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Extraction of Phospholipids from Aldehyde-Fixed Membranes*

Extracción de fosfolípidos de membranas fijadas con aldehídos

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The effect of aldehyde fixation on membrane lipids and proteins has been studied. Purified fractions of toad retinal receptor outer segments were treated with a nonionic detergent after aldehyde fixation. Glutaraldehyde-fixed material retained proteins and phosphatidyl serine while other phospholipids were solubilized and extracted by detergent. Since phosphatidyl ethanolamine is extracted it is proposed that the mechanism of phosphatidyl serine retention does not depend on its amine groups.

Formaldehyde fixed material was not able to retain its proteins when treated with detergent. The difference in protein retention between formaldehyde and glutaraldehyde may be due to the formation of a supramolecular network in the presence of the two reactive groups in glutaraldehyde.

Although the mechanism of glutaraldehyde fixation is not well understood, it has been suggested that it depends mainly on the formation of intermolecular bridges by condensation of aldehyde groups with reactive groups present in the tissue (Sabatini *et al.*, 1963). The ability of glutaraldehyde to cross-link proteins has been demonstrated both is whole tissues (Hopwood, 1970) and in tissue fractions (Ellar *et al.*, 1971; Steck, 1972). Free amino groups of isolated proteins, such as the ϵ-amino group of lysine, play a major role in protein-aldehyde reactions (Tomimatsu *et al.*, 1971; Ottesen, 1971; Hopwood, 1973). In retinal rod outer segments (ROS) Brown (1972), Cone (1972) and Poo and Cone (1974) showed that glutaraldehyde fixation produces a restriction in rotational movements and lateral diffusion of rhodopsin. These authors proposed that glutaraldehyde cross-links rhodopsin molecules thus hindering movements in the plane of the membrane.

On the other hand, the nature and extent of lipid fixation by aldehydes is less clear. Roozemond (1969) has shown that the extraction of lipids from rat hypothalamus by a chloroform-methanol mixture can be reduced by previous fixation in formaldehyde or glutaraldehyde. Furthermore, these studies show that phosphatidyl serine and phosphatidyl ethanolamine are the lipids most retained by the aldehyde. The author proposed that the retention was mediated by their amino groups.

A simple experimental model, the retinal receptor outer membranes (ROS), was chosen to study the effect of aldehydes on the components of biological membranes. This system is particularly suitable for these experiments because the membranes can be easily isolated and partially purified in an homogeneous fraction. The system provides a large number of membranes of well known structure and chemical composition and, as already mentioned, the

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effect of glutaraldehyde on their main protein, rhodopsin, has been reported.

Rhodopsin can be directly detected with the naked eye under appropriate illumination conditions. By direct observation we have noticed that unfixed or formaldehyde-fixed retinas lose their rhodopsin when placed in a solution containing a non-ionic detergent such as Triton X-100. Conversely, glutaraldehyde-fixed retinas keep their characteristic color after several hours in the detergent solution.

We have analyzed the lipid and protein content of formaldehyde-fixed, glutaraldehyde-fixed ROS's and of unextracted controls to ascertain the extent of protein and lipid fixation by aldehydes. Furthermore, the lipid fraction was fractionated in order to analyze the reactivity of the amino groups of phosphatidyl ethanolamine and phosphatidyl serine with glutaraldehyde.

MATERIALS AND METHODS

Toads *(Bufo spinulosus),* collected during the spring season, were kept overnight in total darkness. They were decapitated under deep red light (Philips lamp PF 704 E). The room was lighted by two safety yellow-green bulbs (Osram 4543) placed at more than 1 m from the animals. We used also opaque shades to protect the eyes from direct exposure to room light. All the following steps were carried out under these conditions. The flasks containing retinas were always kept in light-tight containers between steps of the procedure.

The eyes were excised inmediately after decapitation, washed briefly in cold 0.9% NaCl and the retinas dissected out, washed and fixed for 1 hr in 6% cold glutaraldehyde or 5% formaldehyde in 0.9% NaCl (pH 7.2).

