are purified, a considerable reduction in phosphatidylcholine will be observed.

If phosphatidylcholine is degraded at an enhanced rate in phospholipase C-treated cells, and the total cellular level of phosphatidylcholine is not dramatically altered, then the rate of synthesis of this lipid must be considerably enhanced. When we measured incorporation of ^{32}Pi into total lipid in the absence and presence of phospholipase C, we did see a considerably enhanced rate of incorporation in the presence of the enzyme (Figure 3-3). When radioactivities of the individual phospholipid classes were analyzed after such an incubation, the vast majority of the increased phosphate was found in phosphatidylcholine. A similarly increased rate of incorporation was observed when [^3H] choline was the precursor (Sleight and Kent, 1980). Thus it appears that this is a model regulatory system when we can remove a lipid from the

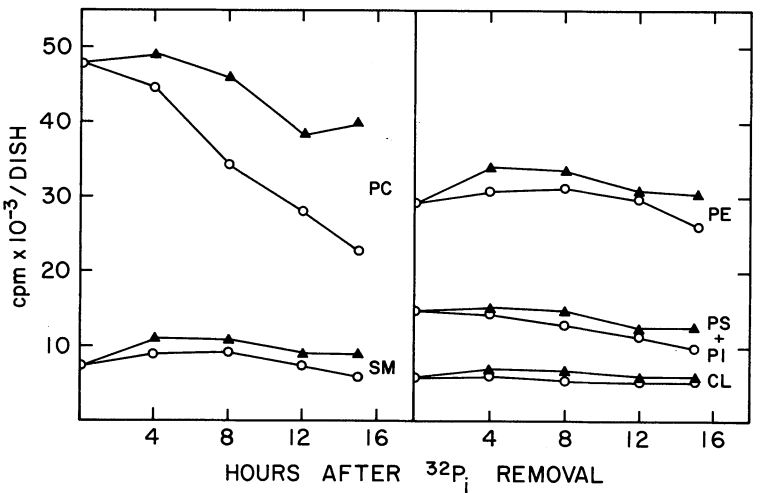


Figure 3-2: Degradation of phospholipids in the presence and absence of phospholipase C. All cells for this experiment were grown in the presence of phospholipase C. ^{32}Pi, 1.5μ Ci/ml, was present from the time of plating. Three days after plating the radioactive medium was removed, and the cells were washed and then incubated in nonradioactive medium in the presence of (●) or absence (▲) of phospholipase C.

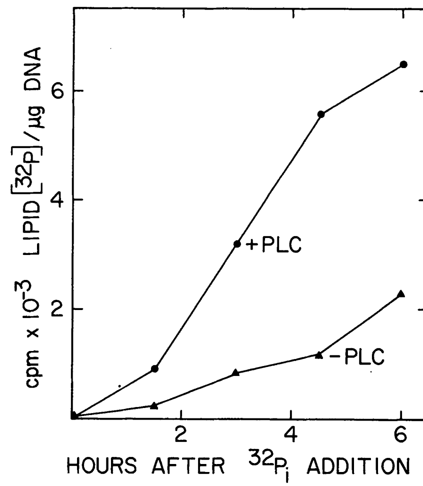


Figure 3-3: Effect of phospholipase C on ^{32}P incorporation into phospholipids. On the third day of culture, ^{32}Pi, 8µ Ci/ml, was added to cells that had been grown in the presence (●) or absence (▲) of phospholipase C. To normalize for variations in cell density, the total cpm in lipid from each dish was divided by the total micrograms of DNA from each dish.

membrane and the cells respond by putting more of that lipid back into the membrane. We have begun to ask, "What enzyme in the pathway of synthesis of phosphatidylcholine is sensitive to the condition of the cell membrane?

The Regulation of Choline Incorporation

When crude extracts of cells grown in the presence and absence of phospholipase C were assayed for activities of the enzymes involved in incorporation of choline into phosphatidylcholine, the only activity that was significantly increased was that of the CTP: phosphocholine cytidylyltransferase (Table 1) (Sleight and Kent, 1980). The activity of this enzyme was enhanced three-fold over that in control cells. We saw slightly increased activity in some of the enzymes responsible for incorporation of glycerol into phosphatidylcholine, but the increase was only about 20 percent. The enhanced activity of the cytidylyltransferase suggests that this enzyme might be regulating incorporation of choline in these cells.

DR. ENGEL: How long does it take to get these changes?

DR. KENT: The data in Table 1 were obtained with cells that were maintained in the presence of phospholipase C for two days, but increased activity of cytidylyltransferase is observed within three hours after addition of phospholipase C. We haven't looked at earlier times.

If the cytidylyltransferase is regulating choline flux in these cells, we should be able to see differences in the levels of the substrates for this enzyme. Table 2 shows an analysis of the metabolites in phosphatidylcholine synthesis. There is a considerable decrease in phosphocholine in phospholipase C-treated cells, and an increase in CDP-choline. One rule of enzyme regulation holds that if levels of the substrate for a non-equilibrium reaction change in a direction opposite to the pathway flux, then that reaction is regulating the pathway. It can be calculated from these data that the cytidylyltransferase is not at equilibrium in these cells (Sleight and Kent, 1980). So the cytidylyltransferase satisfies the criteria of this rule. We can conclude, therefore, that the cytidylyltransferase is regulatory for choline incorporation.

The next question that arises is, how does the cytidylyltransferase know what is happening in the membrane? We have obtained some evidence along these lines that suggest a working model for how the cytidylyltransferase can sense the condition of the membrane. Table 3 shows two important properties of the cytidylyltransferase. First, the enzyme activity is stimulated by phospholipids. The data presented in Table 1, where we saw a three-fold increased cytidylyltransferase activity in the cells grown in the presence of phospholipase C, were obtained in the absence of lipids. When

TABLE 1

Enzymes OF Phospholipid Synthesis in Homogenates

	-PLC	+PLC
	nmol/min/mg	Protein
Choline kinase	1.00	0.89
Cytidylyltransferase	0.37	1.03
Choline phosphotransferase	2.6	2.6
Glycerol-3-P dehydrogenase	5.7	7.7
Glycerol-3-P acyltransferase	1.0	1.2
Acyl G-3-P acyltransferase	0.83	0.78
Phosphatidic acid phosphatase	38.2	48.9

TABLE 2

Levels of Metabolites of the Pathway For Phosphatidylcholine Synthesis

	-PLC	+PLC
	nmol/10^{-7}	Nuclei
Choline	1.0	1.0
Phosphocholine	34.1	13.0
CDP-Choline	0.25	0.63
Phosphatidylcholine	90	88
ATP	52.2	54.3
CTP	15.0	15.2
Diacylglycerol	4.8	9.1

TABLE 3

Activity of Cytodylyltransferase in Cytosolic and Particulate Fractions (Assayed in the Presence of Lipids).

	-PLC	+PLC
	nmol/min/mg	Protein
Homogenate	0.39 (1.05)	1.09 (1.14)
Cytosol	0.34 (2.27)	0.21 (1.31)
Particulate	0.46 (0.43)	1.71 (1.72)

the crude homogenates are assayed in the presence of exogenous lipid, the same activity is obtained in phospholipase C-treated and control cells. That is, there appears to be the same total amount of enzyme in the two cells, but more of it is in an active form in the phospholipase C-treated cells. Lipids that stimulate cytidylyltransferase activity include phosphatidylethanolamine, phosphatidylserine, and a total chick lipid extract. Phosphatidylcholine and diacylglycerol do not appreciably stimulate enzyme activity. The inability of phosphatidylcholine to stimulate is consistant with a regulatory role for lipid stimulation, in that if there is enough of the end product of the pathway, then the rate-limiting enzyme should be inactive. On the other hand, if the enzyme is exposed to excesses of other phospholipids, as it would be if it were exposed to a membrane deficient in phosphatidylcholine, it would be activated.

In conclusion, we would like to postulate that the cytidylyltransferase activity can be governed by the concentration of phospholipids in the membrane. According to our working model, the cytidylyltransferase exists in the cytosol in an inactive form, and when it is exposed to membranes with sufficient phosphatidylcholine, it remains inactive. When the enzyme is exposed to a membrane that is deficient in phosphatidylcholine, it becomes attached to the membrane and is activated. Thus, since the cytidylyltransferase is rate-limiting, phosphatidylcholine synthesis is stimulated. We are now trying to purify the enzyme and study the effect of lipids on the pure enzyme. In addition, we are setting up other systems in culture where cells can be forced to make membranes deficient in phosphatidylcholine.

DR. L. GLASER: What happens if you mix small quantities of phosphatidylcholine with phosphatidylethanolamine?

DR. KENT: You don't lose activity until you have a fairly high percent phosphatidylcholine. As the percent phosphatidylcholine is increased, the activity remains high up to about 60% phosphatidylcholine, then it falls off sharply. We don't want to be too quick to interpret these results because the enzyme is not clean and because the different lipids may not be forming the same structures when dispersed in water.

DR. BRANTON: What do you mean by 100% phosphatidylethanolamine? What form do you suppose it is in?

DR. KENT: That is the problem I mentioned. One hundred percent phosphatidylcholine forms liposomes and 100% phosphatidylethanolamine should form a hexagonal phase.

DR. de KRUIJFF: The phospholipase C is probably producing a deficiency in the plasma membranes but phosphatidylcholine is being synthesized in the endoplasmic reticulum. How do you go from a deficiency in the plasma membrane to a deficiency in the endoplasmic reticulum?

DR. KENT: The only answer I can give at this time is that the cytidylyltransferase does not have to be in the endoplasmic reticulum. We could envision that the production of diacylglycerol in the plasma membrane induces a rapid equilibration across that membrane (Sundler et al., 1978), so that the deficiency in phosphatidylcholine is also manifested on the cytoplasmic surface. The cytidylyltransferase could bind to the plasma membrane and its product, CDP-choline, would diffuse to the choline phosphotransferase in the endoplasmic reticulum.

DR. STRITTMATTER: Have you studied this in non-fusing myoblasts?

DR. KENT: We have done experiments with cells that were in culture for only one day. When phospholipase C is added to these cells, incorporation of choline is enhanced within thirty minutes. At three hours after addition of the enzyme, the cytidylyltransferase is activated and more of it is found in the particulate fraction.

DR. SANDRA: Could you comment on the purity of the phospholipase C?

DR. KENT: We see these effects with enzyme that is at least 95% pure by gel electrophoresis. This brings up a problem with respect to studying myoblast fusion. I'm not yet convinced that the inhibition of fusion is due to the phospholipase C activity. The possibility remains that inhibition of fusion is due to a minor contaminant in the enzyme preparation.

DR. WILLNER: Is the soluble cytidylyltransferase a different enzyme than the one that is membrane-bound?

DR. KENT: We haven't been able to test that rigorously because we don't have an antibody to the enzyme. In the liver system, the cytosolic and microsomal enzymes have been shown to be the same antigenically (Choy et al., 1978). In our system, we can see the redistribution of activity in cells that are treated with cycloheximide to prevent protein synthesis, which suggests that the enzymes in the two compartments are the same.

DR. STRITTMATTER: A number of papers from Dr. Julius Axelrod's laboratory in the last few years have demonstrated the enzymatic synthesis of membrane phospholipids and the interaction between the enzymatic pathway and hormone receptors. I would like to comment on the synthesis of phosphatidylcholine by the sequential transmethylation of phosphatidylethanolamine. I will give one example, in the β-adrenergic receptor system, of the interaction between this enzymatic methylation and membrane receptors. The overall scheme of phospholipid transmethylation is shown in Figure 3-4. Phosphatidylcholine is predominantly found on the outside portion of the bilayer and can be synthesized by several different routes. This figure shows the sequential transmethylation of the polar head group of phosphatidylethanolamine to phosphatidylcholine (Hirata et al. 1978). This enzymatic transmethylation is mediated by two enzymes which are themselves asymmetrically located in the cell membrane (Hirata, F. and Axelrod, J., 1978). The first enzyme, methyltransferase 1, is found on the inner portion of the bilayer and catalyzes a methyl group transfer from the donor S-adenosyl-methionine to form phosphatidyl N-Monomethylethanolamine. This enzyme has a high affinity for S-adenosyl-methionine (K_m 2 μM) and requires Mg^{++}. This monomethyl derivative is the substance for the second enzyme, methyltransferase 11, located on the outer portion of the bilayer, which transfers two more methyl groups to form phosphatidylcholine. Because these enzymes are asymmetrically located in the cell membrane they are involved in the asymmetric distribution of the phospholipids by converting phosphatidylethanolamine on the inner portion of the bilayer, to phosphatidylcholine on the outer portion of the bilayer (Figure 3-5).

Membrane Receptors and Transmethylation

The methylation of phospholipids is regulated by the β-adrenergic receptor (Hirata et al., 1979). Incubation of rat reticulocytes with increasing concentrations of isoproterenol increased the amount of methlyation in a dose-dependent manner. In a series of β-adrenergic agonists, the ability of the agonists to stimulate methylation closely matched their ability to bind the β-adrenergic receptor (Figure 3-6a). The addition of GTP enhanced the ability of isoproterenol to stimulate methylation, similar to its effect of adenylate cyclase (Figure 3-6b). Finally, the ability of isoproterenol to stimulate methylation could be blocked by the β-adrenergic antagonist propranolol, but not by the α-adrenergic agonist phentolamine (Figure 3-6c). These data suggest that β-adrenergic receptor agonists stimulate methylation. The next question was whether this stimulation was mediated by the activation of adenylate cyclase and synthesis of cAMP, or was due directly to agonist occupancy of the receptor site. Direct stimulation of adenylate cyclase by fluoride or cholera toxin, or the addition of dibutyl cyclic AMP, did not stimulate methlyation. It

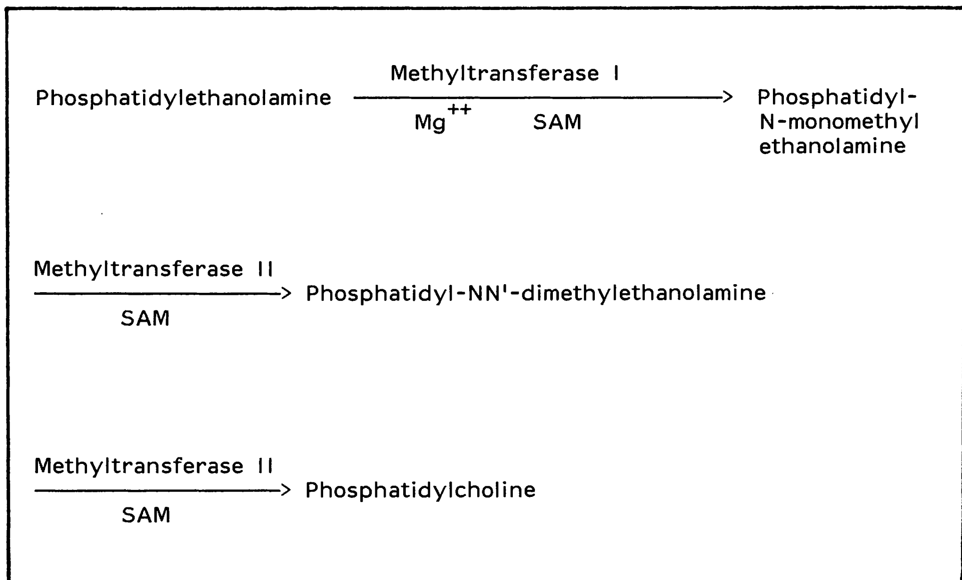


Figure 3-4: Enzymatic methylation of phosphatidylethanolamine to phosphatidylcholine.

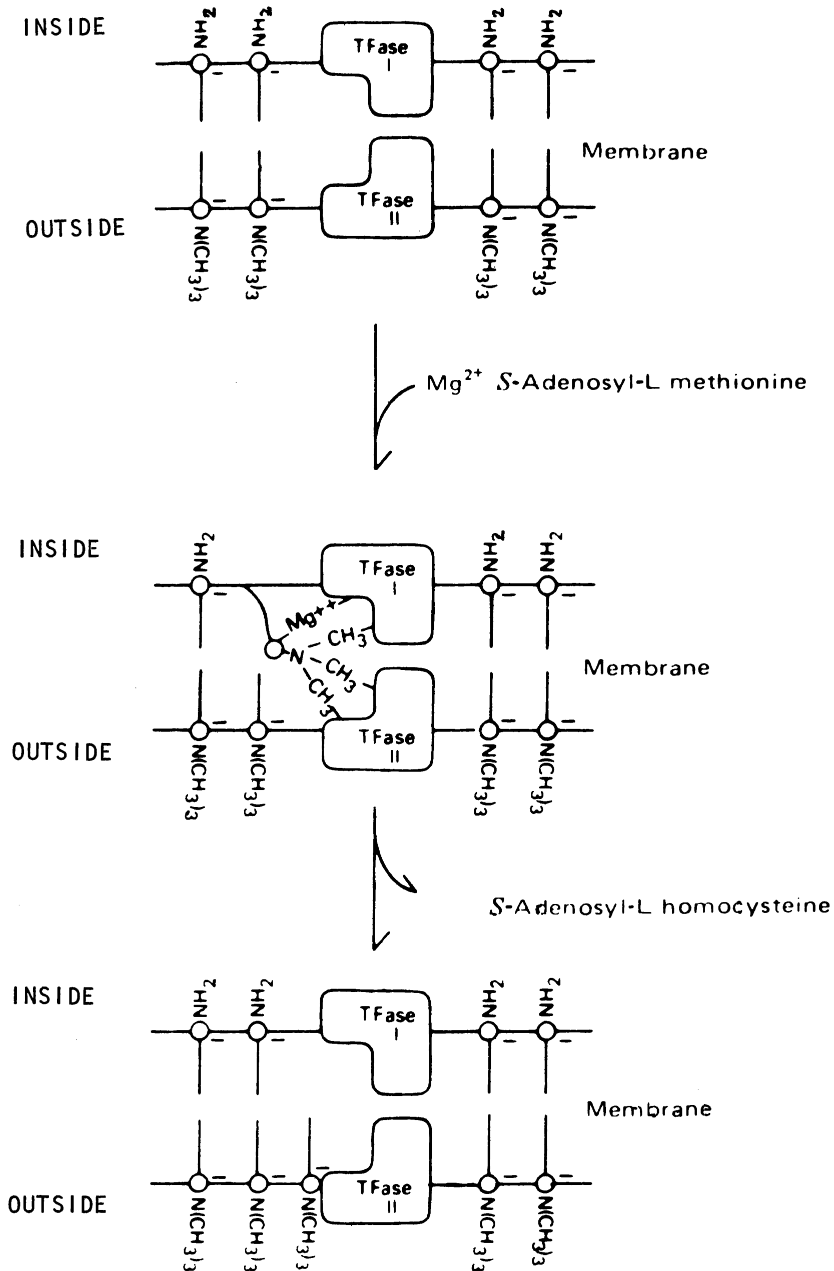


Figure 3-5: Mechanism of enzyme facilitated translocation of phospholipids (Hirata and Axelrod, 1978).

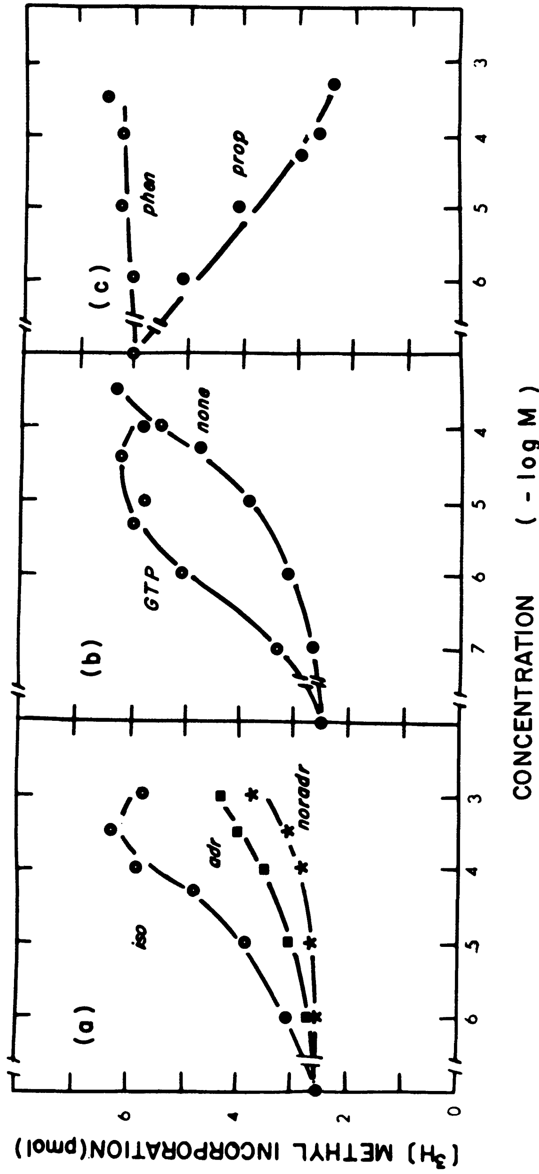


Figure 3-6: Effects of adrenergic agonists and antagonists on phospholipid methylation. (A) Effects of adrenergic agonists. (B) Effect of GTP on the stimulation of phospholipid methylation by iso proterenol. (C) Effect of adrenergic antagonists on isoproterenol stimulated phospholipid methylation (Hirata et al., 1979).

appears that agonist occupancy of the β-adrenergic receptor site itself enhances phospholipid methylation.

Relationship of Methylation to Membrane Fluidity

Dr. Fusao Hirata had previously shown that membrane fluidity was increased in association with the synthesis of phosphatidyl-N-monomethylethanolamine (Hirata F. and Axelrod, J., 1978). Because this methylation reaction increases membrane fluidity, we examined whether β-adrenergic receptor-adenylate cyclase coupling might be enhanced by lipid methylation. Rat reticulocyte ghosts were incubated in the presence of varying concentrations of S-adenosyl-methionine, challenged with isoproterenol, and the accumulated cAMP measured (Figure 3-7). More cAMP appeared in the presence of S-adenosyl-methionine, and paralleled the appearance of phosphatidyl-N-monomethylethanolamine. In summary, it appears that agonist stimulation of the β-adrenergic receptor stimulates the methylation of membrane phospholipids, which in turn increases membrane fluidity in the vicinity of the receptor, and thereby enhances coupling between the β-adrenergic receptor with adenylate cyclase (Figure 3-8).

We have also examined the regulation of the β-adrenergic receptor by lipid methylation, and have found, in reticulocytes, that the number of β-adrenergic receptor sites increases with the gradual accumulation of phosphatidylcholine. The mechanism of this effect is not known but may involve "unmasking" of cryptic receptors by changes in the membrane environment in the region surrounding the receptor (Strittmatter et al., 1979). Similar change in β-adrenergic receptor number occur when phospholipid hydrolysis by phospholipase is pharmacologically manipulated (Mallorga et al., 1980).

In summary, the phospholipid methyltransferases appear regulated by the β-adrenergic receptor-adenylate cyclase coupling. The methylation of membrane phospholipids appears to alter the membrane environment in the region of the receptor and appears to be involved in the regulation of receptor binding sites.

DR. de KRUIJFF: I noticed in one of the X's where you have methylation that there is maximal incorporation of methyl groups of six picomoles. Could you give a little bit of indication of how much of the PE is involved in your incubation mixture. How much of your PE is methylated.

DR. STRITTMATTER: Quantitatively, the amount of lipid synthesized by this pathway is very small. If you compare the synthesis of phosphatidylcholine by transmethylation compared to the synthesis of either CDP or base exchange pathways, it is in the neighborhood of 1/50. But it appears to be tightly regulated

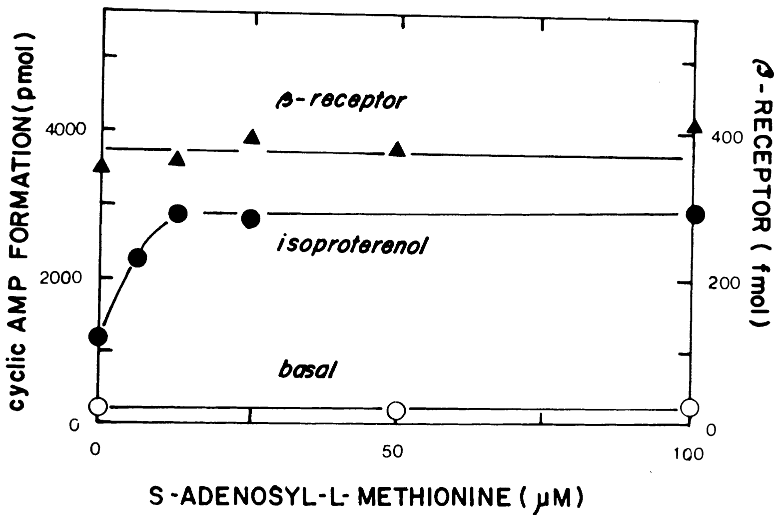


Figure 3-7: Effect of phospholipid methylation on coupling of β-adrenergic receptor with adenylate cyclase. Reticulocyte ghosts were prepared containing various concentrations of S-adenosylmethionione, 10 mM ATP and 1 mM GTP. Hormone sensitive adenylate cyclase was measured in the absence (O) or presence (●) of 500 μM isoproterenol (Hirata et al., 1979).

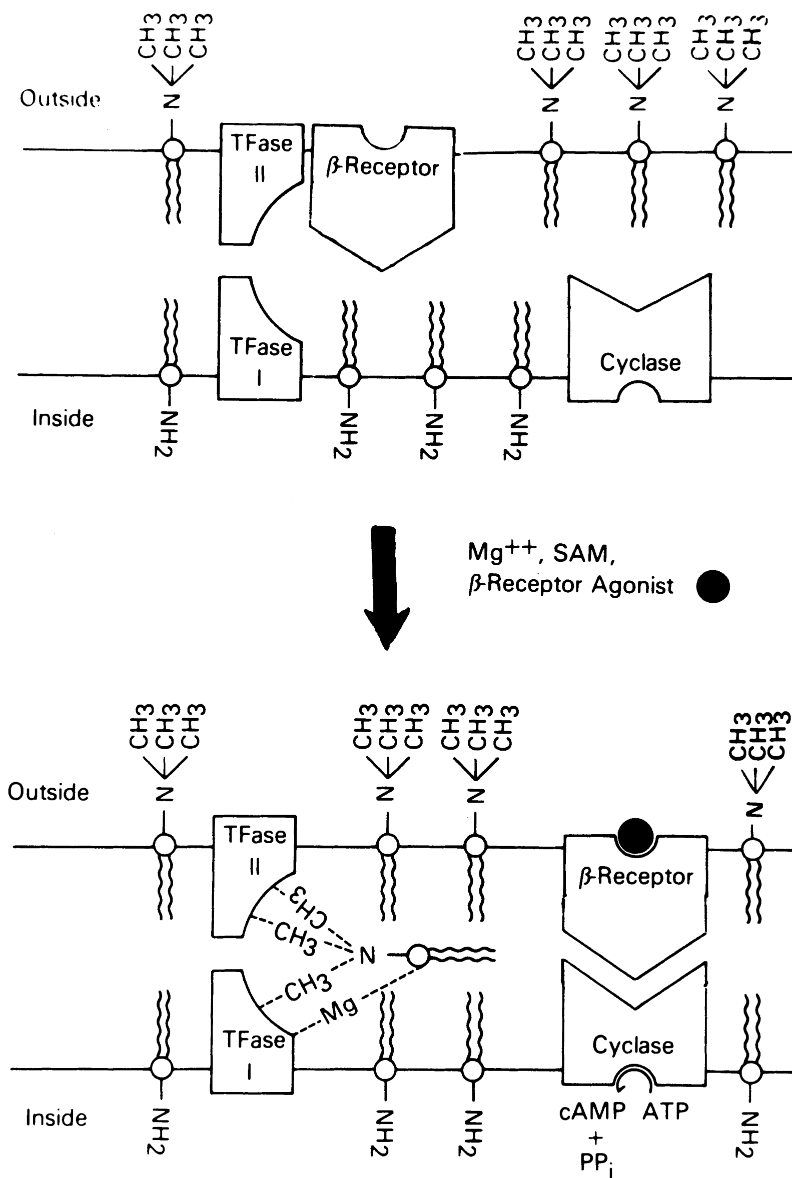


Figure 3-8: Proposed mechanism of enhanced β-adrenergic receptor-adenylate cyclase coupling by isoproterenol stimulation of phospholipid methylation (Hirata et al., 1979).

by a variety of different receptors and therefore may have an effect on membrane function out of proportion to its simple bulk concentration.

DR. de KRUIJFF: We did some calculations on your data and that of the Axelrod group and found only a 1000th of a percent of PE in the erythrocyte associated with a two-fold increase in viscosity. Therefore, I was interested in the work of Michael Glaser with huge changes in the polar head groups.

DR. STRITTMATTER: It may not be the bulk concentration but rather the location of the phosphatidyl monomethylethanolamine molecules in the matrix. We have data demonstrating that this derivative is not accessible to phospholipases applied to either the outside or inside of the membrane, suggesting that it is somehow in transit within the bilayer.

DR. de KRUIJFF: I know the enzyme in the lipid erythrocyte is about 1000th percent. In the endoplasmic reticulum retained reticulocytes the enzyme concentration is higher. Therefore when you see a very low activity in the cell can you exclude the possibility that the preparation has been contaminated with reticulocytes. Since the amount of activity you see in those studies is extremely small, one has to be careful in assigning a localization of the enzyme to the membrane.

DR. STRITTMATTER: The level of methylation occurring by this pathway is about two times higher in the reticulocyte than it is in the mature erythrocyte.

DR. WILLNER: Do you find any changes in activity in this enzyme if you change coupling so as to expose cells for a long time to catecholamine agonists when you have a refractory adenylate cyclase?

DR. STRITTMATTER: We haven't looked.

Acetycholine Receptors

DR. GONZALEZ-ROS: Rather than give you the details of all the experiments we have recently carried out (Gonzalez-Ros et al., 1980 a and b), I am going to summarize them very briefly, and more interesting, I will discuss some of the projects that we have going on right now.

As you know the acetylcholine receptor (AcChR) is an intrinsic membrane protein located at the postsynaptic face of the neuromuscular junction and in the electric organs of certain fish. It is a rather large protein, with a molecular weight of about 270,000 (Martinez-Carrion et al., 1975), which is composed of four different

polypeptide chains (40,000, 50,000, 60,000 and 65,000 molecular weights) known as α, β, γ and δ subunits.

Figure 3-9 very schematically indicates the two fundamental processes which occur during postsynaptic depolarization. First, there is neurotransmitter binding, acetylcholine being the naturally occurring effector, and afterwards, ions are rapidly translocated through the postsynaptic membrane affecting depolarization. One of the key issues in the receptor field has been to establish the possible role(s) of the AcChR protein in those two fundamental processes. In this regard, different affinity labeling techniques (Weill et al., 1974; Damle et al., 1978; Moore & Raftery, 1979) demonstrated the direct responsibility of the receptor protein for neurotransmitter binding with the effector binding site(s) located within the a subunit. On the other hand, neither the identity of the membrane components involved in ion translocation, nor the role of the AcChR protein in such events were established by that time. Since most functional aspects of AcChR, especially ion translocation, are typically membrane phenomena, reconstitution experiments involving the reintegration of the purified receptor protein into a lipid membrane is a very reasonable approach to the study of the molecular aspects of receptor function. In the case of AcChR, it should be possible to combine separate pools of purified AcChR and total lipids and reconstitute membrane vesicles that should exhibit known properties of native AcChR-enriched membranes related to (1) cholinergic ligand building, (2) AcChR-membrane "desensitization" and (3) ion translocation events. Nevertheless, previous attempts to reconstitute AcChR membranes did not demonstrate all those known functional features, whether purified (Michaelson & Raftery, 1974; McNamee et al., 1975; Hazelbauer & Changeux, 1979) or partially purified (Briley & Changeux, 1978; Schiebler & Hucho, 1978; Epstein & Racker, 1978; Wu & Raftery, 1979) AcChR was used. Evidence for the direct involvement of purified AcChR protein in ion translocation has become available only recenlty (Gonzalez-Ros et al., 1980a). Our experimental design allows for reincorporation of purified AcChR protein into Torpedo electroplax lipid vesicles which exhibit most functional properties of native AcChR-enriched membranes.

The procedure we have followed to achieve AcChR reconstitution (Figure 3-10) is based on the detergent dialysis method first used by Riker's group at Cornell. As indicated in Figure 3-10, we purified the AcChR protein by affinity chromatography on α-cobratoxin-Sepharose 4B. Prior to the affinity chromatography step, solubilization of the electroplax membranes is achieved by using a non-ionic detergent, β-D-octyl glucoside, which has been recently introduced in the membrane field as Dr. Glaser stated before. This detergent has a number of properties that are of interest to us, i.e. it is completely transparent as to spectrophotometric or spectrofluoro-

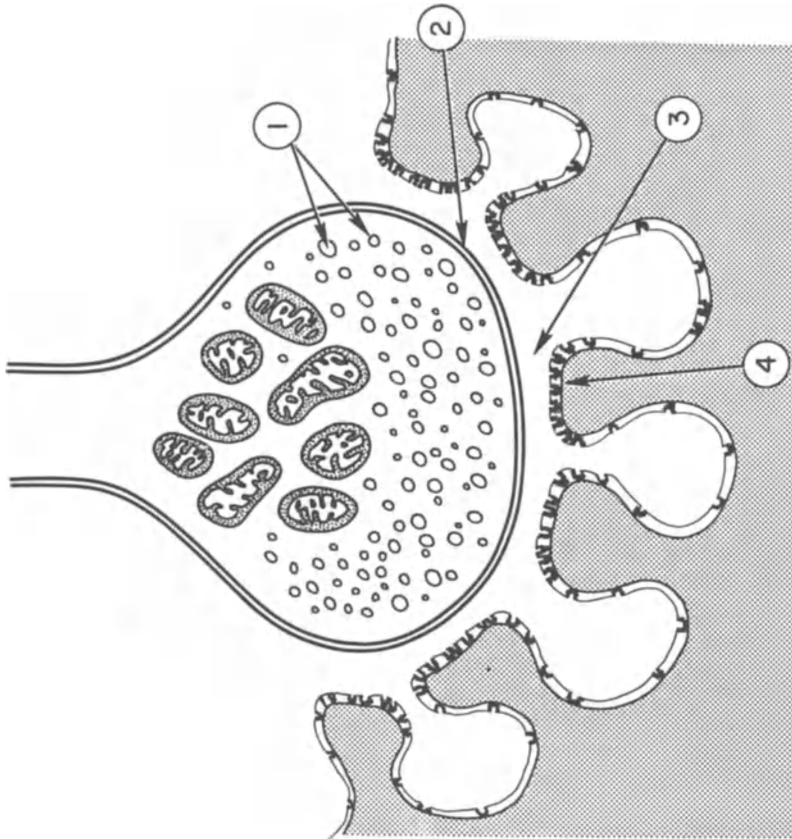


Figure 3-9: Schematic representation of the neuromuscular junction. (1) Presynaptic vesicles containing acetycholine, (2) Presynaptic membrane, (3) Synaptic cleft, (4) Folds in the post-synaptic membrane of the muscle where AcChR is concentrated at the evaginations.

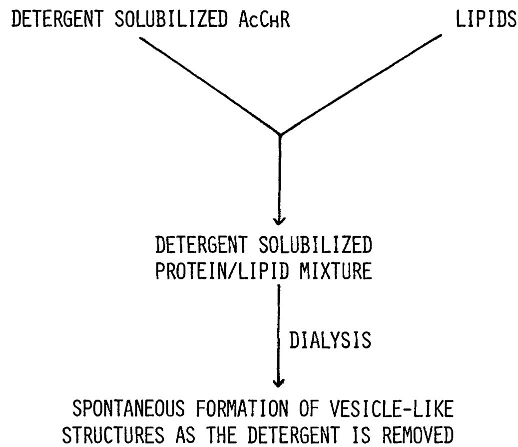


Figure 3-10: Fundamental processes during postsynaptic depolarization at the neuromuscular junction.

metric methods and has a very high critical micelle concentration which facilitates its easy removal by dialyzing out the solubilized preparations. Simultaneously, total lipids are extracted from the electroplax tissue and added at a given lipid to protein ratio to the purified AcChR protein, being the mixture solubilized to clarity by increasing the octylglucoside concentration in the reconstitution medium. Removal of the detergent from the lipid/protein solubilized mixtures results in the spontaneous formation of vesicle-like structures. The vesicular material is further separated from unreconstituted soluble AcChR by sucrose density gradient centrifugation.

