Effect of light on extraction of lipid from retinal rods

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ABSTRACT Chloroform–methanol 2:1 removes a significantly greater quantity of lipid from bleached bovine retinal rods than from a dark-adapted counterpart. The extracts contain phosphatidyl serine, phosphatidyl choline, phosphatidyl ethanolamine, sphingomyelin, and an unknown substance which, it is proposed, may be a combination of phospholipid and retinaldehyde. The difference between extracts of light- and dark-adapted rods is quantitative rather than qualitative.

The data tend to confirm a model of rhodopsin suggested by Kropf and Hubbard in which isomerization of the retinaldehyde chromophore causes its displacement and opens a path to the interior of the molecule.

KEY WORDS bovine · retinal rods · light- and dark-adapted · extraction · chloroform–methanol · phospholipid · retinaldehyde · rhodopsin

PHOSPHOLIPIDS HAVE BEEN SHOWN (1) to contribute about 30% to the dry weight of bovine retinal rods and can be inferred to be an important constituent of the retina. The role of this lipid in the retinal rod elements is not clear and even its exact location is in doubt. However, some interesting facts about retinal lipid are known which bear further investigation with modern techniques.

Bovine retinal rods, exhaustively extracted with petroleum ether (Soxhlet) in the dark, release additional phospholipid upon extraction with additional solvents following exposure to light (2). More recently, the same effect was detected when rods that had been extracted with diethyl ether at −70°C in the dark (3) were shown to yield more phospholipid after illumination than could be extracted from a dark-adapted control preparation.

Abbreviations: DNP, dinitrophenyl; TLC, thin-layer chromatography.

The mechanism that prevents extraction of total lipid from dark-adapted retinal rods until after they are exposed to light is effective in solvent systems which do not "bleach" rhodopsin (i.e., after treatment with these solvents, rhodopsin does not show the spectral shift that accompanies its destruction by heat or light). The extraction of lipid by a solvent known to destroy rhodopsin has not been adequately investigated.

This work describes the effects of chloroform–methanol 2:1 on light- and dark-adapted bovine retinal rods.

EXPERIMENTAL PROCEDURE

Bovine retinae were either obtained from the Hormel Institute, Austin, Minn., dark-adapted and frozen, or dissected from eyes of freshly killed cattle from a local abattoir. Retinae from the second source were also frozen and stored before use. In no case were any of the retinae used after storage for more than 6 months, although there was no detectable difference between extracts from 6-month old retinae and those only 24 hr old.

All buffer and sucrose solutions were prepared the day of use and their pH values checked. Deoxygenation of these solutions was deemed inessential.

Rods were prepared for extraction in a 5°C cold-room in dim red light (ruby darkroom lamp). 50–100 frozen retinae were ground to a paste in a large mortar and slurried by the addition of 0.66 M phosphate buffer (Na_2HPO_4–KH_2PO_4) at pH 6.8–7.0, 2 ml of buffer per retina. Centrifugation at 200 *g* for 5 min eliminated blood cells, nuclei, retinal debris, and other gross contaminants. The supernatant fraction was then centrifuged at 5,000 *g* for 10 min as a means of concentrating the rods, mitochondria, and microsomes.

Rods were isolated by suspension of the solids in 40% sucrose in phosphate buffer and recentrifugation at 10,000 *g* for 15 min to remove any residual blood cells

and heavy contaminants. The supernate was then diluted with phosphate buffer 4:1; centrifugation at 10,000 *g* for 15 min precipitated the rods as a button, leaving the microsomes and mitochondria in suspension. The button of rods was thoroughly broken up and washed twice with distilled water (centrifuged at 5,000 *g* for 10 min) and once with phosphate buffer, unless it was to be immediately treated with chloroform–methanol 2:1 in which case the third wash was with distilled water.

Examination of the centrifuged solutions under the microscope showed the preparation to be free from extraneous material and to consist almost entirely of whole or broken rod outer segments.

The rods so prepared were divided into two, roughly equal parts, one of which was kept dark-adapted. The other was placed in an ice bath and stirred while being illuminated for 15 min by a 75 watt incandescent bulb 60 cm away. Subsequent extractions of all preparations were made in the dark.

Rods in experiments 1 and 2 (Table 1) were dried by lyophilization, then extracted for 12 hr with petroleum ether (bp 40–60°C) in the dark cold-room. After removal of the petroleum ether, chloroform–methanol 2:1 (20 ml/ml of packed rods) was added and extraction continued for 4 hr. A second extraction for 1 hr with fresh solvent seemed sufficient, since additional solvent or increased time produced negligible amounts of lipid.

