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SEGMENT FRAGMENTS.

The Ohio State University, Ph.D., 1975
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STUDIES ON CYCLIC NUCLEOTIDE PHOSPHODIESTERASES
ASSOCIATED WITH BOVINE RETINAL OUTER
SEGMENT FRAGMENTS

DISSERTATION
Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By
Cyril Marston Manthorpe, Jr.

* * * * *

The Ohio State University
1975

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PUBLICATIONS


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FIELDS OF STUDY

Major Field: Retinal Enzymology

Studies in the Biochemistry of Retinal Outer Segments. David G. McConnell, Ph.D.
Studies in Molecular Photobiology. Elizabeth L. Gross, Ph.D.

Studies in Membrane Biochemistry. Gerald P. Brierley, Ph.D.
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LIST OF SYMBOLS

ROS  retinal outer segments
A_280  optical density at 280 nm
ΔA_498  A_498 of unbleached ROS-A_498 of bleached ROS
cAMP  cyclic-3',5'-adenosine monophosphoric acid
cGMP  cyclic-3',5'-guanosine monophosphoric acid
CNPDE  cyclic nucleotide phosphodiesterase
CAPDE  cAMP-phosphodiesterase
cGPDE  cGMP-phosphodiesterase
^3_H  tritium
Ci  curies
mmol  millimole
I^125  iodine-125
EDTA  ethylenediamine tetraacetic acid
popop  p-bis-[2-(5-phenyloxazolyl)]-benzene
ppo  2,5-diphenyloxazole
SDS  sodium dodecyl sulfate
cpms  counts per minute
nmole  nanomole
ATPase  adenosine triphosphatase
M.W.  molecular weight
^32_p  phosphorus-32
dpms  disintegrations per minute
Retinal ROS are very specialized neural organelles responsible for sensory transduction, i.e., conversion of photic stimuli into transient plasma membrane hyperpolarizations. The molecular system responsible for sensory transduction in the vertebrate retina must be capable of amplifying the energy of one photon by a factor of at least $10^4$ and perhaps as high as $10^7$. Although the molecular processes underlying such a function are obscure, two major theories, originally proposed by Wald (1,2,3), still exist. One theory states that rhodopsin, the visual pigment, is a light activated ionophore which is capable of redistributing charge across ROS membranes. The other theory maintains that rhodopsin is an enzyme or coenzyme which directly or via an alternative series of enzymatic or ionophoric sequences causes membrane permeability changes. Although the evidence for either theory is presently not unequivocal elucidation of the true mechanism is precluded by a good understanding of ROS components. Investigators have reported a variety of molecular species, some of which may interact with rhodopsin, to be associated with isolated ROS preparations (see Table 1).
Table 1.—Components and light induced effects reported in association with isolated ROS preparations.

<table>
<thead>
<tr>
<th>System</th>
<th>Reported Effect (if any)</th>
<th>Animal</th>
<th>ROS Purity OD$<em>{280}$/OD$</em>{498}$</th>
<th>Ref(s)</th>
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<td>Rhodopsin</td>
<td>Bleaches to opsin + chromophore</td>
<td>Cow</td>
<td>2.2-2.6</td>
<td>4</td>
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<td>Glycolytic Enzymes</td>
<td>--</td>
<td>Cow</td>
<td>2.5-3.5</td>
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<td>Mg$^{+2}$ or Ca$^{+2}$</td>
<td>Specific activity decreases 15% upon illumination of ROS</td>
<td>Frog</td>
<td>N.G.</td>
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<td>ATPase</td>
<td>DPMs $^{32}P$ bound to rhodopsin increases from 6 to 10 fold upon illumination of ROS</td>
<td>Cow</td>
<td>N.G.</td>
<td>13</td>
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<td>Protein Kinase (rhodopsin phosphorylation)</td>
<td>16% of particulate Ca$^{++}$ pool is transferred to soluble pool upon illumination</td>
<td>Cow</td>
<td>N.G.</td>
<td>14</td>
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<td>Increase in &quot;soluble calcium&quot;</td>
<td>1.6 times more Na$^+$ is released upon illumination of ROS than in dark controls</td>
<td>Cow</td>
<td>N.G.</td>
<td>15</td>
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<td>Sodium ion release</td>
<td>Variable: from 5 H$^+$ bound to 2 taken up per molecule of rhodopsin bleached</td>
<td>Cow</td>
<td>2.5-3.5</td>
<td>17</td>
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$^1$N.G. = not given.
It is possible that each of these reported systems in Table 1 could have a physiological function. However, several points recommend caution in reaching this conclusion. First, rhodopsin has been reported to represent at least 80% of purified ROS protein (4,48,51) and therefore only permitting at best 20% of the total protein for the other reported components. Second, the retinas from which the ROS are isolated contain about a half dozen different cell types and numerous organelles which, if ruptured during ROS purification, could conceivably contribute contamination. Third, the only criterion of ROS purity, rhodopsin concentration (which is generally expressed either as $A_{280}/A_{498}$ or nmoles unbleached rhodopsin per mg), is not reported in most studies of ROS components and in any case is not an absolute standard of ROS purity. The purest ROS preparations so far reported are modifications of the original method of McConnell (26) involving sucrose density gradient centrifugation and having $A_{280}/A_{498}$ values below 3.5 (4,5,26).

Sensory transduction, however, is not the only physiological function an ROS component might have. Alternative processes thought to occur in association with the ROS are rhodopsin regeneration (6,7), ROS membrane renewal

1The smaller ratio indicates greater purity of rhodopsin. Purified rhodopsin preparations typically have values of about 1.6.
(8,41) and dark membrane ion flux (9,10). It is possible that photon absorption by rhodopsin could affect these processes.

Recently attention has been directed towards the presence and role of cyclic nucleotides associated with vertebrate ROS preparations. This concern was initiated by Bitensky et al. (18-24) who reported the existence of adenylate and guanylate cyclase activity, associated with both cow and frog ROS preparations, which was inhibited almost an order of magnitude by bleaching the visual pigment, rhodopsin. Speculation was abundant that cyclic nucleotides might play a role in visual sensory transduction by somehow altering charge permeability of the ROS plasma membrane in a manner analogous to reported cAMP-induced increases in sodium, potassium and calcium movement across liver, muscle and epithelial cell plasma membranes (27, 28,29). Subsequently, however, our laboratory (30; copy of reprint is in Appendix) and two other laboratories (25,31) failed to reproduce this light inhibition of cyclase activity. Although all three laboratories reported measurable adenylate cyclase associated with ROS preparations, this activity was an order of magnitude less than that previously reported by Bitensky's group.

This discrepancy was apparently methodological in that those laboratories reporting no light effects on adenylate cyclase activity in ROS (25,30,31) used adequate
CNPDE inhibitory systems in their cyclase assays. This was significant since it was subsequently reported by Bitensky's group (32) that their observed light-induced decrease in cyclic nucleotide concentration was attributable not to a light-inhibited cyclase but to a light-activated CNPDE associated with their ROS preparations. The reported characteristics of the CNPDE associated with frog ROS are as follows (32):

1. Both CAPDE and CGFPE associated with the ROS are activated almost an order of magnitude by illumination only when an optimal concentration of 0.75 mM ATP is present during assay.

2. The $K_m$ for cAMP is 8 mM; the $K_m$ for cGMP, 0.16 mM.

3. Full light activation of CNPDE results from bleaching only 2% of rhodopsin.

4. Homogenization of ROS (20 strokes) eliminates light stimulation.

5. Storage for 4 days at 4° eliminates light activation.

6. Storage at -20° does not alter light stimulation.

Prior and subsequent to this latest report of a light-activated CNPDE a number of other laboratories reported significant CNPDE activity associated with ROS preparations (33-37) but none of these substantiated significant light activation.
Statement of the Problem

This thesis addresses itself to the following questions: Is there in fact CNPDE activity associated with more rigorously pure ROS preparations? Is the CNPDE activity cryptic (i.e., is the enzyme located within substrate impermeable membranous systems)? Is this CNPDE activity affected by bleaching rhodopsin? Is it possible for CNPDE activity, arising from other retinal cells, to contaminate our ROS preparations? Which substrate is preferred?

The studies comprising this thesis have been divided into two parts. The first part includes studies done on CAPDE of bovine ROS prior to the Bitensky report of a light-activated CNPDE in frog ROS (32 and personal communication) and also studies on CNPDE assay difficulties that may have prevented this investigator and other groups (33-37) from reproducing the reported light activation of CNPDE (32). The second part includes studies on CNPDE involving an alternative assay that eliminates previous difficulties in reproducing light stimulation. This second part substantiates the light activation of CGPDE and provides new data which significantly modify previous interpretations of the ROS-CNPDE system.
METHODS AND MATERIALS

Chemicals and Reagents

$8^{-3}\text{H}\text{cAMP (17.5 C_i/mmol), } 8^{-3}\text{H}\text{cGMP (14.3 C_i/mmol)}$

and $^{125}\text{Iodine (17 C_i/mg)}$ were purchased from International

Chemical and Nuclear; ultrafiltration membranes from

Amicon; sodium phosphate from Baker; EDTA, hydroxylamine

hydrochloride, MgCl$_2$, naphthalene, ammonium acetate and

ammonium persulfate from Mallinckrodt; bisacrylamide and

acrylamide from Canalco; popop and ppo from Research

Products International; p-dioxane from Eastman; sucrose

from Fischer; SDS from Pierce Chemical. All other reagents

and enzymes were purchased from Sigma, and chromatography

paper (Whatman 3MM) from Scientific Products.

