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DIGUANOSINETETRAPHOSPHATE GUANYLOHYDROLASE IN *ARTEMIA SALINA*

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SUMMARY

1. The diguanosinetetraphosphate guanylohydrolase (EC 3.6.1.17) of *Artemia salina* has been found to be located in the cytosol while its substrate, diguanosine tetraphosphate, is in the 700 × *g* sediment.

2. Two spectrophotometric methods have been developed to study this enzyme. One is based in the evaluation of one of the products, GTP, coupled to the auxiliary enzymes phosphoglycerate kinase/glyceraldehyde-3-phosphate dehydrogenase. The other method is based on the hyperchromicity observed at 252 nm by the splitting of diguanosine tetraphosphate.

3. With a partially purified preparation, the following enzymatic properties have been found: K_m for diguanosine tetraphosphate, 5 μM. GMP, GDP, GTP and ATP were competitive inhibitors of the reaction with K_i values of 24, 56, 14 and 30 μM respectively.

4. Even lower values were obtained with the guanosine 5′-tetraphosphate and adenosine 5′-tetraphosphate, which were 0.006 and 0.13 μM, respectively. These rather low K_i values suggest a possible role for these compounds as metabolic regulators.

INTRODUCTION

Artemia salina is a crustacean of the order Anostraca whose embryos may become encapsulated at the gastrula stage. The cysts are viable for years in a dry environment [1]. When placed in a suitable saline medium, the eggs resume their development and differentiate into free swimming larvae in about 24 h, without cell division [2], or synthesis of DNA [3]. This characteristic development and the commercial availability of *Artemia* eggs make this organism particularly suitable for studies on differentiation.

Warner and collaborators have made important contributions to the biochemistry of both the resting and developing *Artemia* embryo. They discovered diguanosine tetraphosphate (Gp_4G) in *Artemia* extracts [4]. Sillero and Ochoa found that Gp_4G is present in the 700 × *g* sediment suggesting a nuclear localization of this compound [5]. Later, Warner and collaborators [6], while confirming the presence of

Gp_3G in the 700 × *g* sediment, presented data in favour of the localization of Gp_4G in the yolk platelets, which are also found in the 700 × *g* sediment.

An enzyme capable of hydrolyzing Gp_4G, was described by Warner and Finamore [7] in *Artemia* extracts. This enzyme has been recently named as diguanosine-tetraphosphate guanylohydrolase (EC 3.6.1.17) by the Enzyme Commission. Warner and Finamore found a K_m of 1 mM for Gp_4G with a partially purified enzyme [7].

We have investigated the subcellular localization of this enzyme and found that it is present in the 150 000 × *g* supernatant. Using methods more sensitive than the one described previously [7], a K_m of about 5 μM for Gp_4G has been obtained. Guanosine tetraphosphate (Gp_4) and adenosine tetraphosphate (Ap_4) are very potent inhibitors of the reaction. Other kinetic properties of Gp_4G guanylohydrolase, together with a discussion on the degradation of Gp_4G during development of *Artemia* cysts are also reported here.

MATERIALS AND METHODS

Preparation of subcellular fractions

Artemia eggs were from Longlife Fish Food Products, Ontario, Canada. All operations were conducted at 0-4 °C. Immediately before use, the eggs were washed with water and NaClO as described previously [8], resuspended in 10 ml of Buffer A (0.035 M Tris–HCl, pH 7.7, 0.07 M KCl, 0.009 M $MgCl_2$, 0.25 M sucrose) per g of original dry eggs and stirred for 2.5 h with a magnetic stirrer. The resulting brei was passed through glass wool. The filtrate will be referred to as the total homogenate. This homogenate was centrifuged at 400 × *g* for 5 min to collect nuclei and yolk platelets. Mitochondria were prepared from the resulting supernatant by centrifugation at 6500 × *g* for 20 min. Further centrifugation of the supernatant at 150 000 × *g* for 75 min yielded the microsome and the high-speed supernatant fractions.

For the purification of the Gp_4G guanylohydrolase, 50 g of *Artemia* eggs were washed as described above and stirred overnight in 500 ml of glass-distilled water. The clarified homogenate (obtained by filtration through glass wool) was centrifuged at 12 000 × *g* for 15 min, and the supernatant fraction was centrifuged again at 150 000 × *g* for 75 min.

