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DNA polymerases in embryos of Artemia sp.

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DNA POLYMERASES IN EMBRYOS OF *ARTEMIA* sp.

by

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DEDICATION

In fond memory of my late grandparents, Shriman. V. Chinnaswami Iyengar, whose advice was to 'hitch my wagon to a star', and to Shrimathi. V.C. Thangammal, who was a standing example of a 'free-spirited woman' in her time.

ABSTRACT

The profile of the DNA-dependent DNA polymerases of Artemia sp. has been determined at selected stages of development and the polymerases purified and characterized from dormant cysts (0h) and nauplii (36h) stages. A reliable purification procedure using DEAE-cellulose chromatography has been established for the first time. Various biochemical parameters including pH optima, ionic requirements, primer-template preferences, regulation by endogeneous dinucleoside polyphosphates, sensitivity to specific inhibitors and sucrose gradient sedimentation analyses were used to characterize the cyst and naupliar DNA polymerases.

The DNA polymerases in the post-mitochondrial fractions from the cysts and nauplii separated as two distinct fractions (PMF-1,PMF-2) upon DEAE-cellulose chromatography. Moreover, the nuclear fraction from dormant cysts eluted as a single peak at the position of fraction 2 (NF-2) while the nuclear enzyme from nauplii fractionated as two fractions (NF-1, NF-2). The results of this study have shown that the cyst DNA polymerase (PMF-1) and naupliar DNA polymerase (PMF-1) probably have different functional roles in development, namely repair and replication, respectively. The cyst DNA polymerase appears to resemble the low molecular

weight β-polymerase of mammalian cells while the naupliar polymerase exhibited several characteristics similar to the α-polymerase from mammalian cells. The endogenous dinucleoside polyphosphates, especially Gp_2G and Gp_3A unique to eggs and embryos of Artemia, showed a marked influence in modulating the DNA polymerase activity suggesting that these nucleotides function in the expression of the repair and replication enzymes of Artemia. The functional role of the cyst DNA polymerase suggests that this enzyme which is akin to β-polymerase of mammalian cells is conserved.

ACKNOWLEDGEMENTS

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Ap_3A	adenosine (5') triphospho (5') adenosine
Ap_4A	adenosine (5') tetraphospho (5') adenosine
ATP	adenosine 5'-triphosphate
βME	β-mercaptoethanol
BSA	bovine serum albumin
dATP	deoxy-adenosine 5'-triphosphate
dCTP	deoxy-cytosine 5'-triphosphate
dGTP	deoxy-guanosine 5'-triphosphate
dTTP	deoxy-thymidine 5'-triphosphate
d2TTP	dideoxythymidine 5'-triphosphate
DEAE-	diethylaminoethyl-(cellulose)
DTT	dithiothreitol
DMSO	dimethylsulfoxide
DNase	deoxyribonuclease
EDTA	ethylenediamine tetraacetic acid
Gp_3A	guanosine (5') triphospho (5') adenosine
Gp_2G	guanosine (5') diphospho (5') guanosine
Gp_3G	guanosine (5') triphospho (5') guanosine
Gp_4G	guanosine (5') tetraphospho (5') guanosine
GTP	guanosine 5'-triphosphate
Hg	homogenate
NEM	N-ethylmaleimide

NF	nuclear fraction
PMF	post-mitochondrial (or cytosol) fraction
POPOP	1,4-bis [2-(5-phenyl oxazolyl)]-benzene
PPO	2,5-diphenyloxazol
RNase	ribonuclease
S.R.M.	stop reaction mixture
TCA	trichloroacetic acid

Chapter I

INTRODUCTION

In recent years, the brine shrimp, Artemia sp. has gained importance as an useful model system for biochemical studies of development (1). During embryogenesis, gastrulae of Artemia follow either one of two developmental routes. They may give rise to swimming nauplii directly or they may encyst and become dormant. In the latter case, development resumes if dormant cysts are hydrated and incubated under aerobic conditions at 15 - 30°C. At 30°C pre-nauplii (E1 and E2) emerge from the cysts after 10 - 12 hours of incubation in sea water. On further incubation, the pre-nauplii give rise to free-swimming nauplii by a process called 'hatching' (2). During the development of Artemia, considerable morphogenesis takes place during the pre-nauplius stage in the absence of cell division and DNA synthesis. At the end of cleavage, the embryo enters the stage of dormancy. Following dormancy there is resumption of RNA and protein synthesis, but only after the hatching of the nauplii is DNA synthesis activated (3 - 6). In other words, DNA synthesis is 'switched - off' during pre-naupliar development and then 'switched - on' as the embryo hatches into a free-swimming larva (Fig.1). Encysted embryos of Artemia have several re-

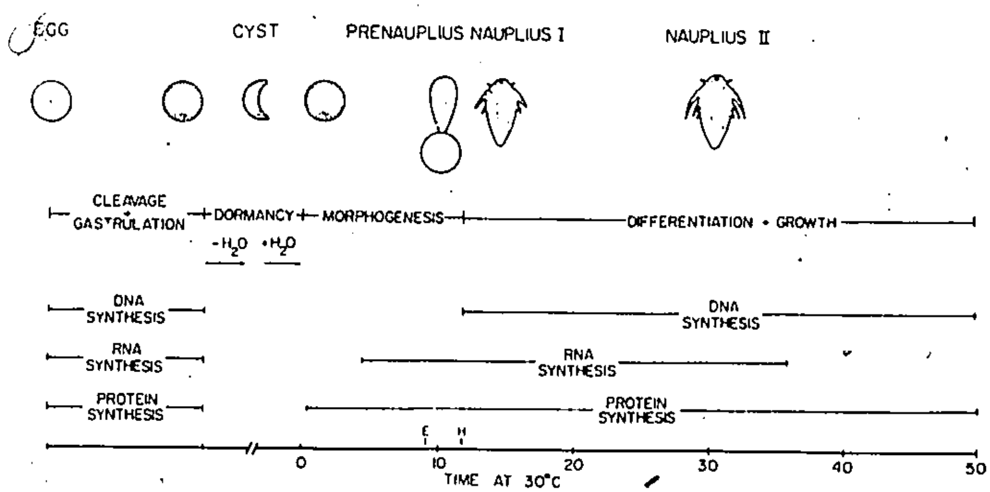


FIG.1. Profile of the major morphological and metabolic changes in encysted embryos of Artemia salina following fertilisation. E and H refer to the time of emergence and hatching, respectively. (from: Warner, A.H. 1979 (9)

markable features which make them particularly attractive as a developmental model for molecular biology studies. Their state of dormancy is a remarkable physiological situation not observed in mammalian cells or tissues. The process of development in Artemia is thus characterized by a complex series of changes in the pattern of selective gene expression. Regulation of DNA synthesis could therefore be the result of stage-specific gene expression. In Artemia, the most direct approach to unravel this problem biochemically would be to measure the levels of the DNA replication enzyme, viz. DNA-dependent DNA polymerases and to characterise this enzyme(s) at different stages of development. In this thesis the DNA-dependent DNA polymerase profile has been determined at selected stages of Artemia development and the polymerases characterized at two stages, dormancy and nauplii.

The molecular basis of genetic information transfer resides in the 'central dogma' that defines three major processes (namely, replication, transcription and translation) in the preservation and transmission of genetic information. Replication, the copying of DNA to form identical daughter molecules, involves the DNA-directed DNA polymerases. These enzymes catalyse DNA replication by adding deoxyribonucleotide units to a DNA strand. The DNA polymerases catalyse the formation of a phosphodiester bond between the 5'- α phosphate group of the incoming purine or pyrimidine deoxyribonucleotide and the 3'- hydroxyl end of a 'primer'. The incorporation of these purine or pyrimidine deoxyribonucleotides is complementary to the 'template' (Fig.2). In DNA synthesis, the term 'primer' refers to the DNA (or RNA) chain from which growth occurs at its 3'-hydroxyl terminus, and the term 'template' applies to the DNA chain that furnishes directions for the sequence of nucleotides. Thus, polymerization of the deoxyribonucleotides occurs via the nucleophilic attack on the α- phosphate of the incoming nucleoside triphosphate by the 3'-hydroxy terminus of the primer, yielding a new 3'-5' phosphodiester bond and inorganic pyrophosphate.

DNA-dependent DNA polymerases have been exhaustively studied in several prokaryotic systems including E.coli (7-9), B.subtilis (10), M.luteus (11), and S.typhimurium (12) to name a few. Bacteria contain three DNA polymerases which are referred to as DNA polymerase I (pol I) (7), DNA polymerase II (pol II) (13,14) and DNA polymerase III (Pol III) (14,15). The three enzymes are distinct gene products which differ with respect to their structure and 'in vivo' function(16-18). Despite their differences (cf.19) each DNA polymerase catalyses essentially the same basic synthetic reaction as described schematically in Fig.2. Considerable progress has been made in establishing the function of multiple DNA polymerases in replication and repair of prokaryotic chromosomes. This has been mainly possible due to

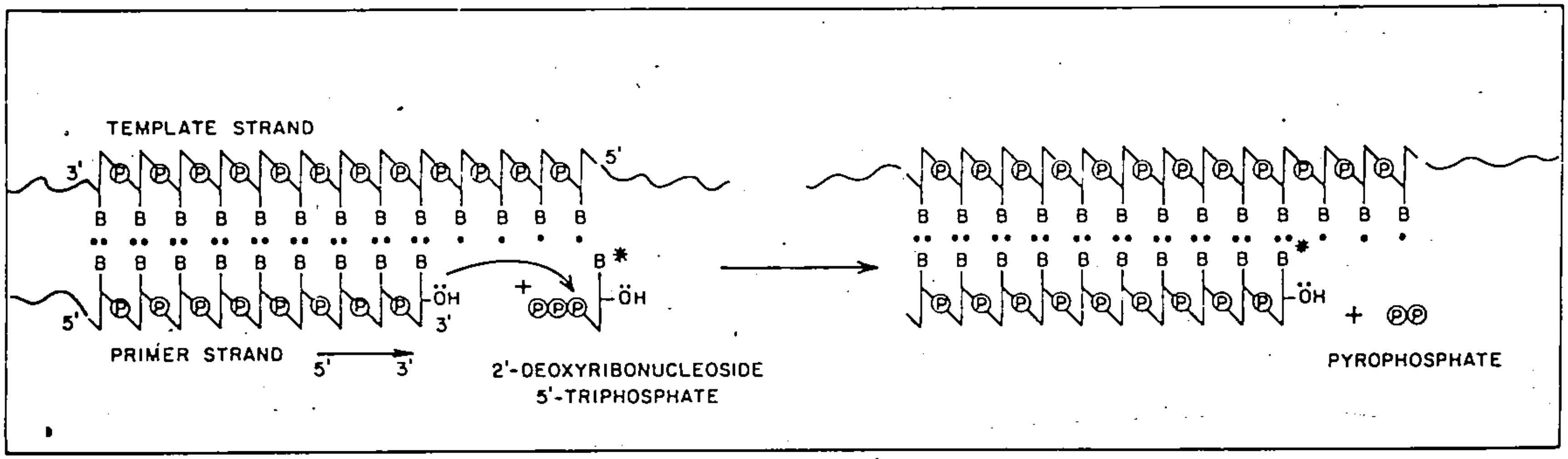


FIG.2. Schematic representation of the DNA polymerase reaction.
(from: Gallo & Sarin, 1980 (79)

the relatively large amounts of these enzymes available in these systems and the ease with which DNA polymerase - deficient mutants can be obtained in prokaryotes (20-24). Studies on the mechanisms of DNA replication have been limited to lower eukaryotes or virus-infected mammalian cells (25 - 28). Studies on lower and unicellular eukaryotes were undertaken since nuclear and mitochondrial events, and the influences of the cytoplasm, may be easier to analyse (as in unicellular fungi and protozoans) when genome sizes are only a few times larger than that of E.coli and can be grown in kilogram quantities (29 - 32). Emphasis on animal cells and tissues that grow and differentiate would provide information on the nature and control of DNA replication. Thus, the existence of multiple species of DNA polymerases came to be recognized in a wide variety of eukaryotic cells. Four classes of DNA polymerases can be distinguished by the differences in their properties (33- 35), and recently a common nomenclature has been assigned to these DNA-directed DNA polymerases (36). There seems to be general agreement that a high molecular weight enzyme (6-8 S), usually derived from the cytoplasmic fraction of cells is DNA polymerase α. A homogeneously sedimenting low molecular weight enzyme (3-4.5 S), usually identified in the nuclei, is designated as DNA polymerase β. A polymerase activity identified in both the nuclear and cytoplasmic fractions that shows preference for the replication of oligonucleotide-primed homopolymeric ribonucleotide templates particularly [(dT)15.(A)n], is DNA polymerase γ. The DNA polymerases isolated from various eukaryotic cells will be referred to as DNA polymerase α, β or γ based on their similarities in properties to the well characterized DNA polymerases of HeLa cells (35, 37, 38) and calf thymus (34). This classification includes comparative studies to determine the subcellular location and quantitative changes of each DNA polymerase in proliferating versus non-proliferating cells and in different phases of a single cell cycle (39 - 41). These three DNA polymerases have also been identified in several other eukaryotic systems such as D.melanogaster embryos (42), sea-urchin embryos (43,44), Euglena (45), X.laevis oocytes (46) human lymphoblastoid cell line NC 37 (47), rat liver and spleen (44-50), human leukemic leukocytes (51,52), human KB cells (53), developing avian erythrocytes (54), mouse myeloma cells (55), chick embryo (37), and soybean (56) and wheat germ (57). In addition, another class of DNA polymerases has been isolated from the mitochondria of several mammalian cells and is termed , DNA polymerase (58,59). Hence, it was of interest to study the types of DNA polymerases (on the basis of the above classification) present in Artemia embryos and their possible role(s) in the regulation of development in this organism.

The building blocks of DNA synthesis are actually deoxyyribonucleoside monophosphates (deoxynucleotides) which are derived from deoxyribonucleoside tri-phosphates (dNTPs). In virtually all cells, two fundamentally different kinds of pathways are used for the synthesis of nucleotides (Fig. 3). One is the 'de novo' pathway in which ribose phosphate, certain amino acids, CO_2 and NH_3 are combined in successive reactions to form the nucleotides. Neither the free purine or pyrimidine bases (adenine, guanine and cytosine, uracil or thymine) nor the corresponding nucleosides (deoxyribonucleosides) are intermediates in the de novo pathway. By contrast, cells also possess various mechanisms for making use of the free bases and nucleosides ultimately produced by the breakdown of nucleic acids (Fig. 3). By various routes, components of nucleotides (bases and nucleosides) are converted back to nucleotides themselves by way of the 'salvage pathway'. Recent findings from our laboratory indicate that _Artemia_ embryos are extremely rich in dinucleoside polyphosphates such as Gp_2G, Gp_3G, Gp_4G and Gp_3A (60,61). The former two have been reported to comprise approximately 2% of the dry weight of embryos (60). The presence of Gp_2G and Gp_3A was discovered for the first time in 1978 and as of this time , they appear to be unique to eggs and embryos of _Artemia_ (61). It is pertinent to note that embryos and adults of _Artemia_ are unable to synthesize purines de novo while they possess the de novo pathway for

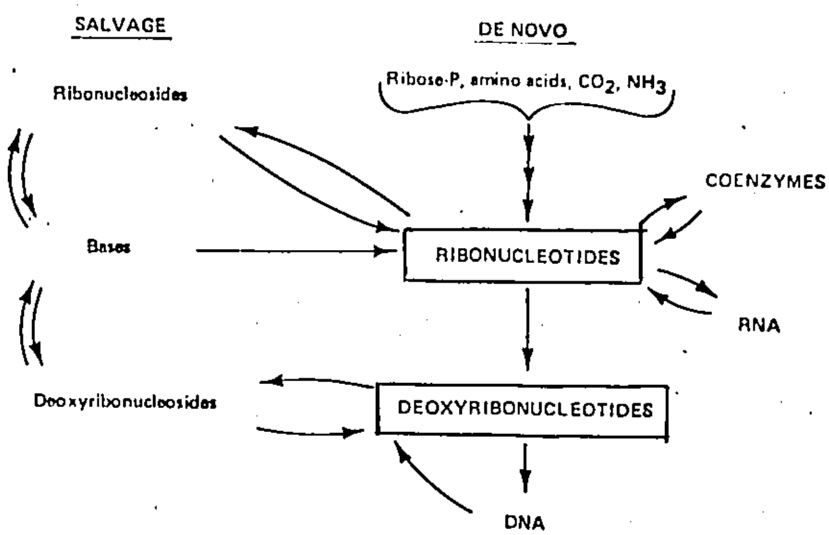


FIG.3. Salvage and de novo pathways of nucleotide biosynthesis.
(from: Kornberg, A. 1980 (41)

pyrimidine biosynthesis (62). It has also been shown that Gp_3G and Gp_4G are the major or primary sources of all nucleic acid purines in Artemia embryos including DNA-adenine (63,64). The role of these dinucleoside polyphosphates in Artemia development has been recently reviewed (65,66). In several other eukaryotic systems the dinucleoside polyphosphate Ap_4A has been shown to be present rather than Gp_4G (67). Moreover, Grummt et al. have shown that Ap_4A is directly involved in the stimulation of DNA synthesis in baby hamster kidney (BHK) cells (68,69). In these cells DNA synthesis is apparently triggered by the specific binding of Ap_4A to DNA polymerase α (70). The capacity of DNA polymerase to bind to Ap_4A is lost in neuronal cells during brain maturation concomitant with the loss of mitotic activity in these cells (71). Since Artemia embryos are rich in several dinucleoside polyphosphate compounds and display a period of discontinuous DNA synthesis, we have become interested in a possible role of the diguanosine nucleotides in DNA synthesis regulation.

The only previous reports on the DNA polymerases of Artemia deal with some aspects of the nuclear enzyme activity in relation to growth rate, but to this author's knowledge, no conclusive or definite role of DNA polymerases in DNA synthesis regulation of Artemia has been reported (72,73). This study is the first major effort to characterize the DNA polymerases in Artemia sp. This thesis examines the DNA-dependent DNA polymerase enzyme profiles in <u>Artemia</u> embryos in the dormant encysted stage (0h) and in one day old nauplii (36h). The distribution of the various DNA polymerases in the nuclear fraction (NF) and post-mitochondrial fraction (PMF) of 0h and 36h embryos was also studied. In Ehrlich ascites cells DNA polymerase α was found to be polymorphic and represented 77 - 80% of the total cell DNA polymerase content in rapidly proliferating cells (34). The nature of its location is still controversial in that earlier reports indicated only a cytoplasmic localization (48,74). Novel methods of experimentation (using enucleation techniques) that prevent leakage from the isolated nucleus have shown that DNA polymerase α is of nuclear origin (75,76). By comparison, there has been little or no controversy in the nuclear localization of DNA polymerase β (77) which is present predominantly in quiescent cells. Many reports have indicated the conservation of this enzyme through evolution (78). The nuclear location of any enzyme can be established only if methods of isolation and/or analysis are used which prevent the leakage into the cytosol. These studies were undertaken on the DNA polymerases of <u>Artemia sp.</u> to determine; 1) the profile of the DNA polymerases at various stages of development; 2) the nature of activation of DNA synthesis following hatching; 3) the role of dinucleoside polyphosphates (the only source of purines) in this activation; and 4) some properties of the cyst and naupliar

DNA polymerases. Interestingly, studies on the DNA polymerases of *Artemia* described herein, indicate that these enzymes are comparable to the mammalian enzymes in their properties. The multiple roles of the *Artemia* enzymes and their possible sequestration could provide clues to the nuclear cytoplasmic interactions. This study has attempted to define the role of *Artemia* DNA-dependent DNA polymerases during development and to characterise them for the purpose of studying DNA synthesis regulation in these embryos as compared to the mammalian DNA polymerases.

Chapter II

MATERIALS

Supplies

Encysted embryos of Artemia sp. were from the San Franscisco Bay salterns in California and supplied by Metaframe Corp., Newark, California. Penicillin G (sodium) was from Ayerst in Montreal, Quebec., Streptomycin sulfate from Schwarz/Mann (Orangeburg, N.Y.), soybean trypsin inhibitor, β-mercaptoethanol, dithiothreitol and glycerol were from Sigma (St. Louis, Mo.). Liquid scintillation fluors 2,5-diphenyloxazol (PPO) and 1,4-bis [2-(5-phenyl oxazolyl)] -benzene (POPOP) and tissue solubilizer (NCS) were from Amersham (Oakville, Ont.). Omnifluor was obtained from New England Nuclear (Boston, Mass.). Diaflo membranes (PM 10) were from Amicon (Lexington, Mass.). Glass fiber filter discs (GF/C, 2.1 cms dia.) were from Whatmann obtained from Mandel Scientific (Montreal,Que.). Eppendorf tubes (1.5 ml capacity) were from Bio-Rad (Missisauga, Ont.) and dialysis tubing was from Fischer Scientific (Toronto, Ont.).

For protein determinations the Bio-Rad protein reagent was used (Bio-Rad, Missisauga, Ont.). For column chromatography Sephadex G-25 (medium) was purchased from Pharmacia (Montreal, Que.); Ultrogel AcA-34 is a product of LKB

(Fischer Scientific, Toronto, Ont.); DEAE-cellulose (microgranular, DE-32, Whatmann) was from Mandel Scientific (Montreal, Que.) and hydroxylapatite was from Clarkson (Williamsport, Pa.). For the DNA polymerase studies, calf thymus DNA, salmon testes DNA, and deoxy-nucleoside 5'-triphosphates (dATP, dGTP, dCTP and dTTP) were from Sigma (St.Louis, Mo.). Dinucleoside polyphosphates Gp_2G, Ap_3A and Ap_4A were from P-L Biochemicals (Milwaukee, Wisconsin), whereas Gp_3G, Gp_4G and Gp_3A were purified fractions from our laboratory (compliments of Sarah Gilmour and Nancy Morrison). Dideoxythymidine 5'-triphosphate (d2TTP) was from P-L Biochemicals (Milwaukee, Wisconsin) and deoxy-thymidine 5'- triphosphate [methyl-^{3}H] (sodium salt, specific activity 71.6 Ci/mmol) was purchased from New England Nuclear (Boston, Mass.). N-Ethyl maleimide (NEM) was from Calbiochem (Missisauga, Ont.) and aphidicolin was a gift from Dr. A. H. Todd of Imperial Chemical Industries (Macclesfield, England).

Media and Buffers

The following media and buffers were prepared for use in the experiments described herein.

Hatch Medium

The medium used to hatch nauplii from the cysts contained the following components: 422 mM NaCl, 9.4mM KCl, 25.4mM $MgCl_2$, 1.4mM $CaCl_2$, 0.5mM $NaHCO_3$, 250 units/ml penicillin G (Na) and 25 ug/ml streptomycin sulfate.

Homogenization Buffer

The homogenization buffer consisted of the following components: 0.01M Tris-HCl, pH 8.0 (0-4 C), 0.005M $MgCl_2$, 0.001M EDTA (Na2), 0.001M β-mercaptoethanol (βME), 0.005M KCl, 25% glycerol (v/v) and soybean trypsin inhibitor (100ug/ml). In subsequent purification experiments, soybean trypsin inhibitor was employed at 400ug/ml (in the homogenization buffer) and 0.001M dithiothreiotol was substituted for BME.

Glass fiber filter soaking solution

The following solution was used to prepare glass filters for collecting the components of the reaction mixture: 0.20M sodium pyrophosphate, 0.10M EDTA (Na2) and unlabelled TTP at 0.4 mg/ml.

Stop reaction mixture (S.R.M.)

The solution used to stop the enzyme reaction contained 0.10M sodium pyrophosphate, 0.001M EDTA (Na_2) and denatured salmon testes DNA (0.5mg/ml).

Buffer A

Buffer A contained 10mM potassium phosphate, pH 7.5, 1mM EDTA (Na_2), 2mM BME and 10% (v/v) glycerol.

Buffer B

Buffer B contained 10mM potassium phosphate , pH 7.5, 1mM EDTA (Na_2), 2mM βME and 15% (v/v) glycerol.

Buffer C

Buffer C was like Buffer B but it contained 1.0M KCl.

Buffer D

Buffer D contained 0.10M NaCl and 0.20M EDTA (Na_2), pH 8.0.

Buffer E

Buffer E contained 0.15M sodium chloride and 0.015M sodium citrate (standard saline-citrate).

Buffer F

Buffer F contained 0.03M potassium phosphate and 0.10M NaCl, pH 8.0.

Buffer G1-G3

Buffer G contained 0.10M NaCl with either 0.06M potassium phos phate (G1), 0.12M potassium phosphate (G2) or 0.25M potassium phosphate (G3) at pH 8.0.

Buffer H

Buffer H contained 1.0M $MgCl_2$, 50uM Tris-HCl, pH 7.0, and 1 mg/ml bovine serum albumin.

Scintillation fluids

The scintillation fluid used in the experiments contained 0.4% PPO, 0.015% POPOP in toluene (or) 4 g/liter of Omnifluor in toluene.

Chapter III

METHODS

Preparation of Artemia embryos and incubation Procedures

Hydrated cysts of Artemia and embryos at different stages of development were prepared to study the activity and properties of DNA-dependent DNA polymerases from these embryos.

Encysted dormant embryos were hydrated in ice-cold 0.5M NaCl, treated with 0.02 % zephyran chloride for 30 minutes, and at the end of two hours hydration washed with ice-cold double distilled water to remove floating cysts and debris as described by Warner et al. (80). The washed cysts were rinsed with cold homogenization buffer and extracted directly (0h embryos). When developing embryos were required for analysis the cysts were rinsed with hatch medium and incubated in hatch medium at 30°C with gentle shaking. The embryos were collected after various incubation periods, washed with homogenization buffer or cold distilled water and either processed directly or stored at -70°C.

Two incubation procedures were used. When crude enzyme extracts were needed, 2 grams (wet weight) of fully hydrated cysts were incubated in glass storage dishes (100 x

80 cms) containing 150 mls of sterile hatch medium for each developmental stage studied. (1 gram of hydrated cysts contains about 100,000 embryos). For enzyme purification studies, 20 grams of fully hydrated cysts were dispensed in 5 gram quantities and each was incubated with 250 mls of sterile hatch medium in Fernbach culture flasks (2800 ml). In this case, the percent emergence and hatching was determined after various incubation periods by counting the pre-nauplii (E1 and E2) and nauplii in an aliquot from selected culture dishes as described previously (80).

All glassware and reagents were sterilized before use and zephyran chloride was added to inactivate the microorganisms attached to the cyst chorion (80). Unless otherwise indicated, the same batch of *Artemia* cysts was used throughout the course of this study.