ROS Preparations

With the exception of one experiment, ROS frag­

ments were isolated by the method of McConnell (26,30) and

the procedure is outlined in Figure 1. These preparations

consistently exhibit $A_{280}/\Delta A_{498}$ ratios of from 2.5 to 3.5

and contain ≤ 1% mitochondrial contamination as deter­

mined by cytochrome oxidase assays (26). The yield from

400 fresh bovine retinas is typically from 100-150 mg Lowry

protein (38) of ROS. Typical electron micrography of such

preparations (Figure 2) reveal fragments which contain a
Fig. 1.—ROS isolation procedure of McConnell (26). All procedures were performed at 0-4° in dim red light. Generally the procedure was initiated with 400 bovine retinas within 48 hours after slaughter. All sucrose solutions are buffered with 0.01 M Tris·HCl (pH 7.5). Six sucrose gradients were necessary for each batch of 400 retinas and the ROS yield is typically 100-150 mg Lowry protein (38) and $A_{280}/ΔA_{498}$ is typically 2.5 to 3.5.
ROS ISOLATION METHOD OF McCONNELL (26)

Bovine retinas at 4°C, lightly homogenized one stroke (teflon)

Retinal homogenate in 1.32 M Sucrose-Tris

\[ 1100 \times g \]

**Pellet**

**Super**

Dilution to 0.44 M Sucrose with 0.1M Tris

Diluted Super

\[ 2100 \times g \]

**Crude ROS Pellet**

**Super**

Resuspended in \( \rho = 1.10M \) Sucrose and homogenized 1-5 strokes (teflon)

Sucrose density

\[ 1.12 \]

\[ 1.14 \]

\[ 90,000 \times g, \text{ } 1\frac{1}{2} \text{ hours} \]

**Figure 1**
Fig. 2.—Electron micrography (x 24,000 diameters) of isotonically washed frozen-thawed ROS. ROS were prepared for electron microscopy as described in "Methods," and the micrograph was taken at 10,000 electron optical magnification by D. G. McConnell. Fresh, non-washed ROS have a similar truncated appearance.
largely intact plasmalemma surrounding stacks of discs. Various derivative fractions were prepared by methods detailed in the text and in captions to figures and tables. Unless otherwise indicated, these fractions were frozen and stored at -76° or -196° in light-tight containers.

In one experiment an evaluation of the sucrose flotation procedure used by Bitensky et al. (18) was used and followed by continuous sucrose density gradient centrifugation as outlined in Figure 3. The first two flotations in this alternative procedure yield an "ROS" preparation having a $A_{280}/A_{498}$ ratio above 5.0.

**Dim Red Light**

Unless otherwise indicated, ROS preparation and CNPDE assays were carried out under two 25 W, 115 vac GE red bulbs.

**Electron Microscopy**

ROS preparations were suspended in 10 volumes of buffered 0.25 M sucrose and pelleted at 100,000 X g for 30 min. The supernate was decanted and the pellet loosened from the bottom of the centrifuge tube. It was fixed on ice for at least 2 hrs. in 1% OsO$_4$, 0.25 M sucrose, 0.1 M PO$_4$ at pH 7.4. After removal of the fixative, the ROS was rinsed with 0.1 M PO$_4$ at pH 7.4. Rinsing with water, and dehydration through ascending concentrations of ethanol followed. Embedding and sectioning were carried out and
Fig. 3.—Sucrose flotation procedure for isolation of ROS. All procedures were performed at 0–4° in dim red light. Data in Table 3 are derived from this procedure using 100 fresh bovine retinas. The continuous sucrose density gradient is the same as in Figure 1.
Retinas shaken in 1.39 M Sucrose-Tris (RS)  

$100,000\, \text{xg, 1 hour}$

- **Pellet (P-1)**
- **Middle (M-1)**
- **Top (T-1)**

**Top (T-1)** Resuspended by shaking in 1.39 M Sucrose-Tris  

$100,000\, \text{xg, 1 hour}$

- **Pellet (P-2)**
- **Middle (M-2)**
- **Top (T-2)**

**Top (T-2)** Resuspended by shaking in 0.25 M Sucrose-Tris  

$100,000\, \text{xg, 1 hour}$

**Pellet (TW)** Resuspended by shaking in 1.10 $\rho$ Sucrose

Placed on a continuous (1.12 to 1.14 $\rho$) Sucrose gradient

Figure 3
ultrathin sections were double-stained with lead citrate and uranyl acetate. Micrographs were taken at electron optical magnification of 10,000 diameters.

**CNPDE Assays**

Two different CNPDE assays were used in these studies. In the early work (Part I of Results) CNPDE was assayed by the method of Rutten et al. (39) employing a two-stage assay. In the first stage a reaction volume of 150 μl included 30 mM Tris·HCl pH 7.5, 3 mM MgCl₂, 2 mM 8⁻³H-cAMP (100-200 cpms/nmole), 0.25 M sucrose (unless otherwise indicated) and from 0.1 to 0.25 mg protein. The assay was initiated by adding substrate, all ingredients having been preincubated at 30°, and the reaction run for 20 min. at 30° and terminated by placing in a boiling water bath for 2 min. The mixture was cooled and a second stage incubation for 30 min. at 30° followed initiation with 25 μl of a solution containing 40 μg 5'nucleotidase (6000 u/mg) in 50 mM MgCl₂. This amount of 5'nucleotidase will convert all of the 8⁻³H-AMP to 8⁻³H-adenosine in this reaction time (Figure 4). The second stage was terminated by placing the reaction vessel in a boiling water bath for 2 min. After cooling on ice, the mixture was centrifuged at 2000 x g 10 min., and 100 μl of the supernate was placed on a 7 X 15 mm column containing Dowex-1 (1 X 2-400) pre-equilibrated in 0.1 M NaHCO₃. The column was then eluted...
Fig. 4.—Determination of the amount of 5' nucleotidase for use in second stage incubation step of coupled CAPDE assay procedure (see "Methods"). 5' nucleotidase assay was as described in "Methods."
nmol cAMP hydrolyzed or
Adenosine formed per 30 min
per 50µl ± SD (n=3)

mg/ml of 5' Nucleotidase in
second stage incubation

Figure 4
with 10 ml 0.1 M NaHCO$_3$ which is enough to recover 99% of the 8-$^3$H-adenosine that could form if all of the 2 mM 8-$^3$HcAMP is hydrolyzed in the first stage by commercial CAPDE (see Figure 5). This same assay can be used for CGPDE but then it becomes necessary to elute with 30 ml NaHCO$_3$ in order to recover an equivalent percentage of 8-$^3$H-guanosine (see Figure 6). Two ml of the NaHCO$_3$ eluate is placed in 10 ml Brays solution (40) (see Figure 7) and counted in a Packard Tricarb Liquid Scintillation Spectrometer. Specific activity was calculated using the original specific activity (100-200 cpms/nmole) and subtracting cpms 8-$^3$H-adenosine recovered from boiled controls using the following formula:

\[
\frac{\text{u/mg/min}}{= \frac{\text{nmoles cAMP hydrolyzed/mg/min}}{\text{[(cpms Sample)-(cpms Blank)](5) 175 \mu l}}{\text{100 \mu l}}{\text{(20 min.)(cpms/nmole)(mgs per rxn)}}
\]

All assay times were chosen to correspond with the linear portion of time curves for the particular retinal fraction assayed. Percent substrate hydrolyzed during any particular assay never exceeded 40%. Unless otherwise indicated, all assays in Part I of Results were performed on bleached ROS preparations.

In the second part of these studies (Part II of Results) CNPDE was assayed in a volume of 150 \mu l including 30 mM Tris-HCl (pH 7.5), 3 mM MgCl$_2$, 0.25 M sucrose,
Fig. 5.—Recovery of $8^{-3}H$-adenosine from Dowex-1 (1 x 2-400) exchange columns after assay as described in "Methods." One ml fractions were eluted into vials containing 10 ml Bray's solution (40). Assays were performed on (○) preboiled CAPDE in first stage incubation, 5' nucleotidase in second; (□) unboiled CAPDE (enough to hydrolyze all the $8^{-3}HcAMP$ present in the assay) in first stage, but no 5' nucleotidase in second stage; (Δ) unboiled CAPDE in first stage and 40 μg 5' nucleotidase (6000 u/mg) in second stage. Recovery of cpms $8^{-3}H$-adenosine in fractions 1-10 for (Δ) is 99% of initial cpms $8^{-3}HcAMP$ present.
153,377 cpms
8-3H-cAMP
initially present

Figure 5
Fig. 6.—Recovery of $^{3}H$-guanosine from Dowex-1 (1 x 2-400) exchange columns after assay as described in "Methods." One ml fractions were eluted into 10 ml Brays solution (40). Assays were performed on (o) preboiled CAPDE in first stage incubation, 5' nucleotidase in second; (□) unboiled CAPDE (enough to hydrolyze all the $^{3}H$cGMP present in the assay) in first stage, but no 5' nucleotidase in second stage; (Δ) unboiled CAPDE in first stage and 40 μg 5' nucleotidase (6000 u/mg) in second stage. Recovery of cpms $^{3}H$-guanosine in fractions 1-30 for (Δ) is 99% of initial cpms $^{3}H$cGMP present.
Figure 6

1,282,000 cpms
$^8$H cGMP
initially present

one ml Fraction number

$cpms \times 10^4$

$^8$H
Fig. 7.--Tritium counting efficiency of Bray's solution (40) versus various amounts of the Dowex column eluant, 0.1 M NaHCO₃. Precipitation of components in Bray's solution occurs if more than 4 ml NaHCO₃ is present per 10 ml Bray's solution.
Figure 7

- Percent counting efficiency
- ml 0.1M NaHCO\textsubscript{3} per 10ml Bray's solution
2 mM $^{3}$H-cyclic nucleotide (100-200 cpm/nmole) with or without 1 mM ATP and 0.1 to 0.25 mg ROS protein, determined by the method of Lowry et al. (38). Assay temp. was 30°. Assay was initiated by addition of either ROS or cyclic nucleotide, in either case preincubated at 30°, as was the mixture of other components, in a Dubnoff shaker. After a predetermined time in the shaker, the assay, or aliquots thereof, was terminated by boiling for 2 min. Cooling and precipitation by clinical centrifugation followed. Aliquots (50 or 100 µl) of the supernatant fluid were spotted on 23 x 57 cm Whatman 3 MM chromatography paper and developed for 18-20 hrs. with 95% ethanol/1 M ammonium acetate: 73/27: v/v in a descending manner. This technique yields the following $R_f$ values: ATP- 0.071; AMP- 0.187; A- 0.662; cAMP- 0.496; GTP- 0.030; GMP- 0.074; G- 0.566 and cGMP- 0.407. The cyclic nucleotide spots were visualized by ultraviolet light, cut into small pieces, incubated for 1 hr. with shaking in 2 ml $H_2O$ to elute the cyclic nucleotide (Figure 8), placed in 10 ml Bray's solution (40) and counted in a Packard TriCarb liquid scintillation system. Two control assays were run on boiled ROS for every three experimental assays, making a total of five spots per chromatography sheet. Specific activity was therefore calculated after subtracting experimental counts from the control counts. In this way the disappearance of cyclic nucleotide was measured directly.
Fig. 8.--Counting efficiency of free $^3$HcAMP (○) and $^3$HcAMP absorbed to Whatman 3 MM chromatography paper (●) as a function of amount of water present during preincubation prior to the addition of 10 ml Bray's solution (40). Either 100 μl of free $^3$HcAMP or 100 μl of $^3$HcAMP adsorbed and dried on Whatman 3 MM paper and cut into small pieces was incubated 1 hour at r.t. with the indicated amounts of (d.d.) H$_2$O. As the graph shows, at least 2 ml H$_2$O must be added in order to elute enough $^3$HcAMP from the paper (●) to give a counting efficiency comparable to that with free $^3$HcAMP (○).
Figure 8
Units are expressed as nmoles cyclic nucleotide hydrolyzed per min. and calculated as follows:

\[
u/mg/min = \frac{cpms \ blank - cpms \ sample}{(\text{time-min.})(\text{mgs/Rxn})(\text{cpms/nmole})}
\]

