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ISOLATION, PURIFICATION, AND SOME PROPERTIES OF P^1,P^4-DIGUANOSINE 5'-TETRAPHOSPHATE asymmetrical-PYROPHOSPHOHYDROLASE FROM BRINE SHRIMP EGGS (Thesis)

A. H. Warner

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ISOLATION, PURIFICATION, AND SOME PROPERTIES OF P^1,P^4-DIGUANOSINE 5'-TETRAPHOSPHATE asymmetrical-PYROPHOSPHOHYDROLASE FROM BRINE SHRIMP EGGS

A. H. Warner

Submitted as a dissertation to the Department of Physiology in the Graduate School of Southern Illinois University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

OCTOBER 1964

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ACKNOWLEDGEMENTS

The author wishes to express his thanks to Dr. F. J. Finamore for valuable criticism during the preparation of this dissertation. Thanks are also due Drs. E. Volkin and A. Hollaender of the Biology Division at Oak Ridge National Laboratory where the experimental work was performed and to the National Institutes of Health for financial support.

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INTRODUCTION

Encysted embryos of Artemia salina have been studied extensively by classical embryologists and geneticists but unfortunately very little biochemical information on their embryonic development has come forth. Unlike most organisms, these embryos can be maintained anabiotically for several years under the most adverse environmental conditions and then resume normal development upon immersion in sea water.

The recent finding that large quantities of a unique pyrophosphate ester, P^1,P^4-diguanosine 5'-tetraphosphate (di-GDP), occur in encysted embryos of Artemia salina (1) prompted the search for an enzyme system in the undeveloped egg capable of attacking this compound.

The present paper describes the isolation and purification of a new pyrophosphohydrolase capable of hydrolyzing di-GDP. This enzyme, P^1,P^4-diguanosine 5'-tetraphosphate asymmetrical-pyrophosphohydrolase (asym-di-GDPase), apparently has an absolute substrate specificity and produces equimolar quantities of guanosine monophosphate and guanosine triphosphate from diguanosine 5'-tetraphosphate.

METHODS

ISOLATION AND PURIFICATION

Preparation of 23,500 x g fraction. Thirty-two grams of dry cysts of the brine shrimp, *Artemia salina* (Wardley and Co.), were ground unmoistened with sand in a motor-driven mortar and pestle for 30 minutes. Enough cold 0.30 molar NaCl containing 0.05 M TRIS-HCl*, pH 7.4, was added to make a thick slurry and the grinding continued for an additional 10 minutes in the cold. 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, pH 7.4, and the extraction carried out for 30 minutes with stirring. The homogenate was centrifuged for 30 minutes at 23,500 x g and the supernatant fluid decanted through several layers of cheesecloth to remove floating debris.

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

*The following abbreviations have been used: TRIS-HCl, Tris(Hydroxymethyl) aminomethane; EDTA, ethylenediamine tetraacetate; DEAE, diethylaminoethyl; CM, carboxymethyl; GMP, guanosine 5'-monophosphate; GDP, guanosine diphosphate; and GTP, guanosine triphosphate.

Treatment with Diethylaminoethyl-cellulose. DEAE-cellulose (Matheson, Coleman and Bell Co.) was prepared according to Peterson and Sober (2) and equilibrated with 0.05 M TRIS-HCl, pH 8.5, prior to use. The enzymatically active fraction was percolated through a DEAE-cellulose column (2 x 20 cm) and washed with two bed volumes of cold buffer. Under these conditions nucleic acid components are retained by the cellulose whereas the enzyme fraction passes through and can be recovered from the effluent by saturation with ammonium sulfate, followed by centrifugation. The precipitate was dissolved in 10-15 ml of 0.03 M NaCl containing 0.025 M TRIS-HCl, pH 8.5.

Filtration on Sephadex. G-100 Sephadex (Bead form, Pharmacia Fine Chemicals, Inc.) was prepared in the same manner as DEAE-cellulose and equilibrated with 0.30 M NaCl containing 0.025 M TRIS-HCl, pH 8.5. Following DEAE-cellulose treatment and ammonium sulfate concentration the active enzyme fraction was layered over a Sephadex column (3.5 x 75 cm) and filtration was performed using 0.30 M NaCl containing 0.025 M TRIS-HCl, pH 8.5. Fractions of 10-12 ml were collected with an automatic fraction collector at a rate of 0.75 ml per minute and read at 260, 270, 280, and 290 millimicrons in a model DB Beckman Spectrophotometer. Each fraction was assayed for protein according to the method of Lowry et al (3) and for asym-di-GDPase activity as described under assay methods. Figure I depicts the protein elution profile from G-100 Sephadex. 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, pH 4.0.

Fractionation on Carboxymethyl-cellulose. The carboxymethyl-(CM) cellulose (Brown Co.) used in all experiments was prepared in the same manner as DEAE-cellulose and Sephadex. The dialyzed protein solution was adjusted to pH 4.0 with 5 M acetic acid, applied to a CM-cellulose column (2.5 x 18 cm) and washed with 200 ml of 0.025 M sodium acetate, pH 4.0. Elution was carried out stepwise with increasing amounts of sodium chloride as shown in Figure II. Fractions of 8-10 ml were collected automatically, 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°C.

ASSAY OF ACTIVITY

Liberation of orthophosphate. Early experiments on the characterization of di-GDP showed that a bacterial alkaline phosphatase preparation (Worthington and Co.) was inactive towards di-GDP but released orthophosphate (Pi) 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 Pi indicated the presence of asym-di-GDPase activity.

During the purification of asym-di-GDPase all assays were performed in the following manner: the desired fraction was incubated with 0.5 micromole di-GDP, 4.0 micromoles $MgCl_2$, 100 micromoles TRIS-HCl, pH 8.0, and with an excess of the alkaline phosphatase preparation at 40°C. for 20 minutes. In all cases the final volume was 3.0 ml. After incubation the reaction was stopped by the addition of 0.5 ml cold 10 N H_2SO_4 and Pi was determined according to Ernster et al (4). When Pi^{32} was to be determined aliquots were removed, plated, and counted in a SC-88 Tracermatic scaler.

