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PROTEIN INHIBITOR OF CYCLIC ADENOSINE 3′:5′-MONOPHOSPHATE PHOSPHODIESTERASE IN RETINA

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Summary

A protein acting as inhibitor of cyclic 3′:5′-nucleotide phosphodiesterase (EC 3.1.4.1.) activity was found in the ox retina tissue. An inhibitor from one tissue (ox retina) effectively cross-inhibited a phosphodiesterase from another tissue (rat brain), indicating a lack of tissue specificity.

Kinetic analysis showed that inhibition was independent of the time of preliminary incubation of the inhibitor with enzyme but dependent on its concentration in the reaction mixture. An inhibitor decreased the V of the enzyme and had no effect on its K_m for cyclic adenosine-3′:5′-monophosphate. The inhibitory effect was more pronounced with cyclic adenosine-3′:5′-monophosphate than with cyclic guanosine-3′:5′-monophosphate used as substrates of the reaction.

The extractable form of the phosphodiesterase of the retina rod outer segments was much more sensitive to the inhibitory action than the membrane-bound one.

The binding of labeled cyclic adenosine-3′:5′-monophosphate to the inhibitory protein was shown not to occur.

The inhibitor was sensitive to trypsin treatment, indicating that it was a protein which was resistant to boiling at pH 1.5; boiling at pH 11 obliterated all activity.

An attempt was made to purify the inhibitory factor. Gel filtration indicated that the inhibitor had a molecular weight of 38 000.

Introduction

Adenosine 3′:5′-monophosphate (cyclic AMP) has been shown to regulate a great variety of cellular processes. Recently some evidence appeared in favour of the role of cyclic AMP on the early stage of the photoreceptor process. The question was asked whether the enzymes which support cyclic nucleotide meta-

bolism might be present in photoreceptor outer segment membranes, and also, whether light might influence the regulation of cyclic nucleotide levels in this structure [1—3]. These questions seemed reasonable because photon capture by rhodopsin results in a change in the sodium conductance of the photoreceptor membrane, and cyclic AMP is known to alter cell membrane properties.

Due to these facts of fundamental importance the problem concerning regulation of the cyclic nucleotide content in the photoreceptor cell becomes evident. Cyclic nucleotide phosphodiesterase (EC 3.1.4.1) is the only enzyme that catalyzes the hydrolysis of cyclic AMP, thereby terminating its action. Thus, this enzyme (or enzyme system) plays an important role in determining the extent and duration of cyclic AMP action, and a study of the factors affecting phosphodiesterase activity assumes significance.

As mentioned in some recent works [4—7], low molecular proteins referred to as activators of phosphodiesterase were isolated from a number of tissues, such as brain, liver, heart, testis. The nature, properties and mechanism of action of these phosphodiesterase protein activators, has recently attracted much attention. This interest is fully justified since these proteins probably fulfil the function of nature regulators of phosphodiesterase activity. It should be noticed too that in some cases the activator proteins were recently shown to act as Ca^{2+}-binding proteins, and there is some evidence to indicate that the active form of the protein activator is a Ca^{2+}-activator complex [8—10].

We have discovered in animal tissue (ox retina) a protein fraction acting as a phosphodiesterase inhibitor. Up to now phosphodiesterase protein inhibitor has only been found in the slime mold *Dictyostelium discoideum* [11] and in plant tissue [12].

This communication describes experiments indicating the presence of the inhibitor in the ox retina. Some properties of the inhibitor and its nature are given.

Materials and Methods

Cyclic AMP and cyclic GMP were obtained from Boehringer Mannheim, Sigma and B.D.H. Chemicals, Ltd (Poole, England). Cyclic [^{14}C] AMP (ammonium salt, 209 mCi/mmol) was purchased from the Radiochemical Centre (Amersham). Lyophilized snake venom (*Echis carinatus*) was a product of the Institute of Zoology and Parasitology (Tashkent, U.S.S.R.). Acrilamide, *N,N'*-methylenebisacrylamide, *N,N,N',N'*-tetramethylenediamine, trypsin, soybean trypsin inhibitor, horseradish peroxidase, cytochrome *C*, bovine serum albumin and RNAase were from Reanal; Sephadex, G-75 and G-150 were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden); microgranular DE 32-cellulose was purchased from Whatman.

