LIGHT SENSITIVE ZINC CONTENT OF PROTEIN FRACTIONS FROM BOVINE ROD OUTER SEGMENTS[1]

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Summary: Bovine retinas, isolated rod outer segments and emulphogene extracts of rod outer segments have been shown to contain appreciable amounts of Zn^{2+}, Ca^{2+} and Mg^{2+} when isolated in the absence of added metal ions. Chromatography of emulphogene extracted rod outer segments in Sephadex G-25 showed virtually all the Ca^{2+}, Zn^{2+} and protein to elute with the void volume. Levels of Zn^{2+} but not Ca^{2+} were light sensitive. The Zn^{2+} contents of protein fractions were about 60% higher when samples were bleached. Under optimal conditions protein fractions contained 1.4 - 1.8 g atoms Zn^{2+}/mole rhodopsin for dark adapted samples and 2.1 to 3.2 g atoms Zn^{2+}/mole of rhodopsin for bleached samples.

In recent years the biochemical mechanism of visual excitation has been greatly elucidated, but one of the most central mechanisms, intracellular communciation between the ROS[3] disc and the plasma membrane, has evaded description. Undoubtedly, photoinduced molecular transitions occur in the disc membrane and induce hyperpolarization at the plasma membrane representing a surprisingly high signal amplification (1). A number of independent reports suggest Ca^{2+} to be involved in some manner in the amplification mechanism (2,3) since this ion has been repeatedly shown to be released from ROS disc preparations when exposed to light (4-7). Others have reported light-dependent flux changes of Ca^{2+} using ROS disc (8). While these experiments lend support for the hypothesis that Ca^{2+} is the mediator between the ROS and the plasma membrane, the influence of photo-

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3 Abbreviations: ROS, rod outer segments.

induced isomerization of rhodopsin on Ca^{2+} transport in the ROS disc remains obscure.

As the isolation of ROS disc from bovine retinas by conventional techniques allows ample opportunity to wash out native metal ions we undertook to measure metal ion compositions of retinas, ROS and different fractions of detergent extracted ROS. These studies demonstrated clearly that in addition to Ca^{2+} and Mg^{2+}, Zn^{2+} is a prevalent metal ion in ocular tissue and that its content in protein fractions is not only stoichiometrically related to rhodopsin but is also light dependent.

Materials and Methods: Fresh bovine eyes were obtained in lots of 100 to 200 from American Stores Packing Company, Lincoln, Nebraska 68522. Eyes were collected in a black polyethylene bag and kept dark at ambient temperature for 1-2 hr, then cooled with crushed ice. Retinas were excised under dim red light (Nalco ruby 4 cp, Nalco Company, St. Louis, Missouri) and stored in the dark at 4° until used, but not for more than 24 hr.

Imidazole was purchased from Sigma Chemical Corp., St. Louis, Missouri and was recrystallized from benzene to remove photooxidation products. Emulphogene BC-720 was purchased from General Analine Film Corp., 2459 Wilkinson Blvd, Charlotte, North Carolina 28201. Sephadex G-25 was purchased from Pharmacia Fine Chemicals, Piscataway, New Jersey.

ROS was isolated from retinas by the method of Anderson and Maude (9). Rhodopsin was extracted from the ROS from 100 retinas with 10 ml of 1% emulphogene in 50 m*M* imidazole buffer pH 7.35 by incubation at 4° overnight, and sonicated gently for 5, 1 sec intervals in a melting ice bath. The debris was removed by centrifugation at 48,200 x *g*. The supernatant fluid was filtered through a Millipore 0.45 μ filter before use. The 498 nm to 278 nm absorbance ratio of detergent extracted rhodopsin was usually 5 when absorbance measurements followed Beers Law. Higher absorbancies gave ratios which approached 3.

Sephadex G-25 used for column chromatography was swelled overnight in 1% emulphogene in 50 m*M* imidazole buffer pH 7.35, then packed to a height of 35 cm in a 2.5 x 45 cm column and equilibrated with the same buffer. Prior to the addition of samples to the column, 20 ml of 0.5 *M* EDTA, pH 9.0 - 9.5 were passed through the gel-bed to remove possible trace levels of divalent metals. The columns were then washed with at least 2 column-bed volumes of 1% emulphogene in 50 m*M* imidazole, pH 7.35 prior to adding the sample. All buffers and solutions were prepared with deionized distilled water. Samples (4-5 ml) of rhodopsin were loaded into the column and 4 ml fractions were collected for a total of 1 column-bed volume. In some experiments rhodopsin samples were bleached after being loaded in the column, by means of fluorescent lamp placed adjacent to and aligned with the column (600 lux at the column surface). All Sephadex G-25 columns were eluted at ambient temperature 23-26°.

