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BIOCHEMICAL ASPECTS OF THE VISUAL PROCESS

XXIV. ADENYLATE CYCLASE AND ROD PHOTORECEPTOR MEMBRANES: A CRITICAL APPRAISAL*

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SUMMARY

Adenylate cyclase was found to be present in rod outer segment preparations, but its specific activity was only about 1% of activities reported in earlier studies. In frog the activities ranged from 0.015 to 1.1 nmoles 3′,5′ cyclic AMP/mg protein per 10 min depending on the method of preparation and homogenization. In cattle, the rod outer segment layer obtained after sucrose density gradient centrifugation, had an activity of 0.22 nmole 3′,5′ cyclic AMP/mg protein per 10 min. Furthermore a second (more dense) layer obtained in this procedure possessed a 10 times higher specific activity.

Light decreased the adenylate cyclase activity in the rod outer segment suspensions of both frog and cattle, but the maximal inhibition was about 50% at extensive illumination. Light did not affect the activity in the second layer, unless rod outer segment layer material was present, indicating that an inhibitory diffusible factor is released from outer segments during illumination. Evidence that either Ca^{2+} or free all-*trans* retinaldehyde constitutes this factor could not be obtained.

The activities of some marker enzymes in the two layers and in whole retina homogenates from cattle were determined. Comparison of some properties of the adenylate cyclase activities in the two layers and consideration of these enzyme activities do not exclude the possibility that the activity in the rod outer segment material is due to contamination with other retinal material.

The available evidence does not support a direct role for 3′,5′ cyclic AMP in the visual excitation process.

INTRODUCTION

Recently Bitensky *et al.*[1–4] reported a high adenylate cyclase activity in rod outer segments, which was nearly completely inhibited by illumination. On the basis of these results it was suggested that 3′,5′ cyclic AMP might act as a mediator between

Abbreviation: EGTA, ethyleneglycol-bis-(β-aminoethyl-ether)-*N*,*N*′-tetraacetic acid.

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the primary, light-induced events in the rod disk membrane, involving rhodopsin and the decrease in sodium permeability of the plasma of the rod outer segment.

In repeating and extending these experiments we are unable to confirm the results of Bitensky *et al.* We find a relatively low adenylate cyclase activity in rod outer segments, which is only partly inhibited by light, and which may even be due to contamination by other retinal structures. These findings make it unlikely that 3′,5′ cyclic AMP acts as a mediator in visual excitation in the rod cell.

METHODS

Frog rod outer segments were prepared in two different ways: by the flotation method used by Bitensky *et al.*[1] (Method II) and by shaking off the outer segments in saline (Method I). The latter method is much faster and produces less contaminating cellular debris. In both cases the frogs (*Rana temporaria* or *Rana esculenta*) were dark-adapted during periods varying from 2 to 18 h. All subsequent operations, except deliberate illumination, were performed under dim red light. After decapitation the retinas were extruded through a slit in the cornea and treated according to one of the following methods. Method I: the retinas were collected in ice-cold modified Ringer solution (containing 112 mM NaCl, 3 mM KCl, 3 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose, 3 mM ATP sodium salt and buffered to pH 7.4 with 10 mM Tris–HCl), gently shaken for 1 min and filtered over a stainless steel wire screen (60 mesh). In some cases the suspension was centrifuged for 5 min at $4000 \times g$ and the sediment suspended in Ringer or buffer solution.

Method II: the retinas were collected in ice-cold 47.6% sucrose (0.1–0.2 ml per retina) and agitated vigorously during 1 min on a Vortex mechanical vibrator. The suspension was centrifuged at $100000 \times g$ for 90 min at 4 °C in a Spinco SW 39 rotor. The viscous paste of rod outer segments on top of the solution was collected with a spatula.

These suspensions were freshly prepared before each assay and homogenized in various ways indicated under Results.