The main part of the present work was carried out on the isolated fraction of cattle retina rod outer segments, used as a source of enzyme system. The fractions of the rod outer segments were obtained according to the method worked out in our laboratory and reported previously [13].

A heat-stable, nondialyzable activator of phosphodiesterase was prepared according to the procedure described by Cheung [4].

Several grams of whole rat brain were homogenized in 3 vols. of cooled dis-

tilled water and the pH was adjusted to 5.9. The suspension was centrifuged for 30 min at 13 000 × *g*. The supernatant fluid was placed in a boiling water bath for 5 min. After cooling, the preparation was again centrifuged for 30 min at 13 000 × *g* and the supernatant fluid was subjected once more to heating and centrifugation. The supernatant fluid was finally concentrated with Sephadex G-150 and the samples were diluted to 5 mg protein per ml and were stored frozen. The same procedure was used for isolating a similar fraction from ox retina and rod outer segments.

Subsequent purification and determination of molecular weight were carried out using the technique of gel filtration. A Sephadex G-75 column (1.4 × 75 cm) was calibrated according to the method of Andrews [14], using insulin, cytochrome *c*, RNAase, soybean trypsin inhibitor, horseradish peroxidase and bovine serum albumin as markers. The column was equilibrated with 20 mM Tris-chloride, pH 7.5, containing 10 mM NaCl. 2-ml fractions were collected at a flow rate of 20 ml per h.

Another type of column was used to obtain the purified inhibitor fraction. The column (2 × 40 cm) with DE32-cellulose was equilibrated with 25 mM imidazole buffer, pH 6.5, containing 1 mM $MgSO_4$ and 0.2 M NaCl. The elution was carried out using the step-gradient of NaCl (from 0.2 to 0.7 M). 5-ml fractions were collected at a flow rate of 45 ml per h.

Analytical acrylamide gel disc electrophoresis was conducted according to Maurer [15].

The ability of protein fractions to bind cyclic AMP was investigated using the method of equilibrium dialysis [16].

Phosphodiesterase activity was determined by converting its reaction product, 5′-AMP into adenosine and inorganic phosphate by snake venom 5′-nucleotidase and then measuring the inorganic phosphate release [17,18]. The reaction mixture (total vol., 1 ml) consisted of 40 mM Tris chloride, pH 8.0; 0.1 mM $MnCl_2$; 100 μg snake venom; 1 mM cyclic AMP, and an appropriate concentration of the enzyme. The reaction was started by adding cyclic AMP. After 10 min incubation at 37°C the reaction was terminated by adding 0.1 ml of 55% trichloracetic acid.

The inorganic phosphate was measured by the method of Eibl and Lands [19]; protein content was assayed by the method of Lowry et al. [20].

Results and Discussion

At the early stage of the work the method for isolating protein fraction activating phosphodiesterase from rat brain tissue was followed. This fraction was shown to increase phosphodiesterase activity of rat brain homogenate and of isolated retina rod outer segments (Fig. 1). The relation between the degree of stimulation of phosphodiesterase of rod outer segments and the concentration of activator is depicted in Fig. 2a.

Thus, the above experiments offer more proof of the presence of phosphodiesterase protein activator in rat brain tissue and lack of tissue specificity of its effect, as has been shown by other investigators [4].

