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Perturbation of Membrane Fluidity

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Introduction

It is well recognized that amphipathic lipids in biomembranes are in a liquid–crystalline arrangement. It is becoming apparent, however, that biomembranes are plastic, changeable structures which may be noticeably different in different areas in the same membrane and under changing physiological or experimental conditions. There are several observations concerning the consequences that modifications in the lipid physical state may have on membrane functions, but little attempt has been made to search for the reasons for such correlations. Since membrane functions are mainly linked to the protein components, it

* *Abbreviations.* ANS, 1-anilinonaphthalene-8-sulfonate; BHM, beef-heart mitochondria; BSA, bovine-serum albumin; CD, circular dichroism; CS, cerebroside sulfate; CTAB, cetyl trimethylammonium bromide; DCCD, dicyclohexyl carbodiimide; DSC, differential scanning calorimetry; EDTA, ethylene diamino tetra acetate; ESR, electron spin resonance; F_1, coupling factor 1 of oxidative phosphorylation (= oligomycin insensitive ATPase); Hb, hemoglobin; NCCD, *N*-(2,2,6,6-tetramethyl-1-oxypiperidyl)-*N'*-cyclohexyl-carbodiimide; NMR, nuclear magnetic resonance; ORD, optical rotatory dispersion; OSCP, oligomycin sensitivity conferring protein; PA phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidyl ethanolamine; PI, phosphatidyl inositol; PMS, phenazyme methosulphate; PS, phosphatidyl serine; SDS, sodium dodecyl sulfate; TEMPO, 2,2,6,6-tetramethylpiperidine-1-oxyl; T_f, temperature corresponding to the onset of solidification of fluid lipids; T_s, temperature corresponding to completion of solidification.

follows that lipids may affect membrane function by changing protein conformation. This review attempts to give experimental evidence and experimental ideas on this working hypothesis; rather than to cover all the literature on the subject of membrane fluidity, it is meant to represent a somewhat personal interpretation of the problem in the light of the most recent literature and of the experimental work which is being carried out in our laboratories.

For this reason, whenever possible we will make reference to more comprehensive papaers and reviews, and put the main emphasis on the following questions. What is the evidence for heterogeneity of membrane fluidity? What is the role of membrane proteins on the lipid microenvironment? How can lipid fluidity be changed by external agents? Will these changes affect protein conformation and functions? And what physiological and pathological implications will result from the above considerations?

Of course, in the present state of research not all questions will receive a straightforward answer; certain conclusions will be tentative and should be viewed with caution; we will indicate so when appropriate. It is, however, our aim to present the problem in many of its different facets and we hope that specific experimental ways will be soon outlined in order to achieve better knowledge of this interesting area of membrane research, the importance of which is more and more recognized in view of its outstanding biological implications.

I. *Lipid Protein Interactions in Membrane Structure*

Nearly all agree that lipids in membranes are in bilayer arrangement. There is now overwhelming evidence that hydrophobic interactions play a major role in the association of proteins to the lipid bilayer. Some reviews are available related to the experimental evidence for this important conclusion (Fourcans and Jain, 1974; Lenaz, 1973a; Singer, 1974; Rothfield and Romeo, 1971; Chapman, 1972; Triggle, 1970; Vanderkooi, 1974). In addition, there is evidence that in most membranes several proteins penetrate into the lipid bilayer (Lenaz et al., 1973), and at least a few proteins span the thickness of the membrane. This has been verified for the main erythrocyte membrane glycoprotein (glycophorin) (Marchesi et al., 1972; Segrest et al., 1973; Steck, 1972; Morrison et al., 1974; Shin and Carraway, 1974) and for mitochondrial cytochrome oxidase (Tinberg et al., 1974; Schneider and Racker, 1971; Schneider et al., 1972; Lenaz et al., 1973; Harmon et al., 1974), using different experimental methods, and both structural and functional approaches.

The independent notions of hydrophobic interactions and protein

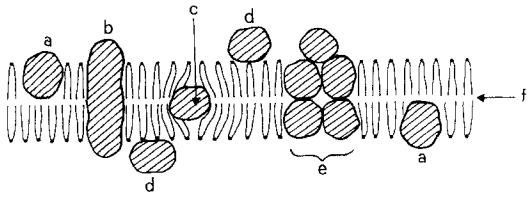


Figure 1. Schematic representation of a membrane according to the fluid mosaic model, with different kinds of proteins. (a) Intrinsic proteins localized asymmetrically in the membrane; (b) intrinsic protein spanning the membrane continuum; (c) intrinsic protein buried in the bilayer; (d) extrinsic proteins; (e) a multienzyme complex spanning the membrane and composed of intrinsic and extrinsic subunits; (f) lipid bilayer.

penetration greatly strengthen the validity of membrane models of the fluid-mosaic type of Singer (Glaser et al., 1970; Singer, 1972; Singer and Nicolson, 1972) and of that proposed by Vanderkooi and Green (1970) (Fig. 1).

Extensive penetration into the lipids is a characteristic shared by those proteins which usually have a higher content of nonpolar amino-acid residues (Capaldi and Vanderkooi, 1972; Capaldi and Green, 1972) and a nonpolar surface, and are called *intrinsic* or integral proteins. There are several other proteins in membrane which are easily detachable and water soluble when detached; they are called *extrinsic,* peripheral, or membrane-associated proteins, and their linkage with the membrane could be due mainly to polar interactions with either intrinsic proteins or phospholipids. It is clear that different membranes may differ in the quantitative ratio of extrinsic to intrinsic proteins, as well as in the nature and function of the different types of components.

Most of the discussion in the following sections will concern the intrinsic protein components, since they are considered more directly in contact with the portions of lipids which undergo physical changes. It is therefore pertinent to define here the characteristics of hydrophobic associations mainly in relation to membrane lipids and proteins. It is well known that the "hydrophobic bond" (Nemethy, 1967) results from the gain in entropy obtained by the squeezing out of nonpolar components from water, as a consequence of their low affinity for water itself. Membranes will therefore be very stable systems if both lipids and intrinsic proteins are, as they appear to be, amphipathic molecules (Singer and Nicolson, 1972; Capaldi and Green, 1972; Green, 1972). Part of the intrinsic protein will protrude from one or both membrane surfaces. Only the buried moieties are predicted to contain a high percentage of nonpolar amino acid residues: the primary structure of the intramembrane fragment of glycophorin confirms the above predictions (Segrest and Kohn, 1973).

Each component of a membrane (lipids, intrinsic and extrinsic proteins) will affect the properties of the surrounding molecules. Extrinsic proteins, for example, could impose restrictions on the mobility of the intrinsic components in the lipid bilayer, as in the case of "spectrin" on the internal surface of the erythrocyte membrane (Nicolson and Painter, 1973). As we will discuss later on, both lipids and intrinsic proteins will reciprocally affect their physicochemical properties upon interaction. Indeed, this will be the principal aspect of our review.

II. *The Physical State of Membrane Lipids*

Amphipathic lipids undergo an important phase transition with increase in the system temperature; when the hydrocarbon chains of the component fatty acids "melt," the system changes from a crystalline to a liquid-crystalline state, where the fatty acyl chains are liquid-like, whereas the polar ends are still experiencing strong mutual attractions (cf. Luzzati, 1968).

We will briefly review the intrinsic properties of lipids which affect the transition temperatures as well as discuss the validity of the physical methods employed to assess lipid fluidity.

A. *Methods employed in assessing lipid fluidity*

Differential scanning calorimetry (DSC)[°] detects the endothermic transition which accompanies "melting" of the lipid hydrocarbon chains (Chapman, 1969; Chapman and Dodd, 1971). The transition is abrupt in the case of a pure lipid component containing only one fatty acid species; for example hydrated dipalmitoyl lecithin has a transition temperature of 41.5° C. Hydrated lipid mixtures in the form of liposomes (Bangham, 1963) or of vesicles obtained by sonication (Huang, 1969), which are often used as the most practical bilayer systems, show a very wide endothermic peak which is centered near 0° C in the case of liver mitochondrial phospholipids (Blazyk and Steim, 1972). Such a large range of melting temperature is considered to result from the heterogeneity of the lipid molecules and their melting at different temperatures, causing phase separations (Chapman et al., 1974) (see below). For pure phosphatidylcholine (but not for phosphatidylethanolamine) a pretransition at lower temperature has been observed by DSC (Chapman et al., 1974): such an endothermic peak is probably associated with an increase in mobility of the polar head prior to the main transition.

Phase transitions are also detected by means of other techniques such as *x-ray diffraction* (Shechter et al., 1974; Cain et al., 1972): a crystalline packing characterized by a sharp 4.2 Å diffraction band and a fluid state identified with a broad diffuse band at 4.5–4.6 Å are

separated by broad gradual transitions in the case of heterogeneous lipids or membranes.

Transitions in the state of lipids are also detected by *spin labeling* methods (Smith, 1972) Spin-labeled fatty acids or phospholipids, having paramagnetic nitroxide groups at different positions in the fatty hydrocarbon chain are used as probes of the lipid motion in the surrounding environment (Jost et al, 1971; Mehlhorn and Keith, 1972). The shape of the paramagnetic spectra is very sensitive to probe motion, and rotational correlation times can quantitate the rotational mobility of the lipids at least for times not higher than 10^{-9} sec (Kivelson, 1960). The lipid mobility decreases with decreasing temperatures, and abrupt discontinuities or breaks are found at well-defined temperatures in the Arrhenius plots of the rotational correlation times or other motion parameters (Raison et al, 1971). These sharp breaks however *do not* correspond to the wide endothermic transitions detected by DSC, although they usually lie in the range of such transitions.

The reasons for such discrepancies are debatable, but it is clear that the two methods detect different aspects of the same phenomenon. Raison and McMurchie (1974) have found two breaks for the motion of spin-labeled fatty acids in mitochondrail membranes, one in the upper and the second in the lower range of the calorimetric phase transition. Uncertainty in data interpretation arises when using stearic acid spin labels because of their preferential disposition in the more fluid areas of a bilayer having a heterogeneous physical state (Oldfied et al., 1972). Such preferential affinity will tend to squeeze out the labels into fluid bilayer areas whenever crystalline and liquid–crystalline domains coexist in the same bilayer. A discontinuity in the upper range can be related to *phase separation* of crystalline from liquid-like domains when certain lipids begin crystallization at decreasing temperatures, while a discontinuity in the lower range will indicate completion of solidification. A spin label like TEMPO (2,2,6,6-tetramethylpiperidine-1-oxyl) (Shimshick et al., 1973) which has much more favorable partition coefficient for fluid than for rigid lipids has been used as a probe for phase separation; the upper field line in the paramagnetic spectrum has a different position in polar (water) and nonpolar (lipid) environments (Shimshick and McConnell, 1973), so that a clear splitting is evident when the label is present in both environments at the same time. By plotting the spectral parameter f, given by the ratio of the two peaks, against temperature, discontinuities are observed at the upper and lower transition point (Shimshick et al., 1973); the region between the two discontinuities corresponds to the temperature range where solid and liquid regions coexist. By changing temperature in lipid binary mixtures it has been possible to build phase diagrams where the fluid (F-phase) and solid (S-phase) regions are separated by an area of heterogeneous state (Fig. 2).

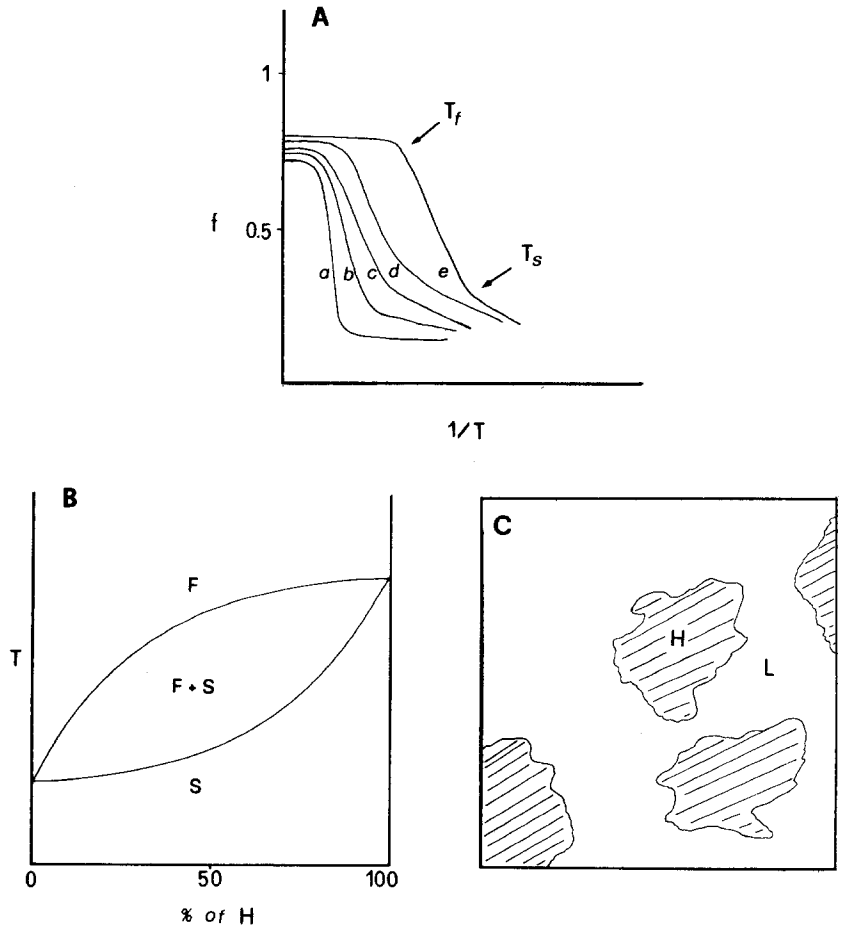


Figure 2. Phase diagrams of binary mixtures of phospholipids. H represents a high-melting component (e.g., distearoyl lecithin) and L a low-melting component (e.g., dimyristoyl lecithin). Freely adapted from Shimshick et al. (1973). (A). The TEMPO solubility parameter f (fraction of label dissolved in the membrane: see text) is plotted as a function of reciprocal temperature for mixtures of H and L, (a) being pure H, (e) pure L, and (b), (c), and (d) intermediate mixtures thereof. The changes in slope define the onset (T_f) and the completion (T_s) of phase separation. (B). The two temperature values corresponding to the changes in slope (T_f and T_s) are reported in a phase diagram for each phopholipid mixture examined. F = fluid; S = solid; F + S = region of the diagram where solid diagram for each phospholipid mixture examined. F = fluid; S = solid; F + S = region of the diagram where solid and fluid phases coexist. (C) Schematic representation of solid clusters containing a majority of H molecules in the fluid phase consisting mainly of L molecules, in the intermediate F + S region of the phase diagram.

Butler et al. (1974) have carefully considered the potentialities and drawbacks of different spin labels, and have reached the conclusion that a cholestane probe (the *N*-oxyl-4′,4′,-diemethyloxazolidine derivative of cholestane-1-one) is much more suited for investigating *absolute* values of fluidity, in comparison with the fatty-acid labels, since it is not segregated into fluid areas of the bilayer. Experimental and theoretical spectra for the cholestane probe in lipid mixtures in different proportions are coincident, whereas large deviations occur for stearic acid spin labels when fluid and rigid areas coexist. Thus, labels showing higher affinity for fluid areas may be more useful as sensitive indicators for phase separation.

A possible drawback for all spin labels is that they are extrinsic probes, in other words foreign molecules which are likely to induce some perturbation in a lipid bilayer (Cadenhead and Müller-Landau, 1973).

Also, *fluorescent probes* may be used as indicators of membrane properties and fluidity (Chapman and Dodd, 1971; Radda, 1971). There are two main kinds of fluorescent probes which can be used in membrane studies. One kind is represented by hydrophobic chromophores which are located in the nonpolar core of a lipid bilayer, and their spectral properties are sensitive to microviscosity of the environment represented by the fatty acyl chains. For example, polarization of the fluorescence of perylene or 9-methylanthracene (Cogan et al., 1973; Faucon and Lussan, 1973) increases as a function of viscosity and has been used as a sensitive probe for phase changes in biomembranes.

On the other hand, amphipathic molecules like 1-anilino naphthalene-8-sulphonate (ANS) are located in the polar region of the lipid bilayer near the water interface (Lesslauer et al., 1972). ANS is strongly fluorescent in hydrophobic media, but its fluorescence is quenched in aqueous environments with a shift of emission maximum to higher wavelength. Also, ANS fluorescence is sensitive to the viscosity of its microenvironment, and it has been used to detect phase changes in membranes (Traüble and Overath, 1973).

The interpretation of data obtained using ANS in studying membrane structure and functions is still the subject of some controversy. ANS interaction with biological membranes is usually described in the literature as a process of adsorption on particular binding sites. Dissociation constants and number of sites are evaluated according to Scatchard (1949) or with more precise methods for multiple equilibria (Rosotti and Rosotti, 1961). Studies carried out in our laboratories (Silvestrini et al., 1973; Marinangeli et al., 1976) indicate that the probe interactions with the membranes are due to a partition phenomenon; such an interpretation allows us to overcome both theoretical and experimental criticisms recently raised to the former interpretation. Also the effect of neutral salts such as Na_2SO_4, NaCl, NaSCN, and KCl on

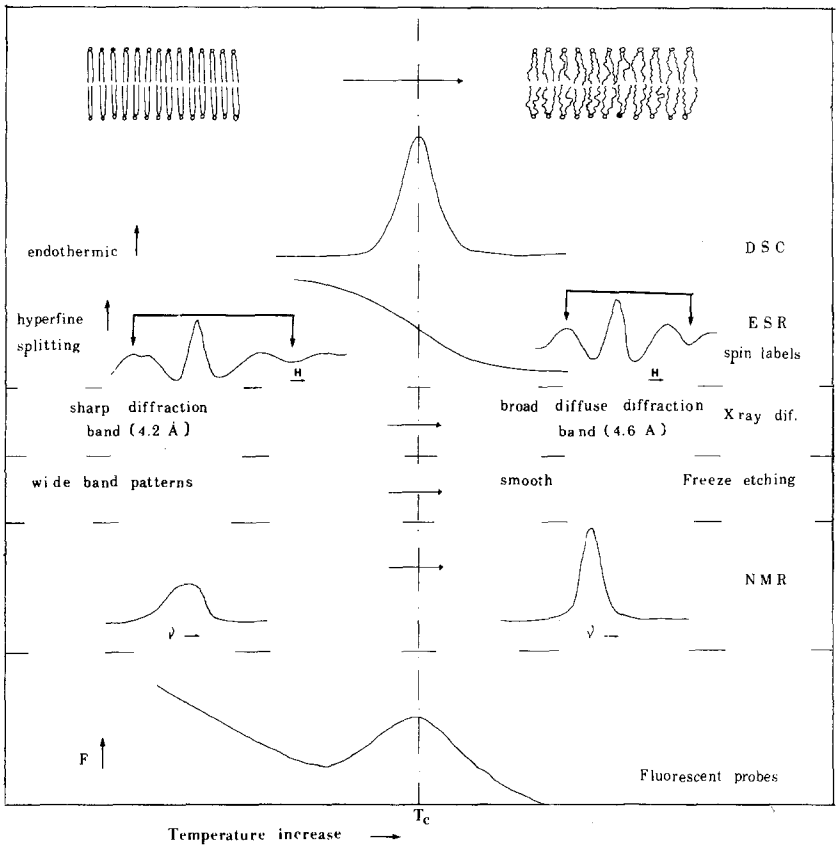


Figure 3. Highly schematic representation of changes detected by different probes at the lipid-phase transition. DSC detects the heat absorption associated with melting of the fatty acyl chains. Spin labeling shows the narrowing of the hyperfine splitting associated with increased mobility. X-ray diffraction detects the transition from the sharp band associated with the stiff crystalline chains to the broad diffuse band characteristic of the fluid state. Freeze-etching electron microscopy shows wide bands with a spacing of 192 Å when the sample is quenched from below phase transition, and smooth indistinct areas when the sample is quenched from above the transition. Proton magnetic resonance detects a large increase in spectrum intensity above transition (cf. Chapman and Dodd, 1971). Fluorescent probes like ANS or *N*-phenyl-1-naphthylamine detect a discontinuity at the transition in the fluorescence (F) decrease associated with increasing temperature (Traüble and Overath, 1973). T_c = transition temperature.

ANS fluorescence can be readily explained in terms of partition. It has been experimentally found that the natural logarithm of the ratio of the partition coefficient of ANS between the membrane and water to the partition coefficient of ANS between the membrane and the saline is a

function of the ionic strength of the solution and is independent on the nature of the salt. If octanol is chosen as a standard reference system, the data indicate that the interactions of ANS with octanol are of the same nature as in the membrane.

The main criticism that can be advanced against fluorescent probes is again that they are foreign molecules which are likely to perturb the lipid bilayer. However, the extreme sensitivity of fluorescence spectroscopy allows use of the probes at extremely low concentrations which may not affect the original packing of the membranes.

