TOPOLOGY OF PHOSPHOLIPIDS IN ROD DISK MEMBRANES

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ERIK HENDRIKUS SIKKO DRENTHE

geboren te Gennep

Krips Repro Meppel

Parts of this study have been or are about to be published: de Grip et al. (1979), Bonting et al. (1980), Drenthe et al. (1980a, 1980b, 1981), Drenthe and Daemen (1981).

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Aan mijn vader

CONTENTS

BHT	butylated hydroxytoluene; 2,6-di-*tert.*-butyl-4-methylphenol
DFDNB	1,5-difluoro-2,4-dinitrobenzene
EAI	ethylacetimidate
EDTA	ethylenediaminetetraacetate
ESR	electron spin resonance
FDNB	1-fluoro-2,4-dinitrobenzene
IAI	isethionylacetimidate
MAI	methylacetimidate
Mops	4-morpholinepropanesulphonic acid
NMR	nuclear magnetic resonance
PA	phosphatidic acid
PC	phosphatidylcholine
PE	phosphatidylethanolamine
P_i	inorganic phosphate
PI	phosphatidylinositol; 1-monophosphoinositide
PI-bisP	1-PI 4,5-diphosphate; 1,4,5-triphosphoinositide
PI-P	1-PI 4-phosphate; 1,4-diphosphoinositide
PS	phosphatidylserine
TNBS	2,4,6-trinitrobenzene-1-sulfonate
Tris	tris(hydroxymethyl)aminomethane

The vertebrate retina is a thin layer of tissue in the back of the eye, which contains the light-sensitive photoreceptor cells. The vertebrate photoreceptor cell is an elongated cell, consisting of an inner and an outer segment. The inner segment contains the nucleus and the metabolic machinery of the cell and has a synaptic end. Attached to the other side of the inner segment through a narrow cilium is the outer segment, which is specialized in light absorption. There are two kinds of vertebrate photoreceptor cells: rods and cones. The short cone outer segment has a continuously infolding plasma membrane. The longer rod outer segment consists of a stack of several hundreds of parallel, flat disks, surrounded by a plasma membrane (Fig. 1). The plasma membrane is infolded a few times at the base of the rod outer segment. The disks are free-floating and are probably not directly connected with the plasma membrane. The cones are responsible for color vision in

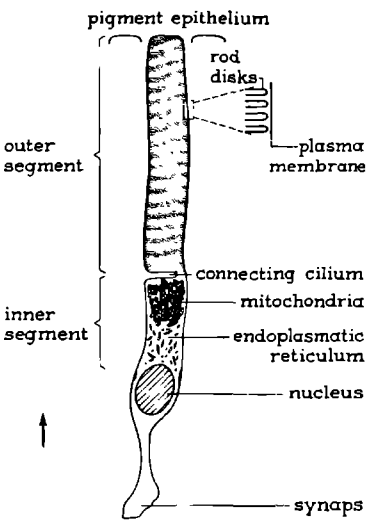

Fig. 1. Schematic diagram of a rod cell. The direction of the incident light is given by the arrow. Modified after Young (1976).

bright light, the rods for black and white vision in dim light.

Since in most vertebrate retinas rods greatly outnumber cones, and since the rod outer segments can be easily separated from the rest of the retina by gentle homogenization, which also permits isolation in large amounts, this study is concerned with rod outer segments only, and particularly with the disk membrane, the photoreceptor membrane.

The photoreceptor membrane consists of a bilayer of mainly phospholipids, between which protein molecules are interspersed. More than 85% of these membrane proteins are formed by the visual pigment, rhodopsin. Rhodopsin consists of a chromophore, 11-cis retinal, bound to a glycoprotein called opsin. The rhodopsin molecule most likely spans the entire disk membrane with the C-terminal end of opsin exposed to the cytosol and the carbohydrate bearing N-terminal end of opsin to the intradiscular space. The chromophore is buried in the rhodopsin molecule and is linked as a protonated Schiff-base to the ε-amino group of a specific lysine residue of opsin.

The phospholipids in the disk membrane have a large number of highly unsaturated fatty acids. This makes the disk membrane highly fluid and allows rhodopsin great freedom of movement. The rhodopsin molecules rapidly diffuse in the membrane and also rotate around an axis perpendicular to the plane of the membrane. These movements of rhodopsin and the orientation of the chromophore in the plane of the membrane contribute to a highly efficient photon absorption.

Upon illumination rhodopsin undergoes a series of conformational changes, beginning with the isomerization of 11-cis retinal to its all-trans conformation and finally resulting in the release of free all-trans retinal from opsin. Only the first step in this photolytic sequence is light-dependent. The intermediates have been characterized spectroscopically by their particular visible-absorption spectrum. Fig. 2 shows the characteristic absorption spectra of rhodopsin before and after illumination. A light-independent adaption process allows the generation of rhodopsin from opsin and newly formed 11-cis retinal.

Following photon capture by rhodopsin, the rod cell becomes hyperpolarized within 200 msec, the underlying mechanism of which is only partially elucidated. In the dark an electric current, consisting of sodium ions, runs from the outer segment to the inner segment. The sodium ions are pumped out of the inner segment and enter the outer segment passively through some kind of Na^+ channels in the plasma membrane. Illumination causes closure of Na^+

channels and consequent reduction of the dark current and hyperpolarization of the plasma membrane. The hyperpolarization spreads over the entire plasma membrane and reaches the rod cell synapse, thus exciting the connecting neurons. These eventually send an elaborated signal through the optic nerve to the brain.

It is generally accepted that the sodium permeability of the plasma membrane must be regulated by an internal transmitter (or transmitters), that mediates between the photolysis of rhodopsin in the disk membrane and the decrease in sodium conductance of the plasma membrane (see, however, Schnetkamp, 1980: evidence for a direct communication between disk membranes and the plasma membrane). Although the exact nature of the transmitter(s) and of the process of closing and opening of the Na^+ channels has not yet been clarified, two general mechanisms are currently under consideration, involving Ca^{2+} and/or cyclic GMP (Hubbell and Bownds, 1979):

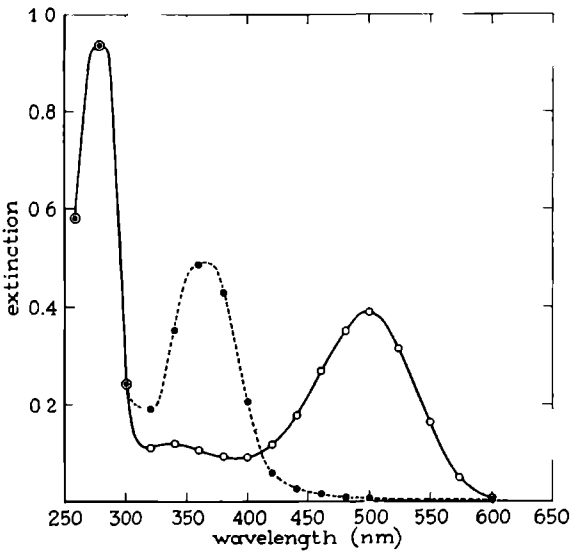

Fig. 2. Absorbance spectra of detergent-solubilized rhodopsin before (o——o) and after (●---●) illumination in the presence of hydroxylamine (NH_2OH). Hydroxylamine allows complete 'bleaching', but shifts the characteristic absorbance of all-trans retinal (380 nm) towards lower wavelength (about 365 nm) due to oxim formation.

(1) *Calcium as internal transmitter.* It is assumed that the photolysis of rhodopsin in the disk membrane causes the release of many calcium ions, which diffuse to the plasma membrane and there close Na^+ channels. However, the light-activated release of a large number of Ca^{2+} ions has yet to be demonstrated;
(2) *Cyclic GMP as negative transmitter.* Upon illumination a very fast and large decrease in the cytoplasmic cyclic GMP concentration has been observed. This led to the hypothesis, that in the dark the level of cyclic GMP is sufficiently high to keep the Na^+ channels open, and that upon illumination a cyclic GMP phosphodiesterase is activated resulting in decrease of the cyclic GMP concentration and closure of the Na^+ channels. The role of Ca^{2+} in this model is restricted to activation of the cyclic GMP phosphodiesterase, which is sensitive to changes in cytoplasmic Ca^{2+} concentration.

Thus, the coupling between the photolysis of rhodopsin in the disk membrane and the changes in sodium conductance of the plasma membrane, possibly mediated by a transient transmitter mechanism, remains largely unanswered. Some indirect evidence suggests that the coupling events are associated with the metarhodopsin I-metarhodopsin II transition: (1) it is the last step in the photolytic sequence which is fast enough (msec); (2) it is accompanied with large conformational changes in the rhodopsin molecule and is pH-dependent (proton uptake), and (3) it is sensitive to changes in the lipid environment of rhodopsin (e.g., it is markedly slowed down in the absence of the disk membrane or in a bilayer of saturated lipids; Daemen and de Grip, 1980). There is no direct evidence that rhodopsin itself acts as a transmembrane (Ca^{2+}) channel (Hubbell and Bownds, 1979).

It is clear that a detailed knowledge of structure and composition of the photoreceptor and plasma membrane will contribute to a full understanding of the visual excitation process.

The composition of the photoreceptor membrane, including the plasma membrane*, has been known for some time (Daemen, 1973). The three major phospholipids of this membrane, comprising about 95% of total phospholipids, are: phosphatidylcholine (PC), phosphatidylethanolamine (PE) and

*The plasma membrane comprises only a small fraction (1-3 wt %) of the total membrane content of the rod outer segment. An isolation procedure, that separates disks from the plasma membrane, is not yet available.

phosphatidylserine (PS). They are arranged in a bilayer, with the hydrophobic fatty acyl chains pointed towards each other and the polar head groups located on the outer faces of the membrane. The question still open is the distribution of the phospholipids and their fatty acyl chains over the two leaflets of the disk membrane. This study has been aimed at answering this question for the three major phospholipids.

CHAPTER 1

CURRENT CONCEPTS ON STRUCTURE AND FUNCTION OF THE ROD PHOTORECEPTOR MEMBRANE

This chapter deals primarily with rod disk (phospho)lipids, possible functions of which are discussed in relation to their metabolism and to some known properties of rhodopsin and the disk membrane.

1.1 Chemical composition

Rhodopsin and phospholipids are the main components of vertebrate rod outer segments. Rhodopsin accounts for 85 wt % of the total protein (which is 38 wt %) and phospholipids for 81 wt % of the total lipid (which is 51 wt %) in water-washed and lyophilized bovine rod outer segment preparations, which contain 5-10 wt % of water (de Grip et al., 1980). These values refer mainly to water-insoluble, membrane-bound constituents. (In intact rod outer segments about 30% of the total protein is soluble, non-membrane protein; Godchaux and Zimmerman, 1979). From molecular weights of 38,000 for rhodopsin and 800 for phospholipid, it can be calculated that the phospholipid content is about 61 mol/mol rhodopsin. The amounts of protein and lipid may vary somewhat depending on the isolation procedure used and the quality criteria applied (Daemen, 1973). Their values are not very much different from those for rod outer segments from frog and rat (Anderson et al., 1975).

The remaining part of the total protein represents most likely membrane-bound enzymatic activities (Zimmerman et al., 1976; Shichi and Somers, 1980) and that of the total lipids mainly cholesterol (about 4 wt %, or 6 mol/mol rhodopsin; Hendriks et al., 1976). Also present are minor amounts of retinal, α-tocopherol (vitamin E), diglycerides, free fatty acids and glycolipids, but except for retinal (0.47 wt %, or 1 mol/mol rhodopsin; de Grip et al., 1980) no exact figures are available for these compounds. Values of 0.07 mol α-tocopherol/mol rhodopsin (Farnsworth and Dratz, 1976), 2 mol diglycerides/mol rhodopsin and 5 mol free fatty acids/mol rhodopsin (Dratz et al., 1979) have been reported for bovine rod outer segments. They may vary considerably when no extensive precautions against oxidative degradation of the lipids have been employed (Farnsworth and Dratz, 1976). Glycolipids make up less than 1 wt %

of total lipids in bovine rod outer segments (de Grip et al., 1980).

The phospholipid classes observed in rod outer segment preparations are phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI) and sphingomyelin, the former three of which are by far the most abundant ones (Table 1.1). Minor amounts of phosphatidic acid (PA), lysophospholipids and diphosphatidylglycerol appear to be present, but their determination must be carefully executed as they may derive in part from contaminants and (enzymatic) degradation. The structure of some of these phospholipids is shown in Fig. 1.1.

Analysis of the fatty acid composition of rod outer segments reveals a very high content of polyunsaturated fatty acids, notably docosahexaenoic acid (22:6). The saturated fatty acids are mainly palmitic (16:0) and stearic (18:0) acid. Some published data are collected in Table 1.2. As pointed out by Stone et al. (1979), a number of different factors could contribute to the observed differences in polyunsaturated fatty acid content, such as contaminants having a polyunsaturated fatty acid content different from that of pure rod outer segment preparations, differences in precautions against lipid autooxidation and differences in genetic factors and dietary history (see also section 1.2.3).

The fatty acid analyses of the individual phospholipids (see the References in Table 1.2) show that PE and PS are enriched in docosahexaenoic acid (22:6) relative to PC and PI. All these phospholipids have roughly the same proportional content of palmitic (16:0) and stearic (18:0) acid with the exception of PS. PS hardly contains palmitic acid (16:0) and has, in bovine rod outer segments, also appreciable amounts of relatively long-chain fatty acids (i.e., 24:4-24:5).

The position of the various fatty acids in individual phospholipids of bovine rod outer segments has been investigated by Anderson and Sperling (1971). They used *Crotalus adamanteus* phospholipase A_2 to hydrolyse the fatty acyl chains at the 2-position in the glycerol backbone of the phospholipids. Their results indicate that the polyunsaturated fatty acids are mainly located at the 2-position as normally found in nature, but that the 24-carbon polyunsaturated fatty acids of PS are predominantly located at the 1-position. The positional distribution of the fatty acids has been approximated by Miljanich et al. (1979) in an indirect way. They used thin-layer chromatography to separate the three major phospholipid classes of bovine rod outer segments into subfractions, which differ markedly in fatty acid composition.

TABLE 1.1

PHOSPHOLIPID COMPOSITION OF PHOTORECEPTOR MEMBRANE EXPRESSED AS MOL PERCENT OF TOTAL PHOSPHOLIPIDS

SPH, sphingomyelin; nd, not determined

	PC	PE	PS	PI	SPH
cattle					
Borggreven et al. (1970)	34.7	39.2	11.2	5.7	1.0
Anderson and Maude (1970)	40.2	38.6	13.3	2.3	3.6
Nielsen et al. (1970)	38.8	47.2	13.8	1.7	1.5
Anderson et al. (1975)	35.7	45.0	15.8	1.5	0.8
Miljanich et al. (1979)	39	42	16	nd	nd
frog					
Mason et al. (1973)	44.6	26.1	15.1	2.1	6.4
Anderson and Risk (1974)	45.3	34.6	12.8	2.2	1.9
rat					
Anderson and Maude (1972)	41.0	36.6	12.6	2.2	3.9

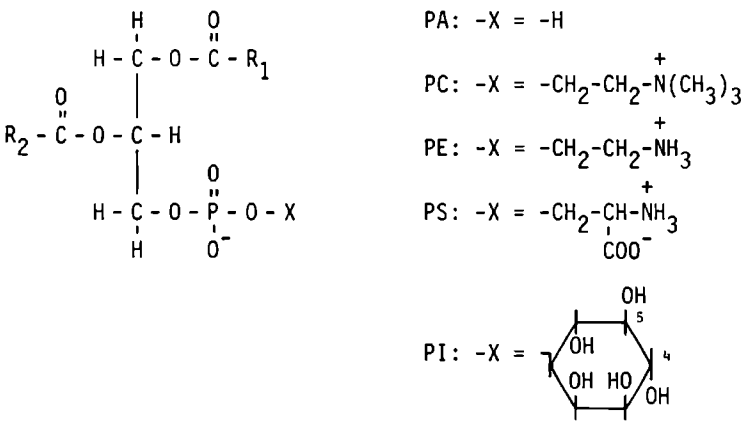

Fig. 1.1. Structure of some 3-*sn*-phosphoglycerides (at pH 7). R_1 and R_2, fatty acyl chains; X, alcohol or base group, which determines the name of the phospholipid.

TABLE 1.2

FATTY ACID COMPOSITION OF PHOTORECEPTOR MEMBRANE EXPRESSED AS MOL PERCENT OF TOTAL FATTY ACIDS

	Fatty acid (C atoms: double bonds)								
	<16	16:0	18:0	18:1	20:4	22:4/5	22:6	>22	PUFA
cattle									
Borggreven et al. (1970)**	0.7	22.1	23.9	6.7	5.8	2.4	30.9	nd	41
Poincelot and Abrahamson (1970b)**	0.2	21.5	24.6	8.0	6.9	-	20.6	5.6	38
Anderson and Maude (1970)*	2.6	18.0	18.1	7.4	4.1	6.4	24.7	1.9	39
Nielsen et al. (1970)**	2.1	15.3	19.9	4.6	8.4	3.3	34.1	3.6	47
Anderson et al. (1975)*	0.3	20.5	24.9	4.0	3.0	5.9	34.8	2.3	47
Hendriks et al. (1976)**	-	15.6	20.9	3.5	5.5	7.7	45.6	nd	60
Miljanich et al. (1979)*	-	17.5	20.6	3.7	3.0	2.7	44.1	3.8	53
Stone et al. (1979)	1.7	16.3	20.2	4.7	3.6	2.7	47.1	2.7	57
frog									
Mason et al. (1973)**	17.1	15.2	9.2	4.8	1.2	7.1	14.6	3.3	42
Anderson and Risk (1974)*	1.3	18.1	17.2	6.5	3.3	6.8	34.3	-	45
Stone et al. (1979)	2.2	14.3	13.8	4.2	2.6	8.5	46.9	2.9	63
rat									
Anderson and Maude (1972)*	0.5	17.0	26.3	8.8	4.9	1.1	31.3	-	38
Stone et al. (1979)	1.9	9.8	24.3	3.8	5.3	5.6	43.3	4.4	60

PUFA, polyunsaturated fatty acids; nd, not determined.
*Calculated from the author's data on the fatty acid composition of the major phospholipids.
**Calculated from the author's data, which are in wt %, by using the molecular weights of the fatty acid methylesters.

They calculated that in 43 mol% of the PS, in 24 mol% of the PC and in 24 mol% of the PE two unsaturated fatty acids are present, which are mainly polyunsaturated, and that 18 mol% of the PC must contain two saturated fatty acids. The other part of each phospholipid class contains one saturated and one unsaturated fatty acid, which are probably distributed in the way Anderson and Sperling (1971) suggest.

The high content of polyunsaturated fatty acids makes the photoreceptor membrane very fluid. The rates of rotational movement (Brown, 1972; Cone, 1972) and translational diffusion (Liebman and Entine, 1974; Poo and Cone, 1974) of rhodopsin in the plane of the membrane are consistent with a membrane viscosity in the range of 1-10 poise, i.e., like that of olive oil. The photoreceptor membrane exhibits a reversible phase transition around - 20^{o} C in frog, as studied with differential scanning calorimetry (Mason and Abrahamson, 1974). This transition can be interpreted as a gel-to-liquid crystalline change, and confirms that the disk membrane has a fluid nature at physiological temperatures. The photoreceptor membrane of cattle also has a fluid nature at such temperatures. While a reversible phase transition is observed around 7^{o} C with differential scanning calorimetry (Miljanich et al., 1978), ESR studies (Watts et al., 1979) and fluorescent measurements (Stubbs et al., 1976) give no indications for lipid phase transitions or lateral phase separations* between 3^{o} and 37^{o} C.

In summary, vertebrate rod outer segments contain rhodopsin and phospholipids as their major constituents. The three major phospholipid classes are PC, PE and PS with palmitic (16:0) and stearic (18:0) acid as the major saturated fatty acids and with docosahexaenoic (22:6) acid as the predominant polyunsaturated fatty acid. PS contains also relatively long-chain 24-carbon polyunsaturated fatty acids. The polyunsaturated fatty acids are mainly located at the 2-position, but appreciable amounts, including the 24-carbon polyunsaturates, are also present at the 1-position in the glycerol backbone of the phospholipids. The higher contents of polyunsaturated fatty acids found in recent investigations are probably due to the improved isolation procedures used for rod outer segments.

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*Phase separation: formation of domains, which differ in lipid fluidity and/or composition.

1.2.1 *Renewal of rod outer segment disks and rhodopsin*

Rod outer segment membranes are continually renewed by a process which involves both membrane and molecular replacement, as demonstrated by autoradiographic and biochemical techniques (reviewed by Young, 1976). Rhodopsin, after synthesis on the endoplasmic reticulum of the inner segment, is transported through the myoid part of the inner segment and the connecting cilium to the infoldings of the plasma membrane at the base of the outer segment. The repeated infoldings of the plasma membrane pinch off and form free-floating disks. Once they are incorporated into the disk membrane (Hall et al., 1969; Papermaster et al., 1975), radioactive rhodopsin molecules are displaced to the apical end of the outer segment as a single band without significant turnover. At the apical end small groups of disks are shed, phagocytized and destroyed by the pigment epithelium (Young and Bok, 1969). Complete outer segment renewal requires 9-12 days in warm-blooded animals like rat, mouse and monkey, and 6-9 weeks in cold-blooded animals like frog (Young, 1967, 1971; Hall et al., 1969). Unlike rhodopsin, the water-soluble outer segment proteins appear to be replaced diffusely, i.e., on a molecular basis (Bok and Young, 1972).

1.2.2 *Phospholipid renewal*

In view of the high metabolic activity of the retina and the continuous renewal of photoreceptor membranes, there must also be an adequate synthesis of membrane lipids. The pathways generally operating for the biosynthesis and interconversion of phospholipids in vertebrates (Fig. 1.2) also function in the photoreceptor cell (Swartz and Mitchell, 1970; Dreyfus et al., 1978; Giusto and Bazan, 1979). Experiments with labeled phospholipid precursors demonstrate that the phospholipids are synthesized *de novo* in the inner segment and then become incorporated into newly formed disks. However, unlike rhodopsin, the phospholipids are subject to random, molecular replacement, the nature of which depends on the particular phospholipid precursor used.

In continuation of the earlier work of Bibb and Young (1974b) with labeled glycerol, Anderson and coworkers (1980a-d) show that the three major phospholipids turn over with a half-life of 18-23 days in frog outer segments.

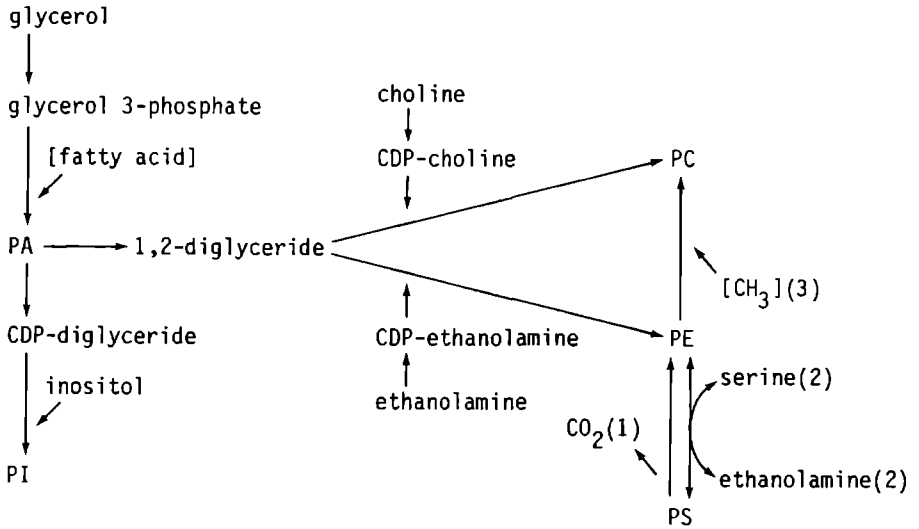

Fig. 1.2. Simplified scheme for the biosynthesis and interrelationships between the major classes of phospholipids in vertebrates. Interrelationships: (1) decarboxylation of PS to PE; (2) base exchange reactions between PS and PE, and (3) methylation of PE to PC. [fatty acid], fatty acid activated to acylCoA thioester; [CH_3], methyl group from S-adenosyl-L-methionine.

In addition, their results suggest a conversion of PS to PE in the outer segment. Synthesis and turnover of PI is much faster than for the other phospholipids. The half-life of PI turnover is 3-5 days in frog. There appears to occur a rapid hydrolysis of PI into 1,2-diglycerides. A rapid PI turnover is often recognized in various cell types in response to external stimuli (Michell, 1975, 1979). Its function in photoreceptor cells is not known, but the rapid hydrolysis of PI may be involved in Ca^{2+} release from the disks upon illumination (see also section 6.2). In addition, the resulting diglycerides may be involved in the fusion events associated with the formation of new and the shedding of old disks (Anderson et al., 1980d).

The rapid turnover of PI and the conversion of PS to PE are also observed with labeled phosphate (Anderson et al., 1980a, 1980d; Hall et al., 1973). However, as compared to glycerol, phosphate is slowly incorporated into the phospholipids of the outer segment and, once incorporated, turns over slowly. This is due to storage of phosphate in the retina, so that its specific radioactivity remains high for several weeks.

A similar observation is made for choline in relation to PC. Choline is mainly stored as choline phosphate and acetylcholine in the rabbit retina. With time, choline phosphate is used for the biosynthesis of phospholipids, primarily PC (Masland and Mills, 1979). The kinetics of PC incorporation into disk membranes is nearly identical to that of rhodopsin, *in vivo* (Hall et al., 1973), as well as in vitro (Basinger and Hoffman, 1976). However, as PC incorporation continues when rhodopsin synthesis is inhibited by puromycin, their pathways for incorporation must be partly independent. Unlike that of rhodopsin and other phospholipids, incorporation of PC is significantly inhibited by light exposure and in the presence of the pigment epithelium (Basinger and Hoffman, 1976; Mizuno, 1976). Basinger and Hoffman (1976) suggest that disk shedding at the tip of the outer segment is initiated by a change in PC composition in disks, which in turn may be mediated by illumination of the retina.

Compositional changes in the disk membrane are also reported for cholesterol. By making use of the freeze-fracture technique and the sterol-specific antibiotic filipin, Andrews and Cohen (1979) tentatively attributed the preferential location of cholesterol to particle-free patches in the plasma membrane and in the basal disks of outer segments in rods of mice and frog, but not in the older disks farther from the inner segment. They suggested that disk membranes undergo changes in cholesterol composition during their replacement towards the pigment epithelium.

Like phosphate, ethanolamine is slowly incorporated into outer segment PE, but it is found to be a poor lipid precursor in the retina (Anderson et al., 1980c; Mizuno, 1976). Thus, PE synthesis by a base exchange mechanism and *de novo* from CDP-ethanolamine appears to play a minor role. There is an active conversion of PE to PC by methylation. Experiments with labeled serine show that PS is synthesized *de novo* in the inner segment and that it serves as a precursor for PE by decarboxylation, in both inner and outer segment (Anderson et al., 1980a). This confirms the observations with glycerol and phosphate, but appears to contradict the generally accepted concept that base exchange is the principle route of PS biosynthesis in vertebrates (van Golde et al., 1974).

The random, molecular replacement mechanism is clearly demonstrated with labeled palmitic (16:0), stearic (18:0) and arachidonic (20:4) acid (Bibb and Young, 1974a). In stead of initial concentration of label in the endoplasmic reticulum of the inner segment, as found with other lipid precursors, the

whole photoreceptor cell becomes quickly labeled, in particular the oil droplets of the pigment epithelium, which are storage sites for vitamin A esterified with fatty acid. The experiments indicate a rapid turnover of fatty acids in outer segment phospholipids with exchange reactions between outer segment and pigment epithelial cells.