Bovine rod outer segments were prepared according to de Grip *et al.*[5]. Bovine eyes (30–40), placed immediately after death at room temperature in a light-tight container, were dissected within 2 h and the retinas were mildly homogenized in 15 ml ice-cold Tris–HCl buffer (0.16 M, pH 7.1) by means of a loosely fitting Potter–Elvehjem homogenizer. The homogenate was filtered through 120-mesh stainless steel wire screen under cautious stirring. The residue was washed with the same Tris–HCl buffer and the combined filtrates represented whole retinal homogenate. For isolation of outer segments this homogenate was mixed with 66.7% aqueous sucrose to a final concentration of 0.42 M and a final volume of 52 ml. With this suspension and an equal volume of 40% aqueous sucrose solution, two continuous gradients with a density range of 1.05–1.18 (0.42–1.38 M) were prepared. After centrifugation in a swing-out rotor (1 h, $27000 \times g$, 10 °C) a heavy sediment and two layers were obtained with densities of approximately 1.10 and 1.13. The upper layer at $d \approx 1.10$ contains the rod outer segments. Both layers were collected and stored at −20 °C. After thawing the suspensions were routinely sonicated (3 times for 1 s with a Branson B 12 sonifier at maximal output for use with microtip).

Illumination

The outer segment preparation was illuminated either in ice during 10 min by a 100 W tungsten lamp through 3 mm thick GG3 and OG2 filters (Schott–Jena) or by 1–3 flashes from a Rollei Strobofix E60 flash lamp through an OG2 filter. In the latter case the light intensity was regulated by varying the distance between lamp and suspension.

Before and after illumination 150-μl aliquots were taken to determine the rhodopsin content. The 150-μl aliquot was mixed with 125 μl Ringer of buffer solution and 30 μl 10% Triton X-100. After centrifugation (10 min, 8000 $\times g$) 200 μl supernatant was transferred to a cuvet with 1-cm light path and 10 μl hydroxylamine (1 M) was added. The 500-nm absorbance was determined before and after illumination during 5 min by a 100 W tungsten lamp through GG3 and OG2 filters. Comparison of the 500-nm absorbance change (ΔA_{500nm}) of an non-illuminated sample with the ΔA_{500nm} of the bleached samples yields the percentage bleaching at a given light intensity.

Enzyme assays

Adenylate cyclase activity was assayed in dim red light at 30 °C with a reaction volume of 25 μl and a standard incubation time of 10 min. The medium contained 1.6 mM ATP, 5.3 mM aminophylline, 2.9 mM $MgSO_4$, 32 mM glycylglycine (pH 7.4) and an ATP regenerating system (2 mM phosphocreatine and 80 μg/tube creatine-phosphokinase). The reaction was stopped by addition of 350 μl ice-cold 7% trichloroacetic acid. The saturation analysis of Brown *et al.*[6], as modified by Cooke *et al.*[7], was used for the determination of 3′,5′ cyclic AMP formed during incubation. Immediately after termination of the incubation 50 nCi, ^{3}H-labelled 3′,5′ cyclic AMP was added. The sample was centrifuged and the protein washed with 5% trichloroacetic acid and water. The combined supernatants were chromatographed and assayed as described by Cooke *et al.*[7]. Recovery of ^{3}H-labelled 3′,5′ cyclic AMP was usually 70–80% and never fell below 50%. The formation of 3′,5′ cyclic AMP during incubation proved to be linear with time for at least 20 min. The adenylate cyclase activities are expressed in units (1 unit = 1 nmole 3′,5′ cyclic AMP/mg protein per 10 min). The relative standard error for triplicate determinations was 8%.

(Na^+–K^+)-ATPase and Mg^{2+}-ATPase activities were determined as described by Bonting[8]. The tissue preparation was lyophilized and before assay the dry material was reconstituted with distilled water. Aliquots of 20 μl were mixed with 300 μl of either one of two media and incubated for 15–60 min at 37 °C. The activity in Medium A (2mM ATP, 55 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 100 mM Tris, 0.1 mM EDTA) represents total ATPase activity, while activity in Medium E (2 mM ATP, 60 mM NaCl, 2 mM $MgCl_2$, 100 mM Tris, 0.1 mM EDTA and 0.1 mM ouabain) represents the Mg^{2+}-ATPase (Na^+- and K^+-insensitive) activity. The difference between these two activities gives the (Na^+–K^+)-ATPase activity.

