Guanosine Monophosphate Reductase from *Artemia salina*: Inhibition by Xanthosine Monophosphate and Activation by Diguanosine Tetraphosphate†

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ABSTRACT: In the course of studies on the metabolic role of diguanosine tetraphosphate during development of *Artemia salina*, a guanosine monophosphate (GMP) reductase has been found and partially purified from the 150 000*g Artemia* cysts supernatant. From Lineweaver–Burk plots, two apparent K_m values of 5 and 50 μM were obtained for GMP. Xanthosine monophosphate (XMP) is a very strong inhibitor of the reaction. In the presence of 1.5 μM XMP hyperbolic kinetics are found. Diguanosine tetraphosphate counteracts very effectively the inhibition of the activity by XMP, concomitantly changing to hyperbolic the kinetics of the enzyme, with a unique K_m value of about 5 μM. The complex kinetic and the existence of allosteric effectors at physiological concentrations, together with our lack of success in resolving two isoenzymes, makes it very likely that GMP reductase presents negative cooperativity towards its substrate. The effect of diguanosine tetraphosphate on the enzyme is very specific; other structural analogues, diadenosine tetraphosphate and diguanosine triphosphate, tested at micromolar concentrations had no detectable effect on the enzyme. Guanosine triphosphate (GTP) (mM) was also able to counteract the inhibition of guanosine monophosphate (GMP) reductase by XMP. The properties of the *Artemia* GMP reductase are here compared with those of the similar enzyme from calf thymus and *Escherichia coli*. As a consequence, the regulation of eukaryotic GMP reductase is resulting to be quite different from that of the reductase from prokaryotes.

Part of the work of our laboratory has been focused on the metabolism and function of diguanosine tetraphosphate (Gp_4G[1]) in *Artemia salina*. This nucleotide is especially abundant (10 mM) at the gastrula stage and its level decreases during development to larva (Warner and Finamore, 1967; Sillero and Ochoa, 1971). During this period, *Artemia salina* does not synthesize purines de novo (Clegg et al., 1967) and uses Gp_4G as a source of both guanine and adenine nucleotides. The first step in the metabolism of Gp_4G is, most probably, its hydrolysis to GTP and GMP carried out by diguanosinetetraphosphatase (EC 3.6.1.17) (Warner and Finamore, 1965; Vallejo et al., 1974, 1976). Searching for the metabolic fate of GMP, a specific reductase was found in *Artemia* extracts which transforms GMP into IMP (Renart and Sillero, 1974).

Simultaneously, GMP reductase was also characterized in calf thymus (Stephens and Whittaker, 1973). Previously, a GMP reductase activity had been detected in human and rabbit erythrocytes (Hershko et al., 1963). With these reports, the existence of this enzyme in eukaryotes is firmly established. The K_m value described for the GMP reductase from *Artemia* and calf thymus was similar, around 5 μM. In addition, the thymus enzyme was reported to be competitively inhibited by xanthosine 5′-monophosphate (XMP) with a K_i value of around 10 nM (Stephens and Whittaker, 1973).

In the course of studies on the effect of XMP on the *Artemia* GMP reductase, the enzyme was assayed in the presence of a wider range of GMP concentration and found to present negative homotropic kinetics towards its substrate GMP. XMP and Gp_4G, at nanomolar concentrations, were negative and positive effectors of the reaction, respectively. The object of this paper is to present these findings together with other properties of the enzyme. A preliminary account of these results has been published (Renart et al., 1975).

Experimental Procedures

Artemia eggs were from Longlife Fish Products, Ontario, Canada. All operations were conducted at 0–4 °C. Immediately before use, the eggs were resuspended in water and washed several times, followed by treatment with 1% NaClO for 3 min at room temperature. After decantation, the eggs were thoroughly washed with glass-distilled water to remove residual NaClO. The eggs were resuspended in 10 ml of ice-cold buffer A (0.035 M Tris-HCl, pH 7.7, 0.07 M KCl, 0.009 M $MgCl_2$, 5 mM mercaptoethanol, 0.25 M sucrose) per g of original dry eggs. In a typical experiment of enzyme purification, aliquots of 25 g each were stirred overnight in buffer A. The homogenate was filtered through glass wool to remove cysts walls, and centrifuged at 5000*g* for 10 min. The supernatant was centrifuged again at 20 000*g* for 2 h and the supernatant filtered through glass wool to remove the lipid layer formed during the centrifugation.

