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THE EFFECTS OF DRUGS ON MEMBRANE FLUIDITY

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INTRODUCTION

Drugs will find their way into membranes. Any foreign molecules will distribute into the hydrophobic regions of cells according to their lipid solubilities, and it is reasonable to predict that their presence in adequate concentrations will disrupt the structure of the bilayer. Some drugs never reach effective concentrations in membranes because they are intercepted by chemical binding to sites in the aqueous phase. Such drugs may have dramatic pharmacological effects at doses much too low to affect membranes, so their membrane effects can only be observed in vitro. Drugs that are simple chemical molecules are likely to disrupt membranes because they have little effect in the aqueous phase and will do no harm until they partition into membranes in sufficient amounts. Some of these drugs are highly lipid soluble, but great lipid solubility is not a prerequisite for membrane action. All that is required is that the membrane effect not be preempted by stronger effects at lower doses.

This review will deal primarily with anesthetics and similar drugs, such as alcohols, for which we have long-standing evidence of action in some lipid phase as well as new information about the nature of the interaction. Anesthetics are the best known members of this class of drugs but they are not the only such agents.

It may be premature to take sides in the controversy about whether membrane lipids or membrane proteins are the site of drug action. The change that concerns us is physical and can occur in any hydrophobic phase. Drugs with high lipid solubility will enter hydrophobic regions of both proteins and lipids in the core of the membrane. Although it is too early to explain exactly how the hydrophobic interaction affects function, it is reasonable to predict that disrup-

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tion of protein function will result from disorder in any of the hydrophobic regions. Some proteins presumably will be more affected than others.

Drugs that have receptors may have hydrophobic actions as well. Benzodiazepines and neuroleptics, for example, have high lipid solubilities that must affect their availability at the site of action. It is not yet known whether the actual binding sites are in the aqueous phase or in the membrane, although the natural ligands, such as GABA or dopamine, must bind in the aqueous phase since they are highly hydrophilic. The intramembrane concentrations of the lipophilic drugs are often hundreds of times higher than the aqueous concentration, a fact that is not always considered in binding experiments. Some of the equations for dealing with these drugs have been worked out (1), but the field is largely undeveloped.

Meaning of Membrane Fluidity

The concept of membrane fluidity lacks a precise definition but it can nevertheless be useful. In general, the term means a combination of different types of mobility of membrane components. These include the following: flexibility of acyl chains, lateral diffusion of molecules in the plane of the membrane, transverse diffusion of molecules from one monolayer to the other, and phase transitions leading to lateral phase separations. These are all anisotropic motions and cannot be considered measures of viscosity, which is a characteristic of isotropic resistance to flow. Any of these measures of fluidity may be affected by drugs.

The motion of phospholipid acyl chains in membranes can be observed with several commonly used techniques, such as nuclear magnetic resonance (NMR) and electron spin resonance (ESR). This motion is the basis for most pharmacological work on membrane fluidity. The acyl chains can be selectively deuterated at different positions, allowing the use of deuterium NMR to observe their motion. Spin labels, with nitroxide groups attached at different positions along a fatty acid chain, are used for the same purpose. Results of tests using both techniques agree that there exists a gradient of flexibility in the chains from the surface toward the core of the bilayer, with increasing freedom of motion near the center. However, the shape of the gradient appears different with each technique (2, 3). Order parameters used in NMR and ESR techniques are related to the angle of a particular chain segment to the membrane surface. The cumulative motion of segments produces the flexibility gradient. Trans-gauche isomerization about carbon-carbon bonds causes evanescent "kinks" in the chain (4) and *cis* double bonds produce permanent kinks. In this way, spaces are created and parts of adjacent chains can move in and out. Drugs can affect the probability of trans-gauche isomerizations (as cholesterol does, for example) and thus affect the fluidity gradient.

Lateral diffusion of phospholipids along the surfaces of the bilayer occurs rapidly, with diffusion constants on the order of 10^{-8} cm^2 sec^{-1} for phospho-

lipids (5). Proteins also diffuse laterally; their rates are slower and more variable than those of lipids and some proteins are anchored in place by the cytoskeleton (6). Lateral diffusion of proteins is important for coupling receptors to enzyme catalytic units and in the ability of bivalent ligands (such as calcium or antibodies) to attach to the membrane surface. We have few experimental data on the effects of drugs on lateral diffusion in membranes, but it is reasonable to expect that drugs will alter diffusion rates and that membrane function will be affected.

Transverse diffusion from one surface of the bilayer to the other is extremely slow in model membranes (7) but may be faster in biomembranes (8). Since the different phospholipid head groups are of different size and charge, flipflop might serve to change the membrane properties substantially. A limited amount of flipflop is mediated by the phospholipid methyltransferases described by Hirata & Axelrod (9) that convert phosphatidylethanolamine to phosphatidylcholine and move it from the inner to the outer monolayer. The change in membrane fluidity reported to accompany this activity is discussed below.

