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LIGHT-ACTIVATED HYDROLYSIS OF GTP AND CYCLIC GMP IN THE ROD OUTER SEGMENTS

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SUMMARY

1. The hydrolysis of guanosine triphosphate (GTP) and the consequent formation of guanosine diphosphate (GDP) and phosphate (P_i) are activated by light in a suspension of broken retinal rods: the hydrolysis rate with GTP in the μM concentration range is 2·5–3·5 n-mole/min per mg of rhodopsin in the preparation.

2. The ionic composition of the medium suspending the rods is not critical: the hydrolysis is present in NaCl saline solution with Mg^{2+} as well as in Tris-HCl buffer solution, and with the chelating agent EDTA.

3. The ionic strength is critical: the effect is reduced when the broken rods are suspended in a low salt mannitol solution, and is altogether abolished when they are separated from the mannitol solution; it reappears when the mannitol solution is added again in the presence of salts. An element essential for the effect is thus reversibly released in the mannitol solution. No hydrolytic activity on GTP, however, is found in the mannitol soluble fraction.

4. The cyclic nucleotide phosphodiesterase is eluted from the rods in the mannitol solution, and is reaggregated to the rods in the presence of salts; once recombined with the rods, it can be activated by light.

5. The activation of the phosphodiesterase by light is present in the absence of added nucleotide triphosphates.

INTRODUCTION

Previous studies have shown that (i) the illumination of isolated frog rods induces the hydrolysis of high energy triphosphates, (ii) this reaction is sensitive to stimuli that bleach a small amount of photopigment, (iii) the reaction is rapid and within 1 sec a sizeable fraction of the nucleotides is hydrolysed and (iv) after weak illuminations the level of these compounds is restored in darkness (Robinson, Yoshikami & Hagins, 1975; Carretta & Cavaggioni, 1976; Bignetti & Cavaggioni, 1977).

These findings suggest that the hydrolysis of triphosphates may have a physiological role in the mechanism of photoreceptor excitation and justify pursuing the study of the effect. The two earlier studies were carried out in suspensions of isolated rods with the natural nucleotides and the intracellular medium could not be controlled. In the Bignetti & Cavaggioni (1977) study the effect of light was present also in fragmented rods with added triphosphates. The possibility of studying the effect in a medium of definite composition had prompted the present study. In particular,

it became interesting to investigate the relationships of the hydrolysis of the high energy triphosphates with the nucleotide phosphodiesterase since both activities share the common property of being activated by light (Miki, Keirns, Marcus, Freeman & Bitensky, 1973; Miki, Baraban, Keirns, Boyce & Bitensky, 1975).

METHODS

The preparation

The frog rods were isolated from the retinae of bullfrogs (*Rana catesbeiana*) in Ringer solution, and sedimented by the method described elsewhere (Carretta & Cavaggioni, 1976). The pellet was resuspended in 300 μl. of saline or mannitol solution (see Table 1), and broken by forcing the solution to flow 20–30 times through a syringe needle (22 gauge). The broken rods were separated from the soluble fraction by sedimentation (25,000 *g*, 15 min). The rhodopsin content was determined by the differential absorption of light (λ = 500 nm) of an extract in 3% Triton X-100, assuming for rhodopsin a molar absorbance of 40,500 M^{-1} cm^{-1} (Daemen, 1973). A medium size bullfrog yielded 0·4–0·6 mg of rhodopsin. In some cases we used a suspension of bovine rod outer segments purified by a flotation in sucrose (35%, w/w) and by a sucrose continuous gradient (25–35%, w/w) without salts; the ratio of the absorbances measured at 280 and 500 nm was 2·25. Soluble fractions were recombined in several ways with frog broken rods or bovine rod outer segments, as shown in Fig. 1 and as will be explained in detail in the text.

Radioactive materials were from Amersham; non-radioactive nucleotides were purchased from Boehringer and used without further purification. In one experiment cyclic GMP was purified of traces of GTP by absorption on aluminium oxide (Merck).

TABLE 1. Composition of solutions used

	Solutions (mM)			
Salts	Ringer	Saline	Mannitol	EDTA*
NaCl	102	110	—	110
KCl	2·7	—	—	—
$MgCl_2$	2·1	1	—	—
$CaCl_2$	1·8	—	—	—
$NaHCO_3$	2	2	2	2
NaH_2PO_4	0·36	0·36	0·36	0·36
Mannitol	—	—	220	—
EDTA	—	—	—	10
pH	7·3	7·3	7·3	7·3

* Ethylenediaminetetraacetic acid (sodium salt).

Assay for hydrolysis of GTP and ATP

All the reactions were carried out in dim red light and at room temperature. Adenosine 5′-[α-^{32}P] triphosphate, guanosine 5′-[α-^{32}P]triphosphate or guanosine 5′-[γ-^{32}P]triphosphate, sodium salts, were used as tracers. The reaction was initiated when the triphosphate nucleotides, with 10^7 d.p.m. of the tracer, in 100 μl. 175 mM-Tris (hydroxymethyl)amino-methane-HCl (Tris-HCl) buffer (pH 7·4) were added to 300 μl. of the preparation. A part of the preparation received a flash of light while the rest remained in darkness and was used as a control. 25 μl. samples were withdrawn at intervals of 30 sec and mixed with 8 or 16 μl. of a 20% solution of trichloroacetic acid (TCA) to stop the reaction. A control was made by mixing 25 μl. of the preparation with the TCA solution, and adding successively the radioactive solution. The samples were centrifuged (200 *g* for 5 min) and 1 or 0·5 μl. of the supernatant of each sample were absorbed over an aluminium sheet precoated with a layer (0·10 mm thick) of polyethylene imine cellulose (Merck), and stepwise developed with sodium formiate buffer (pH 3·4) 0·25 M for 30 sec, 2 M for 4 min and 4 M for about 1 h. The chromatography sheet was dried and placed to autoradiograph on an X-ray film with the inter-position of a paper sheet to avoid chemography. The film was developed after about 24 h exposure and the spots containing

