Phospholipids of Bovine Rod Outer Segments*

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ABSTRACT: The Schiff base all-*trans*-retinal and dipalmitoylphosphatidylethanolamine (*N*-RPE) was synthesized and its spectral properties studied. Molar extinction coefficients were determined to be 31,300 ± 2100 for the protonated compound (λ_{max} 455) and 38,800 ± 2800 for the free Schiff base (λ_{max} 365). The *N*-RPE concentration of bovine rod outer segments (ROS) was determined by two procedures, one, a spectrophotometric technique employing the molar extinction coefficient and the other a thin-layer chromatography, phosphorus assay technique. The molar concentration of *N*-RPE was less than that of rhodopsin, and it was concluded that the Schiff base does not serve as the chromophore of rod visual pigment. Phosphatidylethanolamine (PE), phosphatidylcholine (PC), and phosphatidylserine (PS) are the major phospholipids of bovine ROS, making up 39, 40, and 13%, respectively, of the lipid phosphorus. The major fatty acids in PS and PE are 18:0 and 22:6, while PC is made up predominantly of 16:0 and 22:6. All of the phospholipid classes examined contain high levels of polyunsaturated fatty acids; phosphatidylcholine has 30%, phosphatidylserine has 60%, and phosphatidylethanolamine 52%.

In 1958, Krinsky, discussing the lipoprotein nature of rhodopsin, stated: "Possibly in rhodopsin itself retinene is combined with the amino group of a lipid rather than of an amino acid." Nine years later Adams (1967) tentatively identified the Schiff base of retinal and phosphatidylethanolamine in bovine ROS.[1] Poincelot *et al.* (1969) also identified this compound in bovine ROS and, quoting the earlier work of Krinsky, claimed that *N*-RPE serves as the chromophore of rod visual pigment. They recently published a series of papers supporting this contention (Poincelot *et al.*, 1970; Kimbel *et al.*, 1970; Poincelot and Abrahamson, 1970a). The presence of *N*-RPE in ROS was confirmed by Daeman and Bonting (1969), and Akhtar and Hirtenstein (1970) recently showed that labeled retinal was incorporated *in vitro* into *N*-RPE.

However, it has been claimed by three laboratories that phosphatidlyethanolamine does not participate in the binding of retinal to opsin. Heller (1968) found no phospholipid in purified rhodopsin. Hall and Bacharach (1970) injected ^{32}P into frogs and found that the phospholipids from the ROS were highly labeled, whereas purified visual pigment was not. In a preliminary communication, Anderson (1970) stated that the molar concentration of *N*-RPE was less than that of rhodopsin when both were expressed as micromoles per milligram of dry ROS.

In the present report, we describe the isolation and quantification of *N*-RPE from bovine ROS. In addition, the phospholipid class composition and the fatty acid distribution in the major phospholipids are given.

Materials and Methods

Dark-adapted bovine retinas were obtained from the Hormel Co., Austin, Minn. Solvents were purchased from Mallinckrodt. Silica gel G and HR were from Brinkmann Instruments. Dipalmitoylphosphatidylethanolamine was purchased from Mann Research Laboratories and was purified prior to use by column chromatography on silicic acid. All-*trans*-retinal was purchased from Eastman. Acetic acid and hexane were distilled before use in the thin-layer solvent systems. All other chemicals were reagent grade and were used without further purification.

Preparation of ROS. Approximately 25 dark-adapted bovine retinas were gently homogenized by hand in an all glass homogenizer in 40 ml of 0.067 M phosphate buffer (pH 7.0). The milky solution was spun at 15,000*g* at 4° for 30 min. The resulting pellet was suspended in a 37% sucrose solution (in phosphate buffer) and spun for 45 min at 15,000*g*. A float formed which was removed with a Pasteur pipet and diluted approximately threefold with the phosphate buffer. The crude ROS suspension was spun at 15,000*g* for 20 min, the supernatant was discarded, and the pellet was suspended again in sucrose buffer. After spinning for 1 hr at 15,000*g*, the float was removed and diluted with phosphate buffer, and spun for 20 min at 15,000*g*. The resulting pellet was washed with phosphate buffer followed by deionized water. After the last sedimentation, the ROS pellet was suspended in deionized water, transferred to a round-bottom flask, and lyophilized. The rod outer segments were stored at −20° in a light-tight jar in a desiccated container. All operations were performed under dim red light. This isolation procedure is essentially that of Heller (1968).

