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Biochemical Characterization and Developmental Behavior of *Artemia* Embryonic and Nauplial Deoxyribonucleases†

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ABSTRACT: Deoxyribonuclease (DNase) activities have been studied during larval development of *Artemia*. The low DNase activity levels detected in extracts of dormant gastrulae were found to increase markedly in the nauplial stages. Partial purification and characterization of this activity in extracts of developed nauplii indicate the presence of three enzymic forms (A-I, A-II, and A-III). These DNase activities of *Artemia* are endonucleases, degrading supercoiled substrates mainly by a single-stranded attack mechanism. They differ in pH profiles, anion sensitivity, reactivity toward NH_2 group reagents, molecular weight, and chromatographic behavior. DNase activity A-I is the only activity detected in extracts of dormant embryos and appears associated with the yolk granules, in a "masked" form. The increase of activity around hatching is mainly due to unmasking of DNase activity A-I during yolk granule metabolism while the further increase during nauplial development correlates with the appearance of DNase A-III.

Artemia is a small crustacean, class *Anostraca*, which has evolved the ecological adaptation of laying dormant gastrulae which can remain in this state for very long periods of time. Upon exposure to the appropriate environmental conditions (Vallejo et al., 1980), development is resumed. After about 20 h, fully formed swimming nauplii hatch from the thick shells which envelop the dormant gastrulae. An interesting feature of this system is that cleavage, i.e., the subdivision of

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the oocyte cytoplasmic territory into the very much smaller embryonic cells, is temporarily dissociated from the subsequent differentiation and morphogenesis, since it has been shown that *Artemia* larval development can occur in the absence of cell division and DNA synthesis (Nakanishi et al., 1962; Olsen & Clegg, 1978). The role of procaryotic deoxyribonuclease (DNase)[1] activities is well understood (Kornberg, 1980). They are involved in many crucial cellular activities such as replication, recombination, and repair of nucleic acids. However, the functions and mechanisms of action of eucaryotic DNases are still not established in detail (Sierakowska & Shugar, 1976), although some of these enzymes have been known for decades (Feulgen, 1935). Thus, it seemed interesting to characterize the major DNases found in developing *Artemia*, since this information could provide new insight into the functional role of these enzymes in eucaryotic systems. In addition, it would be helpful in preventing the side effects of these enzymic activities in studies of the nucleic acid organization during *Artemia* nauplii differentiation.

Materials and Methods

pBR322 DNA was prepared as described (Maniatis et al., 1982). High molecular weight calf thymus DNA and pancreatic DNase were obtained from Worthington Millipore. Agarose, phosphodiesterase, pancreatic RNase, S_1 nuclease, micrococcal nuclease, snake venom phosphodiesterase, bovine serum albumin (BSA), soybean trypsin inhibitor (STI), dithioerythritol (DTE), and diethyl pyrocarbonate (DEPC) were purchased from Sigma Chemical Co.

Cysts. Dormant cysts of *Artemia* were from San Francisco Bay (Metaframe Menlo Park, CA 94025). Treatment of cysts, culture conditions, synchronization, and counting of nauplii were carried out as described elsewhere (Vallejo et al., 1979).

Culture of Embryos. Artemia cysts were hydrated overnight at 4 °C, dechorionized (Vallejo et al., 1981), and added to a flask containing hatching medium (Vallejo et al., 1979). They were incubated with continuous agitation at 30 °C during the time of development indicated in the figure legends. The percentage of hatching was determined by counting the nauplii and prenauplii vs. unhatched cysts under the dissection microscope.

Extraction Buffers. Three different homogenization media were used that varied in their properties with respect to the stabilization and/or disruption of the yolk granules (Vallejo et al., 1979): a sucrose medium, a yolk-stabilizing Ficoll medium, and a yolk-disruptive medium. The components of the sucrose medium were 0.7% sucrose, 5 mM EDTA, and 25 mM HEPES buffer adjusted to pH 7.5. The yolk-stabilizing Ficoll medium was composed of 15% Ficoll 400 (Pharmacia), 0.3 M sucrose, 5 mM EDTA, and 25 mM HEPES buffer adjusted to pH 7.5. In certain experiments, the more drastic, yolk-solubilizing medium, modified from Ohlendorf et al. (1977), was used. This Monol-containing alkaline medium is composed of 50 mM 2-amino-2-methyl-1-propanol (Monol, Sigma) and 30% glycerol, pH 11.