Determination of Gp_4G guanylohydrolase

In crude extracts, the method used was a modification of the one described [7] previously in which the hydrolysis of Gp_4G was coupled to alkaline phosphatase as auxiliary enzyme. The standard assay conditions were: 50 mM Tris–HCl buffer, pH 8.0, 2 mM $MgCl_2$, 0.3 units (Boehringer) of alkaline phosphatase (EC 3.1.3.1), *Artemia* extract, and 0.6 mM Gp_4G in a total volume of 0.5 ml. The mixtures were incubated for 20 min at 37 °C and the reaction stopped by adding 1 ml of 10% trichloroacetic acid. The samples were centrifuged and the inorganic phosphate was determined in the supernatant by the method of Fiske and SubbaRow [9]. *Artemia* extract was added to the reaction mixture to produce up to 0.3 μmole of inorganic phosphate. In these conditions the reaction was linear with respect to both time and amount of extract.

For the assay of the Gp_4G guanylohydrolase in partially purified extracts, two spectrophotometric methods were developed. One was based on the evaluation of the GTP formed in the reaction; in the other we availed ourselves of the fact that an in-

crease in absorbance at 252 nm takes place when Gp_4G is degraded to GTP and GMP. Details of these methods are under Figs 2 and 4, respectively.

Determination of glucosephosphate isomerase (EC 5.3.1.9)

The reaction mixture contained in a final volume of 1 ml the following components: 50 mM imidazole buffer, pH 7.0, 2 mM fructose 6-phosphate, 0.25 mM NADP, 2 units of glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and *Artemia* extract in a suitable amount to give an activity of 0.005–0.01 units.

Purification of nucleotides

Isolation of diguanosine polyphosphates was carried out essentially as described [5] previously. The 700 × *g* sediment, which contains practically all the diguanosine polyphosphates present in the total homogenate [5], was treated with 0.2 M $HClO_4$ (2 ml per g of eggs), and centrifuged after standing for 10 min at 4 °C. This operation was repeated twice: the washings were combined, diluted 10 times and applied to a DEAE-cellulose column previously equilibrated with 0.1 M $(NH_4)_2CO_3$. Nucleotides other than Gp_3G and Gp_4G were eluted with 0.16 M $(NH_4)_2CO_3$. The diguanosine polyphosphates were eluted with 0.4 M $(NH_4)_2CO_3$. Gp_3G and Gp_4G were separated by chromatography on DEAE-cellulose, using a linear gradient of NH_4HCO_3, pH 8.6. The two diguanosine polyphosphates so obtained were characterized with snake venom phosphodiesterase as described previously [5].

Commercial Ap_4, from Sigma, was purified as follows: 4 $A_{260\ nm}$ units of Ap_4 were incubated, at 30 °C for 3 h, with 40 mM Tris–HCl, pH 8.0, 5 mM $MgCl_2$, 4 mM glucose and 4 units of hexokinase in a final volume of 240 μl. The reaction mixture was then heated at 70 °C for 5 min and Ap_4 purified by electrophoresis [5] in 20 mM citrate buffer, pH 5.0, using Whatman III MM paper.

GDP, GTP and Gp_4 were obtained from a preparation of commercial GTP (Sigma) through chromatography on Dowex-1 (Fig. 1).

ATP (Sigma) was purified as described by Small and Cooper [10] and concentrated with charcoal as specified in Fig. 1.

Other materials

The auxiliary enzymes were from Boehringer, GMP was purchased from Pharma Waldhof, DEAE-cellulose from Serva, Sephadex G-100 from Pharmacia, and Dowex-1 from Sigma.

RESULTS

Subcellular localization of Gp_4G guanylohydrolase

Artemia subcellular fractions were obtained as indicated in Materials and Methods. Glucosephosphate isomerase, a marker for the soluble fraction, was determined in parallel with Gp_4G guanylohydrolase. As seen in Table I, the distribution of Gp_4G guanylohydrolase follows the pattern of the enzyme marker. The assay of the Gp_4G guanylohydrolase is not reliable in the total homogenate and, for this reason, no activity data have been entered for this fraction in Table I. The percentage distribution of this enzyme was calculated from the sum of the activities found in the different fractions. Moreover, the sum of the activities of glucosephosphate isomerase in the

