Properties of a Magnesium- or Calcium-Dependent Adenosine Triphosphatase from Frog Rod Photoreceptor Outer Segment Disks and Its Inhibition by Illumination†

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ABSTRACT: Isolated frog retinal rod photoreceptor disk membranes were found to possess an ouabain-insensitive adenosine triphosphatase (ATPase) which was dependent on the presence of Mg or Ca in the incubation medium. Several tests showed the activity was not of mitochondrial origin, although the level of mitochondrial contamination was about 5%. The level of ATPase activity was optimal at an Mg:ATP ratio of 1:1. Ca supported a lower level of activity in the absence of Mg, showed no optimum Ca:ATP ratio in the range tested, and inhibited ATPase activity when added in the presence of Mg. The released inorganic phosphate was not due to an adenylate cyclase and pyrophosphatase activity. When the suspension of rod disks and fragments was illuminated before ATPase activity was assayed, subsequent activity was reduced by 15% in light-exposed material compared to dark controls when assayed at 37 and 45°. This light-induced reduction in ATPase activity was found with monochromatic 500-nm light as well as with white light. It seems that the state of rhodopsin in the photoreceptor disk membrane is capable of modifying the activity of another membrane protein, the Mg- or Ca-dependent ATPase.

The retinal photoreceptor outer segment is the site of the absorption of light in vision. The rod outer segment contains freely floating membranous organelles in the form of a stack of flattened disks. The membranes of these disks contain, as an integral part, the visual pigment rhodopsin which absorbs light and initiates vision. It has been shown previously that the disks contain a distinct internal space, separate from the intracellular space (Heller *et al.*, 1971) and that the membranes are semipermeable (Heller *et al.*, 1970).

As a part of the biochemical study of these membranous organelles, we performed an electron microscopical and a chemical analysis of the isolated rod outer segment preparation, and then reinvestigated the known adenosine triphosphatase (ATPase)[1] activity of the preparation. This paper deals with the characteristics of the (Mg–Ca)-dependent, ouabain-insensitive ATPase first described by Bonting *et al.* (1964) and Frank and Goldsmith (1965).

In most respects our results confirm and extend those already described. However, we found a distinct change in ATPase activity upon illumination, in contrast to the results of either Bonting *et al.* (1964) or Frank and Goldsmith (1965).

Methods and Materials

Rod outer segments (ROS) were prepared fresh each day from *Rana pipiens* which were dark adapted overnight, as described previously (Heller *et al.*, 1971). For experiments in imidazole buffer, the purified outer segments were washed three times, after the sucrose isolation procedure, in 60 mM KCl, 20 mM NaCl, and 10 mM imidazole buffer of the pH to be used in the subsequent experiment. For most experiments in maleate, or in other buffers, the rod outer segments were washed in the above salts with no added buffer. Experiments were performed in 30 mM KCl, 10 mM NaCl, 0.1 mM ouabain, and noted amounts of $CaCl_2$, $MgCl_2$, NaATP (pH 7.2), and buffer. Sonication was performed with a Bronwill Biosonick III Sonifier, for 2 min at 0° at the top energy of the 4-mm probe.

ATPase activity was assayed by the method of Marsh (1959), modified by reducing all volumes by one-third. Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard. Unless otherwise noted, experiments were run at 37 ± 0.2°. The reaction was stopped by placing the incubation tubes on ice and immediately adding trichloroacetic acid (40%) to a final concentration of 6.7%.

Illumination with white light was from a 100-W incandescent lamp for 1 min, which was more than adequate to "bleach" the sample. Subsequent operations on the illuminated samples were carried out under dim red light along with "dark" samples. Temperature rise during the illumination was less than 0.5°, and subsequent incubation was controlled at 37°.

Illumination at 500 or 400 nm was carried out in a Beckman DU spectrophotometer with a slit width of 2.0 mm, which gives a half-intensity bandwidth of 20 nm at 400 and 40 nm at 500 nm. Both parts of the sample were kept at ice temperature during illumination. The 400- and 500-nm samples received equal amounts of energy. Dark samples were exposed to the normal dim red illumination of the working lights (cutoff at 650 nm). The white light and 500-nm illuminated samples were "bleached" after illumination, while the sample illuminated at 400 nm lost about 10–15% of the absorption at 500 nm.

Mitochondria were purified from all the material remaining after isolation of the ROS. This material was homogenized

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[1] Abbreviations used are: ROS, rod outer segment(s); ATPase, adenosine triphosphatase; $CetMe_3NBr$, cetyltrimethylammonium bromide; ADP, adenosine 5′-diphosphate.

in 0.4 M sucrose with a Tri-R homogenizer (New Jersey) with a Teflon pestle as described for isolation of ROS (Heller *et al.*, 1971). After homogenization the material was centrifuged at 2000*g* for 15 min and the supernatant was removed. The supernatant was centrifuged for 15 min at 13,000*g* and the pellet thus formed (mitochondria) was washed in the same media as the ROS being prepared.