Lateral phase separations must be critical to events occurring within membrane structures, but precise evidence on this topic is limited to model membranes of known composition. Shimshick & McConnell (10) demonstrated the coexistence of solid and fluid phases in binary mixtures of pure phospholipids at suitable temperatures, and there is evidence from freeze fracture studies (5) and fluorescence lifetimes (11) for patches of gel in otherwise fluid biomembranes. Exogenous compounds can increase the proportion of fluid lipid without changing the rate of lateral diffusion in the fluid phase (11). Since proteins are probably excluded from the gel domains, any increase in the amount of fluid lipid will decrease the effective concentration of proteins, which must affect cooperative interactions and coupling.

Thus the evidence to be discussed below that drugs "fluidize" or "disorder" membranes can be interpreted to mean that the drugs decrease the packing density of phospholipids, allowing all components of the membrane to jostle each other more often but less strongly than in the drug-free state. A quite different interpretation is that the frequency and vigor of such collisions is unaffected in the fluid domains but more of the membrane is free to engage in them. Either effect would have important implications for the function of membrane-bound proteins.

ENTRY OF DRUGS INTO MEMBRANES

Bulk Solvents

The lipid solubility of drugs is measured as their partition at equilibrium between aqueous and organic phases. A nonpolar or amphiphilic solute disrupts the network of hydrogen bonds in water and as a result is driven into a more favorable energy state in the organic solvent (12). In an initial approach to an

investigation of drug concentrations in membranes, octanol is a useful model solvent; it accommodates both hydrogen-bonding and nonpolar solutes (13). The solubilities of hundreds of compounds in various solvents have been converted to octanol:water partition coefficients by Leo et al (14) using generally applicable equations. Lindenberg provides an unusually complete set of oil:water partition coefficients for alkanols (15). Solubility data can be predicted to some extent, because each moiety of a solute molecule contributes an incremental factor to the partition coefficient (16). A factor of two or three is usually observed for each additional methylene group (17), but the effect of additional hydroxyl groups varies widely in different solvents.

Model Membranes

The solvent properties of phospholipid bilayers resemble those of octanol, although lecithin is more like the lower alcohols than like octanol, at least for a series of hydrogen-bonding solutes (17). The measured solubility of any compound in a bilayer must be an average of its concentrations at different distances from the surface. Furthermore, the structured bilayer (unlike octanol) differentiates among solutes of different molecular shapes. For example, lecithin discriminates against branched solutes more than a bulk solvent does (17).

Biomembranes

Measurement of the solubility of drugs in biomembranes is more complex. Roth & Seeman (18) measured membrane:buffer partition coefficients for several alcohols and phenols in red cell ghosts and in some nerve and muscle membranes and found them to be about 20% of the corresponding octanol:water partition coefficients. Two negatively charged drugs, valerate and pentobarbital, were about ten times less soluble in membranes than in octanol. According to Diamond & Katz (17), the erythrocyte membrane is similar to dimyristoylphosphatidylcholine as a solvent for drugs, although the biomembrane is slightly more hydrophobic than lecithin and its additional surface charge (sialic acid and protein) must be taken into account. The partition coefficient of benzyl alcohol in erythrocytes increases at high drug concentrations (contrary to the definition of the partition coefficient) and also increases slightly with temperature (19), suggesting that a more fluid membrane may accommodate more drug.

The octanol:water partition coefficient of charged compounds is usually measured for the undissociated drug at least 4 pH units away from pK (14), and the overall partition at physiological pH can be calculated on the assumption that only the uncharged form of the drug enters the membrane. However, Miller & Yu (20) found that the charged form of pentobarbital has an appreciable solubility in egg phosphatidylcholine vesicles.

Amphiphilic drug molecules that have both polar and nonpolar moieties may

accumulate at the surface of the bilayer, where they may act in both the aqueous and the membrane phase. The studies of Bienvenüe et al (21) illustrate the factors that affect the activity of such drugs. The potency of spin-labeled long-chain acylcholines in displacing acetylcholine from its membrane-bound receptor depends on the availability of bulk lipid to take up the drug as well as on its binding affinity to the receptor. Seeman's studies of the dopamine receptors (22) illustrate the same point.

Conrad & Singer (23) were unable to detect any entry of amphiphilic drugs into biomembranes under conditions where partition into liposomes was easily measurable. They used a gentle filtration technique rather than centrifugation. They concluded that a large internal pressure in biomembranes prevents uptake of drugs. However, there has been no confirmation of this surprising finding, and other laboratories have since reported contradictory results (24, 25). The known uptake of amphiphilic spin labels and fluorescent dyes, which can be measured in biomembranes without separating the phases, argues against the internal pressure concept.

Rottenberg et al (26) reported much higher partition coefficients of ethanol in mitochondrial and synaptosomal membranes than predicted from Seeman's conversion factor or from the known partition of longer-chain alkanols.

From the Meyer-Overton relation of 40 mmoles of anesthetic per kg of membrane to produce local anesthesia (27), one can calculate a mole fraction of 0.05 for the drug in membrane lipid (assuming a membrane that is half protein and half lipid by weight and a cholesterol/phospholipid molar ratio of 0.5) General anesthesia occurs at 10- to 20-fold lower membrane concentrations (27), about one drug molecule per 200 lipid molecules. Many studies of drug effects in membranes have been done at drug concentrations that are orders of magnitude higher than this.