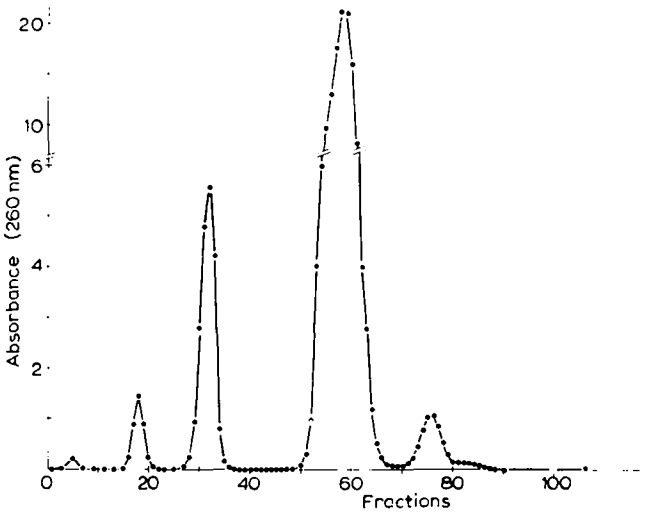

Fig. 1. Fractionation of commercial GTP. 800 μl of 0.1 M GTP were applied to a Dowex-1 (1 × 8-400, 200–400 dry mesh, 8% cross linked) column (1.7 cm × 26.5 cm) in Cl^- form. The different nucleotides were eluted with 700 ml of a linear gradient (0.15–0.5 M) of NaCl in 0.01 M HCl. Fractions of 6.6 ml were collected. Fractions under each peak were pooled and after addition of charcoal (15 mg per ml) the mixture was stirred for 10 min and centrifuged. The nucleotides were eluted from the precipitate with 40% ethanol–0.14 M NH_4OH and, in each case, the washings were combined and taken to dryness in a flask evaporator. Gp_4 was characterized by analysis of guanine P and the other nucleotides by suitable enzymatic assays. The percentage of each of the nucleotides present in commercial GTP are indicated inside the brackets: GMP (3.3), GDP (13.0), GTP (81.3), Gp_4 (2.5).

TABLE I

SUBCELLULAR DISTRIBUTION OF Gp_4G GUANYLOHYDROLASE IN *ARTEMIA SALINA*

Subcellular fractions were obtained from 5 g of cysts as described in the text. The 400 × *g*, mitochondrial and microsomal pellets were resuspended up to 12, 6 and 8 ml with Buffer A, respectively. Aliquots (1.5 ml) of each of the subcellular fractions and of the total homogenate were dialyzed jointly against 2 l of Buffer A for 1 h followed by an overnight dialysis against 4 l of the same buffer. Enzymatic activities were determined as shown in Materials and Methods. For the determination of Gp_4G guanylohydrolase, alkaline phosphatase was used as auxiliary enzyme. In both cases 1 unit is the amount of enzyme able to transform 1 μmole of substrate per min at 37 °C. Observe that glucosephosphate isomerase is 1000-fold more active than Gp_4G guanylohydrolase in *Artemia* extracts. Proteins were measured by the method of Lowry et al. [11].

Fractions	Protein (mg/g)	Gp_4G guanylohydrolase		Glucosephosphate isomerase	
		munits/mg	%	units/mg	%
Soluble	45	1.5	89	1.15	84
Microsomal	12	0.3	4	0.24	5
Mitochondrial	12	0.5	7	0.16	3
400 × *g* precipitate	121	0.1	-	0.10	8

subcellular fractions coincided with the value found in the total homogenate, thus showing a total enzymatic recovery. These results (Table I) can be presented as evidence for the soluble localization of the Gp_4G guanylohydrolase.

The presence of the Gp_4G guanylohydrolase in the cytosol, and the localization of Gp_4G almost exclusively in the 700 × *g* sediment [5], together with the high K_m (1 mM) for Gp_4G reported previously [7] made difficult to explain the role of this enzyme in the metabolism of Gp_4G.

Partial purification of Gp_4G guanylohydrolase

To investigate the metabolic role of this enzyme, we undertook its purification as an essential requirement for subsequent kinetic studies. A 150 000 × *g* supernatant, in a volume of 420 ml was obtained from 50 g of cysts as described in Materials and Methods. The 150 000 × *g* supernatant was brought up to 50% saturation with $(NH_4)_2SO_4$ and stirred for 30 min. The precipitate was collected by centrifugation at 27 000 × *g* for 30 min. Solid $(NH_4)_2SO_4$ was added to the supernatant to bring the solution to 90% saturation and, after stirring for 30 min, the precipitate was collected as above. The precipitate containing the Gp_4G guanylohydrolase activity was dissolved in 20 ml of 0.35 M Tris–HCl buffer, pH 7.7, 0.05 M KCl, 0.009 M $MgCl_2$ (Buffer B), and applied to a column of Sephadex G-100 (2.8 cm × 90 cm). Elution was with the same buffer, and fractions of 8.7 ml were collected. Fractions 39–50, containing the major portion of the Gp_4G guanylohydrolase activity, were pooled and concentrated with $(NH_4)_2SO_4$ (90% saturation). The precipitate was collected by centrifugation as described above, resuspended in 4.1 ml of Buffer B and dialyzed to remove residual $(NH_4)_2SO_4$. The purification procedure is summarized in Table II. The experiments described below were carried out with this preparation.

