Isolation, Purification, and Characterization of P^1,P^4-Diguanosine 5′-Tetraphosphate *Asymmetrical*-Pyrophosphohydrolase from Brine Shrimp Eggs*

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ABSTRACT: A new enzyme, P^1,P^4-diguanosine 5′-tetraphosphate *asymmetrical*-pyrophosphohydrolase (*asym*-di-GDPase), has been isolated from undeveloped eggs of the brine shrimp, *Artemia salina*, and purified over 200-fold. This enzyme is specific for P^1,P^4-diguanosine 5′-tetraphosphate (di-GDP), has a *p*H optimum of approximately 8, and requires Mg^{2+} for optimal activity. In the presence of the enzyme di-GDP is completely hydrolyzed to guanosine monophosphate and guanosine triphosphate in equimolar quantities.

The reaction is inhibited extensively by heat, chymotrypsin, *p*-mercuribenzoate, ethylenediaminetetracetate, and fluoride, whereas guanosine 5′-mono-, di-, and triphosphates, and diguanosine triphosphate, a homolog of di-GDP (A. H. Warner and F. J. Finamore, 1965, *Biochim. Biophys. Acta* (in press)), fail to inhibit the reaction.

Encysted embryos of the brine shrimp, *Artemia salina*, have been studied extensively by embryologists and geneticists but, unfortunately, little biochemical information on their embryonic development has come forth. The recent finding that large quantities of a unique pyrophosphate ester, P^1,P^4-diguanosine 5′-tetraphosphate (di-GDP),[1] occur in encysted embryos of *A. salina* (Finamore and Warner, 1963) prompted the search for an enzyme system in the undeveloped egg capable of attacking this compound. The present paper describes the isolation, purification, and characterization of a new pyrophosphohydrolase capable of hydrolyzing di-GDP.[2] This enzyme, di-GDP *asymmetrical*-pyrophosphohydrolase (*asym*-di-GDPase), apparently has an absolute substrate specificity and produces equimolar quantities of GMP and GTP from diguanosine 5′-tetraphosphate.

Experimental

Enzyme Isolation and Purification

Preparation of 23,500 × g Fraction. Dry cysts (32 g) of the brine shrimp, *A. salina* (Wardley and Co.), were ground, unmoistened, with sand by means of a motor-driven mortar and pestle for 30 minutes. Enough cold 0.30 M NaCl containing 0.05 M Tris-HCl, *p*H 7.4, was added to make a thick slurry, and the grinding was continued for an additional 10 minutes in the cold (3°). The thick slurry of ground eggs was then brought to 350 ml by the addition of cold 0.30 M NaCl containing 0.05 M Tris-HCl, *p*H 7.4, and the extraction was carried out for an additional 20 minutes in the cold. The preparation was centrifuged at 23,500 × *g* for 30 minutes and the supernatant fluid was decanted through several layers of cheesecloth to remove floating debris.

Ammonium Sulfate Fractionation. Sufficient solid ammonium sulfate was added to the 23,500 × *g* soluble fraction to make the solution 50% saturated. The solution was stirred for 15 minutes and centrifuged, and the insoluble material was discarded. To the clear solution was added enough solid ammonium sulfate to completely saturate the mixture. The precipitate that formed after 15 minutes of stirring was removed by centrifugation and dissolved in a small amount of cold 0.05 M Tris-HCl, *p*H 8.5.

Treatment with DEAE-Cellulose. DEAE-cellulose (Matheson Coleman and Bell, Inc.) was prepared according to Peterson and Sober (1956) and equilibrated with 0.05 M Tris-HCl, *p*H 8.5, prior to use. The enzymatically active fraction was percolated through a DEAE-cellulose column (2 × 20 cm) and washed with two bed-volumes of the cold buffer. Under these conditions nucleic acid components are retained by the cellulose whereas the enzyme fraction passes through

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[1] The following abbreviations have been used: di-GDP, P^1,P^4-diguanosine 5′-tetraphosphate; *asym*-di-GDPase, P^1,P^4-diguanosine 5′-tetraphosphate *asymmetrical*-pyrophosphohydrolase; GMP, guanosine 5′-monophosphate; GDP, guanosine 5′-diphosphate; and GTP, guanosine 5′-triphosphate.

[2] A preliminary report of these studies was presented at the 148th annual meeting of the American Chemical Society, Chicago, Ill., Aug. 31–Sept. 5, 1964.