Assay of Endonuclease Activity. Assay 1. Specific DNA endonuclease activity on double-stranded DNA was assayed as described by Smith (1974) under conditions that allow quantification (Cervera, 1978). One clot assay unit corresponds to the amount of enzyme necessary to cleave 30 μg of double-stranded DNA under standardized conditions (20 min at 37 °C in 0.2 mL volume). After addition of 0.1 mL of 0.1% BSA and 0.2 mL of 10% trichloroacetic acid, the high molecular uncleaved DNA from Worthington would clot into a compact aggregate. After DNase cleavage, the DNA becomes transformed in a uniform suspension of small pieces.

Assay 2. Assay 2 was performed with 0.1 μg of supercoiled pBR322 DNA in 10 μL of reaction buffer, and the digested products were analyzed by electrophoresis in 0.7% agarose slab gels. This assay is specific for endonucleolytic activities, and the time course of the reaction reveals the attack mechanism (single or double stranded attack) of the enzyme (McKenna et al., 1981).

Assay 2 is more sensitive but less reliable concerning quantitation. Therefore, both assays have been used in parallel.

Artemia DNases were assayed in the presence of 5 mM Cl_2Mg and 10 mM Tris, pH 7.5, unless specifically indicated otherwise.

Subcellular Fractionation. Homogenates obtained with the different extraction buffers in Dounce homogenizers were fractionated by differential centrifugation. After filtration through a nytex screen, the homogenates were centrifuged at 500*g* × 10 min. The pellet was resuspended in the Ficoll medium diluted by half with distilled water and centrifuged again at 500*g* × 10 min, producing the 500*g* × 10 min pellet that, as shown before (Vallejo et al., 1981), is made of pure yolk granules. The pooled supernatants are centrifuged at 2000*g* × 10 min, producing a pellet composed of the *Artemia* nuclei mixed with slightly damaged yolk granules (Vallejo et al., 1981). The corresponding supernatant is pelleted at 15000*g* × 20 min to produce a sediment containing the free mitochondria. For some experiments, whole particulate and soluble fractions were prepared by centrifugation of the homogenate at 105000*g* × 90 min.

Sucrose Gradient Fractionation. The 500*g* × 10 min and the 2000*g* × 10 min pellets were resuspended in the diluted Ficoll homogenization medium and sedimented through a sucrose discontinuous gradient (layers of 1, 1.3, 1.5, 1.7, and 2 M sucrose) in an SW 27 at 26 000 rpm for 12 h.

Polyacrylamide Electrophoresis. Analysis of the protein samples was carried out in 1 mm thick 10% polyacrylamide–SDS slab gels using a 4% stacking gel, prepared as described by Laemmli (1970). Gels were stained with Coomassie Blue in ethanol/acetic acid.

Purification of the DNase Activities. (1) Hydroxylapatite Chromatography. Hydroxylapatite, prepared essentially according to Bernardi (1971), was stored in 10 mM phosphate, pH 6.8. After being loaded with the sample, which was extensively dialyzed against this buffer, the column was washed with 3 volumes of buffer and developed with a gradient of 10 mM to 1 M sodium–potassium phosphate pH 6.8. Soybean trypsin inhibitor (STI) was included in the homogenization medium and during the elution of the column.

(2) Chromatography on Phosphocellulose and DEAE-Sephadex. Columns were equilibrated in 10 mM phosphate, pH 6.8, and samples were extensively dialyzed against this buffer. After being loaded, the columns were washed with buffer and developed with a gradient of 0 to 1 M KCl in the same buffer.

(3) Chromatography on G-100 Sephadex. The column was equilibrated in 10 mM potassium phosphate, pH 6.8, 50 mM KCl, and 10 μg/mL STI. After the column was loaded the sample, the activity was eluted with the same buffer.

Results

Changes in DNase Activities during Artemia Development. The DNase activity detected in extracts of Artemia embryos

[1] Abbreviations: DNase, deoxyribonuclease; RNase, ribonuclease; EDTA, ethylenediaminetetraacetic acid, HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; SDS, sodium dodecyl sulfate.

Table I: Purification of DNase A-III from Developed Nauplii (48 h)[a]

	volume (mL)	total protein (mg)	total activity	specific activity	yield (%)
homogenate	78	6820	49920	7.3	100
particulate	29	1070	9280	8.6	19
soluble	72	4540	46080	10.2	92
hydroxylapatite	50	920	40320	43.5	81
phosphocellulose	134	900	48380	53.7	97
DEAE-cellulose I (A-I)	129	124	3860	31.3	8[b]
DEAE-cellulose II (A-III)	24	134	19200	81.8	38
Sephadex G-100 (A-III)	70	31	11140	366.6	22[b]

[a] The details of the purification procedure for the *Artemia* DNases and the definition of units are given under Materials and Methods. Activity was measured by using assay I. The electrophoretogram of the DNase A-III activity is given in Figure 3. [b] Since the total activity of the homogenate corresponds to 75% of DNase A-III and 25% of DNase A-I, the actual yield of the two forms is higher, 32% for A–I and 29% for A-III.

