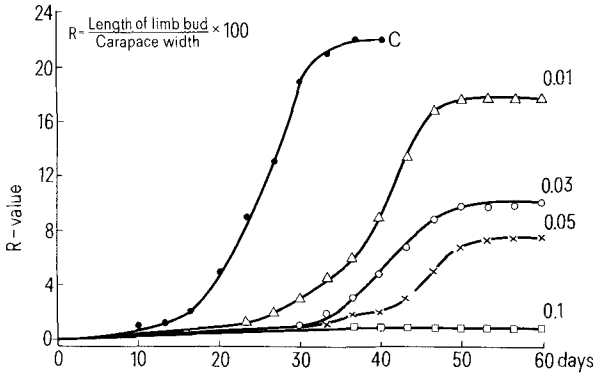
Materials and methods. Sumithion (Technical grade 99% W/V) obtained from Rallis India Ltd was used as a test chemical. Only adult, healthy male specimens were used. The crabs were intermolt individuals, having a body wt 30–32 g; carapace width 32–35 mm. The animals were kept singly each in 1000 ml medium at 28 ± 1 °C. 300 crabs were divided into 5 equal groups. The left 4th walking leg was removed from all the crabs. One of the groups served as control while the others were exposed to 0.01, 0.03, 0.05 and 0.1 ppm sumithion from the day of limb removal until the animals completed at least 1 ecdysis or until the termination of the experiment (60 days). The media for control and experimental crabs were replaced with fresh solutions daily. The crabs were fed with frog muscle on alternate days for the duration of the experiment. The limb bud growth rate (R-value) of animals were determined after[6].

Results and discussion. No mortalities occurred at either of the 4 sumithion concentrations or in the control group during the experiment. The crabs in normal water lacking a left 4th walking leg that served as controls regenerated limbs at a rapid rate (fig.). However, the crabs that were in sumithion regenerate limbs at a much slower rate than the control group. The degree of inhibition increased with the concentration of sumithion exposed. Depending on the concentratiaon used, sumithion caused a complete inhibition of regeneration, a delay of initiation of limb bud development, or a reduction of limb bud growth (fig.). Similar results in limb bud growth rate was also recorded in fiddler crab[2-4] and shrimp[5] after exposing a sublethal concentrations of mercury, DDT, pentachlorophenol.

The results in this investigation suggests, that both delay in crustacean limb bud initiation and inhibition of growth rate are sensitive parameters and can also be used for monitoring toxic responces of chemical pollutants in aquatic ecosystem without sacrificing the animals.



Effect of sumithion on limb regeneration of *Oziotelphusa senex senex* Fabricius. Number on each curve indicates concentration of sumithion in mg/l.

1 Acknowledgments. We are thankful to Rallis India Ltd (Bangalore) for providing us technical grade sumithion. We wish to express our gratitude to CSIR, New Delhi for providing financial support to PSR. Reprint requests should be addressed to RR.

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0014-4754/83/121380-02$1.50 + 0.20/0

Protamine inhibits adenylate cyclase activity: a possible reason for the toxicity of protamine

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Summary: Protamine is an effective inhibitor of the various activated forms of adenylate cyclase of liver plasma membranes. Inhibition of adenylate cyclase may account for its toxic but not its antitumor effects.

Protamine is an inhibitor of the growth of certain tumors[2-5]. This property is probably related to its tumor angiogenesis-inhibitory activity[6], the mechanism of which is unclear. Unfortunately, systemic administration of protamine is limited by its toxicity[6]. The reason for the toxicity of protamine is not known. Clearly, knowledge of the mechanism(s) which is (are) responsible for either of the action of protamine may help in the development of more specific antitumor agents.

We report here that protamine strongly inhibits the adenylate cyclase system of liver plasma membranes.

Materials and methods. ATP, GPP(NH)P(guanyl-5′-yl imidodiphosphate), glucagon, NaF, protamine (free base; prepared from salmon), creatine phophate and creatine phosphokinase were purchased from Sigma (St. Louis, Mo., USA). (α-^{32}P) ATP (500 Ci/mmole) was prepared by the Isotope Institute of the Biological Research Center, Szeged. Preparation of liver plasma membranes and the assay of adenylate cyclase activity was performed as described earlier[7] with the only difference that 10μg plasma membrane proteins were used.

For the solubilization of adenylate cyclase, 1.7 mg plasma membrane proteins were incubated at 4 °C for 30 min in the presence of 5.1 mg Lubrol PX, 20 mM Tris/HCl pH 7.5 and 2 mM $MgCl_2$ (volume 1.5 ml). In some cases the solubilization mixture also contained 10 mM NaF ± 0.5 mM ATP. The solubilized cyclase was recovered in the high speed supernatant (centrifugation at 4 °C for 1 h at 105,000 × g in a Beckman L 50 ultracentrifuge).

Protein was determined according to the method of Lowry et al.[8] using bovine serum albumin as standard.