In some experiments, ROS were incubated for 2 hr at room temperature in solutions of buffered cations. These solutions were modified Ringers solutions in which the principle modification was the substitution of imidazole for phosphate. The imidazole-Ringers solution pH 7.4 contained in m*M*: NaCl, 119; KCl, 6: $CaCl_2$, 1.7; $MgSO_4$, 2.0: and imidazole, 25. In other experiments $ZnSO_4$ was added to imidazole Ringers solution at 0.5 m*M*.

Divalent cations were measured using a Perkin-Elmer Model 303 atomic absorption spectrophotometer. In all experiments, metal contents of buffers

were monitored and were below 0.3 μM for Ca^{2+}, 0.07 μM for Mg^{2+} and Zn^{2+} was undetectable when using deionized-distilled water as the instrument blank.

Phosphate analysis were performed by the method of Bartlett (10), which was modified by adding 2 drops of 30% H_2O_2 to the digestion mixture at three 1 hr intervals after acid digestion to prevent the formation of charred carbonaceous material.

Rhodopsin concentrations were measured spectrophotometrically at 498 nm using a molar absorptivity of 41,000 (11). Protein concentration was measured at 280 nm and expressed as absorbance per ml.

Results and Discussion: The implication of Ca^{2+} as the mediator of photostimulation between ROS disc and the plasma membrane led us to characterize the native ion content of isolated retinal fractions. The Ca^{2+}, Mg^{2+} and Zn^{2+} compositions of samples of retinas, ROS and detergent extracted ROS are shown in Table I. These data demonstrate that ROS as well as detergent extracted ROS possess appreciable levels of all three cations relative to rhodopsin. At least a portion of the native metal ions are retained through the ROS isolation procedure and some ions are extractable with emulphogene.

Table I

Metal Ion Composition of Retinal Preparations

	Retina[1]	ROS Disc[2]		Emulphogene Extract
	µg atoms / g dry wt	µg atoms / g dry wt	g atoms / mole rhodopsin	g atoms / mole rhodopsin
Ca^{2+}	5.7	7.3	6.7	1.4
Mg^{2+}	37.2	3.8	3.6	1.4
Zn^{2+}	10.1	2.3	2.1	0.8

[1] Retinas and ROS were suspended in 50 mM PO_4 buffer and sonicated for 2 min. The solutions were refluxed for 3 hrs in 2 mM EDTA and 1% emulphogene, then centrifuged. Supernatant fluids were collected and diluted with deionized water. Divalent ions were determined as described in the text. Ca^{2+} and Mg^{2+} are determined in the presence of 1% Lanthanum oxide.

[2] 1.07 µmoles rhodopsin/g dry wt ROS.

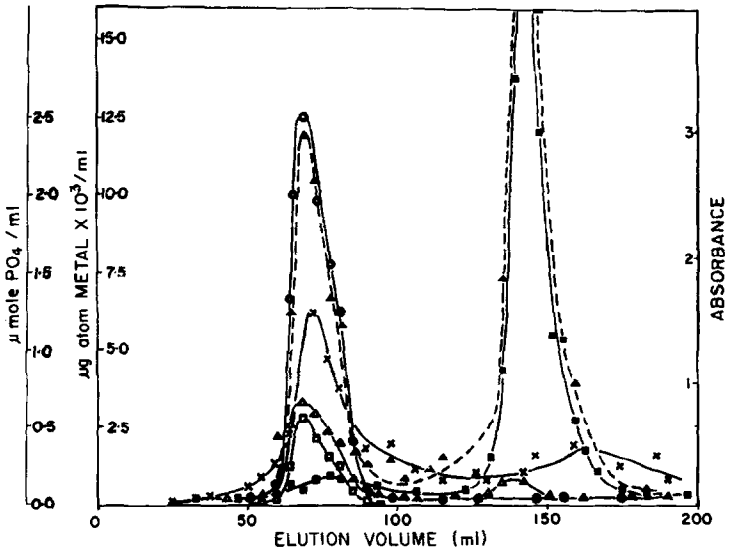

Fig. 1 Chromatography of dark adapted emulphogene extracts of ROS from 25 retinas in Sephadex G-25. Sample was not exposed to added metal ions, but was exposed to inorganic phosphate during isolation of ROS. Absorbance at 280 nm (○), (▲) μmoles total phosphate/ml, μg atoms/ml of (X) Ca^{2+}, (△) Zn^{2+} and (■) Mg^{2+} and absorbance at 498 nm (□).