Determination of lipids. The retinas of 20 toads were pooled during fixation, homogenized with 5 ml of 0.9% NaCl in a Potter-Elvejehm homogenizer and placed at the bottom of a discontinuous sucrose gradient (0-35-60%) in saline. They were centrifuged at 18.000 g for 90 min at 4°. The layer between 35% and 60% sucrose containing mostly ROS was washed in saline and centrifuged three times. After resuspension of the pellet they were extracted for 1 hr in 1% Triton-X-100 (Hartmann, Ledder & Co. Philadelphia) in 0.9% NaCl except for an aliquot that was left out in 0.9% NaCl and served as a control. Triton extracted and control specimens were centrifuged and washed three times in saline solution. Phospholipid phosphorus was determined after thin layer chromatography according to Rouser *et al.* (1969). Results are referred to protein determined by the method of Lowry *et al.* (1951).

Rhodopsin determination in detergent extracts. The light absorption at 495 nm was determined spectrophotometrically in an aliquot of the Triton extract before and after 20 min bleaching under white light. An aliquot of the buffer in which control retinas were kept after fixation was also measured.

Glutaraldehyde Purification. Glutaraldehyde (J.T. Baker Chemical Co., Philipsburg,) was purified by vacuum distillation according to Fahimi *et al.* (1965).

Formaldehyde. Fixative solutions were prepared from paraformaldehyde according to Karnovsky (1965).

RESULTS

Proteins

The small difference in protein content between extracted and control ROS after glutaraldehyde fixation (Table I) suggests that this aldehyde is a good cross-linking agent for proteins. No rhodopsin was detected spectrophotometrically in the Triton extract.

In formaldehyde-fixed retinas the amount of proteins left in the ROS fraction after detergent extraction was below the sensitivity of our technique. In these retinas the amount of rhodopsin in the detergent extract was measured in two experimental series. In one case the differential absorbance was 0.022 and in the other, 0.042. No differential absorbance was detected in the controls. Rhodopsin extracted in the detergent could be estimated using a molar extinction coefficient of 43,000 (Borggreven *et al.*, 1971) and after correction for sample volume we obtained values

of 2.55 and 5.35 nmoles of rhodopsin extracted per retina or about 1 fmol of rhodopsin per ROS. This value is about the same order of magnitude as the value given by Daemen (1973) for frog ROS (3.55 fmoles).

TABLE I

Effect of detergent treatment on glutaraldehyde fixed specimens*

	Fixed	Fixed and extracted
Protein (mg)	1.12	1.10
Lipid phosphorus (mg)	0.029	0.0044
μg Lipid phosphorus/mg protein	25.6	4.0

* Results correspond to the average of duplicate determinations in two experimental series of 20 pooled retinas each. Values were corrected for sample volume.

Lipids

Lipid extraction by detergent is extensive (Table I). About 85% of lipids of control ROS were absent in treated ROS. Since control ROS were kept in NaCl solution while experimental ROS were extracted in Triton, the difference in lipid content can be ascribed to the detergent. The lipids present in control ROS may be linked with membrane proteins through hydrophobic bonds which may be broken by the detergent.

Table II shows that major phospholipids species of ROS decrease in detergent-treated ROS except for phosphatidyl serine. If absolute values (not shown) are considered, the differences are even more marked for all lipid species except phosphatidyl serine which can be considered unchanged. The amount of phosphatidic acid increased both in relative and absolute terms. The increase of the substances found at the origin of thin-layer chromatograms can be explained by oxidation which is enhanced by the detergent.

TABLE II

Relative proportion of phospholipids species in glutaraldehyde-fixed ROS lipid fractions*

	Fixed	Fixed and extracted
	% of phospholipid phosphorus	
Origin	6.4	10.2
Phosphatidyl serine	4.9	35.3
Phosphatidyl inositol	0.7	0.4
Sphingomyelin	0.9	0.2
Phosphatidyl choline	26.2	14.5
Phosphatidyl ethanolamine	60.2	31.5
Other (including phosphatidic acid)	0.7	7.9
Total	*100.0%*	*100.0%*

* Results correspond to the average of duplicate determinations in two experimental series of 20 pooled retinas each.

DISCUSSION

Proteins

Cross-linking of ROS proteins by glutaraldehyde seems to be quite complete, thus confirming earlier results (Brown, 1972; Jost *et al.*, 1973). The resulting supramolecular network could be demonstrated by electron microscopy (not shown). Freeze-etching (but not conventional transmission electron microscopy) show that extracted ROS have a supramolecular structure and that this structure follows closely the original membrane distribution (Doggenweiler and Elías, manuscript in preparation).

