

EFFECTS OF SOME CYCLIC NUCLEOTIDES ON THE WOUND HEALING AND THE REGENERATION OF THE SEA-ANEMONES, *CEREUS PEDUNCULATUS* PENN. AND *METRIDIDIUM SENILE* L.

P. M; LENICQUE, J. P. FÉRAL and D. DOUMENC
Laboratoire de Biologie des Invertébrés marins du Museum National
d'Histoire Naturelle, 57 rue Cuvier, 75005 Paris et Faculté de
Pharmacie, Chatenay Malabry, France

(Received 24 April 1978)

Abstract-1. The variation of the synthesis of-soluble collagen were studied during cicatrization and regeneration in sea-anemones treated with cyclic nucleotides and in non-treated animals.

2. Monobutyl cAMP, dibutyl cAMP, dibutyl cGMP, 8-Br cAMP and 8-Br cGMP were tested at different doses and at different times during the development of this material.

3. Mono- and dibutyl cAMP had a promotive effect while the other tested nucleotides had no effect or a toxic one.

4. The results obtained were compared with those observed on animals belonging to different zoological phyla.

INTRODUCTION

In previous work it has been demonstrated that some biogenic amines and their precursors have a promotive effect when applied during the cicatrization and the regeneration of the sea-anemones *Cereus pedunculatus* and *Metridium senile* (Lenicque & Féral, 1977). Similar results have been observed on another invertebrate: the planarian worm, *Dugesia tigrina* (Lenicque, 1971, 1974). These substances, NA and 5-HT, act in the same manner on adenylate and/or guanylate-cyclases. It is thus logical to perform studies on the regulatory action of some cyclic nucleotides on the regeneration of these animals. Research on *Dugesia tigrina* has shown that the 8-Br cGMP has a highly significant promotive effect on regeneration while the DBC had a weak inhibitory effect (Lenicque, 1976).

The aim of this present work is to observe the effects of some cyclic nucleotides on the wound healing and on the regeneration of the sea-anemones, i.e. on animals belonging to a very different phylum.

MATERIALS AND METHODS

The sea-anemones employed were *Cereus pedunculatus* Penn. collected on the shore of Dinard (the French coast of the English Channel) and *Metridium senile* L., collected in the Gullmarfjord (Swedish west coast). The experiments were performed at the marine biology laboratories of Concarneau and Roscoff (France) and of Kristineberg

(Sweden). *Cereus pedunculatus* has a good capacity for regeneration and *Metridium senile* a tremendous one (Polteva & Lenicque, 1970). *Cereus pedunculatus* with a diameter of about 1 cm was divided into equal parts, following the siphonoglyph, from the oral to the pedal disc (Fig. 1). One half was utilized as a control for the other which was treated by the nucleotides to be tested. The pedal disc of *Metridium senile* was separated from the body by means of iris scissors and divided into pieces of about 3 mm³. The mucus was discarded and groups of 12-15 pieces were cultivated in Petri dishes. After the operations the animals were maintained in running seawater at about 12°C. During the daily treatment of 6 hr, they were kept in vessels containing the solutions of nucleotides. During the time of the treatment the control animals were kept in similar vessels, with non-running seawater, in order to have the same conditions of pH, oxygenation and temperature.

The wound healing of *Cereus pedunculatus* took 24-30 hr. After a week, the regeneration progressed very well and a few tentacles had formed. The cicatrization of the small pieces of *Metridium senile* took 16-20 hr, then the regeneration began with the formation of the column after 6 days. At the end of the first week, the mouth and some tentacles had formed at the top of the column (Lenicque & Féral, 1977).

The substances to be tested were N⁶MBC and the DBC prepared at the Laboratory of the Cyclic Nucleotides of the Faculty of Pharmacy (University Paris XI). The 8 Br cGMP, the 8 Br cAMP and the DB cGMP were provided by Sigma Chemicals (St Louis, U.S.A.). The cyclic nucleotides were dissolved in filtered seawater which is a slightly basic buffer. The doses varied from 0.1 mg to 2 mg/100 ml, i.e. 2 x 10⁻⁶ to 5 x 10⁻⁵ M.

