

# Influence of Light and Calcium on Guanosine 5'-Triphosphate in Isolated Frog Rod Outer Segments

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**ABSTRACT** Frog rod outer segments contain  $\approx 0.25$  mol of GTP and 0.25 mol of ATP per mol of rhodopsin 3 min after their isolation from the retina. UTP and CTP are present at 10-fold and 100-fold lower levels, respectively. Concentrations of GTP and ATP decline in parallel over the next 4 min to reach relatively stable levels of 0.1 mol per mol of rhodopsin. Illumination reduces the concentration of endogenous GTP but not ATP. This light-induced decrease in GTP can be as large as 70% and has a half-time of 7 s. GTP is reduced to steady intermediate levels during extended illumination of intermediate intensity, but partially returns to its dark-adapted level after brief illumination. The magnitude of the decrease increases as a linear function of the logarithm of continuous light intensity at levels which bleach between  $5 \times 10^2$  and  $5 \times 10^8$  rhodopsin molecules/outer segment per second. This exceeds the range of intensities over which illumination causes decreases in the cyclic GMP content and permeability of isolated outer segments (Woodruff and Bownds. 1979. *J. Gen. Physiol.* **73**:629–653). Thus, over 4 log units of light intensity, a sensitivity control mechanism functions to make extended illumination less effective in stimulating a GTP decrease. GTP levels in dark-adapted outer segments are sensitive to changes in calcium concentration in the suspending medium. If the external calcium concentration is reduced to  $10^{-8}$  M, GTP concentration is lowered to the same level caused by saturating illumination, and the GTP remaining is no longer light-sensitive. Lowering calcium concentration to intermediate levels between  $10^{-6}$  and  $10^{-8}$  M reduces GTP to stable intermediate levels, and the GTP remaining can be reduced by light. Restoration of millimolar calcium drives synthesis of GTP, but not of ATP, and GTP lability towards illumination is again observed. These calcium-induced changes in GTP are diminished by the addition of the divalent cation ionophore A23187. Lowering or raising magnesium levels does not influence the GTP concentration. These data raise the possibility that light activates either a calcium transport mechanism driven by the hydrolysis of GTP, or some other calcium-sensitive GTPase activity of unknown function. Known light-dependent reactions involving cyclic nucleotide transformations and rhodopsin phosphorylation appear to account for only a small fraction of the light-induced GTP decrease.

## INTRODUCTION

This paper examines changes induced by light in GTP and ATP in suspensions of intact frog rod outer segments. These nucleoside triphosphates most likely serve as energy sources for the visual transduction mechanism and the maintenance of ionic gradients. They function as substrates in the phosphorylation of rhodopsin (Bownds et al., 1972; Kühn et al., 1973) and other proteins (Polans et al., 1978; Polans et al., 1979), and GTP is both the precursor for cyclic GMP and a necessary cofactor for light-activation of outer segment cyclic GMP phosphodiesterase activity (Wheeler and Bitensky, 1977). Previous studies have demonstrated that ruptured outer segments display light-activated nucleotidase activity (Robinson and Hagins, 1977; Wheeler and Bitensky, 1977; Thacher, 1978; Caretta et al., 1979), and a recent abstract has reported that the GTP content of intact outer segments is reduced by illumination (Robinson and Hagins, 1979). Bignetti and Cavaggioni (1977) have also reported a change in high energy phosphates in disrupted outer segments.

In the experiments presented here, high pressure liquid chromatography (HPLC) has been used to analyze endogenous levels of several nucleotides simultaneously in the same suspension of freshly isolated and intact rod outer segments. Outer segments have been treated gently and maintained in a modified serum-containing Ringer's solution which preserves their osmotic integrity and permits expression of light-induced permeability changes (Bownds and Brodie, 1975). The intact outer segments have been quenched with acid to extract nucleotides, and the light-dependent nucleotide changes have been studied at the low levels of illumination at which rod cells function. Similar conditions have been used by Woodruff et al. (1977) and Woodruff and Bownds (1979) to demonstrate that cyclic GMP levels can decrease very rapidly upon illumination.

These experiments have also examined the effects of varying external calcium concentration on nucleoside triphosphate levels within the outer segment. It has been found in sarcoplasmic reticulum vesicles that nucleoside triphosphate levels can be influenced by the direction and magnitude of transmembrane calcium gradients (de Meis and Carvalho, 1974). Schnetkamp et al. (1977) have suggested that outer segments contain an energy-dependent calcium transport mechanism. Calcium-sensitive chemistry also is of interest since this ion is thought to play an important role in outer segment physiology (for review, see Hubbell and Bownds, 1979).

## MATERIALS AND METHODS

Retinas were removed from dark-adapted bullfrogs (*Rana catesbeiana*) under infrared illumination using the procedures described in Woodruff et al. (1977) and Woodruff and Bownds (1979), and gently rinsed in 15 ml of modified Ringer's solution: 115 mM NaCl, 2.5 mM KCl, 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 10% (vol:vol) dialyzed and heat-inactivated calf serum, and 0.1 mM dithiothreitol, pH 7.5. The serum was dialyzed against 10 mM HEPES until free of HPLC-detectable nucleoside diphosphates and triphosphates. (The calcium level in this solution was determined by atomic absorption spectroscopy to be 0.09 mM;

magnesium concentration was 0.04 mM.) Within 3–13 min after dissection retinas were transferred to 500  $\mu$ l Ringer's solution and slowly shaken for 2 min to detach the outer segments. After settling for 30 s at 1 *g*, the middle portion of the resulting rod outer segment suspension, containing  $\sim 10^6$  outer segments, was removed. This partitioning minimized contamination from retinal and pigment epithelial cells. (In several experiments it was determined that pigment epithelium contamination much greater than the amount present in these experiments did not alter nucleoside triphosphate levels.) From this suspension 100- $\mu$ l portions were withdrawn for determination of nucleotide content and one or two 50- $\mu$ l portions for determination of rhodopsin content by difference spectroscopy as described in Brodie and Bowns (1976). All of these manipulations were performed at room temperature (22°C) under infrared illumination. Approximately 90% of the isolated outer segments present were judged to be osmotically intact by the fluorescent staining procedure of Yoshikami et al. (1974). Within 3–10 min after isolation of the outer segments from the retina, some 100- $\mu$ l portions of the suspension were illuminated for varying periods of time with the calibrated light source previously described (Brodie and Bowns, 1976) while others were kept dark. Samples were then quenched with cold 10% trichloroacetic acid (TCA, 200  $\mu$ l) added either successively to individual portions at appropriate intervals or simultaneously to a set of three or four samples. Acid-treated samples were kept chilled to avoid degradation of ATP and GTP. After centrifugation for 10–20 min at 1,500 *g*, supernates were neutralized (Khym, 1975) by the addition of an equal volume (300  $\mu$ l) of a 20% (wt:vol) solution of tricaprylyl tertiary amine (Alamine 336, McKerson Corp., Minneapolis, Minn.) in 1,1,2-trichloro-1,2,2-trifluoroethane (Freon TF, E.I. duPont de Nemours & Co., purchased from Wedor Chemical Co., Milwaukee, Wis.). The upper aqueous phase (pH 4–5) was stored (–20°C) until analyzed by HPLC. Recovery of added radioactive nucleotide standards during these manipulations was >95%. Further acid extractions of the TCA-precipitated outer segments yielded no additional ATP or GTP.

