THE ULTRASTRUCTURE OF LIPID-DEPLETED ROD PHOTORECEPTOR MEMBRANES

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ABSTRACT

The structure of lipid-depleted retinal rod photoreceptor membranes was studied by means of electron microscopy. Aldehyde-fixed retinas were exhaustively extracted with acetone, chloroform-methanol, and acidified chloroform-methanol. The effect of prefixation on the extractability of lipids was evaluated by means of thin-layer chromatography and fatty acid analysis. Prefixation with glutaraldehyde rendered 38% of the phospholipids unextractable, while only 7% were unextractable after formaldehyde fixation. Embedding the retina in a lipid-retaining, polymerizable glutaraldehyde-urea mixture allows a comparison of the interaction of OsO_4 with lipid-depleted membranes and rod disk membranes which contain all their lipids. A decrease in electron density and a deterioration of membrane fine structure in lipid-depleted tissue are correlated with the extent of lipid extraction. These observations are indicative of the role of the lipid bilayer in the ultrastructural visualization of membrane structure with OsO_4. Negatively stained thin sections of extracted tissue reveal substructures in the lipid-depleted rod membranes. These substructures are probably the opsin molecules which are the major protein component of retinal rod photoreceptor membranes.

The frog rod photoreceptor membrane consists of about 40% lipid and 60% protein (8, 18). Of the protein, about 80–90% is opsin (5, 14). Thus the extraction of lipids from the photoreceptor membrane will result in a special situation in which the remaining components will consist mainly of a single protein—opsin. The ultrastructural investigation of lipid-depleted photoreceptors should therefore reveal the contribution of the opsin molecules to the structural organization of the rod photoreceptor membranes.

In order to visualize the hydrophobic opsin molecules in lipid-depleted membranes, we have used a technique of embedment in a water-containing, polymerizable glutaraldehyde-urea mixture. This hydrophilic embedment has been successfully used in combination with ionic stains for the visualization of hydrophobic substructures in membranes (12, 21, 22, 23).

The retention of lipids by the glutaraldehyde-urea-embedding mixture makes possible the evaluation of the role of OsO_4 in the visualization of retinal membranes. During conventional preparation, which involves the use of organic solvents, there is a loss of lipids, even after treatment of the tissue with OsO_4 (16). Thus, only by comparing lipid-depleted sections with controls in which the membranes retain all their lipids, is it possible to evaluate accurately the role of lipids in the visualization of membranes by OsO_4.

It was previously reported that depletion of lipids from tissue prefixed with glutaraldehyde did

not change the membrane structure as revealed by en bloc osmication (20). However, it has also been shown that glutaraldehyde is capable of binding certain phospholipids to the membrane proteins (11). We decided, therefore, to accurately evaluate the effect of prefixation of the retina with aldehydes on the extractability of its phospholipids and the subsequent stainability with OsO_4. Thus, a careful lipid analysis of unfixed and aldehyde-fixed retinas was performed so as to relate a given structural organization to its actual lipid content.

MATERIALS AND METHODS

Extraction of Lipids

10 frogs were dark adapted for 2 h. The eyes were enucleated and the retinas were removed from the eye cup. Adhering pigment epithelium and choroid were dissected away. Any portion of the retina from which the pigment epithelium could not be removed was cut off and discarded. 10 dissected retinas were dropped into cold 1% glutaraldehyde, and 10 into cold 4% formaldehyde, in 75% and 50% Earle's balanced salt solution, pH 7.5, respectively. The retinas were fixed in the dark at 4°C for 24 h. At the end of this period, 10 additional dark-adapted frog retinas were placed in cold 75% Earle's solution, pH 7.5. This group served as a control from which lipids were extracted without prefixation with aldehydes. Extraction of lipids was carried out essentially according to Napolitano et al. (20). These three sets of retinas were washed with three changes of cold 75% Earle's solution, and dehydrated at room temperature with graded acetone in water—30%, 50%, 70%, 85%, 95%, and 100% (10 min per step). The acetone extracts were pooled and stored under nitrogen at −15°C. The retinas were then allowed to stand in 10 ml of chloroform-methanol (2:1) for 24 h at room temperature. The solvent was removed after centrifugation and combined with the acetone extract. The retinas were extracted with 10 ml of chloroform-methanol-concentrated HCl (200:100:1) for 1 h. The solvent was again removed by centrifugation and combined with the previous extracts.

Thin-Layer Chromatography

The combined lipid extract from each group of retinas was dried in a rotary evaporator, and the residue was dissolved in chloroform:methanol (2:1). Any insoluble residue was removed by centrifugation. The supernates were partitioned against 0.2 vol of 0.1 M NaCl. The chloroform layer was removed and aliquots were taken for determination of total phosphorus (24). The remainder of the chloroform extract was blown to dryness under nitrogen and redissolved in 0.5 ml of chloroform. 50-μl aliquots of these samples were subjected to two-dimensional thin-layer chromatography (TLC) on silica gel H (Merck Chemical Div., Merck and Co., Inc., Rahway, N. J.) according to the method described by Anderson et al. (2). After drying, the plates were sprayed lightly with a solution of Ninhydrin to visualize the amino-containing phospholipids, phosphatidyl ethanolamine, phosphatidyl serine, and the lyso-derivatives of these compounds. The plates were then sprayed with a solution of potassium dichromate in sulfuric acid and charred at 120°C. The spots thus revealed were scraped off the plates and analyzed for total phosphorus (24).