In summary, experiments with radioactive phospholipid precursors indicate that the phospholipids are synthesized *de novo* in the inner segment and are then incorporated into newly formed disks at the base of the outer segment. But, unlike rhodopsin, the phospholipids are in addition subject to random, molecular replacement. In the frog, the half-life for the turnover of PC, PE and PS is 18-23 days and 3-5 days for PI, while rhodopsin in the disks turns over much slower (6-9 weeks). Decarboxylation of PS to PE and methylation of PE to PC take place in both inner and outer segment. There is a rapid hydrolysis of PI into diglycerides and a fast exchange of fatty acids between outer segment and pigment epithelial cells.

These findings strongly suggest the existence of specific enzymes of lipid metabolism in outer segments for modification reactions. Although phospholipases (Swartz and Mitchell, 1973), acyl transferases (Swartz and Mitchell, 1974), phospholipid exchange proteins (Dudley and Anderson, 1978) and base exchange activities (Mizuno, 1976) have been demonstrated in whole retina, their presence in outer segments has not been proved.

1.2.3 *Essential fatty acids*

The photoreceptor membrane has an unusually high content of polyunsaturated fatty acids (see Table 1.2), of which the function is not well understood. The two major polyunsaturated fatty acids, docosahexaenoic acid (22:6ω3) and arachidonic acid (20:4ω6), belong to the ω3 and ω6 families, of which γ-linolenic acid (18:3ω3) and linoleic acid (18:2ω6) are the respective precursors. Since vertebrates cannot synthesize these precursors, they are called essential fatty acids and must be derived from dietary sources. Deficiency of the essential fatty acids usually results in compensatory production of eicosatrienoic acid (20:3ω9), as its precursor, oleic acid (18:1ω9), can be synthesized by vertebrates (see Fig. 1.3). However, in rats raised during 2-11 months on a fat-free diet with extremely low levels of essential fatty acids, this compensatory production of 20:3ω9 occurs in

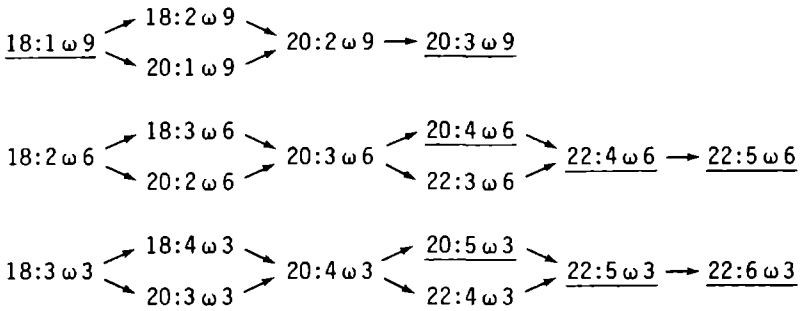

Fig. 1.3. The three fatty acid families with chain elongation and desaturation pathways. First number, carbon atoms; second number, double bonds; number after ω, the position of the first double bond from the methyl terminus (ω-end) of the fatty acid. Since vertebrates cannot introduce double bonds between existing double bonds and the methyl terminus, the fatty acid family remains the same regardless of the number of added carbon atoms and/or double bonds. Underlined fatty acids are commonly found in photoreceptor membranes.

tissues such as liver, kidney, lung and red cells, but hardly at all in brain, retina (Futterman et al., 1971; Forrest and Futterman, 1972) and rod outer segment phospholipids (Anderson and Maude, 1972). Likewise, there is hardly any change in rhodopsin content, phospholipid to rhodopsin ratio and protein composition of rod outer segments, in photoreceptor ultrastructure and electroretinogram (Dudley et al., 1975). Thus, the retina must possess an efficient mechanism to maintain its high level of essential polyunsaturated fatty acids. Apparently, the essential fatty acids are recycled after phagocytosis by the pigment epithelium, while the retina may also be able to produce these fatty acids or to extract small amounts of them from the blood. Slowing down of photoreceptor renewal by a decrease in the rate of new disk formation does not seem to occur (Anderson, 1978).

Only after depriving rats of essential fatty acids for two generations on a diet containing only minor amounts of 18:2ω6, a decreased 22:6ω3 fatty acid content of rod outer segments is observed (Anderson et al., 1974). A decrease in retinal 22:6ω3 by 90% has been obtained in the second generation by Tinoco et al. (1977). Under those circumstances the a-wave of the electroretinogram is significantly reduced (Anderson et al., 1974). Wheeler and Benolken (1975) demonstrated that the electrical response of photoreceptor cell

membranes appears to be a function of the position as well as of the total number of double bonds in fatty acid supplements. They found the a-wave amplitude of the electroretinogram to increase linearly with increasing ω3 concentration. The importance of the polyunsaturated fatty acid content for the electroretinographic response is also shown by the observation, that induction of lipid peroxidation by the Fe^{2+}-ascorbate system in the frog retina decreases the amplitude of the electroretinographic waves (Shvedova et al., 1979).

1.3 Bilayer structure

1.3.1 *The fluid mosaic model*

The basic structure of biological membranes is currently presented in the fluid mosaic model of Singer and Nicolson (1972). The lipid components are thought to be organized as bilayers, with the hydrophilic moieties exposed to the aqueous medium and the hydrophobic hydrocarbon chains of the inner and outer molecules meeting at the center of the bilayer. The ionic lattice at both inner and outer surfaces stabilizes the structure and presents an electrostatic barrier to penetration, whereas the interior of the bilayer is an extremely unfavourable environment for water-soluble compounds. The lipids are thought to be fluid at physiological temperatures, with intramolecular motion of the liquid crystalline hydrocarbon chains and relatively high mobility of the molecules within the plane of the membrane. Membrane proteins may either span the membrane or be partially embedded in it (intrinsic proteins), or be associated with the inner or outer monolayer only (extrinsic proteins). There is no reason to assume that the various lipid components are uniform in their characteristics. It is quite possible that different degrees of molecular packing and mobility occur in different regions of the membrane. In particular, lipid associated with proteins may be relatively restricted in its molecular motion.

The fluid mosaic model holds also for the photoreceptor membrane. The lipid bilayer, fluid at 37^{o} C (see section 1.1), is the major structural frame of the rod disk membrane, as evidenced by birefringence (Liebman et al., 1974), X-ray diffraction (Dratz et al., 1979), neutron diffraction (Yeager et al., 1980) and freeze-fracturing (Corless et al., 1976; Olive, 1980) studies.

These and other techniques reveal rhodopsin as an intrinsic, amphipathic

glycoprotein. It appears to span the lipid bilayer of the disk membrane and to be partly exposed to the aqueous environment of the disks. Rhodopsin is an intrinsic membrane protein since it cannot be removed from the disk membrane without the use of detergents (de Grip et al., 1980). Physical studies have demonstrated that rhodopsin displays rapid lateral and rotational movements in the plane of the disk membrane, aided by the fluidity of the lipid environment in which rhodopsin is partly embedded (see also section 1.1). This indicates that the lipids and rhodopsin are hold together in the disk membrane by weak forces. Rhodopsin is also partly exposed to the aqueous environment of the disks. Specific parts of the molecule can react with water-soluble reagents like proteolytic enzymes (Daemen and de Grip, 1980), chemical modifying reagents (ibid.; Chen and Hubbell, 1978; Hubbell and Bownds, 1979) and the lectin concanavalin A (Adams et al., 1978).

Other membrane proteins (i.e., cyclic GMP phosphodiesterase, GTPase and rhodopsin kinase) may be extrinsic, associated with the external surface of the disk membrane, as shown by their easy removal from the intact disk membrane by low ionic strength or in the absence of divalent cations (Shichi and Somers, 1980).

1.3.2 *Non-bilayer structures in biological membranes*

It has recently been demonstrated that the lipid bilayer, while still the basic feature of biological membranes, is not the only conformational state in which the lipids of membranes can occur. ^{31}P NMR and freeze-fracturing studies have presented strong evidence that in the microsomes and the inner mitochondrial membranes of rat liver the phospholipids can undergo isotropic motion, which suggests that inverted micellar structures may be present in conjunction with the bilayer (de Kruijff et al., 1978; Cullis et al., 1980).

Model studies of fully hydrated preparations of individual (membrane) lipids show that the preferred phase depends on the type of lipid (reviewed by Cullis and de Kruijff, 1979). Under most conditions (at 37^{o} C) PC, PS, sphingomyelin and saturated PE adopt the bilayer, while lysophospholipids prefer the micellar phase, and unsaturated PE and PA-Ca^{2+} and diphosphatidylglycerol-Ca^{2+} mixtures the hexagonal (H_{11}) phase (see Fig. 1.4). Of particular importance are bilayer-to-hexagonal (H_{11}) phase transitions, induced by changes in temperature, divalent cation concentration and pH. While saturated PE prefers the bilayer, unsaturated PE undergoes a bilayer-to-hexagonal (H_{11})

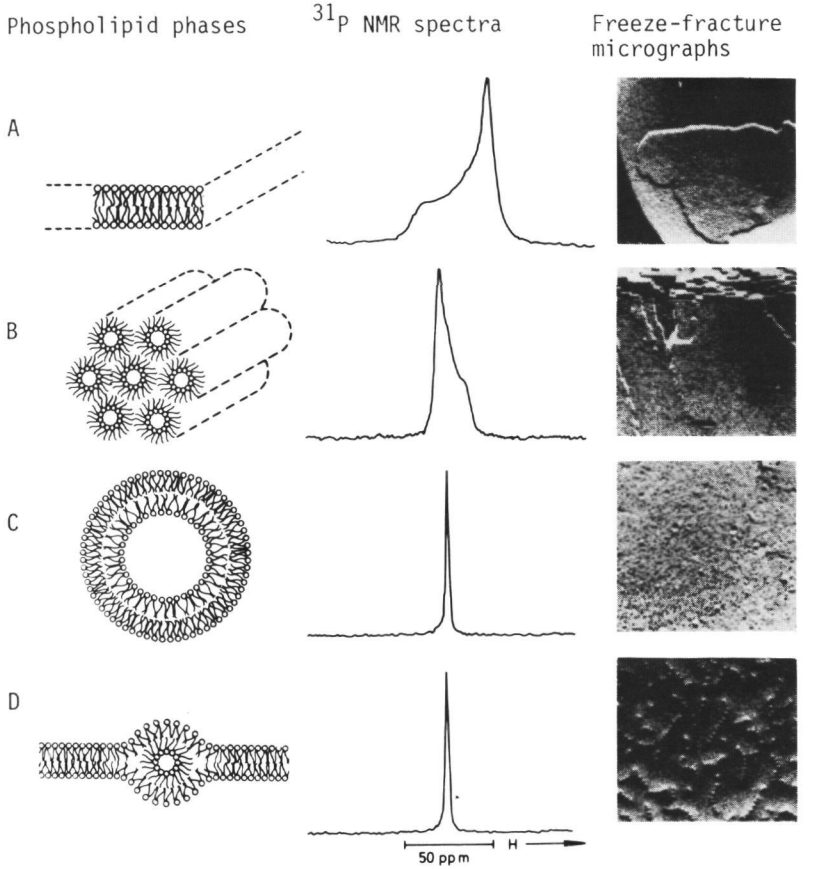

Fig. 1.4. Polymorphic phases available to hydrated liquid crystalline phospholipids and corresponding (36.4 MHz) ^{31}P NMR spectra and freeze-fracture micrographs (150,000 x). A, bilayer phase (egg PC, 30°C); B, hexagonal (H_{11}) phase (soya PE, 30°C); C and D, phases which show isotropic motion spectra; C, sonicated vesicle (dipalmitoyl PC, 50°C); D, inverted micelle sandwiched between the monolayers of the bilayer phase (diphosphatidylglycerol-PC-Ca^{2+}, 30°C). Adapted from Cullis and de Kruijff (1979).

phase transition as the temperature is increased through a characteristic value, which depends in part on the fatty acid composition. This temperature is about 5° C in dioleoyl (18:1/18:1), about 10° C in erythrocyte, 25-30° C in egg and 55-60° C in *Escherichia coli* PE, i.e., values which are 10-20° C above the gel-to-liquid crystalline phase transition temperature. This means that in general unsaturated PE adopts the hexagonal (H_{11}) phase at physiological temperatures. Unlike PE, PC has always the bilayer structure,

independent of the fatty acid composition or other biologically relevant conditions. Diphosphatidylglycerol and (unsaturated) PA undergo a bilayer-to-hexagonal (H_{11}) phase transition when the Ca^{2+} concentration increases. Unsaturated PS prefers the bilayer at physiological pH, but below pH 4.0 (i.e., below the pK of the carboxyl group) it adopts the hexagonal (H_{11}) phase. Calcium is able to immobilize the polar head group of PS strongly and specifically.

The preference of a lipid species for a given phase may reflect the dynamic molecular shape assumed by the lipid. The larger polar head group of PC dictates a cylindrical shape. The smaller polar head group of PE in combination with the unsaturation of its acyl chains, which leads to decreasing chain order at higher temperatures, may result in a cone shape with the polar head group at the top. Saturated fatty acids do not require as much space and so the bilayer phase of saturated PE is stable, even at temperatures well above the gel-to-liquid crystalline phase transition temperature. A lysophospholipid may have an 'inverted cone' shape, as its polar region is larger than that of the single acyl chain. The same way of reasoning can be applied in relation to the negatively charged phospholipids, of which the area per molecule at the lipid-water interface is also sensitive to the net charge in the polar region (e.g., depending on pH and Ca^{2+} concentration).

Most interesting is the behavior of hydrated, mixed lipid systems, consisting of bilayer and hexagonal (H_{11}) phase phospholipids (Cullis and de Kruijff, 1979). Egg PC is able to stabilize bilayer structure in the presence of (equimolar) unsaturated PE, which prefers the hexagonal (H_{11}) configuration upon hydration. At intermediate PC concentrations, isotropic motion is indicated by ^{31}P NMR spectra. This has also been demonstrated in other mixtures of bilayer and hexagonal (H_{11}) phase lipids, such as PC-diphosphatidylglycerol-Ca^{2+} and PC-PE-cholesterol systems. Freeze-fracturing studies of lipid systems exhibiting 'isotropic' NMR spectra reveal numerous small particles and pits on the fracture faces, indicating the presence of 'lipidic particles' (possibly inverted micelles* or small hexagonal (H_{11}) structures) sandwiched between the two faces of the lipid bilayer (see Fig. 1.4.D) or at attachment sites of two lipid bilayers.

*Inverted micelles can be considered as very small hexagonal (H_{11}) fragments, i.e., very small cylinders of the type water-in-paraffin.

The data discussed so far have clearly established that several lipid components of biological membranes can adopt the hexagonal (H_{11}) phase upon hydration. Moreover, mixed lipid systems containing bilayer and hexagonal (H_{11}) phase lipids often exhibit inverted micellar structures. Recently, this has also been demonstrated for the total lipid extract of *Escherichia coli*, which contains 74% PE of total phospholipids (Burnell et al., 1980). This total lipid extract gives rise to ^{31}P NMR spectra, which indicate bilayer, isotropic and hexagonal (H_{11}) phases. These phases are also detected by freeze-fracturing, suggesting the presence of inverted micelles associated with bilayer structure. However, lipid extracts of microsomes (which contain mainly PC and PE) and of inner mitochondrial membranes (which contain mainly diphosphatidylglycerol, PC and PE) of rat liver adopt the bilayer with only minor structure giving rise to isotropic motion in ^{31}P NMR spectra. Addition of Ca^{2+} results in some hexagonal (H_{11}) phase formation as well as lipidic particles as detected by freeze-fracturing. Thus, the observed lipidic particles in natural intracellular membranes of rat liver, suggesting inverted micellar structures in conjunction with the bilayer, may be partly due to the presence of protein (or endogenous Ca^{2+}). Nevertheless, the possible occurrence of transitory non-bilayer structures in such dynamic membrane systems may have important functional implications, which are difficult to reconcile with purely bilayer structures. These include membrane fusion (e.g., phagocytosis) and transbilayer transport processes (e.g., transbilayer movement of lipids and facilitated transport).

Since photoreceptor membranes have a large portion of highly unsaturated PE (see Table 1.2), (transitory) non-bilayer structures may be present. This may offer a new insight on fusion and transport mechanisms of the photoreceptor membrane.

Recently, we have undertaken a ^{31}P NMR and freeze-fracturing study of both the isolated intact photoreceptor membrane and aqueous dispersions of its extracted lipids (de Grip et al., 1979). The results indicate a bilayer configuration for the photoreceptor membrane, and inverted micellar structures for the extracted lipids, associated with the bilayer below 25^{o} C or the hexagonal (H_{11}) phase at 37^{o} C. This confirms the fluid mosaic model, but with rhodopsin as the major structuring component for the photoreceptor membrane. However, Deese et al. (1981) showed that the ^{31}P NMR spectra are indicative of the bilayer phase for both the rod outer segment membrane and

its extracted (phospho)lipids. We cannot presently account for the discrepancy between our results of the extracted lipids and those of Deese et al. (1981), which are obtained under slightly different experimental conditions.

1.4 Interaction between phospholipids and rhodopsin

As mentioned in section 1.3.1, the rod disk membrane is a fluid, bilayered structure, in which rhodopsin-lipid interaction is probably not too strong as to allow high rotational mobility. Several approaches have been used to determine specific rhodopsin-lipid interactions. In most studies these interactions have been deduced from alterations in the properties of rhodopsin after modification or removal of the lipids normally present in the disk membrane. Following delipidation, rhodopsin can be incorporated into lipid bilayers of known composition (Hong and Hubbell, 1972; van Breugel et al., 1977). The complexity of the natural disk membrane is greatly reduced in such model systems, permitting better control of the properties of rhodopsin.

The properties of rhodopsin, which are frequently used to study the influence of an altered lipid environment on rhodopsin, are: its characteristic 500 nm absorbance, the photolytic sequence upon illumination, the regeneration capacity (i.e., the ability of bleached rhodopsin to combine with 11-cis retinal to the visual pigment), the thermal stability, and the accessibility of functional groups within the rhodopsin molecule to certain reagents (Daemen and de Grip, 1980).

Evidence that lipids may have a functional role in rod outer segments comes from earlier work on the extractability of lipids under different conditions. When rod outer segments are treated with non-polar organic solvents which do not bleach rhodopsin (hexane, Borggreven et al., 1970; Poincelot and Abrahamson, 1970a; petroleum ether, Shichi, 1971; Ishimoto and Wald, 1946; diethyl ether, Poincelot and Abrahamson, 1970a; Krinsky, 1958), PE is preferentially removed at the expense of PC, which appears to be more tightly bound to the membrane. Borggreven et al. (1970) found no further release of phospholipids upon illumination, but others (Poincelot and Abrahamson, 1970a; Ishimoto and Wald, 1946; Krinsky, 1958) observed a large release of residual phospholipid, mainly during the conversion of rhodopsin to metarhodopsin I (Poincelot and Abrahamson, 1970a). When rod outer segments are extracted with chloroform-methanol, which bleaches rhodopsin, all lipids

are removed (Borggreven et al., 1970; Poincelot and Zull, 1969). Thus, the experiments with the milder non-polar organic solvents show the presence of a loosely bound and a more tightly bound phospholipid pool, and could suggest that native rhodopsin binds phospholipids in a different manner than does bleached rhodopsin.

Borggreven et al. (1971, 1972) and Shichi (1971) used phospholipases A_2 and C to modify photoreceptor membrane phospholipids. Phospholipase A_2 hydrolyses phospholipids into lysophospholipids and free fatty acids, phospholipase C produces diglycerides and phosphate esters. Borggreven et al. (1971) could hydrolyse 95% of the rod disk phospholipids with phospholipase C from *Bacillus cereus* without loss of 500 nm absorbance but with a decreasing regeneration capacity of rhodopsin. Hexane extraction of the diglycerides caused no further change in the regeneration capacity. Lipid analysis revealed that phospholipase C treated-hexane extracted disk membranes contain 0.2 mol PE and 1.6 mol PS per rhodopsin molecule. Treatment of this preparation with phospholipase A_2 from *Crotalus adamanteus* and subsequent removal of the released lysophospholipids with serum albumin (Borggreven et al., 1972) did not further lower the regeneration capacity of rhodopsin and only slightly reduced the 500 nm absorbance. The final preparation contained only 0.1 mol PE and 0.1 mol PS per rhodopsin molecule. These results indicated that PS and PE are not involved in binding the chromophore at its active center, but they did show the dependence of the regeneration capacity of rhodopsin on the lipid environment. Shichi (1971) found a complete loss of the regeneration capacity of rhodopsin upon treatment with phospholipase A_2 from *Naja naja*, which is restored after addition of phospholipids to the partially hydrolyzed disk membranes. Lysophospholipids, which are potential detergents, may contribute to the observed effect, since detergents like Emulphogene have a similar effect.

Van Breugel et al. (1978) also modified rod disk phospholipids by means of phospholipase C from *Bacillus cereus*, which hydrolyses up to 90% of the phospholipids. Electronmicroscopic observations showed that aggregation of rhodopsin occurs, when more than 20% of the phospholipids are hydrolyzed. This is due to removal of diglycerides from the disk membrane and their coalescence into lipid droplets (Olive et al., 1978). Extraction with hexane is thus not needed to produce partly delipidated disk membranes. Van Breugel et al. (1978) also isolated lipid-free rhodopsin and incorporated purified rhodopsin into lipid bilayers of unsaturated egg phospholipids. The characteristic 500 nm absorbance was unaffected in all cases, again indicating that the chromophoric

center is not influenced by the altered lipid environment. However, the photolytic sequence after the formation of metarhodopsin I is markedly slowed down and the thermal stability and the regeneration capacity of rhodopsin are reduced in phospholipase C treated membranes and in delipidated rhodopsin. The changes, observed in phospholipase C treated membranes, start after 40% hydrolysis of the phospholipids and gradually reach maximal levels at 90% hydrolysis. The indicated properties of rhodopsin are completely restored upon its reconstitution in lipid bilayers (at 25^{o} C). However, when unsaturated egg PC (which contains about one double bond per molecule) or saturated PC (O'Brien et al., 1977) is used for reconstitution, the photolytic sequence after metarhodopsin I formation is again slowed down (at 18^{o} C). It thus appears that rhodopsin can only function properly, when it is dispersed in a lipid bilayer consisting of at least 37 (i.e., 40% of 62) phospholipids per pigment molecule. The lipid bilayer must be sufficiently fluid, i.e., more than one double bond per lipid molecule should be present. The results also suggest that no specific, polar lipid-rhodopsin interactions are involved in keeping rhodopsin structurally and functionally intact (as judged from the properties of rhodopsin presently known).

1.4.1 *Specific phospholipid-rhodopsin interactions*

Some observations, however, suggest specific lipid-rhodopsin interactions, the function of which is not always understood. Experiments on the extractability of phospholipids with non-polar organic solvents suggest that PC is tightly associated with the membrane of rod outer segments, whereas the bulk of PE belongs to a loosely bound lipid pool (see above). It is not clear, whether this effect is caused by stronger interaction of rhodopsin with PC, or is due to better solubility of PE in a particular non-polar organic solvent. In addition, the experiments of Borggreven et al. (1972) with phospholipases indicate that it is difficult to remove PS from the disk membrane. It can be removed completely only after extensive sequential treatment with phospholipase C, hexane, phospholipase A_2 and serum albumin. Thus, PS might be closely associated with rhodopsin. However, it cannot be excluded that this effect is caused by the substrate preference of these phospholipases.

Upon modification of the primary amino groups in disk membranes with methylacetimidate (MAI), de Grip et al. (1973) found 0.2 mol PS, 0.4 mol PE and 1.4 mol lysine/mol rhodopsin unmodified in darkness. After illumination

these values became 0.9, 0.5 and 0.5, respectively. This suggests that the chromophore is released from the ε-amino group of lysine and moves largely to PS. After illumination in the presence of NADPH the observed values are 0.1, 0.2 and 0.4, respectively, indicating that the chromophore is liberated and reduced to retinol by the retinol dehydrogenase system, which requires NADPH as cofactor. The results could suggest a role of PS in chromophore binding after illumination, but could also indicate the relative inertness of PS towards modification with methylacetimidate upon illumination.

Upon illumination of disk membranes, rhodopsin undergoes a series of conformational changes. Metarhodopsin II is formed within milliseconds at physiological temperatures. It decays in the course of several minutes into three stable products: opsin, free retinal and metarhodopsin III. The latter substance consists of a mixture of all-trans retinal bound to opsin and to PE. In the case of bovine disks, illuminated in suspension, retinal is distributed as follows: 30-35% free, 51% opsin-bound, 15% PE-bound (van Breugel et al., 1979). The fraction of retinal, bound to PE, could be increased up to 34% at the expense of free retinal, when purified rhodopsin was reconstituted with egg PE into bilayers. Reconstitution of rhodopsin into bilayers of PC or PS shows no lipid-bound retinal fraction. These observations suggest that in the later, slow part of the photolytic sequence PE is involved in chromophore displacement during metarhodopsin III formation, at least under the experimental conditions used in this study.

The purification of rhodopsin is most conveniently achieved through affinity chromatography on immobilized concanavalin A in the presence of detergent (de Grip et al., 1980). All common detergents are capable of removing the lipids from rhodopsin, except digitonin and dodecylmaltose. In the presence of 2% digitonin, 3 mol PC, 3 mol PE and 7 mol PS remain bound per mol rhodopsin (de Grip, unpublished result), suggesting again that PS could be more tightly associated with rhodopsin than the other lipids. However, the incomplete delipidation of rhodopsin may also reflect the limited solubilization capacity of this mild detergent.

Bifunctional chemical reagents, i.e., with two reactive groups, can be used to introduce both inter- and intra-molecular cross-links. Under suitable conditions a nearest neighbor analysis of membrane components can be performed. Crain et al. (1978) were able to cross-link primary amino groups in photoreceptor membranes with the membrane-permeable reagent difluorodinitrobenzene (DFDNB). Under maximal cross-linking conditions (pH 8.5, 21-37^{o} C,

75-100 μM reagent), 6-11% of PE and 35-39% of PS were found to be cross-linked to protein, i.e., 2-3 mol PE and 3-4 mol PS per rhodopsin molecule (lower values correspond to lower temperature and reagent concentration). Light exposure did not appreciably influence the cross-linking of aminophospholipids to protein. Fatty acid analysis of the various reaction products indicates that PE, not cross-linked to protein, contains the same fatty acids as PE in the original membrane preparation. The aminophospholipids, cross-linked to protein, are slightly enriched in docosahexaenoic acid (22:6). The results suggest that a minor part of PE and PS is closely associated with rhodopsin, but that the aminophospholipids are randomly distributed in the plane of the disk membrane with respect to their fatty acids. However, it is difficult to make a distinction between links formed between membrane components which show static, long-term interactions, and links between membrane components, which accidentally collide during the course of diffusion in the membrane (Peters and Richards, 1977).

Various physical techniques have recently been used to investigate lipid-rhodopsin interactions in disk membranes. Results obtained with photoreceptor membrane are usually compared with those of its extracted lipids in order to detect lipid populations, which are immobilized by rhodopsin. Stubbs et al. (1976) found no evidence for different lipid populations, when using the fluorescent probe diphenylhexatriene. Since the disk membrane has a (fourfold) higher effective microviscosity than the extracted lipids, they concluded that almost all lipids in the disk membrane are immobilized by rhodopsin and behave as a single pool, with rapid exchange between boundary and bulk lipid populations. Lower temperatures resulted in decreasing fluidity of the disk membrane (1.4 poise at 40^{o} C to 15 poise at 0^{o} C), which was not affected by bleaching of rhodopsin. From ESR studies with spin-labeled fatty acids, Favre et al. (1979) concluded that the lipids are only slightly affected in their mobility by rhodopsin. Other ESR studies with a number of lipid spin-labels (Watts et al., 1979) suggested that a large part of the lipids (33-43%) is immobilized by rhodopsin, but that all lipids can diffuse in the plane of the disk membrane with rapid exchange between boundary and bulk lipid populations. The results indicated a limited preference for the immobilized region by PS molecules. A critical assumption in the interpretation of these results is that the distribution of membrane lipids be identical with that of spin-labels of the same type. In addition, the lipid extract is probably not a good reference preparation, since it adopts an isotropic phase. The isotropic phase

represents possibly inverted micellar structures, associated with the bilayer below 25° C or the hexagonal (H_{11}) phase at 37° C (de Grip et al., 1979; but see Deese et al., 1981). ^1H and carbon-13 NMR studies with rhodopsin-egg PC recombinants also indicate that the lipids exchange rapidly ($<10^{-3}$ sec) between boundary and bulk lipid populations, but the saturated *sn*-1 acyl chains of the phospholipids are significantly more immobilized than the polyunsaturated *sn*-2 acyl chains (Zumbulyadis and O'Brien, 1979).