The Meyer-Overton concept that anesthetic potency varies with lipid solubility has been repeatedly confirmed (27). Recently, McCreery & Hunt (28) and Lyon et al (29) used the Hansch equations and Seeman's factor to calculate membrane:buffer partition coefficients for many drugs and reported good correlations of central nervous system (CNS)-depressant activity with solubility in biomembranes.

DRUG EFFECTS MEASURED IN VITRO

Many different physicochemical techniques have been used to provide evidence that anesthetic agents and similar drugs have a biophysical action on cell membranes that can often be described as a disordering or fluidizing effect.

Nuclear Magnetic Resonance

Nuclear magnetic resonance (NMR) is a versatile but insensitive technique that has not yet made its main contributions to pharmacology. The first experiments

on membrane disorder were done with proton NMR to study the effect of benzyl alcohol on red cell membranes (19). No probe was needed; the signal was derived from the drug itself. Rapid isotropic motion of the benzyl alcohol in solution produced a narrow signal line. On addition of erythrocytes the line broadened, indicating that the motion of the drug was constrained in the red cell membrane. However, when progressively higher concentrations of the drug were added, the line narrowed again, showing that the alcohol had increased the fluidity of its environment in the membrane. The membrane proteins did not contribute to this effect, as it occurred equally well in extracts of the membrane lipids. At very high drug concentrations, however, the membranes became stiffer, apparently because of irreversible changes in the proteins. Metcalfe et al (19) were the first to recognize that membrane disorder is the result of the addition of anesthetic agents.

In lecithin liposomes with selectively deuterated acyl chains, deuterium NMR can be used to locate a drug effect. The disordering effect of benzyl alcohol is maximal at midchain, even though benzyl alcohol is a fairly water-soluble compound and might be expected to remain near the membrane surface (30). The ordering effect of cholesterol is also maximal at midchain. The NMR data allow calculation of the orientation of each C-C segment of the acyl chain, from which it can be seen that benzyl alcohol decreases the thickness of the bilayer and cholesterol increases it. Drug concentrations above 0.5 mole of drug per mole of lipid were used in this study; no effects could be seen at anesthetic concentrations.

Vanderkooi et al (31) used proton NMR to show that anesthetic drugs increase the proportion of fluid lipid in phosphatidylcholine bilayers and in sarcoplasmic reticulum. Addition of halothane or chloroform increased the resolution of peaks attributed to the fatty acid chains of melted phospholipids, indicating increased mobility of the chains.

^{31}P NMR is a suitable technique for observing the presence of nonbilayer forms of phospholipids. Cullis and co-workers (32) have described the formation of hexagonal H_{II} forms of phosphatidylethanolamine and cardiolipin. It is not known whether these forms exist in vivo, but if they do they may promote membrane fusion. Calcium facilitates the formation of H_{II} and dibucaine (used here at a 1:1 molar ratio with phospholipid) antagonizes the calcium effect, stabilizing the bilayer form.

Electron Spin Resonance

Because of its sensitivity and its versatility, electron spin resonance (ESR) has been the most useful technique for studying drug effects on the physical properties of membranes. For excellent discussions of ESR principles and techniques in biological work, see McConnell (5), Griffith & Jost (33), and Smith & Butler (34). Suitable spin labels are derivatives of natural membrane

components, such as fatty acids, phospholipids, or sterols, to which a nitroxide group is attached. Hydrophilic probes and those that bind to proteins have been less often used in pharmacological studies. The orientation and mobility of the probe are measured from the spectra by the amplitude of the peaks or the distances (splittings) between them. A commonly used measure is the order parameter of Hubbell & McConnell (35), which is related to the time-averaged angle that the probe nitroxide axis makes with the bilayer surface and is thus a measure of the flexibility of the probe. By definition, order parameters vary from 0 (completely free to move) to 1 (total rigidity).

GENERAL ANESTHETICS Gaseous anesthetics reduce the order of pure lipid bilayers and biomembranes. Halothane and methoxyflurane have equal potencies at equivalent intramembrane concentrations (36) and are equally effective at different depths in phospholipid bilayer, as measured by phospholipids spin-labled at the 6 and 10 position of the acyl chain. The disordering can be measured at surgical concentrations (37), is not stereospecific (38), and can be partially reversed (as can anesthesia itself) by high hydrostatic pressure (39). In synaptic membranes, a greater disordering effect of halothane was seen deep in the bilayer (16-doxylstearic acid probe) than near the surface (5-doxyl) (40). These biomembranes were more sensitive to disordering by halothane than were lipids extracted from them. Concentration-related disordering of phosphatidylcholine bilayers and synaptic membranes is caused by other anesthetic agents, including trichloroethanol, α- (but not β-) chloralose, urethane, and ketamine (40, 41).

