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Diadenosine 5',5'''-P^1, P^4-tetraphosphate in developing embryos of *Artemia*

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ABSTRACT

Diadenosine 5',5'''- P^1, P^4 - tetraphosphate (Ap_4A) has been detected in cysts and developing embryos of the brine shrimp Artemia in amounts 10^4 -10^6 times lower than that of the guanine analogue, Gp_4G. The unexpectedly high level of Ap_4A in dormant cysts of 2.37 pmol/10^6 cells can be reduced to 0.03 pmol/10^6 cells by decapsulation and storage in saturated NaCl. When development is reinitiated, the Ap_4A content of the decapsulated embryos undergoes a rapid 125 -fold increase, reaching a maximum of 3.79 pmol/10^6 cells at the point of emergence when DNA replication begins. If replication is delayed by hypoxia, the Ap_4A level is adjusted in order to reach the same maximum value when replication finally begins. As replication proceeds, the level of Ap_4A declines again. Unlike mammalian cells, Ap_4A in Artemia is less metabolically labile than ATP. These results are consistent with the suggested role of Ap_4A in the initiation of DNA synthesis.

INTRODUCTION

Under adverse environmental conditions the female brine shrimp Artemia sheds its developing embryos from the ovisac into the surrounding medium in the form of desiccated gastrular cysts (1). These cysts are protected by a tough impermeable capsule and retain typically 2 - 5% of the cellular water of the embryo. They can remain in a metabolically quiescent state for many years but upon reintroduction to seawater the cysts hydrate and development is reinitiated. Protein synthesis resumes within 10 min of rehydration and RNA synthesis shortly afterwards (2).

During the latter stages of this "pre-emergence" development, the embryo undergoes extensive differentiation culminating in its emergence from the shell at about 10 - 12h and its release from the hatching membrane as a nauplius larva after 14 16h. Remarkably all this achieved in the complete absence of DNA replication and with no increase in cell number (3,4). DNA synthesis resumes only at the time of emergence with a doubling of the DNA content and cell number occurring during the subsequent 60h (5).

We are interested in determining the factors responsible for controlling the reinitiation of DNA replication in Artemia embryos. One possible candidate is a

nucleotide of considerable current interest, diadenosine 5',5''' - P^1,P^4 -tetraphosphate (Ap_4A). Ap_4A is produced in vitro, and presumably in vivo, by the back reaction of an activated aminoacyl adenylate with ATP, a reaction catalysed by the aminoacyl - tRNA synthetases and strongly stimulated by Zn^{++} (6,7,8). It has been detected in several mammalian cells and its intracellular content found to vary inversely with the cell doubling rate within the range 0.05 to 7.5 pmol/10^6 cells (9). It has also been shown to stimulate DNA synthesis and cause the formation of replication eyes when added to permealysed Gl cells (10,11). Its intracellular target in calf thymus and HeLa cells is a subunit of a complex form of DNA polymerase - α (12,13). These findings suggest a possible role for Ap_4A in DNA replication, possibly at the level of initiation.

A particular reason for wishing to detect and study this nucleotide in Artemia is the fact that this organism contains large quantities of the guanine analogue, Gp_4G, as well as substantial amounts of other dinucleoside polyphosphates e.g. Gp_3G, Gp_2G and Gp_3A which total 2% of the total dry weight of the cyst (14,15). It has been suggested that Ap_4A and Gp_4G may have related roles as regulators of nucleic acid metabolism in mammalian cells, but as yet Gp_4G has not been detected in such systems (16,17). In Artemia, Gp_4G serves a dual role as a source of purines for DNA and RNA synthesis and as a high energy compound from which ATP can be derived (15). These nucleotides may also have a role in the regulation of protein synthesis (15). Since the catabolism of Gp_4G is enhanced at the onset of DNA replication (4,15), it may prove fruitful to search for a relationship between Ap_4A and Gp_4G in the control of macromolecular syntheses and in particular DNA synthesis in this organism. As a first step we report here on the identification of Ap_4A in Artemia embryos and on changes in its intracellular concentration during development which correlate with the onset of DNA replication.