Material for experiments 3 and 4 (Table 2) were extracted with chloroform–methanol 2:1 without prior lyophilization or petroleum ether treatment. The preparation used in experiment 5 was hydrolyzed by the method of Getz and Bartley (4) with KOH–methanol in an 80°C oven for 12 hr. After cooling and neutralization with 50% H_2SO_4, lipids were extracted with three changes of petroleum ether, and the extract was dried over anhydrous Na_2SO_4.

All extractions with chloroform–methanol were done under N_2; the solvents had been deoxygenated by passing N_2 through them for several hours. Extracts were evaporated under N_2 in tared containers, weighed, diluted with chloroform–methanol to about 100 μg/ml, and stored under N_2 in a desiccator at −20°C.

Thin-layer chromatography (TLC) served to partially identify the components of lipid extracts. Spots of 1–10 μg of lipid in chloroform–methanol 2:1 were placed upon 250 μ Silica Gel G plates (Analtech, Wilmington, Del.) under a stream of dry nitrogen, and the chromatograms were developed in unlined glass jars with chloroform–methanol–28% ammonia 70:30:4 to the 15 cm level. A synthetic mixture of phosphatidyl ethanolamine, phosphatidyl choline, phosphatidyl serine, and sphingo-

TABLE 1 DRY WEIGHT OF EXTRACT EXPRESSED AS PERCENT OF THE DRY WEIGHT OF RETINAL RODS

Expt. No.	Chloroform–Methanol: Dark-Adapted Rods	Chloroform–Methanol: Light-Adapted Rods	Petroleum Ether after Hydrolysis: Light-Adapted Rods	Total*
1	28.90	32.30	not done	
2	27.37	36.93	6.47	42.40
3	29.13	34.90	0.70	35.60
4	24.46	31.40	not done	
5				38.80†
Mean	27.46	33.88‡		38.93§

* Values of chloroform–methanol extracts from light-adapted rods combined with those from hydrolyzed residues.

† These rods were hydrolyzed without prior extraction with chloroform–methanol and lipids were taken up with petroleum ether.

‡ Fisher's "*t*" tables show $P < 0.02$ for difference between averages of dark- and light-adapted extracts.

§ The "*t*" test for unpaired variables gives a value $P < 0.07$ for the difference between light-adapted and hydrolyzed extracts.

TABLE 2 IDENTIFICATION OF COMPONENTS OF CHLOROFORM–METHANOL EXTRACTS OF BOVINE RETINAL RODS BY PAPER CHROMATOGRAPHY

	R_f	Ninhydrin*	Choline*	2,4-DNP Hydrazine*	P*	Tentative Identification
S (Sphingomyelin)†	0.38	−	+	−	+	S
PS (Phosphatidyl serine)†	0.46	+	−	−	+	PS
PC (Phosphatidyl choline)†	0.45	−	+	−	+	PC
PE (Phosphatidyl ethanolamine)†	0.50	+	−	−	+	PE
Extract of dark-adapted rods	0.29	+	−	−	+	Lyso PE
	0.32	−	+	−	+	S
	0.38	−	+	−	+	PC
	0.46	+	−	−	+	PS
	0.50	+	−	−	+	PE
	0.67	−	−	+	+	Retinaldehyde-lipid
Extract of light-adapted rods	0.31	+	−	−	+	Lyso PE
	0.34	−	+	−	+	S
	0.40	−	+	−	+	PC
	0.47	+	−	−	+	PS
	0.50	+	−	−	+	PE
	0.67	−	−	+	+	Retinaldehyde-lipid

* A description of these testing procedures appears in the text.

† All standards are from Applied Science Laboratories Inc., State College, Pa. and contain dipalmitate as the fatty acid moiety, except PE, which is bovine natural extract.

myelin was placed on either side of the unknown for identification purposes.

After they had dried, chromatograms were sprayed with 2% ninhydrin in *n*-butanol–acetic acid 95:5 and the plate was again allowed to dry for 1 hr. Heating the dry plates in a 100°C oven for 10 min produced pink spots on a white background for amine-containing phospholipid. Additional plates were sprayed with 50% sulfuric acid and heated for 10 min in a 250°C oven to the point of charring, as a confirmation of spot positions.

Results obtained with the paper chromatographic techniques described by Marinetti (5) gave additional information, as well as confirmation of the TLC results. Lipids applied as spots to silicic acid-impregnated paper were separated in unlined cylindrical jars by development with diisobutyl ketone–acetic acid–water 40:20:3; complete separation required about 3.5 hr. Dried chromatograms dipped into Rhodamine 6G were rinsed with distilled water and observed, both wet and dry, under UV light for fluorescent spots representing lipid. Additional chromatograms were sprayed with ninhydrin (2% in acetone–lutidine 9:1) for amine-containing lipids, or immersed in 2,4-dinitrophenyl (2,4-DNP) hydrazine (150 mg/ml in 3 N HCl) (5), rinsed, dried, and viewed under UV for detection of plasmalogens. Other chromatograms were run which showed the location of phosphorus-containing lipids when sprayed with Hanes–Isherwood (6) solution and developed for 2 hr under strong UV light (blue spots appeared with phospholipid standards). The test for choline (7) consisted of immersing washed chromatograms in 1% phosphomolybdic acid (aqueous) followed by three washes of distilled water and addition of 1% stannous chloride (aqueous); this produced blue spots for phosphatidyl choline and sphingomyelin controls.