Rosenberg and coworkers reported an unusual ordering effect of halothane at low concentrations in lecithin vesicles or synaptic membranes (42, 43), reversing to disordering at higher (clinically irrelevant) concentrations. The ordering was detected by increased order parameters of 5-doxylstearic acid and by a decreased rate of lateral diffusion of the probe, measured by spin exchange. Boggs et al (44), using relatively insensitive methods, were unable to detect an effect of halothane, chloroform, or diethyl ether on order parameters of 5- , 8- , or 12-doxyl fatty acid spin labels.

LOCAL ANESTHETICS The membrane order of oriented lipid films that contain very little cholesterol increases dramatically on addition of low concentrations of local anesthetics (45, 46). Calcium potentiates the ordering. The extent of ordering by tetracaine is greater at low than at high pH, suggesting that the ionized form of the drug orders the lipid. By contrast, at high drug concentrations and high pH, the undissociated drugs disorder the membranes (45). Hubbell et al (47) found that tetracaine and xylocaine had a mild disordering effect in red cell membranes, and Rosenberg et al (43) did not observe an effect of lidocaine on synaptic plasma membranes or liposomes. Ionizable local

anesthetics probably will not show the same relation of membrane disorder to anesthetic potency as do neutral drugs—some specificity of binding is to be expected since these drugs interact with anionic sites on the cytoplasmic surface of the membrane (48).

Chlorpromazine has a local anesthetic action at concentrations far above those required for binding to dopamine receptors. At 0.1 mM it disorders erythrocyte membranes (49) but orders low-cholesterol brain lipids as other local anesthetics do (46). Much higher concentrations disorder the lipids. These findings agree with the observations of Pang & Miller (50), who reported that the ordering effect of chlorpromazine in low-cholesterol liposomes reversed to a disordering effect at higher cholesterol concentrations.

Propranolol is a cationic drug with a local anesthetic action independent of its adrenergic blocking effect. It dissolves in (or binds to) acidic phospholipids extremely strongly and has an ordering effect in liposomes of phosphatidylserine or phosphatidic acid, but it is much less soluble in neutral phospholipids and does not perturb them (51). Red cell membranes are slightly disordered by propranolol and some weakly immobilized protein sites are converted to strongly immobilized sites by the drug. The ordering effect is apparently not seen in red cells because of their high cholesterol content and low amounts of acidic phospholipids (52).

STEROID ANESTHETICS These compounds show dramatic stereospecificity in their anesthetic potencies and corresponding differences in their effects on order parameters in high-cholesterol lecithin liposomes (53). The orientation of the 3-OH is critical; 3α-hydroxy-5α-pregnane-11,20-dione is a potent anesthetic and has a strong disordering effect, but the corresponding 3β isomer is inactive in both systems. (Other pairs of steroid isomers show less striking differences.) A convulsant steroid lacks disordering activity. These findings add weight to the hypothesis that hydrogen bonding is an important component of membrane perturbing action of some drugs (54) and that the disordering potency may depend partly on the orientation of a hydrogen bond.

CANNABINOIDS The psychoactive cannabinoids disorder high-cholesterol phosphatidylcholine bilayers (55), but the inactive cannabinol is reported to order (55) or disorder (50) them.

BARBITURATES It is not yet established whether barbiturates have relevant membrane-disordering actions. An ordering effect of pentobarbital in low-cholesterol phospholipids (46, 50, 56) or thiopental in synaptic membranes (43) has been reported. Unlike the effect of local anesthetics, this was not potentiated by calcium (46). However, the lipid composition of the bilayer strongly affects its response to barbiturates, and the ordering effect of barbitu-

rates, like that of chlorpromazine and cannabinol, reverses to disordering when a high-cholesterol membrane is used (50, 56). Increasing the phosphatidic acid content of the vesicles from 4% to 10% changed the action of barbiturates from disorder to order. Three barbiturates were among the drugs that Pang et al (41) found to disorder spin-labeled lipid vesicles that contained 33 mol% cholesterol. In that study thiopental and pentobarbital seemed to be more potent as anesthetics in vivo than could be accounted for by their disordering potency in vitro, as would be expected if the drugs act via a specific receptor.

BENZYL ALCOHOL Benzyl alcohol is often chosen as a prototype disordering drug for studies of membrane function. It consistently disorders membranes, but function may not change in parallel. Low concentrations of benzyl alcohol reduce order parameters and stimulate adenylate cyclase and sodium-potassium ATPase activities in liver plasma membranes (57). Higher concentrations inhibit the enzymes, whereas the disordering is stronger than ever. A problem arises when enzymes are inhibited by a disordering drug. For example, benzyl alcohol decreases order and inhibits glucose uptake in adipocyte membranes (58). It is unlikely that disorder causes the inhibition of glucose transport because an increase in temperature has the opposite effect. Drug-induced disorder can explain enzyme malfunction only if the enzyme is stimulated by the drug. Enzymes that are inhibited by disordering drugs are almost always stimulated by warming. For such enzymes the disorder hypothesis fails.

ALIPHATIC ALCOHOLS The normal alcohols of length 3 to 8 carbon atoms increase the mobility of a spin label in phosphatidylserine vesicles (59), in lipids extracted from erythrocytes or from brain white matter (60), and in mitochondrial (61) or synaptic (29, 40) membranes. The log of the potency of the alcohols, like the log of their partition coefficient, is directly proportional to the chain length. Butanol is more potent in whole membranes (whether synaptic or mitochondrial) than in extracted phospholipids (40, 61).