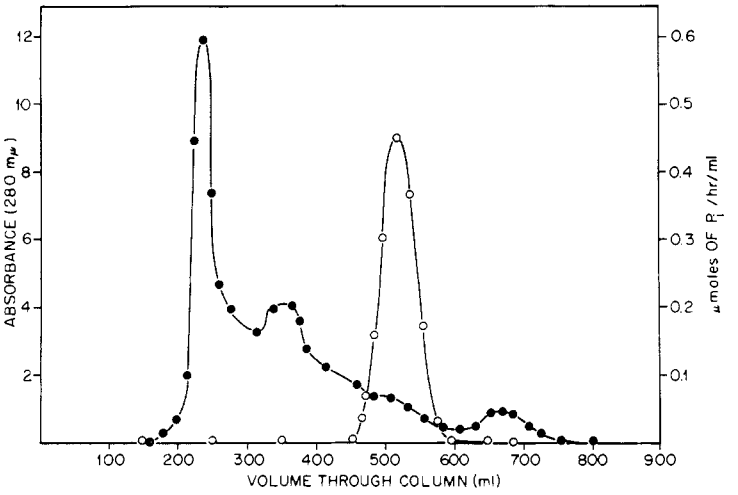

FIGURE 1: Fractionating on G-100 Sephadex column. Ten ml of the concentrated protein solution was applied to a 3.5- × 75-cm Sephadex column. Elution of *asym*-di-GDPase was carried out in the cold with 0.30 M NaCl containing 0.025 M Tris-HCl, *p*H 8.5. ●—●, optical density at 280 mμ; ○—○, *asym*-di-GDPase activity.

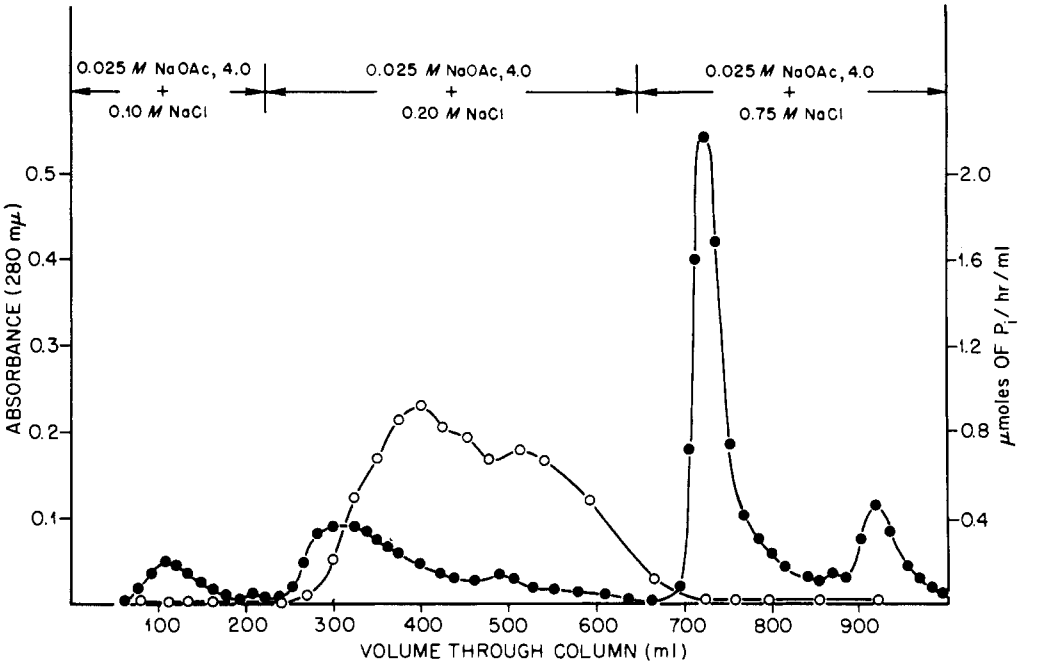

FIGURE 2: Fractionating on CM-cellulose. The dialyzed enzyme preparation was adjusted to *p*H 4.0 by addition of acetic acid and applied to a 2.5- × 18-cm column. The column was washed with 200 ml 0.025 M sodium acetate, *p*H 4.0, and the active enzyme was eluted in the cold as indicated. ●—●, optical density at 280 mμ; ○—○, *asym*-di-GDPase activity.

and can be recovered from the effluent by saturation with ammonium sulfate and subsequent centrifugation. The precipitate was dissolved in 10–15 ml of 0.30 M NaCl containing 0.025 M Tris-HCl, *p*H 8.5.