MATERIALS AND METHODS

Great Salt Lake Artemia cysts were supplied by the Sanders Brine Shrimp Co., Ogden, Utah, USA and stored as described (18). [^{3}H] Ap_4A (24 Ci/mmol) was synthesised from [^{3}H]ATP (Amersham) and adenosine 5' -phosphomorpholidate (19) and purified by chromatography on DEAE -cellulose. Incubation medium was as previously described (18).

Preparation and harvesting of cysts

For most experiments, cysts were hydrated overnight at 0° in incubation medium and the floating empty shells and sand removed. The remaining viable cysts were sterilised and decapsulated with NaOCl (20) including final washes with 1% Na_2SO_3 and 1% acetic acid to remove residual traces of adsorbed chlorine. The cysts were

then dehydrated again by washing and storing in saturated NaCl in the dark at room temperature until required. Viability was unaffected for up to 3 months.

1g portions of washed, decapsulated cysts were incubated in 100 ml medium in baffled Erlenmeyer flasks in an illuminated (2l) orbital shaker at 28°C. Under these conditions embryos began to emerge at 12h with hatching commencing some 2h later as determined by the method of Warner et al. (22). Final hatch rate was 85%. Cysts and larvae were harvested at the appropriate times by filtration under suction, washed with ice-cold water and frozen in liquid N_2 (total harvesting time = 8 sec).

Preparation of extracts and high performance liquid chromatography

Frozen cysts harvested as above were broken with a Potter-Elvehjem homogeniser in 5 ml 0.4M TCA containing 0.5 µCi [^{3}H]Ap_4A (24 Ci/mmol). For the determination of Ap_4A in decapsulated dry cysts, 1g portions were broken in TCA by two passages at 40MPa through a French pressure cell. After 30 min at 0°, homogenates were centrifuged (1000g, 10 min) and the supernatants neutralised by shaking for 5 min with an equal volume of 0.6M tri-n-octylamine in Arcton (I.C.I.) (23). After centrifugation (500g, 10 min) the upper aqueous layer was removed, recentrifuged (16,000g, 4 min) and the supernatant mixed with 0.25 vol of 0.5M potassium phosphate, pH 7.2, 25mM tetrabutylammonium hydroxide and passed through a 0.22 µ Millipore filter. Samples were taken for scintillation counting and ATP, ADP and AMP determinations.

200 µl samples of extract were immediately chromatographed at 2ml/min on a Waters Radial-Pak C_{18} 10µ cartridge in 0.1M potassium phosphate, 12% (v/v) methanol, 5mM tetrabutylammonium hydroxide using an LKB 2150 HPLC pump and Uvicord SII detector. 0.6ml fractions were collected and samples taken for scintillation counting and Ap_4A determinations. Recoveries of nucleotides were calculated from dilution of the [^{3}H]Ap_4A and were of the order of 50%. Recoveries calculated with [^{14}C] ATP were identical.

Thin layer chromatography

Pooled Ap_4A - containing fractions from several HPLC runs were diluted 4-fold with H_2O and applied to a 1ml column of DEAE - cellulose equilibrated in 50mM NH_4HCO_3 pH 8.6. The Ap_4A was eluted with 0.3M NH_4HCO_3 and freeze-dried three times. The residue was dissolved in 50µl of water and 5µl samples applied to a PEI - cellulose thin layer plate (20 x 20 cm, F1440-PEI, Schleicher and Schüll). The plate was run for half its length in 1.6M LiCl, washed with anhydrous methanol and dried. It was then re-run to its full length in 0.8M LiCl, 0.8M CH_3 COOH and washed with methanol. Spots corresponding to nucleotide markers were scraped off, eluted for 30 min with 4M NH_4OH, neutralised with HCl and centrifuged (16,000g, 3 min).