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[1] Abbreviations used are: Ap_4A, diadenosine 5′,5‴-(P^1,P^4-tetraphosphate); Ap_4G, adenosine, guanosine 5′, 5‴-(P^1,P^4-tetraphosphate); Gp_4G, diguanosine 5′,5‴-(P^1,P^4-tetraphosphate); Gp_3G, diguanosine 5′,5‴-(P^1,P^3-triphosphate); p_4G, guanosine 5′-tetraphosphate; ATP, UTP, GTP, adenosine, uridine, and guanosine, 5′-triphosphates; GMP, IMP, XMP, guanosine, inosine, and xanthosine 5′-monophosphates; DEAE, diethylaminoethyl; EDTA, (ethylenedinitrilo)tetraacetic acid.

TABLE I: Purification of *Artemia salina* GMP Reductase.[a]

Step	Vol (ml)	Protein (mg) × 10^{-2}	Act. (units)	(Sp. Act. (milliunits/mg)	Recovery (%)	Purification Factor
Supernatant 20 000*g*	2432	148	10.6	0.7	100	1
45–70% $(NH_4)_2SO_4$	114	74.4	12.0	1.6	112	2
Heating at 55 °C	85	23.6	8.6	3.7	81	5
DEAE-cellulose	26	1.09	3.6	33	34	23
Sephadex G-200	99	0.22	2.7	120	26	168
Phosphocellulose	70	0.016	0.9	564	9	795

[a] Two hundred grams of *Artemia* cysts was used. One unit is the amount of enzyme able to transform 1 μmol of GMP/min at 30 °C. Proteins were measured by the method of Lowry et al., 1951.

Assay of GMP Reductase. Unless otherwise stated, the reaction mixture contained the following components in a final volume of 1 ml: 50 mM Tris-HCl, pH 7.5, 5 mM mercaptoethanol, 0.15 mM NADPH, 0.1 mM GMP, and extracts in a suitable amount to give an activity of 0.005–0.01 unit. Effectors were added to the reaction mixture when indicated. Decrease in absorbance at 340 nm was followed either in a Gilford 2400-S or in a Varian Techtron Model 635 spectrophotometer.

Other Materials. Nucleotides were purchased from Boehringer Mannheim and Pharma Waldhof. Gp_4G was obtained from *Artemia* cysts and Gp_3G and Ap_4A by chemical synthesis as previously described (Lobatón et al., 1975).

Results

Purification of Enzyme. The analysis of the negative homotropic kinetics described below forced us to develop a new method to further purify this enzyme. The modifications introduced to the method previously described (Renart and sillero, 1974) were as follows. In order to shorten the time of manipulation, the starting material was the 20 000*g* supernatant and the order of the DEAE-cellulose and Sephadex columns was interchanged. A purification step through a phosphocellulose column was introduced. Mg^{2+} ions were removed and 5% glycerol was added to the buffers. The purified enzyme was kept in 50% glycerol, at −20 °C.

The actual purification procedure was as follows. The 20 000*g* supernatant obtained from 200 g of cysts, as described under Experimental Procedures, was fractionated with ammonium sulfate as previously described (Renart and Sillero, 1974). The precipitate obtained between 0.4–0.75 $(NH_4)_2SO_4$ saturation was resuspended in buffer B (10 mM Tris-HCl, pH 7.5, 50 mM KCl, 0.1 mM EDTA, 0.1 mM dithioerythritol, 5% glycerol) and dialyzed overnight against the same buffer. The final solution, in a volume of 114 ml, was heated at 55 °C (Renart and Sillero, 1974) and centrifuged at 150 000*g* for 1 h. The precipitate obtained was washed with 10 ml of buffer B and centrifuged as above. Both supernatants were pooled and applied to a DEAE-cellulose column (58.5 × 2.5 cm) previously equilibrated in buffer B. The column was then washed with 300 ml of the same buffer. GMP reductase was eluted with 1800 ml of a linear gradient (0.05–0.3 M) of KCl in buffer B. Fractions of 7.5 ml were collected. Fractions 149–180 containing the major portion of MP reductase activity were pooled and concentrated with ammonium sulfate (90% saturation). The precipitate was resuspended in buffer B up to a final volume of 11 ml. This concentrate was applied to a Sephadex G-200 column (84 × 2.8 cm) previously equilibrated in buffer B, and eluted with the same buffer. Fractions of 7.9 ml were collected. The fractions 20–31 containing the GMP reductase activity were pooled (95 ml) and brought to pH 6.7 by addition of 0.1 M citric acid, and applied to a phosphocellulose column (8.8 × 1.8 cm) previously equilibrated in buffer C (10 mM sodium citrate, pH 6.5, 0.5 mM dithioerythritol, 5% glycerol). Upon application of the sample, the column was successively washed with 23 ml of the same buffer and 50 ml of buffer C supplemented with 0.15 M KCl. GMP reductase was eluted with 0.2 M KCl in buffer C. Fractions of 4 ml were collected. Fractions containing the major portion of GMP reductase activity were pooled, brought up to pH 7.5 with KOH, and concentrated using an Amicon P-30 membrane. The same volume of glycerol was added to the concentrate and the enzyme preparation was kept at −20 °C. With this method, a purification of 800-fold with a recovery of 9% was obtained (Table I).