Fig. 1 shows a typical chromatogram of 2.5 ml sample of emulphogene extracted ROS (100 retinas 10 ml). As expected, rhodopsin eluted with the protein fraction in the void volume of the column. Organic phosphate (phospholipid) was also present in this fraction. The mole ratio of phospholipid to rhodopsin in emulphogene has been reported to be 85-95 (11). Calculation of this ratio from Fig. 1 gave a ratio of 161. However, when isolated ROS were washed once with phosphate free Ringers solution before extraction with emulphogene to remove inorganic phosphate left from the isolation procedure, the ratio was 85-110. A second phosphate component was detected at 140 ml and presumably contained phospholipids. Significant concentrations of Mg^{2+} were also detected at 140 ml and Ca^{2+} at about 160 ml, Ca^{2+} and Zn^{2+} were also at significant levels in the fractions containing rhodopsin. Virtually all the Zn^{2+} extracted from ROS by emulphogene chromatographed with rhodopsin.

Fukami *et al.* (12) demonstrated rhodopsin not to be a metalloprotein, as rhodopsin extracts from frozen retinas, passed through a mixed bed resin of IR-120 and IR-400 did not possess trace levels of metal ions. When the experiment, shown in Fig. 1, was performed with ROS preparations, which had been frozen at any step of the procedure, the rhodopsin fraction did not possess discernible levels of Ca^{2+}, Zn^{2+} or Mg^{2+}. The association of metal ions with the protein fraction was concluded to be cold sensitive.

The levels of Ca^{2+} and Zn^{2+} present in the protein fraction could be elevated by incubating ROS preparations in imidazole-Ringers solution containing $ZnSO_4$ or $CaCl_2$. Preincubation of ROS in imidazole-Ringers containing 1.7 m*M* $CaCl_2$ elevated the Ca^{2+} content and depressed but did not abolish the Zn^{2+} content of the protein fraction. Conversely, if 0.5 m*M* $ZnSO_4$ was added to the imidazole-Ringers solution containing 1.7 m*M* $CaCl_2$, Zn^{2+} was the predominate ion in the protein fraction. Due to the

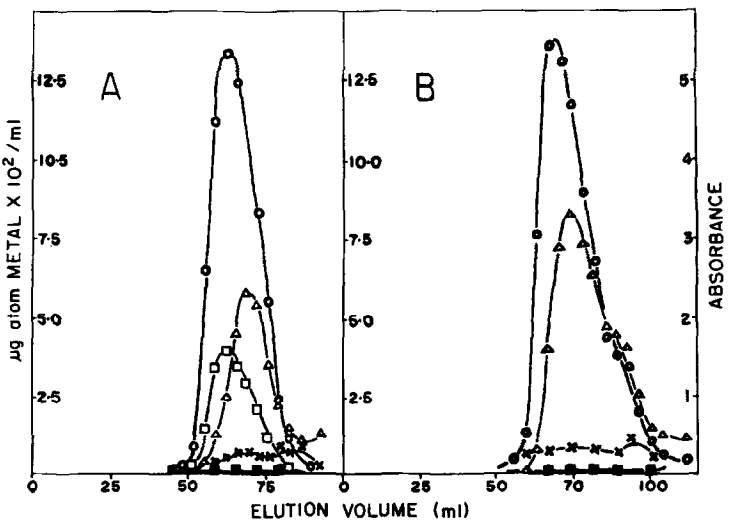

Fig. 2 Chromatography of ROS extracts in Sephadex G-25. A. Dark adapted; B. Bleached. The procedure was the same as in Fig. 1 except that ROS from 50 retinas were incubated in imidazole-Ringers which contained 0.5 m*M* $ZnSO_4$ for 2 hr at room temperature prior to being treated with emulphogene. (○) absorbance 280 nm, (△) Zn^{2+}, (□) absorbance 498 nm, (×) Ca^{2+} and (■) Mg^{2+}.

differences in the two ion concentrations it appears that the selectivity of the protein fraction is greater for Zn^{2+} than Ca^{2+}.