Nucleotide assays

Ap_4A was determined by a coupled phosphodiesterase - luciferase assay (24) using

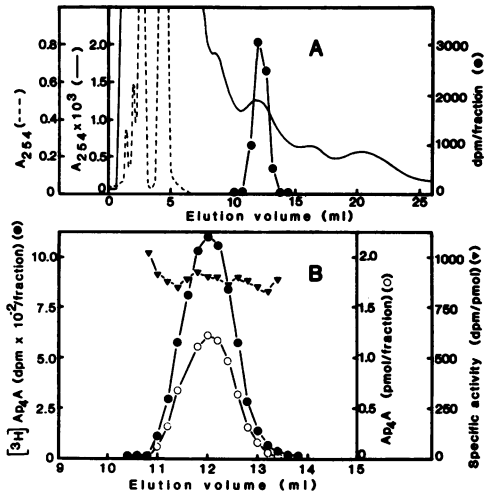

Fig.1 : High performance liquid chromatography of TCA-extracts of Artemia. (A) 200µl of an extract prepared from whole, dormant cysts was injected and run as described in Materials and Methods. 0.6 ml fractions were collected. Standards eluted at the following volumes : AMP, 3.2 ml; ADP, 4.2 ml; Gp_3G, 4.4 ml; Gp_4G, 4.4ml; ATP, 4.6 ml; Ap_3A, 9.8ml; Ap_5A, 11.6ml; Ap_4A; 12.0ml.
(B) as above but 0.2ml fractions collected between 10 and 14 ml.

an LKB 1250 Luminometer and LKB ATP monitoring reagent. ATP, ADP and AMP were also determined by bioluminescence assays (25).

RESULTS

Identification of Ap_4A in Artemia

In order to perform the multiple determinations described here with ease, it was necessary to establish a rapid and simple method for separating Ap_4A from both ATP and the large quantities of diguanosine polyphosphates which are present in acid-soluble extracts of Artemia cysts and larvae and which would interfere with the assay of Ap_4A. This was achieved by isocratic reversed phase ion-paired HPLC. When a TCA-soluble extract prepared from dry, dormant cysts to which a tracer of [^{3}H] Ap_4A (52,000 dpm/pmol) had been added before homogenisation was analysed, the [^{3}H] Ap_4A eluted well after those compounds which were expected to interfere with the Ap_4A assay (Fig.1A). An absorbance peak corresponding to the Ap_4A could be seen but was rarely sufficiently well resolved to allow quantitation by peak integration. The Ap_4A eluted with a constant specific activity of 880 dpm/pmol which strongly suggests that the endogenous material responsible for the reduction in specific activity of the tracer was Ap_4A (Fig. 1B).

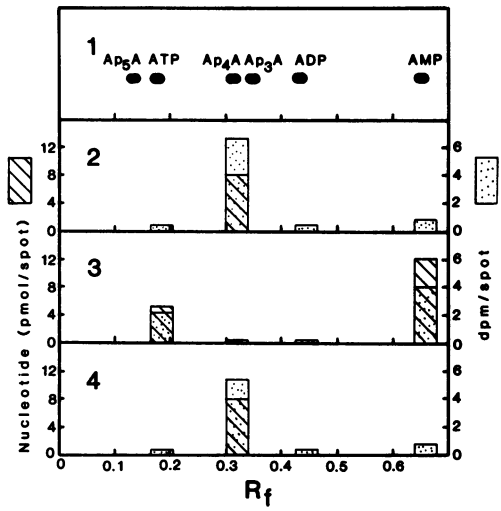

Fig.2 : Thin layer chromatography of Ap_4A from pooled HPLC fractions. The PEI - cellulose plate was run as described in Materials and Methods. Lane 1, standards; lane 2, Ap_4A from HPLC fractions; lane 3, as 2 but pretreated with 0.2mU snake venom phosphodiesterase for 2 min; lane 4, as 2 but pretreated with 0.2mU alkaline phosphatase for 2 min.