The criteria that we have used to establish the sealed vesicular nature of the reconsituted sample are: osmotic activity, loading and retention of ^{22}Na, and electron microscopic examination. The osmotic activity was monitored by light scattering upon dilution of the vesicles with hypotonic and hypertonic buffers. Unlike free AcChR solutions, light scattering of the reconstituted preparation dramatically increased upon dilution in 1 M NaCl. In addition, the vesicles could be loaded with ^{22}Na. The permeability coefficient for ^{22}Na in the vesicles appeared to be low compared to native (freshly isolated) AcChR-enriched vesicles because longer periods of time were required for equilibration.

Actually, quasi-linear increases in ^{22}Na uptake were detected in the first 72 hrs. for the reconstituted vesicles. In agreement with these results, the retention of ^{22}Na in efflux assays was higher in the reconstituted vesicles than in the native vesicles. In other words, the reconstituted preparation was less "leaky" to Na^+ than native AcChR-enriched vesicles. Upon addition of octyl-glucoside at concentrations below the critical micelle concentration, or upon preincubation with gramacidin A, the amount of retained ^{22}Na was dramatically reduced. Finally, the vesicular nature of the reconstituted samples was explored by electron microscopy after negative staining. The micrographs reveal a heterogeneous population of vesicle-like structures with an average diameter of approximately 2500 Å similar to those obtained with native <u>Torpedo</u> membrane vesicles.

In order to determine the orientation of the AcChR molecules within the reconstituted vesicles, α-Bgt-binding assays were conducted on untreated vesicles (to label available external toxin sites) and on vesicles after their solubilization with 1% Triton X-100. The results indicated that 90-100% of the total α-Bgt-binding sites are located on the external surface of the reconstituted vesicles, resembling the "right-side-out" orientation detected for this receptor in native AcChR-enriched vesicles from <u>Torpedo</u> electroplax.

We conclude that the reconstituted vesicles are functionally similar to AcChR-enriched membranes, because the vesicles exhibited the following well-recognized characteristics of the membrane-bound AcChR: (1) an ability to undergo reversible affinity state transitions as a consequence of prolonged exposure to agonists, and (2) an increase in ion permeability in response to the presence of agonists. Unlike solubilized receptor, the reconstituted vesicles exhibited the ability to undergo agonist-induced transitions from low to high ligand affinity states. This phenomenon resembles pharmacologic desensitization of postsynaptic membranes induced by cholinergic agonists. The existence of this affinity state transition can be examined by measuring the binding of ^{125}I-α-Bgt to both lipid-bound and solubilized receptor. Samples were incubated with or without (control) 10 μM carbamylcholine at time zero or were preincubated with 10 μM carbamylcholine for 30 min. The results indicate that the "sensitized" state (low affinity) is absent in the solubilized AcChR, but reappears when the receptor is reintegrated into a lipid environment, thus resembling the behavior of "sensitized" native AcChR-enriched membranes.

Reconstituted Vesicles

Finally, the excitability of the reconstituted vesicles was examined by measuring the efflux of trapped $^{22}Na^+$. As expected for sealed vesicles, a small $^{22}Na^+$ efflux was observed upon a 1:25 dilution of the vesicles in the absence of agonists.

A similar baseline efflux of $^{22}Na^+$ was obtained from plain *Torpedo* lipid vesicles that contained no protein. The retention of $^{22}Na^+$ was greatly reduced in both types of vesicles when they were either preincubated with the ion-pore-forming polypeptide gramicidin A or solubilized with octylglucoside at concentrations below the critical micelle concentration. Conversely, only the vesicles containing AcChR were stimulated to release Na^+ in response to 0.5 μM carbamylcholine present in the dilution buffer. Furthermore, this response was selectively blocked by preincubation of the vesicles with an excess of either α-Bgt or d-tubocurarine, suggesting that the AcChR is responsible for agonist induced increases in ion permeability.

In conclusion, we have described the preparation and characterization of reconstituted AcChR vesicles that exhibit most of the inherent qualities of native AcChR-enriched membranes. The nature of the reconstituted vesicles is schematically shown in a cartoon fashion in Figure 3-11. Whether the reconstituted AcChR is present as a monomeric, dimeric or higher aggregate forms is not yet known. Our results strongly suggest that the receptor protein, isolated by conventional purification methods, contains all of the molecular determinants necessary for specific ligand binding and for ion translocation.

DR. ENGEL: About the orientation of the AcChR molecules within the reconstituted vesicles. Why should they be all "right-side-out"?

DR. GONZALEZ-ROS: I do not know. There is something that I would like to study in more detail.

DR. ENGEL: Well, they have a lot of carbohydrates.

DR. GONZALEZ-ROS: Yes. Actually the α and δ subunits (40,000 and 65,000 molecular weight) have, not a lot, but enough carbohydrates to assume that they might be able to induce some asymmetry in the AcChR placing during the formation of the vesicle. It must be either that or else, the reconstituted lipid phase by itself is inducing it. The latter could be assessed, for instance, by labeling of phospholipids containing free amino groups with 2,4,6-trinitrobenzenesulfonic acid (TNBS) (Litman, 1975) under non-penetrating conditions in both intact reconstituted membranes to determine the availability from the outside of the membrane and detergent solubilized ones.

DR. ENGEL: Do you have proof that all vesicles will go "right-side-out"?

DR. GONZALEZ-ROS: Not all of them individually, but the reconstituted system as a whole is "right-side-out."

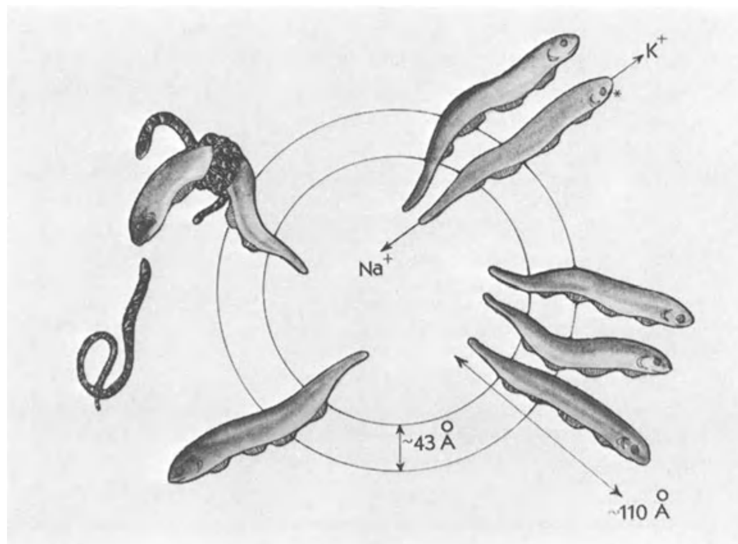


Figure 3-11: Schematic representation of reconstituted AcChR vesicles. Electric eels represent AcChR molecules in different states of aggregation (monomer, dimer, etc.). Snakes represent α-neurotoxin molecules.

Actually, as I said before, Raftery's group at Cal Tech has estimated the "right-side-out" distribution of AcChR in native membranes as the same figure that we get with our reconstituted system which, therefore, resembles the "in situ" results.

DR. BRANTON: How much lipid is retained by the protein upon purification?

DR. GONZALEZ-ROS: I did not make that measurement personally, but it has been described that there is no lipid associated with the purified protein.

That was the summary of our more recent contributions. Let me now make some comments about a few of the problems we are presently considering. As a first approach one of the objectives of our research is the establishment of any possible dependance between membrane-bound AcChR functionality and the physical and/or chemical features of the surrounding liquid matrix. The existence of such hypothetical dependence appears likely on the basis of several unsuccessful attempts at reconstitution where the presence of residual detergent tightly bound to the purified protein and/or the utilization of non-native phospholipids hindered the reappearance of membrane-bound AcChR features. In addition, the presence of active phospholipase A and/or its fatty acid hydrolysis products, as well as other general membrane perturbants such as local or

general anesthetics have been shown to produce altered functionality, i.e. the membrane bound receptor is locked in a pharmacological "desensitized"-like state. Our own recent findings (Gonzalez-Ros et al., 1980a) and others illustrate how the presence of lipid is required for the maintenance of AcChR in a native "sensitized" state. In this regard, systematic alterations of the liquid matrix should allow for the establishment of any possible dependence between AcChR function and the physical or chemical properties of the liquid phase in the reconstituted vesicles.

Another important issue in this field is to assess the native state of aggregation (monomer, dimer, etc.) of AcChR which gives rise to functionally active membrane-bound molecules. Our reconstituted system could provide a reasonable approach to this problem by using purified fluorescent labeled monomer populations as a reconstituting material. Fluorescence energy transfer studies in the reconstituted vesicles should address the question of whether monomers, dimers, or even high aggregates are formed as molecular entities responsible for AcChR function. Finally, the reconstituted system will also be used in the design of a new spectrofluorometric assay for accurate determination of cation fluxes within the physiological time-scale (milliseconds). The assay will be based on the trapping and further quenching of fluorophores contained within the reconstituted vesicles as they collide with incoming cations.

DR. ENGEL: Can you be sure that all four subunits of AcChR are incorporated into the reconstituted vesicles?

DR. GONZALEZ-ROS: Yes. The SDS gel patterns of the starting purified AcChR are identical to the ones obtained from AcChR isolated from purified reconstituted vesicles.

DR. ENGEL: Do you think all four subunits are essential for functional activity?

DR. GONZALEZ-ROS: Well, the controversy at this point is due to the fact that there are a number of groups, especially Changeux's, which have been claiming a different subunit pattern for the purified receptor. However, I do not think that is a widely accepted idea now. Actually, most groups working in the receptor field accept the idea of having purified AcChR preparations containing the four different subunits to which I referred above, all of them being essential to receptor function. In addition, it is known that the 40,000 molecular weight subunit is the only one implicated in ligand binding. We published a paper in Biochemistry about a year ago (Sator et al., 1979) in which we used a photoactivatable hydrophobic probe to label those subunits of AcChR that are accessible from within the membrane environment. Those results indicated that the

β and γ subunits (50,000 and 60,000 molecular weight) are in contact with the hydrophobic surrounding whereas the α subunits, are exposed to the aqueous environment and the δ subunit seems to be the least exposed to either the aqueous or hydrophobic environment. Nevertheless, the functional role of those β, γ and δ subunits is yet to be determined as well as their possible implication in ion translocation events.

DR. SANDRA: The approximately 5% of your AcChR that are not "right-side-out" oriented, are they confined to a separate population of vesicles or are they just distributed randomly?

DR. GONZALEZ-ROS: I don't know.

EDITORIAL SUMMARY:

Some of the regulators of cell growth were described including the growth inhibiting effect of cell contact in fibroblasts in culture and the stimulating effect of neuronal processes on the growth of Schwann cells. Further discussion centered on models for the study of cellular lipid synthesis and the relationship between the enzymatic synthesis of phospholipids and β adrenergic receptor function. A study of acetylcholine receptor function was reported using reconstituted vesicles to clarify the mechanisms of ligand binding and ion translocation.

CHAPTER 4

THE LIPID BILAYER, MECHANISMS OF FUSION AND TRANSMEMBRANE TRANSPORT

Functional Significance of Non-Bilayer Lipid Structure

DR. CULLIS: Fusion is among the most important events that takes place in the cytoplasm including, for example, the blebbing off of various secretory granules to fuse with the membrane surface, the fusion processes involved in interorganelle transport, the virus blebbing off process, on endocytotic processes, such as occur for coated vesicles, which then fuse to form secondary lysosomes. In addition to fusion events, there are situations where you appear to have continuity between two organelles (Morré et al., 1978). This may be between rough endoplasmic reticulum and the outer mitochondrial membrane or between the golgi and the smooth endoplasmic reticulum as well as various secretory vesicles. These observations are of interest from the point of view of lipids that have very different properties from what we normally think they should have in a membrane. In particular, we are concerned with the roles of lipids which prefer non-bilayer arrangements in isolation.

I will discuss fusion processes first. There are two basic problems of fusion. One of these concerns bringing two membrane-bound bodies into close apposition. The second, and most interesting event results in formation of a single membrane-bound body. We are interested in what happens during this process. It is our basic contention that it is very difficult to imagine this event occurring while the lipids in those membranes maintain a bilayer organization.

Why should we think in the first place that fusion processes and bilayer or bilayer structures could have any relation to each other? Dr. de Kruijff in his presentation showed that lipids such as phosphatidylethanolamine can undergo a transition from a bilayer organization to hexagonal H_{II} organization.

In this latter organization aqueous channels of lipids are surrounded by the head groups (Luzzati et al., 1968). You can induce these transitions as a function of temperature in PE (Cullis and de Kruijff, 1979). We will address the problem of how these transitions occur to see whether a similar kind of process could be involved in fusion events. This can be approached by an NMR experiment shown in Figure 4-1. This is a very simple minded diagram indicating that if we have bilayer phospholipids oriented so that the bilayer is perpendicular to the magnetic field, then we will see a ^{31}P NMR resonance with a chemical shift characteristic of the so-called low field shoulder of our "bilayer" line shape. Alternatively, if we have the cylinders of the hexagonal H_{II} phase oriented parallel to the plane of the bilayer from which they are formed, a resonance at a different chemical shift position will be observed. This actually occurs experimentally as shown in Figure 4-2 for egg PE (Cullis et al., in press). This tells us that the H_{II} phase cylinders lie parallel to the phase of membranes from which they are formed, allowing us to suggest the model shown in Figure 4-3.

In this model we suggest that cylinders actually form as an inter-bilayer event. Full conversion to the H_{II} phase is shown in a slightly more artistic fashion in Figure 4-3 The main point is that the bilayer H_{II} transition appears to occur as an inter-bilayer event, which we suggest could be related to other inter-bilayer events such as occur in fusion processes.

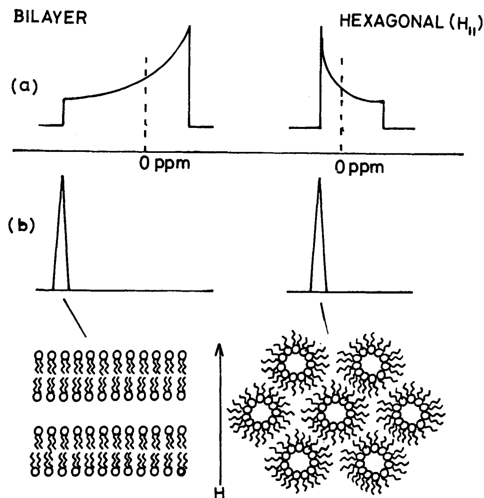


Figure 4-1: Theoretical ^{31}P NMR spectra expected from (a) unoriented phospholipids and (b) oriented phospholipids in either the bilayer or hexagonal H_{II} phase. The direction of the magnetic field (H) is perpendicular to the plane of the bilayer or perpendicular to the axis of the aqueous cylinders of the H_{II} phase. 0 ppm corresponds to the resonance position of the sonicated vesicle systems.

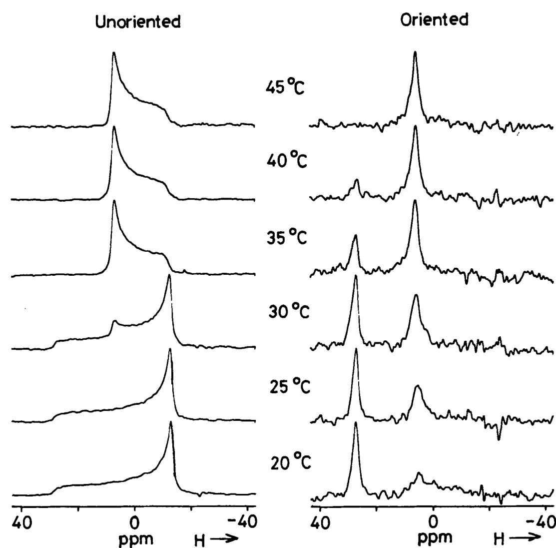


Figure 4-2: 81.0 MHz ^{31}P NMR spectra at various temperatures arising from (unoriented) aqueous dispersions of egg phosphatidylethanolamine and egg phosphatidylethanolamine oriented between glass plates.

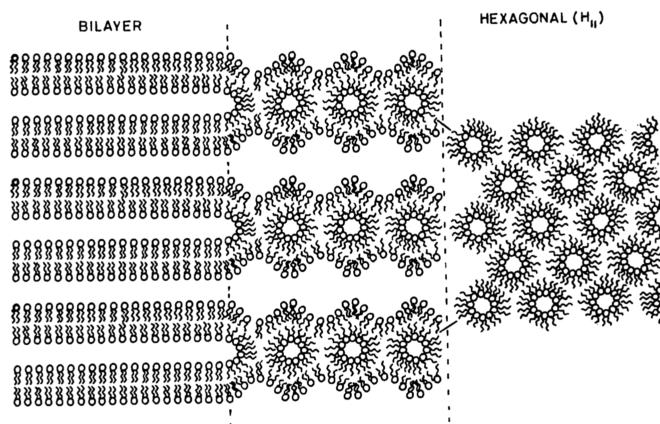


Figure 4-3: Proposed mechanism of the bilayer-hexagonal (H_{II}) transition for closely opposed bilayers. The long aqueous cylinders characteristic of the H_{II} phase are envisaged as forming from the two opposed monolayers, which results in an orientation of the axis of H_{II} cylinders which is parallel to the plane of the bilayers from which they are formed. The right hand side of this figure shows complete transition from the bilayer to H_{II} phase.

DR. BRANTON: Could you explain how the hexagonal phase is more likely to explain fusion?

DR. CULLIS: I think it would probably be best if we wait a little bit longer until some more models and data are presented.

Role of Fusogens

We became interested, in particular, in fusion not only from these theoretical considerations but also when we examined the influence of membrane lipids. We found that equimolar oleic acid (which is one of the more common fusogens) generates hexagonal phase formation in previously bilayer PC systems. The next stage, of course, was to examine the effects of fusogen on intact biological membranes and so we tried on the erythrocyte ghost membrane as shown in Figure 4-4. The ^{31}P NMR in the absence of fusogen indicates that we start off with the phospholipids in a bilayer (I should say this represents 98% at least of the phospholipids in the erythrocyte ghost membrane).

Incubation in the presence of oleic acid results in progression from a bilayer organization to hexagonal organization as the concentration of oleic acid is increased (Cullis and Hope, 1978). This is an interesting observation but it is only interesting if it also correlates with the amount of fusogen one needs in order to actually induce fusion between ghosts. Some of the correlations we observe for certain well known fusogens, glycerol monooleate also

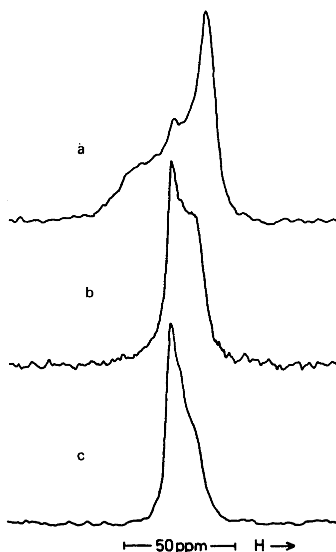


Figure 4-4: 36.4 MHz ^{31}P NMR spectra at 37° from (a) erythrocyte ghosts; (b) erythrocyte ghosts and oleic acid, where the oleic acid/phospholipid ratio is 1.3; (c) erythrocyte ghosts and oleic acid where the oleic acid/phospholipid ratio is 2.4. (Cullis and Hope, 1978).

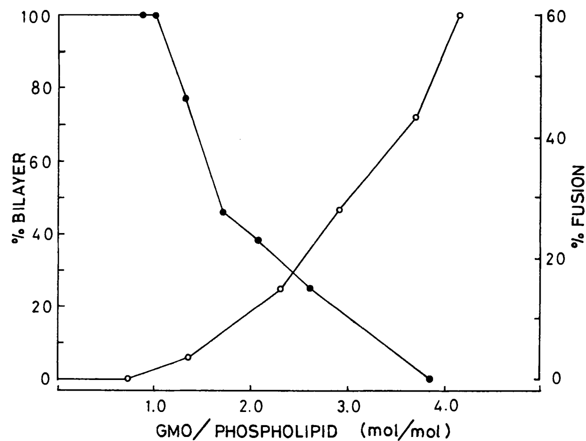


Figure 4-5: A comparison of the extent of fusion between erythrocytes and the amount of bilayer phase remaining in erythrocyte (ghost) membranes at various membrane concentrations of glycerolmonoleate (GMO): (0) - the percentage of membrane phospholipids in extended bilayers; (□) - the percentage fusion of erythrocytes following incubation with fusogen (Hope and Cullis, 1980).

for oleic acid are shown in Figure 4-5. We see as the amount of glyceromonooleate increases in the intact membrane, the amount of lipid that assumes an hexagonal H_{II} organization increases as the amount of fusion increases.

In other words when fusion occurs, we have enough fusogen in the membrane to generate non-bilayer structures. This makes us feel fairly strongly that somehow these non-bilayer structures must be involved in fusion processes. Our early model end (Cullis and Hope, 1978) of this is shown in Figure 4-6. In this model we have two closely opposed regions of membrane and subsequently at this point generate a cylinder of lipids (hexagonal phase) or possibly the other inverted structures (lipidic particles). This gives us a view of how non-bilayer lipids might be involved in fusion processes. However, it is pretty artificial because we just don't have equimolar concentrations of fusogen in membranes so we really can't make a strong case that perhaps this is a naturally occurring mechanism of fusion _in vivo_. However, we can go a little bit further and say that we do have PE in membranes and we know that PE will adopt these hexagonal phases as will cardiolipin (Cullis et al., 1978). An important question is whether we can stimulate and control non-bilayer structure appropriate to fusion processes making use of these endogeneous non-bilayer lipids. One of the first things one thinks of, of course, is calcium because it is well known that calcium is a primary trigger of fusion processes (Poste and Allison, 1973).

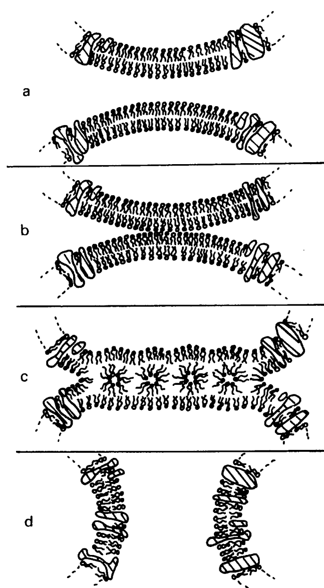


Figure 4-6: Proposed mechanism of membrane fusion. The slashed areas traversing the membrane indicate integral membrane protein. The intermediate structures noted in part c correspond to short aqueous channels (H_{II} phase structure) or inverted micelles.

In this context it is interesting that we can trigger a bilayer to hexagonal transformation for cardiolipin by addition of calcium (Cullis et al., 1978). This gives us a feeling that two things are possible. We have a lipid there that is able to adopt these phases which may be involved in fusion, and we have a possibility that calcium can induce those structures. So we are starting to satisfy two primary things in fusion processes.

There is another criticism, however, and that is that most membranes contain very little cardiolipin, the only exception being the inner mitochondrial membrane. Most plasma membranes and other organelle membranes contain appreciable amounts of PE and PS, however, and these systems also have interesting properties in that you can generate H_{II} phase formation by addition of Ca^{2+}. The involvement of hexagonal or non-bilayer structure and the fusion process is beautifully given in Figure 1 of Verkleij et al., 1979). We see that we start off with very small vesicles and we end up with very large ones. These are all at the same magnification, so fusion has occurred. We see, more interestingly, that some of the sites which correspond to fusion interfaces exhibit little bumps which correspond to "lipidic particles" (Verkleij et al., 1979 and de Kruijff et al., 1979) occurring in those regions.

For the purpose of this discussion, we can consider lipidic particles to be a short region of hexagonal phase cylinders (inverted micelles). This makes a fairly strong case for the involvement of non-bilayer structures in fusion where those non-bilayer structures are induced by the calcium present. The behavior of systems containing phosphatidylserine and phosphatidylethanolamine are particularly interesting, as shown in Figure 4-7. This shows that for a bilayer arrangement of a phosphatidylethanolamine-phosphatidylserine mixture a transition from the bilayer to the hexagonal arrangement is observed as we add calcium (Cullis and Verkleij, 1979). So in these systems, we do have the possibility of triggering the bilayer to hexagonal transition. The next thing we examined was the behavior of selected analogues to biological membranes - particularly the erythrocyte membrane. One interesting system is provided by the inner monolayer of the erythrocyte membrane, which contains 50 mol % phosphatidylethanolamine (which prefers the H_{II} phase in isolectin) as well as 25 mol % PS (Zwaal et al., 1977). Again, as shown in Figure 4-8 Ca2+ induces H_{II} phase formation in such a lipid mixture, behavior which is not observed for "outer monolayer" phospholipids. So we are gradually getting closer and closer to some real situations. How can this actually relate to something that we see as occurring either *in vitro* or maybe even *in vivo*? One event that occurs *in vitro* that is well characterized (Allan et al., 1976) for erythrocytes is that on incubation for longer times (hours) they "bud off" membrane in the form of small vesicles, losing up to 20-30% of their membrane as time goes on. This event seems to be correlated to the entry of calcium into the cell. This intrigues us because if calcium gets inside the cell it may induce a tendency to form non-bilayer lipid structures in the inner monolayer, and this might be involved in the fusion event that is of course vital to the "blebbing off" process. A possible model of this is shown in Figure 4-9. In Figure 4-9, in regions where we have closely opposed inner monolayers, we might expect that those regions would be very susceptible to adopting an intermembrane non-bilayer organization. This is drawn in schematic terms as a short hexagonal or inverted micelle arrangement. This gives us again an intermediary structure, allowing us to see how the "blebbing off" process might be facilitated by the lipids of the inner monolayer and by the entry of calcium into the erythrocyte membrane. We took this again one step further and asked "how could events like this be related to other fusion processes such as secretory processes?" We were particularly interested in the mechanism whereby chromaffin granules approach the inner monolayer of plasma membrane, fuse with it and then release their contents, a process which seems to require the presence of calcium (Edwards et al., 1974). In particular, can the lack of stability of the inner monolayer in such situations be expressed by an enhanced fusion of the chromaffin granule with the plasma membrane which, of course, is vital to the release of the secretory contents? So we did a simple experiment, the results of which are shown in

Figures 4-10 and 4-11. The chromaffin granules were incubated in the presence of vesicles composed of phosphatidylethanolamine and phosphatidylserine. On addition of calcium to these vesicles we get the hexagonal configuration. The results of incubating the chromaffin granules with this model membrane system and then adding calcium are given in Figure 4-10, which shows that the PE-PS vesicles act as adjuncts for Ca^{2+} stimulated release of chromaffin granule contents. We see such effects only for lipid adjuncts which undergo a structural transition, such as a bilayer to H_{II} transition, on addition of Ca^{2+}.

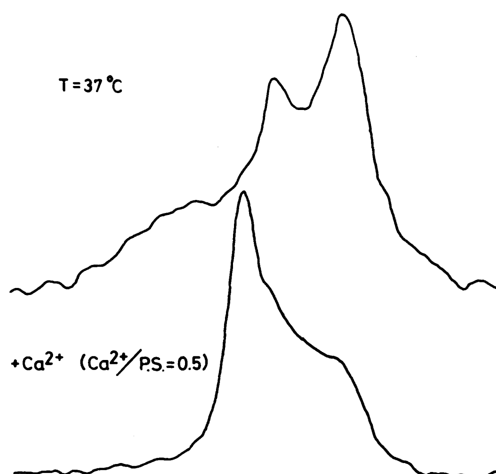


Figure 4-7: 36.4 MHz ^{31}P NMR spectra at 37°C of an aqueous dispersion of 20 mol % bovine brain phosphatidylserine and 80 mol % hen egg yolk phosphatidylethanolamine: (a) in the absence of Ca^{2+} and (b) in the presence of Ca^{2+} to achieve a Ca^{2+} phosphatidylserine ratio of 1.0 (Cullis and Verkleij, 1979).

DR. BRANTON: What concentration of calcium do you use?

DR. CULLIS: In these experiments we are using 5 millimolar calcium. On the basis of these results we can suggest another model for the release process and that is shown in Figure 4-11. In this model the calcium coming in induces an instability in the inner monolayer lipids, which may tend to adopt a non-bilayer organization. This is really the previous model turned upside down. This is about as far as I want to take the fusion work, as I would like to move on to discuss certain aspects of membrane morphology and their relation to non-bilayer capabilities of lipids.

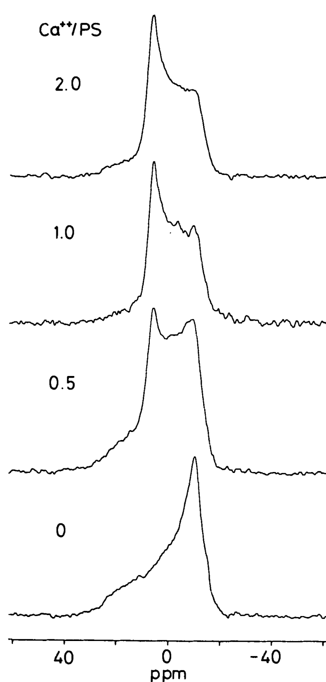


Figure 4-8: 81.0 MHz ^{31}P NMR spectra of erythrocyte inner monolayer phospholipid at 37°C in the presence of varying amounts of Ca^{2+} (Hope and Cullis, 1979).

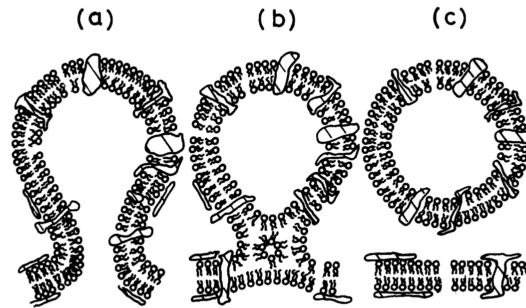


Figure 4-9: Proposed model of the blebbing off process for erythrocytes. This model can also be suggested to apply to secretory processes occurring via exocytosis.

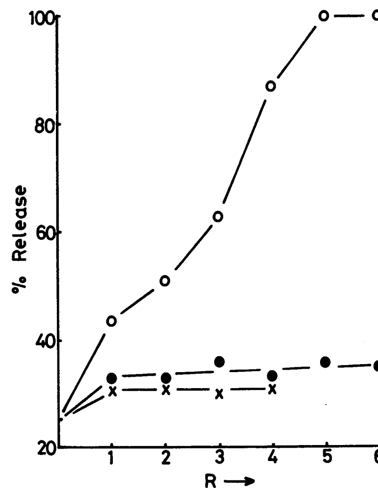


Figure 4-10: Release of chromaffin granules after incubation in the presence of phospholipid vesicles of varying lipid composition, followed by the introduction of 5 mM Ca^{2+}: 0 - PE-PS vesicles; 0 - PE-PS vesicles, no Ca^{2+}; X - PC-PS vesicles (Nayer et al., 1980).

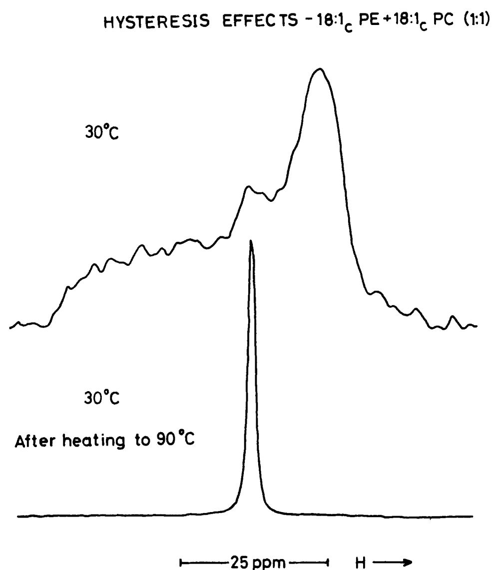


Figure 4-11: 36.4 MHz ^{31}NMR spectra from an equimolar mixture of dioleoyl phosphatidylethanolamine and dioleoyl-phosphatidylcholine at 30°C: (a) before and (b) after heating to 90°C for 30 min (Cullis et al., 1978). R refers to the molar ratio of exogenous (vesicular) phospholipid to endogeneous (chromaffin granule) phospholipid.

DR. M. GLASER: Do you have any idea how pure that hexagonal phase lipid composition is from model studies? How much PS can you put into PE before it refuses to act as an adjunct?

DR. CULLIS: Pure PS can induce the same effect.

DR. BRANTON: Do you know what the time scale is for that sort of thing?

DR. CULLIS: It appears to be instantaneous ($\ll$ 1 min). However, the measurement of release is taken sometime afterwards (15 min).

DR. PARK: It is interesting because things like insulin which act on cells now are thought to move transport proteins from something like the Golgi apparatus to the plasma membrane. That kind of thing has a 5 minute time scale before you get maximum stimulation so that even if you don't see this sort of instantaneous type of thing, if you had it within a 5 minute period you are still within a good physiological range of what might be happening in the cell. It's kind of nice. But that 5 minute time period is just about allowable.

DR. L. GLASER: If you up the calcium concentration inside the red cell, do you randomize the lipid composition of the inner and outer monolayers?

DR. CULLIS: I think you do. I know that as you ATP deplete the cells you certainly get more PE appearing in the outer monolayer. So you are randomizing it to some extent.

The next few things I want to talk about concerning membrane continuity are hopefully appropriate to this meeting because they are very speculative. Figure 11 shows the ^{31}P NMR spectra of an equimolar mixture of phosphatidylethanolamine and phosphatidylcholine which was heated to 90° for about 5 minutes then brought back down to the same temperature. A very narrow lineshape is seen in that situation, certainly suggesting that something has happened to the bilayer structure. A new structure is present that allows isotropic motion that certainly wasn't there before. In order to get rid of this we have to freeze-thaw and then we come back to our original bilayer situation. The possible nature of that "isotropic" structure we find fascinating, particularly from the membrane compartmentalization point of view. In Figure 6 of Cullis and de Kruijff, 1979 shows the lipidic particles formed in mixture of bilayer and Hexagonal H_{II} phase lipids (de Kruijff et al., 1980). One thing I want to point out is that these particles often occur in rows. In addition, you may also remember that in the cardiolipin-phosphatidylcholine systems fused by addition of calcium, that lipidic particles (inverted micelles) appear to be occurring at the fusion interface. A logical case could be made that what we are seeing here is a fusion between two bilayers of these multilamellar systems. As a result of those kinds of considerations, as well as the narrow ^{31}P NMR lineshape we came up with a model which is shown in Figure 4-12. The first part of this figure gives the usual representation of a multilamellar liposomal system which is an aqueous dispersion of lipids. This is an idealized model so we thought we would draw another idealized version which indicates that in some regions you are going to have regions in which bilayers are in close proximity. So you get the tortoise shell arrangement. We then suggest that in these regions (for systems in which H_{II} phase lipids are present) you could generate fusion or partical fusion between these various bilayers. This is shown here and we have a little dot indicating the presence of inverted micelles or perhaps a short region of the H_{II} structure. The main point here is that we are left with interstitial regions which are potentially very small. Returning to the comments made this morning, if these regions are on the order of say 500 angstroms or 1000 angstroms in diameter, they will give rise to isotropic motional averaging. This will result in the NMR characteristics observed, and also they will result in a kind of freeze fracture pictures obtained. It also accounts for some of the permeability characteristics.

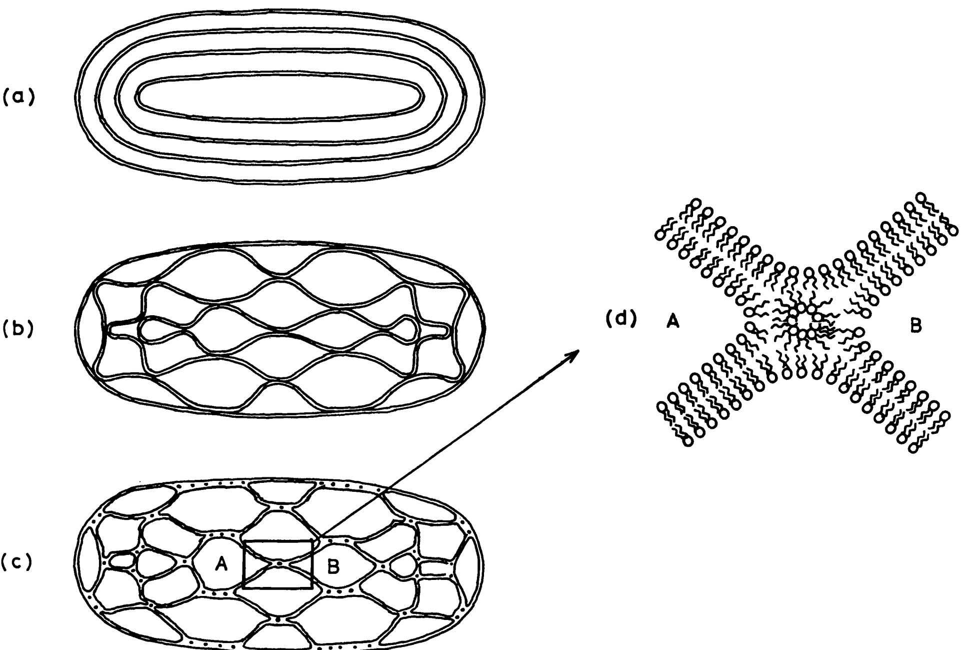


Figure 4-12: Mechanism of formation of a "honeycomb" structure from multilammelar PE-PC-cholesterol systems and other systems containing mixtures of "bilayer" and hexagonal (H_{II}) phase lipids. In (a) the usual depiction of multilamellar liposomes is given, however, it is clear that in general, regions will exist where the layers are in close apposition. A stylized version of this is given in (b). In (c) it is postulated that these regions will undergo (partial) fusion with associated formation of lipidic particles at the interface. Compartmentalization in a continuous membrane structure then results, as indicated for Compartments A and B in the expanded diagram of part (d).