Kinetic Properties of GMP Reductase. Effect of GMP Concentration. In previous studies on the influence of the GMP concentration on the initial velocity of the enzyme, a hyperbolic kinetics was observed and a K_m of about 5 μM was calculated (Renart and Sillero, 1974). However, upon studying the effect of XMP on GMP reductase (see below), a wider range of GMP concentrations was used and two apparent K_m of 5 and 50 μM were detected (Figure 1). From a Hill plot, two n_H values of 0.3 and 1.2 were obtained at concentrations of GMP below and above 50% saturation, respectively. The theory of kinetics of this type has been worked out by Koshland and co-workers (Koshland, 1970) and two main causes have been presented as responsible for this enzymatic behavior: the existence of a type of interaction of the substrate with the enzyme termed negative cooperativity, or the presence of two forms of enzyme with different K_m values. The following experiments were conducted in order to detect the possible existence of isoenzymes of *Artemia* GMP reductase. (a) From experiments of inactivation of GMP reductase from the DEAE-cellulose step, after heating at 57 °C, a straight line was obtained in a plot of the log of the remaining activity vs. time of incubation. (b) Apparent K_m, V, and coefficient of Hill values for GMP reductase were calculated when the $(NH_4)_2SO_4$ precipitate (see above) was subjected to the following further consecutive purification steps: chromatography on DEAE-cellulose; heating at 55 °C and chromatography on DEAE-cellulose; heating at 55 °C, gel filtration on Sephadex G-200, chromatography on DEAE-cellulose; heating at 55 °C, chromatography on DEAE-cellulose, gel filtration on Sephadex G-200, and chromatography on phosphocellulose. In every case, the K_m values found were of about 5 and 50 μM, the Hill coefficients were 1 and 0.3 and the ratio of the two V values, obtained from Hofstee plots, was 1.3. Taken together, these results tend

