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## Synthesis of adenine nucleotides during opercular compensatory regeneration in the polychete Hydroides norvegica

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**Zoologia.** — *Synthesis of adenine nucleotides during opercular compensatory regeneration in the polychete* Hydroides norvegica (*). Nota di EGIDIO PUCCIA, MARIA DURANTE e CONCETTA MAZZOLA, presentata (**) dal Corrisp. G. REVERBERI.

RIASSUNTO. — L'adenosina 5' monofosfato (5' AMP) è noto, tra l'altro, per inibire la rigenerazione opercolare nel polichete *Hydroides norvegica*. La sintesi di questo composto (e di correlati nucleotidi) è stata studiata in quattro stadi di rigenerazione, e nell'opercolo funzionale. Lo stadio H, quello che per forma e funzioni è simile all'opercolo funzionale, è caratterizzato dalla più alta sintesi dell'inibitore 5' AMP e del più basso valore di sintesi di un composto non ancora identificato, e qui chiamato «Composto X».

I risultati ottenuti suggeriscono che lo stadio H rappresenta il momento in cui l'opercolo raggiunge il massimo di attività inibente; tale attività decresce con l'ulteriore maturazione dell'opercolo.

## INTRODUCTION

The polychete *Hydroides norvegica* has two opercula which differ in their degree of structural organization. The more highly developed one is as long as the gills. It consists of a long stalk topped by two daisy-like whorls. This operculum is defined as " functional " since it has the function of closing the calcareous tube in which the animal lives. The second operculum is positioned symmetrically with respect to the first. It is a club-like structure without the daisy-like whorls mentioned above. This second operculum is defined as " rudimentary " because the removal of the functional operculum induces it to develop into a well-organized structure which resembles the functional operculum in size, shape and function. This type of regeneration was first described by Zeleny (1920) who called it " compensatory regeneration ". Regeneration of the rudimentary operculum takes place in about 8 days at 18 °C. Durante and Puccia (1970) have defined 8 consecutive stages of regeneration and assigned to them the letters " A " through " H ". In this series stage A indicates the rudimentary operculum, stage B the first stage of regeneration and stage H the completely regenerated operculum.

In a preceding paper (Puccia and Durante, 1973) it was shown that regeneration of the rudimentary operculum is inhibited by an adenine nucleotide (adenosine 5' monophosphate: 5' AMP) whose pool is located mainly in the functional operculum.

A rudimentary operculum subjected to this inhibition will be capable *in vivo* of opercular inhibition once it is regenerated and becomes a functional one itself.

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(**) Nella seduta del 10 aprile 1976.

*In vitro* this capacity is present (although in a different degree) in all the stages of regeneration, as determined by testing the inhibitory capacity of extracts made with opercula at different stages of regeneration. The earlier the stage of regeneration, the higher the opercular concentration required to obtain the same degree of inhibition. Furthermore, the more organized functional opercula very often show less inhibitory activity than those at stage H of regeneration. It is not difficult to find rudimentary opercula regenerating in just those animals which show the most developed functional operculum (bi-operculate animals).

These findings suggest that the amount of inhibitor, that is the rate of inhibitory synthesis by the operculum, may change during and after regeneration.

An investigation of stages B, D, F and H of regeneration and of the functional operculum (fo.) was undertaken. Synthesis of adenosine 5′ diphosphate (5′ ADP), and adenosine triphosphate (5′ ATP) were also studied because of the metabolic relationship known to exist between these 3 phosphorylated nucleosides.

## MATERIALS AND METHODS

*Hydroides norvegica* individuals fresh from the sea were removed from their calcareous tubes, repeatedly washed, and then cultured in pasteurized sea water containing 0.1 (mg/ml) of chloromycetin.

After several days the functional opercula were removed and the animals were allowed to regenerate their rudimentary opercula up to the desired stage. Lots of 120 animals at the chosen opercular stage (B, D, F, H) were cultured for 10 hours at 18 °C in the presence of 10 μCi/ml of $^3$H-adenosine (Radiochemical Center, Amersham; spec. act. 5000 mCuries/mMole).

After five washes with sea water containing 1 μmole/ml of Adenosine the opercula were removed for the preparation of acid soluble fractions. Intact animals were similarly cultured and the functional opercula (fo) removed.

After the removal of a sample for the determination of $^3$H-adenosine uptake, the acid soluble fraction was obtained as previously described (Durante and Puccia, 1970).