Release of guanosine 5'-monophosphate. 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°C., made slightly alkaline with dilute NH_4OH and applied to small Dowex-1-Cl^- (0.9 x 1.5 cm.) columns. The columns were washed with water and 0.01 M NH_4Cl; then GMP was eluted with 125 ml of 0.010 N HCl. In many cases GTP was also determined to corroborate results obtained with GMP measurements.

DISC ELECTROPHORESIS

For all electrophoretic experiments highly purified acrylamide monomers (Products Distillation Co.) were used. The acrylamide polymers were prepared according to Reisfeld et al (5) and all electrophoretic runs were carried out with the Model 12 Disc Electrophoresis Apparatus (Canal Industrial Co.) using 0.5 x 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 angstroms (6). The spacer and sample gels were composed of 6% and 3% acrylamide respectively. In all experiments the sample gel contained between 150-225 micrograms protein. All experiments were performed at 5°C. and at pH 4.3 using 0.175 M ß-alanine-acetate buffer. Satisfactory results were obtained when a current of 7 milliamperes 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 Analine-Naphthol Blue Black) while the remaining unstained gels were frozen over solid CO_2. Destaining of the gels was carried out under the influence of 10 milliamperes per column until clear distinct bands were apparent. For the asym-di-GDPase assay the frozen gels were cut into 1 millimeter cross sections and eluted for 3 hours at 37°C. with a TRIS-HCl buffered substrate solution containing an excess of bacterial alkaline phosphatase.

The presence of asym-di-GDPase activity in the gel segments was determined as the release of inorganic orthophosphate.

Nuclease activities were determined on 1 millimeter gel sections by incubating one-half of each segment with thymus DNA and the other half with yeast RNA at pH 8.0 for 2-16 hours. Under these conditions nuclease activity was determined by measuring the appearance of acid-soluble, ultraviolet absorbing compounds 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 from the preparation. In all experiments recovery of active enzyme from Sephadex was greater than 95%. In Figure II 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. Table I summarizes the course of asym-di-GDPase purification. One difficulty encountered during the course of purification is the inability to completely "salt-out" the active material after DEAE 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.

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 III shows the presence of six distinct protein bands, only one of which shows asym-di-GDPase activity. In the bottom half of Figure III the solid line shows the location of asym-di-GDPase activity in the gel while the two broken lines 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 towards di-GDP.

Products of hydrolysis. Hydrolysis of di-GDP by the partially purified enzyme described above 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 pre-treatment of the enzyme preparation with heat or chymotrypsin and partially inhibited when assayed in the presence of p-hydroxymercuribenzoate.

pH optimum. The maximal rate of di-GDP hydrolysis occurs about pH 8 as shown in Figure IV.

Activation energy. The effect of temperature on the hydrolysis of di-GDP is shown in Figure V. The optimal temperature of hydrolysis is 45°C. and the activation energy is approximately 6,000 calories $mole^{-1}$.

Heat inactivation. The partially purified asym-di-GDPase preparation appears to be heat labile in the absence of substrate. Incubation of the enzyme preparation for 5 minutes at 37°C. and 45°C. shows 18% and 69% inactivation respectively while heating to 80°C. 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 maximum activity. Table II summarizes the results 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} molar) plus TRIS-HCl buffer, pH 8.0 and enzyme. When mercaptoethanol and EDTA were used, 10 micromoles of each were added. The effect of $MgCl_2$ and $MnCl_2$ on the rate of di-GDP hydrolysis was also determined over a wide range of concentrations. In the presence of Mg^{+2} optimal activity occurs when (Mg^{+2})/(di-GDP) equals 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 molar) show a marked inhibition of the reaction.

Substrate specificity. Under conditions suitable for asym di-GDPase activity, phosphorylated compounds were assayed for substrate activity with the partially purified enzyme preparation. Inorganic pyrophosphate, fructose 6-phosphate, fructose 1,6-diphosphate, glyceraldehyde phosphate, phosphoenolpyruvate, adenosine mono-, di-, tri-, and tetra- phosphates, cytidine mono-, di-, and triphosphates, uridine mono-, di-, and triphosphates, guanosine mono-, di-, and triphosphates, ribonucleic acid, and deoxyribonucleic acid were tested as substrates. Notwithstanding the activity towards di-GDP, the results indicate the enzyme preparation to be slightly active towards RNA and DNA while completely inactive towards all other compounds tested.

Action of asym-di-GDPase on di-GDP. Figure VII shows the course of di-GDP hydrolysis as determined by the release of guanosine monophosphate. Thirty-five percent hydrolysis occurs in 15 minutes while the reaction goes to completion in

75 minutes. Further addition of di-GDP, 90 minutes after the onset of the reaction, shows the enzyme still to be active; however, the rate is only 65% of that initially observed. When small amounts of guanosine triphosphate are present initially the reaction rate is inhibited markedly but the reaction is complete in about 2.5 hours. Furthermore, when hydrolysis is carried out in the presence of bacterial alkaline phosphatase, the reaction velocity is increased by about 12%, presumably because GTP is removed from the reaction mixture.

The reaction rate has been studied in the presence of varying amounts of enzyme and between 0.17-2.1 milligram protein the reaction rate is linear.

Effect of substrate and hydrolytic products on the reaction rate. From the data in Figure VI it is apparent that one of the products, namely GTP, is acting as an inhibitor of the reaction. Under all conditions tested GMP failed to inhibit the reaction while GTP was effective in micromolar quantities. When the data in Figure VI are plotted in the double reciprocal manner described by Lineweaver and Burk (7) Figure VIII results. These data suggest that GTP inhibits the reaction non-competitively.

DISCUSSION

Maximal extraction of asym-di-GDPase from Artemia salina eggs is obtained when the eggs are ground in 0.3 M NaCl containing 0.05 M TRIS-HCl, pH 7.4; lower sodium chloride concentrations incompletely solubilizes the enzyme. Although the homogenate is buffered at pH 7.4 with TRIS-HCl, the crude enzyme preparation is stable at pH's ranging from 5 to 10.5. The use of DEAE-cellulose does not elevate significantly the specific activity of the preparation, but proved invaluable in removing contaminating nucleic acid components while permitting the recovery of virtually all of 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 carboxymethylcellulose suggests the presence of multiple forms of asym-di-GDPase, electrophoresis on acrylamide shows only one protein band active towards 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 (1), asym-di-GDPase is inactive towards GTP and all other phosphate compounds tested. 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.