The synthesis of collagen was taken as a test of regeneration: The sea-anemones have a high content of this protein, specially in the mesoglea. Some preliminary histological studies have shown that the collagen plays an important role during the cicatrization and the regeneration. As this substance is easily isolated from the other proteins of the tissues in hot TCA, we studied the variations of the ratio soluble collagen/total proteins (S.C./T.P.) at different stages during the week following the operation. We

Abbreviations-8-Br cAMP-8-bromo-cAMP; 8-Br cGMP-8-bromo-cGMP; cAMP-adenosine-3,5-monophosphate; cGMP-guanosine-3,5-monophosphate; DBC-dibutyl cAMP; DB cGMP-dibutyl cGMP; 5-HTP-5-hydroxytryptophan; 5-HT-5-hydroxytryptamine; NA-noradrenaline; N⁶MBC-monobutyl cAMP; S.C soluble collagen; T.P.-total proteins; T.C.A.-trichloroacetic acid.

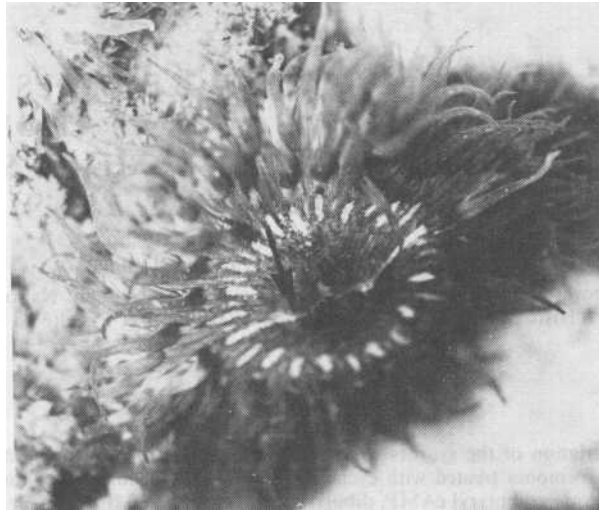


Fig. 1. *Cereus pedunculatus*. The arrow shows the syphonoglyph.

used a modification of the method of Lukens (1965). The animals were removed from the freezer and minced with scissors, then they were poured into an equal volume of ice-cold 10% TCA and blended for 20 sec in an ultraturax mixer. The insoluble material was recovered by centrifugation at 10,000g at 4°C and washed several times with cold 5% TCA by resuspension and centrifugation. The washed pellet was extracted by 5% TCA at 90°C for 75 min. The supernatant, constituting the collagen fraction, was extracted with diethyl ether to remove the TCA. The insoluble material, constituting the other proteins, was removed by centrifugation and dissolved in NaOH 0.1 N. The method of Lowry *et al.* (1951) was used to determine the quantity of hot TCA soluble proteins in the supernatant (S.C.) and the insoluble ones (non-collagen protein fraction) in the precipitate. Lukens (1965) pointed out that the hot TCA soluble fraction is polluted by some proteins or peptides and substances derived from nucleic acids. In some experiments, the non-collagen protein fraction was measured directly by spectrophotometry at 260 Å in a solution of formic acid.

We observed the variations of the ratio S.C./weight and S.C./T.P. in populations of treated animals and of control

animals during the wound healing and the regeneration. Incorporations of [14 C]proline from CEA, France, (sp. act. = 235 mCi/mM) were done in order to follow the synthesis of collagen. The animals at each stage were incubated in a 1 μ Ci/ml concentrated solution of [14 C]proline for 2 hr. They were first washed in a solution 100 times more concentrated of [12 C] proline three times and then in filtered seawater. Liquid scintillation countings were performed on aliquots of different fractions. They were dropped into counting vials containing the scintillant mixture (PPO, 12g; POPOP, 0.3 g; Toluol, 31, Triton X 100, 1.5 l). After stabilization of the temperature, countings were performed with the measure channel of an Intertechnique SL 32 spectrometer at a maximum opening. The time was chosen to totalize 2000-15,000 imp for each sample.