Table II: Properties and Effect of Different Reagents on the Activity of the Three Partially Purified DNases of *Artemia*

	A-I	A-II	A-III
M_r by G-100 Sephadex (A-I)	46000	68000	34000
M_r by Bio-gel A-1.5 (A-I*)	1.1×10^6		
pH optimum (Figure 2)	8.8	7.5	9.1
residual activity in 5 mM EDTA	–	+	–
inhibition by tetraborate at high pH	+	+	+++
sensitivity to modification of different functional groups			
–SH	++	++	–
$-NH_2$	++	++	–
competitive inhibition by RNA	–	++	–

and larvae at different times of development showed the changes summarized in Figure 1. The levels of the activity were quite low until the emergence of the swimming larvae and increased about 15-fold during early larval development. This increase was quite similar whether it was expressed in terms of the total activity per animal or in terms of activity per cell, because it is known that, in *Artemia*, the number of cells remains constant at the dormant gastrula stage (Nakanishi et al., 1962) and does not increase markedly during the early nauplial development (Olson & Clegg, 1978). The increase during development (Figure 1) can be divided into two phases: One around hatching, in which there was a sharp increment of 3–4-fold and a second of 5–10-fold which occurred progressively during the following 24 h of nauplial development.

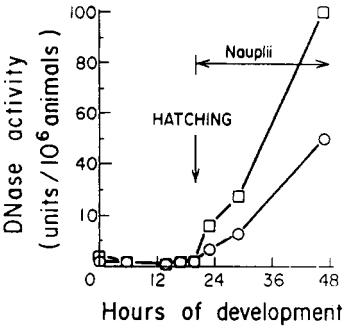

FIGURE 1: Changes in the levels of the total and soluble DNase activity during development. Total homogenates (squares) and soluble fractions (circles) after homogenization in a standard sucrose medium.

Characterization of DNase Activities from Artemia Nauplii. To further characterize the molecular basis of these developmental changes, the DNase activity detected in soluble extracts from 48-h developed *Artemia* nauplii was subjected to different types of chromatographic fractionation. As seen in Figure 2, it is resolved into four peaks when chromatographed in a DEAE-cellulose column: A-I, A-II, A-I*, and A-III, one which appears in the flow through and the other three which elute at 0.1, 0.2, and 0.4 M NaCl, respectively. A similar separation into four peaks is achieved when the nauplial extract was fractionated by gel filtration in Sephadex G-100, but the order of elution was now A-I*, A-II, A-I, and A-III (as discussed later). A-I* came together with the major peak of protein in both types of chromatography. This major peak of protein was excluded in the G-100 filtration column and was of yolk origin, as ascertained by polyacrylamide gel electrophoresis (data not shown). Upon rechromatography in the same or in a different type of column (for example, first by gel filtration and second by DEAE-cellulose), the A-I* peak always gave rise to two peaks with A-I and A-I* chromatographic characteristics, while the other three activities (A-I to A-III) maintained the initial chromatographic properties. These results and the ones summarized later strongly suggest that A-I* and A-I correspond to two molecular forms of the same enzymic activity.

In Table I (Figure 3), the partial purification of the enzyme forms A-I and A-III from extracts of developed nauplii is presented. The major activity, form A-III, was purified 50-fold. The major contaminating peak visible in the SDS–polyacrylamide electrophoretogram is the STI added to the buffers in all the purification procedures, since in its absence further artifactual chromatographic peaks, particularly in hydroxylapatite, were detected (Cervera et al., 1980).

The molecular weights of the different enzymes after partial purification (see Table II) are the following: A-I*, 1 100000; A-I, 46000; A-II, 68000; A-III, 34000.

The three forms of DNase activity are endonucleases capable of transforming supercoiled substrates into open circles, indicating that the attack is mainly, if not exclusively, haplotomic, i.e., nicking one of the two strands in the double helix. As the enzymes continue working on the substrate, a relatively small proportion of linear forms accumulates probably due to the introduction of another nick in the opposite strand, sufficiently close to a previously existing one to ensure the linearization of the circle. Eventually, a large smear of degraded DNA accumulates at longer digestion times and slowly decreases in molecular weight. Enzyme A-III markedly stimulates the activity of *E. coli* DNA polymerase I on supercoiled substrates, indicating that A-III cleaves on the 3′ side of the polynucleotide phosphodiester bond (data not shown).

Although the low activity of peak A-II (see below) has hampered the study of its enzymic properties, it is the only activity whose action on DNA is inhibited by high concentrations of RNA (yeast tRNA).