TABLE II

PURIFICATION OF *ARTEMIA SALINA* Gp_4G GUANYLOHYDROLASE

50 g of cysts were used. The purification was followed by measuring the enzymatic activity using alkaline phosphatase as auxiliary enzyme.

Step	Volume (ml)	Protein (mg)	Specific activity (munits/mg)	Recovery (%)
Supernatant 150 000 × *g*	420	2394	1.5	100
50–90% $(NH_4)_2SO_4$	20	780	2.6	52
Sephadex G-100	97	82	20.0	51
Concentrate	4.1	54	23.6	41

Spectrophotometric coupled method for the assay of Gp_4G guanylohydrolase

As reported by Warner and Finamore, the products of the hydrolytic cleavage of Gp_4G are GMP and GTP. We developed a spectrophotometric method to measure Gp_4G guanylohydrolase activity by coupling the formation of GTP to the phosphoglycerate kinase (EC 2.7.2.3)/glyceraldehyde phosphate dehydrogenase (EC 1.2.1.12) system. With this method, the reaction is linear in relation to both time and amount of extract and is strictly dependent on the addition of Gp_4G (Fig. 2).

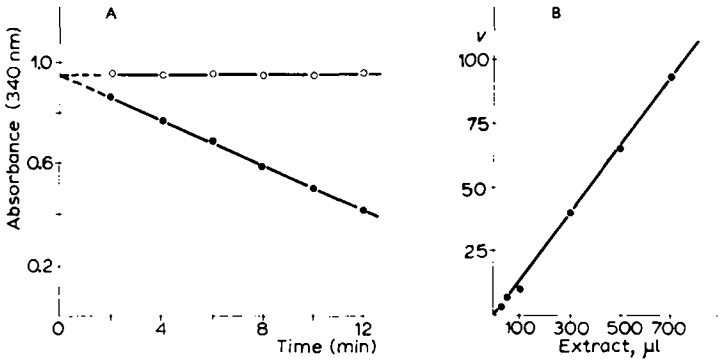

Fig. 2. Gp_4G guanylohydrolase coupled assay. The reaction mixture contained in a final volume of 1 ml the following components: 50 mM Tris–HCl buffer, pH 7.5, 7 mM $MgCl_2$, 5 mM EDTA, 12 mM 3-phosphoglycerate, 0.2 mM NADH, 0.13 mM Gp_4G, 0.9 units of phosphoglycerate kinase, 1.8 units of glyceraldehyde-3-phosphate dehydrogenase and varying amounts of purified extract. For the time curve (A), 300 µl of the Sephadex G-100 step (Table II) were used. ●—●, complete system; ·····, without Gp_4G.

Michaelis constant for Gp_4G

With the spectrophotometric method described above, a K_m of about 5 µM was calculated for Gp_4G (Fig. 3). This result markedly differs from the value of 1 mM reported by Warner and Finamore [7]. An examination of the method and data reported by these authors to obtain the *K*m of the enzyme for Gp_4G provided an explanation for this discrepancy. The assay method used by them measured the GMP formed, by ion-exchange chromatography, after incubation at 45 °C for 30 min. From their Fig. 6 [7], it can be inferred that the amount of enzyme used in this experiment was such that at the lower Gp_4G concentrations (less than 1.25 mM) all of the substrate was consumed, thus yielding GMP in stoichiometric amount to Gp_4G in the reaction mixture. At higher Gp_4G concentration (more than 1.25 mM), the GMP formed during the reaction time remained constant. Under these assay conditions, a plot of

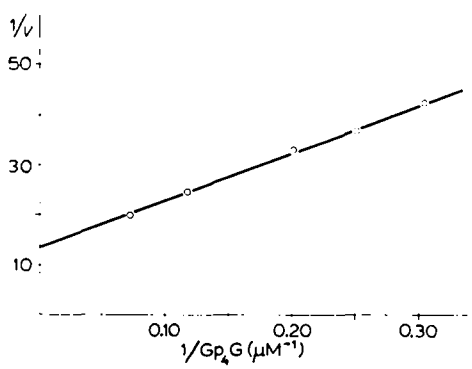

Fig. 3. Effect of the concentration of Gp_4G on the initial velocity of the Gp_4G guanylohydrolase activity. The assays were carried out as described in Fig. 2 with 25 µl of the concentrate (Table II), using a Gilford model 2400 spectrophotometer.