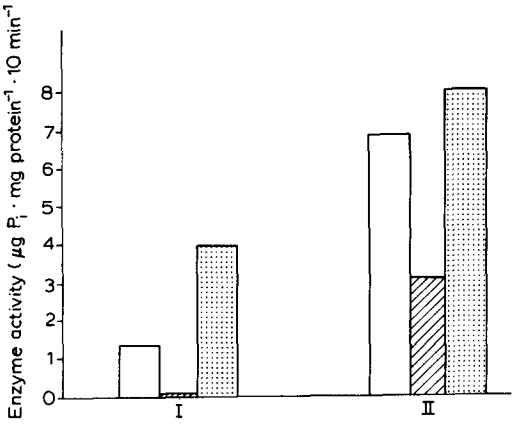

Fig. 1. Effect of brain activator and retina inhibitor on phosphodiesterase activity of rod outer segments and rat brain homogenate. I, Isolated rod outer segments served as a source of phosphodiesterase; II, Rat brain homogenate served as a source of phosphodiesterase; □, control activity of enzyme; ▨, phosphodiesterase activity in the presence of fraction from brain; ▩, phosphodiesterase activity in the presence of fraction from retina; assay was performed as described in Materials and Methods. The boiled pH 5.9 supernatants were used as a source of the activator and inhibitor.

An attempt to isolate a similar activator from ox retina tissue showed that this fraction, when added to the incubation mixture, evoked the inhibition of phosphodiesterase activity. The inhibitory effect of this retina fraction on phosphodiesterase activity was observed not only in rod outer segments, but in the brain homogenates also (Fig. 1), i.e. there was no tissue specificity for inhibition or activation.

Inhibition was directly proportional to the protein concentration (Fig. 2b).

The following difference should be noticed between the effect of the activator obtained from the brain tissue and the inhibitor isolated from the retina. It is evident from the data presented in Fig. 3, that in contrast to the activator the substrate reaction K_m does not change with the inhibitor.

The effect of inhibitor was recorded not only in the case of cyclic AMP as

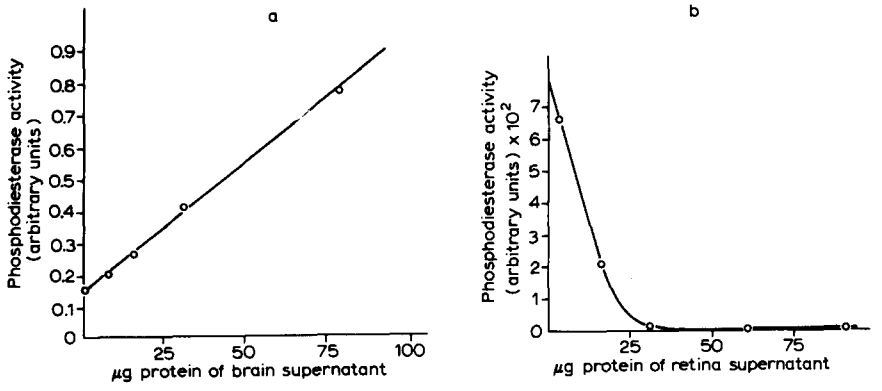

Fig. 2. Effect of activator (a) and inhibitor (b) concentrations on phosphodiesterase activity of rod outer segments. The phosphodiesterase activity was assayed as described in the text.

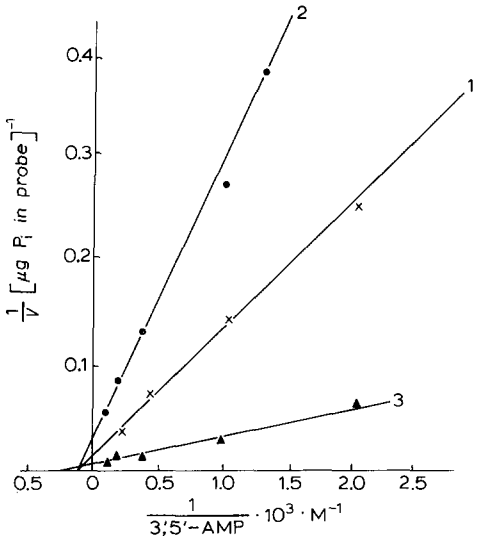

Fig. 3. Mechanism of inhibition (2) and stimulation (3) of phosphodiesterase activity by retina inhibitor and brain activator. The effect was studied by the double reciprocal plots. 1, control.

reaction substrate. It is well known that phosphodiesterase of rod outer segments also catalyzes the decay of cyclic GMP [21]. Comparing the influence of the inhibitor on the decay process of the two substrates, cyclic AMP and cyclic GMP, the inhibitory effect was shown to take place in both cases (Table I). However, the inhibitory action was more pronounced with cyclic AMP than with cyclic GMP, i.e. the inhibitor changes the relative rate of hydrolysis in favor of cyclic AMP.