DNA content during development in *Artemia*

The DNA content of *Artemia* embryos was determined at various stages of development between 0 and 60 hours. Embryos of *Artemia* (2 grams, starting cysts wet weight) were homogenized with 5 mls of 0.5N perchloric acid ($HClO_4$) and centrifuged to remove the acid-soluble materials. The acid-insoluble pellet was recovered, washed twice with 5 mls of 0.5N $HClO_4$, then neutralised with 0.5mls of ammonium acetate. Delipidation was carried out using equal volumes of an ethanol -ether mixture (3:1,v/v) with constant stirring at 40°C, 30 min. The insoluble proteins were collected by centrifugation and the delipidation step repeated until the supernatant became almost colorless. The precipitate was air dried, then suspended in 2 mls of 2M NaCl-0.01M Tris-HCl, pH 8.0, and incubated for one hour at 90°C with constant stirring to solubilize the nucleic acids. The soluble fraction was recovered by centrifugation and the pellet re-extracted twice with hot NaCl-Tris solution. The supernatant fractions from the latter two steps were pooled filtered through glass fiber filters, then tested for DNA content using the method of Ceriotti (81).

Preparation of Artemia DNA for use in polymerase studies

Artemia DNA was isolated from 36h embryos following the procedure of Vaughn (82) with slight modifications, and the DNA was activated for use as a primer-template in the DNA polymerase assay.

Fresh tissue (10 grams wet weight, nauplii) was homogenized with 5-6 volumes of ice-cold buffer D. and centrifuged to remove cell debris. Sodium dodecyl sulfate was then added to the homogenate to 2.5% (w/v) final concentration. Next, DNase-free pronase prepared according to the method of Hotta and Basel (83) was added to a final concentration of 100 ug/ml and the mixture incubated at 37°C with vigorous shaking. At the end of two hours, the solution was made 5M with respect to sodium chloride, then an equal volume of a chloroform ($CHCl_3$)-isoamylalcohol (IAA) mixture (24:1, v/v) was added. The mixture was agitated thoroughly for 30 min using a wrist-action shaker. The aqueous phase obtained by centrifugation was separated using a large bore pipet and the $CHCl_3$-IAA extraction repeated until little or no denatured protein was present at the interface. The aqueous phase was dialysed against buffer E, then ribonuclease (rendered DNase -free by heating at 80°C for 30 min) was added to a final concentration of 50 ug/ml. The mixture was stirred for 60 min at 37° C, followed by the addition of pronase (50 ug/ml) and two additional hours of incubation at 37°C. Solid NaCl was then added to a final concentration of 5M and the CHCl -IAA extraction repeated as before. The aqueous phase was recovered and dialysed overnight against buffer E, then concentrated by vacuum dialysis. The DNA was 'spooled out' following the addition of ice-cold 100% ethanol and resuspended in buffer F. The DNA was loaded onto a hydroxy-apatite column (1 x 16cms) previously equilibrated with buffer F and the column washed with two bed volumes of buffer F. The bound DNA was eluted sequentially with buffers G1 to G3, concentrated by vacuum dialysis, dialysed against buffer E and stored at -70°C. The DNA was quantitated by the method of Ceriotti (81) and activated for use as a primer-template.

Activation of DNA for use as primer-template

The method of Aphosian and Kornberg (84) was used to activate calf thymus DNA and *Artemia* DNA for use as primer-template in the DNA polymerase enzyme assays. Activated calf thymus DNA was used in all the regular procedures for enzyme assays except where indicated otherwise.

Calf thymus DNA or *Artemia* DNA (in 8mg quantities) was suspended in 2 ml of buffer H containing 400 ug of pancreatic DNase I (made RNase free as described earlier). Digestion of the DNA was allowed to proceed by incubation at 37°C for 35 min. The reaction was stopped by heating for 5 min at 77°C then chilled in an ice bath. The extent of DNA activation was estimated by comparing UV-absorbance ratios at 235, 260 and 280 nm according to the method of Fansler and Loeb (85). The activated DNA was quantitated according to the method of Ceriotti (80) then diluted to 400 ug/ml and stored in small aliquots at -70°C. The primer-template stock solution of DNA thus prepared was used throughout the course of this work.

Preparation of crude enzyme extracts

Particulate (nuclear) and non-particulate (soluble) fractions were prepared from *Artemia* embryos at different developmental stages to determine the distribution and profile of DNA polymerase activity with development.

Embryos at different stages of development were homogenized in a glass tissue grinder (Duall type) to a thick paste with 6mls of homogenization buffer. The homogenate was filtered through a cheese cloth-glass wool-cheese cloth filter and one ml of this filtrate (as total homogenate, Hg) was stored at $-70^{\circ}C$. The remaining filtrate was centrifuged at 1000 x g for 15 min. The resulting nuclear fraction or pellet (NF) was resuspended in 1 ml of the homogenization buffer and stored at $-70^{\circ}C$. The 1000 x g supernatant fluid was centrifuged at 2500 x g for 15 min and the supernatant fluid was collected free of the floating orange lipid layer ~~and~~ used as the post-mitochondrial or cytosol fraction (PMF). The Hg, NF and PMF were dialysed against three changes of homogenization buffer (250 mls each) up to a maximum of 6 hours. The dialysed enzyme samples were used as crude enzyme fractions in the assay for DNA polymerase activity.

Assays of DNA polymerase activity

Method I: Glass fiber filter method.

Enzyme assay conditions were essentially identical for both crude extracts and purified fractions. The main variation was in the collection of the acid-insoluble precipitate and its subsequent processing for assay of DNA polymerase activity. The reaction mixture contained the following:

0.10M potassium phosphate pH 7.4, 40 mM $MgCl_2$, 5mM βME, 50 ug activated calf thymus DNA or Artemia DNA, 500 uM unlabelled deoxynucleoside 5'-triphosphates (dATP, dGTP, dCTP) and 50 uM [methyl-^{3}H] -thymidine 5'-triphosphate Enzyme was added to a final volume of 0.25 ml and aliquots of 0.05 ml were sampled at the desired time intervals for measurement of polymerase activity.

All assay mixtures were prepared at 0°C, and transferred to 37°C to start the reaction at the time of enzyme addition. (Zero time samples indicative of non-specific radioactive incorporation were sampled initially at 0°C.) At the desired time intervals the reaction was stopped by transferring 0.05 ml to 1 ml of ice-cold stop-reaction mixture (SRM). This mixture was heated at 100°C for 2 min, cooled then mixed with 0.5 mls of ice-cold 15% trichloroacetic acid (TCA). After standing at 0-4°C for 10 min, the acid-insoluble precipitate was collected on a pre-soaked glass fiber filter using a micro-Millipore filter assembly. The precipitate was washed with five 10 ml portions of ice-cold 5% TCA, followed by 2 mls of 95% ethanol and dried either under an infra-red lamp or at 110°C for 5 min. When all samples were collected they were counted with 5 mls of scintillation fluid in a Beckman (LS-3150P) or Nuclear Chicago (Mark II) Liquid Scintillation Counter.

Method II Using NCS tissue solubilizer.

In some of our studies, method I using glass fiber filters to collect the acid-insoluble radiolabelled product was unsuitable due to color quenching with the nuclear fractions (NF) and therefore an alternative procedure was used.

Samples at various time points in the enzyme reaction were transferred directly to Eppendorf tubes containing 1 ml SRM in an ice bath. The tubes were capped, vortexed and transferred immediately to a boiling water bath for 2 min. After cooling the precipitate was allowed to form in the cold for 10 min following the addition of 0.5 mls of cold 15% TCA. The precipitate was recovered by centrifugation and the supernatant fluid was removed with a disposable pipet. Since the precipitate has a tendency to stick to the walls of the Eppendorf tubes, 1.5 mls of cold 5% TCA (wash) was added to each tube. The precipitate was suspended using a glass spatula as a policeman. The coloured precipitate was pelleted and the procedure repeated twice. At each step, care was taken to ensure complete removal of the supernatant without disturbing the pellet. To the precipitate (in the Eppendorf tubes) was added 0.2 mls of 95% ethanol and the mixture dried at 110°C (with caps open) for 15 min. After cooling, 0.5 mls of 90% NCS was added to each tube and the mixture was heated at 55°C for a minimum of 3 hours.

The solubilized pellet was then transferred quantitatively into counting vials and 10 mls of scintillation fluid was added. The amount of radioactivity was determined after allowing the vials to stand in the dark overnight. The counts were repeated over a period of 1-2 days to ensure stability of the counting vessel. Each sample was counted for 10 min and corrected for quenching using a previously established quench correction curve for tritium samples.

Biochemical characterization of Artemia DNA polymerases

The purified DNA polymerase fractions from DEAE-cellulose chromatography were treated with several agents as outlined below. Each enzyme fraction was assayed in the regular assay mixture with the treatment as indicated. At the desired time intervals the enzyme reaction was stopped and the acid-insoluble precipitates processed using assay method II.

pH studies

Potassium phosphate buffer was used for assays between pH 6.5 - 8.0 and Tris-HCl at 37°C, for pH 7.0 - 8.5. Each buffer was tested at a final concentration of 10mM. Controls in these studies contained potassium phosphate at 3 mM final concentration as the enzyme incubation buffer.

Effect of rNTPs and dinucleoside polyphosphates on DNA polymerase activity

The effect of dinucleoside polyphosphates, namely Gp_2G, Gp_3G, Gp_4G, Gp_3A, Ap_3A and Ap_4A, were tested at 0.1 mM final concentration. ATP or GTP was tested at 1 mM final concentrations.

Ionic requirements

Anions (Cl) as KCl and (PO_4) as potassium phosphate were added to the regular assay mixture. The KCl concentrations ranged from 0.5 to 4.0M and the potassium phosphate was tested at concentrations of 10mM, 50mM and 100mM. The divalent cation Mn^{2+} (as $MnCl_2$) was tested as a substitute for Mg^{2+} regularly used in enzyme assays) at equimolar concentrations (40 mM).

Effect of inhibitors on polymerase activity

The sulfhydryl group inhibitor NEM was used to determine the dependence of Artemia DNA polymerases on the presence of free sulfhydryl groups. In these studies, both the crude enzyme extracts (NF and PMF) and purified DNA polymerase fractions of the cysts and nauplii were subjected to NEM treatment. Each enzyme sample was pre-incubated with NEM in a salt-ice bath mixture. At the end of 30 min, the treated samples were transferred to reaction vessels to give final concentrations of NEM of 1 mM (for crude enzyme samples) and 6 mM (for purified fractions) respectively. The enzymatic reaction was allowed to proceed by incubation at 37°C and at suitable time intervals, the acid-insoluble radioactivity was estimated as described earlier. In other inhibitor studies, the chain terminator inhibitor dideoxy thymidine 5'-triphosphate (d2TTP) was included at a ratio (d2TTP/dTTP) of 4 x10^{-2}. Aphidicolin, an inhibitor of α-DNA polymerase activity, was dissolved in dimethly sulfoxide and tested at a final concentration of 5 ug/ml in the enzyme reaction.

In all studies, suitable controls for each treatment were used.

<u>Sucrose-density gradient analysis of Artemia polymerases</u>

Sucrose-density gradient analyses were performed on fraction 1 (obtained from DEAE-cellulose columnn) from the post-mitochondrial fraction (PMF) of cysts and nauplii to determine the sedimentation coefficients (S values) of the DNA polymerase(s).

Linear sucrose-density gradients of 5-20% were prepared in buffer B (excluding the 15% v/v glycerol) using an automatic gradient former (ISCO model 570, Instrument Specialities Co., Lincoln, Nebraska). The gradients were made in cellulose nitrate tubes and the volume in the tube was 12.6 mls including a layer of glycerol-free buffer B (0.4 ml) placed on top of the sucrose gradient. The samples to be analysed (0.4mls each) were layered under the glycerol-free buffer and centifugation was then carried out in an SW 41 rotor (Beckman) at 39,000 rpm for 22 hours. After centrifugation, fractions of 0.4 ml were collected using an ISCO Model 640 gradient fractionator set at 0.4 mls/min. Individual fractions were assayed for protein content using the Bio-Rad reagent and the enzyme activity was determined using Method II. Bovine serum albumin (4.6S) was added to a separate tube as a marker protein and run under the same experimental conditions. The sedimentation coefficients were calculated by comparing the migration position of the DNA polymerase activity to bovine serum albumin (protein standard) using the following relationship of Martin and Ames (86).

$$\frac{\text{'S'unknown}}{\text{'S'marker}} = \frac{\text{Distance unknown}}{\text{Distance marker}}$$

Estimation of protein content of all fractions

The protein content of all enzyme fractions was measured by the method of Bradford (87). To 0.1 ml of suitably diluted protein samples 5 mls of the dilute Bio-Rad reagent (stock diluted 1:5 in double distilled water) was added, vortexed gently to prevent frothing and allowed to stand for 1 hour. Absorbance measurements were taken at 595 nm and compared to samples containing bovine serum albumin as the protein standard.

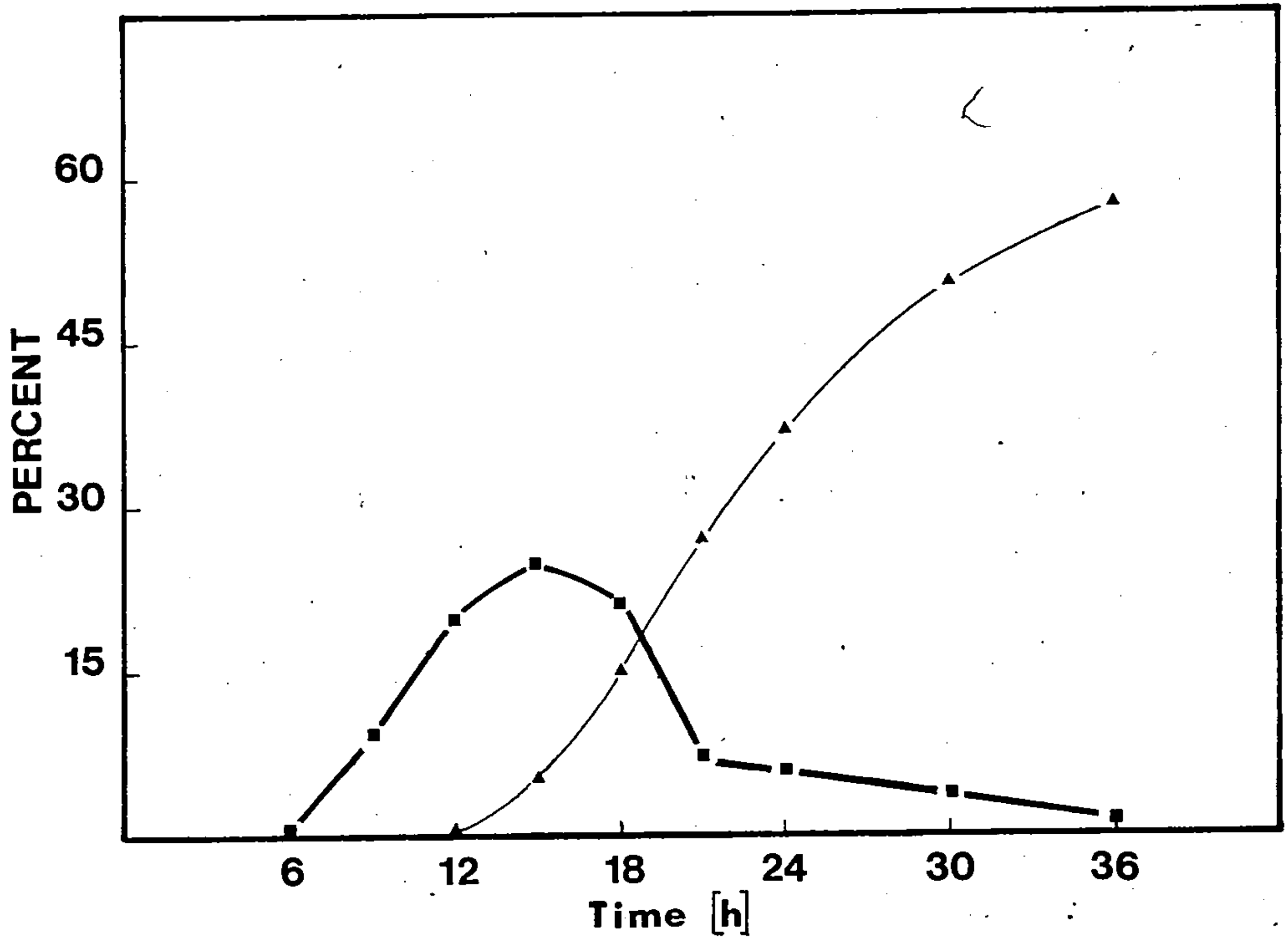
Chapter IV

RESULTS

DNA synthesis and developmental growth of *Artemia sp.*

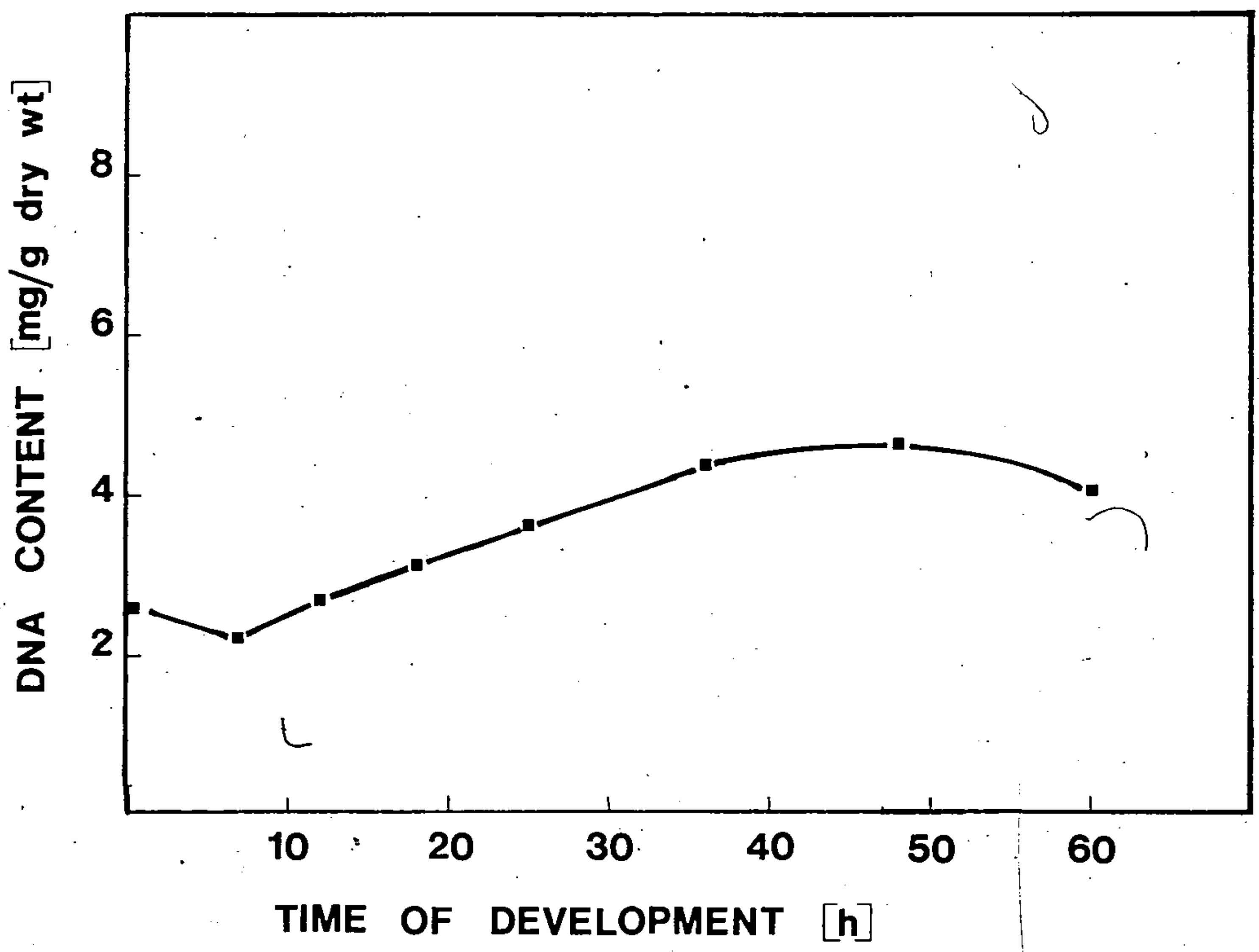
In order to investigate the DNA-dependent DNA polymerase profile at different stages of developmental growth of *Artemia sp.* it was first essential to establish the viability of the San Francisco cysts used in all these studies. The developmental profile of *Artemia* sp. shown in Fig.4 was obtained starting with dormant cysts incubated aerobically at 30°C as described in the Methods. The embryos were scored as cysts, pre-nauplius larvae or emerged embryos (E1 and E2) and free-swimming nauplii. The sum of the unhatched cysts and empty shells was taken as 100%. [No empty shells were found prior to incubation since only fully hydrated cysts with a density greater than 1.0 were used]. Hatching began at about 12h incubation and reached 56% at 36h. To assess the DNA content of *Artemia* sp. during development, measurements of DNA content were made at selected developmental stages as shown in Fig.5. DNA synthesis was initiated around 12 hours incubation reaching a maximum by 48 hours. The total DNA content exhibited nearly a two-fold increase between the cyst and nauplius stage. These data were in accordance with the values obtained by MaClean and Warner (5).

Figure 4: Developmental profile of Artemia sp. The percent nauplii hatched (▲——▲) and embryos emerged (at stages E1 and E2) (■——■) is shown at various times of development. These values were calculated as indicated in the Methods.



PERCENT
60
45
30
15
6
12
18
24
30
36
Time [h]

Figure 5: DNA content with development in <u>Artemia sp.</u> The total embryo DNA content (mg/g dry weight starting cysts) was determined as described in the Methods. Each point is an average of two experiments.


DNA CONTENT [mg/g dry wt]
8
6
4
2
10
20
30
40
50
60
TIME OF DEVELOPMENT [h]

DNA polymerase studies with crude embryo extracts

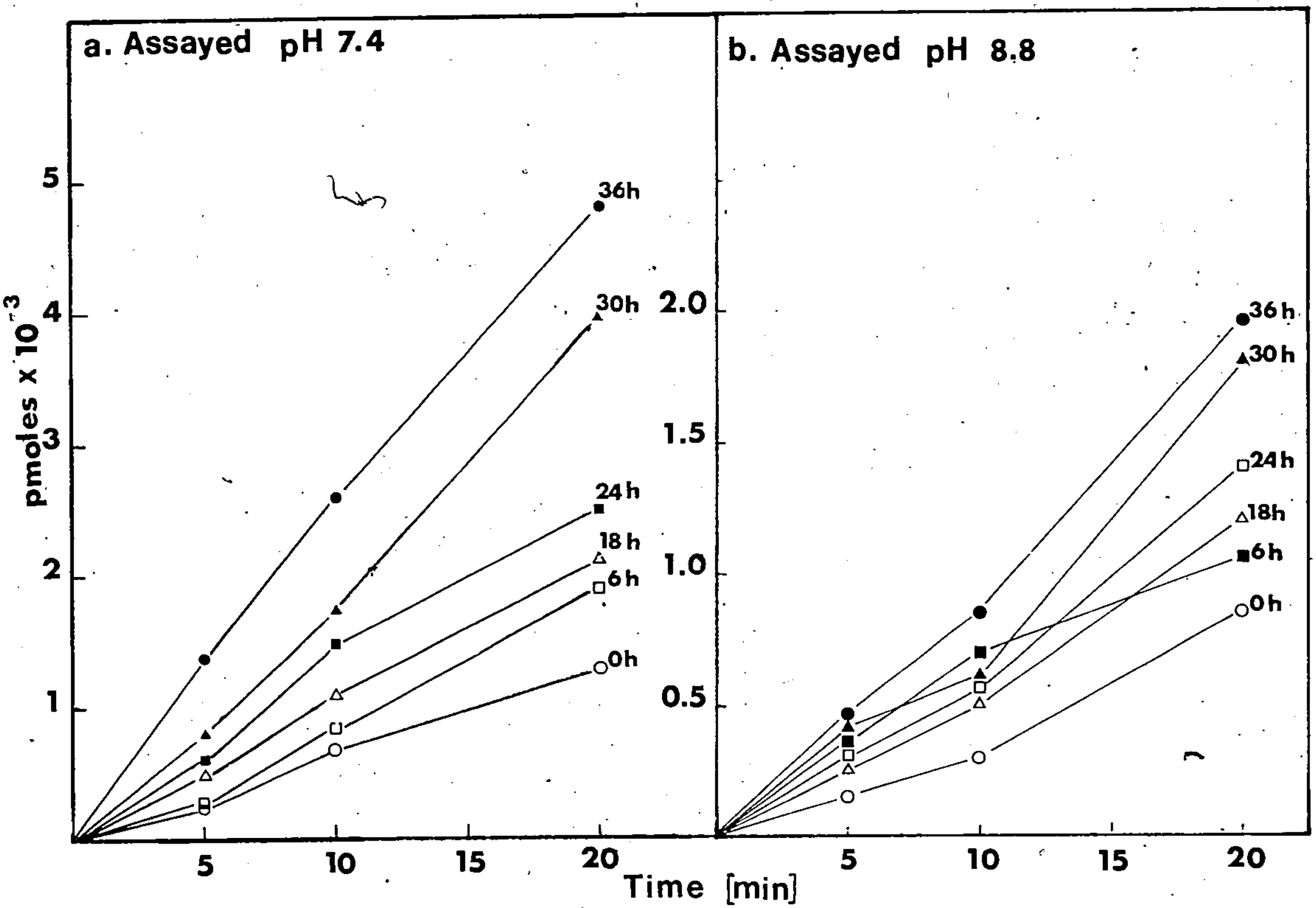
The amount of DNA polymerase activity in *Artemia* was determined initially using crude enzyme preparations from embryos at various developmental stages. The enzyme activity was determined in both the nuclear (NF) and the post-mito chondrial fractions (PMF). One Enzyme Unit (E.U.) in the crude extract is defined as the pmoles of ^{3}H-TMP incorporated as acid-insoluble product in 10 min at 37°C. [The specific activity is obtained by expressing the enzyme units per gram wet weight starting cysts].

When the nuclear fraction (NF) from each developmental group of embryos was assayed at pH 7.4 and 8.8, the data shown in Fig. 6a and 6b were obtained. These data show that the activity of DNA polymerase from the NF is about 2 times higher at pH 7.4 than at pH 8.8 at all stages studied. Similar observations were recorded for the DNA polymerase activity from the cytosol fraction. (see Fig. 7a and 7b).

When the total E.U.'s in the NF and PMF were compared, it was observed that the DNA polymerase activity of the dormant cysts is 2-fold higher in the PMF than in the NF and this observation was independent of the pH of the assay.