the Gp_4G concentration versus the amount of GMP formed (their Fig. 6 [7]), gives an apparent Michaelis–Menten saturation kinetic curve, that can not be used to calculate the K_m of the enzyme. As a matter of fact, the lowest concentration of Gp_4G used in this experiment (0.5 mM) was 100 times higher than the real K_m of the enzyme.

Other properties of the Gp_4G guanylohydrolase

In agreement with previous results from Warner and Finamore [7], one mole of GTP was produced per mole of Gp_4G hydrolyzed, Gp_3G was not a substrate of the enzyme, and optimal pH for the Gp_4G guanylohydrolase was about 8 (results not shown).

As the coupled spectrophotometric method described above is not suitable for assaying of the effect of nucleoside triphosphates on the Gp_4G guanylohydrolase reaction, a new method was developed taking advantage of the fact that the cleavage of Gp_4G to GTP and GMP involves an increase in absorbance at 252 nm. The extinction coefficient of Gp_4G is 26.3 at 252 nm. This figure is calculated from the value previously reported [12] (ε = 22.3 at 260 nm) and the ratio $A_{252\ nm}/A_{260\ nm}$ = 1.18 at pH 7.0. The extinction coefficient for both GTP and GMP is 13.7 at 252 nm, pH 7.0. The hydrolysis of 1 mole of Gp_4G to GTP and GMP is associated with an increase in absorbance of 1.1 $A_{252\ nm}$ units. Experimental details of this method are under Fig. 4.

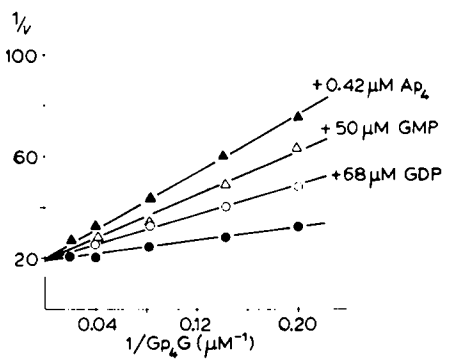

Fig. 4. Gp_4G guanylohydrolase hyperchromicity assay. Effect of GMP, GDP and Ap_4 on the initial velocity. The reaction mixture contained, in a final volume of 1 ml the following components: 50 mM Tris–HCl buffer, pH 7.5, 2 mM $MgCl_2$, 10 μl of the concentrate described in Table II and varying amounts of Gp_4G. Concentration of nucleotides as indicated in the graph. Increase in absorbance was followed in a Varian Techtron Model 635 spectrophotometer at 252 nm.

The method is linear with both time and amount of extract (results not shown). A K_m of 4 μM for Gp_4G was obtained (Fig. 4), in good agreement with the result presented above (Fig. 3). Preliminary experiments had shown that commercial GTP was a very potent inhibitor of the reaction. However, GTP generated in situ from GDP, using pyruvate kinase as auxiliary enzyme, turned out to be much less inhibitory. This result pointed to a contaminant present in the commercial preparation as the actual potent inhibitor of the Gp_4G guanylohydrolase reaction. The fractionation of GTP showed the presence of GMP, GDP and Gp_4 as contaminants (Fig. 1), and Gp_4 was indeed more inhibitory than GTP (see below). Based on the previous findings, ATP and Ap_4 were assayed as effectors. For this kind of experiments all the nucleotides were purified

before use, except for commercial GMP which was employed as such. When assayed with the hyperchromicity method, all of the nucleotides tested were found to be competitive inhibitors with the following K_i values (μM): GMP, 24; GDP, 56; GTP, 14; Gp_4, 0.006; ATP, 30; Ap_4, 0.13 (Figs 4 and 5). It is interesting to note the striking inhibition by Gp_4 and Ap_4 with K_i values several orders of magnitude lower than the K_m of the enzyme for Gp_4G.