Fatty Acid Analysis of Retinal Residues

The extracted retinas were dried to constant weight *in vacuo*. To each residue was added 500 μg of arachidic acid as an internal standard and 2 ml of 15% boron trifluoride in methanol. The tubes were sealed with a Teflon-lined cap and heated at 90°C for 15 min (19). Gas chromatography and identification of the liberated fatty acid esters was carried out according to Anderson et al. (3).

Electron Microscopy

Electron microscope observation was carried out on sections of lipid-extracted and unextracted retinas. The extracted tissue was taken from the same group of retinas which were analyzed for their lipid content. After the last extraction step, the retinas were divided into two groups. One group was embedded in a glutaraldehyde-urea mixture without en bloc osmication. The second group was osmicated before embedding according to the procedure described by Napolitano et al. (20). The tissue was rinsed for 10 min in CCl_4 and then exposed to 5% OsO_4 in CCl_4 for 20 min at room temperature. After osmication, the tissue was rinsed for 10 min in CCl_4, followed by 10 min in acetone. At this point, part of the tissue was transferred to 50% glutaraldehyde for subsequent embedment in polymerizable glutaraldehyde-urea mixture. The other part was rinsed in propylene oxide for 20 min, then embedded in Epon (17). The glutaraldehyde-urea embedding procedure has been described elsewhere (21, 22, 23). In the present study, the embedding mixture consisted of one part of a saturated solution of urea in 50% glutaraldehyde and two parts of 50% glutaraldehyde, with the pH adjusted to 4.3. Unextracted controls were fixed for 24 h with 1% glutaraldehyde in 75% Earle's solution and were then prepared for electron microscopy by using the same procedures described for extracted tissue.

Thin sections of osmicated tissue were treated with uranyl acetate and lead citrate for further contrast enhancement. Membranes in tissues which were embedded in the glutaraldehyde-urea mixture without en bloc osmication possess very low contrast. In order to visualize these membranes with positive contrast, the sections were exposed to OsO_4 vapors for 60 min at room temperature. Negative contrast was obtained by treating other thin sections with uranyl acetate and lead citrate. The sections were examined in a Siemens Elmiskop 1A electron microscope.

RESULTS

Phospholipid Analysis of Fixed and Unfixed Retinas

Table I shows the phospholipid analysis of the control, formaldehyde- and glutaraldehyde-fixed retinas. The large quantities of lyso-phospholipids are the result of breakdown of the parent phospholipids during the long extraction procedure used, which is carried out at room temperature. Previous experiments have shown that retinas extracted in the cold immediately after dissection contain negligible quantities of lyso-phospholipids (1, 13). While the lyso-derivatives of phosphatidyl ethanolamine (PE), phosphatidyl choline (PC), and phosphatidyl serine (PS) have been listed separately in Table I they have also been added to the parent phospholipids to give the total PE, PC, and PS originally present in the retinas, and will be discussed as such in this paper. It is apparent from this table that, in comparison to the unfixed retinas, the amount of PE and PS which can be extracted by the procedure used is dramatically reduced by glutaraldehyde fixation, and only slightly reduced by fixation with formaldehyde. Furthermore, fixation with glutaraldehyde or formaldehyde results in an increase in the amount of phosphorus-containing material which migrates to the solvent front in TLC. Those phospholipids which do not contain primary amino groups [PC, and sphingomyelin (Sph)] in their structure are not affected by the fixation procedure and remain freely extractable. However, the amount of extractable phosphatidyl inositol (PI) does seem to be reduced by formaldehyde fixation. Also shown in Table I is the quantity of total phospholipid extracted per milligram dry weight of retina. Thus, after formaldehyde and glutaraldehyde fixation, 7% and 38%, respectively, of the total phospholipid is unextractable. In order to account for all of the extracted phospholipid, the formaldehyde and glutaraldehyde solutions used during fixation were analyzed. Less than 0.5% of the total phospholipids was extracted during this step.

Fatty Acid Analysis of Fixed and Unfixed Retinas

In order to estimate the amount of phospholipid bound into the tissue by the fixation procedure, the retinal residues were subjected to methanolysis with boron trifluoride in methanol. The methyl esters of the fatty acids thus liberated were separated by gas-liquid chromatography. Table II

TABLE I

Quantitative Analysis and Composition of Phospholipid Extracts from Fixed and Unfixed Retinas of Rana pipiens

	Unfixed		Formaldehyde		Glutaraldehyde	
Total phospholipid extracted*	362.4		336.8		226.0	
Lipid‡						
PC	30.1	46.8§	44.6	52.9§	56.0	72.6§
LPC	16.7		8.3		16.6	
PE	24.0	35.2	21.1	27.8	7.7	7.7
LPE	11.2		6.7		0.0	
PS	8.6	11.2	9.2	10.2	1.8	1.8
LPS	2.6		1.0		0.0	
PI	2.8		1.2		2.1	
SPH	2.3		2.4		3.4	
SF	1.3		6.3		11.2	
OR	0.0		0.3		1.2	
Total (PE + PS + SF)	47.7		44.3		20.7	

* Data are expressed as nanomoles of phospholipid per milligram dry weight of retina and are calculated from the total lipid phosphorus in the extracts before thin-layer chromatography.