the products labelled with ^{32}P were localized. All the radioactive spots were cut, eluted with 10 ml. 1 M-formic acid in glass scintillation vials and the Cherenkov radiation was measured in a liquid scintillation counter with gain and energy window at maximum. The counts (2% precision) of the radioactivity of the GTP, GDP and GMP spots of each sample (*A*, *B* and *C* respectively) were expressed in per cent of the over-all radioactivity of the sample after correcting the counts of GDP and GMP by the value of the control (*b* and *c*):

$$\text{GTP} = \frac{A}{A+B+C-b-c} \times 100,$$

$$\text{GDP} = \frac{B-b}{A+B+C-b-c} \times 100,$$

$$\text{GMP} = \frac{C-c}{A+B+C-b-c} \times 100.$$

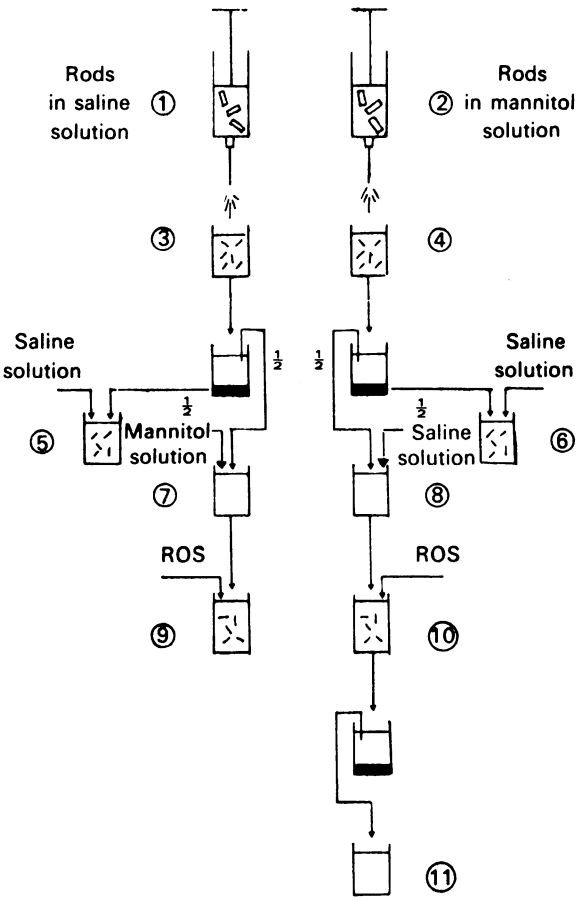

Fig. 1. Recombinations of the sedimentable and soluble fractions of the rods. Isolated rods in saline and mannitol solution are shown in 1 and 2 respectively. They are broken by forcing them through a syringe needle, 3 and 4. The suspensions are sedimented and the sediments resuspended in saline solution, 5 and 6. The composition of the supernatants is equalized by adding saline to the mannitol supernatant and *vice versa*, 7 and 8. Bovine rod outer segments (ROS) washed in sucrose solution are added to the equalized supernatants, 9 and 10. The supernatant is separated from the rod outer segments by sedimentation, 11.

Assay for hydrolysis and formation of cyclic GMP

[8-^{3}H] guanosine 3′, 5′-cyclic phosphate was used as radioactive tracer and the reaction was carried out in the same way as described for GTP with 10^7 d.p.m. of the tracer, except that the reaction was stopped by boiling the samples for 1 min. Protamine sulphate (Sigma, Grade I) 0·5 mg/ml. has been used in a few experiments. 5 μl. of the samples were deposited on a thin layer chromatography aluminium sheet Silica Gel 60 F_{254} (Merck), and developed for 3 h with a solution containing butanol, acetone, acetic acid, 5% ammonia solution and water (35:25:15:10 v/v). The spots were visualized under U.V. light (λ = 254 nm), cut, and left in 2 ml. 0·5 M-ammonia solution to elute the nucleotides for approximately 1 h. The eluate was neutralized with HCl, and 7 ml. Bray scintillation solution were added (Bray, 1960). The counting was done in the tritium channel of a liquid scintillation counter with 2% precision. For the cyclase assay the method of Zimmerman, Daemen & Bonting (1976) has been used but without theophylline and using [α-^{32}P]ATP. The reaction was stopped, and the products of the reaction were determined by the methods described for the phosphodiesterase assay.

Assay of the high energy phosphate with the luciferin-luciferase system

Some early experiments were carried out with this method that allows rough estimates of the amount of the high energy compounds in equilibrium with ATP in the preparation. The volume of the preparation was 3 ml., and the test was carried out in a stopped-flow apparatus after addition of ATP as previously described (Bignetti & Cavaggioni, 1977).

Stimulation

The illumination of the suspension was by means of a photographic flash apparatus with a broad band interference filter (λ = 500 nm), Balzer K3 and NG4 neutral density filters (Schott) and bleached 10% of the rhodopsin.

RESULTS

1. *GTP hydrolysis*

In the suspension of broken rods, GTP was hydrolysed in darkness at a rate of about 0·2–0·5 n-mole/min per mg of rhodopsin. The hydrolysis was accelerated when the suspension was illuminated; Fig. 2 illustrates the spontaneous hydrolysis of GTP in darkness (continuous lines), as well as the enhancement of the hydrolysis after a flash that bleached 10% of the rhodopsin (interrupted lines). After the flash the rate of hydrolysis rose to 2·5–3·5 n-mole/min per mg of rhodopsin. The fraction of GTP hydrolysed after the flash was greater with μm concentrations of GTP in the experimental conditions, and became relatively smaller at higher concentrations. In three experiments, the preparation was divided in two parts, one half received ATP and the other GTP; the effect of light was observed only with GTP; it was also seen that ATP was hydrolysed with a fast rate in darkness suggesting the presence of non-specific ATP-ases. In other experiments [γ-^{32}P]GTP was used rather than [α-^{32}P]GTP, and the radioactive product of the hydrolysis was chromatographically identified as $^{32}P_i$.

