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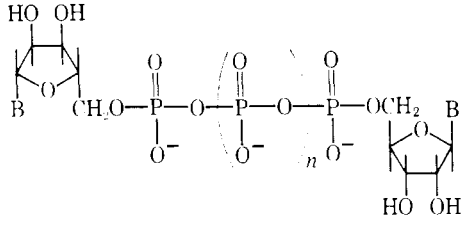
Nucleotides in the Encysted Embryos of *Daphnia magna**

T. G. Oikawa and M. Smith

ABSTRACT: The encysted embryos of *Daphnia magna* contain guanosine-5′ phosphate, guanosine-5′ diphosphate, P^1,P^3-diguanosine-5′ triphosphate, and P^1,P^4-diguanosine-5′ tetraphosphate. Ion-exchange chromatography on diethylaminoethylcellulose provides a very satisfactory method for separation of these nucleotides.

The α,ω-dinucleoside-5′ polyphosphates (I) are a class of compounds which have recently attracted attention. P^1,P^2-Adenosine-5′ tetraphosphate (I, B =



I

adenine, $n = 2$) was suggested as an intermediate in the reaction of adenosine-5′ diphosphate with dicyclohexylcarbodiimide (Smith and Khorana, 1958), and this nucleotide together with its homologs are produced in dismutation reactions of adenosine-5′ polyphosphates (Verheyden *et al.*, 1965; Wehrli and Moffatt, 1965). General procedures for synthesis of α,ω-nucleoside-5′ polyphosphates have been devised (Reiss and Moffatt, 1965). The accolade of natural occurrence followed from the demonstration of P^1,P^3-diguanosine-5′ triphosphate (I, B = guanine, $n = 1$) and P^1,P^4-diguanosine-5′ tetraphosphate (I, B = guanine, $n = 2$) as major constituents of the encysted embryos of the brine shrimp, *Artemia salina* (Finamore and Warner, 1963; Warner and Finamore, 1965a).

The present study was directed at the nucleotides of the encysted embryos of *Daphnia magna*. Both P^1,P^2-diguanosine-5′ triphosphate and P^1,P^4-diguanosine-5′ tetraphosphate were present but in amounts differing from those found in *A. salina*. Ion-exchange chromatography on diethylaminoethylcellulose, using ammonium bicarbonate as eluting agent, provided a convenient method for isolation of α,ω-diguanosine-5′ polyphosphates. This procedure was also applicable to the isolation of these nucleotides from encysted embryos of *D. magna* and *A. salina*.

Experimental Methods and Results

Analytical Methods. Paper chromatography was carried out on Whatman 40 paper, using descending technique, in solvent 1, isobutyric acid–concentrated ammonia–water (66:1:33); solvent 2, isobutyric acid–1 M ammonia–0.1 M sodium ethylenediaminetetraacetate (100:60:1.6); solvent 3, 1 M ammonium acetate, pH 3.8–ethanol (7:3). Chromatography using ascending technique was carried out in solvent 4, 0.1 M sodium phosphate, pH 6.8–solid ammonium sulfate–1-propanol (100:60:2). Authentic nucleotides were always chromatographed alongside unknowns. Nucleotides were detected by viewing under an ultraviolet light, and mobilities are recorded in Table I. Ribose was determined by the orcinol method (Mejbaum, 1939). Total phosphate was determined after digestion in 70% perchloric acid and labile phosphate after hydrolysis in 1 M hydrochloric acid (7 min at 100°) using King's procedure (1932). Guanosine was determined from its ultraviolet absorption using a Beckman DU spectrophotometer assuming a molar extinction coefficient of 13,700 at 252 mμ at pH 7.0.

The hydrolysis of nucleotides by snake venom diesterase (Worthington) was carried out using published procedures (Razzell, 1963; Finamore and Warner, 1963), the products being examined chromatographically after removal of magnesium by treatment with a cation exchanger (Dowex 50) in the ammonium form.