Filtration on Sephadex. Sephadex G-100 (bead form, Pharmacia Fine Chemicals, Inc.) was prepared in the same manner as DEAE-cellulose and was equilibrated with 0.30 M NaCl containing 0.025 M Tris-HCl, *p*H 8.5. Following DEAE-cellulose treatment and ammonium sulfate concentration, the active enzyme fraction was layered over a Sephadex column (3.5 × 75 cm) and filtration was performed using 0.30 M NaCl containing 0.025 M Tris-HCl, *p*H 8.5. Fractions of 10–12 ml were collected with an automatic fraction collector

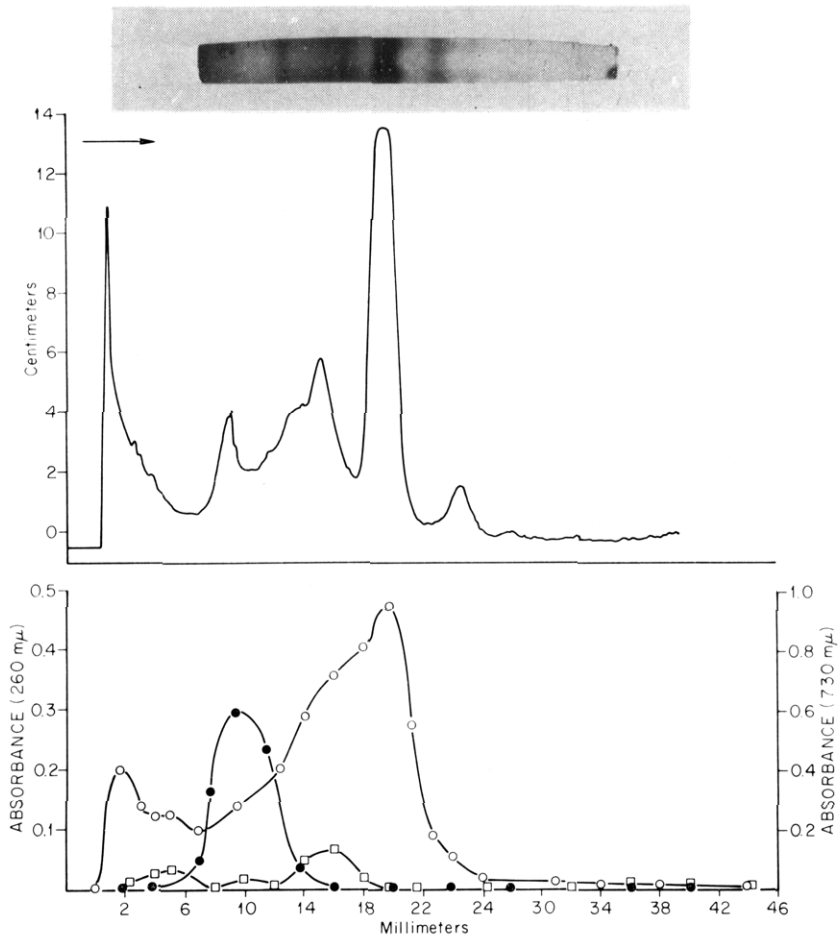

FIGURE 3: Disc electrophoresis. The Model 12 disc electrophoresis apparatus was used in separating the active protein fraction into six discrete bands. All runs were carried out at *p*H 4.3 for 75 minutes at a current of 7 ma per column. The photograph at the top shows the protein bands in the acrylamide gel as they appear after staining. The middle figure represents a densitometric analysis of a photographed gel using a Spinco film densitometer with the Beckman Analytrol. The lower three curves show the result of enzyme analysis along the gel. For *asym*-di-GDPase analysis, liberation of P_i as described under Methods was used as the assay. For nuclease assays 1 mg of either yeast RNA or thymus DNA in 0.5 ml 0.1 M Tris-HCl, *p*H 8.0, containing 0.01 M $MgCl_2$ was added to tubes containing the gel segments. RNAase assay was carried out for 2 hours at 37°, and DNAase activity was determined after 16 hours at 37°. At the desired time interval 0.5 ml of cold 1 N $HClO_4$ was added to each tube. The precipitates were removed by centrifugation, and samples were taken from the acid-soluble fraction for optical density measurements at 260 mμ. ———, densitometer curve; ●—●, *asym*-di-GDPase activity; ○—○, RNAase activity; □—□, DNAase activity.

at a rate of 0.75 ml/min and read at 260, 270, 280, and 290 mμ in a Beckman Model DB spectrophotometer. Each fraction was assayed for protein (Lowry *et al.*, 1951) and for *asym*-di-GDPase activity as subsequently described. The contents of all tubes containing *asym*-di-GDPase activity were pooled and dialyzed for 2–3 hours against 50 volumes of cold 0.025 M sodium acetate, *p*H 4.0.

