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Diguanosine tetraphosphate pyrophosphohydrolase in the development of the brine shrimp Artemia salina.

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DIGUANOSINE TETRAPHOSPHATE PYROPHOSPHOHYDROLASE IN THE DEVELOPMENT OF THE BRINE SHRIMP *ARTEMIA SALINA*

BY

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ABSTRACT

In order to study the importance of P^1, P^4 -diguanosine 5' -tetraphosphate asymmetrical-pyrophosphohydrolase (asym-diGDPase) during the development of the brine shrimp, Artemia salina, an earlier purification procedure (Warner and Finamore, 1965a) has been modified so that contaminating proteases found in the developmental stages of this crustacean are removed. Asym-diGDPase so purified has been examined with respect to the hydrolysis of two dinucleotides, diguanosine triphosphate (Gp_3G) and diguanosine tetraphosphate (Gp_4G), and the optimal conditions for hydrolysis of these two dinucleotides were found to differ markedly with respect to the metal ion Mg^{2+}. In addition, the molecular weight of this enzyme was estimated at $2.0 \times 10^4 \pm 0.10$ using G-200 Sephadex. Finally, the activity of asym-diGDPase increases markedly during development and is related to the utilization of these two dinucleotides during the early embryogenesis of Artemia salina.

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INTRODUCTION

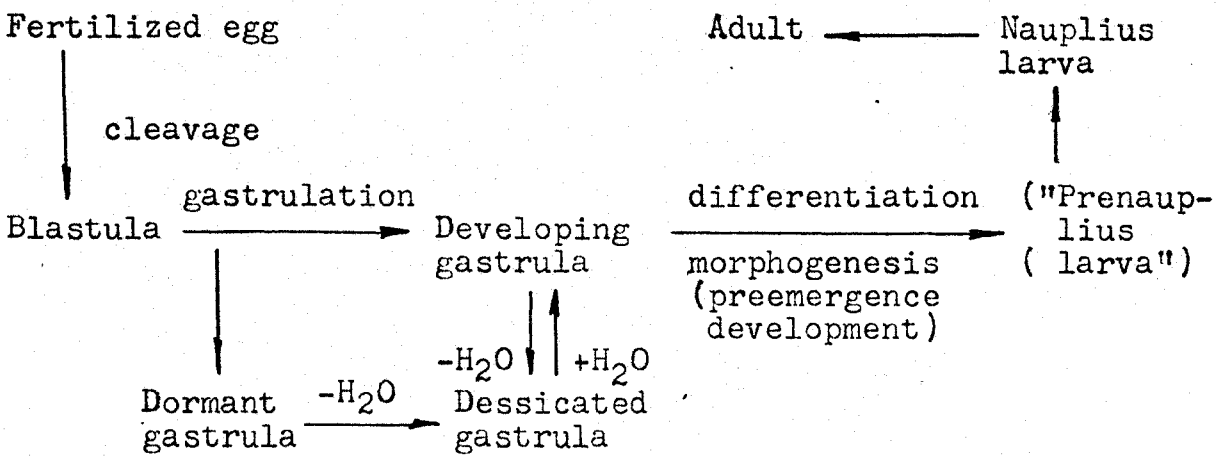
The relationship between a given enzyme and the overall development of a multicellular organism is highly complex (Moog, 1965). With regard to enzyme studies in developing systems, most of the significant literature is only 30 years old. Joseph Needham in his three volume summary of chemical embryology up to 1931 suggested that much of the research involving enzyme studies in embryos is limited by the techniques available. Notwithstanding this lack of information, however, he had this to say regarding enzyme activity in developing organisms: "In any case, it seems likely that the embryonic body starts life with an assortment of fundamental enzymes, or a collection of fundamentally active surfaces, to which, as development goes on, certain others are added, and from which possibly others are subtracted" (Needham, 1931). It is true that this statement by Needham is a general one, but it seems that studies undertaken since 1931 have proven it valid. For example, experimentation on sea urchins and amphibians have shown that the egg at the time of fertilization contains, in addition to its nucleic acids a variety of structural proteins and a large number of enzymes (Boell, 1955; Wallace, 1961). In the chick it is clear that enzymes or other specific proteins are identifiable in the early blastoderm (Moog, 1952; Ebert et al., 1955). Sensitive immunological techniques, however, indicate that some of these enzyme proteins are

absent at very early stages and must be the result of synthesis (Ebert et al. 1955; Holtzer et al., 1957). Finally, recent studies involving RNA synthesis in amphibians and sea urchins (Spirin, 1966; Gross, 1967; Brown, 1967) have indicated that informational RNAs are present in the unfertilized egg and are required for synthesis of both structural and catalytic proteins during early development. The informational RNA and its functional complex (polysomes) may be interpreted as the "active surfaces" to which Needham referred some thirty years ago.

Although enzymes involved in development have been studied mainly in embryos of amphibians and in marine invertebrates, particularly the sea urchins, several studies have been reported using embryos of the brine shrimp, Artemia salina. In 1957 Bellini reported changes in dipeptidase and protease activities during the 90 hours immediately following cessation of dormancy, and Urbani and Bellini (1958) studied changes in acid and alkaline ribonucleases during a similar period of Artemia development. For virtually all enzymes analysed it appears that following cessation of dormancy a gradual but slight increase in enzyme activity occurs during prenauplius development followed by a marked increase immediately after hatching. However, activities of all enzymes studied heretofore have been shown to decline sharply with age in swimming nauplii. It is difficult to attribute much significance to the studies on Artemia particularly during the period of post-hatch development, because in all cases the enzyme activities

were determined using extracts from crude homogenates which often contain considerable amounts of protease activity. Protease contamination is of major importance in all but very short term incubation studies and must be considered in all assays carried out on extracts of swimming nauplii. Furthermore, newly hatched nauplii must be maintained in as aseptic an environment as possible and both the temperature and osmotic pressure of their environment must be carefully regulated.

Before proceeding further, and in order to render subsequent discussion clearer, a brief outline of brine shrimp development is in order. Eggs are produced in the ovaries of the female then transported to the ovisac where fertilization takes place. After the eggs are fertilized, development proceeds to the blastula stage. At this stage development may be suspended and the embryos encysted in a tough chitinous shell or development may continue uninterrupted. The encysted embryos are dessicated by the environment and can remain dormant for several years. Upon hydration dormant embryos will resume development through the instars characteristic of this crustacean to a free-swimming adult. If development of the embryo is not arrested, then metamorphosis proceeds normally into a free-swimming nauplius with either ovoviviparous or viviparous parturition. The overall embryology of Artemia can be summarized diagrammatically as follows: (Finamore and Clegg, 1969, p.250)



In 1963, a unique dinucleotide, P^1, P^4-diguanosine 5'-tetraphosphate[1] was reported to be present in large quantities in the encysted embryos of Artemia salina (Finamore and Warner, 1963). In 1965 the same investigators discovered that a similar compound, P^1, P^3-diguanosine 5'-triphosphate is present in lesser amounts in these embryos (Warner and Finamore, 1965b). Since the discovery of these two dinucleotides, several studies have been undertaken to ascertain their role in development. At first, since Artemia salina is a cryptobiotic organism, Gp_4G was thought to be a molecule which stored energy during dormancy (Finamore and Warner, 1963; Clegg, Warner and Finamore, 1967). Subsequent research, however, demonstrated that Gp_4G is present in both encysted and nonencysted embryos and suggested that this important nucleotide anhydride may not

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[1]Abbreviations used in this paper will follow the IUPAC-IUB rules. In addition the following abbreviations will be used: Gp_4G, P^1, P^4-diguanosine 5'-tetraphosphate, Gp_3G, P^1, P^3-diguanosine 5'-triphosphate; asym-diGDPase, P^1, P^4-diguanosine 5'-tetraphosphate asymmetrical pyrophosphohydrolase (E. C. No. Pending).

simply be a protective mechanism of phosphate bond energy during encystment (Warner and McClean, 1968). This same study also indicated the following: (1) Artemia cannot synthesize purines de novo but depends on the environment for its source of purines; (2) Gp_4G is synthesized only in the female; (3) Gp_3G is not synthesized at the same time as Gp_4G but appears after cleavage; (4) both Gp_4G and Gp_3G disappear rapidly during post-hatch development, and both compounds incorporate ^3H guanosine but Gp_3G only slightly during this period of development. Finally, during the period of Gp_4G and Gp_3G utilization, there is little fluctuation in the total purines. However, the concentration of total guanine decreases during this period and is accompanied by an increase in total adenine which matches the guanine decrease (Clegg, et al., 1967). Thus it seems that Gp_4G and Gp_3G are storage forms of purines required for early development of Artemia, and that "most" of the hydrolytic products of these two compounds are converted into adenine compounds.

In an earlier study Warner and Finamore (1965a) demonstrated that the encysted embryo of Artemia contains an enzyme that functions in the metabolism of diguanosine tetraphosphate during development. Their studies indicated that the enzyme hydrolyses Gp_4G into equimolar amounts of GMP and GTP and called the enzyme, P^1, P^4-diguanosine 5'-tetraphosphate asymmetrical-pyrophosphohydrolase (asym-diGDPase). However, these authors were unable to demonstrate that this enzyme is active in the hydrolysis of Gp_3G. Since both of these

dinucleotides are involved in the early development of Artemia, the purpose of this study was: (1) to determine the enzyme(s) responsible for the hydrolysis of Gp_3G; and (2) to correlate the activity of asym-diGDPase with the disappearance of Gp_4G and Gp_3G during early development.