Fig. 2 shows the effect of photobleaching on the metal ion content of the protein fraction from ROS preincubated in imidazole-Ringers containing 0.5 m*M* $ZnSO_4$. In these experiments, dark adapted rhodopsin was added to the column and after the sample had just entered the gel, it was illuminated with 600 lux flourescent lamp. As shown in Fig. 2, light treatment completely abolished the 500 nm chromophore associated with the protein fraction and increased the Zn^{2+} content of this fraction by about 60% from a value of 1.4 to 1.8 g atoms of Zn^{2+} per mole of rhodopsin in the dark to 2.1 to 3.2 g atoms of Zn^{2+} per mole of rhodopsin in the light. Experiments were also performed under conditions where retinas were bleached just prior to being removed from the eye cup and without subsequent incubation in imidazole-Ringers solution (Fig. 3). As shown in Table II the Zn^{2+} content of the protein fraction from bleached retinas

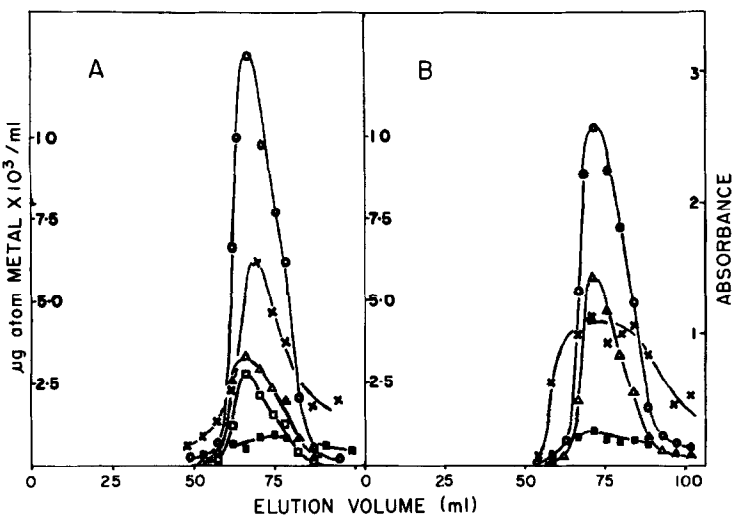

Fig. 3 Chromatography of ROS extracts in Sephadex G-25. A. Dark adapted; B. Bleached. The method was the same as for Fig. 2 except in B, the retinas were bleached in intact eyes prior to being dissected in the light. ROS were not incubated in zinc-imidazole-Ringers. (○) absorbance 280 nm, (×) Ca^{2+}, (△) Zn^{2+}, (□) absorbance 498 nm and (■) Mg^{2+}.

Table II

Stoichiometry of Zinc Relative to Rhodopsin

ROS Incubation Medium	μg atoms/μmole Rhodopsin[1]	
	Dark	Light
No Ringers	0.28	0.45
Zn Ringers[2]	1.77 1.41	3.21 2.11
Ca Ringers	0.18	0.24

[1] Calculations are based on the zinc contents of emulphogene extract of ROS which co-chromatographed with rhodopsin in Sephadex G-25.

[2] These values represent typical variability in repetitive experiments. The experiment with Zn Ringers was performed 6 separate times with similar results.

was 60% higher than the protein fraction from dark adapted retinas. The lack of stoichiometry of Zn^{2+} to rhodopsin presumably resulted from losses of metal ions during the isolation of ROS from retinas, but the data strongly suggest that the increased Zn^{2+} content of the protein fraction occurred *in situ*.

Whether Zn^{2+} actually binds to rhodopsin or to another component or complex in the protein fraction has not been demonstrated but the fact that the data reported here cannot be obtained with frozen preparations reopens the question of whether rhodopsin is a metalloprotein. We do not question the validity of the hypothesis that light induced Ca^{2+} released from ROS may play a role in vision. However, in protein fractions of emulphogene extracts, photobleaching does not effect Ca^{2+} concentrations relative to rhodopsin or total protein, whereas Zn^{2+} concentrations are markedly influenced. If Zn^{2+} ion levels do play a role in vision, it seems

reasonable that they are more intimately associated with the primary photochemical event, i.e. the photoisomerization of rhodopsin, than is Ca^{2+}.

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