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MEMBRANE LIPIDS AND DRUG ACTION

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I. INTRODUCTION

In a recent paper Ansell (13) commented that "direct relationship between the lipids of the nervous system and physiological-pharmacological activity has rarely been demonstrated." This statement remains true and applies not only to nervous tissues but to non-nervous tissues as well. It might be considered therefore that a review on "membrane lipids and drug action" is somewhat premature. However, there are reasons for supposing that such a review is useful. First, membranes, together with affixed enzymes and special components, comprise 60% to 90% of the total cell substance (226). Second it is agreed that membranes are composed mainly of protein and lipid, even though the basic arrangements of these two components are not clear, and finally the cell membrane is an important locus of drug action.

The range of drug types that exert their pharmacological effect on the cell membrane is impressive. Groups as diverse as the neurohumors and their antagonists, local and general anaesthetics, some of the diuretics, some antibiotics,

some steroids and fat soluble vitamins, together with many cations have supposed sites of action on the cell membrane. There is also good reason to suppose that many drugs can cause relatively major changes in the properties of cell membranes. Two examples will suffice to illustrate this point. During the action of the transmitter the end plate of skeletal muscle is converted to an ion "sink" (56, 111) by an increase of permeability to sodium, potassium, and calcium ions but not chloride ions (347–349). In denervated skeletal muscle acetylcholine causes a 5-fold increase in permeability to potassium (4). In smooth muscle, agonist drugs cause large increases in ion permeability. For instance, the maximal rate of loss of Rb^{86} from smooth muscle preloaded with this isotope in the presence of cholinergic drugs is many hundred times the resting release (48). If the Davson-Danielli model (69) of the cell membrane is accepted, it seems likely that these dramatic changes of permeability reflect a substantial upheaval of the membrane ultrastructure. Permeability changes of these magnitudes must involve the opening, enlargement or creation of pores or time-averaged free space through which lipid-insoluble ions can pass. It is one aim of this review to examine the hypothesis that these changes in membrane ultrastructure may involve the membrane lipids.

Even if a case for the involvement of membrane lipids in drug action can be made this does not imply that they are directly involved. Generally it is believed that drugs active on the cell membrane bring about a conformational change in a membrane protein, the receptor, which acts as a trigger for subsequent events which may involve the lipids. It is interesting to speculate that a second order involvement of membrane lipids in drug action provides another dimension for the diversity of drug action. For instance, the structure-action relationships for drugs, and the specificity of antagonists, affecting the muscarinic receptors in the heart and intestine are similar (29); yet, the permeability changes caused by muscarinic agents are dissimilar in the two tissues. In the heart there is a selective increase in potassium permeability (49) whereas in smooth muscle there is an increase in permeability to sodium, potassium (50), calcium (307), and chloride ions (104). One explanation of this may be that identical receptor events trigger different ultrastructural changes, because of differences in the composition of membrane lipid, which reflect themselves in the different permeability changes.

II. CHEMICAL CONSTITUTION

The lipids found in cell membranes consist of compound lipids and cholesterol (fig. 1, VIII). Compound lipids are essentially esters of fatty acids with an alcohol that contain an additional group. Phospholipids contain phosphate as an additional group whereas gangliosides and cerebrosides contain a carbohydrate residue. The nomenclature is confusing, particularly amongst the glycolipids, and seems to require authoritative definition. There is a wide variation in structure, space-filling properties, and charge location among membrane lipids.

A. Phospholipids

Naturally occurring phospholipids are L-α-compounds. Ansell and Hawthorne (16) sensibly suggested the nomenclature should be changed to L-3-compounds

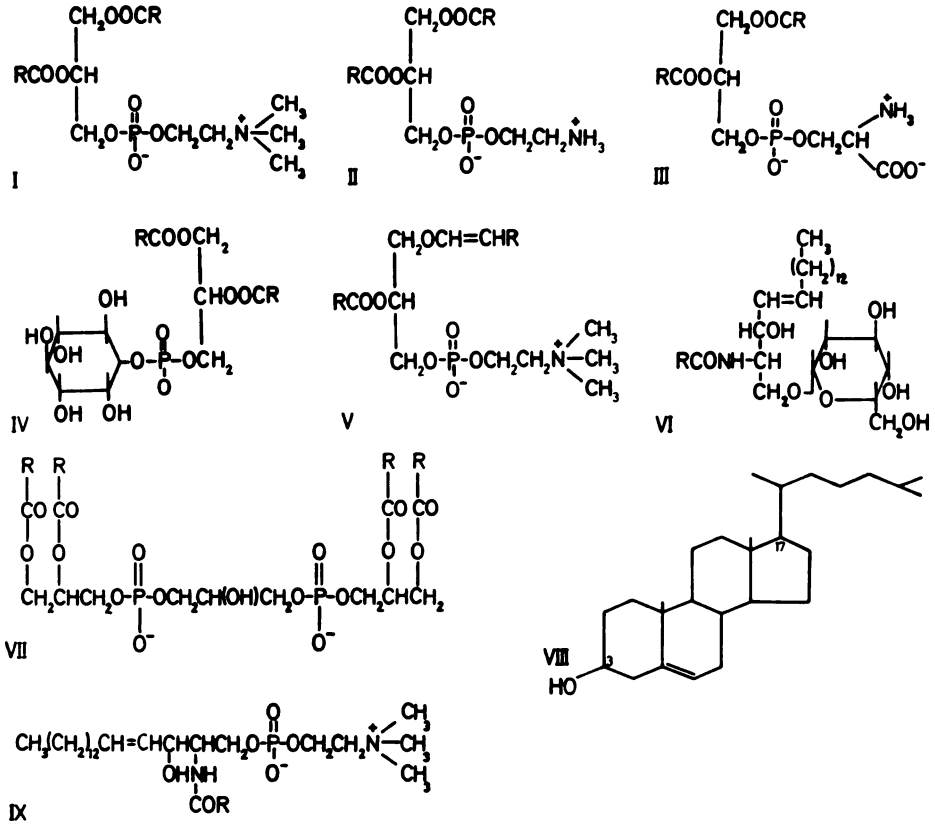

FIG. 1. Formulae of some common lipids.

since it is not clear whether α refers to the 1-OH or 3-OH of glycerol. The most common phospholipid is L-3-phosphatidyl choline (I) or lecithin. "Phosphatidyl" corresponds here to "diacylglycerophosphoric acid." Phosphatidyl choline is a mixture of compounds because of the variety of fatty acids esterified in positions 1 and 2. Phosphatidylcholines have no net charge. The structural formulae of phosphatidylethanolamines (II), phosphatidylserines (III), and phosphatidylinositols (IV) are shown in figure 1. Of the various isomeric inositols, only myoinositol has been found in natural phospholipids. Di- and triphosphoinositides are also found, their structures being 1-phosphatidyl-L-myoinositol 4-phosphate and 1-phosphatidyl-L-myoinositol 4,5-diphosphate respectively. The phospholipids that contain serine, ethanolamine, and inositol are often collectively called cephalin. Diphosphatidyl glycerol (cardiolipin) (VII) is found in some tissues.

Some phospholipids release fatty aldehydes instead of fatty acids on acid hydrolysis. These are called plasmalogens. Plasmalogens have been found containing choline, serine, and ethanolamine. Choline plasmalogen (phosphatidal choline) may have the structure shown in (V). In sphingolipids (sphingomyelins and cerebrosides) glycerol is replaced by 1,3-dihydroxy-2-amino octadec-4-ene (sphingosine). Sphingomyelins (IX) have a phosphoryl choline group at C1. At-

tachment of a fatty acid residue at C2 gives an amide. Ansell and Hawthorne (16) have written a comprehensive treatise on phospholipids.

B. Glycolipids

Cerebrosides (VI) and gangliosides are found in nervous tissues. Cerebroside is a general term for ceramide-monohexosides (ceramide is *N*-acyl sphingosine), the prefix gluco- or galacto- indicating the sugar moiety. Cerebrosides are similar therefore to sphingomyelins. Gangliosides have a lipophilic ceramide unit and a hydrophilic oligosaccharide chain. The monosialoganglioside has the form,

$$\text{GAL}(1 \rightarrow 3)\ \text{GALNAc}\ (1 \rightarrow 4)\ \text{GAL}\ (1 \rightarrow 4)\ \text{GLU}\ (1 \rightarrow 1)\ \text{Ceramide}$$

$$\begin{pmatrix} 3 \\ \uparrow \\ 2 \end{pmatrix}$$

$$\text{NANA}$$

where GAL is galactose, GLU is glucose, GAL NAc is *N*-acetyl galactosamine and NANA is *N*-acetylneuraminic acid (a sialic acid). Disialo- and trisialo-species of gangliosides containing respectively one and two additional NANA have been described. Brain gangliosides have been implicated in transmitter release in the CNS (51). The chemistry of the glycolipids has been reviewed recently (54, 224).

III. DISTRIBUTION

It is the intention in this section to indicate the wide variation in lipid composition of tissues and membranes and to show where possible its relevance to drug action. Two important points must be remembered when the lipid composition of membranes is discussed. First, the membrane cannot be regarded as homogeneous (130, 135, 144, 279). Islets of a particular membrane lipid or a particular grouping of membrane lipids may occur in a specified membrane, apart from the differences in composition of the various membranes (plasma, mitochondrial, endoplasmic *etc.*) within a single cell. In addition, the membrane must be regarded as dynamic (6, 79) so that particular structures or groupings of lipid molecules have only a transient existence during the lifetime of the membrane. Secondly, the relative proportion by weight of various types of lipid in membranes does not indicate the membrane composition because of the variation in fatty acid composition in each lipid class. It is more meaningful to state the molar percentage of lipids (264) although this requires a further stage of analysis, namely the hydrolysis of lipid esters and the estimation of the resulting fatty acids and aldehydes.

The distribution of lipids has been determined for a great number of tissues, but there have been no analyses of plasmalemma composition, although many drugs act on this membrane. One exception to this is the myelin lipids, which are nothing but plasmalemma lipid. Unfortunately this plasma membrane is of little pharmacological interest except as a barrier or drug depot.

The proportion of phosphatidylcholines in red cell ghosts of various species is rather variable, whereas the content of phosphatidylserines is similar (80). Non-

ruminants have three times (31.7%) as much phosphatidylcholine as ruminants (9.1%). Ruminants have a corresponding excess of sphingomyelin and choline plasmalogen. In omnivores (man and pig) the percentage of phosphatidylethanolamine (20.4%) in relation to the total phospholipids is three times that in herbivores (7.1%) (horse, cow, sheep, and goat). These figures illustrate the variation between the same cells of different species. The permeability of red cells of various species to glycerol and glucose has been correlated with lecithin content (134) and with the ratio of palmitic to oleic acids in the fatty acid residues (374) found in red cell ghosts.

Normal rat liver cell membranes contain 30.3% lipid, of which 73.6% is neutral lipid and phospholipid (326). Phosphatidylcholine is the main phospholipid (14.3%) then phosphatidylethanolamine (6%) and sphingomyelin (7.2%) with smaller amounts of phosphatidylserine (3.2%) and phosphatidylinositol (2.8%). Thus the phosphatidylcholine content of rat liver cell membrane lies between that of the red cells of ruminants and non-ruminants, whereas the content of phosphatidylethanolamine is similar to that found in the red cells of herbivores but not ominivores. These examples are sufficient to show the variation of membrane lipid composition of tissues.

O'Brien and Sampson (264) investigated the lipid composition of white matter, grey matter, and myelin from normal human brains of various ages. The total lipid from these three tissues was grey matter 36 to 40%, white matter 49 to 66%, and myelin 78 to 81%. Grey matter had a higher molar proportion of ethanolamine-containing phosphatides and serine-containing phosphatides than did myelin. The latter on the other hand had a much higher percentage of cerebrosides and cerebroside sulphates and a slightly higher molar percentage of cholesterol than did grey matter. In a subsequent paper (265) the authors analysed the fatty acid composition of grey matter, white matter, and myelin. Ethanolamine-containing phosphatides and serine-containing phosphatides from grey matter were low in octadecanaldehyde whereas the same phosphatide fractions from white matter were rich in this fatty acid aldehyde. Both contained a large proportion of C20 to C22 polyunsaturated fatty acids whereas choline-containing phosphatides had little of these fatty acids. In general each glycerophosphatide from grey matter had 3 to 6 times the proportion of unsaturated fatty acid as the same glycerophosphatide from myelin. Thus not only does the type of lipid composition vary from tissue to tissue in the central nervous system, but the fatty acid and fatty aldehyde composition in individual lipid fractions varies according to the source. In contrast to this some tissues show a more constant composition. Myelin has a remarkably constant composition both within and between species. The ratios of cholesterol:ethanolamine glycerophosphatides: serine glycerophosphatides:choline glycerophosphatides:sphingomyelin:cerebroside:cerebroside SO_4 were similar for myelin from man and ox (262, 264). Yet the ratio phospholipid:cholesterol in myelin is higher in peripheral nerve than in brain (110).