‡ Data are relative mole percent. Values are averages of triplicate analyses from two separate experiments. Abbreviations are: PC, phosphatidyl choline; LPC, lyso-phosphatidyl choline; PE, phosphatidyl ethanolamine; LPE, lysophosphatidyl ethanolamine; PS, phosphatidyl serine; LPS, lysophosphatidyl serine; PI, phosphatidyl inositol; SPH, sphingomyelin; SF, solvent front; OR, origin.

§ These values are the sum of the parent phospholipid and its lyso-derivative.

shows the composition of the fatty acids liberated from each retinal residue. Assuming an average molecular weight of the liberated fatty acids to be that of eicosanoic acid (MW 312), it is possible to calculate the nanomoles of fatty acid liberated per milligram dry weight of retina. These results are also shown in Table II. Only negligible amounts of fatty acids are liberated from the control retinas, which proves the efficiency of the extraction procedure. The formaldehyde- and glutaraldehyde-fixed retinas, however, contain 16 and 62 times as much bound fatty acid, respectively, as does the control tissue.

TABLE II

Fatty Acid Content and Analysis of Fixed and Unfixed Retinas after Lipid Extraction

	Unfixed	Formaldehyde	Glutaraldehyde
Total fatty acids liberated*	1.2	19.5	77.8
Fatty acid analysis‡			
16:0	72.8	9.0	6.9
16:1	26.2	0	0
18:0	0.9	9.9	9.7
18:1	0	7.8	8.7
20:4	0	4.6	5.3
20:5	0	3.2	0.7
22:4	0	0.9	2.8
22:5	0	4.1	5.0
22:6	0	60.5	61.0

* Data are expressed as nanomoles of fatty acid per milligram dry weight of retina and are calculated from the total acids liberated after methanolic boron trifluoride treatment of the lipid-extracted retinas. See text for details.

‡ Data are relative weight percent composition.

Electron Microscopy

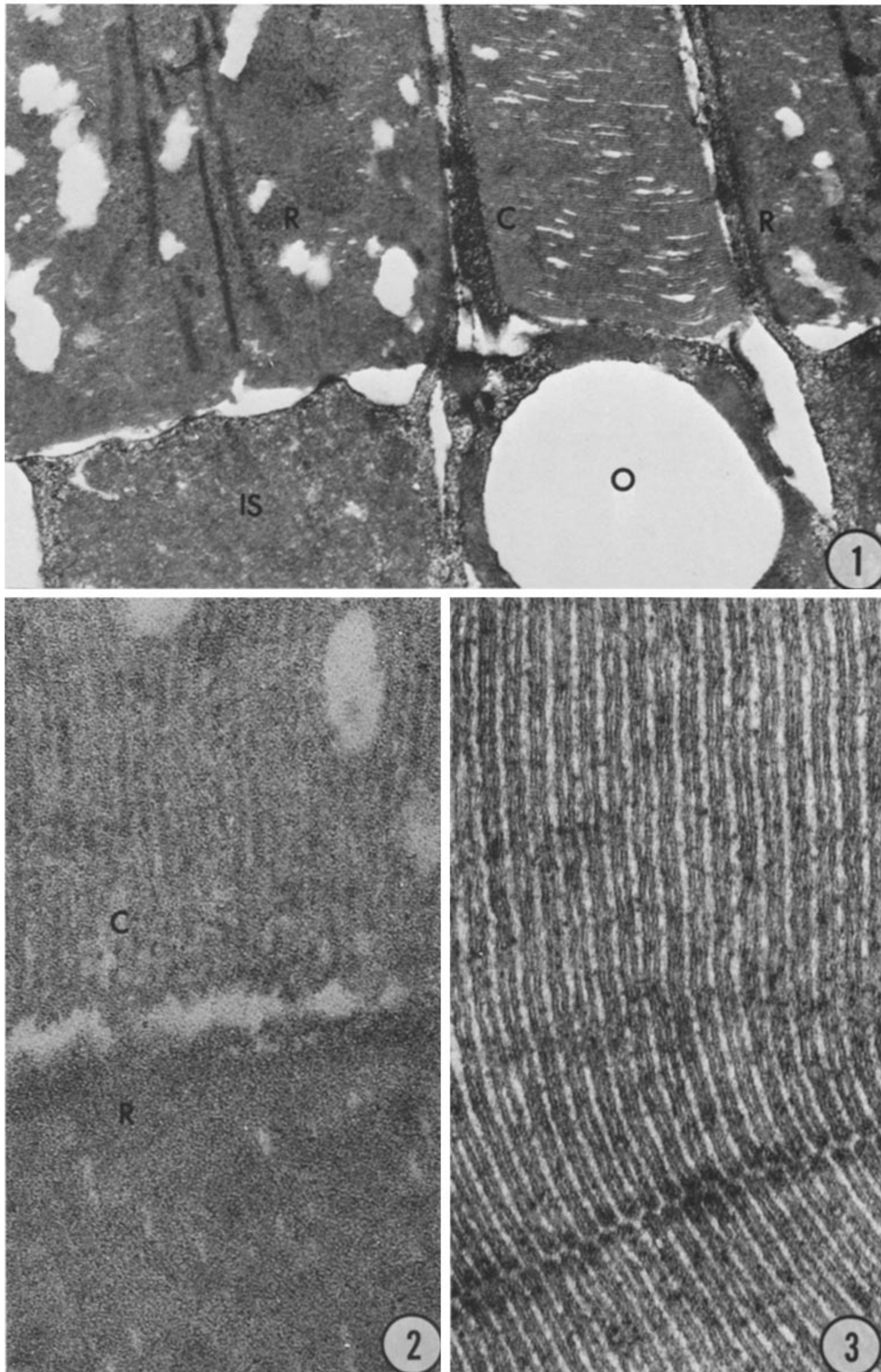
PREFIXATION WITH GLUTARALDEHYDE: For comparison with other studies (15, 20, 27), a procedure of glutaraldehyde prefixation, lipid extraction, en bloc osmication, and embedding in Epon was initially used. The general morphology of such preparations is shown in Fig. 1. Parts of rod and cone inner segments can be identified. The general features of the photoreceptors are preserved. The electron-transparent oil droplets in the cone reflect the effect of extraction with organic solvents. Higher magnification of the outer segments (Fig. 2) reveals considerable structural change, the extent of which varies between the rods and cones. This change is always evident in the rods where the membrane system is essentially destroyed. In the cones, membrane continuity persists, but there is no evidence of the trilaminar arrangement of unit membranes. This trilaminar arrangement can be clearly visualized in unextracted rod or cone membranes which were fixed with glutaraldehyde and OsO_4 before embedding in glutaraldehyde-urea or Epon (Fig. 3).