Fractionation on CM-cellulose. The CM-cellulose (Brown Co.) used in all experiments was prepared in the same manner as DEAE-cellulose and Sephadex and equilibrated with 0.025 M sodium acetate, *p*H 4.0. The dialyzed protein solution was adjusted to *p*H 4.0 with 5 M acetic acid, applied to a CM-cellulose column (2.5 × 18 cm), and washed with 200 ml of 0.025 M sodium acetate, *p*H 4.0. Elution was carried out stepwise with increasing amounts of sodium chloride. Fractions of 8–10 ml were collected, read in the spectrophotometer, and assayed for *asym*-di-GDPase activity. The contents of the tubes containing *asym*-di-GDPase activity were pooled and dialyzed overnight against 50 volumes of cold distilled water without loss of activity. The enzyme solution was concentrated by lyophilization with about 25% loss in activity and stored at −20°.

Enzyme Assay Procedures

Liberation of Orthophosphate from Di-GDP. Early experiments on the characterization of di-GDP showed that a bacterial alkaline preparation (Worthington

and Co.) was inactive toward di-GDP but released orthophosphate (P_i) from GMP, GDP, and GTP. For this reason the alkaline phosphatase preparation proved useful in assaying for *asym*-di-GDPase activity in crude fractions. Regardless of the products of hydrolysis, release of P_i indicated the presence of enzymatic activity toward di-GDP.

During the purification of *asym*-di-GDPase all assays were performed in the following manner. The desired fraction was incubated for 20 minutes at 40° with 1.0 μmole di-GDP, 4.0 μmoles $MgCl_2$, 100 μmoles Tris-HCl, *p*H 8.0, and with an excess of the alkaline phosphatase preparation. In all cases the final volume was 3.0 ml. After incubation the reaction was stopped by addition of 0.5 ml cold 10 N H_2SO_4 and P_i was determined colorimetrically (Ernster *et al.*, 1950). When $^{32}P_i$ was to be determined, aliquots were removed, plated, and counted in an SC-88 Tracermatic scaler.

Release of GMP. After the products resulting from di-GDP hydrolysis had been ascertained to be GMP and GTP (see Results), it became necessary to determine either one or both products in order to obtain meaningful kinetic information on *asym*-di-GDPase activity. Since the appearance of either product could be used as a measure of activity, it was decided to measure the release of GMP using an ion-exchange technique. All reactions were terminated at the desired time interval by addition of an equal volume of cold 1 N HCl, allowed to remain in acid for 5 minutes at 0°, made slightly alkaline with dilute NH_4OH, and applied to small Dowex 1 (chloride) (0.9 × 1.5 cm) columns. The columns were washed with water and 0.01 M NH_4Cl; then GMP was eluted with 125 ml of 0.01 N HCl. In many cases GTP was also determined (by eluting with 0.01 N HCl containing 0.10 M NaCl), to corroborate results obtained with GMP measurements.

Test of Enzyme Purity

Disc Electrophoresis. For all electrophoretic experiments highly purified acrylamide monomers (Distillation Products Co.) were used. All electrophoretic runs were carried out with freshly prepared acrylamide polymers (Reisfeld *et al.*, 1962) in the Model 12 disc electrophoresis apparatus (Canal Industrial Co.) using 0.5- × 7.0-cm glass columns. The lower gel was prepared by diluting a 30% acrylamide solution with an equal volume of freshly prepared 0.5% ammonium persulfate solution. The resulting polymer contained 15% acrylamide with an average pore diameter of about 22 A (Raymond and Nakamichi, 1962). The spacer and sample gels contained 6 and 3% acrylamide, respectively. In all experiments the sample gel contained between 150 and 225 μg protein. All experiments were performed at 5° and at *p*H 4.3 using 0.175 M β-alanine acetate buffer. Satisfactory results were obtained when a current of 7 ma per column was applied for 75 minutes.

At the conclusion of each run, two gels were stained routinely for 1.5 hours with Canalco's protein stain (National Aniline-Naphthol Blue Black) and the remaining unstained gels were frozen over solid CO_2. Destaining of the gels was carried out with 10 ma of current per column until clear, distinct bands were apparent. For *asym*-di-GDPase assay the frozen gels were cut into 1-mm cross sections and eluted for 3 hours at 37° with 3.0 ml of 0.1 M Tris-HCl, *p*H 8.0, containing 1.0 μmole di-GDP, 4.0 μmoles $MgCl_2$, and with an excess of bacterial alkaline phosphatase. At the end of the 3 hours' incubation, the presence of *asym*-di-GDPase activity in the gel segments was determined as the release of inorganic orthophosphate. No pyrophosphohydrolase activity toward P^1,P^3-diguanosine 5′-triphosphate could be determined under these conditions.

Nuclease activities were determined on 1-mm gel sections by incubating one half of each segment with thymus DNA and the other half with yeast RNA at *p*H 8.0 for 2–16 hours. Under these conditions nuclease activity was determined by the appearance of acid-soluble ultraviolet-absorbing material in the reaction mixture.