Nuclear magnetic resonance (NMR) methods (with either ^{1}H, ^{2}H, ^{31}P, ^{13}C) have been also used to probe lipid fluidity (Chapman and Dodd, 1971). They have a major advantage of not introducing foreign molecules in the membrane; the interpretation of NMR studies can, however, be very difficult, especially in natural membranes containing proteins. Moreover, controversies on the significance of the increased line widths in unsonicated preparations in comparison to sonicated vesicles (Finer et al., 1972; Sheetz and Chan, 1972) are not yet fully resolved, although the differences are probably to be ascribed to structural molecular changes rather than to different tumbling rates of small or large vesicles as a whole.

One major ambiguity in studies of membrane fluidity is that different probes do not necessarily detect the same types of physical changes. The terms *mobility, disorder, fluidity*, etc. are not used with the same meaning by investigators using different techniques. For example, can a "phase change" as detected by calorimetry be compared with a change in mobility detected by spin labeling? Spin labeling has shown that cholesterol induces a very viscous state in lipids, endowed with slow molecular motion, yet, this state will be detected by DSC as a fluid phase, which does not undergo any more the transition to the "solid" state. X-ray diffraction has shown that several mesomorphic phases, which are not liquid–crystalline, are nonetheless characterized by various extents of long-range or short-range disorder and are not comparable with a true crystalline packing (Ranck et al., 1974; Caron et al., 1974; Tardieu et al., 1973).

A summary of some of the methods employed to probe fluidity is shown in Fig. 3.

III. *Endogenous Agents Affecting Fluidity*

A. *Lipid composition and heterogeneity*

Van der Waals attractions between fatty acids decrease with decreasing chain length and increasing number of *cis* double bonds. It is not surprising therefore that the more unsaturated a lipid mixture is, the

lower is the transition temperature. It is less directly obvious why different lipid classes, even having the same fatty-acid composition, will have different transition temperatures: in this case restrictions must be exerted by the polar heads of the phospholipids onto the hydrocarbon chains. Several studies related to this problem have been accomplished with monolayers (Kézdy, 1972), by investigating the different condensing effects of phospholipids of differing composition (Hayashi et al., 1973; Ghosh et al., 1973; De Kruijff et al., 1973a).

The existence of restrictions induced by the polar heads is made quite evident by studying the rotational mobility of stearic acid spin labels having the nitroxide at varying distance from the carboxyl groups and hence at varying depths in the lipid bilayer (Jost et al., 1971). Figure 4

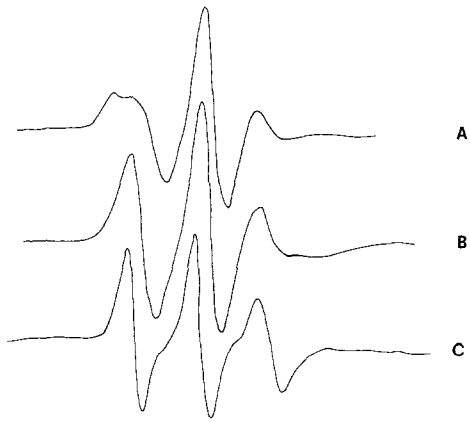


Figure 4. The fluidity gradient observed with stearic acid spin labels in phospholipid vesicles. (A). 5-doxyl-stearic acid. (B). 12-doxyl-stearic acid. (C). 16-doxyl-stearic acid.

shows the ESR spectra of three different labels having the paramagnetic group at the 5, 12, and 16 position in the fatty acyl chain (Lenaz et al., 1974). The rotational mobility is highest in the center of the bilayer, and rapidly decreases approaching the polar surfaces. Clearly, there is an immobilizing effect of the polar groups on the nearest methylene groups (Seelig and Hasselbach, 1971; Godici and Landsberger, 1974; Hegner et al., 1973).

The fluidity gradient detected by spin labels has not been fully confirmed by other methods: deuterium magnetic resonance shows that a fluidity gradient exists, but is not so evident and gradual as indicated by ESR (Seelig and Neiderberger, 1974a, b; Seelig and Seelig, 1974). The difference has been ascribed to the perturbing effect of the nitroxide in the case of spin labels.

The lipid composition of a membrane may also be heterogeneous in space. This represents an additional factor affecting fluidity of individual membrane microenvironments.

It has been found in model systems that dioleyl and dibehenoyl lecithins, widely differing in the properties of the component fatty acids, do not form homogeneous mixtures but rather islands of crystalline dibehenoyl lecithin in a sea of fluid dioleyl lecithin (Oldfield et al., 1972). The same finding has been confirmed and extended for a variety of lipid mixtures; for lipids of similar fatty acid composition, only one intermediate transition is observed (Chapman et al., 1974); however, for lipids with differing fatty-acid content, when the chain length difference is above four CH_2 groups (like dimyristoyl and distearoyl lecithin), two transitions clearly indicate a biphasic behavior, with immiscibility below the phase transition of the more rigid component. A similar immiscibility may be observed when lipids of different classes (e.g., PE, PC) even having fatty acids of the same length, are mixed (Chapman et al., 1974); clusters of gel and liquid–crystalline lipids can coexist in the same bilayer. As already pointed out, phase diagrams of these mixtures define three areas characterized by solid and liquid lipids separated by a region where solid and liquid phases coexist. Even for a pure lipid like dioleyl lecithin (Lee et al., 1974), the partition of TEMPO has shown the formation of quasicrystalline clusters at 30° C, well above the phase transition (−22° C). This phenomenon is not uncommon for many organic liquids; monomeric molecules exist only at very high temperatures. If the lifetime of the cluster is sufficiently long, it behaves as a colloidal particle; thus the group of molecules involved behaves as a whole, as detected by spin labeled fatty acids.

An analogous situation could exist in natural membranes having a very heterogeneous lipid and fatty acid composition; for instance it would be difficult to understand why only certain very specific lipids are effective in restoration of certain membranes' enzymic activities [e.g., PS or other acidic phospholipids (sulfatides) for Na^+–K^+–ATPase (Wheeler and Whittam, 1970; Karlsson et al., 1974; Roelofsen and Van Deenen, 1973)] if the same lipids (often present in minor quantities) are not preferentially found in the microenvironment of that specific enzyme. In one case a specific type of lipid heterogeneity has been detected in a membrane. By use of specific attack with different phospholipases on intact erythrocytes and in red-cell ghosts it was found that the two monolayers composing the lipid bilayer of the membrane have different compositions, in that the whole PS and most PE is present in the inner face and choline-containing phospholipids in the outer face (Verkleij et al., 1973) (Fig. 5). Pioneering work of Casu et al. (1968) had reached similar conclusions. The reason for this kind of hetereogeneity may be either metabolic, as in the erythrocyte membrane (Zwaal et al., 1973), or related to bilayer curvature in small or invaginated membranes and

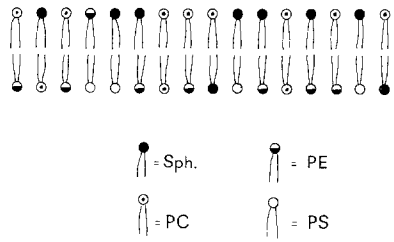


Figure 5. Asymmetric distribution of phospholipids in the human-erythrocyte membrane. After Zwaal et al. (1973). Proteins and cholesterol are not shown.

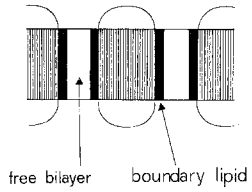


Figure 6. Boundary lipid and bilayer in cytochrome oxidase according to Jost et al. (1973). Free layer (0.5 mg/mg protein); boundary lipid (0.2 mg/mg protein).

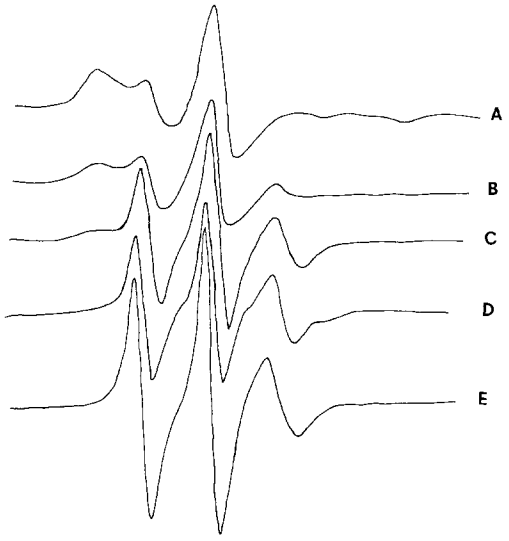


Figure 7. ESR spectra of 16-deoxyl-stearic acid in lipid-depleted mitochondria and after addition of progressive amounts of phospholipids. Asolectin added: none (A), 1.6 μg P/mg protein (B), 3.2 μg P/mg protein (C), 4.8 μg P/mg protein (D), 12.8 μg P/mg protein (E).

vesicles (Litman, 1974; Huang et al., 1974; Michaelson et al., 1973). In small sonicated vesicles asymmetry was found to be derived merely from the necessity to accomodate bulky or charged components.

B. *Cholesterol*

The cholesterol content is an important factor modulating membrane lipid fluidity (Chapman, 1973). In model systems cholesterol abolishes the endothermic transition from a crystalline to a liquid–crystalline state. In general, cholesterol appears to induce an intermediate degree of fluidity in bilayers (Chapman, 1973). Such an effect of cholesterol can be of utmost importance in the physiological properties and pathological deviations of plasma membranes which are rich in this compound (Papahadjopoulos, 1974). The effect is specific: it is related to several features of the cholesterol molecule and requires an appropriate fatty-acid composition of the interacting phospholipids (De Kruijff et al., 1973a; De Kruijff et al., 1974). The irregular surface of the sterol molecule prevents an ordered packing of the hydrocarbon fatty-acid chains and tends to abolish the disorder–order transition (Phillips and Finer, 1974). At low cholesterol concentration, and above the transition temperature of the surrounding phospholipids, viscous islets of cholesterol–phospholipid complexes exist in the more fluid phospholipid bilayer. At high cholesterol concentration, all the lipid molecules are immobilized, yet in a more disordered array than below the phase transition. The transition itself is only slightly shifted to lower temperature but its heat content is progressively lowered (Oldfied and Chapman, 1972), till it disappears when the cholesterol content is higher than 30%.

C. *Proteins*

Of extreme interest is the effect of proteins on lipid fluidity, since proteins together with lipids are the main membrane constituents.

Basic proteins, which bind electrostatically anionic groups on the polar heads of phospholipids, reduce label mobility only in proximity of the polar surface. On the other hand, comparison of rotational mobility of spin labels in membranes has shown that membrane proteins exert a strong immobilizing effect on lipids at all depths in the bilayer (Tourtellotte et al., 1970; Rottem and Samuni, 1973; Lenaz et al., 1974). The immobilizing effect of proteins on the carbons near the methyl ends of the fatty acyl chains can be therefore taken as a further indication that membrane proteins penetrate deeply into the lipid bilayer. It can be concluded that extrinsic and intrinsic proteins affect lipid mobility as measured by spin labeling, each acting within their sphere of influence. The order parameter S of 5-doxylstearate in *Tetrahymena* membranes (Nozawa et al., 1974) is markedly higher than

in the corresponding lipid extract only at temperatures lower than 18° C. This difference, shown as a loss of a discontinuity in the Arrhenius plot of S, has been ascribed to the presence of proteins inducing a higher packing in otherwise very mobile lipids.

Immobilization by proteins has been directly shown in an elegant study by Jost et al. (1973a,b) on isolated cytochrome oxidase. A stearic acid spin label becomes progressively more mobile as the lipid content of the enzyme complex is increased; computer calculations show that the mobility at any lipid content is the average of mobility of the label in the lipid-poor oxidase and in free bilayer, suggesting the existence of a strongly immobilized layer of phospholipids interacting with the enzyme proteins, while the surrounding lipids experience the fluidity of a protein-free bilayer (Fig. 6). However, since spin labels prefer the most fluid areas in a heterogenous environment (Oldfield et al., 1972), the increased mobility resulting from increasing the lipid content in cytochrome oxidase could also be the result of progressive partition of the label into the lipids situated farther away from the protein and progressively less immobilized. This idea underlines the possibility of a cooperative effect of the immobilized phospholipid molecules on their neighbours. A similar effect has been found also in lipid-depleted mitochondria (LDM) (Lenaz et al., 1974); as shown by Awasthi et al. (1971) for cytochrome oxidase, a layer of cardiolipin tightly bound to lipid-depleted membranes appears strongly immobilized (Landi et al., 1974); progressive addition of phospholipids results in increased mobility until the spectra resemble those of intact mitochondrial membranes (Fig. 7). Also in this case we can face two possible interpretations: (1) the label experiences a mobility intermediate between that in a strongly immobilized lipid layer and that in fluid surrounding bilayer; (2) the label moves to the more mobile areas but they are not as fluid as free bilayer due to cooperative effects among the lipid chains.

Steroid spin labels are better suited to probe all types of lipid environments (Butler et al., 1974); with cytochrome oxidase the indications given by the 3-doxyl-5α-androstane-3β-ol spin label confirm the stearic probe studies (Jost et al., 1973a) suggesting that interpretation (1) may be correct. If this is the case, lipids "immobilized" by proteins do not exclude stearic acid spin labels as do crystalline lipids. Immobilization by proteins therefore may not be a real phase change but rather an increase in viscosity of otherwise "fluid" (disordered) lipids.

This interpretation is stengthened by DSC and x-ray data. DSC has been used to detect the effect of different kinds of proteins on lipid transitions. Cytochrome *c*, which interacts with phospholipids ionically, lowers the lipid transition by 10° C (Chapman and Urbina, 1971; Chapman et al., 1974). The thermal transitions of lipids strongly shifted by proteins are probably related to the reorganization of the polar groups brought about by ionic interaction with the charged groups in

fixed positions of the proteins, leading to less efficient packing of the lipid chains (Chapman et al., 1974). This finding again appears to contradict ESR results. As already pointed out, lysozyme binding to lipid vesicles restricts the mobility of 5-doxyl-stearate (but not of 12- or 16-doxyl stearate); it has not been possible to test the effect of cytochrome *c* due to spin–spin interactions (Lenaz, Bertoli, Masotti and Spisni, unpublished data). The possibility exists that basic proteins immobilize the neighboring segments of lipid chains, but at the same time decrease the overall packing of the fatty chains in the bilayer, leading to the lowering of transition temperature. Some effects of extrinsic and intrinsic proteins are reported in Table I (Lenaz et al., 1974).

Bertoli et al. (1976) have found an increase in phase transition temperature (measured by DSC) in promitochondrial membranes from *S. cerevisiae* in comparison with the lipid extracts; they have related these differences to "polar" interactions, leading in this case to more efficient packing of the lipids; such differences do not usually occur in membranes (Chapman et al., 1974), giving evidence against the importance of polar interactions in most membranes. However, a similar effect can be seen in calorimetric scans of rat-liver mitochondria in the study of Blazyk and Steim (1972).

Intrinsic membrane proteins do not appear to change the transitions temperatures significantly (Chapman et al., 1974). In a study of the effect of different perturbants on phase transitions in model phospholipids, the peptide gramicidin A was shown to behave like cholesterol, inducing a dramatic decrease in heat content of the endothermic peak (Chapman et al., 1974). It is likely that intrinsic proteins behave similarly, although their influence may be less pronounced. Shechter et al. (1974) by x-ray diffraction of *E. coli* membranes found that at low temperatures the amount of ordered paraffin chains is smaller for a membrane than for the corresponding lipid extract. For example, the degree of order for membranes of elaidic-acid-grown cells is 50%, but it is 100% in the corresponding lipid extract; the interaction with proteins prevents ordering of lipids which potentially are able to "crystallize" in absence of proteins.

Thus the possibility exists that the strongly immobilized layer of lipids surrounding the intrinsic proteins and detected by spin-labeling techniques may not undergo the endothermic transition accompanying melting of the lipid chains; it may then behave as a disordered, yet highly viscous lipid region. Although in an average membrane such a layer may account for 25–30% of total phospholipids (Jost et al., 1973a), quantitative differences in heat content (as in the case of cholesterol) would not be easily observed, due to the very broad transitions in natural membranes. It is significant that Steim et al. (1969) and Blazyck and Steim (1972) found that approximately 25% of *A. laidlawii* phospholipids do not undergo any calorimetric transition.

An additional complication in detecting transition associated with intrinsic proteins has been pointed out by Bertoli et al. (1976): cooling of membranes induces phase separation (Shimshick et al., 1973), as already pointed out. When the higher-melting lipids crystallize in the membrane, the chain's crystallization will exclude some proteins from that region into the remaining fluid region. Protein aggregation is observed (as shown by freeze–fracture studies) and total exclusion of protein from the bilayer structure may occur (see following sections). If this process does occur, then the subsequent heating curves of the membrane will reveal phase transition behavior of the membrane lipids from which the protein has been excluded. This important phenomenon may also obscure any effect of the proteins on the phase behavior of lipids as detected by any other means.

A general picture of membrane fluidity can now be drawn. The overall fluidity of a membrane is the result of the summation of a number of microenvironments having varying ranges of fluidity resulting from the following factors: (1) Intrinsic lipid microheterogeneity in the plane of the membrane; (2) presence and localization of cholesterol; (3) amount, localization and nature of intrinsic and extrinsic proteins; (4) possibly, the presence of other components like quinones, chlorophyll, etc., and the presence of bound water and ions; (5) finally, the lipid mobility increases with the distance from the bilayer surface. Such a situation itself may be locally perturbed by presence of proteins or other molecules.

D. *Lateral mobility and flip-flop*

The picture of the previous conclusion is necessarily static; membranes, however, rapidly change their physical state with time in any given region (cf. Gitler, 1972). To this purpose, lateral motion of the phospholipids has been shown to occur very rapidly (Kornberg and McConnell, 197,) with diffusion coefficients in the order of 10^{-8} cm^2/sec, calculated by three independent methods (McConnell et al., 1972), whereas the jump of a lipid molecule from one to the other side of a bilayer (flip-flop) occurs at much lower rate (Kornberg and McConnell, 1971) with diffusion coefficients in the order of

There are now means to investigate protein mobility in a bilayer and to define rotational correlation times for membrane-bound proteins. Although proteins immersed in a fluid bilayer are theoretically free to move and undergo statistical collisions with other free components, the motion of an intrinsic protein in the plane of the membrane may actually be restricted or abolished so that the microanatomy of the membrane may quite well be fixed and might be changed only after well-defined stimuli (Edidin, 1972). Extrinsic proteins might represent a factor preventing lateral motion of intrinsic proteins; this may be the function of spectrin on the inner surface of erythrocyte membranes

upon the mobility of the major glycoprotein, glycophorin (Nicolson and Painter, 1973). Also in mitochondria, it was found in our laboratory that removal of extrinsic proteins by dilute acids allows increased lipid incorporation by removing the restrictions normally exerted and imposing a fixed distance among intrinsic proteins (Lenaz et al., 1970). Further implications of the lateral mobility of membrane components will be discussed later on.

IV. *Exogenous Agents Affecting Mobility*

A number of agents can modify the fluidity of membrane lipids by either binding on the membrane surface or penetrating into the membrane continuum; in the latter case either cohesion between different lipid molecules or lipid–protein interactions may be affected. According to their chemical nature we may divide exogenous agents capable of affecting fluidity into three classes: polar, amphipathic, or nonpolar. The first two agents will simply bind to the surface of the bilayer and can affect fluidity by modifying the surface charges of the membrane; it would be predicted that their effects will be mainly confined locally to the mobility of the most superficial methylene groups in the fatty acyl chains. Amphipathic molecules, having a polar end and a nonpolar moiety will become intercalated among the lipid molecules and will be able to affect membrane fluidity at depths in accordance with the size of the nonpolar moiety. Finally, agents of little or no polar character, will become included into the membrane continuum and will be able to affect the overall lipid fluidity of a given membrane.

A. *Polar compounds*

The effect of metal cations on the rotational mobility of lipids in model systems has been recently investigated. An immobilizing effect has been shown by addition of Ca^{2+} or other divalent or multivalent cations. The effect is larger near the polar heads of the phospholipids, as shown by spin labeling of cardiolipin lamellae with the different labels, but is exerted even in the hydrophobic core of the lipid bilayer (Hegner et al., 1973), while for PI the effect is restricted to the more polar regions of the membrane (Schnepel et al., 1974).

Ca^{2+} and other cations significantly increase the transition temperature of anionic phospholipids, as observed by calorimetric studies, but zwitterionic lecithin is also affected by UO_2^{2+} (Chapman et al., 1974). An increase of the transition temperature is also induced by lowering the pH of the medium, an indication that binding of hydrogen ions to anionic groups also induces a better packing of the fatty acyl chains (Verkleij et al., 1974; Tocanne et al., 1974).