Further study has shown that the total destruction of lipid-depleted rod membranes can be avoided. When the extracted retina was embedded in glutaraldehyde-urea, and OsO_4 was applied as a vapor to the thin sections, discrete membrane continuity could be observed (Fig. 4). It seems that the combination of en bloc osmication and Epon embedding causes the extensive damage to the extracted rod membranes. It should be emphasized that after the staining with OsO_4 vapors the photoreceptor membranes become electron dense and the typical disk structure can be clearly identified in unextracted photoreceptors (Fig. 5).

FIGURE 1 Retina fixed with glutaraldehyde. Lipid-extracted tissue was postfixed with OsO_4 and embedded in Epon. Thin sections were stained with uranyl acetate for 5 min and lead citrate for 3 min. The general morphology of the photoreceptor cells is preserved. (*R*, rod outer segment; *C*, cone outer segment; *IS*, inner segment; *O*, oil droplet). × 17,500.

FIGURE 2 The same as Fig. 1 but at higher magnification. In the rod outer segment (*R*), in the lower part of the figure, the membrane system is completely destroyed. In the cone outer segment (*C*), in the upper part of the figure, membrane structure can be distinguished, but trilaminar arrangement of the unit membrane leaflets cannot be resolved. × 105,000.

FIGURE 3 Unextracted control retina fixed in glutaraldehyde and postfixed in OsO_4 solution before embedding in glutaraldehyde-urea mixture. Thin sections were stained with uranyl acetate for 2 min and lead citrate for 1 min. Trilaminar arrangement of membrane leaflets is clearly visible. × 135,000.


R
C
R
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O
1
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2
3

In the extracted retina, lipid-depletion affects the stainability of the membranes by OsO_4 vapors. In Fig. 4, therefore, the photoreceptor membranes are seen with lower density and are poorly resolved in comparison with the unextracted photoreceptor membranes (Fig. 5).

Prefixation with Formaldehyde

Glutaraldehyde is usually the fixative of choice for the preservation of ultrastructure (25). However, in view of the fixation of certain lipids by glutaraldehyde (Table I), it was necessary to use prefixation with formaldehyde in order to explore the effect of almost total lipid extraction upon the structural integrity of the photoreceptor membranes. After fixation with formaldehyde and extraction with organic solvents, the tissue was embedded in glutaraldehyde-urea, and thin sections were stained with OsO_4 vapors. As already noted, this preparation procedure was found to