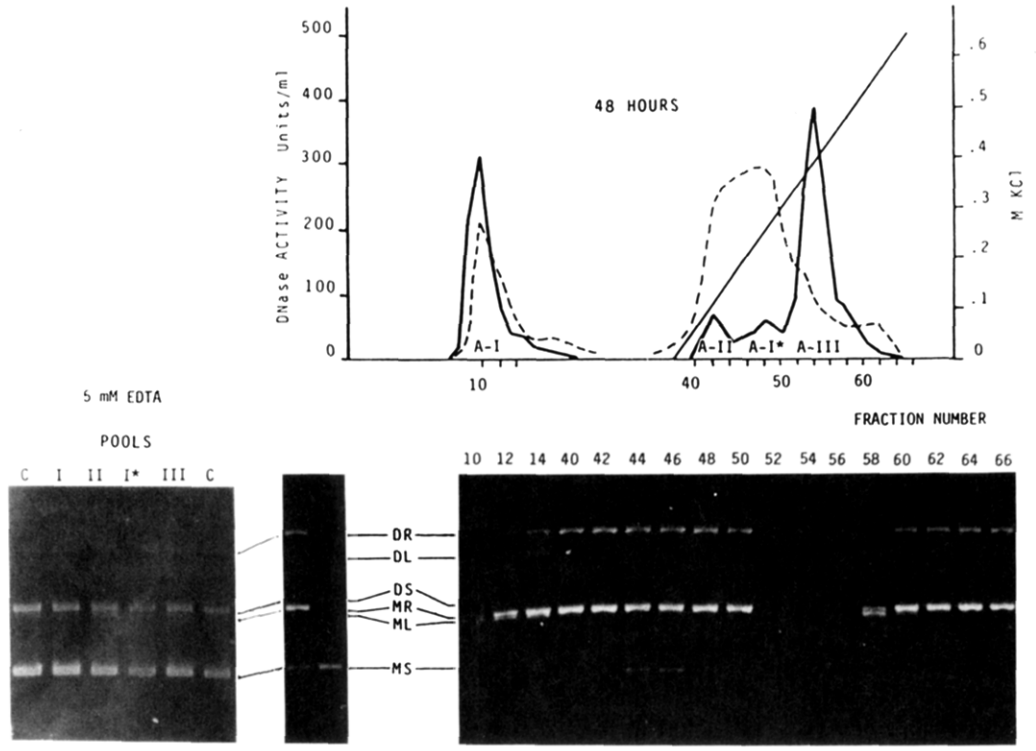

FIGURE 2: DEAE-cellulose chromatography of a developed nauplii soluble fraction. DNase activity is shown by using assay 1 and assay 2 as described under Materials and Methods. The lower central panel shows the electrophoretic migration of the different molecular forms of pBR322: supercoiled, covalently closed circles of monomer, MS, and dimer, DS; relaxed, open circles of monomer, MR, and dimer, DR; linear monomer, ML, and dimer, DL.

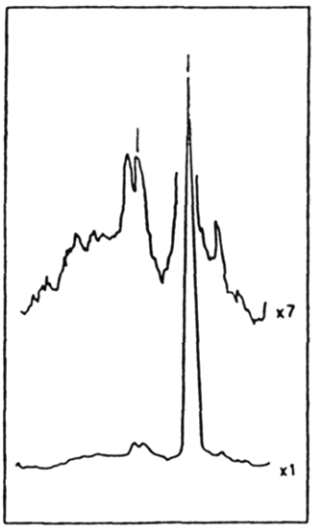

FIGURE 3: Electrophoretogram of the DNase A-III activity after the Sephadex G-100 is presented including a 7× magnification. The large peak corresponds to the STI included in all the purification steps to prevent further modification of the enzyme (Cervera et al., 1980).