Pre-incubation of the rod outer segments with the inhibitory factor 5 for 60 min did not affect the inhibition of the enzyme activity, suggesting that the in-

TABLE I

INFLUENCE OF INHIBITOR ON PHOSPHODIESTERASE ACTIVITY WITH CYCLIC AMP AND CYCLIC GMP AS SUBSTRATE

The phosphodiesterase activity was assayed as described in the text, using 1 mM cyclic AMP or 0.1 mM cyclic GMP as substrate of reaction. At these concentrations of substrates the levels of enzyme activity in control probes in the cases of cyclic AMP and cyclic GMP were close to one another.

Exp. No.	Inhibitor (μg protein)	Inhibition (%) Substrate	
		cyclic AMP	cyclic GMP
I	10	16	8
	20	23	13
	45	54	21
	90	100	100
II	5	9	7
	12	27	17
	50	100	70
III	50	41	13

hibitory effect is reversible. The absence of any affect of the time of preliminary incubation of phosphodiesterase with the activator on enzyme activity has been noted by other investigators [4].

The degree of the inhibitory effect on phosphodiesterase activity depended on the form of the enzyme. As a result of a number of experiments it was shown that about 50% of phosphodiesterase of rod outer segments is extractable and enters the solution after the rod outer segments precipitate has been treated with 0.9% NaCl [22]. On comparing the influence of the inhibitory fraction on the extracted phosphodiesterase and that bound to the rod outer segments membranes it was shown that the former is much more sensitive to the inhibitory action than the latter (Fig. 4). The 50% inhibition of the extracted phosphodiesterase was observed with as little as 6 μg of the inhibitory protein in the probe whereas to achieve the same degree of inhibition for the bound phosphodiesterase 40 μg of protein was needed, in which case phosphodiesterase failed to be inactivated completely. In this respect the properties of retina phosphodiesterase inhibitor were similar to those of the inhibitor isolated from slime mold [11].

When studying the mechanism of the observed inhibition the following question had to be solved first: whether or not the inhibitory effect is due to the nonspecific binding of the reaction substrate to the proteins present in the fraction. Such a mechanism of inhibition was described for phosphodiesterase of various sources [23]. However, in our case such a mechanism was not observed. By the method of equilibrium dialysis the binding of labeled cyclic AMP to the inhibitory protein was shown not to occur.

As shown, the action of isolated inhibitory factor ceases completely as a re-

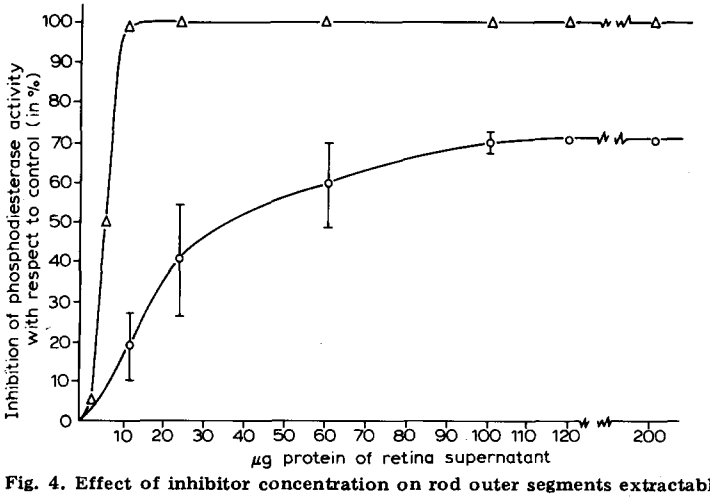

Fig. 4. Effect of inhibitor concentration on rod outer segments extractable and membrane bound phosphodiesterase activity. To reveal the extractable form of phosphodiesterase of rod outer segments the precipitate of the latter was homogenised two or three times in 0.9% NaCl, each time the mixture being incubated for 2 h at 4°C, centrifuged for 30 min at 12 000 × *g* and the enzyme activity being determined both in the precipitate (membrane bound form of enzyme) and supernatan (extractable form). After the third treatment of precipitate the supernatant revealed no enzyme activity. △——△, Extractable form; ○——○, membrane bound form.