Cytochrome oxidase activity was assayed by the method of Wharton and Tzagoloff (1967). In some experiments the mitochondria were either sonicated or suspended in 0.1% Emulphogene BC-720 before assaying. Malic dehydrogenase activity was assayed according to Ochoa (1955).

Nucleotide triphosphates (Sigma) were used without further purification except in low Ca concentration experiments where ATP was decalcified by a passage through a column (0.9 × 13 cm) of Dowex 50-X8 (Na form) which was eluted with H_2O. Ouabain and mersalyl (Salyrgan) were from Sigma. $CetMe_3NBr$ was from Eastman and Emulphogene BC-720 from GAF Industries. Cytochrome *c* was from Sigma and prepared according to Wharton and Tzagoloff (1967). DPNH was from Sigma.

To test if pyrophosphate ($Na_2P_2O_7$) might be recorded as orthophosphate by the assay used, $Na_2P_2O_7$ was added to a typical medium (30 mM KCl, 10 mM NaCl, 1 mM $MgCl_2$, 0.1 mM ouabain, and 30 mM maleate, pH 7.6) in the presence or absence of ATP and/or ROS. Incubation was for 10 and 45 min in darkness at 37°. For sucrose gradient density centrifugation, ROS and mitochondria were prepared as usual, washed in 10 mM $CaCl_2$ and 10 mM $MgCl_2$, and then applied in 0.2 ml of the same to the gradient. The gradients were linear from 45 to 30% sucrose, 45 to 25% sucrose, or 45 to 10% sucrose, with 10 mM $MgCl_2$ and 10 mM $CaCl_2$ added. Volume was 5.7 ml. All operations were performed at 4° and material was kept in darkness until after centrifugation. Material was centrifuged for 2 hr in an S39 rotor in a Beckman L2 ultracentrifuge at 40,000 rpm. Fractions were collected from the bottom of the tube and protein was determined on all fractions. Cytochrome oxidase was measured in those fractions with significant amounts of protein.

For electron microscopy, purified ROS or homogenized retinas were prepared for analysis according to the method of Worsfold *et al.* (1969). This method yields a sample adsorbed onto a Millipore filter, which is random in its distribution of constituents in both horizontal and vertical directions. Thin sections were cut from several areas of the samples and stained with lead and uranium. Fifteen different areas, each including the full thickness of the pellet, were photographed with a Siemens Elmiskop 1A electron microscope and printed at the same magnification (19,500×). The part of each print which showed tissue was cut out, and from this cutout the areas identified as mitochondria were removed. The two fractions (tissue minus mitochondria and mitochondria) were weighed, and from these weights the percentage contamination of the sample by mitochondria was calculated. Care was taken to pick areas which were within the same section, from at least 5 μ away in the block, or from different blocks. This prevented the same area from being counted twice.

Results

Purity of ROS Preparations. The experiments reported in this paper are concerned with an enzymic activity (ATPase) which is distributed in varying amounts throughout almost all tissues. Therefore it was of some concern to establish the degree of contamination of ROS preparations by other particulate tissue fractions, primarily mitochondria. Two approaches were used to establish the extent of contamination of ROS preparations by mitochondria. (1) A study by electron microscopy of ROS pellets prepared in a way that assures complete randomness of all constituents was used. In other words, a section taken through any particular area of the pellet would be a true representation of the pellet as a whole. (2) A study of enzymic activities of ROS preparations was used. Cytochrome oxidase and malic dehydrogenase were chosen because they are probably found only in mitochondria (Schnaitman and Greenawalt, 1968) and, thus, would give a quantitative estimate of mitochondrial contamination of the ROS preparation.

An analysis of the electron micrographs has shown that purified ROS pellets consist predominantly of membranes that can be identified as fragments of the disk membranes (Figure 1). To obtain a quantitative estimate of the degree of contamination of ROS preparations by material other than the disk fragments, the areas of the electron micrograph that contained elements identified as mitochondria were cut out, and their weight was compared to the weight of the areas which showed only disk fragments. Using this approach the degree of contamination of ROS preparation by observable mitochondrial material was found to be 0.3%.

When specific activity of cytochrome oxidase of retinal mitochondria purified by sucrose gradient was compared to the specific activity of cytochrome oxidase in ROS preparations, assuming that all the cytochrome oxidase activity is of mitochondrial origin, the degree of mitochondrial contamination of ROS was of the order of 5%. Similar results were obtained when the specific activity of malic dehydrogenase was compared in the ROS and mitochondrial fractions.

ATPase Activity of ROS. When purified ROS were incubated in a medium containing ATP, Mg, Ca, K, Na, a buffer, and ouabain (to inhibit (Na–K)-ATPase activity), P_i was released into the medium. This release was linear for at least 60 min at 37° and was directly proportional to the amount of ROS added (Figure 2). Sonication had no noticeable effect on the release of P_i (Figure 2).