Results

Purification of asym-di-GDPase. Figure 1 shows the importance of filtration through G-100 Sephadex in removing contaminating protein(s) from the preparation. In all experiments recovery of the active enzyme from Sephadex was greater than 95%. In Figure 2 the degree of purification obtained from chromatography on CM-cellulose is shown. The most striking features of this profile are the heterogeneous appearance of the activity curve and the observation that very little protein is associated with this activity. The course of *asym*-di-GDPase purification is summarized in Table I. One difficulty encountered during the course of purification is the inability to completely "salt out" the active material after DEAE-cellulose treatment. The 34% loss in activity after Sephadex treatment is not a result of the filtration process but reflects the inability to concentrate the active protein by conventional "salting out" techniques.

TABLE I: Course of *asym*-di-GDPase Purification.

Purification Steps	Specific Activity[a]	Per Cent Total Activity
Crude homogenate	0.05–0.09	100
50–100% $(NH_4)_2SO_4$	0.48	95
DEAE-cellulose treatment	0.51	96
G-100 Sephadex filtration	2.45	62
CM-cellulose fractionation	12.5	49

[a] Specific activity is defined as the number of enzyme units/mg protein where one enzyme unit is equal to that amount of enzyme which releases 1 μmole P_i per hour.

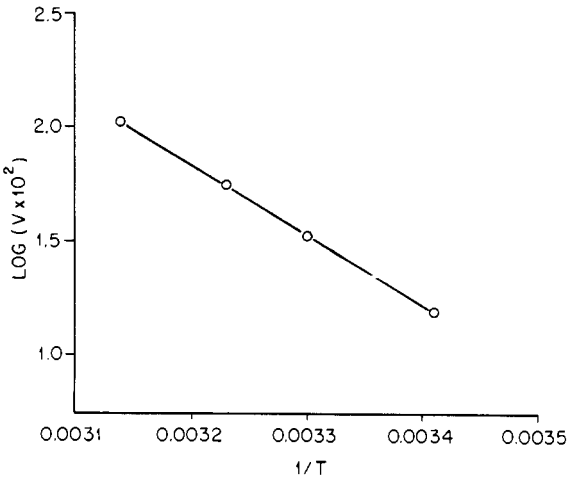

FIGURE 4: Influence of temperature on reaction velocity. Each reaction vessel contained 2 μmoles di-GDP, 4 μmoles $MgCl_2$, 50 μmoles Tris-HCl, *p*H 8.0, and 690 μg enzyme, final volume 2.0 ml. All reactions were carried out for 20 minutes at the indicated temperatures, and GMP was measured by ion-exchange chromatography.

Electrophoresis. The heterogeneous appearance of *asym*-di-GDPase activity during fractionation on CM-cellulose and the fact that all preparations showed slight nuclease activity prompted the use of acrylamide gel electrophoresis to determine whether multiple forms of *asym*-di-GDPase are present and to assess the significance of the endogenous nuclease activity. Figure 3 shows the presence of six distinct protein bands, only one of which shows *asym*-di-GDPase activity. In the bottom half of Figure 3 the closed circles show the location of *asym*-di-GDPase activity in the gel; the open markers show the position of nuclease activities as measured by the appearance of acid-soluble material in the reaction mixture. Most of the observed nuclease activity appears to be associated with protein bands that are inactive toward di-GDP.

Products of Hydrolysis. Hydrolysis of di-GDP by the partially purified enzyme described releases GMP and GTP in equimolar quantities, as can be shown by column chromatography. Under no conditions tested thus far has the reaction been found to be reversible. Di-GDP hydrolysis can be completely inhibited by treatment of the enzyme preparation with heat or chymotrypsin or assaying in the presence of fluoride ion; it is partially inhibited when assayed in the presence of *p*-mercuribenzoate (hydroxy).

Effect of pH. Maximal rate of di-GDP hydrolysis occurs about *p*H 8. No "buffer effect" is observed at *p*H 8 when the enzyme is assayed in maleate, Tris, ammonium bicarbonate, or glycine buffers at 0.05 M concentrations.

Activation Energy. The effect of temperature on the hydrolysis of di-GDP is shown in Figure 4. The optimal temperature of hydrolysis is 45°, and the activation energy is approximately 6000 cal $mole^{-1}$.

Michaelis Constant (K_m). The K_m value for *asym*-di-GDPase was calculated from the double-reciprocal plot in Figure 6b (Lineweaver and Burk, 1934). The value obtained in Tris-HCl buffer, *p*H 8.0, at 45° and at a constant ratio of 2 for $(Mg^{2+})/(\text{di-GDP})$ is 1.03×10^{-3} M.