MATERIALS AND METHODS

Preparation, Hatching and Collection of *Artemia* Embryos

Encysted embryos of the brine shrimp, *Artemia salina*, used throughout these experiments were obtained from the Great Salt Lake area (Sanders Co., Ogden, Utah). Prior to use, the cysts were sterilized by gentle stirring in 7% antiformin solution for 15 minutes at 5°C (Nakanishi *et al.*, 1962). After the antiformin treatment and removal of cracked cysts and debris, 2.0 gram portions were introduced into glass petri dishes (2 x 14 cm) and covered with 50 ml of sterile artificial sea water.[2] These embryos were maintained by gentle shaking at 30°C until needed. Under these conditions prenauplii emerged between 9 and 14 hours and swimming nauplii appeared between 13 and 18 hours. Variations in this schedule have been noted by McClean and Warner (1970) and were due to developmental divergencies of commercially available encysted embryos. Prenauplius larvae were obtained as follows. At 12 hours incubation the contents of 20 dishes were combined in a large culture dish (8.5 x 24 cm) and swirled gently for a few minutes. Due to differences in buoyant density between cysts and

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[2] The salts used in this sea water are given by Warner and McClean (1968), p. 279.

prenauplii[3] a ring of these larvae formed near the periphery of the dish. These animals were harvested, washed in distilled water, introduced into large glass culture dishes (6 x 20 cm) containing 250 ml of freshly prepared sterile sea water, and maintained with gentle shaking at 30°C for 12 additional hours. At the end of this period (24 hours total), the swimming nauplii were harvested and washed with distilled water. They were then either frozen or transferred to fresh, sterile sea water to permit further development. Swimming nauplii were maintained until needed at 20-22°C in sea water supplemented with 0.05 gram/ml of NaCl[4] and antibiotics [1000 IU/ml of penicillin G (sodium citrate buffered, Squibb & Sons, Montreal) and 100 ug/ml streptomycin sulphate (Mann Research Laboratories, New York)] to supress background growth. Under these

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[3] The encysted embryo of Artemia possess two membranes. The outer membrane is hard and unyielding and composed chiefly of chitin. The inner membrane is very thin and yielding. When Artemia cysts hatch the prenauplii emerge still attached to the outer capsule by the inner membrane, but hang down from it, as Spangenberg says "wie die Gondel am Bollon" (Spangenberg, 1875).

[4] Swimming nauplii vary considerably in the molarity of salt required to achieve optimum development in vitro (Boone and Baas-Becking, 1931; Dutrieu, 1960; Fautrez-Firlefyn, 1961; and D'Agostino and Provasoli, 1968). Nauplii maintained at room temperature can develop through the first two instars, 4-7 days after hatching, at the expense of stored yolk protein (Provasoli and Shiraishi, 1959). We have found that encysted embryos from the salterns of Utah hatch more readily in a medium which closely resembles the molarity and mineral composition of the Great Salt Lake. However, nauplii beyond 36 hours of development require 0.05 gram per ml additional NaCl in the medium to insure optimal development.

conditions, nauplii can be maintained for 4-5 days without feeding. At the desired developmental stage, sufficient nauplii were collected in a 2000 ml beaker and stirred to distribute evenly. Three 1.0 ml aliquants were removed and streaked on 3 x 47 cm strips of Whatman #1 filter paper. The animals on each strip were counted twice and the counts averaged. From these data and the volume of sea water the total population was determined within ±7% (McClean and Warner, 1970). After counting, the nauplii were collected on a silk screen filter, washed three times with distilled water and once with 0.3 M NaCl containing 0.05 M Tris-HCl, pH 7.4, and stored at -20°C.

Pulse Labelling With ^{14}C-Bicarbonate

Aseptically maintained embryos at several stages of development were counted, washed several times with bicarbonate-free sea water, then placed into petri dishes (9 x 5 cm) containing 47.5 ml of sterile bicarbonate-free sea water fortified with streptomycin, penicillin and additional NaCl as previously described. To each dish was added 2.5 ml of sea water containing 0.1 mc/ml ^{14}C-bicarbonate (S. A., 54.5 mc/mM, Nuclear-Chicago) and each dish was sealed with saran wrap and a glass cover. The embryos were maintained in the radioisotope medium for three hours at 30°C then the contents of each dish were decanted into a liter beaker, diluted with sea water containing unlabeled NH_4HCO_3 (10 umoles/ml), and washed with distilled water on a nylon screen prior to storage at -20°C.

Preparation and Purification of Asym-DiGDPase from Encysted Embryos

The purification procedure outlined here is a modification of an earlier procedure by Warner and Finamore (1965a). Dried cysts were ground in a motorized mortar and pestle, (F. Kurt Retsch) and the powder was then extracted in 5 volumes of 0.3 M NaCl containing 0.05 M Tris-HCl, pH 7.5, for one hour at 5°C.[5] The extract was centrifuged at 23,500 g for 20 minutes and the supernatant fluid decanted through several layers of cheese cloth and a cellulose pad (CF-1) to remove suspended debris and lipids. The clear amber-coloured extract was treated with solid ammonium sulfate and the fraction insoluble between 50 and 95% saturation collected by centrifugation. The ammonium sulfate precipitate containing 85-90% of the total activity was dissolved in 25 ml of 0.05 M Tris-HCl, pH 8.5, and applied to a G-100 Sephadex column (2.5 x 100 cm) previously equilibrated with the same buffer. This step removes residual ammonium sulfate and nucleotides and separates the enzyme(s) from a considerable amount of protein. The active fractions were combined and applied to a DEAE-cellulose column (1.0 x 15 cm) equilibrated with the same buffer. The enzyme was washed on the column with 250 ml of the Tris buffer, and the elution was carried out using a linear gradient of NaCl in the Tris buffer from 0.05M (125 ml) to 0.4M (125ml). The

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[5] Unless otherwise indicated, all enzyme purification steps were carried out in the cold room at 5°C.

active fraction from this column was concentrated through a Diaflo membrane (Um-10) at a pressure of 80 p.s.i. of N_2 then stored at -20°C. Protein determinations were made using the method of Lowry et al. (1951). Since crude homogenates contain considerable folin positive material such as amino acids, lipids etc., protein values were obtained on acid-insoluble protein from which lipid material was removed by incubation at 40°C with acetone or an ethanol-ether mixture (3:1).

Preparation of Enzyme from Developing Embryos

Artemia salina nauplii in the early stages of development have been shown to contain considerable amounts of protease activity (Coromaldi & Urbani, 1959; Bellini, 1957; Coromaldi, 1961). Since the presence of protease activity in extracts from developing embryos presented serious difficulties not encountered in extracts from undeveloped cysts, the purification scheme used for preparation of cyst enzyme had to be modified. Each frozen sample was homogenized in a Duall-type homogenizer using 5 volumes of 0.3 M NaCl buffered with 0.05M Tris-HCl, pH 7.5, and the 50-95% ammonium sulfate fraction obtained exactly as before. The 50-95% ammonium sulfate fraction was dissolved in 10-15 ml of 0.05 M Tris-HCl, pH 8.5, and percolated through a G-25 Sephadex column (2 x 50 cm). The active fraction from the Sephadex column was treated with 10.0 mg of chicken ovomucoid (Worthington Biochemical, Montreal) and the total fraction applied to a DEAE-cellulose column (1 x 15 cm) previously equilibrated with 0.05 M Tris-

HCl, pH 8.5. The column was washed with 250 ml of the starting buffer and the active protein was eluted from the column using a linear gradient of NaCl in the Tris buffer from 0.05M (125 ml) to 0.4M (125 ml). The active fractions were pooled and reduced to a paste through a Diaflo membrane (Um-10) at 80 p.s.i. of N_2. Finally the enzymatically active fraction was dissolved in 1.0 - 1.5 ml of 0.05M Tris-HCl, pH 8.5, and stored at -20°C.

Polyacrylamide Gel Electrophoresis

Polyacrylamide gels were prepared from a stock solution containing 0.3 g/ml acrylamide, 0.002 g/ml bis-acrylamide, 0.0046 ml/ml of N, N, N^1, N^1, tetramethylenediamine (Eastman Kodak, Rochester, N.Y.), and 50 umoles/ml of Tris-HCl, pH 8.5. This stock solution was diluted with distilled water to give the desired gel concentration and ammonium persulfate was added to a final concentration of 2.3×10^{-4} g/ml to catalyse the polymerization.

Electrophoretic studies were carried out using 10% or 15% gels. For separation of ^{14}C-labelled protein, 10% gels were used at 300 volts and 0.8 to 1.6 ma/cm current from a constant voltage power supply (E.C. Model 454). To illustrate separation of protein from different stages of development 15% gels were used at 300 volts and 4-6 ma/cm current. Gel slabs to be stained were treated for one hour with a 0.25% aqueous solution of amido black (E. Merck A.G., Darmstadt, Germany) and destained in an electrophoretic destainer (E-C model 479). Records of

stained protein were obtained using a recording densitometer (Photovolt, model 542).

The location of asym-diGDPase activity was determined on unstained gels using the following procedure. The gel was frozen, sliced into 2 mm strips and one half of each strip was assayed for asym-diGDPase and the other half was counted in a liquid scintillation system (Nuclear-Chicago, Mark II), using the method of Basch (1968).

Isolation and Purification of Naturally-Occurring Substrates

Isolation and purification of Gp_3G and Gp_4G were routinely carried out by a modification of the method of Warner and Finamore (1965b). Artemia cysts in 100-gram batches were ground dry in a motorized mortar and pestle then homogenized in a Waring blender with five volumes of ice-cold 1.0 N $HClO_4$ for five minutes. The slurry was stirred for one hour and centrifuged at 23,500 g for 20 minutes. The gold-coloured supernatant fluid was deacidified through charcoal (Cohn, 1965) and 50,000 units (O.D. 260) were applied to a Dowex-1-formate column (2 x 50 cm) and fractionated using the elution scheme of Warner and Finamore (1965a). The Gp_3G and Gp_4G fractions were deacidified through charcoal and rechromatographed on separate Dowex-1-formate columns (2 x 25 cm). The diguanosine nucleotide fractions were deacidified as before then chromatographed on separate DEAE-cellulose columns (2 x 50 cm) using a linear gradient of NH_4HCO_3 at pH 8.6 according to Warner and Finamore (1967). The NH_4HCO_3 was removed by vacuum evaporation then

the dinucleotides were passed through a Dowex 50-H^+ column (1 x 6 cm) and neutralized with sodium or ammonium hydroxide.