RESULTS

1. *Cereus pedunculatus*

Figure 2 shows that the quantity of S.C. per unit of weight decreases during the first hours following the operation. This stage was reached in about 24 hr

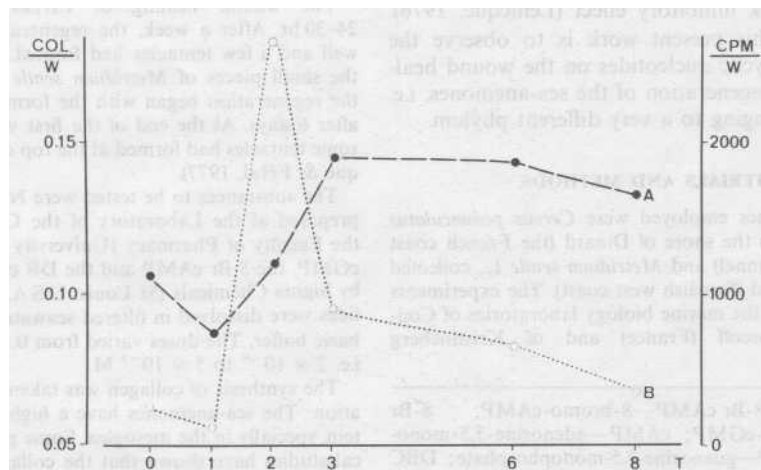


Fig. 2. A = Variations of the quantity of soluble collagen per unit of weight at different stages during the wound healing and the regeneration of the sea-anemone *Cereus pedunculatus*. Y-axis: soluble collagen (μ g)/weight (mg). X-axis: time (days). The sequence of the observed variations is characteristic for cicatrization of vertebrates and of invertebrates. B = Variations of the rate of incorporation of [14 C]proline during the same time. Y-axis: imp/min/weight (mg).

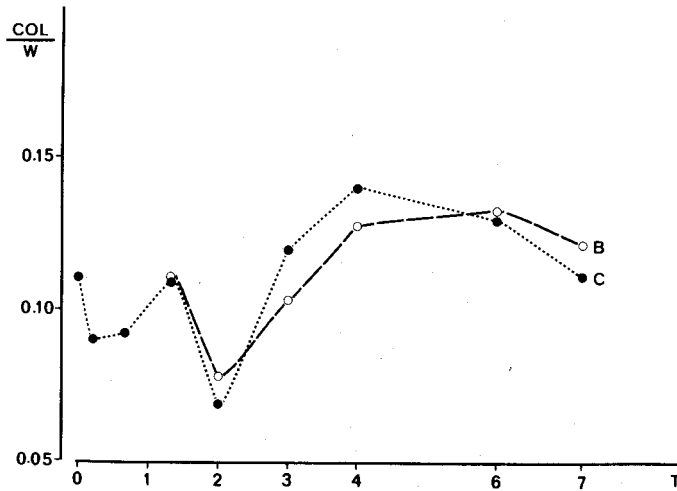


Fig. 3. Effect of 8 Br-cGMP on the cicatrization and regeneration of *Cereus pedunculatus*. Y-axis: soluble collagen (μg)/weight (mg). B = Bromo cGMP-treated animals; C = Control animals.

with the conditions of this experiment. After 48 hr, the value of the quantity of S.C. was similar to the initial one. During day 3-day 6, it even increased above the normal level and later decreased to a normal value. The incorporation of [^{14}C]proline, as measured by the liquid scintillation spectrometer, took place the second day. At the dose of 2.1×10^{-5} M, 8-Br cGMP tested for 1 week, had no effect (Fig. 3). The action of the other cyclic nucleotides of the synthesis of collagen was tested 66 hr after the operation, i.e. during a phase of a high synthetic activity. DBC had a promotive effect, especially at the highest tested doses, 4×10^{-5} M (Fig. 4). DB-cGMP had no significant effect; but at very low doses, 8-Br cAMP had an inhibitory or a toxic effect (Fig. 4). Finally, our study tried to determine at what moment(s) the cells of the developing animals were receptive to the impulses of the tested cyclic nucleotides. The used concentration was 2×10^{-5} M for the DBC and 2.4×10^{-5} M for the N⁶MBC. The animals

were treated in groups of seven at 6 hr after the operation and then at 18, 24, 30, 55, 78 and 96 hr. Figure 5 shows that the addition of N⁶MBC or DBC had a slight positive effect during the wound healing and a very strong one at different stages of the regeneration (30 and 78 hr). At 24 hr, we did not detect any effect on the synthesis of collagen.