RESULTS

Chloroform–methanol 2:1 extracted more lipid from the half of a bovine retinal rod preparation that had been illuminated than from the other half that remained dark-adapted. Table 1 shows mean values for four such experiments which, when examined by the "*t*" test, are significantly different ($P < 0.02$). However, in neither case is all the lipid removed, as shown by the fact (Table 1) that petroleum ether extracts additional lipid from the hydrolysate of the residue.

TLC gave spots corresponding to phosphatidyl ethanolamine, phosphatidyl serine, phosphatidyl choline, and sphingomyelin. Two other spots, one of which traveled near the solvent front, were unidentified although the one nearest the front was assumed to be retinaldehyde since it was pale yellow.

Paper chromatography (Table 2) confirmed the tentative identifications by positive tests for choline, amines, and phosphorus at appropriate locations. A positive 2,4-DNP hydrazine spot, R_f 0.67, indicated the presence of an aldehyde group, which strengthened the suspicion of retinaldehyde; unexpectedly, this spot also showed a positive phosphorus test. The unidentified lower spot (R_f 0.29–0.31) which was ninhydrin positive and DNP hydrazine negative, may be lysophosphatidyl ethanolamine, which often accompanies phosphatidyl ethanolamine in tissue extracts.

DISCUSSION

The data indicate that the interior of the rhodopsin molecule is available to chloroform–methanol 2:1 only after bleaching. Although other lipoprotein complexes have been shown to resist lipid extraction (8), none has been shown to give up more lipid after exposure to visible light.

The results support the model proposed by Kropf and Hubbard (9). Rhodopsin is pictured as having its protein moiety folded so that the 11-*cis*-retinaldehyde chromophore fits as a plug which prevents access to the interior of the lipoprotein complex. Light isomerizes the retinaldehyde and destroys the fit; the remaining structure is unstable and is readily hydrolyzed by thermal processes. Such a reaction would presumably make the interior of the molecule accessible to solvents. Extensive thermodynamic data recently published by Ostroy, Erhardt, and Abrahamson (10) were interpreted to indicate that after exposure to light, rhodopsin in solution unfolds, refolds, and finally unfolds again. Either of the two schemes, which are not mutually exclusive, would fit the data found in the present work. Ostroy et al. also consider that hydrogen bonds break and re-form in the folding process. This suggests that further experiments concerned with the extraction of lipid in the presence and absence of water and at various pH values are in order.

The lipids extracted from light- and dark-adapted rods, though different in amount, are not very different in kind: only slight differences in the R_f values are evident (Table 2). Individual phospholipids have not yet been examined, but determination of total fatty acids (as methyl esters) by gas–liquid chromatography (2) shows differences in composition between extracts of light- and dark-adapted retinal rods. The assignment of the differences to specific phospholipids is being pursued in this laboratory and will be the subject of a later report.

It is of interest to consider the spot on paper chromatograms (R_f 0.67) that contains P as well as giving a positive test for aldehyde. Since chloroform is known to remove retinaldehyde from rhodopsin, this compound must have been present in the extract and would account

for the yellow color of the spot, which also seems to contain phospholipid (Hanes-Isherwood and Rhodamine 6G stains). Wald has shown (11) that retinaldehyde extracted from bleached rhodopsin with petroleum ether still exhibited pH indicator properties. Krinsky (3) thought that perhaps the Schiff-base linkage which occurs in rhodopsin between retinaldehyde and some unknown amino acid group could involve an amino phosphol pid such as phosphatidyl serine or phosphatidyl ethanolamine. Possibly, then, the unknown spot represents a complex between retinaldehyde and one of these phospholipids.

Attempts to recover the suspected complex from thin-layer plates were only partially successful. The eluate showed a broad absorption band (300–400 mμ) but no peak; this neither supports nor eliminates the presence of retinaldehyde. No further attempt at identification was made. Higher concentrations of the complex might be obtained in extracts of rhodopsin rather than of rods.

Obviously more problems have been uncovered than solved, and further efforts need to be made using rhodopsin. This is not easy since the only known method of obtaining rhodopsin depends on the use of detergents such as digitonin, which make extraction with organic solvents difficult. However, I have made some progress in this by precipitation of digitonin as the ergosterol complex, which seems to leave much of the rhodopsin molecule intact. This work will shortly be submitted for publication.

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