Normal, branched, and secondary alcohols up to 8 carbons in length all disorder biomembranes, with potencies determined by their lipid solubilities (29). The magnitude of the effect is linearly related to the drug concentration, as would be expected for drugs that act by partitioning into their site of action. This is in contrast to the logarithmic dose response curve of drugs that act by binding to a specific receptor. For alkanols up to hexanol, the disordering potencies correlate well with hypnotic doses in mice. Heptanol and octanol are less potent; they may be too water-insoluble to be delivered to the brain in effective concentrations by intraperitoneal injections.

The alkanols up to decanol uniformly disorder membranes and are anesthetics (62, 63). Longer chains, however, are weak fluidizers (59, 63) or increase the order of membranes (62). They are not anesthetics in tadpoles (63). They

do block nerve conduction in the system used by Richards et al (62) but with latencies of several hours. Pringle et al (63) and Lyon et al (29) explain the cutoff in anesthetic potency simply as an inability to achieve adequate membrane concentrations in vivo because of the very low water solubility of these drugs. Alternatively, Richards et al (62) conclude that anesthetic molecules (which are of many different chemical types) occupy multiple receptor sites in the hydrophobic regions of proteins. No data are presented to demonstrate a direct interaction with proteins.

Long-chain alkenols are more potent anesthetics than their saturated counterparts, perhaps because they perturb the bilayer more strongly. Alkenols with different orientation of the double bond differ in disordering potency in liposomes made of egg lecithin with 33 mol% cholesterol but they have the same anesthetic potency in tadpoles (63). This discrepancy may be explained by the observation that the isomers are equipotent in disordering liposomes with 50 mol% cholesterol.

ETHANOL Because of its medical importance, ethanol has received extra attention in studies of membranes. Chin & Goldstein (64) showed that sublethal concentrations of ethanol reduce the order parameter of spin-labeled membranes. The effect is significant with mouse erythrocyte membranes and synaptosomal plasma membranes (but not with myelin) and is also seen in egg lecithin bilayers (65). The effect is stronger in the core of the bilayer than near the surface, as shown by comparing the motion of the 5- and 12-doxylstearic acid probes in model membranes (65) and in synaptosomal membranes (66), as previously reported for butanol (40). However, this does not tell us where the drug molecules actually reside, since we can only see where their effects are strongest. Ethanol is probably held near the membrane surface by hydrogen bonding. Its relatively slight disordering effect at that site may be magnified along the flexible acyl chains. Cholesterol, known to be located near the membrane surface (67), also has a greater effect on order parameters with 12-doxyl than with 5-doxylstearic acid (65).

In general, the more fluid regions in the membrane core are more easily perturbed by any agent than are the stiffer surface regions (65). This holds for the disordering effect of increased temperature and of ethanol and for the ordering effect of cholesterol (65). Furthermore, cholesterol, an ordering agent, blocks the ability of ethanol to disorder a lecithin bilayer (65). These observations suggest that ethanol has its greatest effect in regions of the membrane that are already relatively fluid, and also that the lipid composition of local regions of membranes may determine their vulnerability to ethanol.

Correlations between disorder and intoxication suggest a causal relation. Genetic correlations carry the idea further. Inherited traits greatly affect the sensitivity of mice to acute effects of ethanol, and the differential sensitivity is

expressed in their membranes. For example, the most ethanol-sensitive individual mice in a genetically heterogeneous population have brain membranes that are disordered to a greater extent by ethanol in vitro than are membranes from ethanol-resistant mice (68). Furthermore, two lines of mice that have been selectively bred for differential sensitivity to the hypnotic effects of ethanol also show differential sensitivity of their membranes to ethanol in vitro (68). The correlation between disorder and intoxication also holds up when it is shown (see below) that ethanol-tolerant mice have brain membranes that are resistant to the disordering effects of ethanol in vitro.

Fluorescence Polarization

The polarization of fluorescence emitted by a membrane-bound dye reflects the mobility of the probe in its hydrophobic environment. Dye molecules immobilized in a rigid matrix and excited by polarized light emit fluorescence that is polarized parallel to the exciting light. To the extent that they move during the few nanoseconds' lifetime of the excited state the polarization of the emission is reduced (69). Thus, a decrease in fluorescence polarization (sometimes expressed as fluorescence anisotropy) is a measure of disordering. This technique is comparable to ESR in sensitivity to the membrane-disordering effects of drugs. It has been less used, perhaps because the available probes cannot be as accurately directed to exact locations in the bilayer. The most commonly used probe is 1,6-diphenyl-1,3,5-hexatriene (DPH), a rod-shaped molecule. Whether it remains oriented parallel to acyl chains or can "lie down" in the core of the membrane is unknown, and it is not yet certain whether DPH is distributed evenly throughout the bilayer. Stubbs et al (70) have shown that DPH partitions equally between liposomes and rod outer segment disk membranes, where essentially all of the lipid is associated with proteins, suggesting that this probe monitors different hydrophobic regions equally well. Klausner et al (71), in studies of the heterogeneity of fluorescence lifetimes of DPH, suggested that 10 or 20% of the signal comes from the edges of gel domains in biomembranes.