DR. BRANTON: Is it true?

DR. CULLIS: I just said I wanted to present something that is very speculative.

DR. BRANTON: This is a model that is not worth drawing because you can look in the electron microscope and determine right away whether it is true or not. It's a 5 minute experiment.

DR. CULLIS: If you could tell us how to do that, we would be quite pleased because we have been worried as to exactly how you can prove that those structures exist.

DR. BRANTON: I don't understand what the problem is. You can see the liposomes in the upper configuration by electron microscopy; you can see these others also by electron microscopy, or are you talking about dimensions that I'm not familiar with? Your're talking about small vesicles?

DR. CULLIS: The sizes are on the order of 100 Å or larger.

DR. BRANTON: So what is the problem here?

DR. CULLIS: I don't know, I'm not an expert on the freeze-fracture side of this, but from what I know it appears to be difficult to say unequivocaly that what you have is a sponge-like structure such as I have suggested. If this is a 5 minute experiment, I think we would appreciate a 5 minute experiment being done.

DR. WOLF: Could you elaborate a bit, Dr. Branton? Your point is that one can get direct morphologic evidence of the existence or non-existence of these two models by transmission electron microscopy.

DR. BRANTON: By standard transmission electron microscopy in the length of time it took to prepare this figure. It's not 5 minutes, but no longer than it took you to prepare that beautiful figure.

DR. CULLIS: Are you saying if they were there you would have seen them by now?

DR. BRANTON: No, I'm not implying that because we might not have looked under the right conditions. I was just wondering whether you looked.

DR. CULLIS: No, we haven't looked. We don't even have access to an electron microscope at the moment.

DR. M. GLASER: What would the right conditions be?

DR. CULLIS: One system, for example, would be one in which you had phosphatidylethanolamine and phosphatidylcholine which you would incubate at a temperature in which the phosphatidylethanolamine would prefer a non-bilayer arrangement.

DR. BRANTON: At 90°.

DR. CULLIS: No, not necessarily at 90°. It could equally well be done for longer time at lower temperature or it could be done for cardiolipin phosphatidylcholine system where we add the calcium to transform the cardiolipin to a non-bilayer species.

DR. SANDRA: I have a problem regarding what you would call fusion in one of the vesicles where you saw the vesicles appearing in various stages of fusion. I don't understand why they look like a bunch of grapes. I would think that they would have fused if they had a smooth surface. How do you distinguish between fusion and aggregation?

DR. CULLIS: One thing we would see is that aggregation would be reversible on addition of, for example, EDTA. Our thesis is that if you have induced the presence of a non-bilayer lipid in a liposomal system, this basically induces the presence of the lipidic particles. If you have a situation where that occurred then you will facilitate fusion processes.

DR. L. GLASER: How many areas of contact do you need to see in order to produce this model. In other words, if Dr. Branton looks at such liposomes how frequently would you expect to see such an arrangement?

DR. CULLIS: We would have to have something that gave us in effect compartments that were smaller than 2000 angstroms in diameter in order to account for our NMR observations.

DR. WOLF: We assume that your temperatures are centigrade.

DR. CULLIS: Yes.

Figure 4-13 shows some of the potential manifestations of non-bilayer lipids in membrane function and structure. We call it the "metamorphic mosaic" model at the risk of being rather pretentious. I think it gives us a rather different feel for some of the things that membranes can do in a particular organelle. One of the things we have been particularly concerned about is what really is the morphology of the endoplasmic reticulum and also what is the real morphology of the inner mitochondrial membrane. These are questions which are difficult to answer.

For example, in the case of the endoplasmic reticulum, they are always isolated in the form of relatively small fragments which appear to form spontaneously. Our feeling is definitely that those don't really reflect what was originally there. But what is originally there? Is it a network where you have lots of various compartments performing various functions or is it really just what we are used to thinking of as a big bag sitting there in the cytoplasm? It is really those kinds of questions that we are coming to, and I feel that the ability of lipids to go to adopt alternative structures and the variety of agents which will make them adopt one structure or another gives us some confidence that they may play a role in both the morphology as well as directly in the function of these membranes.

DR. STRITTMATTER: I wanted to point out that the problem with these models is that they all lack specificity with regard to natural fusion processes.

DR. CULLIS: What do you mean exactly?

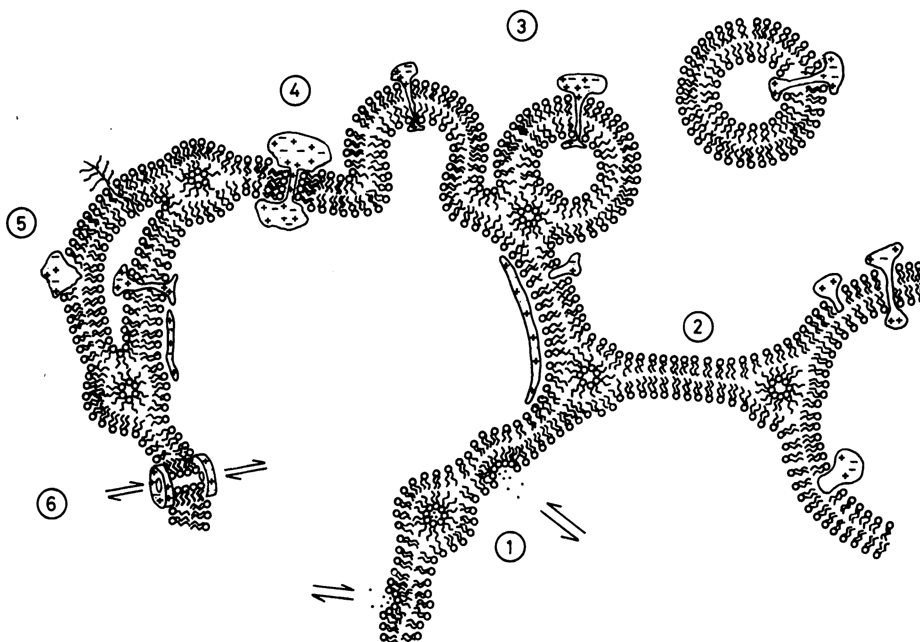


Figure 4-13: A metamorphic mosaic model of biological membranes illustrating various structures and processes suggested by the ability of lipids to adopt non-bilayer structures.

DR. STRITTMATTER: Well, fusion in myoblasts, myotubules, etc. all depend on calcium.

DR. CULLIS: Our systems can also depend on Ca^{2+}.

DR. STRITTMATTER: But can't you get the same effect with magnesium?

DR. CULLIS: No. Not in the case of PS. PS effects are specific for calcium, not magnesium. There is a remarkable difference in the interaction. For example, you have pure phosphatidylserine, you add calcium to it, you induce so-called cochleate structures which is a crystalline arrangement. With magnesium for the exact same system you do not see this.

DR. BRANTON: What about the calcium concentration that you are using. Does it worry you that they are 1, 2 and sometimes 3 orders of magnitude higher than cytoplasm?

DR. CULLIS: Not necessarily, particularly in systems where the effects are produced by an influx of Ca^{2+} - i.e. the "blebbing off" process for erythrocytes, or the extracellular release of chromaffin granule contents.

DR. BRANTON: It was my understanding that the levels of calcium in most cells in the cytoplasm as opposed to the exoplasmic phases was in the micromolar region. It is a large jump from there to the millimolar, several orders of magnitude. That's what I meant.

DR. CULLIS: In the case where it would worry us or does worry us would be if your talking about a "blebbing off" process from endoplasmic reticulum.

DR. ENGEL: If what you say is correct, you would expect to see the H_{II} organization during fusion, is that correct?

DR. CULLIS: Well, or inverted micelle organization at fusion sites, yes.

DR. ENGEL: Therefore, if you cross-fracture you should see the intramembrane lipid.

DR. CULLIS: Yes.

DR. ENGEL: We have looked at new spots in fusing myoblasts, myoblasts and myotubes and also looked at liposome lipids. I don't see them. I possibly overlooked them.

DR. CULLIS: There wouldn't have to be very many. I agree with you though. We certainly had a very good look through literature particularly at the bare patches in regions of fusion to see why they are not there as particles which we could say correspond to intrabilayer inverted micellar structures. We can't see anything we could identify unequivocally.

*DR. MURRAY: Targeting of exogenous administered enzyme to non-reticuloendothelial tissues is one of the primary obstacles to successful enzyme replacement therapy in hereditary metabolic diseases. de Barsy and co-workers (de Barsy et al., 1973) administered α-glucosidase purified from human placenta to patients with Pompe's disease (α-glucosidase deficiency) and demonstrated uptake of the enzyme primarily by liver and to a negligible extent by muscle. The infused enzyme disappeared from the plasma with a half-life of 10 minutes, accumulated in the liver to levels 10-20% of normal, and was degraded with a half-life of 4-5 days. Their experience is similar to that of others who have administered exogenous enzymes to patients and found the liver to be the primary site of intracellular localization (Desnick et al, 1976).

Enzyme Transport by Linkage to Lipoprotein

One approach to the problem of enzyme targeting is to attach the desired enzyme to a physiologic carrier molecule which is taken up by specific receptors on multiple cell types. The system of our choice is the low density lipoprotein pathway described by Goldstein and Brown (Goldstein and Brown, 1977). Low density lipoprotein (LDL), the major cholesterol carrying particle in the circulation, is bound by specific receptors on the cell surface, internalized and subsequently degraded in lysosomes. LDL is primarily metabolized outside the liver (Sniderman et al, 1974) permitting the delivery of enzyme-LDL complexes to extrahepatic tissues.

The fluid endocytosis rates were measured for Pompe, familial hypercholesterolemic and control cell lines using ^{3}H-sucrose (Wagner et al., 1971). The rates of sucrose internalization were linear with time up to 15 hours, and with concentration (5μg and 25μg/ml) and the same for all three cell lines, 19ng/mg cell protein/6 hr at 25μg/ml (Table 1). The rate of endocytosis or uptake of ^{125}I-albumin was linear with concentration (1 to 100 μg/ml) for any given time of incubation. The combined rates of ^{125}I-albumin internalization and degradation, i.e. uptake, are shown for all three cell lines in Figure 4-14. The rate is 20-24 ng/mg cell protein/6 hr at an albumin concentration of 25 μg/ml.

*Work done in collaboration with J.C. Williams, U. of California, San Diego & Allen K. Murray, U. of California at Irvine. Dr. Williams is presently at U. of Texas, Houston; Dr. Murray is presently at MDA in New York.

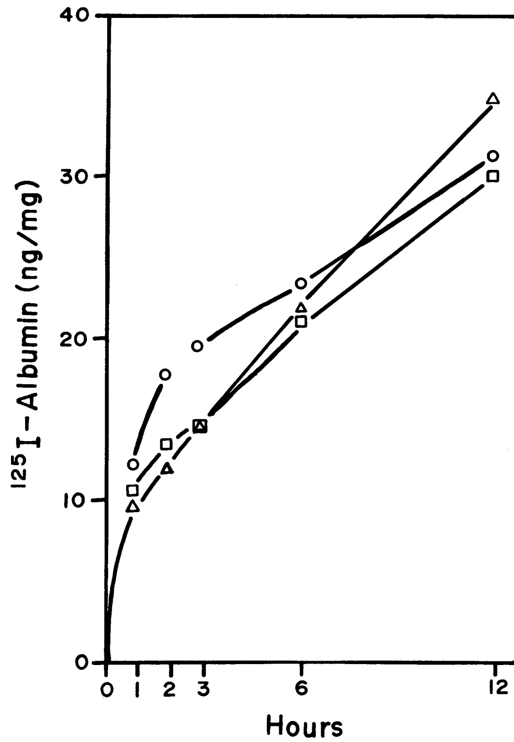


Figure 4-14: The combined internalization and degradation of ^{125}I-albumin of control (□), Pompe (Δ), and familial hypercholesterolemic (O) cells. The concentration of ^{125}I-albumin in the media was 25μg/ml.

Several lysosomal enzymes have been postulated to exist in high uptake forms as a result of binding to high affinity sites on fibroblast cell surfaces (Hickman et al., 1971). We have measured the uptake of the lysosomal α-glucosidase by all three cell lines. In order to overcome problems inherent in measuring an increased α-glucosidase activity over the high endogenous levels in the control and familial hypercholesterolemic cells, ^{125}I-labeled α-glucosidase was used for all three cell lines in the initial experiments. Later experiments measured the uptake α-glucosidase by enzyme activity in Pompe (enzyme deficient) fibroblasts. The rates of uptake of ^{125}I-α-glucosidase were similar for all three cell lines in the range of 150-200 ng/mg cell protein/6 hr and 300-400 ng/mg cell protein/6hr at a concentration of 5 μg/ml and 15 μg/ml respectively. (Table 1). Uptake of free α-glucosidase is 25 times the albumin endocytosis rate which is 14 ng/mg cell protein/6 at 15 μg/ml. For comparative purposes it can be extrapolated that at 25 μg/ml α-glucosidase uptake would be approximately 450-600 ng/mg cell protein/6 hr compared to 20-24 ng/mg cell protein/ 6 hr for albumin. This may indicate that the α-glucosidase is entering the cells via a receptor mediated and/or a high affinity pathway. The rates of uptake of sucrose, albumin and free α-glucosidase are summarized in Table 1. The data also suggest that the α-glucosidase enters cells via a different pathway from that of the LDL receptor as the enzyme enters the familial hypercholesterolemic cells (LDL receptor deficient) at the same rate as the other cells as described above. Also unlabeled α-glucosidase does not compare with ^{125}I-LDL for uptake in normal cells (results not shown). The effect of concentration on the rate of unlabeled α-glucosidase uptake by Pompe cells as determined on an activity basis is shown in Figure 4-15. The uptake appears to reach saturation near 80-100 μg/ml. This is also indicative of a pathway different from that of LDL as high affinity binding of LDL to its receptor reaches saturation at a much lower level, approximately 20-25 μg/ml (Goldstein et al., 1976).

The time course of α-glucosidase activity taken up by Pompe cells and the inactivation of α-glucosidase in culture media is shown in Figure 4-16. These data suggest that fllowing inactivation in the media the enzyme is reactivated upon uptake of the cells. The apparent difference in the rates of uptake by ^{125}I-α-glucosidase and α-glucosidase activity taken up by Pompe cells (Table 1) may be due to the inactivation of glucosidase in culture media and incomplete reactivation in the cells. As the reactivation process may not be complete, the appearance of α-glucosidase activity in Pompe cells may represent a lower limit of the mass of enzyme taken up assuming the enzyme is stable in the lysosomal environment during the time course of these experiments. This assumption is valid as the half-life of α-glucosidase in liver is 4-5 days (de Barsy et al., 1973), while that of albumin, a non-lysosomal protein, is on the order of hours. Also, in the albumin

Table I: Summary of uptake rates for ^{3}H-sucrose, ^{125}I-albumin, ^{125}I-α-glucosidase and α-glucosidase by Pompe cells.

Substance Taken UP	Concentration in Culture Medium	Uptake Rate
^{3}H-Sucrose	25μg/ml	19ng/mg cell protein/6hr
^{125}I-Albumin	25μg/ml	24ng/mg cell protein/6hr
α-glucosidase	25μg/ml	150ng/mg cell protein/6hr [1]
^{125}I-α-glucosidase	25μg/mg	150-200ng/mg cell protein/6hr
^{125}I-α-glucosidase	15μg/mg	300-400ng/mg cell protein/6hr

[1] Uptake determined by α-glucosidase activity

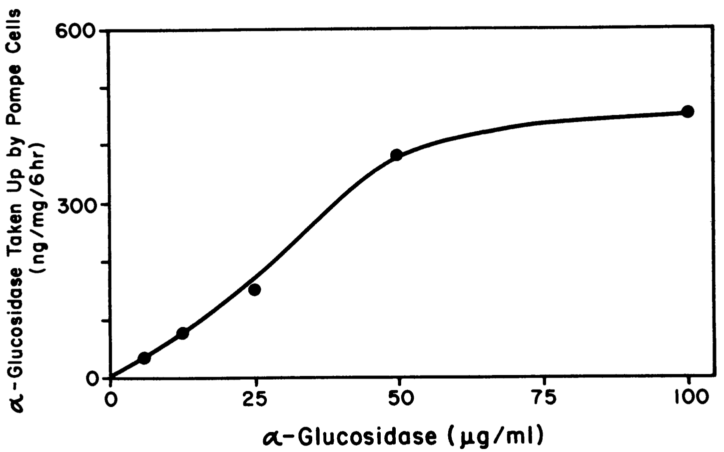


Figure 4-15: The effect of concentration on the uptake of unlabled α-glucosidase by Pompe cells, as determined on an activity basis.

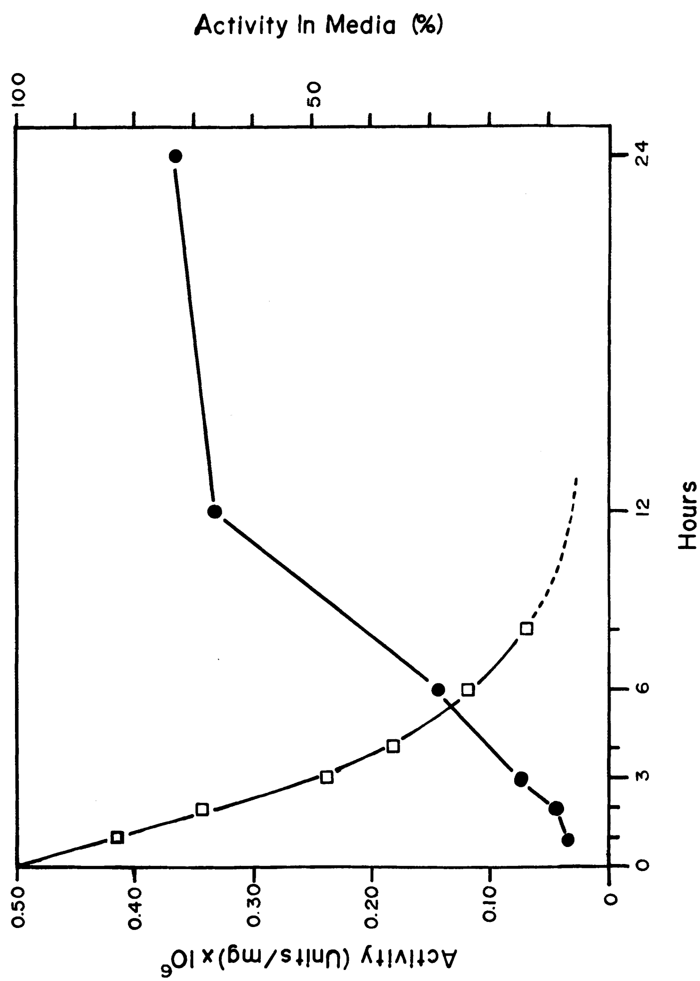


Figure 4-16: Time course of the uptake of α-glucosidase activity (●) by Pompe cells at 25μg/ml (780x10^{-3}U/mg) as measured by activity and inactivation of α-glucosidase activity (□) in cell free media.

endocytosis experiments only 25% of the albumin internalized was degraded in 6 hours suggesting that degradation of lysosomal α-glucosidase would be insignificant in this time period. An alternative explanation of the discrepancy between uptake of α-glucosidase by mass (as measured by ^{125}I-α-glucosidase) and by enzyme activity may be that the liver enzyme preparation consists of several forms, one of which is a high uptake form with diminished enzyme activity. But isoelectric focusing of the purified enzyme from human liver demonstrates four protein bands, all of which have the same specific activity (Murray et al., 1978). This also rules out the possibility that the difference in uptake measurements was due to selective iodination of one isoelectric focusing band with a low specific activity.

The purified α-glucosidase (Sp.Ac.780 x 10^{-3} units/mg) was linked to human LDL, isolated by ultracentrifugation (Havel et al., 1955), using 1-(3-dimethylaminopropyl)-3-ethylacarbodimide. Summation of the enzyme activity in the enzyme-LDL fractions and the free enzyme fractions indicated that all of the initial α-glucosidase activity was recovered. The α-glucosidase-LDL (Sp.Ac. 17.7 x 10^{-3}U/mg) was separated from unlinked α-glucosidase by chromatography on Sephadex G-100, an affinity absorbent of the unlinked enzyme, as shown in Figure 4-17. The decreased specific activity of α-glucosidase in the complex indicates a α-glucosidase protein to LDL protein ratio of 1:40 as there was no loss of enzyme activity. The recovered α-glucosidase which was not linked was unaltered on the basis of specific activity, antiserum inhibition and uptake by Pompe cells. The efficiency, based on recovery of α-glucosidase activity, of the linking reaction is approximately 30% which is presumably due to the fact that the linking is done at a suboptimal pH. The pH used for the linking was necessitated by the inactivation of the α-glucosidase above pH 7.3 and the precipitation of LDL below pH 6.5. The α-glucosidase LDL complex has not been subjected to rigorous characterization. However, in addition to the co-chromatography of the α-glucosidase activity with LDL on Sephadex G-100, the α-glucosidase activity co-electrophoreses with LDL in polyacrylamide gels and the α-glucosidase activity linked to LDL is 3-fold less sensitive to antibody inhibition than the free α-glucosidase. Also the half-life of α-glucosidase activity linked to LDL is 2.5 fold longer than that of free α-glucosidase in culture media without cells indicating a stabilizing effect of linkage to LDL. These properties may be advantageous for in vivo studies.

The uptake, as measured by enzyme activity, of α-glucosidase-LDL and free α-glucosidase by Pompe cells is shown in Figure 4-18.

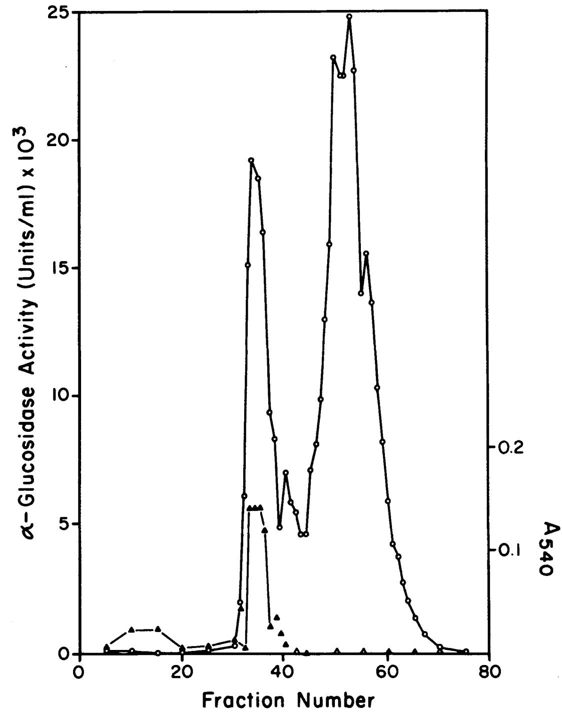


Figure 4-17: The separation of α-glucosidase-LDL and unlinked α-glucosidase on Sephadex G-100. The column (1.5 x 40 cm, Vo = 35 ml) was eluted as a flow rate of 2.0 ml/hr. α-glucosidase activity (0) is resolved into two peaks while cholesterol (Δ) is present in the first peak. Fractions containing α-glucosidase activity and cholesterol were pooled.

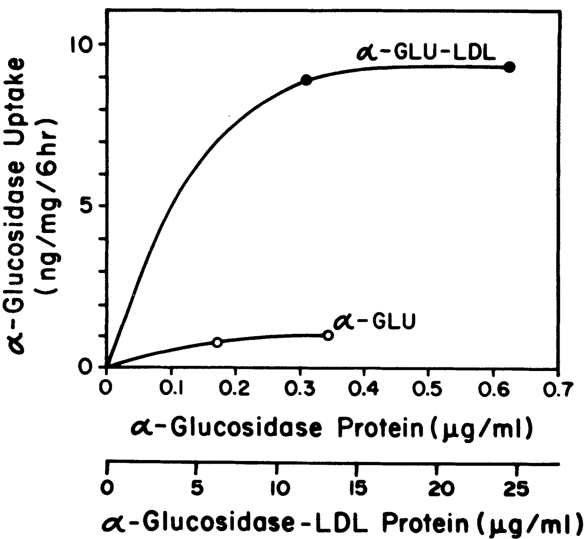


Figure 4-18: Comparison of the uptake of α-glucosidase (O) and the α-glucosidase-LDL comples (●) by Pompe cells. α-glucosidase protein represents the media concentration of both free enzyme and the α-glucosidase moiety of the α-glucosidase-LDL complex (α-glucosidase:LDL protein in the complex = 1:40). α-glucosidase-LDL protein represents the total protein concentration of the α-glucosidase-LDL complex. The α-glucosidase uptake was determined by enzyme activity.

The α-glucosidase-LDL complex is taken up at a rate 9 times that of free enzyme at low concentrations. The greater stability of the α-glucosidase-LDL complex compared to the free enzyme may bias the uptake data in favor of the complex. But this bias would only be a fact of two ($T_{1/2}$ free = 3 hr, $T_{1/2}$ complex = 8 hr, time course of experiment = 6 hr) and does not take into account the reactivation process suggested above. As the uptake of α-glucosidase LDL appears to saturate at 20-25 μg/ml and as the uptake of free enzyme does not saturate until it reaches a concentration of 80-100 μg/ml (Figure 2), one would expect that high enzyme concentrations the uptake of free enzyme may surpass that of α-glucosidase-LDL. But high concentrations of α-glucosidase are not feasible for in vivo replacement regardless of targeting, stability or immune inactivation considerations. By use of the enzyme-LDL complex, α-glucosidase can be delivered to cells more efficiently, i.e. greater uptake at lower concentration. We have treated one patient who became available to us (Williams and Murray, 1980). This was a tremendous undertaking to isolate enough enzyme but we felt it was a unique opportunity for us to learn something. Muscle biopsy 2 days after the first infusion or 5 days after the second infusion did not demonstrate an increase in α-glucosidase activity. Unfortunately, the patient expired 26.5 days after the second infusion. Autopsy tissues were assayed for α-glucosidase activity with 12.7%, 6.3% and 0.5% of normal activity present in muscle, heart and liver respectively. No evidence of antibody formation or immunologic reaction against the enzyme or its carrier could be detected. Although the increases in enzyme activity appear to be real the questions of variability in enzyme activity within an organ may be raised. Resolution of this problem can only be obtained by conducting detailed studies of the α-glucosidase-LDL complexes uptake, distribution and degradation in animals. This work is now continuing in conjunction with Drs. John McC. Howell and Peter Dorling at Murdoch University in Western Australia utilizing their α-glucosidase deficient cows.

DR. de KRUIJFF: In Pompe's disease, is the enzyme not made or is it made and degraded?

DR. MURRAY: The question you are asking is if anyone has demonstrated cross-reacting material to antibodies to the purified enzyme in patient material. I am aware of about six reports in the literature which address this question and only one group has reported the presence of cross-reacting material while the others, including myself, have not been able to detect cross-reacting material.

DR. de KRUIJFF: Is it only the α-glucosidase which is lacking in Pompe's disease?

DR. MURRAY: It is an enzyme with both α-1,4, and α-1,6 glucosidase activities.

DR. de KRUIJFF: Is this the only lysosomal enzyme which is missing in Pompe's disease?

DR. MURRAY: Yes.

DR. BLOBEL: Can you remove the cholesterol and reconstitute the particles?

DR. MURRAY: Brown and Goldstein's group has shown that they can remove the lipid and they have put other lipids in without affecting the binding to the LDL receptor. The nice thing about it is that in theory it could be used as a double carrier.

DR. BLOBEL: And when you said one group had found that there was some inactive α-glucosidase in Pompe's disease. How do you explain that?

DR. ENGEL: The study was on a single patient, but may still be significant. It is possible that one out of 5 patients has residual enzyme activity and it is certainly not inconceivable that there should be no immunological cross reacting material even in infants.

DR. MURRAY: We know that in many other systems proteins that are aberrant are more subject to proteolysis. If you had a defective protein that was synthesized and was not catalytically active, it could also be much more susceptible to degradation and consequently it might never be detected. The other thing is, the heterogeneity within a genetic disease. If you have a genetic disease and you know different patients have the same enzyme defect, unless you know your patients are related, you don't know that they have the same genetic lesion. One could imagine an enzyme containing 100 amino acids and then ask the question of how many of those amino acids are crucial for catalytic activity. I might point out one interesting thing. The α-glucosidase deficient cows do mimic the later onset form of the human disease more so than the infantile form because they don't have the terribly severe cardiac involvement. However John Howell tells me that now that their veterinary cardiologists are interested and involved they are seeing cardiac alterations in these cows much earlier. They've had two animals die quite a bit younger than the others. It looks like those two animals have represented the infantile form of the disease and the others represent the later onset form.

DR. L. GLASER: Did these animals have enzyme when they were young or did they never have enzyme?

DR. MURRAY: The animals are deficient from the beginning.

DR. ENGEL: I don't think one can really answer that question in humans because, if the onset is diagnosed later in life, and at that time they are found to be deficient in the enzyme it is unlikely that they had it earlier.

DR. MURRAY: This is why cows are such a nice model for the study of the onset of the disease in a preclinical situation. One could never do that kind of a study with a human because except in a rare family situation one could never identify a patient prior to some clinical manifestations. The earliest stages that there are any clinical signs in the cows is about 3 months of age with some very subtle cardiac aberrations. The cows are biopsied on the day of birth so that the α-glucosidase deficient ones are identified and can be followed closely.

DR. BLOBEL: Has anyone observed in the lysosomal disease something similar to α-1-antitrypsin deficiency in which you may get storage in the rough ER and not in the packaging of lysosomes? I don't know how you would detect that. Would it stay in the rough ER very much like the α-1-antitrypsin deficiency? Is there any lysosomal disease that has this sort of picture?

DR. MURRAY: Not that I'm aware of.

DR. ENGEL: I really can't say that a precursor is accumulating in the rough ER.

DR. WILLNER: Do patients with these diseases have a lack of the enzyme protein or do they also have cross reacting material?

DR. MURRAY: The cows have cross reacting material. One point on the biosynthesis of these enzymes. In some of the work that Andre Hazilik did in Elizabeth Neufeld's lab with the precursors in fibroblasts, the α-glucosidase does not fit the story as nicely as do the other lysosomal enzymes.

DR. BLOBEL: There is something previous to their precursor. The precursor which we see is already gone when they look because their work is done in vitro.

DR. MURRAY: What I am saying is the α-glucosidase story is not as straightforward as it is for other lysosomal enzymes.

DR. BLOBEL: Is this because they have a high molecular weight?

DR. MURRAY: The molecular weight of the α-glucosidase is about 115,000. An unusual thing about the α-glucosidase is that it can dissociate on SDS gels into two subunits and the sum of the molecular weights of the subunits is greater than that of the native enzyme. We don't know why this is so.

DR. ENGEL: What is the fate of LDL?

DR. MURRAY: As much as 70% of the total LDL pool may be degraded by extrahepatic tissue.

DR. SANDRA: I would like to briefly describe some of the work we are doing in terms of liposome cell interactions. As you know, liposomes are useful as model membranes. Investigators have used liposomes to point out important attributes of membrane structure and function. In addition, liposomes have also been used for a wide variety of other applications. For example, you are probably familiar with their potential use as a pharmacological capsule to entrap drugs and other biologically active materials (Poste et al., 1976 and Liposomes and their uses in Biology and Medicine, 1978). Liposomes have also been useful to modify certain membrane lipids and M. Glaser talked about using fatty acids in this regard. Workers have also used the low density lipoprotein (Cooper, R.A., 1977) especially to modify lipids in red blood cells. Liposomes, as an additional technique to modify membrane lipids have, in principal, the advantage of introducing intact phospholipid molecules rapidly and specifically into the plasma membrane. We're interested in looking at the interaction of liposomes with cells and this is a rather complicated problem. A summary of the kinds of major interactions unilammelar liposomes can undergo with cells (Pagano and Weinstein, 1978) include, first of all, stable absorption. This absorption can be either non-specific, just random adherence of vesicles on to the cell membrane or specific adherence, i.e., to specific sites on the cell membrane. Another mechanism of interaction is endocytosis, in which vesicles can be simply endocytosed and processed like other foreign material. Fusion is a third mechanism of interaction in which the lipids become at least initially incorporated into the cell membrane and the contents of the liposome are introduced into the cytoplasm of the cell. A fourth mechanism is a transfer phenomenon in which individual lipid monomers are transferred between cells and liposomes without any further interaction between the two entities. We have been working on developing various methods of distinguishing which pathway takes place under defined conditions and I could generally summarize what we have found in this regard. This is work that was initiated with R.E. Pagano and colleagues (Huang & Pagano, 1975; Pagano & Takiechi, 1977; Sandra & Pagano, 1979) at the Carnegie Institution. If vesicles are incubated at low temperatures (below their phase

Effect of Lipids on Cell Fusion

transition) with cells, absorption or exchange predomates. For instance, if DPPC dipalmitolphosphatidylcholine, vesicles with a transition temperature of about 36°, are incubated with cells at low temperatures, 4°, the adhesion process will tend to predominate. The fusion process will predominate at higher temperatures with fluid vesicles; for instance, with DOPC dioleoylphosphatidylcholine vesicles, egg yolk phosphatidylcholine vesicles, or binary vesicles containing combinations of such lipids. Fluid vesicles that are incubated at low temperatures, however, tend to undergo an exchange process. Endocytosis can occur at elevated temperatures and therefore it is sometimes difficult to distinguish between fusion and endocytosis.

One type of experiment that we've been carrying out is designed to essentially double label vesicles before interacting them with cells. We can get the internal contents labeled either with a radioactive water-soluble material or a flourescent dye. We can study the introduction into the cell of both the internal contents and the vesicle lipid itself. In a transfer process, only the lipids will be associated with the cell and not the internal contents. We can use this criterion, at least initially, as evidence for this mechanism. In all the other mechanisms, of course, the content with the vesicle lipid will be incorporated into the cell. In endocytosis, however, metabolic poisons may inhibit this process and we can account for this portion of the vesicle uptake. It is possible to build up various criteria to distinguish between these mechanisms of interaction. The pathways I will discuss here primarily involve vesicle-cell fusion and the absorption.

I would also like to discuss the applicability of this vesicle-cell fusion process to myoblast fusion. Little is known about how myoblasts fuse, especially in the living state. The process of cell-cell-fusion is not really a trivial matter, and hasn't been extensively studied in a direct manner because it is difficult to probe this process in a non-perturbing manner (Lipton & Konigsberg, 1972; Kalderon, 1980). We think the following may be a useful way of looking at this phenomenon. One of the things we're in the process of doing is interacting a population of prefusion myoblasts with vesicles that fuse with the cells and thereby introduce their contents into the cell. In this case we were using a flourescent dye, namely 6 carboxyflouresein. It is a water soluble flourescent dye which can be prepared and trapped within the vesicles (Weinstein et al., 1977). The vesicles may then be separated from the dye by appropriate gel chromatography. Having populations of myoblasts which have their internal contents uniformily labeled with a water soluble flourescent dye which is essentially sequestered in the cell, we interact these cells with other pre-fusion myoblasts which are either non-labeled or marked by some other method. This technique should allow us to analyze the kinetics of fusion with respect to the internal tents of myoblasts.

In addition, it's possible to conjugate certain lipids to appropriate hapten groups and thus render them antigenic (Schroit & Pagano, 1978). By using lipid antibodies to haptenated lipids, we can also look at the spread of lipids or surface components over fusing myoblasts in which one population of cells has been preincubated with vesicles composed of such haptenated lipids. For example, we may ask, do they spread at equal rates over the fused myoblasts, are they sequestered, i.e., does a mosaic myotube structure, apply to all components intermixed during the process of fusion (Figure 4-19). These are the kinds of questions that we are looking at presently.