The metal requirement of asym-di-GDPase is not surprising in view of the fact that many phosphohydrolases require metals for their hydrolytic action (8, 9, 10, 11). It is interesting, however, that Mg^{+2} and Mn^{+2} behave differently in their activation patterns. Whereas magnesium shows optimal activity at a metal-to-substrate ratio of 2, manganese shows optimal activity at 1.

If the assumption is to be made that di-GDP represents a primary source of energy for embryonic development of Artemia, it then becomes necessary to study the metabolic mechanism involved in regulating the organism's energy requirements. The data in Figures VI and VII show that guanosine triphosphate has a marked inhibitory effect on di-GDP hydrolysis in vitro and suggest the presence of an effective energy regulating mechanism in these eggs. Conversely, guanosine monophosphate has no effect on the reaction rate which further supports the idea of an endogenous regulatory mechanism rather than a simple product-reactant equilibrium phenomenon.

Figure VI also shows the reaction rate not to be proportional to inhibitor (GTP) concentration and that inhibition can be partially reversed at high substrate concentrations. When these data are plotted in the double reciprocal manner previously described the inhibition appears to be non-competitive in nature. Furthermore, if the hydrolysis is allowed to proceed normally in the absence of added GTP the reaction goes to completion (100%) in 75 minutes although GTP is constantly being generated in the reaction. However, if GTP is added initially to the reaction mixture, as shown in Figure VII, the reaction rate is markedly inhibited

although the reaction does go to completion. If additional di-GDP is added 90 minutes after the onset of the reaction, hydrolysis of di-GDP will continue but at a reduced rate. Whether this decreased rate of hydrolysis is due to GTP inhibition, enzyme denaturation, or some other rate limiting step has not yet been ascertained.

The isolation and purification of a new enzyme capable of hydrolyzing di-GDP *in vivo* offers a system by which the metabolism of this compound in the developing egg can be studied.

SUMMARY

A new enzyme, P^1,P^4-diguanosine 5'-tetraphosphate asymmetrical-pyrophosphohydrolase (asym-di-GDPase), from the undeveloped eggs of the brine shrimp, *Artemia salina*, has been isolated, purified and partially characterized. The enzyme is specific for di-GDP, has a pH 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 by heat, chymotrypsin and p-hydroxymercuribenzoate and insensitive to ß-mercaptoethanol. It appears that an excess of guanosine triphosphate inhibits non-competitively the hydrolytic reaction while an excess of guanosine monophosphate shows no effect.

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Table I. Course of asym-di-GDPase purification.

Purification Steps	Specific Activity*	Percent 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

*Specific activity is defined as the number of enzyme units per milligram protein where one enzyme unit is equal to that amount of enzyme which releases 1 micromole Pi per hour.

Table II. Effect of cations on asym-di-GDPase activity.

Reagent	Relative Activities
$MgCl_2$	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
$HS\text{-}CH_2\text{-}CH_2\text{-}OH$	0
$HS\text{-}CH_2\text{-}CH_2\text{-}OH + MgCl_2$	100

All reaction vessels contained the following: 2.0 micromoles di-GDP, 2.0 micromoles cation, 100 micromoles TRIS-HCl, pH 8.0, and 0.2 ml asym-di-GDPase (3.4 milligrams per ml). The total volume was 2.0 ml. All reactions were run for 15 minutes at 40°C.

EXPLANATION OF FIGURES

Figure I. Fractionating on G-100 Sephadex column. 10 ml of the concentrated protein solution was applied to a 3.5 x 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, pH 8.5. ●——●, optical density at 280 mμ; O——O, asym-di-GDPase activity.

Figure II. Fractionating on Carboxymethyl-cellulose. The dialyzed enzyme preparation was adjusted to pH 4.0 by the addition of acetic acid and applied to a 2.5 x 18 cm column. The column was washed with 200 ml 0.025 M sodium acetate, pH 4.0, and the active enzyme eluted in the cold as indicated. ●——●, optical density at 280 mμ; O——O, asym-di-GDPase activity.

Figure III. 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 pH 4.3 for 75 minutes at a current of 7 milliamperes 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 Pi as described under Methods was used as the assay. For nuclease assays 1 milligram of either yeast RNA or thymus DNA in 0.5 ml 0.1 M TRIS-HCl, pH 8.0, containing 0.01 M $MgCl_2$ was added to tubes containing the gel segments. RNase assay was carried out for 2 hours at 37°C. while DNase activity was determined after 16 hours at 37°C. 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 aliquots were taken from the acid-soluble fraction for optical density measurements at 260 mμ. ——, densitometer curve; O——O, asym-di-GDPase activity; ●——●, RNase activity; □——□, DNase activity.

Figure IV. Effect of pH. Each reaction vessel contained 2.0 μmoles di-GDP, 4 μmoles $MgCl_2$, 50 μmoles buffer and 0.1 ml enzyme preparation containing 345 μgrams protein, final volume: 2.0 ml. The buffers used were maleate-NaOH, TRIS-HCl, and glycine-NaOH at 0.05 M concentrations. All incubations were carried out at 40°C. for 15 minutes. In all cases GMP liberation was measured by ion exchange chromatography.

Figure V. Influence of Temperature on Reaction Velocity. Each reaction vessel contained 2 μmoles di-GDP, 4 μmoles $MgCl_2$, 50 μmoles TRIS-

HCl, pH 8.0, and 690 μgrams 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.

Figure VI. Influence of Substrate and Products on Reaction Velocity. All reaction vessels contained 50 μmoles TRIS-HCl, pH 8.0, 690 μgrams enzyme preparation, $MgCl_2$ in a 2:1 ratio with respect to di-GDP and GMP and GTP as indicated above, final volume 2.0 ml. All reactions were carried out for 15 minutes at 45°C. then stopped by addition of an equal volume cold 1 N HCl. GMP liberation was determined by ion exchange chromatography.

Figure VII. Time Course Study of Di-GDP Hydrolysis. Initially, all vessels contained 1.85 μmoles di-GDP, 100 μmoles TRIS-HCl, pH 8.0, 4.0 μmoles $MgCl_2$, and 690 μgrams protein, final volume 2.0 ml. Ninety minutes after the onset of the uninhibited reaction vessel an additional 1.85 μmoles di-GDP and 4 μmoles $MgCl_2$ were added. In the inhibited reaction vessels, 0.43 μmole GTP was added. All reactions were carried out at 45°C. O——O, di-GDP alone; ●——●, di-GDP + 0.43 μmole GTP; ▲——▲, di-GDP + additional di-GDP.