2. *Metridium senile*

The DBC was tested at a concentration of 10^{-5} M and 10^{-7} M. Two series of experiments were performed on 143 pieces of animals. The treatments were done on days 3 and 4 i.e. after cicatrization and during a period characterized by the lysis of the endodermal cells and of the mesoglea (which is very rich in collagen). By day 6 the formation of the column with a new mesoglea had begun. By day 7, nearly all the animals had a well-developed column. Figure 61 shows that the stage of destruction of the collagen was first reached by the DBC-treated animals (D) on

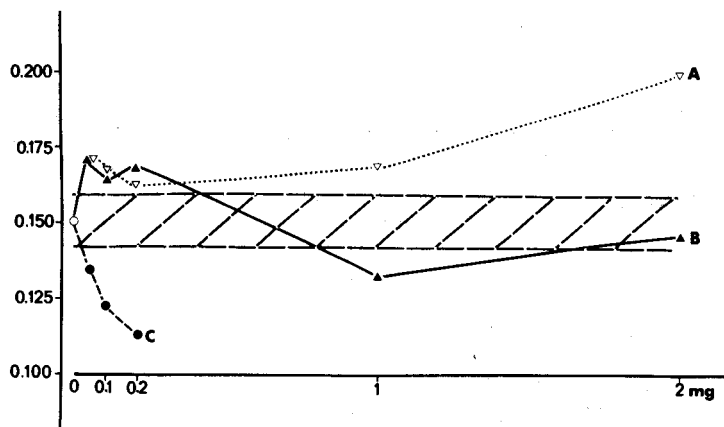


Fig. 4. Effects of DBC, 8 Br cAMP and DB cGMP on the synthesis of soluble collagen 66 hr after the operation. Y-axis: variations of the quotient soluble collagen/total proteins. X-axis: doses (mg) for 100 ml seawater. The striated area is the zone of variations for the control. A = DBC; B = DB cGMP; C = 8 Br cAMP; n = 78 treated animals (pool of 6 per point) and 48 for the controls (*Cereus pedunculatus*).

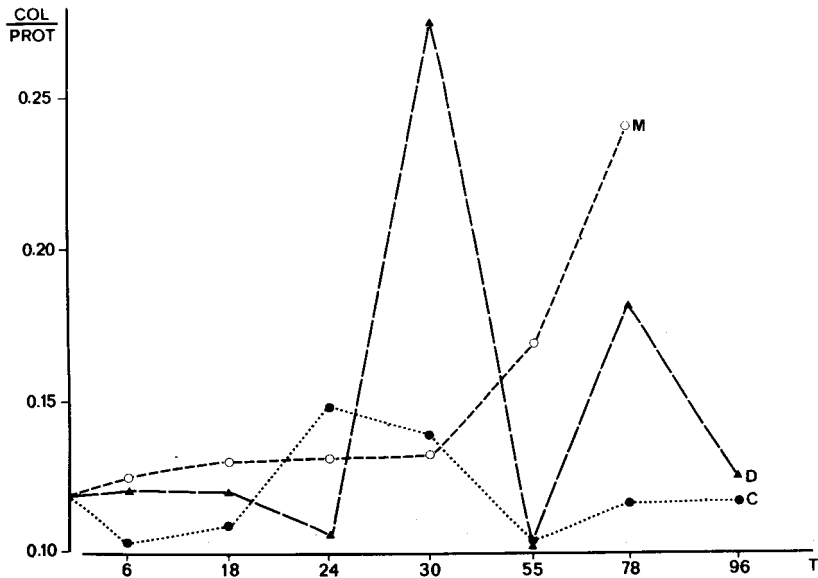


Fig. 5. Effects of DBC and N⁶MBC on the synthesis of soluble collagen at different stages during cicatrization (6 and 18 hr after the operation) and regeneration (> 30 hr) of *Cereus pedunculatus*. Y-axis: soluble collagen/total proteins, X-axis: time (hr). $n = 259$ animals.