In summary, there are in general no indications for a strongly immobilized lipid population by rhodopsin in the disk membrane. Biochemical evidence indicates that rhodopsin needs a minimal fluid lipid environment to function adequately, at least in so far as revealed by its properties presently known. The dependence of rhodopsin activity on the lipid environment is not so much an expression of the need for a particular lipid as it is the need for a suitable fluidity in the overall lipid phase. Specific rhodopsin-lipid interactions are only apparent during the final stages of the photolytic sequence involving chromophore displacement, and appear to involve both PE and PS.

During the last years, progress has been made in the characterization (and isolation) of soluble outer segment proteins. which can be activated by light-excited rhodopsin (Shichi and Somers, 1980; Kühn, 1980). It is not yet clear whether the presence of a wide variety of phospholipids with remarkably high degree of unsaturation has any relevance for such or other still unknown activation processes.

1.5 Transverse distribution of phospholipids

Biological membranes, consisting of a bilayer in which proteins are (partially) embedded, serve not only as barriers separating two compartments and allowing bidirectional passive diffusion, but also maintain various unidirectional active transport processes. Thus, biomembranes must be asymmetrically organized to some extent in order to maintain the latter processes. Asymmetrical insertion of membrane proteins has been demonstrated in many instances. Asymmetrical distribution of membrane lipids has also been claimed in many instances, most firmly for the erythrocyte plasma membrane (op den Kamp, 1979).

The photoreceptor membrane also consists of a lipid bilayer, in which rhodopsin is asymmetrically inserted (see section 1.3). The involvement of PS

and PE in chromophore binding upon illumination, and the possibility that PS binds the (proposed) transmitter Ca^{2+} at the disk membrane, suggest a specific transbilayer distribution of (part of) PE and PS. A fully or partly asymmetric lipid distribution may also be needed for attachment (orientation) and full activity of some extrinsic membrane proteins.

Before this study was started, little was known about the transbilayer distribution of the phospholipids. The transbilayer distribution of the primary amino group containing phospholipids, i.e., PE and PS, has been studied in bovine rod outer segment preparations by means of amino group modifying chemical reagents (see further). In principle, the use of membrane-impermeable reagents, like trinitrobenzenesulfonate (TNBS) and isethionyl-acetimidate (IAI), allows selective labeling of primary amino groups which are located in the outer leaflet of the membrane. The use of membrane-permeable reagents, like ethylacetimidate (EAI) and fluorodinitrobenzene (FDNB), or the use of membrane-impermeable reagents under conditions at which all amino groups from both leaflets become accessible, gives an estimation of the total amount of primary amino groups available to the reagents.

In biochemical studies on the effect of amino group modification on the properties of rhodopsin, de Grip et al. (1973) determined that 52 primary amino groups/mol rhodopsin are present in bovine rod outer segments, consisting of 16 ε-amino groups of lysine, 27 amino groups of PE and 9 amino groups of PS. They observed that at pH 8 and 40^{o} C a 15-fold molar excess of TNBS labels all primary amino groups present, in darkness as well as in light and also after addition of Triton X-100 (final concentration, 1 wt %). These conditions led to complete denaturation of rhodopsin, i.e., complete loss of characteristic 500 nm absorbance. At 20^{o} C, in darkness at pH 8, a final level of 70% modification is observed without loss of 500 nm absorbance, which has been confirmed by the experiments of Litman (1974). At a 3-fold molar excess of TNBS at pH 8.5 and at 20^{o} C in darkness, Litman (1974) also found a plateau level of about 60% modification. The complete labeling of the primary amino groups at elevated temperatures was attributed to a change in the permeability of the membrane as a direct consequence of the thermal denaturation of rhodopsin by reagent labeling.

Raubach et al. (1974) observed that 4-6 ε-amino groups of lysine and 26 amino groups of the aminophospholipids are labeled by excess IAI at pH 9 and 20^{o} C. These numbers are increased to 10-11 and 38, respectively, by excess EAI under the same conditions, which labels the total amount of the amino-

phospholipids. Hence, they concluded that about 70% of the aminophospholipids (i.e., 26 of 38) are located at the outside of the disk membrane.

This comprises our knowledge of the transverse distribution of phospholipids at the time when this study was started: a suggestion that the aminophospholipids predominate at the outside face of the disk membrane.

Two papers about transverse distribution of aminophospholipids have been published during the course of this study (Smith et al., 1977; Crain et al , 1978). The results of Smith et al. (1977) indicated complete asymmetry of aminophospholipids, since all lipid primary amino groups react with a 2-fold molar excess of TNBS at pH 8.5 and 20^{o} C, while arginine, which also contains reactive amino groups and which is trapped inside the disks, does not react at all. The most detailed information on reactivity of lipid primary amino groups in disk membranes is given by Crain et al. (1978), who used various chemical reagents under different conditions. At 21^{o} C and pH 8.5 an excess of TNBS, but also of FDNB, labels 94-86% of PE and 43-63% of PS very quickly. At 0^{o} C only 63% of PE and 25% of PS react relatively fast with TNBS, as compared to the remaining fraction of these lipids. In the presence of valinomycin, which makes the erythrocyte permeable to TNBS (Marinetti and Crain, 1978), 86% of PE and 50% of PS react quickly. The authors concluded that 63% of PE and 25% of PS are located outside, that 23% (i.e., 86-63%) of PE and 25% (i.e., 50-25%) of PS are located inside, and that the remaining fraction of these lipids is not readily available. The fractions, not readily available, are consistent with corresponding fractions, cross-linked to protein (see section 1.4.1). The authors suggested that TNBS at 21^{o} C, or at 0^{o} C in the presence of valinomycin, penetrates the membrane. Experiments with excess IAI, applied at pH 8.5 and 21^{o} C, confirmed the results of TNBS reacting at 0^{o} C.

The data of Crain et al. (1978), together with those of the other investigators, are summarized in Table 1.3, and suggest that 60-100% of the aminophospholipids are located at the outside face of the photoreceptor membrane. It is clear from these results that the reaction conditions greatly affect the accessibility of the amino groups to the reagents. The influence of the reaction conditions will be evaluated in more detail when we have presented our results regarding the transverse distribution of the phospholipids.

TABLE 1.3

ACCESSIBILITY OF PRIMARY AMINO GROUPS IN DARK-ADAPTED BOVINE ROD OUTER SEGMENTS

NH_2, total primary amino groups; NH_2-PL, total primary amino groups of aminophospholipids.

		Incubation temperature		
	Reagent	0^o C	$20\text{-}21^o$ C	$37\text{-}40^o$ C
De Grip et al. (1973)*	TNBS		70% of NH_2	100% of NH_2
Litman (1974)*	TNBS		60% of NH_2	100% of NH_2
Raubach et al. (1974)*	IAI		70% of NH_2-PL	
	EAI		100% of NH_2-PL	
Smith et al. (1977)*	TNBS		100% of NH_2-PL	
Crain et al. (1978)**	TNBS	63% of PE, 25% of PS	94% of PE, 43% of PS	
	TNBS + valinomycin	86% of PE, 50% of PS		
	FDNB		86% of PE, 63% of PS	
	IAI		72% of PE, 31% of PS	

*Final modification level.
**Fast-reacting phospholipid pool.

1.6 Aim of this study

The aim of this study was to elucidate the transbilayer distribution of the three major phospholipids and their fatty acyl chains over the two leaflets of the disk membrane. The study has been performed with rod outer segments isolated from bovine retina. Bovine rod outer segments contain phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylserine (PS) as their major phospholipids (respectively, 36, 44 and 15 mol% of total phospholipids; Table 1.1). In these preparations the plasma membrane is still present, but only as a minor impurity (1-3 wt %).

Knowledge of the transverse distribution of phospholipids in the disk membrane is obviously needed to obtain a detailed picture of the membrane structure and to understand its functioning on a molecular scale. Because of conflicting results, reported for the transverse distribution of the aminophospholipids PE and PS in rod disk membranes (section 1.5), there is a need for further investigation. Moreover, neither the transverse distribution of PC is known, nor the transverse distribution of the acyl chains in individual phospholipid classes.

In addition to the use of amino group modifying reagents like trinitrobenzenesulfonate (TNBS) we have used three different, specific phospholipid hydrolyzing enzymes (phospholipases A_2, C and D) and combined treatment with TNBS and phospholipase D. Considerable effort has been expended on determining correct conditions for these experiments, which guarantee complete accessibility of only externally located phospholipids (e.g., to minimize membrane inversion upon isolation, inaccessibility of externally located phospholipids, redistribution of phospholipids upon modification, and reagent penetration).

CHAPTER 2

PHOSPHOLIPASES AND TRINITROBENZENESULFONATE AS TOOLS FOR STUDYING THE STRUCTURE OF PHOTORECEPTOR MEMBRANE

In this chapter a description is given of materials, procedures and determinations, which have been used throughout this study. In the introductory part some characteristics of phospholipases and trinitrobenzenesulfonate (TNBS) are described, as well as prerequisites for the correct use of these reagents in determining the phospholipid distribution across the photoreceptor membrane.

2.1 Introduction

Phospholipases and group-specific reagents like TNBS have been widely used as mild agents for the modification and removal of phospholipids of biological membranes. They have been used as probes for the transbilayer organization of phospholipids in erythrocyte membranes (Zwaal et al., 1975; Marinetti and Crain, 1978) and in many other biological membranes (Bergelson and Barsukov, 1977; Rothman and Lenard, 1977; op den Kamp, 1979; Etémadi, 1980). In addition, phospholipases have been useful in studying the dependence of membrane-bound enzymatic activities on phospholipids (Gazzotti and Peterson, 1977; Bonting and de Pont, 1980). Group-specific reagents are less suitable for the latter purpose, since they can also react with functional groups of proteins.

Phospholipases have been used to investigate the influence of photoreceptor membrane phospholipids on the properties of rhodopsin (section 1.4; see also Cohen, 1973; Farnsworth and Dratz, 1976; Baroin et al., 1979; Favre et al., 1979). The transverse distribution of phospholipids in photoreceptor membranes has previously been studied only with amino group reagents (section 1.5). These reagents could thus only provide direct information on the position of amino-containing phospholipids like phosphatidylethanolamine (PE) and phosphatidylserine (PS). Hence, we have undertaken a study with three different phospholipases, which in principle can attack all available phospholipids in photoreceptor membranes. These phospholipases are: phospho-

lipase A_2 from porcine pancreas, phospholipase C from *Bacillus cereus*, and phospholipase D from Savoy cabbage. To corroborate the results of our work with phospholipases, we have also used TNBS, which appears to be a very useful tool, particularly in combination with phospholipase D.

2.1.1 *Some characteristics of phospholipases*

Phospholipases are lipolytic enzymes, classified according to the type of bond they hydrolyse in phospholipids (see Fig. 2.1; not shown is their action on phosphate-containing sphingolipids like sphingomyelin). Phospholipase A_2 (EC 3.1.1.4) specifically removes the fatty acid from the 2-position of the glycerol backbone to leave a lysophospholipid. Phospholipase C (EC 3.1.4.3) hydrolyses the bond between phosphoric acid and glycerol, yielding a phosphate ester and a diglyceride, while phospholipase D (EC 3.1.4.4) removes the polar group leaving a phosphatidic acid (PA). Phospholipase D can also catalyze a transferase reaction by which the base group of the phospholipid substrate is replaced by an other acceptor alcohol. This transferase reaction may be considered the general reaction, with hydrolysis representing a specific case in which the acceptor alcohol is water. Phospholipase A_1 (EC 3.1.1.4) will not be considered here, since we have not made use of it.

Each phospholipase (A_2, C and D) is available from different sources (Brockerhoff and Jensen, 1974) and may exhibit variations in substrate specificity and in the rates of hydrolysis for individual substrates. The phospholipases A_2 from pancreas and venoms act on all common types of phosphoglycerides, although they are not all hydrolyzed at the same rate.

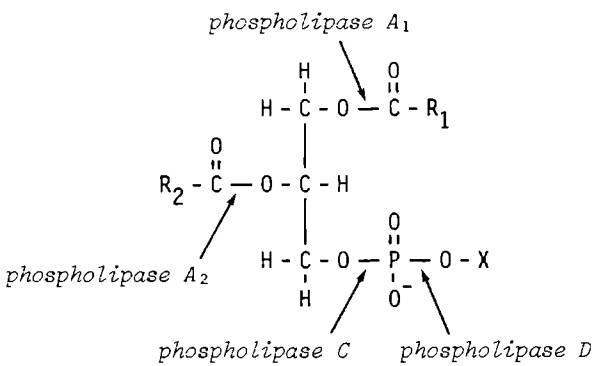

Fig. 2.1. Sites of action of different phospholipases on phosphoglycerides.

Porcine pancreatic phospholipase A_2 prefers negatively charged phosphoglycerides like PA and PS, whereas the enzyme from the venom of *Naja naja* is more active towards PE and phosphatidylcholine (PC; de Haas et al., 1968; Roberts et al., 1978).

The situation is more complex with phospholipases C. The enzyme from *Bacillus cereus* degrades only phosphoglycerides, except phosphatidylinositol (PI), but PC and PE faster than PS (Zwaal et al., 1975; Otnaess et al., 1977; Roberts et al., 1978). It may, however, hydrolyze sphingomyelin under certain conditions (Otnaess, 1980). On the other hand, the *Staphylococcus aureus* sphingomyelinase C is strongly specific for sphingomyelin, whereas the *Clostridium perfringens (welchii)* phospholipase C can attack sphingomyelin and zwitterionic phosphoglycerides but not acidic ones like PS (Zwaal et al., 1975). PI-specific phospholipases C have been purified from bacterial sources (see Ohyabu et al., 1978)

Phospholipases D from plants like peanut and cabbage show a broad substrate specificity. These enzymes are able to hydrolyse various phosphoglycerides, but not PI. Under certain conditions the cabbage enzyme may hydrolyse sphingomyelin (Heller, 1978).

The activity of a particular phospholipase is dependent on several factors, in particular on the physical properties of the lipid-water interface at which catalysis occurs. This will be illustrated for phospholipases A_2.

Porcine pancreatic and bee-venom phospholipases A_2 display maximal activity on multilamellar liposomes of saturated PC only when structural defects, disorder or more space are induced in the membrane, e.g., by sonication yielding small unilamellar vesicles, by osmotic shock and by temperatures near the gel-to-liquid crystalline transition of the lipids. Apparently, conditions which introduce structural defects (free space) in the membrane favour penetration of the enzyme into the interface. Similarly, unsaturated PC is hydrolyzed at temperatures above the transition temperature, which has been ascribed to an increased distance between the lipid molecules allowing penetration of the enzyme (op den Kamp et al., 1975; Wilschut et al., 1978; Upreti and Jain, 1980). With *Naja naja* phospholipase A_2 no specific enhancement of hydrolysis rate has been observed in sonicated PC vesicles at the transition temperature (Kensil and Dennis, 1979). The action of various phospholipases A_2 is also stimulated at temperatures just above the transition temperature of the substrate, after a certain concentration of products (free fatty acids) has been reached. This may be attributed to an increase in the

phase transition or in the free space, caused by the free fatty acids (Wilschut et al., 1978; Upreti and Jain, 1980).

Porcine pancreatic phospholipase A_2 is unable to attack intact erythrocyte membranes and resealed ghosts, but produces breakdown of proper substrates when it is trapped inside resealed ghosts or these are lysed (Zwaal et al., 1975). Hydrolysis also occurs in monolayers of palmitoyl-oleoyl (16:0/18:1) PC at a surface pressure below 16.5 dynes/cm, and in monolayers of dioleoyl (di18:1) PE/PS mixtures at a pressure as high as 39 dynes/cm (Demel et al., 1975). The outer membrane surface of the erythrocyte consists mainly of PC and sphingomyelin, whereas the inner leaflet contains mainly PE and PS. This indicates that the action of pancreatic phospholipase A_2 is dependent on the state of compression of the membranous lipid layer upon which it acts. *Naja naja* phospholipase A_2 belongs to the group of lipolytic enzymes, which are able to hydrolyse their respective substrates in intact erythrocyte membranes (Zwaal et al., 1975; but see Adamich and Dennis, 1978; Roelofsen et al., 1980). It fails to hydrolyse monolayers of choline-containing phospholipids at a surface pressure above 35 dynes/cm (Demel et al., 1975). Thus, the *Naja naja* enzyme may penetrate the interface more easily than the pancreatic enzyme. However, Roelofsen et al. (1980) report the existence of several isoenzymes from *Naja naja*, which exhibit phospholipase A_2 activity, but only one of which produces optimal hydrolysis (68%) of PC in intact erythrocyte membranes. The observed differences in final PC hydrolysis could be the result of a slight increase in lateral surface pressure in the outer monolayer, caused by the production of lyso-PC and free fatty acid and making the less potent isoenzymes unable to penetrate the interface after a certain hydrolysis level is reached. This phenomenon can be expected to occur in biological membranes with a surface pressure close to the limit above which the phospholipase can no longer attack the membrane phospholipids (e.g., 31-35 dynes/cm for the erythrocyte membrane and 35 dynes/cm for the *Naja naja* enzyme; Demel et al., 1975).

The presence of other lipids, who need not be cosubstrates, may stimulate the activity of a given phospholipase A_2 towards a particular substrate, but it may also alter the apparent substrate specificity. In individual phospholipid-Triton X-100 mixed micelles, the *Naja naja* enzyme strongly prefers PC above PE, but in mixtures of PE and PC the rate of PE hydrolysis is greatly enhanced. PE is also the preferred substrate in erythrocyte ghosts (Adamich and Dennis, 1978; Roberts et al., 1979) It is clear that the apparent substrate

specificity of a given phospholipase is markedly affected by many factors.

2.1.2 *Some characteristics of trinitrobenzenesulfonate*

The negatively charged reagent TNBS is now widely used to modify primary amino groups in proteins and aminophospholipids. It introduces a large, uncharged trinitrophenyl group (Fig. 2.2). The reaction is strongly pH-dependent and is usually carried out at alkaline pH, since TNBS reacts only with unprotonated amino groups (Means et al., 1972). TNBS can also react with sulfhydryl groups, but the resulting S-trinitrophenyl groups are unstable at alkaline pH (ibid.; but see Haest et al., 1981). Sulfite ion, liberated in the reaction of TNBS with amino groups, readily complexes with trinitrophenyl products above pH 7, giving a characteristic visible absorption peak. This peak can be abolished by acidification, which dissociates the sulfite complex (Habeeb, 1966). The modification reaction can then be followed spectroscopically by measuring the absorbance at 340 nm against appropriate blanks (ε_{340} = 13,100 $M^{-1}cm^{-1}$; de Grip et al., 1973).

Since the reaction of TNBS with amino groups is dependent on the pH of the medium and the pK_a of the amino groups, differences in reaction rate of individual amino groups can be expected to occur. In a $CHCl_3/CH_3OH/H_2O$ medium of pH 8.5 at 23° C PE reacts more rapidly with TNBS than does PS (Gordesky and Marinetti, 1973). A similar pattern is observed in photoreceptor membranes solubilized in Triton X-100 at pH 8 and 40° C (de Grip et al., 1973). Likewise, the extent of the reaction with PE of intact erythrocyte membranes

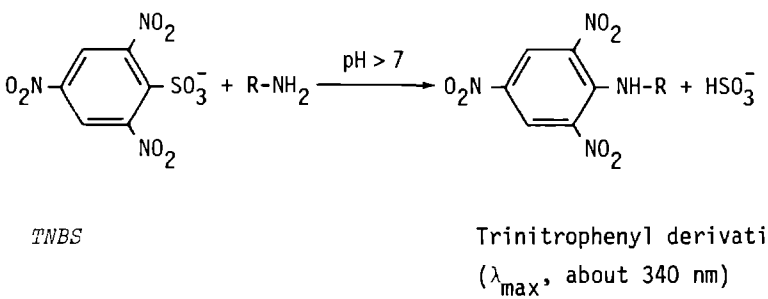

Fig. 2.2. Reaction equation for the amino group modification by 2,4,6-trinitrobenzene-1-sulfonate (*TNBS*).

for 0.5 h at 23° C increases with the pH between pH 6 and 8. It does not further increase above pH 8, probably because all outside-located PE has been modified during that period (Gordesky et al., 1975; Marinetti and Crain, 1978; but see next paragraph).

Studies with phospholipid monolayers demonstrated that introduction of the bulky trinitrophenyl group during amino group modification can cause a significant increase in the surface pressure of the monolayer, leading to incomplete reaction. The increase in surface pressure is greater for unsaturated PE and PS in the liquid crystalline state than for saturated PE and PS in the gel state. At an initial surface pressure of 25 dynes/cm, only 80% of PE from *Bacillus subtilis* could be trinitrophenylated. Incorporation of negatively charged phospholipids resulted in a further decrease in the labeling of PE. These and other results clearly indicate that a high initial surface pressure as well as negative surface charge inhibit the reaction and that both parameters become more effective as more aminophospholipids are modified (Bishop et al., 1979).

2.1.3 *Requirements for phospholipid localization*

Incubation of photoreceptor membranes with a selected probe leads to modification of natural substrates as mentioned in sections 1.4 and 1.5. In all cases residual, unmodified phospholipids can be quantitatively analyzed after extraction and two-dimensional thin-layer chromatography. Analysis of the products of the action of the reagent allows an independent control determination of the degree of modification. In order to establish the phospholipid distribution across the bilayer unambiguously, it is necessary to select reaction conditions, which guarantee complete accessibility of outside-located phospholipids only. Such conditions should minimize inaccessibility of externally located phospholipids, redistribution of phospholipids upon modification, and reagent penetration. In addition, the isolation procedure of the membrane vesicle should not induce membrane inversion. Naturally occurring transbilayer movement of phospholipids (flip-flop) should be negligible during the period of treatment. These requirements and their fulfilment are discussed below and at appropriate places in the next chapters.

Membrane inversion. Freshly prepared stacked disk preparations show very little membrane inversion. This is indicated by the lack of binding of concanavalin A, which would react with sugar residues of rhodopsin if inversion

occurs. When applied on top of a concanavalin A-Sepharose column, essentially all disks are recovered in the non-adherent void volume, and none of them are collected after subsequent elution with α-methylmannoside, the inhibitor of concanavalin A binding (Adams et al., 1978). Drastic treatments like exposure to a hypotonic medium or sonication give disk vesicles, which also fail to bind concanavalin A. However, the reduced yields of disks frequently observed after these treatments may indicate that sonication and hypotonic disruption of disks result in a mixture of inverted and uninverted vesicles, and that small inverted vesicles cannot be dissociated from the concanavalin A beads by elution with α-methylmannoside. When intact disks are frozen and thawed, the membrane shows inversion and becomes capable of binding concanavalin A (Adams et al., 1978, 1979). Freeze-fracture studies indicate that membrane inversion is a rare event in the case of intact disks and water-washed vesicles. They reveal, however, that upon lysis by water the disks rupture and that only the uninverted disks tend to reseal (Barry et al., 1980). However, this does not exclude that packing and mobility of the lipids are subtly altered, which appears to occur upon preparing ghosts of erythrocytes (Bloj and Zilversmit, 1976; Adamich and Dennis, 1978). Thus, the original, right-side-out configuration of the disk membrane is best assured by using freshly prepared stacked disk preparations, which have not been washed with strongly hypotonic solutions.

Inaccessibility of externally located phospholipids. Reduced reactivity of phospholipids, leading to incomplete reaction, may be caused by several factors such as lipid-protein interactions, sterical unavailability of the lipids to large reagents (phospholipases), differences in local packing of the lipids, differences in local pH and ion concentrations, and changes in state of compression of the lipids upon modification (op den Kamp, 1979; preceeding sections). Such factors should not constitute a serious drawback in the case of the photoreceptor membrane, since its lipids are highly unsaturated and thus have a fluid nature, at least above 7^{o} C (no space limitations), and there are no strong interactions with rhodopsin (section 1.4). Open, leaky membrane systems, in which all lipids from both membrane leaflets will be available to the reagent, can be used to trace masked lipid populations. Membrane structure and lipid reactivity should not be altered too much in open systems in order to allow comparison with the native situation. In any case, when all lipids become modified, e.g., in the presence of detergent, the reagent has been applied under conditions which in principle guarantee complete

reactivity.

Redistribution of lipids upon modification. Modification of phospholipids inevitably disturbs the membrane structure to some extent. This may lead to lipid redistribution and reagent penetration. Induced transbilayer movement of lipids has been demonstrated in unilamellar vesicles of egg PC upon treatment with cabbage phospholipase D, where part of the outside-produced PA rapidly exchanges with inside-located PC, but without disturbing the barrier properties of the bilayer (de Kruijff and Baken, 1978). Sundler et al. (1978) reported hydrolysis of inside-located PE in mixed PC/PE vesicles by phospholipase D, when the vesicles were first made asymmetric by modification of outside-located PE with isethionylacetimidate (IAI). They suggested that aggregation of PA with Ca^{2+} ions present in the medium occurs with subsequent loss of vesicular structure and penetration of the enzyme. On the other hand, when unilamellar vesicles of rat liver PC are treated with cabbage phospholipase D in the presence of Ca^{2+}, or are subjected to exchange with mitochondrial PC by means of beef heart exchange protein, it appears that the hydrolyzed fraction and the exchangeable fraction are the same. This indicates that no lipid reorganization occurs (Johnson et al., 1975). Similarly, production of asymmetric vesicles by removing PI from the outer surface of unilamellar PC-PI-diphosphatidylglycerol vesicles by means of exchange protein or PI-specific phospholipase C, did not induce rapid phospholipid translocation (Low and Zilversmit, 1980). Obviously, the findings in these model systems are contradictory, but may be caused by different reaction conditions.

Phospholipases A_2 from *Crotalus adamanteus* and *Naja naja* and phospholipase C from *Bacillus cereus* give nearly complete hydrolysis of natural substrates in photoreceptor membranes (section 1.4), indicating that the action of these probes does not leave the membrane structure intact. Diglycerides, produced by phospholipase C, tend to aggregate into discrete droplets after hydrolysis of 20% of the disk phospholipids. This probably induces gross disorganization of the membrane, although membranous structures are still present after 90% phospholipid hydrolysis (Olive et al., 1978). Phospholipase A_2 produces lysophospholipids and free fatty acids, which form micelles when separately dispersed in aqueous buffer, and thus disruption of the bilayer structure may occur. However, model studies demonstrated that an equimolar mixture of palmitoyl (16:0) lyso-PC and fatty acid forms a stable bilayer phase (Jain et al., 1980). Palmitoyl (16:0) lyso-PC, added to dioleoyl (di18:1) PC vesicles, is incorporated in the outer monolayer of the

vesicles, and shows appreciable transbilayer movement only when the vesicles contain glycophorin (de Kruijff et al., 1977; van Zoelen et al., 1978). Rhodopsin may thus induce transbilayer movement of the products of phospholipase action, leading to almost complete hydrolysis of phospholipids.

Reagent penetration. Penetration of the reagent into the membrane interior can be demonstrated by the modification of cytoplasmic proteins. The reaction conditions should allow adequate modification of these proteins. Reagent penetration may also be indicated indirectly, when inside-located proteins (enzymes) become detectable in the external medium or when they express their activity on presumed impermeable substrates. Thus, whenever hemoglobin in the interior of the erythrocyte becomes modified by TNBS, or detectable in the external medium upon phospholipase treatment, penetration of the reagent most likely occurs. Penetration of TNBS into the erythrocyte leads, however, to modification of sulfhydryl groups of the tripeptide glutathione rather than of amino groups of hemoglobin (Haest et al., 1981). Thus, conclusions with respect to the absence of binding of TNBS to amino groups of inside-located proteins must be drawn with caution.