5′-Nucleotidase was determined according to Heppel and Hilmoe[9]. A total volume of 150 μl, containing 1 M glycine–NaOH buffer, pH 8.5, 0.1 M $MgCl_2$, 2.4 mM 5′-AMP and enzyme, was incubated for 15 min at 37 °C. The reaction was terminated by addition of 50 μl 20% trichloroacetic acid. Inorganic phosphate was determined according to Bonting[8].

Alkaline phosphatase was assayed according to Wöltgens *et al.*[10] and succinate

dehydrogenase according to King[11] using phenazine methosulphate as an artificial electron acceptor.

Protein was measured by the method of Lowry *et al.*[12] using bovine serum albumin as a standard.

Materials

Adenosine 5'-triphosphate (ATP), adenosine 3',5'-monophosphate (3',5' cyclic AMP), creatinephosphate and creatinephosphokinase (rabbit muscle) were obtained from Boehringer Mannheim, Germany, bovine serum albumin from Behringwerke AG, Marburg/Lahn, Germany, Dowex 50Wx8, 200–400 mesh from Fluka AG, Germany, sucrose organic analytical standard from B.D.H. chemicals Ltd, Poole, England and all-*trans* retinaldehyde from Eastman Kodak Company, Rochester, New York. Adenosine-8-^{3}H-3',5'-monophosphate, ammonium salt (24.1 Ci/mmole) was received from the Radiochemical Centre, Amersham, Nottingham, England.

All other reagents were of analytical grade.

RESULTS

Adenylate cyclase activity in rod outer segments

With suspensions prepared according to Method I, the adenylate cyclase activity in frog rod outer segments depended to a large extent upon the method of homogenization (Table I, Column 2). While all activities were very much lower than those reported by Bitensky *et al.*[3], the highest activity (0.11 unit) was obtained after sonication. With suspensions prepared according to the method of Bitensky *et al.*[1] (Method II), we obtained somewhat higher activities (Table I, Column 3), which again depended on the method of homogenization. The highest activities were obtained by sonication in 40% sucrose and by glass on glass homogenization, 0.52

TABLE I

ADENYLATE CYCLASE ACTIVITY IN FROG ROD OUTER SEGMENTS, PREPARED AND TREATED IN DIFFERENT WAYS

Method I: outer segments isolated by gentle shaking in Ringer, followed by filtration through wire screen and centrifugation. Method II: outer segments isolated by vigorous shaking, followed by sucrose flotation.

Homogenization method	*Activity in nmole 3',5' cyclic AMP/mg protein per 10 min*	
	Method I	*Method II*
Repeatedly passing through injection needle in water	0.067	0.041
Glass on glass in glycylglycine buffer	0.022	0.49 ± 0.085 (4)
Glass on glass in Tris buffer	0.015	—
Glass on glass in Tris buffer, followed by freezing and thawing	0.024	—
Sonication in Ringer	0.11	0.31
Sonication in 40% (w/v) sucrose	—	0.52 ± 0.13 (7)
Range	0.015–0.11	0.041–0.52

± 0.13 and 0.49 ± 0.085 unit, respectively, representing only 0.8% of the average activity for frog reported by Bitensky *et al.*[3]. There was a considerable variation in activity between different samples. For seven samples isolated by Method II and treated by sonication in 40% sucrose the relative standard error was 25%, much larger than the 8% relative standard error for triplicate assays. In these suspensions a high sucrose concentration was necessary during the assay in order to obtain maximal activity. When the samples were diluted with water before assay, the activity decreased to 20% of that obtained by dilution with sucrose.

Most experiments on frog rods were done with sonicated suspensions obtained by Method II because these gave the highest activities and also because during the other homogenization procedure, which gave satisfactory activities (glass on glass homogenization), the inhibition by light was lost (see next section).

The adenylate cyclase activity was also determined in cattle rod outer segments prepared according to a method developed in our own laboratory (de Grip *et al.*[5]). In this method the gradient centrifugation of a retina homogenate yields two layers in addition to a pellet of heavy material. The two layers each contain about 3% of the protein present in the pellet. The upper (purple) layer contains the rod outer segments (rhodopsin), the lower layer contains no rhodopsin. Table II shows the distribution of the adenylate cyclase activity over both layers and the whole retina, the specific activity in the outer segment layer being only 1/10 as high as in the lower layer and 1/4 as high as in the whole retina.