Further evidence for the identity of this material was provided by thin layer chromatography on PEI - cellulose of the pooled material eluted from several HPLC runs (Fig. 2). The initial specific activity of the Ap_4A (720 dpm/pmol) remained essentially unchanged after chromatography (770 dpm/pmol). Furthermore it was unaltered by treatment with alkaline phosphatase prior to t.l.c. whereas treatment with snake venom phosphodiesterase yielded both ATP and AMP of roughly half the original specific activity (390 and 326 dpm/pmol respectively). This was the expected result since the Ap_4A was labelled in only one adenine moiety. Given that 1g of cysts contains 2.5×10^5 embryos and each embryo comprises 3,800 cells, the Ap_4A content of dormant cysts was calculated to be 2.37 ± 0.36 (n = 3) pmol/10^6 cells. The concentrations of adenine mononucleotides were found to be : ATP, 0.49 nmol/ 10^6 cells; ADP, 1.20 nmol/10^6 cells and AMP, 1.09 nmol/10^6 cells, in agreement with published values (14). Hence Ap_4A has been positively identified in ametabolic Artemia cysts at a level more characteristic of proliferating cells. The significance of this will be discussed later.

Changes in Ap_4A during embryonic and early larval development

In order to determine whether the level of Ap_4A could be correlated with the processes of DNA, RNA or protein synthesis, cysts and larvae were examined at

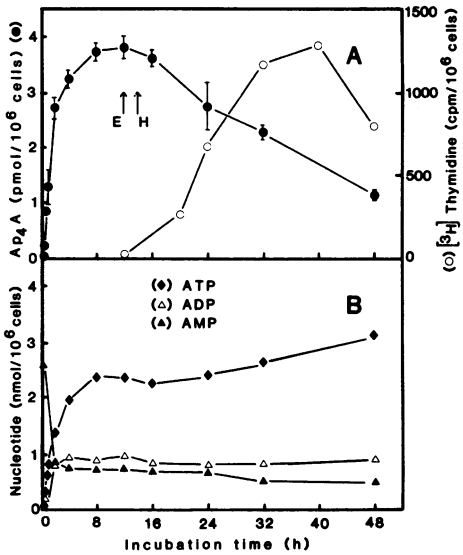

Fig.3 : Changes in (A) Ap_4A and (B) adenine mononucleotides after reinitiation of development. Values for Ap_4A are the means of 3 or 4 determinations and the error bars represent ± one S.D. Values for ATP, ADP and AMP are the averages of duplicate determinations. Arrows mark the times of emergence (E) and hatching (H) of the embryos. For the measurement of Ap_4A at 0h, 0.05 µCi [^{3}H] Ap_4A was added to the homogenate and the material from 8 runs concentrated by chromatography on DEAE -cellulose and freeze-drying as described for t.l.c. (see Materials and Methods).

different times after the reinitiation of development. Since Ap_4A has been reported to be particularly sensitive to extraction conditions (9,26) this study employed decapsulated cysts which had been dehydrated again and stored (96 days) in saturated NaCl solution. These can be homogenised much more rapidly than whole cysts (see Materials and Methods) and contain about 1.5 x10^5 embryos/g.