For experiments in which outer segments were exposed to differing calcium concentrations ethyleneglycol-bis-( $\beta$ -aminoethyl ether)*N,N'*-tetraacetic acid (EGTA) was used as the calcium buffer, and levels of free calcium were calculated by the method of Caldwell (1970). The apparent affinity constants used were  $K'_{Ca} = 4.70 \times 10^7$  and  $K'_{Mg} = 176$ . For the experiment of Fig. 7, 1 mM  $Ca^{++}$  and 2 mM  $Mg^{++}$  were added to the modified Ringer's solution (with serum omitted) and EGTA was added as follows: for  $10^{-6}$  M  $Ca^{++}$ , 1.03 mM EGTA; for  $10^{-7}$  M  $Ca^{++}$ , 1.29 mM EGTA; for  $10^{-8}$  M  $Ca^{++}$ , 3.68 mM EGTA; for  $10^{-9}$  M  $Ca^{++}$ , 23.9 mM EGTA. All solutions were maintained at pH 7.5. In those preparations containing the divalent cation ionophore A23187 (Eli Lilly and Co., Indianapolis, Ind.), the presence of its solvent (<1% ethanol vol:vol) was shown not to influence ATP and GTP levels.

In 14 separate experiments designed to evaluate the possible contribution of mitochondria-rich inner segment material to the ATP and GTP content of outer segment suspensions, the number of free and attached inner segment saccules was counted after staining with Janus Green B. This dye is oxidized and made visible by the mitochondrial electron transport system (Gabe, 1976). The procedure used was modified from that of Conn (1969). Outer segment suspensions were applied to microscope slides which had been previously sprayed lightly and evenly with an ethanolic solution of the dye (0.5 mg/ml). After 20 min inner segments were stained blue and could be counted. Preparations in which the number of inner segment saccules varied from 10 to 70% of the number of outer segments present were analyzed. Papain treatment of single retinas was used to obtain outer segment suspensions with high inner segment content. These suspensions were obtained after first exposing

retinas for 30 s to the modified Ringer's solution with serum omitted and papain (0.25 mg/ml), 3 mM EGTA, and 10 mM dithiothreitol added. After three 5-s rinses in 6 mM  $Mg^{++}$  Ringer's solution without dithiothreitol, papain, and EGTA, the retinas were shaken successively for 30 s, 30 s, and 60 s into three separate vials, to yield suspensions in which the number of inner segments varied from 40 to 70% of the number of outer segments present. Papain treatment freed all but 1% of the inner segments from their attachment to outer segments.

#### *High Pressure Liquid Chromatography (HPLC)*

Nucleotide levels were measured by HPLC (Hartwick and Brown, 1975) using a Waters system (Waters Associates, Inc., Milford, Mass.) equipped with two model 6000A solvent delivery modules. Samples were injected through a model U6K septumless injector onto a 4.6-mm  $\times$  25-cm Partisil-10 SAX column (Whatman Inc., Clifton, N.J.) consisting of a strong (quaternary ammonium) anion exchange resin bound to a microparticulate silica matrix. Gradients were generated by a model 660 solvent programmer from reservoirs containing 0.01 M  $NH_4H_2PO_4$ , pH 3.7 (buffer A) and 0.5 M  $NH_4H_2PO_4$ , pH 5.0 (buffer B), respectively. Buffer salt was freed of most of its UV-absorbing contaminants by recrystallization and removal of the first crystalline crop. Buffers were filtered (Millipore, type HA, 0.5- $\mu$ m pore size, Millipore Corp., Bedford, Mass.) prior to use. After equilibration of the column with 40 ml buffer A, optimal resolution of the nucleoside di- and triphosphates could be obtained with the following gradient program (1.5 ml/min): 5 min A, to elute relatively low charged or cationic species, including the purine and pyrimidine bases and nucleosides, as large unresolved peaks; 15 min convex gradient to 30% B, to elute the nucleoside monophosphates as sharp but incompletely resolved peaks followed by the nucleoside diphosphates; 24 min after injection, a 3-min linear gradient to 43% B, to elute UTP, CTP, and ATP; 33 min after injection a 3-min linear gradient to 58% B, to elute GTP. The highly charged nucleoside triphosphates, retained longest on the strong anion exchange column, eluted as well-resolved peaks (see *insets*, Fig. 1). A model 440 absorbance module monitored effluent absorbance at 254 nm at 0.005 absorption units full scale (AUFS) sensitivity. Output from the detector was recorded with a Houston Instrument Div., Bausch & Lomb, Inc. (Austin, Tex.) strip chart recorder. Areas of recorded peaks were determined by integration with a Hewlett-Packard 9820 calculator (Palo Alto, Calif.) interfaced with a Numonics Corp. (Lansdale, Pa.) model 264 electronic graphics calculator. Measurements were made in the range in which peak area (or in some cases peak height) varied linearly with concentration as determined by calibration with known standards. Though increases in base-line absorbance owing to residual buffer absorbance could not be avoided, in all the gradients used nucleotide peaks eluted in regions of stable base-line absorbance (see *insets*, Fig. 1). Reproducibility of HPLC analyses and recovery from the column exceeded 95% as determined by injections of standards. The largest systematic error ( $\pm$  5%) was introduced during pipetting of the outer segment suspension rather than through the assay procedure. (Larger errors shown in some of the figures resulted from variation among animals.) Two or three HPLC analyses routinely could be obtained using outer segments prepared from one retina. The identities of the various peaks were established by comparison of both retention times and bandwidths with those of known standards (Kyhm et al., 1977). The absence of components coeluting with ATP and GTP was indicated by the following: a degree of correspondence of elution peak bandwidths with those of standards exceeding 90%; the absence of peak distortions attributable to any such components under a variety of flow and gradient conditions; and the failure to find any other compounds with the same retention times.

### *Isotope Experiments*

In several experiments either [ $^{14}\text{C}$ ] ATP (0.5  $\mu\text{Ci}$ , 438 mCi/mmol) or [ $^{14}\text{C}$ ] GTP (0.5  $\mu\text{Ci}$ , 360 mCi/mmol), obtained from ICN Pharmaceuticals, Inc., Irvine, Calif., was added to two portions (500  $\mu\text{l}$ ) of an outer segment suspension in modified Ringer's solution with carrier added to give a final nucleotide concentration of 4.5  $\mu\text{M}$ . After aliquots (50  $\mu\text{l}$ ) were withdrawn for determination of rhodopsin content, one portion was exposed to illumination bleaching  $5 \times 10^5$  rhodopsin molecules/outer segment per second. From both the dark and illuminated portions aliquots (100  $\mu\text{l}$ ) were withdrawn at appropriate intervals, quenched, and prepared as described above for HPLC analysis.

In other experiments in which endogenous pools of ATP and GTP were made radioactive, excised retinas were incubated for 2.5 min in 500  $\mu\text{l}$  modified Ringer's solution containing  $^{32}\text{P}_i$  (0.5 mCi), obtained from New England Nuclear, Boston, Mass. Retinas were then gently rinsed in 10 ml modified Ringer's solution for 20 s before outer segments were removed by shaking. After 7 min in the dark, portions of this outer segment suspension were exposed to 30 s of illumination bleaching  $10^6$  rhodopsin molecules/outer segment per second, while others were kept dark, and all samples were then quenched with TCA. After centrifugation supernates were neutralized and analyzed by HPLC as described above. The TCA precipitates were washed with 200  $\mu\text{l}$  modified Ringer's solution, solubilized with sodium dodecyl sulfate in a buffer suitable for electrophoresis, and analyzed on gradient polyacrylamide gels as described in Polans et al. (1979). The excised rhodopsin regions of the gel stained with Coomassie Brilliant Blue, were solubilized in 1 ml  $\text{H}_2\text{O}_2$  with heating, mixed with Aquasol (10 ml; New England Nuclear), and their radioactivity was determined with a Searle Mark II liquid scintillation counter (Searle Radiographics, Inc., Des Plaines, Ill.). In all radioactive tracer experiments HPLC effluent was collected in 1.0-ml fractions with an UltroRac LKB 7000 fraction collector (LKB Instruments, Inc., Rockville, Md.). Aliquots (0.5 ml) from each fraction were mixed with 5.0 ml Aquasol and radioactivity was determined.