Fig.8 represents a summary of the DNA polymerase activity of both the nuclear (NF) and the post-mitochondrial fractions (PMF) between 0h and 36h of development. The lev-

Figure 6: Kinetics of nuclear DNA polymerase activity at pH 7.4 and 8.8. The nuclear fraction (NF) was obtained from embryos at selected stages of development (0-36h). The DNA polymerase activity in each extract was assayed at pH 7.4 (a) and at 8.8 (b) as described in the Methods. These values represent the average of two experiments.



a. Assayed pH 7.4
b. Assayed pH 8.8
pmoles x 10^{-3}
5
4
3
2
1
2.0
1.5
1.0
0.5
Time [min]
5
10
15
20
36h
30h
24h
18h
6h
0h

Figure 7: Kinetics of cytosol DNA polymerase activity at pH 7.4 and 8.8. At selected stages of development (between 0 and 36 h), the cytosol fraction (PMF) was obtained from embryos as described in the Methods. The DNA polymerase activity in each extract was assayed at pH 7.4 (a) and 8.8 (b) as described in the Methods. These values represent the average of two experiments.

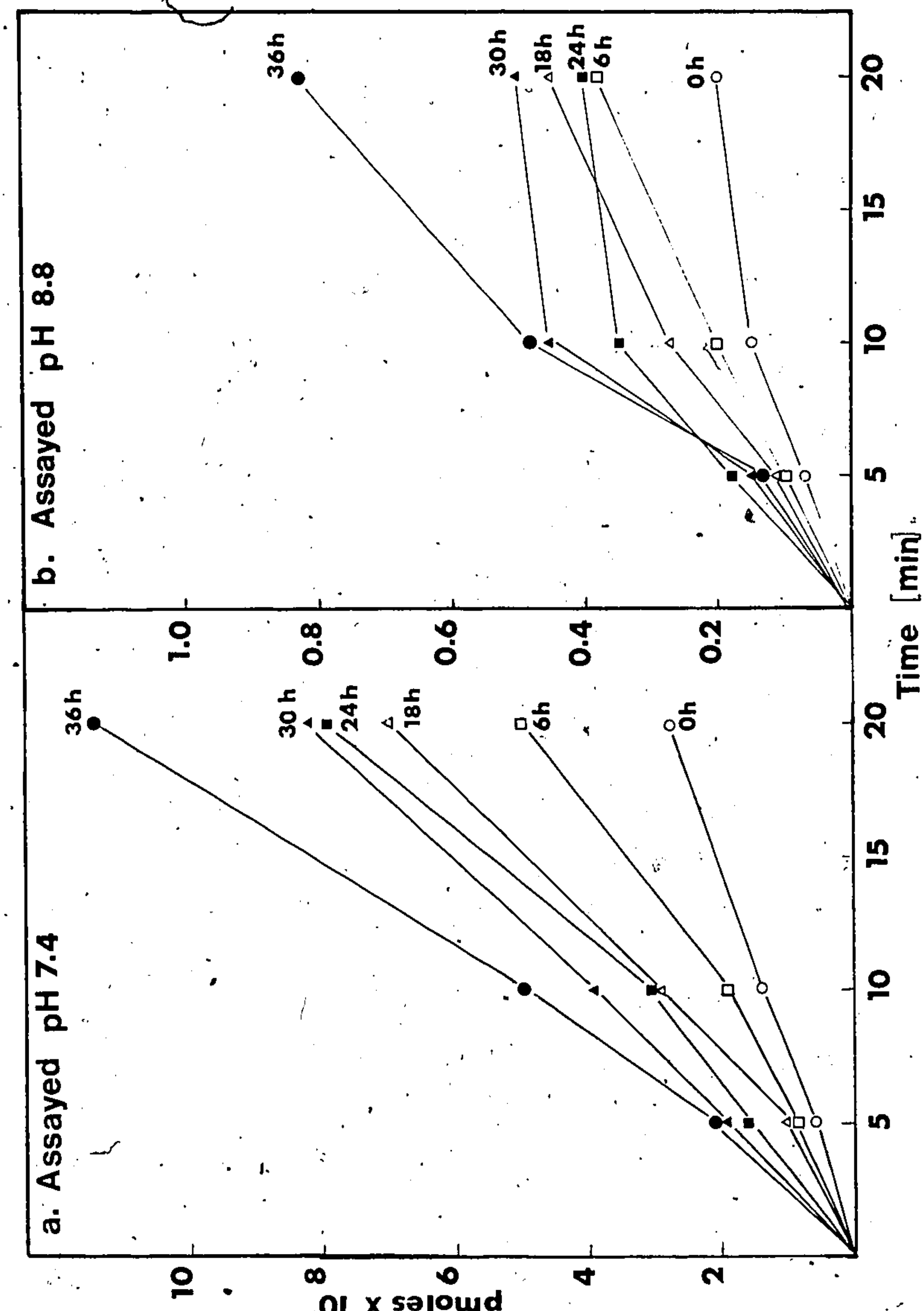
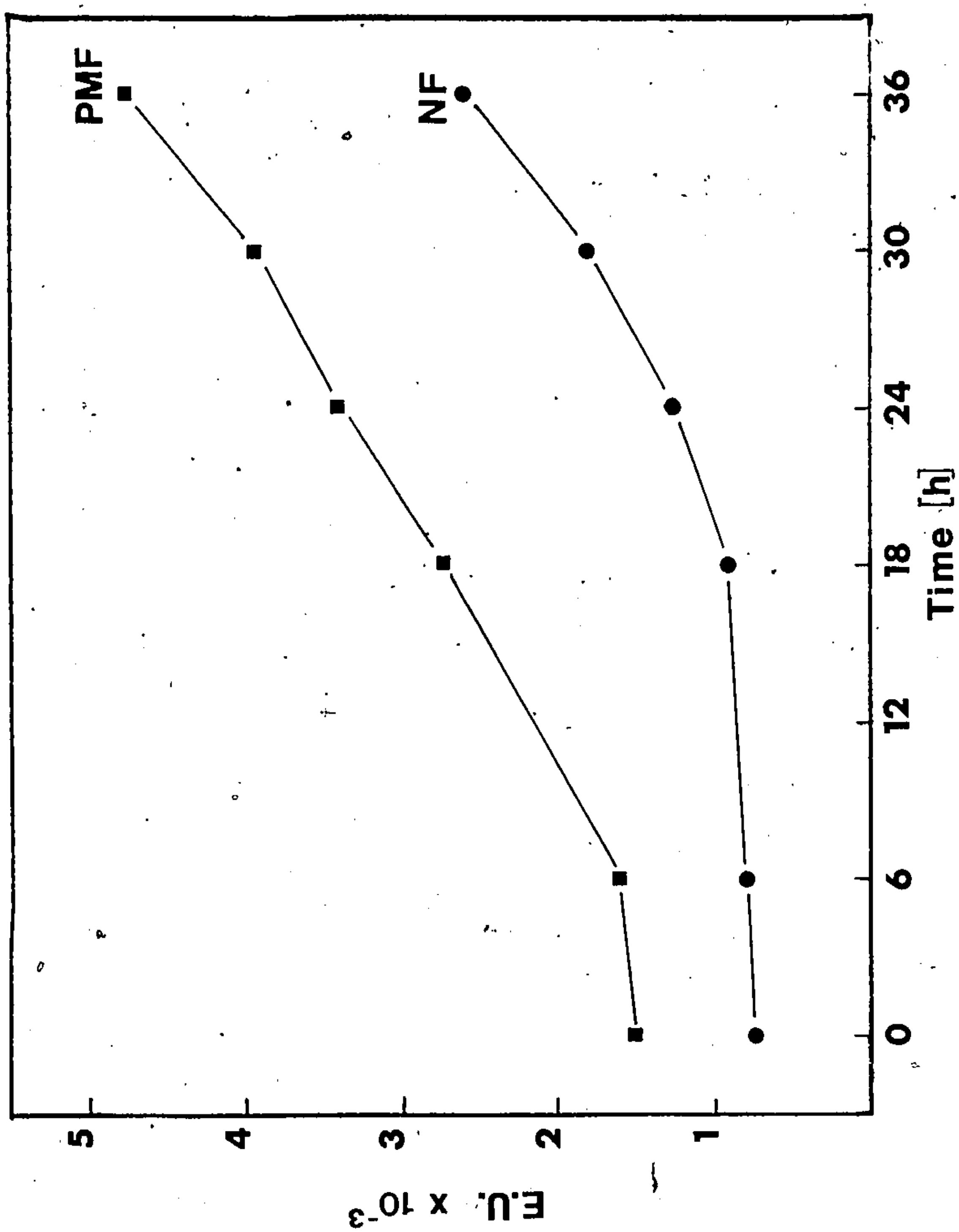

a. Assayed pH 7.4
b. Assayed pH 8.8
pmoles x 10^-3
Time [min]
36h
30 h
24h
18h
6h
0h
36h
30h
18h
24h
6h
0h
10
8
6
4
2
1.0
0.8
0.6
0.4
0.2
5
10
15
20

Figure 8: DNA polymerase activity in the post-mitochondrial (PMF) and nuclear fractions (NF) of developing embryos. The nuclear (NF) and cytosol fractions (PMF) were obtained from embryos at selected stages of development as described in the Methods. The DNA polymerase activity in each extract was assayed at pH 7.4 as described in the Methods. Each point is an average of two experiments.



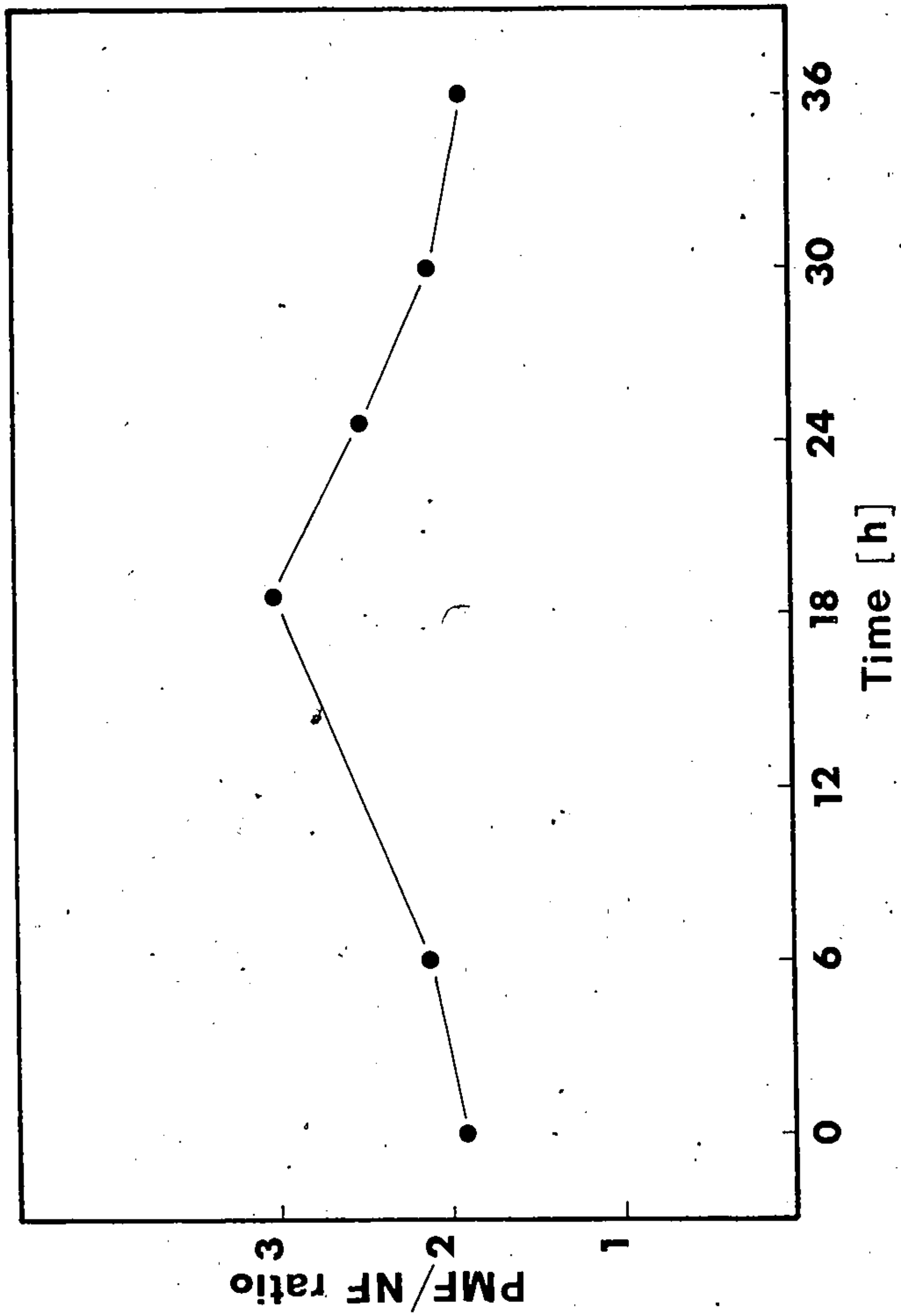
PMF
NF
0
6
12
18
24
30
36
Time [h]
1
2
3
4
5
E.U. x 10⁻³

el of DNA polymerase activity in the NF is relatively constant between 0h and 18h development, after which time it increases at a rate similar to that in the PMF. In contrast to the changes observed in the NF, the activity in the PMF increases soon after immersion of dormant cysts in hatch medium (at 30°C). The increase in DNA polymerase activity in NF between 18 and 36 hours of development correlates well with the mitotic activity observed by other workers (88-89).

The relative distribution of DNA polymerase activity between the nuclear and cytosol fractions is shown in Fig.9. These data show that the ratio of DNA polymerase activity in the PMF to NF is initially 1.9. This increases to 3.1 at the onset of nuclear division, then returns to a level of 1.8 following cessation of DNA synthesis (36-40 hours). Since DNA polymerase in the PMF of the nauplii (36h) exhibits significantly higher levels of activity than the enzyme from early embryos, subsequent studies on the characterization of larval DNA polymerase were confined to these embryos and compared to DNA polymerase partially purified from dormant cysts.

To exclude the possibility that increased enzyme activity in the post-mitochondrial and nuclear fractions of developing embryos is due to an artifact of hydration, DNA polymerase measurements were made from embryos incubated at 0-4°C. The results of these experiments are shown in

Figure 9: Ratio of DNA polymerase activity in post-mitochondrial (PMF) and nuclear fractions (NF) during development of *Artemia*. The data in Fig 8. was used to calculate the ratio of DNA polymerase activity in the post-mitochondrial and nuclear fractions at different times of development. Only the values obtained by assay at pH 7.4 were used in these calculations.



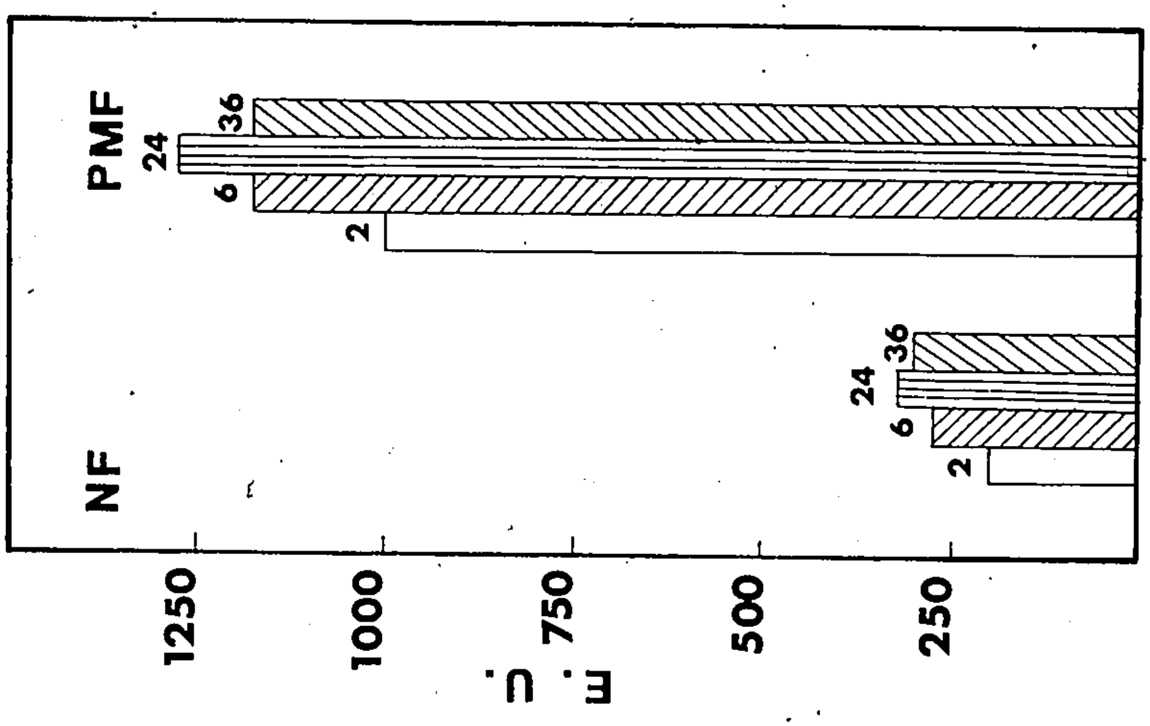
PMF/NF ratio
1
2
3
0
6
12
18
24
30
36
Time [h]

Fig.10. Dormant cysts (2 gms, wet weight) were hydrated at 0°C for up to 36 hours, the NF and PMF were obtained as usual and the DNA polymerase activity analysed in the standard polymerase assay. The results showed that the enzyme activity in both fractions increased only slightly (less than 15%) even after 24-36 hours of hydration, which was considerably less than that observed using embryos incubated at 30°C for similar periods of time.

Stability of the crude embryo extract

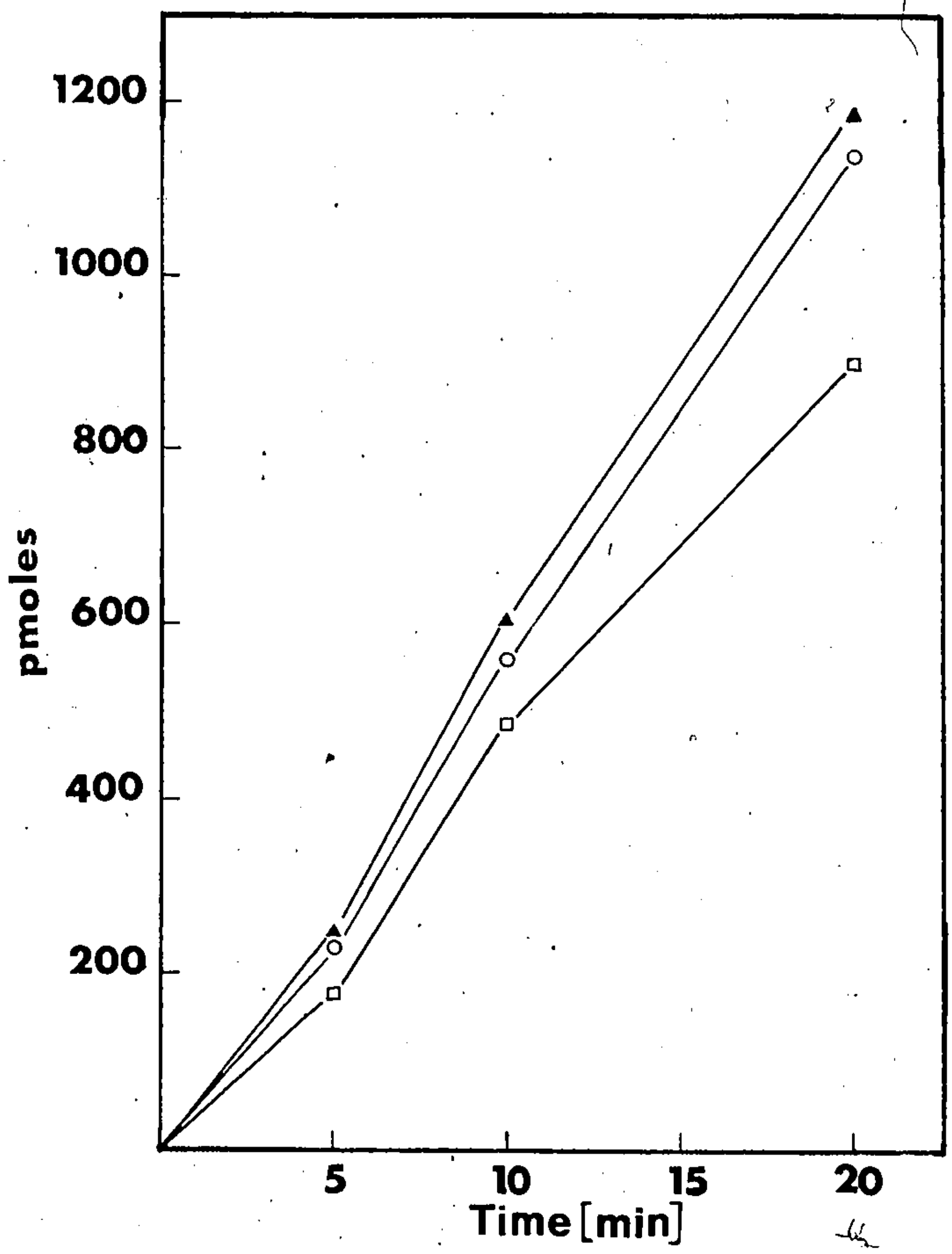
Prior to experiments on the purification of the DNA polymerase, it was necessary to establish the stability of crude extracts to various routine purification treatments, especially the extracts from 36h embryos which contain significant protease activity (88-90). The effect of regular dialysis on DNA polymerase activity was studied using extracts from the nuclear fraction (NF) and the results of these experiments are shown in Fig.11. These results show that dialysis for 12 hours resulted in a 21% decrease in enzyme activity. Similar findings were observed with extracts from the post-mitochondrial fraction (PMF).

Figure 10: Effect of varying periods of cyst hydration on DNA polymerase isolation and activity measurement. Dormant cysts were hydrated in 0.5M NaCl at $0^{\circ}C$ for the times indicated, then processed for the isolation of DNA polymerase as described in the Methods.



NF
PMF
2
6
24
36
1250
1000
750
500
250
E. U.

Figure 11: The effect of dialysis on 36h nuclear DNA polymerase activity. The nuclear DNA polymerase fraction from 36 h nauplii was concentrated by pressure dialysis and the activity measured prior to (▲——▲) and after 6 h (○——○) and 12 h (□——□) of regular dialysis. The enzyme activity was measured as described in the Methods.



1200
1000
800
600
400
200
pmoles
5
10
15
20
Time [min]

Purification of the DNA polymerase from cysts and nauplii

In order to study the activity and subcellular distribution of DNA polymerase between the nuclear (NF) and post-mitochondrial fractions (PMF) of the dormant cyst (0h) and nauplii (36h), and to characterize this enzyme in Artemia embryos enzyme(s) were isolated and purified as described below. All treatments were carried out at 0-4°C unless otherwise stated.

Processing of the post-mitochondrial supernatant

Twenty grams (wet weight) of 36h nauplii (separated by their photo-tactic response) were ground in small portions in the Duall-type glass tissue grinder with homogenization buffer in a total volume of 35 mls. For dormant cysts, an electric mortar (Torsion, model MG-2) was used for homogenization purposes. The nuclear pellet obtained by centrifugation for 15 min at 1000 x g was stored at -70°C and processed separately. The post- mitochondrial fraction which is rich in proteases, was processed immediately. Following concentration of the PMF under N_2 pressure (Diaflo membrane, PM-10) at 60 psi, the concentrate (8mls volume) was filtered through a Sephadex G-25 column (3.5 x 40 cms) using buffer A as the eluent. The UV-absorbing material which eluted in the void volume was retained and concentrated by pressure dialysis to approximately 4mls. The DNA polymerase enzyme was purified further by gel filtration on a column of Ultrogel AcA-34 (2.0 x 52 cms) previously equilibrated with buffer A and eluted with the same buffer.

The contents of the active enzyme samples from Ultrogel AcA-34 were pooled and applied to a DEAE-cellulose column (2.5 x 40 cms) equilibrated with buffer B as described by Warner and Finamore (64). The bound polymerase was eluted using a 440 ml linear gradient of KCl to a final concentration of 1 molar (buffer C). Each column fraction was analysed for UV-absorbance and enzyme activity as described above. Column fractions showing enzyme activity were pooled, concentrated by pressure dialysis and the concentrate dialysed against buffer B for 6 hours. The salt-free concentrates were dispensed into aliquots of 0.5 mls and stored at -70°C.

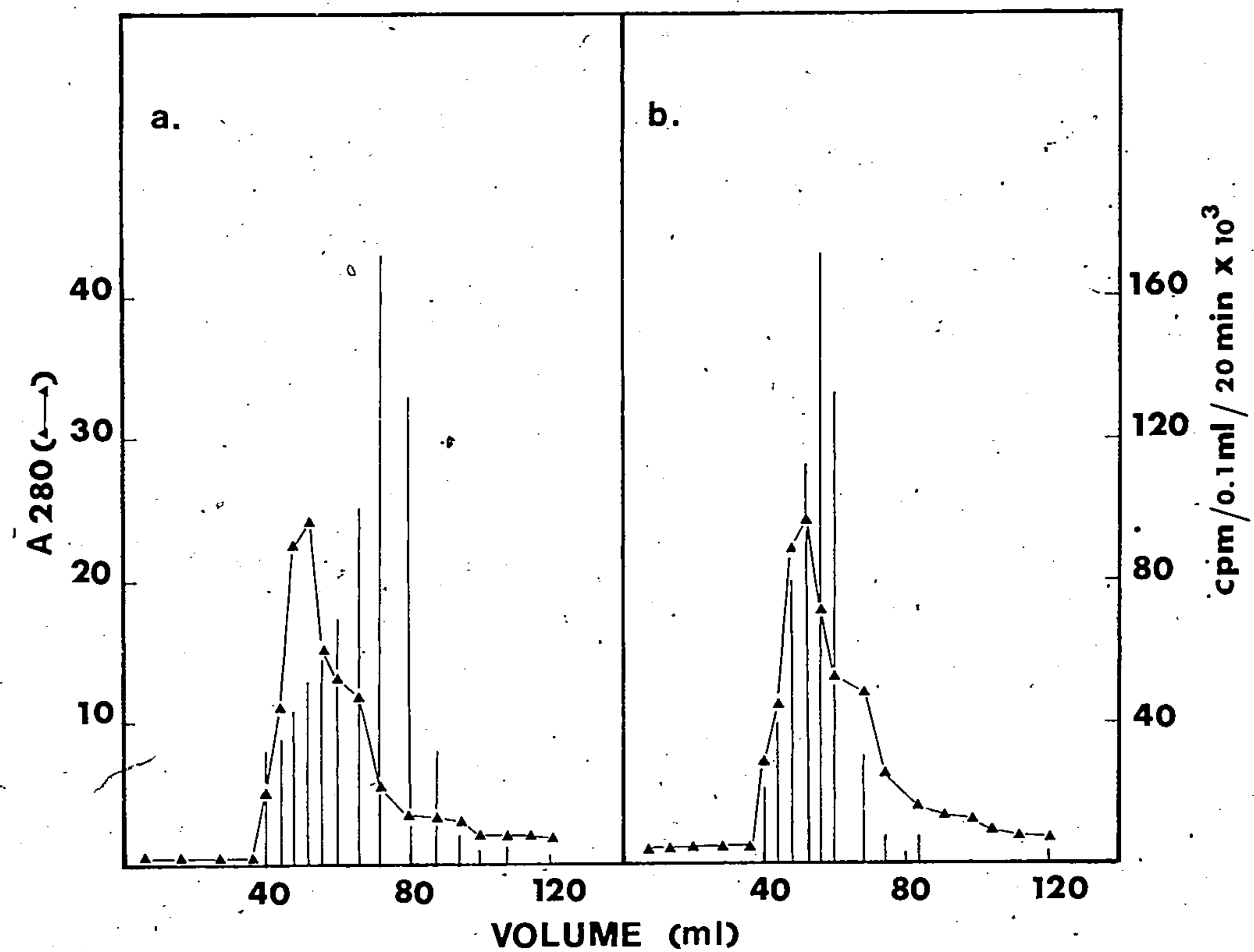
In subsequent studies, the enzyme preparations were frozen and thawed not more than twice. It should be noted that *Artemia* DNA polymerases are very unstable at 0-4°C, therefore all procedures were carried out as rapidly as possible. *Artemia* DNA polymerase fractions requiring storage for two weeks or longer were stored in 50% glycerol at -70°C. This treatment ensured minimal loss in activity for up to 2 months. The purification procedure outlined above was followed for both the nuclear and cytosol enzymes from 0h cysts and 36h nauplii.