Influence of Ionic Strength (μ) *on Reaction Velocity.* Table II summarizes the effect of several electrolyte solutions on *asym*-di-GDPase activity. In all cases tested no significant ionic effects were observed below $\mu = 0.25$, whereas solutions above $\mu = 0.50$ showed significant inhibitory effects on *asym*-di-GDPase activity. Lithium and bicarbonate ions appear to be the most inhibitory at all concentrations tested.

TABLE II: Effect of Various Electrolytes[a] on *asym*-di-GDPase Activity.

	Per Cent Inhibition at Ionic Strength (μ):			
	0.125	0.250	0.50	1.00
NaCl	7	10	33	51
KCl	0	0	14	32
LiCl	4	25	60	80
NH_4Cl	0	0	26	60
NH_4HCO_3	8	30	73	96
Tris-HCl	0	0	18	38

[a] All reaction vessels contained the following: 2.0 μmoles di-GDP, 4.0 μmoles $MgCl_2$, 50 μmoles Tris-HCl, *p*H 8.0, 0.2 ml *asym*-di-GDPase (3.4 mg/ml), and the indicated electrolyte. NH_4Cl and NH_4HCO_3 stock solutions were adjusted to *p*H 8. The final volume was 2.0 ml. All reactions were run for 15 minutes at 45°. Liberation of GMP was determined by ion-exchange chromatography.

Heat Inactivation. The partially purified *asym*-di-GDPase preparation is irreversibly inactivated by heat in the absence of substrate and Mg^{2+}. Prior to *asym*-di-GDPase assay, incubation of the enzyme preparation for 5 minutes at 37 and 45° shows 18 and 69% inactivation, respectively, and heating to 80° for 5 minutes completely inactivates the enzyme.

Effect of Cations. At all stages of *asym*-di-GDPase purification Mg^{2+} or Mn^{2+} is required for maximal activity. Table III summarizes the results obtained when other cations or reagents are substituted for magnesium in the reaction mixture. All reaction vessels contained equal molar quantities of di-GDP and cation (10^{-3} M) plus Tris-HCl buffer, *p*H 8.0, and enzyme. When mercaptoethanol and EDTA were used, 10 μmoles of each was added. The effects of $MgCl_2$ and

TABLE III: Effect of Cations on *asym*-di-GDPase Activity.[a]

Reagent	Release Activity
$MgCl_2$	100
$MnCl_2$	68
$ZnCl_2$	25
$FeCl_2$	8
$FeCl_3$	8
$CaCl_2$	<1
$CdCl_2$	0
$CuCl_2$	0
KCl	0
NaCl	0
EDTA	0
HS—CH_2—CH_2—OH	0
HS—CH_2—CH_2—OH + $MgCl_2$	100

[a] All reaction vessels contained the following: 2.0 μmoles di-GDP, 2.0 μmoles cation, 100 μmoles Tris-HCl, *p*H 8.0, and 0.2 ml *asym*-di-GDPase (3.4 mg/ml). When EDTA or mercaptoethanol was tested, 10 μmoles of each were added. The total volume was 2.0 ml. All reactions were run for 15 minutes at 40°. Liberation of GMP was determined by ion-exchange chromatography.

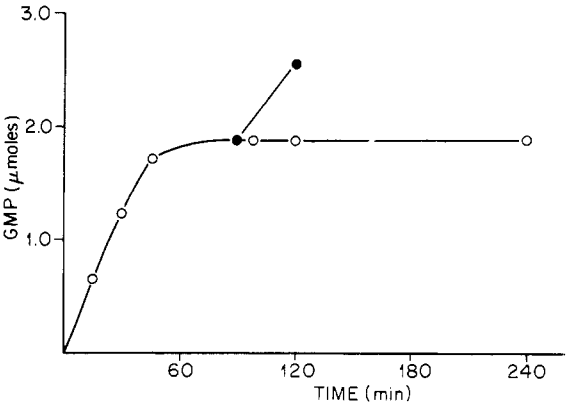

FIGURE 5: Time course study of di-GDP hydrolysis. Initially, all vessels contained 1.85 μmoles di-GDP, 100 μmoles Tris-HCl, *p*H 8.0, 4.0 μmoles $MgCl_2$, and 680 μg protein, final volume 2.0 ml. Ninety minutes after the onset of the reaction an additional 1.85 μmoles di-GDP and 4 μmoles $MgCl_2$ were added. All reactions were carried out at 45°. O—O, di-GDP alone; ●—●, di-GDP + additional di-GDP.

$MnCl_2$ on the rate of di-GDP hydrolysis were also determined over a wide range of concentrations. In the presence of Mg^{2+} optimal activity occurs when (Mg^{2+})/(di-GDP) = 2, whereas optimal activity for Mn^{2+} occurs when the ratio is 1. It is also apparent that high cation concentrations (0.01–1.0 M) show a marked inhibition of the reaction.