A lower molecular ratio of phospholipids:cholesterol was found for the plasma membranes from liver, kidney, and intestinal mucosa cells of the guinea-pig than

for other membrane systems found in each tissue (63). The distribution of plasmalogens has been the subject of several studies. Ethanolamine analogues were found to be the principal plasmalogens in invertebrates (146). Adult mammalian cardiac tissue is unusual in having a large amount of choline plasmalogen (120) even though this lipid is scarce in embryonic hearts of man, cats, and dogs (147). Certainly the embryonic heart responds differently to acetylcholine than the adult heart (291, 292) and a study correlating sensitivity with membrane lipid composition might prove worthwhile.

It is likely that variations in total lipid composition reflect variations in the membrane lipid composition. The examples given indicate the infinite variety of the lipid make-up of cells. How does the lipid composition of cell membranes affect their properties and in particular their responses to drugs? Clear examples are difficult to find. Cytoplasmic membranes from brain neoplasms have a high phosphatidylcholine:phosphatidylethanolamine ratio compared with normal brain tissue (60). This probably results in an increased surface electronegativity (9) and failure of normal contact inhibition (1). It might be thought that phosphatidylcholine would not confer surface electronegativity because of the equal numbers of positive and negative charges. However, Finean (121) has presented evidence that the trimethylammonium head can withdraw into the lipid phase, leaving the negatively charged phosphate groups exposed to the surface. High phosphatidylcholine:phosphatidylethanolamine ratios are found in most cells with low cellular differentiation and organisation, and this ratio is low in embryonic cells (61). Drugs that can reduce surface electronegativity present a challenge to the chemical pharmacologist. Permeability properties of cells are affected by their lipid content. For instance, the permeabilities of skins of several amphibia to water are related inversely to their lipid content (310). Adrenal medullary chromaffin granules are unusual in being rich in lysolecithin (34) and it may be more than coincidence that lysolecithin is a potent catecholamine releaser (116). The polyene antibiotic amphotericin complexes with sterols (113, 379). If these are present in significant amounts in a cell membrane then these agents can be expected to influence the properties of the membrane. On this basis the antifungal effects, the actions on permeability, and possibly the toxic effects of this agent may have a unitary explanation.

IV. CELL MEMBRANE ULTRASTRUCTURE

A brief account of membrane ultrastructure is included here so that the part played by membrane lipids in membrane structure can be appreciated. The classical picture of plasma membrane ultrastructure described by Davson and Danielli (69) was based on biological properties shown by cells. They concluded that a bimolecular lipid leaflet, with the polar groups orientated to the outside, was covered on both sides with a layer of protein. From permeability studies it was suggested that this structure also contained polar pores. A similar "unit membrane" structure was proposed by Robertson (293–296) as a result of studies with electron microscopy, X-ray diffraction and optical polarisation. As seen with the electron microscope, two layers of 20 Å thickness, perhaps representing protein,

separated by a lighter layer of 35 Å thickness, perhaps representing lipid, give the unit membrane a thickness of some 75 Å. In 1958 Lehninger *et al.* (227) proposed that the respiratory assemblies were arranged in a planar array attached to or embedded in the protein of the unit membrane. Electron microscope studies by Green *et al.* (145), Stoeckenius (341), and Fernandez-Moran (119) of mitochondrial membranes have shown the presence of "elementary particles," which are thought to be enzyme "packets." Green considered these an integral part of the membrane structure, although they can be dissociated from it. In addition, Sjöstrand (325) has demonstrated a periodicity in the actual substance of the mitochondrial unit membrane. This has led some to believe that the granularity represents a micellar rather than a laminar arrangement of the unit membrane lipids. Recently, Branton (43) has demonstrated globular subunits in cell (plant) plasma membranes by the freeze-etching technique and concluded that the biological membrane is organised in part as an extended bilayer and in part as globular subunits. Ultrastructural membrane changes from a laminar to micellar form and *vice versa* have become a popular concept. Kavanau (203) has explored the theoretical possibilities of this. Space limitations do not allow a detailed discussion of the theoretical arguments concerning the stereospecific arrangements of lipids and proteins in cell membranes or the part played by water in cell membrane organisation. Most of the theories are concerned with the molecular parameters of myelin and not of cell membranes. Interested readers are referred to the following sources: the structure of myelin (123, 263, 357, 370–372); lipid protein interactions (373); and membrane water (157, 158, 232).

V. BIOLOGICAL CONSIDERATIONS

The involvement of membrane lipids in drug action, and the changes in lipid organisation, composition, and synthesis caused by various agents, will be considered in this section. The headings used serve only to collect the information so that the most logical discussion can be made within each subsection.

A. The effects of drugs on the incorporation of phosphate into phospholipids

Several drugs increase the incorporation of P^{32} (as phosphate) into one or more of the phospholipid fractions of target tissues. Furthermore the physiological effects and the effects on P^{32} incorporation are parallel in a number of important ways, for instance, in the behaviour of these different responses to antagonists and to ions.

Acetycholine, carbachol, adrenaline, and noradrenaline all caused mucin secretion and P^{32} incorporation in rat submaxillary glands and amylase secretion and P^{32} incorporation in rabbit and guinea-pig parotid glands. The effects of cholinergic drugs were blocked by atropine but dibenamine blocked only the secretory and not the phospholipid effects of catecholamines (179). Pancreazymin stimulated amylase secretion and P^{32} incorporation in slices of pigeon pancreas (184). Similar effects with pancreas slices were obtained with acetylcholine, carbachol, and pilocarpine. These effects were blocked by atropine (182). Removal of calcium abolished the secretion caused by acetylcholine from pancreas

slices but incorporation of P^{32} was inhibited by only 30% (170). Stimulation of amylase synthesis by amino acids had no effect on P^{32} incorporation (182). In the adrenal medulla adrenaline secretion and P^{32} incorporation were caused by acetylcholine (181). Corticotrophin-releasing factor caused ACTH release and P^{32} incorporation in the rat adenohypophysis (188). An exception to this pattern was noted for the adrenal cortex, where ACTH released corticoids but had no effect on P^{32} incorporation (188). Recently it has been shown (245) that corticoid release by ACTH from the rat adrenal cortex is not accompanied by membrane depolarisation whereas depolarisation of the cortical cells by potassium is not accompanied by secretion. Thus the failure of ACTH to stimulate P^{32} incorporation may be only an apparent exception and it would be interesting to know the effects of potassium on phospholipid metabolism in the adrenal cortex. Insulin stimulates P^{32} incorporation into phospholipids in rat epididymal fat pads (368) and into skeletal muscle of the rat diaphragm (240). Thyroid-stimulating hormone and acetylcholine cause P^{32} incorporation into the phospholipids of thyroid gland slices (7, 128, 216, 250). Atropine blocks the effects of acetylcholine but not those of thyroid-stimulating hormone (7).

Similar data have been obtained for nervous tissues. Phosphorus-32 incorporation into brain slices was stimulated by acetylcholine and this effect was blocked by atropine (45, 172, 173). Potassium ions also caused P^{32} incorporation in brain slices, and the effects of both potassium and acetylcholine were shown to be sodium dependent (45). Acetylcholine and preganglionic stimulation of various peripheral ganglia caused P^{32} incorporation (189, 221, 222). This effect was not produced when antidromic stimuli were used or when synaptic transmission was blocked by tubocurarine (222). No effect on P^{32} incorporation was seen with electrical stimulation of nerve trunks (222).

Three important questions must now be considered. First, into which phospholipid fractions is P^{32} incorporated; second, what is the locus of this effect and finally what is the significance of these results? In general, P^{32} incorporation takes place into phosphatidic acid and phosphatidylinositol (173, 181, 189). Other phosphatides may be involved in various tissues, for instance, diphosphoinositide in brain (173) and phosphatidylglycerol in adipose tissue (368). There is some disagreement between various groups as to whether or not P^{32} incorporation in ganglia takes place exclusively into phosphatidylinositol or whether phosphatidic acid is also involved (189, 221, 222). Glycerol-1-C^{14} is also incorporated into glycerophosphatides, in, for instance, pancreas slices, but this is not stimulated by acetylcholine (183). These results indicate that drugs and other agents do not simply increase the levels of the components required for phospholipid synthesis.

The kinetics of P^{32} incorporation has been investigated by Hokin and Hokin (178, 185, 187) in the avian salt gland. This organ secretes inorganic ions, mainly Na^+ and Cl^-, in response to acetylcholine (311). Acetylcholine caused P^{32} incorporation into phosphatidic acid in the salt glands of the goose, albatross, and gull. The level of labelled phosphatidic acid reached a plateau after 1 to 2 min exposure to acetylcholine (185). Thereafter there was a small but continued

increase in the radioactive phosphatidic acid levels. It is the rapidly labelled fraction of phosphatidic acid which is of interest. The slowly labelled fraction does not lose its P^{32} when the stimulus is removed and other phospholipid fractions, such as phosphatidylcholine, show this low rate of labelling. The size of the rapidly labelled fraction was maximally 0.12 μmoles of phosphatidic acid/gram of fresh tissue and depended on the acetylcholine concentration (178). The appearance of P^{32} in phosphatidylinositol during the stimulation of salt gland slices with acetylcholine was minimal. However, on removal of the stimulus (178) or addition of atropine (174) the fall in activity of the phosphatidic acid fraction was equivalent to the rise in activity of the phosphatidylinositol fraction (187). If P^{32} was not introduced until 90 min after the acetylcholine stimulus there was still a rapid increase in the radioactivity of phosphatidic acid, which reached a maximum in 1 to 2 min (185). This suggests there is a fraction of phosphatidic acid which is rapidly turning over even 90 min after the introduction of the stimulus.

On the basis of these results Hokin and Hokin (175, 177, 187) and Wolfe (388) suggested that lipoprotein bound (176) phosphatidic acid acted as the sodium carrier, binding and then releasing sodium as it was hydrolysed to a diglyceride. This diglyceride is then rephosphorylated by ATP (175) to reform the phosphatidic acid. When ion transport is not being stimulated the phosphatidic acid is converted to phosphatidylinositol, probably *via* a cytidine diphosphate diglyceride. Phosphatidic acids have certainly been shown to transfer cations from an aqueous to an ethereal phase (376) and the enzymes necessary to cause the biochemical changes described above have been described (187). Initially it was suggested that membrane bound ATPase might be the specific lipoprotein of the phosphatidic acid cycle (180). Ouabain inhibits the acetylcholine-induced secretion of avian salt glands but not the effects on the phospholipids (187). Later Hokin and Hokin (186) concluded it was unlikely that phosphatidic acid participated in the $(Na^+ + K^+)$-dependent ATPase activity of avian salt gland homogenates. It was estimated that 4.7 sodium ions are secreted by salt glands per ATP cleaved (180). The maximal secretion rate of salt glands is 420 μEq./g fresh-weight/min which is equivalent to the cleavage of 91 μmoles ATP/g fresh-weight/min. Values of not more than 30 μmoles ATP cleaved/g fresh-weight/min were obtained experimentally. Glynn *et al.* (138) could find no evidence for phospholipid involvement as intermediates in the "transport adenosine-triphosphatase" system of the electric organ. The possibility of an undetectable amount of intermediate turning over at an enormously high rate could not be excluded but powerful arguments against this were presented.