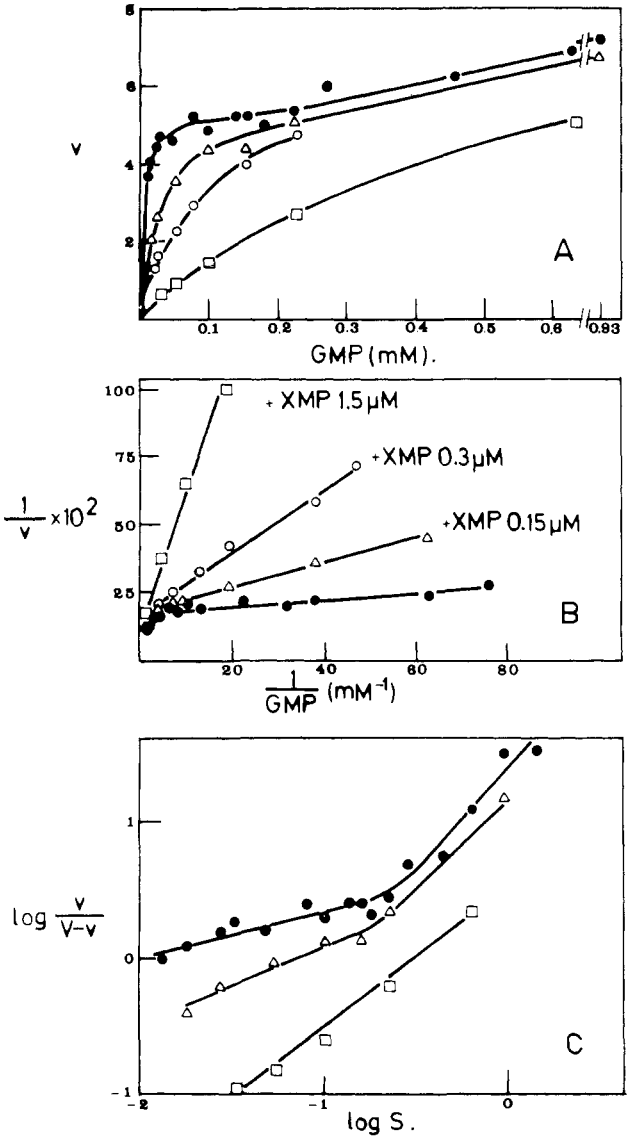

FIGURE 1: Effect of GMP concentration, in the presence or absence of xanthosine 5'-monophosphate, on the initial velocity of *Artemia* GMP reductase. The assay conditions were as described under Experimental Procedures. When indicated (see part B of the figure), the reaction mixture was supplemented with XMP. The results are represented in Michaelis-Menten (A), double-reciprocal (B), and Hill plots (C). In Figures 1 and 4 (cases B and C), the best fitting curves were obtained using the method of least-squares. (●-●) GMP reductase assay mixture in the absence of XMP; (△-△) plus 0.15 μM XMP; (○-○) plus 0.3 μM XMP; (□-□) plus 1.5 μM XMP.

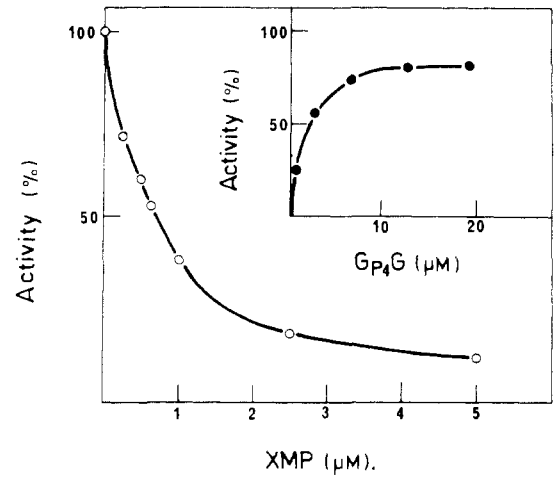

FIGURE 2: Inhibition of *Artemia* GMP reductase by xanthosine 5'-monophosphate and reversion of the inhibition by diguanosine tetraphosphate. The concentration of GMP in the reaction mixture was kept at 0.1 mM in all cases. The concentration of XMP and Gp_4G were as indicated in the figure. The effect of increasing concentrations of Gp_4G on the inhibition caused by 5 μM XMP is shown in the inset.

to disprove that the kinetic behavior of GMP reductase is due to the presence of two forms of the enzyme and point to the existence of negative homotropic interactions between GMP reductase and its substrate.

Effect of Xanthosine 5'-Phosphate on Artemia GMP Reductase. Stephens and Whittaker had previously shown that XMP was a very potent competitive inhibitor (K_i = 10 nM) of GMP reductase from calf thymus. This finding led us to explore the effect of this nucleotide on the *Artemia* enzyme. When *Artemia* GMP reductase was assayed in the presence of 0.1 mM GMP and variable concentrations of XMP, more than 50% inhibition was obtained with 1 μM XMP (Figure 2). At fixed concentrations of XMP and variable concentrations of GMP, the saturation curve with the intermediary plateau obtained in the absence of the effector (Figure 1) became hyperbolic in the presence of 1.5 μM XMP. Double-reciprocal plots of the same data clearly show that the concave obtained in the absence of effector is displaced towards a straight line as the concentration of XMP is increased. The inflection points of the concaves occur at the same GMP concentration. From the Hill plots, it can be seen that the n_H value (0.33), obtained at low GMP concentration, rises as the concentration of XMP is increased and, at 1.5 μM XMP, a unique value of n_H = 1.1 is obtained (Figure 1).