**5'Nucleotidase Assays**

Assays of 5' nucleotidase were performed by using a medium identical to the CNPDE assays described above except for (a) ATP concentration, and (b) substitution for ROS of commercial (Sigma) phosphodiesterase in the medium (4 mg/ml, specific activity = 180 u/mg). This was enough enzyme to convert all the 8-\(^3\)HcAMP to 8-\(^3\)AMP during the 20 min. incubation period. After termination of the reaction by boiling, cooling and centrifugation, 5'nucleotidase (Sigma), dissolved in 50 mM MgCl\(_2\) and 30 mM Tris-HCl pH 7.5 as in CNPDE assay, was added to the supernatant fluid to a final concentration of 0.25 mg/ml. Specific activity of this enzyme was 6000 u/mg. After a second 20 min. incubation at 30\(\^\circ\), the product, 8-\(^3\)H adenosine, was isolated by the paper chromatographic procedure described above. Control assays were carried out using preboiled 5'nucleotidase.

**Rhodopsin Assays**

Unbleached ROS preparations (0.3 ml containing 1.5 to 3 mg protein, determined by the method of Lowry et al. (38)) were delivered to a 3 ml silica cuvette
which included 0.1 M \( \text{NH}_2\text{OH} \cdot \text{HCl} \) neutralized with KOH, 1.0% Triton X-100, and water to a total volume of 3.0 ml. The absorption spectrum was recorded between 180 and 600 nm in the Cary 14 Spectrophotometer, using all of the above except ROS in the blank cuvette. The sample was bleached at room temperature for 3 min. with a 40 W fluorescent light situated 12 in. above the cuvette, with intermittent shaking. After the bleached spectrum was recorded the difference (\( \Delta A \)) in absorbance at 498 nm between unbleached and bleached preparations was compared by ratio to the molar absorption of 40,600 (42) in order to calculate the number of nmoles of unbleached rhodopsin per mg protein. 

\( A_{280}/\Delta A_{498} \) ratios were determined from the same spectra. 

\( A_{280} \) did not undergo appreciable change upon bleaching of rhodopsin.

**Cyclic Nucleotide Binding or Permeability Assays**

Cyclic nucleotide binding or permeability was determined using a reaction volume of 1.5 ml, including 30 mM Tris-HCl (pH 7.5), 3 mM \( \text{MgCl}_2 \), 2 mM \( ^3\text{H} \)-cyclic nucleotide, 1.0 mg ROS protein, 0.25 M sucrose, plus or minus 1.0 mM ATP. This mixture was incubated 20 min. at 30° in the dark (the ROS was prebleached on ice when indicated) and the assay terminated by placing it on ice. Centrifugation for 20 min. at 100,000 x g ensued. Pellets were resuspended in 1.5 ml 0.25 M sucrose and counted in
10 ml Bray's solution (40) as was 1.5 ml of each supernatant fluid. Total tritium counts per nmole cyclic nucleotide were calculated from addition of supernatant and pellet counts, the total being then divided by the nmole c-nucleotide (3,000) in the initial reaction medium. The number of nmole cyclic nucleotide bound or taken up by the ROS was then calculated from the reciprocal as
follows: \[
\frac{3,000 \text{ nmole}}{\text{total counts}} \times \text{ROS counts.}
\]
Triplicates were run and standard deviations calculated.

**Iodination**

Ten mg commercial (Sigma) cyclic nucleotide phosphodiesterase (180 u/mg) was labeled by \(^{125}\text{I}\) in a final reaction volume of 0.86 ml including 30 mM Tris-HCl, pH 7.5, 1 mg lactoperoxidase, \(^{125}\text{I}\) (4 x 10\(^{7}\) cpm), 0.01 mM KI, 0.1% butylated hydroxytoluene to prevent lipid peroxidation, and 0.006% H\(_2\)O\(_2\) introduced in five 0.005 ml aliquots 1 min. apart. The reaction was initiated with H\(_2\)O\(_2\) and terminated after 6 min. incubation at 25\(^{\circ}\) by pipetting the mixture onto a Sephadex G-50 column inside a 10 ml pipette. This separated \(^{125}\text{I}\) and other components of the reaction mix from the labeled phosphodiesterase which was eluted in a 2 ml volume of high activity (3 x 10\(^{7}\) cpm) (see Figure 9). Of this volume, 1.3 ml was added to a sample of crude ROS, a final volume of 3.0 ml of this then being layered on top of the linear density gradient.
Fig. 9.---Sephadex G-50 elution pattern of components in iodination procedure (see "Methods"). One ml fractions were eluted with Tris-HCl pH 7.5. Fractions 4 and 5 were pooled and used as the $^{125}$I-CAPDE in Figure 23.
Figure 9
(ρ = 1.12 to 1.14) used to purify the ROS from mitochondrial and other contaminants. The remainder of the 2.0 ml of labeled phosphodiesterase was brought to a final volume of 3.0 ml with density 1.10 sucrose (the crude ROS was also in density 1.10 sucrose (Figure 1)) and layered onto a second, identical gradient. Both gradients were centrifuged at 100,000 x g in the Beckman Sw 25.1 rotor for 1 hr. Fractions were removed by hypodermic needle and counted for \(^{125}\)I in a Beckman gamma counter. Fractions were also assayed for rhodopsin.

**Analytical Disc Gel Electrophoresis**

Gel tubes 11 x 0.5 cm were acid cleaned, siliconized in 2% (v/v) dimethyldichlorosilane/CCl\(_4\) and oven dried. Stacked gels were prepared according to the procedure of Lammli (49) using 8.75% polyacrylamide in the lower gel and 3% in the upper. Before electrophoresis, protein samples were diluted with a protein solvent containing 0.05% bromphenol blue, mercaptoethanol and buffer, and the mixture was boiled 15 min. Forty to 50 µg of sample was applied to each gel and the gels were electrophoresed 16 hr. at 10 mA per gel. The gels were then removed from the tubes, fixed 25-35 hrs. in methanol/acetate acid/water (23:7:70, v:v:v) with three changes, stained in 0.2% Coomassie blue in methanol/acetate acid/water (5:1:5) for 1 to 3 hrs, destained and stored
in the fixing solution. Gels were scanned at 540 nm in a Gilford scanning transport coupled to a Beckman DU Spectrophotometer and Gilford absorbance recorder.
RESULTS: PART I

Distribution of CAPDE Activity in Retinal Fractions

The data in Table 2 reflect the distribution of CAPDE activity in various fractions derived from retinal homogenates as outlined in Figure 1. Proceeding from the retinal homogenate to the final ROS, rhodopsin, a marker for ROS membranes, is purified about 30-fold whereas CAPDE specific activity is purified approximately 10-fold. High specific activity also appears to precede and follow the ROS fraction on the gradient (see gradient top and middle, Table 2).

This relationship of CAPDE activity and the ROS was further examined by analysis of the final continuous sucrose density gradient (Figure 10) on another preparation. Figure 10 shows there to be a reasonably close correspondence between CAPDE activity and rhodopsin. CAPDE activity peaks with fractions 4-6 whereas rhodopsin peaks at fractions 5-9. CAPDE activity also precedes and follows the ROS band (fractions 1-3 and 10-30, respectively) as it did in Table 2. The gradient pellet, which contains microsomes, mitochondria and cell debris, also has some CAPDE activity.
<table>
<thead>
<tr>
<th>Fraction</th>
<th>mgs Total Protein</th>
<th>CAPDE&lt;sup&gt;a&lt;/sup&gt; Activity u/mg/min. ±SD (n=6)</th>
<th>CAPDE Activity Total Units</th>
<th>Rhodopsin nmoles/mg</th>
<th>Rhodopsin Total nmoles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retinal Homogenate</td>
<td>13833</td>
<td>3.2±0.9</td>
<td>44266</td>
<td>0.232</td>
<td>3209&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>1100 x g Pellet</td>
<td>9576</td>
<td>2.8±1.1</td>
<td>26813</td>
<td>0.216</td>
<td>2068&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>1100 x g Supernate</td>
<td>3640</td>
<td>8.1±0.6</td>
<td>29484</td>
<td>0.511</td>
<td>1860</td>
</tr>
<tr>
<td>2100 x g Pellet</td>
<td>520</td>
<td>11.1±0.9</td>
<td>5772</td>
<td>2.81</td>
<td>1461&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>2100 x g Supernate</td>
<td>3280</td>
<td>5.7±3.9</td>
<td>18696</td>
<td>0.03</td>
<td>98</td>
</tr>
<tr>
<td>Gradient Top</td>
<td>58</td>
<td>45.1±1.2</td>
<td>2616</td>
<td>1.43</td>
<td>83</td>
</tr>
<tr>
<td>ROS</td>
<td>70</td>
<td>33.9±2.9</td>
<td>2373</td>
<td>7.35</td>
<td>515</td>
</tr>
<tr>
<td>Gradient Middle</td>
<td>85</td>
<td>37.7±10</td>
<td>3205</td>
<td>1.72</td>
<td>146</td>
</tr>
<tr>
<td>Gradient Pellet</td>
<td>304</td>
<td>6.5±0.4</td>
<td>1961</td>
<td>0.46</td>
<td>140&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>Assays were performed on sonicated, prebleached fractions.