Figure VIII. Non-Competitive Inhibition by GTP. Each reaction vessel contained 50 μmoles TRIS-HCl, pH 8.0, $MgCl_2$ at a 2:1 ratio with di-GDP, 690 μgrams enzyme and di-GDP and GTP as indicated above, final volume 2.0 ml. All reactions were run for 15 minutes at 45°C. and GMP liberation was determined by ion exchange chromatography.

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FIGURE I

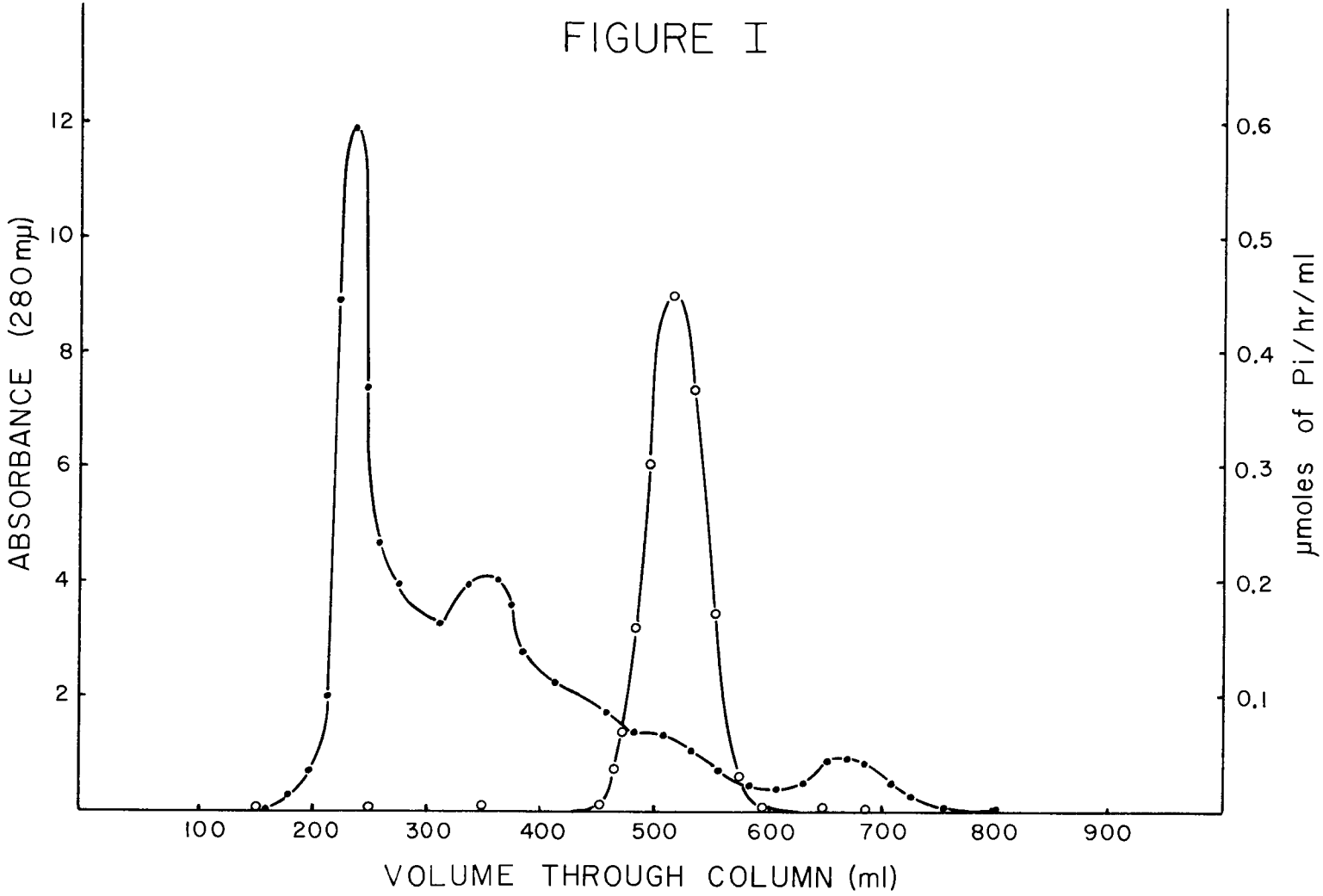

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FIGURE II