2. *Ionic requirements*

The hydrolysis of GTP was studied with broken rods suspended in the 10 mM-EDTA solution and thus virtually free of Ca^{2+} or Mg^{2+}. The rate of hydrolysis in darkness was slower than usual (0·045 n-mole/min per mg of rhodopsin) and there was no sizeable formation of GMP, but the hydrolysis was raised by light to 0·45 n-mole/min per mg of rhodopsin (see Fig 3). The effect was also present in

a solution of Tris-HCl buffer with 1 mM-((ethylene dioxy) diethylene-dinitrilo) tetra-acetic acid (EGTA) where the sodium concentration was estimated to be less than 10 mM after suspending the broken rods.

The effect was smaller when the broken rods were suspended in the mannitol solution with low salt concentration than in saline solution. This point is illustrated in Fig. 4 from an experiment in which the luciferin-luciferase system was used.

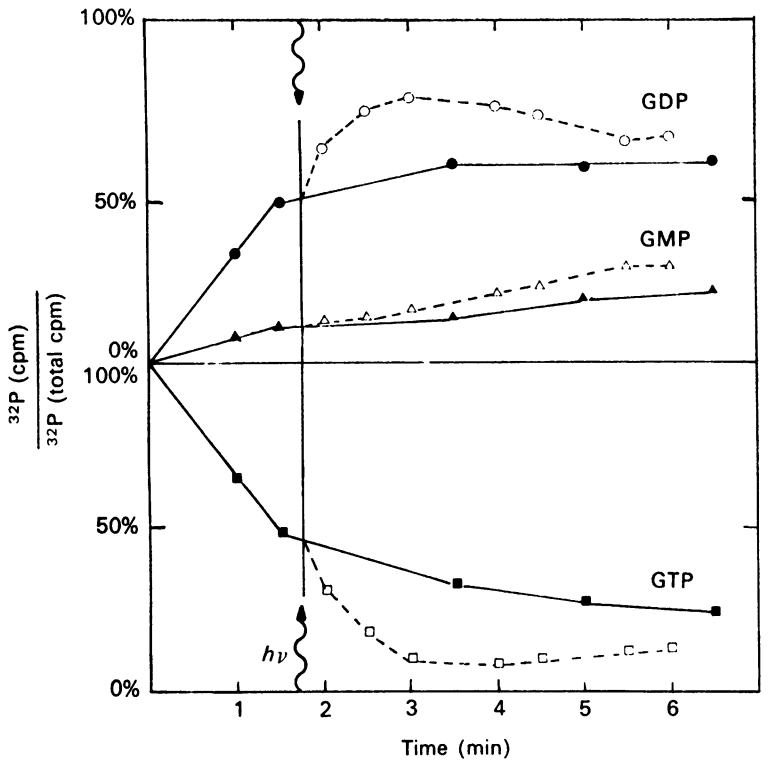

Fig. 2. Effect of light on the hydrolysis of [α-^{32}P]GTP in broken rods. Filled symbols and continuous curves show hydrolysis in darkness, empty symbols and interrupted lines show hydrolysis after the flash (arrow). See 3 of Fig. 1. The radioactivity of GTP, GDP and GMP are in per cent of the total radioactivity of the samples. Initial GTP concentration 1·6 μM, preparation containing *ca.* 0·08 mg of rhodopsin.

3. *Need for a factor soluble in the mannitol solution*

An effect was also present when the cytoplasm was washed away by successively breaking the rods in the saline solution, sedimenting, discarding the *saline-supernatant*, and resuspending the rods in new saline solution. An effect was absent, however, when the mannitol solution was used instead of the saline solution, and the rods were resuspended in saline solution after discarding the *mannitol supernatant* (see Fig. 5*A* and *B*). Bovine rods washed in sucrose solution without salts during purification were also inert.

The effect was brought back when the inert cattle or frog rods were treated with the mannitol supernatant in the presence of salts; the saline supernatant did not

restore the effect. Fig. 6 shows the results of an experiment with the luciferin-luciferase system, and Fig. 7 the hydrolysis of GTP in bovine rods with frog mannitol supernatant. The saline and mannitol supernatants have been compared for GTP hydrolysis; no difference was found and the rate of hydrolysis was even slower than that seen in the presence of discs in darkness (Fig. 8).

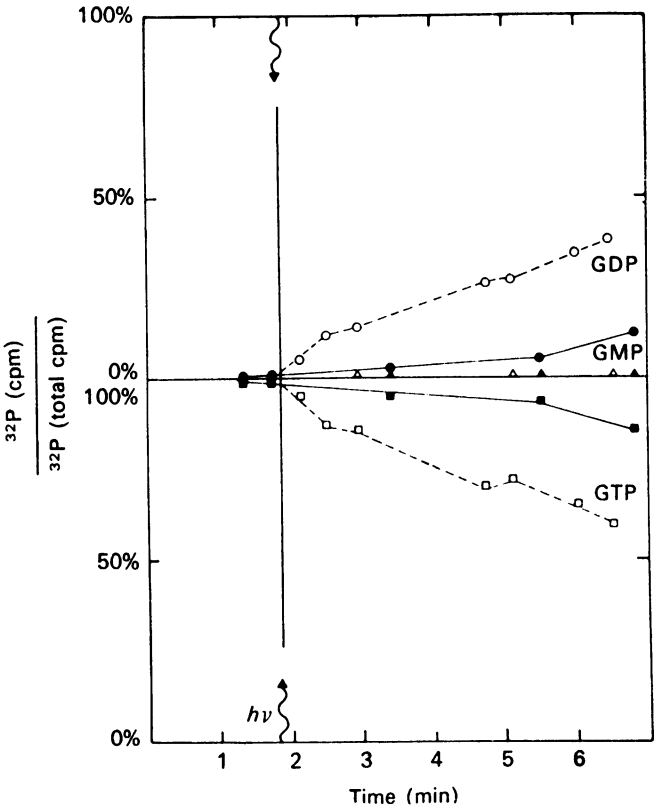

Fig. 3. Hydrolysis of [α-^{32}P]GTP in a solution with 10 mM-EDTA. Symbols as in Fig. 2. Initial GTP concentration 3 μM, preparation containing *ca.* 0·24 mg rhodopsin.