The release of P_i, or ATPase activity, was completely dependent on the presence of Mg or Ca in the assay medium (Figures 3 and 4). The optimal concentration ratio of Mg to ATP was 1:1. Higher concentrations of Mg relative to ATP were inhibitory (Figure 3). Ca could also support ATPase activity, although less effectively than Mg at ratios of divalent cation: ATP of about 1:1 or less. However, while Mg was inhibitory at concentration ratio of Mg:ATP greater than 1:1, higher Ca concentrations caused increased P_i release throughout the range of concentrations employed (Figure 3).

Using a Mg:ATP concentration ratio of 1:1 and varying the concentration of both from 10^{-5} to 10^{-2} M, a linear relationship between $1/v$ and $1/[S]$ (Lineweaver–Burk plot) was obtained, yielding a K_m of 4×10^{-4} M and V_m of 4.8 μmol of P_i released per mg of protein per hr at 37°.

Effect of Buffers and pH. The ATPase activity was dependent on pH, increasing from pH 6.0 up to about pH 8.3. The ATPase activity was influenced by the buffer system used. As shown in Table I, maleate gave the highest ATPase activity and was therefore used exclusively in later experiments.

Activity in the Presence of Various Nucleotide Triphosphates and ADP. As shown in Table II, the P_i released by ROS was highest in the presence of ATP. The other nucleotide triphosphates served as poorer substrates compared to ATP. This series of substrate effectiveness in the ATPase reaction is similar to the one reported by Frank and Goldsmith (1965).

FIGURE 1: Electron micrographs of crude retinal homogenate and typical pure ROS. See Methods for details. (A) Homogenized whole retina. (B) Purified ROS membranes. Abbreviations usedare: M, mitochondria; N, nucleus; D, disks. Scale = 1 μ. Magnification = $\times$19,500.

Added ADP (ADP:ATP, 0.4:1.0) reduced P_i release to about 30% of uninhibited levels (*i.e.*, ATP only (Table II).

Dependence on Temperature. No P_i was released in 60 min at 0°, while at 45° release was virtually linear for 1 hr at a rate of about 5.6 μmol of P_i released per mg of protein per hr (Figure 5). Using these data, an activation energy for ROS ATPase of about 9.6 kcal/mol can be calculated from an Arrhenius plot (in 30 mM sodium maleate, pH 7.6). Kadota *et al.* (1967) found that the relative efficacy of equal concentrations of Mg or Ca in stimulating ATPase activity in brain

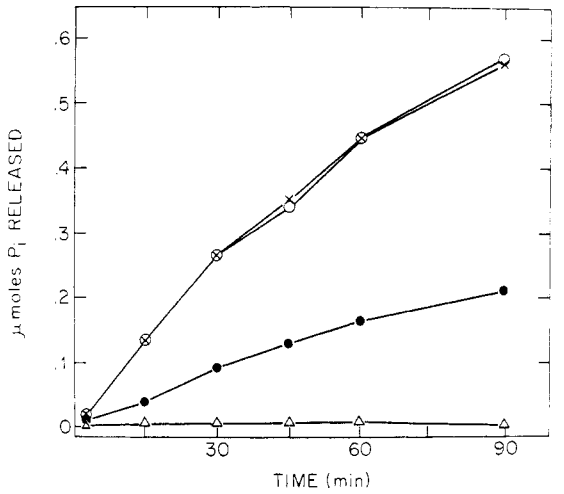

FIGURE 2: ATPase activity of ROS; phosphate released as a function of incubation time. Conditions of incubation were 2 mM ATP, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 80 μM ouabain, 30 mM KCl, 10 mM $NaCl_2$, and 30 mM sodium maleate (pH 7.6) at 37°. (△) No ROS; (●) 38 μg of protein/ml; (×) 152 μg of protein/ml; (○) same as (×) but sonicated ROS.

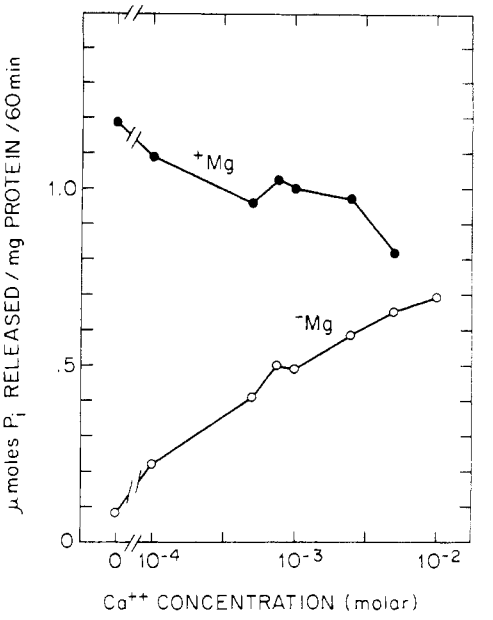

FIGURE 4: ATPase activity of ROS as a function of $CaCl_2$ concentration in the presence or absence of 2 mM $MgCl_2$. Conditions of incubation were 2 mM ATP, 0.1 mM ouabain, 30 mM KCl, 10 mM $NaCl_2$, and 30 mM imidazole buffer (pH 8.0) at 37°. (○) No $MgCl_2$ added; (●) 2 mM $MgCl_2$ present.

synaptic vesicles was temperature dependent; *i.e.*, while at 37° Mg was much more effective in stimulating activity than was Ca, at 20° the two were equal. Comparison of ATPase activity at 20 and 37° in this ROS preparation showed no such dependence on temperature; Mg remained more effective than Ca at both temperatures, and to about the same degree.