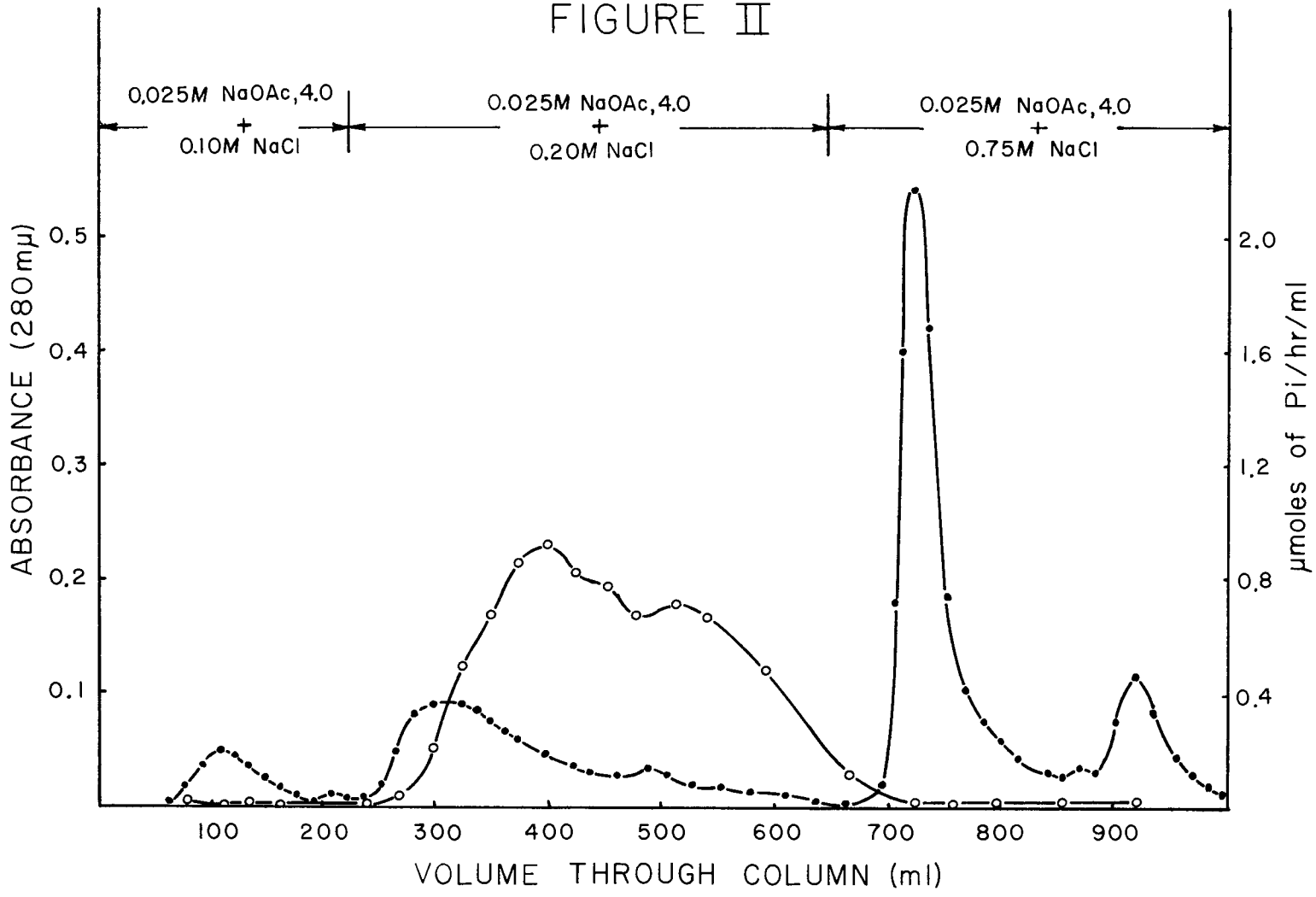

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FIGURE III

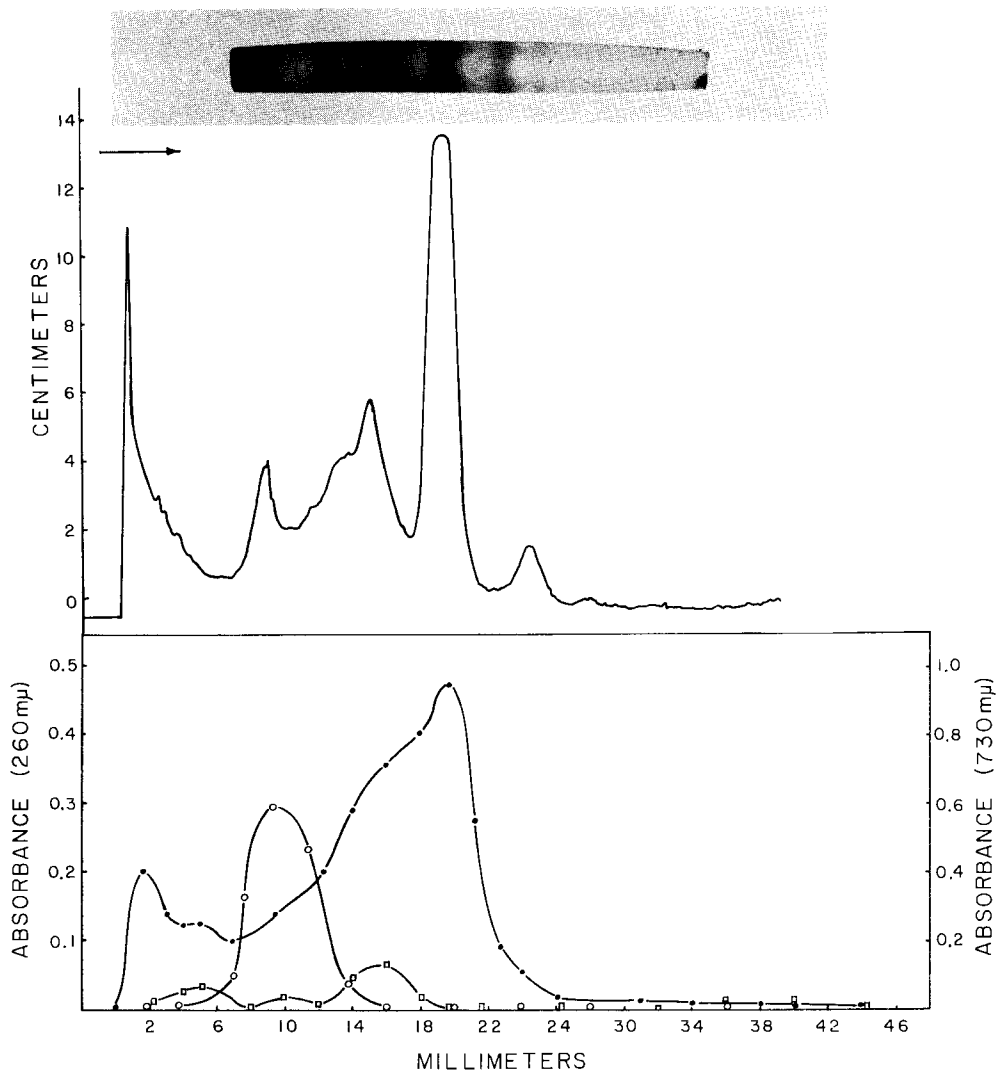

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FIGURE IV

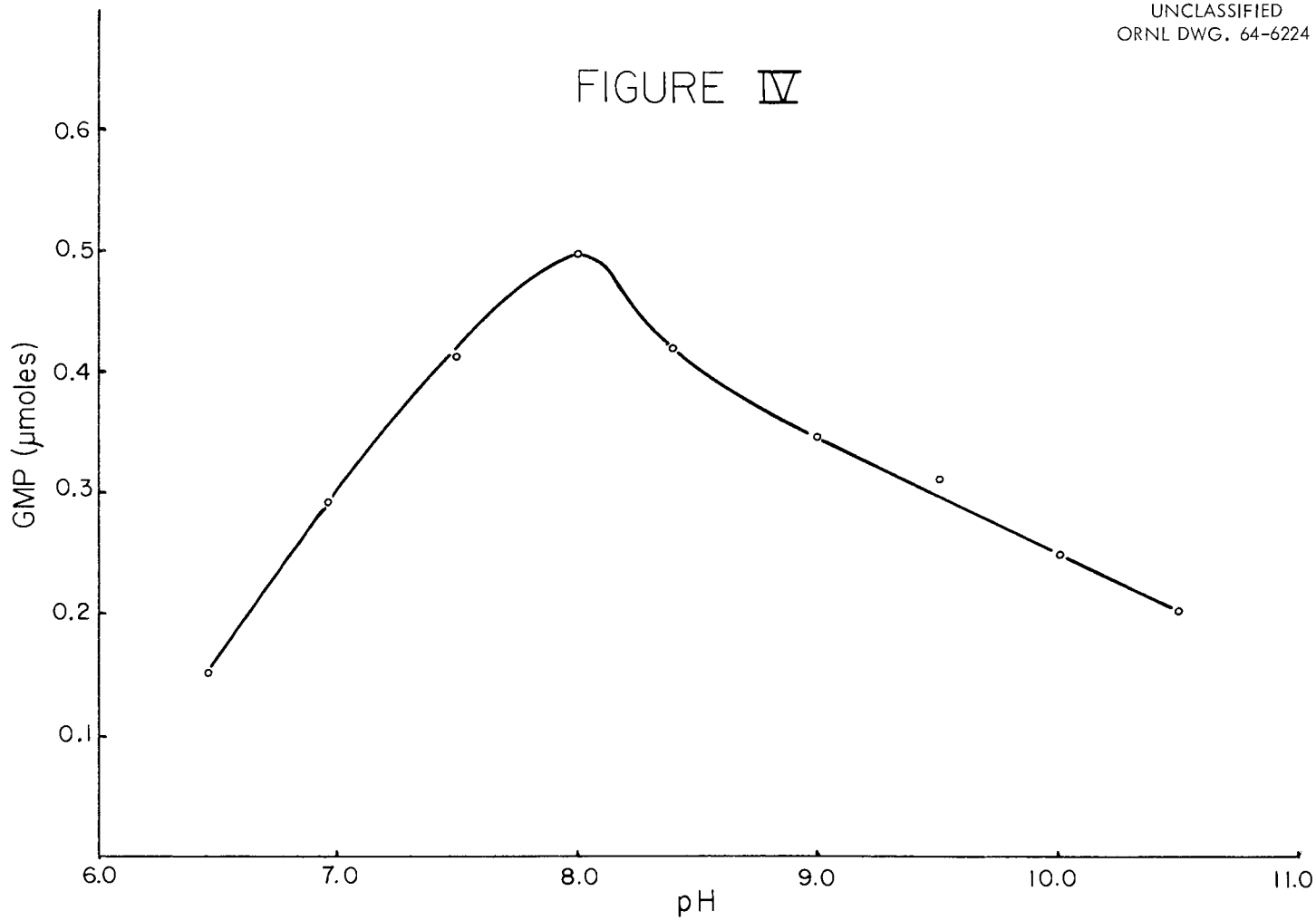