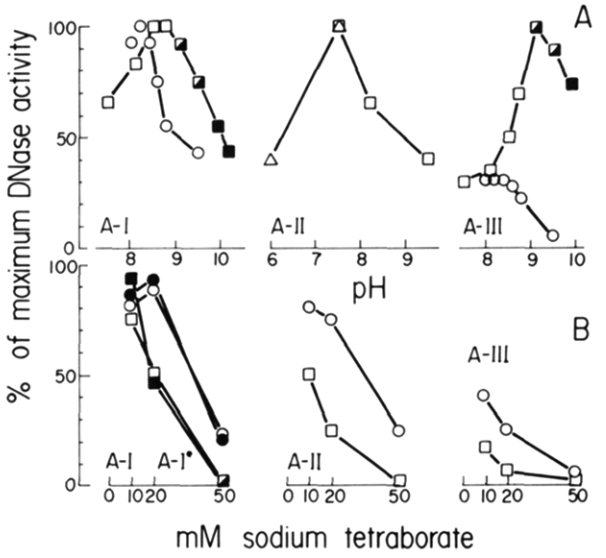

FIGURE 4: Effect of pH and tetraborate inhibition with the different *Artemia* DNase activities. The activity represented is the percent of the maximum activity for each DNase enzyme form. (A) The assay mixtures were prepared by using different buffers: Tris-HCl (open squares) from pH 7.5 to pH 9.5; Gly–NaOH (closed squares) from pH 9.1 to pH 10.1; cacodylate (triangles) from pH 6.0 to pH 7.5. The pH profile of DNase activities A-I and A-III using 20 mM tetraborate from pH 8.2 to 9.5 (open circles) is also represented. (B) The tetraborate inhibition was measured at pH 8.2 (circles) and 9.5 (squares); DNase activity A-I* (closed symbols); A-I, A-II, and A-III (open symbols).

The three enzyme peaks A-I to A-III differ in their pH optimum and sensitivity against certain anions, like, for example, tetraborate and ATP, particularly at alkaline pH. In Figure 4A, the pH curves are presented. While the pH optimums of A-I/A-I* and A-III are in the alkaline range (around pH 9), the peak A-II has maximum activity at pH 7.5. The presence of the anion tetraborate (Figure 4B) strongly inhibits these enzyme activities, particularly in the case of enzyme A-III. The same effect is visible in the presence of physiological concentrations of ATP (2 mM) which is strongly inhibitory to enzyme A-III at alkaline pH, while it is less inhibitory to the enzyme A-I. These inhibitory effects result in shifts of the assayable optimum pH toward more physiological pHs (data not shown).

The three enzyme activities require Mg^{2+} for activity (K_m = 20 μM) and are inactive in the presence of 5 mM EDTA, with the exception of enzyme A-II which shows a residual but significant activity in the presence of this metal chelator (Table II). Ca^{2+} does not affect the activity of any of the enzyme forms. In the absence of added divalent cations, the enzyme

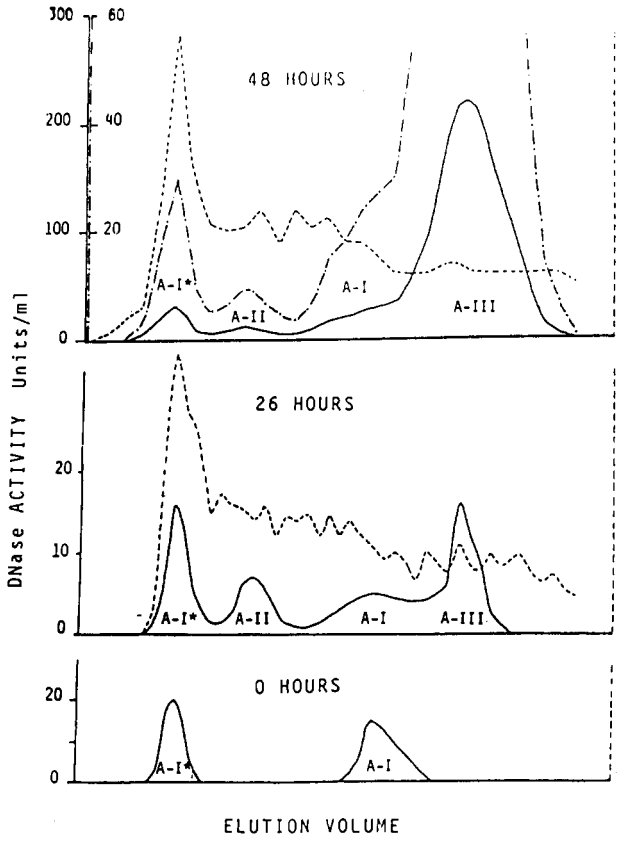

FIGURE 5: Separation of DNase activities during development. Soluble fractions of dormant cysts (0 h), newly hatched nauplii (26 h), and developed nauplii (48 h) were chromatographed in Sephadex G-100. Identification of the four distinct DNase forms is shown. DNase activity (solid line); DNase activity × 5 (–·–); proteins (broken line) were determined by measurement of UV absorption at 280 nm.

activities degrade the DNA due to the divalent cations bound to the substrate in commercial samples. If DNA is pretreated with EDTA, the DNase activities A-I and A-III become absolutely dependent on the addition of Mg^{2+} cations.

The presence of SH group protective reagents like DTE had no significant effect on any of the three forms. Treatment of the partially purified enzyme forms with iodoacetate or *p*-(hydroxymercuri)benzoate did not affect enzyme A-III, while it produced a 60% decrease in enzyme A-I/A-I* and A-II activities. Amino groups reagents like fluorescamine strongly inhibited the type A-I/A-I* activity with very little effect on the type A-III activity. On the other hand, DEPC, a reagent of amine and imidazolic groups (Means et al., 1971), strongly inhibited the type A-III activity, suggesting the presence or involvement of a histidine group in its active site (Table II).