Other evidence also argues against the phosphatidic acid cycle being responsible for sodium transport. For example oxytocin stimulates sodium transport in the toad bladder but does not cause P^{32} incorporation into phospholipids. Acetylcholine, on the other hand, stimulates P^{32} incorporation but does not cause sodium transport in this preparation (159). Incorporation of P^{32} into phosphatidic acid was too slow in red cells to account for sodium extrusion (212). Finally antidromic stimuli applied to the postganglionic trunk of sympathetic ganglia

do not cause changes in the phospholipids of the ganglia although the action potential spikes are certainly accompanied by Na^+ movement (222), and similarly stimulation of nerve trunks does not increase phospholipid labelling. It would seem that more data are required before the role of phosphatidic acid in the membrane transport of ions can be finally assessed. Nevertheless a great number of compounds, whose actions are presumed to be on the cell membrane modify incorporation of P^{32} into phospholipids. It is pertinent to consider where these phospholipids are situated in the cell. Larrabee and Leicht considered the phospholipid effect in sympathetic ganglia to be at the postsynaptic membrane (222), whereas Hokin (169), who studied the acetylcholine-stimulated incorporation of myoinositol-2-H^3 in ganglia, claimed that autoradiography showed the effect to be throughout the cell soma. In a subsequent paper he (171) showed that at least part of the phospholipid effect was presynaptic and thought that this may reflect the cholinergic control of acetylcholine release from presynaptic terminals as suggested by Koelle (215). Nuclear, mitochondrial, microsomal, and supernatant fractions of various tissues show increased P^{32} incorporation after stimulation (172, 176) and it was suggested (284) that the phospholipids involved may be located in intracellular membranes.

No mention has been made of the significance of the phospholipid changes which accompany the glandular secretions. Fawcett (112) argued that protein secretion is by reversed phagocytosis and involves the membrane lipids. Stimulation of phagocytosis in leucocytes is accompanied by increased P^{32} incorporation into phosphatidylinositol, phosphatidylserine, and phosphatidic acid (200, 201), but incorporation of P^{32} into pancreatic phospholipids can occur without secretion in the absence of calcium (170).

It is difficult therefore to summarise the physiological and pharmacological significance of this section. Evidence continues to accumulate suggesting that the pharmacological and phospholipid effects of drugs are closely coupled. For instance, it was found that adrenaline, but not glucagon, stimulated P^{32} incorporation in the liver and that this effect was blocked by ergotamine (367). It is well known that both adrenaline and glucagon have anti-anabolic effects in the liver and that the effects of glucagon are not antagonised by ergotamine. Surprisingly adrenaline did not affect P^{32} incorporation into ATP in the liver although it is agreed that most of the metabolic actions of adrenaline and glucagon are mediated by cyclic AMP (281, 345). It is also known that adenylcyclase is limited to an insoluble (membrane?) structure in the cell (346) and that the solubilised enzyme does not respond to adrenaline (214). The interesting suggestion has been made (367) that an initial effect of adrenaline on phospholipids may be the stimulus for adenylcyclase activation and cylic AMP formation. The effects of some other groups of drugs (anticholinesterases, barbiturates, and chlorpromazine) on phospholipid metabolism have been reviewed by Ansell (12). Of particular interest is the observation that, in phenobarbitone induced synthesis of drug metabolising enzymes in rat liver, increased incorporation of P^{32} into microsomal phospholipids is detectable before increased enzyme levels appear in the "rough" endoplasmic reticulum (266, 267). Finally a phosphatidic

acid has been implicated as a carrier for phosphodissacharidepentapeptide units in bacterial cell wall synthesis (11, 93) a result that may be relevant to the action of some antibiotics.

B. The effects of hydrolytic enzymes and organic solvents on physiological and pharmacological responses

Hydrolytic enzymes provide the obvious tools for causing selective destructive changes to cell membranes. They have been used to alter the membrane properties of a great variety of cells, for instance, red cells (17), protozoa (261), and sea urchin eggs (148). Unfortunately, there have been relatively few attempts to study the effects of hydrolytic enzymes on electrically or chemically excitable cell membranes.

Tobias studied the effects of proteases (papain, trypsin, and chymotrypsin) and phospholipases (A and C) on lobster giant axons (362–364). The ability to conduct action potentials was not affected by externally applied proteases, whereas phospholipase enzymes produced depolarisation, reduced excitability, decreased the conduction velocity and spike height, and eventually caused complete inexcitability. The results with phospholipase C are more reliable as the products of hydrolysis with phospholipase A (lysolecithins) are themselves lytic. Tobias concluded that the integrity of the lipids of the membrane was necessary for electrical excitability and conduction. The protein component of the cell membrane was, he concluded, less essential for impulse conduction. However, as only phospholipase treatment produced convincing changes in axon ultrastructure as seen with the electron microscope (363), it is not proven that the membrane protein was available to proteolytic attack. Similar findings were obtained for the nodes of Ranvier in frog nerve (259). Proteases perfused through giant squid axons caused depolarisation, increased permeability to Na^+ and K^+, and eventually led to inexcitability (299, 300). Thus the inner protein component of the cell membrane appears to be necessary for impulse conduction.

Hydrolytic enzymes have also been used in the study of drug responses. Neuraminidase plus EDTA selectively inhibited the action of 5-HT on rat stomach strips (391). The response to 5-HT was restored by adding a mixture of crude lipids extracted from rat stomach. The suggestion was made that the 5-HT receptor was a neuraminidase-sensitive ganglioside (391). Both phospholipase C and α-chymotrypsin depolarised the smooth muscle cells of the guinea-pig taenia coli. With a suitable degree of phospholipolysis membrane potentials remained normal but the response to acetylcholine disappeared. These preparations responded to mechanical stretch with an action potential discharge and an increase of tension. With a suitable degree of proteolysis, the preparations remained responsive to acetylcholine by showing a normal electrical discharge but failed to contract (67). Since the action of phospholipase C on ileal muscle is prevented by adding lecithin to the bathing fluid (233), the effects of the enzyme are unlikely to be mediated by hydrolysis products. The increase in short-circuit current and conductivity of the frog skin caused by vasopressin is abolished by phospholipase C treatment even though the resting short-circuit current and

conductivity are normal. In contrast the effects of vasopressin on the frog skin are not abolished by α-chymotrypsin (68).

The conclusions which may be drawn about the nature of drug receptors from the observations described above are subject to so many reservations that it would be idle to pursue them here. However, there is hope that this approach to the nature of drug receptors can be made more specific. As far as the involvement of membrane lipids in the drug-receptor interaction is concerned, whether as a primary event or as a secondary change, the use of lipolytic enzymes that attack specific phospholipids may be worthwhile. For instance, a bacterial phospholipase C is known which preferentially attacks phospholipids containing linoleic rather than arachidonic acid esters (143); different phospholipases vary in their ability to attack the same phospholipids (64, 196); and there is evidence that some phospholipase A enzymes show a positional specificity in that they attack esters only in the 1 or 2 position in phospholipids (41). The actions of phospholipases on nerve and muscle has recently been reviewed (247).

Organic solvents have been used to remove membrane lipids in a few cases. For instance Dikstein and Sulman (95) extracted frog rectus and rabbit uterus preparations for 2 min with 25% acetone. After this treatment responses to the usual agonists were lost but could be restored by adding phosphatidylserine or phosphatidylethanolamine, or less effectively by adding lecithin. It would be interesting to know what effect solvent extraction has on membrane ultrastructure as seen by the electron microscope. Caution is required when attempts are made to reverse the destructive effects of enzyme or solvent treatment by addition of lipids. Finean and Martonosi (124) found that when muscle microsomes were treated with phospholipase C a diglyceride separated from the membranes and appeared as dense drops under the electron microscope. The total membrane area was reduced in proportion to the lipid loss. Addition of lecithin dispersed the dense droplets without increasing the total membrane area of the system. Lecithin does not therefore cause a structural reconstitution of the microsomal membrane.

C. Ions

The interactions of divalent cations with cell membranes are considered to influence the permeability and electrical properties of the latter (167, 321). Also, at least one theory (365) of cell membrane excitation requires cation binding by phospholipids. There is much evidence (Sections VI A and B 2) for the association of metallic ions with the lipids of model membranes. However, there have been few studies on the interactions of ions with lipids within cell membranes. More studies of this kind might increase our understanding of the functions of lipids within the membranes of living cells.

Membrane fragments from bullfrog skeletal muscle bound radio Ca^{++} from a 1 mM solution, and the binding of Ca^{++} was 50% inhibited by 112 mM Na^{+} or K^{+} (217). After extraction with a mixture of chloroform and methanol the membrane fragments no longer bound Ca^{++}; this was taken as evidence of binding to a lipid or lipoprotein. Ca^{++} and Mg^{++} were bound equally well to red cell

ghosts (55), possibly to the phosphate groups of phospholipids. Membrane fragments from various mycoplasma (pleuropneumonialike organisms), red cells and bacterial protoplasts (283), and also from myelin (131) can be prepared by treatment with detergents such as sodium lauryl sulphate and sodium deoxycholate. The fragments prepared from mycoplasma are extremely pure. After removal of the detergent they can be reaggregated by 1 mM Ca^{++} or Mg^{++} to a unit membrane structure. Microscopically the reaggregated membranes resemble the original membranes (283). At junctional surfaces between cells (92) rather free diffusion of ions can occur from one cell to the next. Removal of calcium from the bathing fluid decreases the conductance across the junctional surfaces between cells before cell adhesion is affected (256, 272). By contrast tumour cells lack intercellular conduction across junctional surfaces (234). Also it is known that the lipids of tumour cell membranes may be different from those of normal cells (16, 60). It is interesting to speculate that perhaps the lipids of tumour cells do not have a normal Ca^{++}-binding capacity.

D. Insulin

The plasmalemma is now considered to be the primary site for the action of insulin. The multiple actions of this hormone thus have a unitary explanation (229). Some hydrolytic enzymes mimic the action of insulin on isolated fat cells (35, 36, 297, 298). Low concentrations of phospholipase C stimulate glucose and amino acid transport in isolated fat cells (35, 297). High concentrations of phospholipase C inhibit glucose utilisation and mimic the actions of cortisol (35). Phospholipase C, like insulin, was found to inhibit the release of free fatty acids from fat cells stimulated by ACTH, adrenaline, glucagon, or theophylline (298). Similarly phospholipase A mimics the actions of insulin on isolated fat cells (36). 3-O-Methylglucose competitively inhibits the effects of both insulin and phospholipase A on glucose entry.

Rodbell (297) considered that both insulin and phospholipases cause a change in membrane ultrastructure from a laminar to a micellar or globular form. He considered the effect of the enzymes to be due to hydrolysis of membrane phospholipids, but Blecher (35, 36) found that detectable hydrolysis did not occur and suggested that insulin, cortisol and phospholipases affect the configuration of phospholipoproteins. An enzyme-substrate complex formed between phospholipase, Ca^{++}, and the phospholipid substrate would, he thought, produce the same change in membrane ultrastructure as the insulin-Zn^{++}-lipoprotein complex of Krahl (218) It is of interest that changes in membrane ultrastructure associated with membrane excitation also produce an insulin-like effect. For instance, contraction of frog sartorius muscles and insulin act on the same system for the transport of 3-methylglucose across the cell membrane (190).

E. Lysins: steroids, vitamin A, detergents, and saponins

There is a wealth of evidence (22, 24, 85, 86, 156, 275, 276, 286–288, 313, 314) that the primary lesion which precedes cell lysis involves membrane lipids. It is the purpose of this section to consider the part played by steroids, vitamins,

detergents, and saponins in lysis. The examples chosen are those in which the nature of the reaction between lytic agent and the cell membrane is best understood. Seeman (316) has pointed out that most lysins actually stabilise the membrane when tested in low concentration. Hence, it cannot be assumed that the substances under discussion here, such as steroids and vitamins, have lytic activities of physiological importance.

Steroids. Some natural steroids (for example, cholesterol) are constituents of cell membranes. It is therefore pertinent to ask if other steroids can be incorporated into cell membranes, and if so what effects this has on membrane properties. Finean (121) proposed that the bases of the phospholipids turn inward into the hydrocarbon layer (like a walking stick) and provide a point for the loose attachment of the 3C-OH of cholesterol. Willmer (387) presented ideas for predicting the effect of phospholipid or cholesterol incorporation into membranes on cell shape. He proposed that the physiological activity of steroids is controlled by the groups at C-3 and C-17. The steroid molecules are assumed to pack into the membrane so that the polar groups are exposed to the surface. "Pools of hydrophilia" are thus formed, and these modify permeability properties of the membrane. The theory predicts correctly that 5β-H-compounds are inactive whereas 5α-H-compounds are active. If the theory is correct it is surprising that 5β-H-steroids cause lysis, a process involving an initial permeability increase, whereas 5α-H-compounds do not (380, 381, 384). To this reviewer it would seem that some of the effects obtained with steroids in high concentration (10^{-3} to 10^{-4} M) may be explained by a mechanism similar to that proposed by Willmer. High concentrations of steroids cause lysis of red cells (268, 353, 384), activity being most pronounced in the 5β-H series. 5α-H steroids and those oxygenated at C11 or with α-OH at C17 are non-lytic. Non-lytic steroids augment the actions of lytic compounds (384). Steroids in high concentration also lyse mitochondria (37) and lysosomes (106, 380, 381), and in general those steroids with a 5β-H configuration are most active. Anti-inflammatory steroids (cortisone and cortisol) with polar groups in the C11 and C17 positions protect red cells against immune lysis (197) and lysosomes against lysis by ultraviolet irradiation (383). Finally of 75 steroids examined for anaesthetic properties all those showing activity had polar groups at the C3 and C17 or C3 and C20 or 21 positions (318). It would be interesting to know if steroid anaesthesia is a result of stabilisation or labilisation of the membrane.