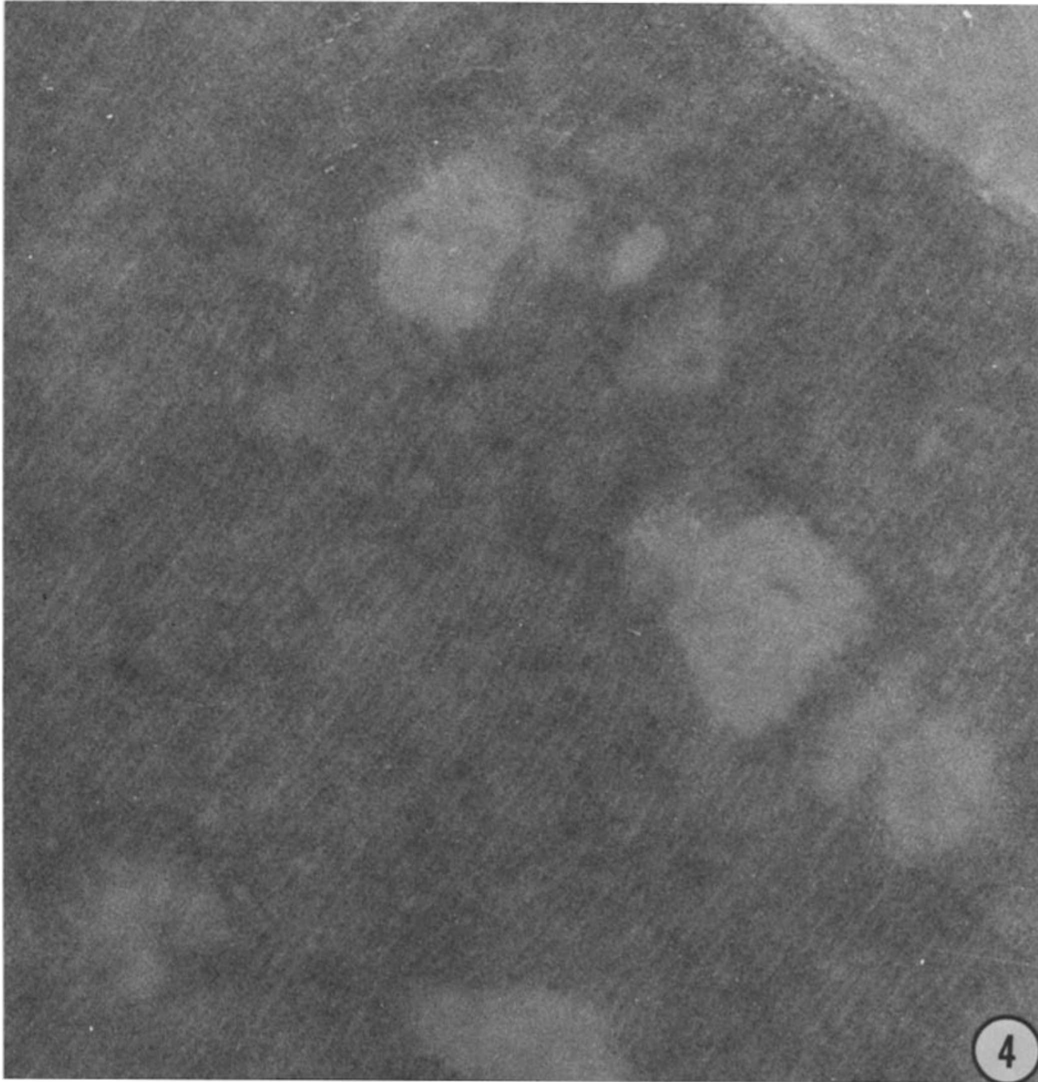


FIGURE 4 Retina fixed in glutaraldehyde. Lipid-extracted tissue was embedded in glutaraldehyde-urea mixture. Thin sections were exposed to OsO_4 vapors for 60 min at room temperature. Discrete electron-dense membrane continuity can be seen in the rod outer segment (compare with Fig. 2, where rod membranes cannot be seen in identically extracted tissue which was embedded in Epon after en bloc osmication). × 88,000.