Characterization of DNase Enzyme Activities at Earlier Developmental Stages. The data presented so far indicate that in 48-h developed nauplii there are three different DNase activities characterized by chromatographic and enzymic criteria. In soluble extracts prepared from dormant gastrulae under disruptive conditions, only form A-I/A-I* was detected, both by gel filtration (Figure 5) or by DEAE-cellulose chromatography (data not shown). This result has also been confirmed by partial purification and enzymic characterization of the activity from undeveloped cysts. In newly hatched nauplii (24-h total development), forms A-I/A-I* and A-II and only small amounts of A-III activity were detected. Thus, the increase in activity upon further development is clearly associated with the expression of *Artemia* DNase A-III.

DNase A-I/A-I in the Dormant Gastrulae Is Associated with the Yolk Granules in a Masked Form.* Two main lines

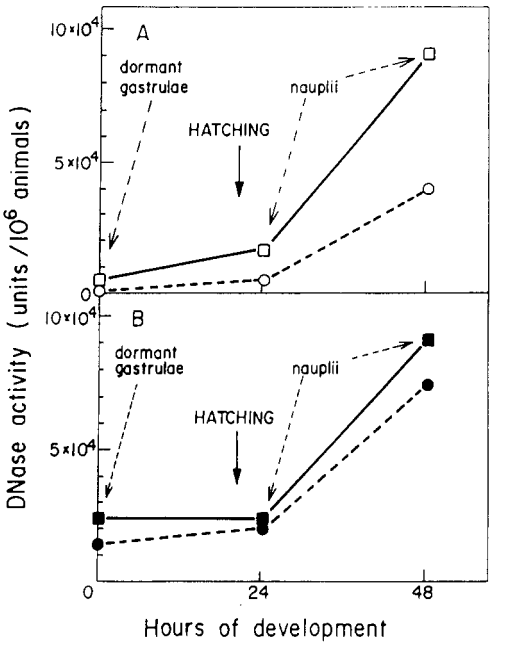

FIGURE 6: Effect on the DNase activity of extraction with a yolk-disrupting medium during *Artemia* development. Total homogenates (squares) and soluble fractions (circles) (A) obtained in the yolk-stabilizing Ficoll medium (see Materials and Methods) and (B) in the yolk-disrupting Monol medium (see Materials and Methods).

Table III: Unmasking of the DNase A-I Activity in Extracts of *Artemia* Dormant Cysts[a]

	DNase activity (%)
solubilizing conditions	
homogenate	100
soluble fraction	72
nondisruptive conditions	
homogenate	21
particulate[b]	72 (A)
soluble fraction[c]	14 (B)
	86 (A + B)

[a] Particulate fraction corresponds to the total precipitate after 150000*g* × 90 min centrifugation of the homogenate. Soluble fraction is the corresponding supernatant. [b] Particulate resuspended in solubilizing medium and assayed in the solubilized fraction obtained after centrifugation. [c] If solubilizing medium is added, no increase in activity is detected. The solubilizing medium is described under Materials and Methods.

of evidence indicate that DNase A-I/A-I*, the form found in the dormant gastrulae, is associated with the yolk granules: (1) the use of yolk disrupting media and (2) subcellular fractionation experiments.

(1) The DNase activity detected in extracts of dormant *Artemia* gastrulae, using homogenization conditions that preserve the integrity of the yolk granules (Vallejo et al., 1979), was much lower than the activity found at later stages of development and appeared mainly associated with the particulate fractions (Figure 6A).

On the other hand, when the homogenates were obtained by using the yolk granule solubilizing medium, the assayable activity increased (4–5-fold, depending on the experiments), appearing mainly in the soluble fraction (Figure 6B). Under stabilizing conditions, the DNase activity that remained associated with the particulated fraction showed a similar increase in activity when solubilized with the yolk granule solubilizing medium. After this treatment the majority of the assayable activity appears now soluble (Table III).

When these experiments were repeated with young nauplii immediately after hatching, the total activity found (Figure 6A) was equivalent to that detected in the dormant gastrulae

Table IV: Subcellular Distribution of the DNase A-I Activity in Particulate Fractions of *Artemia* Dormant Cysts[a]

	%	stimulation by resuspension in disruption medium
yolk granule pure fraction, 500*g* × 10 min sediment	42	2.5
yolk granule and nuclear fraction, 2000*g* × 10 min sediment	50	1.3
mitochondrial fraction, 15000*g* × 20 min	8	1.1

[a] The percentages shown correspond to the percentages of activity in the particulated fractions and are the mean of three fractionation experiments. The Polyacrylamide gel electrophoresis of equivalent aliquots of the different fractions is shown in Figure 7.