<sup>b</sup>These values are expected to have large error in rhodopsin determinations due to interference by light scattering and the presence of other pigments, hemoglobin, cytochromes, etc.
Fig. 10.—Analysis of final continuous sucrose density gradient shown in Figure 1. One ml fractions were collected from the bottom of the centrifuge tube under dim red light and stored in light-tight vials at -20°. Protein (Δ), rhodopsin (▲) and CAPDE activity (●) were assayed as described in "Methods."
Figure 10

--- mg Lowry protein per ml

--- cAMP PDE activity units/ml x10^-2

--- Unbleached Rhodopsin Concentration nmoles per ml x10^-2
Table 3 presents data derived from the ROS isolation procedure shown in Figure 3. T-2 in Figure 3, the final ROS preparation used by Bitensky's laboratory in their studies of adenylate cyclase and CNPDE (18,32), contains about 1.3 nmoles rhodopsin per mg which compares with 7.3 nmoles per mg using the McConnell (26) ROS preparation (Figure 1, Table 2). When this T-2 fraction is placed on a continuous sucrose density gradient (Figure 3) CAPDE activity decreases (from 15.9 to 8.4 u/mg/min.) while rhodopsin concentration increases (from 1.3 to 9.8 nmoles/mg). It should be remembered that in Figure 3 this T-2 fraction is isotonically (0.25 M sucrose in 0.01 Tris•HCl pH 7.5) washed and centrifuged 100,000 x g 1 hr. prior to gradient application whereas the McConnell crude ROS is not prewashed before gradient application. The procedure outlined in Figure 1 is used for ROS purification in all subsequent studies in this thesis.

Crypticity Studies of ROS-CAPDE

The inconsistent copurification of CAPDE and ROS in the two ROS isolation schemes (Figures 1 and 3) prompted crypticity studies analogous to those routinely used in quantitatively monitoring erythrocyte rupture (43). The theory is that if an enzyme or other component is within membranous vesicles and the vesicles are ruptured the enzyme or component will be released. If the membrane is
Table 3.—Flow chart data corresponding to ROS isolation procedure in Figure 3.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>mgs Total Protein</th>
<th>CAPDE Activity u/mg/min. ±SD (n=3)</th>
<th>CAPDE Activity Total Units</th>
<th>Rhodopsin nmoles/mg</th>
<th>Rhodopsin Total nmoles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retinal Shakenate</td>
<td>5850</td>
<td>10.4±1.2</td>
<td>60840</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>P-1</td>
<td>2550</td>
<td>6.6±2.6</td>
<td>16830</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>M-1</td>
<td>1977</td>
<td>7.6±1.4</td>
<td>15104</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>T-1</td>
<td>1210</td>
<td>16.5±3.5</td>
<td>19965</td>
<td>1.0</td>
<td>1210</td>
</tr>
<tr>
<td>P-2</td>
<td>46</td>
<td>18.9±2.4</td>
<td>869</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>M-2</td>
<td>208</td>
<td>19.7±4.4</td>
<td>4098</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>T-2</td>
<td>879</td>
<td>15.9±0.5</td>
<td>13976</td>
<td>1.3</td>
<td>1150</td>
</tr>
<tr>
<td>TW</td>
<td>566</td>
<td>13.7±3.4</td>
<td>7752</td>
<td>1.9</td>
<td>1075</td>
</tr>
<tr>
<td>W</td>
<td>146</td>
<td>40.5±10</td>
<td>5913</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Gradient Top</td>
<td>27</td>
<td>15.2±7.1</td>
<td>410</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>ROS</td>
<td>85</td>
<td>8.4±1.4</td>
<td>715</td>
<td>9.8</td>
<td>833</td>
</tr>
<tr>
<td>Gradient Middle</td>
<td>180</td>
<td>20.1±6.0</td>
<td>3618</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Gradient Pellet</td>
<td>260</td>
<td>10.7±2.8</td>
<td>2782</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>
substrate impermeable the rupture should result in an increased specific activity. Such an experiment using sonication (Figure 11) of ROS at 0-4°C shows no initial increase in CAPDE activity. Using an analogous procedure hemoglobin is seen to be released from red blood cells (43). A characteristic electron micrograph of sonicated ROS (2 min. at 0-4°C) or ROS suspended hypotonically is shown in Figure 12. After 250 seconds only about 60% of the CAPDE activity remains (Figure 11). Table 4 shows that under assay conditions cyclic nucleotides (cAMP and cGMP) are neither bound to nor taken up by the ROS enough to account for the amount of cAMP hydrolyzed in the assay. The gradual decrease in CAPDE specific activity upon prolonged sonication could be due to localized thermal denaturation effects and to increased entrapment of the enzyme in the resultant substrate-impermeable vesicles.

Since the ROS is obtained from the final sucrose density gradient as a 0.9 M sucrose suspension (Figure 1) the addition of an adequate amount of hypotonic buffer will cause immediate rupture and loss of the typical ROS structure which can easily be monitored by light microscopy. Figure 13 shows that hypotonic (<0.15 M sucrose), isotonic (~0.15-0.4 M sucrose), and hypertonic (>0.4 M sucrose) washing of the ROS remove CAPDE to the same extent. Also, if isotonically washed ROS is resuspended
Fig. 11.—Crypticity of the ROS to CAPDE as determined by sonication. Intact, bleached ROS fragments suspended in 0.9 M sucrose-Tris were sonicated at 0-4°C for indicated times and assayed in 0.25 M sucrose as described in "Methods" within one hour. Figure 12 is an electron micrograph of ROS similarly sonicated 2 min. at 0-4°C and Figure 2 is unsonicated ROS. Sonication was performed using the Fisher Ultrasonic Probe at maximum power setting.
Figure 11

nmoles cAMP hydrolyzed per mg per min ±S.D. (n=6)
Fig. 12.—Electron micrograph (x24,000 diameters) of ROS sonicated 2 min. at 0-4° using full power of Fischer Ultrasonic Probe. ROS was prepared as described in "Methods." Micrograph was taken at 10,000 electron optical magnification by D. G. McConnell. ROS hypotonically lysed has similar vesicular appearance.
Table 4.—Permeability of ROS to cyclic nucleotides. Assay procedure is described in "Methods." 3000 nmoles/mg protein cyclic nucleotide were initially present.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Nmoles cyclic nucleotide recovered from ROS pellet per mg protein per 20 min. at 30° (±S.D.; n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No ATP Present 1 mM ATP</td>
</tr>
<tr>
<td>Preboiled ROS</td>
<td>54±1.2 50±0.8</td>
</tr>
<tr>
<td>Unbleached ROS</td>
<td>44±0.4 52±0.6</td>
</tr>
<tr>
<td>Less Preboiled Control Value</td>
<td>-10 2</td>
</tr>
<tr>
<td>Prebleached ROS</td>
<td>54±0.6 58±0.6</td>
</tr>
<tr>
<td>Less Preboiled Control Value</td>
<td>0 8</td>
</tr>
</tbody>
</table>
Fig. 13.--Effects of washing bleached ROS unfrozen (o) and frozen -20° for 1 day (●) using different concentrations of sucrose in 0.01 M Tris•HCl pH 7.5. Seven 8 mg aliquots of each type of ROS (fresh and frozen) were washed with 10 volumes of the indicated sucrose concentrations and pelleted at 100,000 ×g 1 hr. The pellets were resuspended in 0.25 M sucrose and then assayed according to "Methods. ROS washed in sucrose concentrations ≤ 0.05 M have the same appearance as the sonicated preparation in Figure 12.
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and assayed in a variety of osmolarities of sucrose the CAPDE activity still does not appear cryptic (Figure 14). If one assumes that the plasmalemma and discs are rigorously intact in these ROS preparations during washing so that the CAPDE cannot be removed from within the ROS, then the previous data indicate an external location for CAPDE.

**Recovery of CAPDE and Other Proteins from Isotonically Washed ROS**

The ROS was washed isotonically following the procedure in Figure 15 and the distribution of CAPDE in the various derived fractions is shown in Table 5. Almost all of the CAPDE activity associated with the ROS was removed and recovered with a ten-fold increase in specific activity (Table 5). Figure 2 is an electron micrograph of the intact ROS structure following isotonic washing.

SDS-polyacrylamide disc gel electrophoresis was performed on the fractions derived from the procedure shown in Figure 15 and are shown as densitometric scans (Figure 16) and photographs (Figure 17). The electrophoretic scans in Figure 16 show that four major bands are removed from the ROS upon washing and are concentrated after ultrafiltration (Figure 16c). Bands 2, 3 and 4, present in the ROS (Figure 16a), are seen to be almost absent from the washed pellet (b) but band 1 appears to originate from a trough between two major non-removable bands in the M.W. range 30,000-40,000. Rhodopsin, reported to represent 80% of ROS
Fig. 14.—Crypticity of CAPDE in isotonically washed and resuspended ROS pellets as determined by assaying in the presence of various sucrose concentrations. 12-10 mg aliquots of ROS were first washed in 10 volumes 0.25 M sucrose in 0.01 M Tris-HCl pH 7.5 and pelleted at 100,000 xg 1 hr. Two pellets were each resuspended separately in each of the sucrose concentrations in which they were subsequently assayed. One set (i.e. pellets resuspended in 0, 0.06, 0.12, 0.25, 0.45 and 0.87 M sucrose) was sonicated 2 min. at 0-4° and assayed for protein and CAPDE (Δ) as described in "Methods." The other set was not sonicated (o) and assayed for protein and CAPDE.
Figure 14

Molar Sucrose in Assay

nmoles cAMP hydrolyzed per mg per min ±SD (n=6)
Fig. 15.—Flow chart for isotonically washing ROS and subsequent recovery of eluted protein. All procedures were carried out in dim red light at 0-4° except the ultrafiltration step which was performed at 0-4° in room light. There were no visible pigments in the supernate or concentrated supernate. The Diaflo PM-10 ultrafiltration membrane was designed to concentrate only those components with molecular weights ≥ 10,000.
Unbleached ROS in .9 M Sucrose in 0.01 M Tris HCl pH 7.5 (S/T) + 10 volumes 0.25 M S/T 100,000 xg 1 hour

Unbleached pellet → Supernate

Resuspended in 0.25 M S/T

Concentrated via Diaflo PM-10 ultrafiltration

Concentrated supernate, Filtrate

Figure 15
Table 5.—Flow chart data corresponding to ROS treatment in Figure 15.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total Protein mgs</th>
<th>CAPDE Activity&lt;sup&gt;a&lt;/sup&gt; u/mg/min.±SD(n=3)</th>
<th>Total Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>ROS</td>
<td>179</td>
<td>19.2±2.3</td>
<td>3437</td>
</tr>
<tr>
<td>Pellet</td>
<td>144</td>
<td>3.4±1.5</td>
<td>440</td>
</tr>
<tr>
<td>Concentrated Super</td>
<td>14</td>
<td>252±67</td>
<td>3528</td>
</tr>
<tr>
<td>Filtrate</td>
<td>&lt;1</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>

<sup>a</sup>All values assayed on unbleached fractions.
Fig. 16.—Stacked 1% SDS analytical disc gel electrophoretic pattern for fractions derived from flow chart in Figure 15. (a) unwashed ROS, (b) isotonically washed ROS, (c) concentrated supernatant. M.W. standards were β-galactosidase--130,000; BSA--68,000; trypsin--23,300; γ globulin--50,000.
Fig. 17.—Photograph of actual gels a, b and c derived from procedure in Figure 15 and scanned in Figure 16. a', b' and c' are same fractions electrophoresed 3 hours less than a, b and c to show apparent non-separation of proteins around M.W. region 30-40,000. 40 µg protein were applied to gels a, b, a', b' and 30 µg to c and c'.
protein (4,48), has a M.W. in this region. The intriguing possibility is presented that the ROS contains more than one protein of M.W. 30,000 to 40,000. This may not have been observed by previous investigators due to over-denaturation procedures (4), overloading, overstaining, or insufficient running time (Figure 17). This possibility is being examined more closely using longer gels and the rhodopsin labelling procedure of Papermaster (4). An alternative explanation is that both proteins in this 30,000 to 40,000 M.W. range are rhodopsin each containing different amounts of bound lipid.