Two preparations of ROS were used in this study and are designated ROS-1 and ROS-2.

Determination of Rhodopsin Content of ROS. A known weight of dry ROS was mixed with a known volume of 2% aqueous digitonin and left to stand at 4° in the dark. After 2–3 hr (with intermittent shaking), solubilization of the ROS was obtained, and any digitonin that precipitated in

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[1] Abbreviations used are: *N*-RPE, Schiff base of retinal and phosphatidylethanolamine; ROS, rod outer segments.

the cold was removed by centrifugation. The absorbance spectrum of rhodopsin was determined from 240 to 650 nm, after which the solution was exposed to room light for 30 min and the scan was repeated. Difference between the absorbance at 500 nm was taken as the absorbance due to rhodopsin. Rhodopsin concentration was then determined from Beer's law: $A = \epsilon \cdot c \cdot l$, where A = absorbance units from the difference spectrum, ϵ = molar extinction of rhodopsin, c = molar concentration of rhodopsin, and l = light path (1 cm). This equation was solved for c, and the millimicromoles of rhodopsin per milligram of dry ROS was calculated.

Synthesis of N-RPE and Determination of Its Molar Extinction Coefficient. *N*-RPE was synthesized by allowing phosphatidylethanolamine (2.5 μM) to stand overnight at room temperature with all-*trans*-retinal (10 μM) in 1.0 ml of chloroform–methanol (2:1, v/v, 95%–concentrated NH_4OH, 5%. (Better yields have recently been obtained by substituting triethylamine for the ammonium hydroxide and using 30-min reaction times.) The pure compound was isolated by preparative thin-layer chromatography on 0.25-mm layers of silica gel G using chloroform–methanol–acetic acid (80:10:12.5, v/v) as developing solvent. *N*-RPE was eluted from the plate with chloroform–methanol (1:2, v/v) and scanned immediately. Reaction products were applied to only one-half of the plate; the other half was left blank. An equivalent amount of silicic acid from the *N*-RPE region of the blank side of the plate was washed with chloroform–methanol (1:2, v/v), and served as the blank for the absorption spectra determined for the synthetic material. The Schiff base eluted from the plate in the protonated form; the nonprotonated form was made by adding a known volume of methanolic KOH to each cuvet.

After scanning, a known volume of the *N*-RPE solution was removed for phosphorus determination, and the micromoles of *N*-RPE per milliliter was calculated. The molar extinction coefficient of *N*-RPE was then calculated from Beer's law.

Extraction of Lipids from ROS. Lipids were extracted from known weights of dry ROS with chloroform–methanol (2:1, v/v), according to the procedure of Folch *et al.* (1957). After water partitioning, the chloroform layer was made to a known volume and aliquots were removed for the determination of lipid–phosphorus. The remaining material was used for phospholipid class and fatty acid composition determinations. For the extraction of *N*-RPE, the chloroform–methanol solvent was made 0.00032 N with anhydrous HCl gas and the entire procedure was carried out in the dark. Details of this extraction procedure are given in the section describing the quantification of *N*-RPE.

ROS phospholipids were separated by two-dimensional thin-layer chromatography on 0.5-mm layers of silica gel HR. The following solvent systems were used: $CHCl_3$–MeOH–HOAc–0.9% saline (v/v), direction 1, 100:50:16:8; direction 2, 100:15:16:4. Qualitative identification of retina phospholipids has been outlined in a previous publication (Anderson *et al.*, 1970a). Pictures of the separations in these solvent systems have also been given (Anderson *et al.*, 1969, 1970b).

After development, the thin-layer plates were sprayed with 55% sulfuric acid containing 0.6% potassium dichromate, and charred at 190°. The darkened areas were removed and lipid–phosphorus determined by the procedure of Rouser *et al.* (1966).

