The Acid-Soluble Nucleotides of the Copepod *Euchaeta japonica* Marukawa*

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ABSTRACT: The embryos of *Euchaeta japonica* Marukawa contain, as major components, the ribodinucleotides, adenylyl-(5′→3′)-adenosine 5′-phosphate, cytidylyl-(5′→3′)-adenosine 5′-phosphate, adenylyl-(5′→3′)-cytidine 5′-phosphate, uridylyl-(5′→3′)-adenosine 5′-phosphate, adenylyl-(5′→3′)-uridine 5′-phosphate, adenylyl-(5′ → 3′)-guanosine 5′-phosphate, cytidyl-(5′→3′)-cytidine 5′-phosphate, and cytidylyl-(5′→3′)-guanosine 5′-phosphate. The total amount of these ribodinucleotides is 30 μmoles of nucleotide/g, that is, 60-fold as much as the ribonucleic acid content. Additionally the embryos contain the ribonucleotides adenosine 3′,5′-bisphosphate and guanosine 3′,5′-bisphosphate (approximately 1.0 and 2.0 μmoles per g, respectively). In the parent females of this organism, adenosine monophosphate, inosine monophosphate, adenosine diphosphate, and adenosine triphosphate are the major components. The ribodinucleotides are synthesized during oogenesis and stored in the prehatching embryos. Large amounts of deoxyribonucleoside 5′-monophosphates, 2′-deoxycytidine 5′-monophosphate, 2′-deoxythymidine 5′-monophosphate, 2′-deoxyguanosine 5′-monophosphate, and 2′-deoxyinosine 5′-monophosphate, about 2 μmoles/g of each, and lesser amounts of 2′-deoxyadenosine 5′-monophosphate and 2′-deoxyuridine 5′-monophosphate are present in the embryo before hatching.

Recent studies have shown that the encysted embryos of brine shrimp, *Artemia salina*, contain large quantities of the unusual nucleotide P^1,P^4-diguanosine 5′-tetraphosphate, and that the tetraphosphate is the major and perhaps sole source of purines in the developing embryos (Finamore and Warner, 1963; Warner and Finamore, 1967; Clegg *et al.*, 1967; Warner and McClean, 1968). Other species of the crustacean subclass Branchiopoda contain these nucleotides in considerable amounts (Oikawa and Smith, 1966; Warner and McClean, 1968).

The present investigation has been undertaken to characterize the free nucleotides present during the development of *Euchaeta japonica* Marukawa, a member of the crustacean subclass Copepoda. The experiments show that the diguanosine tri-, or tetraphosphate is not detectable in this species. However, a new group of acid-soluble nucleotides, in the main simple ribodinucleotides, is present in large quantity in the embryos. Some of these results have been presented in a preliminary communication (Hepner and Smith, 1967). These ribodinucleotides are synthesized during oogenesis and are stored in the prehatching embryos. The deoxyribonucleotides dCMP, dTMP, dIMP, and dGMP also accumulate in major amounts during oogenesis. The ribonucleotides pAp and pGp,[1] which are present in the embryos, accumulate later than the other nucleotides.

Materials and Methods

Organisms. All experiments were carried out on the marine calanoid copepod, *E. japonica* Marukawa (Campbell, 1934). Females having blue ovaries during oogenesis and females each with an attached embryo cluster were collected, using standard oceanographic plankton sampling equipment, from a depth of 200 m at 49° 42.7′ N, 122° 52.3′ W in Indian Arm, a fjord-like inlet of the Straits of Georgia in southern British Columbia. The embryo clusters (about 15 embryos in a cluster) were detached from the parent females, using forceps. These parent females contained ovaries at a very early oogenesis stage. The average weight of an embryo cluster was 0.76 mg and the average weight of a female at an advanced stage of oogenesis was 7.45 mg. Some embryo clusters were cultured in covered glass dishes (20 × 20 cm) containing sea water at 10°, and newly hatched nauplii were collected with an eye dropper every other day. Organisms at each stage were frozen immediately and stored at −80°. Sea water was collected at the same depth as the organisms.