Compared to a reagent like TNBS, the phospholipases have large molecular weights: 13,800 for porcine pancreatic phospholipase A_2 (de Haas et al., 1968), 23,000 for *Bacillus cereus* phospholipase C (Otnaess et al., 1972), at least 100,000 for Savoy cabbage phospholipase D (Allgyer and Wells, 1979) and 22,000-200,000 for the peanut enzyme (Heller, 1978). Penetration of phospholipases would only be expected, when the membrane structure is severely damaged, whereas penetration of the relatively small TNBS molecules has been observed in many instances, depending on temperature, type of buffer and reagent concentration (Gordesky et al., 1975). In photoreceptor cells no adequate internal marker for detecting reagent penetration is presently known.

Naturally occurring transbilayer movement of phospholipids. In model systems the spontaneous transbilayer movement of phospholipids under equilibrium conditions is slow, with halftimes in the order of days (Johnson et al., 1975; Low and Zilversmit, 1980). Incorporation of protein may enhance the transbilayer movement (van Zoelen et al., 1978; but see Dicorleto and Zilversmit, 1979). Halftimes in the order of minutes have been observed in bacterial membranes (Rothman and Kennedy, 1977) and microsomes (Zilversmit and Hughes, 1977; but see van den Besselaar et al., 1978). The transbilayer movement of phospholipids is rather slow in some other biological membranes, in the order of hours in erythrocytes (Bloj and Zilversmit, 1976; Renooij et

al., 1976), vesicular stomatitis virus (Shaw et al., 1979) and chromaffin granules (Buckland et al., 1978), and in the order of days in influenza virus (Rothman et al., 1976), inner mitochondrial membranes (Rousselet et al., 1976) and LM cell plasma membranes (Sandra and Pagano, 1978). Membranes capable of phospholipid synthesis and growth, like microsomal and bacterial membranes, appear to have rapid rates of transbilayer movement of the phospholipids, representing a special mechanism used in membrane assembly (Rothman and Kennedy, 1977). This mechanism may be partially lost or absent in membranes incapable of growth, like erythrocyte and viral membranes. At present, no evidence for naturally occurring transbilayer movement of phospholipids in rod outer segment membranes is available (see section 1.2.2).

In summary, it will be difficult to meet all requirements for correct localization of phospholipids across the disk membrane. Although membrane inversion may be small in freshly prepared stacked disk preparations, and all phospholipids freely accessible in the outer surface of the disk membrane (no strong interactions with rhodopsin), an internal marker to establish the moment of reagent penetration is lacking, while a naturally occurring flip-flop of phospholipids is not known. Yet, the treatment with phospholipases A_2 and C will lead to nearly complete modification of disk phospholipids.

The degradation of individual phospholipids in disk membranes by phospholipases A_2 and C can be followed in time and be compared with that of a reference preparation, in which the same phospholipids are present in random distribution. Differences in initial hydrolysis rate of individual phospholipids in either preparation should then reveal differences in phospholipid availability, provided that the disk membrane disturbance is small in the early stages of modification (compare Sundler et al., 1977, 1978).

2.2 Preparations and their treatments

2.2.1 *Modifying agents*

Highly purified, lyophilized phospholipase A_2 (EC 3.1.1.4) from porcine pancreas is a gift of Professor G.H. de Haas (Department of Biochemistry, University of Utrecht, The Netherlands). It has been purified according to the method of Nieuwenhuizen et al. (1974) and has a specific activity of about 800 units per mg of protein with egg-yolk lipoprotein as substrate (ibid.).

The enzyme is dissolved in H_2O before use (100 units per ml). It has an absolute requirement for Ca^{2+} and can be inactivated by EDTA.

Phospholipase C (EC 3.1.4.3) is purified from cultures of *Bacillus cereus* according to the method of Otnaess et al. (1972). It has a specific activity of about 1950 units per mg of protein with egg-yolk lipoprotein as substrate (Zwaal and Roelofsen, 1974). The enzyme is dissolved in 50% glycerol (by volume) containing 1 mM Zn^{2+} (100 units per ml). It needs Zn^{2+} for activity and can be inactivated by EDTA or o-phenanthroline.

Phospholipase D (EC 3.1.4.4) is isolated and partially purified from Savoy cabbage according to the method of Davidson and Long (1958), or obtained commercially (Boehringer, Mannheim, F.R.G.). Both preparations give the same results. The enzyme has a specific activity of about 1 unit per mg of dry weight with egg-yolk PC as substrate (Davidson and Long, 1958). The preparation is dissolved in water before use (20 units per ml). Partially purified preparations from Savoy cabbage are contaminated with phosphatidate phosphohydrolase activity (EC 3.1.3.4), which causes some additional hydrolysis (maximally 20%) of PA into diglycerides and P_i (Davidson and Long, 1958). The enzyme is stimulated by Ca^{2+} and inhibited by EDTA. The three phospholipase preparations used do not have proteolytic activities.

2,4,6-Trinitrobenzene-1-sulfonic acid tetrahydrate has been purchased from BDH Chemicals Ltd, Poole, U.K. TNBS is added as a freshly prepared solution in the same buffer as used in the experiments.

2.2.2 *Rod outer segment and lipid preparations*

Three different types of bovine rod outer segment preparations and a retinal lipid extract have been used, always starting from fresh retinas.

'Stacked disks' are prepared in a sucrose-Ficoll 400 medium according to the method of Schnetkamp et al. (1979). Rod-like structures are clearly visible under the phase-contrast microscope. Electron-microscopic observation reveals stacked disks, partially surrounded by plasma membrane.

'Disk vesicles' are isolated rod outer segments according to the method of de Grip et al. (1972), omitting the enrichment procedure, which are finally washed three times with distilled water. Electron-microscopic observation shows that after washing with distilled water the flat disk structure has been converted to globular unilamellar vesicles. The term disk membranes is used to imply both stacked disk and disk vesicle preparations.

'Solubilized disks' are made by dissolving disk vesicles in 0.16 M Tris-maleate buffer (pH 6.0) containing 20 mM β-1-nonylglucose. The non-ionic detergent β-1-nonylglucose was prepared by Dr. W.J. de Grip in our laboratory (de Grip and Bovee-Geurts, 1979).

'Retinal lipid suspension' is prepared by extraction of the lipids from whole bovine retina by a modification of the procedure of Bligh and Dyer (1959). After evaporation of the organic solvent, the lipids are suspended in 0.16 M Tris-HCl buffer (pH 7.4) and subjected to sonic vibration at 0^{o} C (Branson Sonifier B-12; three 1-min periods at half-maximal output with a 1 min delay between each sonic burst), and finally centrifuged (10 min at 10,000 x g) in order to remove metal (titanium) contamination and larger lipid particles. When retinal lipid suspensions are applied on top of a Sepharose 4B column and eluted with 0.16 M Tris-HCl buffer (pH 7.4), they are collected near the void volume of the column. This indicates that retinal lipid suspensions consist of large particles, in agreement with similar observations for dispersions of rod outer segment phospholipids (Brown et al., 1977).

The phospholipid composition of the retina (Anderson et al., 1970) very much resembles that of rod outer segments (Tables 1.1 and 1.2). Hence, a retinal lipid suspension can be used as a reference which reflects the substrate specificities of phospholipases A_2 and C for different phospholipids, present in a given ratio and randomly available. In the case of phospholipase D, which requires 40 mM Ca^{2+} for good activity (section 2.2.3) and which Ca^{2+} concentration causes aggregation and flocculation of the retinal lipid suspension, solubilized disks are used as a reference for the substrate specificity of the enzyme.

2.2.3 *Treatment with modifying agents*

Phospholipase treatment. The most suitable reaction conditions for treatment with phospholipase D have been determined by varying pH, amount of Ca^{2+}, amount of enzyme and temperature, and measuring the extent of total hydrolysis in disk vesicles after 60 or 120 min (see Fig. 2.3). Optimal activity has been observed at pH 6.0, 40 mM Ca^{2+}, at 30^{o} C and about 1 unit/ml phospholipase D in a disk vesicle suspension containing 30-40 μM rhodopsin. At temperatures above 32^{o} C the contaminating phosphatidate phosphohydrolase activity increases significantly (data not shown), but at 30^{o} C and the other conditions selected, less than 20% (av. 10%) PA is converted to diglycerides

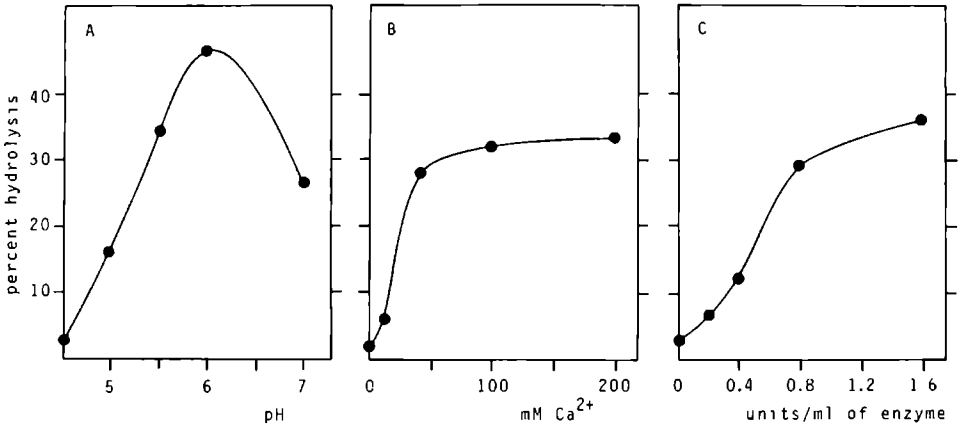

Fig. 2.3. Dependence of phospholipase D catalyzed hydrolysis of disk vesicles on pH, Ca^{2+} and enzyme concentration. Results are expressed as percent hydrolysis of total phospholipids.
A, pH-dependence (40 mM Ca^{2+}; 1.6 unit/ml phospholipase D; 120 min); B, Ca^{2+}-dependence (pH 5.6; 1 unit/ml phospholipase D; 60 min); C, dependence on enzyme concentration (40 mM Ca^{2+}; pH 5.6; 60 min). Disk vesicle concentration 30-40 μM rhodopsin, incubation temperature 27°C.

and P_i. Suitable reaction conditions for phospholipases A_2 and C have been derived from Borggreven et al. (1971) and van Breugel et al. (1978).

The stacked disks are suspended in a medium containing 600 mM sucrose, 5% (w/w) Ficoll 400 and 20 mM Tris-HCl buffer, pH 7.4 (for treatment with phospholipases A_2 and C), or 20 mM Tris-maleate buffer, pH 6.0 (for treatment with phospholipase D). Disk vesicles are suspended in 0.16 M Tris-HCl buffer, pH 7.4 (for treatment with phospholipases A_2 and C), or 0.16 M Tris-maleate buffer, pH 6.0 (for treatment with phospholipase D).

Incubations with phospholipase C are carried out at 20° C, with phospholipase A_2 at 20° C in the presence of 10 mM $CaCl_2$, with phospholipase D at 30° C in the presence of 40 mM $CaCl_2$, all in darkness under N_2. The reactions are started by adding an appropriate amount (1-2 units/ml) of solubilized enzyme at disk concentrations equivalent with 30-40 μM rhodopsin. The reactions are stopped by adding an excess of ice-cold buffer containing 10 mM EDTA.

Retinal lipid suspensions and detergent-solubilized disk membranes are incubated as described for disk vesicles, and the reaction is stopped by adding the $CHCl_3/CH_3OH$ extraction mixture to the incubation medium.

The entire procedure, starting with the isolation of rod outer segment membranes, takes less than 6 h. Freshly isolated rod outer segment membranes have been used throughout. Control incubations, omitting phospholipase, are always carried out and demonstrate the absence of endogenous phospholipase activity under the conditions used.

Treatment with trinitrobenzenesulfonate. Optimal conditions for the application of TNBS as an impermeable reagent with respect to disk membranes have first been established (see section 4.3.2). In the routinely adapted procedure, the membrane preparations are suspended in 40 mM Mops buffer (pH 7.4), 2 mM $CaCl_2$, 3 mM $MgCl_2$, 140 mM NaCl to a final rhodopsin concentration of 3-4 µM. TNBS is added as a freshly prepared solution in the same buffer to a final concentration of 1 mM, representing a 5-fold excess with regard to the number of primary amino groups present in the membrane preparations. Incubation is performed at 20° C under N_2. The reaction is stopped by lowering the pH through addition of excess ice-cold 0.2 M sodium acetate buffer (pH 5.5). Spectral assay procedures for determining the extent of modification are described in section 2.3.3.

Combined treatment with phospholipase D and trinitrobenzenesulfonate. The incubations with phospholipase D and TNBS are carried out as described above. When the first treatment is with TNBS, the reaction is stopped by addition of an excess of ice-cold buffer (pH 6.0), the mixture is centrifuged (0° C, 10,000 x g, 30 min), and the pellet is resuspended in the buffer used for the phospholipase D treatment. When the first treatment is with phospholipase D, the incubation mixture can be diluted directly by addition of the medium used for the modification with TNBS (e.g., without prior centrifugation).

2.3 Assay methods

The methods, described here, include the electron-microscopic procedures and the spectral determinations of rhodopsin and trinitrophenyl compounds. In addition, methods are given for the quantitative extraction of the phospholipids and their separation by two-dimensional thin-layer chromatography. In the spots the phospholipid content can be determined as P_i after destruction or the fatty acid composition can be measured by gas-liquid chromatography. All procedures are carried out under an N_2 atmosphere wherever possible, and all solutions are bubbled with N_2 before use, in order to avoid oxidation of the highly unsaturated phospholipids.

2.3.1 *Electron microscopy*

Stacked disk and disk vesicle suspensions are fixed in glutaraldehyde (final concentration 2%, v/v) in the same buffer as used for stopping the phospholipase treatment of disk vesicles (section 2.2.3), and post-fixed in 1% OsO_4. The samples are then dehydrated and embedded in Vestopal W. Thin sections are stained with uranyl acetate and lead citrate and examined in a Philips 300 or 301 electron microscope (Olive et al., 1978).

2.3.2 *Rhodopsin determination*

Rhodopsin is determined spectrophotometrically by measuring the 500 nm absorbance of a disk membrane suspension before and after illumination in the presence of 50 mM NH_2OH and 1% (w/v) Triton X-100 (van Breugel et al., 1978). By means of the molar absorbance of 40,500 $M^{-1}cm^{-1}$ at 500 nm (Daemen et al., 1972), the 500 nm absorbance difference can be converted to the molar rhodopsin concentration.

2.3.3 *Spectral determination of trinitrophenyl compounds*

The total number of modified amino groups after treatment with TNBS is determined by stopping the reaction through addition of 1 M HCl (final pH 2.5) and Triton X-100 (final concentration 1%, w/v) and reading the 340 nm absorbance. Appropriate blanks are used to correct for scattering, rhodopsin and TNBS absorbance and TNBS hydrolysis. They include: (1) buffer, to which are added HCl, Triton X-100 and TNBS just before reading; (2) TNBS in buffer, to which are added HCl and Triton X-100, and (3) disk membranes in buffer, to which are added HCl, Triton X-100 and TNBS. Molar contents are obtained by using the molar absorbance value ε_{340} = 13,100 $M^{-1}cm^{-1}$ (de Grip et al., 1973).

The total number of amino groups present is determined by a modification of the method of Habeeb (1966). The reaction with TNBS is then performed in the presence of 1% (w/v) Triton X-100 for 3-4 h. After addition of 1 M HCl (final pH 2.5), the 340 nm absorbance is read against appropriate blanks and converted to molar content.

2.3.4 *Lipid extraction*

Aliquots of the diluted suspensions (1 ml, containing about 2 µmol phospholipid; section 2.2.3) are centrifuged (30 min, 100,000 x g, at 4° C). The supernatant, which contains neither rhodopsin nor phospholipids, is collected. When water-soluble products of the enzymatic hydrolysis (phosphate esters and P_i) are expected, aliquots of the supernatant are taken to determine the P_i content. In this way the balance of phospholipid breakdown can be checked. The pellet is resuspended in an equal volume of CH_3OH. This suspension is extracted with 20 volumes of $CHCl_3/CH_3OH$ (2:1, v/v), containing 50 mg/l butylated hydroxytoluene (BHT) as antioxidant, by vigorous shaking during 30 min at room temperature (modified after Folch et al., 1957). After centrifugation (5 min at 3000 x g) the supernatant is removed and extraction is repeated. The pooled extracts are washed with 0.2 volumes of acidified 0.1 M KCl (Palmer, 1971) by gentle shaking. After centrifugation the lower layer is removed and concentrated by evaporation under reduced pressure at room temperature. During washing and centrifugation minor amounts of lyso-PS are lost into the upper aqueous layer in spite of the use of the acidified salt solution. The concentrated extract is diluted with 4 volumes of $CHCl_3/CH_3OH$ (1:1, v/v), and taken to dryness in a stream of N_2. The residue is dissolved in 0.5 ml $CHCl_3/CH_3OH$ (1:1, v/v) for direct analysis or stored in 0.5 ml toluene/ethanol (4:1, v/v) at - 20° C under N_2.

Lipid dispersions and detergent solutions can be extracted by a modification of the procedure of Bligh and Dyer (1959), in certain cases after addition of EDTA (5 mg to 1 ml aliquots containing about 2 µmol phospholipid) and cooling to 0° C. The aliquots (1 ml) are mixed with 3 ml $CHCl_3/CH_3OH$ (1:2, v/v) containing 50 mg/l BHT, so that a monophasic system results. The mixture is filtered through a sintered glass funnel and the filter is washed with 1 ml $CHCl_3$. The combined filtrates are washed with 1 ml acidified 0.1 M KCl. After centrifugation, the upper and lower layers are separated, and analyzed for P_i and phospholipids, respectively.

2.3.5 *Thin-layer chromatography of lipids*

Two-dimensional thin-layer chromatography of phospholipids. Lipid extracts, containing about 0.8 µmol phospholipid, are applied to thin-layer plates as a single spot by means of a micropipette. The plates (20 x 20 cm)

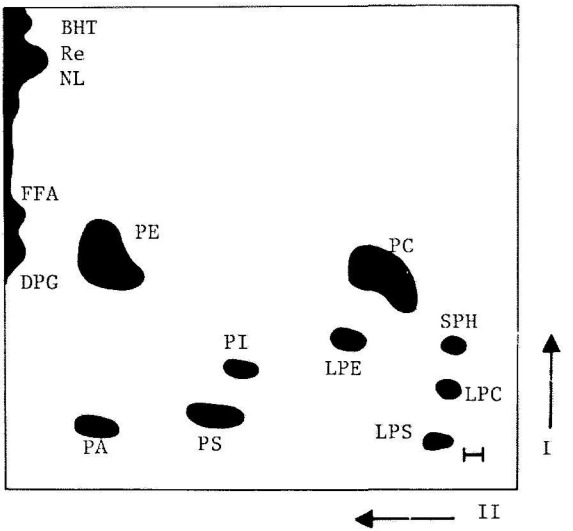

Fig. 2.4. Schematic two-dimensional thin-layer chromatogram of a total lipid extract of bovine rod outer segments. Development in two dimensions (I and II) on plates prepared as described in the text. Staining with iodine vapour. Identity of the spots: SPH, sphingomyelin; DPG, diphosphatidylglycerol; FFA, free fatty acids; Re, retinal; NL, (other) neutral lipids; LPC, LPE and LPS, lyso-compounds of PC, PE and PS, respectively.

are coated with 0.30-0.25 mm silica gel and preactivated for 1 h at 110° C. Either, precoated plates (e.g., DC-Fertigplatten Kieselgel 60, Merck, Darmstadt, F.R.G.), cleaned by a prerun in $CHCl_3/CH_3OH/H_2O$ (65:25:4, v/v), are used or else plates prepared from a slurry of purified silica gel (Silicagel 60 HR, Merck) containing 3% (w/w) alkaline Mg silicate (Woelm, Eschwege, F.R.G.). The latter plates give better separation of acidic phospholipids and do not contain any phosphorus (Broekhuyse, 1968).

After evaporation of the solvent in a stream of N_2, the chromatogram is developed in the first dimension with solvent I and in the second dimension with solvent II. Solvent I contains $CHCl_3/CH_3OH$/14 M ammonia/H_2O (90:54:5½:5½, v/v) and solvent II $CHCl_3/CH_3OH$/acetic acid/H_2O (90:40:12:2, v/v). After each dimension the plates are dried for 1 h under reduced pressure over concentrated H_2SO_4. Trinitrophenylated aminophospholipids are identified by their yellow color (Gordesky et al., 1975), the other phospholipids are localized by staining with iodine vapour.

When fatty acid analysis is to be performed, precoated plates are

preferred and 50 mg/l BHT is added to the elution solvents. The lipids are then localized by spraying with 0.001% (w/v) 1-amino-2-hydroxy-4-naphthalene-sulfonic acid (ANSA) and detection in ultraviolet light.

A schematic chromatogram is given in Fig. 2.4. The trinitrophenylated aminophospholipids are located near the solvent front next to their respective mother compounds PE and PS.

The spots are scraped off and transferred to test tubes (16 x 160 mm) for phosphate determination, or to screw-cap vials (5 ml) for fatty acid analysis. Aliquots of the plates, containing no spots, are also scraped off to serve as (phosphate) blanks.

Thin-layer chromatography of neutral lipids. Neutral lipids are collected by applying the total lipid extract in $CHCl_3$ to a silicic acid column (Bio-Sil HA, 325 mesh; Bio-Rad Labs., Richmond, USA), prepared and eluted according to the procedure of Kates (1972). As internal standard heneicosanoic acid (21:0; Supelco, Inc., Bellefonte, USA) is added. The neutral lipids are removed by elution with 7 volumes of $CHCl_3$. No phosphorus can be detected in this fraction. Alternatively, the neutral lipids are collected from the front of precoated silica gel plates after elution in $CHCl_3/CH_3OH/H_2O$ (65:25:4, v/v). The neutral lipids are separated by thin-layer chromatography on silica gel plates in hexane/diethylether/acetic acid (80:30:1, v/v) and visualized as before. Identification is achieved by co-chromatography of reference compounds.

2.3.6 *Phospholipid phosphate determination*

Samples containing 0.02-0.2 µmol phospholipid are transferred to thoroughly steamed test tubes (16 x 160 mm), topped with glass marbles, and dried at 110° C. The organic material is digested with 0.2 ml concentrated H_2SO_4/70% $HClO_4$ (5:1, v/v) for 1 h at 180° C. The tubes are then cooled below 50° C. Usually, especially when sucrose is present in the sample, the destruction is incomplete. In that case 0.1 ml 30% H_2O_2 is added and the mixture is heated again for at least 1 h at 180° C. This step is repeated until the mixture has become colorless. After cooling, 4.75 ml of the color reagent (Broekhuyse, 1968) is added. The tube is mixed and incubated for 20 min in a boiling water bath. After cooling with tap water and standing for 30 min in darkness, the 820 nm absorbance is measured against water as blank. In each determination reagent blanks and a series of standard P_i samples (0.02-0.2 µmol) are similarly treated. Tubes, containing samples scraped off

from thin-layer plates, are centrifuged (5 min at 3000 x g) prior to measuring the extinction in order to remove silica gel. Since the method is quite sensitive to changes in final pH, the concentration of acid (H_2SO_4) in the final mixture is critical. The relative standard error in the phosphate determination of phospholipids, scraped off from thin-layer plates, varies from 5 to 10%, depending on the amount of lipid analyzed.

2.3.7 *Fatty acid analysis*

Lipid extracts are transferred to screw-cap vials (5 ml). For fatty acid analysis of disk preparations, the total lipid extract is evaporated after addition of heptadecanoic acid (17:0; Supelco, Inc.; 15 µg dissolved in 50 µl n-pentane) as internal standard. The vials are sealed with a Teflon/rubber septum after addition of 0.5 ml CH_3OH containing either 14% (w/v) BF_3 (Morrison and Smith, 1964) or 10% (v/v) CH_3COCl. Both reagents give the same results. The vials are heated for 15 min at 100° C to allow lipid hydrolysis and methylation of the fatty acids. Equal volumes (0.5 ml) of n-pentane and water are added. The mixture is vigorously shaken (15 min) and the methyl esters are extracted into the upper pentane layer by centrifugation (5 min at 3000 x g). The aqueous lower layer is re-extracted once with n-pentane (0.5 ml). The pooled pentane layers are washed by shaking with 1 volume of H_2O (15 min) and centrifugation (5 min at 3000 x g). The upper pentane layer is removed, dried over anhydrous Na_2SO_4 and centrifuged. The methyl ester solution is evaporated in an N_2 stream and the residue is taken up in 50 µl n-pentane.

In the case of fatty acid analysis after thin-layer chromatography, the spots of the various lipid classes are scraped off and treated as above. Methylation of the fatty acids is then carried out with CH_3COCl/CH_3OH, since the use of BF_3/CH_3OH makes silica gel stick to the glass wall.

The methyl esters are analyzed at 210° C in a Pye Unicam gas-liquid chromatograph model 204, equipped with an all-glass sample stream and flame ionization detector. Injection of the sample (1 µl) and detection are performed at 250° C. A 6 ft x 4 mm inner diameter column, containing 10% SP-2330 on 100/120 Supelcoport (Supelco, Inc.), is used to separate the methyl esters. Identification is by comparison with reference methyl ester mixtures PUFA No. 1, PUFA No. 2, GLC 50 and GLC 60 (Supelco, Inc.). Relative molar concentrations of each fatty acid in the sample are calculated with a

Hewlet Packard integrator model HP 2280 A. The internal standard method is used and the integrated area of each peak is divided by the molecular weight of the corresponding methyl ester.

The overall recovery is always better than 95% as estimated by using heneicosanoic acid (21:0; Supelco, Inc.), added as internal standard to the suspensions prior to lipid extraction. The error in the fatty acid analysis appears to be 5% or less (standard deviation of the mean).

CHAPTER 3

TRANSBILAYER DISTRIBUTION OF PHOSPHOLIPIDS IN PHOTORECEPTOR MEMBRANE STUDIED WITH VARIOUS PHOSPHOLIPASES*

3.1 Introduction

The question as to whether membrane lipids are distributed symmetrically or asymmetrically over the two faces of biological membranes is currently much studied because of its obvious significance for our understanding of the role of lipids in membrane function. It has recently been claimed that asymmetrical distribution of phospholipids occurs in various biological membranes (Rothman and Lenard, 1977), although in some cases controversy still exists (van den Besselaar et al., 1978; Nilsson and Dallner, 1977; Sundler et al., 1977; Higgins and Dawson, 1977).

Various independent methods are available to obtain information on the lipid arrangement in biological membranes and artificial bilayered systems. These involve the use of chemical modifying reagents, of lipolytic enzymes like phospholipases, of phospholipid exchange proteins and of physical techniques like NMR spectroscopy (Bergelson and Barsukov, 1977).

Relatively little is known in this respect about the photoreceptor membranes of rod outer segments, which contain 36% phosphatidylcholine (PC), 45% phosphatidylethanolamine (PE) and 16% phosphatidylserine (PS), together 97% of total phospholipid (Anderson et al., 1975; see also Table 1.1). So far, chemical labeling experiments, mainly with trinitrobenzenesulfonate (TNBS), have suggested that at least PE is preferentially located at the cytoplasmic (outer) face of the rod outer segment membranes (see section 1.5).

It is, however, generally felt that more than one approach should be used before reliable conclusions can be drawn. Hence, we have carried out a study of the phospholipid distribution of rod outer segment membranes by means of three different phospholipases. The results suggest a nearly symmetrical distribution of the phospholipids.

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*Adapted from Drenthe et al. (1980a).

3.2 Materials and methods

A description of the experimental procedures is given in Chapter 2 (sections 2.2 and 2.3).

Two approximations have been applied in order to establish the transbilayer distribution of phospholipids in photoreceptor membranes. In the first approximation the final levels of phospholipid hydrolysis after prolonged treatment with phospholipases A_2, C and D are determined. In the case of treatment with phospholipases A_2 and C, nearly complete hydrolysis of natural substrates has previously been observed, suggesting that the phospholipid hydrolysis is not restricted to the outer leaflet of the disk membrane (see sections 1.4 and 2.1.3).