Inhibition of ATPase Activity. Mersalyl, an organic mercurial, inhibited the ATPase up to about 70% (in maleate buffer). The inhibition by mersalyl was proportional to the mersalyl concentration up to about 0.5 mM (Table III). This inhibition was not seen in imidazole buffers. Azide also inhibited ATPase activity.

One percent (final concentration) Emulphogene destroyed about 90% of the ATPase activity, and 1% $CetMe_3NBr$ abolished 94% of it. One percent cholate reduced activity by 94%, digitonin by 80%. Freezing and thawing the ROS three times within approximately 30 min led to a 53% loss of activity, while sonication, shown in Figure 2, had no noticeable effect (also Table III).

Product of ATPase Reaction. To ascertain whether the assay employed in this study was specific for orthophosphate, a known amount of pyrophosphate was added to a standard reaction mixture. When 2 μmol of pyrophosphate was assayed, less than 0.01 μmol (0.5%) was recorded as orthophosphate (part or all of which might actually be due to contamination of the added pyrophosphate by orthophosphate). In addition, added orthophosphate standard was quantitatively recovered in the presence of pyrophosphate.

A test was performed to exclude the possibility of a pyrophosphatase activity being present; *i.e.*, to confirm that only the loss of a *terminal* phosphate from ATP would be recorded as orthophosphate. When pyrophosphate was added to a standard ATPase assay of ROS, no additional phosphate release was observed. This was true irrespective of whether ATP was present in the incubation mixture. (The conditions for assay in the absence of ATP included 0.2 mM pyrophosphate and 1 mM $MgCl_2$ (pH 7.6), assuring an excess of Mg

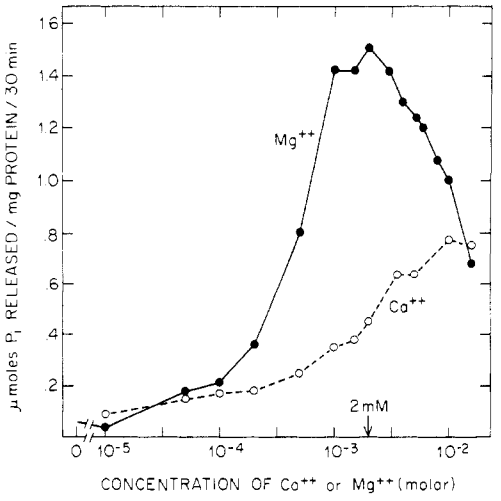

FIGURE 3: ATPase activity of ROS as a function of either $CaCl_2$ or $MgCl_2$ concentration. Conditions were as in Figure 2 except for Ca and Mg concentrations (ATP = 2 mM). (○) Varied $CaCl_2$ concentration in the absence of $MgCl_2$; (●) varied $MgCl_2$ concentration in the absence of $CaCl_2$.

TABLE I: Release of P_i after 60-min Incubation with Various Buffers (30 mM, pH 6.9, 2 mM ATP, 2 mM $MgCl_2$, 0.5 mM $CaCl_2$, and 0.1 mM Ouabain, 37°).

Buffer	μmol of P_i/mg of Protein per hr
Imidazole	3.3
Barbital	3.5
Tris	4.6
Histidine	5.0
Cacodylate	5.1
Maleate	7.8

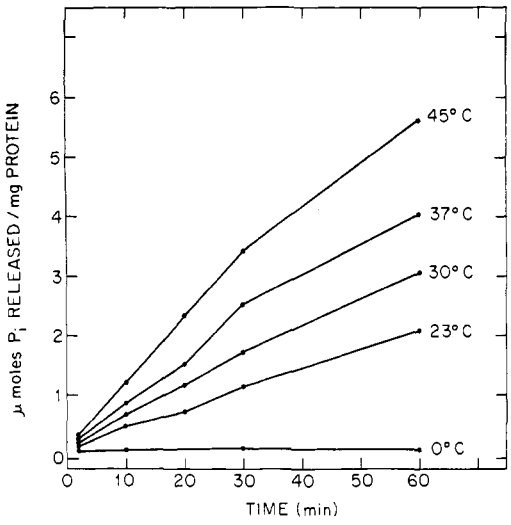

FIGURE 5: ATPase activity of ROS as a function of temperature. Conditions of incubation were 2 mM ATP, 0.5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 30 mM KCl, 10 mM $NaCl_2$, 80 μM ouabain, and 30 mM sodium maleate (pH 7.6).