Gas–Liquid Chromatography of the Methyl Esters of ROS Phospholipids. Phosphatidylserine, phosphatidylethanolamine, and phosphatidylcholine were separated by two-dimensional thin-layer chromatography as described above. The spots were detected under ultraviolet light after a heavy spray of 0.001% aqueous rhodamin 6G and transferred to a small tube. Three milliliters of 15% BF_3 in methanol was added and methyl esters were prepared by heating the phospholipid at 80° for 10 min (Morrison and Smith, 1964).

Gas chromatography was carried out on a Barber-Colman Series 5000 gas chromatograph. A 6 ft × 4 mm i.d. glass U column packed with 15% EGSS-X on 100–120 mesh Gas Chrom P was used for all analyses. Injector and column temperatures were maintained at 250 and 185°, respectively. Qualitative identification of the various peaks was made on the following bases: (1) plots of log retention time *vs.* carbon number, (2) relative retention times given in the literature (Ackman and Burgher, 1965), (3) cochromatography with esters of known structure, and (4) peak areas before and after catalytic hydrogenation.

Quantification of N-RPE. *N*-RPE was determined by two procedures. In the first, the acidic solvent extract of dry ROS was immediately hydrogenated over platinum oxide, converting the unstable Schiff base into a stable secondary amine. After a water wash to remove the acid, the fully saturated phospholipid was separated from other phospholipids by one-dimensional thin-layer chromatography on 0.25-mm layers of silica gel G. The developing solvent was $CHCl_3$–MeOH–HOAc (80:5:12.5, v/v). The lipid–phosphorus content of the saturated *N*-RPE was compared to the total lipid–phosphorus applied to the plate, and the millimicromoles of *N*-RPE per milligram of dry ROS was calculated.

In the second procedure, a known weight of dry ROS was homogenized by hand in a small glass homogenizer in a known volume of the acidic extracting solvent. The homogenization tube was placed in a centrifuge and the insoluble protein sedimented. The clear supernatant was transferred to a cuvet and its absorbance spectrum was determined immediately. The molar concentration of *N*-RPE was then calculated from Beer's law.

Results and Discussion

Phospholipid Content of ROS. ROS-1 and ROS-2 contains 0.377 and 0.315 μmole of phospholipid per mg of dry tissue, respectively. This corresponds to 30.2 and 25.3%, respectively, assuming an average molecular weight of 800 for ROS phospholipids. Published values for the percentage of phospholipids in bovine ROS vary from 30 (Collins *et al.*, 1952) to 59.8% (Fleischer and McConnell, 1966). The latter value is nearly double that most commonly reported (30–38%) (Adams, 1967; Poincelot and Zull, 1969; Sjöstrand, 1959). Our values are slightly lower than those previously given, and probably reflect differences in the manner in which the ROS were prepared. Some procedures may result in the loss of more protein than others and thus vary the relative lipid content.

Phospholipid Composition of ROS. Phosphatidylcholine and phosphatidylethanolamine are the major phospholipids

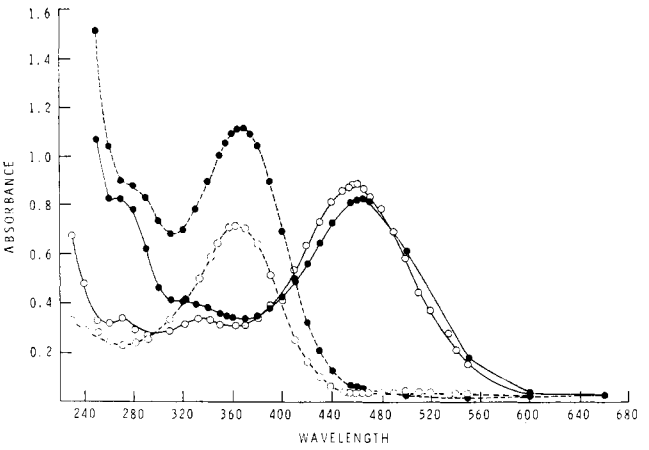

FIGURE 1: Absorption spectra of *N*-RPE. (O—O) Synthetic and (●—●) ROS extract. Dashed lines, nonprotonated form; solid lines, protonated form.

in the ROS and account for 80% of the lipid–phosphorus (Table I). Phosphatidylserine makes up 13% of the ROS lipids; phosphatidylinositol, sphingomyelin, and solvent front lipids make up the rest. Lipids in the solvent front are probably phosphatidic acid, phosphatidylglycerol, diphosphatidylglycerol, and *N*-RPE. These combined lipids contribute less than 3% of the total lipid-phosphorus. The values in parentheses in Table I are the mole percentages of *N*-RPE, determined on the hydrogenated lipid extract.