Adenosine and adenine nucleotides 5′AMP; 5′ADP; 5′ATP (Sigma Chem. Comp.) in a mixture of 3 mg each were added to each soluble fraction as optical density markers. The solutions were adjusted to pH 10 with $NH_4OH$ and then chromatographed on 1 × 12 cm columns of the anionic exchanger resin Dowex 1 × 2–200 Cl form.

Elution was carried out at a flow rate of 0.5 ml/min, in fractions of 20 ml/tube with the following solvents: $H_2O$ ($E_0$); 2mN HCl ($E_1$); 3mN HCl ($E_2$); 20 mM NaCl in 10 mN HCl ($E_3$); 90 mM NaCl in 10 mN HCl ($E_4$).

Optical density was tested in each fraction with a Zeiss spectrophotometer at 257 nm (adenosine peak in the UV region st pH 2). Moreover, aliquots were assayed for the cpm (counts per min.) of $^3$H-adenosine and its nucleotide derivatives, in a liquid scintillation counter. (Scintillation medium: PPO, POPOP; naphthalene, ethylene glycol, methanol, dioxane).

The $^3$H-adenosine uptake was determined by pouring the homogenated samples on 45 HA millipore filters. The discs were then dried and read for radioactivity with a scintillation medium made of Toluene/PPO/POPOP.

## Results

Column chromatograms of the acid soluble fractions for all stages of regeneration studied are shown in fig. 1.

As can be seen, there is synthesis of the adenine nucleotides 5′AMP, 5′ADP, 5′ATP but their relative ratios are different in the four regeneration stages. The first peak that appears in the chromatogram is adenosine which has not been phosphorylated to nucleotide. Between adenosine and 5′AMP there appears a peak of a yet unidentified compound which has been named "Compound X",

Samples belonging to each nucleotide peak were collected and mixed. The total radioactivity (as count per minute: cpm) was measured and referred to the operculum. With regard to the functional operculum (fo) the chromatogram is not represented since the elution patterns of the nucleotides do not differ from those of the regenerating opercula, the only difference being in the height of the peaks.

Table I summarizes the data relative to these determinations for each stage of regeneration and for the functional operculum. It can be seen that the incorporation of $^3$H-adenosine in 5′ AMP increases from stage B up to stage H. A sharp decline is observed between stage H and fo. 5′ADP increases from stage B and remains at an almost constant rate up to stage H. The 5′ ATP has the highest rate at stage D, with a sharp decline at stage F and an increase at H.

Table I

*Incorporation of* $^3$H *adenosine into adenine nucleotides per operculum.*

| Stage | (*) cpm × $10^2$ | | | |
|---|---|---|---|---|
| | 5′AMP | 5′ADP | 5′ATP | « Compound X » |
| B | 9.44 ± 0.70 | 18.80 ± 1.00 | 8.20 ± 0.50 | 11.27 ± 0.65 |
| D | 21.00 ± 0.90 | 58.00 ± 4.00 | 27.46 ± 1.65 | 48.90 ± 2.45 |
| F | 89.17 ± 5.20 | 50.22 ± 3.00 | 3.75 ± 0.35 | 32.59 ± 1.95 |
| H | 250.00 ± 20.00 | 59.50 ± 5.01 | 4.34 ± 0.42 | 28.00 ± 2.60 |
| fo | 14.31 ± 1.00 | 29.55 ± 2.07 | 2.18 ± 0.20 | 230.00 ± 20.01 |

(*) Values are the average of 4 determinations.