Another area that we are interested in is the vesicle absorption phenomenon and this particular phenomenon actually constituted a preliminary experiment for the above questions concerning cell fusion which we are now doing with flourescent and haptenated liposomes.

Figure 4-20 indicated that myoblasts deprived of calcium in culture do not fuse. With calcium readdition fusion occurs rapidly as a burst of over a period of only about 6 hours; thus cells can be assayed for fusion shortly after the addition of calcium. The first

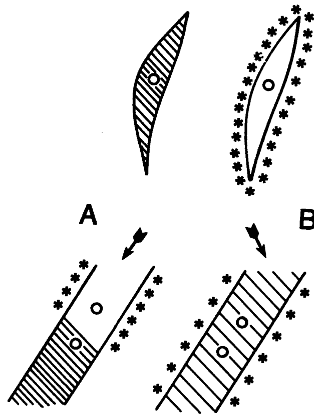


Figure 4-19: Schematic representation of the problem of determining the distribution of cell membrane and cytoplasmic components during myoblast fusion. In the extreme cases, specific components are segregated (A), or randomly dispersed (B) following fusion.

question which needs to be addressed is to determine if vesicles themselves interact, affect cell fusion to alter or inhibit it. We sought a vesicle system which fused with myoblasts and did not adversely affect subsequent fusion of myoblasts with one another so that this could be used as a probe. We therefore interacted a series of phosphatidylcholines to determine if in fact we could utilize any that were essentially neutral in a sense that it didn't affect the subsequent processes we were interested in.

Figure 4-21 shows the consequences on cell fusion of incubating such phosphatidylcholine vesicles with chick embryo myoblasts.

Myoblast Fusion

Figure 4-21a shows cells which are normally fused. In Figure 4-21b, there are myotubes which formed from cells preincubated with DOPC, dioleoylphosphatidycholine. Figure 4-21c shows unfused cells which have been preincubated with DPPC, the solid vesicles. If these cells are either treated lightly with trypsin or incubated briefly at higher temperature (41°), shorter myotubes form, Figure 4-21d. Therefore, this process which is brought about by DPPC vesicles is reversible.

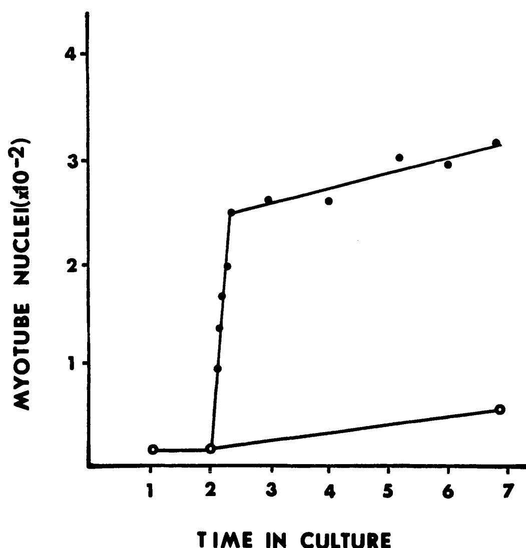


Figure 4-20: The rapid burst of myoblast fusion following readdition of calcium to culture medium. Cells were grown for 48 hours in serum-containing medium formulated without calcium. At this time, medium was changed to either complete medium (●) or fresh low calcium medium (0). Fusion is scored morphologically by determining the number of nuclei within myotubes in equivalent microscopic fields.

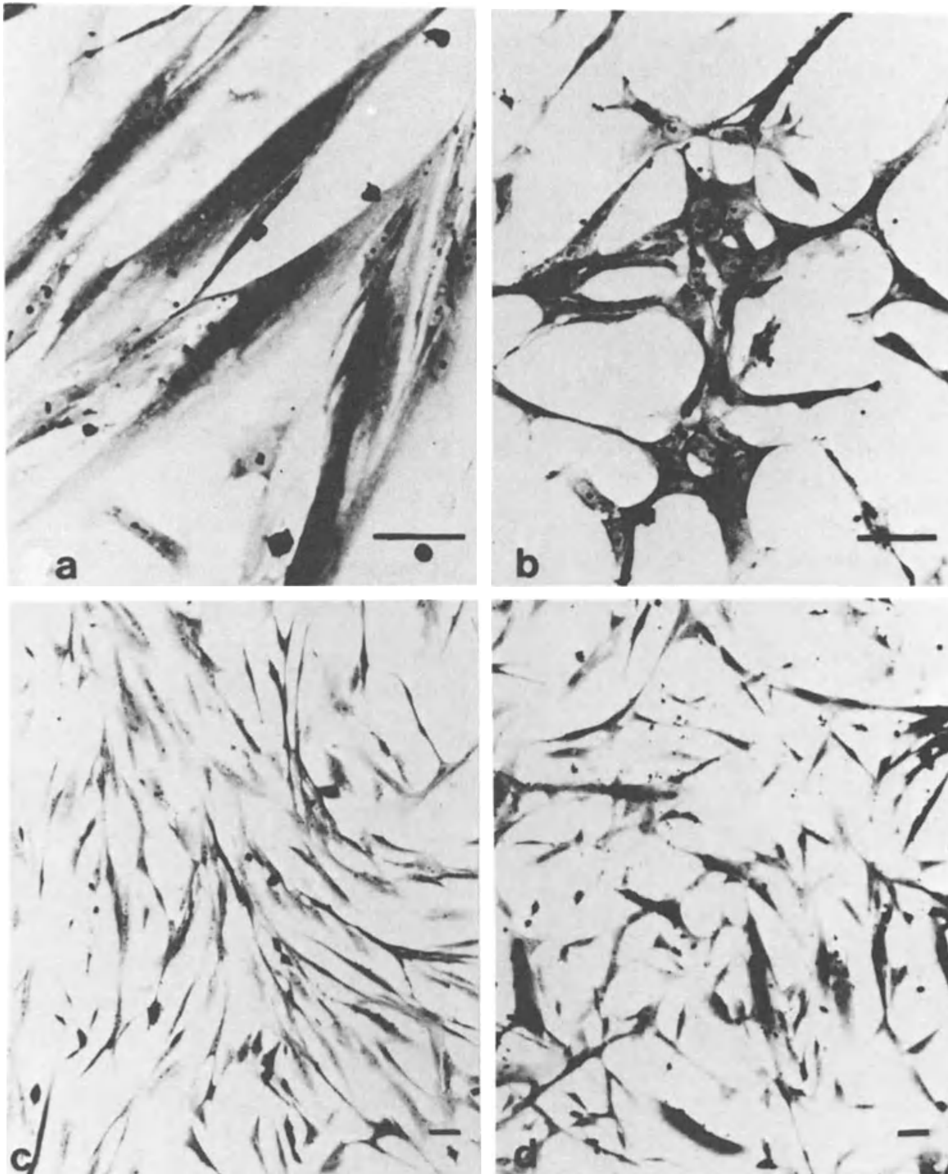


Figure 4-21: Photomicrographs demonstrating the effects of various phospholipid vesicle treatments on muscle cell development. All treatments were made at 48 hours. The cells were changed to complete medium and fixed 24 hours later. Bar is 25 μM. (a) control culture; (b) culture treated with DOPC at 4°C; (c) culture treated with DPPC at 4°C; (d) culture treated with DPPC at 4°C and incubated at 41°C, instead of 37°C.

Table 2 shows the results of incubating four different kinds of vesicles with prefusion myoblasts. DPPC inhibited fusion when the vesicles were incubated with cells at 4° and at 22°, however as previously mentioned, at temperatures slightly above the phase transition 37° this inhibition is alleviated and at 41° it is almost non-existent.

In other words, the inhibition of myoblast fusion is a temperature dependent phenomenon consistent with the notion that the temperature influences the mechanisms of vesicle-cell interaction. On the other vesicle types, DOPC has no effect and is in fact slightly stimulatory of cell fusion as is egg yolk lecithin. DMPC dimyristoylphosphatidylcholine has a transition temperature of 22° and at 4° tends to be inhibitory. At 22° and above, this lipid does not affect the myoblast fusion system.

We are now interested in determining if the DPPC vesicles which inhibit fusion do so by a general sticking all over the cell surface or by sticking to specific points, receptors, or areas of the cell membrane which are perhaps hydrophobic and have an affinity toward these particular lipids. Therefore we have prepared high specific activity [^{3}H] DPPC which was synthesized from radioactive palmitic acid and are interacting ^{3}[H] DPPC vesicles with cells. Previous studies from Pagano's lab have shown that such vesicles interact with other types of cell by adhesion and that the vesicle-cell membrane component can then be isolated on polyacrylamide gels and visualized by autoradiography. In this particular case, specific proteins still bind to radiolabeled lipid, suggesting that perhaps DPPC vesicles bind to discrete proteins. Our plan is to do similar experiments to determine if DPPC vesicles can be used as a probe for potential fusion recognition sites since myoblast fusion is a cell-cell specific interaction process. If we are fortunate and only 1 or 2 discrete proteins are implicated, this would be a feasible approach to consider. Additional evidence of vesicle-cell sticking is derived from other kinds of experiments utilizing scanning electron microscopy in which adherence was directly visualized (Figure 4-22).

Figure 4-22a depicts myoblasts which were treated with DPPC vesicles of 4°C. They are covered round vesicles which are about 600 ångstroms in diameter. The DOPC vesicles are devoid of vesicular structures (Figures 4-22b). This evidence also supports the idea that DPPC vesicles stick to the myoblast surface.

Finally, another line of evidence that DPPC vesicles adhere to the myoblast cell surface is derived from fluorescence microscopy.

Table 2: Effect of phosphatidylcholine vesicles and temperature of vesicle-cell incubation on myoblast fusion.

Vesicle composition	% Fusion			
	Temperature of vesicle-cell interaction			
	4°	22°	37°	41°
DOPC	98	119	117	104
EYPC	93	89	88	109
DPPC	30	32	59	85
DMPC	49	84	102	104

Results are the means of three independent experiments. Variation among experiments was less than 10%. Fusion is expressed as the ratio of the number of myotube nuclei in vesicle treated cultures to that of corresponding non-treated cultures. Culture conditions and treatments were performed as described in Methods.

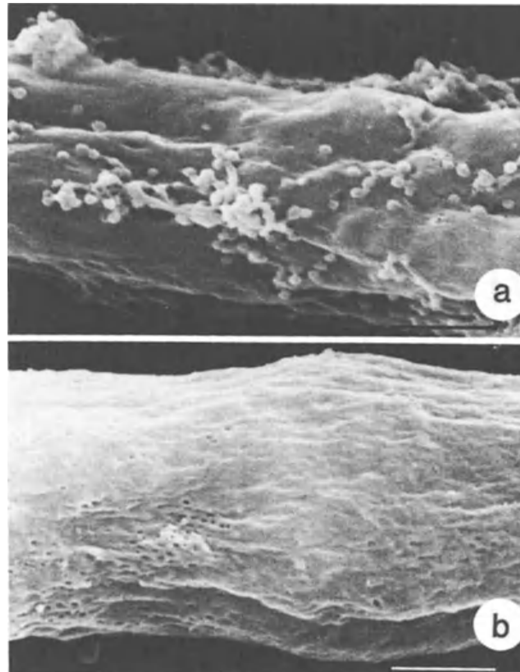


Figure 4-22: Scanning electron photomicrographs of vesicle treated muscle cells. Cells were incubated with vesicles as described in the text and fixed and processed for SEM. Bar is 1 μM. (a) cell treated with DPPC at 4°C; (b) cell treated with DOPC at 4°C.

Myoblasts treated in suspension with DOPC vesicles having their internal aqueous space filled with the fluorescent dye 6-carboxy-fluorescence (Figure 4-23). When such cells are incubated with dye-filled DPPC vesicles at 4°C, only the periphery of the cell is labeled, again suggesting that DPPC vesicles stick to the cell periphery (Figure 4-23).

In summary, there are several observations from different lines of evidence which suggest that DPPC vesicles stick to myoblasts. Upon doing so, cell fusion is adversely affected. This process is reversible and is potentially useful as a probe to study specific sites which may be involved in the cell-cell interaction of myoblast fusion. We hope to also use this approach to study normal fusion because certain fluid vesicles (e.g. DOPC) do not inhibit fusion. These vesicles may be useful non-perturbing marker probes.

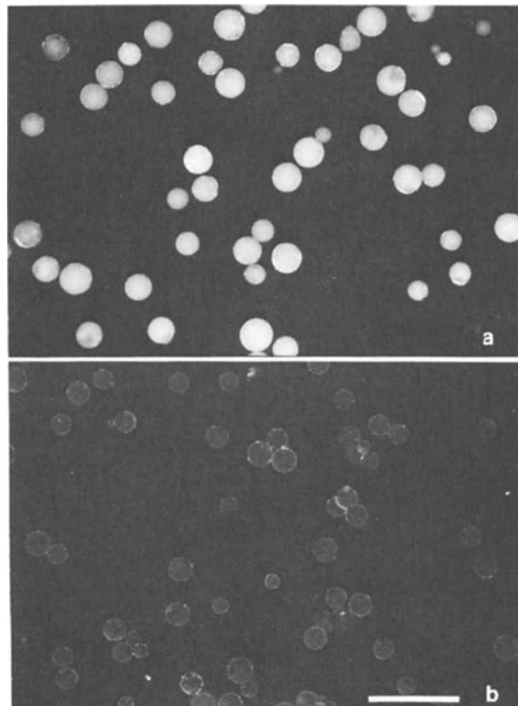


Figure 4-23: Fluorescence micrograph of myoblasts prepared as above and incubated with 6-CF filled DPPC vesicles. Fluorescence is distributed generally throughout the cell interior. B. Fluorescence micrograph of isolated chick myoblasts incubated at 37° for 15 min. with 6-CF filled (20 mM) DPPC vesicles. Vesicle fluorescence is limited to cell periphery in all focal planes. Magnification bar = 20 μM.

Intracellular Protein Topogenesis

DR. BLOBEL: A cell contains millions of protein molecules. These are steadily being synthesized and degraded. At homeostasis, a given species of protein is represented by a characteristic number molecules that is kept constant within a narrow range. Very little is known about the cell's accounting procedure, i.e., how it balances and controls biosynthesis and biodegradation.

An important aspect of biosynthesis (Blobel et al., 1979; Blobel, 1980) as well as biodegradation (Blobel, 1978) is the intracellular topology of proteins. Many protein species spend their entire life in the same compartment in which they are synthesized, others have to be translocated across the hydrophobic barrier of one or two distinct cellular membranes in order to reach the intracellular compartment or extracellular site where they exert their function. Numerous protein species have to be integrated asymmetrically into distinct cellular membranes. For many proteins this requires partial translocation, i.e., selective transfer of one or several distinct hydrophilic or charged segments of the polypeptide chain across the hydrophobic barrier of one or two intracellular membranes. Following complete or partial translocation across a translocation-competent membrane(s), sub-populations may undergo further "post-translocational" traffic. Soluble or membrane proteins may be shipped in bulk or by receptor-mediated processes from a translocation-competent donor compartment to a translocation-incompetent receiver compartment. This post-translocational traffic may be unidirectional (in which case the protein ends up as a permanent resident of a particular cellular membrane) or may follow a cyclic pattern between distinct cellular membranes (e.g., recycling of receptors).

The collective term "topogenesis" has been introduced (Blobel, 1980) to encompass protein translocation (partial or complete) across membranes as well as subsequent post-translocational protein traffic. Not included in these processes that define topogenesis are distinct traffic patterns that may be required for protein degradation. Theoretical considerations on the topology of protein degradation have been presented elsewhere (Blobel, 1978) and will not be dealt with here: in essence, these considerations argue for the existence of three (animal cells) or even four (plant cells) separate compartments for protein degradation, each containing a distinct set of proteases. Detailed proposals have been made also for protein topogenesis (Blobel, 1980). The essence of these proposals is that the information for intracellular protein topogenesis resides in discrete "topogenic" sequences that constitute a permanent or transient part of the polypeptide chain. The repertoire of distinct topogenic sequences was predicted to be relatively small because many different proteins would be topologically equivalent, i.e., targeted to the same intracellular address.

The information content of topogenic sequences would be decoded, each by a distinct effector. Four types of topogenic sequences were distinguished: (1) *Signal* sequences initiate translocation of proteins across specific membranes. They would be decoded and processed by protein translocators that, by virtue of their signal sequence-specific domain and their unique location in distinct cellular membranes, effect unidirectional translocation of proteins across specific cellular membranes. *Stop-transfer* sequences interrupt the translocation process that was previously initiated by a signal sequence and, by excluding a distinct segment of the polypeptide chain from translocation, yield asymmetric integration of proteins into translocation-competent membranes. (3) *Sorting* sequences would act as determinants for post-translocational traffic of subpopulations of proteins, originating in translocation-competent donor membranes (and compartments) and going to translocation-incompetent receiver membranes (and compartments). A specific example for sorting, namely the separation of secretory from lysosomal proteins is discussed. (4) *Insertion* sequences initiate unilateral integration into the lipid bilayer without the mediation of a distinct protein effector.

An attempt is made here to amplify some of these previous proposals (Blobel et al., 1979; Blobel, 1980) and to discuss some of the recent experimental data that are relevant to these proposals.

Signal Sequences

Table 3 lists the biological membranes or membrane pairs (see h and i) that have been proposed (Blobel, 1980) to be endowed with a transport system (translocator) for unidirectional translocation of nascent or completed polypeptide chains. The conjecture was made (Blobel, 1980) based on evolutionary relationships between various cellular membranes (Figure 4-24), that present translation-coupled (co-translational) translocation systems (Table 3, a-d) are derived from a common ancestral system and that they might be highly conserved (Blobel, 1980). A high degree of conservation has indeed been demonstrated for the RER translocator within the animal and plant kingdoms (Shields and Blobel, 1977). Recently it has been demonstrated (Talmadge et al., 1980) that a signal sequence addressed to the RER can be decoded by the putative signal receptor of the prokaryotic plasma membrane and be cleaved correctly by signal peptidase of the prokaryotic plasma membrane.

It should be emphasized that signal sequences only initiate the translocation process and that they do not guarantee its completion nor do they assure completion of subsequent post-translocational traffic. In fact, chain translocation can be prematurely terminated by a distinct "stop-transfer" sequence.

Table 3

Cellular membranes proposed to be endowed with a transport system (translocator) for the unidirectional translocation of nascent or newly synthesized proteins (Blobel, 1980).

Mode of Translocation		Membrane	Code
co-translational	a.	prokaryotic plasma membrane	PPM
	b.	inner mitochondrial membrane	IMM
	c.	thylakoid membrane	TKM
	d.	rough endoplasmic reticulum	RER
post-translational (across one membrane)	e.	outer mitochondrial membrane	OMM
	f.	outer chloroplast membrane	OCM
	g.	peroxisomal membrane	PXM
post-translational (across two membranes)	h.	mitochondrial envelope	MEN
	i.	chloroplast envelope	CEN

Each of the translocation-competent membranes (1) listed here (a-i) is proposed to contain only one distinct "translocator" (in multiple copies). Each translocator responds to one type of signal sequence. Translocation can proceed across a single membrane (a-g), or two membranes (h-i), co-translationally (a-d), or post-translationally (e-i). Suggested abbreviations for these translocation-competent membranes might serve as useful codes. For example, a signal sequence (Si) addressed to the rough endoplasmic reticulum (RER), to the chloroplast envelope (CEN), etc., might be designated Si(RER, Si(CEN), etc. Likewise, a particular signal receptor (SiR), or signal peptidase (SiP) could be classified as SiR(RER), SiR(CEN), or SiP(RER), SiP(Cen), etc.

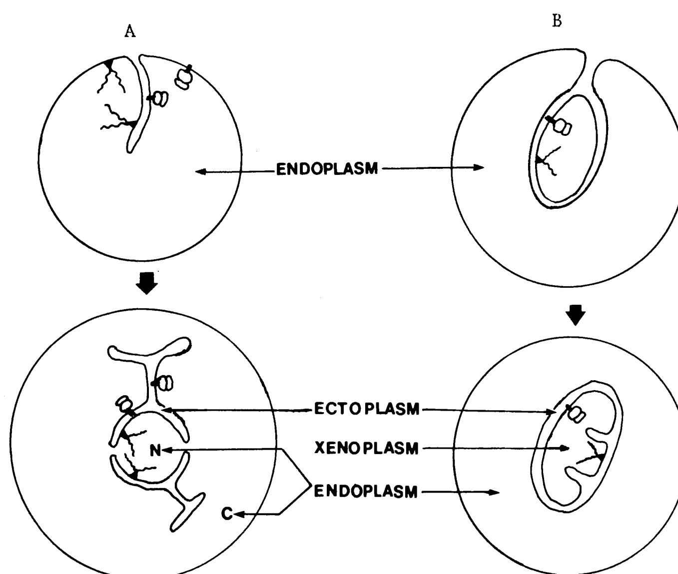


Figure 4-24a-b: Schematic illustration of the evolution of intracellular membranes and compartments (Figure 4-25). (A) Aggregation of certain membrane functions in the plane of the pluripotent plasma membrane; nonrandom removal of these functions from the plasma membrane by invagination and fission resulting in the formation of a nuclear envelope (pore complexes omitted) continuous with the endoplasmic reticulum (rough and smooth) and generating an ectoplasmic compartment. The endoplasmic compartment is thereby subdivided into nucleoplasm (N) and cytoplasm (C). Note, however, that N and C remain connected via nuclear pores that do not present a membraneous barrier. Other intracellular membranes that are distinct from the endoplasmic reticulum, such as lysosomal, peroxisomal and Golgi complex membranes, could have also developed by invagination from the plasma membrane or could be outgrowth of the endoplasmic reticulum. (B) Symbiotic capture of another cell generating an additional xenoplasmic compartment. Green plant cells have two such xenoplasmic compartments (mitochondrial matrix and chloroplast stroma. Only the inner mitochondrial membrain. and the inner chloroplast membrane (including derived thylakoid membrane) would be of xenoplasmic origin, whereas the outer mitochondrial and chloroplast membrane would be of orthoplasmic origin, like all other cellular membranes. The proposed terminology may be useful for describing the precise topology of IMPs (Figure 4-25). For example, monotopic IMPs of the thylakoid membrane may be exposed ectoplasmically (toward the intradisc space) or xenoplasmically (toward the stroma); bitopic IMPs of the outer mitochondrial membrane have an ectoplasmic and endoplasmic domain.

It might also be prematurely terminated by other less specific sequence constellations which are not normally translocated (e.g., those occurring in "hybrid" proteins (Emr et al., 1980).

It has been assumed that the boundary for an amino terminal signal sequence is more or less defined by its signal peptidase cleavage site. This, however, has recently been called into question in the case of the signal sequence addressed to the prokaryotic plasma membrane. It has been argued that the information necessary for translocation extends beyond the cleavage site into the mature portion of the protein (Moreno et al., 1980) or may require additional information that is expressed only by the completely synthesized chain (Koshland and Botstein, 1980). However, the experimental evidence is not definitive in either of these two studies. Evidence in the first case (Moreno et al., 1980) rests on data with a hybrid protein for which the translocation process may have been properly initiated by a normal signal sequence but subsequently may have been aborted by some other unspecific sequence that is not permissive with the translocation process. Evidence in the second case (Koshland and Botstein, 1980) rest on _in vivo_ experiments with UV-radiated bacteria. Thus, the conjecture that the signal peptide portion of the nascent chain contains all the information necessary to function as a signal sequence to initiate translocation has not been invalidated by these experiments. In fact, the question could be raised whether the amino terminal cleaved signal peptide contains more sequence information than is necessary to initiate chain translocation and whether some of the signal peptide's amino terminal or carboxy terminal residues could be trimmed without affecting its interaction with the putative signal receptor to initiate chain translocation.

Elegant genetic studies (Talmadge et al., 1980; Emr et al., 1980) on the signal sequence addressed to the prokaryotic plasma membrane have provided strong support for the concept that the information for initiating the translocation process does indeed reside in the signal peptide. Furthermore, these studies have also revealed (Emr et al., 1980) that the apparent degeneracy in the primary structure of the signal sequence has its limits: replacement of hydrophobic residues (that are clustered in the center region of the signal sequence) by charged residues prevents translocation; or, in the case of _E. coli_ pre-lipoprotein, substitution by Gly by Asp in position 14 of the 20-residue-long signal peptide results in an abolition of cleavage but not of translocation (Lin et al., 1978).

There are numerous examples of proteins that are translocated without cleavage of a signal sequence (Bonatti, Blobel, 1979). In the case of hen ovalbumin it has been shown that the uncleaved signal sequence might be located not at the amino terminus but in the central region of the molecule (Lingappa et al., 1979).

The primary structural features have been determined and exemplified by numerous proteins (Blobel et al., 1980) in the case of Si (RER) and Si (PPM). The primary structure for Si (CEN) has been elucidated so far only for one protein (Schmidt et al., 1979). Primary structure information is not yet available for signal sequences addressed to IMM, TKM, OMM, OCM, PXM and MEN (Table 1).

As already mentioned, the primary structure of the signal peptide for RER and PPM reveals common features, a finding that supports the evolutionary relationship between these two membranes (Figure 4-25). Although there is variability in the length of these signal peptides (total number of amino acid residues varies 15-30) their common feature appears to be a stretch of hydrophobic residues in the central region with charged or hydrophilic residues on either side of this hydrophobic core. Moreover, the penultimate residue, in all cases, is a small side chain amino acid (Gly, Ala, Cys, Ser, Thr). As expected the primary structure of the signal sequence addressed to CEN (Schmidt et al., 1979) differs considerably from that addressed to RER or PPM. However, the primary structure of other examples needs to be established before the distinct features of Si (CEN) can be delineated.

Although detailed models for the mechanism of co- and post-translational translocation have been formulated (Blobel, 1980), little is known about the various translocators (Table 1). Attempts to isolate and characterize them have so far been reported only for the RER translocator. Salt extraction (Warren and Dobberstein, 1978; Jackson et al., 1980; Walter and Blobel, 1980) as well as controlled proteolytic digestion (Walter et al., 1979; Meyer and Dobberstein, 1980; Meyer and Dobberstein, 1980) of isolated microsomal vesicles has been shown to abolish their protein translocation activity either partially or completely. Translocation activity can be fully restored to the salt- or protease-extracted vesicles upon readdition of a salt or protease extract (Warren and Dobberstein, 1978; Jackson et al., 1980; Walter and Blobel, 1980; Walter et al., 1979; Meyer and Dobberstein, 1980; Meyer and Dobberstein, 1980). The active components of the protease extract (Meyer and Dobberstein, 1980) as well as of the salt extract (Walter and Blobel, 1980) have been purified. It remains to be investigated, however, how these components function in initating the translocation process.

Stop-transfer Sequences

A "stop-transfer" sequence was proposed to contain the information required to interrupt the process of chain translocation that was previously initiated by a signal sequence (Blobel, 1977; Lingappa et al., 1978; Chang et al., 1979). Because translocation of the polypeptide chain (Blobel, 1980) is likely to proceed sequentially and asymmetrically in both

co-translational and post-translational translocation, stop-transfer sequences are effective means for asymmetric integration into the membrane of certain integral membrane proteins (IMPs) by either mode of translocation (Table 1).

The sequence features that constitute the stop-transfer sequence remain to be defined. The stop-transfer sequence may not simply be that stretch of ∿25 primarily hydrophobic residues which is typical of the transmembrane portion of bitopic IMPs and which might be envisioned to act as a stop-transfer sequence by virtue of being non-permissive with the translocation process. There are, e.g., viral bitopic IMPs which possess stretches of at least 28 hydrophobic residues in their ectoplasmic domain (Scheid et al., 1978; Gething et al., 1978). Since this domain is translocated it is clear that a long stretch of hydrophobic residue *per se* is not sufficient to stop the translocation process.

There could exist as many translocator-specific stop-transfer sequences as there are translocator-specific signal sequences. On the other hand, there could be only one stop-transfer sequence addressed to one component common to all translocators, e.g., phospholipid molecules.

It has been known for a long time that certain IMPs can insert into a lipid bilayer spontaneously, i.e., by a mechanism that is not receptor mediated. The existence of specific insertion sequences has therefore been postulated (Blobel, 1980). These insertion sequences function to anchor proteins to the hydrophobic core of the lipid bilayer (Figure 4-25). This anchorage is not accompanied by the translocation across the membrane's lipid bilayer of charged domains of the polypeptide chain. The latter can be achieve presumeably only by a signal sequence in a receptor-mediated process (Blobel, 1980).

Insertion Sequences

At this point it is necessary to comment on an alternative proposal (Wickner, 1979) that argues that the cleaved amino terminal sequence extension of bacteriophage fl (or M 13) coat protein (CP) functions not as a signal sequence, as proposed earlier (Chang et al., 1979) but functions essentially as an insertion sequence that "folds" this bitopic IMP into the lipid bilayer of PPM by a receptor-independent mechanism. Although the cleaved amino terminal sequence extension of the nascent CP is structurally analogous to all the other RER or PPM signal sequences it will be possible to validate or to invalidate the receptor-independent mechanism of integration of CP into PPM in a definitive manner only after identification and characterization of the putative PPM translocator has advanced to the same analytical level as that achieved for its RER counterpart.

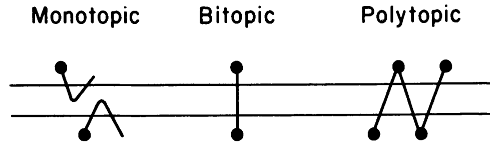


Figure 4-25: Classification of integral membrane proteins (IMPs) as monotopic, bitopic and polytopic (Blobel, 1980). The hydrophobic boundary of the lipid bilayer is indicated by two parallel lines. Dots on polypeptide chains indicate major hydrophilic domains. The hydrophilic domain of a monotopic IMP is exposed only on one side of the lipid bilayer. A hydrophobic domain is indicated to anchor the polypeptide chain to the hydrophobic core of the lipid bilayer. A monotopic IMP may contain several hydrophilic and hydrophobic segments alternating with each other (not indicated here). All hydrophilic domains, however, are unilaterally exposed. The polypeptide chain of bitopic IMPs spans the lipid bilayer once and contains a hydrophilic domain on opposite sides of the membrane. In variants of biotopic IMPs (not indicated) the bilateral hydrophilic domains could be further subsegmented by interspersed hydrophobic domains that are capable of monotopic integration. The polypeptide chain of polytopic IMPs spans the membrane more than once and contains multiple hydrophilic domains on both sides of the membrane. The existence of polytopic IMPs remains to be demonstrated. Two structurally monotopic IMPs located on opposite sides of the membrane could interact via their hydrophobic anchorage domain and form a functionally bilateral ensemble.

As is the case for the stop-transfer sequence, the structural features of an insertion sequence remain to be defined. It is conceivable that there are several unique insertion sequences that can distinguish lipid composition and therefore insert only into specific membranes. On the other hand, the specificity of insertion into distinct membranes may be largely dictated by protein-protein interaction (i.e., by an affinity of a protein to be inserted to another IMP).

Sorting Sequences

Sorting sequences were defined as those discrete portions of the polypeptide chain that act as determinants for post-translocational traffic and that are shared by proteins with an identical travel objective (Blobel, 1980). The structural features and the number of distinct sorting sequences remain to be defined. The cell's machinery which decodes sorting sequences and effects displacement remains to be identified. In eukaryotic cells, the RER is one of the most important origins for post-translocational traffic of a large variety of soluble and integral membrane proteins. Let us consider here the case only of soluble proteins. Recent data (Erickson and Blobel, 1979; Hickman and Neufield, 1972) strongly suggest that at least two sub-populations of soluble proteins are found mixed within the cisternae of the RER, namely lysosomal and secretory proteins. Thus, lysosomal enzymes have been shown (Erickson and Blobel, 1979; Erickson et al., in preparation) to be synthesized with a signal sequence addressed to the RER that is structurally and functionally indistinguishable from that common to secretory proteins. Besides secretory and lysosomal proteins the intracisternal space on the RER may contain other soluble proteins that are neither lysosomal nor secretory but that are either permanent residents or in transit to be routed to other membrane-bounded compartments. Would each species of these groups of soluble proteins possess a common sorting sequence? And, would there be a corresponding number of sorting sequence-specific receptors in the RER that effects the distribution of these proteins.

In considering the problem of sorting of soluble protein from the cisternae of the RER into other compartments it might be useful to search for precedents and analogies. The plasma membrane, for example, is able to "sort" extracellular proteins into intracellular compartments by a variety of routes and mechanisms. There is unspecific uptake of soluble protein (pinocytosis), probably as part of an exchange of liquid quanta between the extracellular compartment and a distinct intracellular vesicular compartment. The only requirement for a protein to be transported this way might be for it to be water soluble rather than to possess a sorting sequence. Liquid phase transport between these two compartments is probably accomplished by a set of one or several distinct IMPs which, by fission-fusion processes, shuttle between two distinct membranes

and which in the process of doing so, may not mix (by lateral diffusion) with other IMPs of the two membranes.

Analogous bidirectional transport systems for water soluble proteins may exist between the RER and specific cisternae of the Golgi, between specific cisternae of the Golgi and the extracellular space or between other distinct intracellular compartments. Each of these transport systems would likely be represented by a unique set of IMPs. If such a transport system would indeed exist between the cisternae of the RER and a specific "downstream" cisterna (which, unltrastructurally, could be part of the Golgi complex) then any water soluble protein in the cisternae of the RER can potentially undergo downstream transport, unless it is immobilized by binding to a constitutive IMP (permanent resident) of the ER membrane. It is proposed here that secretory proteins might be displaced from the RER by such a liquid phase transport system.

How about lysosomal proteins? Some time ago, Neufeld (Hickman and Neufeld, 1972; Neufeld et al., 1977) proposed that lysosomal enzymes are endowed with a recognition marker that is common to all lysosomal enzymes and that functions in receptor-mediated sorting. Considerable evidence has since accumulated in support of this concept (Hasilik and Neufeld, 1980; Fischer et al., 1980). The recognition marker appears to be phosphorylated mannose residues of the Asn-linked core-sugars (Kaplan et al., 1977; Sando and Neufeld, 1977; Tabas and Kornfeld, 1980). Because many secretory proteins also contain Asn-linked core sugars (but so far have not been detected to contain phosphorylated mannose residues) and because both secretory and lysosomal proteins are co-segregated (Erickson and Blobel, 1979; Erickson et al., in preparation) in the cisternae of the RER, it is likely that the mannose modifying enzyme(s) recognize a protein sequence feature that is common to all (and unique for all) lysosomal enzymes. This sequence feature therefore would be an example of a sorting sequence. More recently (Fischer et al., 1980; Kaplan et al., 1977; Sando and Neufeld, 1977; Tabas and Kornfeld, 1980; Sly and Stahl, 1978) has proposed that receptor-mediated sorting of lysosomal proteins from secretory proteins occurs primarily upstream, at the level of the RER.

If secretory and lysosomal proteins are displaced from the RER, each into a separate and unique downstream compartment, secretory proteins by liquid phase transport and lysosomal proteins by receptor-mediated transport, one could conceive of a variety of possibilities for malfunctioning. For example, mutations in secretory proteins resulting in their having a lower solubility product might cause accumulation of the secretory protein within the cisternae of the RER; mutations in the sorting sequence of a lysosomal enzyme might result in its secretion. Alterations in the receptor might result in secretion of all lysosomal enzymes, etc.

How about other soluble proteins that may be permanently mobilized by binding to constitute IMPs of the RER or of further downstream compartments. Immobilization may occur by protein-protein interaction, distinct in each case and therefore not mediated by a sorting sequence, at least not as defined here. On the other hand, it is conceivable that a permanently immobilized recpetor could act as a ligand for several distinct species of proteins by virtue of a sequence feature that they share. This common sequence feature would constitute a sorting sequence.

Multiple Topogenic Sequences

The occurrence of multiple topogenic sequences, such as a signal sequence and a sorting sequence for a given species or group of proteins has already been described above in the case of bitopic IMPs (signal sequence and stop-transfer sequence). Figure 4-26 illustrates a further application of the concept of multiple topogenic sequences to the problem of how to achieve asymmetric integration of the polypeptide backbone of the IMPs into the lipid bilayer. Although the precise orientation of the polypeptide backbone with respect to the lipid bilayer is unknown for most species of IMPs, the proposed (Blobel, 1980) hypothetical schemes of multiple topogenic sequences can explain any one orientation by what essentially are a limited number of highly redundant mechanisms.