The second layer was further characterized by determining the activities of five other enzymes in both layers and in whole retina (Table II). The rod outer segment layer is not contaminated with mitochondria as shown by the absence of the mitochondrial marker enzyme succinate dehydrogenase. The lower layer contains, in addition to mitochondria, plasma membrane fragments as indicated by the relatively high activities of the last four enzymes, which are all marker enzymes for the plasma membrane (De Pierre and Karnovsky[13]). It is as yet not clear which retinal cell types contribute to this layer. However, based on the tentative conclusion of Hagins[18], that a large dark current of Na^+ "is carried into the outer segments and extruded

TABLE II

ENZYME ACTIVITIES DETERMINED IN CATTLE RETINAS AND TWO FRACTIONS OBTAINED BY GRADIENT CENTRIFUGATION

	Whole retina	*Upper layer (rod outer segments)*	*Lower layer*
Adenylate cyclase*	4.8	1.3 ± 0.3 (5)	13.8 ± 1.8 (4)
Succinate dehydrogenase**	4.9 ± 1.3 (2)	0 (4)	12.7 ± 2.1 (5)
(Na^+-K^+)-ATPase***	15.4 ± 4.6 (2)	6.6 ± 0.2 (4)	30.6 ± 1.3 (4)
Mg^{2+}-ATPase***	8.7 ± 0.7 (2)	4.0 ± 0.2 (4)	13.8 ± 1.2 (4)
Alkaline phosphatase***	3.0 ± 0.6 (4)	1.2 ± 0.1 (6)	6.9 ± 0.6 (6)
5′-Nucleotidase†	3.8 ± 0.7 (4)	1.8 ± 0.1 (5)	8.7 ± 1.2 (6)

* Expressed as nmoles 3′,5′ cyclic AMP/mg protein per h.
** Expressed in arbitrary units.
*** Expressed in μmoles ATP/mg protein per h.
† Expressed in μmoles 5′-AMP/mg protein per h.

again by an ouabain-sensitive sodium pump, located somewhere along the inner segments and cell bodies", one could speculate that the very high (Na^+–K^+)-ATPase activity of the lower layer indicates the presence of a large amount of inner segment plasma membrane.

Effect of illumination

The effect of illumination on the adenylate cyclase activity of rod outer segment suspensions was determined at different light intensities. All illumination experiments were performed with sonicated suspensions, since it appeared that glass on glass homogenization destroys the light dependence, as was also reported by Bitensky *et al.*[3].

Both in frog and cattle rod outer segments there was a significant inactivation by light, especially at higher intensities. Since there was no basic difference between the light effects on the two species, the combined results of all illumination experiments, grouped according to the degree of bleaching of rhodopsin, are shown in Fig. 1. Complete inhibition upon total bleaching of rhodopsin was never observed. The average maximal decrease at extensive illumination was 50% of the original activity. Only one preparation of the twelve tested (included in the 21–40% bar) showed a larger inhibition (80%). At low bleaching levels (5% or less) we found no significant decrease in activity.

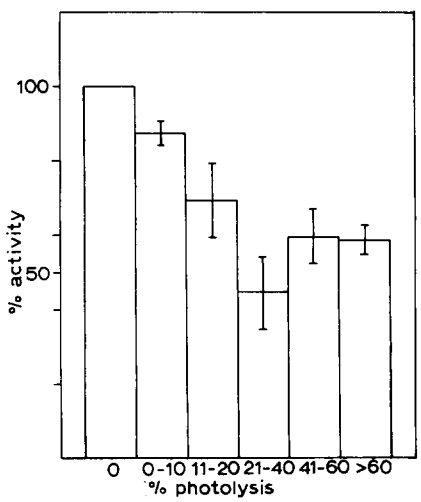

Fig. 1. Inhibitory effects of illumination on adenylate cyclase activity of rod outer segment suspensions. Combined results of all experiments (total 36) on frog and cattle.