## RESULTS

### *Decay of ATP and GTP Levels after Detachment of Outer Segments from the Retina*

GTP and ATP are present in equimolar concentrations in suspensions of freshly isolated frog rod outer segments. Approximately 0.25 mol of both GTP and ATP per mol of rhodopsin are present 3 min after outer segments are shaken from the retina. Fig. 1 summarizes data from 36 experiments that demonstrate that these initial levels of GTP and ATP decay and reach a fairly stable level within 10 min after detachment from the retina. The effects of illumination reported in this paper are measured during this stable period 7–13 min after isolation of the outer segments, when levels of GTP and ATP have each fallen to a concentration of  $\sim 0.1$  mol per mol of rhodopsin present. UTP and CTP are present at 10-fold and 100-fold lower levels, respectively, and are not considered in detail.

### *Illumination Decreases GTP but Not ATP*

The data of Fig. 2 show the effect of continuous illumination that bleaches  $5 \times 10^5$  rhodopsin molecules/outer segment per second. This is the amount of light that causes maximum suppression of cyclic GMP levels and permeability

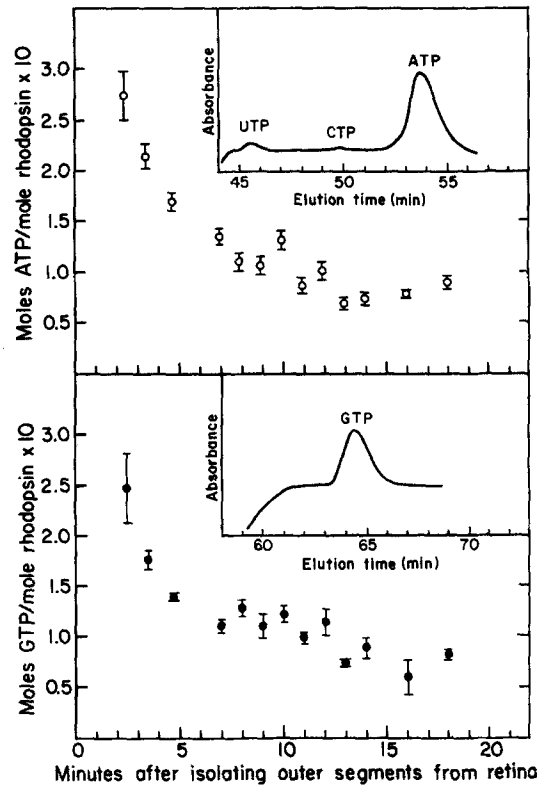


FIGURE 1. Decay of ATP and GTP levels in the dark after isolation of outer segments from the retina. Upper panel: levels of ATP (○), expressed as moles of ATP per mole of rhodopsin, were obtained in 36 experiments involving 115 separate determinations. In each experiment outer segments from four retinas were combined in 1 ml of modified Ringer's solution and nine 100- $\mu$ l portions were removed. A portion of the remainder was used for determination of rhodopsin content. After differing periods of time, single aliquots or sets of two to four samples were quenched by the addition of 200  $\mu$ l of 10% trichloroacetic acid (see Methods). Data obtained within 1-min intervals about the times shown were averaged. Mean values ( $\pm$  SE) for each interval are indicated. Lower panel: levels of GTP (●), expressed as moles of GTP per mole of rhodopsin, were obtained in the same experiments. *Insets*: resolution of nucleoside triphosphates by HPLC as described in Methods. Relative absorbances (254 nm) of UTP, CTP, and ATP (upper *inset*) and GTP (lower *inset*) extracted from a typical dark-adapted outer segment preparation are shown as a function of elution times in minutes. The rises in absorbance preceding UTP (upper) and GTP (lower) represent steps in the elution gradient described in Methods.

in isolated outer segments (Woodruff et al., 1977). GTP levels decrease by 57% within 30 s of the onset of illumination, with a half-time of  $\sim$ 7 s. No further decrease develops as illumination continues for several minutes (see below). A 10% decrease in GTP is observed after 1 s of illumination. This

corresponds to a net decrease of  $\sim 85$  molecules of GTP per molecule of rhodopsin bleached. After 30 s of illumination, 50% of the GTP decrease is accounted for by GDP formation and the remainder by formation of compounds with lower charge (data not shown). In contrast, no change in ATP or ADP is observed. Levels of UTP also show no change in response to illumi-

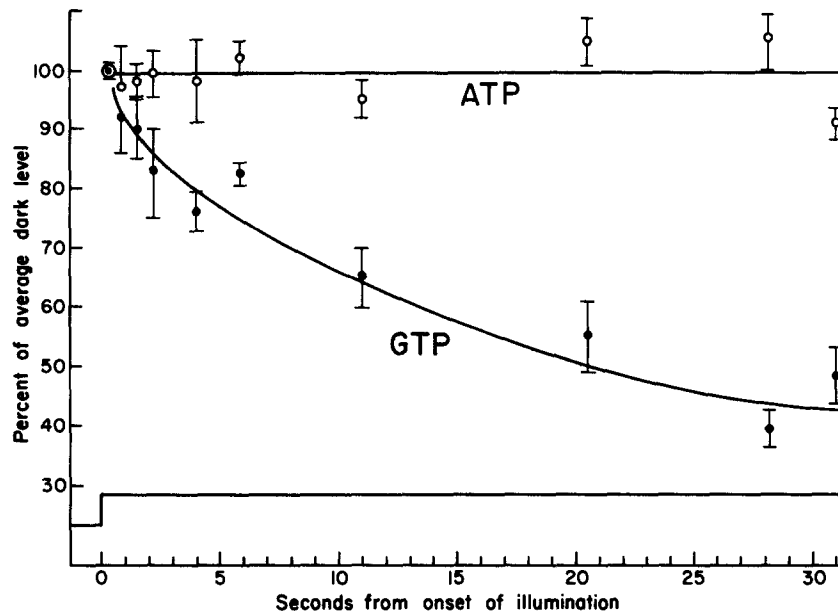


FIGURE 2. Light-induced decrease in GTP, but not ATP, in response to continuous illumination. Levels of GTP (●) and ATP (○), expressed as percent of their respective average dark levels, are plotted against time in seconds from onset of illumination. Between 9 and 12 min after isolation of outer segments from the retina, unilluminated (control) samples were quenched, as described in Fig. 1. Remaining samples were then exposed to illumination bleaching  $5 \times 10^5$  rhodopsin molecules/outer segment per second. Within the initial 6 s of illumination, individual samples were quenched in succession; between 10 and 31 s, triplicate or quadruplicate samples were quenched simultaneously at the times indicated. In most experiments sets of unilluminated samples masked from the light were quenched immediately after the illuminated samples. Determinations from at least three experiments were partitioned into groups spanning 1-s intervals about the times shown. Mean values ( $\pm$  SE) of each group are indicated. The upper solid line is drawn through the mean of the combined ATP values; the lower solid line is drawn by hand.

nation, and levels of CTP are too low for the effects of illumination to be accurately measured.