Experiments on CAPDE Binding to ROS

Attempts were made to evaluate the possibility that a contaminant CAPDE originating from other retinal cell types might adventitiously bind to the ROS during the purification procedure. Figure 18 and Figure 19 show the effects of isotonically washing the ROS with various concentrations of EDTA or MgCl₂. Although the various EDTA concentrations (0-10 mM) appear to have little effect on the washing pattern (Figure 18) MgCl₂ concentrations above 5 mM cause about 60% of the CAPDE activity to remain with the ROS during the one hr., 100,000 xg centrifugation period (Figure 19). Prebleaching the ROS did not affect CAPDE activity (Figure 19). Such applied variations in ionic strength have been used in the past to remove or bind
Fig. 18.—Effect of washing ROS in 0.25 M sucrose-0.01 M Tris·HCl pH 7.5 containing various EDTA concentrations. Six 13 mg aliquots of unbleached ROS were washed with 10 volumes 0.25 M sucrose-tris containing indicated concentrations of EDTA and pelleted at 100,000 xg for 1 hr. Pellets were resuspended in 0.25 M sucrose-tris containing no EDTA and assayed as described in "Methods."
Figure 18

Unwashed ROS activity

nmol cAMP hydrolyzed per mg per min ±SD (n=3)

mM EDTA in 0.25M Sucrose wash
Fig. 19.—Effects of washing ROS in 0.25 M sucrose-0.01 M Tris·HCl pH 7.5 containing various MgCl$_2$ concentrations. Sixteen 5 mg aliquots of unbleached ROS were taken: 8 aliquots were bleached (○) and 8 left unbleached (●), and each was washed in 10 volumes 0.25 M sucrose-tris containing indicated MgCl$_2$ concentrations and pelleted at 100,000 xg 1 hr. Pellets were resuspended in 0.25 M sucrose-tris containing no MgCl$_2$ and assayed for protein and CAPDE as described. CAPDE assays were performed under dim red light.
Lowry protein on washed, resuspended ROS contained 5 mg/mL

ROS-PDE sp activity before treatment

nMoles cAMP hydrolyzed/mg/min ±SD (n=2)
plasmalemma-bound ATPases (44,45), and other enzymes (50) although little success has been obtained in rebinding 100% of the removed enzyme.

That the observed increase in CAPDE activity of the MgCl$_2$-washed ROS cannot be due simply to an activation of the CAPDE itself is shown in Figure 20. The fact that the ROS CAPDE does not increase with increasing concentration of added MgCl$_2$ points to the possibility of adequate levels of endogenous MgCl$_2$. Hendricks et al. (16) recently found millimolar concentrations of both Ca$^{++}$ and Mg$^{++}$ in frog ROS. Also Russell and Pastan (46) found CNPDE activity in fibroblasts in the absence of added Mg$^{++}$ which could be removed by EDTA and restored by adding back Mg$^{++}$. Inhibition of CAPDE by EDTA concentrations greater than 0.05 mM (Figure 20) seems to attest to the requirement by the enzyme for a divalent cation. The EDTA and MgCl$_2$ have little effect on the 5'nucleotidase (Figure 21) which was used as a coupling enzyme in the CAPDE assay (see "Methods").

Experiments were performed to see if commercially available CAPDE could be added to the crude ROS (Figure 1) before gradient application and shown to associate with the gradient ROS band in a way similar to what one might expect for a naturally occurring contaminant. One gradient was assayed for CAPDE, rhodopsin and protein without additions (Figure 22); a second contained added CAPDE (Sigma) (Figure 23); and a third contained added $^{125}$I-CAPDE
Fig. 20.—Effect of various concentrations of EDTA and MgCl$_2$ on ROS CAPDE. Assays were performed as described in dim red light on unbleached ROS. Assays contained either EDTA with no added MgCl$_2$ (○) or MgCl$_2$ with no EDTA (●).
Figure 20

nmoles cAMP hydrolyzed per mg per min ± SD (n=3) vs. mM EDTA or MgCl$_2$ in reaction.
Fig. 21.—Effect of EDTA and MgCl$_2$ on commercial 5'nucleotidase. 5'nucleotidase was assayed as described in "Methods." EDTA (△) and MgCl$_2$ (■) were present only in the second stage. As described in "Methods" 5'nucleotidase was always introduced into the second stage incubation mixture in the presence of 50 mM MgCl$_2$. 
Figure 21

nmoles of $^3$H Adenosine formed/mL/20 min $\times 10^{-2}$ (n=3)

mM reagent in second stage incubation
Fig. 22.--Analysis of final continuous sucrose density gradient with no additives. Fractions were collected and assayed as in Figure 10.
Figure 22

--- mg Lowry protein per ml

--- cAMP PDE activity units/ml x 10^-2

--- Unbleached Rhodopsin Concentration, nmoles per ml x 10^-2
Fig. 23.--Gradient analysis as in Figure 22 except that 10 mg commercial CAPDE was added to the crude ROS (Figure 1) before homogenization and gradient application.
(Figure 24). A comparison of the three gradients shows that the added CAPDE (Figure 23) and $^{125}$I-CAPDE (Figure 24) tend to concentrate in fractions preceding and overlapping the ROS-containing fractions through not in precisely the same pattern as the CAPDE found associated with the isolated ROS (Figure 22). Figure 24 shows that about 50% of the added $^{125}$I-CAPDE remained associated with the ROS fractions (3-10) after centrifugation but only 25% of the $^{125}$I-CAPDE descended to the same point on a blank gradient containing no crude ROS. These data point to the possibility that any soluble enzyme released during ROS isolation (Figure 1) could contribute contamination to the ROS band on the gradient although this possibility is also dependent upon the soluble contaminants centrifuging with the 2100 xg pellet preceding gradient application (Figure 1).

The Effects of Light and ATP on CAPDE

Efforts were directed at reproducing the reported ATP-dependent light activation of CNPDE (32). Initial attempts to show light activation in various ROS-containing preparations were negative (Figure 25). ATP seems to inhibit CAPDE at concentrations greater than 0.5 mM, a result consistent with other neural CAPDE (47) and partially consistent with the results of Chader et al. (36,37) who reported slight stimulation of bovine ROS CAPDE by light in the presence of 0.1 mM ATP but complete inhibition in the dark and light by millimolar ATP.
Fig. 24.—Gradient analysis as in Figure 21 except for addition of $^{125}$I-CAPDE as prepared in "Methods." Data are also included for distribution of $^{125}$I-CAPDE added to a blank gradient.
Cyclic Phosphodiesterase $^{125}$I on gradient with crude ROS

Cyclic Phosphodiesterase $^{125}$I on blank gradient

Unbleached rhodopsin concentration

Figure 24
Fig. 25.—Apparent effect of ATP on CAPDE activity on bleached (open symbols) and unbleached (closed symbols) preparations containing ROS. Assays are as described in "Methods" using indicated ATP concentrations present in both stages of assay; T-2 from Figure 3 (Δ, Δ); retinal shakenate from Figure 3 (■, □); ROS from Figure 1 (●, ○). All fractions were sonicated 2 min. at 0-4° prior to assay. Results similar to above were obtained on ROS-containing fractions assayed as unsonicated or hypotonically lysed.
Figure 25

The graph shows the effect of different mM ATP concentrations on the nmoles cAMP hydrolyzed per mg per min ± SD (n=3). The concentration of ATP ranges from 0 to 10 mM, while the nmoles cAMP hydrolyzed per mg per min ± SD values are depicted on the y-axis.
The interpretation offered by Bitensky (32 and personal communication) of the inability of this investigator and others to demonstrate light effects on ROS-CAPDE is that bovine eyes (since they cannot be dark adapted overnight prior to enucleation as can laboratory-kept frogs) contain at least 2% bleached rhodopsin which results in a full light activation of CAPDE (see "Literature Review"). Bitensky further claims that this disproportionality of rhodopsin bleaching and CAPDE activation necessitates use of infrared illumination in ROS isolation and assay since traditionally used dim red illumination bleaches significant amounts (i.e. > 2%) of rhodopsin. Table 6 shows, however, that no significant amount of rhodopsin is experimentally bleached even by 24 hrs. exposure to dim red light.

The light stimulation of CAPDE reported by Bitensky et al. (32) requires ATP. Therefore, the effects of ATP on the coupling enzyme, 5'-nucleotidase, which is used in the CAPDE assays was studied (Figure 26). Bitensky's group would not have had this problem since they had been using thin layer chromatography of unhydrolyzed cyclic nucleotide (52). Inhibition of 5'-nucleotidase at ATP concentrations greater than 0.5 mM was discovered. This apparently explains difficulties in reproducing light stimulation of CAPDE in the presence of ATP. It was at this point that the alternative assay (see "Methods") was
Table 6.—Bleaching of ROS as a function of time in dark, dim red light or Kodak Safelight. Spectra were recorded in the Cary 14 Spectrophotometer, using the procedure in "Methods" described for determining unbleached rhodopsin. ROS concentration was 6.9 mg/ml as prepared, but was diluted to 2.07 mg per 3 ml total vol. in the cuvette, in the presence of 0.1 M NH$_2$OH•HCl, pH 7.0, and 1% Triton X-100. A$_{498}$ was derived from difference spectra of bleached and unbleached rhodopsin, and a value of 40,600 was used for the extinction coefficient of rhodopsin.