Conspicuous changes of transition temperatures induced by variations in the ionic environment of the membrane can be of the uttermost physiological importance; in fact, transitions *at constant temperature* may be obtained by changing the ionic composition or the pH even within limited ranges (Traüble and Overath, 1973). It may not be unreasonable that physiological variations of the ionic conditions near certain membrane surfaces may induce large modifications of membrane fluidity. For example (Ohnishi and Ito, 1974) Ca^{2+} at 2 mM or higher concentration brings about a rapid, reversible two-dimensional phase separation of PS and PC bilayers into solid PS aggregates, bridged by Ca^{2+} chelation, and a fluid phase consisting of PC alone. Mg^{2+} and local anesthetics have antagonist effects on such phase separations. A fluidizing action of local anesthetics could be the result of Ca^{2+} displacement from phospholipid anionic sites. Similar clustering is observed in bilayers of phosphatidic acid PA and PC (Ito and Ohnishi, 1974), but not in the case of PI, PE, or cardiolopin mixtures with lecithin. Ca^{2+}-induced clustering can have implications in nerve conduction, and also in cell adhesion, since the Ca^{2+}-chelated surfaces become more hydrophobic (Ohnishi and Ito, 1974).

B. *Amphipathic compounds*

Detergents, which are known to disrupt membranes by intercalating between lipid molecules or by binding to intrinsic proteins, increase membrane lipid mobility. For example Triton-X-100 converts lipid vesicles into mixed micelles (Dennis and Owens, 1973). Waggoner et al. (1969) had shown that 12-doxyl-stearic acid tumbles more rapidly in micelles (SDS in this case) than in aqueous dispersions of egg lecithin at the same temperature. Deoxycholate decreases ANS fluorescence in lipid vesicles and membranes. This effect may be ascribed to fluidification of the lipid phase (unpublished studies from our laboratory).

Systematic studies of the direct action of drugs on membrane lipid fluidity are not available. Membrane expansion is however a direct result of lipid fluidification as shown by monolayer studies at the air/water interface; among the different types of experiments indicating membrane expansion, the protection from hypotonic hemolysis is one of the best known (Seeman, 1972). A large number of neurotropic drugs, many of which are amphipathic in chemical structure, are anti-hemolytic; they include hallucinogens, anti-inflammatory drugs, detergents, fatty acids, tranquillizers etc. Seeman (1972) reports references and antihemolytic concentrations for a number of such drugs.

Cater et al. (1974) have directly studied by DSC the effect of certain compounds like morphine derivatives and antidepressants similar to desipramine on the fluidity of dipalmitoyl lecithin liposomes. For both types of drugs there is a shift of phase transition to lower temperature

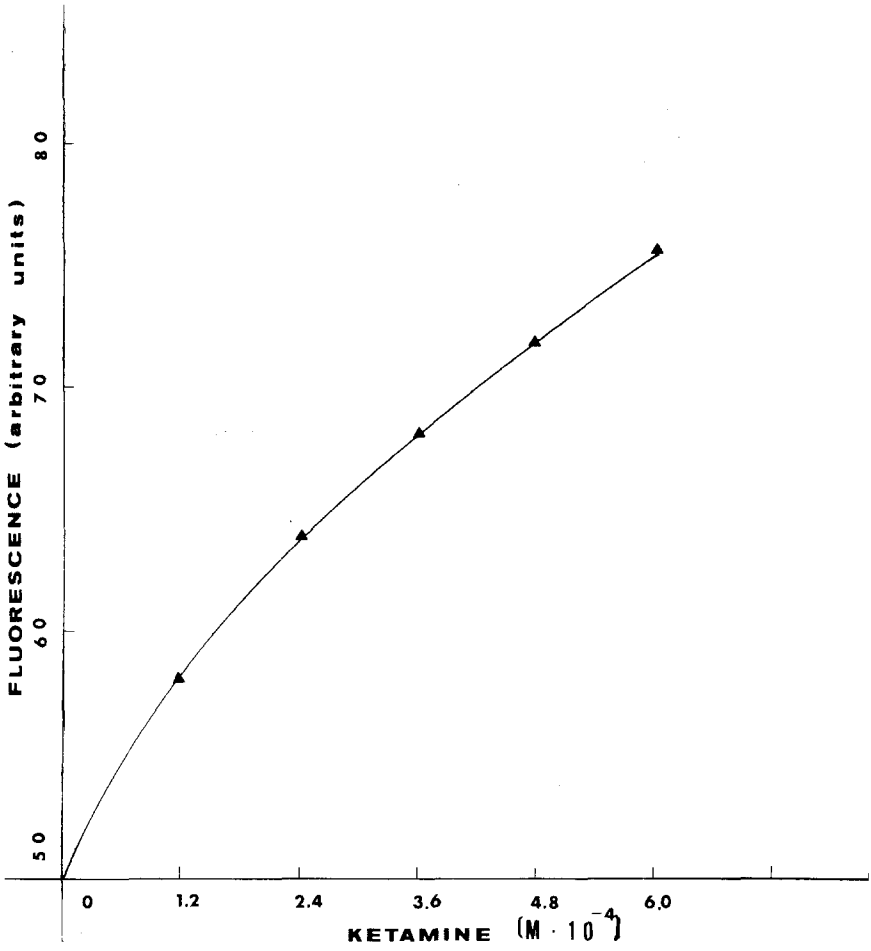
with increasing drug concentration, but the variation of heat transition is not continuous with the change in concentration. At low concentrations the drugs abolish the pretransition, an effect shared by cholesterol, decane, and gramicidine A. These drugs do not appear to penetrate deeply within the fatty acid chains, otherwise they would be expected to decrease the heat of transition (Chapman et al., 1974).

Ketamine (2-chlorophenyl, 2-methylamino cyclohexanone) is a general anesthetic of amphipathic structure. Spin labels show a negligible effect of this compound on the mobility of lipids in the depth of mitochondrial and erythrocyte membranes, while some effect is seen with the 5-doxylstearic acid spin label (G. Lenaz, G. Curatola, L. Mazzanti, and A. Bigi, in preparation). Ketamine very strongly enhances the fluorescence of ANS in mitochondrial and other membranes, including pure bilayer lipids; the effect is due to an increase of F_{max} (fluorescence at infinite membrane concentration) (Fig. 8) and not to a changed partition coefficient of ANS for the membrane. The fluorescence increase is not accompanied by a shift of the emission maximum, and has been therefore ascribed to increased packing in the glycerol region of the bilayer (G. Lenaz, G. Curatola, L. Mazzanti, and A. Bigi, in preparation).

C. *Alcohols and nonpolar compounds*

The effect of aliphatic primary alcohols on lipid fluidity in mitochondrial membranes and erythrocytes ghosts is related to their hydrophobicity (*viz.* their chain length) (Lenaz et al., 1976b; Paterson et al., 1972). Accordingly, the concentration required for 50% antihemolysis decreases from 2.7 M down to 1.10^{-5} M for the series methanol–decanol (Seeman, 1972). The effect of alcohols in promoting lipid extraction or changes in certain enzymic activities (Lenaz et al., 1971; 1972a) is also dependent on alcohol chain length.

In this respect *n*-pentanol is more effective than *n*-butanol and so forth in lowering ANS fluorescence (Seeman, 1972; Lenaz et al., 1976b), or in increasing rotational mobility of both 16- and 5-doxyl stearic acids in membranes (Lenaz et al., 1976b) (Fig. 9). The effects are significant for all probes, indicating that the action of alcohols is exerted at any level in the lipid bilayer in biomembranes. It may be of value to analyze the effect of an alcohol like butanol on a membrane such as the inner membrane of beef-heart mitochondria in comparison with a model liposome system. Figure 10 shows the mobilization of 5-doxylstearic acid in liposomes and mitochondrial membranes induced by *n*-butanol, as apparent from the decrease of the height ratio of middle-field to high-field peak or by decrease of the hyperfine splitting. The effect on liposomes is moderate in comparison with the dramatic increase in mobility induced in mitochondrial membranes. Indeed, it appears that



butanol makes the more immobilized mitochondrial membrane more similar to the protein-free liposomes; in other words the alcohol abolishes the immobilization induced by mitochondrial proteins on the phospholipids. The effect of alcohols may therefore be primarily on lipids–protein interactions. Metcalfe et al. (1968) studied the proton relaxation of benzyl alcohol in erythrocyte membranes and found that at low concentrations the anesthetic is strongly immobilized but its mobility increases at anesthetizing concentrations.

Several nonpolar molecules used as general inhalation anesthetics affect membrane fluidity as alcohols do. Diethyl ether, halothane,

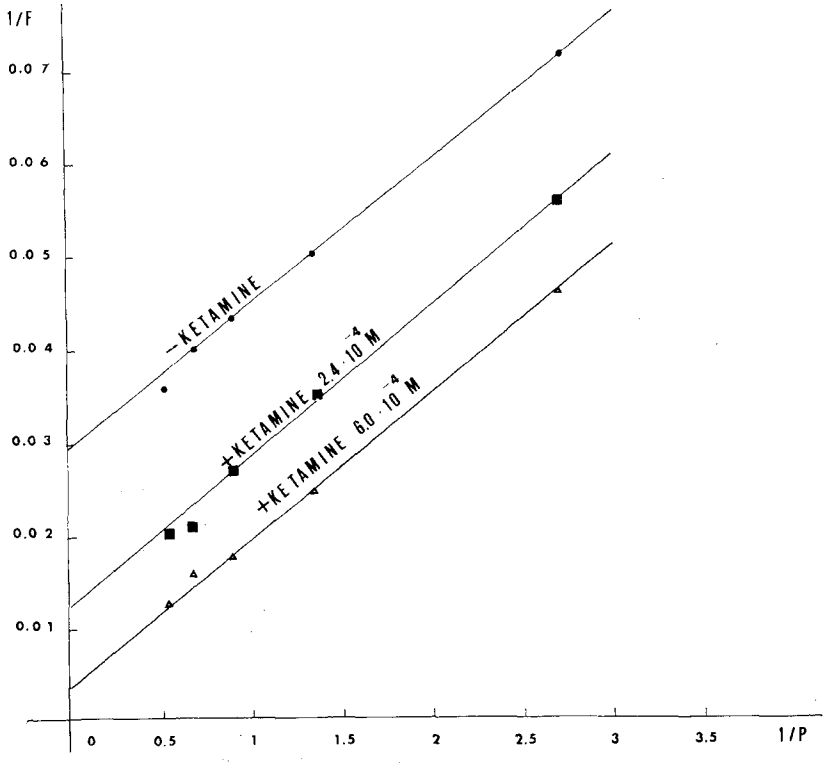


Figure 8. Effect of ketamine on ANS fluorescence in BHM. (A, facing page) Effect of ketamine concentration. (B, above) Double reciprocal plot of fluorescence as a function of membrane protein concentration. ●—●, no ketamine; ■—■, ketamine, $2.4 \cdot 10^{-4}$ M; △—△, ketamine $6.0 \cdot 10^{-4}$ M.

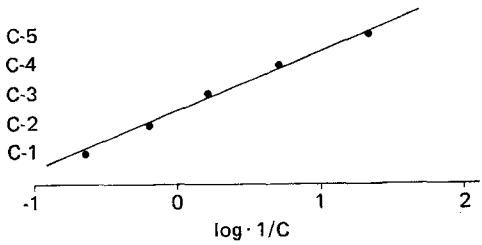


Figure 9. Effect of alcohol chain length on the mobility of 5-doxyl-stearic acid in mitochondria. The logarithm of the concentration of alcohol required to decrease the hyperfine splitting from 56.2 gauss (control value) to 55 gauss is plotted against alcohol chain length.

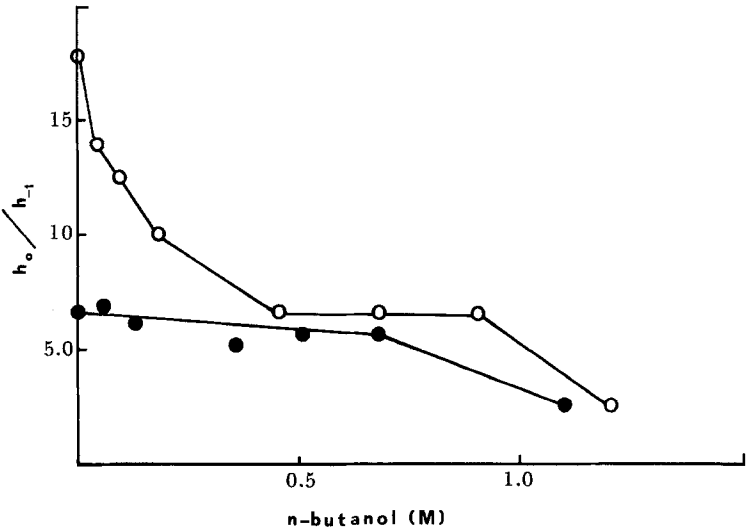
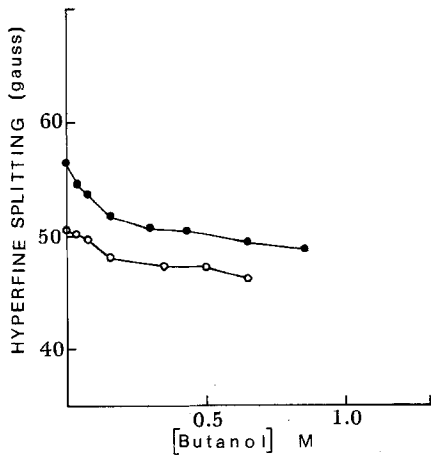
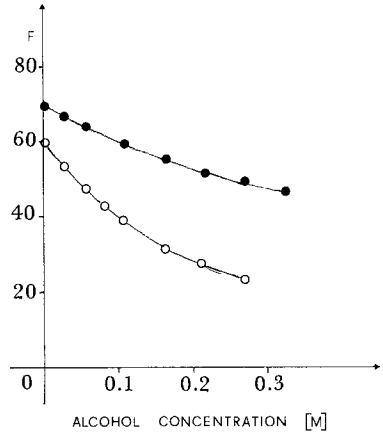


Figure 10. Effect of *n*-butanol on spin labels mobility and ANS fluorescence in lipid vesicles and mitochondrial membranes. (A, above) Mobility of 5-doxyl-stearic acid measured from the ratio h_0/h_{-1}. ●—●, lipid vesicles; ○—○, mitochondria. (B, below) Mobility of 5-doxyl-stearic acid measured from the hyperfine splitting. ○—○, lipid vesicles; ●—●, mitochondria. (C, top, facing page) ANS fluorescence in lipid vesicles (○—○) and mitochondria (●—●). (D, middle, facing page) Double reciprocal plot of ANS fluorescence against mitochondrial protein concentration. ○—○, no addition; ●—●, butanol 0.11 M; ■—■, butanol 0.22 M; △—△, butanol 0.32 M. (E, bottom, facing page) Effect of *n*-butanol on the emission spectrum of ANS in lipid vesicles and mitochondria. Curve (a): ANS in buffer (sensitivity x 10); (b) ANS in asolectin (sensitivity x 3); (c) ANS in asolectin + butanol (0.1 M) (sensitivity x 3); (d) ANS in mitochondria (sensitivity x 10); (e) ANS in mitochondria + butanol (0.1 M) (sensitivity x 10).

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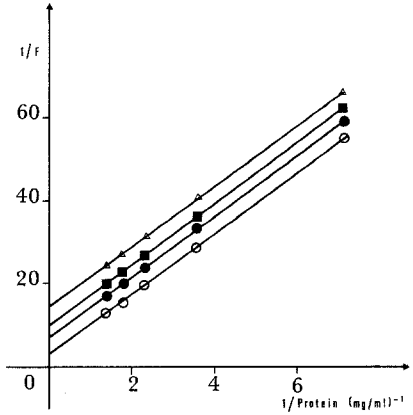
ALCOHOL CONCENTRATION [M]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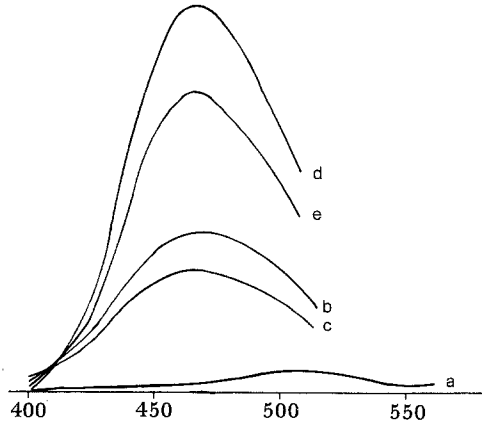
d
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550

TABLE I. Effect of proteins on the mobility of spin labels. Mobility is measured by the empirical ratio h_0/h_{-1}

Spin-label	Asolectin	Asolectin + lysozyme	ETP	ETP + pronase digestion	LDM + asolectin	LDM control
N-16	1.8–2.5	1.8–2.5	3–4	3–4	2.5–4	∞
N-12	4–5	4–5	9–10	6–8	–	–
N-5	6–7	10–12	15–20	9–12	15–20	∞

pentrane or methoxyfluorane, ethrane, and chloroform, all increase lipid mobility although to different extents in two different membranes (G. Lenaz, L. Mazzanti, G. Curatola, and A. Bigi, in preparation) (the differences between mitochondria and erythrocyte ghosts may be due to the high cholesterol content of the latter. Table II reports changes observed in BHM.

Inhalation anesthetics were shown to increase lipid mobility also in lipid membranes (Paterson et al., 1972; Hill, 1974; Trudell et al., 1973a,b; Johnson et al., 1973). According to Trudell et al. (1973a,b) general anesthetics cause a generalized fluidization of PC vesicles rather than a disorder localized in a particular region of the bilayer. We have confirmed the same effect on mitochondrial and erythrocyte membranes in a spin label study with different volatile anesthetics (G. Lenaz, L. Mazzanti, G. Curatola, E. Bertoli, and A. Bigi, in preparation). Measurements of the isotropic coupling constants also show a *decrease* in polarity of the environment with increasing anesthetic concentrations (Trudell et al., 1973a).

TABLE II. Effect of anesthetics on mobility of stearic acid spin labels in BHM

Solvent (0.1 M)	5-NS		16 N-S	
	$2T_{\parallel}$		h_0/h_{-1}	
n-Pentanol	51.0	(54.5)[a]	3.2	(4.5)
Ethrane[b]	53.0	(57.0)	2.7	(4.0)
Pentrane[c]	55.5	(59.5)	2.0	(4.15)
Chloroform	51.5	(58.5)	2.4	(4.05)
Halothane[d]	52.5	(58.0)	2.0	(4.15)
Ether (1 M)	54.5	(58.3)	3.55	(4.0)

[a] Numbers in parenthesis refer to controls without addition.
[b] 2-chloro-1,1,2-trifluoroethyl-difluoromethylether (CHF_2-$CCl\,F$-O-CHF_2).
[c] 2,2-dichloro-1,1-difluoroethylmethylether (CH_3-O-CF_2-$CHCl_2$).
[d] 2-bromo-2-chloro-1,1,1-trifluoroethane (CF_3-$CHClBr$).

Nonpolar compounds also generally share the property of decreasing ANS fluorescence in membranes. In the case of alcohols, ether, and halothane, it was shown that there is a decrease of F_{max} (at infinite membrane concentration) while the partition coefficient of ANS for the membrane is not changed (G. Lenaz, A. Bigi, G. Curatola, and L. Mazzanti, in preparation) (Fig. 10). The decrease of ANS fluorescence in the membrane is not accompanied by shifts in the emission peak; therefore it must represent decreased viscosity rather than increased polarity, in accord with the findings of Trudell et al. (1973a) that anesthetics *decrease* the polarity of their membrane environment. In a study of intact and lipid-depleted membranes of sarcoplasmic reticulum, Augustin and Hasselbach (1973) have suggested that halothane and ether affect primarily the protein components. However, analysis of their data, and our studies of the effect of alcohols on lipid vesicles, mitochondria, lipid-depleted mitochondria, and control proteins, indicate that the primary effect detected by ANS fluorescence must be on the membrane lipids. In fact, F_{max} of ANS is decreased by alcohols in lipids and membranes, but is unaffected in lipid-depleted mitochondria and in serum albumin. On the other hand, Hoss and Abood (1974) have used quenching of native tryptophan fluorescence to assess the fluidity of intact synaptic membranes and calculated that the viscosity of membrane proteins was at least one order of magnitude lower than the values reported for lipid bilayer regions of nerve membranes. Consequently, quenching of tryptophan fluorescence by chloroform was taken as an indication that membrane proteins are the locus for the anesthetic action of chloroform. However, such a conclusion is based on indirect evidence and does not account for effects of proteins on the lipid bilayer in intact membranes.

The study of Hill (1974) is the only one relating the effect of nonpolar solvents to changes in lipid phase transitions, studied by decrease in absorbance at 450 nm of dispersions of dipalmitoyl lecithin. All anesthetics measured gave a decrease in transition temperature of 6 deg at a 15% saturation of the anesthetic solution. This effect is compared with the freezing-point depression induced in a pure liquid by the presence of foreign molecules. The well-known effects in bulk solutions are applied to membranes as two-dimensional solutions.