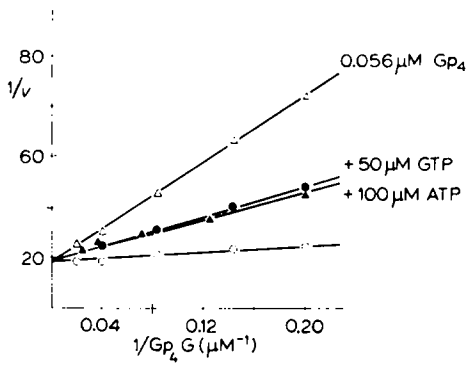

Fig. 5. Effect of ATP, GTP and Gp_4 on the initial velocity of the Gp_4G guanylohydrolase activity. Assay conditions as described in Fig. 4. Concentration of Gp_4G and nucleotides as indicated in the graph.

DISCUSSION

Artemia cysts contain about 10 μmoles of Gp_4G per g located almost exclusively in the 700 $\times$ *g* sediment; only around 2% is present in the 150 000 $\times$ *g* supernatant [5]. Its location remains the same as in the cysts [5], at least up to the third day of development, when about two-thirds of Gp_4G has been metabolized.

As shown in this paper, the activity of the Gp_4G guanylohydrolase in the *Artemia* cysts is about 80 munits/g; the enzyme is located in the 150 000 $\times$ *g* supernatant and has a K_m of 5 μM towards Gp_4G. The concentration of Gp_4G found experimentally in the cytosol is about 0.2 mM.

Concerning the situation in vivo, it is known that when *Artemia* is grown at 27 °C half of its Gp_4G content is metabolized in about 2 days [5]. One can easily calculate that the average speed of degradation of Gp_4G is around 2 nmoles $\cdot m^{-1} \cdot g^{-1}$ (5 μmoles/2 $\times$ 24 $\times$ 60 min).

From the kinetic data obtained in vitro, and allowing a V of around 40 munits/g at 27 °C (usual temperature at which *Artemia* eggs are cultured), the enzyme present in the cysts would be able to transform around 40 nmoles $\cdot m^{-1} \cdot g^{-1}$, one order of magnitude higher than the rate of degradation of Gp_4G in vivo. As the cysts are not able to synthesize Gp_4G during development [13], and we have not observed a decrease in Gp_4G guanylohydrolase up to the third day of incubation, the discrepancy cited above could be accounted for by the following factors: (a) the actual concentration of Gp_4G in the cytosol is lower than that found experimentally, the reason being that Gp_4G comes out of the 700 $\times$ *g* sediment during the extraction procedures; (b) the enzyme is partially inhibited in vivo. The more potent inhibitors are Gp_4 and

Ap_4. Ap_4 has been detected in muscle preparations [14]. The physiological significance of nucleoside tetraphosphates is largely unknown. In addition to the effect reported here on Gp_4G guanylohydrolase, actomyosin is split into actin and myosin by Ap_4 [15]. Although the presence of these nucleotides has not been reported in *Artemia* extracts, the concentrations at which they can act as effectors are so low that, if actually present at these concentrations, its occurrence would not be detected in a routine screening of the nucleotides present in *Artemia*.

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REFERENCES

1 Dutrieu, J. (1960) Arch. Zool. Exp. Gen. 99, 1–134
2 Nakanishi, Y. N., Iwasaki, T., Okigaki, T. and Kato, H. (1962) Annot. Zool. Jap. 35, 223–228
3 Bellini, L. (1960) Res. Sci. 30, 816–822
4 Warner, A. H. and Finamore, F. J. (1963) J. Biol. Chem. 238, 344–348
5 Sillero, A. and Ochoa, S. (1971) Arch. Biochem. Biophys. 143, 548–552
6 Warner, A. H., Puodziukas, J. G. and Finamore, F. J. (1972) Exp. Cell. Res. 70, 365–375
7 Warner, A. H. and Finamore, F. J. (1965) Biochemistry 4, 1568–1575
8 Renart, M. F. and Sillero, A. (1974) Biochim. Biophys. Acta, 341, 178–186
9 Fiske, C. H. and SubbaRow, Y. (1925) J. Biol. Chem. 66, 375–400
10 Small, G. D. and Cooper, C. (1966) Biochemistry 5, 14–26
11 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
12 Clegg, J. S., Warner, A. H. and Finamore, J. F. (1967) J. Biol. Chem. 242, 1938–1943
13 Warner, A. H. and McClean, D. K. (1968) Develop. Biol. 18, 278–293
14 Small, G. D. and Cooper, C. (1966) Biochemistry 5, 26–33
15 Winand-Devigne, J., Hamoir, G. and Liebecq, C. (1967) Eur. J. Biochem. 1, 29–32