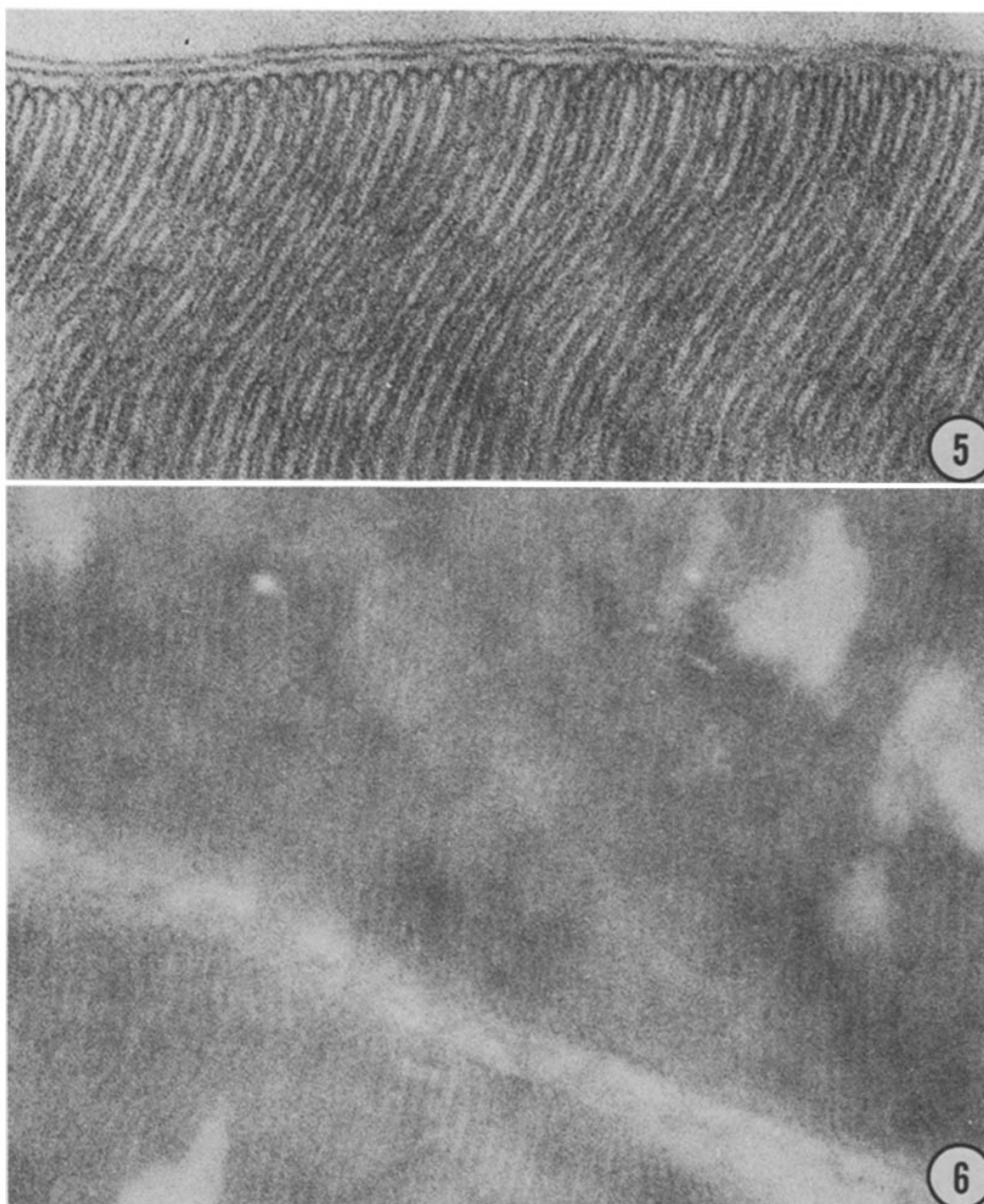


FIGURE 5 Unextracted control retina fixed with glutaraldehyde and embedded in glutaraldehyde-urea mixture. Thin sections were stained with OsO_4 vapors for 60 min at room temp. The typical rod photoreceptor disks are visualized as electron-dense structures. The electron density of the membranes represents maximum stainability of the retinal rod membranes under these experimental conditions. Note that this section was stained simultaneously and under identical conditions as were the sections of extracted tissue shown in Figs. 4 and 6. The comparison of Figs. 4 and 6 with Fig. 5 demonstrates, therefore, the effect of lipid extraction on the reaction of OsO_4 vapors with the retinal membranes. $\times$ 100,000.

FIGURE 6 Retina fixed in formaldehyde. Lipid-extracted tissue was embedded in glutaraldehyde-urea mixture. Thin sections were stained with OsO_4 vapors for 60 min at room temperature. The residual electron density represents the interaction of OsO_4 with retinal rod membranes which are almost completely depleted of lipids. $\times$ 105,000.

improve the preservation of the extracted retinal membranes. The structure of the rod outer segments is shown in Fig. 6. The visibility of the membranes is further reduced, but the capability of membrane substance to interact with OsO_4 is not altogether eliminated, as can be judged from the presence of electron-dense structures. As these membranes are very low in lipid, the residual staining with OsO_4 can be attributed to the interaction of OsO_4 with residual lipids and with proteins. A comparison of Fig. 6, in which the membranes are almost totally lipid-free, with the control membranes shown in Fig. 5, provides a clear indication of the contribution of lipids to the visualization of the retinal membranes with OsO_4.

Because the visualization of the retinal membranes with OsO_4 is largely dependent upon the presence of lipids, alternative procedures should be used for adequate visualization of the structure of extracted membranes. Satisfactory visualization of membrane structures in extracted retina was achieved by using ionic stains such as uranyl and lead salts (Fig. 7). The application of ionic stains to an hydrophilic embedment such as the glutaraldehyde-urea mixture used in this study visualizes the membranes as electron-transparent structures (21, 22). In unextracted retina, the typical rod disk structures can be clearly identified (Fig. 8). After lipid extraction the membranes appear fragmented and, at higher magnifications, irregular electron-transparent substructures can be seen (Fig. 9). In some areas (see arrows in Fig. 9), electron-transparent globules can be resolved. As the extracted membranes are almost completely depleted of lipids, we assume that the visualization of these membranes with ionic stains is largely due to the protein component of the membranes.