TABLE II

EFFECTS OF TRYPSIN AND THERMAL TREATMENTS ON INHIBITORY ACTION

The incubation mixture contained the usual components as described in the text, and where applicable, 50 μg of trypsin. After incubation for 0.5 h at 22°C the tubes containing trypsin received 50 μg of soybean trypsin inhibitor. The thermal treatment was performed as follows: the supernatant fluid pH 5.9 was adjusted to desired pH with concentrated HCl (Nos. 7 and 8) or with 6 M NaOH (Nos. 9 and 10). Tubes were heated in a boiling water bath for 5 min and then cooled. The solutions were adjusted to pH 7.4 with solid $NaHCO_3$ or 6 M HCl. Denatured proteins precipitated during these treatments were removed by centrifugation. An aliquot of these neutral solutions was tested for its ability to inhibit the phosphodiesterase activity.

Exp. No.	Treatment	Control activity	Phosphodiesterase activity: ($\mu g\ P_i \cdot mg\ protein^{-1} \cdot 10\ min^{-1}$)	
			In the presence of untreated inhibitor	In the presence of treated inhibitor
1	Trypsin treatment	2.1	0	3.0
2	Trypsin treatment	1.1	0	1.8
3	Trypsin treatment	1.8	0	1.7
4	Trypsin treatment	4.3	0	5.0
5	Trypsin treatment	5.7	0	5.5
6	Trypsin treatment*	7.0	3.4	7.0
7	Boiling at pH 1.5	4.9	2.0	3.2
8	Boiling at pH 1.5**	11.3	4.6	6.5
9	Boiling at pH 11	4.9	2.0	4.5
10	Boiling at pH 11**	11.3	4.6	11.3

* The purified inhibitor (received from Sephadex 6-75 column) was treated.

** A high level of the phosphodiesterase activity in the control probes in experiments Nos. 8 and 10 may be due to the previously observed season alterations in the enzyme activity.

sult of trypsin treatment (Table II). This experiment demonstrated that the inhibitory activity was associated with a protein.

Other properties of the inhibitor obtained from retina appeared to be quite close to the activator isolated from brain [4]. The inhibitory factor turned out to be fairly thermostable; when boiled for 5 min at pH 1.5 it did not lose its activity, but boiling at pH 11 obliterated all inhibitory effect (Table II).

In the next series of experiments an attempt was made to purify the inhibitory factor and to determine its nature. Fig. 5 depicts the elution profile of a pH 5.9 supernatant fluid from Sephadex G-75 column. The peak of inhibitory activity is clearly seen to correspond to the fraction 17. It is of interest that after passing through the column a fraction activating phosphodiesterase was revealed (fraction 22). It should be noticed that no activator was observed in a non-purified pH 5.9 supernatant.

The molecular weights of purified fractions affecting the phosphodiesterase activity were as follows: activation protein 15 000, and inhibitor protein 38 000. Calibration of the Sephadex G-75 column and determination of the molecular weights were performed accoding to Andrews [14]. With respect to the activator protein this result fully confirms the data obtained in the experiment with other tissues [6]. The molecular weight of the inhibitory factor was found to be almost the same as that of the protein inhibitor isolated from the slime mold *Dictyostelium discoideum* [11].