Surprisingly these cysts now displayed a very low level of Ap_4A of 0.03 ± 0.01 pmol/10^6 cells. Upon rehydration however this increased rapidly, reaching a maximum value of 3.79 ± 0.21 pmol/10^6 cells at the time of emergence (12h). This peak coincided with the onset of DNA replication as measured by the incorporation of [^{3}H] thymidine into pre-nauplius and nauplius larvae (18) (Fig. 3A). Once replication had begun, the Ap_4A level fell, reaching 1.6 pmol/10^6 cells around the peak of DNA synthesis (40h). This was not due to an enhanced lability of the Ap_4A during extraction from larvae since the recovery of the [^{3}H] Ap_4A tracer was the same at all times and, although precautions were normally taken to harvest the cysts and larvae as rapidly as possible, the Ap_4A content of 16h and 48h larvae was unaffected by leaving the packed organisms on the suction filter for up to 5 min before

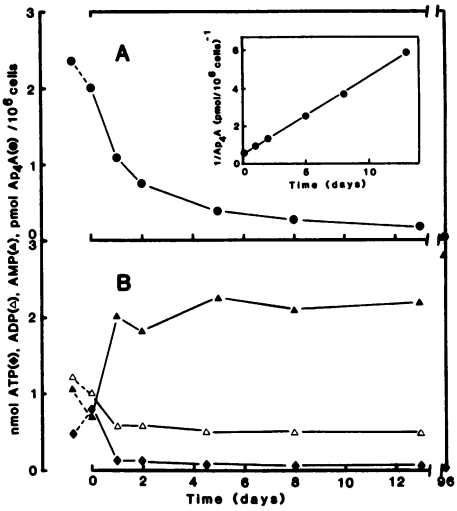

Fig.4. : Changes in (A) Ap_4A and (B) adenine mononucleotides during storage of decapsulated cysts in saturated NaCl. All points are averages of duplicate determinations. The points shown at t = - 0.75 represent the values for dry cysts before decapsulation.

homogenisation (data not shown). Hence the fall in Ap_4A appears to represent true catabolism in vivo.

ATP and ADP were also present at reduced levels in these cysts : 0.016 and 0.10 nmol/10^6 cells respectively, with the ATP rising rapidly during redevelopment to 2.35 nmol/10^6 cells primarily though not totally at the expense of AMP (Fig. 3B). This value is again similar to that previously reported (14). In contrast to Ap_4A, ATP continued to rise during larval growth as a result of Gp_4G metabolism, demonstrating that Ap_4A does not simply reflect the energy state of the cells.

Stability of Ap_4A in vivo

In view of the low levels of Ap_4A and ATP in stored decapsulated cysts compared to untreated embryos, the kinetics of decay of these nucleotides during storage was examined. The processes of hydration and decapsulation which were carried out primarily at 0°C caused a slight loss of Ap_4A and a small rise in ATP, probably as a result of the heat generated during decapsulation. On transfer to saturated NaCl (t = 0) at room temperature, ATP and ADP were rapidly dephosphorylated during the first 24h and thereafter declined very slowly (Fig. 4B). Such a loss of ATP has been previously described in cysts which contain less than 40% water, the level necessary for redevelopment (27). The brine-stored cysts used here retained 30% water. By contrast, the kinetics of Ap_4A disappearance were quite different (Fig. 4A) and

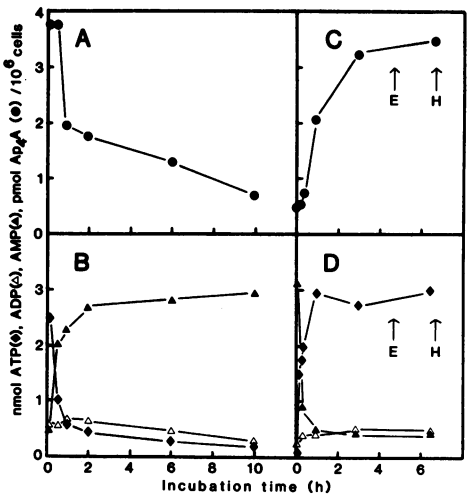

Fig.5 : Changes in Ap_4A and adenine mononucleotide levels during anoxia and reaeration. Portions of cysts were incubated for 10h in aerated seawater then transferred to seawater that had previously been purged with pure N_2 for 1h. Purging was continued for 30 min before sealing the flasks. Measurements of (A) Ap_4A and (B) adenine mononucleotides were made at various times after transfer. After 24h under N_2, samples were transferred back to aerated seawater for various times and measurements made of (C) Ap_4A and (D) adenine mononucleotides. Points are the averages of duplicate determinations.

appeared to approximate a 2nd order decay with a rate constant of 5.5 l/mol/sec (inset, Fig 4A). Using this rate constant the predicted Ap_4A content of cysts stored for 96 days was 0.025 pmol/10^6 cells which compares well with the measured value of 0.03 ± 0.01 pmol/10^6 cells. The significance of this finding remains to be determined.