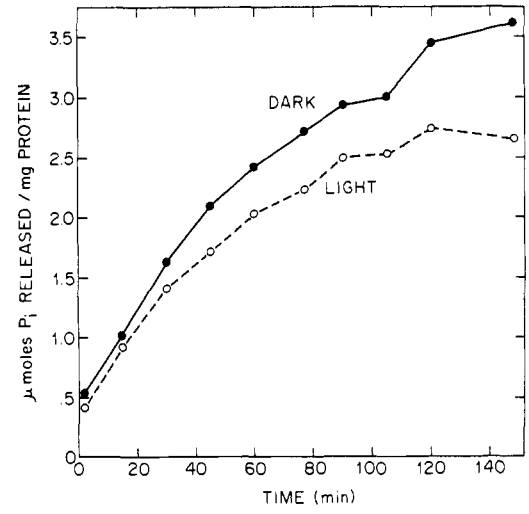

FIGURE 6: ATPase activity of ROS before and after exposure to 500-nm light. Conditions of incubation were 2 mM ATP, 0.5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 30 mM KCl, 10 mM NaCl, 0.1 mM ouabain, 30 mM imidazole buffer (pH 7.6) at 37°. Before incubation was begun, prepared ROS were divided into two parts: one exposed to 500-nm light (see Methods), while the other remained in "dark," *i.e.*, under dim red illumination. After exposure to 500-nm light, ATPase activity was assayed in parallel for 2.5 hr under dim red illumination. (●) No 500-nm exposure; (○) after 10-min exposure to 500-nm light.

which is known to be required for optimal activity by some pyrophosphatases (Irie *et al.*, 1970).)

This series of experiments shows that (a) the assay which was employed in the present study is specific for orthophosphate and (b) that the ROS preparations do not contain a pyrophosphatase under our conditions of assay.

Light Sensitivity. When a preparation of ROS was divided in two, and one half was exposed to white light long enough to "bleach" the rhodopsin present while the other half was kept in the dark, the subsequent ATPase activity of the illuminated fraction was reduced compared to that of the one in the dark. Figure 6 shows the results obtained in a typical experiment in which the illuminating light was a monochromatic light of 500-nm wavelength. This type of experiment was performed under different experimental conditions where the nature of the buffer (imidazole, maleate), the Ca and Mg concentrations, the Mg:ATP ratio, and the wavelengths of the illuminating light were varied.

The light-induced decrease in ATPase activity was observed in the presence of both imidazole and maleate buffers and in media containing both Mg and Ca or Mg alone. The presence of calcium was not necessary in order to observe the difference between illuminated and "dark" ROS ATPase activities. The same difference between illuminated and dark ROS preparations was observed at Mg:ATP ratio of 1:4, 1:2, and 1:1. Illumination with white light or with monochromatic light at 500 nm was equally effective. On the other hand, illumination of ROS preparations with monochromatic light at 400 nm with an equal amount of energy as the 500-nm light did not

TABLE II: Release of P_i with Various Nucleotide Triphosphates.[a]

NTP	μmol of P_i/mg of Protein per hr
ATP	8.5
GTP	2.9
ITP	3.9
UTP	1.0
CTP	0.2

[a] Assay conditions were 1.0 mM nucleotide triphosphate, 1.0 mM $MgCl_2$, 30 mM maleate buffer (pH 7.6), and 0.1 mM ouabain. The experiments were performed with and without 0.25 mM $CaCl_2$ with identical results.

TABLE III: Inhibition of ATPase Activity under Various Conditions.[a]

	Activity as % of Control
Emulphogene (1%)	11
$CetMe_3NBr$ (1%)	6
Digitonin (1%)	18
Phospholipase C (0.1 mg/ml)[b]	20
Sodium cholate (1%)	6
Freeze–thaw (three times)[c]	53
Sonication	100
Ouabain (1 mM)	102
Mersalyl (0.1 mM)	50
Mersalyl (0.5 mM)	30
Mersalyl (1 mM)	30
Sodium azide (0.5 mM)[c]	36
Oligomycin (2 μg/ml)[c]	26
ADP (0.4 mM)	30
Mn (10 mM)	22

[a] Assays were performed in 30 mM maleate buffer, 37°, pH 7.6 containing 0.1 mM ouabain, 2 mM ATP, 2 mM $MgCl_2$, and 0.5 mM $CaCl_2$. [b] ROS preincubated with phospholipase C for 45 min at 37°. [c] Assay for ATPase performed in imidazole buffer (30 mM, pH 8.0, 37°), in 1 mM $MgCl_2$–1 mM ATP.