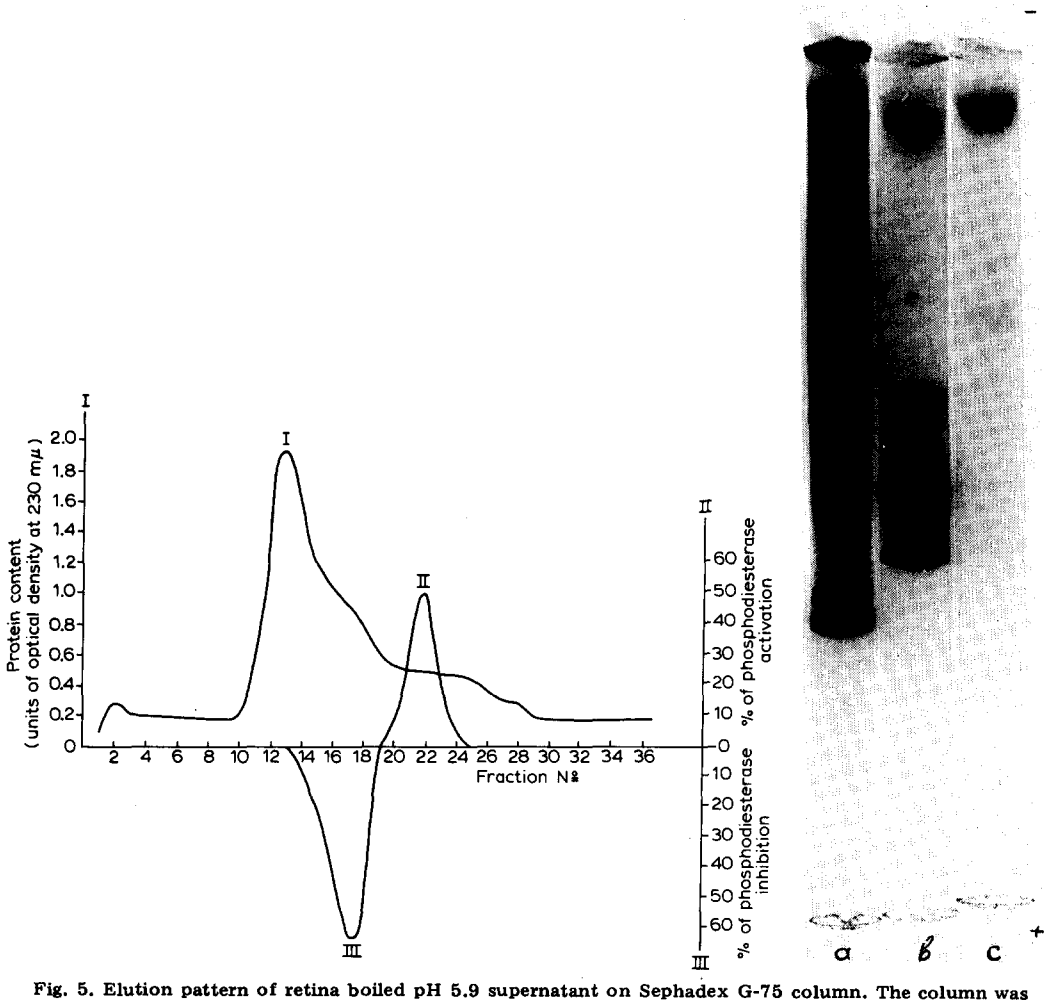

Fig. 5. Elution pattern of retina boiled pH 5.9 supernatant on Sephadex G-75 column. The column was prepared and equilibrated as described in Materials and Methods. A boiled pH 5.9 supernatant fluid served as a source of inhibitor. 0.5 ml of the inhibitor with 5 mg of protein was applied to the column. Protein was followed spectrophotometrically at 230 nm. An aliquot of each fraction was assayed for its ability to affect the phosphodiesterase activity.

Fig. 6. Polyacrylamide gel electrophoresis of a boiled pH 5.9 supernatant (a), purified on Sephadex G-75 column inhibitor (b) and purified activator (c). Disc gel electrophoresis was performed by the method of Maurer [15], using 7.5% polyacrylamide gels. Amido black was used for staining proteins. The amount of protein in each gel was 120 μg.

Electrophoresis of an unpurified inhibitory fraction indicated 7 bands, 4 of which were major and 3 minor (Fig. 6a). After this fraction was allowed to pass through the column, the protein activator was represented by one band (Fig. 6c), whereas the inhibitory fraction by two main and three minor bands (Fig. 6b).