Transfer of developing embryos to a hydrated, anoxic environment also resulted in the rapid conversion of ATP to AMP (28 and Fig 5B) but Ap_4A again lagged behind, showing in fact no change during the first 30 min (Fig. 5A). On reintroduction to aerated seawater, ATP was rapidly reformed reaching 55% of its final level within 5 min (28 and Fig. 5D) while again Ap_4A showed no change for the first 15 min (Fig.5D). Thus, in contrast to what has been reported in other systems, Ap_4A in Artemia responds much more slowly than ATP to metabolic conditions and to stress.

The final level of Ap_4A of 3.8 pmol/10^6 cells attained after reoxygenation coincided once more with hatching and the onset of DNA replication. Further experiments (not shown) where Ap_4A was reduced to different levels by varying degrees of hypoxia show that the same ultimate level of 3.8 pmol/10^6 cells was always reached after 6½h and coincided with the initiation of DNA synthesis. Hence this value is a characteristic of the G1/S boundary in Artemia embryos.

DISCUSSION

Although the resolution of Ap_4A from major cellular nucleotides by HPLC has been described (26,29,30) these systems have involved comparatively slow gradient elution. The isocratic system used here is similar to that in Ref. 31 and was designed to separate Ap_4A from the diguanosine polyphosphates sufficiently well to permit accurate assay. Multiple determinations could therefore be performed every 10 min. The further characterisation of the putative Ap_4A by t.l.c. was necessary since Artemia may also contain minor and as yet unidentified nucleotides such as Gp_4A (32) which would react in the assay.

Grummt and co-workers have reported unpublished work describing a 1000 -fold increase in the Ap_4A pool as mitogenically stimulated G_0 BHK and 3T3 cells progress to the G1/S boundary (33). The data presented here are however the first which follow the size of the Ap_4A pool through what is effectively a partially synchronised complete cell cycle. Such studies are essential for an understanding of the function of Ap_4A. The 125-fold increase observed corresponds to a rise in intracellular concentration from 25nM to about 3.3μM, figures which are in accordance with previously observed levels in resting and proliferating cells (9,24,25,33). In a preliminary communication, we reported the Ap_4A content of decapsulated 0h cysts to be 0.47 pmol/10^6 cells, rising to 5.7 pmol/10^6 cells at emergence (34). These results were obtained using cysts 4 days after decapsulation which had therefore not been fully depleted of stored Ap_4A and by using figures for no. of cysts/g which we now believe to have been underestimates.

The correlation of maximum Ap_4A with the onset of DNA replication and the subsequent fall, at a time when RNA and protein synthesis are continually increasing (2,4,15) are consistent with the suggestion that this nucleotide is involved in the initiation of DNA synthesis. It appears that as Ap_4A serves its function during S-phase it is consumed in the process. Possible modes of action may be either direct, as an initiator of replicons or indirect as an effector molecule to promote, for example, the sequential relaxation of replicon clusters or the spooling of DNA through previously fixed replication sites (35). In this context Ap_4A has the capacity to prime DNA polymerase -α *in vitro* (36). Alternatively the direct correlation may be with another S-phase related process such as histone synthesis (37). Ap_4A has been found to bind to histone H1 and to act in the bound state as an acceptor for ADP-ribose (38).