4. *Phosphodiesterase activity*

It has been described that the nucleotide phosphodiesterase of the rods is eluted in low salt media and can be reabsorbed to the rod membranes in the presence of salts with recovery of the sensitivity to light (Miki *et al.* 1975); the mannitol and the saline supernatants were thus compared for phosphodiesterase activity. Only the mannitol supernatant hydrolysed cyclic GMP: the enzyme extracted from frog rods with 1 mg rhodopsin hydrolysed approximately 2·4 μmole/min, and the activity was not enhanced by protamine. The activity could be removed by reaggregating the phosphodiesterase to bovine rods in the presence of salts and sedimenting away the complex (Fig. 9). Bovine rod outer segments purified in a solution of sucrose without salts (see Methods) did not have phosphodiesterase activity, but in the recombined system the flash enhanced the hydrolysis of cyclic GMP and the Fig. 10 shows the

effect tested in the presence of ATP as recommended by Miki *et al.* (1973); it is seen that part of the radioactivity of cyclic GMP was recovered with the di- and/or triphosphates, a transformation that was absent when ATP was omitted. The activation of the phosphodiesterase was present also in the EDTA solution, but the hydrolysis rate was slower.

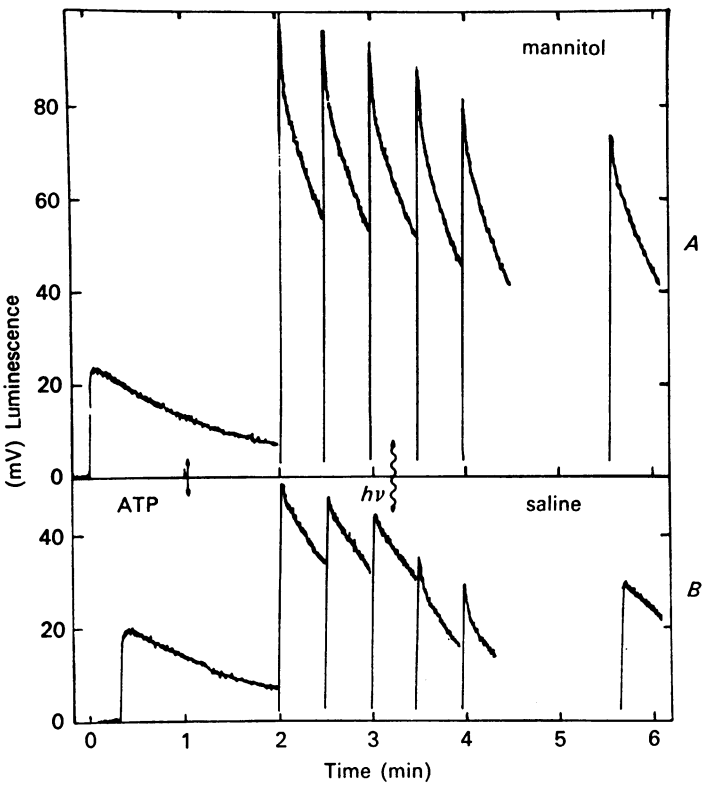

Fig. 4. The effect in mannitol and saline solution. The high energy compounds were tested with the luciferin-luciferase system. *A* and *B* are two halves of the same preparation of rods broken in mannitol and saline solution respectively. See 3 and 4 of Fig. 1. The broken rods were mixed with the luciferin-luciferase system of the firefly in a stopped flow apparatus, first trace of *A* and *B*; successively, ATP was added and the reaction started again three times at regular time intervals; a flash was given to a part of the preparation (arrow) and the reaction repeated twice with the stimulated material, and a final trace was taken with non-stimulated rods. Preparation of two frog retinae. Initial ATP concentration 0·16 μM.

5. *GTP and the phosphodiesterase*

The photic activation of the phosphodiesterase was observed in the presence and in the absence of added GTP. The activation by the flash bleaching 10 % of the rhodopsin was present and the amplitude constant with 10^{-3} M, 10^{-6} M added GTP as well as when GTP was omitted; the hydrolysis rate was 25–29 μmole/min per mg of rhodopsin (Fig. 11). In this experiment the frog broken rods have been washed of the cytoplasm by centrifugation and the purified cyclic GMP has been used, in order to reduce the free triphosphate concentration. In another series of experiments, the