TABLE IV: Statistical Analysis of ATPase Activity of ROS in the Dark and in Light.[a]

Incubn Time (min)	Total No. of Expts (n)	No. of Expts in Which "Dark" ATPase Was Higher Than Light ATPase[b] (x)	P ($X \geq x$)	Confidence Level $(1 - P) \cdot 100\%$	% Change[c]
2	7	6	0.0625	>93	20.2
15	8	7	0.0351	>96	12.6
30	8	8	0.0039	>99	12.8
45	8	8	0.0039	>99	13.9
60	8	8	0.0039	>99	17.0
75	7	7	0.0078	>99	14.4
90	7	7	0.0078	>99	13.1
105	7	7	0.0078	>99	13.9
120	7	7	0.0078	>99	15.1
150	7	7	0.0078	>99	15.1
Average change[d]					14.8 ± 2.2

[a] We assume that the number of experiments in which "dark" ATPase is higher than "light" ATPase (X) is a random variable with a binomial distribution, *i.e.*, X is bin(n, 0.5), where n is the total number of experiments and 0.5 is the probability that represents the assumption that it is as probable that "dark" ATPase activity is higher than "light" as it is that "light" is higher than "dark" (Hoel, 1954). [b] x is the observed value of X. [c] % change = % decrease in ATPase activity in the light compared to dark (dark = 100%). [d] Average change of all (n = 74) experiments ± 1 standard deviation.

lead to any change in ATPase activity as compared to the "dark" controls. When the ouabain-insensitive ATPase activity of the retinal mitochondrial fraction was tested, no sensitivity to illumination with white light was observed.

All the ATPase assays reported so far were performed at 37°. When the rod outer segments were illuminated as described above and the ATPase assay of the illuminated and dark preparation was assayed at either 15 or 24°, there was no difference between the activities of the two preparations.

In order to analyze the significance of the difference between the illuminated and dark ATPase activities a nonparametric test was performed (Hoel, 1954). The principle behind such an analysis is to make the hypothesis that light-exposed and dark ATPase activities are not significantly different; *i.e.*, both belong to the same class. On the basis of this assumption one would expect that at the time of each measurement, namely, at 2 min, 15 min, etc., either the light or the dark ATPase activity would have an equal chance of having the higher value.

The statistical analysis was performed on all experiments on which complete data were available. These experiments included those in maleate and imidazole buffers, those illuminated with white light or with monochromatic light at 500 nm, as well as one sonicated preparation. The results are summarized in Table IV. Thus, this treatment of the data shows that ATPase activity in illuminated ROS preparation at each time point of incubation was *significantly different* (*i.e.*, lower) from the ATPase activity of the dark preparation.

Illuminated ROS preparations released, on the average, at each time point of sampling in ten different experiments, 14.8 ± 2.2% less P_i than that released by unilluminated ROS preparations (Table IV). During the first 45 min of the incubation, while the reaction proceeds at an almost linear rate, the P_i released by the illuminated ROS was also 14.8% less than that released by the dark preparation.

Discussion

Many systems have been shown to contain a (Mg–Ca)-dependent ATPase activity. These systems include among others, bacteria (Muñoz *et al.*, 1969), chloroplasts (Vambutas and Racker, 1965), red cells (Vincenzi and Schatzmann, 1967; Wins and Schoeffeniels, 1966), brain synaptic vesicles (Germain and Proulx, 1965; Kadota *et al.*, 1967), and muscle (MacLennan, 1970). In preparations of photoreceptor ROS, both (Na–K)- and (Mg–Ca)-dependent ATPase activities have been identified and some of their properties have been described (Bonting *et al.*, 1964; Frank and Goldsmith, 1965). The current investigation was undertaken because of renewed interest in the mechanisms of cation transport in ROS as related to vision. Therefore special attention was given to the effects of illumination on the (Mg–Ca)-ATPase in ROS.

Since both mitochondria and ROS are particulate and other studies have shown (McConnell, 1965) that mitochondria constitute the major contaminant of ROS preparations, it was necessary to establish the degree of homogeneity of our ROS preparations. A quantitative evaluation by electron microscopy of the purity of ROS pellets showed a 0.3% mitochondrial contamination (Figure 1). However, assays of cytochrome oxidase and malic dehydrogenase activities indicated a higher value of about 5%. The explanation of this difference between the electron microscopical and enzymic estimation of the mitochondrial contamination probably lies in the fact that fragmented membranes of mitochondria may be indistinguishable from fragmented ROS disk membranes by electron microscopy while still possessing some or all of their enzymatic activity. The possibility that the (Mg–Ca)-dependent ATPase described in this paper was due to mitochondrial contamination seems rather unlikely for two reasons.

One reason is that when the (Mg–Ca)-ATPase activity of mitochondrial fractions purified on sucrose gradients was tested, its specific activity was found to be about four times that of ROS preparations. Assuming a 5% contamination by mitochondria (obtained by our cytochrome oxidase activity measurements), this implies that the ATPase activity derived from mitochondria contributes 20% to the total ATPase of ROS preparations.

An even more convincing reason is the fact that the (Mg–Ca)-dependent ATPase of ROS is sensitive to illumination at 500 nm while that of retinal mitochondria is not.