Jacobson & Wobschall (72) provide a thoughtful analysis of fluorescence polarization in lipid bilayers, including the reasons that it is inappropriate to convert the polarization or anisotropy data into units of microviscosity (poise). Shinitzky and co-workers (73) used the Perrin equation to convert polarization to viscosity, using a standard oil for calibration. However, this concept is only applicable to isotropic motion where the volume of rotation of the probe is the same as in the oil, and not in a bilayer where motion of the probe is anisotropic.

It is important to know whether the drug changes the lifetime of the excited state. The lifetime is the period during which motion of the dye can be detected; if the lifetime is shortened (for example, by energy transfer from the dye to the

drug), the motion will appear to be slower and any disorder caused by the drug will be underestimated.

ANESTHETICS Several inhalation anesthetics can disorder phospholipid vesicles (31). With the exception of halothane, which contains a fluorescence-quenching bromine atom, they do not affect the fluorescence lifetime of the dye pyrene. Dibucaine disorders acidic phospholipids (74); it partitions into acidic phospholipids much more strongly than to neutral phospholipids. Calcium orders the membranes and antagonizes the drug-induced disorder. Dibucaine, itself a fluorophore, quenches fluorescence and shortens the lifetime of DPH. Thus, an increased rate of probe rotation can be demonstrated after correcting for the altered lifetime, whereas the polarization actually increases in the presence of the drug.

BARBITURATES Barbiturates reduce the polarization of DPH fluorescence in mouse synaptosomal membranes, with potencies proportional to their lipid solubilities (75). Both anesthetic and convulsant barbiturates disorder the membranes, and (unlike anesthesia) there is no stereospecificity. Intact synaptosomal plasma membranes are more easily disordered than lipids extracted from them, and a lipid extract containing only phospholipids (no cholesterol) is ordered by the drug, in agreement with Pang & Miller's ESR data on pure phospholipid vesicles (50).

BENZYL ALCOHOL Cherenkevich et al (76) used DPH polarization data to calculate an order parameter in macrophage membranes. The order was reduced by benzyl alcohol in parallel with a change in oxidase activity.

ALIPHATIC ALCOHOLS Kutchai and coworkers have studied a series of normal alkanols in red cells, using DPH polarization to measure disordering and relate it to changes in membrane function such as water permeability (77) or sugar transport (78). As expected, the intrinsic order of the membrane was increased by extra cholesterol, and the disordering potency of alkanols (pentanol through heptanol) increased with chain length. Despite their opposite effects on fluidity, cholesterol and alkanols sometimes had the same effect on function, so it was not possible to explain the relation between fluidity and the particular transport system under study. The alkanols from 4 to 8 carbons reduce the fluorescence anisotropy of DPH in chick embryo heart microsomes, an effect that is proportional to the lipid solubility of the drugs (79).

ETHANOL Using fluorescence anisotropy of DPH, Harris & Schroeder (80) have shown that ethanol disorders biomembranes. In synaptosomal plasma membranes, the effects of 20 mM ethanol are significant, but lipids extracted

from membranes are much less sensitive. Myelin is less affected than the plasma membranes. A probe of the membrane surface, 1-aminopyrene, is not much affected by ethanol, confirming ESR data about the relative sensitivity at different depths in the membrane.

A high concentration of ethanol, 1.8 M, can reduce the fluorescence lifetime of pyrene in phosphatidylcholine vesicles (31), but 0.32 M ethanol does not affect the fluorescence lifetime of DPH in synaptosomal plasma membranes (75).

PHOSPHOLIPID METHYLTRANSFERASES Hirata & Axelrod have described a phospholipid methyltransferase system that converts phosphatidylethanolamine to phosphatidylcholine and transfers the lipid from the cytoplasmic surface of the membrane to the outer surface. This reaction occurs in a great variety of cells in response to many pharmacological stimuli (9). In red cells it is reported to be accompanied by a large change in microviscosity, measured by polarization of DPH fluorescence (81). The increased fluidity is thought to be the mechanism for a number of events triggered by β-adrenergic receptors, mitogens, and chemotactic compounds. The change in membrane order accompanying the enzyme activity has not been found in kidney or brain membranes, however (82, 83). The methyltransferases are inhibited by ethanol in vitro (84).

Phase Transitions

Thermotropic phase transitions between the gel and liquid crystal forms of lipids are easily seen in pure lipid bilayers by a variety of methods such as differential scanning calorimetry, turbidimetry, and the spectra of spin labels that partition only into the fluid portion of a lipid.