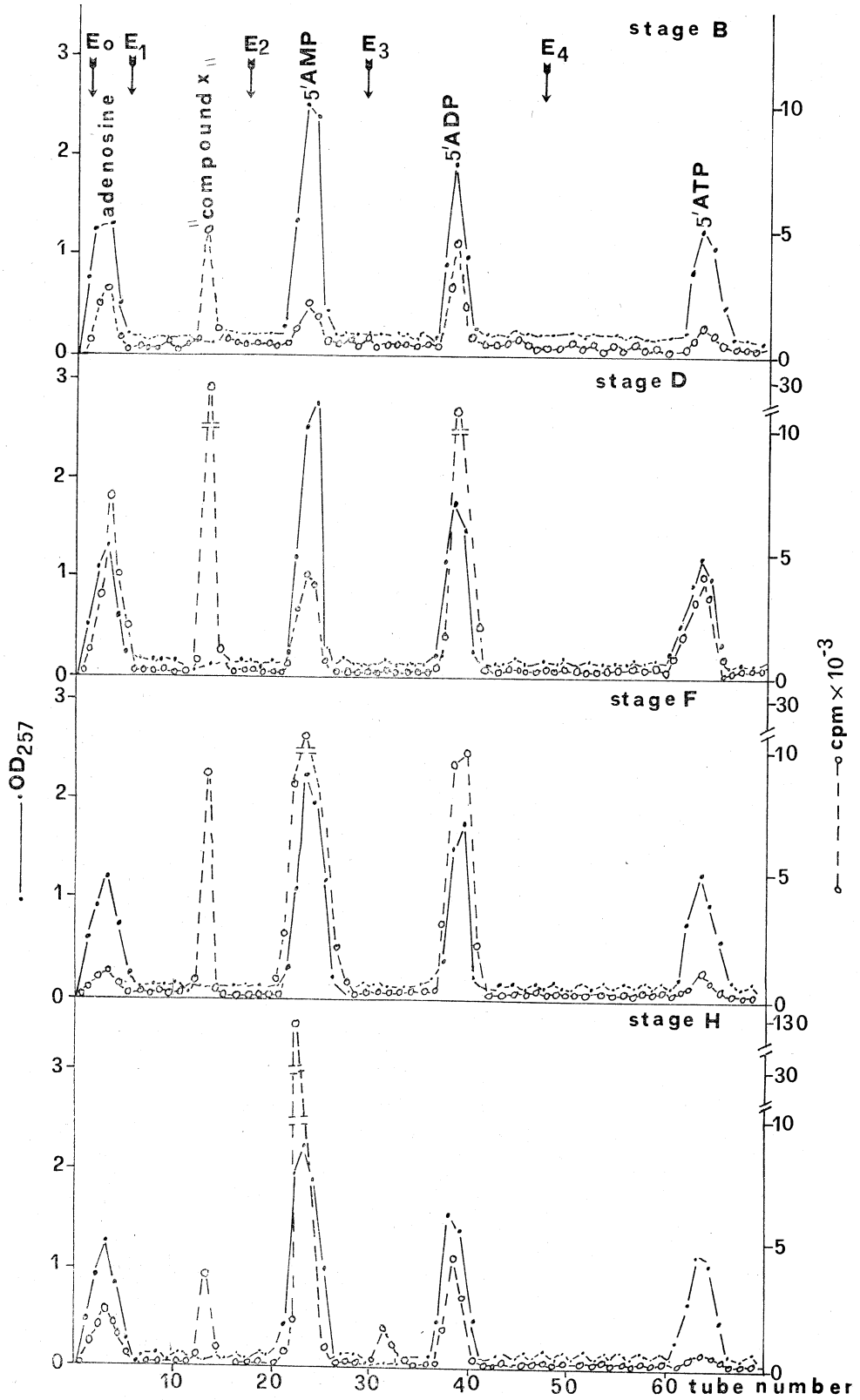


Fig. 1. – Column chromatographies on Dowex 1×2-400 Cl-form of the opercular acid soluble fraction for some stages of opercular regeneration. $E_0 \cdots E_4$: eluents as described in materials and methods.

" Compound X " has a peak at stage D, but the highest rate is in the fo. The uptake of $^3$H-adenosine is shown in Table II. As can be seen, the highest rate occurs at stage H.

TABLE II

*Uptake of* $^3$H *adenosine per operculum.*

| Stage | (*) cpm $\times 10^{-2}$ |
|---|---|
| B | $210 \pm 8$ |
| D | $400 \pm 24$ |
| F | $588 \pm 41$ |
| H | $700 \pm 65$ |
| fo | $353 \pm 15$ |

(*) Values are the average of 4 determinations.

Fig. 2 shows the course of the synthesis of the adenine nucleotides in the operculum during (stage B, D, F, H) and after (stage fo) regeneration. For each stage data are obtained by dividing the values of incorporation taken from Table I by the corresponding value of uptake indicated in Table II. Looking at the profiles it can be seen that 5′ AMP has an increasing rate of synthesis from stage B up to stage H. 5′ADP and 5–ATP profiles have their peaks at stage D. The profile of " Compound X ", after a peak at stage D, has the lowest rate at stage H, and the highest at stage fo.