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FIGURE V

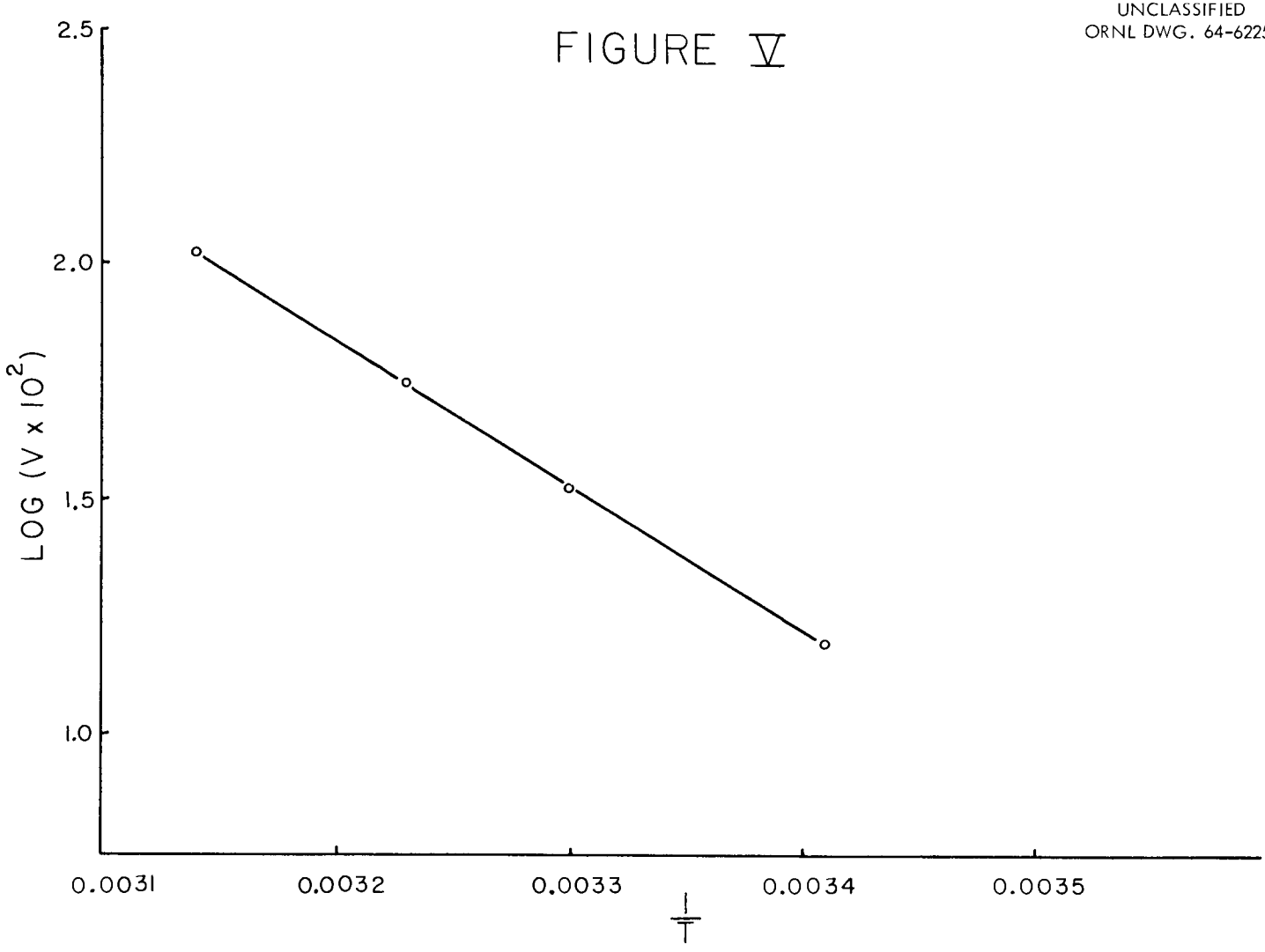

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FIGURE VI

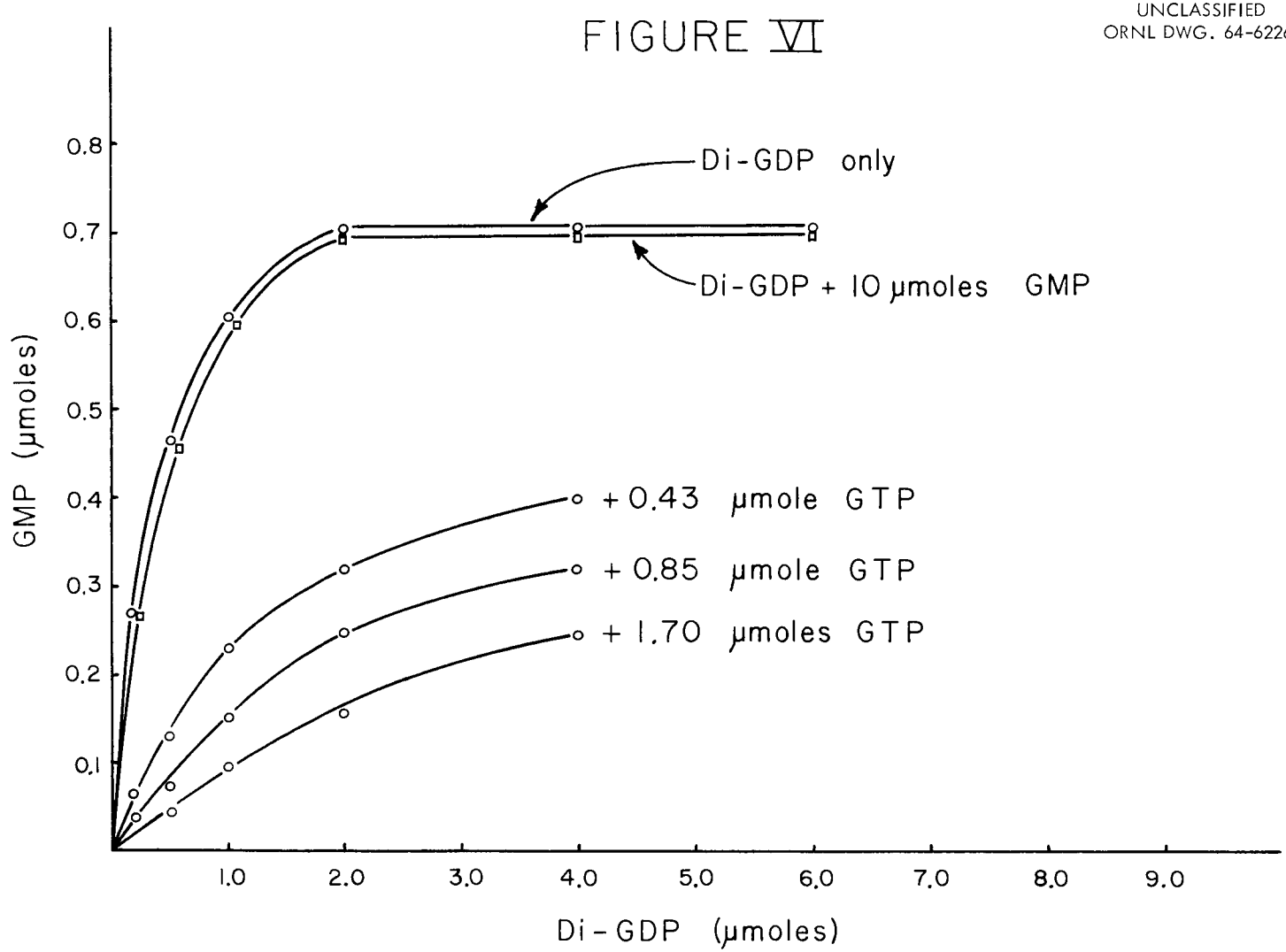