Isotope Incorporation. Organisms, collected with the plankton net and brought back to the laboratory in sea water collected at a 200-m depth were incubated in petri dishes which contained 25 ml of sea water with [^{3}H]adenosine (25 μCi/8.0 nmoles) and [^{14}C]cytidine (5 μCi/0.5 mmole). After incubation at 10° for various periods of time, organisms were collected, washed with 50 ml of sea water on a Millipore filter under mild vacuum, frozen immediately in a Dry Ice–acetone bath,

* From the Department of Biochemistry, Faculty of Medicine, University of British Columbia, Vancouver 8, B. C., Canada. *Received September 9, 1969.* This research was generously supported by a grant from the Medical Research Council of Canada.

† Holder of a National Research Council of Canada Bursary. Died August 24, 1966.

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[1] The abbreviations used are: pApA, adenylyl-(5′→3′)-adenosine 5′-phosphate; pApC, cytidylyl-(5′→3′)-adenosine 5′-phosphate; pCpA, adenylyl-(5′→3′)-cytidine 5′-phosphate; pApU uridylyl-(5′→3′)-adenosine 5′-phosphate; pUpA, adenylyl-(5′→3′)-uridine 5′-phosphate; pGpA, adenylyl-(5′→3′)-guanosine 5′-phosphate; pCpC, cytidylyl-(5′→3′)-cytidine 5′-phosphate; pGpC, cytidylyl-(5′→3′)-guanosine 5′-phosphate; ApA, adenylyl-(5′→3′)-adenosine; CpA, adenylyl-(5′→3′)-cytidine; ApC, adenylyl-(5′→3′)cytidine; pAp, pGp, pCp, and pUp, adenosine, guanosine, cytidine, and uridine 3′,5′-bisphosphate, respectively.

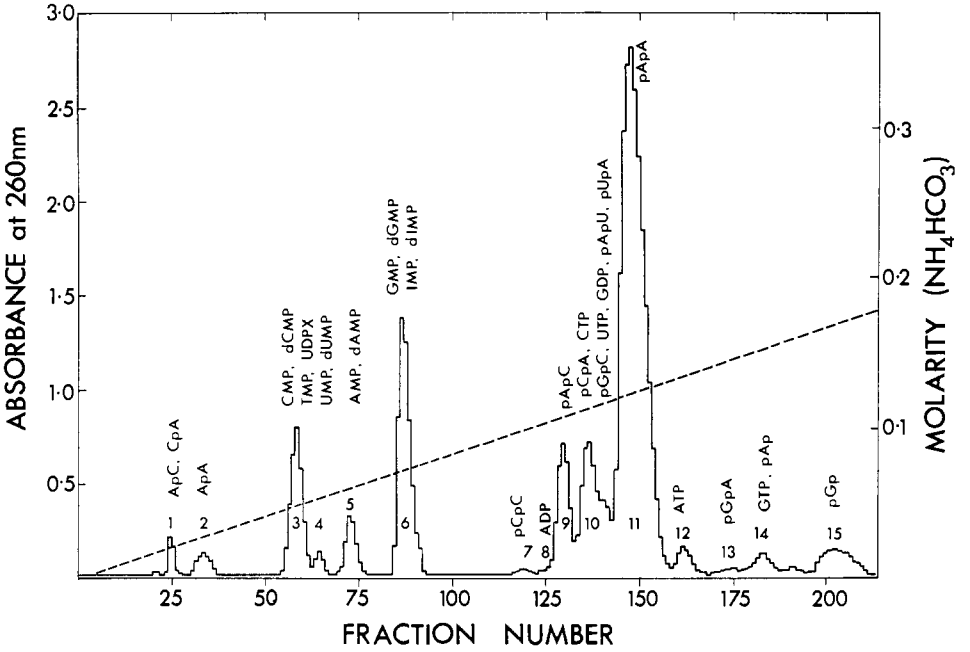

FIGURE 1: Separation of nucleotides from *E. japonica* embryos (0.75 g) on a DEAE-cellulose column (45 × 1.2 cm diameter). Fractions (10 ml) were collected at 10-min intervals.

and stored at −80°. All radioactivity determinations were made with a Nuclear-Chicago liquid scintillation counter (Mark 1).