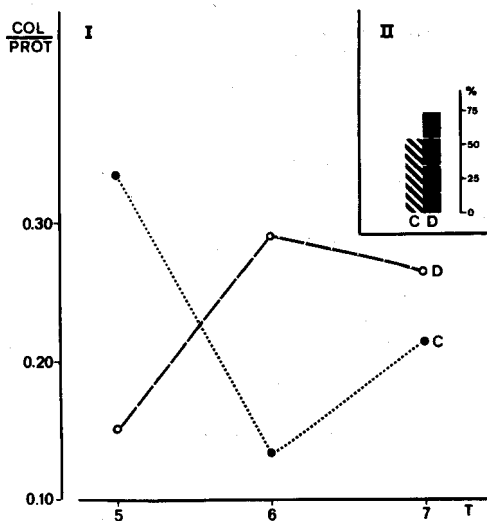


Fig. 6. I-Effects of DBC on the synthesis of soluble collagen during the regeneration of small pieces of the pedal disc of *Metridium senile*. The processus of development worked faster in the populations of treated animals than in the controls. Y-axis: soluble collagen/total proteins. X-axis: time (days). II-Percentage of animals with column at 7 days. C = Control animals; D = DBC treated animals

the fifth day after the operation and that the stage of the new collagen also began first with the treated animals (D). With the control animals (C) the synthesis of collagen first decreased on day 6 and increased again on day 7. These results were confirmed by the morphological analysis.

Figure 611 shows that the proportion of animals with well-developed column was higher in the populations of treated animals (D) than in the control ones (C).

DISCUSSION AND CONCLUSIONS

The rate of synthesis of collagen was taken as an indicator of the progress of cicatrization and of regeneration. Many authors have described that the rate of this protein follows a characteristic curve during these phenomena. The first phase is a diminution of collagen followed by a phase of syntheses of new collagen in granulation tissues (Bailey *et al.*, 1973; Bazin *et al.*, 1976; Delaunay & Bazin, 1968). These workers measured hydroxyproline rate in the collagen fraction because the quantity of this amino acid is a characteristic of this protein. But, in order to apply numerous dosages, we isolated just the hot TCA soluble fraction. In some experiments we measured the [¹⁴C]proline-bound activity of this fraction. It corresponds to a well-known phenomenon. Actinians can absorb and accumulate L-amino acids dissolved in seawater. This fact is attributed to the cytological structure of the epithelia. The concentration can be 10⁵-10⁷ times higher in the tissues than in the medium in which the experiments take place, showing that the uptake is an active process. It depends on temperature and is susceptible to inhibitors (Schlichter, 1975). Gosline (1971), working on incorporating kinetics of [¹⁴C]proline into mesoglea by the sea-anemone *Aiptasia* sp., showed that the free [¹⁴C]proline is incorporated into bound [¹⁴C]proline and bound [¹⁴C]hydroxyproline of proteins which become soluble on autoclaving (collagen precursors and collagen).

The effect of the mono- or dibutyryl-cyclic AMP on cicatrization and regeneration was a promotive one: the quantity of S.C., compared with the quantity of T.P. was higher for the treated animals than for the controls, except at the stage of 24 hr, i.e. a stage of transition between cicatrization and regeneration. At that moment, these nucleotides had no effects. They seemed to act as flashes at determined periods of receptivity of the target cells.

Table 1. Effects of biogenic amines and of cyclic nucleotides on the regeneration of the actinians *Cereus pedunculatus* and *Metridium senile* and of the planarian worm *Dugesia tigrina*

Effect on the regeneration of...	Actinians	
	diffuse nervous system	Planarian worms central nervous system
5-HT (and precursors)	-?	+
NA (and precursors)	+	+
Insulin	/	+
DBC	+	-?
N ⁶ MBC	+	/
8 Br-cAMP	-	/
8 Br-cGMP	-	+

+ = promotive effect; - = inhibitory effect; / = not tested.