The data given in Table I agree with the published values of Daeman and Bonting (as quoted by Bonting, 1969), but differ slightly from those given by Poincelot and Zull (1969) and drastically from those given by Adams (1969). Poincelot and Zull quantified the base portion of the phospholipid molecules, a procedure that is less accurate than phosphorus assay. Adams found large quantities of lysophosphoglycerides, which were not observed in the other studies.

TABLE I: Phospholipid Composition of Bovine Rod Outer Segments.

Lipid	Mole % Phosphorus	
	ROS-1	ROS-2
OR[a]	0	0.3 ± 0.3
SPH	2.9 ± 1.0	4.2 ± 1.4
PC	41.2 ± 0.8	39.1 ± 0.4
PI	2.5 ± 0.4	2.1 ± 0.3
PS	12.9 ± 0.8	13.7 ± 0.5
PE	39.0 ± 1.2	38.1 ± 2.0
SF	1.3 ± 0.5	2.5 ± 0.3
	(0.66 ± 0.09)[b]	(0.47 ± 0.07)
% recovery	99.4 ± 2.1	99.1 ± 1.6

[a] Abbreviations used are: OR, origin; SPH, sphinogmyelin; PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethanolamine; SF, solvent front. [b] Values in parentheses are the percentages of *N*-RPE, determined as described in the text.

TABLE II: Fatty Acid Composition of the Phospholipids of Bovine Rod Outer Segments.[a]

Acid	PS	PC	PE
14:0	3.0 ± 0.8	1.9 ± 0.2	1.3 ± 0.3
14:1 + 15:0	2.0 ± 0.4	1.6 ± 0.2	T[b]
16:0 DMA[c]			1.5 ± 0.2
16:0	3.3 ± 1.0	32.3 ± 1.4	10.3 ± 0.5
16:1	2.3 ± 0.3	2.3 ± 0.2	0.7 ± 0.4
17:0		1.4 ± 0.1	T
17:1 + 18:0 DMA	0.9 ± 0.7	0.6 ± 0.1	3.9 ± 1.1
18:0	19.2 ± 4.1	17.7 ± 0.5	21.9 ± 1.4
18:1	5.4 ± 0.5	10.7 ± 0.2	6.1 ± 0.6
18:2	1.9 ± 1.6	1.8 ± 0.2	2.2 ± 0.2
20:0	1.3 ± 1.3	0.8 ± 0.3	0.9 ± 0.2
20:1	1.0 ± 1.0	0.4 ± 0.4	1.1 ± 0.2
20:2	0.5 ± 0.5	T	1.1 ± 0.3
20:3	1.3 ± 1.3	0.6 ± 0.5	1.5 ± 0.2
20:4	3.0 ± 1.4	4.3 ± 0.2	5.0 ± 0.3
20:5 + 22:2	1.7 ± 1.1		T
22:4	6.8 ± 2.2	1.1 ± 0.2	3.4 ± 1.5
22:5	5.6 ± 1.8	2.0 ± 0.4	5.7 ± 1.8
22:6	31.8 ± 4.0	20.6 ± 1.0	31.7 ± 1.5
24:X[d]	10.0 ± 0.9	T	1.4 ± 0.9

[a] Average plus and/or minus standard deviation of four thin-layer chromatography separations of the same sample. [b] T means trace (less than 0.1%). [c] Dimethyl acetals resulting from the methanolysis of plasmalogens. [d] Identified as a C-24 ester by hydrogenation. Number of double bonds unknown.

However, it is probably that these compounds arose from autolysis during his lengthy extraction procedures.