Pleiotopic Proteins

Pleiotopic proteins have been defined (Blobel, 1980) as proteins which are similar in structure and function but differ in topology. The presence of absence of topogenic sequence or the acquisition of another topogenic sequence would be an effective means to achieve several distinct cellular localizations of a given species of protein. Pleiotopic proteins may be represented by proteins that exist, for example, as a cytoplasmic form (no topogenic sequence) and a secreted form (+Si(RER)); or as a secreted for (+SI(RER)) and a membrane-bound form (several possibilities, see Figure 4-26) or as a cytoplasmic form (no topogenic sequence) and a mitochondrial matrix form (+Si(MEN)) and a peroxisomal form (+Si(PXM)), etc.

EDITORIAL SUMMARY

The significance of the non-bilayer configuration to membrane fusion and transmembrane transport was discussed together with the mechanisms responsible for bringing about the shift from bilayer H_{II} phase and vice versa. A strategy was presented for targeting administered enzyme to peripheral tissues including muscle cells while bypassing the reticuloendothelial system, presented together, with a preliminary report of the administration of α-glucosidase to a patient with Pompe's disease.

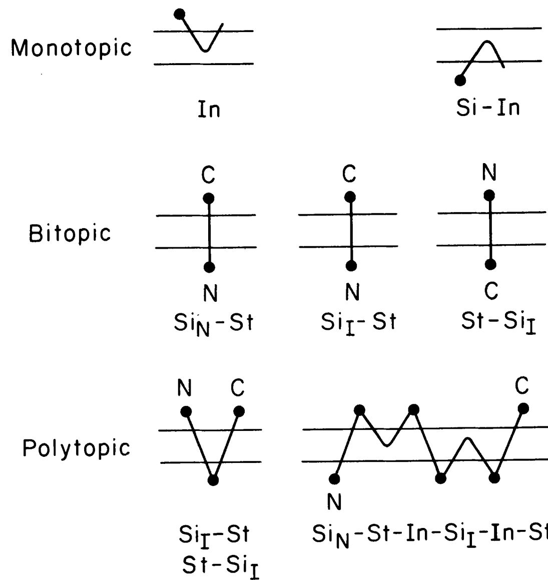


Figure 4-26: Program of topogenic sequences for the asymmetric integration into membranes of some representative examples of monotopic, bitopic and polytopic integral membrane proteins (IMPs) (Blobel, 1980). Hydrophobic boundary of lipid bilayer is indicated by two parallel lines, with upper line facing the protein biosynthetic compartment. Dots represent major hydrophilic domain which, when indicated, contain amino (N) or carboxy (C) terminus of the polypeptide chain. Topogenic sequences are: insertion sequence (In), signal sequence (Si) and stop-transfer sequence (St). Si_N and Si_I indicate aminot terminal and internal signal sequence, respectively. Examples given here (except for monotopic IMP on upper left) are for co-translational integration into PPM, IMM and TKM as well as for post-translational integration into PXM, OMM, OCM, IMM (using Si(MEN)) and ICM/TKM (using Si(CEN)). An attempt has been made to list topogenic sequences in order of their location along the polypeptide chain starting from the amino terminus. The problems encountered in predicing the order relate to uncertainties as to the order of chain translocation. In particular in the case of an internal signal sequence (Si_I) there are several possibilities depending on the order or translocation (Lingappa et al., 1979). The orientation of a polytopic IMP such as indicated at the lower right is entirely hypothetical and is illustrated here only to indicate how such a polypeptide chain could be integrated into the membrane by a program of multiple topogenic sequences.

The role of liposomes in transmembrane transport and in cell fusion was also discussed especially with regard to myoblast fusion. Other aspects of transmembrane transport of proteins were considered from the perspective of the signal sequence theory of Blobel.

CHAPTER 5

MEMBRANE ALTERATIONS IN ERYTHROCYTES AND CULTURED FIBROBLASTS IN NEUROMUSCULAR DISEASES

DR. WILLNER: Studies of erythrocyte membranes in muscular dystrophy have recently been reviewed (Rowland, 1980 and Plishker and Appel, 1980). The variety and number of published studies may be testimony to technical problems only partially solved or not addressed by many of them. Specifically, nearly 30 different abnormalities have been found by at least one laboratory in the Duchenne red cell. (Rowland, 1980). However, many reported abnormalities could not be verified by other workers the number of reported abnormalities verifed in more than one laboratory is limited to 2; calcium ATPase is increased and the properties of cholinesterase are abnormal.

Conflicting Findings of Abnormalities in Erythrocyte Membranes

DR. L. GLASER: Isn't it true you can at least conclude that there is a membrane defect?

DR. WILLNER: Perhaps there is. Certainly surface membranes of muscle (Rowland, 1976), red cells and lymphocytes (Verrill et al., 1977) are likely to be abnormal in Duchenne dystrophy. But the number of apparent abnormalities is confusing, and this confusion obscures the appropriate direction for future research. It is entirely possible that the observed changes in membrane structure and chemistry are all secondary and that these studies are not leading us substantially closer to identification of a genetic defect. Inconsistencies between the laboratories are partly attributable to non-uniformity of methods; the use of calcium chelators may, for example, be an important variable (Roses, 1979).

DR. L. GLASER: Is it clear that there is a single genetic defect in Duchenne dystrophy?

DR. WILLNER: A suggestion of genetic heterogeneity appeared in a paper Alan Emery published last summer (Emery et al., 1979), which reported that in those boys with lower intelligence the course of the myopathy was less rapid and the rate of decline of CPK with age is less than in those who are not mentally retarded, implying there may be genetic heterogeneity. Until we have a reliable biochemical marker, we may not know how heterogeneous Duchenne dystrophy really is. Muscle seems to have a limited number of ways to degenerate.

The situation isn't much better in myotonic dystrophy (Table 1). Dr. Allen Roses now suggests that his observation of decreased experimental phosphorylation of band 3 is secondary to increased band 3 phosphorylation *in vivo*. His most recent work suggests that membrane phospholipid metabolism is abnormal.

Studies of isolated sarcolemma membrane have run into the problems of isolation and characterization of sarcolemma as well as the difficulties encountered in studies of erythrocytes; to my knowledge, no report of an abnormality has been followed by a confirming report.

DR. L. GLASER: Are any changes in myotonic erythrocytes confirmed?

DR. WILLNER: As in the case of Duchenne dystrophy, no observation has been faithfully replicated by another laboratory.

DR. PARK: Nevertheless, it is interesting that the cells themselves are more phosphorylated after their isolation. Even though maybe the interpretation is wrong, the differences are very apparent.

New Biophysical Studies of Erythrocytes

As to the reproducibility of the RBC results, there has indeed been some discrepancy in the data from various laboratories on red blood cells and the red blood cell membranes. This could be due to a number of things: first, it is very hard to get these cells immediately after they are drawn from the patient. There is often a time lag and sometimes samples are not put in the refrigerator. Since cells are handled in different ways, one might predict that the results would be different. As RBC's haven't been used for a long time in this area, the procedures are not uniform. However, if one does things in a systematic way, I think that the results, at least in our own hands, become quite reproducible.

Table 1

CHANGES IN THE MYOTONIC DYSTROPHY ERYTHROCYTE

	Yes	No
Abnormal membrane fluidity	1 - Butterfield et al, 1974	2 - Chalikan & Barch, 1980 Gaffney et al, 1980
Abnormal Na^{+}-K^{+} ATPase or monovalent cation transport	1 - Hull & Roses, 1976	1 - Festoff, 1977
Decreased Ca^{+2}-promoted K^{+} efflux	1 - Appel & Roses, 1976	
Increased Ca^{+2} influx and efflux	1 - Plishker et al, 1978	
Decreased protein kinase	1 - Appel & Roses, 1977	
Abnormal temperature response of membrane phosphorylation	1 - Vickers et al, 1979	
Decreased band 3 phosphorylation	1 - Roses & Appel, 1974	
Increased acyl CoA synthetase	1 - Ruitenbeek & Scholte, 1979	
Increased unsaturated fatty acids	1 - Ruitenbeek, 1978	
Abnormal phospholipid metabolism	1 - Greg et al, 1980	

Roses, Appel and Butterfield made significant contributions by introducing the red blood cell of Duchenne patients into the experimental field of muscular dystrophy. First, they had the idea that muscular dystrophy was not just a muscle disease but that all membranes in the body might be affected. This was a very important notion which was not very popular at the time. I think that it has been a productive concept in terms of the kinds of experiments which people planned. If one could find abnormalities in the red blood cell, it might give you some clues as to the etiology and the pathogenesis of dystrophy. Secondly, red blood cells are readily available for investigation. Moreover, these cells could be used in diagnosis to detect mothers and sisters who might be carriers. One could also look at fetal blood cells in order to detect abnormalities in the child when the mother is a known carrier. All this is very important for research in dystrophy, because it is difficult to get muscle tissue. Biopsies are not taken very frequently. People don't like to have them done and the physicians don't feel that they can do them very often. Moreover, it is hard to get muscle biopsies of normal controls. Thus if the red blood cells continue to be useful, it will be very helpful to the whole field.

The abnormalities we observed in the red cell in Duchenne dystrophy with saturation transfer electron paramagnetic resonance (EPR) (which I think Dr. Butterfield is now also observing with a different spin label) reflect small changes in the membrane. These are possibly related to the fatty acid content of the red cell. The sensitivity of the instrument is so great that we can detect small changes. This is in contrast to the defects in the plasma membrane observed in muscle where the lesions are quite advanced and morphological rather than biochemical.

We have compared the red blood cell from Duchenne dystrophy patients with those of normal age matched boys using electron paramagnetic resonance (EPR) technqiues (Proc. Natl. Acad. Sci., 75, 838, 1978). We inserted into the red blood cell membrane a spin labeled fatty acid (5-NS), which was spin labeled in the 5 carbon position. There was much more intense EPR signal in the normal boys than in the Duchenne patients. However, after 24 hours at 37°C, the spectral intensity of the RBC from Duchenne patients became equal to that of the normal boys. This indicated that in the dystrophic RBC the nitroxide spin labels were initially located in focal regions, interacted with each other, and reduced the intensity of the spectrum by Heisenberg spin exchange. With time, those spin labels dispersed through the membrane, the intensity increased and then the Duchenne patients and normal children looked exactly alike. So you can see from this experiment that it is important to consider the time of the measurements and not to try to do experiments on the next day because you won't see any differences. Such detail proved to be extremely important in

trying to differentiate between dystrophic and normal red blood cells. I might say this work was done with the saturation transfer EPR techniques which were developed by Hyde, Thomas and Dalton, as Dr. Hyde discussed. Saturation transfer EPR measures slower motion than the conventional EPR measurements. In agreement with other workers we found that conventional EPR spectra showed no difference between the normal and the dystrophic red blood cells.

I would like to say that the Muscular Dystrophy Association has been really marvelous in recognizing that very theoretical studies can be translated into more practical projects such as therapeutic testing of drugs. In addition to the drug trials, the Association offers enthusiastic encouragement for a large component of biochemical work in the laboratory involving electron paramagnetic resonance (EPR) studies on free radicals and glyceraldehyde-3-phosphate dehydrogenase, synthesis of more sensitive ^{15}N-deuterated spin labels for membrane studies and computerized spectral simulations for elucidating and interpreting EPR line shapes. Ultimately, it is hoped that this type of information can also be extrapolated into clinical management of patients with muscular dystrophy.

DR. ENGEL: Are the saturation transfer EPR studies quite specific for Duchenne dystrophy?

DR. PARK: We only examined one other disease, Becker's disease, and we did not see any abnormalities in Becker's disease. That was the only disease control which we had available at the time.

Studies of Membrane Components by Electron Spin Resonance

DR. BUTTERFIELD: I share Dr. Park's enthusiasm for the potential of extraneural tissues to help us understand something about the molecular basis for muscular dystrophy and other neurological diseases. I shall tell you about some of the things we have done in the muscular dystrophy area that relate to the question of specificity. Figure 5-1 shows the structural formula for 5-NS and another spin label, the MSL as Dr. Park calls it or as I call it MAL 6. It is a maleimide spin label and it covalently binds to proteins and does not interact with lipids at all. In the case of 5-NS in red cells, one gets the spectrum something like that in Figure 5-2 and using the McConnell formula, can measure certain structural parameters to calculate order parameters. In the case of MAL 6 one gets a spectrum (Figure 5-2) which is not observed in the case of glyceraldehyde-3-phosphate dehydrogenase with the same spin label. It has a single binding site in the latter cell or single binding characteristics but in the red cell proteins there are two different kinds of sites.

So just using that label per se one could not do saturation transfer ESR without first removing this W signal which we now feel we have learned to do and so now we can get ready to go on to saturation transfer ESR work on red cells with protein specific spin probes rather than lipid ones as Dr. Park as elegantly done.

Another way to measure membrane fluidity in addition to the order parameter is the half width at half height of the low field line of the lipid specific spin label.

The spin label MAL 6 is like N-ethyl maleimide which many of you are familiar with. It covalently links to SH groups but also to NH2 groups.

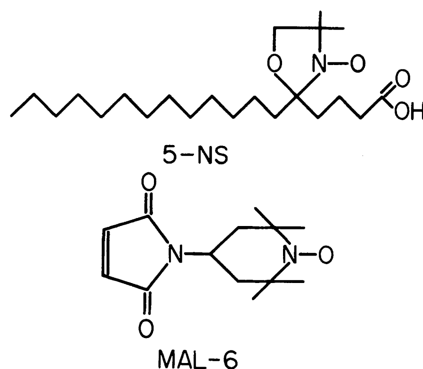


Figure 5-1: Structural formula of 5-NS and MAL-6 spin labels.

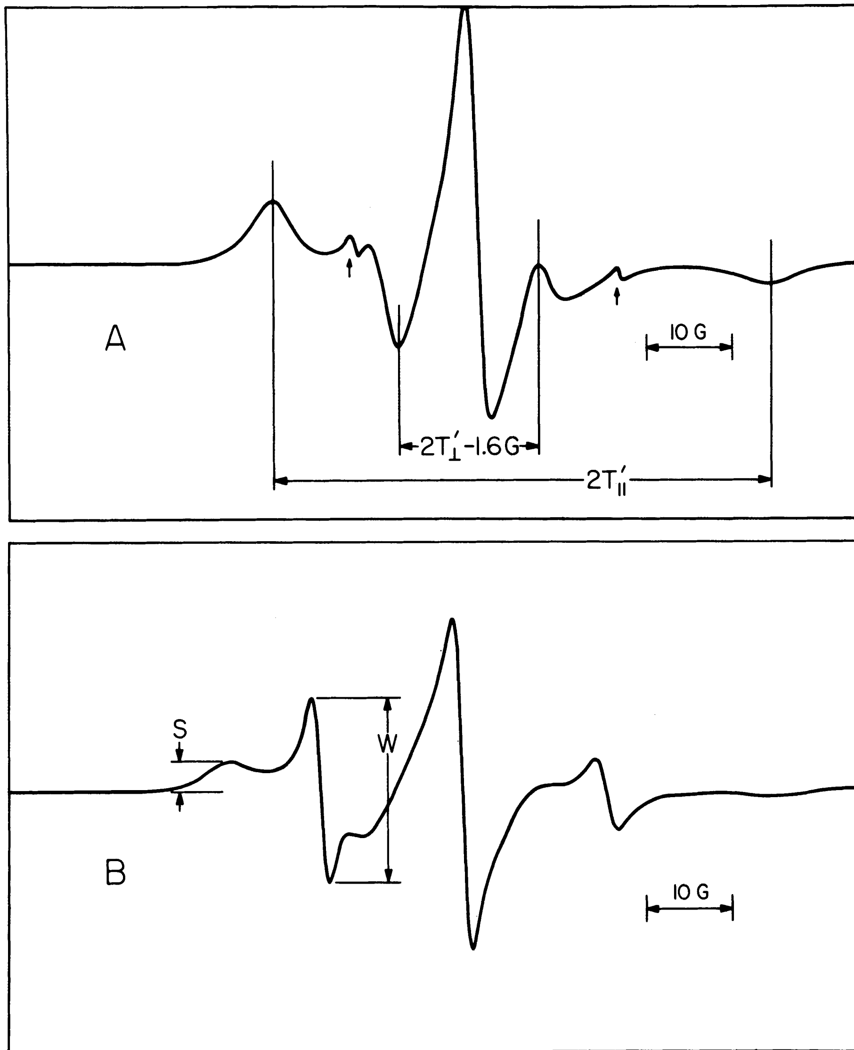


Figure 5-2: (Top) Typical spectrum of 5-NS in control intact erythrocytes. The measured T-tensor parameters from which the order parameter, S, is calculated are indicated:

$$S = \frac{T'_{\parallel} - T'_{\perp}}{(T_{\parallel} - T_{\perp})_{XL}} \cdot \frac{(TrT)_{XL}}{(TrT')}$$

Where XL refers to crystal values of doxyl propane. The smaller the value of S, the more fluid is the local microenvironment of the paramagnetic center of 5-NS. (Bottom) Typical spectrum of MAL-6 attached to membrane proteins in control erythrocytes. The ESR spectral amplitudes of the $M_I = +1$ line of MAL-6 attached to strongly and weakly-immobilized protein binding sites are indicated by S and W, respectively.

Glycophorin is labeled by MAL-6 and glycophorin has no SH groups so it does label NH_2 groups.

To analyze the spectra of the spin label one measures the W height and the S height and computes the ratio W/S. You can't look at the middle lines of these two superimposed spectra because they overlap and the high field lines have relatively low amptitudes so you look at the low field lines, compute the W/S ratio and that turns out to be fairly sensitive monitor of the physical state of proteins within the membrane. (Figure 5-2).

Figure 5-3 shows the effect of a drug called diethylstilbesterol. Diethylstilbesterol is thought to work in Duchenne muscular dystrophy to prevent leakage of soluble muscle enzyme into the serum. Principally CPK levels go down when Duchenne boys are treated with DES. We simply incubated red cell membranes with DES and you see a decrease in the order parameter from the case of no DES to a concentration of 10^{-5} and even 5×10^{-4}, relative to the untreated case. A decrease in the order of parameter implies that the membrane is becoming more fluid as a result of DES and at the same time the physical state of proteins is changing because the W/S ratio is also changing by a fair amount. Dr. Park has shown quite convincingly that the Duchenne membrane lipids are in a more rigid state than are control membranes and we have shown in our laboratory that W/S ratio is significantly increased in Duchenne red cell membrane proteins. DES works to alleviate both situations at low concentrations. It may cause the Duchenne W/S ratio to become more normal by altering the physical state of the membrane proteins and likewise causing the more rigid lipid phase of the membrane to become more fluid and closer to normal.

Modification of Membrane Fluidity

This may be the way that DES would work in muscle to prevent the leakage of soluble muscle enzymes into serum, but that certainly is speculative at the moment. All this has been done in normal cells and needs to be repeated in Duchenne cells and in the muscle systems if possible, once we learn how to label them.

DR. ENGEL: Isn't DES a synthetic compound?

DR. BUTTERFIELD: It's a synthetic hormone, as a matter of fact, which has been used as a growth promoter in cattle and has a number of problems associated with it.

DR. L. GLASER: What kind of circulating levels of DES does one reach with treatment on that scale?

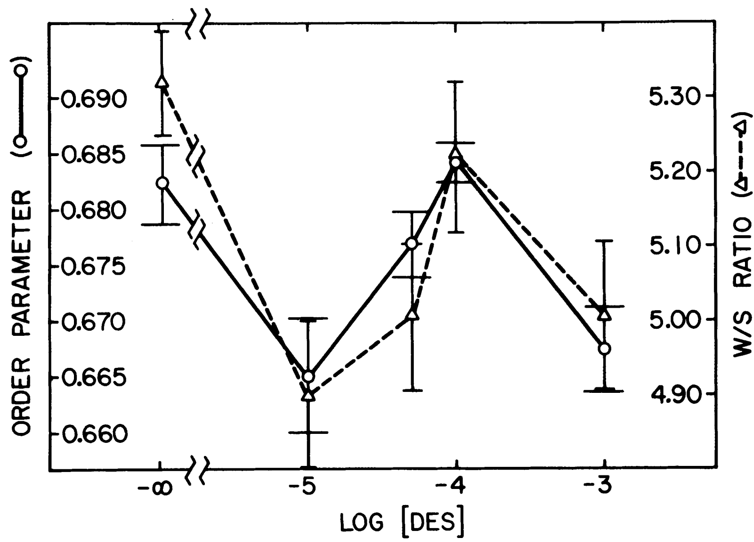


Figure 5-3: Variation of the order parameter of 5-NS (O——O) and the W/S ratio of MAL-6 (Δ--Δ) in erythrocyte membranes with concentration of DES. Standard deviations are indicated.

DR. ENGEL: You might be able to arrive at a rough approximation by dividing the dose used into the calculated extra-cellular volume of the patient.

DR. BUTTERFIELD: I am not sure what that is in making that calculation but I suspect we are at fairly low level, 10^{-5}M.

DR. HYDE: Do you have any rationale for that W/S ratio change on a molecular level in the case of Duchenne?

DR. BUTTERFIELD: No, not yet. We are trying to sort that out via saturation transfer ESR but also by regular ESR by looking at isolated proteins one at a time. But I don't know the answer to why W/S ratio is increased in red cell membrane proteins in Duchenne yet and how it is related to the lipid changes that Dr. Park has described, we don't know that either.

Findings in other Neuro-Muscular Diseases

I want to quickly go over some of the other results. We have looked at the specificity of changes that we have observed in our laboratory in Huntington's disease which, for the uninitiated, is an autosomal dominant inherited disease that affects a certain region of the brain called the basal ganglia but other areas are also affected, but this is the primary pathological change. A different disease, Friedreich's ataxia is an autosomal recessive inherited disease that involves spinal-cerebellar degeneration in mid-teen or so onset and death by age 30 usually. In Huntington's disease, by the way, the onset is in mid-life, 35 to 45 with about a 15 year span of life after that. Alzheimer's disease is acquired but there is a familial form of it. The principal pathological changes are cortical degeneration earlier than normal but somewhere in the 5th or 6th decade. Amyotrophic lateral sclerosis is manifested by anterior cell and corticospinal tract degeneration.

Table 2 shows the W/S ratio of all of the membrane proteins of red cells labeled by MAL-6 and this is the mean ratio. We see an increase W/S ratio in the case of Huntington's disease, in Friedreich's ataxia, in Alzheimer's disease, in myotonic and Duchenne dystrophy but not in the case of amyotrophic lateral sclerosis. There is no change that we can describe in ALS in any parameter that we examined even in the case of people who are quite far along in the disease.

Table 3 shows the fluidity measurements via ESR in these six diseases. There is no change in fluidity in Huntington's disease, or Friedreich's ataxia or Alzheimer's disease or ALS and by ordinary ESR in Duchenne, but as I indicate there is an increased rigidity as observed by Dr. Park's group by saturation transfer ESR. In the case of myotonic dystrophy, we describe about a 5 or 6% increased

Table 2: Comparison of the W/S ESR parameter of MAL-6 attached to membrane proteins in erythrocytes: ratio of disease state to corresponding control values.*

Disease State	Mean Ratio**	S.E.M.	$N_{Control}$	$N_{Disease\ State}$	P***
HD	1.09	± 0.016	41	41	< 0.0001
FAx	1.05	± 0.022	12	12	< 0.05
AD	1.12	± 0.041	14	14	< 0.02
ALS	1.02	± 0.035	6	6	> 0.8, N.S.
MyD	1.10	± 0.021	13	13	< 0.001
DMD	1.17	± 0.059	11	11	< 0.02

* The results of six separate sets of experiments are presented.

** Expected mean value is 1.0 if the physical state of membrane proteins is the same in a particular disease state and its corresponding control.

*** P-value calculated by Student's t-test.

Table 3: Comparison of the order parameter, S^a, of 5-NS in intact erythrocytes: ratio of disease state to corresponding control values.[b]

Disease State	Mean Ratio[c]	S.E.M.	$N_{Control}$	$N_{Disease\ State}$	p^d
HD	0.992	± 0.013	8	8	> 0.5, N.S.
FAx	1.01	± 0.004	7	7	< 0.1, N.S.
AD	0.986	± 0.013	7	7	< 0.5, N.S.
ALS	0.989	± 0.002	6	6	< 0.1, N.S.
MyD[e]	0.944	± 0.011	7	7	< 0.001,↑ fluidity
DMD[e]	1.01	± 0.007	10	10	< 0.2, N.S.
DMD	increased lipid rigidity observed by saturation-transfer ESR[f]				

[a] S is calculated from appropriate T-tensor values by:

$$S = \frac{T_{11}' - T_{\perp}'}{T_{11} - T_{\perp}} \cdot \frac{Tr\ T}{Tr\ T'}$$

where the primed values are obtained experimentally and the unprimed crystal values obtained from the results; Jost et al. (1971). Tr T is the trace of the electron-nuclear hyperfine tensor.

[b] The results of six separate sets of experiments are presented.

[c] The expected value is 1.0 if the lipid fluidity of the microenvironment reported by 5-NS is the same in disease state and corresponding control membranes.

[d] P-value calculated by the Student's t-test.

[e] The methyl ester of 5-NS was used in these experiments.

[f] (Wilkerson et al., 1978).

membrane fluidity. Our results have not been repeated by Dr. Gaffney to whom Dr. Park referred or by a fluorescence measurement. We previously reported a suggestive but not statistically significant increased membrane lipid fluidity in 0-1 day in vitro aged, MyD intact erythrocytes by electron spin resonance methods. In a later study we found a statistically significantly increased membrane fluidity in MyD erythrocytes that had aged two days in buffer at 4°C. The reasons for the delay were very simple: the muscular dystrophy clinic to which MyD patients attended occurred two days prior to access to the ESR spectrometer. Recently, attempts to reproduce our findings of increased membrane fluidity in MyD by spin labeling studies of fresh erythrocytes were unsuccessful. In order to further study the molecular basis of MyD and to attempt to resolve the apparent discrepancies of ESR studies of lipid fluidity, a time course of the alterations in MyD erythrocytes has been performed. The results suggest that increased membrane fluidity in MyD erythrocytes is fully manifested only after two days of in vitro aging in buffer (Table 4). The molecular basis for this result is unknown but it is possible that the metabolic deprivation of the cell with attendant membrane protein, lipid and Ca^{2+} both control and MyD have greatly reduced ATP levels. MyD erythrocytes membranes are reported to have an altered Ca^{2+} promoted K^{+} efflux that is expressed in ATP-depleted cells. Moreover, MyD cells have an altered transport of Ca^{2+}. Abnormaliites in the Ca^{2+} -dependent enzyme, diacylglycerol kinase, is suggested as one mechanism responsible for the large diminution of phophatidic acid in MyD erythrocyte membranes. diacylglycerol is derived from phosphorylated phosphatidylinositol, the diphosphatidyl moiety of which is known to affect red cell shape and flexibility. Moreover, a decrease in phosphatidic acid would decrease electrostatic interaction of the phospholipid near the head group (5-NS reports on an environment only a few angstroms from the head group) which would have the effect of increasing membrane fluidity. In addition, Ca^{2+} accumulation in ATP-depleted cells as a result of the inoperation of the Ca^{2+} -pump resulted in this "extra" calcium being tightly associated with membrane lipids and proteins. Given the diminution of phosphatidic acid in MyD, Ca^{2+} would be less able to cause phase separation of the negatively charged lipids and thus compared to normal controls appear to give an increased membrane fluidity. Additional evidence for an altered physical state of lipids on MyD erythrocytes have been obtained by lipid vesicles prepared from isolated RBC membranes in MyD a reported altered sodium permeability. Previous ESR studies using a protein-specific spin probe demonstrated alterations in MyD erythrocyte membrane proteins in 2-3 day old ghosts. The basis of this finding is also yet unknown but the effect of Ca^{2+} on membrane proteins is well known.

The specificity of the present results is not known; however ESR alterations of the lipid phase of Duchenne muscular dystrophy

Table 4: Comparison of the half-width at half-height of the low field line of 5-NS (Δh_L)§ in intact erythrocyte membranes from myotonic muscular dystrophy (MyD) and controls as a function of time.*

Time †	$\frac{(\Delta h_L)_{MyD}}{(\Delta h_L)_{Control}}$
0	1.04 ± 0.02 (6)
1	1.03 ± 0.008 (5)**
2	1.05 ± 0.01 (5)‡

* Means ± SEM (number of different samples) are presented.

† Number of days after blood was drawn prior to the recording of ESR spectra

** $P < 0.05$

‡ $P < 0.01$

§ The larger Δh_L, the more fluid the local microenvironment of the paramagnetic center of 5-NS (Mason et al., Biochemistry 16:1196-1201, 1977).

were reported to be present immediately after drawing the blood but not one or two days later by Dr. Park and colleagues.

The biochemical and biophysical basis for an in vitro age-dependence on membrane fluidity in MyD erythrocytes is still unclear but the results of the present study suggest that (a) the putative role of Ca^{2+} in the pathogenesis of MyD needs further examination; (b) the relationship of membrane fluidity to myotonia and dystrophy in MyD is still unknown and may be secondary since no alterations are observed in fresh cells; and (c) minor variations in technique from different laboratories may lead to changes in membrane characteristics in fragile systems such as erythrocytes.

Table 5 is a summary of W/S changes by ESR, lipid changes by ESR, scanning electron microscopy studies of unmanipulated red cells, sodium - potassium ATPase activity, protein kinase activity and cell deformability. Over all diseases and all experiments you could separate each disease from the other with the exception of Friedreich's and Alzheimer's. Duchenne is separated from ALS via W/S parameter and by rigidity of the membrane lipids, by stomatocyte formation by scanning EM, by rigidity of the membrane lipids separated from these other techniques although admittedly these others have not been done by saturation transfer. No changes in stomatocyte formation and three of these diseases separate Duchenne from those. There is still some controversy about sodium-potassium ATPase activity in Duchenne, but it is clearly separated from these other three.

DR. ENGEL: The rigidity in Duchenne is done by saturation transfer?

DR. BUTTERFIELD: That's correct.

DR. HYDE: Is there any effort to resolve the controversy between your group and Gaffney's; any plan of cooperation between laboratories or is everyone just going to drop it.

DR. BUTTERFIELD: We are not actively pursuing lipid studies in myotonic dystrophy but could if it were important. In my view, the proteins are really the thing to look at. Dr. Howland has described initially some fatty acid abnormalities in myotonic dystrophy with the net result that one would predict the increased membrane fluidity may be likely.

We did some experiments on protein kinase and ESR using the Roses and Appel protein kinase assay procedure and lined up the phosphorylation of membrane proteins and found it had absolutely no effect on W/S ratio compared to the case where you didn't phosphorylate and so we don't think that protein kinase and W/S ratio alterations are necessarily correlated.

Table 5: Specificity of biophysical and biochemical alterations of erythrocyte membranes in various neurological diseases.

Disease State	Altered Physical State of Membrane Proteins--ESR?	Altered Physical State of Membrane Lipids--ESR?	Increased Numbers of Stomatocytes--S.E.M.?	Na^{+}+K -ATPase Activity Altered	Protein Kinase Activity Altered	Cell Deformability Altered?
HD	Yes (41)*	No (8)	Yes (7)	Yes (10)	No (5)	Yes,↓(11)
FAx	Yes (12)	No (7)	No (7)	No (6)	---	---
AD	Yes (14)	No (7)	No (4)	No (5)	---	---
ALS	No (6)	No (6)	No (4)	No (6)	---	---
DMD	Yes (11)	Yes, small ↑ rigidity	Yes	Stimulated by oubain[c]	Yes, ↑	Yes, ↓
MyD	Yes (13)	Yes, small ↑ fluidity (9)	Yes[b]	No	Yes, ↓	---

* Numbers in parentheses represent number of samples employed in our laboratory.

HD - Huntington's disease
FAx - Friedreich's ataxia
AD - Alzheimer's disease
ALS - Amyotrophic lateral sclerosis
DMD - Duchenne dystrophy
MyD - Myotonic dystrophy
ESR - Electron spin resonance
SEM - Scanning electron microscopy

Table IV: Summary of W/S changes by ESR, lipid changes by ESr, scanning electron microscopy studies of unmanipulated red cells, socium-potassium ATPase activity, protein kinase activity and cell deformability.

I would like to briefly mention a different way to spin label membranes than with MAL-6 or 5-NS and that is the sialic acid specific spin labeling procedure. If you expose the sample to sodium periodate for 10 minutes in the cold adjacent cis hydroxyl groups are cleaved to form an aldehyde between them. The net effect is to split off carbon atoms 8 and 9 and to leave the carbon 7 atom of sialic acid with an aldehyde group which then reacts by reductive amination of Tempamine spin label (which has an NH2 group at the end). Tempamine reacts with the aldehyde to form an intermediate which is reduced by the sodium cyanoborohydride to make a covalently linked sialic acid specific spin label.

DR. BRANTON: How much lipid do you label?

DR. BUTTERFIELD: Something in the order of 30% of the total label taken up is by gangliosides and the 70% by glycoproteins, principally glycophorin.

Some other things we have looked at are prednisone, lithium carbonate, sodium chloride and insulin. Prednisone and lithium carbonate are supposed to affect serum CPK levels and sodium flouride may affect adenylate cyclase. Insulin has been reported to be important, perhaps in the monocytes in myotonic dystrophy having altered receptor.

The bottom line is that insulin may be doing something at very low concentrations, but there certainly is no effect at higher concentration. Again there may be something with lithium that is specific for lithium at very low concentrations but not a higher concentration. With respect to the others, there is just no effect at all. I don't know how one might relate these agents to their purported function in muscle with respect to red cell.

DR. SANDRA: What did you say insulin does?

DR. BUTTERFIELD: We look at the W/S ratio parameter as a function of insulin concentration because of its possible role in myotonic dystrophy monocytes as reported by Festoff.

DR. SANDRA: You said the low concentration.

DR. BUTTERFIELD: Low concentrations seem to affect the W/S ratio in normal cells relative to the untreated case but at higher concentrations there didn't seem to be any effect.

DR. SANDRA: How low is this concentration?

DR. BUTTERFIELD: 10^{-8}M.

DR. HYDE: The MAL-6 label is probably on the hydrophilic side, on the outer side of the plasma membrane on proteins that are somehow sticking out into the water, is that the guess?

DR. BUTTERFIELD: No. MAL-6 is thought to label spectrin, on the cytoplasmic side of the membrane, band 3 which goes all the way through the membrane and which has 5 SH groups per molecule, glycophorin which has no SH groups, and ankyrin and perhaps some of those other minor components in the 2.1 to 2.5 regions are principally labeled by the spin label.

DR. HYDE: Did you try any ascorbate reduction?

DR. BUTTERFIELD: Oh, yes! We have characterized that spectrum very carefully. All the W sites are exposed to solvent, they are completely removed in 5 or 10 minutes by ascorbate. Only some of the S sites are not, but some are. I appreciate that this is a composite spectrum but an approximated coupling constant is also equivalent to MAL-6 dissolved in aqueous solvent. Those W sites are clearly very polar. Some of the sites are inaccessible to ascorbate.

A Strategy in the Search for a Genetic Enzyme Defect

DR. HOWLAND: Considerable discussion thus far has been centered upon ultrastructural abnormalities in the Duchenne sarcolemma and has been based on the unassailable assertion that muscle disease pathogenesis is suitably studied in muscle tissue. Without arguing against the soundness of this strategy, I should like to add, as a sort of a footnote, the suggestion that it is nonetheless possible to gain useful pathogenetic clues using cell types other than muscle. In particular, I suggest that the defective genes underlying Duchenne and myotonic muscular dystrophies are expressed in both cultured fibroblasts and erythrocytes and will provide some evidence for this assertion shortly. Such cells thus may provide opportunities to examine biochemical features of these conditions without the background of catastrophic muscle degeneration, cell population replacement, and other secondary influences of the diseased target tissue.

However, the lack of agreement about the validity of observations regarding "dystrophic" erythrocytes (and to a lesser extent, fibroblasts) has become almost legendary, and has been commented upon frequently in this colloquium. Whereas, the study of intact muscle is hindered by one set of constraints, clearly examination of other cell populations is fraught with a different collection of difficulties. These include the inevitable disagreements between laboratories that isolate and handle cells in a different manner. In addition, difficulties arise in a failure to differentiate between *in vivo* cell abnormalities and those so-to-speak

"dynamic," abnormalities which become increasingly apparent under *in vitro* cell handling and incubation. Considering on one hand such difficulties, and on the other, the apparent utility of non-muscle investigations, I should like to simply enumerate some of the constraints that I believe should be recognized in order that investigations with, say, erythrocytes or cultured skin fibroblasts, provide constructive information about the pathogenesis problem. This will, in effect, be nothing more than a list and I will end it with two concrete illustrations, to offset its necessary generality. One each of these examples will concern Duchenne and myotonic muscular dystrophy. It is hoped that the utility of this list (if any) will be to assist in determining whether an apparent abnormality is trivial (reflecting perhaps a second-order effect of the disease process) or, rather, is close to the primary pathogenetic event.