It is well known that among the vertebrates a variety of developmental mechanisms are controlled by the nervous system. For instance, in amphibian limb regeneration depends on its trophic influence. It seems that the neurotrophic agent could be one substance or a group of substances (Singer, 1974): histo-fluorescent observations have shown the presence of catecholamines in the nerve invading the bud of regeneration. Inhibition of tyrosine hydroxylase delays regeneration in the newt (Taban *et al.*, 1977a, b). As the catecholamine effects are linked to the activation of adenylate cyclase Taban and co-workers measured the fluctuations of AMP at the various stages of regeneration and were able to correlate them with NA sensitivity changes (Taban *et al.*, 1978).

Working with invertebrates as models, we have found that biogenic amines, 5-HT and NA, play a positive role during the regeneration of the planarian worm *Dugesia tigrina* as well as several polypeptides like insulin (Lenicque, 1976). Similar experiments were performed on the actinians *Metridium senile* and *Cereus pedunculatus* (Lenicque & F^oral, 1977). Inhibition of tryptophan hydroxylase delayed regeneration (Lenicque, 1971). The conclusion of this series of works was that the positive biogenic amines are probably 5-HT for *Dugesia tigrina* and NA for the actinians. It is of interest to note that *D. tigrina* is an animal characterized by the presence of a central nervous system, while the sea-anemones have a diffuse one. It is worth noticing that the development of the sea-urchin larva depends on the presence of 5-HT (Gustafson & Toneby, 1968).

Biogenic amines and many polypeptides act on adenylate and/or guanylate-cyclase. Adenylate cyclase was detected in the sea-anemones by Gentleman & Mansour (1974). It is therefore interesting to try to explain the role of the cyclic nucleotides during the regeneration. In a previous work, it has been already noted that in planarian worm the promotive nucleotide was cGMP and that cAMP had no effect or an inhibitive one. In fact, we used synthetic nucleotides, 8-Br cGMP and DBC, because the corresponding physiological nucleotides had no action. In the present work, it is shown that the effects of the derivatives of cGMP and cAMP were different on actinians: N⁶MBC and DBC had a positive effect while 8-Br cAMP and 8-Br cGMP had no effect or were toxic. In both cases, it is possible that the toxicity is imputable to the Br ion. Table I summarizes these results.

Numerous experiments have been performed on cell multiplication, protein synthesis and differentia-

tion in cell cultures and *in vivo* (Berridge, 1975), often with conflicting results depending on doses and materials. However, it is worth noting that DBC at a concentration of 1 mM stimulated the synthesis of collagen by a factor of 20-35% in cultured human embryonic bone fibroblasts (Manner & Kuleba, 1974). Several authors have studied the influence of DBC on proteins synthesis by following the incorporation of [¹⁴C]-leucine. They observed that the DBC at 5 x 10⁻⁶ mM enhanced protein synthesis in regeneration blastema of newt limbs (*in vitro*) or in reagggregates of embryonic organs (Medoff & Parker, 1977).

Mono- and dibutyl cAMP had a promotive effect on the wound healing and the regeneration of the sea-anemones *Cereus pedunculatus* and *Metridium senile*. Dibutyl cGMP, 8-Br cAMP and 8-Br cGMP had no effect or a toxic one.

Acknowledgements-We wish to express our cordial thanks to the authorities and the staff of Kristineberg Marinebiologiska Station, Fiskebuckskil (Sweden) and of the Stations of Marine Biology of Concarneau and Roscoff (France). We are indebted to Dr. Georges Cehovic who provided us with DBC and N⁶MBC and laboratory facilities, and to Charles A. Briant and Professor Paul L. Illg who improved the English text.