Vitamin A. The lipophilic nature of the fat soluble vitamins makes them candidates for pharmacological and possibly physiological actions at cell membranes. Excess vitamin A causes lysis of red cells (100, 136, 237), release of proteolytic enzymes from lysosomes (98, 99), and degradation of the matrix of cartilage (101, 117, 238), and increases the speed of metamorphosis in Xenopus (378). The last two phenomena probably result from the release of lysosomal enzymes.

Vitamin A alcohol and vitamin A aldehyde are equipotent as haemolytics (10–20 μg/ml) whereas the acid has little activity. In this latter case the negative anion may be unable to penetrate the cell membrane. Hydrogenation of the rigid

vitamin A molecule removes haemolytic activity. Lysis by vitamin A of red cells is preceded by an increase in surface area and vacuolisation. The lytic event may involve a transformation from laminar form to micellar form with consequent increase in cell permeability. Monolayers containing high amounts of vitamin A become micellar (156). The lytic action of vitamin A is inhibited by vitamins E and K_1 although the rapid expansion of the cell membrane is not prevented (237). Vitamin E also stabilises monolayers containing vitamin A. Oxidation of vitamin A is thought not to be responsible for its lytic action although this cannot be excluded (237). Since low concentrations of vitamin A have a stabilising effect on cell membranes, it is not possible to say whether vitamin A normally controls the release of lysosomal enzymes.

Detergents. The most serious studies of the lytic actions of detergents have been performed on red cells (235, 236, 287, 288). Both anionic (287, 288) and cationic (235, 236) detergents cause haemolysis characterised by an initial rapid lysis followed by a prolonged phase of slow lysis. With anionic detergents (sodium alkyl sulphates) the degree of rapid haemolysis can be reduced to zero by repeated washing of the cells with saline (287). Cells treated in this way are rapidly lysed on adding the washings from fresh cells or on adding lecithin. This rapid type of lysis is not seen with saponins. The second, slow phase of haemolysis involves successively the adsorption of the detergent by lipoprotein, breakdown of the membrane, increased cation permeability, cell swelling, and finally lysis. Theoretical haemolytic-rate curves have been constructed on the assumption that lysis is a two-stage process (rapid adsorption and slow breakdown of the lipoprotein-detergent complex); they predict the observed behaviour (288). The reaction of the detergent with membrane lipids is thought to involve the phospholipids rather than cholesterol. Whether the rapid and slow phases involve binding with free and bound phospholipids is a matter of conjecture. Love (236) showed that the binding of dodecyl ammonium ions to red cells involved competition by hydrions for two kinds of receptor site. Ponder and Cox (277) showed that haemolysis must be considered as a progressive reaction in a heterogeneous system. The heterogeneity may correspond to red cells with and without free phospholipid.

Saponins. Saponins are known to form a complex with cholesterol and penetrate lipid monolayers only when this is present (139, 242, 313, 314). As most cell membranes contain cholesterol, it is not surprising that saponins are haemolytic. Dourmashkin *et al.* (103) treated a number of different cell membranes (liver cells, red cells, Rous sarcoma cells, and Rous sarcoma virus) with saponin and found a similar pattern of damage in each case. Holes or pits, approximately 80 Å in diameter and 35 to 50 Å deep, were formed; these were in an hexagonal array (140 Å separation), each pit being surrounded by a ring. Digitonin, which also complexes with cholesterol, did not produce this pattern but antagonised the effects of saponin. Pits or holes were not seen after tryptic digestion. These workers thought that the holes were formed by removing cholesterol from the membrane and considered their findings to be evidence for a cell membrane structure composed of cylindrical lipid micelles in a protein meshwork, as pro-

posed by Parpart and Ballentine (270). Treatment of sheep erythrocytes with rabbit Forssman antibody and guinea-pig complement also produces "holes" (194), but unlike the hexagonal holes produced by saponin they are circular. These are thought to be due to a highly localised change in the lipid layer due to the action of an activated C'3 complex (C'3 phase of the complement system causing a membrane lesion which impairs osmotic regulation). Two other groups (23, 137) considered that the patterns were due to addition of saponin to the membrane, rather than removal of cholesterol from the membrane. They could produce similar patterns in cholesterol-containing monolayers by treatment with saponin. Dourmashkin's electron micrographs showed the edges of the pits to have approximately 20 subunits. It was shown (137) that a micelle of 20 molecules of saponin with the hydrophilic sugar chains directed centrally and with the steroid nucleus associated with cholesterol in the lipid phase, would be approximately 80 Å in diameter. Thus it appears the rings seen in electron micrographs of cell membranes are not holes but saponin-containing micelles which cause sufficient disruption of the membrane structure to produce lysis.

It is not proposed to include a section on cell membrane stabilisers in this review. A comprehensive review on this subject has appeared recently (316). One group of membrane stabilisers, the local anaesthetics, have been dealt with under model systems (Section VI B 4). For an account of the biological effects of local anaesthetics the reader is referred to a review by Ritchie and Greengard (290).

F. Polyene antibiotics

There is very good evidence that the antifungal effects of polyene antibiotics, such as nystatin, amphotericin B and filipin, are due to their interaction with sterols present in the cell membranes of yeasts and fungi. The initial effect is an increase in cell permeability (142, 206, 241) leading to a loss of small ions and then of cytoplasmic contents. The binding of nystatin to protoplasts of Neurospora was studied by Kinsky (207). He found that extraction of Neurospora with acetone-ethanol prevented the binding of the antibiotic. Bacterial protoplasts (207) and fungi insensitive to polyene (220) failed to bind nystatin. The action of filipin on a number of polyene-sensitive fungi could be antagonised by sterols such as cholesterol and ergosterol (141). The most convincing evidence for the mode of action of polyenes comes from work on *Mycoplasma laidlawii*. The cell membranes of this organism contain only small amounts of steroids but the organism will incorporate steroids present in the culture medium (282). Only when *M. laidlawii* is grown in a medium rich in cholesterol does it become sensitive to lysis by amphotericin B (113) or by filipin (379).

Mammalian cells containing sterols also react with polyene antibiotics. Erythrocytes show potassium loss and even lysis on treatment with amphotericin B (53,208). Lysis of red cells by filipin is prevented by cholesterol, and to a lesser extent by lecithin (309). Nystatin and amphotericin B increase the permeability of the toad bladder to sodium ions and to urea when applied to the mucosal, but not serosal, surface (230). The bulk flow of water across this mem-

brane is little affected by polyenes. Although after amphotericin treatment sodium transport cannot be further stimulated by vasopressin, this does not imply a common site of action for these two. Lichtenstein and Leaf (230) proposed that the permeability of the bladder is controlled by two parallel membranes. Vasopressin is thought to affect both of these, controlling Na^+ permeability at the first barrier, and amphotericin is thought to remove the first Na^+ controlling barrier. In other actively transporting systems, stimulant effects with polyene antibiotics are not obtained. Active Na^+ transport across frog skin is only depressed by amphotericin (68). This presumably reflects a difference in the sterol composition of the relevant membranes. Mitochondrial membranes which have a low sterol:phospholipid ratio are insensitive to polyene antibiotics (209).

G. Proteolipids

It is now realised that proteolipids are concerned in the activity of a number of particulate enzyme systems and the same may be true of some drug receptors. Proteolipids are lipoproteins whose complex molecular structure is reflected in their solubility parameters; they are soluble in a mixture of chloroform and methanol, 2:1 (225). Treatment of some particulate enzyme systems with either lipolytic enzymes or organic solvents reduces or abolishes enzyme activity. Furthermore, repletion of such systems with lipid may restore activity to the enzyme system.

The membrane-bound ATPase responsible for movement of Na^+ and K^+ across cytoplasmic membranes (327) requires phospholipid for optimal activity (350, 351). The activity of this enzyme is reduced by treatment with phospholipases (278, 308, 334, 354) or neuraminidase (109). It has been suggested that phospholipids are necessary to induce a change in the tertiary structure of the inactive enzyme (351). Some steroids (for instance, progesterone and testosterone) can reduce the potassium loss from the heart caused by strophanthidin (219), and it was suggested that the steroids compete with the steroidal glycoside for association with ATPase. Aldosterone also antagonises cardiac glycosides, but in this case it is thought to stimulate protein synthesis, and hence ATPase formation.

Other enzyme systems are dependent on lipid for their integrity. Extraction of electron transport particles from beef heart mitochondria with iso-octane (65) or their treatment with phospholipase (8) removes succinoxidase activity. Activity could be restored by adding lipid, cytochrome C, and Co Q (65). DPNH oxidase activity was also removed by iso-octane extraction of electron transport particles (386). The nature of the cytochrome C-lipid complex has been investigated by several groups (70–72). Not all phospholipids are equally effective in forming a complex that can restore enzyme activity (70, 72). Phospholipids that contain ethanolamine and inositol form respectively 26:1 and 12:1 complexes with cytochrome C (71). After treatment with nitrous acid, cytochrome C no longer forms these complexes; this suggests that the binding occurs between the 32 free amino-groups of cytochrome C and the phosphate groups of the phospho-

lipids. Lecithin-cytochrome C complexes, which are ineffective in restoring enzymic activities, are still formed from cytochrome C treated with nitrous acid (71).

Other examples of enzyme systems dependent on lipid cofactors for activity are as follows: cytochrome oxidase (285), mitochondrial nicotinamide-adenine-dinucleotide transhydrogenase (273), D-β-hydroxybutyrate dehydrogenase (198), zinc-protoporphyrin chelatase in chromatophores and mitochondria (246), and bacterial galactosyl transferase (302). From the examples quoted it would seem to be a general rule for particulate enzyme systems to depend on lipid for their integrity. A similar dependence by drug receptors has been indicated in other parts of this review (see 67, 68, 95, 391; Section V B).

H. Modification of cell membrane composition

One technique, unexplored by pharmacologists, which would seem to be a powerful tool in exploring the participation of membrane lipids in drug action, is the opportunity to modify the lipid composition of the cell membrane. Modification could include the incorporation in the cell membrane of abnormally high amounts of naturally occurring lipids, or their exclusion; or abnormal lipids could be incorporated into the cell membrane.

There is ample evidence that such modifications are possible. For instance, red cells incubated with fatty acids incorporate these into phosphoglycerides. Unsaturated fatty acids are incorporated into the 2-ester position, whereas palmitic acid forms an ester in the 1 position (255). Abnormal bases [for example, propanolamine (15), dimethylaminoethanol (14) and guanidoethanol (59)] can be incorporated into the phospholipids of brain dispersions by incubation with cytidine-5′-diphosphate esters of these bases. It is of course necessary to demonstrate that such phospholipids occur in cell membranes after incubation.

Ansell and Chojnacki (14) have suggested that the increased susceptibility to seizures produced by pentylenetetrazole in animals fed 2-dimethylaminoethanol is due to the incorporation of this abnormal base into membrane phospholipids. Other examples of the modification of physiological and pharmacological behaviour by modifying membrane composition are rare. Some possible examples are given here. Serum lipids and fatty acids can restore beating in heart cells in culture, without acting as a specific energy source (154). Some fungi normally unaffected by polyene antibiotics become sensitive if grown in media containing cholesterol (113, 379). The phospholipid content, particularly of lecithin and phosphatidylserine, of platelets is reduced in the acquired type of thrombocytopathy and it is proposed that this reduction may account for the haemostatic defect (199).