Therefore, a second approximation has been used in accordance with the method of Sundler et al. (1977, 1978). The hydrolysis of individual phospholipids in disk membranes is followed in time and compared with that of a reference preparation in which the same phospholipids are assumed to be randomly available. These reference preparations are retinal lipid suspensions in the case of treatment with phospholipases A_2 and C, and detergent-solubilized disk membranes in the case of treatment with phospholipase D. Special attention is given to the early stages of hydrolysis, since then disturbance of disk membrane structure may be minimal.

3.3 Results

3.3.1 *Rhodopsin content*

Determination of rhodopsin before and after incubation with any of the three phospholipases never shows a significant loss of rhodopsin absorbance, regardless of the degree of phospholipid hydrolysis reached.

3.3.2 *Phospholipase treatment*

Phospholipase C treatment. During phospholipase C treatment, quantitative agreement exists between the amount of phosphate ester, appearing in the aqueous supernatant and measured as P_i after acid destruction, and the disappearance of phospholipids from the disk membranes and the retinal lipid suspension.

TABLE 3.1

LEVELS OF PHOSPHOLIPID HYDROLYSIS UPON PROLONGED TREATMENT WITH PHOSPHOLIPASE C, A_2 AND D

Results are expressed as percent of total phospholipids hydrolyzed in stacked disks, disk vesicles and reference preparations: retinal lipid suspensions (for phospholipases C and A_2) and detergent-solubilized disk membranes (for phospholipase D). Incubation conditions as in Figs. 3.1-3.3, except that a double concentration of phospholipase D as in Fig. 3.3 has been used; incubation time, 180 min. The results are an average of at least three experiments with a relative standard error of maximally 6%.

Phospholipase	Stacked disks	Disk vesicles	Reference
C	98	97	99
A_2	100	100	100
D	40	53	88

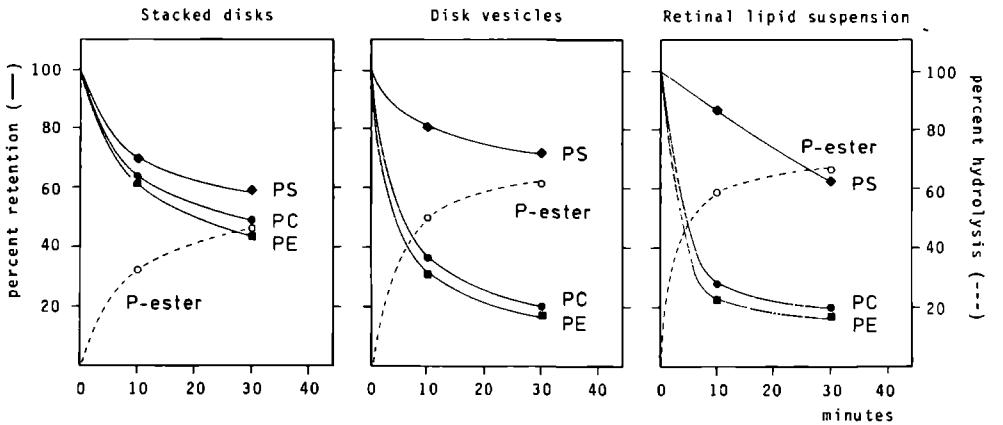

Fig. 3.1. Treatment with phospholipase C. Percent retention of individual phospholipids (solid symbols) and percent hydrolysis (open symbols) upon treatment of stacked disks, disk vesicles and retinal lipid suspensions are shown. Conditions: pH 7.4; darkness; 20°C; about 2 μmol/ml phospholipid and 2 units/ml phospholipase C. P-ester, phosphate ester.

Exhaustive phospholipase C treatment of disk membranes and retinal lipid suspensions results in nearly complete hydrolysis of the phospholipids (Table 3.1). Residual phospholipid is almost exclusively phosphatidylserine, 5-20% of

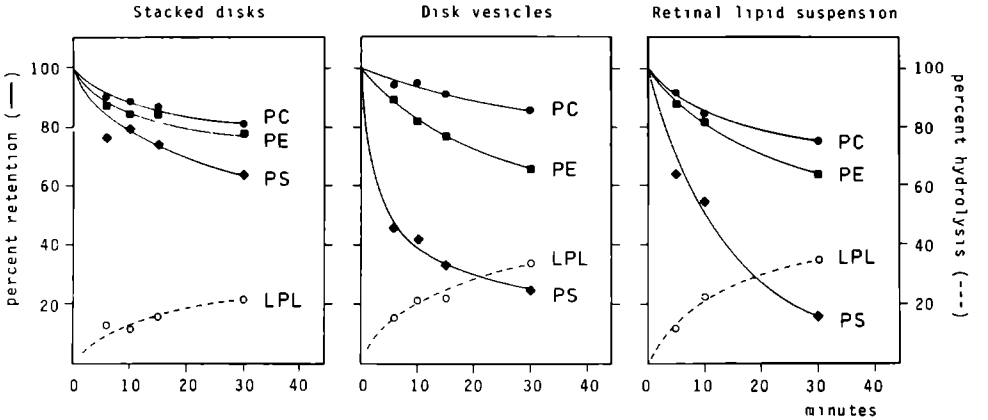

Fig. 3.2. Treatment with phospholipase A_2. Percent retention of individual phospholipids (solid symbols) and percent hydrolysis (open symbols) upon treatment of stacked disks, disk vesicles and retinal lipid suspensions are shown. Conditions: pH 7.4; darkness; 20°C; 10 mM Ca^{2+}; about 2 μmol/ml phospholipid and 0.7 μg/ml phospholipase A_2. LPL, lysophospholipid.

which is resistant to this enzyme.

The substrate preference of phospholipase C, as measured with the retinal lipid suspension, decreases in the order: PE > PC > PS (Fig. 3.1). The same order of preference is found for stacked disks and disk vesicles.

Phospholipase A_2 treatment. During phospholipase A_2 treatment, the decrease for each phospholipid is completely accounted for by the increase in the corresponding lysocompounds, except that some lyso-PS is lost, probably during the washing procedure.

Treatment of disk membranes with high concentrations of phospholipase A_2 completely modifies all phospholipids into their corresponding lysoproducts (Table 3.1).

The observed order of preference of phospholipase A_2 using retinal lipid suspension is PS > PE > PC, which is also found with stacked disks and disk vesicles (Fig. 3.2).

Phospholipase D treatment. The optimal conditions for phospholipase D hydrolysis of phospholipids in rod outer segment disk membranes have been ascertained by varying pH, temperature, Ca^{2+} concentration and amount of enzyme. Maximal hydrolysis is obtained at pH 6.0 and 30° C in the presence of 40 mM $CaCl_2$. Under these conditions no transferase activity of phospholipase D

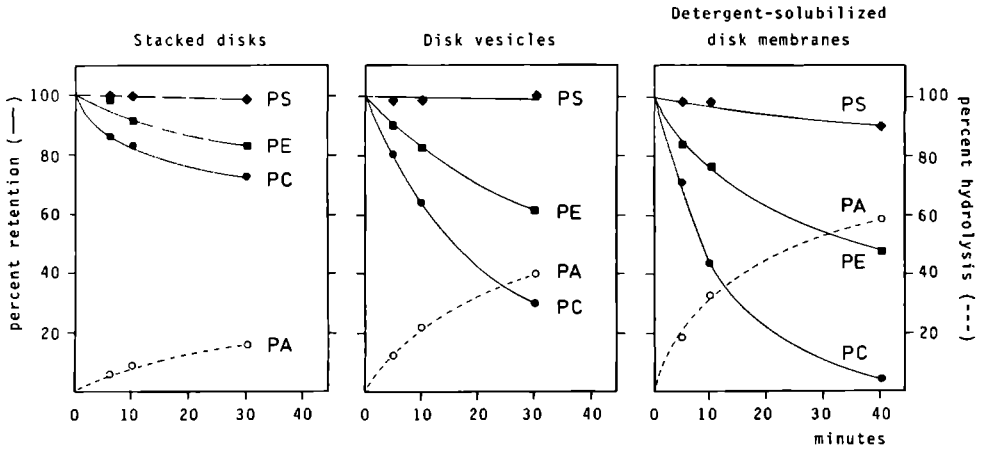

Fig. 3.3. Treatment with phospholipase D. Percent retention of individual phospholipids (solid symbols) and percent hydrolysis (open symbols) upon treatment of stacked disks, disk vesicles and detergent-solubilized disk membranes are shown. Conditions: pH 6.0; darkness; 30°C; 40 mM Ca^{2+}; about 2 μmol/ml phospholipid and 0.7 mg/ml phospholipase D. Disk membranes solubilized in 20 mM β-1-nonylglucose. PA, phosphatidic acid.

is observed (section 2.2.3).

The decrease in each phospholipid after treatment with phospholipase D should be equal to the amount of phosphatidic acid (PA) formed, but we always find less PA than expected. This is due to the presence in phospholipase D preparations of phosphatidate phosphohydrolase (EC 3.1.3.4), which hydrolyzes PA to diglyceride and P_i (Davidson and Long, 1958). Determination of P_i in the aqueous layers gives the contribution of PA breakdown, which is less than 20%. The sum of PA and P_i fully accounts for the amount of phospholipids hydrolyzed.

In contrast to the situation with phospholipases A_2 and C, there is a limited final level of hydrolysis upon treatment of disk membranes with phospholipase D (Table 3.1). Approx. 50% of PC and PE and nearly all PS, together 60% of the membrane phospholipids, are resistant to this enzyme in stacked disks. Removal of the water-soluble hydrolysis products, either by centrifugation followed by addition of fresh phospholipase D or by dialysis of the incubation mixture against fresh buffer, does not result in additional hydrolysis. Thus, inhibition by the water-soluble hydrolysis products does not play a role and the final level seems to represent a true limit to hydrolysis. Also, in disk vesicles an apparent final level of phospholipid hydrolysis is

observed. This level is significantly higher (53%) than in stacked disks and will be discussed later.

When the membranes are solubilized in nonylglucose, phospholipid hydrolysis by phospholipase D proceeds much further, viz., to approx. 90% (Table 3.1). Residual intact phospholipid consists of approximately equal amounts of PE (9% retention) and PS (30% retention). Nearly complete hydrolysis (at least 95%) of the phospholipids can only be obtained at longer incubation times (up to 6 h) and higher concentrations of phospholipase D.

The substrate preference of phospholipase D decreases in the order: PC > PE >> PS in detergent-solubilized disk membranes. The same order of decreasing preference is found in stacked disks and disk vesicles (Fig. 3.3).

3.3.3 *Initial hydrolysis*

Under identical incubation conditions, the rate at which the phospholipids of stacked disks are hydrolyzed in the first 10 min is always lower than that for disk vesicles and retinal lipid suspension. This is shown for phospholipase C in Fig. 3.1 and for phospholipase A_2 in Fig. 3.2. In the case of phospholipase D, this is true for PC and PE, of which the initial rates of hydrolysis decrease in the order: detergent-solubilized disk membranes > disk vesicles > stacked disks (Fig. 3.3). PS is only attacked in detergent solution. The differences are most readily explained by the different degree to which the substrates are directly accessible to the enzymes.

The effects of the enzymes during the initial stage of the incubation are particularly important, since in this early period changes in the membrane organization due to phospholipid hydrolysis can be expected to be minimal. The most relevant results of 10-min incubations are, therefore, analyzed in more detail in Table 3.2. In stacked disks about 30% overall hydrolysis is achieved with phospholipase C during this time and about 10% with the two other phospholipases at the enzyme concentrations used. The results are presented as the percent at which each phospholipid class contributes to the total fraction of phospholipids hydrolyzed in 10 min. In this way the substrate specificities of the phospholipases in the reference preparations are easily compared with those in stacked disks. A lower percentage indicates that the phospholipid is less available to enzyme action than in the randomized sample and vice versa.

The data of Table 3.2 clearly argue against a major asymmetrical distribution of any of the phospholipids in the disk membrane. If the phos-

TABLE 3.2

PHOSPHOLIPID HYDROLYSIS DURING THE INITIAL STAGE OF TREATMENT WITH PHOSPHOLIPASE C, A_2 AND D

Results are expressed as the percent at which each phospholipid class contributes to the total fraction of phospholipids hydrolyzed after 10 min of incubation. Overall hydrolysis is the percent of initially present total phospholipid hydrolyzed at that time. Minor phospholipids (3% of total phospholipid) are neglected. Reference preparations as in Table 3.1. Values are averages of two experiments with a relative standard error of maximally 10%. Ref., reference preparation.

Phospholipase		Stacked disks	Reference	Ratio disks: ref.
C	phosphatidylcholine	38	49	0.75
	phosphatidylethanolamine	47	48	0.98
	phosphatidylserine	15	3	5
	overall hydrolysis	*33*	*59*	
A_2	phosphatidylcholine	30	38	0.79
	phosphatidylethanolamine	45	36	1.25
	phosphatidylserine	25	26	0.96
	overall hydrolysis	*14*	*23*	
D	phosphatidylcholine	54	61	0.89
	phosphatidylethanolamine	44	37	1.20
	phosphatidylserine	2	2	1
	overall hydrolysis	*9*	*32*	

pholipids in the reference preparations are indeed randomly available (see section 3.4.1), PC would appear to occur at a somewhat lower, and PE at a somewhat higher percentage at the outer face of the disk membranes. Even after a 30 min incubation period, these percentages are fairly close to those after enzyme treatment for 10 min. This is particularly true for phospholipases A_2 and D, which have both produced about 20% overall phospholipid hydrolysis at that time.

Corresponding calculations for disk vesicles have been omitted from Table 3.2, since they do not provide additional information.

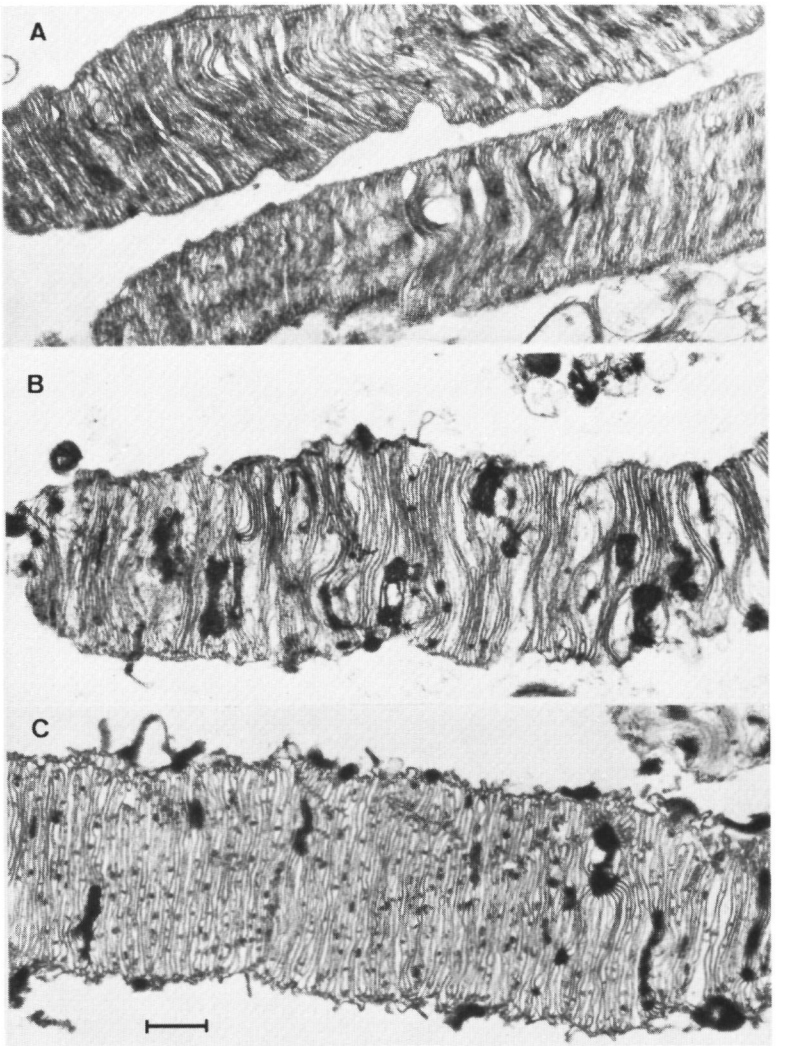

Fig. 3.4. Thin sections of stacked disks treated with phospholipase D. A, control sample incubated for 150 min in the absence of enzyme; B, sample incubated with phospholipase D during 40 min, resulting in 19% phospholipid hydrolysis; C, sample incubated with phospholipase D during 150 min, resulting in 41% phospholipid hydrolysis. The dark spots in B and C are presumably aggregates of phospholipase D (+ Ca^{2+}?), since they are absent if the suspensions are centrifuged and washed prior to fixation. Bar represents 0.4 μm.

3.3.4 *Microscopy*

Phase-contrast microscopy reveals that treatment of the stacked disk preparations with any of the three phospholipases does not seriously change their appearance in the earlier stages of incubation. Electron microscopy of thin sections (Fig. 3.4) convincingly shows that stacked disks retain their original morphology when 19 or even 41% of the phospholipids are hydrolyzed by phospholipase D. Similar results are obtained with phospholipase C and A_2 at relatively low (approx. 30%) phospholipid hydrolysis levels.

3.4 Discussion

3.4.1 *Principles of the approach*

The distribution of phospholipids over the two faces of biomembranes can, in principle, be deduced from their availability to the action of phospholipases on intact cells or cell organelles. However, if valid conclusions are to be drawn, a number of conditions must be fulfilled: (1) the outer membrane face of the intact preparation should be the original outer face and this outer face should remain exposed during incubation with phospholipase; (2) the membranes should not be significantly penetrated by the phospholipase, and (3) the specificity of the phospholipase towards different phospholipids should be taken into account by using random control preparations. In addition, the results with different phospholipases should agree.

Membrane orientation. Studies on the topography of phospholipids in rod outer segment disk membranes have, so far, only been conducted with disk vesicles (Raubach et al., 1974; Smith et al., 1977; Crain et al., 1978). In order to avoid the uncertainty about the maintenance of the original inside-outside arrangement in these structures (section 2.1.3), we have used isolated rod outer segments (stacked disks) as the primary experimental preparation. Their morphology resembles that of outer segments in situ so closely that membrane inversion must be considered to be extremely unlikely. Microscopic and electron-microscopic evidence indicates that the gross morphology of the stacked disks is hardly affected during the early stages of incubations with phospholipases. Therefore, it seems safe to assume that the stacked disk membranes retain a 'right-side-out' orientation under these conditions.

Penetration of phospholipases. Since phospholipases are water-soluble

proteins with molecular weights of at least 14,000, it seems improbable that they would penetrate the intact hydrophobic core of a biological membrane. However, the application of phospholipases results in chemical alteration of part of the phospholipids, which might lead to abolition of the permeability barriers in the membrane. Unfortunately, an endogenous or exogenous internal marker, the release of which could indicate gross permeability of the membrane (as has been applied with erythrocytes), cannot be used very well. A well defined internal marker is lacking, whereas the introduction of an exogenous marker presents unknown risks of loss of the right-side-out orientation in the resulting vesicles. Since, however, secondary effects due to the action of phospholipases should be minimal in the early phase of enzymatic degradation (Sundler et al., 1977), special attention has been given to the initial period of incubation (Table 3.2).

Substantial disturbance of the membrane, leading to almost complete hydrolysis of the phospholipids, clearly happens in the later stages of incubation with phospholipases C and A_2 (Table 3.1). The products of phospholipase C treatment, hydrophobic diglycerides and water-soluble phosphate esters, will not fit very well in a bilayer membrane, while the products of phospholipase A_2 treatment, lysophospholipids, are well known for their lytic activity. Therefore, it is not surprising that these enzymes upon extensive incubation induce serious disturbance of the membrane, resulting in complete accessibility of all phospholipids.

Phospholipase D, which hydrolyses glycerophospholipids to PA, would be expected to present less risk. PA very much resembles the usual phospholipids with respect to its amphiphilic character and, therefore, serious membrane disturbance is unlikely (Berden et al., 1975; Papahadjopoulos et al., 1976). This probably explains the limited final level of hydrolysis by phospholipase D, at least in stacked disks. It also suggests that in this preparation, even up to 180 min incubation, penetration of the enzyme, gross disturbance of the membrane and appreciable transbilayer exchange of phospholipids against PA do not occur. Although it has been shown that phospholipase D is able to induce transbilayer exchange of PA against PC in unilamellar PC vesicles in the absence of Ca^{2+} (de Kruijff and Baken, 1978), this phenomenon was not found in the presence of 6 mM Ca^{2+} (Johnson et al., 1975; see also section 2.1.3). The alternative explanation for the limited final level of hydrolysis, viz., shielding of outer face phospholipids either by their interaction with protein or by accumulation of negative charge, seems unlikely. The unrestricted action

of phospholipase A_2 and, especially, phospholipase C argues against the first possibility and the presence of 40 mM Ca^{2+} during phospholipase D treatment against the second.

The reason for the different final hydrolysis levels in stacked disks (40%) and in disk vesicles (53%) after phospholipase D treatment is not immediately clear. A possible explanation is provided by the observation of Woodward and Zwaal (1972) that resealed ghosts, prepared from intact erythrocytes by a hypotonic lysis procedure, are more easily hemolyzed by treatment with phospholipase C (*Bacillus cereus*) than erythrocytes themselves. This shows that prior exposure to hypotonic conditions makes the membrane permeability barrier for macromolecules more vulnerable to phospholipases, which attack the polar head groups. Disk vesicles, obtained by washing disk membranes with distilled water, may similarly have become partially permeable to phospholipase D. This would imply another argument in favor of preferring stacked disks to disk vesicles in studying phospholipid distribution in the photoreceptor membrane.

Specificity of the phospholipases. The specificity of the phospholipases has been determined by measuring their effect on control preparations of phospholipid composition similar to rod outer segments, but with presumably random orientation of the phospholipids. The ^{31}P NMR spectra obtained with retinal lipid suspensions indicate that the phospholipids in this preparation are present in hexagonal and inverted micellar phases, in which at least PC and PE are randomly available (de Grip et al., 1979; but see Deese et al., 1981). Disks solubilized in nonylglucose above its critical micelle concentration (6.5 mM; de Grip and Bovee-Geurts, 1979) must also have their phospholipids randomly available.

The specificity of phospholipase C decreases in the order: PE > PC > PS (Fig. 3.1). Roberts et al. (1978) reported the same substrate preference of this enzyme in Triton X-100-solubilized phospholipid preparations.

The specificity of phospholipase A_2 decreases in the order: PS > PE > PC (Fig. 3.2). This specificity is in agreement with the preference of pancreatic phospholipase A_2 for negatively charged phospholipids (like PS) compared to neutral ones (de Haas et al., 1968).

The substrate specificity of phospholipase D decreases in the order: PC > PE >> PS (Fig. 3.3). The specificity of this enzyme, which has not been obtained in a pure form so far, is not well established (Davidson and Long, 1958; Roughan and Slack, 1976; Heller, 1978; Allgyer and Wells, 1979).

3.4.2 *Tentative conclusions*

The phospholipid hydrolysis patterns obtained from stacked disks and disk vesicles with phospholipase C (Fig. 3.1), phospholipase A_2 (Fig. 3.2) and phospholipase D (Fig. 3.3) do not substantially deviate from those obtained from randomized control preparations, apart from readily explained differences in reaction rate. They suggest symmetry rather than asymmetry in the distribution of the phospholipids over both faces of the disk membrane.

In view of the discussion in section 3.4.1, more refined conclusions might be derived from the comparison of the effects of a brief (10 min) phospholipase treatment on stacked disks and randomized control preparations (Table 3.2). With respect to PC, all three approaches show a slightly asymmetrical distribution with 40-45% of this phospholipid at the outer (cytoplasmic) face of the disk membrane. For PE, a similar conclusion seems warranted, albeit with 55-60% of this phospholipid at the outer face of the membrane. For PS, we must rely primarily on the results with phospholipase A_2, since its high preference for this phospholipid compensates for the reduced analytical accuracy caused by the low PS concentration. The results obtained with phospholipase A_2 indicate a symmetrical distribution of PS over the two faces of the membrane.

Our present conclusions obtained with three different phospholipases can only have a preliminary character. The occurrence of preferential transmembrane flip-flop of a specific phospholipid and the possibility of shielding of outer face phospholipids against phospholipase D, including inaccessibility due to the piled disk structure, cannot yet be excluded. In addition, our conclusions do not agree with those obtained by studies of the modification of PE and PS by amino group reagents, where preferential (70-100%) location of both PE and PS (Raubach et al., 1974; Smith et al., 1977) or of PE only (63-72%; Crain et al., 1978) on the cytoplasmic side of the disk membrane has been concluded. We shall return to these aspects in Chapter 4, which describes the effects of TNBS alone and in combination with phospholipase D. Such multiple approaches are necessary in order to avoid the many pitfalls that may occur in studies of the phospholipid distribution in biological membranes (section 2.1.3).

3.4.3 *Summary*

The distribution of the three major phospholipids of bovine rod outer segment disk membranes over the two faces of the membrane has been studied by means of treatment with phospholipase C, phospholipase A_2 and phospholipase D.

Two different preparations of rod outer segment disk membranes have been used, which are called 'stacked disks' and 'disk vesicles' on account of their morphological appearance.

The hydrolysis patterns obtained by phospholipase treatment of these preparations have been compared to those of a retinal lipid suspension or detergent-solubilized disk membranes, which serve as control preparations with a similar phospholipid composition but a random availability of the phospholipids.

Special attention is given to the early phase of enzyme treatment in order to eliminate secondary effects on the molecular organization of the membrane due to appreciable phospholipid hydrolysis. Analysis of the hydrolysis patterns for all three phospholipases in stacked disks, as compared to those in randomized control preparations, suggests a slightly asymmetrical distribution of phosphatidylcholine (PC; 40-45% at the outer face) and phosphatidylethanolamine (PE; 55-60% at the outer face) and a symmetrical distribution of phosphatidylserine (PS) in rod outer segment disk membranes.

Extensive treatment with phospholipases C and A_2 leads ultimately to nearly complete hydrolysis of all phospholipids, but with phospholipase D a final level of 40% phospholipid hydrolysis is observed in stacked disk preparations. This suggests that in the latter case the inner face of the membrane is inaccessible to the enzyme.

Further work will be necessary in order to substantiate these conclusions.

CHAPTER 4

TRANSBILAYER DISTRIBUTION OF PHOSPHOLIPIDS IN PHOTORECEPTOR MEMBRANE STUDIED WITH TRINITROBENZENESULFONATE ALONE AND IN COMBINATION WITH PHOSPHOLIPASE D*

4.1 Introduction

In Chapter 3 we have used three phospholipases in a study of the phospholipid distribution over the two faces of the disk membrane in bovine rod outer segments. The results suggested an approximately symmetrical distribution of the three major phospholipids: 40-45% of the phosphatidylcholine (PC), 55-60% of the phosphatidylethanolamine (PE) and 50% of the phosphatidylserine (PS) in the outer (cytoplasmic) face. Our conclusions were conditioned by the consideration that the possible occurrence of preferential transbilayer movement (flip-flop) of a given phospholipid as well as shielding of outer face phospholipids against phospholipase D has yet to be disproved.

The conclusions disagree with those of other investigators (see section 1.5) who had used amino group reagents like trinitrobenzenesulfonate (TNBS). They reported predominant (between 65 and 100%) location of PE in the outer face of the membrane. The location of PS remained unclear with values of 25% (Crain et al., 1978) and 100% (Smith et al., 1977; see Table 1.3) at the outer face. PC, which has no primary amino group, cannot be localized by this method. In order to resolve this discrepancy, we decided to study in more detail the reaction between rod disk membranes and TNBS, alone and in combination with phospholipase D. The results strengthen our earlier conclusion of a nearly symmetrical distribution of the three major phospholipids in the disk membrane and offer a possible explanation for the divergent earlier reports.

4.2 Materials and methods

A description of the experimental procedures is given in Chapter 2 (sections 2.2 and 2.3).

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*Adapted from Drenthe et al. (1980b).