Preparation of ^{32}P-labeled Diguanosine Triphosphate

^{32}P-labeled Gp_3G was synthesized using the method of Adam and Moffat (1961, 1964, 1966) and the ^{32}P-labeled compound was separated from other radiolabeled guanosine nucleotides by chromatography on columns of DEAE-cellulose (Warner and Finamore, 1967). The purity of the compound was determined by chromatography of the substrate and products of snake venom phosphodiesterase hydrolysis, ie., GMP and GDP, on columns of Dowex-1-chloride (1 x 10 cm).

Enzyme Assay Procedures

It has been shown (Warner and Finamore, 1965a) that asym-diGDPase will hydrolyse Gp_4G into equimolar amounts of GMP and GTP. In this study it was observed that the partially purified enzyme hydrolyses Gp_3G into equimolar amounts of GMP and GDP. Since both Gp_3G and Gp_4G are resistant to bacterial alkaline phosphatase (Finamore and Warner, 1963; Warner and Finamore, 1965b), whereas their hydrolytic products are not, liberation of P_i by alkaline phosphatase treatment of the products of asym-diGDPase activity (GMP, GDP or GTP) can be used as an assay for this enzyme.

During the purification of asym-diGDPase and for routine isolation of enzyme all assays were done as follows. Enzyme from the desired fraction was incubated for 20-30 minutes at

40°C with 0.8 umoles Gp_4G, 1.6 umoles $MgCl_2$, 20 umoles of Tris-HCl, pH 8.5, and an excess of alkaline phosphatase (Worthington Biochemical, Montreal) in a final volume of 1.5 ml. The reaction was terminated by the addition of 0.25 ml of 10 N H_2SO_4 and P_i was determined using the method of Ernster et al., (1950). When Gp_3G was used as substrate, it was found necessary to omit the alkaline phosphatase treatment until after termination of the asym-diGDPase reaction because alkaline phosphatase appeared to compete with the enzyme for the available Mg^{2+} ions. Therefore, the Gp_3G assays were carried out as follows. Enzyme from the desired fractions of either columns or gels was incubated with 1.0 umole Gp_3G, 25 umoles $MgCl_2$, 25 umoles Tris-HCl, pH 8.5, in a final volume of 1.5 ml at 40°C. The reaction was terminated by heating the reaction vessel at 90°C for five minutes, and after cooling, an excess of alkaline phosphatase (about 25 ug enzyme protein) was added to each tube and the tubes incubated for the desired time at 40°C. The amount of P_i liberated was determined as described above. (p. 15).

When activity measurements were to be made on enzyme extracts the following procedures were used. Each reaction vessel contained 1.0 umole Gp_4G, 4.0 umoles $MgCl_2$, 25 umoles Tris-HCl, pH 8.5, enzyme protein and an excess of alkaline phosphatase in a final volume of 1.0 ml. All reaction vessels were incubated at 40°C and at the desired times 0.25 ml aliquants were removed and deproteinized with $HClO_4$. The

precipitate was removed by centrifugation and P_i in the supernatant fluid was determined using the method of Ernster et al., (1950). (In all cases P_i liberated from endogeneous monoesterified compounds by the alkaline phosphatase was determined using heat denatured enzyme controls).

Finally, when the assay employing alkaline phosphatase could not be used, e.g. in determination of optimal Mg^{2+} requirements, chromatography on Dowex-1-Cl columns (1 x 2.0 cm) was used. In this assay procedure each reaction vessel contained asym-diGDPase, 2.0 umoles substrate (Gp_4G or Gp_3G), varying amounts of Mg^{2+}, 100 umoles Tris-HCl, pH 8.5, in 2.0 ml final volume. The reaction was initiated by addition of enzyme and the incubation temperature was 40°C. At desired times, 1.0 ml aliquots were removed and the reaction terminated by the addition of 0.20 ml 5N $HClO_4$. After neutralization with N-alamine (Warner and Finamore, 1967), the samples were applied to the Dowex-1-Cl columns and the amount of GMP eluted with 0.01 N HCl was determined.

Molecular Weight Determination of Asym-DiGDPase

Molecular weight determinations were carried out on a G-200 Sephadex column (2.5 x 36 cm). The column was washed well with 0.05 M Tris-HCl, pH 8.5 and the void volume of the column determined using dextran blue. Four homogeneous proteins of known molecular weight (ribonuclease A, 13,7000; chymotrypsinogen A, 25,000; ovalbumin, 45,000; and gamma globulin, 160,000) were chromatographed and the relationship

between elution volume and molecular weight determined. Asym-diGDPase purified through either G-100 Sephadex or DEAE-cellulose was concentrated using a UM-10 Diaflo membrane with a constant nitrogen pressure at 80 p.s.i. The concentrated protein was dissolved in 2.0 ml of the starting Tris buffer and applied to a G-200 Sephadex column previously equilibrated with 0.05M Tris-HCl, pH 8.5. The active protein was located using the Pi liberation method outlined earlier (pg. 14 - 15).

RESULTS

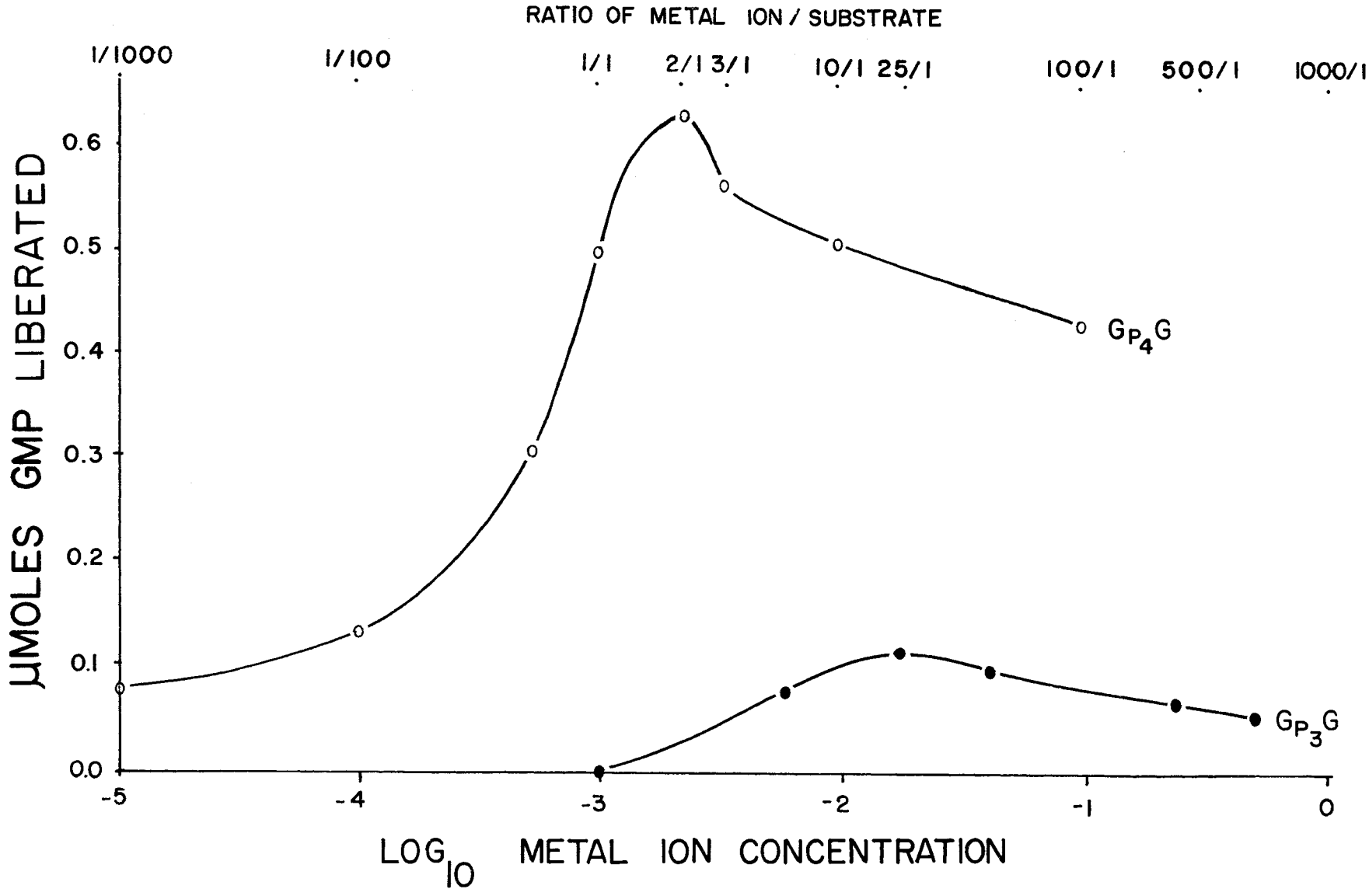
Hydrolysis of Gp_3G by Asym-diGDPase

Previously it was reported (Warner and Finamore, 1965a) that asym-diGDPase is inactive using Gp_3G as substrate under conditions optimal for the hydrolysis of Gp_4G. These investigators, however, did not measure enzyme activity using diguanosine triphosphate under varying Mg^{2+} concentrations. When Gp_3G is used as substrate and enzyme activity determined under varying Mg^{2+} concentrations, the data shown in Figure 1 are obtained. It is evident that very little hydrolysis of Gp_3G occurs at Mg^{2+} concentrations optimal for Gp_4G hydrolysis (.0013M), although higher concentrations of Mg^{2+} (.025M) cause some hydrolysis of Gp_3G. When the ratios of Mg^{2+}/dinucleotide are compared it was observed that the ratios are 2:1 and 25:1 for for Gp_4G and Gp_3G, respectively. In these experiments the primary products of asym-diGDPase hydrolysis were determined by column chromatography on Dowex-1-chloride to avoid difficulties with the P_i assay under high Mg^{2+} concentrations. In separate experiments employing ^{32}P-labeled Gp_3G as substrate, similar results were obtained. In addition, it was determined that asym-diGDPase hydrolyses Gp_3G into equimolar amounts of GMP and GDP. In no case is Gp_3G affected if incubated alone with high concentrations of Mg^{2+} or with heat denatured enzyme protein in Tris-HCl, pH 8.5.