Extraction of Acid-Soluble Nucleotides. The embryo clusters or the hatched nauplii were homogenized, with 10 ml of 1 M $HClO_4$ to each 1 g of organisms at 0°, using a glass–Teflon homogenizer. As the embryos were disrupted, their blue color was replaced by a pink coloration. After 1–2 min of homogenization, the mixture was centrifuged at 10,000*g* for 10 min at 0°. The clear colorless supernatant solution was decanted and the solid pink residue was washed with 1 M $HClO_4$ at 0°. The residue was stored at −30° for determination of RNA and DNA. The combined extract was filtered through a Millipore filter to remove floating particles and then neutralized to pH 7.0 with either cold 1 M NaOH or cold 5 M KOH (see Results section for a discussion of these two procedures). After neutralization with NaOH, water was removed in a rotary evaporator (bath temperature, 30°) and the nucleotides were precipitated by addition of 95% ethanol. The precipitate was collected by centrifugation, washed with 95% ethanol followed by acetone, and then dried *in vacuo* over $CaCl_2$ at 25°. The solid was dissolved in water for application to an ion-exchange column. After neutralization with KOH, the precipitated $KClO_4$ was removed at 0°. The extract was dried in a rotary evaporator, dissolved in a small amount of water, and a further crop of $KClO_4$ was removed by centrifugation. This sample was diluted with a tenfold volume of water, and was applied to an ion-exchange column.

The adult females were disrupted for a few minutes in the frozen state, using a pestle and mortar. They were then extracted in the same way as the embryos.

Ion-Exchange Fractionation of Acid-Soluble Nucleotides. The aqueous solution of nucleotides was fractionated on DEAE-cellulose (1.2 cm diameter × 45 cm column for samples containing more than 20 μmoles and 1.2 cm diameter × 20 cm column for those in small amount) using a linearly increasing concentration of NH_4HCO_3 (pH 8.0) (Oikawa and Smith, 1966). The elution of nucleotides was followed spectrophotometrically. Appropriate fractions were combined and concentrated to dryness in a rotary evaporator (bath temperature 30°). Residual ammonium carbonate was removed by addition of aqueous ethanol and reevaporation. The residual ammonium salt of the nucleotide was dissolved in a small amount of water and the nucleotide was isolated as a solid after lyophilization. Alternatively, the aqueous solution of nucleotides was stored frozen at −30° prior to further fractionation (by repeating the ion-exchange chromatography with a more gradual increase in salt concentration or by partition chromatography) and characterization.

Partition Chromatography. Solvent systems were isobutyric acid–1 M NH_4OH (5:3 ml), 0.1 M sodium phosphate (pH 6.8)–solid $(NH_4)_2SO_4$–1-propanol (100 ml:60 g:2 ml), and isopropyl alcohol–concentrated NH_4OH–water (7:1:2 ml). Chromatography on Whatman No. 40 paper was carried out by the descending technique at 20–25°. Whenever possible, authentic standards were chromatographed alongside unknowns and nucleotides were detected by viewing under a short-wavelength ultraviolet light. Nucleotides were eluted for spectral examination using a standard technique (Heppel, 1967).

Absorption Spectra. These were determined using a Unicam SP800 spectrophotometer equipped with either 10- or 2-mm path-length cells. All samples were examined at pH 2, 7, and 10 and were compared with authentic spectra (Beavan *et al.*, 1955).

Analytical Methods. Total phosphate, phosphate liberated by hydrolysis in 1 M HCl at 100° for 7 min, and phosphate liberated by *Escherichia coli* alkaline phosphatase were determined by the method of Ames (1966). Ribose was determined by the orcinol procedure (Ashwell, 1957) and deoxyribose by the diphenylamine reaction (Dische, 1955). Bases were determined from published absorbance values (Beavan *et al.*, 1955).

Periodate Degradation. This was carried out by the method of Neu and Heppel (1964) with isolation of the degradation products by ion-exchange chromatography essentially as described by Weith and Gilham (1967).