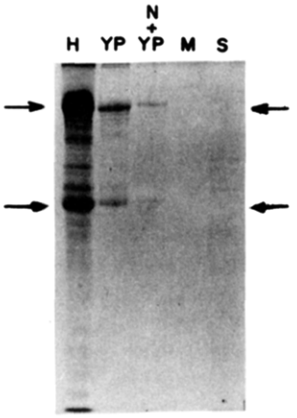

FIGURE 7: Polyacrylamide gel electrophoresis of equivalent aliquots of the different fractions. H, (homogenate) YP (500*g*), YP + N (2000*g*), M (15,000*g*), and S (soluble) fractions prepared after homogenization in the yolk-stabilizing Ficoll medium, obtained as described under Materials and Methods and run in 10% polyacrylamide gels. The two arrows indicate the main yolk *Artemia* polypeptides of M_r 190 000 and 68 000, molecular weights respectively (Chaffoy & Kondo, 1980). The ladder of bands between the two arrows corresponds to the degradation forms of the higher molecular weight lipovitellin as described (Chaffoy & Kondo, 1980).

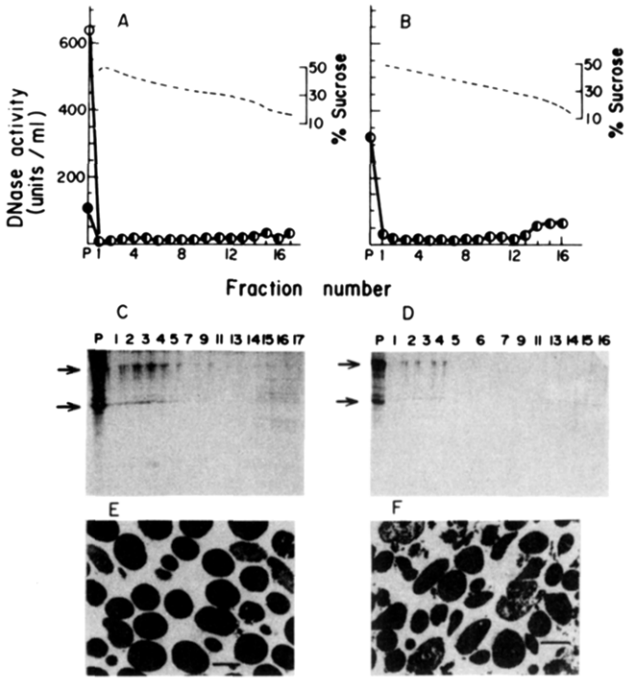

FIGURE 8: Sucrose gradient fractionation of the particulate DNase activity from dormant gastrulae homogenates. The DNase activity of the 500*g* (A) and 2000*g* (B) fractions from cysts (Table V), obtained after homogenization in Ficoll medium, were fractionated in sucrose gradients as described under Materials and Methods. The distribution of the activity is presented, assayed in the presence (open circles) or in the absence (closed circles) of a yolk-disrupting Monol medium. Half-open, half-closed circles correspond to the fractions where both types of symbols coincide. The composition of the pellets obtained from the sucrose gradients and visualized by electron microscopy is shown: (E) 500*g* fraction and (F) 2000*g* fraction. The bar corresponds to 1 μm. Pellets from the sucrose gradients were fixed in 1% glutaraldehyde, treated with 1% OsO_4, embedded in Araldite, sectioned, and stained for electron microscopy (Hayat, 1970). In (C) and (D) the corresponding SDS–polyacrylamide gel electrophoresis of the fractions is shown. The applied sample from the pellet (P) corresponds only to $^1/_{10}$ of the applied sample of the fractions from the gradient. The two arrows correspond to the main *Artemia* yolk polypeptides of M_r 190 000 and 68 000, respectively (Chaffoy & Kondo, 1980).

extracts under solubilizing conditions (Figure 6B). The assayable activity in nauplii extracts was the same in yolk-solubilizing and stabilizing conditions. In whole developed nauplii after 48 h of development, an additional increase in activity was detected, independent of the type of homogenization medium employed. After hatching, the type of homogenization medium used affected only the relative proportion of enzyme activity which appeared soluble (Figure 6).

(2) If the particulate material in yolk-stabilizing Ficoll medium is subjected to subcellular fractionation (Table IV; Figure 7), the DNase activity remains associated with the yolk granule rich fractions as shown by the PAGE electrophoresis. Interestingly, the 500*g* × 10 min pellet, which corresponds to a pure yolk fraction, is the only one that is markedly stimulated when resuspended in a yolk-solubilizing media. Furthermore, if these fractions are further purified in isopycnic sucrose gradients (Figure 8A,B), again the DNase activity sediments in the pellets where the yolk granules are present. The low activity found in the gradients could be explained by the leakage from the yolk granules as shown by the yolk protein profiles in the corresponding polyacrylamide gels in Figure 8C,D. Again the stimulation by the yolk-solubilizing media is essentially confined to the 500*g* × 10 min pellet, where the majority of intact yolk granules sediment (Figure 8E,F).