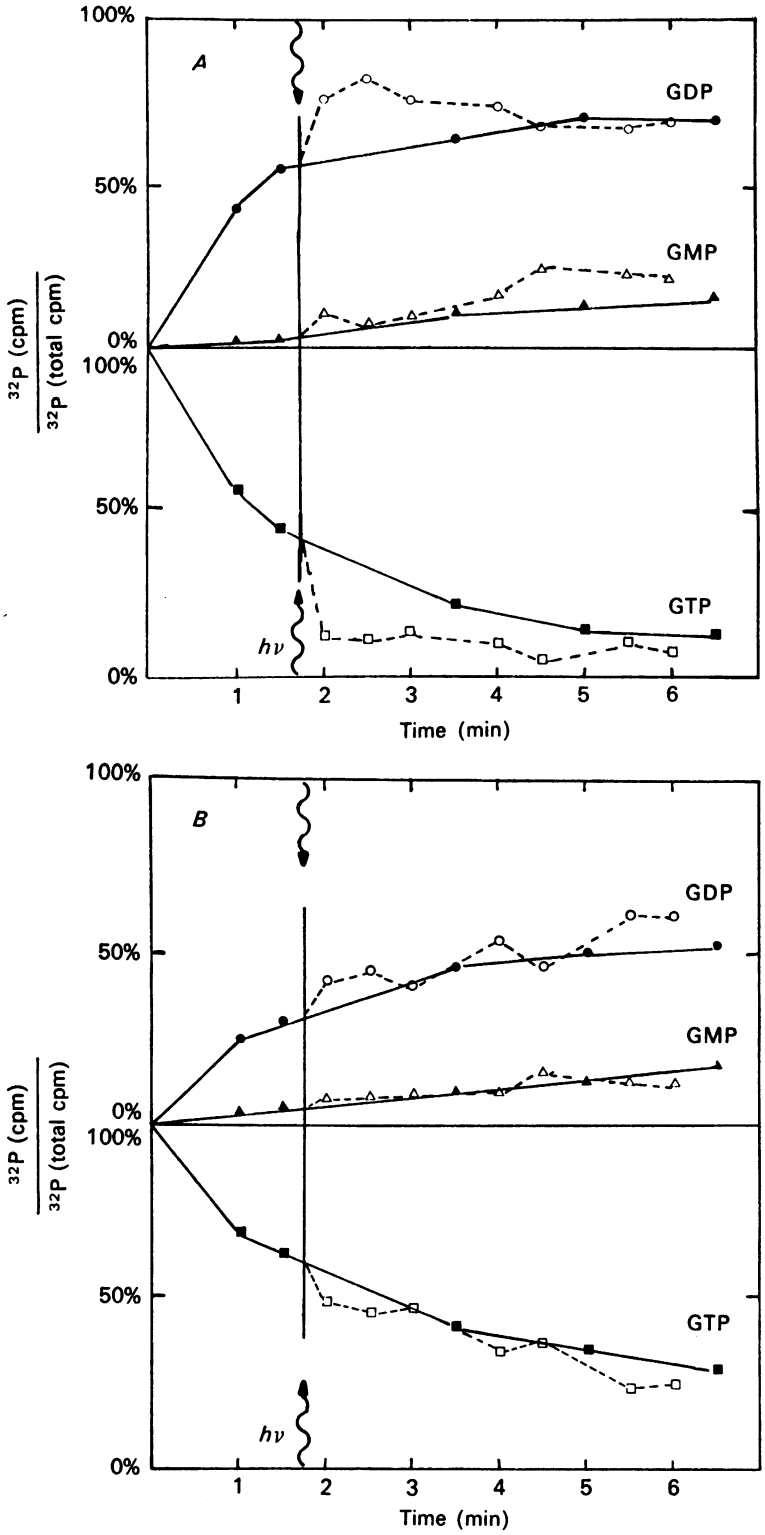

Fig. 5. For legend see facing page.

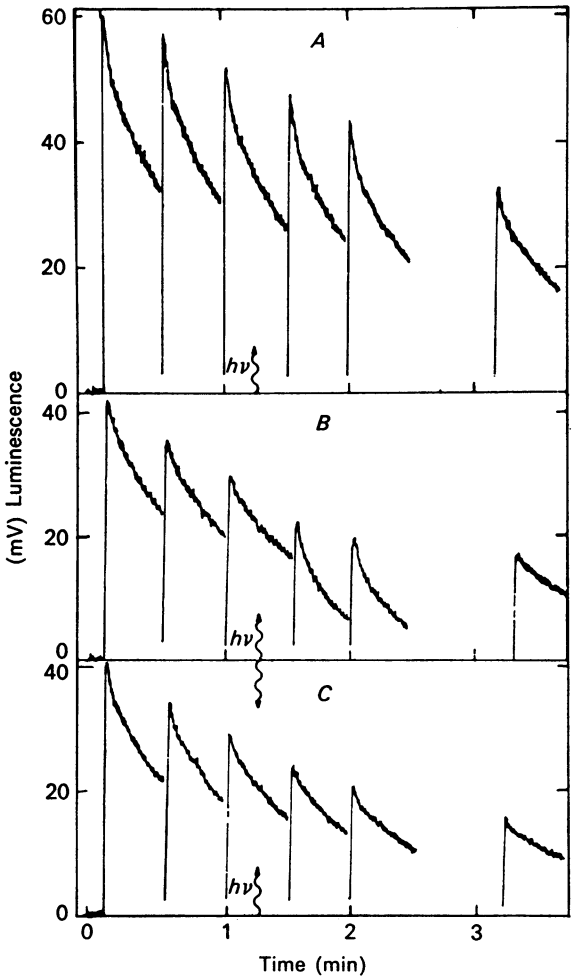

Fig. 6. Effect of light in bovine rod outer segments treated with the mannitol supernatant of a frog. The high energy compounds are tested with the luciferin-luciferase system. *A*: suspension of bovine rod outer segments in a solution of equal parts of mannitol and saline solution; *B*: suspension of bovine rod outer segments in solution obtained by mixing equal volumes of frog mannitol supernatant and saline solution; *C*: same as *B* with equal parts of saline supernatant and mannitol solution. *B* and *C* are 10 and 9 of Fig. 1. The two supernatants were from the same preparation of isolated rods of one frog. Cattle rhodopsin 0·04 mg/ml. ATP initial concentration 0·25 μM in *A* and 0·16 μM in *B* and *C*.

Fig. 5. Hydrolysis of [α-^{32}P]GTP in rods washed in saline or in mannitol solution. *A*: rods broken in saline solution; *B*: rods broken in mannitol solution. The rods were sedimented and resuspended in saline solution. See 5 and 6 of Fig. 1. Initial GTP concentrations: *A*, 5 μM, *B*, 6·5 μM. Symbols as in Fig. 2. Two preparations from two medium size bullfrogs.

phosphodiesterase of frog rods, either bleached or unbleached and washed of the cytoplasm, has been recombined with purified bovine rod outer segments and its activation by light was again constant over a range of concentration of added GTP from 10^{-3} M to about 10^{-10} M (Fig. 12).

Cyclase activity was present in the purified bovine rod outer segments and was not modified by illumination.