FIGURE 1

Magnesium Requirements of Asym-diGDPase with respect to Gp_3G and Gp_4G.

Each reaction vessel contained 2.0 umoles Gp_3G, 200 umoles Tris-HCl, pH 8.5, 435 ug protein in 2.0 ml final volume. At the desired times a 1.0 ml aliquot was taken, treated with cold 5.0 N $HClO_4$ and the precipitate removed. The soluble fraction was neutralized with N-Alamine then chromatographed on columns of Dowex-1-chloride (1 x 2.0 cm). After washing the columns with distilled water and 0.01 M NH_4Cl, GMP was eluted with 0.01 N HCl. The Gp_4G data was obtained in a similar manner by Warner (1964).



In a previous study Warner and Finamore (1965a) demonstrated that asym-diGDPase preparations purified on columns of CM-cellulose contained several other protein bands as evidenced by acrylamide gel electrophoresis. In the present study it was necessary to determine whether Gp_3G was hydrolysed by the same enzyme or by some other component in the preparation. Using ^{32}P-labeled substrate as well as naturally occurring Gp_3G and Gp_4G, it was found that the enzymatic hydrolysis of Gp_3G and Gp_4G was coincident on columns of G-100 Sephadex, CM-cellulose, or DEAE-cellulose. These data are summarized in Figure 2. In addition, when the DEAE-cellulose purified enzyme was further fractionated by acrylamide gel electrophoresis as outlined previously, the protein active using Gp_3G as substrate was found in the same portion of the gel as that for Gp_4G. This is shown in Figure 3. It is evident that asym-diGDPase activity is bimodal when Gp_4G is used as substrate, whereas for Gp_3G this may not be the case. However, the low activity of Gp_3G makes it difficult to fully elucidate the differences at the present time.

Finally, the Km values for Gp_3G and Gp_4G were determined to be $5.9 \times 10^{-3}M$ and $1.1 \times 10^{-3}M$, respectively. However, it should be pointed out that these values were determined on DEAE-cellulose purified enzyme. In an earlier study, Clegg, Warner and Finamore (1967) reported that the rate of disappearance of Gp_4G and Gp_3G during the first day of post-hatch development is 8.8 and 1.4 umoles/mg protein/day, re-

FIGURE 2

Purification of Asym-diGDPase on Various Adsorbents

All assays were carried out using the P_i liberation method described in Materials and Methods. In (a) a 50-95% ammonium sulfate fraction was applied to a Sephadex G-100 column (2.5 x 100 cm) and the protein eluted with 0.05 M Tris-HCl buffer, pH 8.5. Activity using Gp_3G was measured by counting $^{32}p_i$ liberation and activity using Gp_4G as P_i. In (b) a sephadex treated fraction was dialyzed for 4.0 hours against 0.025 M NaOAc pH 4.0 and after pH adjustment to 4.0 with 5N HOAc was applied to a CM-cellulose column (2 x 20 cm) and eluted using a linear gradient of 0.05 M NaCl in 0.025 M NaOAc, pH 4.0 (250 ml) to 1.0 M NaCl in 0.025 M NaOAc, pH 4.0 (250 ml). In (c) a sephadex treated fraction was applied to a DEAE-cellulose column, (1 x 15 cm) and eluted using a linear gradient of 0.05 M Tris-HCl, pH 8.5 (125 ml) to 0.4 M NaCl in 0.05 M Tris-HCl, pH 8.5 (125 ml).

●——●, optical density at 280 nm.
○——○, activity using Gp_4G.
◒——◒, activity using Gp_3G.

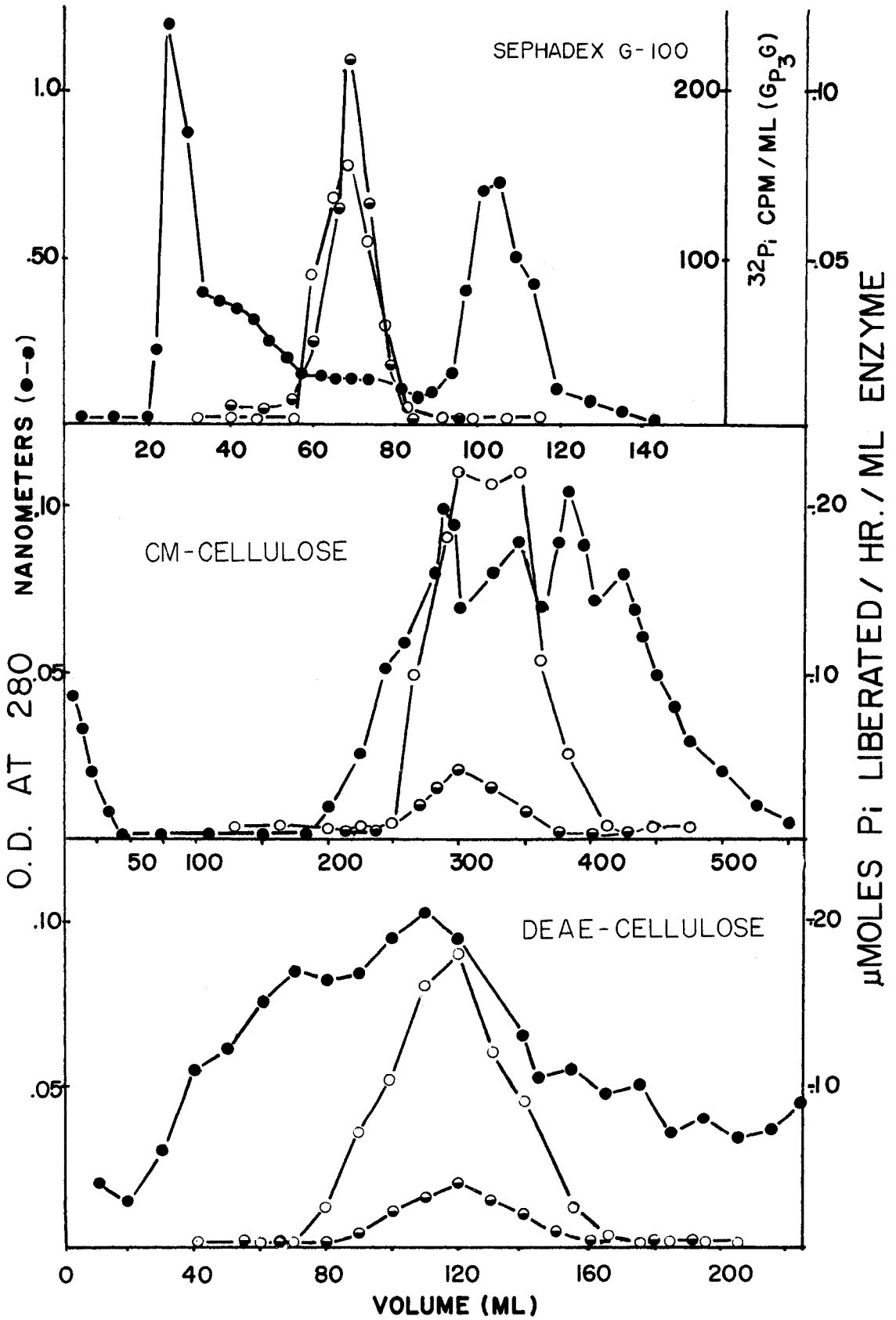


FIGURE 3

Migration of Asym-diGDPase on Acrylamide Gels.

Active protein was purified from both cysts and nauplii and separated on 15% acrylamide gels as described in Materials and Methods. 1.2 mg protein was applied to each gel slot and electrophoresis was carried out at 300 V and 4-6 ma/cm for 2.0 hours. Assays were performed on 2 mm slices cut in half, one half with Gp_3G and the other half with Gp_4G. The densicord tracing is from a separate but similar electrophoretic separation of 300 ug of purified cyst enzyme. All assays were carried out using the P_i liberation method. Activity using Gp_4G, O——O ; activity using Gp_3G, ◒——◒ .