Fluctuations in the intracellular level of Ap_4A are assumed to be the result of the fine control of the balance between synthesis by the aminoacyl-tRNA synthetases and degradation by diadenosine tetraphosphatase (8,39). Specific degradative enzymes have been isolated from several sources (16,33,39,40,41) including Artemia (42). In all our experiments involving delayed harvesting, response to anoxia and storage in brine,

we have found that Ap_4A responds less rapidly than ATP to environmental changes in contrast to the results of other workers (9,25,26). There may be several explanations for this. Firstly, Artemia cysts may be inherently less responsive to stress than mammalian cells with ATP being the exception. Secondly if the Artemia dinucleoside tetraphosphatase is also involved in the mobilisation of Gp_4G (both nucleotides are equally good substrates), the much higher cytosolic concentration of Gp_4G of 1.2mM (15) may affect the rate at which Ap_4A can be degraded. Thirdly it is possible that under certain circumstances Ap_4A may be degraded by an alternative process, either by a reversal of its synthesis or by other means; the unusual kinetics of decay of Ap_4A in brine-stored cysts may lend support to this idea.

Whatever the cause, the low lability of Ap_4A may explain the unexpectedly high level found in whole, dry cysts. While no actual data are available, it is likely that early cleavage embryos undergoing rapid DNA synthesis will contain a constitutively high concentration of Ap_4A. When desiccation is initiated, the Ap_4A may slowly disappear until there is insufficient water to allow further degradation, thus preserving the residual content of 2.37 pmol/10^6 cells.

In conclusion, the identification of Gp_4G and now Ap_4A in Artemia should permit the use of this organism for studies on the possible roles of these nucleotides as regulators of nucleic acid metabolism.

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REFERENCES

1. Clegg, J. S. and Conte, F. P. (1980) in the Brine Shrimp Artemia, Persoone, G., Sorgeloos, P., Roels, O. and Jaspers, E. Eds., Vol. II, 11-54, Universa Press Wetteren.
2. Hultin, T. and Nilsson, O. (1980), in The Brine Shrimp Artemia, Persoone, G., Sorgeloos, P., Roels, O. and Jaspers, E. Eds., Vol. II, pp. 83-103, Universa Press Wetteren.
3. Nakanishi, Y. H., Iwasaki, T., Okigaki, T. and Kato H. (1962) Annot. Zool. Jpn. 35, 223-228.
4. Finamore, F. J. and Clegg, J. S. (1969) in The Cell Cycle, Padilla, G. M., Whitson, G. L. and Cameron, I. L. Eds., pp 249-278, Academic Press, New York.
5. McLean, D. K. and Warner, A. H. (1971) Dev. Biol. 24, 88-105.
6. Zamecnik, P. C., Stephenson, M. L., Janway, C. M. and Randerath, K. (1966) Biochem. Biophys. Res. Commun. 24, 91-97.
7. Plateau, P., Mayaux, J.-F. and Blanquet, S. (1981) Biochemistry 20, 4654-4662.
8. Goerlich, O., Foeckler, R. and Holler, E. (1982) Eur. J. Biochem. 126, 135-142.
9. Rapaport, E. and Zamecnik, P. C. (1976) Proc. Nat. Acad. Sci., U.S.A., 73, 3984-