4.3 Results

4.3.1 *General*

In accordance with previous results from our laboratory (de Grip et al., 1973), analysis of the primary amino groups in disk membranes with TNBS in detergent solution shows the presence of 52 ± 2 mol/mol rhodopsin, consisting of 16 ε-amino groups of lysine, 27 amino groups of PE and 9 amino groups of PS.

4.3.2 *Treatment of disk vesicles with trinitrobenzenesulfonate*

Disk vesicles have primarily been used to establish reaction conditions leading to a limited final level of modification, which would represent minimal reagent penetration and membrane disturbance. These conditions have then been used in our further studies with disk vesicles and stacked disks. In addition, inclusion of disk vesicles in these studies allows comparison of our results with those of other investigators (section 1.5) who have exclusively used this type of preparation.

Previously, it has been observed that temperatures substantially above 20^{o} C and illumination lead to complete modification with TNBS (de Grip et al., 1973; Litman, 1974). Hence, we have chosen to work at 20^{o} C and in darkness. Buffer composition, pH and concentration of TNBS have been varied. The buffer composition hardly influences the final degree of modification, except that the reaction proceeds rather slowly in a medium containing sucrose and Ficoll 400.

The effect of the reagent concentration has been studied by spectrophotometric determination of total amino group modification as a function of time at pH 7.4 and 20^{o} C (Fig. 4.1). The TNBS concentration has been varied from 1 to 5 mM, representing a 5- to 25-fold molar excess with regard to the total number of primary amino groups. Both the course and the final level of modification are greatly influenced by the reagent concentration. The complete modification occurring at 3-5 mM TNBS shows that in principle all amino groups are reactive with the reagent and suggests that at these concentrations TNBS penetrates the membrane. However, at 1-2 mM TNBS the final level of modification remains restricted to 50-60% of the amino groups. Therefore, we have further investigated the course of the reaction at 1 mM TNBS.

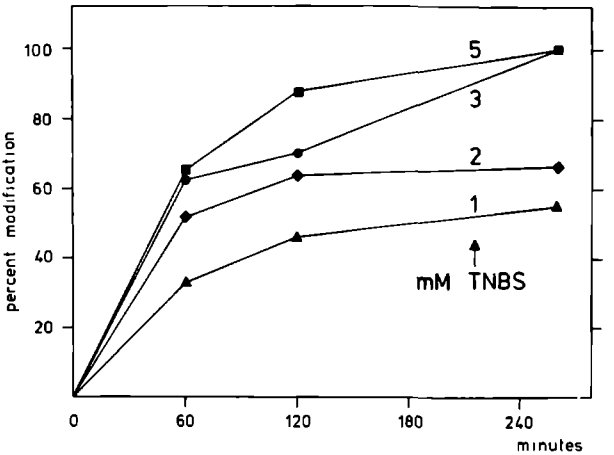

Fig. 4.1. Amino group modification in disk vesicles at different TNBS concentrations. Results are expressed as percent modification of total amino groups, determined by measuring the 340 nm absorbance. Conditions: pH 7.4; darkness; 20°C; 3-4 µM rhodopsin.

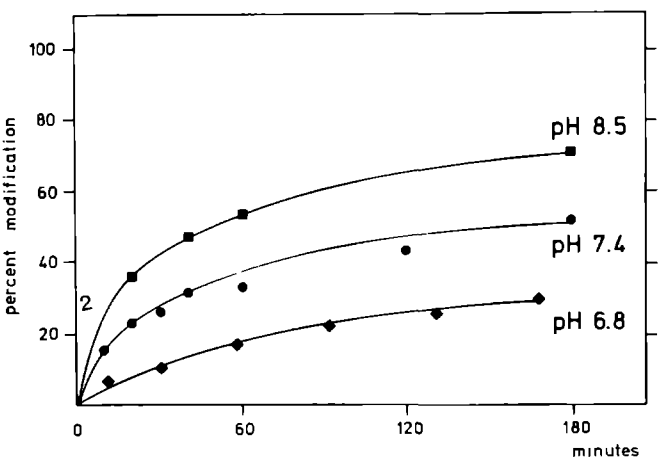

Fig. 4.2. Amino group modification in disk vesicles at three pH values. Results are expressed as percent modification of total amino groups, determined by measuring the 340 nm absorbance. Conditions: darkness; 20°C; 1 mM TNBS; 3-4 µM rhodopsin. Incubations at pH 6.8 and 7.4 are carried out in 40 mM Mops buffer containing 2 mM $CaCl_2$, 3 mM $MgCl_2$ and 140 mM NaCl; incubations at pH 8.5 in 120 mM $NaHCO_3$ and 40 mM NaCl.

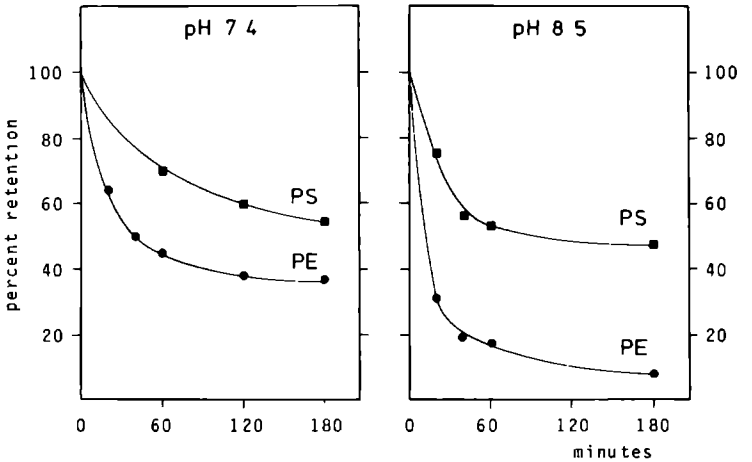

Fig. 4.3. Treatment of disk vesicles with TNBS at pH 7.4 and 8.5. Percent retention of PE (●) and PS (■) is shown. Conditions: see legend of Fig. 4.2.

Variations of pH also appears to have a large influence (Fig. 4.2). After 3 h of incubation, approx. 70, 50 and 30% of the amino groups are found to be modified by 1 mM TNBS at pH 8.5, 7.4 and 6.8, respectively. Analysis of the individual amino groups, modified at pH 7.4 and 8.5, shows that after 3 h of incubation the higher overall modification at pH 8.5 is largely due to almost complete modification of PE: 92% at pH 8.5 as compared to 63% at pH 7.4 (Fig. 4.3). PE is the compound of most interest to us, since in contrast to PS, it is also a substrate of phospholipase D. Considering the limited final level of PE modification at pH 7.4, we have chosen this pH for our further experiments.

Under the standard conditions for limited final modification thus established (20° C, darkness, 1 mM TNBS, pH 7.4), complete modification can be induced within 1 h by addition of Triton X-100 to a final concentration of 1% (w/v). Thus, even at 1 mM TNBS and pH 7.4, all amino groups can in principle be modified.

4.3.3 *Combined treatment of disk vesicles with TNBS and phospholipase D*

We have previously noticed (Chapter 3) that phospholipase D also shows a limited final hydrolysis level of disk membrane phospholipids. Therefore, it

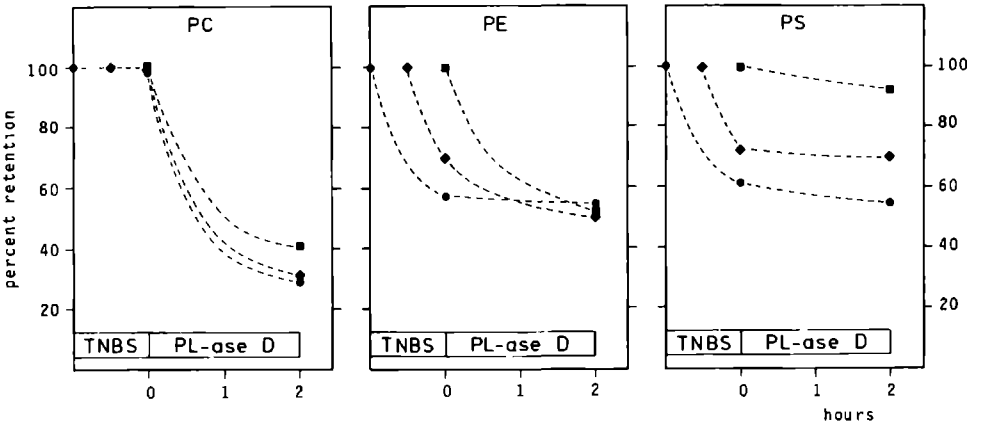

Fig. 4.4. Successive treatment of disk vesicles with TNBS and phospholipase D. Disk vesicles are first treated with TNBS during 0 (■), 30 (◆) and 60 (●) min. After removal of TNBS, each preparation is treated with phospholipase D during 120 min. The results for each phospholipid are expressed as percent retention: 100% - % modified - % hydrolyzed. Conditions: for TNBS treatment, pH 7.4; darkness; 20°C; 1 mM TNBS; 3-4 μM rhodopsin; for phospholipase D treatment, pH 6.0; darkness; 30°C; 40 mM $CaCl_2$; 30-40 μM rhodopsin; 0.7 mg/ml phospholipase D. PL-ase D, phospholipase D.

seemed of interest to test the complementarity of the reactions of TNBS and phospholipase D with disk vesicles.

Aliquots of a single disk vesicle preparation are treated with TNBS for either 30 or 60 min. This leads to trinitrophenylation of 30 or 43% of PE and 28 or 39% of PS, respectively. A third aliquot, incubated for 60 min without reagent, serves as the control. After removal of excess TNBS by centrifugation and washing, these samples are incubated with phospholipase D during 2 h, leading to a plateau level of hydrolysis. The reaction products are analyzed by thin-layer chromatography, and the results are shown in Fig. 4.4. The trinitrophenylated phospholipids are found not to be hydrolyzed by phospholipase D.

With respect to PC (a good substrate) and PS (a poor substrate), the action of phospholipase D appears to be largely independent of the pretreatment with TNBS. The most relevant results concerns PE, for which the

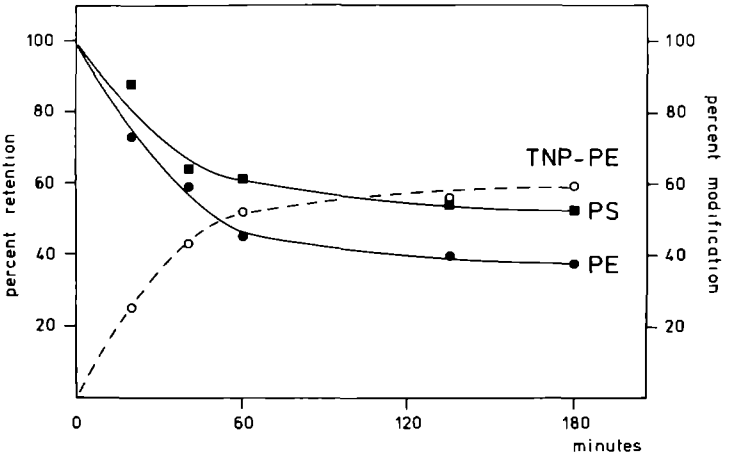

Fig. 4.5. Treatment of stacked disks with TNBS. Percent retention of PE (●) and PS (■) and percent modification (o) of PE are shown. Conditions: pH 7.4; darkness; 20°C; 1 mM TNBS; 3-4 µM rhodopsin. TNP-PE, trinitrophenyl-PE.

cumulative, final level of modification (trinitrophenylation + hydrolysis) is equal for all three preparations.

4.3.4 *Treatment of stacked disks with trinitrobenzenesulfonate*

Reaction of stacked disks with TNBS under our conditions leads to the results shown in Fig. 4.5. Quantitative thin-layer chromatography shows that 50% of PE is rather rapidly modified within 1 h. During the next 2 h an additional 10% is slowly modified. These results are confirmed by analysis of the product: trinitrophenyl-PE. PS reacts rapidly with 40% modification in 1 h and a further 10% reacting slowly during the next 2 h. In this case, analysis of the product, trinitrophenyl-PS, gives unsatisfactory results due to difficulties with the exact location of the less intensely colored spot on the thin-layer plate, leading to recoveries varying from 50 to 100%.

Addition of Triton X-100 (final concentration 1%, w/v) to the incubation mixture leads again to complete modification of both phospholipids, indicating that also in stacked disks all aminophospholipids can in principle be modified with 1 mM TNBS at pH 7.4.

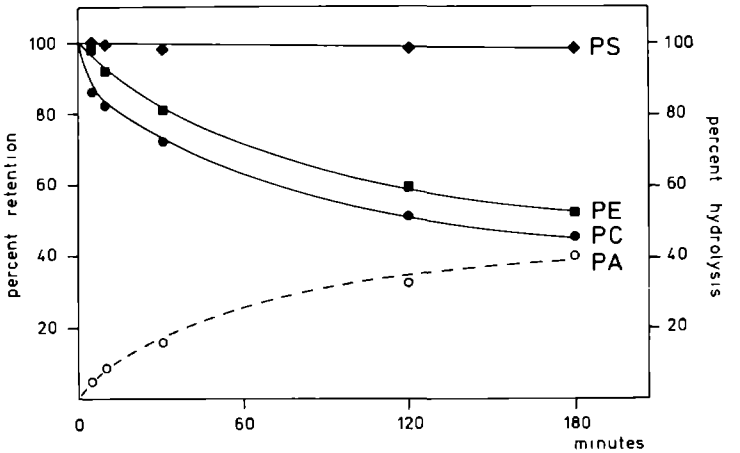

Fig. 4.6. Treatment of stacked disks with phospholipase D. Percent retention of individual phospholipids (solid symbols) and percent hydrolysis (open symbols) are shown. Conditions: pH 6.0; darkness; 30°C; 40 mM $CaCl_2$; 30-40 μM rhodopsin; 0.7 mg/ml phospholipase D.

4.3.5 *Combined treatment of stacked disks with phospholipase D and TNBS*

The complementarity of the action of TNBS and phospholipase D is investigated by treating stacked disks first with phospholipase D, followed by treatment with TNBS. Treatment of stacked disks with phospholipase D alone for 3 h causes maximally 40% phospholipid hydrolysis, comprising 55% hydrolysis of PC and 50% hydrolysis of PE, while PS is almost resistant to the enzyme (Fig. 4.6). This confirms our earlier experiments (Chapter 3).

The results obtained after treatment with phospholipase D, followed by TNBS, are shown in Fig. 4.7. Aliquots of a single stacked disk preparation are incubated for either 30 or 120 min with phospholipase D, leading to the hydrolysis of 20 or 40% total phospholipid, viz., 20 or 50% PE, 1 or 3% PS and 29 or 55% PC, respectively. A third aliquot, incubated for 120 min without enzyme, serves as the control. Subsequently, these preparations are incubated with TNBS during 3 h and the course of the reaction is followed by phospholipid analysis. For PE, the same cumulative, final level of nearly 60% modification (hydrolysis + trinitrophenylation) is obtained, regardless of the degree of previous hydrolysis by phospholipase D. The final level of modification of PS (50-52%, largely trinitrophenylation), and even the rate at which

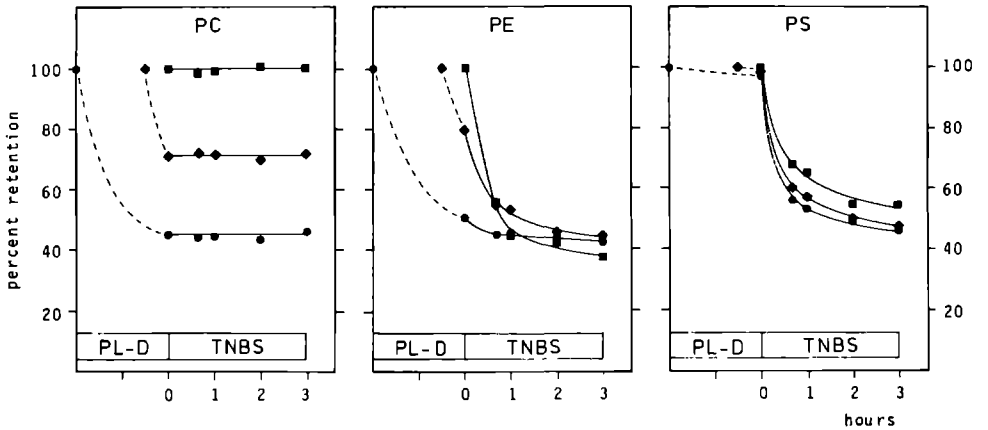

Fig. 4.7. Successive treatment of stacked disks with phospholipase D and TNBS. Stacked disks are first treated with phospholipase D during 0 (■), 30 (◆) and 120 (●) min (-----). After removal of the enzyme each preparation is treated with TNBS during 180 min (———). The results for each phospholipid are expressed as percent retention: 100% - % hydrolyzed - % modified. Conditions: for phospholipase D treatment as in Fig. 4.6, for TNBS treatment as in Fig. 4.5. PL-D, phospholipase D.

this level is reached, appear to be almost independent of the previous treatment with phospholipase D. Hence, successive treatment with the two reagents again does not lead to greater modification than that maximally obtained with either reagent alone, taking into account their different specificities.

4.4 Discussion

During the last 5 years, it has become evident that studies of the transbilayer distribution of phospholipids in biological membranes should be executed and interpreted with great caution (op den Kamp, 1979). Maintenance of the original lipid orientation, exclusive reactivity of the outer face of the membrane and complete accessibility of all outer face lipids to the reagent should be firmly established before valid conclusions can be drawn. We will discuss our results with TNBS and phospholipase D treatment in the light of these conditions.

4.4.1 *Trinitrobenzenesulfonate and disk vesicles*

The conditions under which TNBS has previously been used as a supposedly impermeable amino group reagent vary considerably, especially with respect to reagent concentration and pH. Since differences in membrane permeability of TNBS have been noticed for different membrane systems (op den Kamp, 1979), it is essential to establish conditions under which its permeability is restricted as much as possible. One of the difficulties with disk membranes is the lack of a well defined endogenous marker, becoming detectable in the case of membrane leakage, like hemoglobin in erythrocytes. Insertion of an exogenous marker, if at all possible, presents unknown risks of loss of the right-side-out orientation in the resulting vesicles. Therefore, we have confined ourselves to finding incubation conditions which lead to a clearly limited modification level of the amino groups in disk vesicles.

Figs. 4.1-4.3 suggest that at 1 mM concentration and pH 7.4, TNBS behaves as a non-permeant probe with respect to PE. The lower reactivity of PS at pH 7.4 is probably due to the higher pK_a of its amino group and a possible shielding effect of the carbonyl group. This is suggested by the observation that trinitrophenylation of PS rapidly reaches a plateau level at pH 8.5 only (Fig. 4.3). The reactivity of the lysine ε-amino group is even lower than that of the phospholipid amino groups due to its higher pK_a (de Grip et al., 1973), but this substance can be left out of consideration for our present purpose.

The fact that, even at pH 7.4, all amino groups of the membrane can be modified in the presence of detergent or at high (3-5 mM) TNBS concentration shows that in principle all amino groups are available for modification with TNBS and that appreciable shielding effects due to protein-lipid interaction do not seem to occur.

The effects of combined treatment of disk vesicles with TNBS and phospholipase D (Fig. 4.4) indicate that the pool of PE, susceptible to the action of phospholipase D, coincides with that accessible to TNBS under the conditions used. Since trinitrophenylation of PE and PS does not seem to influence seriously the action of phospholipase D towards residual substrate, indicating little perturbation of the membrane, this strongly suggests that the accessible PE represents the outer face of the disk membrane.

4.4.2 *Trinitrobenzenesulfonate and stacked disks*

In studies of the phospholipid transbilayer distribution, stacked disks are preferable to disk vesicles, since disk vesicles may show membrane inversion (Adams et al., 1979) which is unlikely to occur in the native structure of stacked disks.

Treatment of stacked disks with TNBS under our standard conditions results in modification of PE in a way similar to that in disk vesicles. The reaction course suggests a biphasic reaction, in which about 50% of the total PE belongs to a fast-reacting pool and this PE must almost certainly be located at the outer face of the membrane. The slow second phase of trinitrophenylation could then represent penetration of the reagent or perturbation of the membrane, leading to modification of originally inside-located PE. A similar biphasic pattern of trinitrophenylation is observed for PS, although percentually slightly less PS than PE is susceptible to modification.

As already mentioned, the behavior of TNBS towards disk vesicles is rather similar to that found towards stacked disks. Therefore, it is very unlikely that in stacked disks phospholipids are not accessible due to spatial restrictions like residual plasma membrane and juxtaposition of disks, which are not present in disk vesicles.

4.4.3 *Combined treatment of stacked disks with phospholipase D and TNBS*

In our earlier experiments we have already obtained evidence that phospholipase D does not seem to penetrate the disk membrane (Chapter 3). In agreement with this, we now find that exhaustive treatment of stacked disks with this enzyme shows (Fig. 4.6) that approximately one-half of the PE and PC present is susceptible to enzymatic hydrolysis.

The experiments with combined treatment of stacked disks with phospholipase D and TNBS (Fig. 4.7) show that only the PE, which is susceptible to phospholipase D action, can be modified by TNBS. Although we cannot exclude the possibility of a rapid 1:1 exchange between the pools of non-accessible and accessible unmodified PE, this would not change the overall conclusion of a symmetrical distribution of PE.

The maximal extent of trinitrophenylation of PS is virtually the same, regardless of whether 0, 20 or 40% of the accessible phospholipid pool is hydrolyzed. This indicates that no net transfer of PS occurs from one pool to

the other due to treatment with phospholipase D. The independence of the rate of the PS trinitrophenylation of prior phospholipase D treatment can only mean that the membrane is not seriously disturbed by either reagent.

Thus, we find that the combined action of phospholipase D and TNBS gives the same results as those obtained with these reagents separately: about one-half of the three major phospholipid classes is accessible in stacked disks. For PE we find that both reagents see the same pool, even though they differ very much in molecular size, mode of action and other properties. Since (1) we start with the original right-side-out configuration, (2) disk structure remains essentially intact, and (3) all phospholipids can in principle be modified (no shielding), we conclude that the accessible pool represents the outer face of the disk photoreceptor membrane. This implies phospholipid symmetry in these membranes.

4.4.4 *Comparison with previous studies*

Our conclusions differ from those of earlier studies on the phospholipid distribution in rod outer segment membranes with amino group reagents. Raubach et al. (1974) find 70% of the aminophospholipids on the outer surface when isethionylacetimidate (IAI) is used as a modifying reagent. Smith et al. (1977) find complete modification of all amino groups by TNBS, while more recently, Crain et al. (1978) conclude that 63-72% of the PE is located at the outer surface, 18-27% at the inner surface and that 6-14% is not readily available to labeling with TNBS (see section 1.5). PS was found to be located on the outer face at an amount of 25-31% and on the inner face at 25-35% with 35-50% resistant to labeling.

However, these studies have been conducted under conditions, which in our hands give evidence of reagent penetration and membrane disturbance. In all cases, a pH of 8.5 or higher was used, higher TNBS concentrations (4.9 and 2 mM) were used in two cases, and in all three studies disk vesicles were used, which in the TNBS studies were prepared from frozen retinas. Freeze-thawing of disk vesicles causes inversion of rhodopsin molecules, as detected by concanavalin A-labeling studies (Adams et al., 1979), indicating serious disturbance of the membrane. The results obtained in these experiments agree with our own results for disk vesicles at similar TNBS concentrations and pH (Figs. 4.1-4.3). The low level of PS modification in the experiments of Crain et al. (1978) at 0^{o} C may be due to the difference in reactivity between it

and PE, which may be even more pronounced at low temperature.

Recent experiments of Bishop et al. (1979) with monolayers show that in this case, the rate and extent of trinitrophenylation of PE are greatly affected by the fatty acid composition of this phospholipid. Saturated PE reacts much more slowly than dioleoyl (18:1) PE at the same surface pressure. However, in the highly unsaturated disk membranes (at least six double bonds per phospholipid molecule), the individual phospholipid molecules are probably so far apart that their trinitrophenylation does not cause the spatial restrictions for further reactivity observed in other membranes.

Another factor, which may interfere with trinitrophenylation of membrane aminophospholipids, could be the presence of negatively charged phospholipids (op den Kamp, 1979; Bishop et al., 1979). However, a negative surface charge introduced in stacked disk membranes by prior phospholipase D treatment, leading to up to 40% PA, does not seem to influence the rate and extent of trinitrophenylation of the enzyme-resistant PS molecules (Fig. 4.7). This may be due to the spacing of the phospholipids in the disk membrane, but it is also conceivable that Ca^{2+}, present in high concentration, shields the extra negative charge of the PA.

In Chapter 3, we have stated that our tentative conclusions on the transbilayer phospholipid distribution rested on the assumption that no induced preferential transbilayer movement of a specific phospholipid and no shielding of outer face phospholipids against phospholipase D occur. The results of the combined treatment with phospholipase D and TNBS indicate that this assumption is valid. Hence, the present findings confirm and strengthen our earlier conclusions obtained with three different phospholipases of a nearly symmetrical distribution of the three major phospholipids, viz., 55-60% of the PE, 40-45% of the PC and approx. 50% of the PS located on the outer (cytoplasmic) surface of the disk membrane. The question as to whether this symmetrical distribution represents a static or a dynamic situation remains open. Hence, the rate of transbilayer movement of phospholipids in intact disk membranes needs further investigation.

4.4.5 *Summary*

In a further study of the transbilayer distribution of phospholipids in rod disk membranes, the amino group reagent trinitrobenzenesulfonate (TNBS) and the phospholipid-hydrolyzing enzyme, phospholipase D, have been used alone

and in combination.

Under carefully defined conditions (1 mM TNBS, pH 7.4, 20° C, darkness), TNBS yields limited final levels of modification of phosphatidylethanolamine (PE) and phosphatidylserine (PS), suggesting only minor reagent penetration and membrane disturbance under these conditions.

Treatment of stacked disks with TNBS under these conditions leads to a biphasic modification of the aminophospholipids. Relatively fast (less than 1 h) modification of 50% PE and 40% PS occurs, slowly rising (approx. 3 h) to 60 and 50%, respectively.

Extensive treatment of stacked disks with phospholipase D leads to the hydrolysis of 55% phosphatidylcholine (PC) and 50% PE, while PS is hardly attacked by this enzyme.

Treatment of stacked disks with TNBS after prior treatment with phospholipase D leads to no further modification than that maximally obtained with either reagent alone: about one-half of the three major phospholipid classes is accessible. Although both reagents differ greatly in molecular size, mode of action and other properties, they apparently see the same pool of PE, their joint substrate. Considering that we start with the original right-side-out configuration, that all phospholipids can in principle be modified (no shielding) and that the membrane remains essentially intact, we conclude that the accessible lipid pool represents the outer face of the disk membranes.

These results confirm our earlier conclusions from treatment with three phospholipases that the three major phospholipids are nearly symmetrically distributed over the two faces of the disk membrane.

The divergence with the conclusions of other investigators is most likely explained by their use of disk membranes (disk vesicles) in which the original phospholipid distribution had not been maintained and/or conditions under which TNBS markedly penetrates the membrane.

CHAPTER 5

TRANSBILAYER DISTRIBUTION OF PHOSPHOLIPID FATTY ACYL CHAINS IN PHOTORECEPTOR MEMBRANE*

5.1 Introduction

We have been studying the transbilayer distribution of the phospholipids in the rod disk membranes by means of phospholipases (Chapter 3) and trinitrobenzenesulfonate (TNBS; Chapter 4). Reliable methods were developed, in which phospholipase D and TNBS in principle only attacked the outer leaflet of the bilayer of these membranes. A nearly symmetrical distribution of the three major phospholipids, phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylserine (PS), was concluded.

The same methods have now been used to investigate the transbilayer distribution of the phospholipid fatty acyl chains. The results indicate a symmetrical distribution of the fatty acyl chains of all three major phospholipids.

5.2 Materials and methods

A description of the experimental procedures is given in Chapter 2 (sections 2.2 and 2.3).

5.3 Results

5.3.1 *Lipid composition of outer segments*

The fatty acid composition of stacked disks and of the phospholipids present in these preparations is shown in Table 5.1. The total amount of phospholipid is 62 ± 2 (n = 5) mol per mol rhodopsin. Appreciable amounts of diglycerides and free fatty acids (2 ± 1 and 4 ± 1 mol per mol rhodopsin,

*Adapted from Drenthe et al. (1981).