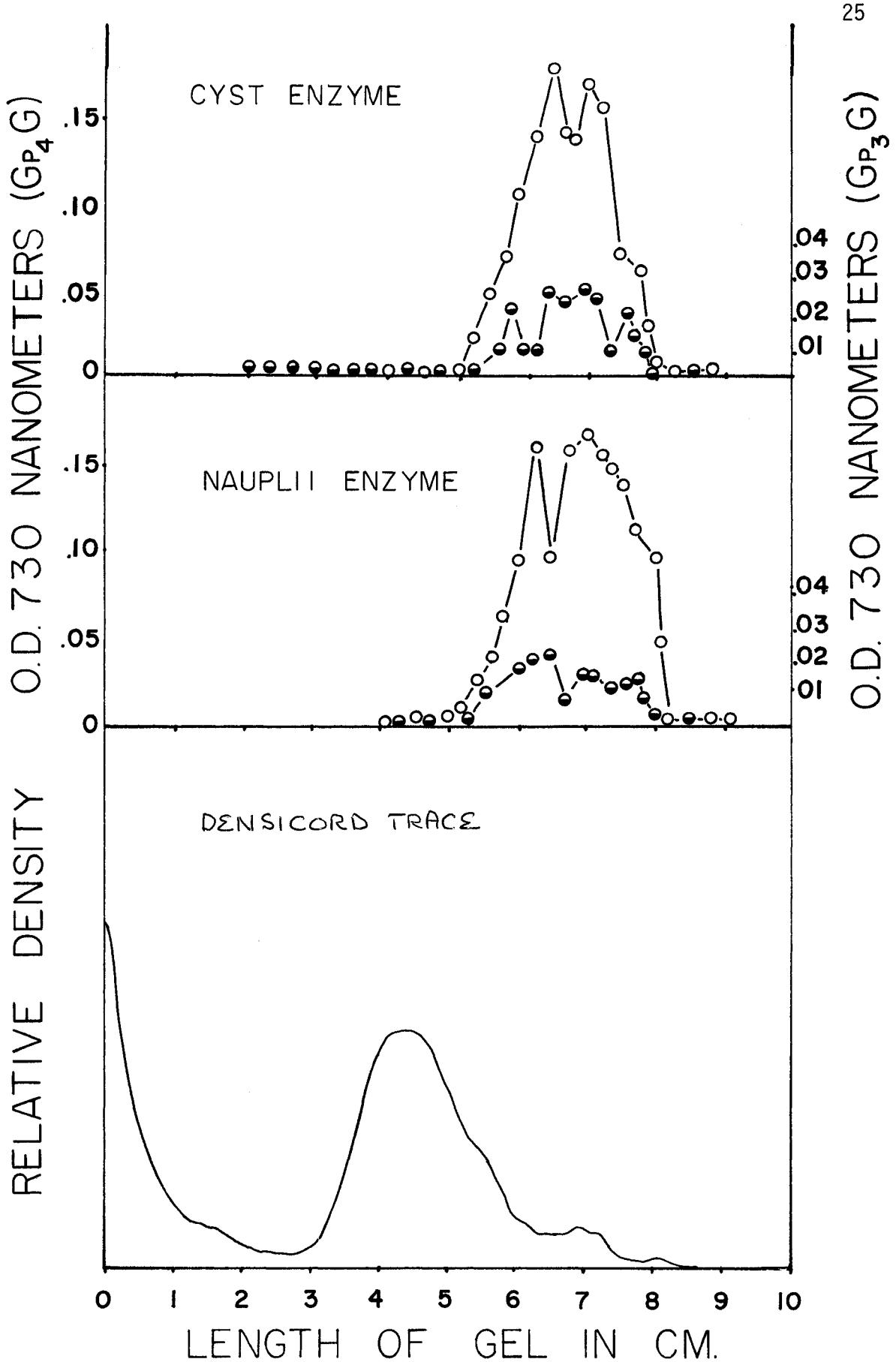


TABLE I

Relationship Between Michaelis-Menten Constants for Asym-diGDPase and the Utilization Rates in vivo of the Diguanosine Nucleotides.[a]

SUBSTRATE	Km	Rate of Utilization of the Diguanosine Nucleotides(umoles/day/mg protein)[b]	Ratio of Km values ($G_{p_3}G/G_{p_4}G$)	Ratio of Rates of Utilization ($G_{p_4}G/G_{p_3}G$)
$G_{p_4}G$	1.02×10^{-3}	8.8	5.7	6.3
$G_{p_3}G$	5.88×10^{-3}	1.4		

[a] All reaction vessels contained 688 ug of partially purified enzyme(G-100 Sephadex), 200 umoles of Tris-HCl,pH 8.5, varying amounts of $G_{p4}G$ and $G_{p3}G$, and optimal Mg^{2+}concentrations in 1.0 ml final volume. The primary products of hydrolysis were assayed on columns of Dowex-1-chloride as described in Materials and Methods.

[b] Data obtained from Clegg,Warner and Finamore(1967) for the first day post hatch development which corresponds with 24-48 hours in this study.

spectively as shown in Table 1. From the *in vitro* studies it is noteworth that the ratio of Km values for these dinucleotides is the inverse of the ratio of disappearance of these two substrates *in vivo*. Whether or not these similarities are fortuitious remains to be ascertained.

Asym-diGDPase Activity During Development of *Artemia salina*

In order to study asym-diGDPase during development, it was first necessary to inhibit all protease activity present in the enzyme preparations. Preliminary experiments with chicken egg ovomucoid, a general protease inhibitor, indicated that this component does not inhibit asym-diGDPase activity at high concentrations (1 mg/ml), but is an effective inhibitor of brine shrimp protease activity. These data are shown in Figure 4. It is apparent that addition of ovomucoid to the Sephadex-treated enzyme preparation from 36 hour embryos prior to chromatography on DEAE-cellulose inhibits virtually all protease activity associated with asym-diGDPase when Gp_4G is used as substrate. Hence, chicken ovomucoid was added to all enzyme preparations from developmental stages to protect against protease activity. With ovomucoid present, asym-diGDPase activity using Gp_4G as substrate was determined in crude enzyme extracts as well as enzyme purified through DEAE-cellulose chromatography from several stages of developing embryos. In all cases, the rate of hydrolysis was determined during the period of zero-order kinetics and these data were used to determine total enzyme activity. When the crude homogenates were

FIGURE 4

Chromatography of Ovomucoid-treated Enzyme on DEAE-cellulose.

The elution conditions were the same as in Figure 2(c). The amount of asym-diGDPase in each fraction was assayed using Gp_4G as a substrate, and P_i liberation was determined as described in Materials and Methods. For the protease assay 2.0 ml of each fraction was added to a reaction vessel containing 2 mg/ml bovine serum albumin and 0.02 M Tris-HCl, pH 8.5, in a final volume of 5.0 ml. Protease activity was determined by the appearance of acid-soluble material absorbing at 280 nm, after 12 hours incubation at 40°C. Asym-diGDPase activity, O——O; optical density at 280 nm, ●——●; protease activity, ◒——◒.

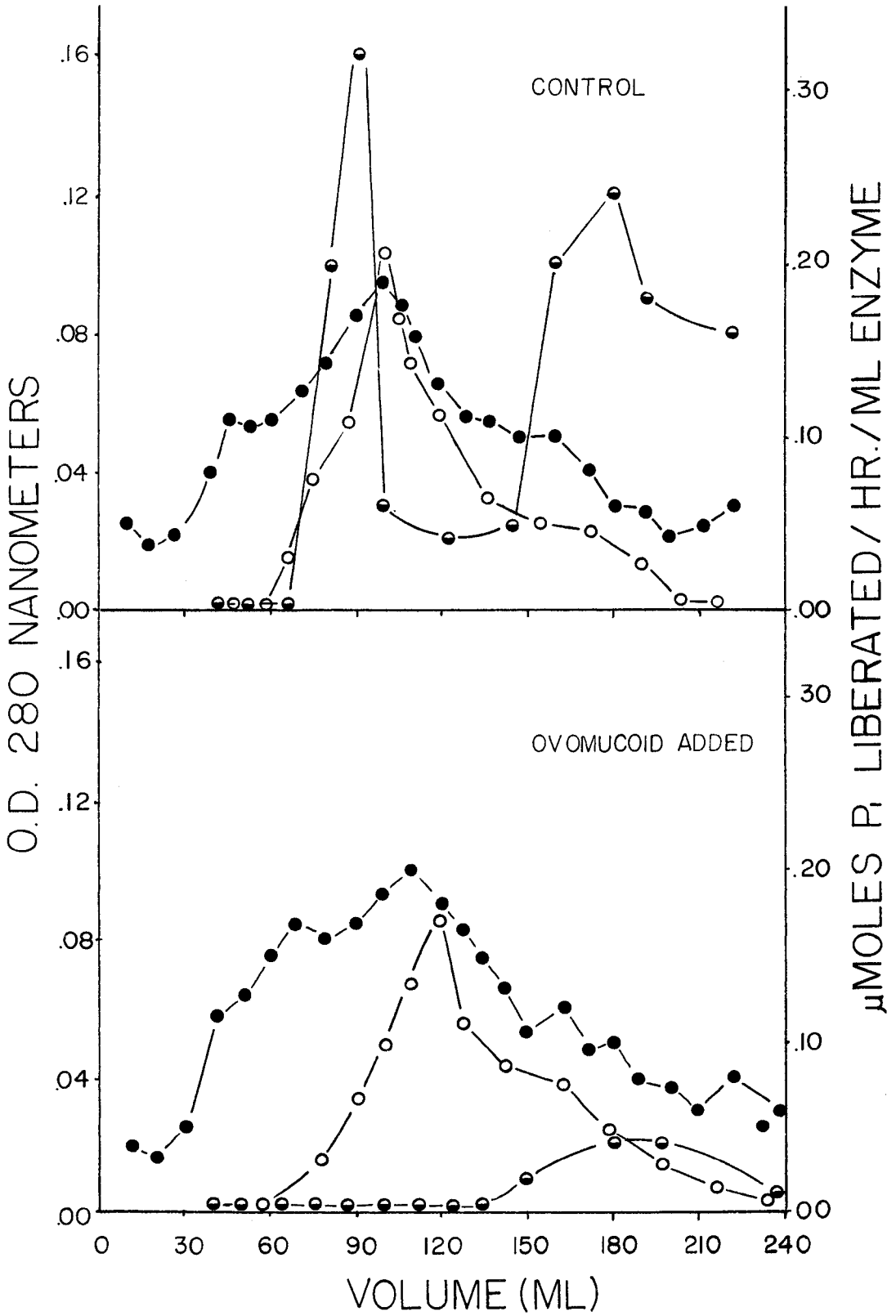


TABLE 2

Activity of Asym-diGDPase During Development of Artemia salina.