The list begins with a negative admonition:

(1) It is probably no longer helpful to add to the catalog of evidence for a generalized membrane defect. To the extent that such a defect is now established (in my view, a considerable extent) one more example of altered enzyme activity or transport function is unlikely to advance our understanding unless evidence can be advanced for a role in primary causation. For instance, debate as to whether Duchenne erythrocytes adopt an echinocyte or stomatocyte configuration will probably not lead to useful resolution, as either condition can not possibly reflect anything but evidence for a general membrane instability, of which there is already ample indication.

(2) In order that a supposed alteration in non-muscle cells from dystrophic individuals be connected with early causal stages of pathogenesis, there are a number of specific tests that may be applied: for the abnormality to be anything but secondary, it must satisfy the important constraint of being attributable to a single defective enzyme. Any proposed connection between a cell abnormality and the disease process should also take into account the systemic character of muscular dystrophies (which, of course, underlies the very strategy of using non-muscle cell types). At the same time, muscular dystrophies are given that name for a good reason, and an important non-triviality test for an observation from say, a fibroblast culture is that one be able to explain why the supposed defect produces particularly grave consequences for muscle. I shall give an example of application of this test in a few moments. In a similar vein, any supposed abnormality arising out of non-muscle investigations should, in principle, explain the slowly-progressive character of muscular dystrophies. A purported defect in the enzyme of such metabolic centrality that it would inevitably produce death *in utero*, represents a singularly bad guess.

(3) For an erythrocyte or fibroblast abnormality to be interesting in a pathogenetic sense it must be disease-specific. Much of the erythrocyte literature is cluttered with abnormalities that are associated with a number of genetically-distinct muscular dystrophies. If one considers the generalized membrane involvement to be established, additional examples are no longer likely to be germane.

(4) Any pathogenetic explanation arising out of non-muscle studies (and perhaps any studies) must also take into account the following seeming paradox: Many investigators now believe that these diseases originate in a systemic abnormality in plasma membranes. This can only reflect an alteration in the functional composition of the membrane. However, in fact the dystrophic plasma membranes appear to be disgustingly normal in both protein and lipid composition. Reported alterations in either class of compounds are, even if valid, of a sufficiently minor character to make it extremely difficult to explain some of the rather impressive changes in known physical properties. (For example, alterations revealed by lipid and protein-bound spin labels come to mind). This apparent normality of composition represents a severe constraint in making up stories about pathogenesis (a good thing) and will be mentioned in a more explicit context in a moment.

(5) Finally, as we said, it is necessary to be clear about the distinction between an intrinsic cellular abnormality and a dynamic abnormality, i.e. one emerging in time, say, after blood is drawn. It is entirely possible that primary pathogenesis may reveal itself in an altered dynamic event (for instance, something that occurs in control cells but not those from dystrophic individuals, or vice versa). However, experience of the past decade suggests that there is a real danger of mistaking dynamic for intrinsic alteration, thus obscuring the real issue of pathogenesis behind a smoke-screen of debate regarding the validity of measurements. As things have turned out, I have been a considerable offender in this regard, having, for example initially failed to realize that apparently diminished palmitoleate (which hereafter will be denoted 16:1 for its 16 carbon atoms and single double bond) in Duchenne erythrocyte membranes was not an intrinsic diminution and having therefore been slow to capitalize on the information contained in the observation.

Let me turn to two examples from our laboratory which I hope will make the discussion a bit more concrete. First, regarding Duchenne dystrophy, I just said that we earlier observed diminished 16:1 in erythrocyte membranes of Duchenne subjects.

This observation became controversial, being confirmed in detail by one laboratory and utterly lacking confirmation at another. This disagreement has turned out to be the result of having failed to consider the palmitoleate (16:1) enrichment in normal (but not Duchenne) cells as a dynamic process, one that occurs after cells are removed form the intact organism. This is evident in progressively increased palmitoleate in control cells incubated in their serum for increasing lengths of time. The increase is completely blocked by compounds that bind calcium and is not observed in Duchenne cells. Therefore, the difference in apparent palmitoleate content probably arises through an *in vitro* event and must be termed artifactual. However, it represents a differential artifact and, as such, may lead to useful information. Indeed, it led us to examine the uptake of labeled, unsaturated fatty acids into erythrocyte phospholipids, a process which occurs, which is stimulated by calcium (in the presence of the ionophore, A23187), and which is substantially depressed (less than 15% of control) in Duchenne cells. The uptake appears to represent the acylation of lysophospholipids, especially lysophosphatidylcholine and lysophosphatidic acid. Such lyso-compounds are noteworthy for being highly disruptive to cellular membranes.

At the same time, we have been observing a second lipid transformation in cultured fibroblasts, the stimulated turnover of phosphatidyl inositol - the so-called "phospholipid effect." Extracellular agents, including neurotransmitters and lectins, stimulate turnover of phosphatidyl inositol (PI), a process that appears to begin with activation of a specific phospholipase C followed by resynthesis via diglyceride, phosphatidic acid (PA), and CDP-diglyceride. There is also likely to be a deacylation followed by reacylation, as the acyl compositions of the intermediates of the cycle are not identical and PI appears to serve as donor of arachidonate (20:4) from its 2-position prior to synthesis of prostaglandins. Measurement of the turnover consists in preincubating cells with inorganic $^{32}PO_4$ followed by stimulation, at which time, label is seen to be lost from PI and increased in PA. For instance, in four normal fibroblasts lines, the labeling of PA increased about 20% and that of PI decreased about 30% upon treatment with Con A or epinephrine. In a similar number of Duchenne cell lines, PA labeling increased by more than 160% and PI decreased by about 45%, an observation suggesting a block in the resynthesis branch of the cycle (i.e. between PA and PI). Having measured the individual reactions leading to turnover of the polar region of these molecules, we have failed to observe any blocked reaction and have now concluded that the defect resides in the deacylation-reacylation event, the reaction leading to acyl composition modification. This event requires the acylation of a lysophospholipid, most probably lyso-PA.

It should be added that the altered turnover is not observed in cells obtained from myotonic dystrophy subjects, thus satisfying the disease-specificity constraint mentioned above. Moreover, the turnover event has been shown to occur at synaptic membranes and has been postulated as an obligatory step in transmitter function. If this is true, and if a supposed defect in acylation of lysophospholipids is involved in pathogenesis, then muscle as a target of the defect becomes less difficult to understand.

A final, indirect indication that this approach may be on the right track comes from examination of the fatty-acyl composition of inositol-containing phospholipids. For example, when diphosphoinositol (diPI) is isolated from control erythrocytes, it exhibits the characteristic acyl composition of PI, namely little palmitate (16:0) and a great deal of stearate (18:0) and arachidonate (20:4). This similarity reflects the fact that diPI is synthesized from PI by a specific kinase. However, Duchenne membranes contain a diPI with a much different composition, suggesting a distinct metabolic origin. Duchenne diPI contains considerable palmitate and practically no arachidonate. This composition is similar to that of PA and is presumed to arise by inability to carry out the reacylation of lyso-PA mentioned above. Again, this compositional abnormality is not observed in membranes from myotonic dystrophic cells.

Therefore, as a working hypothesis (and mostly for the sake of illustrating the general points about strategy made earlier) I suggest that Duchenne muscular dystrophy may arise from an inability to reacylate lysophospholipids, in particular lyso-PA. As an approach to the pathogenesis problem, this suggestion satisfies a number of the constraints mentioned above: it proposes a defect in a single enzyme, is specific for Duchenne (as compared to myotonic) dystrophy, and could give rise to primary muscle involvement by anomalous transmission at the neuromuscular junction. Moreover, lysophospholipids are detergents, and even small increases would be expected to produce important effects, say, on the integrity of the sarcolemma (eg. the focal deterioration observed by A. Engel) and other membrane parameters. Thus, the relative normality of major membrane lipid composition becomes consistent with the variety of changes known to be produced by the Duchenne conditon. In this last connection, it is noteworthy that increased lysophospholipid has been reported in Duchenne erythrocyte membranes.

Having said that myotonic-dystrophic membranes do not exhibit a picture similar to Duchenne, let me finish by telling what we have observed there. Recall that Duchenne red cell membranes contain diPI with an abnormal fatty acyl composition as revealed by gas-liquid chromatography.

In the course of discovering the diPI from myotonic dystrophic membranes exhibited an acyl profile similar to that of control cells, we did observe two rather intriguing alterations. For one thing, odd chain acids were present at about a five-fold increase over normal levels (but were still minor components). These included mostly 17:0 and 19:0 which were subsequently observed at elevated levels in other phospholipids as well. Perhaps more interesting, was a second observation of shifted chromatographic peaks of singly-unsaturated acids. Thus, palmitoleate (16:1) was virtually missing but replaced by a new component with a resolvably-larger effective chain length (but shorter than 17:0). At the same time, oleate (18:1) was present, but with a prominent shoulder not observed in control chromatograms. It should be added that these abnormalities also occurred in other phospholipids, but were particularly noticeable in diPI owing to its low levels of singly-unsaturated fatty acids.

Identification of the anomalous acids was accomplished through the use of capillary columns interfaced to a mass-spectrograph and employing comparison with known standards. The acids turned out to be the _trans_-isomers of the corresponding "normal" _cis_ acids and, as I said, in the case of 16:1 the _trans_-isomer had largely replaced the _cis_. We naturally turned our attention to identifying a single enzyme defect capable in principle of giving rise to both odd-chain and _trans_-acids in myotonic membranes. An interesting candidate is delta--3-cis-delta-2-trans enolyl Coenzyme A isomerase which serves a double function: first it enables cells to oxidize singly-unsaturated fatty acids (which must move a _cis_ double bond one carbon toward the carboxyl group and, at the same time, convert it to _trans_ for subsequent reactions in the oxydation cycle). Secondly, the enzyme is required for the metabolism of dietary _trans_ fatty acids, a class of fatty acids known to interfere with lipid metabolism in a number of ways, including for example, augmenting the effects of essential fatty acid deficiency. We propose that this isomerase is defective in myotonic dystrophy, that the observed _trans_ acids are dietary in origin and that the odd chain acids arise from propionyl CoA chain elongation, this compound, in turn resulting from the inability to move the double bond to the even-carbon position.

Again, this suggested pathogenetic mechanism fulfills the criteria of disease specificity, a single enzyme defect, and membrane dysfunction. The systemic character of myotonic dystrophy is particularly evident and appears consistent with this explanation. Moreover, if dietary _trans_ acids are important in the pathogenetic chain of events, some other observations become explicable, and one can even begin to think about therapeutic approaches. Whereas Duchenne muscular dystrophy exhibits remarkable consistency in age of onset and rate of progression, myotonic dystrophy does not.

Age of onset and pace of deterioration are enormously variable, suggesting at least the possibility that diet, or other environmental considerations, may be significant. Moreover, myotonic dystrophy is associated with an "anticipatory phenomenon" wherein the ages of onset of symptoms have often become progressively earlier during the past three generations in a given family. I would only note here that the last three generations in this country have seen some significant changes in dietary fat intake, wherein vegetable lipids have risen enormously in consumption. These lipids contain large quantities of trans fatty acids whereas animal fats do not. For instance, commercial vegetalbe oils contain up to 17% trans fatty acids, margarines up to 50%, and vegetable shortening up to about 60%. If our premise is correct, a suggestion about diet for myotonic patients is vividly apparent (and should probably be pursued without waiting to see if the premise is, indeed, valid).

I have not touched on the matter of animal models for human neurological diseases; I do so briefly. After finding these lipid abnormalities in myotonic dystrophic membranes, we looked with a fresh gaze at old fatty acid chromatograms of membranes from livers of control and dystrophic mice (dy/dy). In fact, the occurrence of odd-chain (and probably trans) fatty acids there leads us to consider that the dy mouse perhaps should be regarded as analogous to human myotonic dystrophy.

In summary, we have offered a list of constraints that might be used in determining whether a given strategy for examining pathogenesis in non-muscle cells is likely to be constructive. In the course of illustrating some of these constraints, we have advanced provisional approaches to understanding Duchenne and myotonic dystrophy. To be candid we consider these approaches as somewhat more than heuristic in nature and (as one might imagine) we are presently engaged in testing and extending them. In this fashion, we hope that what are now only research strategies may soon be promoted to the status of hypotheses.

EDITORIAL SUMMARY

The discussion focused initially on the lack of successful replication of some of the abnormalities claimed for erythrocytes from Duchenne patients and hence the difficulty of sorting out the leads to be pursued. Newer biophysical studies of erythrocyte membranes by electron paramagnetic resonance (EPR) and electron spin resonance (ESR) were then described in which consistent changes in membrane fluidity were noted among cells from dystrophic animals and patients. Finally a plan was proposed for the use of non-muscle cells including fibroblasts, to elucidate the genetic defect in Duchenne dystrophy.

CHAPTER 6

ABNORMALITIES IN MUSCLE IN DUCHENNE MUSCULAR DYSTROPHY

Synthesis and Speculation

DR. ENGEL: I think it is fair to say that the proper study of muscle should be muscle itself and let us focus on the muscle biopsy findings in Duchenne dystrophy.

There are a few things that one cannot debate:

1. There is progressive destruction and loss of muscle fibers with progressive replacement of contractile elements by fibrous connective tissue, progressive failure of the efficiency of regeneration, progressive weakness and, eventually, tissue or organ failure and death.

2. There is increase in the level of muscle enzymes in serum, particularly of creatine phosphokinase (CPK), and this increase is proportionate to the remaining muscle mass. Thus, ultimately the serum CPK level decreases as the disease leads to progressive loss of muscle bulk. This finding also suggests that there is something wrong with the surface membrane of the muscle fiber since the enzyme must traverse the membrane to find its way into the circulation. Alternatively, the hyperenzymemia could be a consequence of enzyme leakage from those muscle fibers which are undergoing necrosis at any given moment but presumably would not leak from the surface membrane of the non-necrotic muscle cell.

In 1973 Dr. Bahram Mokri and I began a study which was eventually published in 1975 (Mokri and Engel, 1975). We decided to reinvestigate the ultrastructural findings in Duchenne dystrophy in search of possible clues of the etiology and pathogenesis of the disease.

Our first approach was to look at semi-thin epon sections in the phase microscope to locate an abnormality, or something of interest, and then find the same fiber(s) in the electron microscope. Proceeding in this manner we found that a proportion of non-necrotic muscle fibers displayed abnormal regions of a more or less triangular shape, usually with the base of the triangle facing outward and its apex inward. Some of the abnormal regions were irregular in shape or rather small. The rest of the fiber which harbored the lesion seemed to be preserved as adjudged by phase microsocpy. Next, we looked at these fibers in the electron microscope and noted that overlying the abnormal regions there were single or multiple discontinuities, or defects, in the plasma membrane (Figure 6-1). By contrast, the continuity of the basement membrane overlying the defect was intact or, sometimes, it was replicated over the defect. Within the fiber, under the defect sarcotubular profiles were often dilated and the myofibrils were rarified. The latter circumstance was attributed either to loss of myofibrils or to contraction of myofibrils at the border of the lesion resulting in excessive stretching or disruption of sarcomers within the lesion (Figure 6-1). In longitudinal sections the abnormal regions were again more or less wedge-shaped, with apex pointing toward the fiber interior, bordered by a contraction band on either side. Within the wedge-shaped region one could see stretched or fragmented thin filaments. In addition, in these regions we observed small myeloid structures and other evidences of focal cytoplasmic degradation.

We wondered whether what we observed could have been an artifact. Could it be that for some reason the muscle fibers were contracting excessively in these areas, causing mechanical rupture of the surface membrane? Or could the plasma membrane have been damaged at the time of the biopsy? We did not think that the changes arose at the time of the biopsy, or were due to external trauma, because the basal lamina was either preserved or replicated over the defects in the plasma membrane; and because focal cytoplasmic degradation would be unlikely to develop during the brief period which elapsed from the moment the specimen was removed from the patient to the time when it became fixed. We also did not think that fixation in itself was responsible for the changes in the plasma membrane. This was based on the following experiment: We immersed fresh, thin muscle strips from dystrophic patients for a period of 15 minutes into oxygenated mammalian Ringer's solution containing horseradish peroxidase before immersing the tissue into fixative. During this time the extracellular marker diffused around the muscle fibers in the outer tiers or rows in the fascicles. In some of these fibers we again observed the wedge-shaped lesions, this time inundated with the peroxidase marker (Figure 6-2). We felt that this was especially significant when fibers showing these changes were in the second or deeper row because the most superficial fibers might still have been subjected to mechanical trauma.

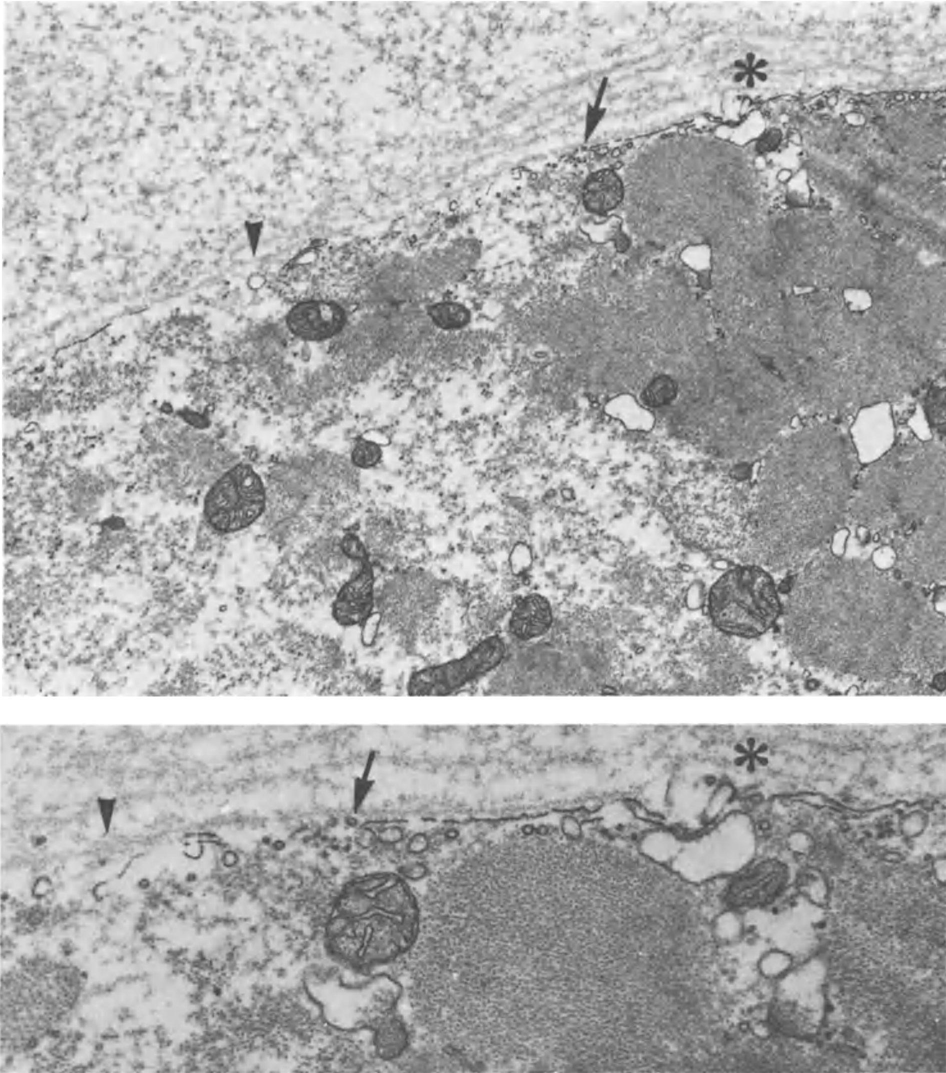


Figure 6-1: Duchenne dystrophy. An extensive defect of muscle fiber plasma membrane is seen to the left of the arrow. Lower panel shows transitional zone between preserved and absent plasma membrane at higher magnification. Basal lamina is intact (arrowhead) in sector from which plasma membrane is absent and is replicated over right upper part of fiber (asterisk). In the underlying fiber region the myofilaments are abnormally sparse, mitochondria are intact, and sarcotubular profiles are dilated. Upper panel, X 25,000; lower panel, X 56,600 (From Mokri and Engel, 1975). (Reduced 22% for reproduction.)

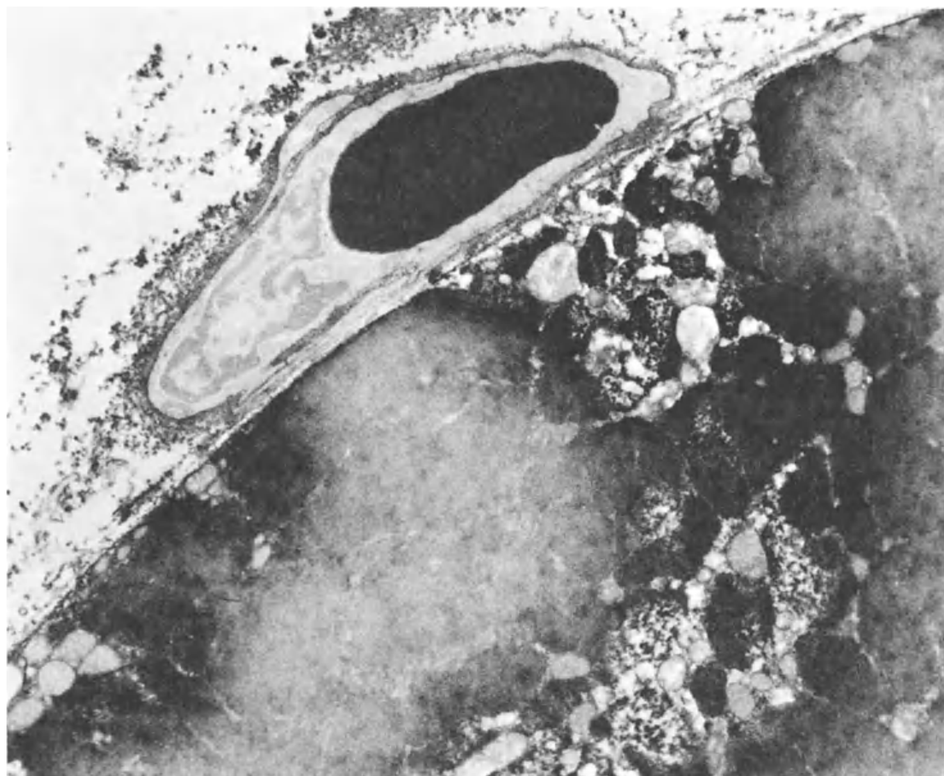


Figure 6-2: Peroxidase loading experiment in Duchenne dystrophy. Peroxidase has entered the fiber in three regions and is distributed in wedge-shaped zones. Zones which were filled with peroxidase also contain swollen mitochondria and dilated sarcotubular profiles. Intact capillary overlies largest lesion. This lesion is contiguous with a deeper one that has also become filled with peroxidase. Unstained section; X9,100. (From Mokri and Engel, 1975). (Reduced 20% for reproduction.)

On surveying a number of biopsies, we found that approximately 5 per cent of the muscle fibers showed such lesions in a given plane of sectioning. This probably represents an underestimate of the total number of fibers which may have defects in their surface at one time.

Our next inference was that if fibers exist with defects in their plasma membrane which allow ingress of the extracellular fluid, then the interior of these fibers would be exposed to harmful concentrations of electrolytes, particularly calcium and possibly sodium. Accordingly, the contraction bands at the side of the lesion could be due to activation of the contractile apparatus by focal calcium excess which the sarcoplasmic reticulum could not sequester. Alternatively, the local cytoplasmic degradation could be secondary to injury to organelles, particularly mitochondria, by calcium. We then said that if this were the case, then we should see excessive calcium in a proportion of non-necrotic dystrophic fibers. To pursue this further, John Bodensteiner and I (Bodensteiner and Engel, 1978) studied the localization of calcium with the von Kossa stain, with alizarin red, and with glyoxal-bis hydroxyaniline in serial fresh-frozen sections in 114 biopsy specimens. The material included Duchenne dystrophy (24 cases), other dystrophies (27 cases), inflammatory myopathies (47 cases), and normal controls (11 cases). Separate counts were made of necrotic and non-necrotic fibers which stained positively for calcium. Essentially all necrotic fibers stained positively for calcium, positive with a mean frequency of 4.83 per cent. For all other groups, the corresponding value was 0.57 per cent with a range of 0.21 per cent (normals) to 1.76 per cent (scleroderma), $p < 0.001$ (Figure 6-3). The calcium excess was often in wedge-shaped regions, but sometimes the entire fiber reacted positively for calcium, or the calcium staining was observed in a crescentic subsarcolemmal region (Figure 6-4). This, of course, is not surprising because calcium may diffuse throughout the cell.

Other investigators have also noted defects in the continuity of the plasma membrane in non-necrotic muscle fibers in Duchenne dystrophy (Schmalbruch, 1975; Carpenter and Karpati, 1979). Carpenter and Karpati emphasize that the defects may also occur in fibers which display no contraction bands adjacent to the defects and assume that the dilated membrane-bound profiles at the borders of the lesions are of transverse tubular origin. In another study, the ingress of extracellular fluid into abnormal dystrophic muscle fibers was shown with the use of Procion yellow (Bradley and Fulthrope, 1978).

Additional morphologic findings have been made by Dr. Schotland and his co-workers.

Figure 6-3: Percentages of calcium-positive non-necrotic fibers in 114 muscle biopsy specimens determined with the glyoxal-bis-hydroxyaniline stain. Columns indicate mean values for each disease.

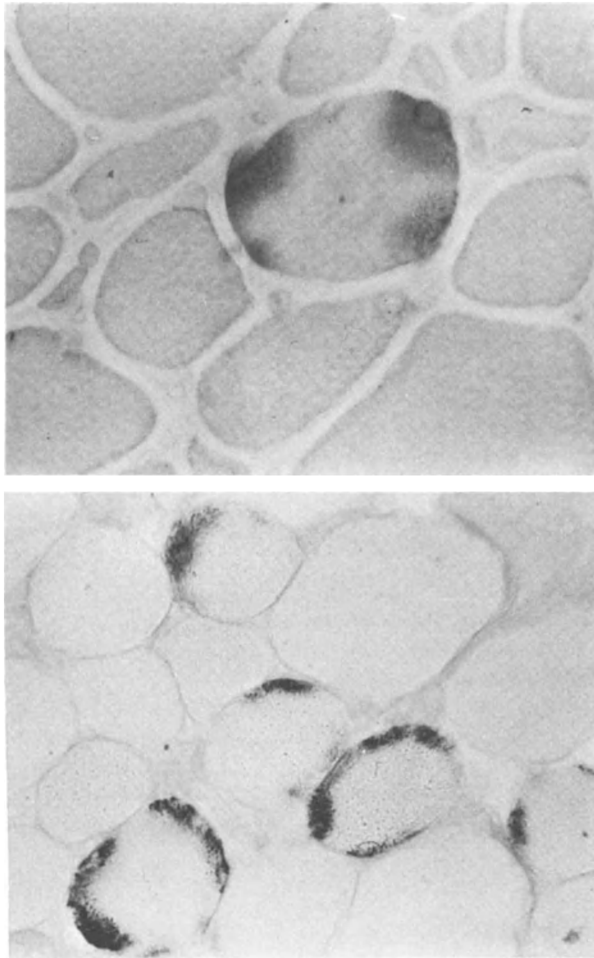


Figure 6-4: Wedge-shaped (top panel) and crescentic or wedge-shaped (bottom panel) calcium-positive regions in non-necrotic muscle fibers in Duchenne dystrophy. Top panel, alizarin red, X 475; bottom panel, glyoxal-bis-hydroxyaniline, X 300 (Bodensteiner and Engel, 1978).

They have noted that in Duchenne dystrophy a proportion of the muscle fibers display regions over their surface membrane which do not bind concanavalin A (Bonilla, Schotland and Wakyama, 1978). An additional membrane abnormality was demonstrated by Dr. Schotland and co-workers in dystrophic muscle (Schotland, Bonilla and Wakyama, 1980). This consists of a decrease in the numerical density of integral membrane particles and, particularly, of particles arranged in orthogonal arrays, in the freeze-fracture plasma membrane.

At this point I would like to ask each of the participants at this meeting what these findings suggest to you and how one might investigate a possible abnormality of the muscle fiber surface membrane. Dr. Branton, could defects in the plasma membrane be related to an abnormality of the cytoskeleton?

DR. BRANTON: Could you describe what is wrong with the membrane? What is it that you see? You have drawn gaps. Is that basically what you see?

DR. ENGEL: This is basically what we see in the non-necrotic fibers, with the membrane lesions overlying focal abnormalities in the cell. Above the gaps in the plasma membrane, the basement membrane is usually preserved or even replicated. The lesions are permeated by extracellular fluid and there is focal calcium excess as determined cytochemically.

DR. SCHOTLAND: In the concanavalin A studies we have seen that in some fibers con A binding sites can be absent without breakdown of the membrane or we see a combination of the two together -- a breakdown of the membrane and loss of concanavalin A binding sites. This suggests that there is a sequential change beginning with loss of concanavalin A binding sites which is then followed by disruption of the plasma membrane.

DR. BRANTON: How do the regions of the lesions appear by freeze-fracture electron microscopy?

DR. SCHOTLAND: We looked at roughly 15 biopsies in Duchenne dystrophy by freeze-fracture electron microscopy. We saw the lesions in only three of the biopsies. There may be a sampling problem in the freeze-fracture studies.

DR. ENGEL: I am not sure that one can say that one does or does not see the lesions in the freeze-fracture specimen because the fracture goes in and out of membrane planes in an unpredictable manner. For example, when one observes cytoplasm it could be because one fractured into it or because one is traversing a region of the plasma membrane which has a gap in it.

There may not be a reliable way of knowing the status of the plasma membrane which overlies a given cytoplasmic region, or that one can really study the focal absence of membrane by freeze-fracturing the remaining membranes.

DR. BRANTON: I would agree that if the lesions you described were associated with disappearance of the lipid bilayer, one could not see it by freeze-fracturing because there are definitions that depend on the presence of the lipid bilayer. If there were selected regions of the membrane that clearly did represent the lesions, that would be significant for it would tell one that the thin sectioning procedure is not truly indicative of the absence of the lipid bilayer, but there might be some combination of factors, as associated with staining, which made it difficult to see the bilayer by transmission electron microscopy, though it was still preserved as determined by freeze-fracture. You said that you do not see the lesions in freeze-fracture, which is what one would expect if the lipid bilayer were truly absent due to localized breakdown. But why do you ask the question in terms of cytoskeletal supporting elements rather than in terms of the lipid bilayer?

DR. ENGEL: The reason for that is that I am going clockwise around the table and you are the expert on the cytoskeleton.

DR. BRANTON: I find the phenomenon very puzzling because it implies that there is a wide open gap in the lipid bilayer which strikes me as extraordinary. It implies that perhaps the bilayer configuration is altered at the edges of the lesion, for a bilayer usually does not remain open. I think that the people concerned with the bilayer configuration of lipids will probably have more to say about that than I do. One cannot say a great deal of cytoskeletal elements because so little is known about the muscle cytoskeleton. Is there any evidence that proteins exist under the muscle cell membrane which are differentiated from the rest of the cell? There are a variety of special staining techniques for cytoskeletal elements and proteins connected with the membrane, for example, tannic acid. Have you tried any of these?

DR. ENGEL: This has not been looked at carefully in differentiated skeletal muscle, but Dr. Schotland has used the tannic acid staining technique. With this he can detect lesions in the plasma membrane very nicely and, in fact, he detects them more frequently than we do. But I am not sure that he is able to visualize the cytoskeleton proper. The cytoskeleton is presumed to provide mechanical stability for the membrane. You commented earlier, Dr. Branton, that if spectrin were lacking or were abnormal in the erythrocyte, the mechanical stability of the membrane would be compromised.

Conceivably a cytoskeletal element could be structurally abnormal in dystrophic muscle and compromise the structural integrity or stability of the muscle fiber plasma membrane. This membrane is subject to a great deal of mechanical stress during contraction. It is not only compressed from side to side but is stretched or thrown into folds myriad times a day. The cytoskeletal supports of the muscle fiber plasma membrane represent an area of ignorance. We need to study carefully so that we can understand it further. What else do you think we ought to learn about the plasma membrane?

DR. BRANTON: A technique that has been useful in visualizing the lamellar layers or meshwork-like arrays that underlie many membranes has been to purposely eliminate the lipid bilayer with a detergent, like Triton-X, and then proceed with electron microscopy. With special staining techniques one may still see a membrane from which the lipid has been extracted. One could also extract the protein from the membrane and it would still look like a bilayer. Both lipids and proteins contribute to the densities which we see in the electron microscope in the normal membrane. One may remove the densities that are attributable to lipids to see if the densities which one would attribute to proteins appear. This way one might discover that discontinuities in the cytoskeleton or some underlying proteins exist close to lesions in the plasma membrane. On the basis of this type of evidence one might be encouraged to ask those detailed biochemical questions which will require an enormous amount of effort. So the first thing to do would be to find out if there really are discontinuities in the underlying protein meshwork. If there were good evidence for this, then one should try to analyze what these proteins are. The problem is particularly difficult because in differentiated skeletal muscle isolated cytoskeletal proteins might be heavily contaminated with other proteins.

DR. FLEISCHER: What is known about the specificity of the cytoskeletal elements? Are there reagents which can distinguish them from ordinary muscle elements?

DR. BRANTON: I do not know of any good differentiation as, for example, how to differentiate alpha from beta from gamma actin. I do not know of any good way except on the basis of small differences in the charge of the molecule, detected by electrophoresis, or by detailed amino acid sequencing.

DR. FLEISCHER: So there is no histochemical selectivity.

DR. BRANTON: I am not aware of any. I think that what you are implying, by the way, is that even though we are dealing with a muscle cell, the kind of actin that underlies the membrane might be different from the kind of actin in the myofilament.

Thus, one would not have to worry so much about contamination, but rather try to measure just that particular kind of actin. In other words, the kind of actin characteristic of non-muscle cells should be the kind of actin in the membrane cytoskeleton.

DR. ENGEL: If the abnormality of the plasma membrane in Duchenne dystrophy were due to some kind of disturbance in the lipid composition of the membrane, could that lead to a focal discontinuity within the membrane?

DR. de KRUIJFF: No one has ever demonstrated open-ended membranes, so if the lesions really exist they must be very unique. I am also particularly intrigued by the stacks of basal lamina on top of the membrane gaps. Conceivably these represent hexagonal H_{II} formations at the gaps. Is there really evidence that open-ended membranes exist, or are there cytoconnections? The latter again would be highly intriguing in terms of the lipid component.

DR. FLEISCHER: Actually you are quite right, one generally does not see open-ended membranes, but there is a system used by Jorgensen for preparing the sodium-potassium pump which gives rise to discs which are open-ended and I must say that I am very puzzled by it. If you really wanted to study open-ended membranes, these discs would be a good system. These are kidney membranes prepared by highly enriching a particular layer from the medulla with regard to the sodium-potassium pump. These are then subjected to negative purification with the detergent sodium dodecyl sulfate (SDS). The sodium-potassium pump seems to be stable to SDS at a certain concentration. The detergent removes contaminating proteins and this is known as negative purification. The orientation of the sodium-potassium pump in the membrane is unidirectional. So there are two unusual aspects of this preparation -- unidirectionality of the pump despite SDS treatment and open-endedness of the discs. I recommend this preparation to anyone interested in studying these rare phenomena.

DR. BRANTON: What is negative purification?

DR. FLEISCHER: One starts out with membranes which have a number of associated proteins. Detergents selectively remove certain proteins but not others from the membrane. Those components not removed represent a higher and higher percentage of the remaining proteins or essentially all remaining proteins so the membranes become homogenous with respect to a single protein. In this preparation there are only two polypeptides which are characteristic of the sodium-potassium pump. Negative purification has been also applied to the calcium pump associated with the sarcoplasmic reticulum, but in this instance one does not obtain open-ended discs.

DR. CHARNOCK: I think it is very fair to point out that there is an incredible concentration of SDS in that preparation.

DR. FLEISCHER: One may wash out a lot of the SDS, but I cannot say how much is left behind.

DR. HYDE: I have never heard of replication of basal lamina. Is it a repair mechanism?

DR. ENGEL: Actually there is a rather nice publication by Vracko and Benditt (1972) on replication of basal lamina in relation to capillaries. They find that whenever capillaries are injured and then regenerate, there is replication of their basal lamina. It is fairly well recognized by pathologists that replication of basal lamina is associated with focal repair.