Processing of nuclear pellet samples.

The nuclear pellet fractions stored at -70°C were diluted (1:5) with homogenization buffer. The diluted sample was then treated with the non-ionic detergent Nonidet P40 to a final concentration of 0.1% and incubated with vigorous stirring for exactly 30 min, in a salt-ice bath, this treatment was found to be adequate in breaking open the nuclei (as visualised under a phase-contrast microscope). The supernatant fluid was obtained by centrifugation, carefully freed of the floating lipid layer and dialysed (to remove the detergent) against buffer A for 6 hours. The dialysate from the processed nuclear pellet was concentrated by pressure dialysis and applied to a column of Sephadex G-25 as the initial step in the purification procedure described earlier with the cytosol fraction.

Since several reports have indicated the presence of high levels of proteases in developed Artemia embryos (88-90) soybean trypsin inhibitor (STI), was added to the homogenization buffer at either 100 ug/ml or 400 ug/ml final concentrations. The profile of the cytosol enzyme from the Ultrogel AcA-34 columns is shown in Fig.12. In all cases, the DNA polymerase containing fractions eluted immediately behind the main protein peak, but the extract containing 400 ug/ml STI eluted from the column as a sharper peak and closer to the main protein peak than extracts prepared with 100 ug/ml STI. [Compare Fig. 12a and 12b].

Figure 12: Effect of soybean trypsin inhibitor on the gel filtration properties of DNA polymerase from 36h nauplii. The UV-absorbing material in the void volume from a Sephadex G-25 column was pooled and concentrated by pressure dialysis to less than 5 mls. The concentrate was applied to a column of Ultrogel AcA-34 (2.0 x 52 cms) previously equilibrated with buffer A and eluted with the same buffer at a flow rate of approximately 15 mls/h. Fractions of 3.0 mls were collected and assayed for UV-absorbance at 280 nm (▲——▲) and for DNA polymerase activity (bars) as described in the Methods. The active fractions were pooled and saved for further purification on DEAE-cellulose. a) DNA polymerase activity when the homogenization buffer contained soybean trypsin inhibitor (STI) at a final concentration of 100 ug/ml. b) DNA polymerase activity when STI concentration was increased to give a final concentration of 400 ug/ml in the homogenization buffer.


a.
b.
A 280 (▲—▲)
40
30
20
10
cpm/0.1ml / 20 min X 10^3
160
120
80
40
40
80
120
40
80
120
VOLUME (ml)

This shift in the elution pattern is probably the result of inhibition of proteases by the additional STI. Also, since the protease activity in the first 100 mls was found to be sufficiently inhibited when STI was included in the homogenization buffer at 400 ug/ml (Fig.13), all subsequent purification experiments contained 400 ug/ml STI in the homogenization buffer.

Ultrogel AcA-34 filtration

The nuclear (NF) and post-mitochondrial fractions (PMF) from 0h cysts and 36h nauplii were filtered through separate Ultrogel AcA-34 columns. A representative elution profile of cyst (0h) and nauplii (36h) cytosol (PMF) DNA polymerases on Ultrogel AcA-34 is shown in Fig. 14a and 14b, respectively. The elution pattern of the dormant cyst cytosol DNA polymerase suggests that this enzyme has a lower molecular weight than DNA polymerase from the cytosol fraction of nauplii. The active fractions from AcA-34 (NF and PMF of the 0h and 36h embryos) were pooled and each was applied to separate columns of DEAE-cellulose for subsequent purification.

Figure 13: The effect of soybean trypsin inhibitor on DNA polymerase activity. Additional soybean trypsin inhibitor (STI) was added at 160ug/ml final concentration to a reaction mixture containing DNA polymerase from the PMF of 36h nauplii. Control samples (▭) contained STI (120ug/ml) present in the homogenization buffer. The assay was carried out as described in the Methods. PMF, post-mitochondrial fraction; Pr.conc, after concentration by pressure dialysis; G-25, DNA polymerase fraction from Sephadex G-25; AcA-34, DNA polymerase fraction from Ultrogel AcA-34.

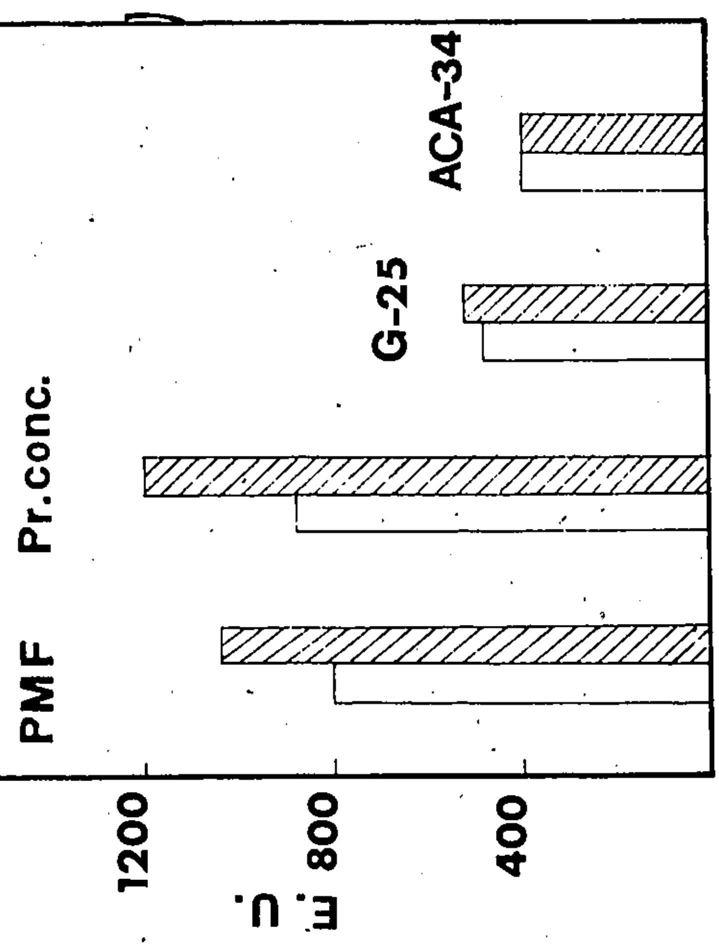
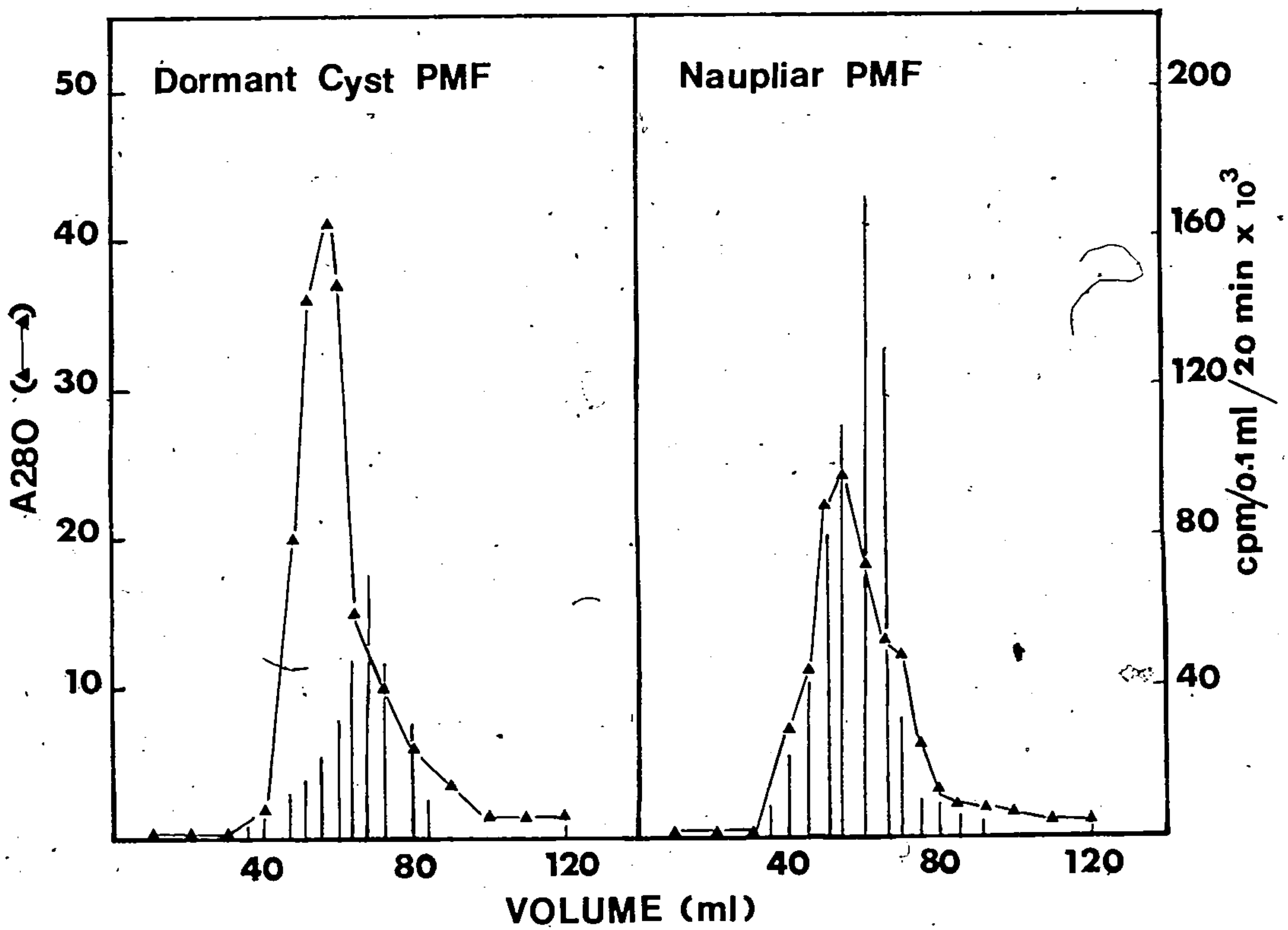

PMF
Pr.conc.
G-25
ACA-34
E. U.
1200
800
400

Figure 14: Chromatography of the post-mitochondrial fraction from 0h cysts and 36h nauplii on Ultrogel AcA-34. The UV-absorbing material in the void volume from a Sephadex G-25 column was pooled and concentrated by pressure dialysis to less than 5 mls. The concentrate was applied to a column of Ultrogel AcA-34 (2.0 x 52 cms) previously equilibrated with buffer A and eluted with the same buffer at a flow rate of approximately 15 mls/h. Fractions of 3.0mls were collected and assayed for DNA polymerase activity (bars) and for UV-absorbance at 280 nm (▲——▲) as described in the Methods. The active fractions were pooled and saved for further purification on DEAE-cellulose. The nuclear (NF) and post-mitochondrial fractions (PMF) of 0h cyst and 36h nauplii were subjected to gel filtration on Ultrogel AcA-34 similarly.



Dormant Cyst PMF
Naupliar PMF
A280 (▲—▲)
50
40
30
20
10
40
80
120
40
80
120
VOLUME (ml)
200
160
120
80
40
cpm/0.1 ml / 20 min x 10^3

DEAE-Cellulose chromatography

Chromatography on DEAE-cellulose of the nuclear and post-mito chondrial fractions of the dormant cyst (0h) and nauplii (36h) following filtration through Ultrogel AcA-34 is shown in Fig.15. The enzyme from the PMF of the dormant cysts eluted as two distinct peaks at 0.2M and 0.38M KCl (Fig.15b). These fractions have been lebelled F1 and F2, respectively. In contrast the nuclear enzyme eluted as a single peak at the position of Fraction 2 (see Fig.15a). On the other hand, the DNA polymerase activity in both the cytosol (PMF) and nuclear fractions (NF) from 36h nauplii eluted as two separate fractions (see Fig. 15c and 15d), at positions in the KCl gradient equivalent to that of F1 and F2 from the dormant cysts PMF. Each enzyme fraction was pooled separately and stored at -70°C as described earlier. Table 1 summarizes the DNA polymerase activity after each step in the purification procedure.

Stability of purified enzymes during storage

The stability of the DEAE-purified cytosol enzyme fraction 1 (PMF-1) from 36h embryos was studied under various storage conditions (F ig.16). The activity decreased by 35% when the enzyme was stored overnight at 0-4°C as compared to storage at -70°C. Storage at -10°C for prolonged periods of time (up to a week) led to a loss in enzyme activity by over 80% (data not included). When the

Figure 15: (a-d). Chromatography of nuclear and post-mitochondrial DNA polymerases from 0h cysts and 36h nauplii on columns of DEAE-cellulose. The fractions from Ultrogel AcA-34 which contained DNA polymerase activity were pooled and applied to separate columns of DEAE-cellulose 32 (2.5 x 40cms) previously equilibrated with buffer B. The columns were eluted using a linear gradient of KCl in buffer B (to 1M KCl) at a flow rate of approximately 30 mls/h and fractions of 5 mls were collected. Column fractions were assayed for absorbance at 280 nm (▲——▲) and for DNA polymerase activity (bars) as described in the Methods. Fraction 1 and Fraction 2 (as indicated) were pooled separately, concentrated (by pressure dialysis), dialysed against buffer B and stored at $-70^{\circ}C$ for further studies. It should be noted that the 0h nuclear enzyme eluted as a single, complex peak at the position of Fraction 2 from the PMF.

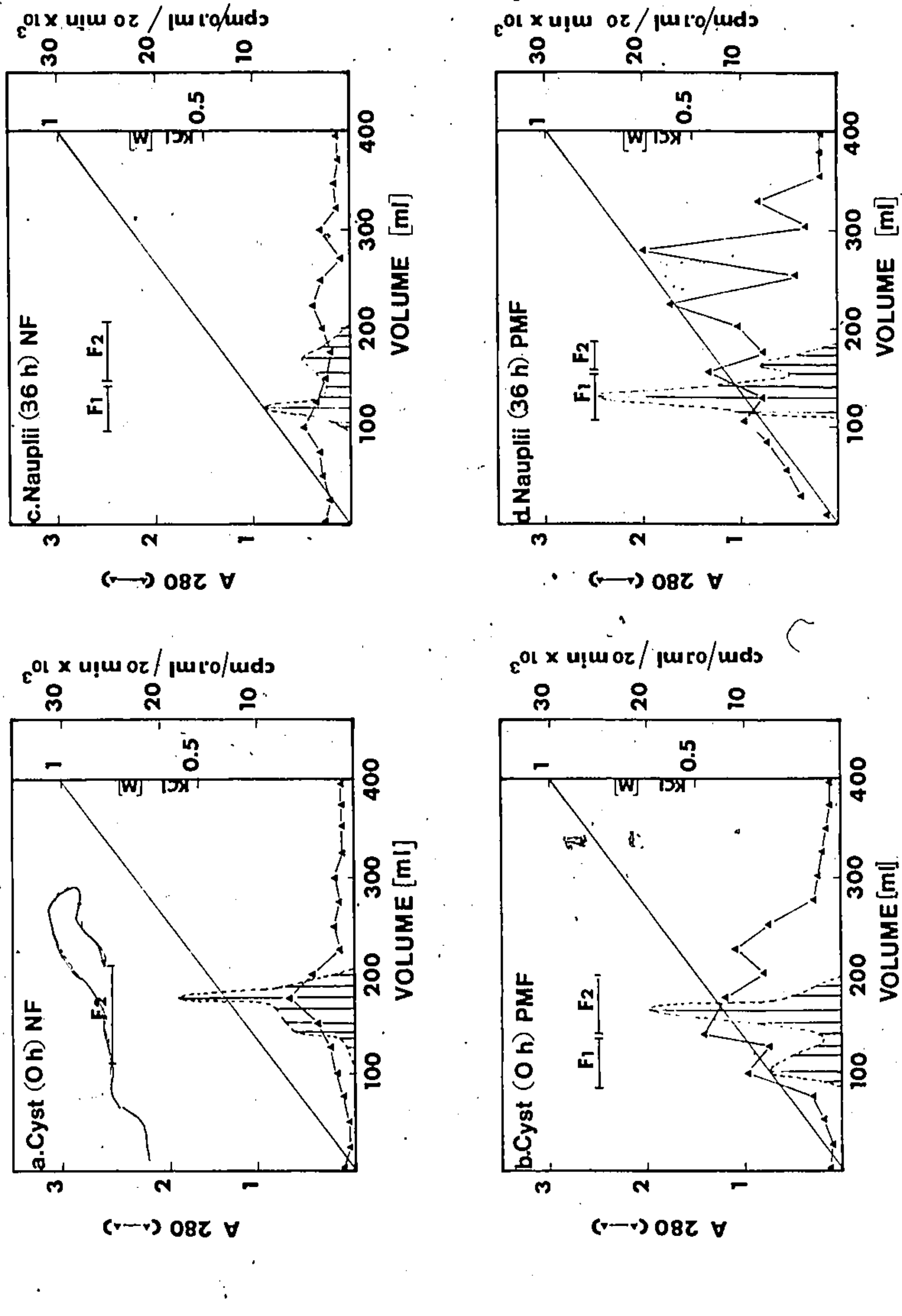

a.Cyst (O h) NF
b.Cyst (O h) PMF
c.Nauplii (36 h) NF
d.Nauplii (36 h) PMF
F1
F2
A 280
cpm/0.1 ml / 20 min x 10³
KCl [M]
VOLUME [ml]

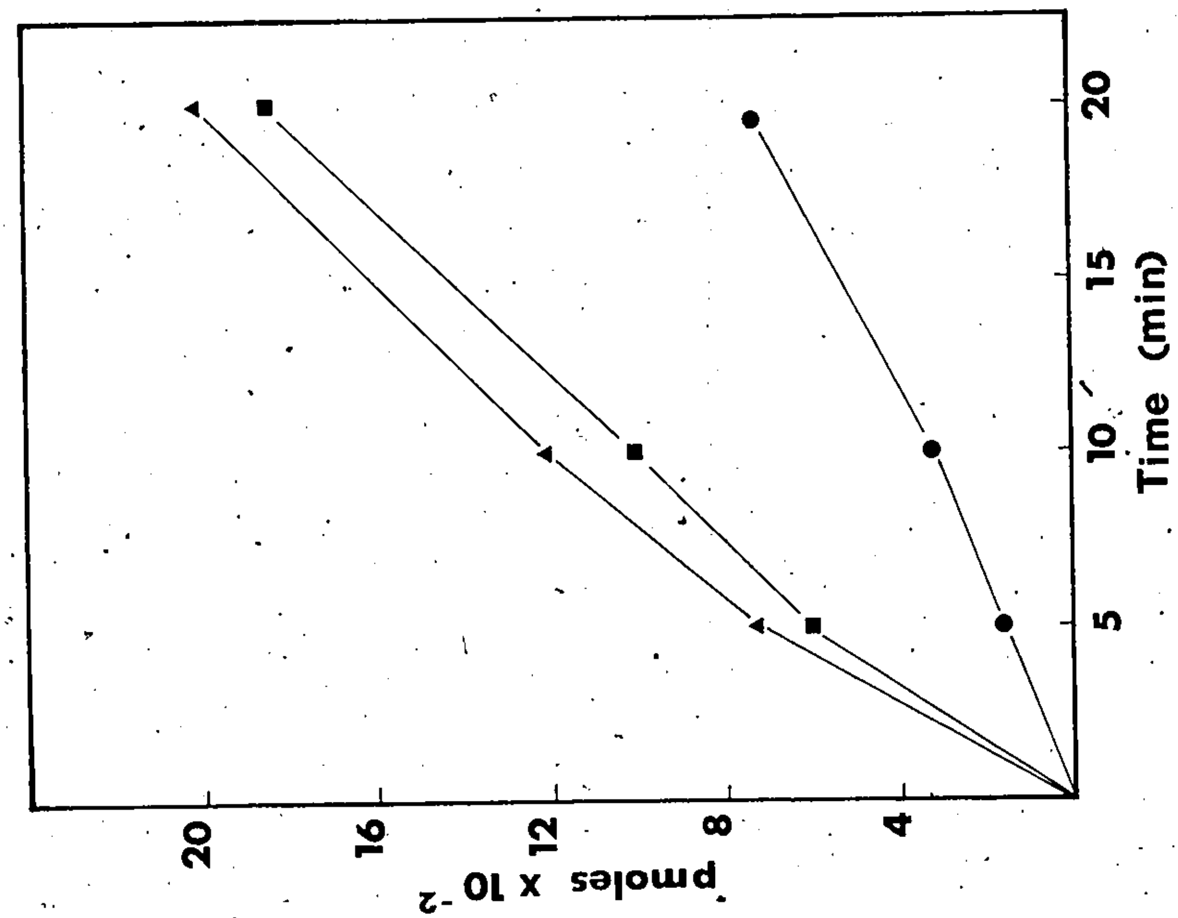
Table - 1

SUMMARY OF DNA POLYMERASE PURIFICATION EXPERIMENTS

Step	Sample Treatment	Enzyme Units[a] (pmoles/ml/10')	Total Volume (mls)	Total Protein (mgs)	Total Activity	Specific Activity E.U/mg protein	Yield (%)
0 h Dormant Cysts							
1.	PMF	17,985	22.0	294.0	395,674	1,346	100.0
2.	Sephadex G-25	5,188	19.0	64.0	98,566	1,540	25.0
3.	Ultrogel ACA-34	1,953	42.0	32.0	82,033	2,564	21.0
4.	DEAE - F1	757	3.0	3.2	2,272	710	5.2
5.	DEAE - F2	4,081	4.5	11.0	18,363	1,669	
0 h Dormant Cysts							
1.	NF	11,877	30.0	443.0	356,309	804	100.0
2.	Sephadex G-25	5,181	20.0	77.0	103,617	1,346	29.0
3.	Ultrogel ACA-34	1,679	31.0	32.0	52,059	1,627	15.0
4.	DEAE - F 2	3,606	4.0	3.5	14,424	4,121	4.1
36h Nauplii							
1.	PMF	20,001	28.0	249.0	560,028	2,249	100.0
2.	Sephadex G-25	16,838	26.5	144.0	446,201	3,099	80.0
3.	Ultrogel ACA-34	7,018	55.2	104.0	387,412	3,725	69.0
4.	DEAE - F1	2,738	5.1	4.6	13,966	1,838	6.1
5.	DEAE - F2	4,110	4.9	3.4	20,064	2,135	
36h Nauplii							
1.	NF	13,989	25.0	404.0	349,717	866	100.0
2.	Sephadex G-25	11,206	20.0	168.0	224,110	1,334	64.0
3.	Ultrogel ACA-34	5,125	42.0	108.0	215,264	1,993	62.0
4.	DEAE - F1	184	6.5	5.6	1,193	213	1.7
5.	DEAE - F2	878	5.5	4.5	4,831	1,074	

a - Enzyme Unit = The pmoles of ^{3}H-TMP incorporated as acid-insoluble product in 10 minutes at 37° C.

Figure 16: Stability of *Artemia* DNA polymerase under different storage conditions. The DEAE-cellulose purified DNA polymerases from 36h embryos (PMF-1) was stored overnight at $-70^{\circ}C$ (▲——▲), at $-10^{\circ}C$ in a regular freezer (■——■) and at $0-4^{\circ}C$, in an ice bath kept in the cold room (●——●) Aliquots of each enzyme preparation were then assayed as described in the Methods.



pmoles x 10 -2
20
16
12
8
4
5
10
15
20
Time (min)

same enzyme preparation was subjected to five freeze-thaw cycles from storage at -70°C the enzyme activity decreased by 53% (see Fig.17). Thus due to the instability of the enzyme small aliquots were stored at -70°C and none were subjected to more than two freeze-thaw cycles before being discarded.

Studies with partially purified DNA polymerase fractions

To characterise the DEAE-purified nuclear (NF) and cytosol (PMF) fractions from 0h cysts and 36h nauplii, the following experiments were carried out. The first was to establish the requirement of a total reaction assay mixture for the purified fractions as indicated in Table 2.

The enzyme shows an absolute requirement for a divalent cation (Mg^{2+}), a DNA-template (activated DNA) and all four deoxy nucleoside 5'-triphosphates. Reaction vessels with only one deoxynucleoside 5'-triphosphate present 26% to 53% lower enzyme activity than the complete reaction mixture. Omission of activated DNA primer-template resulted in over 95% loss in enzyme activity. This also indicates the absence of terminal deoxynucleotidyl transferase (TdT) activity which is usually present in association with mammalian DNA polymerases. Interestingly, the exclusion of dGTP alone (as compared to other dNTP's from the reaction mixture caused a loss in activity of 56%. Deletion of β-mercaptoethanol (βME) reduced the activity of the enzyme nearly 50% indicating a requirement for free sulfhydryl groups.

Figure 17: The effect of freeze-thawing on DNA polymerase activity. The DEAE-cellulose purified DNA polymerase (PMF-1) from 36h embryos was stored at -70°C and subjected to five freeze-thaw cycles over a period of five days. The enzyme activity was measured after each cycle using 50 ul aliquots in the regular enzyme assay as described in the Methods.