Time of Development in Hours (20°C)	Number of Animals x 10^{-5}	Total Protein[a] Extracted(mg) per Animal x 10^5	Crude Homogenates		DEAE Purified Enzyme	
			Specific Activity[b]	Total Activity per Animal x 10^5	Specific Activity x 10^2	Total Activity per Animal x 10^5
Cyst	2.0	73	.22	16	7.5	.70
12	4.6	58	.35	20	18.4	.48
24	4.0	65	.32	20	17.3	.70
36	3.0	65	.38	25	21.5	1.2
48	2.4	58	.38	22	26.1	1.5
60	4.4	67	.31	21	30.2	1.8
72	2.9	52	.62	32	42.4	1.7
84	1.9	64	.65	41	36.0	2.8
108	3.2	52	.64	33	37.0	1.7
132	2.2	38	.69	26	45.0	1.6

[a] Protein determinations were made using the method of Lowrey et al., (1950). Protein measurements of crude enzyme extracts were made on acid soluble protein which was rendered free of lipid material by treatment with acetone or ethanol ether (3:1).

b Specific activity is defined as the number of enzyme units per mg protein.One enzyme unit is equal to the amount of enzyme which releases 1.0 umoles Pi per hour.

assayed it appeared that both specific activity and total activity remained fairly constant up to 60 hours development followed by a general increase in specific activity and only a temporary increase in total activity. In contrast, the DEAE-cellulose purified enzyme showed similar increases in specific and total activities but these events were shown to occur slightly earlier in development. These experiments are shown in Table 2. It is evident that differences exist between the crude homogenate and partially purified enzyme, particularly in the apparent onset of enzyme synthesis, but these differences may be due, in part, to uncontrolled protease activity in the homogenates. It should also be noted that the changes in total protein per animal are similar to that reported earlier by Urbani (1959).

Development Studies Using ^{14}C Bicarbonate

In a recent report, Clegg and Golub (1969) demonstrated that Artemia salina embryos resume protein synthesis immediately following cessation of dormancy. Furthermore, when prenauplii were exposed to ^{14}C bicarbonate, 25% of the radioactivity was incorporated into protein, mainly into glutamic and aspartic acid residues (Clegg, 1966). In the present study Artemia were labeled in 3-hour pulses using ^{14}C-bicarbonate at six stages of development. Incorporation of radioactivity was determined in the crude homogenates (protein fraction only), DEAE-

cellulose purified enzyme, and in acrylamide gel slabs after electrophoresis. The results of these experiments are shown in Table 3 and Figure 5. It should be pointed out that care be taken to group stages in which approximately the same number of animals was used.[6] In the crude extracts, if the 24 hour stage is disregarded, the radioactivity in proteins is constant up to 39 hours development, then gradually declines. With the DEAE purified enzyme, if the 24 hour stage is omitted, the radioactivity in the active fraction, here too, reaches a maximum by 39 hours development then declines. The total activity per 100,000 animals also reaches a maximum by 39 hours development. Finally, when the DEAE-cellulose purified enzyme from three stages of development is further fractionated by electrophoresis on slabs of acrylamide gel, and the distribution of radioactivity compared with the position of the active enzyme, the data in Figure 5 are obtained. In all cases a definite but small amount of radioactivity is associated with the active fraction(s). In the 87 hour sample, and to a lesser extent in the 12 and 39 hour samples, a band very active toward Gp_4G appears but with a totally different electrophoretic mobility. The importance of this active fraction will be dis-

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[6] The low value of the 24 hour sample is probably due to the dilution of the specific activity of the bicarbonate by the large amounts of CO_2 produced during this period of development (Emerson, 1963). Due to the large number of animals in this sample and the resultant isotope dilution effect by endogenously produced CO_2, the 24 hour incorporation data should be viewed with caution.

TABLE 3

Incorporation of $^{14}HCO_3$ Into Soluble Protein and Partially Purified Asym-diGDPase During Development of Artemia salina.

		CRUDE HOMOGENATES				DEAE PURIFIED ENZYME			
Time in Hours	Number of Animals x 10^5	Total Counts Acid Insoluble[a]	Total Counts in Soluble Protein (b) per Animal x 10^5	Total Soluble Protein per Animal x 10^5	Specific Activity x 10^{-6} (cpm/mg. protein)	Total Counts in DEAE Conc.	Total Protein in (mg)	Specific Activity x 10^3 (cpm/mg protein)	Total Enzyme Activity per animal[c] x 10^5
3-6	1.0	935,897	9.4	47	2.0	5,240	2.18	2.4	5.2
9-12	1.0	1,191,522	11.9	56	2.1	36,610	3.86	9.5	1.9
24-27	1.6	1,023,225	6.4	62	1.0	18,300	5.57	3.3	3.6
36-39	.76	886,597	11.7	64	1.8	45,200	3.56	12.7	9.3
60-63	1.3	681,070	5.2	45	1.2	38,100	7.00	5.4	7.8
84-87	.78	248,891	3.2	35	.92	22,400	4.75	4.7	6.8

[a] The radioactivity was determined from 5% TCA precipitates insoluble at 90 for five minutes.

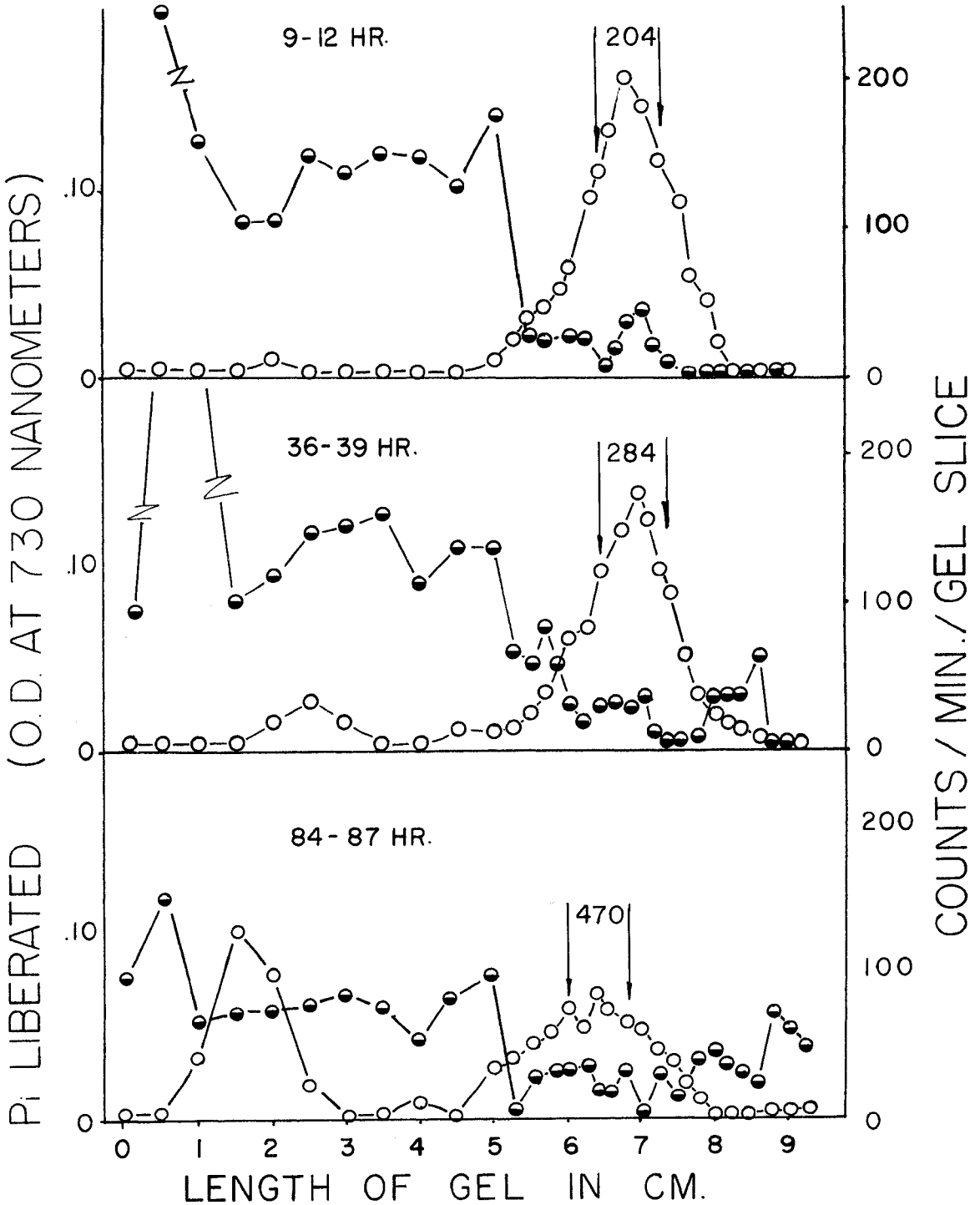
[b] The protein in these samples was solubilized in 0.1 M NaOH.

[c] Total activity was calculated from the DEAE eleuent by assaying a small amount of enzyme and the total umoles of Pi liberated per hour per animal was determined.

FIGURE 5

Separation of ^{14}C-labeled asym-diGDPase from Three Stages of Development on Acrylamide Gels.