#### *Location of ATP and GTP*

Outer segments and inner segment saccules (both free and attached to outer segments) are the major components of the *in vitro* preparation. The following

evidence suggests that most of the ATP and GTP was associated with these organelles, rather than being soluble or associated with lighter membrane fragments:

(a) ATP and GTP added to the preparation were rapidly degraded by nucleotidase activity in the suspension. This was shown by adding radioactive ATP or GTP to the suspension in an amount comparable to the total endogenous pool (see Methods). Approximately 90% of the added nucleotide was degraded with a half-time of 30 s and no effect of illumination on this degradation was observed.

(b) Disruption of outer segments caused decreases in GTP and ATP proportional to the degree of fragmentation, with decreases as large as 70% in GTP and 35% in ATP caused by breaking all outer segments present into smaller fragments.

(c) When crude outer segment suspensions were gently sedimented (12 s at 200 g), > 80% of the remaining GTP and ATP was associated with the pellet.

To estimate the possible contribution of the mitochondria-rich inner segment saccules to the measurements of ATP and GTP, these nucleotides were assayed in 14 different preparations in which the number of inner segments varied from 10 to 70% of the number of outer segments present (see Methods). The total amounts of GTP and ATP in a given outer segment suspension were represented as:

$$\begin{aligned} \text{GTP}_{\text{total}} &= n_{\text{is}} (\text{GTP}_{\text{is}}) + n_{\text{os}} (\text{GTP}_{\text{os}}); \\ \text{ATP}_{\text{total}} &= n_{\text{is}} (\text{ATP}_{\text{is}}) + n_{\text{os}} (\text{ATP}_{\text{os}}), \end{aligned}$$

where  $\text{GTP}_{\text{is}}$ ,  $\text{GTP}_{\text{os}}$ , etc. refer to the number of moles of nucleotide per inner segment or outer segment, and  $n_{\text{is}}$  and  $n_{\text{os}}$  are the number of inner segments and outer segments present. Solutions of these equations for  $\text{GTP}_{\text{is}}$ ,  $\text{GTP}_{\text{os}}$ ,  $\text{ATP}_{\text{is}}$ , and  $\text{ATP}_{\text{os}}$  were obtained by linear regression analysis and indicated that most of the GTP present (50–70%) was associated with outer segments, whereas most of the ATP (65–75%) derived from the inner segments. The light-induced decrease in GTP ( $\Delta\text{GTP}$ ) in a given preparation could be represented as the sum of the products of the number of inner and outer segments and their respective contributions to the light-induced decrease as follows:

$$\Delta\text{GTP} = n_{\text{is}} (\Delta\text{GTP}_{\text{is}}) + n_{\text{os}} (\Delta\text{GTP}_{\text{os}}).$$

No positive (real) solution for an inner segment contribution to the light-induced decrease in GTP was obtained, suggesting that the light-induced GTP decrease is essentially an outer segment phenomenon. This last point was supported by a further observation. In outer segment preparations obtained from papain-treated retinas (see Methods), < 1% of the outer segments present remained attached to inner segments, yet up to 70% of the GTP remained labile towards light. Moreover, a decrease in GTP levels occurred when only several hundred of the  $3 \times 10^9$  rhodopsin molecules in an outer segment had been bleached (see below). It seems unlikely that GTP changes



with such high sensitivity to light could be taking place in membranes or cell contaminants physically separate from the outer segments.

#### *Light Sensitivity of the GTP Decrease*

The magnitude of the light-induced GTP decrease becomes larger as light intensity is increased over four log units, from a level bleaching  $5 \times 10^2$  rhodopsin molecules/outer segment per second to one bleaching  $5 \times 10^6$  rhodopsin molecules/outer segment per second (Fig. 3). The response is maximal at the latter intensity; light bleaching  $2 \times 10^7$  rhodopsin molecules/outer segment per second does not generate a larger decrease. ATP levels remain unaltered even at these higher light intensities (data not shown). At the lowest light level used a 10% decrease in GTP was noted. Experimental uncertainty prevented measuring smaller decreases which might have been

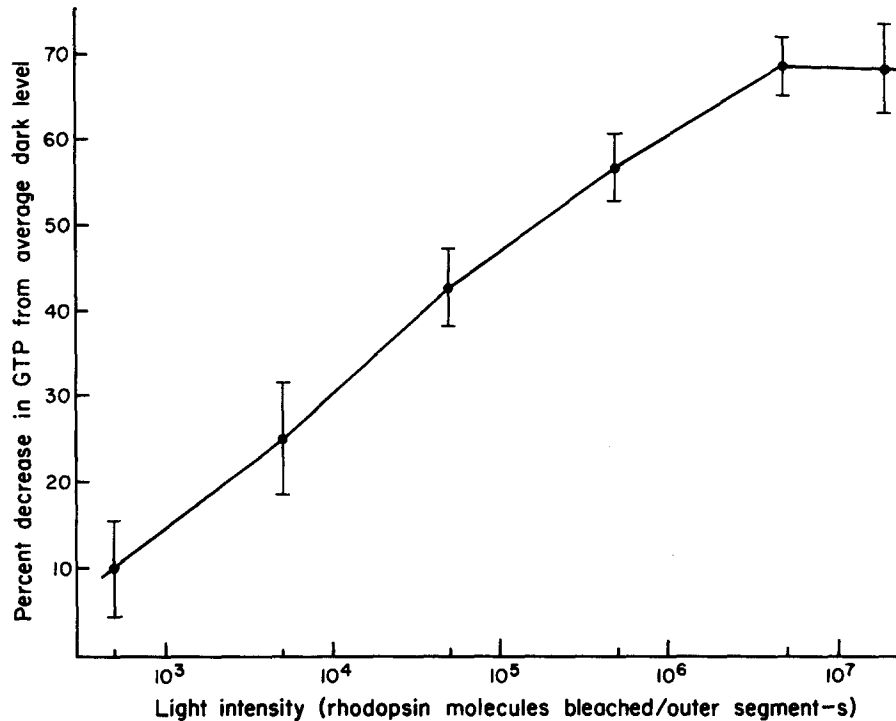


FIGURE 3. Change in magnitude of light-dependent GTP decrease caused by varying the intensity of illumination. The percent decrease in GTP from the average dark level after 30 s of illumination is plotted as a function of intensity of illumination, expressed as rhodopsin molecules bleached/outer segment per second. Outer segments were prepared, and control samples quenched, as in Figs. 1 and 2. Light intensity was attenuated by interposing neutral density filters between the samples and a light source (see Methods). Two to nine experiments, each with several determinations, were done at each intensity and mean values ( $\pm$  SE) are given.

triggered by less illumination. Although Fig. 3 shows the decrease in GTP from its dark levels after 30 s of illumination, continuing the illumination does not cause further decay of GTP. This is shown in Fig. 4, a composite of two experiments in which outer segments are exposed to an intermediate intensity for several minutes. The intensity used, bleaching  $10^4$  rhodopsin molecules/outer segment per second, induces a half-maximal GTP decrease (cf. Fig. 3). At this intensity, as well as with light bleaching  $5 \times 10^5$  rhodopsin molecules/outer segment per second, the light-induced decrease in GTP that occurs within 30 s is maintained in continuous light for at least 10 min, relative to