## DISCUSSION

Adenosine 5′ monophosphate, which has previously been found to inhibit opercular regeneration (Puccia and Durante, 1973), is synthesized by the regenerating operculum according to a pattern which suggests a possible function of this compound in the control of compensatory regeneration.

The progressive rate of increase from stage B up to H-the stage at which the operculum is similar in size and shape to a functional one—and the decline during the H-fo period would seem to indicate stage H as the point in development where the best inhibitory conditions are reached. (During the F-H period about 75% of all the 5′AMP is synthesized). Compensative regeneration is a peculiar kind of regeneration: after the removal of the functional operculum it is not the same organ which regenerates but the rudimentary one located elsewhere. Under experimental conditions the rudimentary operculum

starts to regenerate only after removal of the functional, or stage H operculum. However, *in vivo* it is not rare to find animals with a regenerating operculum, even when the functional operculum is still present. In such a condition we can say that the functional operculum is no longer functional in all senses, as size and shape could suggest. It seems, therefore, that in the functional operculum the reduction of the inhibiting capacity starts much before its spontaneous drop. From this moment on the relationship between functional and rudimentary operculum starts to reverse. Biochemical data of the present study suggest that this moment occurs at stage H.

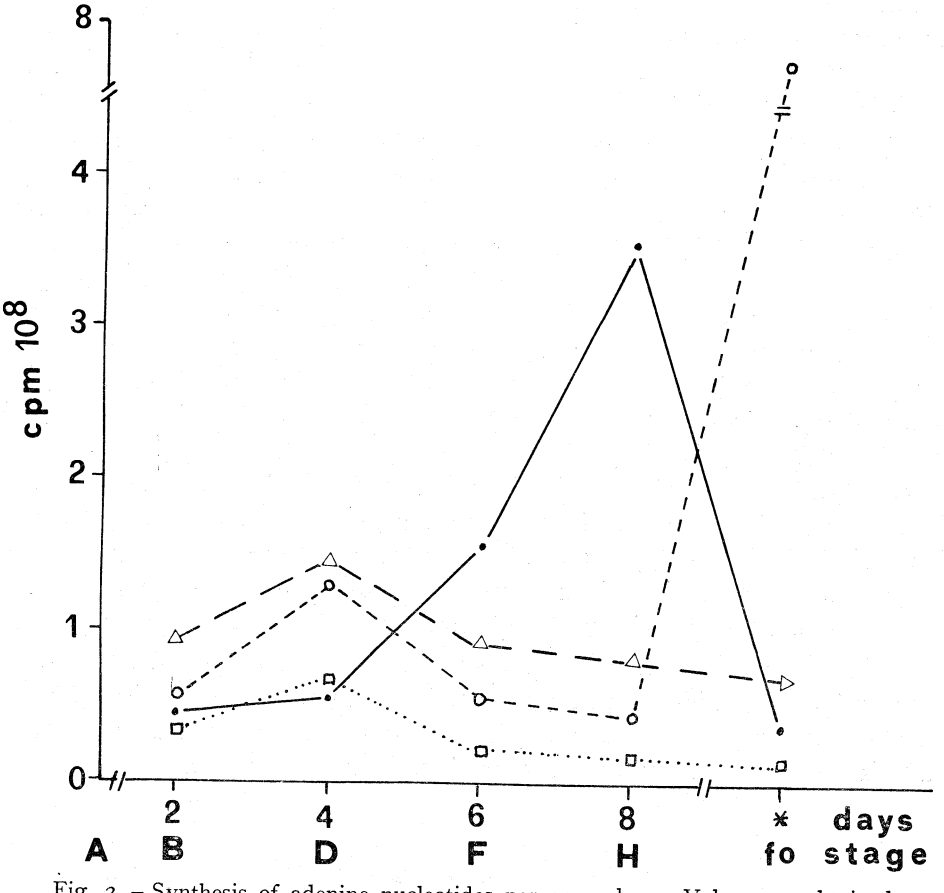


Fig. 2. – Synthesis of adenine nucleotides per operculum. Values are obtained as a ratio between incorporation and uptake. •——• 5'AMP; Δ– –Δ 5'ADP; □···□ 5'ATP; o– – – – –o "compound X". The presumptive age of the functional operculum is about two months.