V. *Consequences of Lipid Fluidity*

In a pure lipid membrane, the main consequence of an increase in lipid fluidity will be an increase of membrane volume, due to expansion of those lipid molecules which have undergone transition to the fluid state. In a natural membrane, however, the presence of proteins embedded in the lipid medium will give rise to a series of modifications related to this

new variable. Changes in protein distribution within the membrane have been described as a consequence of fluidity changes, and alterations of conformation within the individual polypeptide monomers are also to be expected. As membrane functions are linked more directly to membrane proteins, it is largely at these components that we must look in relation to functional modifications as a consequence of lipid fluidity.

A. *Mobility of membrane proteins*

Freeze-etching and freeze-fracturing electron microscopy have been a major advancement in the study of membrane structure (Branton, 1969; Zingsheim, 1972; Oseroff et al., 1973) (Fig. 11). Preferential fracture of

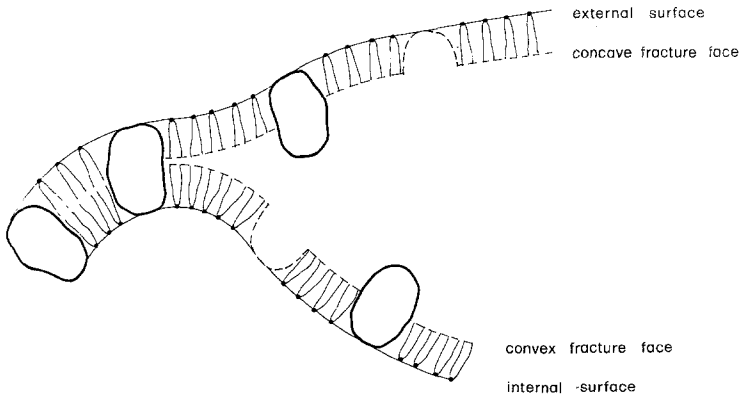


Figure 11. Schematic drawing of the fracture plane in freeze-etching electron microscopy. Freely redrawn from Branton (1966).

the membrane between the two halves of the lipid bilayer has allowed direct visualization of the membrane hydrophonic core. Most membranes (a significant exception being myelin) show particles of ~80 Å diameter in the fracture faces; by reconstruction studies of purified proteins with PL bilayers, the particles are unambiguously demonstrated as being proteins (Segrest et al., 1974; Vail et al., 1974; Hong and Hubbell, 1972). The particle distribution is asymmetric between the two halves: a dramatic example is sarcoplasmic reticulum, where the fracture face of the external monolayer shows a particle distribution 9 times higher than the corresponding face of the internal monolayer (Packer et al., 1974).

It has been shown by combined freeze-fracture and calorimetric (or other) methods that particle aggregation occurs near the lipid phase transition (Haest et al., 1974; Speth and Wunderlich, 1973; James and Branton, 1973), indicating a squeezing out of particles from the solid

into the more fluid domains when both such domains coexist (Grant et al., 1974). Squeezing out of the membrane itself cannot be excluded in certain cases (Kleeman and McConnell, 1974).

Antibodies may also be used to detect rearrangement of plasma-membrane antigens induced by other agents or may themselves be used to remodel the surface of cells; intermixing of surface antigens after fusion of heterokaryons, spreading of a spot of labeled antibody fragment on the cell surface, "cap" formation by antiglobulin reagents in lymphocytes stained with fluorescent antibodies, are all indications of protein movements on the cell membrane (Edidin, 1972).

Agglutination of intramembrane particles may be induced not only as a consequence of thermotropic transitions; in erythrocyte ghosts, not in whole cells, the particles are randomly distributed (Pinto da Silva, 1972), but are reversibly aggregated by pH and ionic-strength changes; the lack of effect in intact erythrocytes may be a consequence of the presence of peripheral proteins which are lost or modified in ghost preparations. In the mitochondrial membrane Packer (1973) has found intramembranous particle aggregation as the pH was lowered. In view of the chemiosmotic hypothesis for energy coupling stating the establishment of an H^+ gradient as the primary event (Mitchell, 1973), this observation is of great interest in bioenergetics.

Plant agglutinins (lectins) (Lis and Sharon, 1973) which bind surface glycoprotein receptors in plasma membranes may also induce agglutination of the same receptors: agglutination requires a fluid membrane in order to occur (Nicolson, 1972). Temperature-dependent agglutinability of concanavalin-A (conA) receptors has been reported for virally and chemically transformed fibroblasts and trypsinized human erythrocytes.

The observations are generally but not unanimously (e.g., Gordon and Marquardt, 1974) interpreted on the basis that agglutination is associated with a temperature-sensitive activity of the cell membrane (Nicholson, 1972); in other words, clustering of lectin receptors involves motion of the glycoprotein receptors in the plane of the membrane; in turn mobility of proteins requires mobility of lipids, as the diffusion of a solute in water requires the mobility of water molecules (Shimshick and McConnell, 1973).

Differences in agglutinability of normal and tumour cells may be related to differences in membrane fluidity and cholesterol content (Rutishauser and Sachs, 1974; Inbar et al., 1974; Shore and Shore, 1974; Huet and Bernhard, 1974; Barnett et al., 1974); in turn, the higher fluidity of several malignant cell membranes has been related to the loss of contact inhibition and control mechanisms for cell replication (Inbar et al., 1974). It has been observed that lymphoma cells treated with cholesterol–lecithin (but not with lecithin) vesicles, are no longer able to kill the animals by ascites tumour after intraperitoneal injection (Inbar

and Shinitzky, 1974); the cholesterol-treated cells raise their cholesterol content to normal, while untreated lymphoma cells have lower cholesterol and lower viscosity in comparison with normal lymphocytes.

Lectin binding and consequent agglutination of lectin receptors have been used to show the existence of transmembrane phenomena (Ji and Nicolson, 1974; Nicolson, 1973). Binding of *Ricinus communis* agglutinin on the outer surface of resealed ghosts results in enhancement of cross-linking of components by the bifunctional reagent dimethyl malonimidate: of these components, two are subunits of spectrin, a protein complex situated on the inner surface of erythrocyte membranes. The transmembrane effect is probably mediated through the glycoproteins spanning the thickness of the membrane.

An opposite effect is the aggregation of outer membrane components induced by spectrin antibodies which cause spectrin aggregation on the inner side (Nicolson and Painter, 1973). Freeze-fracture and labeling techniques have localized conA receptors on the membrane-intercalated particles (Pinto da Silva and Nicolson, 1974). The ConA receptor is a Band-III component (Marchesi and Andrews, 1971); according to Pinto da Silva and Nicolson (1974) the intramembrane particle represents an oligomeric structure containing the principal integral proteins (glycoprotein and Band-III) of the erythrocyte membrane, interacting with spectrin at the inner surface. The particle may represent an amphipathic structure (permeaphore) which topographically and structurally interrupts hydrophobic bilayer membrane domains.

Mobility of the surface cell glycoprotein components is related to such phenomena as contact inhibition, antigen–antibody combination, etc. The functional link between hormone receptors and adenyl cyclase across the cell membrane may involve movement of the receptor protein in a fluid membrane upon hormone binding (Cuatrecasas, 1974).

Rotational mobility (Brown, 1972; Cone, 1972; Cain et al., 1972) besides lateral diffusion (Poo and Cone, 1974) has been found for certain proteins like rhodopsin in retinal rod receptors, and may have functional implications in the process of vision.

B. *Compressibility*

It has been seen that solid and fluid phases may coexist in certain membranes under physiological conditions. The presence of solid and fluid domains within the same membrane confers a high extent of compressibility and extensibility (Kleemann and McConnell, 1974; Linden et al., 1973a). Freezing of a fluid bilayer is accompanied by a large degree of compression. In monolayers the area occupied per lipid molecule decreases from 125 $Å^2$ to 70 $Å^2$ by compression (Demel et al., 1972) until the pressure causes the monolayer to collapse.

The compressibility of a membrane by increasing the content of the

solid phase may allow insertion of new biosynthetic material into the membrane (with morphogenetic implications) and will offer a better possibility of solute translocation in the irregular crevices left between the solid and fluid domains (Papahadjopoulos et al., 1973a). Although transport phenomena require specific proteins, the passive permeability of several molecules and ions is highly increased when solid and fluid bilayer areas coexist (Inoue, 1974).

C. *Fusion*

An interesting phenomenon is the resealing of erythrocyte ghosts formed by hypotonic lysis; resealing partly restores the permeability barrier (Yung et al., 1973); resealing never occurs below 22°C, indicating that lipid fluidity is necessary for this phenomenon.

Resealing is only one aspect of membrane fusion, a phenomenon with important dynamic biological consequences such as phagocytosis, pinocytosis, secretion, cell motion, cell division, etc. (Lucy, 1969, 1970; Poste and Allison, 1973). The rate of fusion increases near the phase-transition temperature (Prestegard and Fellmeth, 1974) and liquid–crystalline lipids are more susceptible to fuse (Papahadjopoulos et al., 1974); cholesterol suppresses the ability of phospholipid vesicles to fuse (Papahadjopoulos et al., 1973a; 1974). The mechanism of fusion is unknown, although it can be different for lyso-lecithin and other fusogenic agents. Lysolecithin (Howell et al., 1974) may induce micelle formation in a bilayer membrane whereas Ca^{2+} may facilitate fusion by bridging adjacent vesicles, thus producing a more hydrophobic surface for contact between two membranes (Papahadjopoulos et al., 1973a; Maeda and Ohnishi, 1974). It is relevant here that local anesthetics, which displace Ca^{2+} from membranes, inhibit virus-induced cell fusion (Poste and Reeve, 1972).

D. *General Anesthesia*

We will now briefly comment on the results decribed in the previous sections in relation to general anesthesia. Further ideas will be developed in the next sections, where membrane protein conformation will be examined in a more direct fashion.

General anesthetics are molecules preventing the propagation of nervous impulses by abolishing the transient depolarization current (action potential) in nerve or muscle cells (Seeman, 1972; Shanes, 1958). According to such a definition, Seeman (1972) has tabulated a wide variety of lipid-soluble compounds including tranquillizers, anticonvulsants, antihistamines, steroids, detergents, antiarythmics, narcotics, vasodilators, and sedatives. Virtually all the drugs studied reduced the sodium conductance at concentrations below those affecting the resting membrane potential.

It has long been recognized that the activity of general anesthetics is related to their hydrophobicity, i.e. it increases with increasing oil/water partition coefficient (Overton, 1901; Meyer, 1937). The Meyer–Overton rule of anesthesia states that narcosis commences when any chemically indifferent substance has attained a certain molar concentration in the lipids of the cell (Meyer, 1937). The idea that narcotics act at the membrane level on the lipid components was therefore advanced a long time ago. Recent work has been reviewed extensively by Seeman (1972). It is recognized that anesthetics increase the fluidity of model lipid membranes (Hill, 1974; Trudell et al., 1973a,b; Johnson et al., 1973), the idea that anesthesia is the result of a change in lipid fluidity of neuronal membranes involved in nerve-impulse propagation has been therefore advanced. It had been postulated very early that the action of anesthetics is a general phenomenon and is not confined to specific nervous membranes, but it is in neuronal membranes that the consequences of such changes will give rise to phenomena resulting in anesthesia. The specific action of narcotis on certain districts of the central nervous system could be the result, among many other reasons for selectivity (Seeman, 1972), of specific lipid composition differences (Balzer et al., 1968).

The fluidification of lipids by anesthetics is accompanied, as expected, by membrane expansion (Seeman et al., 1969; Roth and Seeman, 1972; Machleidt et al., 1972; Seeman and Roth, 1972); expansion is many times higher in biological membranes than in lipid membranes (Seeman, 1974), suggesting that proteins are largely involved in this effect. The role of expansion in anesthetic action is strengthened by the observed relation between pressure reversal of anesthesia and pressure reversal of membrane expansion (Trudell et al., 1973b). If expansion induced by anesthetics is accompanied by decrease of solid lipid areas within the membrane, a decrease of passive solute permeability may be expected.

It is predictable that modifications of fluidity of membrane lipids induce as a consequence changes in those ion movements which are at the basis of neural transmission (i.e., inhibition of the Na^+-conductance "channel"). However, the molecular bases of neural transmission are still obscure, and there is therefore a gap of knowledge between molecular interaction of the anesthetics and the physiological effect, anesthesia. If indeed proteins are largely involved in membrane expansion, these components could be responsible for ion permeability changes in anesthesia. It must be stressed that the problem only constitutes one facet of a more general problem encompassing all membranes: how does membrane fluidity affect membrane functions? If we keep in mind that membrane functions (among them, neural transmission) must be exerted by specific proteins, the problem can be restated as follows: how does membrane fluidity affect the structure of membrane proteins? We will try to answer this specific question in following sections of this review.

VI. *Conformation of Membrane Proteins*

Several techniques have been employed to study protein conformation, e.g., x-ray diffraction, light scattering, infra-red spectroscopy, UV spectroscopy, CD, and ORD. Among these, optical rotation measurements, and CD in particular, stand out as a unique tool to investigate the secondary structure of polypeptides and proteins in dilute solution and of membrane proteins.

Of course, no technique alone can give unambiguous information on the conformation of macromolecules or such large aggregates as membranes; nevertheless the reason for the astoundingly increased application of optical activity measurements to proteins in the last decade lies in the possibility of studying them in solution, under controlled conditions of solvent, pH and temperature, in a very short time and using small quantities of sample. As for biomembranes, intact or fragments, a further advantage is the capacity of observing them directly in active, functional states. In consideration of the increasing importance, of the optical rotation data in understanding biomembranes structure and functions, we shall briefly review the literature available up to date.

The subject will be treated in view of its bearing on the discussion of further aspects of membrane fluidity and agents affecting it, which is to follow.

For extensive discussion of CD and ORD of polypeptides, proteins, and biomembranes, we refer to several reviews available in the literature (Singer, 1971; Urry, 1972a; Sears and Beychok, 1973; Adler et al., 1973; Holzwarth, 1972; Urry, 1974).

When dealing with optical-spectroscopy data of biological membranes it has to be considered that the information yielded is the average protein conformation for all proteins in the membrane. If proteins are not physically isolated, assessment of the contributions of the single protein to the spectra cannot be done. Furthermore, because of their particulate nature, biomembranes yield spectra containing artifacts arising, as first recognized by Urry and Ji (1968), from light scattering, absorption dampening, and differential light scattering. There is now wide agreement that the optical-rotation patterns of biological membranes, namely low magnitude and red-shifted extrema, when compared to model α-helical polypeptides and proteins, are primarily due to the aforementioned artifacts (Urry et al., 1970a,b; Urry and Krivacic, 1970; Schneider et al., 1970; Glaser and Singer, 1971; Glaser et al., 1971; Ottaway and Wetlaufer, 1970; Gordon et al., 1969; Zahler et al., 1972).

Several attempts have been made to correct for the distortions and achieve meaningful CD and ORD data. Some approaches are based on Mie scattering functions (Gordon, 1972; Gordon and Holzwarth,

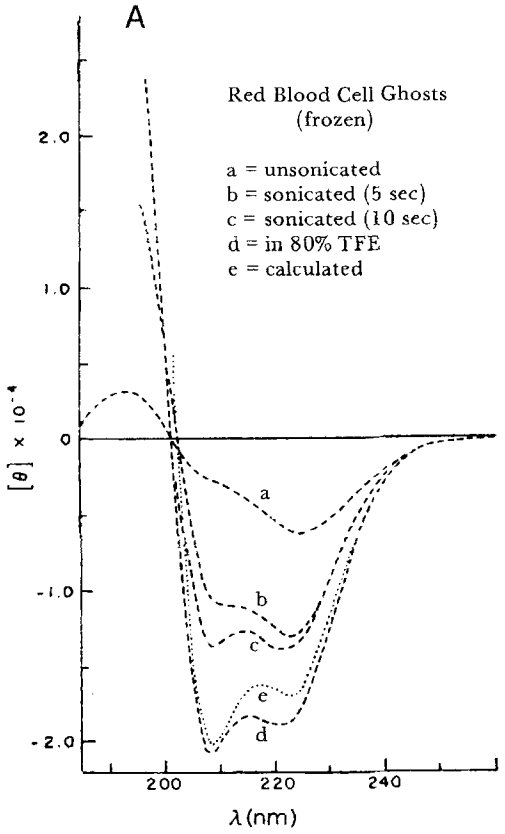
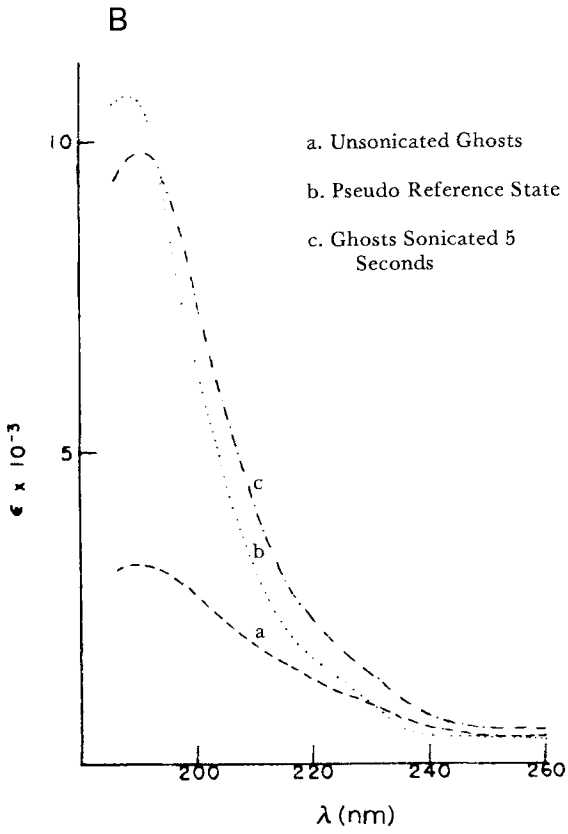


Figure 12. CD curves (A, above) and absorption curves (B, facing page) of red-blood cell ghosts. CD and absorption spectra were recorded simultaneously: curves (a) and (c) in (A) correspond to curves (a) and (c) in (B). 0.1 and 0.2 mm pathlength cells were used. Protein concentration was in the range 1.8–2.2 mg/ml for all the experiments. The CD spectra were recorded on a Cary Model 60 spectropolarimeter with Model 6001 attachment for CD and an accessory for simultaneous measurement of absorption. From Urry et al. (1971b).

1971a,b; Schneider, 1973); Urry and associates (Urry et al., 1970b; Urry et al., 1971b; Masotti et al., 1972a; Urry, 1972a) have proposed a pseudoreference state approach using poly-L-glutamic acid as a model and a suitable dissolved state of the membrane in order to apply the corrections. A comparison of the results obtained by applying the two different analyses to PGA suspensions is reported by Urry and Long (1975). The calculated forms of the distortions to be expected on the basis of Mie scattering theory show a gross similarity with the



experimental curves while the approach adopted by Urry and associates generates distorted curves which resemble the experimental ones very satisfactorily.

The early controversy on the existence of artifacts in the optical rotation spectra (cf., for instance, Urry, 1972a) is now shifted to the feasibility of the application of the corrections to the spectra (Wallach et al., 1973). Caution is called for in order not to introduce, in the process of correcting the distortions, additional artifacts in the spectra, mostly below 233 nm. Indeed, all the authors, applying the calculated corrections to the suspension spectra, have been aware of the inadequacies in the scattering corrections and of the need of better understanding of several features both of model systems and of membranes not as yet well understood.