Further characteristics of cattle retina adenylate cyclase activity

We examined the influence of Ca^{2+} and ethyleneglycol-bis-(β-aminoethyl-ether)-*N,N'*-tetraacetic acid (EGTA) on the adenylate cyclase activity in the rod outer segment layer from cattle retina. Table III shows that addition of Ca^{2+} to the assay medium inhibited the activity, while addition of 0.1 mM EGTA increased the activity. The latter increase could be suppressed by addition of Ca^{2+} ions. When the concentration of Ca^{2+} surpassed the EGTA concentration, stimulation changed into inhibition.

TABLE III

EFFECTS OF Ca^{2+} AND EGTA ON ADENYLATE CYCLASE ACTIVITY IN ROD OUTER SEGMENTS ISOLATED FROM CATTLE RETINA

Activities expressed in percent of the activity in a medium containing 1.6 mM ATP, 5.3 mM aminophylline, 2.9 mM $MgSO_4$, 32 mM glycylglycine, pH 7.4, 2 mM phosphocreatine and 80 μg creatine phosphokinase.

Ca^{2+} added (M)	*− EGTA (%)*	*+ EGTA (10^{-4} M) (%)*
0	100	158
10^{-6}	97	162
10^{-5}	93	158
10^{-4}	88	129
10^{-3}	28	24
10^{-2}	–	4

Addition of EGTA to the second layer also increased the adenylate cyclase activity. Addition of 10 mM NaF, which normally stimulates adenylate cyclase activity in other tissues, inhibited the activity in the rod outer segment layer by 35%, which is a very uncommon phenomenon, but stimulates the activity in the second layer by 40%.

Table IV shows that illumination had no effect on adenylate cyclase activity of the lower layer, but that a 3:1 mixture (on protein basis) of upper and lower layer showed a significantly larger decrease in activity than expected if only the upper layer activity were inhibited by light. This decrease ($35.0-24.2=10.8$) is even slightly larger than the entire upper layer dark activity present (8.3). This means that in the presence of upper layer material the lower activity is also affected by light. It suggests that a diffusible factor, released from the upper layer material, causes the inhibition of the adenylate cyclase activity by light.

The possibility that the diffusible factor might consist of Ca^{2+} was tested by addition of EGTA (0.05, 0.1 or 0.5 mM) before illumination. The light inhibition was not decreased. Another likely possibility is that the diffusible factor might consist

TABLE IV

EFFECT OF LIGHT ON ADENYLATE CYCLASE ACTIVITY IN UPPER AND LOWER LAYERS FROM CATTLE RETINA

Results in pmoles 3',5' cyclic AMP/10 min per tube, averages of three determinations, each with standard errors.

	Upper layer	*Lower layer*	*Mixture*		*P value difference calc'd − obs'd*
			Calculated	*Observed*	
Dark	8.3 ± 0.4	24.3 ± 1.2	32.6 ± 1.3	35.0 ± 2.9	0.5
Illuminated *	5.1 ± 0.7	25.3 ± 0.9	30.4 ± 1.1	24.2 ± 0.1	0.001

* Causing 50% bleaching of rhodopsin.

of all-*trans* retinaldehyde released from rhodopsin upon illumination. However, addition of 5 moles all-*trans* retinaldehyde (15 mM in methanol) per mole rhodopsin, followed by assay in darkness, gave the same adenylate cyclase activity in upper or lower layer as obtained upon addition of the solvent (0.5% final concentration) alone. Neither did addition of NADPH (5 moles per mole rhodopsin), which rapidly reduces free retinaldehyde to retinol, affect the inhibition by light. These findings plead against relase of Ca^{2+} or retinaldehyde as an explanation for the light inhibition of the adenylate cyclase activity.

DISCUSSION

Our experiments confirm the presence in rod outer segment preparations of an adenylate cyclase activity, which is decreased by previous illumination, as first reported by Bitensky *et al.*[1]. Here, however, the agreement ends. The maximal activities observed by us (1.1 units in frog and 0.4 unit in cattle) are only a small fraction of the activities claimed by Bitensky *et al.*[3] (ranging from 33 units for cattle to 78 units for *Rana catesbeiana*).