Effect of Diguanosine Tetraphosphate and Other Nucleotides on GMP Reductase. The strong inhibition of the enzyme by XMP led us to the following reasoning. As outlined in the introduction, GMP reductase, probably, is a key enzyme during development from gastrula to larva. The inhibitory effect of XMP poses a question on the functionality of the enzyme in vivo. To our knowledge, XMP has not been reported in *Artemia* extracts. However, if XMP is present in the micromolar range, the enzyme could be totally or partially inhibited as the concentration of GMP in *Artemia* cysts is in the range of millimolar. It is apparent that a nucleotide present at very low concentration most probably is undetectable in a routine screening of nucleotides present in an extract. Those arguments, together with the complex kinetics observed for the GMP reductase, made probable the existence of a positive effector in *Artemia* extracts.

One of the metabolites tested as effector was diguanosine tetraphosphate. When the enzyme was assayed in the presence of a fixed GMP concentration (10 μM) and variable amounts of Gp_4G, an activation of the enzyme of about twofold was observed (Figure 3); the calculated apparent activation constant for Gp_4G was 30 nM. The result, presented in Figure 3, represents a true activation of the enzyme and is not due to Gp_4G being the substrate of the reductase. Te velocity obtained when Gp_4G was tested as substrate of the reaction at concentrations from 0.001 to 0.1 mM was less than 1% of that obtained with 0.1 mM GMP. Besides being an activator of the reaction, Gp_4G was also very effectively able to counteract the inhibition of the reductase caused by XMP when both effectors were present at the same concentration in the reaction mixture (Figure 2). Finally, the effect on the reductase of fixed amounts of Gp_4G, in the presence of variable concentrations of GMP, is presented in Figure 4. The negative homotropic kinetics is displaced to a Michaelian one; in a double-reciprocal plot, the

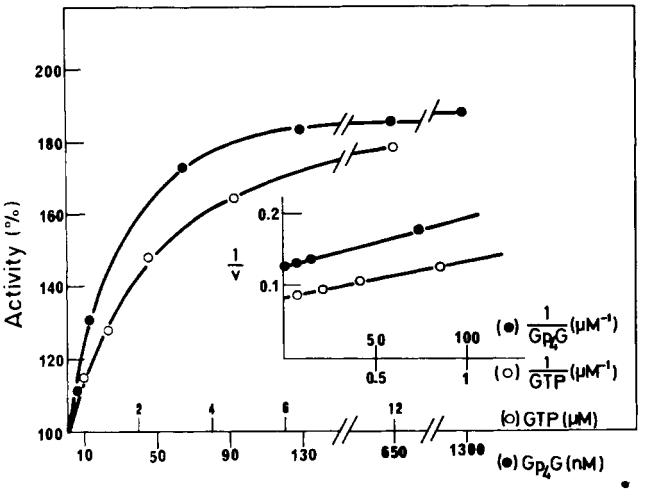

FIGURE 3: Activation of *Artemia* GMP reductase by diguanosine tetraphosphate and GTP. The concentration of GMP in the reaction mixture was 10 μM. The concentrations of Gp_4G and GTP were as indicated in the figure.

TABLE II: Effect of Several Nucleotides on the Inhibition of GMP Reductase by Xanthosine 5'-Phosphate.[a]

XMP 0.3 μM	Nucleotide (μM)	Act. (%)
−	−	100
+	−	21
+	ATP (400)	18
+	GTP (300)	126
+	UTP (400)	21
+	Ap_4A (20)	21
+	Gp_3G (4)	21
+	Gp_4G (3)	86

[a] The reaction mixture contained, in addition to 8 μM GMP, the nucleotides shown in the table.

concave obtained in the absence of Gp_4G is displaced toward a straight line as the concentration of Gp_4G is increased. In the presence of 3.2 μM Gp_4G, unique values for the apparent K_m and n_H of 5 μM and 1.1, respectively, can be calculated (Figure 4).

The effect of other nucleotides on the inhibition caused by XMP (0.3 μM) is presented in Table II. Two structural analogues of Gp_4G, diadenosine tetraphosphate and diguanosine triphosphate, were unable to counteract that inhibition when present at micromolar concentrations in the reaction mixture. Some nucleoside triphosphates (ATP, UTP, GTP) were also tested as effectors at millimolar concentrations; only GTP reverted the inhibition caused by XMP. In view of that, GTP was also assayed as an activator of GMP reductase in a wider range of concentrations, and an apparent activation constant (3 μM), two orders of magnitude higher than that calculated for Gp_4G in the same experimental conditions, was obtained (Figure 3). The effect of Gp_4G on the reductase is then highly specific.