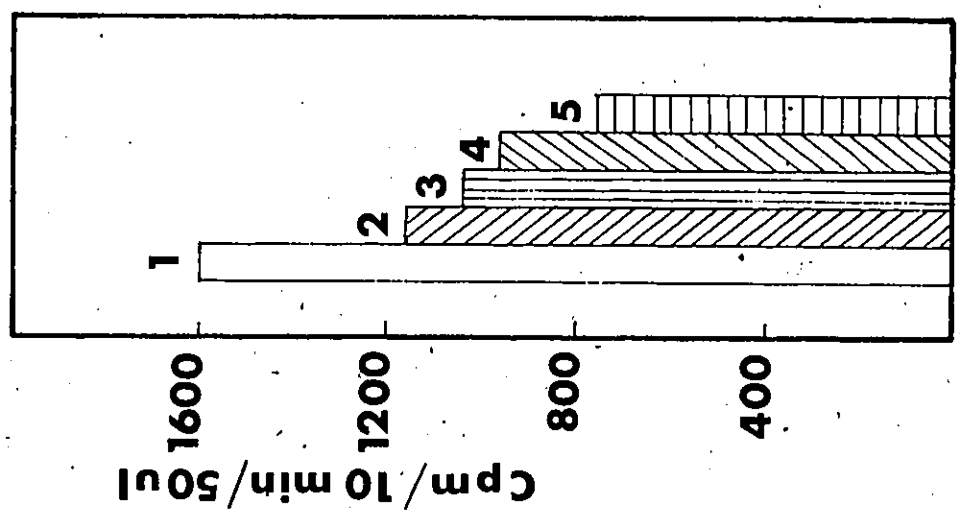

Cpm/10 min/50ul
1600
1200
800
400
1
2
3
4
5

TABLE 2

Requirements of a total DNA polymerase enzyme reaction assay mixture.

Reaction Mixture	Enzyme Units (pmoles/10')	Percent Activity remaining
Complete	1255	100
minus Activated Calf-thymus DNA	55	5
minus Mg	115	9
minus BME	590	47
minus dATP	670	53
minus dCTP	615	49
minus dGTP	555	44
minus dATP, dCTP, & dGTP	330	26
minus enzyme [a]	nil	nil

a : The DNA polymerase preparation used in this experiment was Fraction 1 of the cytosol from DEAE-cellulose of the 0h dormant cyst embryos.

Before further characterizations of the DNA-dependent DNA polymerase enzyme fractions could be carried out, it was necessary to determine the chemical nature of the end-product of the enzyme reaction. This was accomplished using RNase and DNase treatments at 10 units/ml and 40 ug/ml final concentrations, respectively. In one set of experiments the DNA polymerase reaction was allowed to proceed for 20 min in the complete reaction mixture then DNase was added and the incubation was continued at 37°C for a further period of 15 min. Similar experiments were conducted using RNases. The acid-insoluble precipitate was recovered and processed as described in the Methods. The results in Table 3 show that the product of the reaction is sensitive to DNase treatment and only partially to RNase treatment. Also, pre-treatment of the reaction components with RNase A resulted in no loss of enzyme activity. These results suggest that the purified enzyme fractions are DNA-dependent and synthesize product(s) which is(are) sensitive to DNase.

<u>Biochemical characterization of *Artemia* DNA polymerases</u>

The preliminary studies detailed above were essential to establish reliable assay procedures and other requirements for the estimation of the DNA polymerase activity of *Artemia* embryos. In order to study the various DNA polymerase enzyme fractions obtained by DEAE-cellulose chromatogra-

TABLE 3

Effect of RNase and DNase on Primer and Product of *Artemia* DNA Polymerase Activity

Sample Treatment	Specific Activity (E.U./mg Protein)	Percent Loss in Activity
Control	96	NIL
Pre-treatment		
+ RNase A	95	NIL
Post-treatment[a]		
+ DNase (40 ug/ml)	7	93
+ RNase A (10 units/ml)	78	19
+ RNase T1 (10 units/ml)	69	28

[a] These samples were treated at the end of 20 min incubation for comparison with untreated and pre-treated samples.

phy [namely, dormant cyst (0h) PMF-1, PMF-2 and NF-2; and naupliar (36h) PMF-1, PMF-2 and NF-1, NF-2] several biochemical experiments were carried out.

Effect of pH and various buffers

The effect of pH and various buffers on the activity of the nuclear (NF) and cytosol (PMF) fractions from cyst and naupliar embryos is shown in Fig.18 and 19, respectively. The buffers used were potassium phosphate, Tris-HCl at a final concentration of 10 mM each in the reaction mixture. The cytosol fraction PMF-1 showed a pH optimum of 8.0 (see Fig.18), whereas PMF-2 and NF-2 purified from 0h cyst embryos indicated a pH optimum more towards neutral pH, i.e. between pH 7.0 and pH 7.5. Both cytosol fractions (PMF-1 and PMF-2) were not inhibited by Tris-HCl, whereas the nuclear fraction (NF-2) was inhibited up to 50% by Tris-HCl irrespective of pH.
The naupliar enzyme fractions gave results shown in Fig.19 when assayed under similar conditions. All fractions (PMF-1,2 and NF-1,2) showed a marked preference for pH 7.5 in both potassium phosphate and Tris-HCl buffers. However, the activity of these fractions, was reduced considerably (25 - 50%) in the presence of Tris-HCl. This is in marked contrast to the results obtained using enzyme fractions from dormant cysts which showed a preference for Tris-HCl buffer (see Fig.18).

Figure 18: The effect of pH and various buffers on 0h cyst DNA polymerase activity. The partially purified DNA polymerase fractions (1 and 2) from the PMF fraction and DNA polymerase (fraction 2) from the NF fraction of 0h cysts were assayed using 10mM potassium phosphate (●——●), 10 mM Tris-HCl, (37°C) (▲——▲)as indicated.

Each value is an average of two experiments.

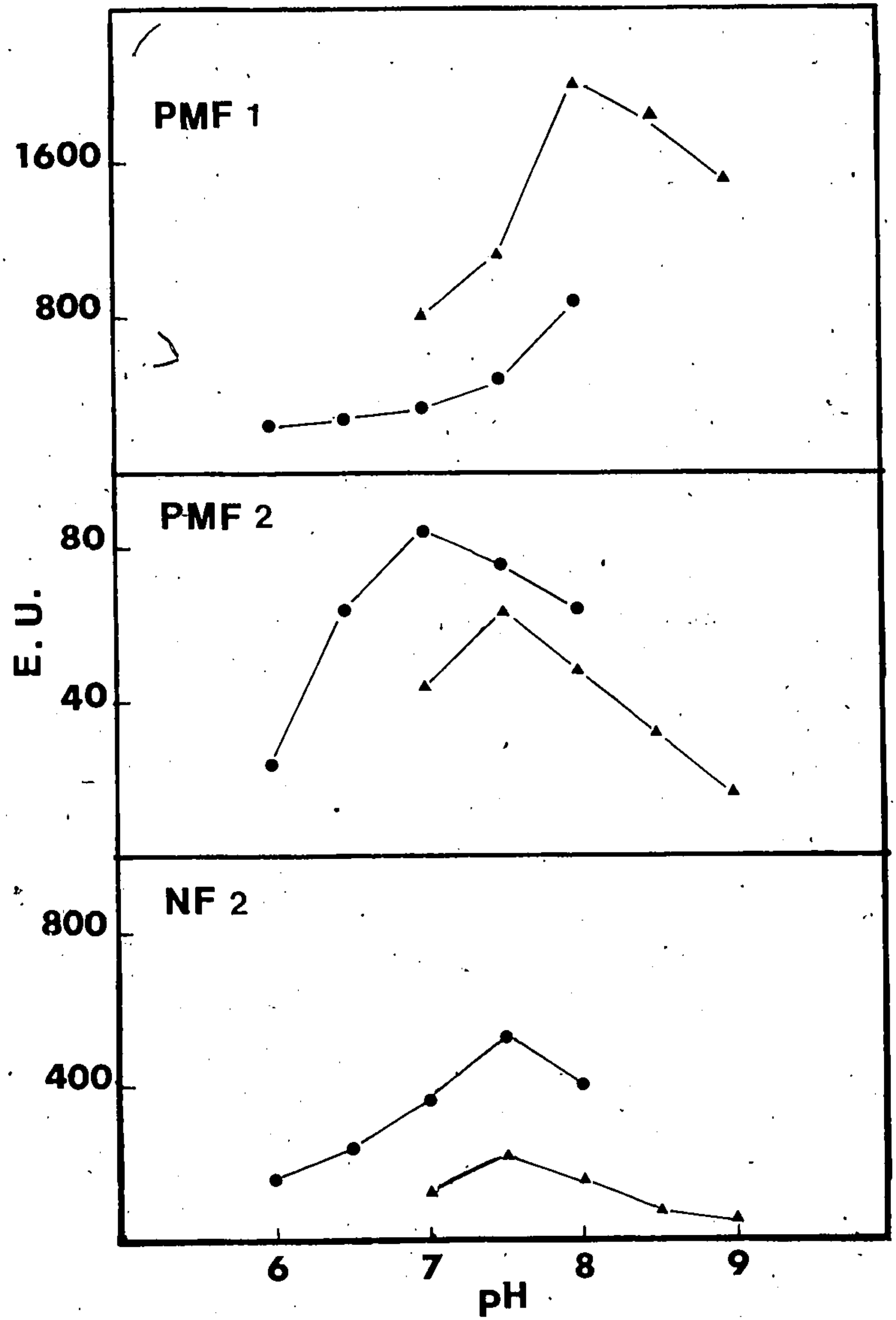
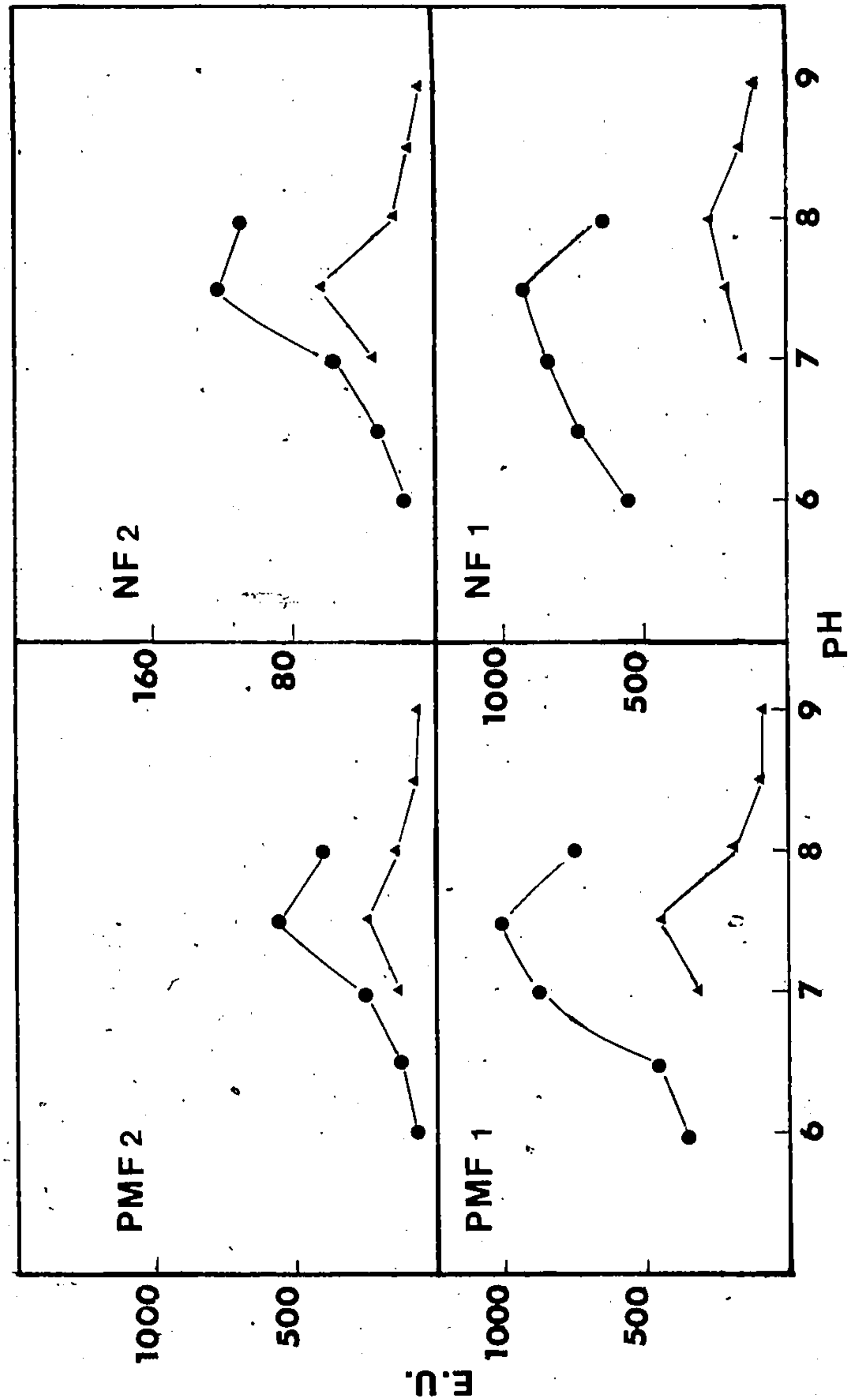

PMF 1
1600
800
PMF 2
80
40
NF 2
800
400
E. U.
6
7
8
9
pH

Figure 19: The effect of pH and various buffers on DNA polymerase activity. The DEAE-cellulose purified post-mitochondrial fractions 1 and 2 from 36h embryos were assayed for enzyme activity using 10 mM potassium phosphate (●——●), 10 mM Tris-HCl (at 37°C) (▲——▲) at the pH's indicated.

Each value is an average of two experiments.

experiments.


PMF 2
NF 2
PMF 1
NF 1
1000
500
160
80
1000
500
E.U.
6
7
8
9
PH

Effect of dinucleoside polyphosphates on enzyme activity

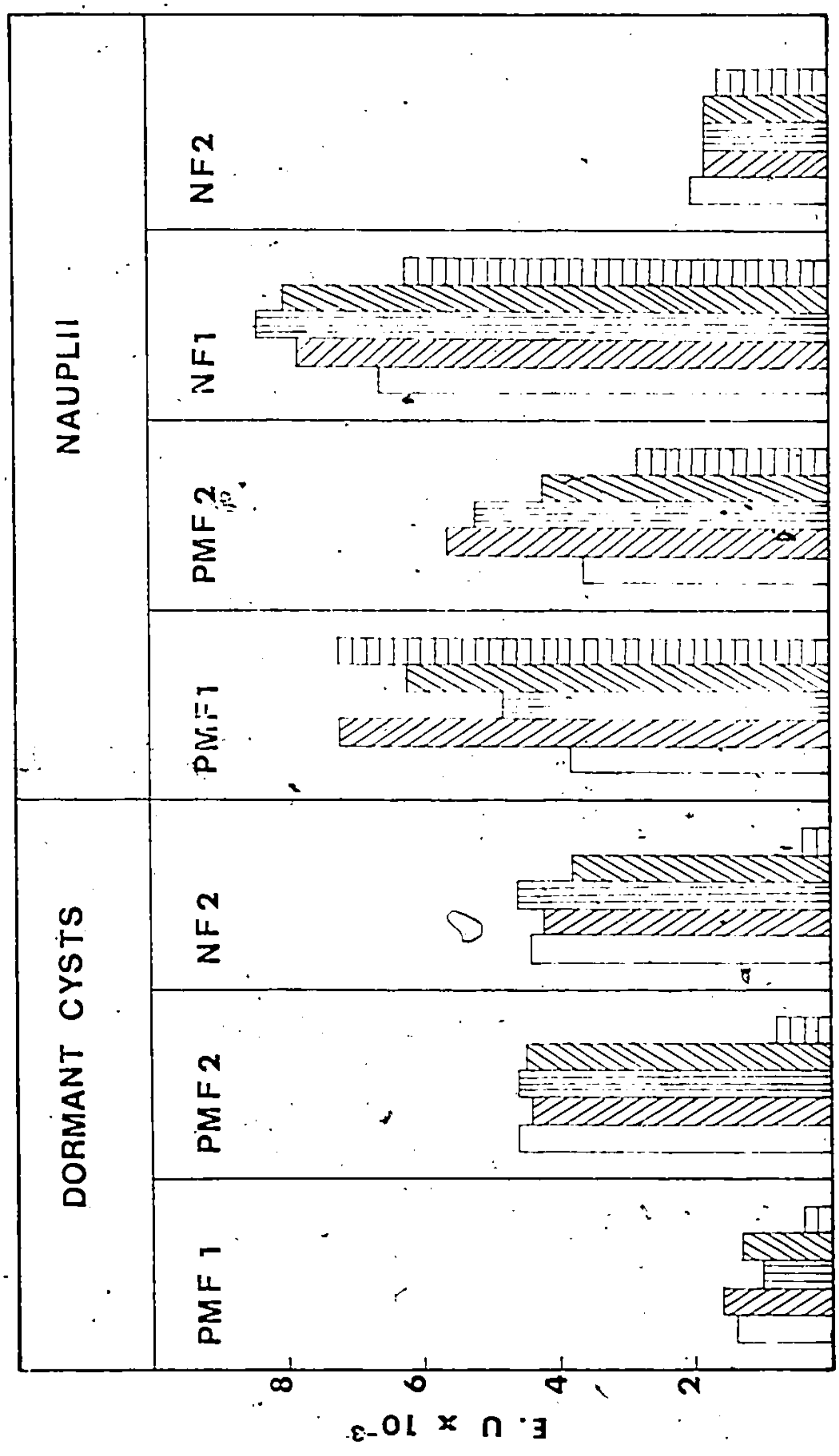
The possible regulation of DNA polymerase activity from dormant cysts and nauplii of *Artemia* embryos by dinucleoside polyphosphates was investigated by assaying for polymerase activity in the presence of each compound at a final concentration of 0.1 mM. The results for dinucleoside polyphosphates containing guanosine are shown in Fig.20.

The nuclear (NF-2) and cytosol fractions (PMF-1,PMF-2) from dormant cysts did not exhibit any stimulation in activity upon addition of Gp_2G, Gp_3G or Gp_4G. However, inclusion of Gp_3A caused a reduction in activity ranging from 83% to 92% compared to the controls.

In contrast, the cytosol fractions (PMF-1,2) from 36h nauplii exhibited stimulation in activity when Gp_2G, Gp_3G and Gp_4G were included. Addition of Gp_2G and Gp_4G increased the activity by 20-80% in both fractions.

When the hybrid dinucleoside polyphosphate (Gp_3A) was tested the activity of PMF-1 increased by 85% which was not observed with the PMF-2 fraction.

Figure 20: The effect of naturally occuring dinucleoside polyphosphates on the DNA polymerase activity of dormant cysts and nauplii. The dinucleoside polyphosphates Gp_2G ( ), Gp_3G ( ), Gp_4G ( ) and Gp_3A ( ) were added to the standard DNA polymerase reaction mixture at 0.01 mM, final concentration. Both control (, no additions) and treated samples were tested for enzyme activity at various times. Each bar represents an average of three measurements from these experiments.



DORMANT CYSTS
NAUPLII
PMF 1
PMF 2
NF 2
PMF1
PMF 2
NF1
NF2
E. U x 10-3
8
6
4
2

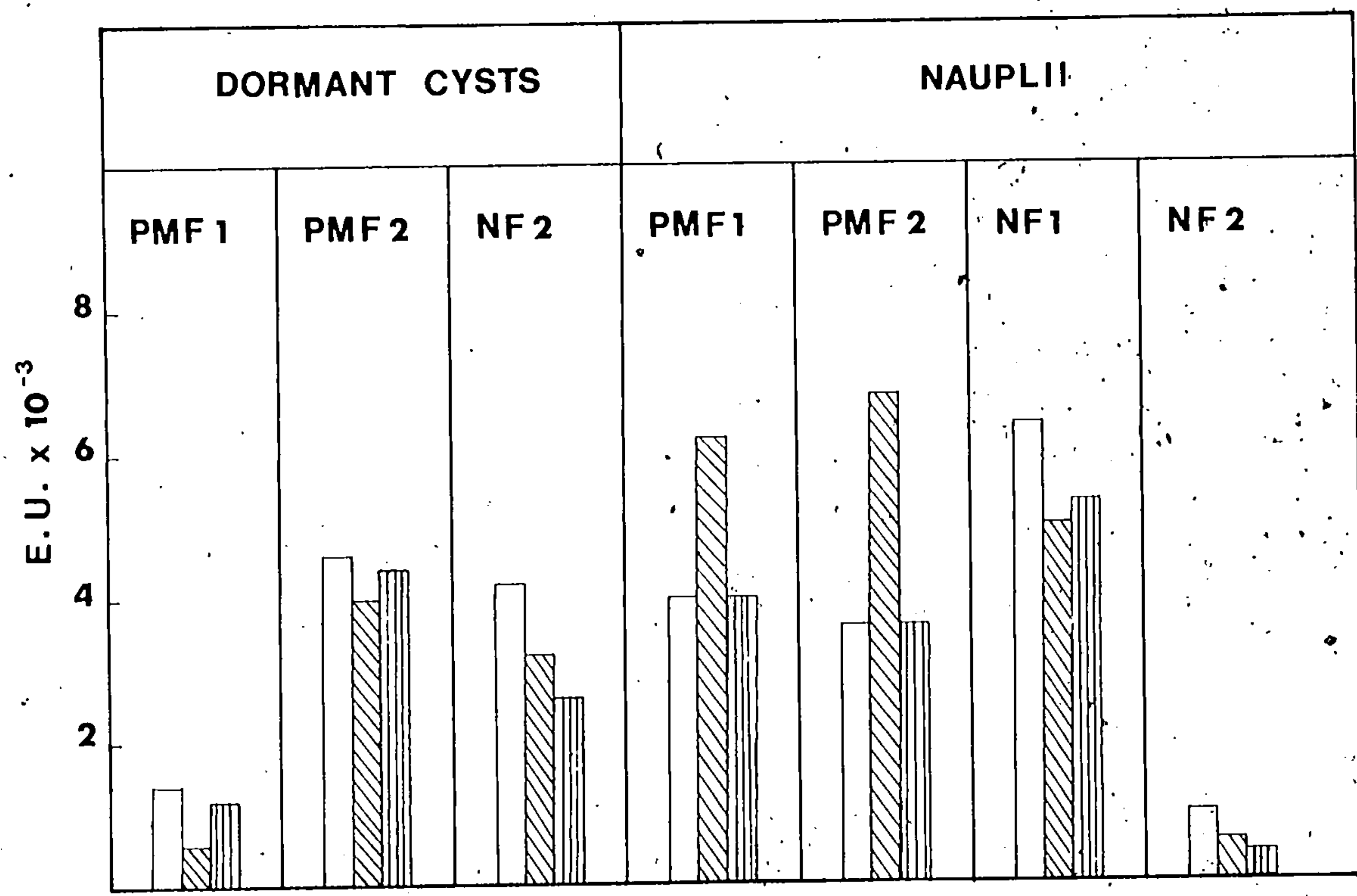
The nuclear fractions (NF-1,2) from nauplii were not affected significantly when assayed in the presence of any of the diguanosine polyphosphates and Gp_3A. In arriving at these conclusions, all data were analysed by the nalyses of Variants (91) using triplicate measurements.

On the other hand, addition of diadenosine polyphosphates (Ap_3A and Ap_4A) to most of the enzyme fractions (Fig.21) did not reveal any statistically significant variation in the activity profile. Only the cytosol fractions (PMF-1, PMF-2) from nauplii showed significant (80%) increases in activity in the presence of Ap_3A. In all other cases, there was no marked change in enzyme activity in the presence of dinucleoside polyphosphates.

On summarizing these data, it was observed that the diguanosine polyphosphates (Gp_2G, Gp_3G and Gp_4G) preferentially stimulate the naupliar DNA polymerases and Gp_3A inhibits markedly the dormant cyst DNA polymerases.

It is pertinent to note that stimulation or inhibition of the DNA polymerase activity was only observed when naturally occuring diguanosine polyphosphates were included in the reaction assay mixture suggesting a possible role for these compounds in DNA polymerase regulation.

Figure 21: The effect of diadenosine polyphosphates on DNA polymerase activity of dormant cysts and nauplii. The dinucleoside polyphosphates Ap_3A ( ) and Ap_4A ( ) were added to the standard DNA polymerase reaction mixture at 0.01 mM, final concentration. Both control (, no additions) and treated samples were tested for enzyme activity at various times. Each bar represents the average from 5 experiments.



DORMANT CYSTS
NAUPLII
PMF 1
PMF 2
NF 2
PMF 1
PMF 2
NF 1
NF 2
8
6
4
2
E.U. x 10^-3

Effect of various ions on DNA polymerase activity

The variable effect of potassium phosphate and Tris-HCl on enzyme activity led to experiments to test the effect of the various ionic components on Artemia DNA polymerase activity.

Varying concentrations of KCl from 0.05 - 0.4M was included in the reaction vessel and the activity was determined (see Fig.22). KCl at concentrations as low as 0.05M inhibited markedly (24 - 44%) the cytosol fractions (PMF-1,2) of the nauplii. In contrast, the DNA polymerase activity of the PMF-1 and NF-2 of the dormant cysts was stimulated by low concentrations of KCl (0.05 - 0.2M). The use of KCl at 0.1M concentrations distinguishes the cytosol fraction (PMF-1) of the dormant cyst from its counterpart of the nauplii.

The effect of phosphate as potassium phosphate in the enzyme reaction mixture is presented in Fig.23. Suitable controls containing potassium phosphate at 3 mM final concentrations were used in these experiments.

DNA polymerase activity of PMF-1, PMF-2 and NF-2 of the dormant cysts was stimulated by 24% to 148% at phosphate concentrations between 10 and 50 mM. Phosphate at 100 mM was slightly inhibitory (see Fig.23).

Figure 22: The effect of KCl on DNA polymerase activity. The activity of various DNA polymerase fractions from 0h and 36h embryos (as indicated) was assayed in the presence of varying concentrations of KCl. ▭, Control with no KCl; ▧, 0.05M KCl; ▥, 0.1M KCl; ▨, 0.2M KCl; and ▤, 0.4M KCl.