The interpretations of optical activity of biomembranes has been attempted in much the same way as for soluble proteins, i.e., resolving

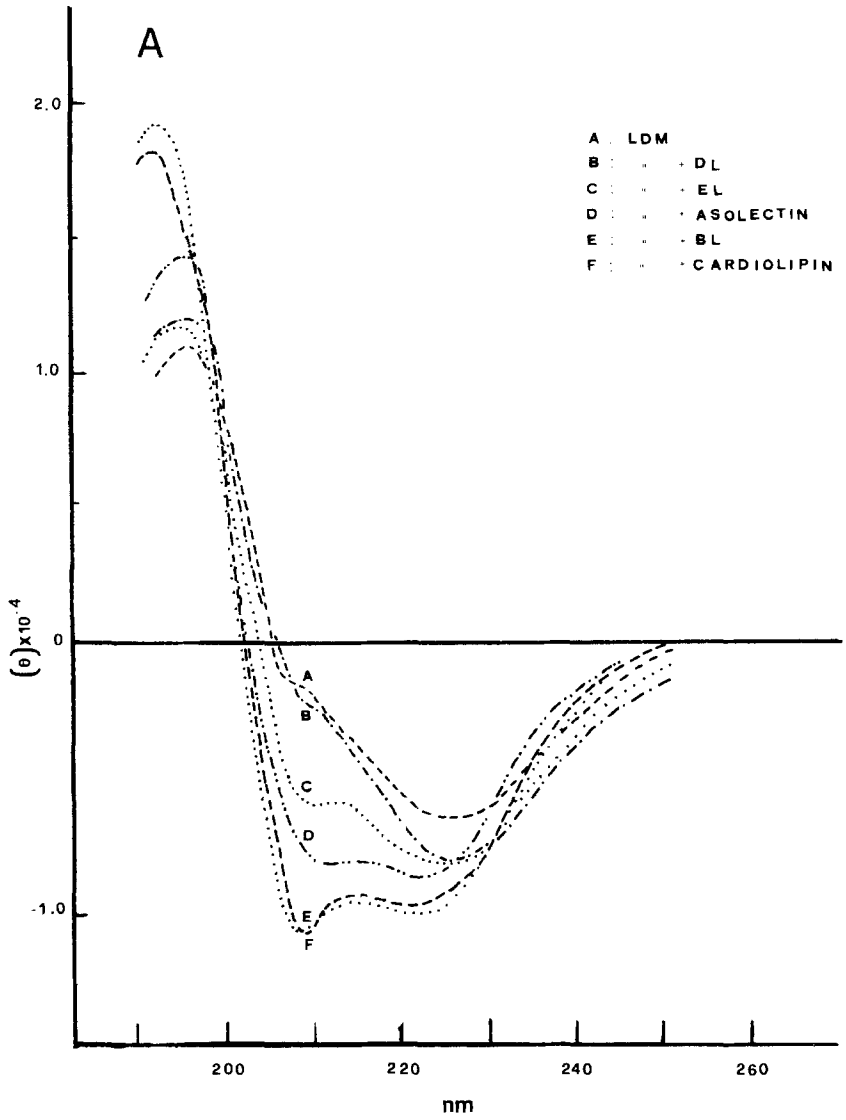
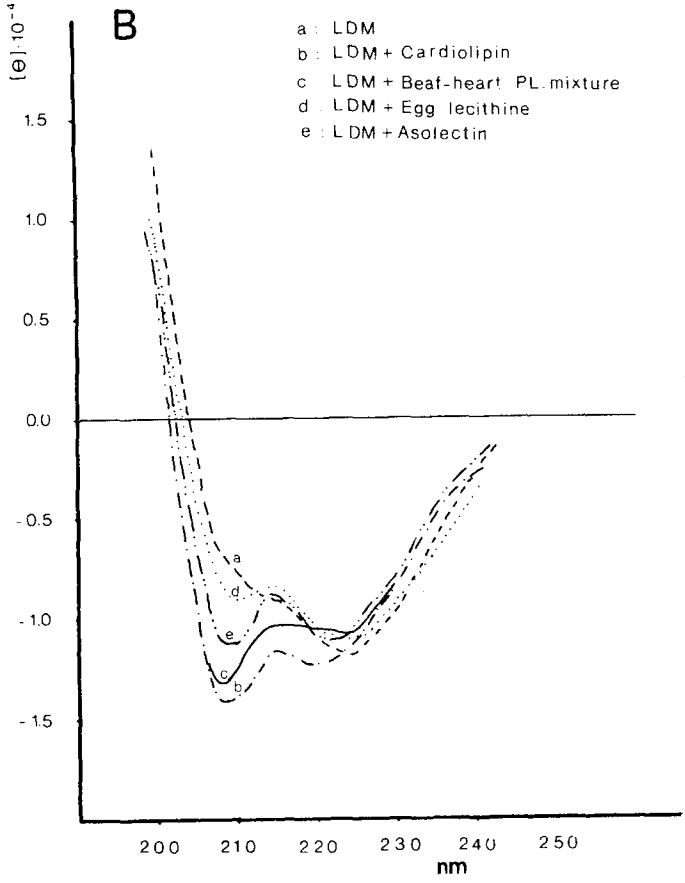


Figure 13. CD of mitochondrial membranes depleted and reconstituted by addition of different PL. (A, above) as prepared and not corrected; (B, facing page) corrected for light-scattering, absorption dampening, and differential light-scattering (see text). Experimental conditions were as in Fig. 12. (From Masotti et al., 1974).



the spectra in contributions due to α-helix, β-structure, and disorder conformation, taking as models polypeptides, for instance, poly-L-lysine (Greenfield and Fasman, 1969) or known structure of RNase, lysozyme, and myoglobin and the experimental CD curves of these proteins (Saxena and Wetlaufer, 1971). This approach is limited, as repeatedly pointed out by many investigators, since, even if it is apparent that CD and ORD of biomembranes originate from the backbone of proteins, with little or no contribution from lipids and carbohydrates, it does not take into account chromophores other than the peptide. Also interactions of segments of proteins in a given conformation with their environment as well as side-chain contributions are not accounted for. Conformations other than the right-handed α-helix, β-type, and random coil should be taken in account: besides the 3_{10}-, π-, and γ-helices

(Donhoue, 1953), also left-handed α-helices may be present in certain conditions which might arise in the membrane (Malcom, 1970), as well as β-helices (Urry, 1971; Urry et al., 1971a) ascribable to specialized moieties involved in specialized membrane functions.

At the present stage of knowledge, by application of the required corrections, there is reasonable hope to achieve meaningful optical-rotation data whose interpretation must be anyway very careful. Impressive advances have been made in interpretation of CD related to the secondary structure of proteins. Many recent theoretical achievements are still to be applied to the analysis of complex biological systems thus making CD, in particular, when employed with other spectrometric techniques, a very powerful tool in studying protein conformation. The optical activity of several membrane systems has been investigated. The CD and ORD curves of rat-liver mitochondria show the typical patterns of optical rotation spectra of membranes, namely, reduced amplitude of the bands and red-shift of the extrema; such features are shown for red-blood cell ghosts in Fig. 12 (Wrigglesworth and Packer, 1968 Urry et al., 1967; Ji and Urry, 1969).

Beef-heart mitochondria, sonicated in order to reduce particle size (Urry et al., 1970b), exhibit CD spectra which closely resemble those of polypeptides in α-helical conformation. The α-helical content has been calculated as being in the range of 50%. Mitochondrial "structural protein" (Steim and Fleischer, 1967) has been investigated by ORD methods. The features of the spectra resemble those of particulate systems: it is unlikely that for this protein, mainly constituted by denatured ATPase (Senior and MacLennan, 1970) α-helical conformation plays a substantial role. CD of sub-mitochondrial particles (ETP) has been reported by Pasquali et al (1973). The corrected spectra show, according to Greenfield and Fasman (1969) an estimation of conformational contributions, a 54% α-helical content, reduced by pronase digestion of the submitochondrial vesicles to 39%. The observed conformational changes might be due to preferential availability of proteins in the α-helical conformation to the enzyme action causing a consequent increase in disordered conformation content; on the other hand, the possibility exists that the enzyme treatment causes a rearrangement of the proteins within the membrane affecting proteins conformation. Very little β structure seems to contribute to the CD spectra of both ETP and pronase-treated ETP. Many data on CD and ORD of human-erythrocyte ghosts have been reported (Gordon et al., 1969; Schneider et al., 1970; Urry et al., 1971b; Lenard and Singer, 1966; Gordon and Holzwarth, 1971a). After correction with the pseudoreference state approach (Urry et al., 1971b) the CD spectra seem to indicate an α-helical content of about 50%. Corrected data using poly-L-glutamic acid (PGA) as a reference, CD of ghosts suspension and CD calculated for spherical shells from SDS-solubilized material, suggest

that the membrane proteins, if solubilized but unaltered in confromation, should be about 40% in α-helical conformation (Gordon and Holzwarth, 1971a).

The CD of rat-liver plasma membranes reveals band shapes and amplitudes very similar to those of red-cell ghosts and mitochondria (Masotti et al., 1972a) both for the suspensions and the corrected values obtained using as a reference state a membrane solubilized with 2% SDS, sonication, and trifluoroethanol.

Sarcotubular vesicles from rabbit-skeletal muscle (Masotti et al., 1972a) are characterized by elliptically lower than that of mitochondria, red-blood cells, or plasma membranes. Sonication, effective in partially removing light scattering and adsorption dampening for the other membranes, does not readily improve their CD patterns nor cause a large increase in absorption near 192 nm. Accordingly, a lower content in α-helix can be calculated for sarcotubular vesicles in comparison with the other membranes so far mentioned: they therefore seem to represent a transition to a class of membranes, as, for instance, axonal membranes (Masotti et al., 1973), characterized by low ellipticities and high absorbances.

The CD and ORD of plasma membranes of Ehrlich ascites carcinoma cells have been reported (Wallach and Zahler, 1966; Gordon et al., 1969). The observed band shapes are very similar but their amplitudes are about one-half of those of red-cell ghosts. Using standards derived from CD and ORD of hemoglobin, myoglobin, and lysozyme (Straus et al., 1969), Gordon et al. (1969) estimated the helix content of the membrane as 58% with an uncertainty of ±20%. Advancing reservation on this method of evaluation, Holzwarth (1972) suggests a much lower helical content, about 25% or less, with an uncertainty, due to the very low value, of about ±15%.

CD studies correlating changes in optical activity with the functionally different states of biological membranes or membrane-bound enzymes have been attempted. Lindenmayer and Schwartz (1970) have presented evidence of CD changes induced in $(Na^+–K^+)$–ATPase particles by addition of ATP. Absorption and CD data of a vesicular preparation derived from oxyntic cells of dog gastric mucosa containing a membrane-bound, HCO_3^--stimulated ATPase has been reported by Masotti et al. (1972b). The ellipticity at 224 nm is comparable with that of axonal membranes, possibly reflecting a considerable amount of disordered or β-structure.

Addition of Mg^{2+}–ATP brought about specifically significant changes in the spectrum, changes only partially originated by the artifacts due to the particulate nature of the vesicles. It seems therefore possible that ATP induces conformational changes in the protein constituents correlated with active transport.

Studies on the correlation of conformational changes with active

cation transport function of brain microsomes were reported by Long et al. (1973). The corrected molar ellipticity of the membrane suspension is in line with the axonal membranes, oxyntic cells, and sarcotubular vesicles. Unlike the oxyntic cells, the CD spectra of brain microsomes in the activated state are indistinguishable from those of the inactivated state. Ca^{2+} and Mg^{2+} cause a significant change in the state of aggregation of the membranes. However, the conformational change correlated with transport translocation does not cause extensive changes in major membrane polypeptides and protein conformations involving probably only localized sites or less than 10% of the protein.

VII. *Effect of Lipids on Protein Conformation and Stabilization*

In the early membrane models the role of lipids seemed to be confined to that of mere structural support of proteins. Green and Tzagoloff (1966) suggested that lipids were necessary in order to prevent tridimensional polymerization of the repeating units, the building blocks of the membrane model they were proposing at the time. But there is increasing evidence that this role should be regarded as being much more complex.

The effect of lipids on protein conformation has been recently studied in lipoproteins. Again, optical spectroscopy has been a very useful tool in revealing the conformational changes of proteins upon removal or addition of PL, or reconstitution of the original lipoprotein.

Several studies (Scanu, 1965; Gotto and Shore, 1969; Lux et al., 1972) have shown that human-plasma HDL has a helical content of about 60%. Delipidation has been proved to decrease the helicity and increase the content in disordered structures (Lux et al., 1972; Scanu and Hirz, 1968). Readdition of lipids restores the native content in the α-helix to different extents, PC being less effective than a mixture of PC and cholesteryl oleate (Scanu, 1969; Hirz and Scanu, 1970; Lux et al., 1972). One of the major components of HDL, ApoLP-Glu-II, has been studied by Jackson et al. (1973a). The original helical content of the lipoprotein estimated as 49% drops at 32% for its performic-acid-oxidized derivative as well as for the COOH terminal cyanogen-bromide fragment (CNBr-III), which also exhibits disordered structures. After reconstitution with PC of the delipidated apoproteins, a significant increase in the magnitude of the negative ellipticity of the 222 nm band in the CD specta is observed, meaning an increase in α-helical content: up to 64% for ApoLP-Glu-II, 57% for the performic-acid-oxidized ApoLP-Glu-II, and 44 for CNBr-III.

All of the apolipoproteins or peptide fragments examined that bind PL exhibit an apparent increase in α-helix content as a consequence of lipid binding. Significantly, no variation is observed in association with

lipid binding in the CD spectra of CNBr-III, the NH_2 terminal fragment which does not bind PL. It is not known if the change in secondary structure is essential for lipid binding or, instead, it is a consequence of lipid–protein interactions indicating a more hydrophobic environment. The selectivity for binding of one fragment seems to indicate the occurrence of lipid-binding sites within the molecule. In this respect, the area of the disulfide linkage in the apolipoprotein has been investigated (Jackson et al., 1973b). In agreement with the findings of Scanu (1970), the reduction of the S–S group affects the secondary structure of the protein in the absence of lipid, but its integrity is not required for binding of PC. Furthermore, ESR and fluorescence studies suggest that the disulfide region is not intimately involved in the binding of PL, a role which seems to be ascribed to the CNBr-III fragment of the protein.

Also, titration of ApoLP-Ala (Morrisett et al., 1973) with a sonicated dispersion of PC increases the calculated α-helical content from 22% to 54%, with maximal helicity at a stoichiometry of PC: ApoLP–Ala = 50:1. Fluorescence studies carried out monitoring intrinsic tryptophan show that the protein can bind up to 50–80 molecules of PC. The binding causes a transition from disordered to helical secondary structure as well as one or more of the three tryptophan residues to experience a more hydrophobic environment. This detail of tertiary structure of the lipoprotein has been taken as originating from increased lipidation. An analysis of the helix-forming ability of each amino-acid residue (Robson and Pain, 1972) leads to the conclusion that while the intrinsic helicity should be 29%, PL provides the forces necessary to increase such a value of about two-fold.

As for membrane proteins, London et al. (1974) have found different lipid affinity for the two major myelin proteins, i.e., Folch–Lees protein and A_1 basic protein. The Folch–Lees protein shows affinity for both charged and neutral lipids. Precisely, it interacts strongly with CS, PS, PI, PE, and cholesterol, and exhibits lower affinity for sphingomyelin, cerebroside, or PC. Affinity for cholesterol is also dependent on the sterol structure, the interaction with the hydrophobic part of the molecule being of critical importance. A_1 basic protein interacts preferentially with negatively charged lipids, more specifically with cerebroside sulfate; it has been demonstrated, anyway, that also hydrophobic interactions with lipids play some role. As a consequence of interaction with lipids, the Folch–Lees protein, which in water has an helical content of 42%, passes to a close to 100% helical content in presence of PC/cholesterol (1:1).

Delipidation to the extent of 1% of the original lipid content treating Ca^{2+}-dependent ATPase of sarcoplasmic reticulum of skeletal muscle (Hardwicke and Green, 1974) with deoxycholate causes its helical content, as judged by CD in the peptide region, to decrease from 35% to 28%. Extraction of large quantities of lipids however does not alter the

CD significantly. Removal of DOC restores the original CD pattern of the ATPase almost completely, but it is difficult to say if the original protein structure is mostly preserved or if new helical structures are formed when DOC is removed. Addition of PL reactivates the enzyme, but the activation extent never exceeds 5% of the original activity. It is suggested that the replacement of lipid by DOC causes in the lipid-free enzyme an irreversible loss of hydrophobic surface.

The possible contribution of lipids to the optical-rotation spectra of biological membranes has also been investigated, but the data available in the literature do not afford unambiguous information. Lenard and Singer (1966) did not find any contribution due to lipids extracted from red-blood cell ghosts at wavelengths longer than 215 nm. PE and L-α-lecithin in trifluoroethanol show a spectrum characterized by a CD positive peak at 218 nm and a negative peak near 192 nm (Urry et al., 1967). However there is not sufficient knowledge in this field as to rule out a different behaviour of lipids in membranes. It is generally recognized though, that membranes UV, CD, and ORD mainly originate from proteins.

Several membranes have been treated with phospholipases in order to assess the contribution of lipids or components of such molecules to their optical-rotation spectra. Hydrolysis of the phosphodiester linkage between L-α-PA and nitrogenous base by treatment of red-blood cell ghosts with phospholipase-C shows that there is no direct contribution of the phosphate groups to the optical rotation of the membrane nor do they affect conformation (Lenard and Singer, 1968). The enzyme, however, causes large enhancement in the methylene mobility of the fatty-acid chains as shown by NMR data (Glaser et al., 1970). Quite different is the effect of phospholipase C on plasma membranes of Ehrlich ascites tumor cells (Gordon et al., 1969). The CD shows, after treatment, an enhancement of the distortions characteristic of membranes, i.e., lower magnitude of the bands and red-shifted extrema. It would therefore seem that the enzyme causes an increase in turbidity or change in the state of aggregation of the plasma membranes. Red-blood cell ghosts treated with phospholipase A or lysolecithin exhibit CD spectra characterized by higher magnitudes of the 208 nm and 192 nm bands, the latter also blue-shifted to 190 nm (Gordon et al., 1969). It seems, consequently, that the effect of these substances is at least a partial removal of the artifacts generated by the particulate nature of the membranes by affecting the binding of the lipids in membranes and possibly by reducing the particle size of the membrane fragments.

In contrast with these findings, Zahler et al. (1972) did not observe noticeable changes in secondary structure of the proteins of submitochondrial vesicles depleted of large amounts of lipids by treatment with phospholipase A. Dramatic changes in the protein conformation of the inner mitochondrial membranes as a consequence of

lipid extraction by means of aqueous acetone and ammonia have been reported by Masotti et al. (1974). When corrected for light scattering, absorption flattening, and differential light scattering, the CD spectra of lipid-depleted membranes showed virtually no 208 nm band, meaning that the differences in the spectra in comparison with the original membranes were due to causes other than aggregation. However, when the membranes were relipidated with different PL, and the spectra corrected also for the heterogeneity of composition of the membranes, the original CD patterns were restored to varying extent by the different PL, cardiolipin being the most effective in restoring CD patterns very close to those of intact mitochondria. The differences in results in comparison with those of Zahler and co-workers may be explained by the difference of procedure used for delipidation as well as to inability of the method employed of extracting PL tightly bound to the membrane proteins and essential to modulate the conformation of the proteins.

The picture emerging from the literature is one of phospholipids playing a definite role in determining the conformation of proteins in the membrane. Even if the bulk of the PL is probably independent of proteins as shown by the experiments with phospholipase C on red-blood cell ghosts or by ESR data discussed in the previous sections, some specific lipids could be required in the regions close to the proteins providing the forces necessary for the proteins to assume the required conformation.

VIII. *Structure of Conductance Channels*

The literature on peptide ionophores, channels, carriers, and isolated membrane systems which regulate membrane conductance has recently become very vast. It is not our aim to fully review and deeply discuss the many interesting facets of such a field. Just some of the most recent work on these topics, therefore, will be reviewed in order to point out the relevance of the hypothesis that bilayer perturbation may affect this vital physiological activity. It is tempting to speculate that the action of anesthetics, drugs, and biogenic amines can be related to their interaction with lipids, thus affecting some of the membrane activities.

Cell conductance in the intact tissue is presumably due to such structures as channels or carriers, molecules that can be abundantly found in bacteria or fungi. The ionophore valinomycin (Lev and Bushinski, 1964) is a cyclic dodecadepsipeptide which can exist in several different conformations. It has the ability of forming stable complexes with K^+, assuming a rigid conformation where the potassium ion appears to reside in a central polar core (Urry and Ohnishi, 1970) forming six ion-dipole bonds with the ester carbonyls. In this

conformation the ionophore, with the hydrophobic groups pointing to the exterior of the molecules, has sufficient lipophilicity as to be able to pass through the internal zone of the membrane. It also shows sufficient surface activity so that it can partition between the surface and the internal zone of the membrane and it can rapidly exchange the potassium ion with another ionophore. Enniatins A, B, and C, cyclic hexadepsipeptides with alternating L and D residues (Ovchinnikov, 1974; Grell and Funck, 1973) also can form 1:1 complexes with alkali-metal ions, and as in the case of valinomycin complexes the ion is coordinated by six carbonyl ligands (Ovchinnikov et al., 1969), originating a disc-like structure in which the ion occupies the center. Such a complex can migrate across the membrane or bind with free ionophores forming "sandwich" complexes with a 2:1 or 3:2 ratio of ionophore to ion. These antibiotics can therefore choose among several different routes of transport across the membrane according to the concentrations, lipophilicity mobility, and stability of the different complexes. Other molecules such as the actins (Ciani et al., 1969) and nigericin (Lutz et al., 1970) share with the antibiotics so far mentioned a common feature in hydrophobic environment, i.e., a central polar region and an outer hydrophobic surface. In artificial bilayers all these compounds induce specific conductance up to 10^{-2} ohm^{-1} cm^{-2}.