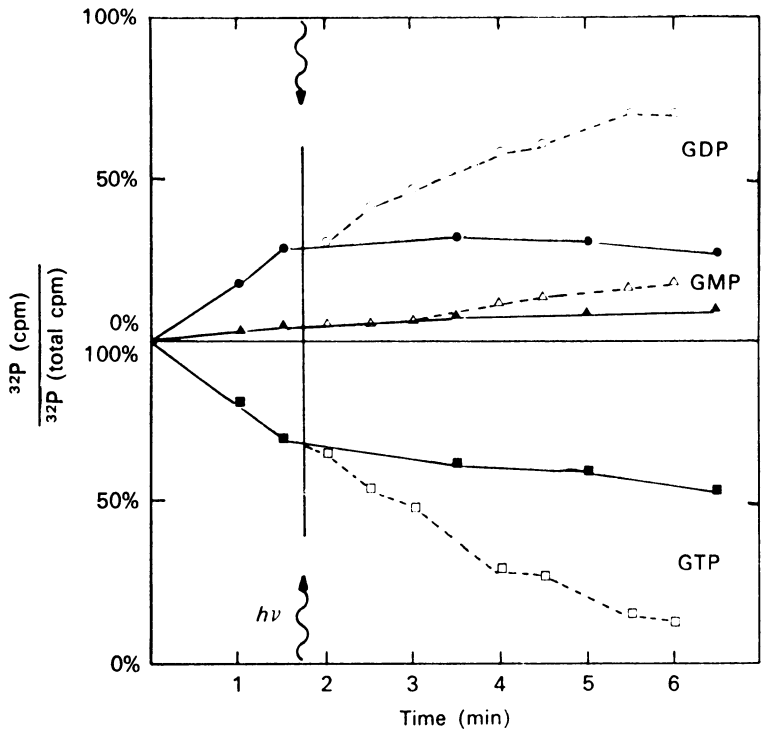

Fig. 7. Hydrolysis of [α-^{32}P]GTP in bovine rod outer segments treated with the mannitol supernatant of one frog. Bovine rod outer segments were resuspended in a solution containing frog rod mannitol supernatant and saline solution in equal parts. See 10 of Fig. 1. Initial GTP concentration 6 μM, cattle rhodopsin. 0·3 mg, supernatant from one frog eye. Symbols as in Fig. 2.

DISCUSSION

The illumination of a suspension of broken rods increases the hydrolysis of GTP. The rate of hydrolysis of GTP, after a flash bleaching 10% of the rhodopsin, 2·5–3·5 n-mole/min per mg of rhodopsin, is sufficiently fast to account for the decrease of triphosphates observed in isolated rods (Robinson *et al.* 1975: Carretta & Cavaggioni, 1976; Bignetti & Cavaggioni, 1977), but small enough to have eluded some previous attempts to measure it. The complexity of this system is documented by the formation of GMP, as a product of hydrolysis of GTP, and, conversely, by the formation of GDP in the presence of kinase in our preparations. The observation that a similar effect has not been detected with ATP does not exclude that a small light induced ATP hydrolysis may be obscured by a fast nonspecific consumption, probably caused by subcellular contaminants in the broken rods.

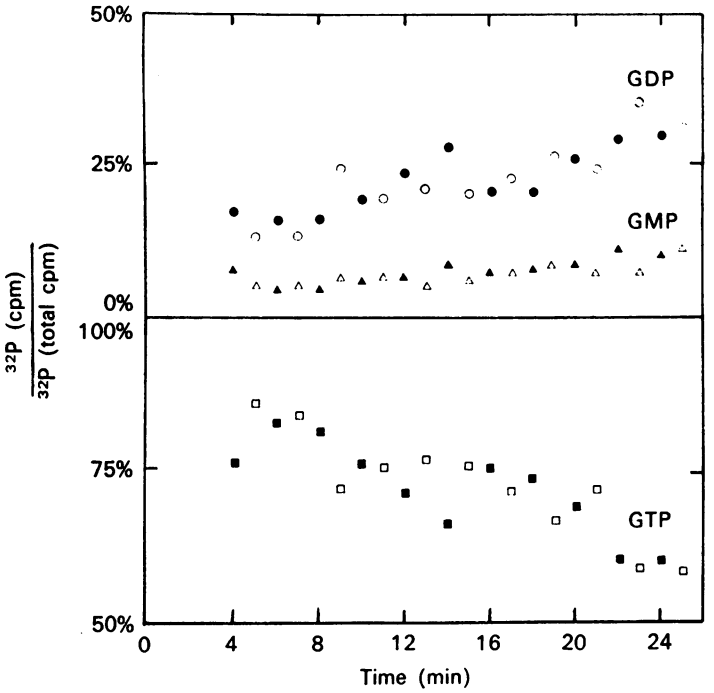

Fig. 8. Hydrolysis of [α-^{32}P]GTP in the mannitol (open symbols) and saline (filled symbols) supernatants. The mannitol and the saline supernatants of the same preparation of rods of one frog were mixed with equal amounts of mannitol and saline solution respectively, to make the composition equal. See 8 and 7 of Fig. 1. GTP initial concentration 5 μM.