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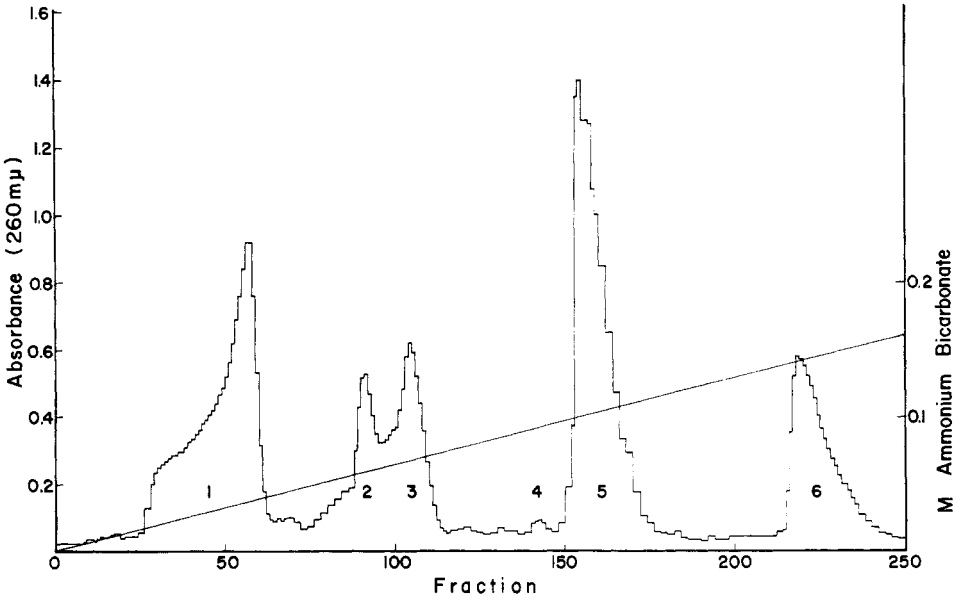

FIGURE 1: Separation of nucleotides from *Daphnia magna* embryos (15 g) on a diethylaminoethylcellulose column (50 × 1 cm diameter). Fractions (12 ml) were collected at 8-min intervals. Peak 1, guanosine-5′ phosphate; peak 2, unidentified; peak 3, guanosine-5′ diphosphate; peak 4, unidentified; peak 5, P^1,P^3-diguanosine-5′ triphosphate; peak 6, P^1,P^4-diguanosine-5′ tetraphosphate.

TABLE I: Chromatographic Mobilities (R_F) of Nucleotides.

Nucleotide	Solvent 1[a]	2	3	4
Adenosine-5′ phosphate	2.41	0.55	0.58	0.20
Uridine-5′ phosphate	1.06	0.32	0.62	
Guanosine-5′ phosphate	1.00	0.31	0.48	0.39
Adenosine-5′ diphosphate	1.30	0.45		0.26
Uridine-5′ diphosphate	0.48	0.20		0.52
Guanosine-5′ diphosphate	0.56	0.21		
Adenosine-5′ triphosphate	0.90	0.35		
Uridine-5′ triphosphate	0.30	0.17		
Guanosine-5′ triphosphate	0.34	0.16		0.59
P^1,P^3-Diguanosine-5′ triphosphate	0.32	0.14		0.23
P^1,P^4-Diguanosine-5′ tetraphosphate	0.23	0.11		0.29

[a] Mobilities in solvent 1 are relative to guanosine-5′ phosphate (1.0).