Fatty Acid Composition of ROS Phospholipids. Table II contains fatty acid compositions of phosphatidylserine, phosphatidylcholine, and phosphatidylethanolamine. The most abundant fatty acid in phosphatidylserine and phosphatidylethanolamine is 22:6. Both lipid classes contain a 24-carbon acid; the chain length was established by hydrogenation, although the number of double bonds was not determined. Phosphatidylserine and phosphatidylethanolamine have only small amounts of 16:0 (3.3 and 10.3%, respectively) compared to 32.3% for phosphatidylcholine. The latter phospholipid has lower amounts of polyunsaturates (30%) than phosphatidylserine and phosphatidylethanolamine (60 and 52%, respectively). These compositions are similar to those we reported earlier for bovine whole retina (Anderson *et al.*, 1970a) and agree for the most part with those recently published by Poincelot and Abrahamson (1970b).

Molar Extinction Coefficient of N-RPE. The open circles in Figure 1 are the absorption spectrum of the protonated and nonprotonated synthetic *N*-RPE. The protonated form has maxima at 455, 330, and 270 nm, somewhat different from the values of 445, 397, and 235 nm reported by Poincelot *et al.* (1969, 1970a). The nonprotonated Schiff base absorbs

TABLE III: Molar Extinction Coefficient of *N*-RPE.

Sample	ε (Acid)	ε (Base)
A	30,200	39,100
B	29,900	35,800
C	30,750	38,350
D	34,500	42,200
Av ± std dev	31,300 ± 2100	38,800 ± 2800

maximally at 365 nm. We have observed a shift in the absorption maximum of all-*trans*-retinal in acidic methanol from 380 to 330 nm. This shift could not be reversed by the addition of base. No shift was observed in acidic chloroform, leading us to tentatively identify the 330-nm peaks in Figure 1 as the dimethyl acetal of retinal.

Absorbances used in the calculations of molar extinction coefficients were taken at 455 nm for the protonated form and 365 nm for the nonprotonated form. Four samples of synthetic *N*-RPE were used to determine the molar extinction coefficients. Individual values are given in Table III along with the average and standard deviation. The coefficient determined for the protonated form was used in the calculation of the *N*-RPE content of the ROS.

The values we report for the molar extinction coefficient of *N*-RPE are lower than those recently reported by Plack and Pritchard (1970). Our lower values may have resulted from loss of extinction at 455 nm due to spontaneous breakdown of the Schiff base in the acid solution. Since the molar concentration of *N*-RPE was based on total phosphorus assay (assuming no decomposition), any decomposition would result in an underestimation of the coefficient. However, a lower value for the molar extinction coefficient, when used in Beer's law, would lead to an overestimation of the concentration of *N*-RPE. Therefore, the values we obtained by the spectral assay are the maximum concentrations of *N*-RPE in bovine ROS.

Rhodopsin Content of ROS. Digitonin solutions of ROS 1 and ROS 2 were scanned from 240 to 650 nm. Average A_{280}:A_{500} and A_{400}:A_{500} ratios were 3.3 and 0.28, respectively, in agreement with the recent values of Shichi *et al.* (1969) for Emulphogene solutions of bovine ROS. We observed no 370-nm peaks in any of our ROS preparations, indicating that the rod visual pigment had been extracted in its native form. However, exposure of the digitonin solutions to room light resulted in a loss of extinction at 500 nm and concomitant increase at 370 nm.

The molar concentration of rhodopsin in the ROS was calculated using the molar extinction coefficients of Wald and Brown (1953) (40,600), Shichi *et al.* (1969) (42,000), and Heller (1968) (23,100). Weight percentages were determined using the molecular weights of rhodopsin given by these authors (40,000, 28,000, and 26,400, respectively). These data are given in Table IV.

The molar extinction coefficients of Wald and Brown and Heller differ by almost a factor of two. However, the calculated weight per cent of rhodopsin is similar because of the almost twofold difference in molecular weights given by these two authors. The molar concentration of rhodopsin differs, reflecting the differences in molar extinction coefficients. Shichi *et al.* calculated the molar extinction coefficient of rhodopsin to be 42,000, in agreement with the value reported by Wald and Brown. However, the molecular weight ascribed to rhodopsin by these authors agrees with the value given by Heller. Because of these unresolved differences, the data in Table IV were calculated using the molar extinction coefficients and the molecular weights given by all three groups.