VI. MODEL SYSTEMS

Advances in the understanding of cell membrane structure and function, together with the work of outstanding physical chemists like Langmuir and Adam, led, not surprisingly, to the construction of membrane "models" and "systems." Examination of the literature suggests that it is a relatively easy task

to construct a model, and furthermore that it is often possible to show effects of ions, enzymes, drugs and the like on such models. While the reviewer does not wish to disparage the results of such investigations he wishes to enter a plea for caution in interpretation of the results of such studies. It can be said at the outset that no model system completely mimics the properties of a cell membrane. It is the purpose of this section to examine how truly the properties of cell membranes are shown by models. All the models contain a single lipid or a mixture of lipids, and often the lipid molecules are arranged in an organised manner, as a monolayer, a bilayer, or a micelle. In some cases protein is adsorbed onto an oriented layer of lipid molecules. Model systems of five types will be discussed: (a) binding and partition systems, (b) monolayers, (c) bilayers, (d) spherulites, and (e) inert membranes impregnated with lipids. This system of classification is convenient here despite the fact that there are close similarities between various systems, for instance, between bilayers and spherulites and between lipid impregnated membranes and triphasic partition systems.

A. Binding and partition systems

The systems described in this section are the least sophisticated of the models but they may be useful in indicating the affinity of certain materials for lipids present in natural membranes. It cannot be concluded from binding studies *in vitro* that the particular binding reaction also occurs *in vivo* or that if it occurs it implies a physiological or pharmacological interaction. The concept of silent or non-effective receptors is well known in pharmacology (58, 108) and a specific but non-effective binding of drug molecules may limit the extent of a drug's effect. In these simple systems significance can be attached to the results only if they show a degree of specificity. Binding processes may be specific for a particular lipid (337) or a particular ion (211) or drug (163). Binding studies on simple models and systems provide only preliminary information for binding studies on complex models and on cell membranes themselves.

In the simplest experiments, lipids dissolved in non-polar solvents are shaken with an imiscible, usually aqueous, solution of drugs or salts. Transfer of materials into the lipid phase is compared with the transfer which occurs in the absence of dissolved lipid (44, 102, 114, 129, 211, 260, 280, 337, 390). Alternatively a three-phase system is used in which the lipid-containing phase separates two aqueous phases. In such systems the transfer of materials between the two aqueous phases can be studied (168). Yet another variant is to use micellar dispersions of a lipid in a continuous aqueous phase. Binding of ions or charged drug molecules to the micellar surface can mask the surface charge or expel H^+ and cause coagulation. In this way binding to lipids can be followed titrimetrically (340) or by nephelometry (2, 114). Finally, binding to lipids can be investigated by examining the crystal structure of complexes formed from lipids and drugs (115) or metallic salts (344).

Lipids with specific binding activities toward the important physiological cations K^+, Na^+, Ca^{++} and Mg^{++} have all been reported, together with competition phenomena between these ions. Phosphatidylethanolamine added to a

butanol phase facilitated the transport of sodium ions between two aqueous phases, but this property was not shown by either phosphatidylcholine or C18 hydrocarbons (168). All four lipid fractions isolated from swine erythrocytes (one was largely phosphatidylserine) bound Na^+ selectively, although some K^+ and Ca^{++} were also bound (211). Binding of Na^+ in these experiments was antagonized by previously incubating the red cells with adenosine before extraction, and it is of interest that adenosine causes Na^+ extrusion from red cells (213). A micellar dispersion of phosphatidylserine shows ion exchange properties with Na^+ resulting in a pH lowering of the continuous phase (2). Calcium ions were 100 times more effective in this respect than Na^+. Evidence for three ionic forms of this phospholipid was deduced from these studies (fig. 2).

Chloroform extracts of several lipids selectively bound K^+ rather than Na^+ when shaken with aqueous solution containing these cations (337). The ratio of the $[K^+]/[Na^+]$ value for the lipid phase to the value for the aqueous phase was greater than unity. Values of this ratio were 14.2 for phosphatidylserine, 7.5 for sphingomyelin, 8.9 for acetal phosphatide and 6.7 for cholesterol.

Calcium occupies a central position in excitation-contraction coupling (38, 304). In view of current ideas on the displacement ot membrane-bound calcium by drugs, the binding of calcium ions by lipids is of immediate pharmacological interest. A biphasic chloroform-methanol-water system was used to show the selective binding of Ca^{++} and Mg^{++} by cerebroside sulphate when low concentrations of Ca^{++}, Mg^{++}, Na^+ and K^+ were present in the hydrophilic phase (44). The calcium and magnesium salts formed were transported to the hydrophobic phase. When the salt concentrations in the hydrophilic phase were raised, water-soluble sodium and potassium lipid salts were formed. Calcium and magnesium ions are preferentially bound also by acidic brain lipids (126) and brain phosphoinositides (339). The binding of divalent metals by micellar dispersions of

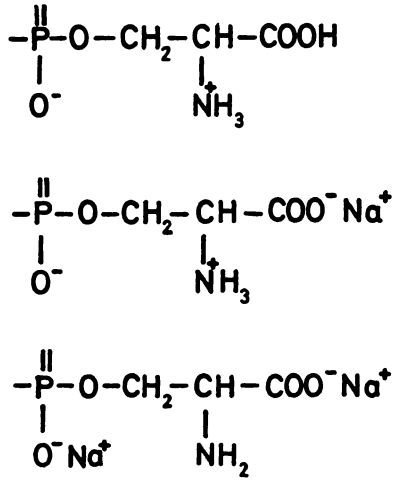

FIG. 2. Forms of phosphatidylserine. Isoelectric or acidic form (HPS), monosodium salt (NaPS), and disodium salt (Na_2PS).

phosphatidylserine and triphosphoinositide has been studied titrimetrically. Calcium, magnesium and nickel ions were bound, the latter forming the most stable complexes (340). It was considered that three of the six co-ordination bonds were involved in the formation of the "complex" (fig. 3). Such a structure is capable of forming mixed chelates with other ligands and may be the basis of metal-dependent lipoprotein interactions, for instance, the calcium-requiring prothrombin activation (204, 269). With triphosphoinositide the divalent metal probably chelates between two monoesterified phosphate groups.

Gangliosides from whole beef brain showed a preferential binding of Ca^{++} over Mg^{++} (129, 280). When shaken in a chloroform-methanol-water biphasic system, the gangliosides remained in the hydrophilic phase. Addition of Ca^{++} (three times the amount required to convert all the gangliosides to calcium salts) caused the movement of the calcium salts of the gangliosides to the hydrophobic phase. Sodium, potassium and magnesium ions could not transport the gangliosides to the hydrophobic phase but Na^{+} and Mg^{++} could antagonise the partitioning effect of Ca^{++}. The range of Ca^{++} concentrations used in this model are similar to those obtaining *in vivo* (10) and it seems possible that the solubility properties of gangliosides (280) and cerebrosides (44) *in vivo* depend upon local ionic concentrations. Gangliosides, cerebrosides and lipid extracts from hogs stomach and spinal cord were partitioned into the non-polar phase by Ca^{++} in a benzene-butanol-water biphasic system (390). Magnesium ions were more potent than K^{+} or Na^{+} in antagonising the Ca^{++} effect in case of tissue lipid extracts, whereas K^{+} was active in this respect for cerebrosides. The examples quoted are sufficient to make a case for the specific binding of ions by various lipids although no comment about their significance can be made from these results.

Fewer studies of the type discussed above have been made with drugs. Butanol-benzene extracts of hog stomach lipids partitioned 5-hydroxytryptamine (5HT) from the aqueous to hydrophobic phase in a biphasic system (102). This property was shared by other acidic lipids such as cholesterol phosphate, α-tocopheral acetate, and cerebroside sulphate. The transport of noradrenaline by this system was 40 times less than for 5HT. There is no reason to suppose that these results

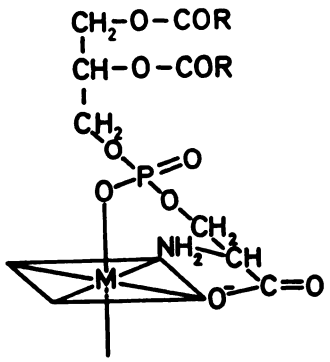

FIG. 3. Complex between metallic ion and phosphatidylserine in which three of the six coordination bonds are used.

represent any more than the combination of an acidic lipid anion with the drug cation to give a non-polar complex. The binding of histamine by lecithin and cephalin has also been reported (231). The transport of Ca^{++45} by stomach lipids into the hydrophobic phase of a benzene-butanol-water system was facilitated by 5HT (389). The effect was seen only when a large excess of Ca^{++} was present; this indicates that only a small fraction of the tissue lipids show this 5HT specificity. Crude lecithin solubilises *dl*-noradrenaline and *l*-adrenaline in an hydrophobic ether phase (260). It would be important to determine whether the biologically active isomers are preferentially transported. The anti-adrenaline drug Dibenamine binds to a cephalin fraction in rabbit aortic strips (96). The fact that this binding was antagonised by adrenaline indicates some degree of specificity of the binding site. The partition of five local anaesthetics between an aqueous solution and pieces of whole ox nerve was measured by Skou (330). There was a fair correlation between the partition coefficients of the local anaesthetics and their relative toxicity, as measured by the concentrations producing irreversible nerve blockade and red cell lysis (329), but correlation with the minimal blocking concentration (328) was poor.

In a series of papers van Heyningen (160–163) investigated the binding of drugs to a ganglioside obtained from nervous tissue. The ganglioside bound tetanus toxin (up to 20 times its weight of toxin), strychnine, brucine, thebaine, morphine, 5HT and chlorpromazine. In addition the binding of 5HT was reduced by lysergic acid diethylamide (LSD) and ergometrine. GABA, β-hydroxy GABA, histamine, adrenaline, noradrenaline and dopamine were not bound (163). Lysozyme and trypsin (161) and also albumin (163) were bound, but the complex dissociated at physiological salt concentrations. Hexosamine (164) and sialic acid residues (162) were necessary for toxin binding activity. Nineteen other glycolipids containing both hexosamine and sialic acid failed to bind toxin. Abnormal brain gangliosides (40) from children with Tay-Sachs' disease failed to bind the toxin although they had the normal complement of sialic acid residues and hexosamine. Thus the chemical requirements for tetanus toxin binding seem fairly specific. It is impossible on the present evidence to state that the effects produced by tetanus toxin result from binding with these gangliosides. Other observations argue that this is not so. Firstly, the binding of toxin is not temperature-dependent, yet, in poikilotherms the toxin is effective only when the temperature is raised (393). Secondly, strychnine, brucine, and thebaine, whose gross effects are like those of tetanus toxin and which are bound by the ganglioside, are considered to act postsynaptically on the inhibitory synapse, whereas the effect of the toxin is presynaptic (107).

Feinstein (114) showed that the actions of local anaesthetics (procaine, tetracaine and butacaine) and of Ca^{++} on phospholipid micelles were similar in that they caused flocculation and pH lowering of the continuous phase. The reaction is thought to involve the phosphate groups of acidic lipids, as it is also shown by phosphoinositides. The stoichiometry of the reaction is one local anaesthetic molecule or calcium ion to two lipid molecules. Although phosphatidylcholine, phosphatidylserine and phosphatidylethanolamine were all able to transport

Ca^{++} from an aqueous to hydrophobic phase of a biphasic system, and although in each case the effect was antagonised by local anaesthetics, these latter failed to coagulate phosphatidylcholine micelles at physiological pH. The internal neutralisation of the basic and acidic groups of phosphatidylcholine probably explains the failure of local anaesthetics to interact with micelles of this phosphatide. Both Ca^{++} and local anaesthetics are membrane stabilisers but their actions differ in detail (323). The inhibition of Ca^{++} transport by local anaesthetics increases as pH falls. This suggests that it is the charged form of the local anaesthetics which combines with the phospholipids. There is evidence that it is the charged form of local anaesthetics which affects excitable cell membranes (91, 289, 342). The potency of local anaesthetics in releasing H^+ from cephalin dispersions corresponded in a general way to their potency as local anaesthetics. Most were less effective in releasing H^+ than was Ca^{++} (115).

Molecular complexes of some local anaesthetics (procaine and phenacaine) with bis-*p*-nitrophenylphosphate have recently been isolated (115) and the crystal structure of these would be worth studying. However, many proteins (pepsin and β casein) also contain phosphodiester groupings which can react with local anaesthetics. It seems important, therefore, to isolate and characterise complexes of local anaesthetics from living tissues to decide the nature of the local anaesthetic "receptor." Recently the crystal structure of the L-α-glycerophosphorylcholine cadmium chloride trihydrate has been examined (344). Studies of this type may indicate the conformations which can be assumed by phospholipids in living tissues. In this instance the glycerol residue had a gauche-gauche and the choline residue a gauche conformation. The structure of this complex is similar to that deduced by Finean (122) for phospholipids.