Isolation of Nucleotides from D. magna. The encysted embryos of *D. magna* (15 g) were ground, dry, using a pestle and mortar at 25°. Grinding was continued until microscopic examination showed that most of the embryos were broken (15 min). Cold 1 M perchloric acid (150 ml) was added and the resultant slurry was then stirred for 15 min at 0°. The mixture was centrifuged at 10,000*g* for 20 min at 0°. The supernatant solution was decanted and some floating embryos removed by filtration through glass wool. The dark colored acidic solution was neutralized to pH 7.0 with 5 M sodium hydroxide at 0°. At *ca.* pH 4.0, a precipitate started to form. The neutralized solution was concentrated to a volume of 50 ml and then diluted with cold 95% ethanol (300 ml) and the precipitate was collected by centrifugation. Nucleotides were extracted by repeated extraction (times three) of this precipitate with distilled water (total volume, *ca.* 150 ml). The aqueous solution was passed through a diethylaminoethylcellulose column (50 × 1 cm diameter) which had been equilibrated with 0.002 M ammonium bicarbonate, pH 8.6. The column was washed with this buffer until elution of material absorbing at 260 mμ was complete. Nucleotides were then eluted using a linear salt gradient with 0.002 M ammonium bicarbonate, pH 8.6 (2.5 l.), in the mixing chamber and 0.3 M ammonium bicarbonate, pH 8.6 (2.5 l.), in the reservoir, fractions (12 ml) being collected at 8-min intervals. Elution of nucleotides was followed spectrophotometrically at 260 mμ (Figure 1). Appropriate fractions were combined and the ammonium bicarbonate removed by treatment with a cation exchanger (Amberlite 1R-120) in the acid form. The resultant solution was neutralized with ammonia and water removal by lyophilization to yield the products as whitish solids. Alternately, ammonium bicarbonate was removed directly by lyophilization. Peaks 1 and 3 were characterized by their chromatographic mobilities and ultraviolet absorption spectra at pH 7.0 as being guanosine-5′ phosphate and guanosine-5′ diphosphate, respectively. The major constituent of peak 2 (R_F 0.37 in solvent 2) did not have

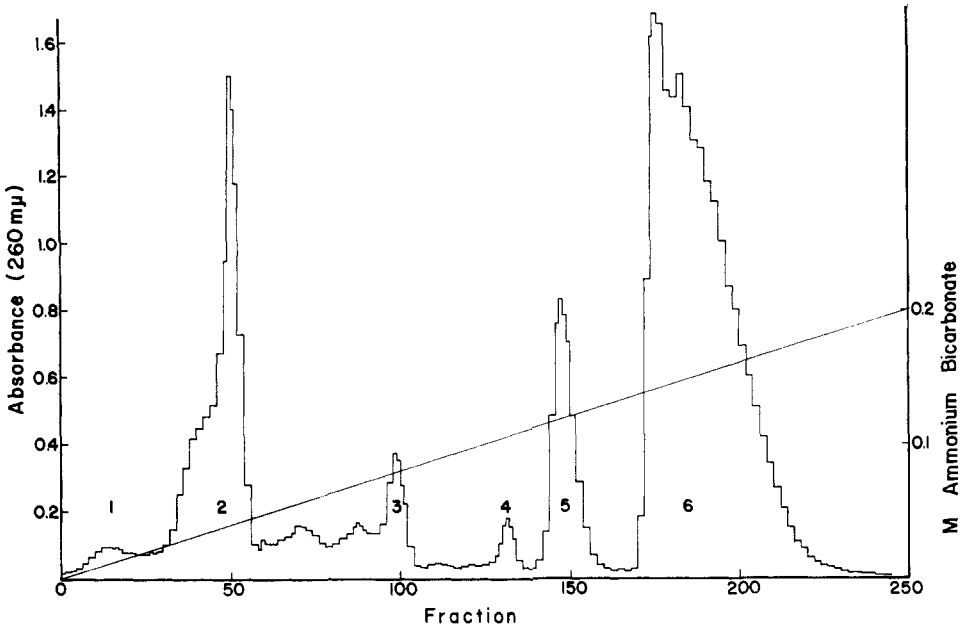

FIGURE 2: Separation of nucleotides from encysted embryos of *Artemia salina* (20 gm) on a diethylaminoethylcellulose column (50 × 1 cm diameter). Fractions (13 ml) were collected at 8-min intervals. Peak 1, adenosine-5′ phosphate; peak 2, guanosine-5′ phosphate; peak 3, guanosine-5′ diphosphate; peak 4, guanosine-5′ triphosphate; peak 5, P^1,P^3-diguanosine-5′ triphosphate; peak 6, P^1,P^4-diguanosine-5′ tetraphosphate.