Substrate Specificity. Under conditions suitable for *asym*-di-GDPase activity, several phosphorylated compounds were assayed for substrate activity with the partially purified enzyme preparation. Adenosine mono-, di-, tri-, and tetraphosphates, cytidine mono-, di-, and triphosphates, uridine mono-, di-, and triphosphates, guanosine mono-, di-, and triphosphates, diguanosine triphosphate, nicotinamide-adenine dinucleotide, inorganic sodium pyrophosphate, fructose 6-phosphate, fructose 1,6-diphosphate, glyceraldehyde phosphate, phosphoenolpyruvate, ribonucleic acid, and deoxyribonucleic acid were tested as substrates. Notwithstanding the activity toward di-GDP, the results indicate the enzyme preparation to be slightly active toward diguanosine triphosphate, RNA, and DNA, but completely inactive toward all other compounds tested.

Action of asym-Di-GDPase on Di-GDP. Figure 5 shows the time course of di-GDP hydrolysis as determined by the release of guanosine monophosphate. Hydrolysis of 35% occurs in 15 minutes, and the reaction goes to completion in 75 minutes. Further addition of di-GDP 90 minutes after the onset of the reaction showed the enzyme still to be active, but the rate was only 65% of that initially observed. The reaction rate has been studied in the presence of varying amounts of enzyme, and between 0.085 and 1.55 mg protein ml^{-1} in the incubation mixture the reaction rate is linear.

Effect of Substrate and Other Compounds on the Reaction Rate. The data in Figure 6 show that substrate inhibition occurs at di-GDP concentrations greater than 2×10^{-3} M, whereas lower amounts give a satisfactory double-reciprocal plot. Small quantities (less than 5×10^{-4} M) of commercial GTP preparations (Pabst Laboratories, Sigma Chemical Co., and Calbiochem) inhibit markedly the enzymic hydrolysis of di-GDP, whereas GTP prepared from hydrolysis of di-GDP by *asym*-di-GDPase shows no inhibitory effect at concentrations as high as 5×10^{-3} M. Commercially available ATP also shows slight inhibitory effects, and comparable preparations of GMP and GDP showed no inhibitory effect at all concentrations tested. When *asym*-di-GDPase is assayed in the presence of optimal amounts of magnesium, the reaction is not inhibited by ions of barium, ferricyanide, pyrophosphate, or orthophosphate at 1×10^{-2} M, while fluoride completely inhibits the reaction under these conditions.

In view of its structural and chemical similarity to di-GDP, diguanosine triphosphate was tested both as an inhibitor and substrate of *asym*-di-GDPase. When the two diguanosine compounds are present in the reaction mixture (in equimolar quantities), less than 5% inhibition is observed as compared with the control containing only di-GDP. Under conditions of this assay, no significant amount of GMP was liberated by diguanosine triphosphate hydrolysis. When diguanosine triphosphate was tested as substrate, the rate of hydrolysis was less than 1% the rate of di-GDP hydrolysis under otherwise identical conditions.