<table>
<thead>
<tr>
<th>Time (hrs.)</th>
<th>Nmoles Unbleached Rhodopsin per mg Folin protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dark$^a$</td>
</tr>
<tr>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>5</td>
<td>12</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>24</td>
<td>9</td>
</tr>
</tbody>
</table>

$^a$ROS were maintained in 15 ml beaker on ice in closed black ice bucket.

$^b$Ice bucket was open, 3 ft. below 2 25 W, 115 vac GE red bulbs.

$^c$Ice bucket was open, 3 ft. below 25 W, 125 vac GE frosted bulb filtered by Kodak Safelight Filter No. 1.
Fig. 26.--Effect of ATP on the commercial 5' nucleotidase used in the second stage incubation in the assay of CAPDE. 5' nucleotidase was assayed as described in "Methods."
Figure 26

nmoles $^3$H Adenosine formed per ml per 20 min $\times 10^2 \pm$ S.D.(n=3)

mM ATP in second stage incubation
developed based upon chromatographic separation of unhydrolyzed cyclic nucleotide. All data in Part II of Results derive from this alternative assay procedure.
RESULTS: PART II

Effect of Illumination on CNPDE Activity

Figure 27 shows the effect of illumination, in the presence of 1 mM ATP, on CNPDE in three different ROS preparations. In one ROS preparation all the cGMP was hydrolyzed within 30 sec. after the light was turned on while the slowest effect seen took about 5 min. to hydrolyze an equivalent amount of cGMP. When cAMP was used as substrate (2 mM) no light stimulation was observed. Bitensky's group (32) reported light stimulation with both cAMP and cGMP in frog ROS. Optimal ATP concentration for the light activation of CGPDE is about 1 mM (Figure 28) which agrees well with the 0.75 mM value reported by Bitensky's group (32) for CAPDE. The light activation is proportional to the amount of unbleached rhodopsin experimentally bleached (Figure 29) which contradicts the results of Bitensky et al. (32) who showed maximal light activation of CAPDE in frog ROS after 2% of the rhodopsin was bleached.

Kinetic Studies on CNPDE

Lineweaver-Burk plots using cAMP and cGMP as substrates for unbleached ROS-CNPDE reveal one $K_m$ of 0.22 mM for cGMP (Figure 30) and two $K_m$'s of 0.05 and 5.0 mM for
Fig. 27.—Effect of illumination on CNPDE activity in 3 different ROS preparations. "Dark" incubation for first 8 min. was performed under dim red light. Assays were conducted as described in "Methods." Reactions were initiated at zero minutes by addition of ROS. Reaction vol. was 5 ml, and aliquots were removed and boiled at the times indicated. Illumination was provided by a 20 W, 118 vac Westinghouse cool white fluorescent light located approximately 15 cm above reactions vessels. o = prep 1, • = prep 2; Δ, △ = prep 3 in the presence of 2 mM cAMP and cGMP, respectively.
Figure 27

nMoles cyclic-nucleotide hydrolyzed per mg at 30°C x 10^-4 ± S.D. (n=3)

Time (minutes)

LIGHT ON

1 mM ATP present

Substrate: 2mM cGMP

Substrate: 2mM cAMP
Fig. 28.—Effect of illumination on CGPDE activity as a function of ATP concentration. In these experiments, illumination (o) of the ROS was carried out for 10 min. on ice before addition of the ROS to the reaction medium. ● = unbleached ROS. Reactions were performed under dim red illumination, unbleached ROS being assayed for 20 min. and bleached ROS for 6 min. (reaction velocity was constant across the assay interval in both cases). The per cent substrate hydrolyzed was from 5 to 60%. 
**Figure 28**

Moles cGMP hydrolyzed per min per mg at 30°C x \(10^{-2}\) ± S.D. (n=3)

**Graph:**
- **X-axis:** mM ATP in reaction
- **Y-axis:** Moles cGMP hydrolyzed per min per mg at 30°C x \(10^{-2}\) ± S.D. (n=3)
Fig. 29.—CGPDE activity as a function of the amount of rhodopsin experimentally bleached. Two aliquots from the same ROS preparation were taken; one was bleached and added in the appropriate proportion to the unbleached aliquot to make the given percentages. Rhodopsin spectra were performed as described in "Methods." Assays were run for 6 min. under dim red illumination. Maximum per cent substrate utilized was 70% for the 100% bleached point. All assay values fall on the linear part of predetermined time curves.
Figure 29

% RHODOPSIN BLEACHED

nMOLES UNBLEACHED RHODOPSIN BLEACHED PER MG PROTEIN

nMOLES cGMP HYDROLYZED/MIN/MG AT 30°C × 10^2

Figure 29
Fig. 30.—Lineweaver-Burk plot of CGPDE activity. Time curves were run for 20 min. on the unbleached ROS for each concentration of cGMP, and the points on the graph plotted by calculating slopes of the time curves.
$K_m = 0.22 \text{ M}$

Figure 30

MOLES cGMP HYDROLYZED/MIN/MG AT 30\(^{\circ}\)C $\times 10^2$
cAMP (Figure 31). This indicates that there may be at least two different CNPDE's associated with bovine ROS preparations. Bitensky's group (32) reported one $K_m$ of 0.16 mM for cGMP and one of 8 mM for cAMP in frog ROS.

**Effects of Freezing, Storage and Sonication on CGPDE**

Freezing and storing the ROS at different temperatures in the dark did not appreciably alter the dark activity or light response of CGPDE provided that the temperature was sufficiently low (Figure 32, lower two panels). At -20°, however, frozen storage gradually diminished both light and dark activity and refreezing completely abolished the light response (Figure 33, top panel). The different curves at -76° as well as -196° reflect typical variability in CGPDE activity encountered with one ROS preparation assayed on different days. This variation is particularly evident for the nine minute point which falls on the steepest portion of the light stimulated curves. This same variability presents a problem in attempting to carry out kinetic or addition studies on light stimulation.

When the ROS were subjected to sonication for 2 min. on ice, morphology was completely disrupted and only small vesicles remained (Figure 12). At the same time sonication dramatically reduced light stimulation of CGPDE activity and even diminished dark activity (Figure 33).
Fig. 31.—Lineweaver-Burk plot of CAPDE activity. Reactions were run for 20 min. in dim red light using unbleached ROS. For the 8 and 10 mM substrate points, MgCl₂ concentration was increased to 8 and 10 mM, respectively. Per cent substrate hydrolyzed in each reaction lay between 20 and 40% and each value falls within the linear portion of velocity curves. mM ATP was present in the assay.
nmole cAMP hydrolyzed per mg per min at 30°C x 10^4 ± SD (n=3)

Figure 31
Fig. 32.--Effect of freezing the same ROS at various temperatures and for various times. Assays were performed as in Figure 27 for cGMP. • = fresh ROS; o = ROS frozen for 4 days; ▲ = ROS frozen for 20 days. □= ROS frozen at -20° for 20 days, thawed and refrozen for 6 days at -20°. △ = ROS frozen for 35 days.
Fig. 33.—Effects of sonication of ROS on the light-stimulation of CGPDE activity. All reactions were performed under dim red light; all reagents were preincubated at 30° for 2 min. prior to initiation of the assay by addition of the ROS. Sonication was carried out for 2 min. on ice with a Fischer ultrasonic probe at maximum setting. ● = sonicated, unbleached; ○ = sonicated, prebleached; ▲ = unsonicated, unbleached; Δ = unsonicated, prebleached.
CAPDE activity decreased only about 20% after the ROS was sonicated for 2 min. (Figure 11.)

**Time Lag in CGPDE Activity After ROS Addition**

When all other components of the reaction mixture had been preincubated, and assay of CGPDE activity was initiated by adding ROS which had been preincubated at the same temperature (30°), a time lag was consistently observed before maximal enzyme activity was achieved. This is apparent in Figure 34, where it can be seen that the time lag applied to both unbleached and prebleached ROS. This effect can also be seen in the lower three curves in Figure 32. Its absence in the top curve may be due simply to insufficient data points at shorter assay times, but may also have been peculiar to the ROS preparation in that experiment. When the ROS was preincubated with other reaction components and the assay was initiated by addition of cGMP, the time lag in CGPDE activity was not observed. The time lag may be associated with osmotic swelling of the ROS, accompanying its transfer from approximately 0.9 M sucrose as it comes off the final isolation gradient, to 0.25 M sucrose in the assay medium. Other explanations, including hysteretic activation of the enzyme, are possible, however.
Fig. 34.—Time curve of CGPDE activity for pre-bleached (○) and unbleached (●) ROS. Reactions were initiated by adding preincubated, bleached or unbleached ROS. Assays were performed in dim red light. Bleaching at 4° preceded preincubation, which was carried out for 2 min. in the Dubnoff shaker at the same temp. (30°) at which assays were subsequently conducted.
Figure 4

nMoles cGMP hydrolyzed per mg protein at 30°C ± SD. (n=3)
Comigration on Density Gradient of CGPDE and Rhodopsin

CGPDE was assayed on fractions taken from the linear sucrose density gradient on which the ROS is finally purified, as was done with CAPDE in Part I of Results (Figures 22-24). In Figure 35 both dark and light-stimulated CGPDE can be observed to peak on the principal rhodopsin fraction, and this is especially dramatic in the case of the light-stimulated activity.