TABLE 5.1

FATTY ACID COMPOSITION OF ROD OUTER SEGMENTS AND THEIR MAJOR PHOSPHOLIPIDS

Fatty acids making up less than 0.3 mol% in any fraction are omitted. Values are averages with standard deviation for five preparations.

	Outer segments	PC	PE	PS
Phospholipid composition		(mol% of total phospholipids)		
		36.0 ± 1.5	44.1 ± 1.6	15.2 ± 0.9
Fatty acids		(mol% of total fatty acids)		
16:0	19.9 ± 0.3	30.6 ± 2.2	12.6 ± 0.3	4.1 ± 0.4
18:0	22.1 ± 0.6	19.4 ± 1.0	25.0 ± 0.4	21.0 ± 1.1
18:1 ω 9	3.3 ± 0.1	4.5 ± 0.5	4.2 ± 0.1	1.5 ± 0.4
18:2 ω 6	<0.1	0.9 ± 0.1	0.9 ± 0.1	<0.1
20:4 ω 6	4.8 ± 0.1	2.7 ± 0.1	2.4 ± 0.1	4.3 ± 0.5
22:4 ω 6	1.6 ± 0.1	0.4 ± 0.1	0.8 ± 0.1	3.0 ± 0.2
22:5 ω 6	2.3 ± 0.1	0.9 ± 0.1	1.5 ± 0.1	1.6 ± 0.3
22:5 ω 3	1.9 ± 0.2	1.4 ± 0.1	1.4 ± 0.1	3.3 ± 0.2
22:6 ω 3	43.0 ± 0.4	35.9 ± 2.2	50.2 ± 0.8	48.1 ± 0.8
24:4	1.2 ± 0.1	<0.1	<0.1	3.9 ± 0.4
24:5		<0.1	<0.1	9.3 ± 0.7

TABLE 5.2

FATTY ACID COMPOSITION OF FREE FATTY ACIDS AND DIGLYCERIDES IN ROD OUTER SEGMENTS EXPRESSED AS MOL PERCENT OF TOTAL FATTY ACIDS

Values are averages of two preparations.

	Fatty acids (C atoms: double bonds)									
	<16	16:0	18:0	18:1	18:2	20:4	22:4	22:5	22:6	24:4/5
Free fatty acids	3.9	21.4	18.6	12.5	3.6	20.4	2.7	4.0	8.5	4.6
Diglycerides	0.9	28.9	20.5	3.6	0.7	33.1	0.8	2.5	1.6	-

respectively) appear to be present consistently, whereas triglycerides are virtually absent. The fatty acid composition of free fatty acids and diglycerides is shown in Table 5.2.

5.3.2 *Treatment with trinitrobenzenesulfonate*

Treatment of stacked disks with TNBS leads to trinitrophenylation of PE and PS (40 and 38% modification, respectively, after 60 min). PC does not react with TNBS. Hence, the fatty acid composition of this phospholipid can serve as a control to indicate the reliability of the method, notably of the retention of acyl chain unsaturation during the experiments. This seems to be satisfactory. After separation of the phospholipids by thin-layer chromatography, the fatty acid composition of the unmodified phospholipids is determined.

Fig. 5.1 summarizes the results after 10, 20, 30 and 60 min of treatment with TNBS. The unmodified phospholipids appear to have the same fatty acid composition as before incubation with TNBS, regardless of the degree of modification attained. For practical reasons only the four most abundant fatty acids are presented, viz., palmitic acid (16:0), stearic acid (18:0), arachidonic acid (20:4) and docosahexaenoic acid (22:6). Clearly, the fatty

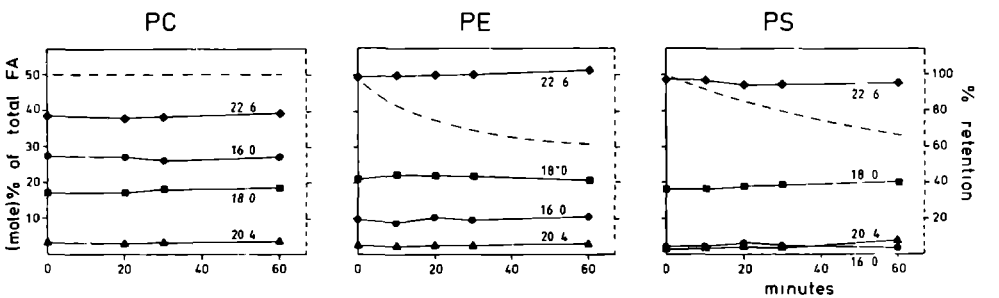

Fig. 5.1. Fatty acid composition of unmodified phospholipids of bovine stacked disk preparations before and after treatment with TNBS. Closed symbols (solid lines) represent content of indicated fatty acids in PC, (residual) PE and PS. Broken lines represent the percent of native phospholipid left.

acid composition of the phospholipids accessible to TNBS is the same as that of the non-accessible phospholipids. Consequently, the products of the reaction, i.e., trinitrophenyl derivatives of PE and PS, must retain the same fatty acid composition as the mother compounds during the course of reaction with TNBS. Analysis of these derivatives indicates that this is indeed the case, but exact calculation is hampered by the presence of the (more saturated) diglycerides and free fatty acids near the spots of trinitrophenyl-PE and trinitrophenyl-PS (close to the solvent front).

The results also show that no phospholipid with a particular fatty acid composition has been preferentially attacked.

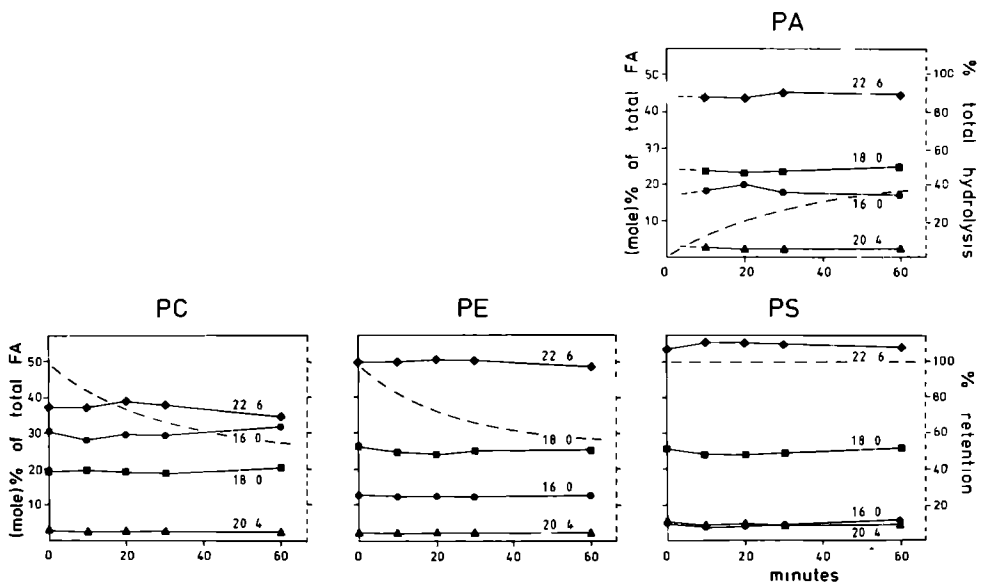

Fig. 5.2. Fatty acid composition of phospholipids of bovine stacked disk preparations before and after phospholipase D treatment. Presentation as in Fig. 5.1. PA, phosphatidic acid, the hydrolysis product. Broken line in top right figure (PA) represents percent of total phospholipid hydrolysis.

5.3.3 *Treatment with phospholipase D*

Treatment of stacked disks with phospholipase D leads to the hydrolysis of 50% PC and 42% PE after 60 min, and gives rise to 37% phosphatidic acid (PA) after this time. A final level of 40% hydrolysis is reached after 180 min. Since phospholipase D hardly hydrolyzes PS under these conditions, the fatty acid composition of this lipid can serve as a control in the same way as that of PC in the TNBS approach.

Fig. 5.2 presents the results obtained with phospholipase D in the same way as those for the treatment with TNBS in Fig. 5.1. Again the unmodified phospholipids have the same fatty acid composition as before the incubation, regardless of the degree of hydrolysis reached. The fatty acid composition of the product, PA, also remains constant during hydrolysis. This composition clearly reflects the derivatives of the PA from roughly equimolar amounts of PC and PE.

There is no indication for preference of the enzyme for phospholipids with a particular fatty acid composition.

5.4 Discussion

5.4.1 *Lipid composition of outer segments*

Our data for the phospholipid composition of rod outer segments are in agreement with those in previous publications (Anderson et al., 1975; Miljanich et al., 1979; Daemen, 1973). The degree of unsaturation of the fatty acids is higher than given in the first reports (see Daemen, 1973, and Table 1.2), but closely resembles that in more recent publications (Hendriks et al., 1976; Miljanich et al., 1979; Stone et al., 1979). This probably reflects the higher purity of the present rod outer segment preparations as compared to about 10 years ago.

Our rod outer segment preparations contain free fatty acids and diglycerides in similar amounts as reported by Dratz et al. (1979). These neutral lipids contain relatively less docosahexaenoic acid (22:6) but more arachidonic acid (20:4) than the three major phospholipid classes; the free fatty acid fraction is also enriched in oleic acid (18:1). In view of the random, molecular replacement of outer segment phospholipids, in particular of phosphatidylinositol (PI), and the fast exchange of fatty acids between outer

segments and pigment epithelial cells (see section 1.2.2), some or all of the free fatty acids may be involved in a deacylation/acylation renewal cycle. The rapid hydrolysis of PI into diglycerides might also explain the approximately close similarity in fatty acid composition of these two lipids (see section 1.2.2 and Anderson et al., 1975).

5.4.2 *Transbilayer distribution of fatty acyl chains*

In Chapters 3 and 4 we have presented considerable evidence for an approximately symmetrical distribution of phospholipids (phospholipid head groups) over the two faces of disk membranes. Under carefully defined conditions, both phospholipase D and the amino group reagent TNBS appeared to behave as probes, which in principle only act on the outer leaflet of the disk membranes. Therefore, these approaches can be used as well for investigating the transbilayer distribution of the fatty acyl chains.

The fatty acid composition of the phospholipid classes and their modification products during the course of action of either TNBS or phospholipase D is constant within experimental error. This leaves little doubt that the fatty acyl chains of each of the major phospholipid classes in the disk membrane are symmetrically distributed over the two faces of this membrane, at least on the time scale of our experiments. Together with the approximately symmetrical distribution of the phospholipid head groups (Chapters 3 and 4), this demonstrates that the lipid part of the disk membrane displays no transverse asymmetry, in contrast to its major protein rhodopsin.

To our knowledge, this is the first time that a symmetrical transverse fatty acyl chain distribution is found in a biological membrane. In all previous reports a higher unsaturation of the inner leaflet has been observed (Perret et al., 1979; Emmelot and van Hoeven, 1975; Renooij and van Golde, 1977; Fontaine et al., 1980; Fong and Brown, 1978; Sandra and Pagano, 1978). It may be significant that, with one exception (mouse brain synaptosomes; Fontaine et al., 1980), these data refer to plasma membranes in which an asymmetrical phospholipid distribution has been found as well. Hence, the number of observations, at least for intracellular membranes, is too small to permit generalization at this time with respect to transverse fatty acyl chain distribution.

Our present results show that no phospholipid with a particular fatty acid composition is preferentially attacked by or protected against TNBS or

phospholipase D, which suggests lateral randomness of the fatty acyl chains in the outer face of the membrane. Recent cross-linking studies of Crain et al. (1978) also 'indicate a random array of lipids with respect to their fatty acid content in the rod disk membrane'.

5.4.3 *Transbilayer movement of phospholipids*

The approximately symmetrical distribution of the phospholipids over the two faces of the disk membrane raises the question as to whether this represents a static situation or a dynamic equilibrium.

From the autoradiographic work of Young and coworkers (Young, 1976), it is well known that in many species the visual pigment rhodopsin, soon after its synthesis in the inner segment, becomes incorporated in the infoldings of the plasma membrane at the base of the rod outer segments. Later they form a package of labeled disks, which gradually moves to the apical end of the outer segment. The specific radioactivity of this rhodopsin remains constant until the labeled disks are scavenged by the pigment epithelium, which in rat takes about 10 days (Young, 1967) and in frog about 40 days (Hall et al., 1969). Clearly, the rhodopsin molecule remains to the end in the disk in which it is originally incorporated. In addition, it remains asymmetrically inserted in the membrane with the carbohydrate-bearing N-terminus at the inside of the disk (Adams et al., 1978). Its mobility in the outer segment is apparently limited to rotation around an axis perpendicular to the disk membrane (Brown, 1972; Cone, 1972) and to lateral diffusion in this membrane (Poo and Cone, 1974; Liebman and Entine, 1974).

The synthesis and fate of the phospholipids of rod outer segments, studied by autoradiographic and biochemical approaches, show a quite different pattern. Most evidence derives from experiments with frog, but in rabbit (Masland and Mills, 1979; Mizuno, 1976) the same general pattern is observed. De novo synthesis of phospholipids takes place in the microsomes of the inner segment and the phospholipids are exported to the outer segment as demonstrated with a wide variety of radioactive precursors: glycerol, fatty acids, choline, ethanolamine and serine (see section 1.2.2). All evidence suggests that these phospholipids are used in the formation of new disks at the base of outer segments, together with newly synthesized rhodopsin (Bibb and Young, 1974a, 1974b; Kinney and Fisher, 1978; Papermaster et al., 1975; Besharse and Pfenninger, 1978; Papermaster et al., 1979). However, autoradiography does not

reveal the banding phenomenon observed with rhodopsin. On the contrary, the radioactivity always diffuses quickly throughout the entire outer segment and any localized radioactivity at the base of the outer segment due to phospholipids is always short-lived (Bibb and Young, 1974a, 1974b; Anderson et al., 1980b). These autoradiographic data are nicely confirmed by recent biochemical experiments of Anderson and coworkers (1980a-d). They find that a pulse label of [2-^{3}H]glycerol is rapidly incorporated into the phospholipids of frog outer segments and, subsequently, the specific radioactivity of all major phospholipids declines exponentially with a halftime of about 20 days.

These observations demonstrate that, in contrast to rhodopsin, the major phospholipids of the outer segments exhibit a high interdiscal mobility, at least in frog and rabbit. This conclusion may probably be extrapolated to vertebrate species other than frog and rabbit, since the phospholipid and fatty acid composition of vertebrate photoreceptor membranes is rather similar (Daemen, 1973; Tables 1.1 and 1.2). The pool of exchanging phospholipids must include the inner leaflet of the disk bilayer, since otherwise more persistent banding of the radioactive label derived from a phospholipid precursor should have been detected. The fact that exchange of fatty acyl chains also seems to take place in outer segments (Bibb and Young, 1974a) only adds an additional dimension to the dynamic behavior of these phospholipids. In other words, there is most likely a transbilayer movement of rod outer segment phospholipids and the halftime of this phenomenon can be expected to be of the order of hours rather than days. Although direct evidence for this transbilayer movement is still not available, it is reasonable to assume that the approximately symmetrical transbilayer distribution of the phospholipids and their fatty acyl chains reflects the high mobility of the entire phospholipid pool of bovine disk membranes *in vivo*.

If this assumption is correct, the question arises why in our experiments only maximally 40% of the total phospholipids (viz., PE and PC) can be hydrolyzed by phospholipase D, and maximally 35% (viz., PE and PS) can be modified by TNBS (see Chapter 4). In the case of a continuous transbilayer movement of phospholipids, ultimately the entire pool of (susceptible) phospholipids would become accessible to the reagents, even when these do not penetrate the membrane at all. One possibility is that the products, PA and trinitrophenyl phospholipids, are not subject to transbilayer movement. The simplest explanation, however, is that the transbilayer mobility of all phospholipids is greatly reduced after isolation and/or under the conditions

of incubation (30^{o} C, 40 mM Ca^{2+} in the case of phospholipase D treatment, 20^{o} C in the case of TNBS treatment). Whereas most experimental evidence (Chapters 3 and 4) is in agreement with this explanation, the strongest argument against transbilayer movement of phospholipids under our experimental conditions is provided by the combination experiments of Figs. 4.4 and 4.7. These show that pretreatment of the disk membranes with phospholipase D or TNBS alters neither the size nor the composition of the accessible phospholipid pool. This seems to eliminate the possibility that we would arrive artifactually at a symmetrical phospholipid distribution during the reagent incubations. However, this does not exclude that an asymmetric distribution of phospholipids would be maintained *in vivo* by a mechanism dependent on active metabolism.

5.4.4 *Concluding remarks*

Many questions regarding the phospholipids of rod outer segments remain to be answered. Which mechanisms are involved in their high mobility, both in interdiscal transfer and in intradiscal transbilayer movements? What is the physiological relevance of a membrane system with a 'static' major protein and very 'dynamic' phospholipids? Is there a relation with the high content of docosahexaenoic acyl chains, not only a highly unsaturated (22:6), but an essential fatty acid as well? The answers to these and other questions on the phospholipid dynamics of disk membranes must clearly await further investigations.

5.4.5 *Summary*

The transverse distribution of the fatty acyl chains of the major phospholipids over the two faces of the photoreceptor membranes has been determined in bovine rod outer segment (stacked disk) preparations. For this purpose, the fatty acid composition of the phospholipids has been analyzed before and after treatment with trinitrobenzenesulfonate (TNBS) and phospholipase D. The latter agents are used under conditions in which they have been demonstrated to attack only the outer (cytoplasmic) face of the membrane.

After treatment with TNBS or phospholipase D, the fatty acid composition of the unreacted phospholipids is the same as that before treatment, regardless of the extent of modification or hydrolysis attained. The fatty acid

composition of phosphatidic acid (PA), resulting from phospholipase D action, also remains unchanged during progressive hydrolysis.

These results indicate that the fatty acyl chains of the major phospholipids have the same composition on either side of the disk membrane. Together with our previously obtained evidence for the distribution of the major phospholipids in rod outer segment disk membranes, this means that both the phospholipids and their fatty acyl chains have a remarkably symmetrical distribution over the two membrane faces.

On the basis of literature data it is concluded that this approximate symmetry reflects the high mobility of the entire phospholipid pool of disk membranes, including appreciable transbilayer movements of the phospholipids.

CHAPTER 6

GENERAL DISCUSSION

6.1 Phospholipid topology in photoreceptor membrane

The objective of our study has been the establishment of the transbilayer distribution of the three major phospholipids and their fatty acyl chains in rod photoreceptor membranes of bovine retina. For this purpose freshly isolated stacked disk preparations (stacked disks) are treated with various lipid-modifying agents, i.e., three different phospholipases (A_2, C and D) and the amino group reagent trinitrobenzenesulfonate (TNBS). Water-washed disk membranes (disk vesicles) are used for determining optimal reaction conditions and allowing comparison with previous studies. Reference preparations, in which the same phospholipids are present in random distribution, are also treated with phospholipases in order to take into account the specificity of each phospholipase towards individual phospholipids of disk membranes.

The results have been presented in Chapters 3-5. It is concluded that the three major phospholipids and their fatty acyl chains are predominantly distributed symmetrically over the two faces of the disk bilayer. This conclusion relies on the assumption that disk membrane treatment is started and prosecuted with a bilayered membrane system of right-side-out orientation, in which all outside-located phospholipids are accessible to the reagents and become modified, and none of the inside-located phospholipids. In our opinion, this assumption is valid for stacked disks, in darkness treated with phospholipase D up to the final level of phospholipid hydrolysis, with TNBS for the fast-reacting phospholipid pool, and with each phospholipase during the early phase of phospholipid hydrolysis when disturbance of disk membrane structure is negligibly small. Most arguments in favour of this statement have been presented in Chapters 3-5. Some arguments will be discussed in relation to requirements for correct localization of membrane phospholipids (see section 2.1.3) and with respect to previous studies.

Bilayered structure of disk membranes. The bilayered structure of disk membranes has recently been confirmed by ^{31}P NMR and freeze-fracture studies

(de Grip et al., 1979). However, a total lipid extract of disk membranes may adopt non-bilayer structures upon hydration (ibid.; but see Deese et al., 1981). Since transient local changes in lipid distribution during the excitation process may escape detection by NMR and thus cannot be excluded during light exposure of disk membranes, the whole procedure, including the isolation of disk membranes, has been performed in darkness.

Right-side-out orientation of stacked disks. The choice of stacked disks is suggested by the close resemblance of their piled disk structure and the configuration in situ. Stacked disks also show very little membrane inversion as judged from the lack of concanavalin A-binding by inside-located sugar residues of rhodopsin (see section 2.1.3).

Inaccessibility of outside-located phospholipids. The presence of the plasma membrane and the juxtaposition of disk membranes in stacked disks may provide a barrier against full access of phospholipids to phospholipases in particular. This hindrance does not play an important role since, apart from the time course, hydrolysis patterns of stacked disks and disk vesicles are fairly similar during the early stages of phospholipase treatment (see section 3.3.3). In addition, nearly all phospholipids are hydrolyzed by phospholipases A_2 and C (Table 3.1), while the effects of phospholipase D treatment are uniformly spread over the outer segment (Fig. 3.4).

The main part of disk vesicles has a right-side-out orientation for the same reason as quoted for stacked disks. The close similarity in hydrolysis patterns of stacked disks and disk vesicles also favours an identical phospholipid accessibility in each preparation. However, more phospholipid, mainly phosphatidylcholine (PC), is eventually hydrolyzed by phospholipase D in disk vesicles (see Table 3.1 and Fig. 4.4). Although the evidence is not absolutely compelling, this difference cannot be attributed to an outside-located phospholipid (i.e., PC) pool, which becomes available after washing disk membranes with water. Since interactions of specific phospholipids with protein have not clearly been demonstrated (section 1.4.1), the availability of more PC in disk vesicles should have led to altered hydrolysis patterns in favour of this lipid. The difference in final hydrolysis level can be explained by penetration of phospholipase D into disk membranes, in which a more open, leaky membrane structure is induced by water-washing (see also section 3.4.1). Due to contaminating phosphatidate phosphohydrolase activity, maximally 10% (av. 5%) phosphatidic acid (PA) is eventually converted into diglycerides and P_i in disk vesicles (section 2.2.3). Although this amount of

diglycerides is probably not large enough to cause its aggregation into discrete droplets (see section 2.1.3), it may be an additional source for making the permeability barrier of disk vesicles more vulnerable for phospholipase D.

Redistribution of phospholipids. The evidence for a symmetrical transbilayer distribution of phospholipids relies on the assumption of their static distribution. However, it can be concluded from metabolic studies that both the lipids and their building blocks are subject to dynamic transfer in the rod disk system *in vivo* (sections 1.2.2 and 5.4.3). For our objective this system is isolated and incubated with lipid-modifying reagents. The driving forces behind the dynamic lipid transfer are probably greatly diminished, at least the metabolic part of it, so that the lipids may have reached a new equilibrium distribution, not necessarily the same one as *in vivo*. On the other hand, the modification of lipids on one side of the disk membrane could result in another unstable situation. This instability arises from lipid alteration due to modification. A compensating movement of (un)-modified lipid can occur and may enhance the transbilayer rearrangement.

It is not unlikely that phospholipase D- and TNBS-modified phospholipids are not longer translocated across the disk bilayer, since otherwise the entire pool of susceptible phospholipid would ultimately become accessible, even for perfectly membrane-impermeable reagents.

In stacked disks maximally 40% of total phospholipid, i.e. 55% PC and 50% phosphatidylethanolamine (PE), is hydrolyzed by phospholipase D. About 35% of total phospholipid, i.e., 50% PE and 40% phosphatidylserine (PS) reacts relatively fast with TNBS. (The slow-reacting lipid pool may arise from TNBS penetration and/or lipid transfer). So, as long as unmodified lipid is present, its translocation may occur. When this transfer is specific, i.e., exchange between lipids of the same class, this will not change the observed distribution of the lipids, provided that the original (e.g., 50/50) distribution of total lipid is maintained. When this transfer is aspecific, i.e., exchange between different lipid classes, this could result in an apparently symmetrical distribution, even if the bilayer was asymmetric. The simplest explanation, in agreement with most experimental evidence of Chapters 3 and 4, would be that the mobility of all phospholipids of crossing the bilayer is seriously reduced upon isolation and/or at the ambient conditions of incubation: 30^{o} C at 40 mM Ca^{2+} in the case of phospholipase D treatment, 20^{o} C in the case of TNBS treatment. The strongest argument against aspecific transfer

of lipid is provided by the successive treatment of disk membranes with phospholipase D and TNBS. These combination experiments demonstrated that pretreatment of disk membranes with either phospholipase D or TNBS does not alter the pool of phospholipid accessible to either reagent nor the rate at which this pool can be modified (see Figs. 4.4 and 4.7). The close similarity in hydrolysis patterns of stacked disks and reference preparations during the early phase (10 min) of phospholipase D treatment also argues against aspecific transfer of lipid. The hydrolysis patterns of stacked disks would be changed by an altered lipid distribution and not longer be comparable with those of the reference. Table 3.2 shows the 10-min data only, but the hydrolysis patterns remain constant at least during the first 30 min of incubation. In this time period about 20% total hydrolysis is reached in stacked disks with phospholipase D.

Finally, a specific transfer of lipid, but aspecific concerning the fatty acyl chains, e.g., saturated lipid against unsaturated lipid of the same class, should lead to a gradual change in fatty acid composition of the unmodified lipids left in the membrane. This does not agree with the results presented in Figs. 5.1 and 5.2, provided that phospholipase D and TNBS do not modify lipids with a particular fatty acid composition. It has been shown that TNBS modifies aminophospholipids without preference for a particular fatty acid composition (Fontaine and Schroeder, 1979). Since the results for both reagents are similar, phospholipase D hydrolyzes phospholipids also without preference for a particular fatty acid composition.

In conclusion, a symmetrical transverse distribution of phospholipids and their fatty acyl chains can be assigned to any moment in time during the reagent treatment. The occurrence of transbilayer movement of specific phospholipids is very unlikely. However, it cannot be excluded that an asymmetrical distribution of phospholipids is occurring *in vivo*, maintained by a mechanism dependent on active metabolism.

Comparison with previous studies. Our conclusion of a symmetrical transverse distribution of phospholipids differs from that of other studies, in which the same reagent (i.e., TNBS) has been used (see section 1.5: Smith et al., 1977; Crain et al., 1978).

Smith et al. (1977) found in intact and sonicated disk membranes, prepared from frozen retinas, that all aminophospholipids are modified by TNBS. Arginine, which is trapped inside sonicated disks, does not react during the same period of incubation. They concluded that all aminophospho-

lipids are located at the outer face of the disk bilayer. However, TNBS penetration leading to complete modification can be expected to occur under the reaction conditions used (i.e., approx. 5 mM TNBS at 20° C and pH 8.5), as shown by us for disk vesicles (Figs. 4.1 and 4.3). Yet, induced transbilayer exchange of trinitrophenylated aminophospholipids against inside-located aminophospholipids without disturbing the permeability properties of the bilayer would be in agreement with the absence of arginine modification. This possibility is very unlikely (see above). In addition, the authors mention no relative reactivity of TNBS on arginine under the reaction conditions applied (compare with section 2.1.3: Haest et al., 1981).

Crain et al. (1978) treated disk membranes, also prepared from frozen retinas, with 2 mM TNBS at pH 8.5 and 21° or 0° C. They performed the reaction at 0° C also in the presence of valinomycin to make the disk membrane permeable for TNBS. They concluded that TNBS strongly penetrates the disk bilayer, at 21° C and at 0° C in the presence of valinomycin. We concluded the same for disk vesicles at pH 8.5 and 20° C, where TNBS modifies nearly all PE (see Fig. 4.3).