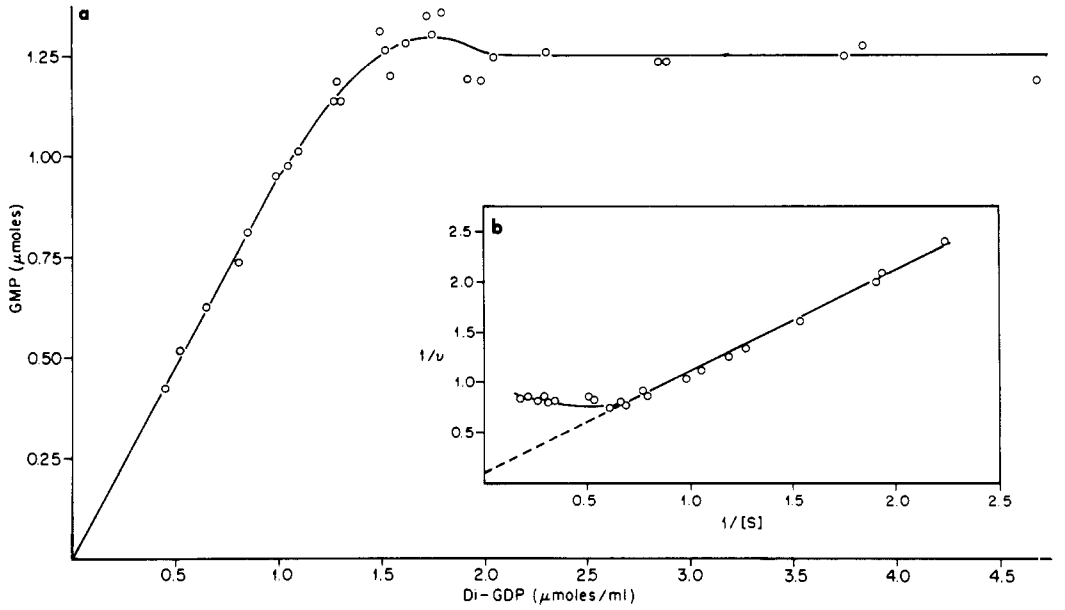

FIGURE 6: Influence of substrate on initial reaction rate. All reaction vessels contained 200 μmoles Tris-HCl, *p*H 8.0, $MgCl_2$ (Mg^{2+} to di-GDP ratio of 2.0), 340 μg protein, and di-GDP as indicated, final volume 1.0 ml. All reactions were run for 30 minutes at 45°, and GMP liberation was determined by ion-exchange chromatography. (a) Substrate curve; (b) double-reciprocal plot of data in (a).

Discussion

Maximal extraction of *asym*-di-GDPase from encysted eggs of *A. salina* is obtained when the arrested embryos are extracted with 0.3 M NaCl containing 0.05 M Tris-HCl, *p*H 7.4; lower sodium chloride concentrations incompletely solubilize the enzyme. Although the homogenate is buffered at *p*H 7.4 with Tris-buffer, the crude enzyme preparation is stable between *p*H 5 and 10. The crude salt-fractionated preparation is stable only 2–3 days at −20°, whereas low ionic strength solutions of the purified enzyme can be stored at −20° for several months without loss of activity.

The use of DEAE-cellulose does not elevate significantly the specific activity of the preparation, but is invaluable in removing contaminating nucleic acid components while permitting the recovery of virtually all the enzyme activity. Additionally, filtration through G-100 Sephadex appears to be the best single step in the purification scheme, combining good resolution and quantitative recovery.

Electrophoresis on polyacrylamide gels indicates the preparation to be contaminated with several proteins. Although the elution profile from CM-cellulose suggests the presence of multiple forms of *asym*-di-GDPase, electrophoresis on acrylamide shows only one protein band active toward di-GDP. Whether or not multiple forms of this enzyme exist remains to be clearly demonstrated.

Unlike snake venom phosphodiesterase, which hydrolyzes di-GDP into GMP and GTP, and then GTP to GMP and inorganic pyrophosphate (Finamore and Warner, 1963), *asym*-di-GDPase is inactive toward GTP. Most of the nuclease activity found in the *asym*-di-GDPase preparation appears to be associated with contaminating proteins and not with *asym*-di-GDPase. Furthermore, when diguanosine triphosphate was tested as substrate for the new enzyme very little activity could be measured after 15 hours of incubation under conditions identical to those employed for optimal di-GDP hydrolysis. However, activity toward diguanosine triphosphate has not been determined under varying Mg^{2+} or Mn^{2+} concentrations. The low level of activity toward diguanosine triphosphate prevented further analysis during gel electrophoresis.

The metal requirement of *asym*-di-GDPase is not surprising in view of the fact that many phosphohydrolases require metals for their hydrolytic action (Garen and Levinthal, 1960; Perry, 1951; Naganna *et al.*, 1955). It is interesting, however, to note the different activation patterns of Mg^{2+} and Mn^{2+}: whereas magnesium shows optimal activity at a metal-to-substrate ratio of 2, manganese shows optimal activity at 1.

Several known enzyme inhibitors were tested against *asym*-di-GDPase with varying results. Neither barium nor ferricyanide ions inhibited *asym*-di-GDPase activity at 1×10^{-2} M, whereas fluoride and EDTA completely suppressed the enzymic activity at this concentration. Inhibition by EDTA is probably the result of chelation of required metal ions, whereas fluoride is thought to form an inactive fluoride-Mg-substrate complex through the phosphate groups on di-GDP.

Although commercial preparations of ATP and GTP are strong inhibitors, exhaustive purification of these

nucleotides by treatment with charcoal as well as anion and cation exchange resins reduces significantly their inhibitory effects. However, no single purification step is sufficient to remove the inhibitor(s). Also, additional magnesium is unable to reverse the inhibition.

In Figure 6 the initial reaction rate is shown to deviate from ideality at substrate concentrations greater than 1.75×10^{-3} M. Since the magnesium concentration is varied in proportion to the added di-GDP, it is unlikely that di-GDP is competing with the "true substrate" for magnesium, an observation previously made for inorganic pyrophosphatase (Bloch-Frankenthal, 1954; Dixon and Webb, 1964). Whether the observed inhibition is due to molecular "jamming" of the active site(s) on the enzyme, substrate aggregation, or some other cause has not yet been ascertained.

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