Glutaraldehyde and formaldehyde have the same reactive groups and will probably react with the same protein groups. However, glutaraldehyde prevents protein displacement and extraction while formaldehyde does not. The difference may correspond to the ability of glutaraldehyde to form a supramolecular network while formaldehyde is unable to do so.

Thus cell and tissue fixation comprises two different processes: alterations in the structure of individual molecules and interactions between independent molecules.

Lipids

Since a certain amount of losses occurs during chromatography of extracted ROS it is safe to limit the discussion only to the most abundant phospholipid species in the ROS fraction, namely phosphatidyl choline, phosphatidyl ethanolamine and phosphatidyl serine.

Two explanations may be offered for the difference in the degree of extraction of

the major phospholipid species by detergent. The first is based in the reaction between amino groups of phosphatidyl ethanolamine and phosphatidyl serine with the aldehyde. These phospholipids will be retained in the specimen as part of the supramolecular network proposed above, while phosphatidyl choline is extracted. Nevertheless, while one half of phosphatidyl ethanolamine or phosphatidyl serine is extracted, no extraction of the latter is detected. Our results disagree with those of Roozemond (1969) but the discrepancy can be explained by differences in materials or methods.

The unique property of phosphatidyl serine is difficult to explain in terms of a reaction of its amine group with the aldehyde (Roozemond, 1969). If this were the case, phosphatidyl ethanolamine should display the same behaviour since it has a similar group. This is more remarkable, as reactions involving aldehydes are known to occur with amino groups of proteins (see above). A differential reactivity of phosphatidyl ethanolamine and phosphatidyl serine to glutaraldehyde cannot be explained as resulting of the lack of a carbonyl group in the vicinity of the amino group in phosphatidyl ethanolamine. This is supported by the strong evidence that ϵ-amino groups of lysine react with glutaraldehyde (Hopwood, 1973).

A second explanation for the retention of phosphatidyl serine requires the presence of strong bonds between it and proteins. It has been shown that some phospholipids are retained after phospholipase and hexane extractions of fresh cattle retinal rods. Furthermore, among the retained phospholipids, phosphatidyl serine is the most abundant (Borggreven *et al.*, 1971). It is then possible that its lack of extraction of the glutaraldehyde-fixed specimens does not reflect a property of the fixative but a situation preexisting in the fresh tissue. According to our results there are 31 molecules of phospholipid per protein molecule in control (fixed) specimens but only about 5 remain after extraction. Since rhodopsin has a diameter of 40 Å and each hydrocarbon chain has a radius of 2.25 Å (Blasie, 1972) more than 15 molecules of phospholipids are needed to make a one-row ring around the protein. Two such rings, each corresponding to a leaflet of the unit membrane, may remain after fixation. Their extraction by detergent suggests that they are held by hydrophobic bonds.

Evidence for such relationship between phospholipids and membrane proteins based on their vicinity has been presented by several authors (see review by Vanderkooi, 1974). Recently, two different lipid-protein associations have been demonstrated in mitochondria using Triton-X-100 (Robinson *et al.*, 1980). Rhodopsin is known to be affected by the phospholipids that surround it (Shichi, 1971; Zorn & Futterman, 1971; Williams *et al.*, 1974), while Pontus and Delmelle and more recently Favre *et al.* (1979) reported a non-fluid fraction of ROS phospholipids using electron spin resonance.

Differences between lipid contents reported for fresh ROS and our values for fixed ROS are difficult to interpret because our control (fixed) specimens stay an extended time in salt solutions after fixation and are subjected to repetitive centrifugations. This may explain the relatively low amount of phospholipids found in fixed tissue compared with those of fresh frog ROS (Daemen, 1973). Lipids retained in control ROS could correspond to those lipids that are bound by exceptionally strong hydrophobic bonds. They are just enough to form two rings around proteins. Phospholipids that are in fluid state will be mostly solubilized by repetitive washings.

The amount of lipids in the formaldehyde fixed, Triton extracted ROS fraction was below the threshold of sensitivity of our technique. This again supports the lack of reactivity of aldehydes with phospholipids.

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