B. Monolayers

Lipid monolayers represent the simplest type of membrane model having an organised structure. If a small quantity of lipid, dissolved in an organic solvent, is dropped onto the surface of an aqueous salt solution, the lipid molecules spread out across the surface with their polar (head) groups in the aqueous phase and their hydrophobic tails above the surface. If the area upon which lipid molecules can "float" is now gradually reduced, the molecules are pushed closer and closer together until finally the surface film collapses. The limiting area per molecule is the same for palmitic, stearic and cerotic acids (that is, for fatty acids with carbon chains of from 16 to 26 atoms). This limiting area is believed to be the cross sectional area of a hydrocarbon chain. The orientation of lipids in a monomolecular film is similar to that proposed for cell membranes.

A discussion of the theory of monolayers is not appropriate in this review and interested readers should consult standard works by Adam (3) and Adamson (5). However, the various parameters which may be measured for monolayers will be mentioned. These are surface concentration, surface pressure, surface tension, surface potential and surface viscosity. Measurements of surface concentration are expressed as area per mole, area per molecule, or perhaps most usefully for biological lipids as area per chain. Surface concentration gives an indication of

the degree of packing and reaches an upper limit at about 20 Å^2 per hydrocarbon chain. Measurements of surface pressure represent the repulsive forces which are tending to disperse the lipid molecules. If the surface pressure becomes very great, lipid molecules may be ejected from the monolayer into the hypophase, or duplex films or lipid lenses may be formed. A substance that becomes concentrated at an interface lowers its surface tension as indicated by the Gibbs Equation.

$$\Gamma = -C/RT\ d\gamma/dc$$

where Γ represents the excess surface concentration, C the bulk concentration and $d\gamma/dc$ the change in interfacial tension with increasing concentration. This equation forms the basis for the theory of penetration of drugs into lipid monolayers. An example is given by Pethica (274). Lipid monolayers represent an extreme case of adsorption at an interface in which the surface tension lowering is equivalent to the surface pressure exerted by the monolayer.

Measurements of surface potential are made between an electrode immersed in the hypophase and a polonium probe suspended above the monolayer. The latter ionises the air above the monolayer sufficiently to make it conducting. Changes in surface potential occur with binding of ions or charged drugs to lipid monolayers. Electrically some monolayers behave as an admittance network, and show "action potentials" when stimulated (249). Surface viscosity is usually measured by the torque necessary to maintain a constant speed of rotation, and gives a measure of the cohesion between the lipid molecules.

When dealing with the permeability of monolayers to drug molecules, it must be remembered that the properties of a monolayer at the point of a particular collision may be very different from the average properties of the monolayer as represented by the overall surface pressure and charge. Consequently the permeability of monolayers is greater than for solids as the former depend on instantaneous rather than average properties. Blank has given a theoretical treatment of this problem (33). The instantaneous surface pressure, π, of an area, A, in a monolayer depends on the average surface pressure, $\bar{\pi}$, and the surface compressibility, C_s. That is, π can vary greatly when the surface compressibility is high. T is the absolute temperature and k the Boltzman constant.

$$\pi = \bar{\pi}\left(1 \pm \sqrt{\frac{kT}{A} Cs}\right)$$

Finally, mention must be made of the states of monolayers. In the gaseous state they are very compressible, obey the equation of state for an ideal gas, and have low surface pressures and large area per molecule. There are several forms of the liquid state, the L1 or liquid expanded state being comparable to the equilibrium existing between a liquid and its vapour. The L2 or liquid condensed state shows a linear relation between surface pressure and area per molecule and corresponds to a monolayer in which the head groups are closely packed. In the solid state, monolayers have a low compressibility and show a linear relation between the surface pressure and area per molecule. The properties conferred on

monolayers, and perhaps on biological membranes too, by lipids of defined chemical structure can now be investigated, since the synthesis of many defined phosphatides has now been achieved. For instance the force-area curves for phosphatidic acid, phosphatidylethanolamine, phosphatidylserine and phosphatidylcholine containing identical fatty acids showed only small differences. In a particular phosphatide class shortening of the chain and the presence of double bonds greatly expanded the films. Force-area curves for isomeric, mixed acid L-α-phosphatides were identical (83).

The implication of monolayer studies to pharmacology will be discussed under the following headings: (1) molecular association in monolayers, (2) interaction with ions, (3) action of phospholipases, (4) membrane stabilisers and labilisers, and (5) miscellaneous drug studies.

1. *Molecular associations in monolayers.* Many reports of molecular associations for certain stoichiometric proportions of the constituents of monolayers are to be found in the literature (87). The formation of complexes or molecular associations between lipid constituents of a monolayer is concluded from measurements of molecular area. If the area per molecule of a lipid in a mixed monolayer is greater or less than its area in a pure monolayer, then molecular association is presumed to have occurred. The condensing effect of cholesterol on lecithin and fatty acid monolayers is well known (81, 223). Associations between pairs of fatty acids, and fatty acids and phospholipids have also been widely reported (32, 88–90). Associations of monolayer lipids may provide information about the organisation of lipids in cell membranes and particularly about the effects upon this organisation of changing the ratio of various lipids within a cell membrane. Willmer (387) has reported both a theoretical and a practical approach to this problem.

One example of a molecular association, that between lecithin and cholesterol (31), will be discussed in detail. Monolayers of cholesterol are only slightly compressible and at a surface pressure of 5 dynes/cm each molecule occupies 38 Å^2. Egg lecithin under the same conditions has an area of 96 Å^2 per molecule. If no association occurs between these two substances, the apparent area per molecule for mixtures of these two lipids should obey a simple additivity rule. However the experimental values are always less than predicted values for lecithin-cholesterol mixtures. Calculation shows that the results are in accord with the formation of two association complexes, lecithin$_2$·cholesterol and lecithin·cholesterol$_2$, the proportions of which depend on the relative molar ratios in the monolayer. The lecithin$_2$·cholesterol complex has a theoretical area of 326 Å^2 and an actual area 284 Å^2, a contraction of 42 Å^2. The lecithin·cholesterol$_2$ complex has a theoretical area of 210 Å^2 and an actual area of 164 Å^2, a contraction of 46 Å^2. Addition of cholesterol or lecithin to a mixed cholesterol-lecithin monolayer thus alters the time-averaged free space in the monolayer. Associations such as these may determine the permeabilities of biological membranes (134, 374).

Molecular associations are thought to represent not chemical complexes with definite dissociation constants but rather the packing patterns of lipids in mono-

layers (88) held together with van der Waals forces. Associations can also occur between monolayer lipids and substances penetrating the monolayer from the hypophase (312). For instance, it is considered that stoichiometric complexes are formed between cholesterol and long chain sulphates. Whether or not stoichiometric complexes can be formed between insoluble lipids and soluble penetrating molecules is a controversy yet unsettled. A recent paper (243) briefly summarises the various points of view.

2. *Interaction with ions.* The binding of metal ions to lipids was dealt with extensively in Section VI A. The remarks on interaction of monolayers with ions will deal mainly with Ca^{++} because of the extreme physiological-pharmacological importance of this ion. Phosphatidylethanolamine and phosphatidylserine monolayers are condensed by Ca^{++}, that is, the surface pressure is reduced. The process is inhibited by high (600 mM) but not low (10 mM) concentrations of Na^{+} and K^{+} (301). It seems possible that charged lipids such as phosphatidylserine and phosphatidylethanolamine act as cation exchangers at cell surfaces. Monolayers of stearic acid, animal lecithin (AL) and synthetic lecithin [PL, L-α-(β,γ-dipalmitoyl) lecithin] reacted to calcium ions, present in the hypophase, in different ways (205, 244). Stearic acid monolayers were found to saturate readily with the formation of calcium stearate. PL and AL monolayers saturated at near physiological Ca^{++} concentration with the formation of a mixed lecithin and calcium lecithin film. Again K^{+} and Na^{+} displace calcium from these monolayers when present in great excess.

Binding of metal ions to lipid monolayers increases their surface potential. The increase in surface potential caused by Ca^{++} depends on the degree of unsaturation of the fatty acid chains in lecithins (319). Comparison between dipalmitoyl, egg and yeast lecithins showed that the highly unsaturated yeast lecithin bound less Ca^{++}. It is thought that the unsaturated lecithins require a greater area/molecule and that this allows internal neutralisation in the polar head group. In closely packed fully saturated lecithin molecules Ca^{++} can chelate with phosphate groups. Pressure-area curves for lecithins and plasmalogen are not affected by Na^{+}, K^{+}, Li^{+}, Mg^{++}, Ca^{++}, Sr^{++}, Ba^{++} or Al^{+++} in the hypophase, but cardiolipin monolayers show a 10 to 13% contraction when treated with Ca^{++}, although the limiting area remains the same (319). Here calcium is thought to decrease the intramolecular distance at low surface pressures (fig. 4). The nature of the chelated cation also contributes to surface pressure. For instance at 120 $Å^2$ per cardiolipin molecule the surface pressure increases in the order Mg^{++}, Ca^{++}, Sr^{++} and Ba^{++}, the order of increasing ionic radii.

3. *The actions of phospholipases.* The hydrolysis by phospholipases of monolayers of phospholipids poses some problems which may be important for understanding drug interaction at cell membranes. The electrical diffuse double layer associated with an orientated lipid monolayer may affect the approach and orientation of the enzyme to the surface. In addition, the local pH at the surface may differ from the bulk pH of the medium and so affect the hydrolytic reaction. The importance of lipid and enzyme orientation is illustrated by the failure of phospholipase C to attack the water soluble phospholipids L-α-diacetyl lecithin and L-α-dibutyryl lecithin (82).

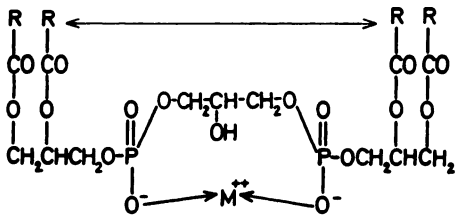

FIG. 4. Complex between a divalent metal cation and cardiolipin. *Arrow* indicates the intramolecular distance.

The actions of phospholipase B on monolayers and emulsions (which can be regarded as multi-monolayers separating aqueous and lipid phases) were studied by Dawson and his colleagues (19, 20, 75, 76). Emulsions and monolayers were attacked only when the surface had a net negative charge. This was achieved by inclusion of monophosphoinositide, cardiolipin or dicetylphosphoric acid. Activity could be abolished by inclusion of long chain cations or charge neutralisation by divalent metal ions such as Ca^{++}. At low surface pressures slow hydrolysis of monolayers was possible even in the absence of a negative charge. Presumably under these conditions the enzyme could easily reach the susceptible acyl ester.

By contrast, phospholipase C hydrolyses lecithin emulsions in the presence of Ca^{++} or if the monolayer is positively charged with stearylamine (21). High pressure films were only attacked in the presence of Ca^{++} but some hydrolysis without Ca^{++} occurred at low pressures. After 60% hydrolysis lecithin emulsions became refractory to phospholipase C; this was believed to be due to charge reversal on the micelles caused by the diglyceride formed. Monolayers of lecithin, phosphatidylinositol and phosphatidylethanolamine had an absolute requirement of Ca^{++} for hydrolysis by phospholipase A no matter what the surface pressure (78). The actions of phospholipases on monolayers has been reviewed by Dawson (77).