When the membrane fluidity is lost by lowering temperature, valinomycin, and nonactin show an abrupt loss of effectiveness in mediating ion conductance as a consequence of reduction of their mobility, but not of solubility. The peptide gramicidin A, in contrast, induces the same conductance in solid and liquid membranes (Krasne et al., 1971). Gramicidin A has been observed to induce step-like variation of conductance in membranes (Hladky and Haydon, 1970; Urry et al., 1971a) indicating formation of channels; other linear peptides such as *N*-formyl(Ala-Ala-Gly)$_4$-OMe (Goodall and Urray, 1973; Goodall, 1973) form channels across bilayers with characteristic discrete increments in conductance of the order 10^{-11}-10^{-10} ohm^{-1}.

The polyene antibiotics, amphotericin (Andreoli and Monahan, 1968) and nystatin (Finkelstein and Cass, 1968) also induce channels in bilayers. It has been suggested in this case that a pore of about 5 Å in diameter is formed by cylindrical stacking of rod-like structures. Cholesterol is specifically required. Hladky and Haydon (1970), however, do not observe conductance fluctuation with nystatin.

In order to explain gramicidin A channels, Urry (1971, 1972a,b) has proposed a new class of helices, the $\pi_{(L,D)}$ with alternating L and D residues, capable of forming hydrogen-bonded dimers and functioning as transmembrane channels (Urry et al., 1971a). The conformation of this $\beta^6_{3,3}$-helix can undergo ion-induced changes, and the ion can be shifted through the structure interacting, with ready exchange of coordination, with the amide carbonyls oriented in the direction of the helical axis. In

mammalian tissues, possible $\pi_{(L,D)}$helices would have Gly residues filling the role of D residues. The interest of these structures is also connected to the possibility that such molecules as biogenic amines or drugs with aromatic groups could control access to the channel by interacting with the two Trp residues at the ethanolamine end and with the negatively charged region provided by the three oxygens. Bamberg and Länger (1974) have provided evidence that the channel consists of a dimer of gramicidin A by performing relaxation experiments as well as single-channel conductance measurements at different temperatures.

Another model of ion transport through an alamethicin channel in lipid membranes has been proposed by Smejteck (1974). It considers a multimolecular array of alamethicin molecules in rod-like conformation, linked to one membrane side by the glutamic groups while the opposite end, containing a metal ion, penetrates the membrane. The model considered is a cylinder bridging the membrane and filled with water. Experimental membrane potentials for NaCl, KCl, and $CaCl_2$ concentration gradients can be reproduced without postulating any specific ion–channel interaction.

Studies on the interactions of polyene antibiotic–sterol in membranes of *A. laidlawii* cells and lecithin liposomes (De Kruijff et al., 1974a) show that the amphotericin–β-cholesterol complex is a circular array of eight antibiotic molecules inter-digitated by eight cholesterol molecules. Again, the hydroxyl groups confer hydrophilicity to the central region of the pore while the peripheral one is hydrophobic. Two such complexes generate an 8 Å diameter pore transversing the membrane. The complex formation is temperature dependent and in liposomes is maximal at 0° C. Below this temperature the formation rate of the pore is decreased (De Kruijff et al., 1974b). Nystatin–cholesterol and etruscomycis–cholesterol complexes are pores while the pimaricin-cholesterol complex cannot function as a conducting channel since its length does not span the membrane thickness. The filipin–cholesterol complex is a rather large aggregate oriented in the hydrophobic core of the membrane, causing membrane fragmentation. That the formation of the polyene antibiotics complex with cholesterol in *A. laidlawii* membranes restricts its interactions with other lipids is demonstrated also by studies on the Arrhenius plots of the ATPase in these membranes. In the absence of cholesterol such plots show a discontinuity at 15.5° C in agreement with the calorimetrically detected phase transition (De Kruijff et al., 1972). Cholesterol shifts the break below 10°C, while all the polyenes shift the discontinuity toward the temperature observed in absence of cholesterol.

De Kruijff et al. (1974c) furthermore demonstrated that not only cholesterol is required for the antibiotics to affect membrane permeability, but also that the structure of the sterol molecule is an important parameter in the polyene antibiotic–sterol interaction. The correlation found between the structural requirements of the

polyene–sterol interaction and lipid–sterol interaction may be explained by the fact that the two interactions are hydrophobic in nature and that lipid–sterol interaction may be necessary to give the sterol the required orientation for interacting with the antibiotic.

Several systems isolated from membranes have been reported to affect conductance of bilayers. High-ionic-strength extracts of rat-brain microsomes or frozen dried electroplax induce a channel activity in bilayers, characterized by Na^+ or K^+ selectivity and voltage dependence (Goodall and Sachs, 1972).

Shamoo and Albers (1973) have reported that the supernatant of precipitated trypsin digest of electroplax membranes added to oxidized cholesterol bilayers lowers the conductance only in presence of Na^+. It has been shown that a Na^+ ionophore is an integral part of Na^+–K^+–ATPase. In experiments in which Na^+ and K^+ were present on opposite sites, conductance changes were observed only when active material was added to the Na^+ side. An ionophore which is dependent on and selective for Ca^{2+} has been shown as being part of the Ca^{2+}–Mg^{2+}–ATPase of sarcoplasmic reticulum (Shamoo and MacLennan, 1974). The enzyme, when added to bilayer, enhanced the conductance several hundred-fold. The Ca^{2+} carrier not only is dependent on Ca^{2+} or Ba^{2+} to increase conductance, but it shows selectivity among divalent cations even at high levels of conductance. Channel- rather than carrier-active material can be extracted from highly purified gastric membranes by nonionic detergents (Sachs et al., 1974). Fractionation be gel electrophoresis of material in several protein bands shows that ion-selective channel activity is concentrated in some of the bands, the anion selective band showing the highest mobility and positive voltage dependence, the cation selective compound being of low mobility because it is subject to aggregation. The channels may aggregate to form larger channels since conductances characteristic of multiples of a single channel event have been measured.

Affinity chromatography with neurotoxins extracts nicotinic receptors from brain (Romine et al., 1974). Also, in this case, discrete increments of conductance have been observed across a NaCl gradient of 1:0.1 M. Large aggregates of receptors appear to activate and deactivate as multiples of basic conductance events. The possibility exists that the basic quanta reflect an average of the open- and closed-channel conductance time rather than the true single-channel conductance.

A divalent cation ionophore has been isolated from a partial digest of mercurial-treated, lipid-depleted mitochondria (Blondin, 1974). The ionophore interacts with both Ca^{2+} and Mg^{2+} and facilitates their transfer across the membrane. Southard et al. (1974) have found that mercurials increase the yield of ethanol-extractable Na^+–K^+ ionophore from mitochondria and induce energized proton transfer against ions. However the ionophore does not normally exist as such.

IX. *Kinetics of Membrane-Bound Enzymes*

Lipids are required for activity of several membrane-bound enzymes (Fourcans and Jain, 1974; Lenaz, 1973a; Triggle, 1970). A manifold role must probably be ascribed for lipids in membrane enzyme function (Fig. 14) (Lenaz, 1973b). As pointed out in the previous section, lipids are certainly involved in the stabilization of membrane-protein conformation; it is therefore likely that membrane lipids induce in enzyme proteins the conformation required for optimal activity. We will review here only those aspects of the role of lipids which can be related to protein conformation.

Scattered information exists in the literature on kinetic differences between lipid-dependent membrane-bound enzymes and solubilized enzymes: in some instances at least, these differences must be the result of different conformational structures induced by changes in the environment.

It should first be pointed out that one reason for a different kinetic behaviour of an enzyme, whether free or membrane-bound, may be due to steric diffusion limitations (Thomas et al., 1974; Goldman, 1973); the behaviour of enzymes in artificial membranes has been reviewed (McLaren and Packer, 1970; Katchalski et al., 1971). It is, however, reasonable that enzymes linked to biological membranes or having a penetrating moiety into the lipid bilayer, assume their optimal conformation (corresponding to the minimum of free-energy content) when the highest number of hydrophobic residues are situated on the exterior of the protein molecule in contact with the hydrophobic hydrocarbon regions of the membrane. Intrinsic membrane proteins are particularly rich in hydrophobic residues; it is increasingly evident that hydrophobic segments of the polypeptide chain deeply penetrate or span the bilayer, as in the case of glycophorin (Marchesi et al., 1972). As for proteins with known catalytic activity, a similar behavior has been found for both cytochrome-b_5 (Spatz and Strittmatter, 1971) and cytochrome-b_5 reductase (Spatz and Strittmatter, 1973), a segment of which is more hydrophobic and penetrates the microsomal membrane, or for the cytochrome oxidase (Vanderkooi, 1974) and ATPase (Racker, 1970) complexes in mitochondria; certain subunits of these complexes are extrinsic or peripheral (more polar), while others (more hydrophobic) are intrinsic or integral to the membrane. It is of interest that the hydrophobic portions or subunits appear devoid of catalytic activity; lipids, therefore, must modulate activity by a long-range cooperative action mediated by the hydrophobic segments or subunits.

A. *Kinetic differences between free and membrane-bound enzymes*

Solubilization of membrane enzymes, delipidation, or also changed lipid composition in a membrane lead to changes in the kinetic properties of membrane-linked enzymes.

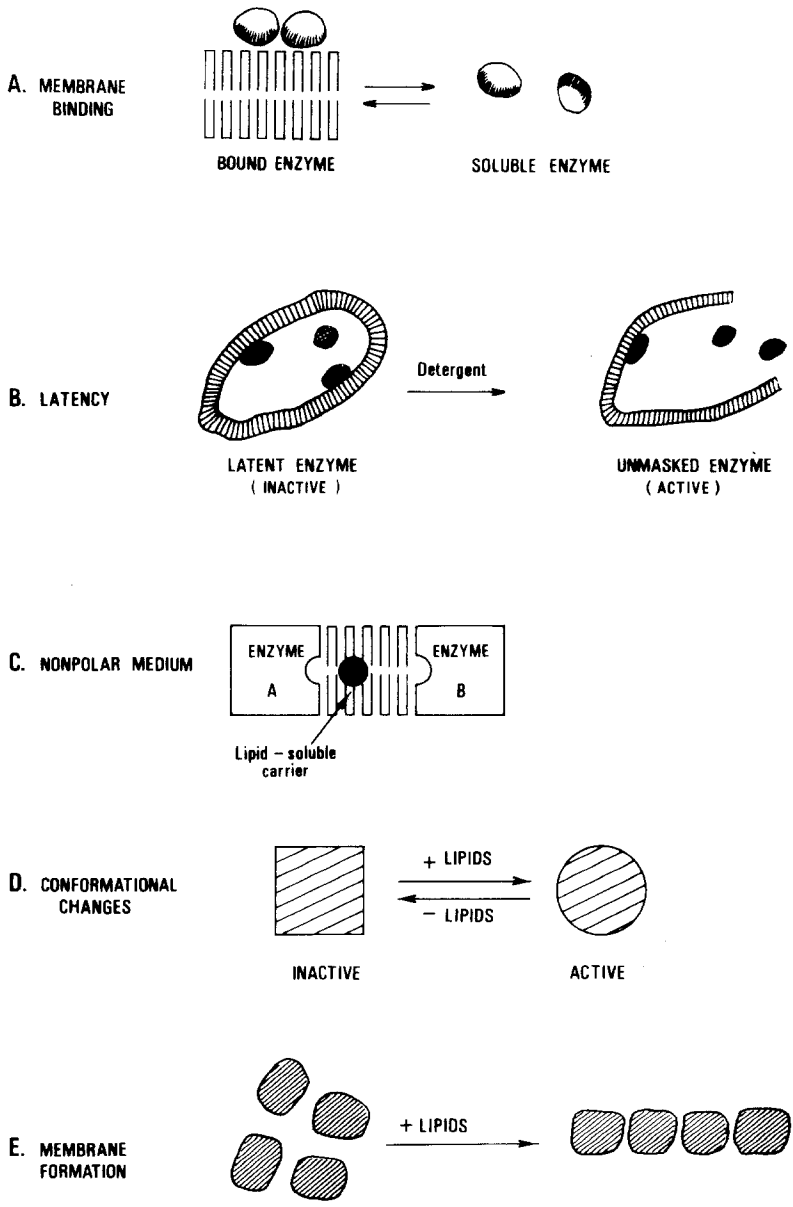


Figure 14. Possible roles of lipids in the function of membrane-associated enzymes (Lenaz, 1973b).

1. Solubilization. Differences have been found in the kinetic behavior between membrane-bound and solubilized enzymes. Affinities for substrates or inhibitors either increase or decrease upon solubilization (Fourcans and Jain, 1974).

For glucose-6-phosphatase (Arion and Wallin, 1973) in intact microsomes, glucose inhibition is accompanied by decrease of both V_m and K_m for glucose-6-P, whereas in microsomes disrupted with taurocholate a mixed type inhibition is apparent, with still a decrease in V_m but an increase of K_m in the presence of glucose.

Treatment of rat-liver microsomes with chaotropic agents such as NaSCN (Vainio, 1973) solubilized considerable protein and showed differential effects on drug-metabolizing enzymes (NADPH– and NADH–cytochrome-*c* reductase, NADPH–cytochrome-P_{450} reductase, and UDP-glucuronyltransferase) with indications that the oxidizing enzymes are more superficial in the membrane than UDP-glucuronyl transferase which must be located behind a hydrophobic barrier. After treatment with NaSCN the activity increased; the K_m for UDP-glucuronic acid (1.4 mM) showed no appreciable change.

A similar effect was found for brain hexokinase (Knuell et al., 1973): the enzyme changes its partition between soluble and mitochondrial fractions of brain according to the energy state of brain itself, perhaps with a physiological significance of priority of the mitochondrial enzyme for using mitochondrially produced ATP. The soluble enzyme has a higher K_m for ATP and a lower K_i for glucose-6-P inhibition. The solubilized enzyme has bound lipid, but behaves as the soluble form, indicating that the two natural forms represent interconversions of the same enzyme.

On the other hand a solubilized acetylcholinesterase from *Electrophorus electricus* (Robaire and Kato, 1973) has a two-fold greater affinity for substrate and exhibits greater inhibition by excess substrate than does the membrane-bound enzyme. Also, CTAB-solubilized glucose-6-phosphatase, inorganic pyrophosphatase, and PPi-glucose phosphotransferase activities show decreases in K_m for glucose-6-P, PPi, and again PPi, respectively (Soodsma and Nordlie, 1969).

In a model study, rabbit-muscle lactate dehydrogenase (Cho and Swaisgood, 1974) was covalently bound to porous glass beads only on one subunit; refolded covalently bound subunits were capable of enzymatic activity and recombination with native subunits in solution. An increase in K_m for NADH of the bound enzyme was suggested to be caused by intrapore diffusion limitation, whereas a large *decrease* in the constant for pyruvate may reflect a conformational change induced by the matrix environment. The K_D for NAD^+ also decreased, suggesting a structural change resulting in more favourable binding.

Glutamate dehydrogenase and malate dehydrogenase are usually considered as soluble mitochondrial matrix enzymes; the two enzymes,

however, strongly bind to certain phospholipid membranes (Dodd, 1973) with both ionic and hydrophobic interactions. Binding of glutamate dehydrogenase to certain phospholipids like cardiolipin and PS is accompanied by substantial inhibition of activity; the interaction and subsequent inhibition is counteracted by high ionic strength. It has been postulated that the enzyme is physiologically bound to the inner mitochondrial membrane and inactive; an intramitochondrial increase of NH_4^+ would activate glutamate formation by detaching the enzyme as a result of weakening ionic interactions. As the NH_4^+ is consumed and the ionic strength becomes normal, rebinding of the enzyme to the membrane occurs.

Reversible binding of enzymes to membranes may be a physiological means to regulate activity *in vivo* and was first postulated by Green and co-workers for the glycolytic enzymes in red-blood cells and yeast membranes (Green et al., 1965).

2. *Delipidation.* Removal of membrane lipids usually results in loss or decrease of membrane enzymic activities (Lenaz, 1973a). Also, in this case, as for solubilization, kinetic parameters change conspicuously. A rather constant decrease of V_m is accompanied by either an increase or a decrease of K_m for substrates or coenzymes (Fourcans and Jain, 1974). Delipidation increases the K_m of pyruvate oxidase for pyruvate (Cunningham and Hager, 1971a,b) but decreases the K_m of glucose-6-phosphatase for glucose-6-P (Zakim, 1970), of succinate-cytochrome-*c* reductase for succinate and PMS (Yu et al., 1973), of cytochrome oxidase for cytochrome-*c* (Zahler and Fleischer, 1971) and of mitochondrial ATPase for ATP (L. Landi and G. Lenaz, unpublished). The K_m for other enzymes are not affected (Fourcans and Jain, 1974). Readdition of phospholipids to the depleted preparations restores the original properties.

A few investigations have also been carried out on the role of lipids in some specific steps of catalysis for lipid-dependent enzymes.

Goldman and Albers (1973) have studied the role of phospholipids in (Na^+–K^+) ATPase of *Electrophorus* electroplax; by use of phospholipases A and C and changing Mg^{2+} concentrations, the effect of phospholipids on the partial reactions postulated for this enzyme was investigated. The hypothetical mechanism proposed for ATPase reaction is as follows:

$$E_1 + ATP \underset{-Mg^{2+}}{\overset{Na^+}{\rightleftharpoons}} E_1P + ADP \qquad (1)$$

$$E_1P \xrightarrow{Mg^{2+}} E_2P \qquad (2)$$

$$E_2P + H_2O \xrightarrow{K^+} E_2 + Pi \qquad (3)$$

$$E_2 \xrightarrow{-Mg^{2+}} E_1 \qquad (4)$$

(where E_1 and E_2 represent two different conformations of the enzyme). The authors, considering the hydrolysis specificity of the phospholipases used, conclude that PS is required for formation of phosphoenzyme as well as its dephosphorylation, and PE acts as a modifier which influences the affinity of the enzyme for Mg^{2+}; phospholipid removal in fact increases the stability of the $E–Mg^{2+}$ complex. In this case, phospholipids would be required for the proper conformations to be attained by the enzyme.

A role of lipids is also evident in hormone action and adenyl cyclase mediation of hormonal activity: membrane-bound cyclase was activated by catecholamines, glucagon, histamine, thyroxine, and triiodothyronine, whereas the solubilized enzyme was not responsive; fluoride activation, thought as a direct interaction with the catalytic site, was not impaired by solubilization. Addition of PS restores activation of soluble cyclase by glucagon and histamine, while PI restores activation by catecholamines. *Binding* of hormones was not impaired by solubilization (cf., also Rubalcava and Rodbell, 1973), suggesting a model for adenyl cyclase consisting of three subunits: a catalytic site in the interior, a regulatory site serving as a binding site for hormones on the exterior, and a coupler site serving to transmit a signal from regulatory of catalytic site, dependent on phospholipids. In an attempt to clarify at a molecular level the effect of phospholipids on glucagon binding to its receptor on rat-liver plasma membranes and its activation of adenylate cyclase, Rubalcava and Rodbell (1973) have used des-histidine-1-glucagon, which is a competitive inactive analog of glucagon. *B. cereus* (but not *C. perfringens*) phospholipase C reduces affinity but not total binding of glucagon for its receptor, but does not affect binding affinity of Des-His-glucagon. This finding suggests that acidic phospholipids, which are hydrolyzed by *B. cereus* phospholipase C, are involved in the liganding of the His residue of glucagon to a regulatory site responsible for glucagon activation. GTP is required for glucagon action on adenylate cyclase and increases glucagon dissociation from its receptor but does not exert these effects on phospholipase-*C*-treated membranes, nor alters the rate of dissociation of Des-His-glucagon. It appears that GTP and the glucagon histidine residue bind to a common site involved in both the activation of adenylate cyclase and the dissociation of glucagon from its receptor, acidic phospholipids being required for these effects.

Also, the unsulin receptor exhibits no phospholipid requirement (Cuatrecasas, 1971), while in the case of thyrotropin-releasing hormone, phospholipid hydrolysis by phospholipases A or C results in a decreased affinity of the receptor for the hormone in plasma membranes of bovine anterior pituitary gland (Barden and Labrie, 1973). It is perhaps pertinent to remind here that the strong effects of extremely low levels of growth hormone on the CD spectra of plasma membranes (Sonenberg

et al., 1973; Postel-Vinay et al., 1974) reflect a highly cooperative conformational change of membrane proteins which may be best envisaged as mediated through the lipid milieu. It would be interesting to study the effect of membrane lipids and their fluidity on such changes.

3. Allotopy. For complex activities like electron transport and ATPase in mitochondrial and other membranes, the picture is increasingly emerging that multiple subunits concur to the overall activity.

One example is cytochrome-*c* oxidase (Vanderkooi, 1974), where subunits synthesized in mitochondria and cytoplasm together form the active complex transferring electrons from cytochrome-*c* to oxygen (Ebner et al., 1973a,b; Rubin and Tzagoloff, 1973). The heme-a-containing subunits are probably the more hydrophilic polypeptide chains, synthesized in the cytoplasm, requiring altogether the presence of very hydrophobic, intramitochondrially synthesized polypeptides, which are intrinsic proteins necessary for correct integration of the catalytic subunits.