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FIGURE VII

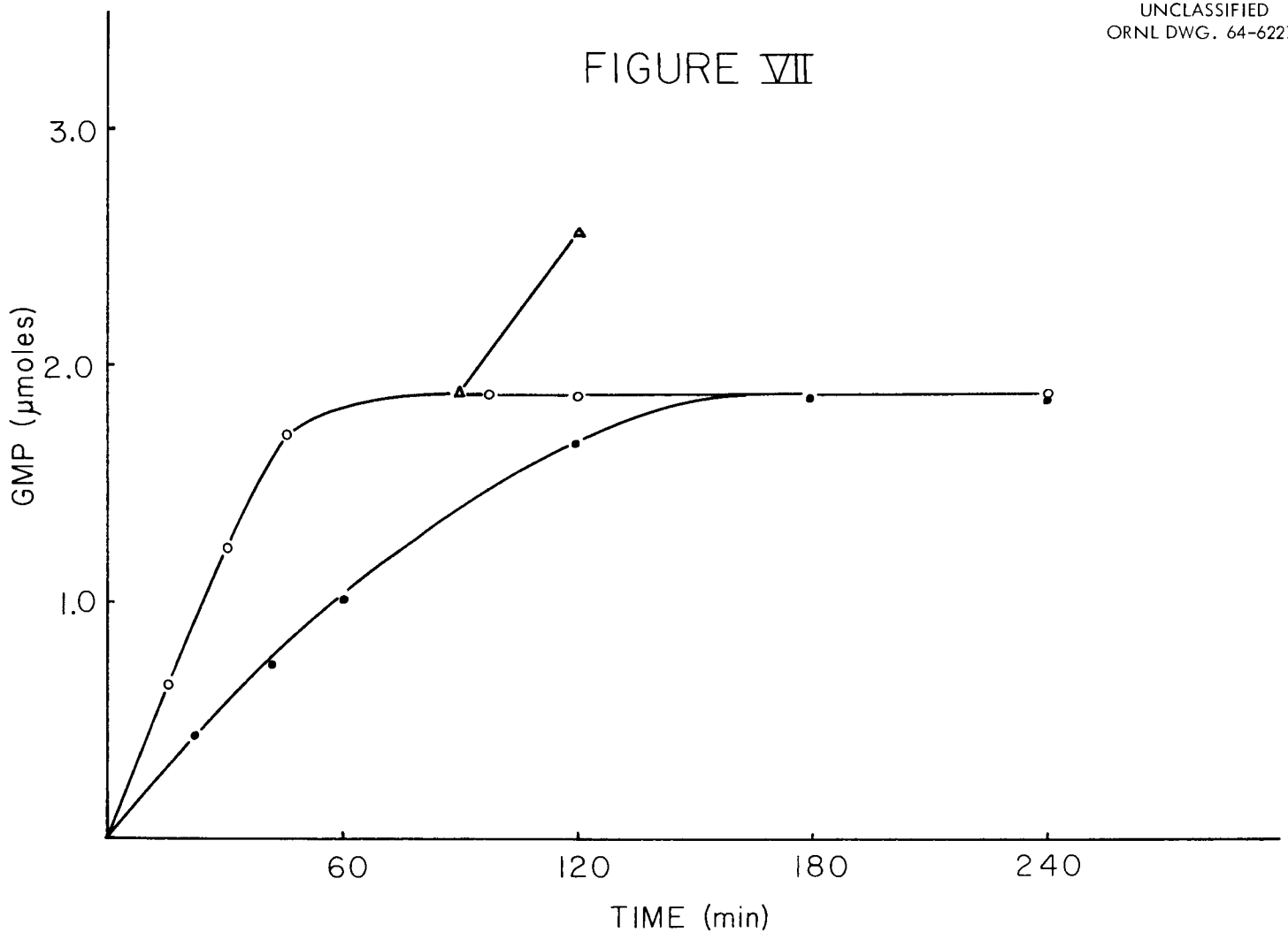

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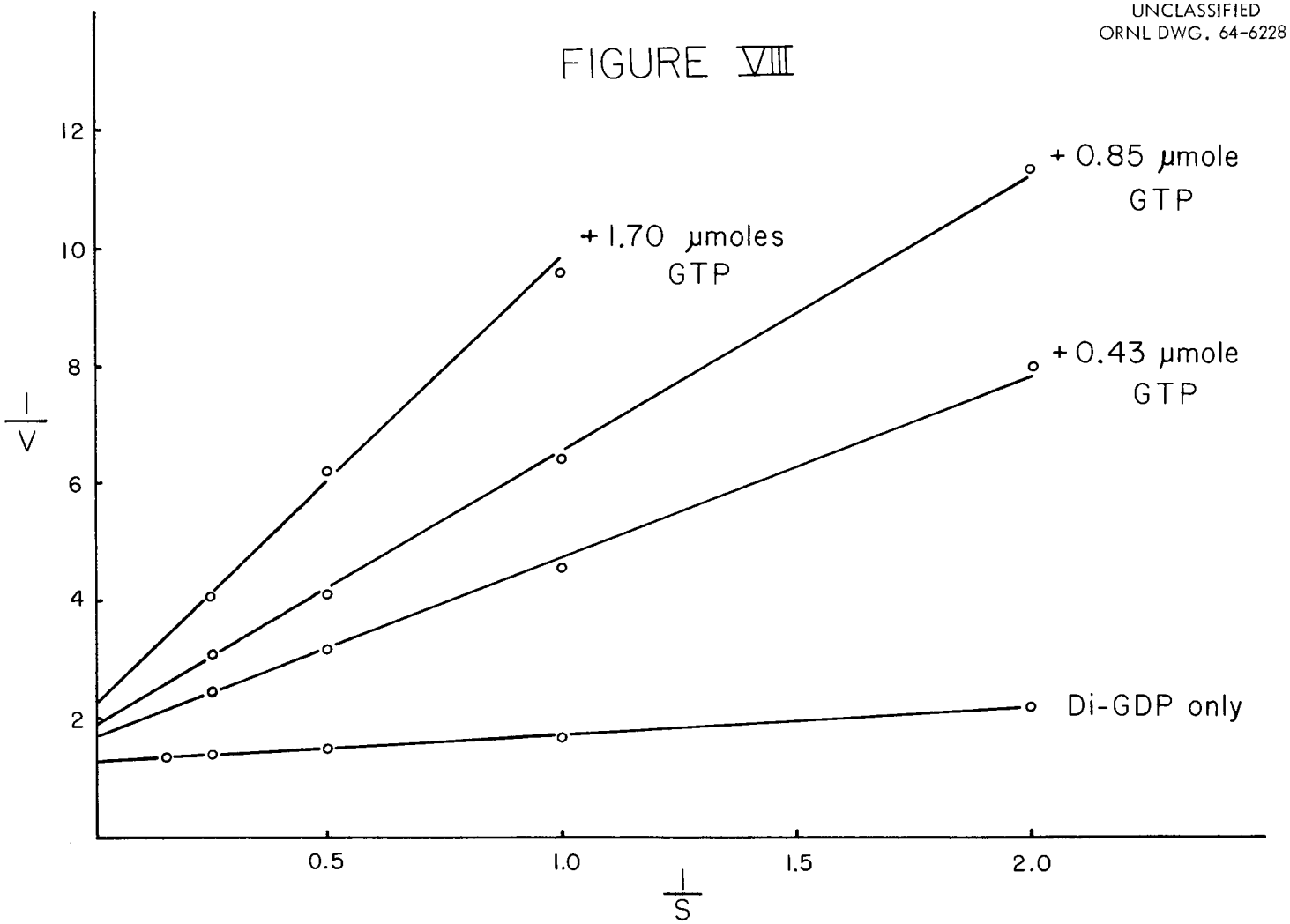
FIGURE VIII



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