an absorption maximum in the ultraviolet and was not examined further. Peak 4 (λ_{max} 240 mμ) contained neither phosphate nor ribose and was not examined further. Peak 5, from its chromatographic mobility, its ultraviolet absorption, and its degradation by snake venom diesterase to guanosine-5′ phosphate and guanosine-5′ diphosphate, was P^1,P^3-diguanosine-5′ triphosphate. *Anal.* Found: guanine, 2.0; ribose, 1.9; total phosphate, 3.14; acid-labile phosphate, 1.07. Peak 6, by its chromatographic behavior, its ultraviolet absorption, and its degradation by snake venom diesterase to guanosine-5′ phosphate and guanosine-5′ triphosphate, was found to be P^1,P^4-diguanosine-5′ tetraphosphate. *Anal.* Found: guanine, 2.0; ribose, 2.0; total phosphate, 4.0; acid-labile phosphate, 1.9. The amounts of nucleotides isolated, estimated spectrophotometrically, are summarized in Table II.

Isolation of Nucleotides from A. salina. The encysted embryos of *A. salina* (20 g) were extracted with 1 M perchloric acid (100 ml) essentially as in the isolation of nucleotides from *D. magna*. The results of diethylaminoethylcellulose ion-exchange chromatography are depicted in Figure 2. Peak 1 was identified as adenosine-5′ phosphate together with a trace of uridine-5′ phosphate by chromatography and ultraviolet absorption spectra. Peaks 2, 3, and 4, using the same criteria,

TABLE II: Nucleotides Isolated from Encysted Embryos of *Daphnia magna*.[a]

Peak	Compd	Embryos (μmoles/ 10 g)
1	Guanosine-5′ phosphate	11.6
3	Guanosine-5′ diphosphate	2.9
5	P^1,P^3-Diguanosine-5′ triphosphate	5.4
6	P^1,P^4-Diguanosine-5′ tetraphosphate	2.4

[a] Numbering of peaks refers to Figure 1. Quantitative estimates were made spectrophotometrically using guanosine-5′ phosphate as standard.

TABLE III: Nucleotides Isolated from Encysted Embryos of *Artemia salina*.[a]

Peak	Compd	Embryos (μmoles/ 10 g)
1	Adenosine-5′ phosphate	0.6
2	Guanosine-5′ phosphate	7.5
3	Guanosine-5′ diphosphate	3.3
4	Guanosine-5′ triphosphate	0.5
5	P^1,P^3-Diguanosine-5′ triphosphate	1.6
6	P^1,P^4-Diguanosine-5′ tetraphosphate	11.6

[a] Numbering of peaks refers to Figure 2. Quantitative estimates were made spectrophotometrically using nucleoside-5′ phosphates as standards.