DISCUSSION

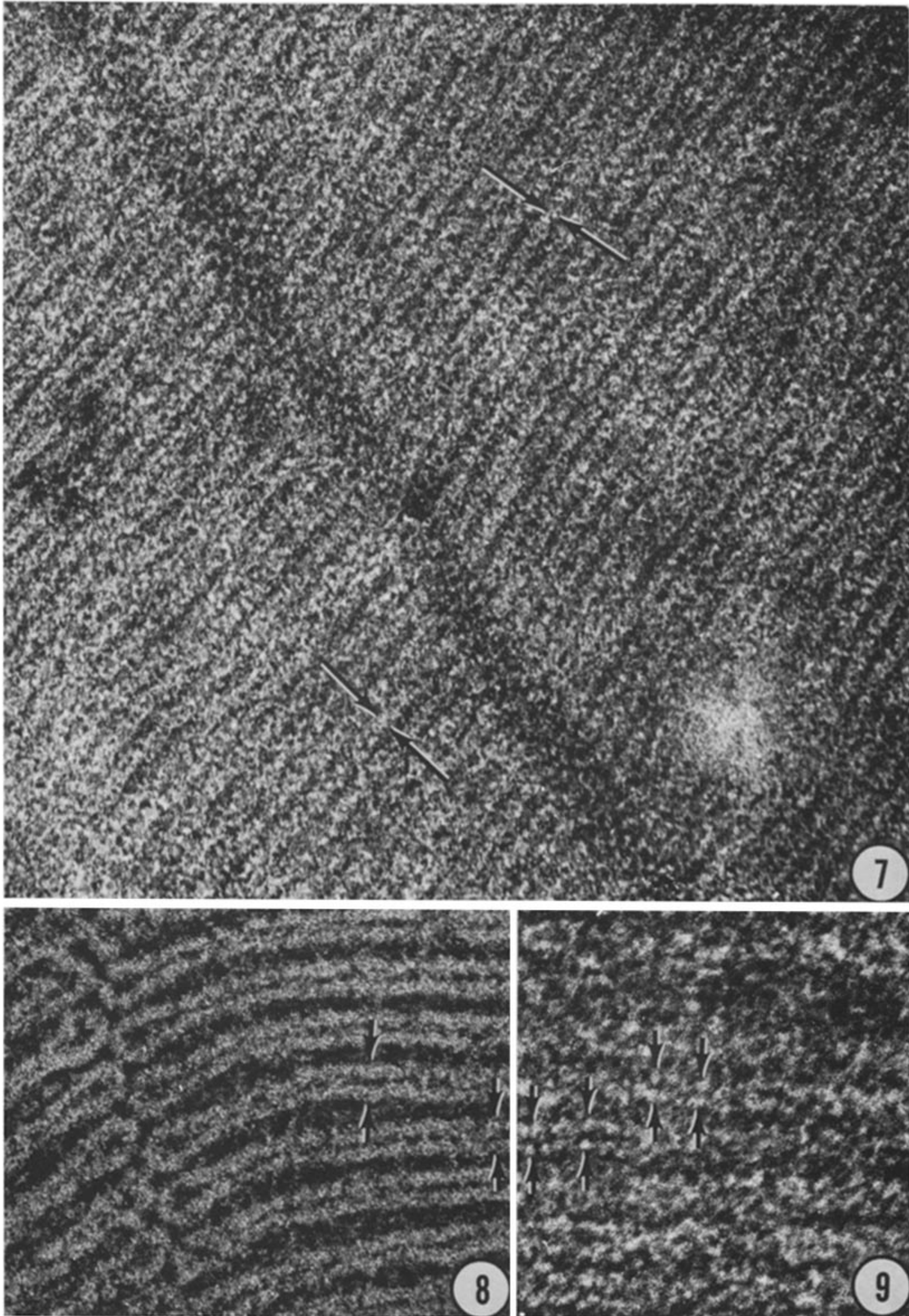
The effect of lipid extraction on the structure of membranes as revealed by electron microscopy has been studied in a variety of biological membranes (4, 7, 10, 15, 20, 27). In some of those studies, a mixture of chloroform-methanol was used to achieve total lipid extraction. For the retention of proteins, and to reduce extensive structural damage to the membranes by organic solvents, prefixation with glutaraldehyde was used.

In the present study, we have established that fixation of retinas with glutaraldehyde renders some of the phospholipids unextractable with chloroform-methanol. Those phospholipids which are most dramatically affected are PE and PS. Formaldehyde fixation appears to have a much lesser effect. After fixation with glutaraldehyde, 38% of the phospholipids are retained in the tissue, while only 7% are fixed into the tissue by formaldehyde. When isolated frog rod outer segments are fixed with formaldehyde and extracted with chloroform-methanol as described above, we have found that 99% of the phospholipids are extracted from these organelles. However, since the entire retina was used for electron microscopy in these studies (for reasons of improved ultrastructural preservation and orientation of membranes), we have reported the figure obtained when whole retinas were fixed and extracted. It should be

FIGURE 7 Retina fixed with formaldehyde. Lipid-extracted tissue was embedded in glutaraldehyde-urea mixture. Thin sections were stained with uranyl acetate for 4 min and lead citrate for 1 min. The electron-transparent lines (delineated by pairs of opposing arrows) are the rod disks in which the intradisk spaces are poorly resolved. The electron-dense lines are the interdisk spaces. × 180,000.

FIGURE 8 Unextracted retina fixed in glutaraldehyde and embedded in glutaraldehyde-urea mixture. Thin sections were stained with uranyl and lead salts as in Fig. 7. The typical rod photoreceptor disks are visualized as electron-transparent structures. The opposing arrows delineate the two membranes of one disk. The narrow gap between the opposing membranes is the intradisk space. The larger electron-dense gap between disks is the interdisk space. × 340,000.