All enzyme preparations used had been purified through DEAE cellulose and all samples were run on the same gel. The gel used contained 10% acrylamide and was run for 2.5 hours at 300 volts and 11-12 ma/cm current. After electrophoresis the gels were frozen then cut into sections indicated in Figure 5 (i.e. 5.0 mm up to 5.0 cm followed by 2.0 mm slices up to 9.0 cm). The amount of radioactivity in each section was determined by counting one-half of each gel section, and asym-diGDPase was assayed on the remaining half using Gp_4G as substrate as described in Materials and Methods. The amount of protein used varied between 157 and 320 ug. and the recovery of radioactivity was at least 85%. The numbers between the arrows refer to the amount of radioactivity in the five peak tubes of the active fraction and are expressed as cpm/O.D. 730 nm. Activity using Gp_4G, o—— o ; cpm/gel slice, ●—— ●.



cussed later. It should be noted, however, that although the radioactivity associated with the asym-diGDPase band is low, there is an increase in both percent counts and specific activity (Cpm per $O.D._{730}$)[7] in the engymatically active region.

Molecular Weight Determinations of Asym-diGDPase Using G-200 Sephades

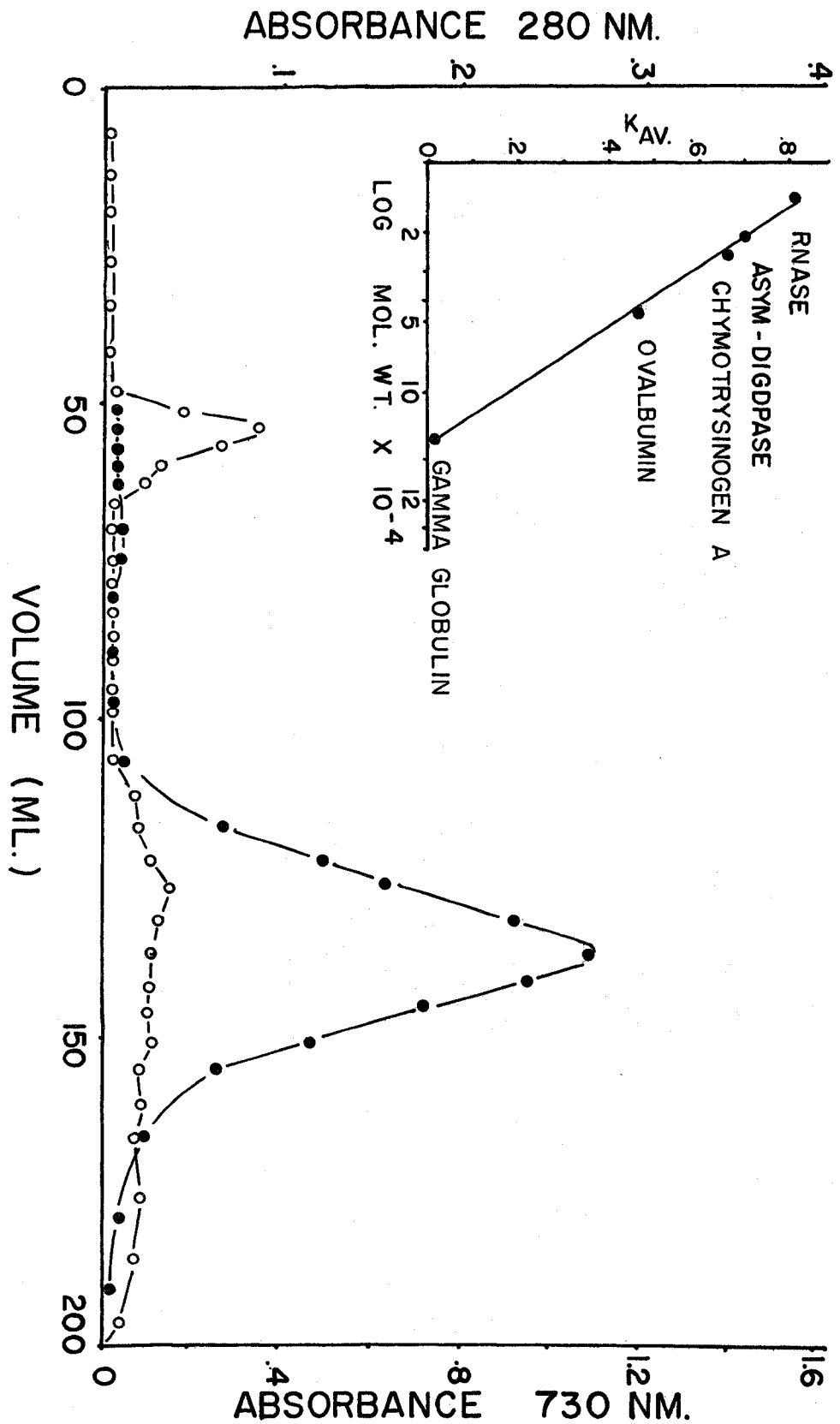
The molecular weight of asym-diGDPase was determined using an ultra filtration procedure and the results of these experiments are shown in Figure 6. Using this method, the molecular weight of this protein was estimated to be $2.00 \times 10^4 \pm .10$.

[7] The reader is referred to Appendix A for additional data from this experiment.

FIGURE 6

Molecular Weight Determinations of Asym-diGDPase Using Sephadex G-200.

Molecular weight determinations and assays for Gp_4G and Gp_3G were conducted according to the procedures outlined in Materials and Methods. The insert shows a standard curve using four purified proteins (Pharmacia, Montreal). Sample volume was 2.0 ml. Activity using Gp_4G, ●——● ; absorbance at 280 mm. o——o



DISCUSSION

The inability of developing embryos of Artemia salina to synthesize purines de novo, and the observation that these embryos contain large amounts of diguanosine nucleotides led Clegg et al. (1967) to speculate that these diguanosine compounds are the sole source of adenine- and guanine- containing nucleotides required for development. In an earlier study, Warner and Finamore (1965a) demonstrated that the enzyme, P^1, P^4 -diguanosine 5'-tetraphosphate asymmetrical-pyrophosphohydrolase (asym-diGDPase) liberates GMP and GTP from diguanosine tetraphosphate, but that asym-diGDPase was inactive using diguanosine triphosphate as substrate. In view of the importance of the diguanosine nucleotides during Artemia development, an investigation was initiated to elucidate the mechanism for diguanosine triphosphate utilization and to elaborate on the importance of asym-diGDPase in regulating/controlling the metabolism of diguanosine tetraphosphate. This study supports the earlier findings that asym-diGDPase is virtually inactive with respect to Gp_3G under conditions optimal for Gp_4G hydrolysis, but indicates that hydrolysis of Gp_3G by asym-diGDPase can occur at Mg^{2+} concentrations 20-30 times higher than that required for Gp_4G hydrolysis. In addition, this study showed that Mg^{2+} alone, or in combination with Tris-HCl buffer and inactivated protein had no effect on Gp_3G hydrolysis. It is

well known that many enzymes require metal ions for optimal activity and that some phosphohydrolases require Mg^{2+} specifically (Dixon and Webb, 1964). In these cases, the metal ion functions either as an integral part of the active site, eg. carboxypeptidase A, or the metal is necessary to provide the substrate with the steric properties required for it to fit into the active site of the enzyme. High molecular weight enzymes such as aldolases are composed of subunits, the association of which can also be a function of metal ions such as Mg^{2+}. However, asym-diGDPase is a small protein. Perhaps the Mg^{2+} needed for optimal activity affects the substrate rather than the enzyme. However, since steric conformational changes in substrate would be expected to occur at a much lower metal ion concentration (Taqui Khan and Martell, 1962), a metal-substrate interaction does not seem to be the answer. Suelter and Melander (1963) reported that when varying amounts of Mn^{2+} and Mg^{2+} were added to the enzyme pyruvate kinase, increasing differences in ultraviolet spectrum were observed until the enzyme was saturated. In a subsequent study these changes in ultraviolet spectra were ascribed to alterations in the solvating environment of protein chromophores which reflect changes in protein conformation (Kayne and Suelter, 1965). Further experimentation appears necessary to determine the precise role of Mg^{2+} in the hydrolysis of Gp_3G by asym-diGDPase and the hydrogen-tritium exchange method of Ulmer (1970) may provide a suitable experimental method to test the

relationship of protein structure to metal ion concentration.

The presence in our preparation of an enzyme specifically active with respect to Gp_3G and different from the Warner and Finamore enzyme has not been ruled out. On acrylamide gels, the best DEAE- or CM-cellulose prepared enzyme is not homogenous, but contains RNAse and DNAse as well as other proteins (Warner and Finamore, 1965a). It may be that a protein isolated along with asym-diGDPase and very similar in molecular weight (such as ribonuclease, MW. 13,700) can catalyse Gp_3G hydrolysis under high Mg^{2+} concentrations. Although Warner and Finamore (1965a) demonstrated that RNAse activity was present in their CM-cellulose prepared asym-diGDPase, the RNAse activity was not coincident with asym-diGDPase when Gp_4G was used as a substrate. However, they did not assay their gels under high Mg^{2+} concentrations or for activity using Gp_3G as a substrate. In the acrylamide gel preparations assayed in the present study, the asym-diGDPase band was active using Gp_3G but the amount of activity was considerably lower than expected when compared to the activity using Gp_4G as a substrate. In view of these inconsistencies two possibilities exist. First, asym-diGDPase is the only enzyme in the preparation able to hydrolyse both Gp_3G and Gp_4G, and that the unexpected differences in the activities measured on the gel fractions result from "physical interference" due to the enzyme being bound in the gel matrix. Second, that Gp_3G and Gp_4G are hydrolysed by different but very similar enzymes. Since we have

observed that pancreatic ribonuclease is inactive with respect to Gp_3G at high Mg^{2+} concentrations, the first possibility seems to be the most favorable. However, Artemia RNAse may be unique, and further experimentation is necessary in order to resolve these difficulties.