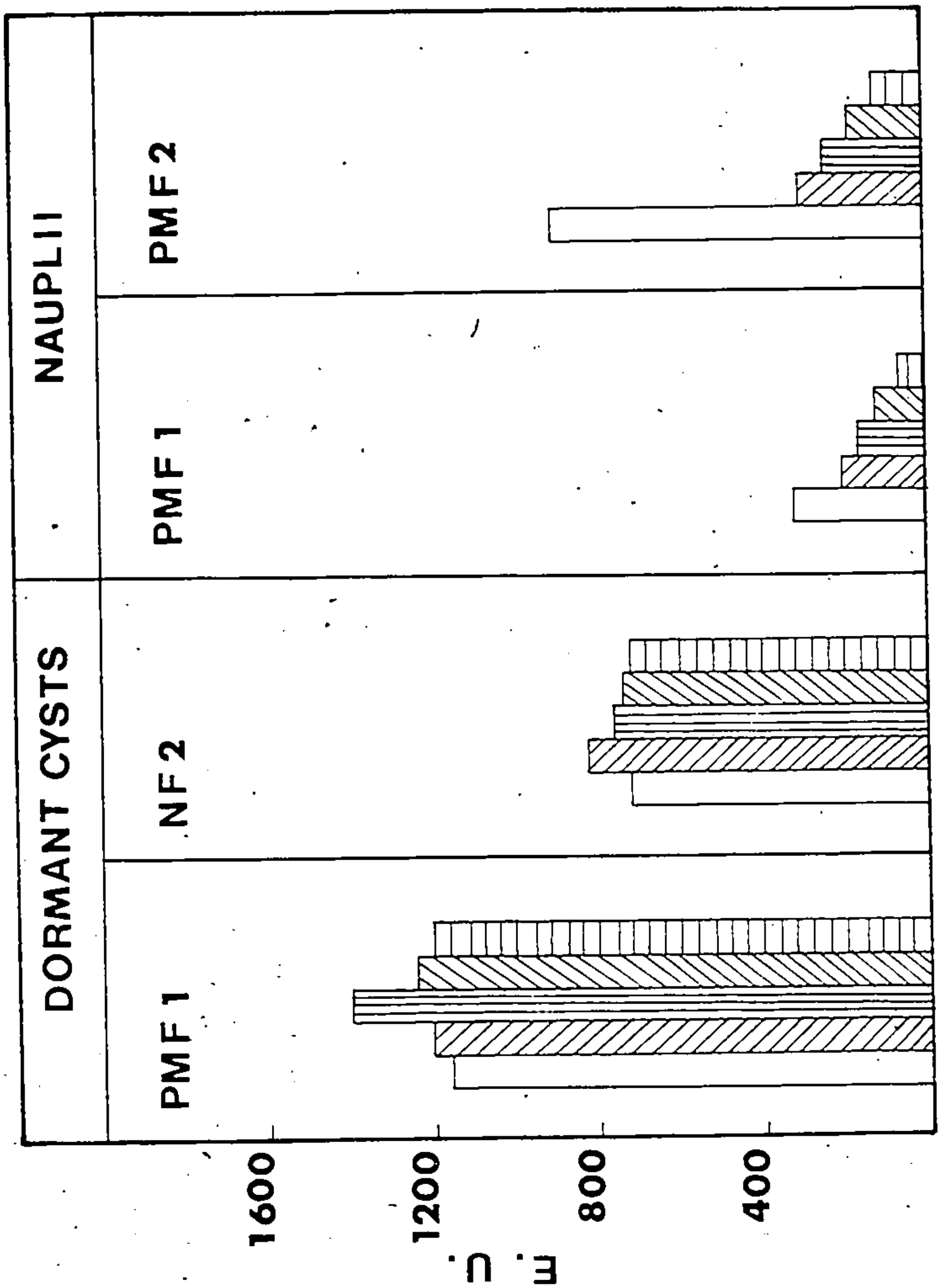
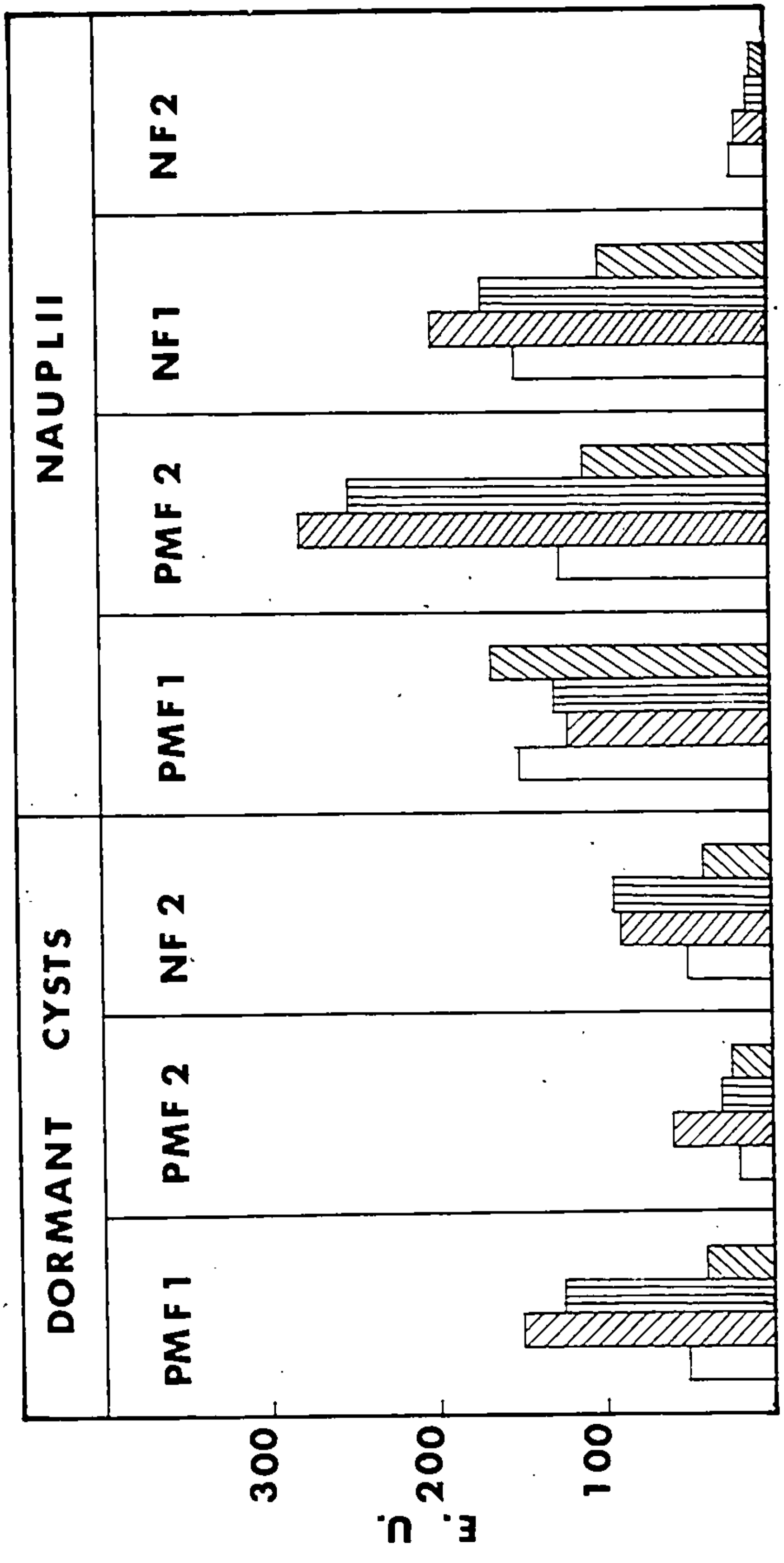

DORMANT CYSTS
NAUPLII
PMF 1
NF 2
PMF 1
PMF 2
1600
1200
800
400
E. U.

Figure 23: The effect of phosphate on DNA polymerase activity. Varying concentrations of potassium phosphate (pH 7.5) were added to reaction mixtures containing various DNA polymerase fractions and the activity was determined as described in the Methods. The concentrations used were 10 mM ( ), 50mM ( ), and 100mM ( ). Controls ( ) contained 3 mM phosphate as the final concentration. DNA polymerase activity in the nuclear and post-mitochondrial fractions from 0h and 36h embryos were assayed as described in the Methods.

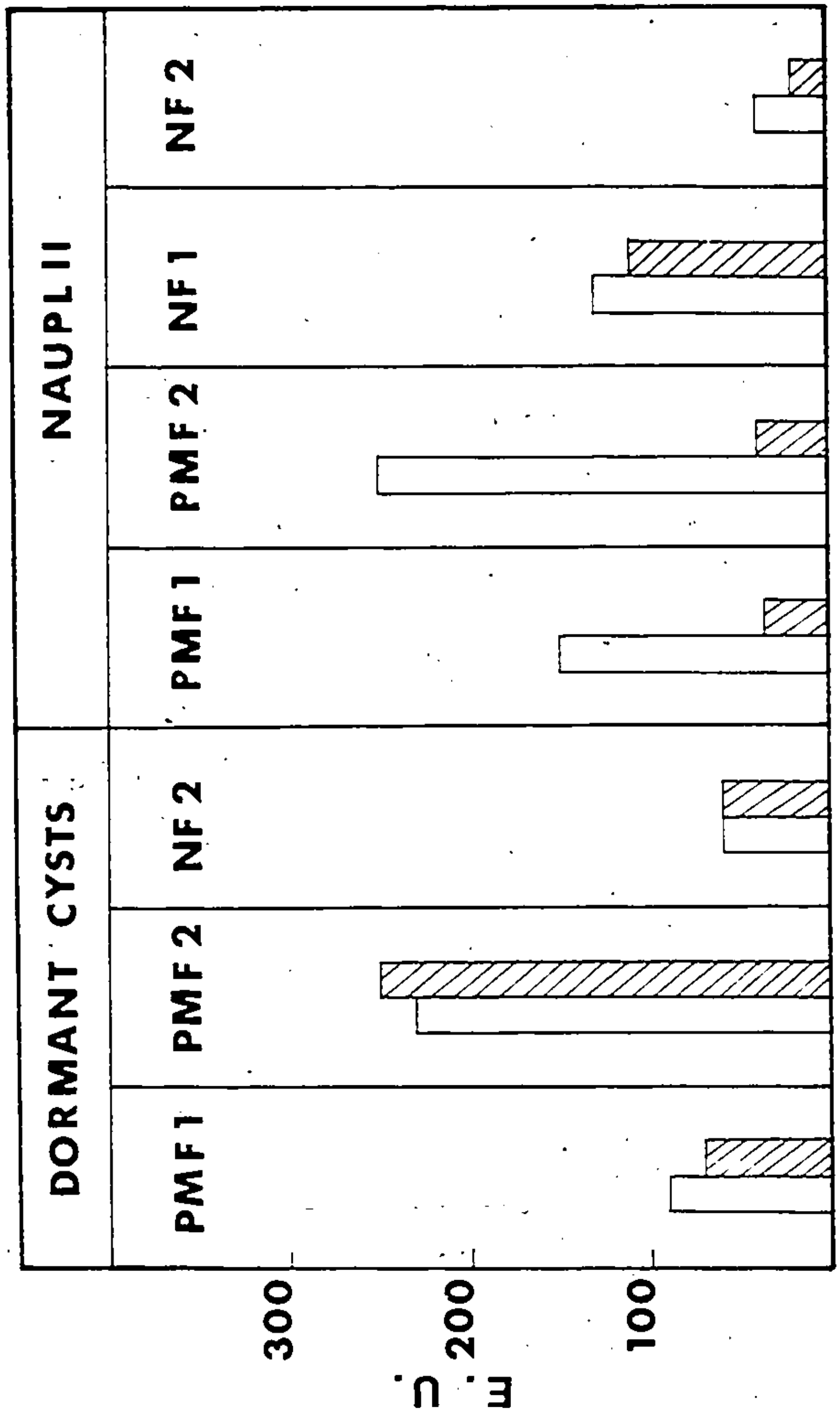

DORMANT CYSTS
NAUPLII
PMF 1
PMF 2
NF 2
PMF 1
PMF 2
NF 1
NF 2
300
200
100
E. U.

Whereas, the naupliar PMF-2 DNA polymerase was stimulated at phosphate concentrations as low as 10 mM, 100 mM phosphate concentration had little effect on the polymerase activity of PMF-1.

The results obtained using the nuclear fractions (NF-1, NF-2) of the naupliar DNA polymerases are shown in Fig.23. NF-1 exhibited a 43% increase in activity 10 mM phosphate which decreases on increasing the phosphate concentration to 100 mM. No effect was observed with NF-2 from nauplii at any of the phosphate concentrations tested.

When Artemia DNA polymerases were assayed in the presence of either $MgCl_2$ or $MnCl_2$, the results shown in Fig.24 were obtained. None of the enzyme fractions from dormant cysts revealed any major difference in activity when either cation was used indicating that these enzymes could utilise Mg^{2+} and Mn^{2+} interchangeably without affecting the acivity of the enzyme. In contrast to this, the cytosol polymerase fractions from nauplii showed high dependency for Mg^{2+} while in the presence of Mn^{2+} the activity was reduced by 78% and 84% while the nuclear enzymes functioned reasonably well with either cation.

Figure 24: The effect of Mg^{2+} and Mn^{2+} on various DNA polymerase fractions. The activity of the various DNA polymerase fractions from dormant cysts (0h) and nauplii (36h) was determined in the presence of 40 mM $MgCl_2$ ( ) or 40 mM $MnCl_2$ ( ) as described in the Methods.


DORMANT CYSTS
NAUPLII
PMF 1
PMF 2
NF 2
PMF 1
PMF 2
NF 1
NF 2
300
200
100
E. U.

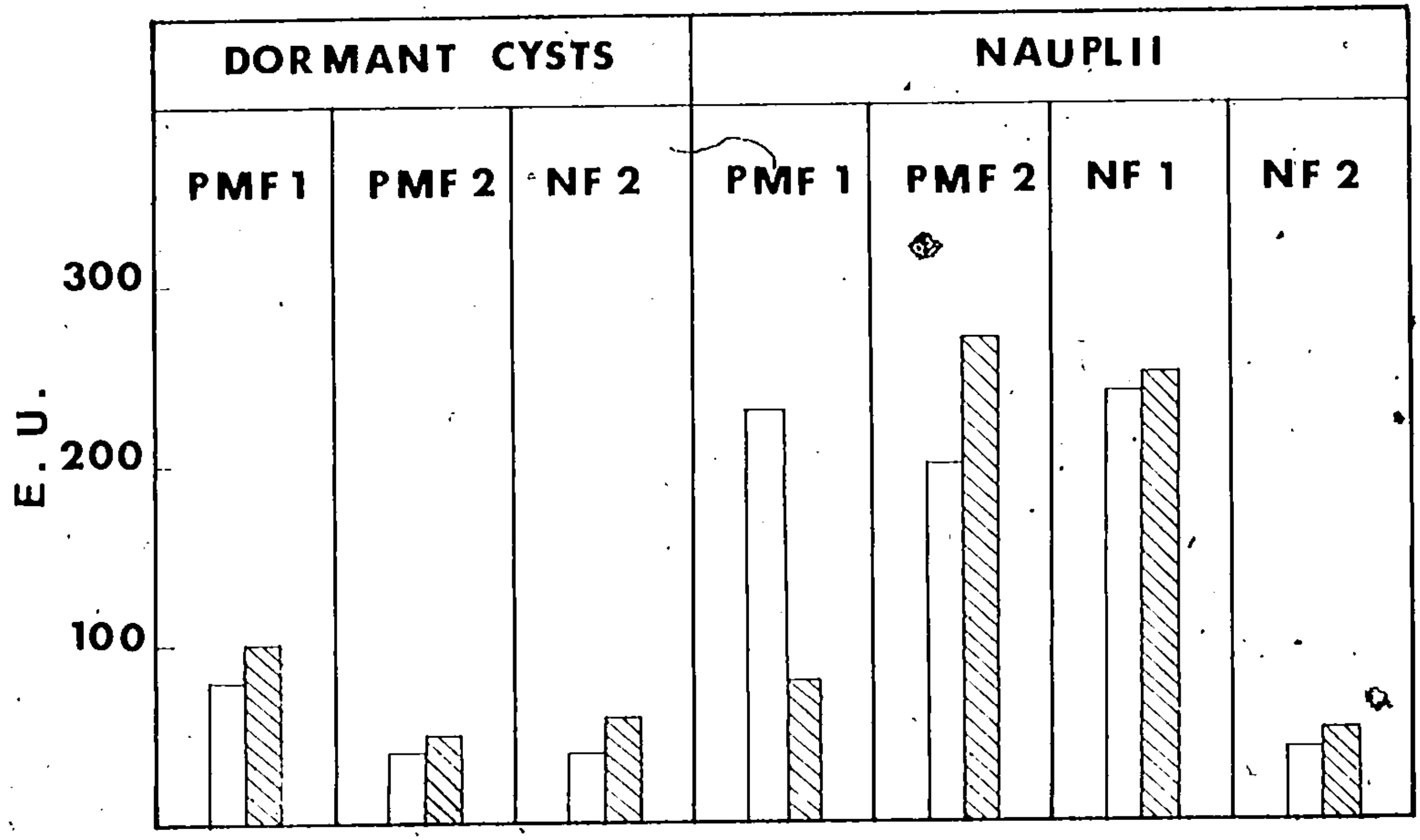
Other biochemical parameters

Studies with different primer-templates (activated) and on the effect of ATP or GTP addition on DNA polymerase activity were also carried out.

Each primer-template was used at a final concentration of 200 ug/ml in the enzyme reaction mixture. Results obtained using activated Artemia DNA compared to Calf thymus DNA are shown in Fig.25. Except for the DNA polymerase in PMF-1 of the nauplii, all polymerase fractions were found to be more effective with Artemia DNA than with calf thymus DNA. Perhaps, the most striking difference was observed with PMF-1 and PMF-2 from nauplii. PMF-1 is most active with calf thymus DNA whereas PMF-2 is most active with Artemia DNA. The reason for these differences is not yet understood.

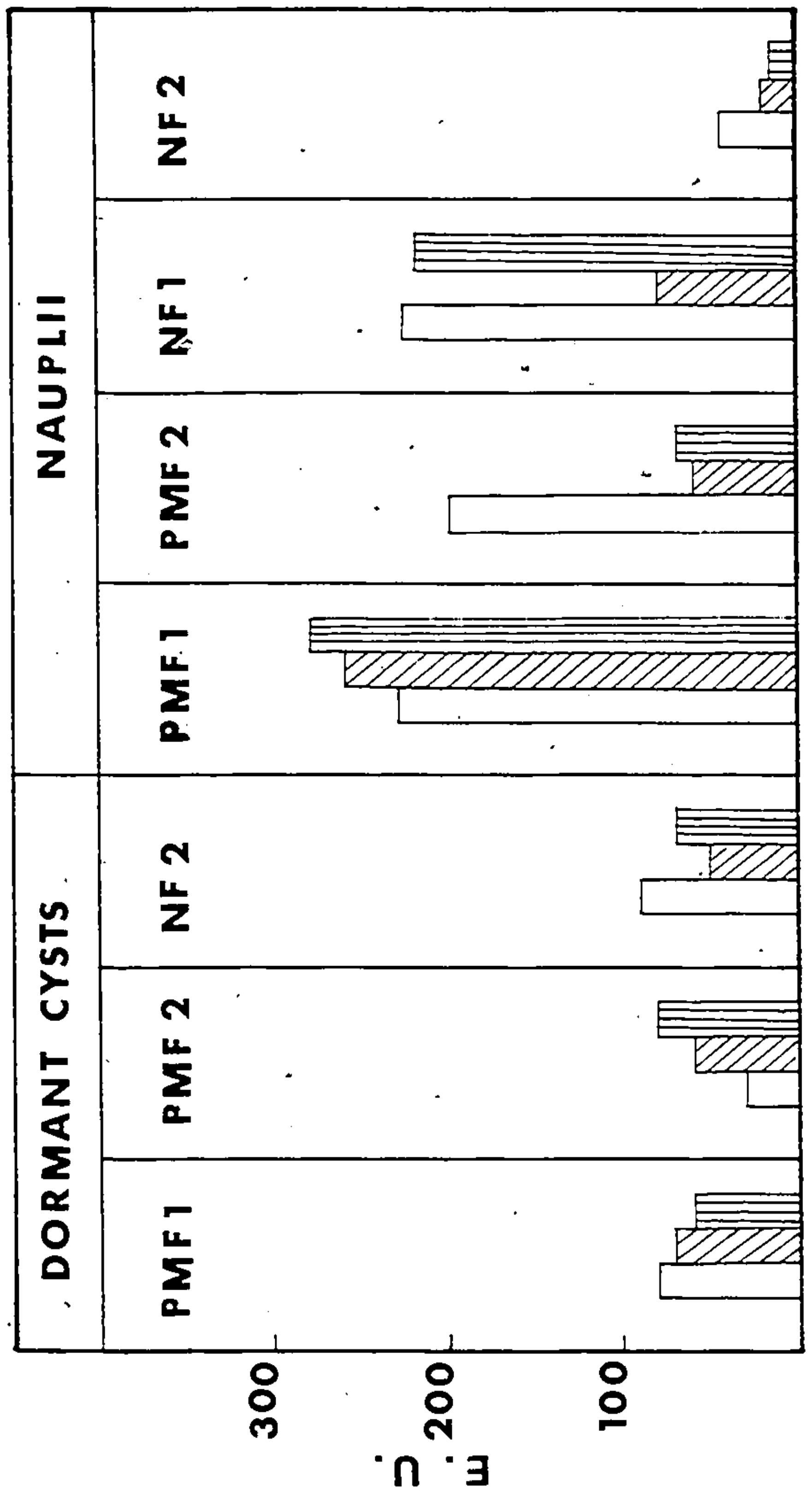
Additional data confirming the differences between the polymerases in nauplii PMF-1 and PMF-2 were obtained when ATP and GTP were included separately in the reaction mixture at a final concentration of 1 mM. The results of these experiments are shown in Fig.26. These nucleotides appeared to have the greatest effect on the soluble polymerases from nauplii. Thus PMF-1 (nauplii) was stimulated 13 to 22% by addition of either nucleotide, whereas PMF-2 (nauplii) was inhibited markedly (70%) when ATP or GTP was included. The reverse results were obtained with their counterparts (PMF-1, PMF-2) from dormant cysts. In

Figure 25: DNA polymerase activity of the cyst and naupliar enzyme fractions in the presence of activated-calf-thymus DNA and activated-*Artemia* DNA as primer-templates. Calf-thymus DNA and *Artemia* DNA were activated for use as primer-templates as described in the Methods. The DNA polymerase activity of the cyst and nauplii enzyme fractions (as indicated) was assayed using the activated calf thymus DNA (▭) or activated-*Artemia* DNA (▨) at a final concentration of 200 ug/ml. Each bar represents the average of three values.



DORMANT CYSTS
NAUPLII
PMF 1
PMF 2
NF 2
PMF 1
PMF 2
NF 1
NF 2
300
200
100
E. U.

Figure 26: The effect of ATP or GTP on DNA polymerase activity. Various DNA polymerase fractions from dormant cysts and nauplii were assayed in the presence of ATP (▧) or GTP (⊞) in the assay mixture at a final concentration of 1 mM control samples (□) without any additions were also run. Each bar represents the average of three values.



DORMANT CYSTS
NAUPLII
PMF 1
PMF 2
NF 2
PMF 1
PMF 2
NF 1
NF 2
300
200
100
E. U.

the latter case PMF-2 was stimulated 25% and 50% in the presence of ATP and GTP, respectively while PMF-1 was inhibited slightly by these nucleoside 5'-triphosphates.

The nuclear fractions (NF-1 and NF-2) respond similarly to added ATP or GTP irrespective of the source of these enzymes. These results indicate that DNA polymerase fractions 1 and 2 from the cytosol and nuclear fractions vary in their biochemical characteristics. However, the possibility that PMF-1 and NF-1 (or) PMF-2 and NF-2 of the cyst (and similarly of the nauplii) are part of the same enzyme cannot be ruled out.

Studies with inhibitors

Various inhibitors namely N-ethylmaleimide (NEM), dideoxy thymidine 5'-triphosphate (d2TTP) and aphidicolin were used to distinguish and possibly define the functional role of *Artemia* DNA polymerases.

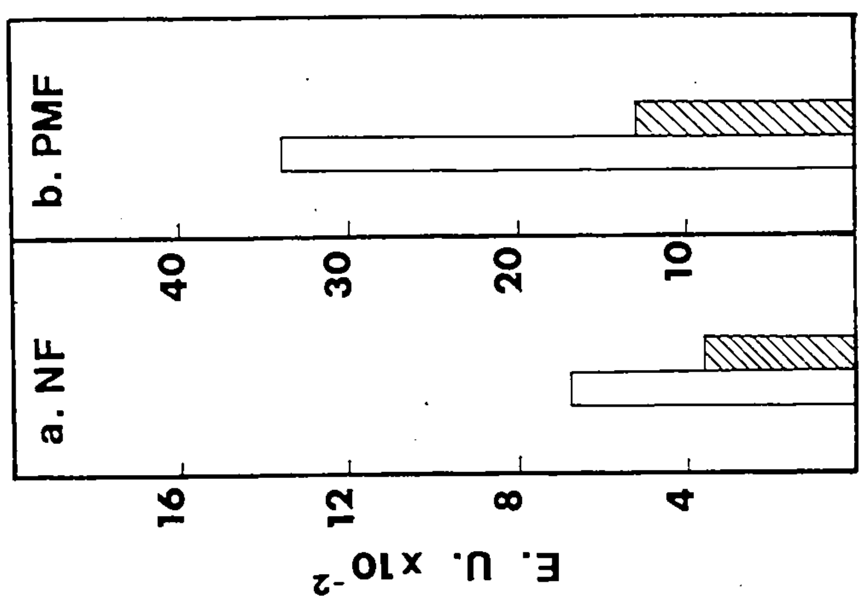
Earlier experiments (see Table 1) indicated the requirement for the presence of free sulfhydryl groups in the reaction assay mixture of *Artemia* DNA polymerases. NEM was used to distinguish the different types of DNA polymerases from *Artemia*.

Preliminary experiments were confined to crude embryo extracts obtained from 36h nauplii. The DNA polymerase activity of the nuclear (NF) and cytosol fractions (PMF) were determined by using NEM at a final concentration of 1 mM. The results with the crude embryo extracts are shown in Fig.27. Treatment with NEM decreased the activity by 48% with the nuclear fraction and by 68% with the cytosol fraction.

Similar studies were carried out using the purified polymerase fractions. The results in Fig.28 summarize the effect of NEM on the various DNA polymerase fractions. Fractions PMF-1 and 2 from nauplii were the most sensitive to NEM treatment. Fractions PMF-1 was inhibited 86% and PMF-2 was inhibited by 68%. Both the cytosol fractions (PMF-1 and 2) from dormant cysts seem unaffected even at concentrations of NEM as high as 6 mM. Nuclear fractions from cyst or nauplii were either not sensitive (cysts) or only slightly inhibited (nauplii) by the NEM pre-treatment. The remarkably high sensitivity exhibited by the soluble DNA polymerases from nauplii indicates these enzymes to be similar to the mammalian DNA polymerase α, whereas PMF-1 from cysts is comparable to the β-polymerase of mammalian cells in its insensitivity to NEM inhibition. DNA polymerase β is thought to be involved in the repair process.