Discussion

Previous results from this laboratory had shown that *Artemia* GMP reductase has a molecular weight of about 250 000 (Renart and Sillero, 1974), and most probably is composed of more than one subunit. Although binding data have not, as yet,

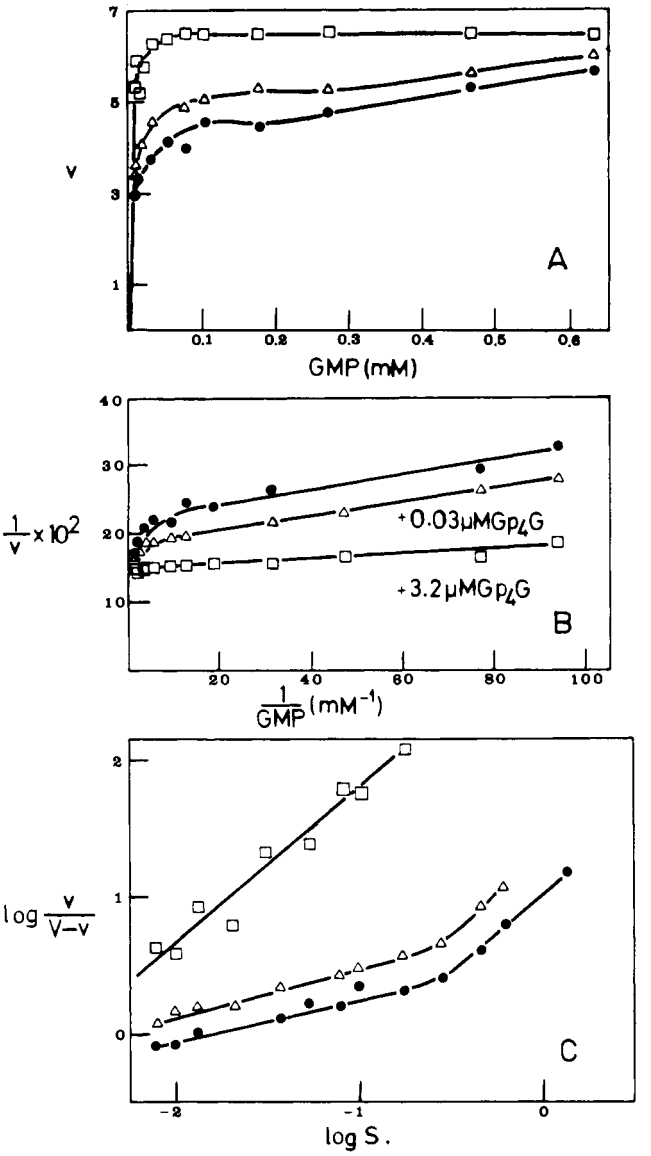

FIGURE 4: Effect of GMP concentrations, in the presence or absence of diguanosine tetraphosphate, on the initial velocity of *Artemia* GMP reductase. The assay conditions were as described under Experimental Procedures. When indicated (see part B of the figure), the reaction mixture was supplemented with Gp_4G. The results are represented in Michaelis-Menten (A), double-reciprocal (B), and Hill plots (C). (●-●) GMP reductase assay mixture in the absence of Gp_4G; (△-△) plus 0.03 μM Gp_4G; (□-□) plus 3.2 μM Gp_4G.

been obtained, the kinetic observations presented here can be interpreted in the following way. Each subunit of the enzyme should contain at least three *loci*, one for GMP, XMP, and Gp_4G. In the absence of effectors, the filling of the substrate site on one subunit hinders subsequent substrate binding to neighboring subunits, as is the case for other well substantiated cases of enzymes exhibiting negative cooperativity (Koshland, 1970). This behavior could explain the existence of two apparent K_m of 5 and 50 μM and two Hill coefficients of 1.2 and 0.3 (Figures 1 and 4). In the presence of high enough concentrations of either Gp_4G or XMP, the enzyme should be conformed in unique forms of high or low affinity for the substrate, respectively (Figures 1 and 4). Moreover, in both cases, binding of successive molecules of substrate should not modify the structure of the enzyme towards less affinity forms. This could explain that, in the presence of either of the two effectors, Michaelian kinetics are observed with only one value for the

apparent K_m and also a unique Hill coefficient of 1. By a similar reasoning, the activation of the reductase, by Gp_4G and GTP, at low GMP concentration, is probably due to a decrease in the apparent K_m value of the enzyme for its substrate (Figure 3).