Isotonic Washing of the ROS

A flow chart describing the procedures for washing the ROS in 0.25 M sucrose appeared in Figure 15. CGPDE activity was assayed on unwashed and washed ROS, as well as on the reconcentrated supernatant fluid from the wash and a combination of washed ROS plus concentrated supernatant. The data presented in Figure 36 and Table 7 make it clear that only the unwashed ROS retained the light-sensitive CGPDE activity, washing having abolished light-sensitivity in the derivative fractions. Data previously shown in Part I indicated CAPDE activity was fully recovered in the reconcentrated supernate but here the data from Table 7 and Figure 36 show that CGPDE activity was only 60% recovered (1680/2812). In both cases, however, activity in the washed pellets was less than 7% of the unwashed ROS. Whether differences in recovery of CAPDE and CGPDE activities reflect different enzymes cannot be evaluated
Fig. 35.—Analysis of density gradient CGPDE distribution. Unbleached ROS was assayed for 20 min., bleached ROS for 3 min. Reaction velocity was linear within these assay times.
Lowry protein per ml

cGMP PDE activity units/ml $\times 10^{-2}$

Unbleached Rhodopsin Concentration, nmoles per ml $\times 10^{-2}$
Fig. 36.—Time curves for various fractions derived from the ROS following the flow chart in Figure 15. CGPDE is plotted as assayed before and after illumination at the indicated points. Assay method was as in Figure 27 for cGMP. Washed ROS were assayed both in isotonic sucrose (●) and in hypotonic sucrose (Δ). Light microscopy of the ROS plus hypotonic (Δ) assay media after the 25 min. incubation showed that the ROS had swelled and burst, leaving only very small membranous vesicles whereas in the other isotonic assays (●, ○, △) the ROS were still intact.
Figure 36

**LIGHT ON**

- Concentrated supernate wash
- ROS untreated
- Washed ROS + concentrated supernate, 1/1 v/v
- ROS washed with 10 volumes 0.25M sucrose in 0.01M Tris HCl pH 7.5
- ROS washed with 10 volumes 0.25M S/T but assayed in 0.04M S/T

nMoles cGMP hydrolyzed/mg protein at 30°C ± S.D. (n=3)

Time in minutes
Table 7.—Data derived from fractions shown in Figure 36.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total mg Protein</th>
<th>Cyclic GMP Phosphodiesterase Activity&lt;sup&gt;a&lt;/sup&gt;</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Cyclic GMP</td>
<td>Phosphodiesterase Activity&lt;sup&gt;a&lt;/sup&gt;</td>
<td>DARK</td>
</tr>
<tr>
<td>ROS</td>
<td>76</td>
<td>37</td>
<td>465</td>
<td>2812</td>
</tr>
<tr>
<td>Pellet</td>
<td>66</td>
<td>0</td>
<td>32</td>
<td>0</td>
</tr>
<tr>
<td>Concentrated Super</td>
<td>4</td>
<td>420</td>
<td>420</td>
<td>1680</td>
</tr>
<tr>
<td>Filtrate</td>
<td>3</td>
<td>0</td>
<td>0</td>
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</tr>
</tbody>
</table>

<sup>a</sup>Activity determined from slopes of time curves shown in Figure 36.

<sup>b</sup>CGPDE was washed off unbleached ROS.
with the present data. The residual activity of CGPDE in the washed pellet was unaffected by assaying it in hypotonic sucrose, as opposed to isotonic sucrose in the other assays.

Failure to reconstitute CGPDE light sensitivity by adding back the concentrated supernatant fraction to the washed ROS pellet can also be visualized in Figure 36. Light microscopy revealed no differences in morphology of the unwashed and washed ROS. Both preparations contained truncated ROS fragments, many rolled into doughnut shape by earlier homogenization. Similarly, electron microscopy revealed no obvious differences, both preparations appearing as in Figure 2. After assay in the presence of 0.25 M sucrose, ROS fragments still appeared as in Figure 2.

It should be pointed out here again that preboiled ROS, as well as unboiled, unbleached and bleached ROS were incubated with tritiated cAMP and cGMP (Table 4, Part I). No consistent differences were observed between boiled and unboiled ROS with respect to labeled nucleotides recovered from ROS pellets after incubation. The greatest recovery was less than 2% of the total nucleotide available, whereas in typical illuminated assays (Figure 27) the entire substrate was hydrolyzed within at most 5 min. This outcome appears to rule out significant cyclic nucleotide transport to the interior of the ROS during the assay period.
DISCUSSION AND CONCLUSIONS

The association with isolated bovine photoreceptor outer segments of cyclic nucleotide phosphodiesterase (EC 3.1.4.17) activity has been confirmed, as has been previously reported by other groups for frog (31,32), human (35) and cow (33,34,37) ROS. That this CNPDE activity associated with bovine ROS is composed of at least two distinct CNPDE's may be implied by the fact that there are two $K_m$ values for cAMP (Figure 30). One of these enzymes may also hydrolyze cGMP.

These ROS preparations have previously been shown to exhibit a linear osmotic response to sucrose, KCl and other solutes in the isotonic concentration range (53), implying that the plasmalemma is intact. The ROS ultrastructure also shows the plasmalemma to be intact (Figure 2) and other studies (54,55) have shown that a soluble protein kinase is retained by the ROS isolated using this procedure. Therefore if one assumes that the plasmalemma is rigorously impermeable to the isotonic washing media, as well as to the CNPDE enzymes, the following facts may be interpreted to mean that the CNPDE enzymes are external to the ROS (i.e., either externally soluble or loosely plasmalemma bound): (1) The ratio of CAPDE-specific activity
to rhodopsin concentration in the ROS varies five fold (Table 2 vs. Table 3), thus implying that the enzyme can move onto or off of the plasmalemma; (2) CNPDE assays can be conducted in isotonic media (0.25 M sucrose) where there is negligible substrate penetration into the ROS (Table 4); (3) CNPDE activity does not appear to be cryptic [i.e., CNPDE activity does not appear to be located within substrate-impermeable membrane enclosures (Figures 11, 12, 13, 14, 33 and 36)]; (4) Added CAPDE can be easily bound to the ROS (Figures 21, 22 and 23); (5) CNPDE activity can be washed off the ROS with isotonic sucrose and quantitatively recovered (Figures 15 and 36; Tables 5 and 7) without observable light microscopic or ultrastructural changes (Figure 2). Whether or not the CNPDE enzymes as assayed are native to the ROS remains to be determined.

On the other hand, if one assumes a perforated plasmalemma it is conceivable that CNPDE might be removed by isotonic washing. Previous studies (53) have shown that the ROS discs in our ROS preparations remain impermeable to sucrose and other solutes under isotonic conditions so that the possibility of an intradiscal location for CNPDE appears unlikely.

One of the most important points in this work is the confirmation for bovine ROS of light-activated, ATP-dependent cyclic GMP phosphodiesterase activity as has been reported by Bitensky's group (32) for frog ROS. Use
of 5' nucleotidase as a coupling enzyme in assaying CNPDE activity is contraindicated because ATP, which is required at 1 mM concentration for demonstration of light-stimulated activity, also inhibits nucleotidase activity (Figure 26). Light sensitivity is found in the cGMP hydrolyzing enzyme but not, to date, in the cAMP enzyme. CGPDE light sensitivity is sigmoidally related to the amount of rhodopsin bleached experimentally, a fact radically different from results of Bitensky's group (32), who claim that a 2% bleach fully activates CNPDE. Structural integrity also appears to be important in determining enzyme activity, since sonication dramatically diminishes both light sensitivity and dark activity.

The fact that an ROS-unrelated soluble enzyme (commercial beef heart CAPDE) shows "binding" to the ROS during our isolation procedure (Figures 23 and 24) suggests that soluble enzymes released from other retinal cells or from the photoreceptor inner segment during ROS isolation might contribute contamination to the ROS. An alternative explanation would be that many CNPDE's share enough common characteristics so that this "binding" may exist physiologically. Extensive experimentation is required to resolve the question of the physiological relevance of the light-stimulated CGPDE activity. Certainly, it is legitimate to speculate that this enzyme plays a role in transducing absorption of photons by rhodopsin in the discs into
subsequent alteration of plasmalemma ion permeability. Such a role has been proposed for $\text{Ca}^{2+}$ (56) and recently for $\text{H}^+$ also (53). Both of these ionic species, however, have been identified internal to the ROS, while the location of CGPDE has not. If outside, its implication in transduction would necessarily involve a novel theoretical mechanism. With the demonstration by De Pont, Rotmans et al. (57,58) that isomerization of all-trans to 11-cis retinal does not require the carotenoid to exit the ROS, it may prove equally challenging to link an externally located CGPDE to regeneration of bleached rhodopsin.

Another most fascinating aspect of this work involves the electrophoretic patterns of the ROS preparations (Figures 16 and 17). It has been reported by several laboratories (4,48,59) that rhodopsin comprises 80% of ROS protein, a fact consistent with its role as the light-trap for sensory transduction. However, the electrophoretic procedure described in "Methods" reveals at least two proteins in the M.W. region of rhodopsin (Figures 16 and 17). Further experiments are in progress to determine if indeed one of these proteins is distinct from rhodopsin. If so, the interpretation of the visual system would be altered.
Adenylate Cyclase in Vertebrate Retina

RELATIONSHIP TO SPECIFIC FRACTIONS AND TO RHODOPSIN

(Received for publication, November 12, 1973, and in revised form, January 2, 1974)

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SUMMARY

Specific activity of adenylate cyclase was examined in various fractions derived from retinal homogenates or retinas shaken in sucrose-Tris, during the isolation of bovine or frog photoreceptor outer segment fragments enriched in rhodopsin. As unbleached rhodopsin concentration rose during fractionation, adenylate cyclase activity declined, although cyclase and rhodopsin were not completely resolved. In those fractions with higher cyclase activity, unbleached rhodopsin concentration was low. Adenylate cyclase activity was confined almost entirely to membranous fractions. Cyclase activity of the outer segment fraction was not affected by bleaching, homogenization, sonication, or freezing at -196°.

A variety of bioenergetic systems have been reported to be present in the retinal outer segment (ROS) including rhodopsin (1), glycerol dehydrogenase (2), NAD-dependent retinol dehydrogenase (3), nicotinamide oxidation-reduction compounds (4-6), nonspecific substrate oxidation (7), both magnesium-activated and Mg + Na + K-activated ATPases (7-9), proteolysis (10), rhodopsin phosphorylation (11-13, 20, 21), calcium translocation (14, 15), protein transfer (16), guanylate cyclase (16), adenylate cyclase (17-19, 22, 23), cyclic nucleotide-dependent phosphodiesterases (17, 25-27), and several proteins (20, 23, 20). Translocation of Na⁺ and K⁺ has also been reported, but as in the case of calcium, difficulties in measurement have prevented agreement on the mechanism or direction of ion movement (42).

The adenylate cyclase system of the ROS has been reported by Hiltunen et al. (17-19, 22, 23) to be unusually active, dramatically inhibited by light, and the per cent inhibition to be proportional to per cent rhodopsin bleached (18, 23). Confirmation of these reports would have important implications for vision, and indeed for sensory transduction in general. The present paper reports the results of our efforts to examine adenylate cyclase activity as a function of fractionation using what Deiter and Karnowsky (43) have called the "analytical approach", which entails making no initial assumptions about the identity of the fractions obtained.