In the presence of 10^{-4} M ouabain the ATPase activity of ROS was completely dependent on the presence of *either* Mg *or* Ca (Figure 3). On a molar basis, Mg was more effective than Ca, and moreover, the activation with Mg displayed a clear optimum at a 1:1 molar ratio of Mg:ATP. On the other hand, the activation with Ca increased continuously with increasing concentrations and failed to show an optimal concentration (Figure 3). The addition of Ca to an incubation mixture which already contained suboptimal or optimal concentrations of Mg, did not lead to further activation of the ROS ATPase. On the contrary, additions of Ca led to some inhibition of the release of P_i (Figure 4). It seems clear that the ouabain insensitive ATPase from ROS is strictly dependent

on the presence of divalent cation, that Mg is more effective than Ca, and that there is no synergism between Ca and Mg in activating the ATPase. Thus the ATPase from ROS can be called a Mg- or Ca-ATPase and in this respect is similar to the ATPase described in brain (Kadota *et al.*, 1967; Germain and Proulx, 1965) and in *Micrococcus lysodeikticus* (Muñoz *et al.*, 1969).

A surprising observation was the effect of various common buffers on the ATPase activity. The reasons for this effect are not clear; it was not due to changes in pH or to lack of pH control. The fact that a common buffer such as imidazole can inhibit ROS ATPase activity by as much as 50% (at 30 mM), relative to the activity in maleate buffer, is quite noteworthy in view of the little concern generally displayed about which buffer system is employed in ATPase experiments. An interesting example of the complex effects of the choice of buffers was the observation that while no inhibition of ATPase activity by mersalyl was seen in imidazole buffer, this same compound caused a 70% inhibition of P_i release in maleate buffer.

The maximum activity of the ouabain-insensitive ATPase shown by ROS preparations under optimal conditions in maleate buffer was about 5–6 μmol of P_i released per mg of protein per hr. The activity in the presence of various buffers was similar to that reported by others, namely, 1–2 μmol of P_i released per mg of protein per hr (Bonting *et al.*, 1964; Frank and Goldsmith, 1965). The pH optimum and the nucleotide specificity observed in this study are similar to those reported by Frank and Goldsmith (1965).

The ATPase activity of ROS is membrane bound. The particulate ROS fraction could be extensively washed with buffers without losing its ATPase activity. The membranes could be disrupted to a degree and still retain their activity as shown by the fact that sonication did not cause any loss of activity. On the other hand, the more extensive disruption caused by detergents or treatment by phospholipase C led to various degrees of ATPase activity loss. Inhibition by phospholipase C indicates that the integrity of the membrane lipids is important in ATPase activity. This is not unexpected, as other (Mg–Ca)-dependent ATPases such as that found in saroplasmic reticulum and mitochondria have a requirement for phospholipids (MacLennan, 1970).

The most interesting aspect of the present work is the demonstration that ATPase activity of ROS is sensitive to light. The decrease in P_i released by ROS was caused by illumination at 500 nm and was not observed when illumination was at 400 nm. This suggests that the effect is mediated by the visual pigment rhodopsin (λ_{max} at 500 nm). Although a detailed action spectrum of this effect would have been most illuminating, for technical reasons (the small magnitude of the effect) this seems to be impossible to do at this time. Although the magnitude of inhibition of ATPase activity is not large, being on the order of 15%, statistically it is highly significant and reproducible.

The specific role of rhodopsin in modulating the Mg- or Ca-dependent ATPase remains undetermined. This study certainly does not claim to show that rhodopsin itself is an ATPase enzyme. It most probably is not. The most that one can say at this time is that since the ATPase is membrane bound and rhodopsin is a known structural component of the rod outer segment disk membrane, it is conceivable that the structural and conformational changes caused in one component (rhodopsin) by illumination do affect the activity (ATPase) of another membrane component.

It is interesting to note that the observed difference in ATPase activity between illuminated and dark ROS is observed only when the ATPase is assayed either at 37° or at 45°, and not when it is assayed either at 15 or 24°. The reasons for this temperature dependence are unclear: the ATPase activity as such is stable at 37 and 45° and shows linear kinetics for at least 60 min. The difference between light and dark ATPase activity does not seem to be due to progressive thermal denaturation of the illuminated sample, since the 15% difference between the two is seen during the whole assay period and they remain at essentially the same percentage throughout. It is quite clear that the effect is specific to illumination of rhodopsin since it is not seen with illumination of mitochondria, or with illumination that is not being absorbed by rhodopsin (400 nm).

Recent publications (Bitensky *et al.*, 1971; Miller *et al.*, 1971) have described a light-sensitive adenylate cyclase in a preparation of ROS. The cyclase activity appears to be almost abolished upon complete bleaching of visual pigment, and the degree of bleaching parallels the degree of inactivation of adenylate cyclase activity. Our results exclude the possibility that the pyrophosphate produced by adenylate cyclase is further hydrolyzed to P_i by a pyrophosphatase present in ROS. Therefore, the assay employed in this study is specific for an ATPase which releases P_i and cannot detect adenylate cyclase activity.