The electrophoretic heterogeneity of this fraction may be a result of bad purification. The inhibitory protein may be also assumed to consist of a number of subunits with weak bonds, which are broken during electrophoresis. It

TABLE III

EFFECT OF SIMULTANEOUS ADDITION OF RETINA INHIBITOR AND ACTIVATOR ON PHOSPHODIESTERASE ACTIVITY OF ROD OUTER SEGMENTS

No. of experiment	Addition of:		
	Inhibitor (fraction 17, 40 μg protein) (inhibition, %)	Activator (fraction 22, 20 μg protein) (activation, %)	and Inhibitor activator (inhibition, %)
1	28	28	70
2	33	35	80
3	40	27	58
4*	43	38	67

* The amounts of inhibitor and activator proteins were 10 μg.

should be mentioned at this point that electrophoresis of the inhibitory component showed the latter to contain a component similar to the protein activator (Fig. 6). It is also interesting that inhibitory and activatory factors, whose effect on the phosphodiesterase activity of rod outer segments is alike with respect to the specific activity but opposite in effect, contributed considerably to the inhibitory effect when added to the incubation mixture simultaneously. The results of these experiments are presented in Table III.

Recently, using another type of column (DE32-cellulose) for purification of the inhibitory factor (the elution profile is shown in Fig. 7), it was possible to

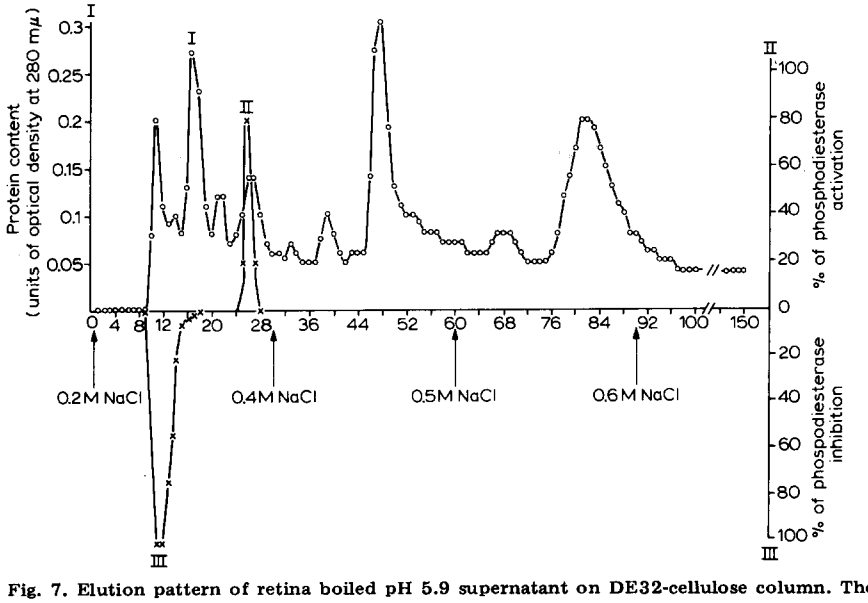

Fig. 7. Elution pattern of retina boiled pH 5.9 supernatant on DE32-cellulose column. The column was prepared and equilibrated as described in the text. Protein was followed spectrophotometrically at 280 nm. An aliquot of each fraction was assayed for its ability to affect the phosphodiesterase activity.

obtain a relatively pure preparation of the inhibitor. It was homogeneous in polyacrylamide gel electrophoresis (Fig. 8). It is interesting that during elution an activator factor, as in the previous series of experiments, was revealed. The inhibitory action of the pure preparation of the inhibitor was evident, but it was increased when the activator was added to this preparation (Table III, Exp. No. 4).

As a result of these experiments the protein activator can be considered as a necessary subunit of the whole complex, i.e. the inhibitor. This explains why no phosphodiesterase activating effect was observed in a non-purified pH 5.9 supernatant from retina, whereas with the complex dissociated into subunits activating protein was detected. However, this supposition needs further experimental support.