4. *Membrane stabilisers and labilisers.* The actions of local anaesthetics on lipid monolayers has been studied by Skou (331–333, 335). Local anaesthetics increase the surface pressure at constant area of a stearic acid monolayer, although the limiting area is unchanged. This would indicate that local anaesthetic molecules can penetrate monolayers of stearic acid but that they are expelled at high surface pressures. The ability to penetrate, blocking potency and toxicity of the drugs were of the same order but the correlation was poor (331). When tissue lipids from sciatic nerves were used, monolayer penetration, calculated from the Gibbs relation, was well correlated with the blocking potency of procaine, cocaine, tropacocaine, tetracaine and diburcaine (332). Similar results were obtained for various alkyl and aryl alcohols (26, 333). The pH dependence of penetration and blocking activity for cocaine was also well correlated, both parameters increasing with increasing pH (332). Thus it appears that the uncharged form of the local anaesthetic is the active moiety. This contrasts with the results of Feinstein (114), who found that the charged form was more effective in inhibiting the phospholipid transport of Ca^{++}. It would seem that two processes are important for local anaesthetic activity, the penetration of the local

anaesthetic to the axonal membrane and the stabilising action on the membrane. The optimal balance of hydrophilic and hydrophobic properties and the lipids involved may be different for these two processes. Certainly the onset and degree of local anaesthesia is facilitated by an alkaline pH (332), that is, when the drugs are in the uncharged form, but there is evidence that it is the cationic form of local anaesthetics that blocks the axon (91, 289, 342). Local anaesthetic activity due to the cationic form of the drug, rate limited by penetration (dependent mainly upon the uncharged form) would seem to provide a unitary hypothesis for the results on model systems. The action of local anaesthetics on model systems has been recently reviewed (290). Shanes (320) has proposed that local anaesthetics block conduction by increasing the "packing" of the lipid molecules in the membrane, and this results in the closure of pores. Bangham (18), however, considered the effect is more likely due to modification of the compositional lipid mosaic of the membrane.

The effects of membrane labilisers on lipid monolayers have been compared with the effects of local anaesthetics (132, 133, 324). Veratrum alkaloids apparently reduced the area per molecule in a monolayer of stearic acid (324). This was shown to be due to interfacial dissolution; that is, both stearate and adsorbed alkaloid leave the surface film and enter the hypophase (133). This effect is antagonised by calcium ions, low pH and procaine. At low pH the ionisation of the lipid is suppressed, and this inhibits the ion-ion interaction between the alkaloid and the lipid. Calcium ions and procaine interfere by competing with the alkaloids for interaction with the lipids. Gershfeld studied the effects of procaine and veratrum alkaloids on monolayers of octodecyl phosphate (132). This latter substance slowly desorbs from monolayers. The desorption is less with increasing surface pressure due to increasing interactions between the hydrocarbon chains at high surface pressures. Both procaine (10^{-4} M) and veratrine increase desorption from octadecyl phosphate monolayers. Procaine (10^{-4} M) causes an initial apparent increase in area/molecule of from 29 $Å^2$ to 100 $Å^2$, the difference, 71 $Å^2$, being the area of a procaine molecule. The horizontal adsorption of procaine thus separates the hydrocarbon chains of the lipid molecules so that the energy required for desorption is reduced. At concentrations greater than 10^{-4} M, procaine adsorbs to the undersurface of the monolayer and prevents desorption. However, the penetration of procaine into the monolayer is stoichiometrically limited, whereas that for veratrine increases with increasing concentration, leading eventually to breakdown of the membrane. The increased Na^+ and K^+ exchange (343) and repetitive activity (385) seen in nerves treated with veratrine alkaloids and the effects these alkaloids have on monolayers probably have a common causation. Membrane stabilisation by drugs has been reviewed recently (316).

5. *Miscellaneous drug studies.* Molecules with both polar and apolar regions become concentrated at interfaces. Therefore it is easy to obtain effects of many compounds on monolayers which may be irrelevant or impossible to interpret in relation to the pharmacology of these compounds. Plant growth regulators, for instance, increase the surface pressure of monolayers (155). As the authors quite rightly point out there is no correlation between their ability to penetrate

a monolayer and their biological activity. The monolayer is therefore an inappropriate model for the investigations of these substances. This type of experiment may be contrasted with that of Bangham, Rees and Shotlander (25), who found a good correlation between the ability of antihistamines to penetrate lecithin-cholesterol monolayers and their ability to antagonise carbon tetrachloride induced liver necrosis in rats. A more difficult type of example is the action of acetylcholine on lecithin monolayers (195). Acetylcholine (1 mM) and K^+ (8 mM) cause an increase in surface pressure at constant area. Does this result have biological significance? If it was shown that the biologically active *d*-isomer of methacholine also behaved like acetylcholine in this system, and that the *l*-isomer was inactive, then the result obtained with acetylcholine would be of greater pharmacological interest. In the remainder of this section, therefore, only results with probable or proven relevance to pharmacological action will be discussed.

Chlorpromazine has been found to penetrate and stabilise insoluble monomolecular films (more than does its sulphoxide) at bulk concentrations which do not have measurable surface activity (394). Chlorpromazine is known to inhibit mitochondrial swelling (338) and red cell lysis (127), whereas its sulphoxide does not have these properties. This type of study is important because there is a good correlation between the biological and physical effects. Progesterone has been found to penetrate monolayers of cholesterol and β,γ-dipalmityl-L-α-lecithin in amounts of 1 to 4% of the total membrane lipid (355). It was not possible to decide whether both or any one of the two polar groups of progesterone was in contact with the hydrophilic phase. The biological effects of penetrating steroid molecules with multiple polar groupings is dealt with elsewhere in this review (387; Section V E). Saponins are related to the steroids but in addition possess a hydrophilic sugar chain. Saponin causes the expansion of monolayers only if these contain cholesterol (139, 242, 313, 314). Recent work suggests that saponins cause a change from a laminar to a micellar structure in monolayers. Evidence from electron microscopy suggests that the cholesterol molecules and saponin molecules form micelles, the diameter of the lipid portion being 35 Å with the hydrophilic sugar moiety of the saponins and the hydroxy group on the cholesterol penetrating the aqueous phase (239) (fig. 5). These micelles are then thought to organise in either an hexagonal, helical or lamellar array (24, 239) to produce the various patterns seen with the electron microscope.

A similar transformation from lamellar to micellar form may underlie the actions of polyene antibiotics on monolayers. Filipin and nystatin both penetrate monolayers of either ergosterol or cholesterol at pressures greater than the collapse pressures of monolayers of the antibiotics. No increase of surface pressure with these agents is seen with phospholipid monolayers (84). By contrast some drugs react preferentially with phospholipid rather than cholesterol monolayers. Vitamin A_1 alcohol, vitamin A_1 and vitamin A_2 alcohol can all penetrate lecithin/cholesterol films at constant area with initial pressures of 30 dynes per cm (22). This pressure is greater than the collapse pressure of monolayers of the vitamin A derivatives. In addition vitamin A_1 alcohol can cause a large increase in area

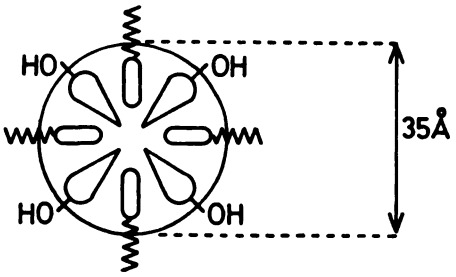

FIG. 5. Mixed saponin cholesterol micelle. (After Lucy and Glauert, J. Mol. Biol. 8: 727–748, 1964). The pear-shaped symbol represents cholesterol. The zig-zag line attached to the steroidal saponin nucleus represents the hydrophilic sugar chain.

at a constant pressure of 30 dynes per cm. Vitamin A_1 alcohol penetrates lecithin/cholesterol or lecithin monolayers at pressures considerably above those for pure cholesterol monolayers suggesting that the interaction of the vitamins is primarily with lecithin. The haemolytic actions of vitamin A_1 may be due to interaction with lecithin in red cell membranes.

C. Bilayers

Lipid bilayers represent the most realistic approach to the problem of cell membrane models. They have been studied by several groups of workers (30, 149–153, 156, 191–193, 251–254) but originally by Mueller, Rudin, Ti Tien and Westcott (252). Lipid bilayers possess an ordered bimolecular array of lipid molecules separating two aqueous phases with the polar groups of the lipids orientated in the aqueous phases. Thus the core of these models consists entirely of hydrocarbon chains. Attempts have been made to adsorb protein onto bilayers and to determine what changes in properties occur (30, 151, 252). Most investigators have concerned themselves with the electrical properties of such bilayers but isolated attempts to study drug effects on such models have been made (395). Essentially the procedure for formation is to brush a solution of lipid (dissolved in a solvent, usually n-decane) across a hole in a Teflon septum separating two aqueous solutions. The lipid material immediately starts to thin, as shown by the formation of interference fringes, the excess lipid forming a torus around the rim of the hole. Sometimes tetradecane is added to the lipid solution to prevent critical turbulence occurring during draining which is liable to rupture the membrane. Interference colours are replaced by a non-reflecting (secondary black) structure which is the bilayer. The area of such membranes is generally between 1 and 10 mm^2 but recently a modified method for the formation of bilayers of 50 mm^2 has been described (30). A technique superior to the "brush technique" for forming bilayers is now available (358).

The thickness of bilayers prepared in this way is usually less than 100 Å. Values of 60 to 90 Å (252), 61 ± 10 Å (191), later corrected to 72 ± 10 Å (356, 359), and 45 to 48 Å (149, 151) have been reported. The variation in values is due to the lipid used and probably the method of determination [by electron micro-

scope (252), capacitance data (149), and optical methods (191)]. However, all the values represent approximately the length of two hydrocarbon chains. Fully extended distearyl phosphatidylcholine for instance has a chain length of 37 Å. Tilting of the hydrocarbon chains from the normal would of course reduce the membrane thickness.

Bilayer membrane capacitances have been reported as 0.46 μF per cm^2 (egg lecithin-cholesterol-n-decane membrane) (152); 0.38 μF per cm^2 (egg lecithin-n-decane membrane) (149, 153); 1.0 μF per cm^2 (split lipids-α-tocopherol-cholesterol membrane) (254). Addition of cholesterol to lecithin produced bilayers of constant capacity until the cholesterol/lecithin ratio reached 0.8, after which the capacitance rose to a limiting value of 0.6 μF per cm^2 (151). The capacitance of the bilayers was not frequency dependent. Membrane conductance values for bilayers, usually separating 0.1 M NaCl, ranged from 10^{-6} to 10^{-9} mho per cm^2 (30, 152, 193, 252, 254). The relation between membrane conductance and ionic strength was not linear (152) and may show a limiting conductance value at high ionic strength. Extreme caution is required with conductance measurements in bilayers as defects at the rim of bilayers increase the conductance values. Both capacitance and conductance are generally lower for bilayers than for cell membranes [0.5 to 1.0 μF/cm^2 and 10^{-3} mho/cm^2 (62, 165, 271)] and furthermore the capacitance of cell membranes may be frequency dependent (315), which is not the case for lipid bilayers (150). How ions cross lipid bilayers (or indeed biological membranes) is an open question. Evidence from bilayers suggests that ion transport involves a lipid-ion interaction. In a bilayer composed of equal parts of egg lecithin and n-decane the interior hydrocarbon region contains 6 ml of empty volume per mole equivalent of —CH_2— (39). Addition of 2,2,4-trimethylpentane instead of n-decane increases the empty volume in the bilayer two times. If the bilayer simply acted as a sieve the conductance values of such bilayers would rise. Incorporation of 2,2,4-trimethylpentane, however, had no effect on membrane conductance (152). The capacitance and conductance of lipid bilayers is considered to be due almost entirely to the hydrocarbon region (150). How then do lipid bilayers compare with cell membranes? If bilayers contained polar pores, occupying 1 % of the membrane area and with a dielectric constant of 80, the membrane would contain an additional parallel capacitance of 0.16 μF per cm^2 and the conductance would rise by a factor of 10^6 (151). Such bilayers would have capacitances and conductances within the biological range.

Permeability coefficients for bilayers with and without an osmotic gradient have been determined. In the absence of an osmotic gradient a value of 4.4 $\pm$ 0.5 μsec^{-1} was found (by diffusion exchange of tritiated water, THO), whereas when accompanied by a net water flux a range of values from 17.3 to 104 μsec^{-1} has been obtained (192). These values are lower than those reported for red cells (375). From the biologist's stand point the most interesting and least understood membrane is the original bilayer described by Mueller *et al.* (252). Addition of some proteins to this membrane lowered the membrane resistance to 10^3 ohms per cm^2, caused the development of a membrane potential and produced electrical excitability, the level of which was determined by the Ca^{++} concentration. The

membrane showed a gating reaction to d.c. electrical stimulation, a threshold stimulus causing a 5-fold increase in conductance. Seufert (317) has described the effects of cationic, anionic and amphoteric detergents on lipid bilayers. All three types of detergent lowered the bilayer resistance and caused the appearance of a membrane potential, but the bilayers failed to show electrokinetic properties. The changes caused by detergents were considered to be due to a laminar-to-micellar transformation in which negatively charged lipid micelles formed cation-selective pores. Proteins may also be able to cause this transition, as suggested by Trams (369). The initiation of a drug effect on an artificial membrane with adsorbed receptor protein would seem to present an intriguing challenge to the molecular pharmacologist. Incorporation of receptor protein models, such as the one recently described by Burgen (47), to lipid bilayers may be a useful approach. Recently (57) bilipid layers have been used to detect antigen-antibody and enzyme-substrate interactions. Lipid bilayers of ox brain lipids together with α-tocopherol and cholesterol were formed and antigens adsorbed onto them. Subsequent addition of the antibody caused a transient increase in the membrane conductance. Thus it appears that the interaction of antigen with antibody was able to induce a secondary change in the structure of the bilayer.