METHODS AND MATERIALS

Detailed methods and materials, including methods for assay for adenylate cyclase and rhodopsin, are given in mimeograph form in the appendix.² Two different methods of retinal fractionation were employed in the present study and for clarity are outlined in Fig. 1. The details of Method I, including electron micrographs and marker enzyme assays, have been previously published (7, 7). The sucrose flotation step in Method II was originally described by Bate (23). To this procedure was added centrifugation at high gravity on a continuous density gradient (11-c) to further purify the sucrose floatate ("top paste," 11-b) which is heavily contaminated with mitochondria and other cell fractions (7, 7). All operations were carried out at 0-4° in dim red light. Prior to all assays, unless otherwise indicated below, 1.5-ml fractions from the steps of each method were stored at -196° in an Xil-16 liquid nitrogen refrigerator (Linde), and retained full cyclase activity for at least 6 months.

Fractions from bovine brain cortes (100,000 X g and 2,000 X g) were prepared using the method of Bradman (33, 34). Activities of these fractions were assayed to determine comparability of the assay method in our hands with the same method used in another laboratory.

RESULTS

Relationship between Adenylate Cyclase Activity and Unbleached Rhodopsin Concentration—As the preparation of purified bovine ROS proceed by Method I, adenylate cyclase specific activity decreased as unbleached rhodopsin concentration increased.

Detailed methods and materials are presented as a mimeograph supplement immediately following this paper. Material published in mimeograph form can be easily read with the aid of a large-field reading glass of a type readily available at most opticians.

References to this paper and the JBC Document Number, the form desired, microfiche or full sized photocopy of 13 pages, and the numbers of copies desired. Orders should be addressed to The Journal of Biological Chemistry, 960 Rockville Pike, Bethesda, Maryland 20014, and must be accompanied by a remittance to the order of the Journal in the amount of $2.50 for microfiche or for photocopy.

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Data reflecting this observation appeared at the stages of the homogenate (Fig. 1, I-A), the 2100 x g pellet (I-D), and the ROS from the gradient (I-F). At these stages, cyclase specific activities were 744, 038, and 373 units per mg, respectively, while unbleached rhodopsin concentrations were 1.2, 2.73, and 0.62 mmol per mg (see Table 1 in the miniprint). If one compares stages between which cyclase specific activities increased, unbleached rhodopsin concentrations at the same stages decreased. Thus, in going from the retinal homogenate (I-A) to the 1100 x g pellet (I-B) cyclase specific activity increased from 744 to 1348, while unbleached rhodopsin concentration decreased from 1.2 to 0.31; in going from the 2100 x g pellet (I-D) to the gradient pellet (I-F), cyclase specific activity increased from 038 to 1107, while unbleached rhodopsin concentration decreased from 2.73 to 0.63. These data indicate that cyclase of higher specific activity and unbleached rhodopsin content did not tend to copurify, though both were found in particulate fractions as the isolation of the ROS progressed. The lowest specific activities of cyclase were measured in the 3100 x g supernatant fraction and in the top fraction of the final gradient. While the 2100 x g supernatant still contained low density membrane fragments (2), the top of the gradient contained only very low density or soluble extracts of membrane fragments. Most of the cyclase activity remained with heavier membranous fragments. These observations support the earlier belief that mammalian cyclases are membrane bound (33).

Method II yielded a "top paste" floatate preferred by some investigators because of a high yield of rhodopsin (see footnote to Table 1 in miniprint) but previously shown in our laboratory to be contaminated with mitochondria and other cell debris (2, 7). Since the method did not entail homogenisation, it could be construed as less likely to disrupt a presumably fragile link between rhodopsin and cyclase activity (33). The red, top paste of Method II contained about one-third of the activity of either the crude "retinal shakenate" or the pinkish brown pellet containing debris (see Table 2 in miniprint). Further separation of the top paste on the continuous sucrose density gradient of Method II caused sedimentation of additional material into a tan pellet at the bottom, the specific activity of which was comparable to the earlier pellet (Fig. 1, II-B) and more than twice as high as that of the ROS at the top of the gradient. Since at no time were these fractions homogenised, that procedure cannot
be held responsible for the observed separation of the more active fraction from the ROS.

Examination of fractions obtained by Method II from dark-adapted leopard frogs revealed higher unbleached rhodopsin content than in bovine ROS (10.4 nmoles per mg of frog ROS, as compared with 0.52 for bovine ROS in Method I). The higher rhodopsin content of the frog ROS is attributable to the fact that frogs are routinely dark-adapted prior to killing, whereas cattle are not. However, essentially the same levels of adenylate cyclase specific activities were observed in frog ROS as in bovine ROS, and once again, a trend was observed toward divergence of fragments with higher cyclase activity from those with greater unbleached rhodopsin content (see Table 3 in miniprint).

Comparison of Adenylate Cyclase Activities among Different Laboratories—Specific activities were generally between 700 and 1600 units per mg for the retinal homogenate or retinal substrate, and between 300 and 800 units per mg for the ROS or top paste. These values are one to two orders of magnitude lower than specific activities reported by Litensky’s laboratory: 7,400 to 78,400 units per mg for frog ROS, and 3,700 to 33,400 units per mg for cow ROS (17). The latter are higher than have been reported in any other tissue known. Gordin et al. (45) reported activities of guanylate cyclase in cow ROS, 23,600 to 41,000 units per mg at 37°, which approach those of Litensky’s group for adenylate cyclase; but Brown and Makman (30) found only 333 units per mg in a calf retinal homogenate. These investigators also found higher activity (31) in the retina of mice which lacked photoreceptors (24). Paunbacker (40) has recently reported adenylate cyclase specific activities for bovine ROS which are comparable to ours, and guanylate cyclase activities an order of magnitude larger. Our values for brain fractions agree closely with those of Braithwaite (33, 35), and were consistently much higher than those determined for the ROS at the same time.

In consideration of the possibility that an activator of adenylate cyclase activity might be removed or an inhibitor unmasked inadvertently during preparative procedures in our laboratory, fractions removed from the ROS fraction during its isolation were added back, one at a time. An expected specific activity was calculated as the weighted mean of the separate activities of the ROS and the added fraction. This procedure resulted in observed activities very close to the activities expected from simple addition of the separate activities to that of the ROS (Table 4 in miniprint).

Effects of Bleaching and Other Treatments—It has been reported by Litensky’s group that adenylate cyclase activity is inhibited 8-fold (17-19, 22, 23) by light and that the decrease in activity is proportional to the per cent of rhodopsin bleached (18). The results of a large number of experiments in our laboratory designed to determine the effects of bleaching and of other possibly related treatments on the adenylate cyclase activity of fresh, unfrozen ROS were essentially negative (Table 5 in miniprint). Bleaching was almost uniformly without effect, either on bovine or on frog ROS, regardless of the method of preparation or the use of sonication, homogenizing, or freezing. Occasional effects of light were never more than those found on fractions of bovine brain. For any fraction, either an “inhibition” or “activation” by light could be expected to occur if a sufficiently large number of experimental replications were conducted. Besides the ROS, the retinal homogenate, the 1100 X g pellet and supernatant fraction, the 1000 X g pellet and supernatant, and the other fractions from the final gradient were also bleached, without effect on adenylate cyclase activity.

Kinetic Analysis—Lineweaver-Burk plots of specific activities (Fig. 6 in miniprint) reinforce the earlier observation that the ROS had uniformly the lowest specific activity of any fraction, and that even the relatively high activity of the initial low-speed pellet was markedly lower than the brain fraction. The kinetic data do not permit differentiation of the cyclases associated with various fractions.

DISCUSSION AND CONCLUSION

The present experiments measured the specific activity of adenylate cyclase and unbleached rhodopsin content associated with various fractions derived from retinal homogenates or shaken preparations, en route to isolation of photoreceptor outer segment fragments enriched in rhodopsin. As unbleached rhodopsin content rose during fractionation, adenylate cyclase activity declined. As is the case in virtually all mammalian adenylate cyclases (39), adenylate cyclase in the principal fractions appeared to be membrane-bound, since only low activity traveled with membrane-depleted fractions. The specific activities of both bovine and frog retinal fractions were generally lower than fractions of bovine brain assayed simultaneously.

These observations applied equally to fresh retinal preparations, to preparations which had been frozen and stored at -190°, and to either fresh or frozen preparations which had been homogenized or sonicated. Adenylate cyclase activity of the ROS was largely unaffected by readdition of fractions removed earlier in the isolation of the ROS. Nor was ROS cyclase activity affected by bleaching.

Although it appears possible that the relatively modest adenylate cyclase activity measured in the ROS is native to the outer segment membrane system (as opposed to a contaminant), it cannot be determined whether the enzyme is associated with ROS discs, plasmalemma, or both. Toward resolving this question, efforts have been initiated in our laboratory to isolate the ROS plasmalemma. Although adenylate cyclase activity is often used as a marker enzyme for plasmalemma fractions (43), this approach is of doubtful value in the case of the ROS, where the rhodopsin-containing disc membranes may represent invaginations of the plasmalemma. Despite these uncertainties it appears possible that the activity measured in the ROS is associated at least with the plasmalemma if not with the discs.

The contention by Litensky’s group (17-19, 22, 23) that adenylate cyclase directly mediates the conversion of light to chemical and electrical energy in the photoreceptor cell is difficult to reconcile with the observations reported in the present study. Not only did bleaching not affect cyclase activity, but the specific activities found in various preparations were as much as two orders of magnitude lower than those reported by Litensky’s group. While one might argue that the present experiments merely failed to capture those conditions which could elicit both high specific activity and light inhibition of an adenylate cyclase activity associated with the ROS, the systematic description of those conditions remains to be provided.

Acknowledgments—Cattle eyes were donated by the Oscar Mayer Company of Madison, Wis. and by the Meat Packing Company of Bay City and Grand Rapids, Mich. The technical assistance of Dr. Blitsensky’s laboratory in the preparation of this manuscript is gratefully acknowledged.

* Cheung (44) has reported such factors for a phosphodiesterase.
assistance of Kofi Amuli, Claire Coley, Hope Crawford, Robert Holmes, Arlene Kovel, James Linnigh, and Linda Stewart is gratefully acknowledged.

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