In summary, the findings of this study show that purified ROS preparations contain a Mg- or Ca-dependent ATPase activity and that this activity is partially inhibited by illumination at 500 nm. It should be emphasized though, *that our results in no way show that rhodopsin itself is an ATPase*, only that the effect of illumination on the ATPase activity is most probably mediated by visual pigment.

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Redox Interactions between Cytochrome Components and Transition Ions and Metals†

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ABSTRACT: Factors influencing phosphate activation of ferricytochrome *c* reduction by Fe^{2+} have been investigated. Rates of ferricytochrome *c* reduction by Fe^{2+} in phosphate-containing system are further increased by (1) high pH, (2) high O_2 tension, and (3) the presence of Fe^{3+} in the medium. In the presence of both phosphate and Fe^{3+}, reduction of cytochrome *c* by Fe^{2+} is activated in synergistic fashion. Involvement of superoxide anion (O_2^-) in the oxygen enhancement of reduction rates is unlikely, based upon lack of inhibitory effect by the following superoxide anion scavengers; superoxide dismutase (erythrocuprein), 3,4-dihydroxybenzoic acid and 1,2-dihydroxybenzene-3,5-disulfonic acid. The effect of O_2 upon reduction of ferricytochrome *c* by Fe^{2+} is also strongly influenced by the anion species in the medium. Thus, in the presence of phosphate, 100% O_2 markedly activates reduction rates, while in the presence of bicarbonate, 100% O_2 markedly inhibits reduction rates. In Tris systems, 100% O_2 exerts a negligible to very slight activation of reduction rates. Ferrocytochrome *c* produced by reduction by Fe^{2+} is reoxidizable by Cu^{2+}, ferricyanide, and by cytochrome oxidase obtained from yeast and beef heart mitochondria, but not by Fe^{3+}. Visible and Soret region absorption spectrum tracings show that yeast and beef heart mitochondria are also reduced by Fe^{2+}, with the apparent difference that in yeast mitochondria, cytochrome *b* and flavoprotein components are significantly less reduced than in heart mitochondria. The direct electrolytic reduction of cytochrome *c* by Zn^0 and Cu^0 dusts and powders is also reported.

A selective role of phosphate in the reduction of cytochrome *c* by the transition ions Cr^{2+} and Fe^{2+} has been reported (Yates and Nason, 1966; Kowalsky, 1969; Zipper *et al.*, 1971; Taborsky, 1972). Yates and Nason (1966), Zipper *et al.* (1971), and Taborsky (1972) found that the reduction of ferricytochrome *c* by Fe^{2+} is activated by phosphate, and that this activation is sensitive to the presence of other anionic species and O_2 in the medium. We now report additional detailed investigations of these phenomena, and also observations of other redox interactions between transition ions and metals (M^0), and cytochrome components.

Materials and Methods

Tris-HCl, Tris base, and cytochrome *c*, type III, horse heart, were obtained from Sigma Chemical Co. (St. Louis, Mo.). N_2, prepurified grade, and O_2, extra dry grade, were from Matheson Gas Products (East Rutherford, N. J.). Bovine erythrocyte erythrocuprein was obtained from Miles Laboratories, Inc., Elkhart, Ind. Pure metals from ALFA Inorganics, Inc. (Beverly, Mass) were: Cu powder, 1 μ, 99.95%; Cu foil, 5 mil, 99.000%; Fe powder, 325 mesh, 99.9%; Fe foil, 5 mil, 99.998%; Co foil, 5 mil, 99.7%; Ni foil, 5 mil, 99.998%; Zn powder, 200 mesh, 99.999%; Zn foil, 10 mil, 99.9%. Foils and strips were stored in 95% ethanol. For experiments, they were cut, dried, weighed, and used immediately. All other chemicals were of reagent grade. To obtain ferrous chloride free from ferric ion, reagent grade $FeCl_2 \cdot 4H_2O$ crystals were rinsed with distilled water and dried rapidly with filter paper under a stream of nitrogen. Other transition metal salts used were $FeCl_3 \cdot 6H_2O$ and $CuSO_4 \cdot 5H_2O$. All solutions were prepared immediately prior to use. Water was triple distilled, once from a tin-lined container and twice from glass.

Absorption spectra were obtained in a Perkin-Elmer Model 450 recording spectrophotometer or in a Process and Instruments Co. Model RS3 recording spectrophotometer. Fe and Cu were determined with a Perkin-Elmer Model 290 atomic absorption spectrophotometer. pH measurements were made with a Radiometer pH meter Model TTT 3C, using a Corning semimicro combination electrode (Type 476020). Rates of cytochrome *c* reduction or oxidation were determined in Thunberg-type cuvets with a 1.0-cm light path by measuring absorbance changes at 549 nm. 4 ml of cytochrome *c* solution was placed in the lower portion of the cuvet. Next, the native metal sample or 0.08 ml of solution containing the transition metal ion was placed in the upper side arm of the Thunberg

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