One clear example of drug action on a lipid bilayer was found in the literature (395). Bilayers containing cholesterol and lecithin in equal molar ratio were disrupted by the polyene antibiotics filipin and nystatin. The antibiotics had no effect on bilayers of pure lecithin or those in which the cholesterol/lecithin ratio was 1/10 or less. Evidence presented elsewhere in this review (Section V F) suggests that filipin and nystatin complex with cholesterol to cause cell membrane dysfunction. Here too the cholesterol/lecithin ratio is important. The low cholesterol content of mitochondria makes them more resistant than cell membranes to lysis by polyene antibiotics (209).

D. Spherulites

Recently Bangham and his group (27, 28) have used a new model system which does not require the inclusion of lipid solvent or filler hydrocarbon. Simply a phospholipid or a mixture of lipids is allowed to swell in an aqueous salt solution. Spherulites consisting of concentric shells of bilipid membranes separated by aqueous compartments are formed. Radioactive salts or other materials can be incorporated in the spherulites by inclusion in the swelling solution. From thermodynamic considerations it was concluded that the bilipid spheres were completely closed. The width of the bilipid layers was found from electron microscopy to be approximately 44 Å (24). The surface charge on the spherulites could be controlled by varying the proportion of positively and negatively charged lipids in the phospholipid mixture before swelling. The distance between the concentric shells of this model was dependent on the surface charge. This model system shows permselectivity (27), being several times more permeable to anions than cations. Chloride and iodide ions had about equal permeabilities and they were not influenced by surface charge. Fluoride, nitrate, and sulphate ions had lower permeabilities than chloride ions. Water was found to exchange as fast as chlo-

ride. Cations (K^+, Na^+, Li^+ and Rb^+) all showed the same permeability, but this could be enhanced by incorporation of negatively charged lipids (*e.g.*, dicetylphosphoric acid) into the membranes, and inhibited with positively charged lipids (*e.g.*, stearylamine). As little as 5 moles per cent of long chain cation was sufficient to completely inhibit cation efflux.

The effect of several groups of drugs on this model have been investigated (26, 28). The increase in cation release from lecithin-cholesterol-dicetylphosphoric acid spherulites caused by some steroids was correlated with their ability to liberate acid phosphatase from lysosomes. This was true of androsterone, desoxycorticosterone, corticosterone, progesterone, etiocholanalone, pregnanoline and diethylstilboestrol. Cortisol and cortisone, however, reduced cation leakage and inhibited the effects of diethylstilboestrol. This effect of the anti-inflammatory steroids correlates with their stabilising effect on lysosomes and red cells. Streptolysin S also caused cation leakage from the model as it did from lysosomes and red cells (382), but this effect was not antagonised by cortisone. Although high concentrations of steroids are required, the close parallelism between their actions here and on biological membranes is encouraging.

Straight chain *n*-alkyl alcohols (n = 4 to 8) caused an increased K^+ efflux from spherulites previously loaded with this ion (26). At constant thermodynamic activity (118) there was an increased efflux with a reduction in chain length. The ratio of activities of different alcohols producing equal K^+ effluxes was well correlated with the ratio of activities causing nerve fibre narcosis. Chloroform and ether also produced K^+ loss at concentrations related to those producing anaesthetic activity. In contrast local anaesthetics were found to reduce K^+ efflux from this model system (26).

E. Inert membranes impregnated with lipid.

Millipore filters impregnated with various lipids have been used as membrane models. They have the advantage of ease of handling over other model systems and can, for instance, be clamped between two aqueous compartments for permeability studies. It is probably correct to regard such models as having two oriented lipid layers, in contact with the aqueous phases, separated by an amorphous lipid phase. The resemblance to a triphasic partition system is obvious. The electrical resistance of millipore filters impregnated with a mixture of animal cephalin and cholesterol was raised by Ca^{++} and lowered by Na^+ and K^+ (366). The resistance was linearly related to the water flux through the membrane and inversely related to the bound Ca/K ratio of the membrane (228, 248). Adsorbtion of protamine onto these models had effects like those of Ca^{++}. Protamine is also known to affect the permselectivity of cellulose membranes (336). The permeability of these membranes to K^+ and Na^+ was reduced by Ca^{++} (248) and phosphatidylserine was shown to be the main phosphatide responsible for these properties (258). This negatively charged lipid is thought to bind reversibly with Na^+, K^+ and Ca^{++} with a preference for Ca^{++}. Thus the oriented surface layer can act as a cation exchanger, with high concentrations of K^+ being required to displace Ca^{++}. Tobias (366) considered that catelectronically propelled

K^+ may displace bound Ca^{++} during electrical activity in excitable biological membranes. The disappointing feature of this model is its failure to differentiate between K^+ and Na^+. Red cell ghosts, for example, behave as a cation exchanger towards Ca^{++} but in addition show a preference for K^+ over Na^+ (305). The lipid-impregnated millipore filter has also been used to show increased electrical resistance associated with binding of local anaesthetics (114).

F. Conclusions

To date model systems have done little to clarify the mechanism of drug action. In a few instances, where it is clear that drugs react with membrane lipids, as do the polyene antibiotics, the evidence is convincing. For other groups such as local anaesthetics, veratrine and corticoids, the evidence for the implication of lipids in drug action is impressive but not conclusive. For yet other drugs the results are interesting but await further appraisal. Model systems certainly can bind, transport and exchange cations and often the models show some specificity. It is not yet possible to say if these processes are important in the cell membrane until the biological processes of ion movement through membranes are better understood.

VII. THEORIES OF DRUG ACTION

There is no adequate unitary theory of drug action on cell membranes. Membrane receptors are generally considered to be proteins which undergo a conformational, allosteric or other change on reaction with the drug. Substantial evidence for this view is not available and it is based more on an intuitive guess made from a knowledge of enzyme-substrate interactions. Several theories of drug action involving membrane lipids have been put forward (73, 97, 105, 377). While the reviewer does not suggest these provide an answer, such speculations provide a framework for the design of new experiments.

Tobias (365) has described a theory of the ultrastructural changes which occur in excitable cell membranes when they change from the resting to excited state. Although this theory was not applied to drug action it may be usefully included here. It was proposed that the outwardly directed catelectronic stimulating current moves potassium ions from the axoplasm to the membrane phase. This has been shown for the squid axon (166). The local increased potassium ion concentration then displaces calcium bound to phosphatidylserine in the membrane. There is ample evidence that calcium is bound at the cell surface in excitable tissues (*e.g.*, 306) and that phosphatidylserine is present in cell membranes (42, 210). Phosphatidylserine will bind calcium as described earlier for monolayer models (301), and the calcium can then be displaced by K^+ (258, 366). Tobias proposed that displacement of Ca^{++} by K^+ was followed by increased membrane hydration together with changes in membrane geometry leading to an increase in the ionic conductance of the membrane. In polarised axons water migrates cathodally (361) and the water content of axons increases after a period of activity (46). A great deal more evidence for this hypothesis will be found in the original paper (365). In many ways the theory is satisfactory although it begs the

question of the precise ultrastructural changes involved. The membrane potential serves to keep the K^+ out of the membrane phase during inactivity; however, depolarised squid axons remain excitable providing the internal potassium concentration is low (257). On this hypothesis excitability depends on the integrity of the phospholipid:Ca^{++} complex. When the membrane potential is low the complex remains intact provided that the [K^+] is low. Tobias extended his hypothesis to explain the prolonged action potentials produced in nerve by tetraethylammonium chloride and nickel ions (352). He supposed that tetraethylammonium chloride or Ni^{++} were unable to displace bound membrane calcium but that once this was displaced by K^+ these agents competed with K^+ for combination with the phospholipids. These stable (340) phospholipid complexes then stabilise the structure in the high resistance state and hinder repolarisation by preventing the rise in K^+ conductance. The theory described by Tobias could be applied equally to drugs which directly displace bound membrane calcium. The theory of permeability change put forward by Shanes (322) is similar to that presented above and based on the fact that permeability of excitable cell membranes depends on the membrane potential (321). Shanes proposes that the large voltage gradients (100,000 v/cm) which exist across membranes affect the distribution of lipophilic anions and cations within the membranes. On depolarisation the gradient is reduced, and changes of distribution and permeability occur. The model was also used to explain inactivation of Na^+ conductance and anomolous rectification. Perhaps the most precise treatment of the excitable cell membrane in terms of phospholipid involvement is that given by Goldman (140). He considered the phospholipids as flexible dipoles in an electric field. Alterations in field strength cause not only a change in configuration but also binding properties. Expressions were developed for the calculation of membrane current components in terms of time, potential and ionic environment. These show good agreement with experimental data.

The simplest theory of drug action involving lipids is that proposed by Davies (73) for odoriferous substances. Essentially it is proposed that odoriferous molecules are adsorbed and penetrate the lipid bilayer. On desorption the 'hole' left by the molecule allows leakage of ions and consequent depolarisation and action potential discharge. Davies' ideas are based on the similarity in size and shape (360) and molecular properties (74) of substances with similar odour. From the formula for desorption at a surface:

$$-\,dn/dt = \frac{10^{28} n e^{-\Delta G/RT}}{n\ total}$$

(where n = number of odoriferous molecules adsorbed at a surface and $n\ total$ = total number of lipid and odiferous molecules), it was calculated that for a molecule with $\Delta G = 7500$ Cal/mole the time for desorption is 10^{-8} sec. A rough calculation of the time for hole healing was made from surface viscosity theory and found to be in the order of 10^{-4} sec, a relatively long time compared with the time for desorption. As stated the theory cannot be applied to drug action. It is difficult to conceive how specific antagonism can be explained, particularly when

pairs of agonist and antagonist molecules are similar in structure and molecular size. However, if the penetrating "molecule" was part of a receptor protein which had undergone a structural change on reaction with a drug the system would show specificity. It is noteworthy that bilipid layers greatly increase their conductance if they contain polar pores occupying a small fraction of their area (151) and that some proteins are able to induce such pores (252). Similar ideas have been pursued by Watkins (377). He was impressed by the fact that many cells in the central nervous system are affected by acetylcholine, GABA and glutamic acid (66, 303) and pointed out the similarity in structure between acetylcholine and the choline moiety of lecithin and sphingomyelin, and between GABA and glutamic acid and the ethanolamine and serine residues, respectively, of phospholipids. He considered the membrane to be a liprotein complex with bound cations and that those portions of the protein which bind the choline, ethanolamine and serine parts of phospholipids can alternatively combine with acetylcholine, GABA, and glutamic acid. When this occurs at polar discontinuities in the membrane the charged groups on the lipid or protein freed by drug interaction can then facilitate ion transfer. To quote from the paper (377), "It is possible that the relative sensitivity of a cell . . . reflects the proportion of the three phospholipids present in the regions of polar discontinuities of the cell membranes." This statement is complementary to the hypothesis concerning the second order involvement of membrane lipids in drug action put forward in the introduction to this review.

Kavanau (202, 203) has collected theoretical, hypothetical and experimental evidence for cell membrane transformation from a lamellar to micellar form and *vice versa*. He has used this to explain such phenomena as protoplasmic streaming, amoeboid movement and various electrokinetic phenomena. Permeability changes caused by drugs resulting from transformation from a laminar (closed configuration) to micellar (open configuration) membrane phase would seem a reasonable model, although Kavanau has not suggested this. The authors of two more recent theories of drug action have supposed that drugs affect a "flavoprotein-regulated ATPase system" (97) or that they influence ATP/ATPase balance (105). Conformational changes in these systems are considered then to alter the membrane properties. It is of interest here that these membrane bound systems depend on a phospholipid component for their activity (125, 308, 334), but it is not known whether or not the phospholipid component has a structural or functional role. The well known theories of drug action, applied usually to anaesthetic gases, such as the Meyer-Overton and Traube surface tension theories, or extensions of these (94, 118), are not dealt with in this review. They have been adequately considered elsewhere (52).

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