GENERAL ANESTHETICS Anesthetic drugs generally lower the temperature at which the phase transition takes place, an effect that increases the overall fluidity of the membrane at temperatures slightly below the original transition temperature. Inhalation anesthetics reduce the transition temperature of phosphatidylcholine vesicles (31, 85, 86); they are equally effective in phosphatidylcholines with chain lengths of 14, 16, or 18 carbons (87). These drugs also increase the width of the transition, which indicates that they reduce the size of the cooperative unit of lipids that participate in the melting. Mountcastle et al (88) suggest that the lipids exist in clusters the size of which influences the interactions between proteins. Both the decrease in transition temperature and the broadening of the transition are reversed by hydrostatic pressure (88–90), and pressure alone raises the transition temperature. Methoxyflurane lowers the transition temperature much further in phosphatidic acid membranes than in phosphatidylcholine (91), and pressure may be able to force the anesthetic out of phosphatidic acid but not out of phosphatidylcholine membranes.

LOCAL ANESTHETICS Barbiturates depress phase transition temperatures of liposomes made of phosphatidylcholine or phosphatidylethanolamine but not phosphatidylserine (92). The uncharged form of the drug seems to be the active form. Lee (92) suggests that barbiturates and other local anesthetics block sodium conductance by melting the boundary lipids that surround the channel protein. In rat liver plasma membranes, phenobarbital depresses the temperature of the transition that has been assigned to the outer monolayer (93). Analysis of Arrhenius plots of enzymes that have known locations in this membrane led to the concept that outer-monolayer enzymes have a phase transition at about 28°. The transition is revealed by a change in slope of the Arrhenius plots and the break is shifted to a lower temperature by phenobarbital. Dibucaine reduces the transition temperature of acidic lipids (phosphatidylserine or phosphatidylglycerol) in which the drug is highly soluble, but does not affect that of phosphatidylcholine (74).

ALIPHATIC ALCOHOLS Short-chain alkanols depress phase transitions of pure phospholipid vesicles (94). Hill (86, 95) and Rowe (96–97) followed phase transitions in liposomes by a simple optical method, and calculated the partition coefficients of alcohols by their ability to lower the phase transition temperature, assuming that the drugs are confined to the fluid phase. Compounds that reduce the transition temperature generally abolish the phosphatidylcholine pretransition.

In addition to reducing the transition temperature, some alcohols affect the width of the phase transition. Short chain alcohols, up to 5 carbons, do not affect the width of the phase transition in phosphatidylcholines (90, 97, 98). Alcohols or fatty acids of 5–10 carbons broaden the transition in phosphatidylcholine vesicles (94, 98) but not in phosphatidylethanolamine (94).

By contrast, long chain alcohols (or fatty acids) with more than 10 carbon atoms raise the transition temperature (94, 99). Alkanols affect long-chain phosphatidylcholines more than short chain phosphatidylcholines (87).

Jain & Wu (98) have classified a variety of drugs according to their ability to shift the midpoint of the transition and/or increase its width in an attempt to localize the drug action within the bilayer.

As a next step from pure lipids toward biomembranes, Lee has studied effects of alkanols on phase diagrams of binary mixtures of phospholipids (94). The drugs shifted downward the temperature range at which gel and fluid lipid coexist. Lee's data provide an illustration of the potential importance of phase transitions in terms of the overall fluidity of a biomembrane. About 40% of the lipid in a particular binary mixture was fluid at 30° C in the absence of drugs, but 63% was fluid in the presence of 0.8 mM octanol.

CHRONIC ADMINISTRATION OF MEMBRANE-DISORDERING DRUGS

Many of the drugs that act on cell membranes are capable of producing tolerance and physical dependence. These phenomena may be adaptive in nature, i.e. homeostatic mechanisms may allow the body to function fairly normally despite the continuous presence of initially toxic drug concentrations. On withdrawal of the drug a dangerous syndrome may ensue. Because these drugs have their primary action on the hydrophobic regions of cell membranes the same regions may be the locale of the adaptation, as was suggested in 1975 by Hill & Bangham (100).

Adaptation to a disordering drug may be analogous to temperature adaptation. Microorganisms and poikilothermic vertebrates compensate for changes in ambient temperature by altering the chemical composition of their membrane phospholipids, a process that Sinensky (101) has called *homeoviscous adaptation*. Bacteria accomplish this by increasing the degree of unsaturation of membrane acyl chains as the temperature falls. The lipids remain at roughly the same fluidity whatever the growth temperature. Homeoviscous adaptation has been observed in many species; even in mammals the degree of unsaturation of depot fats (triglycerides) can change to suit the temperature (102). Thus, it is reasonable to seek evidence that mammalian membrane lipids may adapt to the continuous presence of drugs that mimic warming. At present we have good evidence that chronic exposure to drugs alters the physical properties of membranes but the nature of the underlying chemical change is still in doubt. There may be many ways of accomplishing the same homeoviscous adaptation and they may occur simultaneously or in sequence.

Chronic administration of ethanol alters mammalian membranes both chemically and physically. The chemical composition may be adjusted in several respects simultaneously and the resultant change in membrane order (if any) is difficult to predict. Littleton & John (103) observed an increase in the degree of saturation in the fatty acids of brain phospholipids in mice treated chronically with ethanol. The ratio of linoleic to arachidonic acid increases in many tissues after chronic ethanol treatment of the animals. Similarly, the proportion of linolenic falls while more saturated acids accumulate in mitochondrial cardiolipin from alcohol-treated rats (104). Chin & Goldstein (105) have recently reviewed these changes in more detail than can be included here.