While Crain et al. (1978) performed their final experiments at 0° C (but at pH 8.5), we lowered the pH to 7.4 (but at 20° C), in order to avoid excessive penetration of TNBS. (The additional replacement of disk vesicles by stacked disks in our experiments has hardly any effect on the behavior of TNBS). In the final procedures TNBS penetration is greatly reduced, but is still occurring. However, differences are found in reactivity of individual aminophospholipids. In the experiments of Crain et al. (1978) approx. 13% (i.e., 63% vs. 50%) more PE and approx. 15% (i.e., 25% vs. 40%) less PS belong to the fast-reacting phospholipid pool (compare Table 1.3 with Fig. 4.5). Several factors may account for this discrepancy: (1) Disk membranes prepared from frozen retinas. Membrane inversion may occur in this preparation, so that lipid distribution and availability are altered as compared to those in freshly isolated disk membranes, and (2) pH 8.5 and 0° C as reaction conditions. TNBS penetration and PE reactivity are greater at pH 8.5 than at pH 7.4, while PS reactivity is lower at 0° C than at 20° C. If domains, which differ in lipid fluidity and/or composition, are present at 0° C, lipid distribution and availability may also be altered. This could explain the high level of aminophospholipids, PS in particular, not readily available for labeling with TNBS in the experiments of Crain et al. (1978), even in the presence of valinomycin.

In conclusion, it is difficult to rely on experiments with TNBS alone. In view of our results with phospholipases and particularly those of the combination experiments with phospholipase D and TNBS (Chapter 4), we consider our conditions for TNBS labeling as optimal for assessing the transbilayer distribution of phospholipids in disk membranes.

6.2 Dynamics of photoreceptor membrane phospholipids

The highly unsaturated phospholipids certainly contribute to the dynamic properties of the photoreceptor membrane. They are fluid at physiological temperatures, are subject to molecular renewal mechanisms, and may adopt transient non-bilayer structures.

This study indicates that the three major phospholipids and their fatty acyl chains are randomly distributed over the two faces of the disk bilayer. Evidence has been presented that they may also be randomly distributed in the plane of the disk bilayer. There are no clear indications for strong interactions of specific phospholipids with rhodopsin. Only during the later, slow part of the photolytic sequence PE and/or PS may serve as a hydrophobic sink for the liberated chromophore (section 1.4).

Phospholipid fluidity. The rod cell has an effective mechanism to maintain its high level of unsaturated phospholipids. Yet, all known properties of rhodopsin express their activity fairly well in the presence of less unsaturated phospholipids, which contain at least one double bond per molecule. It has been suggested that the highly unsaturated phospholipids may provide a fluid hydrophobic environment for the highly organized visual pigment system. They enable rhodopsin to display rapid lateral and rotational movements in the plane of the disk bilayer. However, it remains to be proved that these phospholipids are involved in the as yet unknown mechanism, by which light-excited rhodopsin regulates the plasma membrane conductance by means of an internal transmitter.

Molecular renewal mechanisms. Photoreceptor membranes are renewed by membrane replacement, a continuous process of assembly at the base and shedding at the top of outer segments. Phospholipids and their building blocks are, in addition, subject to random renewal mechanisms. It has been suggested that the latter processes are needed for maintaining the high level of unsaturated fatty acids of the phospholipids. Replacement of damaged lipid molecules by new ones may be very effective in cancelling the harmful effects

of light and oxygen (Daemen, 1973).

Molecular renewal mechanisms may also be needed for specialized functions of the visual pigment system. This is suggested by the rapid turnover of phosphatidylinositol (see next paragraph).

Phosphatidylinositol turnover. Phosphatidylinositol (PI) is the major inositol-containing lipid in most cells, with much smaller quantities of PI 4-phosphate (PI-P) and PI 4,5-diphosphate (PI-bisP; compare Fig. 1.1). The concentration of the latter two lipids is greater in tissues, which are enriched in plasma membrane material (e.g., neural white matter and erythrocytes), suggesting that they are located at the plasma membrane. PI is a minor phospholipid in rod outer segments (at most 2 mol% of total phospholipid; Table 1.1). Its location is not known. The presence of PI-P and PI-bisP has so far not been reported, probably because they break down rapidly post-mortem and are difficult to extract quantitatively.

Inositol-containing lipids are interesting minor compounds of cell membranes. They are involved in various active membrane functions, such as (1) selective control of ion movements; (2) packaging and translocation of macromolecules; (3) grouping and orientation of vectorially directed enzyme systems, and (4) transfer of extracellular information to the cell interior (Michell, 1975, 1979).

Involvement of PI in information transfer is indicated by the rapid hydrolysis of PI into 1,2-diglycerides, the PI-response, which can be provoked in various cell types in response to external stimuli. A common feature of external stimuli, which enhance PI hydrolysis, is that their action on cellular metabolism involves Ca^{2+} mobilization and elevates the cyclic GMP concentration.

Involvement of inositol-containing lipids in information transfer is also indicated by the breakdown of PI-P and PI-bisP into diglycerides, which appears to be controlled by a rise in the cytosol Ca^{2+} concentration in response to external stimuli. The function of these cellular reactions is not known. Since PI-P and PI-bisP strongly bind divalent cations, these cellular reactions cause the removal of high affinity binding sites for divalent cations from the cytoplasmic surface of cell membranes.

These processes may also operate in rod outer segments, in which a rapid hydrolysis of PI into diglycerides has been reported (Anderson et al., 1980d). PI hydrolysis may then be involved in the regulation of the extradiscal concentration of the internal transmitter (possibly Ca^{2+} and/or cyclic GMP) in

response to light absorption by rhodopsin. A role of PI hydrolysis in fusion events associated with the assembly and shedding of disk membranes has been suggested by Anderson et al. (1980d).

Transient non-bilayer structures. The bilayered structure of disk membranes has recently been confirmed by ^{31}P NMR and freeze-fracture studies. However, disk membranes contain appreciable amounts of highly unsaturated PE, which adopts non-bilayer structures upon hydration (section 1.3.2). A total lipid extract of disk membranes may also adopt non-bilayer structures (de Grip et al., 1979), although controversy still exists (Deese et al., 1981).

Since transient non-bilayer structures of disk membranes exposed to light may escape detection by NMR, they cannot be excluded. Such structures may offer a new insight on fusion and transport mechanisms of the photoreceptor membrane (e.g., transbilayer movement of phospholipids).

6.3 Perspectives

As indicated in the previous section, there is a need for further investigations to clarify the role of the highly unsaturated phospholipids in photoreceptor membranes. It might be worthwhile to place special emphasis on studies of transbilayer phospholipid movements and transient non-bilayer structures, which might be induced by light-excited rhodopsin. Such studies may offer insight in the dynamic properties of the phospholipids.

Considering the transbilayer distribution of photoreceptor membrane phospholipids, we have demonstrated that under certain conditions TNBS and phospholipases can successfully be used to localize PC, PE and PS. However, the application of chemical and enzymatic agents eventually lead to reagent penetration and membrane disturbance in particular, as shown for TNBS and phospholipases A_2 and C. There are now methods available, which may offer a better approach. These involve the use of phospholipid exchange proteins and NMR spectroscopy (see Bergelson and Barsukov, 1977; op den Kamp, 1979; Etémadi, 1980).

Phospholipid exchange proteins cause phospholipid transfer between different membranes. Only the outer leaflet of the membranes appears to be involved in this process. In principle, the use of phospholipid exchange proteins of different specificities should permit the determination not only of the transbilayer distribution of particular phospholipids but also of their transbilayer movement. The rate of the latter process should not be too

fast in order to allow a distinction between both processes. In the case of photoreceptor membranes, disturbance of membrane structure may occur, if less unsaturated phospholipids are introduced in the outer leaflet of the highly unsaturated disk membranes. On the other hand, introduction of less unsaturated phospholipids may reveal their influence on certain properties of rhodopsin.

NMR spectroscopy has been useful in studying the transbilayer distribution and movement of phospholipids in model systems. Membrane-impermeable paramagnetic ions, affecting the NMR signals of outside-located phospholipids only, are used to distinguish between inner and outer leaflet phospholipids. Although large, complex structures are difficult to study with NMR spectroscopy, this technique has been applied successfully to localize PC in sarcoplasmic reticulum (de Kruijff et al., 1979).

Vertebrate retinas contain photoreceptor cells, capable of light absorption, signal transduction and amplification, eventually leading to vision. Light absorption exclusively occurs in the outer segments of photoreceptor cells. In the rod photoreceptor cell, which is responsible for black and white vision in dim light, the outer segment consists of regularly stacked flat disks, enclosed by the plasma membrane. The membrane of these disks, the photoreceptor membrane, is a lipid bilayer in which the visual pigment rhodopsin is embedded. The primary event in vision is light absorption by rhodopsin in the disk membrane. The next, firmly established event is a modified ion current across the plasma membrane, eventually leading to vision. To link both events, which take place in different membrane systems, a diffusable internal transmitter has been postulated accounting for signal transduction and amplification. The exact nature of the internal transmitter and the regulation of its functioning by light-excited rhodopsin are presently not known.

In the General Introduction current views of the visual excitation process are presented with emphasis on Ca^{2+} and cyclic GMP as possible internal transmitters.

The lipid part of the photoreceptor membrane consists mainly of phospholipids. Phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylserine (PS) comprise more than 95% of these phospholipids.

Some characteristics of the phospholipids are presented in Chapter 1. They contain highly unsaturated fatty acyl chains, so that rhodopsin is embedded in a very fluid lipid environment. Rhodopsin cannot be removed from the disk membrane without disturbing the lamellar membrane structure and altering its properties presently known. Thus, rhodopsin should be dependent on its native lipid environment for full activity. However, there are no clear indications for a lipid population strongly immobilized by rhodopsin. In addition, by modifying photoreceptor membrane phospholipids, by removing them partially, and by reconstitution of delipidated rhodopsin in a lipid bilayer of known composition, it has been shown that at least one double bond per lipid molecule should be present for full activity of rhodopsin. Yet, the

rod cell has an effective mechanism to maintain its high level of unsaturated phospholipids (at least six double bonds per molecule). It has been suggested that these phospholipids may provide a fluid hydrophobic environment for the highly organized visual pigment system.

It is clear that a detailed knowledge of the structure of photoreceptor membranes will contribute to a full understanding of the visual excitation process. The aim of this study is to establish the distribution of the three major phospholipids and their fatty acyl chains over the two faces of the photoreceptor membrane (Chapter 1). For this purpose disk membranes of bovine retina are treated with , in principle impermeable, lipid-modifying reagents, i.e., three different phospholipases (A_2, C and D) and the amino group reagent trinitrobenzenesulfonate (TNBS). Lipid analysis after reagent treatment enables then determination of kind and composition of the phospholipids, either modified or not.

In Chapter 2 a description is given of materials, procedures and determinations, which have been used throughout this study. Prerequisites are given for the correct use of phospholipases and TNBS in determining the transbilayer distribution of photoreceptor membrane phospholipids.

In Chapters 3-5 the results are presented. Considerable effort has been expended on determining correct conditions in order to meet the requirements formulated in Chapter 2. These conditions should minimize membrane inversion upon isolation, inaccessibility of externally located phospholipids, redistribution of phospholipids upon modification, and reagent penetration. In the final procedures disk membranes are used, prepared from freshly isolated bovine retinas and showing the native stacked flat disk structure.

Treatment of stacked disks with phospholipase D leads to a final modification of 55% phosphatidylcholine and 50% phosphatidylethanolamine. Treatment of stacked disks with TNBS leads to a biphasic modification of the aminophospholipids. Relatively fast (less than 1 h) modification of 50% phosphatidylethanolamine and 40% phosphatidylserine occurs, slowly rising (approx. 3 h) to 60 and 50%, respectively. The fast-reacting lipid pool is considered to be located outside. The slow-reacting lipid pool arises from modification of inside-located phospholipids due to TNBS penetration. Treatment of stacked disks with TNBS after prior treatment with phospholipase D leads to no further modification than that maximally obtained with either

reagent alone: about one-half of the three major phospholipids is accessible. Hence, we conclude that the accessible lipid pool represents the outer face of the disk membranes. The results indicate transbilayer symmetry of the three major phospholipids (Chapter 4) and their fatty acyl chains (Chapter 5).

With phospholipases A_2 and C nearly all natural substrates are modified, indicating appreciable disturbance of disk membrane structure (Chapter 3). Therefore, special attention is given to the early stages of phospholipase treatment, since then disturbance of disk membrane structure can be expected to be minimal. The modification of individual phospholipids of stacked disks is compared with that of reference preparations, in which the same phospholipids are present in random distribution. Apart from the time course, modification rates of individual phospholipids of stacked disks and reference preparations are fairly similar, indicating a symmetrical phospholipid distribution across the disk bilayer.

In Chapter 6 it is concluded that the reagents are correctly used to meet all requirements in determining the transbilayer distribution of phospholipids in the photoreceptor membrane. We present evidence that appreciable transbilayer movement of phospholipids (flip-flop) does not occur during treatment of stacked disks with the reagents. *In vivo*, however, photoreceptor membrane phospholipids are continuously renewed by molecular exchange mechanisms, most likely involving their transbilayer movement. Therefore, it cannot be excluded that an asymmetric transbilayer distribution of photoreceptor membrane phospholipids is occurring *in vivo*, maintained by a mechanism dependent on active metabolism. The occurrence of transbilayer exchange mechanisms of photoreceptor membrane phospholipids needs, therefore, further investigation. This and other aspects of photoreceptor membrane phospholipids have to be studied in further detail to enable full understanding of the dynamics of the visual excitation process.

Our results differ from those of earlier studies on the phospholipid distribution of disk membranes with amino group reagents. These studies indicate transbilayer phospholipid asymmetry rather than symmetry. In Chapter 6 we conclude that these studies have been conducted under conditions, which in our hands give evidence for appreciable reagent penetration and membrane disturbance.

De fotoreceptor membraan speelt een belangrijke rol bij de omzetting van een lichtprikkel in een zenuwimpuls, die via de oogzenuw naar de visuele cortex wordt geleid. Het netvlies van vertebraten bezit twee soorten fotoreceptor cellen: staafjes, die functioneren bij schemerlicht (zwart-wit zien), en kegeltjes, die functioneren bij daglicht (kleur zien). Licht absorptie vindt plaats in een daartoe gespecialiseerd deel van de fotoreceptor cel, het buitensegment. In staafjes is het buitensegment opgebouwd uit een groot aantal dicht op elkaar gestapelde platte zakjes, die omgeven worden door de celmembraan. De membraan van de zakjes, de fotoreceptor membraan, is een dubbellaag van lipiden met daarin het visuele pigment rhodopsine. De eerste stap in het proces van het zien is licht absorptie door rhodopsine. Dit leidt tot een verandering in de celmembraan permeabiliteit voor kationen en vervolgens tot een stimulering van het synaptische uiteinde van de fotoreceptor cel.

In het inleidende hoofdstuk wordt een kort overzicht gegeven van de huidige kennis omtrent het proces van het zien. Speciale aandacht is daarbij besteed aan het tot nu toe onopgeloste probleem van de signaaloverdracht van het door licht geactiveerde rhodopsine naar de celmembraan.

De lipiden in de fotoreceptor membraan bestaan voornamelijk uit fosfolipiden, waarvan de belangrijkste zijn: fosfatidylcholine (PC), fosfatidylethanolamine (PE) en fosfatidylserine (PS).

In hoofdstuk 1 worden de voornaamste kenmerken van deze fosfolipiden besproken: hun hoge gehalte aan hoog onverzadigde vetzuren, hun hoge metabole activiteit, en hun invloed op bekende eigenschappen van rhodopsine. Wat dit laatste betreft, het membraan-gebonden eiwit rhodopsine is voor een optimaal functioneren afhankelijk van de fosfolipiden in zijn directe omgeving. Specifieke fosfolipiden blijken echter niet nodig te zijn voor een juiste werking van rhodopsine. Wel dienen de fosfolipiden tenminste één dubbele binding per molecuul te bevatten. Het hoog onverzadigde karakter van de fosfolipiden in de fotoreceptor membraan (tenminste zes dubbele bindingen per molecuul) en de hiermee samenhangende hoge vloeibaarheidsgraad van de membraan worden daarom nog steeds niet goed begrepen.

Voor een beter begrip van de functie der fosfolipiden is een gedetaileerde kennis nodig van de structuur der fotoreceptor membraan. Het onderzoek, beschreven in dit proefschrift, wil hiertoe bijdragen door de verdeling van de drie belangrijkste fosfolipiden over de binnen- en buitenzijde van de fotoreceptor membraan te bepalen met behulp van fosfolipases en het amino-reagens trinitrobenzeensulfonzuur (TNBS). In principe penetreren deze reagentia de membraan niet, zodat hun werking beperkt is tot die fosfolipiden, die aan de buitenzijde van de membraan voorkomen.

In hoofdstuk 2 wordt een beschrijving gegeven van de bepalingen die gedurende het gehele onderzoek zijn toegepast. In dit hoofdstuk wordt tevens vermeld aan welke voorwaarden voldaan moet worden om uit de verkregen resultaten de verdeling van de fosfolipiden over de binnen- en buitenzijde van de fotoreceptor membraan ondubbelzinnig te bepalen. Eén van deze voorwaarden is het gebruik van preparaten, waarin de oorspronkelijke (in situ) verdeling der fosfolipiden gehandhaafd blijft. Vers bereide preparaten van staafjes buitensegmenten met een gestapelde zakjes-structuur benaderen de in situ structuur het beste en worden daarom bij voorkeur gebruikt.

In hoofdstuk 3 zijn drie verschillende fosfolipases (fosfolipase A_2, C en D) gebruikt om de verdeling van de fosfolipiden over de binnen- en buitenzijde van de fotoreceptor membraan te bepalen. Fosfolipase D hydrolyseert ongeveer de helft van fosfatidylcholine en fosfatidylethanolamine. Dit wijst op een symmetrische verdeling van deze fosfolipiden over de fotoreceptor membraan. Fosfolipase A_2 en C hydrolyseren echter vrijwel alle fosfolipiden in de fotoreceptor membraan. Blijkbaar leidt de hydrolyse in deze gevallen tot een ernstige verstoring in de membraanstructuur. Om dit laatste tot een minimum te beperken, is het hydrolyse patroon van de fosfolipiden in de beginfase van de reactie bepaald. Dit patroon is vrijwel hetzelfde als dat van zgn. referentie preparaten, waarin dezelfde fosfolipiden voorkomen als in de fotoreceptor membraan, maar nu vrij toegankelijk voor het fosfolipase. Dit betekent, dat alle fosfolipiden nagenoeg symmetrisch verdeeld zijn over de fotoreceptor membraan.

In hoofdstuk 4 wordt het amino-reagens TNBS gebruikt, alsmede fosfolipase D. Met fosfolipase D wordt maximaal 55% fosfatidylcholine en 50% fosfatidylethanolamine gehydrolyseerd. TNBS reageert betrekkelijk snel (binnen een uur) met 50% fosfatidylethanolamine en 40% fosfatidylserine. De reactie gaat echter

langzaam verder en na 3 uur is van elk lipid ca. 10% extra gemodificeerd. Dit laatste wordt toegeschreven aan penetratie van TNBS, waardoor de fosfolipiden aan de binnenzijde van de fotoreceptor membraan beschikbaar komen voor modificatie. Het maakt geen verschil of de fotoreceptor membraan eerst met fosfolipase D behandeld wordt en dan met TNBS, of omgekeerd: ongeveer de helft van de fosfolipiden is toegankelijk voor hydrolyse en modificatie. Hieruit volgt, dat tijdens de reactie geen verstoring in de oorspronkelijke verdeling der lipiden optreedt. Uit deze experimenten wordt geconcludeerd dat het toegankelijke deel van de fosfolipiden zich aan de buitenzijde van de fotoreceptor membraan bevindt; dit impliceert wederom een vrijwel symmetrische verdeling van de fosfolipiden over de fotoreceptor membraan.

In hoofdstuk 5 worden de experimenten met fosfolipase D en TNBS herhaald, maar nu wordt de vetzuursamenstelling bepaald van dat deel der fosfolipiden, dat niet gehydrolyseerd of gemodificeerd is. De vetzuursamenstelling van deze fosfolipiden is dezelfde als die van de fosfolipiden in het oorspronkelijke preparaat, m.a.w., ook de vetzuurketens van elk fosfolipide zijn symmetrisch verdeeld over de fotoreceptor membraan.

In hoofdstuk 6 wordt toegelicht, dat aan de voorwaarden, gesteld in hoofdstuk 2, om uit de verkregen resultaten de verdeling van de fosfolipiden over de fotoreceptor membraan ondubbelzinnig te bepalen, lijkt te zijn voldaan. Eén nog niet eerder genoemde voorwaarde is het niet optreden van een trans-bilaag beweging van de fosfolipiden (uitwisseling tussen binnen- en buitenzijde van de membraan). Onder de door ons gebruikte experimentele omstandigheden treedt een trans-bilaag beweging van fosfolipiden hoogstwaarschijnlijk niet op. *In vivo*, echter, vertonen de fosfolipiden vrij zeker wel deze beweging. Het mechanisme wat hieraan ten grondslag ligt en de betekenis ervan voor het proces van het zien dienen nader bestudeerd te worden.

De resultaten beschreven in dit proefschrift verschillen met die uit eerdere onderzoekingen, waarin gevonden werd dat met name fosfatidylethanolamine beter toegankelijk is voor modificatie met TNBS. In hoofdstuk 6 wordt geconcludeerd dat deze eerdere onderzoekingen verricht werden onder omstandigheden die leiden tot penetratie van TNBS en verstoring van de membraanstructuur.

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E.H.S. Drenthe is op 1 december 1947 geboren te Gennep. In 1966 behaalde hij het diploma gymnasium-B aan het R.K. Lyceum te Hilversum. In hetzelfde jaar begon hij met de studie scheikunde aan de Rijksuniversiteit te Utrecht. In 1971 legde hij het candidaatsexamen (S1 + S2) af en in 1974 het doctoraalexamen (hoofdvak biochemie, bijvak analytische chemie, met aantekening lesbevoegdheid). Na het vervullen van de militaire dienstplicht, is hij sinds november 1975 werkzaam geweest aan de afdeling Biochemie van de Medische Faculteit der Katholieke Universiteit te Nijmegen, waar het hier beschreven onderzoek is uitgevoerd onder leiding van Prof. Dr. F.J.M. Daemen en Prof. Dr. S.L. Bonting. In 1976 heeft hij meegewerkt aan een pilot studie analytische electronenmicroscopie ter bepaling van calcium in staafjes buitensegmenten (in samenwerking met het Laboratorium voor Submicroscopische Morfologie; hoofd: Prof. Dr. A.M. Stadhouders). Aanvankelijk in dienst van de universiteit, is hij van februari 1977 tot februari 1981 in dienst geweest van de Nederlandse Organisatie voor Zuiver-Wetenschappelijk Onderzoek (ZWO) via de Stichting voor Scheikundig Onderzoek in Nederland (SON).

STELLINGEN

I

Bij de bepaling van de fosfolipide verdeling over de binnen- en buitenzijde van biologische membranen verdient het gebruik van fosfolipase D de voorkeur boven dat van fosfolipase A_2 of C.

Dit proefschrift, hoofdstuk 3 en 4.
Heller, M. (1978) Adv. Lipid Res. 16, 267-326.

II

Het gebruik van een ionofoor om alle aminofosfolipiden in biologische membranen toegankelijk te maken voor reactie met amino-groep reagentia leidt niet tot het gewenste resultaat.

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III

Het gemak, waarmee fosfolipase A_2 uit varkenspancreas fosfolipiden in fotoreceptor membranen afbreekt, illustreert de wijdmazigheid van deze membranen.

Dit proefschrift, hoofdstuk 3.
Demel, R.A., Geurts van Kessel, W.S.M., Zwaal, R.F.A., Roelofsen, B. en van Deenen, L.L.M. (1975) Biochim. Biophys. Acta 406, 97-107.

IV

Wanneer geringe verschillen in lipide- en vetzuursamenstelling van staafjes buitensegmenten grote gevolgen hebben voor de lipide structuur, dan is een goede isolatiemethode met behoud van de oorspronkelijke lipide samenstelling van evident belang.

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V

De waarneming, dat water-oplosbare eiwitten in staafjes buitensegmenten zich na belichting gaan gedragen als extrinsieke (membraan-gebonden) eiwitten, illustreert treffend het dynamische karakter van de fotoreceptor membraan.

Godchaux, W. en Zimmerman, W.F. (1979) Exp. Eye Res. 28, 483-500.
Shichi, H. en Somers, R.L. (1980) Photochem. Photobiol. 32, 491-495.
Kühn, H. (1980) Nature 283, 587-589.

VI

Bij de localisatie van calcium ionen in staafjes buitensegmenten met behulp van röntgen-microanalyse is voorbehandeling van het preparaat met kalium pyroantimonaat niet voldoende om diffusie van ionen tegen te gaan.

Fishman, M.L., Oberc, M.A., Hess, H.H. en Engel, W.K. (1977) Exp. Eye Res. 24, 341-353.
Läuchli, A. (1975) J. Microscopie Biol. Cell. 22, 239-246.

VII

De conclusie van Sen en Ray, dat inactivering van het $(K^+ + H^+)$-ATPase uit de varkensmaag door ethanol het gevolg is van extractie van fosfolipiden uit de onmiddellijke omgeving van het enzym, is ongewettigd.

Sen, P.C. en Ray, T.K. (1980) Arch. Biochem. Biophys. 202, 8-17.
Saccomani, G., Chang, H.H., Spisni, A., Helander, H.F., Spitzer, H.L. en Sachs, G. (1979) J. Supramol. Struct. 11, 429-444.

VIII

Het door Allgyer en Wells gevonden complexe kinetische gedrag van fosfolipase D uit savooiekool is mogelijk mede een gevolg van een nevenactiviteit van het enzym.

Allgyer, T.T. en Wells, M.A. (1979) Biochemistry 18, 5348-5353.
Davidson, F.M. en Long, C. (1958) Biochem. J. 69, 458-466.

IX

Bij de beschrijving van een analytische bepalingsmethode voor nucleosiden dient meer aandacht besteed te worden aan de monster voorbereiding en de gevoeligheid van de methode bij biologisch relevante concentraties der nucleosiden.

Rustum, Y.M. (1978) Anal. Biochem. 90, 289-299.
Pallavicini, M.G. en Mazrimas, J.A. (1980) J. Chromatogr. 183, 449-458.

X

Fosfatidylserine-decarboxylase uit *Escherichia coli* is niet goed bruikbaar om de transbilaag verdeling van fosfatidylserine en de invloed van dit lipide op de binding van opiaten aan membranen te bepalen vanwege zijn experimentele afhankelijkheid van aanwezigheid van het detergens Triton X-100.

Abood, L.G., Salem, N., MacNeil, M. en Butler, M. (1978) Biochim. Biophys. Acta 530, 35-46.
Warner, T.G. en Dennis, E.A. (1975) J. Biol. Chem. 250, 8004-8009.

XI

Bij studies naar de invloed van een dieet, dat deficient is in essentiele vetzuren, op de vetzuursamenstelling van biologische membranen wordt onvoldoende rekening gehouden met de noodzakelijke aanwezigheid van vitamine E om vetzuuroxydatie tegen te gaan.

Anderson, R.E., Benolken, R.M., Dudley, P.A., Landis, D.J. en Wheeler, T.G. (1974) Exp. Eye Res. 18, 205-213.
Tinoco, J., Miljanich, P. en Medwadowski, B. (1977) Biochim. Biophys. Acta 486, 575-578.
Jager, F.C. (1975), in: The role of fats in human nutrition (A.J. Vergroesen, ed.). Academic Press, London, pp 381-432.

XII

De conclusie van Sugden en Lilleyman uit hun experimenten met [^{3}H] thymidine, dat de thymidine inbouw in het DNA van leukemische lymfoblasten geremd wordt door autoloog plasma, is voorbarig bij gebrek aan gegevens omtrent de niet-radioactieve thymidine concentratie in het plasma.

Sugden, P.J. en Lilleyman, J.S. (1980) Brit. J. Haematol. 46, 367-375.

XIII

Bij grasparkieten is sexuele voorlichting onontbeerlijk voor hun voortplanting.

Eigen waarneming.

XIV

De huidige belastingwetgeving is in strijd met de wet op gelijke behandeling van man en vrouw.

Nijmegen, 16 juni 1981

Erik Drenthe