The complexity of the regulatory mechanisms involved in the action of *Artemia* GMP reductase favors the view that this enzyme plays an important role during development of *Artemia* embryos through the conversion of the GMP moiety of Gp_4G into IMP. This pathway, composed most probably of two enzymes acting in sequence, is strongly regulated. GMP is generated by the action of a specific phosphatase, which splits Gp_4G to GMP and GTP (Warner and Finamore, 1965; Vallejo et al., 1974, 1976). Both enzymes have similar V (around 80 mU/g) and K_m values towards their respective substrates. As is the case for GMP reductase, diguanosinetetraphosphatase (Vallejo et al., 1976) is also strongly regulated; a very potent negative effector has been already described, guanosine 5′-tetraphosphate, with a K_i value of 6 nM (Vallejo et al., 1974). We have not investigated the metabolic fate of IMP generated through this pathway; although IMP generated from Gp_4G could serve as a source of adenine nucleotides, data have been presented in favor of an additional pathway in *Artemia*, going from Gp_4G to ATP through the conversion of Gp_4G to Ap_4G and posterior splitting of this molecule to ATP and GMP (Van Denbos and Finamore, 1974). In addition, it is worthy to note that the GMP reductase, studied in this report, is exclusively that of the encysted *Artemia* and that profound changes occur in purine metabolism upon emergence and hatching (Van Denbos and Finamore, 1974).

The low concentration (nM range) at which diguanosine tetraphosphate is active on GMP reductase (a cytoplasmic enzyme) apparently contrasts with its concentration in total *Artemia* cysts extracts (10 mM). This nucleotide is almost totally localized in the 700g sediment (Sillero and Ochoa, 1971), mainly composed of nuclei and yolk platelets. Warner et al. (1972) presented data in favor of the localization of Gp_4G in the yolk platelets. However, and in spite of further work from our laboratory (unpublished results), at present it is not clear to the authors the subcellular localization of this nucleotide, whether in the nuclei, as assumed previously by Sillero and Ochoa (1971), or in the yolk platelets. Experimental difficulties complicate determination of the exact concentration of Gp_4G in the 150 000g supernatant. Although an upper limit of 0.2 mM has been experimentally found (Sillero and Ochoa, 1971), the possibility of artifacts involved in these measurements, due to the liberation of Gp_4G to the cytosol during the preparation of the subcellular fractions, together with considerations based on the kinetic values presented in this paper, make probable that the actual concentration of Gp_4G is even lower tan 0.2 mM.

The presence of GMP reductase in eukaryotic organisms is now well established after characterization of this enzyme in calf thymus (Stephens and Whittaker, 1973) and *Artemia salina* (Renart and Sillero, 1974). The reductase from eukaryotes, in contrast to the one from microorganisms (Mager and Magasanik, 1960), is not inhibited by ATP and has a lower K_m for GMP. The enzyme from thymus and *Artemia* is inhibited by XMP and, as here reported, the *Artemia* enzyme is activated by Gp_4G. The kinetics of the thymus reductase and the type of inhibition, competitive, by XMP are apparently different from those of the *Artemia* enzyme. However, the range of concentrations tested in the case of the thymus enzyme was not wide enough (Stephens and Whittaker, 1973) to detect the possible existence of negative homotropic kinetics and, also, to ascertain whether the effect of XMP is similar to the one reported here on the *Artemia* enzyme. No positive effector of the reductase from thymus has been reported so far. One possible candidate is GTP. Besides that, the physiological implications of the effector(s) being Gp_4G or its structural analogue, diadenosine tetraphosphate (Ap_4A), are clear; that would point to a more general occurrence of Gp_4G or to the existence of a regulatory effect for Ap_4A, respectively. Ap_4A is present in rat liver, ascites tumor cells, and *E. coli* at very low concentrations (Zamecnik, 1969). Diadenosine and diguanosine tetraphosphates are molecules of potential interest in metabolic regulation (Lobatón et al., 1975). Besides the effect here described for Gp_4G, this nucleotide seems to also have a regulatory role on the RNA polymerases from several fungi (McNaughton et al., 1975).

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