TABLE IV: Concentration of Rhodopsin and *N*-RPE in Bovine Rod Outer Segments.

	ROS-1	ROS-2
mμmoles of rhodopsin/mg of dry ROS[a]		
Wald and Brown (1953)	3.1	4.3
Heller (1968)	5.4	7.5
Shichi *et al.* (1969)	3.0	4.1
Wt % rhodopsin (in dry ROS)[b]		
Wald and Brown (1953)	12.6	17.5
Heller (1968)	14.1	19.6
Shichi *et al.* (1969)	8.4	11.5
mμmoles of *N*-RPE/mg of dry ROS		
Hydrogenation–thin-layer chromatography assay	2.5	1.5
Spectrophotometric assay	2.7	1.9
N-RPE:rhodopsin		
Maximum ratio	0.90	0.46
Minimum ratio	0.46	0.20

[a] Values derived using the molar extinction coefficients of rhodopsin given by the cited authors. [b] Values derived using the molecular weights of rhodopsin given by the cited authors.

N-RPE Content of ROS. The *N*-RPE content of ROS-1 and ROS-2 is also given in Table IV. Values from phosphorus assay for ROS-1 are the average of duplicate analyses of eight separate extractions; ROS-2 values are the average of duplicate analyses of three extractions. ROS-1 contains 2.5 mμmoles of *N*-RPE/mg of dry ROS and ROS-2 contains 1.5 mμmoles/mg of dry ROS.

N-RPE values derived by the spectrophotometric method are similar to those determined by phosphorus assay. The dark-circled spectra in Figure 1 are an acid and base scan of a chloroform–methanol (2:1, v/v) (0.00032 N with HCl gas) extract of dry ROS. Absorbancy maxima are nearly the same as for the synthetic *N*-RPE (open-circled spectra). It was assumed that the absorbance at 455 nm was due only to *N*-RPE and the absorbance at this wavelength was used to calculate the *N*-RPE content.

Discussion of the Relationship between N-RPE and Rhodopsin. Poincelot *et al.* (1969, 1970a) claim that *N*-RPE serves as the chromophore of rod visual pigment. They say that upon exposure to light, retinal is transferred from lipid to protein at the metarhodopsin I to metarhodopsin II step (Kimbel *et al.*, 1970). Their contention was recently strengthened when Daeman and Bonting (1969) confirmed the presence of *N*-RPE in bovine ROS and Akhtar and Hirten-

stein (1970) showed that tritiated 11-*cis*-retinal was incorporated *in vitro* into *N*-RPE. However, an important point overlooked by all of these authors is that detergent solutions of ROS are not solutions of pure visual pigment. Rhodopsin makes up at most only 20% of the dry matter of bovine ROS (Table IV). The rest is presumably other protein and lipid. Therefore, the observation that *N*-RPE is present in bovine ROS does not justify the conclusion that it is the chromophore of rhodopsin.

We have shown by two independent assays on two unbleached bovine ROS preparations that the molar concentration of *N*-RPE is less than that of rhodopsin. We therefore disagree with the claims of Poincelot *et al.* that *N*-RPE is the chromophore of rod visual pigment and support the contention of Heller (1968) and Hall and Bacharach (1970) that phospholipid is not involved in the binding of retinal to opsin. In further support of our claim, we (R. E. Anderson and M. O. Hall, unpublished observations) recently purified rod visual pigment from frogs that had been injected with tritiated retinol and found no *N*-RPE. Instead the retinal was bound directly to protein.

There is an overwhelming amount of evidence that *N*-RPE does exist in ROS. Although its function there is not known, it seems reasonable to suggest that the Schiff base is a stored form of the highly reactive aldehyde. Upon decomposition of visual pigment by light, retinal could be transferred from lipid to protein as part of the process of photopigment regeneration. This would explain the observations of Poincelot *et al.* (1969, 1970a) and Kimbel *et al.* (1970) that light-exposed solutions of ROS contain no *N*-RPE.

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