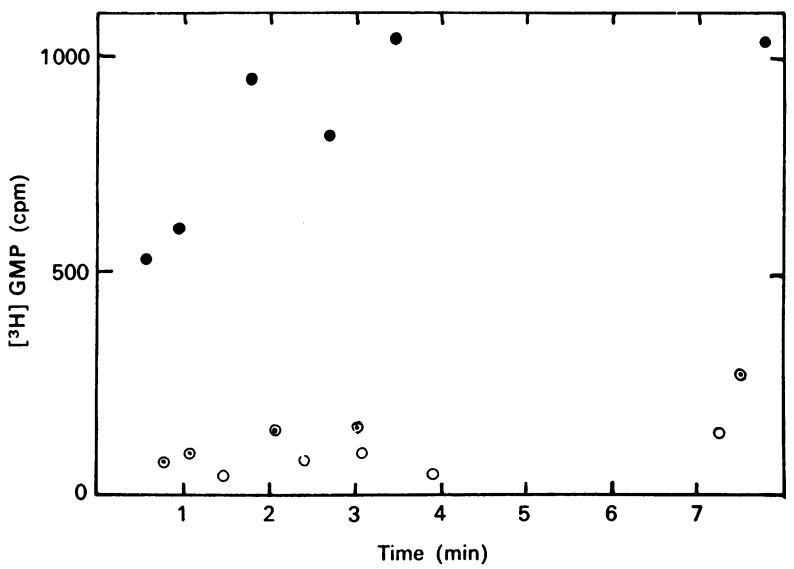

Fig. 9. *Hydrolysis of* [^{3}H]cyclic GMP in the frog supernatants. Mannitol (filled circles) and saline (open circles) supernatants of the same preparation of rods of one frog were equalized in composition, and the formation of linear GMP due to the phosphodiesterase activity was studied. See 8 and 7 of Fig. 1. Circle points refer to the equalized mannitol supernatant cleared of phosphodiesterase activity by adding and sedimenting away bovine rod outer segments (0·20 mg rhodopsin). See 11 of Fig. 1. Initial cyclic GMP concentration 2 mM.

A peculiar aspect of the light induced effect on GTP hydrolysis is that it does not depend on either Mg^{2+} or Ca^{2+} or any other particular cation, while the ionic strength is critical. In fact, the effect requires a *factor* that is released in low salt solution. When this factor is removed from the rod disc membrane, GTP-ase activity is lost;

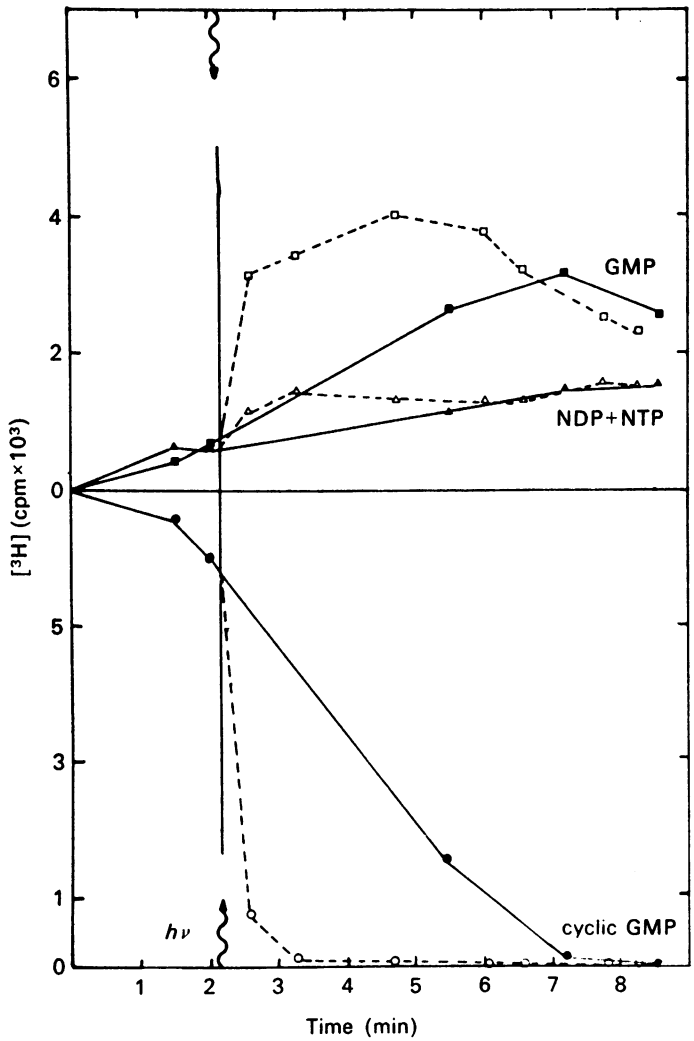

Fig. 10. Hydrolysis of [^{3}H]cyclic GMP by phosphodiesterase in the presence of ATP. Bovine rod outer segments (0·28 mg rhodopsin) suspended in a solution containing frog mannitol supernatant and saline solution in equal amounts and 1 mM-ATP. See 10 of Fig. 1. The disappearance of cyclic GMP and the formation of linear GMP as well as of di- and triphosphates (NDP and NTP) are shown. Initial cyclic GMP concentration 2 mM. Preparation from one eye of a medium size bullfrog.

on the other hand, allowing the membrane to reabsorb the factor in high salt solution, GTP-ase activity and its light induced regulation appear again. At this stage we cannot decide whether the GTP-ase is a component of this extractable fraction that becomes inactive when it is solubilized from the membrane, or rather

a membrane bound enzyme that becomes inactive when this fraction is extracted from the membrane. The rod phosphodiesterase activity can be extracted in low salt media and can be measured in solution; it can be transferred again to the rod membrane in high salt media, resuming photic sensitivity (Goridis *et al.* 1973: Miki *et al.* 1975). Although the phosphodiesterase is a component of the soluble fraction, it cannot be decided whether or not this is the necessary factor for GTPase activity. Also