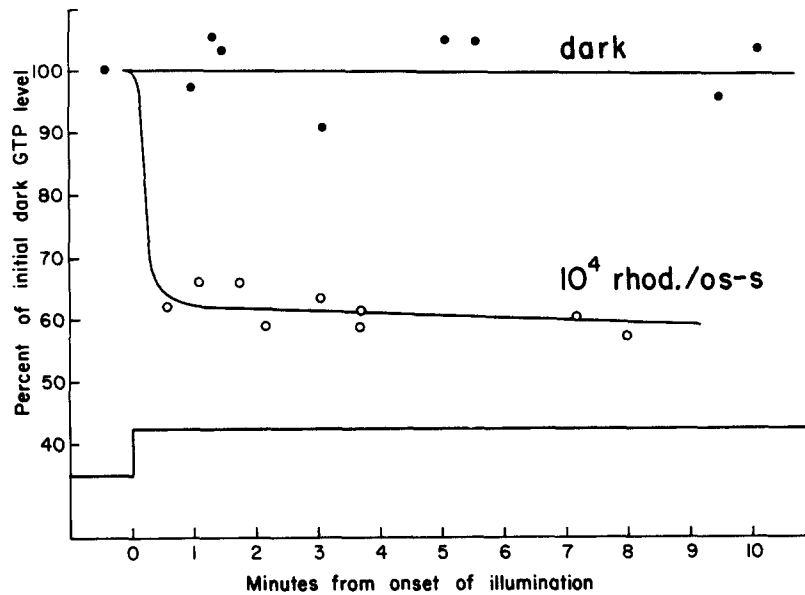


FIGURE 4. Light-induced decrease in GTP in response to extended illumination. Levels of GTP in dark (●) and illuminated (○) samples, expressed as percent of initial dark levels, are plotted against time in minutes from onset of illumination. Samples were exposed to intermediate intensity light bleaching  $10^4$  rhodopsin molecules/outer segment per second. The figure is a composite of data from two experiments in which outer segments were prepared as in Fig. 1 and quenched at the times indicated.

dark-adapted control levels. No change in the level of ATP is noted. Thus it appears that for each level of illumination GTP decays to a characteristic concentration which is then maintained. A similar situation obtains for the light-induced cyclic GMP decrease studied by Woodruff and Bownds (1979), except that it occurs over a narrower range of light intensities than the GTP decrease.

Fig. 5 demonstrates that the GTP decrease triggered by 1 s of illumination bleaching  $5 \times 10^5$  rhodopsin molecules/outer segment per second is similar in kinetics and time-course to the decrease caused by continuous illumination of the same intensity (Fig. 2), but the total decrease is 35% instead of 57%. This

corresponds to a net disappearance of 200 molecules of GTP per molecule of rhodopsin bleached. In two experiments, when the same amount of rhodopsin bleaching was accomplished by illuminating the outer segment preparation for 100 ms (instead of 1 s) with light 10 times brighter, the same 35% decrease in GTP was observed. Thus, it appears that with shorter exposures to light

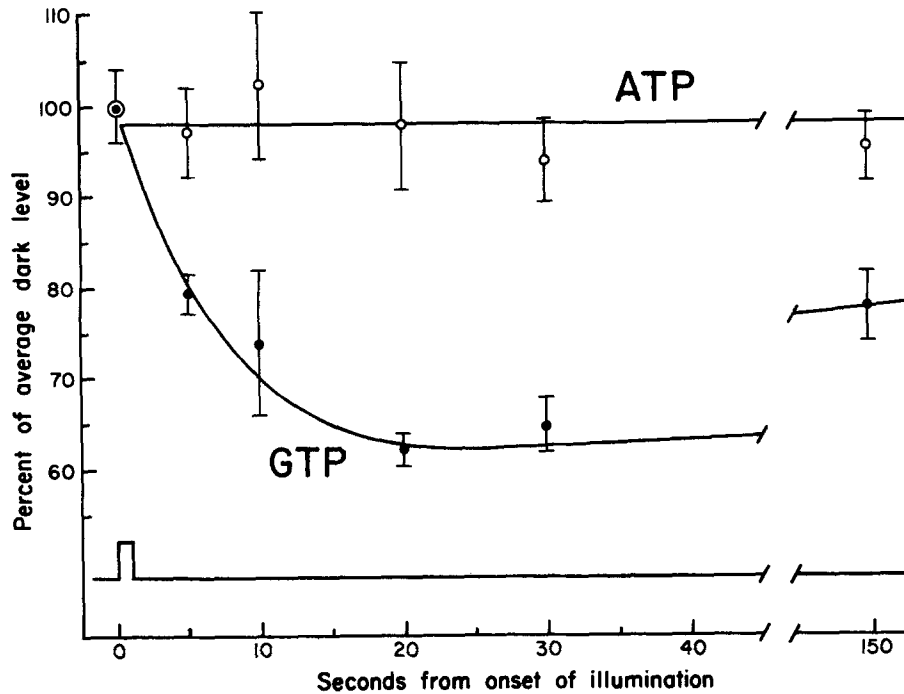


FIGURE 5. Light-induced decrease in GTP, but not in ATP, in response to 1 s illumination. Levels of GTP (●) and ATP (○), expressed as percent of their respective average dark levels, are plotted against time in seconds from onset of illumination. The data is a composite of five experiments in which outer segments were prepared as in Fig. 1. Two or three samples were quenched in the dark and the remainder were exposed to 1 s illumination bleaching  $5 \times 10^5$  rhodopsin molecules/outer segment per second. In three experiments individual samples were quenched successively at 5, 10, 20, and 30 s after illumination. In two experiments triplicate illuminated samples were quenched at 30 s and 2.5 min after illumination, along with triplicate samples which had not been exposed to illumination. The means ( $\pm$  SE) of the combined determinations at each time are indicated.

(1 s and 100 ms) the GTP decrease correlates with the absolute number of rhodopsin molecules bleached, whereas with longer exposures, as in Fig. 3, the rate of rhodopsin bleaching (light intensity) determines the GTP level. Fig. 5 also shows that the light-induced decrease in GTP reverses upon cessation of illumination, and GTP levels partially recover towards their dark-adapted levels. Fig. 5 includes data from two experiments in which 40% of the initial

decrease is restored 2.5 min after the flash. No change in ATP levels is observed during this recovery.