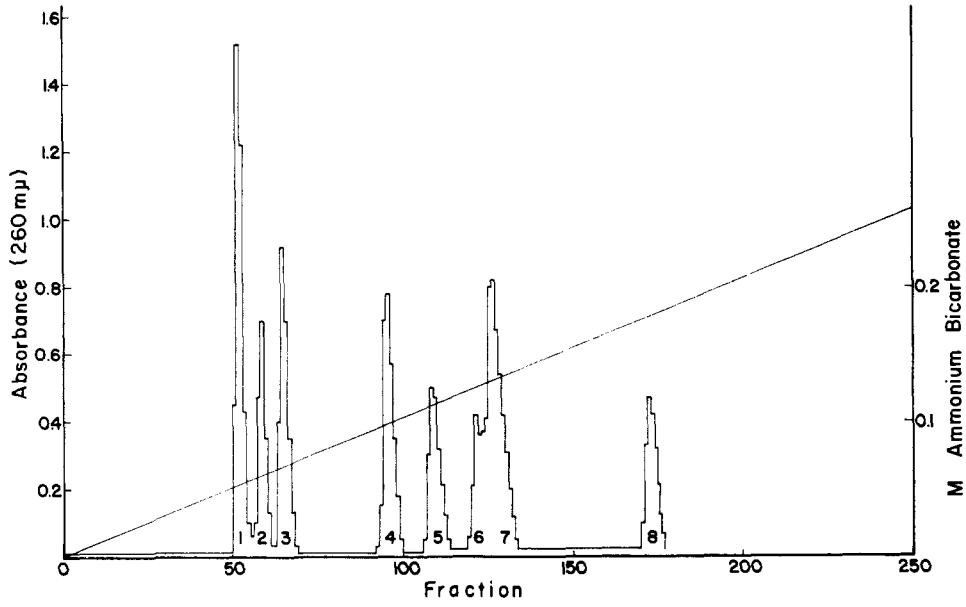

FIGURE 3: Separation of nucleotides on a diethylaminoethylcellulose column (50 × 1 cm diameter). Fractions (11 ml) were collected at 8-min intervals. Peak 1, adenosine-5′ phosphate; peak 2, P^1,P^2-diadenosine-5′ diphosphate; peak 3, guanosine-5′ phosphate; peak 4, adenosine-5′ diphosphate; peak 5, guanosine-5′ diphoshate; peak 6, P^1,P^3-diadenosine-5′ triphosphate; peak 7, adenosine-5′ triphosphate; peak 8, P^1,P^4-diadenosine-5′ tetraphosphate.

were found to be guanosine-5′ phosphate, guanosine-5′ diphosphate, and guanosine-5′ triphosphate, respectively. Guanosine-5′ diphosphate was the only material identified from the region between peaks 2 and 3. Peak 5 was identified as P^1,P^3-diguanosine-5′ triphosphate by the same criteria used in the study of *D. magna*. Similarly, peak 6 was identified as P^1,P^4-diguanosine-5′ tetraphosphate. The amounts of nucleotide isolated, estimated spectrophotometrically, are summarized in Table III.

Discussion

The occurrence of α,ω-diguanosine-5′ polyphosphates in the encysted embryos of *A. salina* (Finamore and Warner, 1963, Warner and Finamore, 1965a) together with an enzyme capable of degrading P^1,P^4-diguanosine-5′ tetraphosphate to guanosine-5′ phosphate and guanosine-5′ triphosphate (Warner and Finamore, 1965b) raises the question of the biological function of these nucleotides. Studies on the embryos of *Balanus nubilis*, a representative of the crustacean subclass Cirripedia, and of *Cancer magister*, of the subclass Malacostraca, showed that these unusual guanosine derivatives were not a general characteristic of crustacean embryos (Oikawa and Smith, unpublished results). The present investigation on the encysted embryos of *D. magna* was undertaken since this genus is a member of the subclass, Branchiopoda, of which *Artemia* is a member. Ion-exchange chromatography (Figure 1) revealed that the major nucleotides in *D. magna* all contained guanine (Table II). Both P^1,P^3-diguanosine-5′ triphosphate and P^1,P^4-diguanosine-5′ tetraphosphate were characterized by analysis of their guanine, ribose, total phosphate, and acid-labile phosphate contents and also by chromatographic comparison with authentic nucleotides. Extraction and purification of the nucleotides of encysted embryos of *A. salina*, using the same procedure as in the isolation of *D. magna* nucleotides (Figure 2), revealed quantitative differences between the nucleotides of the two species (Table III). It is possible that this reflects differing degrees of degradation during extraction of the two species. However, it is notable that P^1,P^3-diguanosine-5′ triphosphate predominates in *D. magna* and P^1,P^4-diguanosine-5′ tetraphosphate in *A. salina*. The presence of these nucleotides in both species may be related to the biological affinity of these organisms or to the arrested development of the encysted embryos. Ion-exchange chromatography on diethylaminoethylcellulose using a linearly increasing concentration of bicarbonate for elution is a convenient procedure for purification of guanine nucleotides from *Artemia* and *Daphnia*. The method offers advantages over separation on a cation exchanger with a polystyrene matrix in allowing operation at neutral or slightly alkaline pH, in ease of salt removal, and in separation of α,ω-diguanosine-5′ polyphosphates from nucleoside-5′ polyphosphates. Studies on the isolation of nucleoside-5′ polyphosphates on diethylaminoethylcellulose indicated that guanosine-5′ derivatives are more strongly bound than those of other ribonucleosides (Staehelin, 1961; Wylie and Smith, 1963). Comparison of the binding of α,ω-diguanosine-5′ polyphosphates (Figure 1) with that of α,ω-diadenosine-5′ polyphosphates (Figure 3) and of α,ω-dithymidine-5′ polyphosphates (Reiss and Moffatt,