Enzymatic Degradations. Degradations using *E. coli* alka-

line phosphatase (Reiss and Moffatt, 1965), snake venom (*Crotalus ademanteus*) diesterase I (Razzell, 1963), spleen diesterase II (Razzell, 1963), ribonuclease T_2 (Uchida and Egami, 1967), pancreatic ribonuclease, and crude snake venom (*Crotalus ademanteus*) as a 5′nucleotidase (Gulland and Jackson, 1938) were carried out on 0.1–1.0 μmole of nucleotide in 100 μl of the appropriate buffer at 37° with sufficient enzyme to complete reaction in times ranging from 5 to 60 min. The products were characterized directly by paper chromatography and following spectral examination. Useful information on the dinucleotides was obtained by following the increase in absorbance at 260 nm when the internucleotide bond was enzymically hydrolyzed. Thus pCpA was readily distinguished from pApC because there was an increase in the absorbance at 260 nm when the former was treated with pancreatic ribonuclease, but not when the latter was treated. Similarly the lack of an increase in absorbance when the dinucleotides were treated with spleen phosphodiestase, and the increase which occurred when they were treated with snake venom diesterase was a clear indication of a 5′-phosphoryl group.

Results

Acid-Soluble Nucleotides in Embryos. When a perchloric acid extract is neutralized with KOH, P^1,P^4-diguanosine 5′-tetraphosphate, which has poor solubility in water, is partly lost. On the other hand, by neutralization of acid extract with NaOH, some nucleotides are partly lost in the alcohol washing to remove $NaClO_4$. Therefore, at first the acid extract from *Euchaeta* embryos was neutralized with NaOH. When P^1,P^4-diguanosine 5′-tetraphosphate was not detected, subsequent preparations of acid-soluble nucleotides were neutralized with KOH. Figure 1 shows the elution profile of the acid-soluble nucleotides on DEAE-cellulose column chromatography. The various components were collected into 15 peaks, and then were subfractionated by paper chromatography in isobutyric acid–1 M NH_4OH (5:3) and where necessary subsequently in 0.1 M sodium phosphate (pH 6.8)–solid $(NH_4)_2SO_4$–1-propanol (100:60:2). Their paper chromatographic mobilities, chemical analyses, and enzymatic degradation are shown in Tables I–III, respectively. The amount of each nucleotide is listed in Table IV, line 3.

Nucleoside 5′-monophosphates were isolated from peaks 3 to 6; CMP, dCMP, dTMP, UMP, and dUMP in peaks 3 and 4; AMP and dAMP in peak 5; GMP, dGMP, IMP, dIMP in peak 6. The nucleotides were characterized using paper chromatography with authentic nucleotides and by spectral examination. In addition dGMP and dIMP were analyzed for their deoxyribose content after rechromatography on DEAE-cellulose. Also dCMP, dTMP, dGMP, and dIMP were treated with crude snake venom in the presence of 0.1 M NH_4HCO_3 and 0.01 M $MgCl_2$. The 5′-nucleotidase converted the nucleotides into nucleosides confirming that they are 5′-phosphate compounds. ATP (peak 12) and GTP (in peak 14) were characterized by chemical analyses and by paper chromatography, and ADP (peak 8), CTP, and UTP (in peak 10) by paper chromatography alone, because of their small quantity.

Various ribodinucleotides were isolated between peak 7 and peak 13. The largest component (peak 11) was further purified by rechromatography on DEAE-cellulose, and was isolated as its solid ammonium salt. The nucleotide was homogeneous on paper chromatography in the three systems mentioned above. The ratios of phosphomonoesterase-released phosphate to total phosphate to ribose to adenosine (estimated spectrophotometrically at 257 nm in 0.01 M HCl) were 0.9:2.0:2.0:1.9. Degradation of the nucleotide by snake venom diesterase I yielded AMP as the sole product. Ribonuclease T_2 gave equimolar amounts of pAp and adenosine. *E. coli* alkaline phosphatase followed by snake venom diesterase I yielded adenosine, AMP, and inorganic phosphate in the ratios of 1:1:1. In addition, oxidation of the nucleotide with sodium

TABLE I: Chromatographic Mobility (R_F) of Nucleotides.