To sum up, one may conclude that in the cells of animal tissues in addition to a phosphodiesterase protein activator, a protein inhibitor is present as well. The question of interrelation between the inhibitory and activatory proteins,

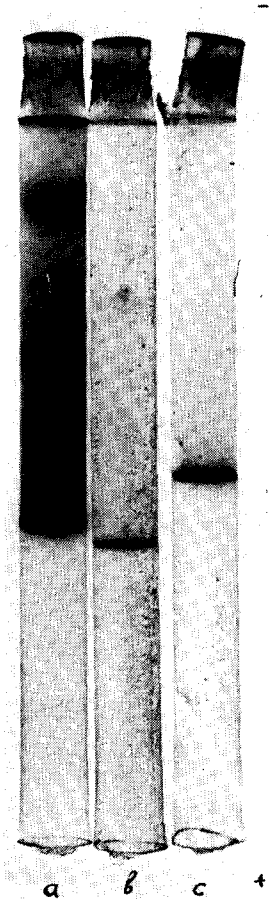

Fig. 8. Polyacrylamide gel electrophoresis of a boiled pH 5.9 supernatant (a), purified on DE32-cellulose column inhibitor (b) and purified activator (c). Disc gel electrophoresis was performed by the method of Maurer [15]. The amount of protein in each gel was: a, 150 μg; b, 50 μg; c, 25 μg.

the functional significance of protein inhibitor in the retina cells and its distribution in other tissues is the subject of further studies.

References

1 Bitensky M.W., Miki N. and Marcus F.R. (1973) Life Sci. 13, 1451—1472
2 Miki N., Keirns J.J., Marcus R. and Bitensky M.W. (1974) Exp. Eye Res. 18, 281—297
3 Etingof R.N. and Dumler I.L. (1975). J. Evol. Physiol. Biochem. 11, 28—38 (in Russian)
4 Cheung W.Y. (1971) J. Biol. Chem. 246, 2859—2869
5 Cheung W.Y., Lin Y.M. and Smoake J.A. (1973) Ninth Intern. Congress of Biochem., p. 102, Abstract Book, AB Egnellska Boktryckereit, Stockholm
6 Smoake J.A., Song S.G. and Cheung W.Y. (1973) Fed. Proc. 32, 609 abs
7 Lin Y.M., Lin Y.P. and Cheung W.Y. (1974) J. Biol. Chem. 15, 4943—4954
8 Teo T.S. and Wang J.H. (1973) J. Biol. Chem. 248, 5950—5955
9 Wolff D.J. and Brostrom Ch.O. (1974) Arch. Biochem. Biophys. 163, 349—358
10 Kakiuchi S., Jamazaki R., Teshima Y., Uenishi K. and Miyamoto E. (1975) Biochem. J. 146, 109—120
11 Riedel V., Gerisch G., Müller E. and Beug H. (1973) J. Mol. Biol. 74, 573—585
12 Brewin N.J. and Northcote D.H. (1973) Biochim. Biophys. Acta 320, 104—122
13 Shukolyukov S.A. and Dumler I.L. (1972) Biokhimiya 37, 339—347 (in Russian)
14 Andrews P. (1964) Biochem. J. 91, 222—233
15 Maurer (1971) Disk-electrophoresis (Levin E.D., ed.), p. 58, Mir, Moscow (in Russian)
16 Weder H.J. and Bickel M.H. (1970) Z. Analyt. Chem. 252, 253—256
17 Cheung W.Y. (1969) Biochim. Biophys. Acta 191, 303—309
18 Dumler I.L. and Etingof R.N. (1973) Biokhimiya 38, 408—411 (in Russian)
19 Eibl H. and Lands W.E. (1969) Anal. Biochem. 30, 51—57
20 Lowry O.H., Rosebrough N.I., Farr A.L. and Randall R.J. (1951) J. Biol. Chem. 193, 269—275
21 Pannbacker R.G., Fleischman D.E. and Reed D.W. (1972) Science 175, 757—758
22 Dumler I.L. (1974) Biokhimiya 39, 1091—1096 (in Russian)
23 Cheung W.Y. (1972) Biochem. Biophys. Res. Commun. 46, 99—105