DR. HYDE: One could immediately make the obvious comment that the lesion is the repair mechanism.

DR. ENGEL: One can only infer that there is focal repair.

DR. FLEISCHER: Can you review again what percentage of lesions you see in dystrophic vs. normal muscle fibers? You see the lesions in both?

DR. ENGEL: Discontinuities in the plasma membrane are not seen by electron microscopy in normal muscle.

DR. FLEISCHER: About 5 per cent of the dystrophic fibers show these lesions in the given plane of sectioning?

DR. ENGEL: Yes.

DR. FLEISCHER: If the lesions are present in 5 per cent of the fibers in a given plane, would they indicate a very high likelihood that all fibers have these lesions?

DR. ENGEL: The frequency is probably considerably higher than 5 per cent but affected fibers tend to show multiple lesions along their length. Thus, the fiber which shows a lesion in one plane of sectioning is more likely to display a lesion in another plane of sectioning. One cannot be sure that all fibers are affected.

DR. FLEISCHER: Have you studied that by cutting longitudinally?

DR. ENGEL: Yes, we have. The percentage affected in longitudinal sections is not particularly different. Incidentally, longitudinal sections again only reveal a very limited segment of a given muscle fiber.

DR. FLEISCHER: In longitudinal sections some fibers display multiple lesions and some fibers show none?

DR. ENGEL: One can see multiple lesions in some fibers whereas others show one or none.

DR. PARK: When there is more serious degeneration and malfunction, do you observe more than 5 per cent of affected fibers?

DR. ENGEL: We are studying lesions in non-necrotic fibers. But for that matter, if one counts necrotic fibers, the percentage of these does not increase as the disease progresses. There are fewer fibers present and there is increasing replacement of contractile elements by connective tissue.

DR. PARK: So the 5 per cent in a sense is a batting average which does not tell anything about fatty infiltration or necrosis, or the general state of the muscle?

DR. ENGEL: About 5 per cent of the remaining fibers display membrane lesions yet are not necrotic in a single plane of sectioning.

DR. BRANTON: Would you even go so far as to say that the low percentage of fibers showing these discontinuities of the plasma membrane suggests that maybe these discontinuities are irrelevant and really not of great concern? When you began the discussion I had the impression that these lesions represented the first stages of the process which leads to cell death and disappearance, but now what I think I am hearing is that the eventual massive loss of muscle fibers is independent of these lesions. Perhaps it is a poor idea to focus on muscle fibers which display these lesions, but instead, we should consider the basic question of why fibers are disappearing.

DR. ENGEL: I did not mean to imply that.

DR. BRANTON: What is the mechanism of fiber disappearance?

DR. ENGEL: The mechanism of fiber disappearance is irreversible destruction of all fiber components followed by removal of the damaged material by macrophages. This can be defined as fiber necrosis.

DR. CHARNOCK: Could you tell me something about the dimensions of the membrane defects?

DR. ENGEL: The dimensions vary from a fraction of a micron to more than one micron in size.

DR. BRANTON: And what precedes necrosis?

DR. PARK: Could the plasma membrane defects really be part of the process of progressive degeneration? At an early stage there is a defect which is observed on the way to necrosis. In other words, fibers which have these defects become more susceptible to necrosis. The healthy fibers, in turn, are still functioning normally. Eventually, when perhaps as many as 50 per cent of the fibers have been destroyed, clinical disabilities become more apparent. It seems to me that the membrane lesions may be on the pathway of fiber degeneration and fragmentation. Big fragmentations would ultimately destroy the fiber.

DR. ENGEL: I am not sure what you mean by big fragmentation. I do think that these fibers are at an increased risk of undergoing destruction. Perhaps one might say that at any given time 5 per cent of the muscle fiber population is at risk of undergoing necrosis and the risk is such that a certain number of fibers will drop out from a unit volume of muscle per unit time. This will be offset by the regenerating potential which will result in the formation of new muscle fibers. If one were able to substitute actual figures for these variables, one might be able to estimate the rate of net decrease in the total number of muscle fibers. Coming back to Dr. Branton's question, I do think that these fibers are at an increased risk of undergoing necrosis. When the defects are very small they could conceivably seal over as suggested by Carpenter and Karpati (1979). These workers suggest that focal plasma membrane loss initiates muscle cell necrosis unless it is repaired. Defects that are not repaired will allow the influx of calcium rich extracellular fluid which, in turn, will have a number of deleterious effects. These effects include activation of proteases which will attack structural proteins; calcium overloading of mitochondria and sarcoplasmic reticulum, which can disrupt the function and structure of these organelles; and the influx of complement components into the muscle fibers. If complement becomes activated then this will result in the release of chemotactic factors which will attract macrophages and opsonize the fiber for removal by the macrophages. Further, the lytic reaction products of the complement cascade will lyse membranous organelles. This may sound like an elaborate scheme, but in the last year or so we have obtained definitive evidence that complement is universally involved in muscle fiber necrosis (Engel and Biesecker, 1980). In serial cryostat sections of 66 muscle biopsies containing necrotic fibers we demonstrated the C5b-9 membrane attack complex (MAC) of the complement reaction sequence. All necrotic fibers and none of the non-necrotic fibers reacted positively for MAC (Figure 6-5). Now then, to come back to Dr. Branton's question, my current thought is that muscle fibers which are beginning to see the extracellular environment are subjected to an alteration of their internal milieu.

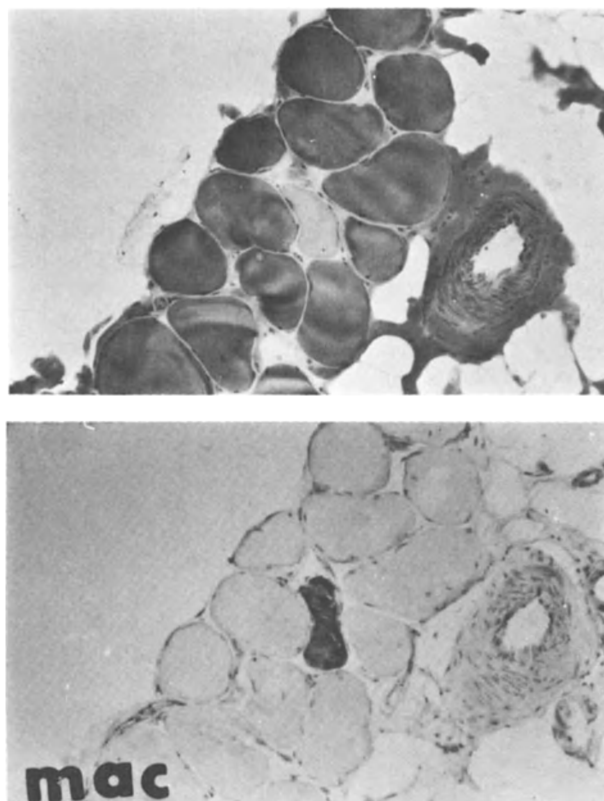


Figure 6-5: Becker type dystrophy. Top panel: trichromatically stained section displays pale necrotic fiber in center. Bottom panel: nonconsecutive serial section reacted for the localization of the neoantigenci determinant(s) of the lytic effect of the C5b-9 membrane attack complex (MAC). The necrotic fiber reacts strongly for MAC. This indicates that complement components entered the fiber during necrosis, that the lytic complement pathway was activated, that MAC was assembled, that MAC bound to target surfaces within the fiber, and that complement split products (C5a, C3b), capable of recruiting macrophages and stimulating phagocytosis, were formed. The cytochemical localization of MAC cannot be due to nonspecific adsorption of complement precursors because the immunoreagent employed was specific for MAC neoantigen(s).

Depending on the ability of the fiber to seal itself off from the outside environment, the muscle fiber may or may not become subject to further injury and eventually necrosis. Interestingly, in serial sections one can observe transitions between fibers that show multiple membrane defects and frank necrosis. Necrotic fibers are usually denuded of their entire surface membrane.

DR. de KRUIJFF: Can you see the size of the lesions enlarging? And are the lesions always approximately 1 micron in diameter?

DR. ENGEL: One cannot make sequential observations on lesions on a single muscle fiber by electron microscopy. What one observes represents what exists at the moment of the biopsy. However, when a fiber is completely necrotic, the membranous organelles are in various stages of breakdown and the contractile elements are hypercontracted. The plasma membrane, in my experience, has nearly completely disappeared.

DR. de KRUIJFF: In the earlier stages, can one see small defects in the plasma membrane?

DR. ENGEL: Yes. These are the ones I have described. They are of the order of a fraction of a micron in size. According to Carpenter and Karpati (1979) such defects may even occur in fibers with no structural damage or focal hypercontraction. Conceivably, there are even smaller defects in the membrane which cannot be resolved by ordinary transmission electron microscopy.

DR. de KRUIJFF: The mechanism producing these changes is unique, and this might give some indication what to look for. It is very unusual to have an open-ended membrane.

DR. ENGEL: Earlier during the symposium you have commented on this and Dr. Fleisher referred to some means of obtaining open-ended membranes. I might say that one can puncture holes in membranes mechanically. If the holes are large enough, the membranes will not seal (Carpenter and Karpati, 1980). Labelizing the membranes by chemical means, as for example by 'chemical skinning' of muscle fibers, can also cause defects in the plasma membrane (Eastwood, Wood, Block and Sorenson, 1978). Muscle fibers can be exposed to detergents, by injection of lysolecithin or Triton X-100 into muscle (Parhad, Pestronk, Price and Drachman, 1980). This will induce light microscopic changes which in some ways resemble the biopsy findings in Duchenne dystrophy. Finally, adding a very small amount of a detergent to a solution bathing an otherwise intact muscle will literally chew up the plasma membrane.

DR. de KRUIJFF: Detergents remove not only cholesterol, but all lipid components from the membrane.

DR. ENGEL: Is it conceivable that an abnormal component of the lipid bilayer would confer increased fragility on the membrane which could cause it to break down when stressed?

DR. de KRUIJFF: I find it remarkable that detergent treated muscle fibers may resemble those in Duchenne dystrophy. Fillipin can complex cholesterol, inducing the formation of 1,000 Å hydrophobic aggregates which disrupt the membrane. So perhaps one could consider the possibility that some factor alters the state of aggregation of cholesterol in the membrane and thus confers lability on it.

DR. ENGEL: One might also be interested in the relative distribution of free and esterified cholesterol.

DR. de KRUIJFF: Cholesterol esters do not form membrane components.

DR. BRANTON: What about lysophosphatides?

DR. ENGEL: Could you elaborate on this?

DR. BRANTON: The appearance of the membrane is reminiscent of detergent treated membranes. Lysophosphatides are detergents which can occur biologically. Is there an increase in lysophosphatides in the dystrophic muscle membrane?

DR. ENGEL: I do not know whether this has been investigated in Duchenne dystrophy.

DR. FLEISCHER: Pursuing Dr. Charnock's suggestion that SDS has something to do with the open-endedness of the sodium-potassium pump discs, I would like to raise the question that biological detergents might exist in these membranes. Lysophosphatides can be generated by internal phospholipases which are present in biological membranes. The mitochondrial membrane can be intentionally digested with phospholipase A which will degrade 70 to 75 per cent of the phospholipids, but does not disrupt the membrane. Addition of albumin removes fatty acids, but the membranes still remain intact. Chromatin granules contain a sizeable amount of lysophosphotides and yet are difficult to breakdown. Therefore, it is not obvious that lysophophotides can be implicated, but these experiments were not done on the plasma membrane of the muscle fiber which may respond to detergents differently.

DR. BUTTERFIELD: I would like to propose an explanation for the findings described by Drs. Engel and Schotland. Let us suppose that a phospholipase results in the formation of a small amount of lysophospholipid within the membrane. An increase in lysolecithin

would make the membrane more rigid. In addition, proteins might be aggregated in the membrane. This would decrease the con A binding ability. Perhaps there might be a decrease in internal membrane particles by freeze-fracture. This, in turn, might decrease the number of square arrays in the plasma membrane as well. Regions of the plasma membrane would have more lipid than usual because the proteins have been aggregated. If one accepts the generalized membrane defect hypothesis in Duchenne and myotonic dystrophy, then the main fault might lie with proteins.

DR. de KRUIJFF: We can degrade 40 per cent of the lipid in the erythrocyte membrane to lysolecithin and fatty acids but do not see any change in the physical aspects of the membrane and its stability remains very good. We do not see aggregation of intramembrane particles. I do not see any reason why the muscle membrane should be any different.

DR. BUTTERFIELD: I only mentioned the lysolecithin aspect because of the previous discussion. I am not sure whether or not it applies. The main point I wanted to make is that if, for whatever reason, protein aggregation occurred, this could cause invaginations in the membrane or could even result in a hole in the plasma membrane.

DR. ENGEL: It does seem to stretch my imagination a little bit but I guess we could keep it in mind. I am not quite sure how aggregation of intramembrane particles could cause collapse of the plasma membrane. I really do not see how this would work.

DR. SCHOTLAND: In the freeze-fracture studies we really do not see aggregation of intramembranous particles.

DR. ENGEL: One of the things that cause aggregation of intramembranous particles is anoxia, yet the plasma membrane remains intact for a period of time even after considerable aggregation of particles has occurred. You can also see aggregation of particles in the course of the capping phenomenon, as when antigenic determinants on the surface of the lymphocyte membrane become aggregated when exposed to cross-linking antibody, but that does not seem to increase the fragility of the membrane. I cannot follow the argument that aggregation of membrane particles can damage the membrane and Dr. Schotland's freeze-fracture studies did not describe aggregation but a decrease in number of membrane particles.

DR. BUTTERFIELD: Are the sizes of the membrane particles seen by you in your freeze-fracture work, Dr. Schotland, indicative of the usual sort one can see in normal membranes?

DR. SCHOTLAND: We have not done histograms of particlular size. On inspection there may be a dropout of 60 Å particles.

DR. PARK: Do type 1 or type 2 fibers show more membrane defects?

DR. ENGEL: I do not think there is selective involvement of histochemical fiber types.

DR. SANDRA: Have you considered the possibility that the plasma membrane defects are related to the way the membrane is sectioned?

DR. ENGEL: By serial sectioning and passing though at least several thousand Å one can trace the defects. The membrane defects are still there. However, you do raise a good point. If the sections are not of good quality, or if they are too thick, or if the membrane is not well stained, or if the section is grazing, one could talk oneself into assuming that the membrane is discontinuous. However, in addition to the discontinuity in the membrane, there are also abnormalities in the immediately underlying fiber regions and there is evidence for ingress of extracellular fluid. So a number of lines of evidence suggest that the membrane defects are not an artifact of the sectioning technique.

DR. SCHOTLAND: When you tilt the electron microscope stage beyond 6 degrees, sometimes you pick up additional defects in the plasma membrane that you would miss ordinarily.

DR. ENGEL: Dr. Schotland has also mentioned previously that after staining with tannic acid he can see the membrane lesions even more clearly. I would now like to ask Dr. Hyde to comment from the standpoint of his expertise. Let us assume that there is an increased membrane rigidity, or decreased membrane fluidity, as perhaps suggested by saturation transfer paramagnetic resonance studies of the red cell in Duchenne dystrophy. Let us assume that one can extrapolate from this to the plasma membrane of the muscle fiber. What does this tell you about the structure of the membrane, or its molecular architecture?

DR. HYDE: This alteration would have to be a consequence of an altered composition of the membrane. There may be an alteration in the membrane cholesterol content. I do not know what else could give rise to altered fluidity. However, the alterations may not be simply a consequence of a change in lipid composition.

DR. FLEISCHER: Dr. Schotland suggested that there is a change in the carbohydrate composition of the plasma membrane of the muscle cell as reflected by con A binding. If there is a change in in sialic acid, or accelerated removal of sialic acid from the erythrocyte, the cell is rapidly destroyed. Of course, muscle is not subject to the same type of filtration and exposure to phagocytic cells as the erythrocyte.

Glycoprotein and Binding of Concanavalin A

DR. BUTTERFIELD: Sialic acid levels have been measured in myotonic dystrophy and possibly in Duchenne dystrophy. Normal results were obtained.

DR. FLEISCHER: Observed levels might be normal, but one would like to know about the rate of removal of sialic acid residues from the membrane.

DR. BUTTERFIELD: I think it is very important that we should look closely at the protein components of the plasma membrane. Lipid analysis is somewhat easier and more sophisticated at the moment, but proteins need to be examined closely, too. The cell surface is obviously quite important and communication between the cell surface and the cytoskeleton should be considered. Alteration in the cytoskeleton could give rise to the defect in the plasma membrane. I am not claiming that this is the case by any means, but it is certainly well worth looking into.

Signifiance of Plasma Membrane Proteins

DR. ENGEL: Dr. Howland, do you have any comments at this point?

DR. HOWLAND: I would like to take the other side of what Dr. Butterfield was saying and suggest that the hydrophobic components of the membrane deserve a lot of attention.

DR. BRANTON: If I understand correctly, there seem to be alterations in lipid in a number of different cells, including the red cells, in muscular dystrophy and yet the evidence to date suggests that there is no great change in the appearance or survival of the red cell. Although we know a great deal about lipids in the red cell, we cannot assign a particular function to any one lipid. In general, what we seemed to have learned over the last few years is that most proteins function equally well in a variety of different environments. The specificity which we looked for in lipids had not been detected or established. However, as regards proteins, differentiated cells express specific proteins.

A certain class of proteins might be found in the red cell, a different class in the muscle cell, yet a different class in another cell. By contrast, in a wide variety of cells the membranes probably contain similar lipids, represented in greater or lesser amount. Thus, if I were going to study muscular dystrophy, I would certainly focus on unique proteins in the muscle cell membrane. Unfortunately, one has to deal with a huge number of proteins and one would not know where to start.

DR. M. GLASER: There are only a limited number of enzymes which act specifically on lipids, but it could be that there are a variety of enzymes which sense the lipid environment and that an altered lipid composition gives rise to different fluidity which influences the activity of a membrane bound enzyme. I would like to ask Dr. Howland if he observed differences between normal and Duchenne fibroblasts, and whether he thinks the fibroblast is suitable for biochemical studies?

DR. HOWLAND: The answer is yes. We do see differences between control and Duchenne fibroblasts, the most apparent being an increased turnover of labeled phosphoinositides under the influence of epinephrine or con A. From a kinetic standpoint the findings suggest a block in the resynthesis of phosphotidylinositol. An abnormality of this kind might have a profound effect on membrane proteins as well as causing a small change in one of the major phospholipids.

DR. ENGEL: Dr. Gonzalez-Ros, what are your thoughts on what might be wrong with the membrane in Duchenne dystrophy?

DR. GONZALEZ-ROS: Before I can speculate about the problem I would need to have much more detailed information. I would search for an experimental system. Perhaps there isn't one thus far. I would like to ask someone else about the possibility of using a culture system, such as myoblasts. Is there a possibility of finding an adequate experimental system?

DR. ENGEL: I think what you say is entirely reasonable and one can only regret that there is not a good experimental or natural model for Duchenne dystrophy in the animal kingdom. None of the animal dystrophies are faithful reproductions of Duchenne dystrophy. As far as the cultured muscle cell is concerned, one is handicapped by the following: (1) Human muscle fibers grow very slowly in culture and are much more difficult to grow than, say, chick embryo cells. (2) We could argue that what goes on in tissue culture represents survival of the fittest, and the cells which grow have been selected against displaying an abnormality. (3) The cultured muscle cell *in vitro* is not subjected to the same mechanical stresses and work demands as the mature muscle fiber *in vivo*.

(4) Cultured human muscle cells do not differentiate fully, and usually do not contract. (5) The proteins expressed in the membrane of mature cells in vivo may not be the same that are expressed in the less differentiated cultured cell in vitro.

Coming back to the elegant studies that you demonstrated extracting membrane proteins, reincorporating them into an artificial lipid bilayer and then studying their function -- could this approach be applied to the study of membrane proteins other than the acetylcholine receptor?

DR. GONZALEZ-ROS: It very well could be, but the problem is that we have no way to assign specific responsibility for dystrophic symptoms to a specific type of membrane protein.

DR. ENGEL: So this approach would be too diffuse.

DR. GONZALEZ-ROS: What one would need would be a specific protein which one could associate with the manifestations of the dystrophic state.

DR. ENGEL: For that type of approach to be fruitful one would have to know specifically the type of protein which is abnormal and how its functional properties can be studied in the membrane. Unfortunately, this situation is much more complicated than it was in the case of the acetylcholine receptor protein, a deficiency of which is associated with myasthenia gravis.

DR. de KRUIJFF: Have there been any studies of serum lipid or lipoprotein composition in Duchenne dystrophy?

DR. ENGEL: It is a fair guess that any routine laboratory test that can be done has been done on patients with Duchenne dystrophy. But specifically, I cannot recall published negative studies on the serum lipid profile. It might be profitable to study cultured muscle cells in media enriched with normal human plasma or Duchenne serum.

Dr. Kent has done some very interesting studies on membranes of cultured muscle cells. She has removed choline and phosphocholine compounds from the membrane by means of phospholipase C digestion and has shown that this turns on the synthetic machinery of the cultured muscle cell. Her findings suggest that if a membrane lipid component is degraded or removed, the cytosol responds by an attempt to replace this component. In terms of this model, is it conceivable, Dr. Kent, that if a membrane constituent could not be synthesized, or if one of the membrane constituents were altered, the stability of the membrane would be compromised?

DR. KENT: It appears that in some conditions we are able to know what is going on in the cultured cell. One would have to ask how does the cell know what is going on on its surface membrane and what lipid it needs to synthesize after exposure to phospholipase C or what protein to produce after exposure to trypsin. It is conceivable that in dystrophy there is a problem with the composition of the surface membrane; perhaps there is also a problem with the signaling mechanism from the membrane to the cell, namely in the transmission of information about the cell surface to the cell interior. However, we know very little about the way the cell repairs its own surface, or how the cell monitors what is going on within the membrane.

DR. ENGEL: So it is conceivably a problem with the signaling mechanism or with the biosynthetic machinery.

DR. KENT: Yes, or it could be a problem with the monitoring process. Also, a lot of membrane goes to the lysosomal machinery. Is it possible that there is a problem with lysosomal enzymes or with proteases in dystrophy? Could these be overly active, eventually damaging the surface membrane?

DR. ENGEL: There is really no good evidence that lysosomal enzymes or proteases play a primary role in the course of dystrophy. Rather careful studies of lysosomal enzymes by Pearson and Kar (1979) have shown that some lysosomal enzymes increase early and some lysosomal enzymes increase late in the course of dystrophy, but it is unlikely that these play a primary pathogenic role in the disease process.

DR. M. GLASER: Does the plasmalemma undergo phagocytosis?

DR. ENGEL: Phagocytosis is a very terminal event and signals cell necrosis. A study of phagocytosis alone would not be an appropriate approach to the cause of the early membrane lesion.

DR. M. GLASER: Does one see an increased number of endocytotic vesicles in the dystrophic plasma membrane which would indicate that the membrane is turning over rapidly?

DR. ENGEL: Endocytosis has not been looked at very carefully but there have been some elegant freeze-fracture studies by Dr. Schotland bearing on this point and perhaps he would like to comment on them.

DR. SCHOTLAND: We just finished a study of caveoli (which are presumed to correspond to pinocytotic vesicles in the freeze-fractured plasma membrane) in Duchenne dystrophy, facioscapulohumeral dystrophy, myotonic dystrophy and normal controls.

In Duchenne dystrophy there was a significantly increased number of caveoli in the surface membrane, but normal results were obtained in the other dystrophies (Bonilla, Schotland and Wakayma, In press).

DR. ENGEL: This might suggest that the turnover of the plasma membrane is increased. Dr. Sandra, have you any thoughts or comments?

DR. SANDRA: There are a number of studies of membrane lipids in Duchenne dystrophy and in animal models. A number of differences have been found. This would suggest that the membrane of dystrophic muscle is indeed somewhat different.

DR. ENGEL: Which particular studies are you referring to?

DR. SANDRA: I am thinking of perhaps three or four papers published on the subject.

DR. ENGEL: Dr. Kunze in Germany has studied this (Kunze, Reichmann, Egger, Leuschner and Eckhardt, 1973; Kunze, Reichmann, Egger, Olthoff and Dohler, 1975). Dr. Willner, I think the studies reported in these papers are still unconfirmed. Is that correct?

DR. WILLNER: Yes. He studied lipids in muscle specimens as well as in erythrocytes.

DR. SANDRA: It would be useful to look at these reports and try to correlate the findings to see if there is anything in the system -- investigators may look at 10 or 15 lipids and they could contradict one another.

DR. ENGEL: Dr. Strittmatter, you have presented some interesting studies on how very small alterations in the composition of the membrane due to methylation of phospholipids can alter membrane function. Do you think that a mechanism of this type might be responsible for altered adenylate cyclase activity, or perhaps a defect in this mechanism could affect the structural integrity of the plasma membrane?

DR. STRITTMATTER: It might be useful and interesting to look at small specific reactions involving membrane lipids instead of looking at the lipid profile of the entire membrane. I would just like to make a point on your data which show the patchy absence of the plasma membrane -- these raise the possibility of a constraint on lateral movement of the membrane, and it might be most interesting to find out what could cause such a constraint over a considerable distance within the membrane. Possibly there is an abnormality in membrane anchoring by the cytoskeleton. One should keep this possibility in mind when studying protein interactions with the membrane. A series of pharmacological tools could be used in studying enzyme transport.

DR. ENGEL: Could you formulate that more precisely? One would like to have a hypothesis before one can walk into the lab. What would you be testing and why?

DR. STRITTMATTER: The hypothesis is whether or not in Duchenne dystrophy an abnormality of the anchoring role of the cytoskeleton and/or of protein mobility in the plasma membrane can provoke the abnormalities seen clinically.

DR. CULLIS: I have a question which may express my basic ignorance in this area. I seem to remember reading somewhere that the density of the muscle fibers in the newborn animals is quite high. This decreases as the animal becomes older with concomitant elargement of individual fibers to make up the difference. Is that correct?

DR. ENGEL: Muscle fibers have a small diameter in infants and there is a regular growth of the muscle fiber with age.

DR. CULLIS: Does the total number stay the same or does it decrease?

DR. ENGEL: Muscle fibers become longer as well as increase in diameter. What are you leading up to?

DR. CULLIS: Perhaps something is wrong with the mechanism which eliminates individual muscle fibers and perhaps this is what is causing muscular dystrophy.

DR. ENGEL: Well, overall, I think that is correct if you say that those people who die are the ones who are not fit to live. But that does not tell us too much about the diseases and the cause of death.

DR. CULLIS: The consensus seems to be that the membrane itself is the locus of the expression of the disease. It might be some other defect.

DR. WOLF: What he is saying is that the normal mechanism to eliminate muscle fibers in the process of development is exaggerated or prolonged. We could argue that in practically every disease a derangement of this type might operate.

DR. ENGEL: In the course of development, amphibian limbs may be resorbed rapidly or may regenerate readily. However, one does not see the same histologic picture as in Duchenne dystrophy.

DR. CULLIS: If one is searching for a model that can be studied in a reproducible manner, one should turn to the cell culture system.

Here one can have a clear idea as to what is happening at a given moment and the material is well defined. This seems to be one of the best approaches.

DR. KENT: I would like to second that motion. We heard that erythrocytes and fibroblasts in Duchenne dystrophy are suitable for study, but cultured cells are not because one does not see deterioration of cultured cells. However, one also doesn't see deterioration of erythrocytes or fibroblasts. Muscle cells might lend themselves to the study of early problems. How much tissue can one obtain for such a study?

DR. ENGEL: Unfortunately, human muscle does not grow as readily as animal muscle, nor are there good clones of human muscle that one can study. Furthermore, one could question whether clones are useful anyway, because they have altered cellular properties. One can get milligram quantitites after several weeks of culturing muscle from a patient.

DR. SANDRA: The culture system, of course, has its advantages but it has certain disadvantages, as mentioned before. The culture medium is not equivalent to the normal environment of the cells. Further, the cells are arranged in a monolayer and not in their usual tissue or organ arrangement. An additional thought would be to do transplantation experiments in certain strains of animals, using particular markers to trace a certain muscle and keep track of it after transplantation. This would be an alternative to the culture situation, but it has obvious limitations. Still it could be used as a probe.

DR. ENGEL: This has been done to some extent in animal models, but it would be extremely difficult to do in the human situation.

DR. GONZALEZ-ROS: Has anyone searched for muscular dystrophy in monkeys?

DR. ENGEL: Not that I know of, but it is an extremely good question. You have to keep in mind that monkeys are studied by relatively few investigators and most of the experimental animals are aggregated in primate centers. The animals are very expensive and difficult to study. Yet this is where we should look for a naturally occurring model of Duchenne dystrophy.

DR. GONZALEZ-ROS: One can induce mayasthenia gravis in monkeys very well, but monkeys differ in several respects from other experimental animals.

DR. ENGEL: Unfortunately, one cannot experimentally induce Duchenne dystrophy the same way one can induce myasthenia gravis.

DR. MURRAY: I would like to ask a few questions of Dr. Engel and Dr. Schotland on the theme that Dr. Strittmatter mentioned regarding studying the cytoskeleton and perhaps alterations in the inner protein components of the membrane. It is hard for me to envision that patches of membrane lipids would be absent without somehow being an associated change in the cytoskeleton. In Dr. Engel's studies on defects in the membrane and Dr. Schotland's studies on decrease in con A binding sites, was there suggestion of asymmetry around the lesion in either the density of lectin binding sites or in anything that would show asymmetrical arrangement of proteins?

DR. ENGEL: Dr. Schotland have you studied lectins other than con A or have you tried to make a systematic map around the lesion, using serial sections, of lectin binding sites?

DR. SCHOTLAND: We are in the process of studying different lectins, but we have not studied the distribution of lectin binding sites in serial sections.

DR. ENGEL: Can one see those patches in which the lectin binding sites are diminished by light microscopy?

DR. SCHOTLAND: Yes.

DR. MURRAY: If you could see something around the most abnormal regions either in excess or in reduced numbers, it could provide some lead on a transmembrane protein, such as Dr. Branton has shown in the erythrocyte. One might then try to isolate such a protein and have a probe that might contribute to solving the puzzle.

DR. ENGEL: The lectin binding sites are extrinsic glycoproteins, are they not? They may or may not tell much about transmembrane proteins.

DR. SCHOTLAND: There are transmembrane proteins that have carbohydrate moiety sticking on them, like glycophorin. Not too many proteins have been studied thoroughly in terms of structural arrangement in the membrane. Most of those glycoproteins that have been studied were transmembrane proteins. Examples are the HLA antigen and glycophorin. As far as con A is concerned, electron microscopy is also useful in studying the topographic distribution of the binding sites.

DR. ENGEL: Dr. Charnock, does the hibernating ground squirrel provide any clues for the study of Duchenne dystrophy?

DR. CHARNOCK: I would like to take a moment to put forward a proposal for discussion which may or may not have any relevance to muscular dystrophy. I think, perhaps, we can consider the concept of lipid modulation of membrane bound enzymes. A unique array of lipids in the membrane is likely to influence the function of an enzyme in the membrane, the function of the membrane bound enzymes will also change. This would be an instance of modulation of a membrane enzyme by its lipid environment. However, if there was a whole series of transmembrane enzymes readily modulated by the lipid composition of the membrane, one would expect to find an incredibly variable biological system and a haphazard state of biology. This, in turn, suggests that the purpose of the membrane lipids closely associated with transmembrane proteins is not so much to modulate enzyme function but to protect enzymes from massive changes, or nutritional alterations, in the environment. Therefore, what we should look for is constancy of environment, not a modulating effect on enzyme function. Whether we feel that that has any bearing on muscular dystrophy itself, I do not know. But we might rethink the concept of lipid modulation of membrane enzymes from the point of view of what roles lipids play in altering or protecting the function of enzymes.

DR. ENGEL: Does anybody react to that?

DR. FLEISCHER: We first showed it about twenty years ago that some enzymes associated with membranes require lipid for function, but in all the work that has followed, there really has not been a definite demonstration that the function of a lipid-requiring enzyme is modulated in a practical way by the fatty acid moeity. The fatty acid composition of the membrane can be changed by the diet, but there has not been a demonstration of an associated change in enzyme properties. One can alter the function of the calcium pump protein by altering the composition of the lipids in the membrane which contain it so that the enzyme does not pump calcium. But it is hard to imagine that this is a physiological or pathological mechanism in the intact organism. In the main, there are only a few enzymes that have a specific requirement of phospholipids for their function. One of these is β-hydroxybutyrate dehydrogenase. This enzyme needs lecithin, but actually it works best in a mixture of mitochondrial phospholipids rather than with lecithin alone, and we have studied the nature of the lipids adjacent to the enzyme in the membrane. We found that the isolated enzyme had no function but the addition of lecithin or mitochondrial phospholipids restored function. The reaction mechanism of the enzyme in the intact membrane, and of the isolated reactivated enzyme is essentially the same. However, the kinetic parameters of the enzyme in the membrane are similar to those of the isolated enzyme reactivated by mitochondrial phospholipids, but very different from those of the isolated enzyme reactivated by lecithin.

In this exceptionally well studied situation, the enzyme does seem to respond to its lipid environment, consistent with the fluid mosaic model of the membrane. However, my own opinion is still that if one is looking for large differences between the normal and dystrophic membrane, one will ultimately have to focus on proteins because they have such exquisite sensitivity and specificity which one cannot envision with lipids. Even if there were some differences between normal and dystrophic lipids, the difference eventually would have to be traced back to a protein.

We have been studying the calcium pump protein in skeletal muscle and more recently have compared the calcium pump protein of skeletal muscle with that of heart. The two proteins are different in an exquisite way.

DR. PARK: It may well be that the changes in the surface membrane are the expression of abnormalities in enzymatic activity of proteins. In the red cell this is expressed in terms of changes in EPR whereas in the muscle cell it is expressed as a gap in the plasma membrane. Previously we have done a lot of work with soluble enzymes of the glycolytic cycle. These decrease in activity in dystrophy, or sometimes appear to become inactive. (Possibly they are decreasing because they are leaking from muscle into serum the same way creatine phosphokinase is leaking). We have been interested in the notion that changes in the redox status of the cell may profoundly affect the cell. Such a change could inactivate crucial enzymes in the glycolytic cycle. It could also result in defects in the plasma membrane, possibly via lipid peroxidation or via inactivation of various enzymes by oxidation of sulfhydril groups. Perhaps this is what Dr. Butterfield is observing when he studies the red cell with his particular EPR label. I think there may be a good correlation between changes in the intracellular redox state, changes in soluble enzymes and defects in the surface membrane of the muscle fiber.

DR. ENGEL: I like your optimism, but there is a flaw in the argument. Not all cells have defects in their surface membrane at any one time. Possibly only a small fraction of the cells are leaky at any one time.

DR. PARK: I don't see that as a problem for not all cells may be at the same stage at the same time in a degenerative disease. Various factors might be affecting individual cells, such as their immediate environment, the circulation, the adjacent tissue cells, i.e. fat cells vs. muscle cells. Some muscle fibers are exercised more than others. So, it is easy to see that different cells are affected to a different extent at a given time.

DR. ENGEL: Conceivably, if some of the lesions are submicroscopic they could allow leakiness of enzymes yet would not be detectable with conventional morphologic methods. Let me ask Dr. Hyde whether the saturation transfer electron paramagnetic resonance studies on the erythrocyte could be extended to cultured muscle cells. Can one study the plasma membrane of cultured muscle cells by this technique? Are there suitable specimen holders? Are there technical problems in studying cells of different shapes and types?

DR. HYDE: We do have a cell culture facility and have just publsihed a paper on membrane fluidity in a line of synchronized, cultured cells. This was possible by using a higher microwave frequency (in the 35 megaherz range). However, we do have to produce a sufficient volume of synchronized cells.

DR. ENGLE: So it would be technically feasible to study the plasma membrane of cultured muscle cells for possible alterations in saturation transfer EPR.

DR. HYDE: The cells are placed in a container which is 10 x 1 x 1 mm or so in size. This volume is packed very closely with cells.

DR. PARK: I would like to say that EPR methods provide a tremendous advantage over ordinary spectrophotometric techniques in that one need not be concerned with turbidity, or other factors, which make spectrophotometric measurements difficult. The EPR methods are more flexible and versatile.

DR. M. GLASER: How many milligrams of lipid or protein does one need to obtain saturation transfer EPR measurements?

DR. HYDE: The best answer I can give is in terms of the dimensions of the container that we need to pack full with cells. The cell is approximately one centimeter long and one to two millimeters thick.

DR. M. GLASER: Are the cells packed in a solution?

DR. HYDE: We pack them.