*Relationship of the GTP Decrease to Rhodopsin Phosphorylation*

To begin an assessment of light-dependent pathways which might draw on the GTP pool in outer segments, endogenous  $\text{GT}^{32}\text{P}$  and  $\text{AT}^{32}\text{P}$  were synthesized by incubating retinas with  $^{32}\text{P}_i$  prior to isolation of the outer segments. Two aliquots withdrawn from the resulting outer segment suspension were exposed to illumination and then acid-quenched along with duplicate dark control samples. After centrifugation the supernate of each sample was analyzed to monitor the effects of illumination on GTP and ATP levels (using HPLC), and the precipitated protein in each sample was analyzed for phosphorylated rhodopsin content by electrophoresis (see Methods). The amounts of radioactivity incorporated into GTP, ATP, and rhodopsin in dark and illuminated samples are shown in Table I, which also lists the nucleotide

TABLE I  
EFFECT OF ILLUMINATION ON  $^{32}\text{P}$  CONTENT AND CONCENTRATIONS OF  
GTP, ATP, AND RHODOPSIN

	GTP		ATP		Rhodopsin
	<i>cpm</i>	<i>moles/mole rhodopsin</i>	<i>cpm</i>	<i>moles/mole rhodopsin</i>	<i>cpm</i>
Dark	3,730	0.133	6,170	0.143	163
	3,620	0.129	6,280	0.130	177
Illuminated	1,910	0.0398	6,130	0.137	237
	1,750	0.0354	5,550	0.135	271

The levels of radioactivity associated with HPLC-determined concentrations of endogenous GTP (as  $\text{GT}^{32}\text{P}$ ) and ATP (as  $\text{AT}^{32}\text{P}$ ) in duplicate dark and illuminated samples are compared with the levels of radioactivity associated with the rhodopsin regions of gradient polyacrylamide-sodium dodecyl sulfate gels from the same samples. Retinas were incubated with  $^{32}\text{P}_i$  for 2.5 min prior to isolation of the outer segments from the retina as described in Methods. Radioactivity is expressed as counts per minute above background with all values normalized to that portion of the outer segment suspension analyzed by electrophoresis. Concentrations of GTP and ATP are expressed as moles per mole of rhodopsin in the sample. Illuminated samples were exposed for 30 s to illumination bleaching  $\sim 10^6$  rhodopsin molecules/outer segments per second. All samples were acid-quenched  $\sim 7$  min after isolation of the outer segments from the retina.

concentrations. The rise in the level of radioactivity associated with the light-activated phosphorylation of rhodopsin is equivalent to  $<5\%$  of the light-induced decrease in  $\text{GT}^{32}\text{P}$ . A normal light-induced decrease in total GTP is observed. No statistically significant light-induced changes in ATP and  $\text{AT}^{32}\text{P}$  are indicated.

*Influence of External Calcium on GTP Levels*

GTP levels in dark-adapted outer segments are sensitive to changes in calcium concentration in the suspending medium. Fig. 6 demonstrates that if outer segment suspensions are made  $\sim 10^{-9}$  M in  $\text{Ca}^{++}$  by adding EGTA, GTP levels decrease rapidly and, within minutes, stabilize at 30–40% of their dark-adapted level. A smaller decrease in ATP is noted.

After GTP levels fall their responsiveness to light is abolished. (This contrasts with the reports of Carretta and Cavaggioni [1976] and Bignetti and Cavaggioni [1977] that endogenous "ATP" is substantially reduced by illumination even in low calcium.) Table II shows that in nine experiments illumination of outer segment suspensions 4–9 min after addition of EGTA caused no further decrease in GTP. Although decreases from 30 to 75% were observed over the

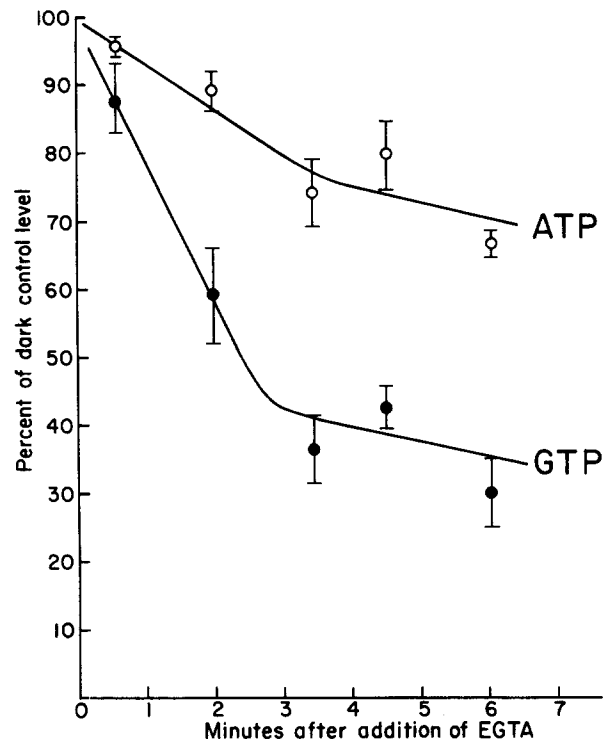


FIGURE 6. Decrease in levels of GTP and ATP in the dark following addition of EGTA. GTP (●) and ATP (○) concentrations, expressed as a percent of their control levels, are plotted against time in minutes after addition of EGTA (3 mM) to the outer segment suspensions. The figure is a composite of eight experiments. In five experiments outer segments were prepared in serum-containing Ringer's medium as described in Fig. 1; in three experiments outer segments were isolated from retinas treated for 30 s with papain (see Methods) prior to isolation. Data are partitioned into groups containing at least three determinations from two to four experiments and spanning 1-min intervals about the times shown. The means ( $\pm$  SE) of the combined determinations at each time are indicated.

series of experiments, within each of the experiments the magnitude of the GTP decrease caused by EGTA addition in the dark was almost the same as that caused by saturating illumination of the control sample. The mean ratio was  $1.02 \pm 0.07$  (SE,  $n = 9$ ). The data of Table II also suggest that the effect of EGTA is related to calcium chelation, for it is abolished by excess  $\text{Ca}^{++}$  but not excess  $\text{Mg}^{++}$ .

The data of Fig. 7 (●) demonstrate that the decrease in GTP within outer segments is a graded function of the external calcium concentration, with a half-maximal decrease in GTP observed when external calcium is  $\sim 10^{-7}$  M. At intermediate calcium concentrations internal GTP levels stabilize at intermediate levels, much like the response to intermediate levels of illumination shown in Fig. 4. Also shown in Fig. 7 is that the light-induced (○) decrease in the GTP remaining after lowering the external calcium concentrations also diminishes. At each of the lower calcium concentrations light decreases GTP to approximately the same level as that caused by light of saturating intensity in control samples. (To insure that the calcium concentrations used here were

TABLE II  
LOSS OF LIGHT SENSITIVITY CAUSED BY EGTA ADDITION BLOCKED BY  
EXCESS CALCIUM BUT NOT MAGNESIUM

	Percent change in dark level caused by indicated medium			Additional change from dark level caused by illumination		
	GTP	ATP	(n)	GTP	ATP	(n)
	%	%		%	%	
Normal medium	-	-		-56±3	0±3	(16)
+ 3 mM EGTA	-55±5	-24±5	(9)	-7±4	-6±4	(9)
+ 4 mM Mg <sup>++</sup>	0±12	2±9	(3)	-54±3	-5±3	(4)
+ 4 mM Mg <sup>++</sup> , 3 mM EGTA	-56	-11	(1)	3±8	4±4	(2)
+ 4 mM Ca <sup>++</sup>	11±2	15±6	(3)	-54±5	-11±5	(3)
+ 4 mM Ca <sup>++</sup> , 3 mM EGTA	-10±1	-13±3	(3)	-57±4	-2±3	(5)

Outer segments in suspension were prepared as described in Fig. 1 using the modified Ringer's medium described in Methods. The combinations of Ca<sup>++</sup>, Mg<sup>++</sup>, and EGTA listed in the left column were then added to separate portions. After 4-9 min two dark samples from each portion were quenched, and two other samples were then exposed to illumination of saturating intensity for 20 s and quenched. Mean percent changes ( $\pm$  SE) for dark samples are indicated in the middle columns. Additional percent changes upon illumination are shown in the right columns. Values were determined in the number of experiments shown in parentheses. The pH of the suspension was maintained at 7.5 throughout.

comparable to those used in studies of calcium effects on rod receptor electrophysiology, the bicarbonate Ringer's medium, pH 7.8, used by Fain et al. [1977] was buffered to " $10^{-7}$  M" Ca<sup>++</sup> using their procedures, and the same intermediate decrease in GTP observed in Fig. 7 was obtained.)