In mammals cholesterol is a major chemical determinant of membrane order. An increase in the membrane content of cholesterol, which is an appropriate response to the disordering effect of an anesthetic drug, sometimes occurs after chronic administration of ethanol. Treatment of mice or rats with ethanol in a liquid diet increases the cholesterol content of their synaptosomal (106–108),

erythrocyte (106), and hepatic (109) membranes. However, when ethanol was administered by inhalation or injection, even when the regimen sufficed to produce changes in fatty acid content of membranes or to produce tolerance and physical dependence, the cholesterol:phospholipid ratio of synaptosomal plasma membranes was unchanged (110, 111).

Ethanol (like an increase in temperature) accelerates the transfer of cholesterol between membranes in vitro without affecting its equilibrium distribution (112). The end result in vivo might be a shift in the steady state distribution of cholesterol among different carriers and membranes. An increased content of cholesterol might account for tolerance, since cholesterol counteracts the disordering effect of ethanol in phosphatidylcholine vesicles (65) and reduces the partition of pentobarbital (and probably other drugs) into lipid vesicles (20). However, these effects are most evident at low concentrations of cholesterol, analogous to intracellular membranes. Liposomes with more than 30 mol% cholesterol, comparable to plasma membranes, are relatively insensitive to further increases.

Membranes isolated from ethanol-treated animals are often found to be resistant to the disordering effects of the drug in vitro. This represents tolerance of the membranes occurring in parallel with behavioral tolerance in the animals. This condition can be elicited in mice either by administration of ethanol in a liquid diet for 8–9 days (113), by a 3-day period of ethanol inhalation (111), or by a series of injections (107). In rats a month of liquid diet treatment accomplishes the same result (104). Membranes of brain, liver, and red cells show similar changes in sensitivity, and phospholipid extracts also show a decreased response to ethanol, indicating that the changes are not restricted to the proteins. Cross-tolerance to ethanol is seen in membranes of mice treated with pentobarbital but not in those treated with morphine (114), and membranes of ethanol-treated mice are cross-tolerant to *t*-butanol (111) and halothane (26).

Less consistently, the membranes from such animals have an increased intrinsic order, an abnormal rigidity that might perhaps mediate the hyperexcitability of the withdrawal reaction, since it is the opposite of the primary (sedative and disordering) effect of the drug. In mice after 3 days of ethanol inhalation we found an increase in intrinsic order in the region monitored by 12-doxylstearic acid but not in the regions monitored by 5- or 16-doxyl probes (111). Fluorescence polarization of DPH also showed increased intrinsic order in these synaptosomal plasma membranes (115), but not in lipid extracts of membranes from alcohol-treated mice or rats (116).

Rottenberg et al (26) reported that the partition coefficient of ethanol (and also that of halothane) was considerably lower in membranes from rats that had been chronically treated with ethanol than in controls. Such a change might explain the decreased slope of the ethanol concentration response curve but could not account for the changed intrinsic order.

SUMMARY

Anesthetics almost always disorder or "fluidize" membranes, i.e. the drugs increase the mobility of spin labels and reduce order parameters. This effect is universal at high concentrations above the clinical range, but in some kinds of membranes low concentrations of drugs have an ordering effect. Drugs that carry charges, including many local anesthetics, often stiffen membranes, as do long-chain alcohols or fatty acids that mimic natural membrane components. The potencies of short-chain alcohols correlate well with lipid solubility, but a cutoff is reached at 10–12 carbons, where pharmacological actions become weak or absent despite a progressive increase in lipid solubility. The cutoff is partly explained by the ordering action of the long chains and partly by the difficulty of administering such water-insoluble drugs in vivo.

The idea of membrane disorder does not exclude some specificity. Closely related drugs may have different molecular shapes and may be capable of forming hydrogen bonds with different orientations, affecting their ability to make membrane more fluid. Perhaps for this reason, there is a remarkable stereospecificity in the disordering effect of anesthetic steroids, chloralose, and long-chain alkenols.

Some specificity is mediated by different membrane environments. The drug action may actually reverse from order to disorder on addition of cholesterol, but in other experimental systems cholesterol blocks a disordering effect, and we cannot yet explain the action of drugs in different biomembranes. Further, drugs may have differential solubilities in membranes of different composition. This cannot always be predicted from octanol:water partition coefficients because branched molecules are differentially excluded from structured bilayers. Charged drugs react quite differently with charged and neutral phospholipids and may have differential actions on the two sides of the bilayer because of the asymmetry of the phospholipid distribution. The deeper reaches of the membrane seem particularly sensitive to disordering, even by drugs that presumably reside near the surface. Thus, proteins whose midregions are sensitive to disordering may be especially disrupted by drugs.

This is a new field of pharmacology, currently applied only to a small group of drugs. But an understanding of the physicochemical actions of drugs in hydrophobic regions of cells will clearly be needed for full understanding of membrane-bound drug receptors, enzymes, and transport systems. This is just a beginning.

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