The same appears true for the mitochondrial ATPase complex (Fig. 15), where the concomitant presence of extrinsic and intrinsic components was recognized much earlier (Kagawa and Racker, 1966). The ATPase contains a catalytic center composed of an oligomycin-insensitive ATPase (F_1) (Pullman et al., 1960) consisting of five types of subunits (Senior and MacLennan, 1970), which is water

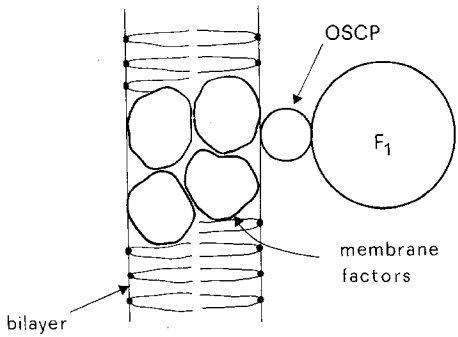


Figure 15. Scheme of the mitochondrial ATPase. F_1 and OSCP are extrinsic proteins, and the four "membrane factors" are intrinsic proteins, responsible for the allotopic properties (lipid dependence, oligomycin and DCCD inhibition, etc.). The intrinsic portion of the complex is shown to span the membrane to account for the H^+-translocating capability of the ATPase system (cf. Ernster et al., 1974). No attempt has been made to reproduce the components in scale with the actual sizes. Although F_1 is an extrinsic water-soluble protein, it cannot be observed on the matrix face by freeze-etching microscopy, suggesting that it can be intramembranous under normal conditions (for discussion cf. Harmon et al., 1974).

soluble and has all the characteristics of a peripheral protein; in addition it contains a membrane factor conferring oligomycin sensitivity for F_1 (Sierra and Tzagoloff, 1973) and a protein called OSCP (oligomycin-sensitivity conferring protein) (MacLennan and Asai, 1968) acting as a link between F_1 and the membrane factor. The membrane factor itself consists of four proteins that in yeast have been recognized as products of mitochondrial-protein synthesis; they are very hydrophobic and soluble in chloroform–methanol. When mitochondrial-protein synthesis is inhibited, cytoplasmic products of ATPase (F_1 and OSCP) are synthesized but not integrated into the membrane (Sierra and Tzagoloff, 1973). As a result of such integration, the overall ATPase activity is modulated by the membrane. Phospholipids are required for ATPase activity in membranes or in the complete ATPase complex (Bulos and Racker, 1968), but not in F_1; acidic phospholipids prevent the inhibition of soluble and particulate ATPase by purified ATPase inhibitor (Dabbeni-Sala et al., 1974). The inhibition of ATPase activity by oligomycin, rutamycin, and DCCD, requires the presence of the hydrophobic membrane factors where the inhibitor binding sites are situated (Stekhoven et al., 1972) and phospholipids are also required for inhibition to occur (Bruni et al., 1971). The change of ATPase properties in the soluble and bound form was first recognized by Racker (1967) and called *allotopy*. It is increasingly clear that this may be a general property of membrane-bound enzymic complexes.

One possibility for the allotopic behavior can be the modulation of different conformational forms of the catalytic subunits by the intramembrane polypeptides upon presence and absence of (specific) phospholipids or upon binding of different inhibitors.

4. Changes in lipid composition. A series of interesting investigations (Farias et al., 1968; 1970; Goldenberg et al., 1972; 1973; Morero et al., 1972; Bloj et al., 1973) have shown changes in the allosteric behaviour of membrane-bound ATPases, *p*-nitro-phenylphosphatase, and acetyl-cholinesterase in rats fed on fat-deficient diets. In rats fed a fat-sufficient diet, for example (Goldenberg et al., 1973), ATPases from heart, kidney and brain microsomes, showed allosteric kinetics for the inhibition by fluoride with values of Hill coefficient $n = -2.0$; in rats fed a fat-free diet, the n values changed to -1.0 in heart and kidney microsomes, but not in brain microsomes. Also, in a bacterial system, modification in fatty-acid composition brings about changes in cooperativity (Farias et al., 1972). In another study of rats fed different fat-supplemented diets (Bloj et al., 1973), correlations were searched for between fatty-acid composition and cooperativity for Mg^{2+}–ATPase $(Na^+–K^+)$–ATPase, and acethylcholinesterase. It was found that the increase in the double bond index/saturation ratio of fatty-acids (indicative of fluidity) is accompanied in an inverse manner by changes in allosteric transitions of $(Na^+–K^+)$–ATPase and acethylcholinesterase whereas Mg^{2+}–ATPase was

not dependent on this ratio. Decrease of fluidity by cholesterol addition *in vitro* confirms this regulatory mechanism (decrease of n): membrane fluidity is a physiological regulator for the allosteric behavior of membrane enzymes; apparently, each enzymes exhibits a particular behavior in this phenomenon. The variations of Hill coefficients are indications of conformational changes giving various extents of cooperativity under different fluidity conditions (Trucco, 1973).

B. *Arrhenius plots of membrane-bound enzymes*

We have already discussed the complex thermotropic behavior of phospholipid bilayers and membranes, and the mutual consequences that proteins and lipids exert in relation to these thermotropic effects. We have also seen in the previous section that fluidity of membrane may affect the Hill coefficients for cooperativity in allosteric membrane enzymes. Among the most striking observations collected in recent years, anomalies in the temperature dependence of membrane enzymic activities have been related to the transitions in the physical state of the membrane phospholipids. Some reviews are now available (Fourcans and Jain, 1974; Raison, 1973; Sechi et al., 1973) and the rich literature on this subject generally agrees that a relation exists between "breaks" in Arrhenius plots of membrane-bound enzymes and membrane lipid phase changes. Figure 16 shows the Arrhenius plot of beef-heart mitochondrial ATPase as an example of membrane-bound enzyme.

The problem of the interpretation of discontinuities in Arrhenius plots has been discussed by Kumamoto et al. (1971), who agree that two independent processes having different energies of activation are required to produce a discontinuity, the process having the higher energy of activation operating exclusively at temperatures below the discontinuity (and vice versa). The proposal of the exclusive operation of the independent processes within the respective temperature ranges is justified on thermodynamic grounds by considering that the system undergoes a phase change at the critical temperature. In turn, the perturbation associated with the phase change in the enzyme environment would be assumed to induce a conformational change in the enzyme protein, so that a conformation with higher activation energy (and therefore a lower catalytic power) exists below the critical temperature.

Much less agreement is found concerning the nature of the "phase change" in the membrane involved in the Arrhenius plot discontinuity; most investigators agree that lipids are usually involved in these changes, but a direct relation with the calorimetric transition temperatures is often not found. An indirect relation however usually exists, and breaks in Arrhenius plots of membrane enzymes are usually higher when higher-melting lipids are present, but without a clearcut correspondence:

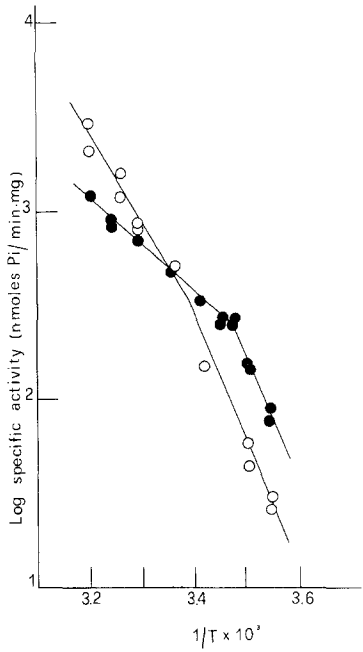


Figure 16. Arrhenius plot of beef-heart mitochondrial ATPase. ●—●,no addition; ○—○, + Triton-X-100 2.5 mg/ml.

this was shown in many investigations where a fatty-acid auxotroph of *E. coli* was grown in presence of different fatty-acids (Esfahani et al., 1969; Wilson and Fox, 1971; Linden et al., 1973a,b; Linden and Fox, 1973; Tsukagoshi and Fox, 1973; Machtiger and Fox, 1973). In a study of Na^+–K^+–ATPase, Kimelberg and Papahadjopoulos (1974) added phosphatidyl glycerols having differing fatty-acid composition to the delipidized inactive enzyme, and compared discontinuities in Arrhenius plots of the reactivated enzyme with lipid transitions observed by DSC. The discontinuity occurs 1–8° C lower than the *initial* rise of the main endothermic peak in the series dimyristoyl, dipalmitoyl, and distearoyl phosphatidylglycerol. Dioleyl phosphatidyl glycerol which does not have transitions within the experimental range activates the enzyme at lower concentrations and the Arrhenius plot shows no discontinuity. On the other hand, PS with heterogeneous fatty-acid composition shows discontinuity 2° C higher than the midpoint of the endothermic peak. The authors suggest that some discontinuities represent the completion of melting, whereas others represent the beginning of melting. This conclusion agrees with the much greater correspondence of breaks in Arrhenius plots of enzymic activities with the transitions of the

spin-labels motion parameters (Inesi et al., 1973; Raison et al., 1971); in fact, a partial clarification is given by analysis of the phase diagrams in the case of mixed phospholipid bilayers (Section III). In a more recent study, Raison and McMurchie (1974) have found that the lipid components of mitochondrial membranes exhibit abrupt changes in molecular ordering at two temperatures, with concomitant changes in activation energy of succinate oxidation. Both the phase changes and breaks were correlated with the temperatures of onset and completion of lateral-phase separation of the mixed lipid phase (T_f and T_s) (Linden et al., 1973b). Several disagreements in the literature could be due to which phase change is actually measured (either T_f or T_s , cf. Fig. 2). However, under other circumstances an apparent lack of any correlation is found between lipid fluidity and activation energy. This is the case of Ca^{2+}-ATPase which was isolated from sarcoplasmic reticulum and reconstituted with pure lipid species (Warren et al., 1974); the enzyme reconstituted with dioleyl lecithin, which has a phase transition at $-22°$ C, shows a clear break at $+29°$ C in the Arrhenius plot of activity; in this case a striking correlation exists with transient cluster formation beginning at about $30°$ C in dioleyl lecithin bilayers as detected by partitioning of the spin label TEMPO (Lee et al.,1974). It has been suggested that breaks around $20°$ C for several membrane enzymes, when the membranes have a high content of low-melting lipids, may correspond to cluster formation rather than to phase transitions or real phase separations. Anyway, for membranes containing heterogeneous lipids, phase separation seem a reasonable event leading to breaks in Arrhenius plots.

In certain studies different enzymic activities from the same membrane show breaks at different temperatures (Esfahani et al., 1971; Lenaz et al., 1972b; Mavis and Vagelos, 1972); microenvironmental heterogeneity, i.e., specific associations of lipids having different fluidities with different enzymes, could explain this observation. The actual fluidity could be in turn modified by proteins (either intrinsic or extrinsic) or by metal ions, etc., leading to a very complex and specialized fluidity environment for each enzyme. We have found (unpublished data) that cytochrome-*c* lowers the discontinuity of ATPase activity in submitochondrial particles from $20°$ to $14°$ C.

Clustering or aggregation of intramembrane particles has often been observed at or below the transition temperature (Grant et al., 1974): aggregation is the result of squeezing of the intrinsic proteins out of the regions undergoing solidification. This effect might be partly prevented for certain enzymes, for example by anchoring to extrinsic proteins, and lead to differences in the discontinuity temperature. Complex changes could also occur by different extents of aggregation (quaternary-structure changes).

Membranes containing cholesterol could also undergo different

behavior due to the previously discussed effects on fluidity. A study by Rottem et al. (1973) on membranes of *Mycoplasma mycoides* var. *capri* adapted to grow with low cholesterol concentrations has shown that the native strain has no lipid transition detectable by either a fluorescent or a spin probe or differential calorimetry, and no break can be demonstrated in the Arrhenius plot of ATPase activity; on the contrary, in the adapted strain, breaks in fluidity parameters at 24° C are accompanied by a sharp discontinuity at 18° C for ATPase activity. The activation energy of ATPase in the native strain is similar to the activation energy of the same enzyme *above* the break in the adapted strain. It was concluded that cholesterol by preventing crystallization of membrane lipids maintains them in a state of (intermediate) fluidity essential for optimal manifestation of several key activities of the membrane. In a study by De Kruijff et al. (1973b) in membranes of *Acholeplasma laidlawii* (which however does not contain cholesterol under physiological conditions), cholesterol decreases the break temperature of ATPase and the temperature of the lower end of the calorimetric phase transitions. The effect is due to lipid–cholesterol interaction since it is reversed by filipin (which is known to disrupt such interactions) and is not given by epicholesterol which is incorporated into the membrane without interacting strongly with other lipids.

In conclusion, the physical state of membrane lipids appears related to the activity of membrane-bound enzymes. Phase transitions of membrane lipids are usually complex events which consist of gradual phase separations. Separation of phases and solidification are accompanied by intrinsic protein aggregation and perhaps extrusion out of the membrane hydrophobic core as shown by freeze-fracture electron microscopy. At the same time increases in activation energies occur for activities of enzymes which have at least certain peptide portions deeply anchored into the bilayer. The change in environment of such enzymes (by aggregation or extrusion) is likely in our opinion to be accompanied by more localized conformational changes near or at the active site, leading to the observed kinetic modifications.

X. *Perturbation of Fluidity and Protein Conformation*

As previously mentioned, a variety of exogenous agents may induce lipid fluidity changes in bilayers and membranes. We wish to discuss here experimental evidence relating to the working hypothesis that such agents induce changes in the conformation and activity of membrane proteins through alterations of the fluidity of membrane lipids.

In order to prove such relation, a major assumption will be the demonstration not only that protein conformational changes accompany the induced changes of fluidity, but that such conformational changes

are the result of the fluidity changes and not of primitive protein alterations. This is a very important but rather difficult issue to prove for enzymic proteins which are native in a lipid environment and require lipids for activity, and no clear answer is yet available.

A. *Effect of solvents on enzyme activities*

1. Allotopic properties. Monohydric alcohols have been widely used to perturb the activity of membrane enzymes (for a review see Fourcans and Jain, 1974). In general, a relation exists between alcohol efficacy in modifying an enzymic activity and alcohol chain length, indicating that the hydrophobicity of the alcohol is the main factor leading to such modifications (cf. Lenaz et al., 1971). In our laboratory we have investigated the effect of alcohols on the mitochondrial ATPase and its oligomycin and DCCD sensitivity (Lenaz et al., 1975), in view of the complex lipid dependence of these phenomena (Section IX). Alcohols make the ATPase oligomycin insensitive with an efficacy depending upon alcohol hydrophobicity. The concentration of *n*-butanol required for this effect corresponds to the concentration which disrupts protein–lipid interactions in mitochondria as probed by spin-labels (Section IVC) (Fig. 17). The ATPase is not detached from the membrane, suggesting that a functional separation of F_1 from the

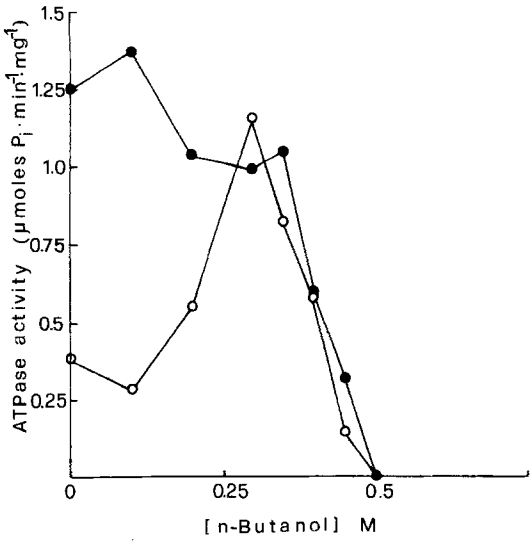


Figure 17. Effect of *n*-butanol on oligomycin sensitivity of mitochondrial ATPase, ●—●, no oligomycin; ○—○, oligomycin, 1 μg/ml.

membrane factors has occurred. Also diethyl ether treatment induces similar changes in submitochondrial membranes (Broughall et al., 1972; Lenaz et al., 1974; 1975). Azzi et al. (1974) have shown, by means of spin-labeling with a paramagentic DCCD analog (NCCD: Azzi et al., 1973) and Mn–ATP, that ether treatment completely abolishes the paramagnetic interaction between the two species, demonstrating that the distance of the ATP-binding site in F_1 from the DCCD-binding protein increases after ether treatment from $\sim$20 Å to $>$35 Å.

The allotopic properties of the membrane-bound ATPase are thus changed by solvents; the effect is shared by a number of general anesthetics, like methoxyfluorane and ketamine (unpublished studies from our laboratory). Since the effects are parallel to lipid fluidity changes, it is tempting to relate the two phenomena on the basis that the allotopic properties of mitochondrial ATPase depend on membrane lipids. The translation of the specific oligomycin or DCCD binding to the membrane factors into a conformational change of the catalytic unit F_1 may be impaired when the lipid environment of the membrane factors is modified by the solvents.

2. K_M. Kinetic parameters of membrane enzymes are modified by alcohols and other solvents. Grisham and Barnett (1973) found that loss of Na^+–K^+–ATPase activity by alcohols is related to the alcohol hydrophobicity in the same way as is the decreased ability to orient an androstane spin-label. In another study Hegyvary (1973) has also shown that several organic solvents including alcohols inhibit Na^+–K^+–ATPase. Inhibition was noncompetitive with respect to ATP; in the presence of the solvents the apparent affinity of the enzyme increased for Na^+ and ATP and decreased for K^+. Solvents interfere with different steps of the transient phosphorylation of the enzyme. By pulse labeling it was shown that solvents enhance interaction of ATP with the enzyme prior to phosphorylation both in absence and in presence of K^+; on the other hand, they decrease the rate of dephosphorylation of P-enzyme in presence of K^+. The author concludes that these modifications are partly due to conformational changes of the enzyme and partly to changes in water structure around the active center which is in a hydrophobic environment. Hale et al. (1972) found that ether and chloroform activate the Na^+ pump without affecting the maximum possible pump rate.

Similar kinetic changes have been shown for the mitochondrial ATPase. In mitochondria (Lenaz et al., 1975) and submitochondrial particles (unpublished), *n*-butanol decreases the K_M for ATP, although a change in V_{max} is not always apparent. An uncompetitive type of inhibition with respect to ATP is given by methoxyfluorane and ketamine (Fig. 18), suggesting an increased stability of the phosphorylated intermediate, which however has not yet been detected for mitochondrial ATPase (Boyer et al., 1972). All of these findings suggest a conformational change of the enzymic protein, but do not

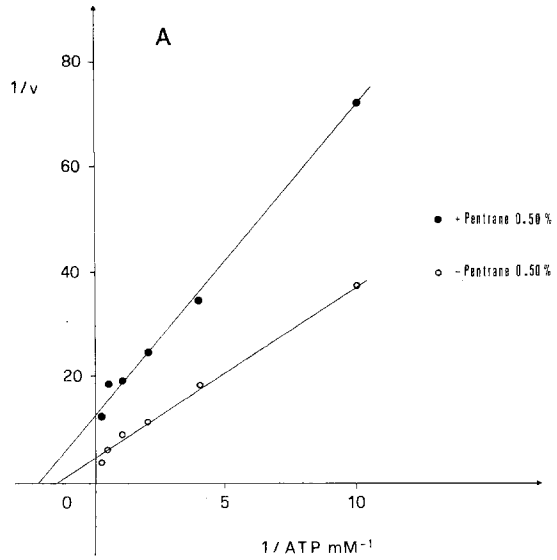
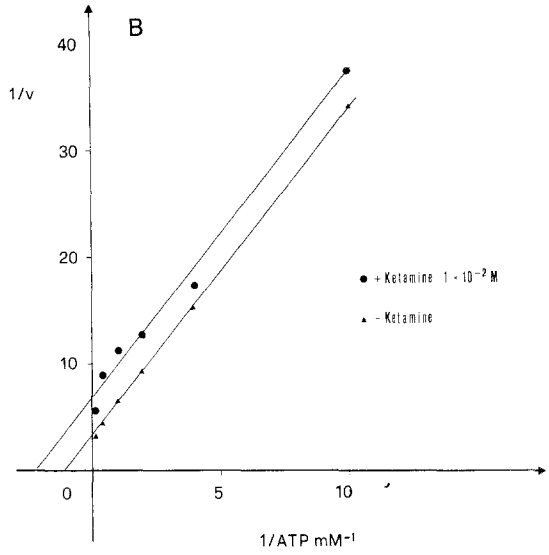


Figure 18. Effect of pentrane (methoxyfluorane) and ketamine on the kinetics of mitochondrial ATPase (Lineweaver–Burk plots). (A, above) Pentrane; (B, facing page) Ketamine. ● Represents + Ketamine 1×10^{-2} M; ▲, No Ketamine.

prove that the effects are mediated through the lipids. Unpublished studies in our laboratory on F_1, the isolated oligomycin-insensitive ATPase, have shown that some anesthetics modify the kinetics of ATPase with an uncompetitive type of inhibition. It is suggested at this stage that nonpolar molecules may affect hydrophobic regions also in lipid-free proteins leading to changes in tertiary or quaternary structure. Some of the effects of general anesthetics could be mediated by lipids, whereas others could be directly exerted on proteins.