Each documented experiment was repeated with 3 different plasma membrane preparations with similar results.

Results. Protamine, tested up to 20 μM, did not inhibit the basal cyclase activity of liver plasma membranes (fig.). It did, however, strongly supress the activatory effect of GPP(NH)P, a nonhydrolyzable analog of GTP. The GPP(NH)P plus glucagon as well as the fluoride activated

Effect of protamine on NaF activation of the solubilized adenylate cyclase

Addition to the solubilization mixture	Addition for the cyclase assay (pmoles cyclic AMP/mg protein/10 min)		Inhibition (%)
	No protamine	+ protamine (20 μM)	
None	501 ± 11*	165 ± 7*	67
NaF 10 mM	1223 ± 43**	481 ± 17**	61
NaF 10 mM + ATP 0.5 mM	2377 ± 112**	840 ± 25**	65
Intact membrane	1749 ± 66*	527 ± 23*	70

Each figure represents mean value ± SEM of 4 assays in 1 representative experiment. * 10 mM NaF was added only for the cyclase assay. ** NaF was omitted from the cyclase assay mixture.
Basal adenylate cyclase activities of intact membranes and the solubilized preparations were 142 and 60 pmoles cyclic AMP/mg protein/10 min respectively.

cyclase activities were inhibited by protamine to a similar extent (fig.). In each case, the inhibitory effect developed in a narrow concentration range of protamine.

We considered the possibility that a nonspecific interaction of protamine with the plasma membrane was responsible for the inhibitory effect. To test this we used solubilized adenylate cyclase for the next experiments. The activatory effect of fluoride on the membrane-bound and the solubilized adenylate cyclase was inhibited to a similar extent by protamine (table). Hence protamine affects the adenylate cyclase system directly.

Preactivation of adenylate cyclase by fluoride or fluoride plus ATP(ATP enhances the activatory effect of fluoride, see Kiss[9]) during the solubilization procedure failed to prevent the inhibitory action of protamine (table). Thus protamine is able to reverse the otherwise irreversible activating effect of fluoride[10]. Similar results were obtained with GPP(NH)P (data not shown), another irreversible activator of cyclase[10].

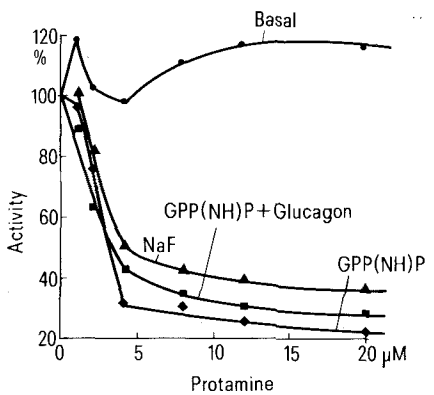
We also considered the possibility that the effect of protamine was merely due to its strong positive charge. However, other positively charged compounds such as spermine, spermidine or lysine-rich histones (H_1, H 2A and H 2B were tested) had no significant inhibitory effect on the cyclase activity (data not shown). Thus, it is likely that the inhibitory effect of protamine depended more on its amino acid sequence than on its charge.

Discussion: Protamine inhibits the variously-activated forms of adenylate cyclase, but not the basal activity, of liver plasma membranes very similarly. This is consistent with a primary action of protamine on the regulatory protein (so-called G/F) which plays a central role in the regulation of adenylate cyclase by guanine nucleotides, hormones and fluoride[10]. Since, a) in intact cells GTP is an important regulator of adenylate cyclase[11] and b) protamine accumulates in some tissues such as lung[6], the present findings are likely to be relevant to physiological situations.

The cyclic AMP system is an important regulator of basic metabolic processes. Thus, a substantial decrease of cyclic AMP synthesis has severe consequences. We suggest that the inhibitory action of protamine on the synthesis of cyclic AMP may well account for its toxicity.

The question arises whether the presently described effects of protamine could also account for its antitumor activity. This is unlikely for the following reasons. First, cyclic AMP is known to inhibit growth[12-17]. Thus, a decrease in the cyclic AMP level would be unlikely to result in the inhibition of growth. Second, various tumor and transformed cells have low levels of cyclic AMP compared to normal cells[18-22]. Therefore, arrest of growth would be expected to be caused by an increase rather than a further decrease in cyclic AMP level. In fact, this has been demonstrated in several cases[23-27].

In summary, we suggest that the inhibition of adenylate cyclase may account for the toxic but not for the antitumor effects of protamine.



Inhibitory effect of protamine on the various activated forms of adenylate cyclase. Basal cyclase activity was 81 pmoles cyclic AMP/mg protein/10 min. 0.1 mM GPP(NH)P, 0.1 mM GPP(NH)P plus 0.1 μM glucagon and 10 mM NaF caused 6.7-fold, 20.8-fold and 18.1-fold increase of basal cyclase activity respectively. Each point represents the mean value of 4 assays in 1 representative experiment. In each case, the experimental error is less than 6%.

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