Discussion

The low DNase levels in extracts of dormant gastrulae were found to increase markedly during early development. This increase could be ascribed to two mechanisms: (A) The first mechanism is the unmasking of an activity (DNase A-I) that is associated with the yolk granules, the major subcellular components of early embryonic systems. This unmasking takes place during the morphogenetic phase before hatching. (B) The second mechanism is the appearance of new DNase activities (DNase A-II and A-III), particularly DNase A-III, during the later development of the hatched nauplii.

The association of A-I/A-I* endodeoxyribonucleases with the yolk granules was confirmed by its subcellular fractionation and chromatographic properties, as well as the stimulatory effect of the yolk-disrupting extraction media.

Also in accordance with this conclusion, the major activity detected in recently hatched (24 h) nauplii was of the type A-I/A-I*, the major difference being that it was then almost fully detectable independent of the type of the homogenization medium, yolk disruptive vs. nondisruptive, although in the latter case it remained associated with the particulated fraction, purifying with the partially metabolized yolk granule fraction (data not shown). It has to be pointed out (Marco et al., 1980) that during development of *Artemia* dormant gastrulae the major part of the initially remaining yolk granules are me-

tabolized. This metabolism occurs abruptly at first, before and around hatching, when major morphogenetic and differentiation events lead to the shaping of the swimming nauplii. The remaining yolk granules are degraded later after hatching, when the differentiation of the internal nauplial tissues is completed (around 48 h of development) allowing the larval animal to feed. In this respect, it is interesting that extracts of *Artemia* gastrulae, which are incubated for 12 h and do not yet show signs of morphogenesis and differentiation, have DNase activities with the same properties as the extracts of the dormant gastrulae; i.e., only the type A-I/A-I* form is detected if yolk granule disruptive conditions are used (data not shown).

Some of the enzymic properties of the three DNase activities are very similar. The three enzymes are endonucleases, active on supercoiled substrates, producing nicking cuts in one of the two DNA strands. The linear and the low molecular weight DNA molecules which accumulate at long incubation times probably reflect the production of second nicks opposite or very close to one already present in the DNA molecule. The type A-III enzyme attacks the double-stranded DNA, leaving free 3′-OH groups, showing a similar degrading mechanism to pancreatic DNase I. Their major differences from DNase I are the absence of a Ca^{2+}-stimulatory effect on the *Artemia* enzymes and their more strict nicking attack than the pancreatic enzyme (Price et al., 1975).

DNases A-I, A-II, and A-III, besides their chromatographic and developmental behavior, differ also in the pH profiles and in other properties, like their sensitivity to anions (tetraborate and ATP at alkaline pHs) and sensitivity to NH_2 group reagents, since A-I/A-I* is sensitive and A-III insensitive. Type A-II is the only enzyme that is active in the presence of EDTA. It is also the only one inhibited by high concentrations of tRNA, similar to endonuclease I of *E. coli*, or from *Proteus mirabilis* and an endodeoxyribonuclease of human KB cells (Tsuoro et al., 1978).

The functions of these enzymes remain unknown. Two alternative hypotheses are currently being investigated in our laboratory: (1) They may be involved in the activation of DNA synthesis, needed for further growth and development of the nauplii. As pointed out in the introduction, *Artemia* initial development can proceed in the absence of DNA synthesis and cell division (Nakanishi et al., 1962; Olsen & Clegg, 1978). (2) The processes involved may include the degradation and further utilization of the yolk DNA. Similar to earlier work in other systems (Hanocq et al., 1972), we have found a significant amount of DNA associated with the highly purified yolk fraction from dormant cysts (Marco et al., 1983); the fate and molecular characteristics of this DNA are being studied now in our laboratory. This second type of role is more likely associated with the type A-I/A-I* yolk granule associated enzyme. On the other hand, the A-II and A-III enzymes are probably involved with DNA metabolism during further nauplial development. In this respect, enzyme A-I/A-I* falls in the category of cellular components of maternal origin stored in the oocyte cytoplasm in an inactive form that are activated and participate in the initial events of zygote, here gastrular development. The properties of DNAse A-III suggest that it is newly synthesized, although our initial attempts to label *Artemia* nauplii with amino acids in the period 24–48-h development have failed to show any significant radioactive incorporation in the partially purified DNase A-III activity (unpublished data).

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