In trying to find an explanation for this discrepancy we tested several possibilities. Our assay method appears to be reliable. Internal 3′,5′ cyclic AMP standards were quantitatively recovered (93–104%). We find the same adenylate cyclase activity in rat pancreas (0.045 unit) as obtained by Rutten *et al.*[14]. For whole cattle retina homogenate we obtained an activity of 0.8 unit against 0.33 unit for whole calf retina reported by Brown and Makman[15]. Varying the ATP and enzyme protein concentrations in the assay does not increase the specific activity observed in rod outer segments.

Since sucrose is present in rather high concentrations during isolation of frog outer segments according to Method II and of cattle outer segments by gradient centrifugation, we investigated the possibility that the sucrose used by us could contain an inhibitory impurity. The activities observed with different reagent grade sucrose preparations were the same, even with the purest sucrose available to us (B.D.H. organic analytical standard).

It is a known fact that the adenylate cyclase activity is rather sensitive to the conditions of tissue preparation. Considerable attention was, therefore, paid to this aspect. On the one hand, we tried to duplicate the rod isolation method used by Bitensky *et al.*[1] (Method II) as accurately as possible, even though their method is not reported very exactly and varies in the different reports. On the other hand, we used a simple and rapid isolation technique, which gives a rather pure frog rod suspension (Method I). In general, higher activities were obtained with Method II, but even those were at best only 1.8% of those reported by Bitensky *et al.*[3].

The influence of the method of homogenization was also studied: glass-on-glass homogenization, repeatedly passing through an injection needle and sonication were used. The combination of isolation Method II with either glass-on-glass homogenization or sonication in sucrose gave the highest activities, but as mentioned before these were still only 0.8% of Bitensky's values on the average.

Another possible cause of low activities could be that the rhodopsin in the isolated outer segments would have been partially bleached. This cannot be the case for our frog rods, since the animals had been dark-adapted for several hours prior

to sacrifice and the retinas have been treated in darkness or safe red light (>610 nm). In the cattle outer segments part of the rhodopsin must have been in the bleached form, since they were normally not treated with 11-*cis* retinaldehyde[5] during isolation. In an experiment, in which the enrichment procedure was applied, no increase in the dark activity was observed as compared to a control, which was treated identically in the absence of added 11-*cis* retinaldehyde. Moreover, solubilization in digitonin did not increase the activity (0.17 ± 0.006 unit before, 0.18 ± 0.0016 unit after solubilization in 2% digitonin), as would have been expected in this case on the basis of the findings of Bitensky *et al.*[3]. Hence, this explanation for our low activities can also be dismissed.

Species differences do not seem to offer an explanation either. Bitensky *et al.*[3] report relatively small differences for different species: from 78 units for *Rana catesbeiana* to 48 units for *Rana pipiens*. In our experiments we do not find a clear difference between the activities in *Rana temporaria* (0.015–0.8 unit) and *Rana esculenta* (0.022–1.1 unit) or even in cattle (0.09–0.4 unit). Therefore, species differences do not seem to be involved.

A point, which casts some doubt on the validity of the high specific activities reported by Bitensky *et al.*[1], is the figure which they cite for the protein concentration of their rod suspensions. Using the eyes of 16 frogs (*Rana pipiens*) they state that in 240 μl rod suspension 720 μg protein was present, which amounts to 23 μg protein/retina. This is less than 1/6 of the amount we find (using *Rana temporaria* or *Rana esculenta*), which amount comes close to that calculated from the rhodopsin content, utilizing a molecular weight of 40000 (Bownds *et al.*[16]) and the fact that rhodopsin forms about 80% of the total rod outer segment protein (Robinson *et al.*[17]). If their protein determination has given erroneously low results, this would explain part of the discrepancy in specific activities of adenylate cyclase between our results and theirs. Furthermore, our finding of a 10-fold higher activity in the lower layer of the cattle retina fractionation than in the upper layer and the presumably lower rod purity in the flotation method used by Bitensky *et al.*[1] leave a further possibility for erroneously high activities in their experiments.