#### *GTP Synthesis Caused by Restoring External Calcium*

If the calcium concentration in outer segment suspensions is shifted from  $\sim 10^{-9}$  M to  $10^{-3}$  M, GTP levels rise and lability to light is restored. In the five experiments shown in Table III, 4 mM Ca<sup>++</sup> was added to outer segment suspensions 3-5 min after treatment with 3 mM EGTA. The mean recovery of GTP towards its dark level was  $45 \pm 6\%$  (SE) corresponding to a net synthesis of 0.034 mol of GTP per mol of rhodopsin present. Illumination after

this recovery again reduces GTP to the same levels observed in illuminated control samples. Recovery of GTP levels was not stimulated by restoration of millimolar  $Mg^{++}$  (data not shown). Levels of ATP were not affected by restoration of external calcium.

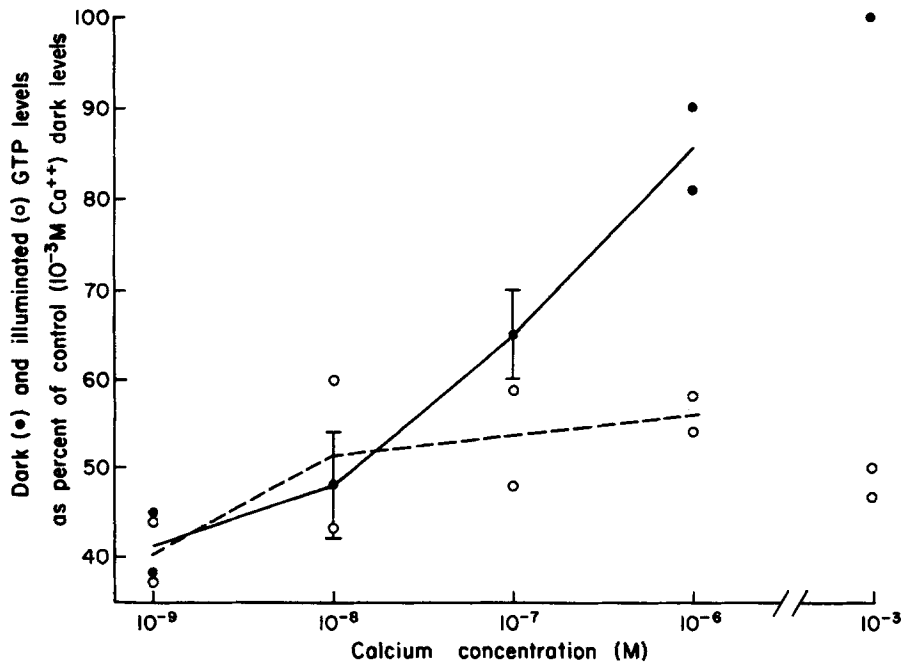


FIGURE 7.  $Ca^{++}$  concentration dependence of the GTP decrease in the dark and decay of the light-induced GTP decrease. Levels of GTP at each  $Ca^{++}$  concentration in the dark (●) and after illumination (○), expressed as percent of control ( $10^{-3}$  M  $Ca^{++}$ ) dark GTP levels, are plotted against the molar concentration of uncomplexed  $Ca^{++}$  in the suspending medium. The figure is a composite of three similar experiments in which outer segments were prepared as in Fig. 1 in serum-free HEPES Ringer containing 1 mM  $Ca^{++}$  and 2 mM  $Mg^{++}$  (pH 7.5). Varying amounts of EGTA were added to separate portions of the suspension, 6–9 min after isolation of the outer segments, to reduce the  $Ca^{++}$  concentration to each of the levels shown (see Methods). The pH of the suspension was maintained at 7.5 throughout. Samples were illuminated for 20 s at saturating intensity 4–7 min after EGTA addition, and all samples were then quenched as in Fig. 1. The solid and dashed lines connect the means of the dark and illuminated values, respectively. Standard error is indicated where three or more determinations were done.

Addition of the divalent cation ionophore A23187 in 5  $\mu$ M concentration decreases the effects of calcium concentration changes on internal GTP levels. In the presence of the ionophore both the GTP decrease caused by lowering calcium concentration from  $10^{-3}$  to  $10^{-8}$  M and the increase caused by returning to  $10^{-3}$  M calcium were approximately half as large as in outer

TABLE III  
SYNTHESIS OF GTP CAUSED BY RAISING EXTERNAL CALCIUM  
CONCENTRATION

Experiment	Dark GTP level			Illuminated GTP level		
	Control	+3 mM EGTA	+3 mM EGTA followed by +4 mM Ca <sup>++</sup>	Control	+3 mM EGTA	+3 mM EGTA followed by +4 mM Ca <sup>++</sup>
	%	%	%	%	%	%
1	100	34	59	33	30	30
2	100	38	57	44	33	32
3	100	48	78	54	50	48
4	100	58	75	59	60	52
5	100	62	84	66	62	64

Outer segments were prepared in five experiments as described in Fig. 1 using the modified Ringer's medium described in Methods. After control samples were withdrawn, outer segments were treated with 3 mM EGTA as described in Fig. 7. After 3 min (experiments 1 and 2) or 5 min (experiments 3-5), during which time EGTA-containing samples were withdrawn, 4 mM Ca<sup>++</sup> was added to a portion of the remaining suspension. The pH of the Ca<sup>++</sup> solution was adjusted prior to addition to maintain suspension pH at 7.5 throughout. After 2.5 min, samples were quenched in the dark, and further duplicate samples from each condition were then exposed to 20 s illumination bleaching ~10<sup>6</sup> rhodopsin molecules/outer segment per second and quenched. Internal controls showed no changes in GTP levels in EGTA-treated samples during this 2.5-min period relative to control levels. Means of the duplicate dark and illuminated GTP levels are shown, expressed as percents of their respective mean dark control levels. Duplicate values were within 5%.

segment suspensions lacking the ionophore. The addition of the ionophore alone to outer segments in 10<sup>-3</sup> M calcium medium causes a 25% decrease in GTP levels.

#### DISCUSSION

Data obtained in more than 100 separate experiments have demonstrated that in isolated frog rod outer segments GTP, but not ATP, is reduced by illumination. This reduction is most easily measured 7-13 min after outer segments have been detached from the retina, a period during which ATP and GTP have declined to a relatively stable level of 0.1 mol of each per mol of rhodopsin present. Woodruff and Bownds (1979) have noted that cyclic GMP, present at 10-fold lower levels than GTP, undergoes a similar decay. The millimolar nucleotide levels reported here agree reasonably well with previous work that has employed the less specific luciferase assay to measure high energy phosphates (Robinson et al., 1975; Carretta and Cavaggioni, 1976; Bignetti and Cavaggioni, 1977), and the decrease in "ATP" measured by these laboratories probably corresponds to the GTP decrease noted here. A preliminary report from Robinson and Hagins (1979) confirms the GTP decrease assayed with HPLC techniques. The light-induced decrease in endogenous GTP reported in this paper should not be confused with the light-activated GTPase enzyme(s) which have been assayed in suspensions of disrupted outer segments by adding exogenous radioactive GTP (Robinson and Hagins, 1977; Wheeler and Bitensky, 1977; Bignetti et al., 1978; Carretta



et al., 1979). That GTPase activity, unlike the endogenous GTP decreases reported here, shows graded activation over only 1–2 log units of light intensity (Wheeler and Bitensky, 1977; Carretta et al., 1979) and functions at micromolar rather than millimolar levels of GTP.