3988.
10. Grummt, F. (1978) Proc. Nat. Acad. Sci., U.S.A., 75, 371-375.
11. Grummt, F. (1978) Cold Spring Harbor Symp. Quant. Biol. 43, 649-653.
12. Grummt,F., Waltl, G., Jantzen,H-M., Hamprecht, K.,Huebscher, U. and Kuenzle, C.C. (1979) Proc.Natl.Acad.Sci.,U.S.A. 76, 6081-6085.
13. Baril,E.,Bonin,P.Burnstein,D.,Mara,K. and Zamecnik,P.Proc.Natl.Acad. Sci.,U.S.A. (1983) 80, 4931-4935.
14. Warner,A.H. and Finamore,F.J. (1967)J.Biol.Chem. 242,1933-1937.
15. Warner,A.H. (1979) in Regulation of macromolecular synthesis by low molecular weight mediators,Koch,G. and Richter,D.Eds.pp 161-177, Academic Press, New York.
16. Cameselle, J. C., Costas, M. J., Sillero, M.A.G. and Sillero, A. (1982) Biochem. J. 201,405-410.
17. Grau, V. P., Sillero, M. A. G. and Sillero, A. (1982) J. Biol. Chem. 257, 11848-11851.
18. Slater, J. M. and McLennan, A. G. (1982) Eur. J. Biochem. 129, 415-421.
19. Reiss, J. R. and Moffat, J. G. (1965) J. Org. Chem. 30, 3381-3387.
20. Bruggeman, E., Baeza-Mesa, M., Bossuyt, E. and Sorgeloos, P. (1979) in Cultivation of fish fry and its live food: European Mariculture Society Special Publication No.4, Styczynska-Jurewicz, E., Backiel, T., Jaspers, E. and Persoone, G. Eds., pp 309-315, EMS, Bredene, Belgium.
21. Sorgeloos, P. (1973) Mar. Biol. 22, 75-76.
22. Warner, A. H., MacRae, T. H. and Wahba, A. J. (1979) Meth. Enzymol. 60, 298-311.
23. Khym,J.X. (1975) Clin.Chem. 21, 1245-1252.
24. Ogilvie,A. (1981) Anal.Biochem.115,302 - 307.
25. Probst, H.,Hamprecht,K. and Gekeler,V., (1983)Biochem.Biophys. Res.Commun. 110, 688-693.
26. Plesner,P., Stephenson,M.L., Zamecnik,P.C. and Buchner,N.L.R. in Specific Eukaryotic Genes, Alfred Benzon Symposium,Vol 13, pp 383-393, Munksgaard, Copenhagen.
27. Clegg,J.S. and Cavagnaro,J. (1976) J.Cell.Physiol. 88., 159-166.
28. Stocco,D.M., Beers,P.C. and Warner, A.H. (1972) Dev.Biol. 27, 479-493.
29. Flodgaard,H. and Klenow,H. (1982)Biochem.J.208, 737-742.
30. Lüthje,J. and Ogilvie,A. (1983)Biochem.Biophys.Res.Commun.115,253-260.
31. Nierenberg,D.W.,Pogolotti,A.L. and Santi,D.V.(1980)J.Chromatog.190,212-216.
32. Van Denbos,G. and Finamore,F.J. (1974)J.Biol.Chem.249,2816-2818.
33. Höhn,M.,Albert,W. and Grummt,F. (1982)J.Biol.Chem.257,3003-3006.
34. McLennan,A.G. and Prescott,M.(1984)in Proteins Involved in DNA Replication, Hübscher,U. and Spadari,S.Eds.,Plenum Press, in the press.
35. Pardoll,D.M.,Vogelstein,B. and Coffey,D.S.(1980) Cell 19, 527-536.
36. Zamecnik,P.C.,Rapaport,E. and Baril,E.F.(1982)Proc.Natl.Acad.Sci.USA 79, 1791-1794.
37. Bagshaw,J.C. (1982) Dev.Genet.3,41-51.
38. Tanaka,Y.,Matsunami,N.,Itaya,A. and Yoshihara,K.(1981)J.Biochem. 90,1131-1139.
39. Ogilvie,A.and Antl,W.(1983)J.Biol.Chem.258,4105-4109.
40. Barnes,L. and Culver,C.A.(1982)Biochemistry 21, 6123-6128.
41. Jakubowski,H. and Guranowski,A.(1983)J.Biol.Chem. 258,9982-9989.
42. Vallejo,C.G.,Sillero,M.A.G. and Sillero,A.(1974)Biochim.Biophys.Acta 358, 117-123