	R_F		
	I[a]	II[b]	III[c]
ApC	0.64		
CpA	0.64		
ApA	0.73	0.02	0.24
CMP	0.48	0.70	0.07
dCMP	0.58	0.63	0.10
UDPX	0.03		
dTMP	0.47	0.50	0.15
UMP	0.33	0.69	0.06
dUMP	0.40	0.60	0.08
AMP	0.54	0.27	0.08
dAMP	0.66	0.21	0.10
GMP	0.29	0.46	0.02
dGMP	0.42	0.37	0.04
IMP	0.32	0.54	0.04
dIMP	0.39	0.49	0.05
CDP	0.30	0.76	
UDP	0.18	0.75	
pCpC	0.42	0.50	
ADP	0.44	0.35	0.04
pApC	0.45	0.24	0.03
pCpA	0.50	0.23	0.02
CTP	0.24	0.77	
UTP	0.12	0.77	
GDP	0.20	0.49	0.01
pGpC	0.20	0.22	
pApU	0.36	0.23	0.02
pUpA	0.36	0.23	0.02
pApA	0.52	0.08	0.03
ATP	0.36	0.38	0.03
pGpA	0.34	0.15	
pAp	0.41	0.32	0.08
GTP	0.14	0.61	0.08
pGp	0.20	0.52	0.00
Cytidine	0.74	0.62	0.45
Uridine	0.55	0.61	0.43
Adenosine	0.84	0.13	0.51
Guanosine	0.55	0.30	0.27
Inosine	0.55	0.41	0.39

[a] Isobutyric acid–1 M ammonium hydroxide (5:3). [b] 0.01 M sodium phosphate–$(NH_4)_2SO_4$–1-propanol (100 ml:60 g:2 ml). [c] Isopropyl alcohol–concentrated NH_4OH–water (7:1:2).

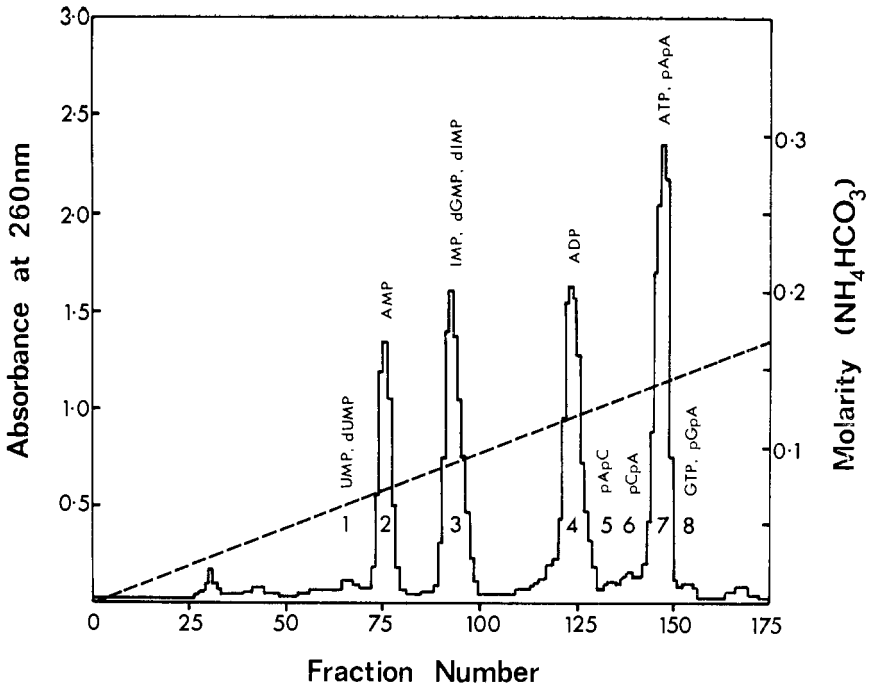

FIGURE 2: Separation of nucleotides from parent females of *E. japonica* (7.5 g) on a DEAE-cellulose column (45 × 1.2 cm diameter). Fractions (8.5 ml) were collected at 10-min intervals.

TABLE II: Chemical Analyses of Nucleotides.