REFERENCES

- BAILEY A. J., BAZIN S. & DELAUNAY A. (1973) Changes in the nature of the collagen during development and resorption of granulation tissue. *Biochim. biophys. Acta* 328, 383-390.
- BAZIN S., LE LOUS M. & DELAUNAY A. (1976) Role of collagenases and collagenolytic cathepsins in granulation tissue from acute and subacute experimental inflammations. *Front. Matrix. Biol.* 3, 234-241. Karger, Basel.
- BERRIDGE M. J. (1975) In: *Advances in Cyclic Nucleotides Research*, Vol. 6. Raven Press, New York.
- DELAUNAY A. & BAZIN S. (1968) Modification of chemical components in inflamed connective tissue. In *Inflammation Biochemistry and Drug Interaction* (Proceedings of the Como Symposium), October 1968.
- GENTLEMAN S. & MANSOUR T. E. (1974) Adenylate cyclase in a sea anemone. *Biochim. biophys. Acta* 343, 469-479.
- GOSLINE J. M. (1971) Kinetics of incorporation of [¹⁴C]proline into mesogleal proto collagen and collagen of the sea anemone *Aiptasia*. In *Experimental Coelenterate Biology* (Edited by Lenhoff H., Muscatine L. & Davis L.). University of Hawaii Press.
- GUSTAFSON T. & TONEBY M. (1970) The role of serotonin and acetylcholine in sea-urchin morphogenesis. *Expl Cell Res.* 62, 102-117.

- LENICQUE P. M. (1971) Effets de substances à noyau indole ou capables de former un noyau indole sur la régénération de *Clava squamata* et de *Dugesia tigrina*. *Thérapie* **26**, 1059-1074.
- LENICQUE P. M. (1974) A mechanism of action of serotonin and noradrenaline on the regeneration of the planarian worm *Dugesia tigrina*. *Acta Zool.* **55**, 163-168.
- LENICQUE P. M. (1976) Contrôle de la régénération de petits morceaux de planaire *Dugesia tigrina* par les nucléotides cycliques AMP et GMP. *C.r. hebd. Séanc. Acad. Sci., Paris* **283**, 1317-1319.
- LENICQUE P. M. & FERAL J. P. (1977) Effects of biogenic amines on the regeneration of small pieces of the pedal disc of the sea anemone *Metridium senile*. *Comp. Biochem. Physiol.* **57C**, 91-93.
- LENICQUE P. M., TONEYBY M. & DOUMENC D. (1977) Demonstration of biogenic amines and localization of monoamine oxydase in the sea anemone *Metridium senile*. *Comp. Biochem. Physiol.* **56C**, 31-34.
- LOWRY O. H., ROSEBROUGH M. J., FARR A. L. & RANDALL A. J. (1951) Protein measurement with the Folin phenol reagent. *J. biol. Chem.* **193**, 265-275.
- LUKENS L. N. (1965) Evidence of the nature of the precursor that is hydroxylated during the biosynthesis of collagen hydroxyproline. *J. biol. Chem.* **240**, 1661-1669.
- MANNER G. & KULEBA M. (1974) Effect of DBC on collagen and non-collagen synthesis in cultured human cells. *Conn. Tissue Res.* **2**, 167-176.
- MEDOFF J. & PARKER N. (1977) Cyclic AMP stimulation of macro-molecular synthesis in reagggregates of embryonic organs. *Proc. Soc. exp. Biol. Med.* **155**, 416-419.
- POLTEVA D. G. & LENICQUE P. M. (1970) Effets des neurotransmetteurs sur l'embryogénèse somatique chez *Metridium senile*. *Thérapie* **25**, 1067-1081.
- SCHLICHTER D. (1975) The importance of dissolved organic compounds in sea water for the nutrition of *Anemonia sulcata* Penn. Coelenterata. In *Proceedings of the European Marine Biology Symposium* (Edited by BARNES H.), pp. 395-405. Aberdeen University Press.
- SINGER M. (1973) *Limb Regeneration in the Vertebrates*, Addison-Wesley Biology Module No. 6. Cummings Reading, Mass.
- SINGER M. (1974) Neurotrophic control of limb regeneration in the newt. *Ann. N.Y. Acad. Sci.* **228**, 308.
- TABAN C. H. (1978) cAMP and noradrenaline sensitivity fluctuations in regenerating newt tissue. *Nature, Lond.* **271**, 470-471.
- TABAN C. H., SCHORDERET M. & CATHIENI M. (1977a) Differential sensitivity of newt limb regenerates to noradrenaline, as revealed by their production of cyclic AMP. *Experientia* **33**, 48.
- TABAN C. H., SCHORDERET M. & CATHIENI M. (1977b), Possible role of catecholamines and cAMP in the regenerating forelimb of the newt. *Experientia* **33**, 832.