To define further the functional role of <u>Artemia</u> DNA polymerases the effect of d2TTP and aphidicolin were tested. The chain terminator, d2TTP was used under conditions described in the legend to Fig.29. The cytosol DNA polymerases from the dormant cysts (PMF-1 and 2) were in-

Figure 27: The effect of N-ethylmaleimide (NEM) on the nuclear and cytosol DNA polymerases from crude extracts. The crude nuclear (a) and post-mitochondrial extracts (b) from 36 h nauplii were treated with 1 mM NEM (final concentration) in the reaction vessel then assayed for DNA polymerase activity as described in the Methods. (▭) Control (▨) NEM treated.



a. NF
b. PMF
E. U. x10^{-2}
16
12
8
4
40
30
20
10

Figure 28: The effect of N-ethylmaleimide (NEM) on DNA polymerase activity of 0h cyst and 36h naupliar enzyme preparations. The activity of various DNA polymerase fractions from the 0h and 36h embryos was assayed without ( ) and with ( ) NEM added to a final concentration of 6 mM in the enzyme reaction mixture as described in the Methods.

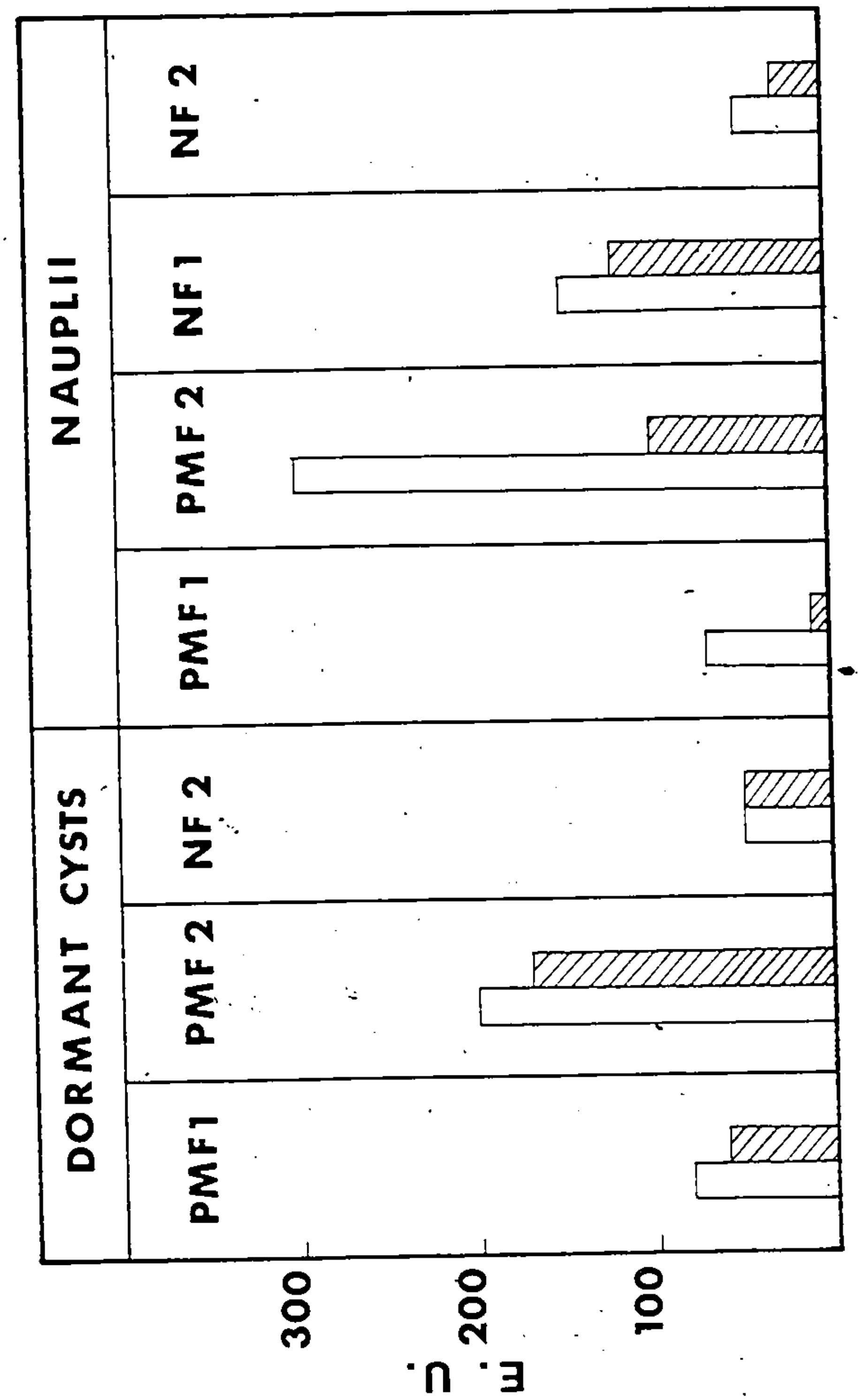
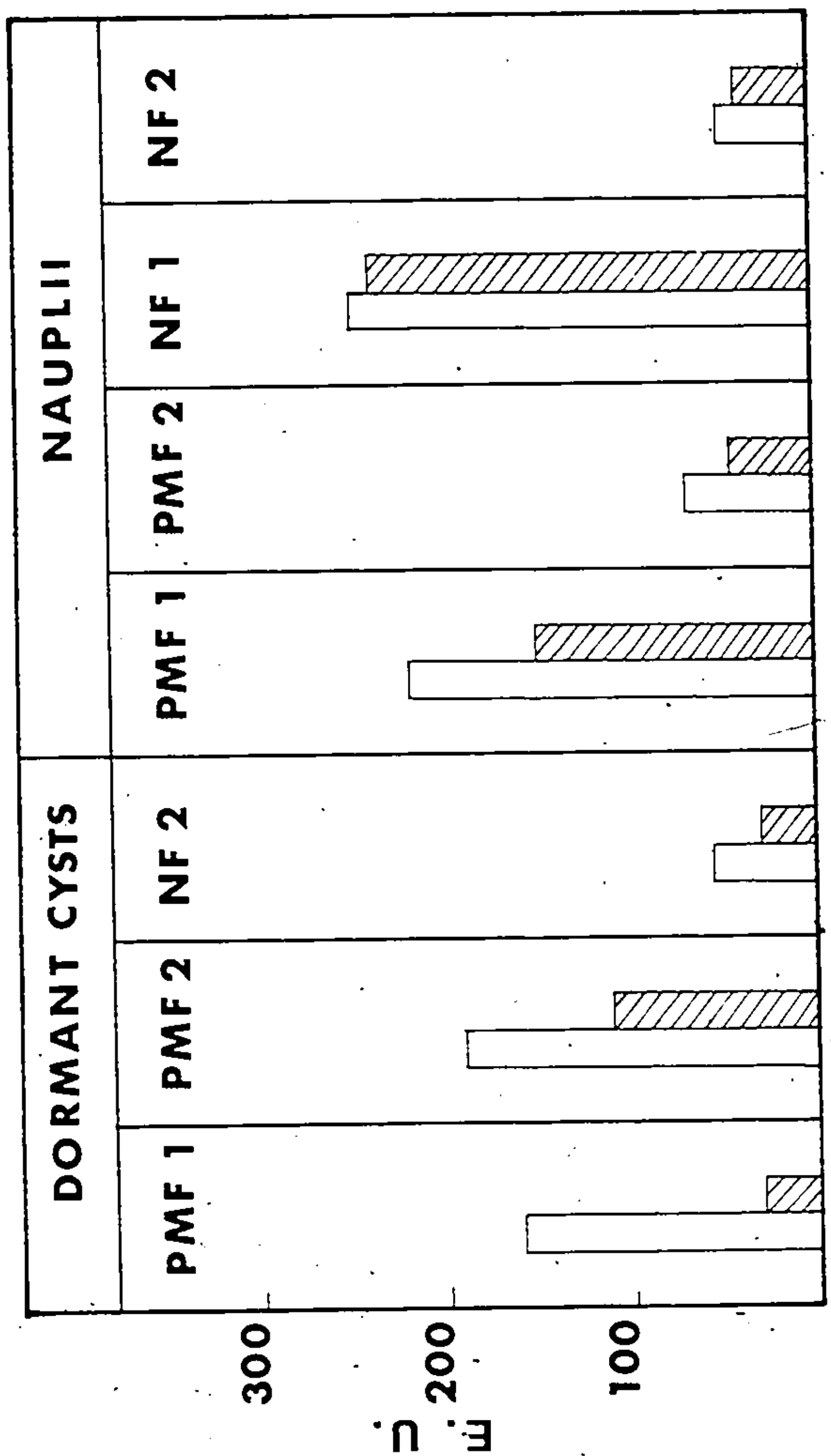

DORMANT CYSTS
NAUPLII
PMF1
PMF 2
NF 2
PMF 1
PMF 2
NF 1
NF 2
300
200
100
E. U.

Figure 29: The effect of dideoxythymidine 5'-triphosphate on the DNA polymerase activity. The DNA polymerase fractions from the dormant cysts and nauplii were assayed for enzyme activity without ( ) and with ( ) dideoxythymidine 5'-triphosphate (d2TTP) in the reaction mixture. The d2TTP/TTP ratio was 4 x 10^{-2} in all cases.


DORMANT CYSTS
NAUPLII
PMF 1
PMF 2
NF 2
PMF 1
PMF 2
NF 1
NF 2
300
200
100
E. U.

hibited 82% and 34%, respectively. When d2TTP was present in the enzyme reaction vessel, the extent of inhibition was not as great with the cytosol fractions 1 and 2 from nauplii which showed only 26% and 36% inhibition, respectively. The nuclear fractions from dormant cysts are inhibited by d2TTP but the nuclear fractions from nauplii appear to be insensitive to d2TTP which is indicative of the α-type polymerase of mammalian cells.

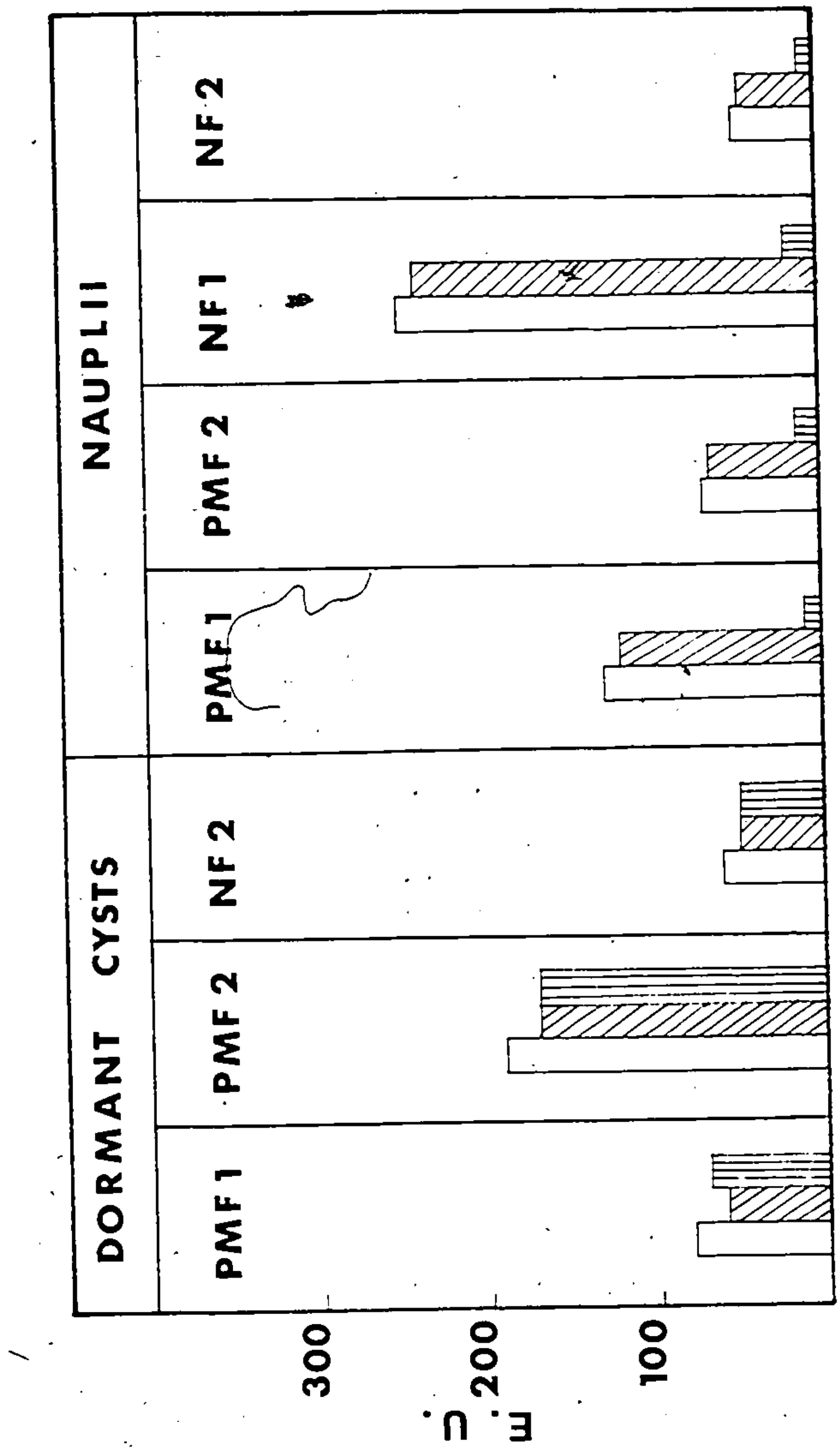
In order to confirm the types of DNA polymerases suggested by the d2TTP and NEM data, aphidicolin, a specific inhibitor of α polymerase was included in the regular reaction mixture. The results shown in Fig.30 show that all enzyme fractions from nauplii are sensitive to this drug at concentrations as low as 5 ug/ml, while the cyst enzymes are refractory to this drug. Dimethylsulfoxide, the solvent used for the solubilization of aphidicolin, was found to have no inhibitory effect on enzyme activity.

This suggests that the enzymes from nauplii are primarily involved in replication and the cyst DNA polymerases are probably involved in reactions other than replication.

Sedimentation studies

Since it is possible to estimate the molecular weight of a protein by its sedimentation coefficient, the cytosol fraction (PMF-1) from the cysts and nauplii was subjected to sucrose density gradient sedimentation as described in the Methods.

Figure 30: The effect of aphidicolin on DNA polymerase activity. Aphidicolin was dissolved in dimethyl sulfoxide (DMSO) and tested under conditions to give a final concentration of 5 ug/ml (▭▭▭) in 0.1% v/v, DMSO. The DNA polymerase activity of the cyst and naupliar enzyme fractions was assayed using a buffer control (▭), and a DMSO control (▨). Each bar represents the average of three values each from two experiments.

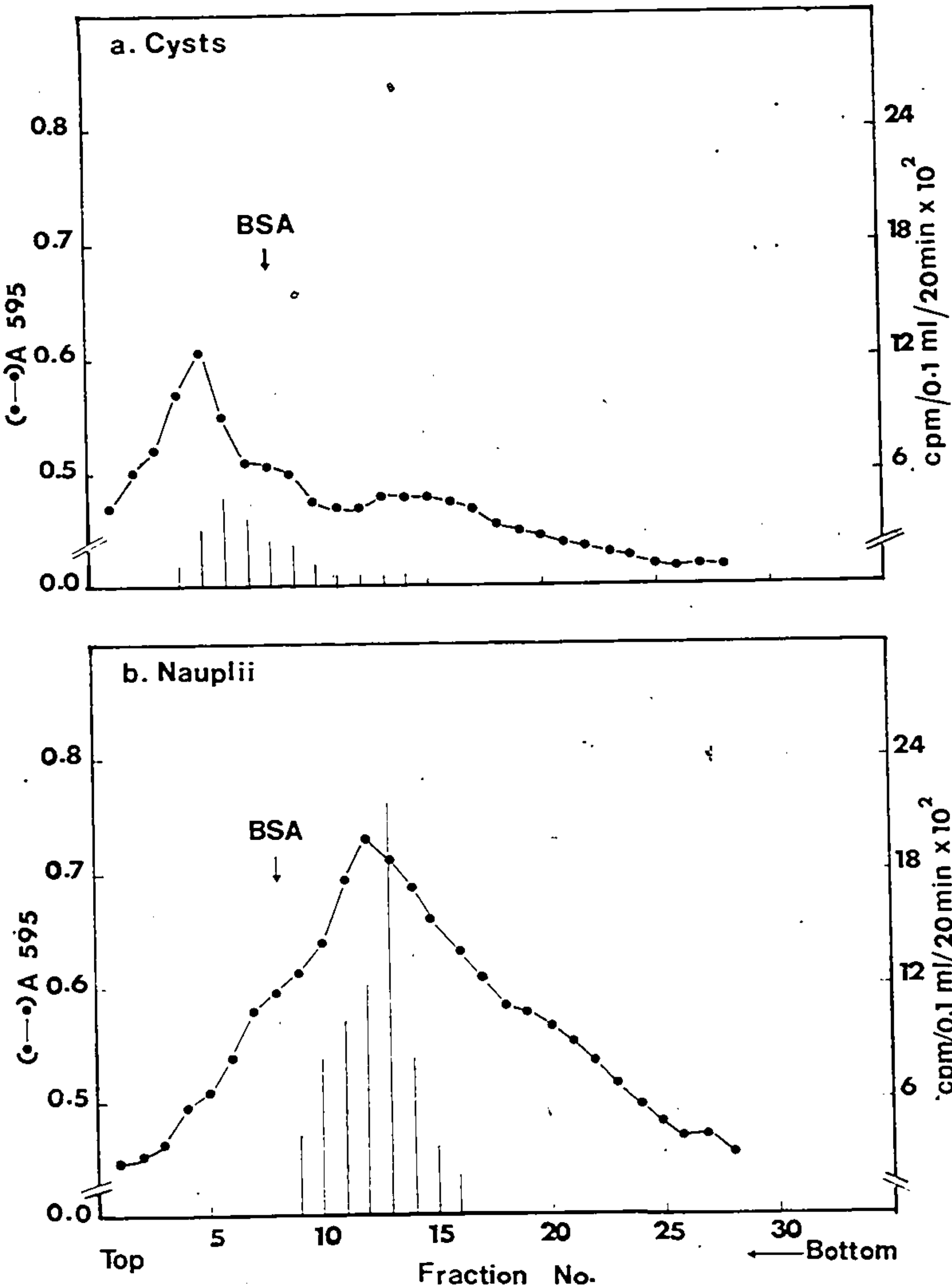


DORMANT CYSTS
NAUPLII
PMF 1
PMF 2
NF 2
PMF 1
PMF 2
NF 1
NF 2
300
200
100
E. U.

These results are shown in Fig. 31a and 31b. Using bovine serum albumin (BSA) as the molecular weight marker sedimentation coefficients of 3.6S and 6.7S were obtained for the cyst (PMF-1) and the nauplii (PMF-1) DNA polymerases respectively. In both cases, maximal enzyme activity was obtained in the gradient fraction immediately following the main protein peak, although the DNA polymerase from nauplii appeared to be distributed broadly (Fig.31b), in contrast to the sharper enzyme peak recorded for the cyst enzyme (Fig.31a).

These data suggest that the DNA polymerase(s) from cysts has(have) a molecular weight in the range of 30,000 - 40,000 M.W., while the DNA polymerase(s) from nauplii exhibit a molecular weight in the range of 150,000.

Figure 31: Sucrose-density gradient analysis of DNA polymerase activity in the post-mitochondrial fraction (PMF-1) of dormant cysts (a) and nauplii (b). Dormant cyst DNA polymerase (and fraction 1 nauplii polymerase) from DEAE-cellulose was layered onto 12.6 mls of a linear sucrose gradient (5-20%) in buffer B (excluding the 15% v/v glycerol) and the preparation was centrifuged for 22 h at 39,000 rpm using a SW-41 rotor. The gradients were fractionated using an ISCO-density gradient fractionator and the protein content (●—●) of each fraction was estimated using the Bio-Rad procedure (as described in the Methods). Each fraction was assayed for polymerase activity (bars). The migration of bovine serum albumin (4.6S, 67,000 M.W.) was determined in a separate centrifuge tube under the same experimental conditions and its sedimentation position is shown by the arrow.



a. Cysts
BSA
0.8
0.7
0.6
0.5
0.0
(•—•)A 595
24
18
12
6
cpm/0.1 ml/20min x 10^2
b. Nauplii
BSA
0.8
0.7
0.6
0.5
0.0
(•—•)A 595
24
18
12
6
cpm/0.1 ml/20min x 10^2
5
10
15
20
25
30
Top
Bottom
Fraction No.

Chapter V

DISCUSSION

Studies on eukaryotic DNA-dependent DNA polymerases have been limited due to the fact that few mutants of any sort are available. Thus, inferences on enzymic functions have been derived mainly by using indirect approaches. Some of these include, 1) a comparison of activity levels of DNA polymerases in cells actively engaged in DNA replication compared to those in resting cells, 2) subcellular localization of the polymerases, 3) biochemical analyses to define the function and role of the DNA polymerases, and 4) studies on the possible regulatory mechanisms that control the expression of these enzymes.

The brine shrimp, *Artemia sp.* exhibits a remarkable separation of morphogenesis from new cell synthesis in the early phase of post-gastrular development. The mechanism that controls DNA synthesis in *Artemia* cysts is still unknown but it appears to be complex and to function at the replication level. Thus, *Artemia* appears to be an ideal system to assess the process of DNA replication ~~in~~ reactivation of the developmental program.

In this thesis, the role of DNA-dependent DNA polymerases in the stage-specific gene expression of *Artemia* was studied. A dual approach has been utilised. One was to determine the changes in the activity of DNA polymerases as development progressed from the 0h dormant cysts to the 36h naupliar stages using crude embryo extracts. The other approach was to isolate and partially characterise DNA polymerase(s) from the nuclear (NF) and post-mitochondrial or cytosol fraction (PMF) of the 0h dormant cyst and 36h nauplii. A method for the purification of *Artemia* polymerase was also devised.

Studies with crude embryo extracts

To understand a process as complicated as DNA replication, it is necessary to work with a system other than the intact cells to overcome the barrier of the cell membrane. Hence assays for the DNA polymerase activities in cell-free extracts have been taken as a measure of DNA synthesis in vivo due to the close correlation between the values obtained in the in vitro assay with the extent of cell multiplication in the tissues examined (92,93).

Earlier attempts to correlate biochemical parameters with growth rate have met with varying degrees of success. Sutcliffe has attempted to predict growth rates in *Artemia* from RNA content but it was later suggested that this relationship worked well only under conditions of exponential growth (94). Dagg and Littlepage concluded that the general positive relationship between growth rate and RNA concentration lacked sufficient specificity to predict growth rates in Artemia salina (95). On the other hand, DNA polymerase activity has been followed in sea urchin development (96,97) and in Xenopus laevis oocytes (98,99) where there is noticeable increase in polymerase activity upon maturation of the egg. Therefore, on the assumption that growth of an organism must be accompanied by cell proliferation, it was shown by Eckstein et al. that the enzyme DNA polymerase shows a pattern of synthesis which coincides with the cell cycle (100). Thus, it should be possible to relate growth and development of an asynchronous culture to DNA polymerase specific activity. Hence, such a relationship between DNA polymerase specific activity and growth rates was investigated in Artemia sp.

Preliminary experiments with crude embryo extracts at various developmental stages of Artemia indicated a parallel increase in DNA polymerase and growth (compare Fig. 4 with 8). Resumption of DNA synthesis upon onset of hatching (Fig.4) was reflected in the sharp rise in specific activity of the cytosol DNA polymerase between 6 and 18 hours development and nuclear polymerase between 18 and 24 hours. Artemia DNA polymerases obtained from crude extracts of 0-36h embryos exhibited maximal specific activity only when assayed at pH 7.4 (Fig.6 and 7). This indicates that Artemia DNA polymerases are dependent on the pH of the ionic environment. This property is similar to the mammalian enzymes described by Chang and Bollum (101) where calf thymus DNA polymerases obtained from crude extracts have been characterized by their pH preferences of the in vitro assay conditions. The nuclear fractions obtained from crude embryo extracts exhibited no increase in DNA polymerase activity between 0-18 hours, which occured between 0-6 hours only with the cytosol polymerase fractions (see Fig.8). Also, the nuclear polymerase activity increased at a slower rate than the cytosol enzyme. Thus it could be hypothesized that translocation of the DNA polymerase from the cytosol (the site of production) to the nucleus (the site of action) may be taking place following hatching.

Studies with crude extracts were useful in establishing the appropriate assay conditions and to distinguish the various types of DNA polymerases. In order to establish the localization of the Artemia DNA polymerases, purification procedures were designed to isolate and characterise the DNA polymerase activity from both the nuclear (NF) and cytosol (PMF) fractions of 0h dormant cysts and 36h nauplii.

The purification of Artemia DNA polymerases

The DNA polymerases of Artemia were purified from dormant cysts (0h) and the nauplii (36h) to distinctly reflect DNA polymerase activity during the state of dormancy versus growth and differentiation. The findings with the crude embryo extracts revealed that a correlation in polymerase activity occured with growth and development; hence a purification procedure was devised using 0h and 36h embryos.

A four-step procedure has been designed for the purification of DNA polymerases from various fractions of Artemia embryos. This procedure permitted the characterization of DNA polymerases from dormant cysts and nauplii in terms of their functional roles in Artemia development. Earlier studies reported that rather large amounts of proteases existed in extracts of swimming nauplii (88- 90). Thus, the inclusion of the general protease inhibitor soybean trypsin inhibitor (STI) was included in the homogenization buffer. This requirement is clearly indicated in the Ultrogel AcA-34 filtration of the cytosol polymerase from nauplii of Artemia (Fig.12). The choice of Sephadex G-25 and Ultrogel AcA-34 as the preliminary gel filtration steps also helped to remove rapidly low molecular weight contaminants thereby avoiding prolonged standing at 0-4°C.

The processing of the nuclear enzymes (as described earlier) from dormant cysts and nauplii involved the isolation of nuclear DNA polymerase relatively free from contaminating yolk proteins (102) without causing severe reduction in enzyme activity. Several procedures using various ionic and non-ionic detergents and including sonication were tested to obtain the most gentle release of the polymerase enzyme from the nuclear pellet (data not included). The choice of the non-ionic detergent, NonIdet P40 at a final concentration of 0.1% lysed the nuclei in a short period of time and permitted the solubilization of the nuclear enzymes.