Peak	Compound	Base[a]	Total Phosphate	Acid-Labile Phosphate	Phosphomonoesterase-Labile Phosphate	Ribose	Deoxyribose
Embryo							
2[b]	ApA	Ade, 1.8	1.0		0.0	2.0	
6	dGMP	Gua, 1.0					1.0
6	dIMP	Hyp, 1.0					1.0
7	pCpC	Cyt, 1.7	2.0		0.9		
11	pApA	Ade, 1.9	2.0		0.9	2.0	
12	ATP	Ade, 0.9	3.0	1.8	2.8	0.9	0.0
13	pGpA	Ade and Gua, 1.7	2.0		1.0	1.7	
14	GTP	Gua, 0.9	3.0	2.2	2.7	1.0	
14	pAp	Ade, 0.9	2.0	1.0[d]	1.8	0.9	
15	pGp	Gua, 1.1	2.0	1.0[d]	2.1	1.0	
Parent female							
2[c]	AMP	Ade, 0.9	1.0		0.9	1.0	
3	IMP	Hyp, 0.9	1.0		0.9	0.9	
4	ADP	Ade, 0.9	2.0	0.8	1.8	1.1	0.0
7	pApA	Ade, 1.8	2.0		0.8	2.3	
7	ATP	Ade, 0.9	3.0	1.8	2.8	1.0	0.0
8	GTP	Gua, 1.1	3.0				

[a] Identified by absorbance spectrum of nucleoside. [b] Numbering of peaks refers to Figure 1. [c] Numbering of peaks refers to Figure 2. [d] Phosphate was liberated in 14 min.

metaperiodate in the presence of lysine at pH 9.0 resulted in equimolar amounts of pAp and adenine. These data unambiguously indicate that the nucleotide is adenylyl-(5′→3′)-adenosine 5′-phosphate (pApA). Dinucleotides pApC (peak 9), pCpA (peak 10), pApU, and pUpA (between peaks 10 and 11) were also isolated (Hepner and Smith, 1967). Other dinucleotides pCpC (peak 7) and pGpA (peak 13) were isolated and characterized in the same procedure. pGpC in peak 10

TABLE III: Products from Enzyme Degradation of Nucleotides.

Peak	Compound	Snake Venom Diesterase I	Phosphomonoesterase Followed by Snake Venom Diesterase I	Phospho-monoesterase	RNase T_2
Embryo					
1[a]	ApC + CpA	A + C + pA + pC (3:4:4:3)			
2	ApA	A + pA (1:1)			
7	pCpC	pC	P_i + C + pC (1:1:1)		pCp + C (1:1)
11	pApA	pA	P_i + A + pA (1:1:1)		pAp + A (1:1)
15	pGpA	pG + pA (1:1)	P_i + G + pA (1:1:1)		pGp + A (1:1)
Parent female					
5[b]	pApC				pAp + C (1:1)
6	pCpA				pCp + A (1:1)
7	pApA			P_i + ApA (1:1)	
8	pGpA				pGp + A (1:1)

[a] Numbering of peaks refers to Figure 1. [b] Numbering of peaks refers to Figure 2.

was separated from pCpA, pApU, pUpA, pApA, and UTP, but not from CTP nor GDP, by paper chromatography in isobutyric acid–1M NH_4OH (5:3). Rechromatography in 0.1 M sodium phosphate–solid $(NH_4)_2SO_4$–1-propanol (100:60:2) separated pGpC from CTP and GDP. The quantity of pGpC, however, was too small to be characterized after the second paper chromatography followed by the removal of $(NH_4)_2SO_4$. Therefore, the ultraviolet spectra of the nucleotide eluated from paper chromatogram in 0.1 M sodium phosphate–solid $(NH_4)_2SO_4$–1-propanol (100:60:2) were confirmed to be the same as those of a GMP–CMP (1:1) mixture at pH 2, 7, and 10. Also pGpC, contaminated with CTP (and/or GDP), was treated with RNase T_2, and the products were determined by paper chromatography to be cytidine, pGp, CTP (and/or GDP), with no detectable pCp or guanosine. Peak 1 contained ApC and CpA, and peak 2, ApA. The extinction coefficient of each dinucleotide was calculated from the absorbance at 260 nm before and after the degradation of the dinucleotides by snake venom diesterase I.