In addition to finding, even under the most favorable conditions, only about 1% of the activity reported by Bitensky *et al.*, the light sensitivity in our experiments differs considerably. While the rod adenylate cyclase activity is also decreased by prior illumination of the rods, the maximal decrease is only about 50% at full bleaching of the rhodopsin. As indicated before, this cannot be explained by partial bleaching of rhodopsin in the non-illuminated samples.

The question now arises whether the low adenylate cyclase activity really is a rod outer segment activity or whether it is due to a contamination with other retinal fragments containing a high activity. Our finding of a 10-fold activity in the lower layer from cattle retina would seem to support the latter possibility, but a second gradient centrifugation of the upper layer did not decrease its specific adenylate cyclase activity. There are, in addition, two differences in properties between the two activities: NaF (10 mM) inhibits the upper layer activity and stimulates the lower layer activity, light inhibits the upper layer activity and does not affect the lower layer activity. The latter difference, however, is only an apparent one as indicated by the results of the combination experiment (Table IV). This experiment suggests that the light effect is due to the release of an inhibitory factor by the photolysis of

rhodopsin. This leaves the effect of NaF as the only difference between the two enzyme activities. The fact that the activity in the rod outer segment layer is inhibited by NaF is very extraordinary. We cannot offer an explanation for this effect, but it does not completely exclude in our opinion the possibility that the adenylate cyclase —at least in cattle— in the rod outer segment fraction indeed derives from a contamination with other retinal material. This would also explain the large variation in the activity, and the fact that, in frog, lower activities are found in Method I, which in our opinion yields a purer preparation than Method II.

Finally, we want to consider the role which adenylate cyclase could play in the visual mechanism. Bitensky *et al.*[1–4] have ascribed an important role to the enzyme, on account of its allegedly high activity in the rods, its inhibition by light which is correlated with the percentage bleaching, and their finding that 3′,5′ cyclic AMP can mimic light. The latter compound might, therefore, play an important role in visual excitation. Several arguments can be advanced against this theory. First, a much lower adenylate cyclase activity is present than claimed by Bitensky *et al.*, and this activity may, moreover, be due to a contamination. Furthermore, the inhibitory effect of illumination in order to have a primary effect in excitation should show a very large decrease in activity at a minimal amount of bleaching. This we do not find and even the curve published by Miller *et al.*[2] does not show this. It appears more likely that the light inhibition is a side-effect of illumination. It could be due to an increase in free Ca^{2+} concentration near the photoreceptor membrane (Hagins[18]) or release of all-*trans* retinaldehyde, although our experiments aimed at elucidating this matter seem to exclude both possibilities. Bitensky *et al.*[1] also state that the occurrence of a 3′,5′ cyclic AMP-dependent protein kinase and a phosphodiesterase in photoreceptor membranes, neither of which they find to be light-sensitive, suggests a direct role of 3′,5′ cyclic AMP in visual excitation. These observations, however, also seem to be questionable. Kuhn and Dreyer[19], Frank *et al.*[20] and Bownds *et al.*[21] describe a light-dependent phosphorylation, which is not dependent on 3′,5′ cyclic AMP[19,21]. Pannbacker and Schoch[22] find three protein kinases in rod outer segments from which only the soluble one is 3′,5′ cyclic AMP dependent. The facts that Ca^{2+} can influence phosphodiesterase activity and that this ion may play a role in visual excitation, which are also cited as arguments by Bitensky *et al.*[4], are in themselves no indication for a role of 3′,5′ cyclic AMP in this process.

Hence, we conclude that there is not enough evidence to accept a direct role for 3′,5′ cyclic AMP in the visual excitation process. Even, if an adenylate cyclase activity exists in rod outer segments at all, it must play another role.

ADDENDUM

At the Symposium on Visual Pigments and Photoreceptors, National Eye Institute Bethesda, Md., U.S.A. June 6–8, 1973, Dr Bitensky revised his earlier high adenylate cyclase activities downward to values approximately equal to those reported in this paper. He also presented evidence that the light inhibition of the enzyme is caused by a diffusible factor, released from rod outer segment membranes upon illumination.

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