Although early attempts at using phosphocellulose or DNA cellulose columns (data not shown) regularly employed by DNA polymerase researchers (16,77,103) were successful to a limited extent, the enzymes purified on these columns lost most of their activity (approximately 90%) upon elution even in the presence of 20-25% glycerol and storage at -70°C. Thus a DEAE-cellulose ion-exchange column was employed and the DNA polymerases were eluted with KCl as shown in Fig.15 (a-d). Previously, Cervera *et al.* had indicated that DNases of *Artemia* do not bind to DEAE cellulose columns (104) and thus the use of such a column was advantageous in ensuring a preparation of DNA polymerase free of DNase activity. Although the use of this procedure resulted in relatively lower yields, it was considered more important to obtain active DNA polymerase preparations, despite low quantities, so that characterization of the enzyme(s) could be carried out.

It is noteworthy that the lability of nuclear DNA polymerase after phosphocellulose chromatography was reported by Cacace *et al.* (73) in their attempt to purify this polymerase from dormant cysts of *Artemia*. It is also pertinent to mention that this study indicated for the first time the potential for using DEAE cellulose in the purification of *Artemia* DNA polymerases.

Studies with purified enzyme fractions

The purified DNA polymerase fractions were stored in 50% glycerol at -70°C. The high percentage of glycerol was found to be necessary to preserve the DNA polymerase activity isolated from such varied sources as yeast cells (25) and bone marrow (30).

One of the early experiments with purified *Artemia* DNA polymerases was to determine the most suitable reaction requirements. Two representative enzymes namely, the cytosol fraction (PMF-1) of cyst and nauplii were tested. The presence of a primer-template (activated DNA), divalent cation, and all four deoxy-NTPs were found to be absolute requirements (Table 2). These enzymes also exhibited a requirement for free sulfhydryl groups. It is interesting to note that 50% of the control activity remained after omission of any one dNTP. As Chang and Bollum have reported, this may be due to the residual properties seen in absence of one to three dNTPs and is probably due to the properties of the template and not a characteristic of the enzyme (105).

To determine if this residual activity could be due to the presence of the enzyme terminal deoxynucleotidyl transferase (TdT), reaction mixtures for measuring TdT activity were set up omitting the DNA primer-template but including one dNTP in large excess to act as the substrate. Each *Artemia* DNA polymerase fraction was assayed using the reaction mixture but no detectable TdT activity was observed with any fraction tested (data not shown). Thus far, TdT activity has been detected only in mammalian cells particularly in calf thymus where it is found associated with DNA polymerase activity (106,107). When all three dNTPs were omitted, Artemia DNA polymerases showed a 80% decrease in enzyme activity. As Wang et al. have reported the removal of three dNTP's resulting in 90% decreased DNA polymerase activity using calf thymus preparations is indicative of the absence of TdT activity (108). Thus, Artemia DNA polymerase preparations appear to be free of significant TdT activity.

The primer required and the chemical nature of the product synthesized using Artemia DNA polymerases in a complete reaction mixture was investigated by use of specific nucleases (see Table 3). Ribonuclease A (which cleaves between Py:Py bonds of ribonucleic acids) and ribonuclease T1 (which cleaves between G:A bonds) had only a slight effect on the measurable incorporation of ^{3}H-TMP into to acid precipitable material, whereas the product synthesized by Artemia DNA polymerases was highly sensitive to pancreatic deoxyribonuclease treatment and therefore probably DNA in nature.

RNase A pre-treatment did not cause any loss in activity whereas post-incubation treatment with either of the RNases caused 19-28% loss in activity. These results suggest that most of the product is DNA-like and that Artemia DNA polymerases are DNA-dependent DNA polymerases.

Eukaryotic DNA polymerases have been reported to use a RNA-primer- initiator template in the initiation of DNA replication (109). Ikeda et al. reported that the addition of RNase A (.2 ug/ml) and RNase T1 (10 units/ml) decreased DNA synthesis in eukaryotic cells but had no effect once DNA elongation had commenced (110). Our results revealed that approximately 20% of the acid-insoluble product probably contains sensitive ribonucleotide phosphodiester linkages. However, this does not preclude the possibility that RNA primers may be involved in *Artemia* DNA replication and that this RNA primer, if present, is complexed or inaccessible to the RNase pre-treatment. Further studies with prolonged incubation periods and highly purified RNases are needed to further clarify the question regarding the need for an RNA initiator.

Regulation of *Artemia* DNA polymerase activity

Several reports have proposed that cytoplasmic factors act as positive regulatory signals and that these factors accumulate gradually during progression through the G1 phase in eukaryotic cells (111-113). Studies by Grummt et al. on growth control in animal cells indicated that purine nucleotides play a major role in the regulation of cellular activities related to DNA synthesis initiation (114,115). In particular, the dinucleoside polyphosphate, Ap_4A, was shown to be directly involved in the stimulation of DNA synthesis in baby hamster kidney cells by Grummt et al. (68,69). Also, they noticed that DNA replication is triggered in these cells by the specific binding of Ap A to DNA polymerase (70).

In Artemia diguanosine polyphosphates, Gp_3G and Gp_4G have been reported to be synthesized during oogenesis and cleavage respectively, and stored in yolk granules (5). Also, Gilmour and Warner have reported the presence of Gp_2G and Gp_3A in dormant cysts of Artemia (60). In the dormant cysts Gp_2G was found to be the predominant nucleotide and present in yolk platelets whereas Gp_3A was found only in the post-mitochondrial supernatant(6).

Our findings using naturally occuring diguanosine polyphosphates suggest that these nucleotides may be involved in a similar way to Ap_4A in regulation of DNA synthesis in Artemia. The cytosol polymerase (PMF-1 and PMF-2) of 36h nauplii exhibited a 20 and 82% increase in activity in the presence of Gp_2G and Gp_4G, respectively (see Fig.20). Since DNA synthesis was found to be maximal at the 36 hour incubation in Artemia development (see Fig.5) the activity of the DNA polymerases at this stage would be modulated according to the cellular requirement. This requirement could be in terms of DNA replication which in turn suggests the presence of several initiation points. Clegg and Finamore proposed that Gp_4G may be involved as a source of DNA-adenine for Artemia development and that Gp_4G metabolism was closely related to the resumption of DNA synthesis in Artemia (2). Adenine nucleotides have been shown to form the major component of eukaryotic DNA replication initiator sites (116). Hence, it appears that mainly Gp_2G (and to a lesser extent Gp_3G and Gp_4G) may be positive regulators of the activity of the cytosol DNA polymerases from 36h nauplii. As DNA synthesis is quiescent during the pre-hatch development of Artemia, such a stimulation in DNA polymerase activity in the presence of Gp_2G, Gp_3G and Gp_4G is unlikely. This was also indicative that Gp_2G and the related diguanosine polyphosphates may be linked to the resumption of DNA synthesis through activation of the cytosol DNA polymerases (probably the enzymes involved in Artemia DNA replication) in much the same way as Ap_4A regulation in baby hamster kidney cell DNA replication.

Gilmour and Warner (60) discovered the presence of Gp_3A in Artemia and proposed its role to be an intermediate in ATP synthesis on hatching. VanDenbos and Finamore had earlier suggested that the hybrid Gp_4A could be an intermediate in the synthesis of ATP in Artemia nauplii (64). In this study, addition of Gp_3A stimulated only the cytosol fraction (PMF-1) of the naupliar DNA polymerases (85%) whereas Gp_3A caused nearly 90% inhibition (see Fig.20) of all DNA polymerases fractions from the dormant cysts of Artemia. These findings suggest that Gp_3A could be an important regulatory molecule which controls the stage-specific expression of DNA polymerases in Artemia. Taken together these results support the view that the endogenous diguanosine polyphosphates are active in the regulation of DNA synthesis in Artemia and provides both for a 'switch-on' and 'switch-off' mechanism in DNA synthesis regulation.

Exogeneously added Ap_3A and Ap_4A on the other hand, did not have any significant effect on Artemia DNA polymerases (see Fig.21) suggesting that the interaction of Ap_4A with DNA polymerase α as reported by Grummt (34) may be confined only to mammalian cell systems. In Artemia other dinucleotide polyphosphates could substitute for Ap_4A in DNA synthesis and regulation.

Biochemical characterization of Artemia polymerases

Various parameters like pH optima, ionic requirements, preferred primer-template and studies with inhibitors were performed to determine some of the properties of the DEAE-cellulose purified DNA polymerases of Artemia.

The use of various buffers indicated that except for cyst cytosol DNA polymerase 1 (PMF-1), all other polymerases exhibited a pH optima of 7.5 (see Figs.22 and 23). Also, the DNA polymerases from 36h nauplii exhibited less tolerance for Tris-HCl than for potassium phosphate. The pH optima and incubation buffers have been used as criteria to discriminate among mammalian polymerases where the 6-8 S DNA polymerases of calf thymus tissue have a pH optima of 7.0 in potassium phosphate buffer, while the 3-4 S enzyme has a pH optima of 8.0 and preferred Ammediol-HCl buffer (101).

Our studies indicated that dormant cyst DNA polymerase 1 (PMF-1) which exhibited a preference for Tris-HCl at pH 8.0 was similar to the low molecular weight mammalian DNA polymerase while its counterpart from nauplii (PMF-1) was similar to the high molecular weight mammalian enzyme in its potassium phosphate tolerance and pH optimum (7.5).

The effect of the ionic environment on Artemia DNA polymerases was also studied using potassium, phosphate, and chloride ions at various concentrations. Studies with DNA polymerases from sea urchin (117) and Xenopus laevis (118) have shown that these enzymes are sensitive to varying concentrations of phosphate and chloride. The high molecular weight DNA polymerase from sea urchin embryos was found to be stimulated by 10-50 mM phosphate. However, the low molecular weight enzyme (also from sea urchin embryos) was found to be sensitive to phosphate concentration of 50 mM, which disappeared when phosphate was increased to 100 mM. These observations were obtained using activated-calf thymus DNA as the primer-template (119).

Our results (see Fig.24) using activated calf thymus DNA as primer- template revealed that DNA polymerases from 36h nauplii more closely matched the properties of the high molecular weight eukaryotic enzyme. However the cyst DNA polymerases were found to be stimulated at high concentrations of phosphate, which indicated a similarity to the low molecular weight eukaryotic enzymes (39) in its susceptibility to phosphate.

Studies with KCl indicated that DNA polymerases from nauplii were inhibited markedly by low concentrations of KCl (0.05M), whereas the cyst DNA polymerase 1 (PMF-1) was stimulated upto 0.2M KCl. (see Fig.25). Joenje and Benbow (118) have described the use of 0.25M KCl concentrations in reaction assays specifically designed to determine the β-polymerases from X. laevis. They have shown that under such conditions the high molecular weight α-polymerases are inhibited over 90% in the presence of 0.2M KCl (118). Our findings also suggest that the DNA polymerases of Artemia nauplii which resemble closely the high molecular weight enzyme of other species could be effectively inhibited using KCl in the 0.1-0.2 M range.

Studies by Kornberg with divalent cations had shown a requirement for the presence of an added divalent metal cation in eukaryotic DNA polymerases reactions (19). Sirover and Loeb (120) using activated calf thymus DNA as template have shown that the sea urchin DNA polymerase α (the high molecular weight enzyme) exhibited only 22% activity in presence of Mn^{2+} when compared to assay mixtures supplemented with Mg^{2+}. On the other hand, the β-polymerase of X. laevis (118) was dependent on Mn^{2+} ions when activated calf thymus was used as a primer-template.

The DNA polymerase in the cytosol fraction (PMF-1) of the dormant cyst tolerates Mg^{2+} or Mn^{2+} equally well, whereas the DNA polymerase (PMF-1) from the cytosol of 36h nauplii preferred Mg^{2+} as the divalent cation (see Fig.26). Thus it can be inferred that DNA polymerases have different requirements for divalent cations depending on the developmental stages from which the enzyme(s) is(are) isolated.

It is known that eukaryotic DNA polymerases cannot initiate DNA replication in the absence of a primer-template. Studies with activated calf thymus DNA and activated *Artemia* DNA (primer-templates) in reactions with *Artemia* DNA polymerases indicated a preference for calf thymus template by the cytosol fraction 1(PMF-1) from nauplii (Fig.27). Moreover, when 1 mM ATP or GTP was included in the reaction assay mixture, this DNA polymerase was stimulated. Such a preference for either the primer-template or the ribonucleotide 5'-triphosphate was not observed using PMF-1 from dormant cysts. Hence it can be concluded that the enzyme from nauplii is markedly different from the cyst DNA polymerase. Further studies using synthetic primer-templates and higher concentrations of ATP or GTP are needed to further elucidate the nature of *Artemia* DNA polymerases.

Studies with inhibitors

The use of several inhibitors to distinguish between the various eukaryotic DNA polymerases have been reviewed recently (121). Inhibitors like N-ethylmaleimide (NEM), dideoxythymidine 5'-triphosphate (d2TTP) and aphidicolin have been used in our investigations to characterise the Artemia DNA polymerases compared to polymerases from other sources.

Studies with NEM have shown that DNA polymerases from nauplii are severely inhibited compared to minimal inhibition observed using cyst DNA polymerases (see Fig.28). Bollum had indicated the use of thiol-blocking agents to be of importance in the classification of eukaryotic DNA polymerases (16). Dube et al. have shown that pre-incubation of the mammalian DNA polymerases with 10 mM NEM for 1 hour at 0°C, was useful in discriminating between the replicative α-polymerase from the β-polymerase which is believed to be involved in repair processes (122)(see Table 4). Wu and Gallo have even shown the inhibition of RNA-dependent DNA polymerases from RNA tumor viruses by thiol-blocking agents (123).

Our studies with crude embryo extracts or partially purified DNA polymerases from nauplii showed a significant loss in activity in the presence of NEM at concentrations as low as 1 mM (see Figs. 12 and 28). In studies with partially purified fractions, (Fig.28) we have used higher amounts of NEM (6mM final concentration) to compensate for the presence of B-mercaptoethanol in the reaction mixture. Smith and Gallo have reported the complete inhibition of α-polymerase from human lymphocytes at 10 mM NEM concentrations at which the β-polymerase from the same tissue was found to be relatively resistant (124)(see Table 4). This finding compares with that of Bollum, who found that calf thymus α-polymerase was almost completely inhibited at 1 mM NEM while the β-polymerases were unaffected even at 10 mM NEM (16). Hence it was concluded that differential sensitivity to NEM by itself may not be an adequate criterion to distinguish between the α and β DNA polymerases.

Despite the above limitations, the DNA polymerases (PMF-1) from Artemia nauplii responded to NEM in a fashion similar to the α-polymerases while the cyst enzyme (PMF-1) was more like the β-polymerases in its resistance to NEM. To study further the functional characteristics in terms of replication and repair for the Artemia nauplii and cyst DNA polymerase fractions, the effect of other more specific inhibitors were tested.

Dideoxythymidine 5'-triphosphate (d2TTP) belongs to the class of indirect inhibitors which include those agents that alter the structure and function of the template, i.e., primer DNA. Several reports have indicated that the addition of d2TTP to the template generates a primer-terminus devoid of the 3'-hydroxyl group rendering the resulting 'primer' incapable of further reaction with an incoming mo-

TABLE 4

Effect of N-ethylmaleimide on Mammalian α and β DNA polymerases[a]

Source	Percent	Inhibition[b]
Acute lymphatic leukemia	99	29-40
Human placenta	98	30-35
Calf liver	98	10-20
Novikoff hepatoma	100	20-30

a. from Dube et al. (1979).

b. Percent Inhibition of DNA polymerases when pre-incubated in the presence of 10 mM NEM for one hour at 37 degrees C.

nomer (41). In effect, this compound acts as a chain terminator. We have used this chain terminator to specifically characterise <u>Artemia</u> DNA polymerases in terms of replication and repair. Our studies indicated that the DNA polymerase (PMF-1) from dormant cysts was inhibited up to 85% in the presence of d2TTP (see Fig.29) whereas the naupliar polymerase (PMF-1) was inhibited only slightly (26%) by d2TTP.

Van der Vliet and Kwant have shown that mammalian DNA polymerase α was the only polymerase not inhibited by d2TTP and they have suggested that the inhibition by d2TTP depended on the number of 3'-ends available, the percent ratio of dTTP/d2TTP and the presence of exonucleases capable of hydrolysing the incorporated d2TMP at the 3'-hydroxyl end (125). The findings of Ono <u>et al</u>. lends support to this suggestion (126). When mouse myeloma α-pol ymerase was tested in the presence of d2TTP no inhibition was noticed upto 50 uM concentration while 5 uM concentrations inhibited the β-polymerase activity by 70%. It was also shown that the strongest inhibition of β-polymerase was observed when the enzyme was assayed at pH 8.5 using Mn^{2+} as the divalent cation. From these findings a competitive mechanism for d2TTP has been proposed for this nucleotide analog. The dormant cyst DNA polymerase (PMF-1) of <u>Artemia</u> has been shown to exhibit a pH optimum of 8.0 and to prefer Mn^{2+} over Mg^{2+}. The nearly complete inhibition observed using d2TTP (even at ratios of d2TTP/dTTP at 4 $\times 10^{-2}$) clearly suggest that PMF-1

DNA polymerase from dormant cysts is the repair enzyme rather than the replication enzyme in _Artemia_.

A number of deoxynucleoside triphosphate analogs have been shown to compete with normal substrates and to be incorporated into DNA in the in vitro DNA-dependent DNA polymerase reactions. Some of these analogs are incorporated at a slower rate and in cells, these may effectively terminate chain growth and cell division. Aphidicolin has been shown by Ikegami _et_ _al_. to be such an inhibitor which specifically inhibits mammalian α-polymerase (127). Dalziel and coworkers reported that aphidicolin prevented mitotic divisions in sea urchin embryos (128). Ohashi _et_ _al_. confirmed this finding and suggested that prevention of cell division by aphidicolin in sea urchin embryos is due to selective inhibition of the DNA polymerase-α activity (129). Further studies by the same group of researchers have shown that DNA polymerase-α from regenerating rat liver is inhibited upto 85% with 15 ug/ml of aphidicolin, whereas DNA polymerase-β or the mitochondrial DNA polymerase-γ is not inhibited even at 75 ug/ml.

Other studies have reported that aphidicolin is not a general inhibitor of all nucleic acid syntheses and that, 1) its action is reversible (130), 2) this drug has no effect on RNA or protein synthesis (132), 3) it did not inhibit repair synthesis in mitotic chromosomes (133) and 4) at 1 ug/ml concentration aphidicolin inhibited over 97% of the

α-DNA polymerase activity of mitotic chromosomes and this inhibition could be observed both under in vivo and in vitro conditions (134-136).

With the above background, aphidicolin was used in our studies to study the functional role of the DNA polymerases (PMF-1) from cysts and nauplii of Artemia. Our results (see Fig.30) revealed a complete cessation of naupliar polymerase activities at concentrations of 5 ug/ml, whereas none of the cyst DNA polymerases were affected under similar conditions. Thus while the naupliar DNA polymerase (PMF-1) was not inhibited by d2TTP, it was severely inhibited by aphidicolin which supports our conclusion that the nauplii enzymes are involved in replication. The cyst enzymes are not inhibited by aphidicolin but by d2TTP which supports the view that their functional role is in repair.

If the naupliar polymerase was indeed the replication enzyme, then it probably should be a high molecular weight enzyme similar to other eukaryotic α-DNA polymerases. Using sucrose density gradient analyses (see Fig.31b) the nauplii enzyme was found to have a sedimentation value of 6.7 S which is in the range (6-8 S) of other eukaryotic α-DNA polymerases. The DNA polymerase activity from the dormant cyst (see Fig.31a) sedimented at 3.5 S or similar to the low molecular weight (3-4 S) β-polymerases of eukaryotes (41).

A summary of the characteristics of the DNA polymerases (PMF-1) from the dormant cysts (0h) and nauplii (36h) of

Artemia is shown in Table 5. These enzymes have also been compared with the mammalian DNA polymerases α and β in Table 6.

The salient features of eukaryotic DNA polymerases α and β are outlined below. DNA polymerase-α has been shown to possess the ability to synthesize primary DNA pieces which are later ligated to large molecular weight DNA moleculea (at concentrations of d2TTP that inhibit β-polymerase) suggesting that it is responsible for initiation, elongation and gap-filling (137). Fisher and Korn have shown that α-polymerase has an effector or allosteric site that is capable of recognizing SS DNA (138). It was also shown that the α-polymerase preferred A-T rich regions (139) and that it acted in a processive fashion (140). Berezney has shown the association of DNA polymerase- within nuclear matrices from actively replicating regenerating rat liver cells (141). Veer Reddy and Pardee have localized mammalian α-polymerase to be perinuclear in origin (142).

DNA polymerase (PMF-1) isolated and partially purified from 36h nauplii of *Artemia* exhibited several characteristics similar to other eukaryotic α-polymerases. It is found in actively dividing cells and increases in amount as growth from cyst to nauplii proceeds. It indicates a preference for potassium phosphate buffer at pH 7.5 and Mg^{2+} as the divalent cation. It is inhibited at high concentrations of chloride but shows stimulation at 100 mM phosphate con-

TABLE 5

Features of the cytosol DNA polymerases in fraction-1 (PMF-1) from dormant Cysts (0h) and nauplii (36h) of *Artemia* *sp.*

	Dormant Cysts	Nauplii
Location	cytosol/nucleus	cytosol
Proposed function	repair	replication
S value	3.5	6.7
pH optimum	8.0	7.5
Preferred buffer	Tris-HCl	Potassium phosphate
Preferred cation (divalent)	Mn/Mg	Mg
Inhibitory Effects:		
Sulfhydryl (NEM)	weak	strong
Salt (0.1M KCl)	no	yes
Phosphate (0.1M)	yes	no
d2TTP	yes	no
Aphidicolin	no	yes
Effect of dinucleotide polyphosphates:		
Gp_2G, Gp_3G, Gp_4G	no stimulation	stimulation
Gp_3A	strong inhibition	weak inhibition
Ap_3A, Ap_4A	no stimulation	slight stimulation

TABLE 6

Comparison of the cytosol DNA polymerases in fraction-1 (PMF-1) of dormant cysts (0h) and nauplii (36h) of *Artemia* sp. with the mammalian α and β DNA polymerases[a]

	α	Nauplii PMF-1	β	Dormant cysts PMF-1
Location	nucleus	cytosol/nucleus?	nucleus	cytosol/nucleus?
Proposed function	replication	replication	repair	repair
S value	6-8	6.7	3-4	3.5
pH optimum	7.2	7.5	8.5	8.0
Preferred divalent cation	Mg	Mg	Mg/Mn	Mg/Mn
Inhibitory Effects:				
Sulfhydryl (NEM)	strong	strong	weak	weak
Salt (0.2M NaCl)	yes	yes	no	no
Phosphate (0.1M)	no	no	yes	yes
d2TTP	weak	weak	strong	strong
Aphidicolin	strong	strong	weak	weak

a from Kornberg, A. (1980).

centration. It prefers the pancreatic DNase-activated calf thymus DNA which is known to contain several SS regions. It is severely inhibited in the presence of NEM and aphidicolin while it is not affected by d2TTP. With the supposition that eukaryotic α poly merases possess an allosteric effector site, it is more likely that the nauplii polymerase (PMF-1) which is akin to the α-polymerases in several aspects, may also possess such a site where positive regulation by the dinucleoside polyphosphates (in particular the diguanosine polyphosphates) could be effective, since the polymerase from nauplii is stimulated in the presence of these compounds. Several prokaryotic and eukaryotic replication sites have been sequenced and these have shown the preponderance of adjacent A-T rich regions (143). It is interesting to hypothesize that the replicative DNA polymerase of <u>Artemia</u> (nauplii) is able to recognize such origins of replication through regulation by the diguanosine polyphosphates at its effector site.

The eukaryotic DNA polymerase-β was reported by Chang to be widely distributed in multicellular animals from sponges to mammals but are not present in bacteria, plants or protozoa thereby indicating a conservation of this enzyme through evolution (144). Yoshida and Masaki indicated the function of β-polymerase was mainly in repair (145) and Tanabe <u>et</u> <u>al</u>. have shown that β-polymerases from various sources have very similar physical and enzymological properties (78). The general characteristics of the β -enzyme include its presence only in quiescent cells of low molecular weight (30-40,000 M.W. range), alkaline pH optima, stimulation by high concentrations of salt (KCl) and not phosphate, a preference for Mn^{2+} and comparative resistance to NEM and aphidicolin while being severely inhibited by d2TTP. The DNA polymerase (PMF-1) from dormant cysts of Artemia exhibits most of these characteristics (see Table 6). Its role in repair is highly probable since it is present in dormant cysts which are metaboli cally inactive unless hydrated and incubated under aerobic conditions. Also, the hydrated cysts are highly resistant to ionizing radiations. The functional role of this enzyme is well suited for the embryonic stage from which it was isolated. The low molecular weight mediator Gp_3A exhibits significant regulation of this enzyme activity under in vitro assay conditions. Since repair is a process triggered only when necessary, a 'switch-on' and 'switch-off' mechanism induced by the presence of Gp_3A could modulate the cyst DNA polymerase activity. Further characterization studies on the DNA polymerases at the 12 hour stage in Artemia development may indicate the regulation of the DNA polymerases by the diguanosine polyphosphates in switching from repair to replication.

In summary, then, a purification procedure for the isolation of Artemia DNA polymerases has been developed for the first time, using DEAE-cellulose chromatography although further improvements for the complete separation of the nuclear and cytosol DNA polymerases need to be developed. Artemia DNA polymerases from two selected stages namely, dormant cyst (0h) and nauplii (36h) have been partially purified and characterized. It has been shown that the cyst DNA polymerase and naupliar DNA polymerase probabaly have different functional roles in development: the cyst enzyme in repair and the naupliar enzyme in replication. They also have many biochemical properties similar to the mammalian systems. It is suffice to state that complete characterization of each DNA polymerase fractions obtained by DEAE-cellulose chromatography was not possible within the scope of this study. However, it can be suggested that the cytosol and nuclear fractions from cysts and nauplii eluting at defined positions from DEAE-cellulose (viz. PMF-1, NF-1) exhibited similar characteristics and therefore could be part of the same enzyme pool. The other active fractions of the nauplii and cysts (namely, PMF-2 and NF-2) which eluted at higher salt concentrations could be either reactive subunits or associated activities of the same enzyme. This could be tested further by mixing experiments which were not carried out in this study. The endogenous dinucleoside polyphosphates appear to influence the expression of the repair and replication enzymes of Artemia. Further studies using defined and synthetic primer-templates would be required to define the nature of the regulation by the low molecular weight mediators present naturally in _Artemia_. The functional role of _Artemia_ cyst DNA polymerase has lent support to the hypothesis that the β-polymerase is conserved through evolution.

Chapter VI

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