Whether one or more enzyme(s) are responsible for the hydrolysis of Gp_3G and Gp_4G is still uncertain, but the fact that these two dinucleotides disappear during the early embryogenesis of Artemia has been definitely established (Clegg et al., 1967; Warner and Finamore, 1967). It is noteworthy, in this respect (cf. Table I), that the ratio of Km values for Gp_4G and Gp_3G is the same as the ratio of rates of disappearance of these two nucleotide anhydrides as reported by these same authors (Clegg et al., 1967). If indeed two enzymes are involved in the utilization of these two compounds, such a correlation would hardly be the case.

In experiments designed to determine whether or not more than one enzyme is present in Artemia embryos for the utilization of the diguanosine nucleotides, it was observed that asym-diGDPase isolated from encysted embryos exists in two molecular forms. The major component has a molecular weight of $2.0 \times 10^4 \pm 0.10$. Whereas the elution profile in some cases seemed to indicate two molecular species (Figure 2), separation of two proteins one for each diguanosine nucleotide was not possible.

In addition to our knowledge of nucleotide metabolism in Artemia alluded to earlier in this work, research has been carried out on protein synthesis, and nucleic acid synthesis and metabolism. Clegg has shown that polysome formation and protein synthesis commence immediately upon cessation of dormancy (Clegg, 1966; Clegg and Golub, 1969), and McClean and Warner have pointed out that RNA synthesis during development in Artemia reaches a maximum at about 36 hours following resumption of development and then is "turned off", whereas DNA synthesis commences after hatching and continues until about 55-60 hours of development (McClean and Warner, 1970). In view of the relationship between changes in the nucleic acid profile and protein synthesis, it is important to examine the activity of a specific protein (asym-diGDPase) during this same period of development. Before such studies could be undertaken, however, a protease inhibitor had to be found. When chicken ovomucoid was tested against asym-diGDPase, that had been isolated and purified from cysts, this chicken egg polysaccharide showed no inhibition toward the hydrolytic activity of asym-diGDPase even when used in concentrations as high as one milligram per milliliter and it was found to be an effective inhibitor of protease activity in Artemia embryos. Since ovomucoid, isolated from chicken eggs, does not adhere to DEAE-cellulose at pH 8.5 (Rhodes et al., 1958), the inhibitor was added to all enzyme preparations prior to chromato-

graphy on DEAE-cellulose in order that it might bind to the proteases present and prevent their adsorption to the cellulose exchanger. As seen in Figure 4, when ovomucoid is added in this manner all the protease activity associated with asym-diGDPase is removed. Using this technique, then, the activity of asym-diGDPase during the development of Artemia was examined in two separate experiments.[8] The results of these two experiments show that the total soluble protein (under these isolation conditions) remains constant up to 36 hours development decreasing by about 50% at 84 hours. These changes in protein are considerably slower at 20°C such that a 50% decline in soluble protein appears at 132 hours. In a similar study Fry and Gross (1970b) reported no net change in protein content during the first five days of development in the sea urchin, Arabacia punctata. These workers attribute this constancy in soluble protein to the utilization of pre-existing yolk proteins in the de novo synthesis of new protein. The pulse-and-chase experiments conducted by these investigators and those by Ecker and Smith with amphibians (1966), argue in favor of this

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[8] In comparing the results of these two experiments it is important to recall that these are two separate studies undertaken at two distinct temperatures as indicated in Materials and Methods.

interpretation rather than rapid turnover of newly synthesized protein. These researchers further point out that uptake of ^{14}C-leucine into the amino acid pool and incorporation of this amino acid and other protein precursors into protein reflect changes in protein synthesis only if the patterns of uptake of these precursors and amino acid pool size are constant during the period in which the observations are made (Fry and Gross, 1970a,b). Although pulse-and-chase experiments were not conducted in this study, it is quite probable that the constancy of soluble protein up to 36 hours is due to the utilization of stored yolk protein present in Artemia yolk platelets (Puodziukas, 1970). The decrease in soluble proteins after 40 hours and the decrease in incorporation of ^{14}C-bicarbonate at the same time argue for a decrease in protein synthesis during this period. This observation is consistent with the RNA pattern reported by McClean and Warner (1970). However, during this period of development changes in incorporation rate could very easily reflect changes in permeability to bicarbonate and/or changes in the utilization of this molecule as a protein precursor. Data regarding permeability to bicarbonate and other protein precursors would be useful in the interpretation of these results. When a specific protein such as asym-diGDPase is examined compared to the overall protein profile a different pattern emerges. Whereas protein synthesis is constant from cessation of dormancy up to 36 hours development, the specific and total activities of asym-

diGDPase begin at a low level, increase gradually up to 36 hours of development and then remain constant during the rest of the period studied. A similar pattern is seen when the radioactivity associated with partially or extensively purified enzyme protein is examined. Notwithstanding the lack of data regarding some of the parameters associated with protein synthesis, the following conclusion can be made. Protein synthesis in Artemia begins immediately upon cessation of dormancy and is maintained at a constant level up to forty hours development. This constancy appears to be due to the utilization of yolk protein in the synthesis of new proteins required for the early development of this crustacean such as asym-diGDPase, a pyrophosphohydrolase necessary to supply purines required for nucleic acid synthesis. After 40 hours (or later if the temperature is lower) the decrease in overall protein content is attributed to the disappearance of yolk but the enzyme asym-diGDPase is maintained at a constant level.

Whereas the data put forth in this study seem to correlate well with the existing knowledge of nucleic acid synthesis and metabolism in Artemia, care must be taken to interpert them in the light of the limitations inherent in in vitro enzyme studies. 1) In embryos, quantitative determinations of enzyme activities are more meaningful if they are paralleled by histological and histochemical studies. An example is provided by the work of Smith and Kissane (1963). By microanalysis of tiny sections of developing kidney these authors have shown that the

glomeruli, proximal tubules, and collecting ducts retain the same predominant form of LDH throughout development, and that the apparent change reported by Markert and Ursprung (1962) must be due to an increase in the relative amount of proximal tissue. 2) The enzyme under investigation should be localized in the embryo. Clearly, an enzyme molecule that is bound to a particulate fraction may perform in a much different manner than when freed by homogenization. For example, the problems which can arise from the breakage of lysosomes have been studied by Fell and her co-workers. They observed that in the chick embryo, limb development responds to an excess of vitamin A with a disintegration of cartilage matrix resulting in a change in the size and shape of the limb bud. It seems that limb bud deformity is the result of the release of acid proteases from lysosomes under the influence of vitamin A (Fell and Dingle, 1963). 3) If the increase in activity of asym-diGDPase is valid, and the in vitro data certainly indicate this, such an increase could be the result of a gain(loss) of dissociable activators or inhibitors or as Wright demonstrated (1960), the same enzyme may change in stability during the course of development. These factors may result in misleadingly low values. 4) Some enzymes can undergo conversions from active to inactive forms under physiological conditions, for example phosphorylase a and b (Krebs and Fisher, 1964). 5) Whereas the overall pattern of in vitro activity of an enzyme (in an homogenate) may show an increase from time A to

time B, the in vivo situation might show an increase, a period of constant activity and then a decrease followed by another increase (Moog, 1952; Boell, 1955). To follow such changes in a single enzyme requires more sensitive techniques. 6) The presence or absence of substrate does not necessarily mean that enzyme activity will change in the same manner. The phosphorylases and other enzymes involved in glycogen deposition in the liver are found several weeks before glycogen appears (Kornfield and Brown, 1963) and Nunnally (1962) has shown that succinic acid dehydrogenase which is involved in supplying energy for the transport of glucose in the chick duodenum, continues to increase in activity beyond the time when glucose absorption has reached a maximum level.

In summary, then, the following conclusions can be made: 1) that asym-diGDPase will hydrolyse Gp_3G but at much higher Mg^{2+} concentration than that required for Gp_4G hydrolysis, 2) that the activity of this enzyme, asym-diGDPase, increases up to 39 hours development (at 30°C) and then is maintained at a constant level while overall protein synthesis decreases, 3) that asym-diGDPase is the only hydrolase which is involved in the release of guanosine nucleotides from diguanosine nucleotides during the early stages of Artemia development. These conclusions, while they are limited by the techniques employed, nevertheless correlate well with existing data on nucleic acid and nucleotide metabolism of Artemia.

SUMMARY

Asym-DiGDPase, a pyrophosphohydrolase isolated from cysts and developing embryos of the brine shrimp, Artemia salina, has been shown to be active in the hydrolysis of two nucleotide anhydrides, diguanosine tetraphosphate (Gp_4G) and diguanosine triphosphate (Gp_3G). These dinucleotides are utilized by Artemia during the early thoracic period of development and appear to be important as a source of purines. It has been demonstrated that the Mg^{2+} requirements for optimal activity using Gp_3G are 25-50 mmolar, whereas Gp_4G hydrolysis requires only 1.3 mmolar. With the use of a general protease inhibitor studies of enzyme activity in crude as well as partially and electrophoretically purified enzyme extracts indicate that total activity increases gradually to about 40 hours development and remains relatively constant thereafter. Maximum activity is attained about one day after hatching. From the ^{14}C-bicarbonate incorporation data it appears that the increase in total activity represents de novo synthesis. It is noteworthy that the onset of maximum enzyme activity corresponds closely to the period of rapid utilization of the diguanosine nucleotides in Artemia nauplii.

APPENDIX A

Additional Data Pertinent to the Experiment Shown in Figure 5.

Time of ^{14}C-HCO_3 exposure	9-12	36-39	84-87
Total counts in the active fraction	292	440	326
Total O.D. at 730 nm.	1.142	.950	.537
CPM per O.D. 730 nm. of the 5 peak tubes.	204	284	470
Total counts recovered	2832	2754	1602
Percent counts in the active fraction	10.3	16.0	20.4

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