3. Temperature dependence. The complex correlation between lipid-phase transitions and breaks in Arrhenius plots of membrane-bound enzymes or transport systems may be the optimal parameter to investigate in searching for drug-induced kinetic changes mediated by lipid fluidity changes. Sullivan et al. (1974) have found that the net rate of transport of *o*-nitrophenyl-β-D-galactopyranoside in *E. coli* is increased below the apparent phase transition in presence of *n*-alcohols. The degree of activation is determined by both concentration and chain length. Arrhenius plots of the kinetic constants indicate that K_M shows discontinuity with increasing temperature, while the slope of V_{max} changes only gradually. 10.5 mM *n*-hexanol increases both K_M and V_{max} at low temperature, but decreases K_M at high temperature.



Alcohols modify the temperature dependence of mitochondrial ATPase. In the presence of 0.35 M *n*-butanol no break is apparent in the Arrhenius plot of ATPase and the activation energy is increased at all temperatures to values in the range of those below the break in unperturbed ATPase, suggesting disorganization of lipid–protein interactions (Lenaz et al., 1975), in agreement with the spin-label studies (Section IVC) (Fig. 19).

Other solvents and anesthetics modify the temperature dependence of ATPase, albeit in different ways (Fig. 19). Diethyl ether decreases the break temperature by about 5° C, without much affecting activation energies (Lenaz et al., 1975); methoxyfluorane both decreases the break temperature and increases activation energies (unpublished). It is possible that different solvents affect lipid fluidity and lipid–protein interactions to different extents. A careful concentration-dependence study is needed for the different drugs, in view also of the biphasic response often found on several membrane functions. For example the biphasic effect of *n*-alcohols on the various membrane processes (cf. Lenaz et al., 1971; Jain et al., 1973; Jain and Cordes, 1973a,b; Sullivan et al., 1974) has been explained by Fourcans and Jain (1974) as an increase in lipid fluidity and interchain distance at low alcohol concentration (as one would induce in bilayers by increasing the temperature), followed by loss of optimal lipid–lipid or lipid–protein interactions at higher alcohol concentrations.

The data available so far suggest that "fluidity" changes, as induced by

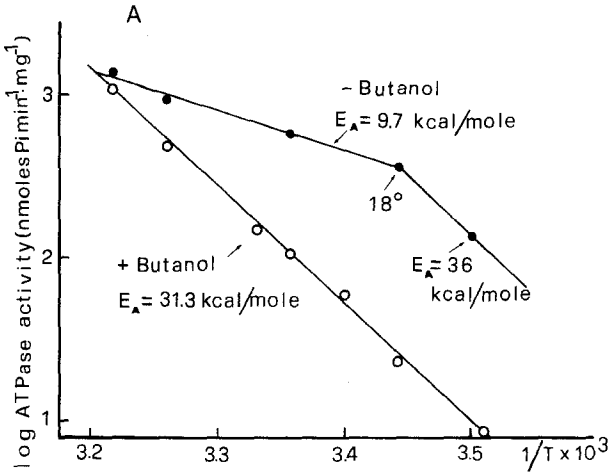
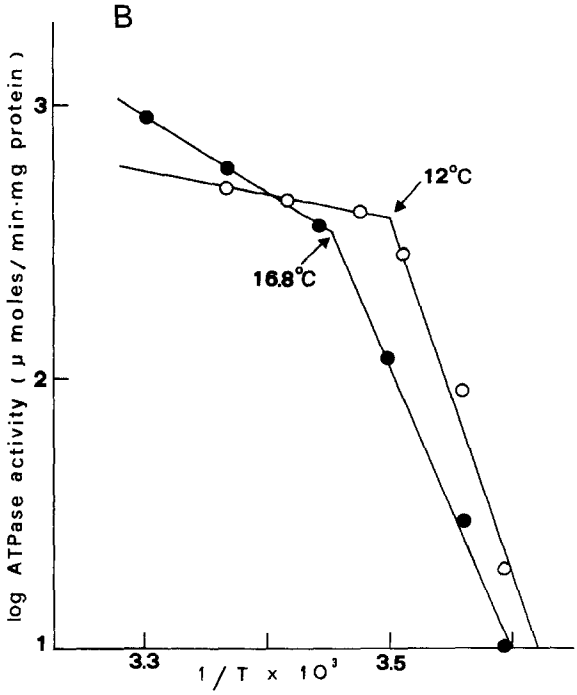
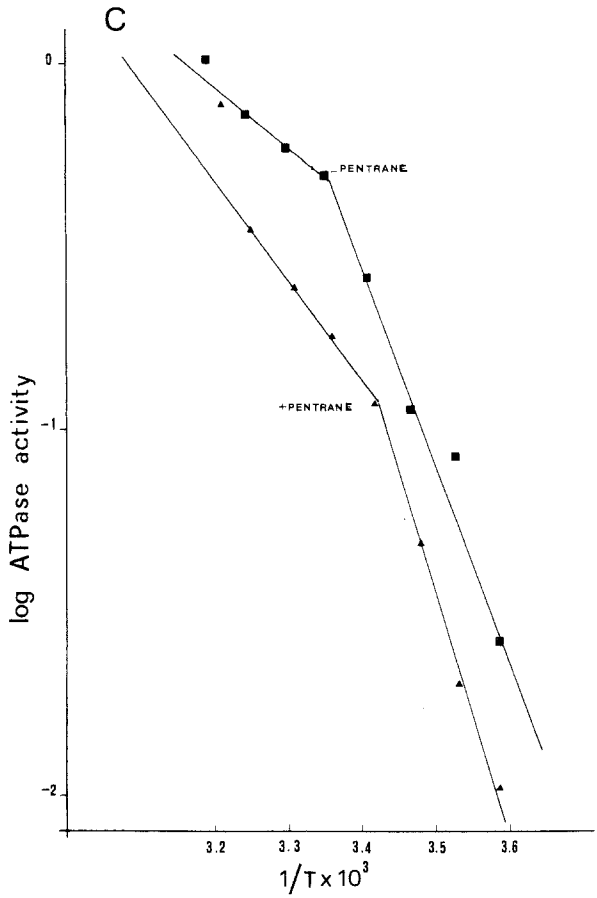


Figure 19. Arrhenius plots of mitochondrial ATPase and the effects of *n*-butanol, methoxyfluorane, and diethyl ether. (A, above) ●—●, no addition; ○—○, butanol, 0.35 M. (B, below) ●—●, no addition; ○—○, diethyl ether (pretreatment according to Broughall et al., 1972); (C, facing page) with and without pentrane.





solvents or drugs, *may* be transferred as conformational changes in membrane functional proteins. We are currently attempting in our laboratory to find direct evidence for this hypothesis.

B. *Effect of solvents and detergents on protein conformation*

Thermodynamics affords us an understanding of the importance of the solvent in determining protein conformation. It is well recognized that when a protein is dissolved in water, nonpolar groups are largely buried in the interior of the molecule, while the polar ones are on the surface. The driving force for such an arrangement is the large increase in entropy of the system resulting from the sequestering of hydrophobic groups from the water environment which causes disordering of the water

formerly somewhat ordered around the hydrocarbon groups. The conformation of the protein is then stabilized by the forming of the best London interactions between the nonpolar groups as well as by hydrogen bonds formed between the possible donor and acceptor groups in the inner regions of the macromolecule. On the other hand, the free energy of burying ion pairs or discharging charged groups in order to place them in the interior of the protein molecule is too unfavorable, which is to say that ionic residues have a strong tendency to be located on the surface of the molecule, in order to be in contact with the aqueous environment. Electrostatic interactions do not seem to play a predominant role in stabilization of protein conformation: the stability of protein is little affected by the variation of NaCl concentration at neutral pH; the addition of nonaqueous solvents, which should strengthen coulombic interactions by lowering the dielectric constant of the medium, does not stabilize the protein conformation; on the contrary, destabilization is observed indicating the predominant role of hydrophobic interactions, as nonaqueous solvents are able to dissolve a larger number of nonpolar groups thus inducing changes in the conformation assumed by the protein in aqueous solution. Such forces are most probably responsible for the fact that protein are only partially α-helical in water. They are far less significant in nonaqueous solvents when α-helix, a conformation where hydrogen bonds are maximized, is predominant. It is therefore possible that the conformation of proteins interacting with lipid bilayers is different from that in aqueous solution and this may be relevant for the effect of anesthetics on protein conformation. To this purpose it may be of interest that the interaction of intrinsic proteins extracted from erythrocyte membranes in water-soluble form requires an initial ionic attraction followed by hydrophobic stabilization of the complex in the form of a reconstituted membrane (Zwaal and Van Deenen, 1970); on the contrary, the insoluble protein skeleton produced by acetone extraction of the lipids from mitochondria directly binds to phospholipid vesicles by exclusively hydrophobic interaction (Lenaz et al., 1970a,b). The best explanation for this difference is the existence of different conformations in the water-soluble and in the insoluble membrane proteins, with polar groups facing the medium in the former. Membrane formation in both cases would induce reorientation of nonpolar groups toward the lipid phase.

Several studies on the effect of nonpolar solvents and anesthetics on protein conformation have been carried out. In several instances the conformational changes were directly exerted on the protein molecules by affecting hydrophobic protein regions and therefore modifying the stability of protein conformation. Bovine pancreatic RNase is only about 15% α-helical in aqueous solvents but its helical content increases up to about 70% in 2-chloroethanol, a less polar solvent where, evidently, hydrogen bonds play a minor role (Singer, 1971). The same behavior has been observed for bovine serum albumin (BSA). The Folch–Lees protein,

42% α-helical in water, or aqueous media, has an helical content close to 100% in chloroform–methanol (1:1, v/v) (London et al., 1974). Such an increase is brought about by the contact of the protein with the hydrophobic nonspecific gaseous phase at the air–water interface when lipids are not present. If a lipid layer is spread at the air–water interface, the behavior of the protein is different. While interaction with lanosterol does not induce the hydrophobic conformation in the protein, such a conformation characterized by a high α-helix content is taken up by the protein in solvents containing hydrogen-bonding groups (Zand, 1968; Sherman and Folch, 1970) and in presence of lipids. In such a conformation, hydrophobic interactions may occur between the hydrocarbon groups of the protein and the alkyl chains of the fatty-acids behaving as a nonpolar solvent. The difference in affinity for the different lipids, as pointed out earlier, may be explained in terms of possibility of hydrogen bonding between the protein and PL.

The induction of helicity in proteins by surface-active molecules has been studied by Jirgensons (1961). While helical proteins, such as serum albumin, are only slightly affected by detergents, nonhelical proteins, such as immunoglobulins, soybean trypsin inhibitor, pepsin, and caseins, show marked effects in the visible and near-ultraviolet CD. Schneider and Edelhoch (1972) have observed that lysolecithin induces helicity in glucagon.

Treatment with detergents results in a partial transition from disordered to α-helical conformation of the proteins, the magnitude of the effect being dependent on the hydrocarbon chain length of the detergent. So tetradecyl sodium sulfate is more effective than dodecyl and decyl sulfates (Jirgensons, 1967). In the interaction between the detergent and the protein, coulombic forces play a significant role as shown by the observation that nonionic detergents are less effective. The charged group of the detergents interact with the positive groups of the protein and hydrophobic interactions link the hydrocarbon tails of protein and detergent. As a consequence of the hydrophobic shield provided by the hydrocarbon tails of the detergent, an ideal environment is generated for the peptide groups to form hydrogen bonds and assume stable helical conformation.

Nonpolar substances induce reversible structural changes in proteins (Wetlaufer and Lovrien, 1964). Interactions of hydrocarbons, such as butane, result in changes in viscosity, H^+ equilibrium, optical rotation, and UV absorption spectra, indicating that nonpolar groups in protein play an important role in the energetics of protein structure. Studies on the solubility of hydrocarbon gases in protein solutions (Wishnia, 1962) show that butane, for instance, is more soluble in BSA than in SDS or Hb. The solubility increments for the effective solute must represent a direct interaction between the gas and the protein or the micelles and are due to solubility in nonpolar material. The ΔH of transfer of gas from H_2O to the macrosolute is close to zero, meaning that the contribution

of hydrophobic interactions toward stabilizing folded protein structures arises primarily on ΔS.

Hydrocarbon gases currently used as anesthetics induce conformational changes, detected by polarimetric techniques, on globular proteins such as β-lactoglobulin and bovine plasma albumin (Balasubramian and Wetlaufer, 1966). The binding of the anesthetics to globular proteins does not apparently involve covalent bonding but seems to be hydrophobic in nature, as suggested by the observation that the effect of binding increases with increasing length of the nonpolar chains. Furthermore a correlation has been found between the potency of a general anesthetic and its effect on the protein structure. Laasberg and Hedley-White (1971) have reported significant reduction in the ORD of ferri-, oxy-, and deoxyhemoglobin as a result of their interaction with halothane, in clinically used concentration. The reversible variations have been observed only for the β chain of the protein, studied at physiological pH and at 23° C. Studies carried out on helical poly-L-lysine and poly-L-glutamic acid in helical and random-coil conformations have shown sensitivity to the anesthetic action only for the former.

ORD of mysin from white skeletal muscle of rabbit shows significant changes in the 230–250 nm region after exposure to commonly used concentrations of the anesthetics halothane, chloroform, and ether (Leuvenkroon-Strosberg et al., 1973). EDTA(K^+)-activated ATPase of myosin is depressed by each of the three anesthetics while Ca^{2+}–ATPase is inhibited only by chloroform.

The effect of nonpolar solvents on protein conformation suggests that very likely the conformation of proteins buried in a lipid bilayer, as in membranes, is different from that in aqueous solutions.

The effect of solvents and detergents on CD and ORD of membranes has been reported by many investigators. Their action certainly removes artifacts by solubilizing the membrane proteins and it is likely to induce helical conformation as well. Addition of glycerol to a ghosts suspension causes a substantial increase of the molar ellipticity at 225 nm, while 2-chloroethanol causes even larger changes (Lenard and Singer, 1966); 2-chloroethanol causes the amplitude of ORD of plasma-membrane fragments to increase two- to three-fold and blue-shifts the extrema (Wallach and Zahler, 1966). Also, trifluoroethanol (TFE) has been used in our laboratory in order to find a suitable dissolved state of the membranes and calculate the appropriate corrections. There is little hyper- or hypochromism at 224 nm caused in the UV spectrum of polypeptides and proteins as a consequence of conformational changes so that the difference between UV of suspension and reference state at this wavelength is due virtually to scatter. The corrected CD of the suspension can then be calculated and a solubilized state can be chosen as a reference state only if its increase at 224 nm as a consequence of solubilization is within 10% of the calculated one. TFE enhances the CD patterns of beef-heart mitochondria, plasma membranes and red-blood

cell ghosts but not those of sarcotubular vesicles because of the formation of large particles. This difference in behavior of the latter membranes along with lower ellipticities probably reflects difference in protein conformation and in membrane structure.

Solubilization of human-erythrocyte ghosts can be achieved by treatment with pentanol (Singer and Morrison, 1972). The lipoprotein preparation shows the CD characteristic of an α-helical conformation: the extremes, though, have been shifted. Treatment with 70% 2-choloroethanol red-shifts the n-π^* transition peak from 219 to 221 indicating that a significant amount of β-structure, possibly generated by pentanol, is present in the pentanol-treated erythrocyte membranes. Also the effect of detergents on membrane protein conformation has been examined, since such substances are used for membrane solubilization (Kagawa, 1972), as well as a reference state for membrane CD studies. Dilute SDS causes an increase of the amplitude of CD and ORD bands of ghosts (Lenard and Singer, 1966) and sarcotubular vesicles of rabbit skeletal muscle (Mommaerts, 1967; Masotti et al., 1972a). There has been observed, in particular, an increase of the 208 nm band. The usual increase in the amplitude of the 192 CD and UV bands is not observed however as a consequence of treatment of oxyntic cell of dog gastric mucosa (Masotti et al., 1972b).

Deoxycholate is effective in giving a suitable reference state for axonal membranes (Masotti et al., 1973), but not for oxyntic cells, as well as Triton-X-100. The latter detergent gives a good reference state for brain microsomes, a soluble state in which furthermore enzymatic activity is maintained (Long et al., 1973).

From the previous discussion it is apparent the importance of hydrophobic bonds in protein conformation and the requirement of a hydrophobic environment for the formation and stability of the hydrogen-bonded secondary structures. Studies on the nature of molecular forces in anesthesia (Schoenborn and Featherston, 1967) show that only weak forces such as Keesom, Debye, and London forces seem responsible of the binding of anesthetics to proteins. It seems therefore that a possibility exists that anesthetics may act on protein conformation perturbing lipid–protein interactions in membranes.

In contrast with the well-documented evidence of the effect of anesthetics on the conformation of nonmembrane proteins, little is known on the effects on membrane proteins, although the larger expansion of biological membranes than of lipid membranes (Seeman, 1974) point to a protein conformational change induced by anesthetics. Unpublished CD studies carried on in our laboratory have shown slight although significant changes induced by n-butanol on the conformation of an oligomycin-sensitive ATPase isolated from mitochondria. Similar changes have been observed in human-erythrocyte ghosts. Another general anesthetic, ketamine at 10^{-4} M, induces important conformational changes in erythrocyte ghosts (Fig. 20). Besides these

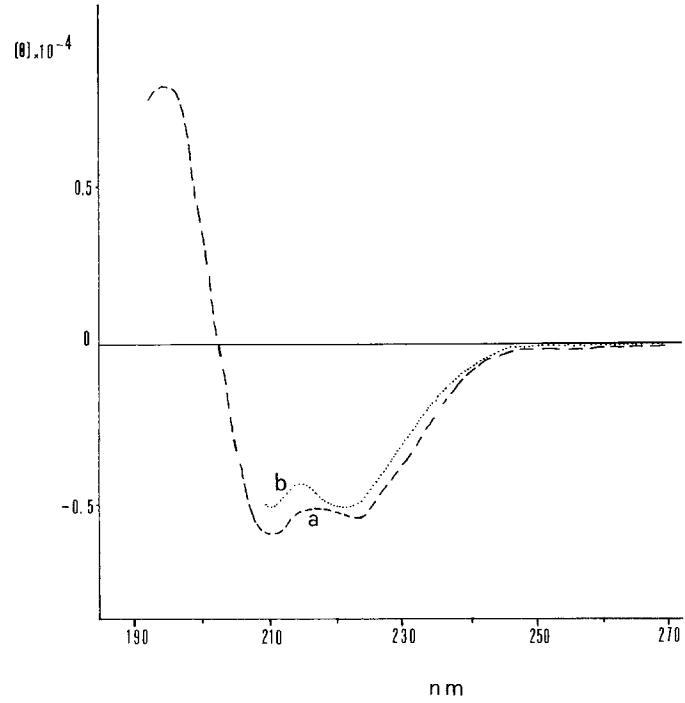


Figure 20. Effect of ketamine on red-blood cell ghosts (Masotti, Curatola and Lenaz, unpublished results). The letter a represents ghosts; b, ghosts + 10^{-4} M Ketamine.

preliminary studies from our laboratory, we are not aware of other studies related to a search for conformational changes induced in membranes by solvent perturbation of the lipid milieu, but we feel that this possibility deserves careful investigation, in consideration of the sensitivity of protein conformation to the nature of their environment.

C. *Conclusions*

The following facts can be stated according to present knowledge in relation to the action of general anesthetics.

1. They increase lipid mobility in artificial and natural membranes, and hence expand the membrane volume, with concomitant possible permeability changes.
2. They affect the conformation of membrane and nonmembrane proteins and modify kinetic parameters of enzyme and transport proteins.

Both effects are related to anesthetic hydrophobicity and are present at anesthetic concentrations.

The unresolved question at this stage is the following: Is general anesthesia the result of lipid changes or of protein changes? Available evidence and theories of nerve conduction suggest that protein changes may be responsible for such a specific phenomenon as the blocking of "Na^+-channel" activity in nerve membranes. However, we have no evidence suggesting either a direct effect on hydrophobic regions of membrane proteins or an indirect effect mediated by lipids. We have collected data showing that both effects are possible, and we would not be surprised if it turned out that both effects are indeed operative, perhaps a direct protein action for certain more specific anesthetics, and an indirect action mediated by membrane lipids in the case of nonpolar solvents. As a tentative target for anesthetic action we would indicate peptides or proteins involved in formation of ion-selective channels in neuronal membranes. All the unresolved questions stated above are open to experimental testing, but the final answers will only be given together with the solution of the molecular basis of nerve-impulse propagation.

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