The results utilized and discussed are those shown in fig. 2. The data concerned with the synthesis of the adenine nucleotides for each stage are obtained as a ratio between adenosine incorporation into nucleotides and adenosine uptake. The inhibitor 5'AMP is synthesized at all stages, even at the beginning of regeneration (stage B). Its rate increases up to the end of regeneration

(stage H), then slows down throughout the opercular aging. For this reason stage H seems to be the moment when the inhibiting relationship between the well organized stage H operculum and the rudimentary one starts to reverse.

With regard to the 5′ ADP and the 5′ ATP synthesis, the same figure indicates the highest rates at stage D. Such a situation could possibly be related to the need of 5′ ATP (and consequently of 5′ ADP) for a higher rate of protein synthesis. Of particular interest is the synthesis of the unidentified " Compound X ". Looking at fig. 2 one can see that the profile of 5′ AMP and of " compound X " show an inverse correlation. Stage H has the highest rate of 5′ AMP synthesis and the lowest rate of " Compound X "; conversely, the functional operculum has the lowest rate of 5′AMP synthesis, and the highest rate of " compound X ". The significance of this inverse correlation is still unknown and remains to be investigated.

Our finding that the inhibitor of opercular regeneration appears to be a very common nucleotide, 5′AMP, raises the question as to how this compound might control regeneration and, more specifically, how it might regulate protein synthesis, which is obviously of extreme importance in regeneration. Recent research has suggested that 5′AMP is able to inhibit many biological processes (for review see: Puccia and Durante 1973; Ortiz *et al.*, 1973) and cellular growth (Pastan, Johnson and Anderson, 1975). In any case no one has yet succeded in elucidating the mechanism underlying these regulatory phenomena. Hilz and Kaukel (1973) found that in the inhibition of cellular growth by 5′AMP as well by adenosine or 3′ 5′ cyclic AMP, there is a depletion of the pyrimidine precursor for DNA and RNA synthesis. On the other hand, the influence of 5′AMP on histon methylation (Burdon *et al.*, 1971), the idea that 3′5′ cyclic AMP acts as a second messenger (Pastan and Perlman, 1971) and the construction of a working model for the lac operon of *E. coli* (Eron *et al.*, 1971), have provided new insights into the regulatory processes.

With regard to " compound X " we would like to suggest that this compound might be an intermediary product in the inhibiting activity of 5′AMP. We plan to isolate it in larger quantities so as to be able to carry out a chemical analysis which hopefully will give additional insight into the problem.

## References

BURDON R. H. and GARVEN E. V. (1971) – *Enzymic modification of chromosomal macromolecules*, II. *The formation of histones* ε-N-*trimethyl*-L-*lysine by a soluble chromatin methylase*, « Bioch. Bioph. Acta », *232*, 371–378.

DURANTE M. and PUCCIA E. (1970) – *Ricerche preliminari sulla natura chimica della sostanza inibitrice dell'opercolo funzionale di* Hydroides norvegica, « Rend. Acc. Naz. Lincei », *48*, 683–689.

ERON L., ARDITTI R., ZUBAY G., CONNOWAY S. and BECKWITH J. R. (1971) – *An adenosine 3′5′-cyclic monophosphate binding protein that acts on the transcription process*, « Proc. Nat. Acad. Sci. », *68*, 215–218.

HILZ H. and KAUKEL E. (1973) – «Mol. Cell Biochem. », *1*, 1–11.

ORTIZ J. R., YAMADA T. and HSIE A. W. (1973) – *Induction of stellate configuration in cultured iris epithelial cells by adenosine and compound related to 3'5'–cyclic monophasphate*, « Proc. Nat. Acad. Sci. », *70*, 2286–2290.

PASTAN I. H., JOHNSON G. S. and ANDERSON W. B. (1975) – *Role of cyclic nucleotides in growth control*, « Ann. Rev. of Bioch. », *44*, 491–522.

PASTAN I. H. and PERLMAN R. L. (1971) – *Cyclic* AMP *in metabolism*, « Nature (New Biology) », *229*, 5–7.

PUCCIA E. and DURANTE M. (1973) – *The compensatory regeneration of the operculum of* Hydroides norvegica: *Identification of the inhibiting substance*, « Journ. Exper. Zool. », *184*, 1–6.

ZELENY C. (1902) – *A case of compensatory regulation in the regeneration of* Hydroides dianthus, « Roux' Arch. », *13*, 597–609.