1965) illustrates that this effect is cumulative.

Acknowledgments

The authors are indebted to Drs. Finamore and Warner for a sample of P^1,P^4-diguanosine-5′ tetraphosphate isolated from *A. salina* and for information on P^1,P^3-diguanosine-5′ triphosphate prior to publication. Dr. J. G. Moffatt very kindly donated chemically synthesized samples of P^1,P^3-diguanosine-5′ triphosphate, P^1,P^4-diguanosine-5′ tetraphosphate, P^1,P^3-diadenosine-5′ triphosphate, and P^1,P^4-diadenosine-5′ tetraphosphate, and Dr. J. W. DeWitt very generously donated the encysted embryos of *D. magna*.

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Synthetic Polypeptides Containing Side-Chain Amide Groups. Water-Soluble Polymers*

Larry H. Krull and Joseph S. Wall

ABSTRACT: Synthetic high-molecular weight polypeptides containing glutamine and glutamic acid residues were employed as model systems to investigate the influence of side-chain amides on protein conformation and aggregation in aqueous solutions. The polypeptides were prepared by converting ester groups of mixed polymers of glutamine and glutamyl esters to free carboxyls.

In some polymers, ester groups were retained to permit evaluation of hydrophobic forces. Solubilities and optical rotatory dispersion measurements at various pH values established that the presence of amide groups increased the minimum pH for solubility and decreased the helical content of the polymers. The minimum pH for solubility of the polymers was lowered with urea. The presence of ester residues also decreased polypeptide solubility but stabilized helical structure. These findings suggest that in high-molecular weight polypeptides or proteins, containing high levels of asparagine or glutamine, conformation and aggregation in aqueous solution may result from hydrogen bonding between amide groups.

In many proteins, glutamine, or asparagine residues occur in high concentration, especially in such prolamines as corn zein or wheat gliadin. The numerous side-chain amide groups may be responsible in part for the tendency toward aggregation and insolubility of such proteins. To investigate this possibility, high-molecular weight synthetic polypeptides, consisting of glutamine residues alone or together with γ-glutamyl ester residues, were synthesized and their properties investigated by Krull and associates (1965). These polymers did not dissolve in water, but when they were dissolved in organic solvents or were in a solid state, pronounced interactions between side-chain amide groups could be demonstrated. This effect was indicated by a decrease in polymer solubility and helical content in organic solvents as the ratio of glutamine–γ-glutamyl ester was increased. X-Ray and infrared studies established that the conformation and crystalline structure of polyglutamine were markedly different from those of poly-γ-ethyl-L-glutamate.

Since the normal environment of proteins is water, it was essential to evaluate the behavior of glutamine-containing synthetic polypeptides in aqueous solution.

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