The amplitude of the GTP decrease observed in these experiments is a linear function of the logarithm of light intensity at levels that bleach between  $5 \times 10^2$  and  $5 \times 10^6$  rhodopsin molecules/outer segment per second. Light-induced decreases in the cyclic GMP level and permeability of outer segments (Woodruff et al., 1977; Woodruff and Bowns, 1979), and light activation of cyclic GMP phosphodiesterase<sup>1</sup> occur within the same range, but become maximal with light which bleaches  $\sim 5 \times 10^4$  rhodopsin molecules/outer segment per second. Once initiated, all of these reactions stabilize at intermediate intensities of continuous illumination, suggesting that they may be regulated within the intact outer segment by mechanisms which desensitize them to continued illumination. Such desensitization mechanisms might function by resetting the balance between activation and inactivation of the reaction in question. GTP utilization, for example, might proceed rapidly after the onset of illumination but then slow down as illumination continues. This is consistent with the observation that brief illumination causes a GTP decrease proportional to the number of rhodopsin molecules bleached (cf. Fig. 5), but with continuous illumination the steady amplitude of the GTP decrease correlates with the rate of rhodopsin bleaching rather than with the total number of rhodopsin molecules which have been bleached (Fig. 3). A similar situation is observed with cyclic GMP (Woodruff and Bowns, 1979). (It seems unlikely that the lowered effectiveness of continuous illumination in producing a GTP decrease is caused by stimulation of GTP synthesis to balance its degradation. The synthesis of  $\text{GT}^{32}\text{P}$  from  $^{32}\text{P}_i$  added to these suspensions requires minutes and is not influenced by illumination.<sup>2</sup> Further, the recovery of GTP levels after cessation of illumination [Fig. 5] is slow and incomplete, suggesting that synthesis does not proceed rapidly.)

The light-induced decrease in GTP cannot readily be accounted for by utilization in either rhodopsin phosphorylation or the cyclic GMP pathway. The data of Table I show that rhodopsin phosphorylation, assayed under conditions where dephosphorylation is very slow (Miller and Paulsen, 1975), uses only a small fraction of the GTP degraded upon illumination. Several observations also suggest that the light-induced cyclic GMP decrease is not tightly linked to GTP levels. Cyclic GMP is present in 10-fold lower levels than its precursor GTP and undergoes a more rapid ( $t_{1/2} \sim 125$  ms) decrease on illumination than GTP ( $t_{1/2} \sim 7$  s). More important, the light-induced GTP decrease can be completely abolished in low calcium media while the cyclic GMP decrease is maintained (Woodruff and Bowns, 1979). Raising calcium concentration from  $10^{-9}$  to  $10^{-3}$  M raises GTP levels (Table III) but lowers the level of cyclic GMP (Woodruff and Bowns, 1979).

<sup>1</sup> Robinson, P. R., S. Kawamura, B. Abrahamson, and M. D. Bowns. Controls of the light sensitivity of cyclic GMP phosphodiesterase in frog photoreceptor membranes. Manuscript submitted for publication.

<sup>2</sup> Biernbaum, M. S., and M. D. Bowns. Unpublished observations.

GTP concentration can fall to the same level as that caused by saturating illumination if outer segments are kept dark but exposed to low-calcium ( $10^{-8}$  M) media, and restoration of millimolar levels of calcium causes net synthesis of GTP. Nucleotide synthesis caused by reversing membrane calcium flux is characteristic of calcium pumps (Makinose and Hasselbach, 1971; also references cited in de Meis and Carvalho, 1974), and thus the data raise the possibility that calcium and GTP may be linked by a calcium pump mechanism. The fact that GTP in outer segments decreases to stable intermediate levels as external calcium is lowered in steps between  $10^{-6}$  and  $10^{-8}$  M suggests an equilibrium relationship between internal GTP and external calcium. This may be analogous to the steady states observed in other calcium transport systems in which uptake balances efflux (de Meis and Carvalho, 1974). To explain the data by a reversible calcium pump one assumes that calcium gradients occur under these conditions, most likely across the plasma membrane, and that most of the calcium that crosses the membrane in response to these gradients does so by means of a carrier mechanism linking calcium movement to GTP hydrolysis or synthesis. Thus, the lowering of external calcium to  $\sim 10^{-9}$  M, by addition of EGTA to a suspension of outer segments whose internal calcium levels are  $\sim 10^{-6}$  M (Hagins and Yoshikami, 1977), should drive the exit of calcium and GTP hydrolysis, whereas adding back calcium after the EGTA exposure should cause calcium entry and synthesis of GTP. Proof of a calcium pump will require direct measurement of fluxes mediated by the pump, and such experiments have not yet been reported for physiologically intact outer segments.

In the present experiments, addition of the divalent cation ionophore A23187 had the effect of diminishing the GTP changes caused by altering external calcium. This is the result expected on adding an additional calcium permeability mechanism alongside a carrier mechanism linking calcium movement with either the hydrolysis or synthesis of nucleoside triphosphate. A serious problem in interpreting this data, however, is that A23187 probably influences the properties of the disc as well as the plasma membrane system. This discussion has emphasized the plasma membrane, but one does not know the possible contribution of the disc membrane system to transport processes involving calcium or GTP. Although there is some evidence for an energy-dependent calcium sequestering mechanism in disc membranes (Daemen et al., 1977; Schnetkamp et al., 1977), the point is disputed (Szuts, 1979).

The sensitivity of internal GTP to external calcium also might be accounted for by an outer segment GTPase enzyme which is normally inhibited by calcium. Thus, GTP hydrolysis might occur if calcium is removed by lowering external calcium levels. This suggestion, however, conflicts with the observation that GTP levels decrease if an attempt is made to increase the internal calcium concentration by incubating outer segments in  $10^{-3}$  M calcium medium containing the ionophore A23187.

Both light and lowering external calcium levels can reduce GTP by as much as 70%. Also, lowering external calcium to intermediate levels ( $10^{-6}$  to  $10^{-7}$  M) and exposure to intermediate intensities of illumination have similar effects on GTP. This raises the possibility that lowering external calcium

activates the same process normally activated by light, perhaps an outward movement of calcium across the plasma membrane.<sup>3</sup> One cannot exclude the possibility, however, that the "calcium-sensitive GTPase" and "light-sensitive GTPase" are separate systems which have in common only their utilization of the same pool of GTP. It is also possible that the light-dependent degradation of GTP reflects the activity of a GTPase enzyme(s) which requires calcium as a cofactor.

An important aspect of the light-induced decrease in GTP is that it appears sufficient to yield large amounts of free energy rapidly to drive endergonic processes in rod excitation. Within 1 s of the onset of illumination, at least 85 molecules of GTP disappear for each rhodopsin molecule bleached (Fig. 2). Although some of this GTP is utilized by the pathways discussed above, one must leave open the possibility that it also contributes to energy-dependent steps in the transduction machinery which have not yet been specified.

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<sup>3</sup> This interpretation is strengthened by the recent work of Gold and Korenbrot who have observed a light-dependent active extrusion of calcium from rod cells into the extracellular space in intact functional retinas. (Gold, G., and J. Korenbrot. Personal communication.)

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