DR. ENGEL: That does not sound like a prohibitive number of cells. One could begin to do measurements on muscle cells which grow readily.

DR: HYDE: It is a big breakthrough based on the use of higher frequency of the signal. This also enables us to detect rather anisotropic motions and analyze them in greater detail.

DR. ENGEL: The study of the cultured muscle cells by this technique will be interesting. However, the defects in the surface membrane have not yet been detected in cultured muscle cells.

DR. de KRUIJFF: A diseased membrane may allow breakdown of the membrane barrier. It might be a very minor breakdown. If one wants to analyze an abnormality of the membrane one has to be very sure that the spin label sits within the plasma membrane and does not become internalized.

DR. PARK: That in itself might be interesting because it might reflect on the transport of fatty acids. Again, a difference between the normal and the control would be of interest.

DR. HYDE: People who do these experiments spend lots of time with the electron microscope.

DR. M. GLASER: I will only make a few comments. A small amount of phospholipid degradation can give rise to lysocompounds which can induce a big change in membrane fluidity. Secondly, alteration of fatty acids in the membrane can affect enzyme activity. For example, changes in fatty acids in the membrane can cause large changes in adenylate cyclase activity.

As far as future research is concerned, in my opinion, the gaps in the plasma membrane must involve degradation of membrane components. We know little about proteases or lipases associated with membrane breakdown. This area needs to be explored further and we must learn more about regulation of the mechanisms which control membrane degradation.

An additional point, already mentioned, is that in order to make progress in biochemical experiments we need good material to work with. One needs a metabolically active cell which can be isolated in large amounts. If, for example, there were an abnormality in membrane lipid metabolism, one could approach this by isolating large amounts of plasma membrane from such cells. The difficulty with myoblasts is that they cannot be obtained in sufficient quantity whereas fibroblasts can be. If there is a genetic defect, it should be also expressed in fibroblasts. Without suitable material for study, we will not make definitive progress.

Finally, I wonder about another thing. Can one keep muscle fibers alive long enough to follow the evolution of the lesions *in vitro*? You mentioned that the lesions in the plasma membrane were big enough to be seen under the light microscope.

DR. ENGEL: We do not see the plasma membrane lesions in the light microscope. We see changes in the underlying muscle fiber regions.

The light microscopic changes have simply guided us to the electron microscopic study of the surface membrane.

DR. M. GLASER: Could one isolate the muscle fiber from a dystrophic animal and study it sequentially?

DR. ENGEL: This would be extremely difficult technically. Carpenter and Karpati (1980) have punctured holes in muscle fibers _in vivo_ with very fine needles guided by a micromanipulator and then studied the ultrastructure of the lesions. These workers feel that the size and number of the lesions inflicted on a muscle fiber determines whether or not the fiber survives. Another experimental study is the injection of detergents, such as Triton X-100 or lysolecithin, into the soleus muscle of the rat (Parhad, Pestronk, Price and Drachman, 1980). These workers concluded that damage to the muscle fiber plasma membrane reproduced characteristic features of the pathology of Duchenne dystrophy. However, I am not entirely sure that there is a way to do the experiment which you suggest.

DR. FLEISCHER: Eventually one must find a protein which corresponds to the genetic defect in the disease.

Enzyme Candidate for Pathogenic Significance of a Genetic Error

DR. WILLNER: To illustrate the difficulties in relating changes in a membrane bound enzyme to a muscle disease, I shall focus on adenylate cyclase. I wish Michael Glaser had discussed adenylate cyclase; he would have done it better and made my task easier. A minimal model of the enzyme includes a receptor on the cell surface, a nucleotide binding subunit, and a catalytic subunit. Activity of adenylate cyclase depends on mobility of these subunits in the membrane, influenced by lipid composition, saturation and methylation and the binding, activation or amount of cytoplasmic proteins, including calmodulin, the cytoplasmic protein which is involved in the effect of cholera toxin on adenylate cyclase, and at least one other still unnamed. Identification of a genetic alteration in activity of adenylate cyclase is the beginning of a research problem, not its conclusion. Components of adenylate cyclase can be dissected and studied individually; these studies are necessary to an understanding of the molecular biology of a genetic error. Abnormalities of enzyme activity which I shall cite should be understood in that context.

In Duchenne dystrophy there have been six studies of adenylate cyclase in crude homogenates of muscle (Table 1). These studies agree that enzyme activity is abnormal, but on important details, such as whether basal activity as well as catecholamine-activated activity are abnormal, there is no consensus. Duchenne muscle contains a heterogeneous, inconstant population of cells, all of

Table 1: Adenylate cyclase in muscle homogenates

First Author	(Ref.)	Number of Subjects	Addition		
			None	Catecholamine * (10^{-4} M)	NaF (10^{-2} M)
Mawarari	1974 & 1976a-b	Normal (7)	2.9	16.1	52.3
		Duchenne (6)	2.6	4.3	20.5
Susheela	1975	Normal (3)	-	1.08	0.85
		Duchenne (5)	-	0.31	0.25
Canal	1975	Normal (16)	5.4	-	42.8
		Duchenne (4)	2.3	-	26.9
Takahashi	1978	Normal (1)	20.0	29.0	53.8
		Duchenne (2)	15.5	18.3	48.4
			21.0	28.6	31.8
Willner	1978	Normal (8)	10.4	33.6	58.5
		Duchenne (8)	5.2	6.6	21.5
Khoklov	1978	Normal (7)	4.1	256.0	1000.0
		Duchenne (6)	3.0	26.0	196.0

Published values,reduced to common denominator, expressed as picomoles/mg prot/min. Individual results given for Takahashi et al; means for the others. Mawatari et al, Canal et al, and Willner et al used non-collagen protein as reference; Susheela et al and Takahashi et al used total protein. Khoklov and Malakhovsky used non-collagen protein in reference, but used a membrane fraction rather than whole homogenates.

*) Mawatari et al, Takahashi et al, and Khoklov and Malakhovsky used epinephrine; Susheela et al and Willner et al used isoproterenol.

which have enzyme activity. Contamination with fat cells and fibroblasts has not been quantified, and has limited the value of these studies. This problem has been approached in studies of myotubes and erythrocytes. In the one study of cells cultured from Duchenne muscle (Mawatari et al., 1976) and in three of four studies of the erythrocyte enzyme (Mawatari et al., 1976; Wacholtz et al., 1979; Lane et al., 1978; Fischer et al., 1978), basal adenylate cyclase was high and catecholamine stimulation of enzyme activity reduced. It seems likely, therefore, that activity of this enzyme is abnormal in Duchenne cells. The next task, as mentioned, for those who believe in the significance of this, among other observations of the Duchenne erythrocyte, is to study the molecular biology of adenylate cyclase in Duchenne dystrophy. For this study the erythrocyte, which has a paucity of beta-receptors and catalytic subunits, offers theoretical advantages.

I have looked recently at yet another disease which may affect the adenylate cyclase in muscle. I thought I would briefly present that.

DR. ENGEL: Could you tell us what problems you have as far as adenylate cyclase is concerned?

DR. WILLNER: That requires some back tracking. For the last year, we have reexamined the assay of adenylate cyclase in muscle homogenates. Two immediate concerns were method of homogenization and enzyme lability. With optimal conditions for each, an all glass homogenizer provides significantly lower adenylate cyclase activity than a teflon-glass homogenizer or a mechanical homogenizer, such as a Polytron. Adenylate cyclase was known to be labile in isolated membranes (Snyder and Drummond, 1978), but we were surprised by how labile it was in muscle homogenates at 4°C. In the absence of a chelator of divalent cations, a delay of 30 minutes between homogenizing and assaying resulted in more than 30% loss of basal activity. We established conditions which avoided loss of enzyme activity during homogenization or prior to or during assay. An immediate question with Duchenne muscle, then, was, might lower activity of adenylate cyclase merely be due to greater lability? The answer, though data are still preliminary, indicates that in crude homogenates enzyme activity indeed is not normal: both basal activity and the capacity of isoproterenol to activate the enzyme are lower than in control muscle.

DR. MURRAY: In Duchenne muscle tissue, with the lability of adenylate cyclase, it is worth attempting the assay in the presence of protease inhibitor.

DR. WILLNER: Following the example of Drummond in studies of cardiac sarcolemma (Snyder and Drummond, 1978), we examined

the effect of a protease inhibitor, pepstatin. It had no influence on lability of the enzyme in control muscle. We found that to prevent enzyme lability it was necessary to have a calcium chelator in the homogenate. However, addition of exogenous calcium in excess to the capacity of the chelator did not cause lability to recur. Neither did addition of Mn^{++}, which also would be chelated by EDTA. Perhaps, vesicle formation in the homogenate blocks Ca^{2+} or Mn^{++} in membrane sites causing lability, but that is purely conjecture.

DR. ENGEL: Having made this observation, what do you think it means?

DR. WILLNER: I really don't know. One interpretation is that abnormal adenylate cyclase is another reflection that the surface membrane of muscle is abnormal, as you and others have shown morphologically and biochemically. I find it difficult to blame contamination with fat or connective tissue for a reduced response to beta-agonists, because these tissues normally are more responsive to catecholamines than is muscle. But the basis of these abnormalities is elusive.

Another important consideration is malignant hyperthermia a syndrome which occurs in human beings but, probably, in other animals as well. A very similar syndrome in pigs has been called the fulminant-hyperpyrexia stress syndrome. Important manifestations are a sustained state of skeletal muscle contraction and hyperthermia. Mortality is in the range of 70%. General anesthesia usually precipitates the syndrome in man, but it has been reported to occur outside the operating room in susceptible individuals who are emotionally or physically stressed (Wingard and Gatz, 1978; Jardon et al., 1979). Susceptible individuals have congenital myopathies (Denborough et al., 1970; Isaacs and Barlow, 1970), usually acquired by autosomal dominant inheritance (Isaacs and Barlow, 1970). This genetic pattern, elevated serum creatine kinase, and abnormal *in vitro* muscle physiology (Kalow et al., 1978), have been used to identify susceptible individuals within families known to be at risk, because of a case of adverse response to anesthesia.

The commonest test for the myopathy of malignant hyperthermia is to determine the response of isolated, intact strips of muscle to caffeine, halothane, or a combination of these drugs (Kalow et al., 1978). Caffeine works by releasing calcium that has been stored in the sarcoplasmic reticulum. Dr. Donald Wood, working at Columbia, found with chemically skinned fibers, using controlled conditions of calcium loading, that sarcoplasmic reticulum in malignant hyperthermia muscle is more sensitive to caffeine than normal, while the response of contractile proteins to directly applied calcium is normal (Wood et al., 1979). One of the

possible explanations for the increased sensitivity to caffeine was that there was more calcium in the SR to be released by the caffeine stimulus.

In 1974 Kirchberger and Tada identified the protein phospholamban in cardiac sarcoplasmic reticulum (1974), in 1976 identifying the identical protein in SR of slow but not fast skeletal muscle (Kirchberger and Tada, 1976). Phosphorylation of phospholamban by cyclic AMP-dependent protein kinase resulted in acceleration of calcium transport from myofilament space into SR and activation of calcium ATPase.

We speculated that increased phosphorylation of phospholamban might be the cause of the apparent acceleration of Ca^{2+} uptake by SR of some fibers from malignant hyperthermic muscle. This speculation was appealing because stressor anesthetic-induced release of adrenal epinephrine could provide a mechanism for exacerbation of abnormal SR function, through activation of sarcolemmal adenylate cyclase. In addition, halothane had been shown to stimulate adenylate cyclase in smooth muscle (Yang et al., 1973).

Using two different methods for assay of muscle adenylate cyclase, we found that basal activity in malignant hyperthermic muscle was increased, and the enzyme was more responsive to activation by isoproterenol. With the limitation that kinetic studies were performed with homogenates, increased activity apparently was due to increased V_{max} with no change in the Km for MgATP (Willner et al.,1979). Activity of cyclic AMP phosphodiesterase, determined in whole homogenates against a substrate concentration of 1 mM, was found to be reduced in homogenates of MH muscle, but the difference and number of patients were small. Increased content of cyclic AMP in MH muscle therefore was attributable to the increased activity of adenylate cyclase (Table 3). Our most recent data on adenylate cyclase, obtained with the above method which stabilized enzyme activity, confirm initial experiments (Figure 6-6).

Phosphorylase kinase is a substrate of cyclic AMP-dependent protein kinase: in phosphorylation results in its activation and catalysis of the phosphorylase b to a conversion. We reasoned that if cyclic AMP were increased, we might get activation of this enzyme pathway. The content of phosphorylase in muscle of both survivors of malignant hyperthermia, and relatives classified as susceptible by physiological testing, was increased; total phosphorylase activity was unchanged (Table 2) (Willner et al., 1980).

Table 2

Biochemical Studies in Malignant Hyperthermia

	Control (Number)	MH (Number)	P
Cyclic AMP Content Picomoles/Mg NCP	2.94 ± 0.80 (8)	9.95 ± 3.23 (5)	< .05
Adenylate Cyclase Picomoles/Mg NCP/min			
Basal	5.54 ± 1.77 (8)	16.7 ± 5.67 (5)	< .005
10^{-4} Isoproterenol	9.26 ± 4.25 (8)	41.1 ± 10.1 (5)	< .001
Cyclic AMP Phosphodiesterase Nanomoles/Mg/10 Min	3.63 ± 1.84 (8)	2.88 ± 1.39 (5)	< .05
Phosphorylase a/Total %	2.63 ± 2.02 (34)	45.6 ± 26.2 (8)	< .0005

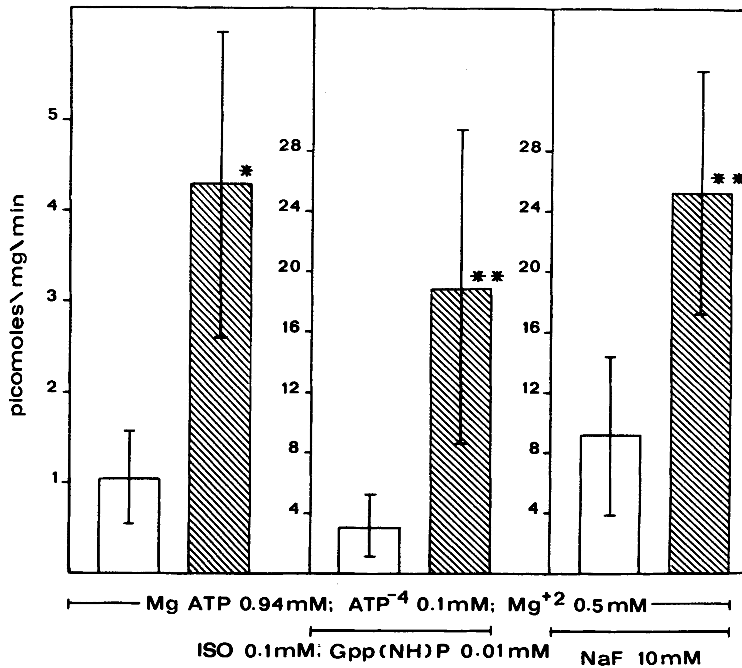


Figure 6-6: Adenylate cyclase activity was determined under basal (left), catecholamine-activated (center) and fluoride activated (right) conditions. Patients - hatched; controls - clear. Means ± standard deviations are diagrammed. * P<.001, ** P<.005.

To summarize, we found increased activity of adenylate cyclase increased content of AMP and activation of at least 1 or, if the SR defect is secondary to phosphorylation of phospholamban, perhaps two substrates of cyclic AMP-dependent protein kinase.

DR. ENGEL: I thought I had a notion of the dependence of age?

DR. WILLNER: I hadn't really carefully analyzed this yet, my impression is that the higher phosphorylase contents in muscle occur in children rather than adults.

DR. ENGEL: What about the adenylate cyclase?

DR. WILLNER: Activity of adenylate cyclase in control muscles we have analyzed is not different between ages 5 and 50. These control ages overlap the ages of the patients we have studied, but at this time it would be overinterpreting limited data to claim independence of age. More control muscles need to be studied.

DR. ENGEL: So you actually haven't systematically prepared or analyzed as to age.

DR. WILLNER: Susceptibility to malignant hyperthermia seems to decline with age. It would be interesting if children who are at risk have higher enzyme activities than adults who are at risk, but we have not studied enough muscle to attempt to establish this correlation.

DR. L. GLASER: What happens to the glycogen in these same muscles?

DR. WILLNER: Interestingly, it is not changed. I have to assume that glycogen synthetase is also more active, but I haven't measured it.

DR. L. GLASER: To generate all of this heat, there must be a breaking down of ATP somewhere.

DR. WILLNER: No one knows the mechanism of thermogenesis in this disease, and it is a fascinating question. Activation of cyclic AMP-dependent protein kinase may result in increased activity of transport ATPase's which would be exothermic. But the mechanism of increased thermogenesis in this syndrome is simply not known.

DR. STROHMAN: What did you say the evidence was that the caffeine effect was showing you a defect in the SR and rather not in contractile protein?

DR. WILLNER: One can expose the myofilaments directly to buffered calcium concentrations and look at the tensional response. With that experiment Don Wood found that muscle of survivors of malignant hyperthermia contracts at the same concentrations of calcium as does normal muscle.

DR. STROHMAN: I was wondering about the ability of the experiment to resolve small differences in intracellular calcium concentration.

DR. WILLNER: One measures small differences (Wood, 1978). However, it is true that the skinned fiber is a model preparation. If, for example, calmodulin had an effect on contractile protein function, you would not detect it because it would have been solubilized and washed out.

DR. STROHMAN: How do dantrolene and caffeine work on the SR?

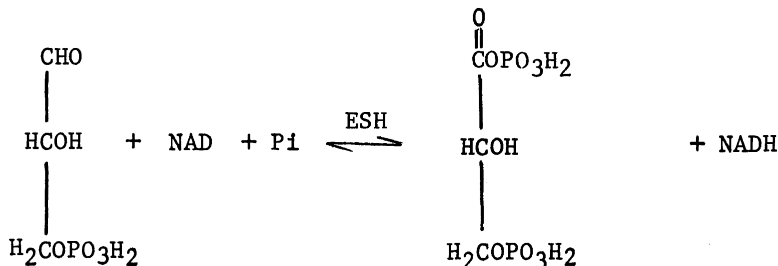
DR. WILLNER: A concept of dantrolene action is that it inhibits excitation-contraction coupling (Ellis and Bryant, 1972). What that means biochemically is not known, at least by me. It has been reported to impair calcium release from sarcoplasmic reticulum (Van Winkle, 1976). Caffeine causes calcium release. The mechanism for that effect also is unknown. It is not due to generation of cyclic AMP.

Modification of the Course of Dystrophy in an Avian Model

DR. PARK: I think that there is really a very good possibility that when the various methods are standardized, one will get a consistent picture of the membrane abnormalities. At that time we will be in a position to discuss pathogenesis.

Altered Metabolism in Dystrophic Muscle and Relationships to Therapeutic Programs

In Duchenne and avian dystrophy there is a decrease in the level of activity of the enzymes in the glycolytic and Krebs cycles. The enzyme which is most rapidly inactivated is glyceraldehyde-3-phosphate dehydrogenase. This dehydrogenase produces a portion of the high energy phosphate in glycolysis and is, therefore, critical for contraction and relaxation. During contraction, glucose is transformed by a series of phosphorylation and isomerization reactions to the triose, glyceraldehyde-3-phosphate. As shown below, this substrate is oxidized by glyceraldehyde-3-phosphate dehydrogenase to the high energy compound 1,3-diphosphoglyceric acid. The high energy phosphate on carbon-1 can then be transferred to ADP to give ATP.

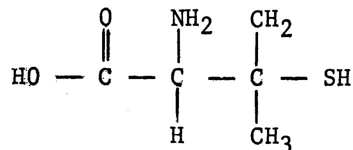


Dehydrogenase Reaction

Glyceraldehyde-3-phosphate dehydrogenase may be so readily inhibited in dystrophy because it contains the most reactive cysteine residue of all the glycolytic enzymes and is extremely sensitive to inactivation by inhibitors or intracellular oxidants such as H_2O_2, peroxides, free radicals, or metals. In addition, this dehydrogenase is more abundant in white fibers which are more severely affected by the disease than red fibers. This

suggested that the inhibition of the glycolytic pathway in dystrophy may be due to oxidation of the essential sulfhydryl group in the active site of this essential dehydrogenase. Sulfhydryl compounds such as cysteine, glutathione and penicillamine are known to protect the dehydrogenase against oxidation and thereby promote the formation of 1,3-diphosphoglyceric acid which supplies high energy phosphate for ATP and subsequently phosphocreatine. By the same mechanism, sulfhydryl compounds with reducing properties might protect or activate other thiol enzymes in the glycolytic cycle and also important enzymes in anabolic pathways.

For a treatment program with a rationale of preserving SH groups, the reducing reagent penicillamine appeared to be the most suitable drug. Unlike cysteine or glutathione, it is not readily autooxidizable and therefore persists in the serum in the reduced (-SH) form shown below:



Moreover, the side effects and toxicity of the drug are well known from the studies of Wilson's Disease and cystinuria. Thus we began a treatment program using the dystrophic chicken as the animal model.

The chicken was selected for therapeutic testing because genetic avian dystrophy has been considered as a good experimental model for inherited Duchenne dystrophy in humans. During the course of both avian and human dystrophy, there is a progressive destruction of muscle fibers with necrosis, phagocytosis, and replacement of muscle with adipose and collagenous connective tissue. The degeneration is accompanied by a rise in serum levels of muscle enzymes such as, creatine phosphokinase (CPK). The onset of symptoms in dystrophic chickens occurs in the second month after hatching. Chickens are unable to right themselves when placed on their backs, and their wings become excessively stiff and eventually cannot be elevated beyond a horizontal plane.

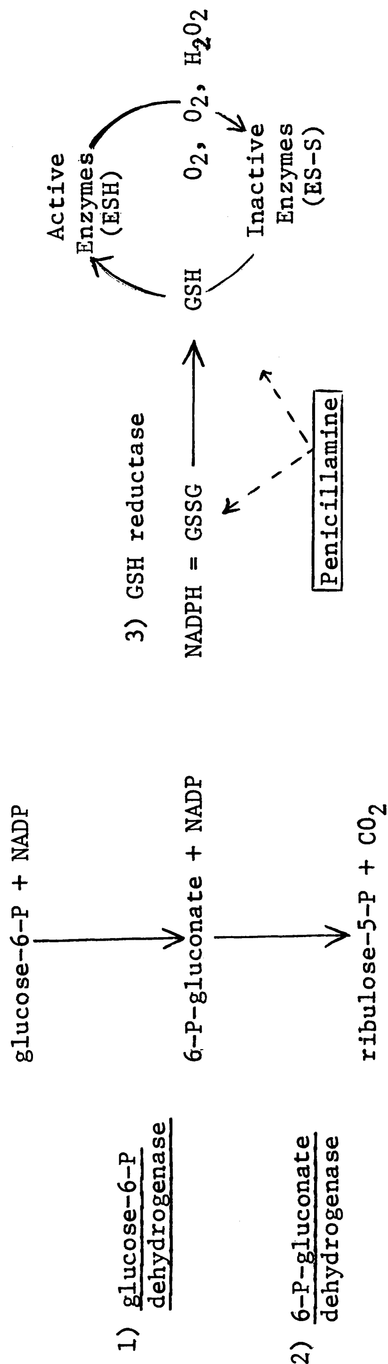
Penicillamine treatment was started on the ninth day after hatching. The drug delayed the onset of symptoms and partially alleviated the debilitating aspects of the disease. Penicillamine produced three major improvements: (a) suppression of plasma creatine phosphokinase activity; (b) better righting ability when birds were placed on their backs; (c) greater wing flexibility (J. Clin. Invest., 56, 842, 1975).

Since the beneficial therapeutic effect of penicillamine may be related to the intracellular oxidation state in dystrophic muscle, we examined the activity levels of glyceraldehyde-3-phosphate dehydrogenase and the reduced sulfhydryl content of soluble proteins. Penicillamine treatment afforded partial protection against the loss of both dehydrogenase activity and sulfhydryl groups in proteins of white dystrophic muscle (Ann. N.Y. Acad. Sci., 317, 356, 1979). These data are consistent with improved muscle function and morphology.

By contrast, the activities of several enzymes in the pentose shunt, namely, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, are significantly increased in the dystrophic muscle. The explanation may be a simple adaptive response of the muscle to increased levels of substrate, glucose-6-phosphate. If glycolysis were blocked at the triose phosphate level by inhibition of glyceraldehyde-3-phosphate dehydrogenase, hexose phosphate would accumulate. A second adaptive response might explain the increases in glutathione reductase activity observed in dystrophic mice and chickens. The pentose shunt enzymes would provide excess NADPH, which could be used by the reductase in a reaction with oxidized glutathione (GSSG) to produce reduced glutathione (GSH). In turn, GSH would afford some but not complete protection for sulfhydryl enzymes (ESH), for example, glyceraldehyde-3-phosphate dehydrogenase shown on next page.

In accordance with our proposed mechanism of drug action, penicillamine treatment of avian dystrophy has been shown to lower the abnormally high 6-phosphogluconate dehydrogenase, and GSH reductase. The drug may regenerate GSH by disulfide interchange and provide sufficient reducing power which partially suppresses the adaptive enzymatic responses.

It has been proposed that the oxidation of critical sulfhydryl groups of enzymes and structural proteins may in part account for the degenerative process in dystrophic muscle. Increased concentrations of deleterious substances, such as peroxides and free radicals, could readily inactivate sulfhydryl of enzymes or attack membrane proteins. Several enzymes that destroy these highly reactive and damaging substances are elevated in dystrophic muscle, namely, glutathione peroxidase, catalase, and superoxide dismutase. These enzymatic activities may also increase as an adaptive response to high concentrations of peroxides and free radicals in dystrophy. Penicillamine, which reduces free radicals, has been shown to suppress the elevated levels of glutathione peroxidase and superoxide dismutase. Thus the drug tends to correct abnormalities in the activity levels of a variety of enzymes and thereby restore a more normal concentration of enzymes for various metabolic pathways.


1) glucose-6-P dehydrogenase
glucose-6-P + NADP
6-P-gluconate + NADP
2) 6-P-gluconate dehydrogenase
ribulose-5-P + CO_2
3) GSH reductase
NADPH = GSSG
GSH
Penicillamine
Active Enzymes (ESH)
O_2, O_2, H_2O_2
Inactive Enzymes (ES-S)

The above scheme shows the reactions involved in the formation of gluthathione (GSH) and on the right hand side the interaction of GSH with sulfhydryl-sensitive enzymes. The postulated effects of penicillamine in potentiating enzymatic activation and maintenance of GSH are indicated by dashed lines.

In summary, all these experimental findings are consistent with our current proposal for the mechanism of action of penicillamine in the treatment of avian muscular dystrophy. The beneficial effects of penicillamine involve; (1) maintenance of intracellular levels of reduced sulfhydryl compounds; (2) protection of sulfhydryl enzymes and membrane proteins; (3) removal of deleterious oxidizing agents, free radicals, and peroxides; and (4) the well known effect of penicillamine in solubilization of collagen thereby relieving contractures.

Although the exact mechanism of action of penicillamine has not been defined, these studies have been useful in the designing of new therapeutic programs. For example, the majority of our findings offer supportive evidence for a role of sulfhydryl groups in the pathogenesis and treatment of hereditary muscular dystrophy. Therefore, we are currently engaged in drug trials that involve other sulfhydryl compounds or supplementation of penicillamine with antioxidants, such as vitamin E. Like cysteine and penicillamine, vitamin E can also act as a chain breaker to decompose free radicals and peroxides of fatty acids which otherwise lead to membrane dysfunction. In three separate trials with dystrophic chickens, vitamin E has been used as adjunct therapy and found to potentiate the beneficial effects of penicillamine. Thus a combination of penicillamine and vitamin E may be the preferred combination for any future clinical trials.

DR. HOWLAND: Two questions. Did you look at ATP levels in the chick muscle cells? Did you look at possible consequences for oxidation in the actual fatty acid composition of the membrane?

DR. PARK: No, we have not assayed ATP levels in chick muscle cells. We have not looked at peroxidation of the membrane, but Tappel has found that there is indeed increased peroxidation of lipids in the muscle membranes of dystrophic chickens. We have investigated a number of the enzymes which remove peroxides and apparently those activities are increased, namely, non-specific peroxidases, glutathione peroxidase, catalase, and superoxide dismutase. The best explanation is induction of enzyme synthesis by increased concentration of substrates.

DR. L. GLASER: Do chicken erythrocytes have abnormalities like the Duchenne RBC?

DR. PARK: The chicken erythrocyte is a nucleated cell, and we have not in our preliminary studies been able to show the same

differences between normal and dystrophic chickens as we have in humans. However, we have only carried out very preliminary investigations.

DR. SCHOTLAND: What is the effect of penicillamine on chickens at various stages of the disease?

DR. PARK: That is a very interesting question. We have administered penicillamine to chickens 30 days after they were hatched. There was some beneficial effects. That is just about the age when they began to show symptoms of weakness and not being able to right themselves. When you start penicillamine treatment on the ninth day after hatching, then you see very dramatic results. We have not started any earlier than that. We really should go back to day 1 or in the embryo. That might be even more beneficial.

DR. BRANTON: I don't think anyone has mentioned the dissociation constant of glyceraldehyde-3-phosphate dehydrogenase.

DR. M. GLASER: Would you consider it a soluble enzyme or a membrane bound enzyme.

DR. BRANTON: Well, it is easily solubilized as the salt concentration falls off. You have to raise the salt concentration higher. It is rather a mystery actually because band 6 glyceraldehyde-3-phosphate dehydrogenase falls off at isotonic salt concentration in the 150 millimolar salt which makes you ask whether it is really bound in vivo in the first place and what relevance the binding has.

DR. PARK: This dehydrogenase is really a marvelous enzyme to investigate because you can study it as a soluble enzyme and as a membrane bound enzyme. Since so much is known about band 3, it is also possible to study the various segments of the transmembrane band 3 protein.

DR. M. GLASER: Are there changes in the kinetic properties?

DR. PARK: Actually, it is my impression that not a great deal has been done to determine whether this dehydrogenase is more active when it is membrane bound or soluble. We are actually looking into that right this moment. It is somewhat difficult because of the salt problem that Dr. Branton pointed out, and I think that's why the studies haven't been done.

DR. SCHOTLAND: Is glyceraldehyde-3-phosphate dehydrogenase similarly decreased in Duchenne dystrophy?

DR. PARK: Yes, there is a parallel decrease in the level of the dehydrogenase activity in Duchenne and avian dystrophy. A

number of years ago, Dreyfus and Shapiro showed that glyceraldehyde-3-phosphate dehydrogenase is the enzyme which disappears first and most rapidly in Duchenne dystrophy.

DR. ENGEL: Is phosphorylase decreased?

DR. PARK: Yes, phosphorylase is also decreased.

DR. ENGEL: There are a whole host of other enzymes. One could argue that the changes are secondary to the degenerative process.

DR. PARK: Oh, yes, I think it is highly possible. The dehydrogenase activity begins to decrease first and other glycolytic enzymes decline subsequently. A number of enzyme activities increase, such as glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, so that not everything goes down. In general, the activity levels of glycolytic enzymes and enzymes with reactive SH groups tend to decrease. Enzymes with S-S groups, such as RNAse, DNAase, and proteases, tend to increase. Then it occurred to us that stable enzymes with disulfide bridges (S-S) are catabolic enzymes. The sulfhydryl (SH) enzymes as well as the DNA, RNA and protein synthesizing enzymes, those SH sensitive enzymes are often inhibited in dystrophic muscle, so it is the anabolic enzyme which tends to disappear. Now you can think of exceptions to that generalization. However, it seemed logical when you consider that in muscular dystrophy there is a decrease in anabolic processes and an increase in catabolic degenerative processes. We used this type of rationale when we were thinking about what kind of therapy one might employ to reverse enzyme imbalances which are so prevalent in dystrophy.

DR. WOLF: To follow the implication of Dr. Engel's earlier question, would you say something about the order of involvement of enzymes in other degenerating muscle situations, such as denervation of muscle.

DR. PARK: I am sorry, I have never made that kind of detailed study.

DR. WOLF: You worked with other cells beside the red blood cells. Would you say a word about that.

DR. PARK: Yes, we have looked at liver and found minimal changes in comparison to muscle. Also the red muscle was much less affected than white muscle. We never have really tried to compare our results with those of denervation, that is severing a nerve and observing preferential enzyme loss or increase. However, there is a rather interesting observation in this connection. Vitamin E deficiency produces a degeneration of muscle. If you administer vitamin E you can reverse the dystrophy. These

facts led us to think about vitamin E as adjunct therapy to potentiate the beneficial effects of penicillamine. There is still another unusual dystrophy which is produced in thyrotoxicosis. In the hyperthyroid patients one sees a degeneration of the muscles and weakness especially through a shoulder girdle. However, we have not looked at the glycolytic processes in thyrotoxic patients or animals given excess hormone.

CODA

The aim of the colloquium as stated in the Preface, was to bring into focus data relating to muscle cell membranes that might contribute to understanding the pathogenic mechanism of Duchenne muscular dystrophy. Because of the complexity of muscle cells and the relative paucity of information on muscle cell membranes, there has been a good deal of work done on the red cells of dystrophic patients. These studies have yielded evidence of abnormalities in the erythrocyte plasma membranes of patients with various forms of muscular dystrophy.

After a brief discussion of the phylogency of mammalian cell membranes, available data on the cytoskeleton of the red cell was reviewed. Later in the meeting there were speculations as to how a defect in the cytoskeleton of a contracting muscle cell might produce breaks in the plasmalemma, thereby allowing ingress and potentially destrictive effects of calcium rich extracellular fluid.

After a discussion of the cytoskeleton, the biophysics of cell membranes was considered. The nature and arrangement of lipids in the membranes of cells and the characteristics of their behavior were then dealt with. There followed a consideration of the regulation of membrane functions including the influence of lipid composition on membrane "rigidity" or "fluidity" and on the activity of membrane bound enzymes. The organization of lipids within membranes was shown to occur not only in the familiar bilayer configuration but also in a hexagonal H_{II} phase, a state dependent upon the phospholipid composition of the membrane, calcium concentration and ambient temperature. The H_{II} phase appears to be important in divalent cation transport and membrane fusion. Alterations in membrane enzyme activity were shown to vary depending on the nature of the lipids in the vicinity. The effects of methylation of lipids in membranes on membrane fluidity and on adenylcyclase activity were described.

A major impediment to progress in understanding the pathogenesis of such a disease as muscular dystrophy has been the failure so far to identify the basic genetic defect. Thus it has been necessary to explore the myriad morphological and biochemical manifestations associated with muscle degeneration, most of which are probably secondary to the degenerative process itself.

Pending the identification of the genetic lesion in Duchenne dystrophy and, in view of the scattered but persistent indications of a basic membrane disturbance, attention has been directed to whatever aspects of membrane morphology and chemistry that are accessible to study. The functions of the cell surface membrane as related to adhesion to substrates and to interaction between cells were discussed and in hibernating animals a change in lipid composition and in membrane bound enzyme function were described in response to a change in environmental temperature. The removal of phosphatidylcholine from a membrane by exposure to phospholipase C was shown to stimulate synthesis of more phosphatidylcholine through increased activity of cytodyl transferase which is apparently the rate limiting enzyme.

Mechanisms governing various membrane receptor functions were commented upon and there was an extended discussion of mechanisms involved in transmembrane translocation of polypeptides and other molecules.

In an effort to determine whether or not some of these membrane phenomena might be pertinent to the pathogenic mechanisms of muscular dystrophy, biophysical and biochemical studies of red cells and fibroblasts from patients and from dystrophic animals were reported and related to attempts at pharmacotherapy of dystrophic animals.

In order to be understandable to all participants the language of the presentations and discussions avoided the jargon of each special field insofar as possible.

Apart from providing a review of widely varying approaches to the study of the composition and behavior of cell membranes the discussions brought together current thinking on strategies of approach to the study of the pathogenesis of muscular dystrophy and out of the personal contacts made at the colloquium inter-institutional collaborative investigations have evolved.

EDITORS' POSTSCRIPT

In the absence of an understanding of genetic defects in the muscular dystrophies, evidence on pathogenesis has been sought in various aspects of muscle and other tissues including cell membranes whose structure, biophysical features and metabolism were reviewed in this colloquium.

Investigators have been hampered by the difficulty of obtaining uniform material from human muscle biopsies in amounts sufficient for their studies. Skin fibroblasts in culture, as they would presumeably share the defective gene or genes may satisfy the need

for large quantities of a uniform tissue that can be shared among numerous investigators. Already several laboratories are investigating this possibility in the hope of accelerating progress in research on Duchenne muscular dystrophy and other muscle diseases.

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