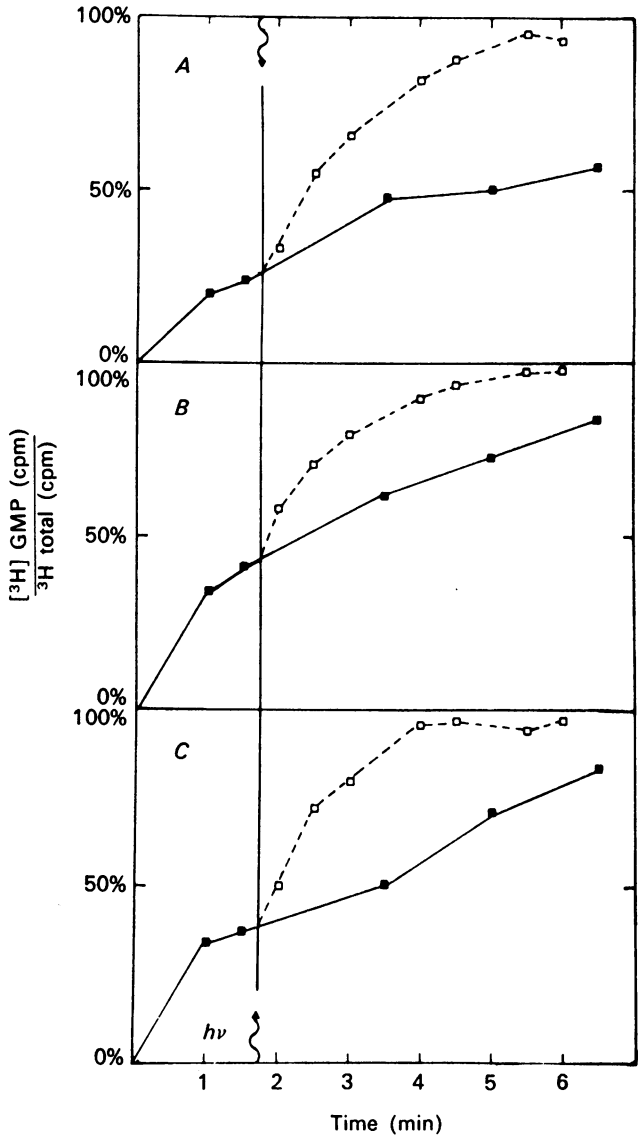

Fig. 11. Light activation of frog rod phosphodiesterase. Broken frog rod outer segments washed out of the cytoplasm by centrifugation in NaCl saline solution (6500 *g* × 15 min). *A*, without added GTP; *B*, with 10^{-6} M-GTP; *C* with 10^{-3} M-GTP. In *A*, cyclic GMP was purified (see Methods). Initial cyclic GMP concentration 4 mM; $MgCl_2$ 10 mM. The percent recovery of the radioactivity of cyclic GMP is reported. 0·025 mg rhodopsin per test.

the opsin kinase, another enzyme of the rods has been shown to be soluble in low salt solutions (Weller, Goridis & Mandel, 1975). Experiments are needed with the purified components rather than with an ill defined mannitol extract in order to define the role of all the soluble components. By contrast, cyclase activity was found in the membrane also after washing in low salt solutions, and was not modified by light, in agreement with some reports (Miki *et al.* 1973, Goridis *et al.* 1973) but not with others (Bensinger, Fletcher & Chader, 1973; Pannbaker, 1973; Krishna *et al.* 1976).

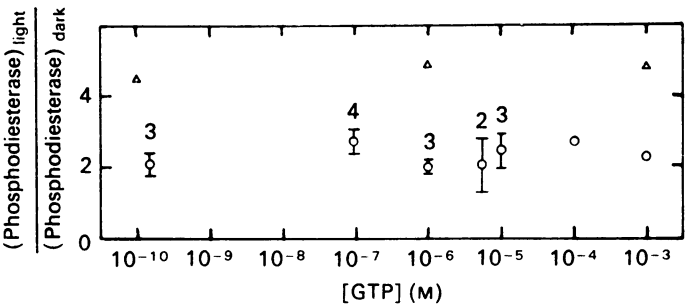

Fig. 12. Light activation of phosphodiesterase and GTP concentration. Ordinate: ratio of the initial phosphodiesterase rates after the flash and in darkness. In some cases the mean, the standard deviation and the number of experiments are shown. Abscissa: concentration of added GTP assuming that the cyclic GMP/GTP ratio is 10^4 in the commercial preparation of cyclic GMP. Triangles refer to broken frog rods and circles to frog phosphodiesterase extracted from rods (0·030 mg rhodopsin) and recombined with bovine purified rods (0·25 mg rhodopsin). Initial cyclic GMP concentration 4 mM.

The present experiments showing GTP and cyclic GMP hydrolysis both activated by light, raise the question as to the structural and functional relationships of the two activities. The only requirement found in our work is that the *whole* system has to be bound to the membrane of the rod. No temporal sequence is evident for these metabolic processes since the requirement of a triphosphate in the mM concentration range for the photic activation of the phosphodiesterase (Miki *et al.* 1973) has not been confirmed, and the effect of light remains constant in our conditions when the GTP concentration is varied between 10^{-3} M and *ca.* 10^{-9} M. The reason of the discrepancy is not clear: (i) the hydrolytic activity of the phosphodiesterase bound to the frog rods measured without added triphosphates after the flash is 25–30 μmole/min per mg of rhodopsin, and should be compared with 4 μmole/min per mg of protein reported by Miki *et al.* (1973) with added ATP; (ii) the hydrolysis rate in solution can be deduced from the present data and is a few hundred μmole of cyclic GMP/mg of enzyme per min, on the basis of the molecular weight (240,000 daltons) and of the stoichiometric ratio between rhodopsin and phosphodiesterase (900/1) (Miki *et al.* 1975), a value in agreement with the rate of the purified phosphodiesterase, 185 μmole/mg per min after protamine activation reported by the same authors (1975). In the present experiments, however, protamine does not increase the activity suggesting that the phosphodiesterase is in a different state in our conditions. It cannot be excluded that the phosphodiesterase has very tight triphosphate

nucleotides binding sites saturated by naturally occurring nucleotides throughout the preparation procedure, nor that GTP at very low concentrations plays a role in the regulation of cyclic nucleotide metabolism by analogy with other membrane-receptor systems (e.g. Salomon, Lin, Londos, Rendell & Rodbell, 1975). For the time being, the relationship between the two enzymes remains problematic.

Note added in proof. A report has been recently published on this subject by G. L. Wheeler, Y. Matuo & N. W. Bitensky (1977: *Nature, Lond.* **269**, 822–824).

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