FIGURE 9 The same as Fig. 7 but higher magnification. For orientation purposes, Fig. 8 and Fig. 9 are so aligned as to assist in identification of the sites of disk membranes and interdisk spaces in the extracted tissue where regularity of membranes is disturbed during the extraction procedure. The disk membranes consist of irregular electron-transparent substructures. The arrows delineate pairs of electron-transparent globules which are part of the two membranes of one disk. The electron-dense gap between disks is the interdisk space. The intradisk spaces (between opposing globules) are poorly resolved. × 360,000.



noted, however, that the figure of 7% retention of phospholipids after formaldehyde fixation is an upper limit when considering the rod outer segments. Clearly, glutaraldehyde, with two active aldehyde groups, could conjugate a molecule of PE or PS to a molecule of protein, rendering an insoluble complex. Presumably, two molecules of PE or PS which are conjugated by glutaraldehyde would still remain extractable by chloroform-methanol, but would migrate to a different position in thin-layer chromatography, as shown by Gigg and Payne (11). This would explain why large amounts of phosphorus are detected in the solvent front in TLC of the chloroform-methanol extract of glutaraldehyde-fixed tissues (Table I). Similarly, the complex formed between PE, or PS, and formaldehyde might also migrate to the solvent front in TLC. Those phospholipids which were not recovered after fixation were not lost during the fixation. Measurements show that less than 0.5% of the phospholipids is extracted by the fixatives.

In order to estimate the amount of bound phospholipid, the residues left after extraction with various organic solvents were treated with boron trifluoride in methanol. This method liberates ester fatty acids as free methyl esters, which were then identified and quantified by gas-liquid chromatography. The predominant fatty acid liberated from the formaldehyde- and glutaraldehyde-fixed tissue is highly unsaturated docosahexenoic acid (22:6). This fatty acid comprises more than 40% of the total fatty acids of PE and PS from *Rana pipiens* rod outer segments (3). Thus, these fatty acid analyses prove that the reduction in the quantity of PE and PS found in TLC of the extract from glutaraldehyde-fixed material is due to the binding of these phospholipids into the tissue by the fixative. Not all of the phospholipid is recoverable from the fixed retinas. A number of explanations are possible. (*a*) Some 31% of the PE, PC, and PS in the control retinas are converted to the lyso-derivatives during extraction. Assuming that an equal amount of the bound phospholipids is also in the lyso-form, the fatty acid analyses would yield a figure for bound phospholipid lower than that actually present in the fixed tissues. (*b*) It is possible that the methanolic boron trifluoride treatment does not quantitatively release the fatty acids from the bound phospholipids.

Considering the almost complete lipid extraction after formaldehyde fixation, we suggest that this fixative should be the fixative of choice whenever a compromise between structural preservation and effective lipid extraction if desired. As the outer segment protein consists of 80–90% opsin, it is apparent that in formaldehyde-fixed material the lipid-depleted membranes consist largely of opsin. We suggest, therefore, that the electron-transparent structures revealed by staining of extracted membranes with uranyl and lead salts are mainly opsin molecules (Fig. 9). We can appreciate that, in the lipid-depleted membranes, these structures are arranged in continuous arrays and that in essence they now form the photoreceptor membranes.

The total disintegration of the rod membranes after lipid extraction which was described by Hirosawa (15) is apparently only a secondary result of the method by which the tissue was prepared for electron microscopy. However, the fact that only rod disks became susceptible to destruction after lipid extraction is indicative of basic chemical and structural differences between cone and rod membranes. It is worth noting that differential destruction of rod and cone membranes after osmication and treatment with Tris-buffer was described by Falk and Fatt (9).

The extraction of lipids with chloroform-methanol before osmication has been used previously for evaluation of the extent of the OsO_4 reaction with membrane components in different tissues (20, 27). In view of our findings on the effect of glutaraldehyde on the extractability of amino-containing phospholipids from the retinal membranes, and similar results obtained by Gigg and Payne (11) in a variety of tissues, the structural observations obtained from tissue prefixed with glutaraldehyde should be interpreted cautiously. In the retinal membranes we found a reduction in the stainability of membranes with OsO_4 as a result of lipid extraction with chloroform-methanol. After prefixation with glutaraldehyde, the extracted membranes still retain relatively high affinity for OsO_4. This can be explained by the retention of PE and PS in the glutaraldehyde-fixed tissue. These two phospholipids have a composition in which greater than 60% of their fatty acids are unsaturated and thus will be available for interaction with OsO_4. It is also possible that PE and PS will be available for polar interaction with OsO_4 at their amino-containing groups (6, 26).

After prefixation with formaldehyde, there is a further reduction in the staining of the membranes with OsO_4. The residual stainability of such membranes which are almost completely devoid of

lipids should be attributed primarily to the interaction of OsO_4 with protein, which is the major component left in the membrane. Together with the decreased stainability, there is a pronounced loss in the visualization of fine structure by OsO_4. This is evident in the loss of trilaminar structure of the "unit" membrane and the reduction in the visibility of clearly defined membranes. We conclude, therefore, that although staining with OsO_4 can be observed in lipid-depleted retinal membranes, the presence of the lipid bilayer is of prime importance for the visualization of membrane fine structures with OsO_4.

We are very grateful to Dr. Daniel C. Pease for valuable discussions, and to Ms. Maureen Maude and Dr. Robert E. Anderson, Baylor College of Medicine, for carrying out the fatty acid analyses reported in this paper.

This investigation was supported in part by United States Public Health Service Grant NS-284 to Dr. D. C. Pease, Department of Anatomy, University of California, Los Angeles, and in part by Grant EY 00046 from the National Institutes of Health.

Received for publication 14 March 1974, and in revised form 26 June 1974.

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