Two other ribomononucleotides were present (in peaks 14 and 15) in the *E. japonica* embryos. Their spectral characteristics showed that they contained adenine and guanine nucleosides, respectively. Pentose and phosphate analyses, phosphomonoesterase-released phosphate, and the rate of release of phosphate in 1 M HCl at 100° suggested that the nucleotides were nucleoside 2′,5′- or nucleoside 3′,5′-bisphosphates. Comparison of their partition chromatographic properties with those of authentic samples confirmed the structures as adenosine 3′,5′- and guanosine 3′,5′-bisphosphates.

One, incompletely characterized, uracil nucleotide was isolated from peak 3.

As may be seen in Tables IV and V (line 3), the quantity of the dinucleotides in the prehatched embryos is 30 μmoles of nucleotide/g of fresh weight (23 nmoles of nucleotide/embryo cluster including about 13 to 15 embryos), that is, more than 60-fold as much as the RNA content, and 79% of total ribose-containing material. It is also noteworthy that *Euchaeta* embryos have a big pool of deoxyribose nucleoside 5′-monophosphates, dCMP, dTMP, dGMP, and dIMP at the level of about 2 μmoles each/g. A lesser amount of dUMP was present, however, only a trace of dAMP was detected.

Acid-Soluble Nucleotides at Various Stages of Development. The parent females, from which the embryo clusters had been detached, contained ovaries at a very early stage of oogenesis. These were analyzed for acid-soluble nucleotides using the procedure mentioned above. Properties of the nucleotides on paper chromatography, chemical analyses, and enzymatic degradation are listed in Tables I to III, respectively. As shown in Figure 2, the elution profile of the nucleotides from these adults on DEAE-cellulose is completely different from that of embryos (Figure 1). Major components are AMP (peak 2), IMP (peak 3), ADP (peak 4) and ATP (91% of peak 7), and small amounts of ribodinucleotides, pApA (9% of peak 7), pApC (peak 5), pCpA (peak 6), and pGpA (in peak 8) were

TABLE IV: Nucleotides Isolated from *Euchaeta* at Different Stages (μmoles of nucleotide/g[a]).

Compound	Parent Female	Female at Oogenesis	Embryo	Nauplius
dCMP		0.19	2.05	0.30
dTMP		0.23	2.12	0.38
dUMP		0.01	0.18	0.05
dAMP			0.04	
dGMP	0.01	0.12	2.14	0.21
dIMP	0.03	0.27	2.71	0.68
CMP		0.01	0.41	0.17
UMP	0.02	0.02	0.33	0.22
AMP	0.38	0.31	0.89	0.66
GMP		0.13	1.63	0.38
IMP	1.19	0.90	0.17	0.12
CDP		0.01		
UDP		0.02		0.10
ADP	0.68	0.82	0.07	0.37
GDP		0.03		0.03
CTP		0.01	0.17	0.16
UTP		0.02	0.16	0.14
ATP	0.98	0.82	0.62	0.63
GTP	0.03	0.03	0.10	0.11
pCpC		0.04	0.42	
pApC	0.04	0.32	3.66	1.38
pCpA	0.04	0.46	3.92	1.44
pGpC		0.16	0.14	0.01
pApU		0.08	0.70	0.28
pUpA		0.08	0.70	0.28
pApA	0.10	1.56	19.44	9.88
pGpA	0.02	0.30	0.40	
pAp		0.04	0.85	0.66
pGp		0.01	2.02	0.26
UDPX	0.06	0.14	0.28	0.33
RNA	0.36	0.30	0.49	0.48
DNA	1.00	0.97	1.36	10.51

[a] Quantitative estimates were made spectrophotometrically.

detected. The amounts of the nucleotides isolated are recorded in Tables IV and V (line 1). As the oogenesis proceeds, the ovaries become blue with eggs. The ion-exchange profile of the acid-soluble nucleotides from females containing ovaries at this more advanced stage of development was similar to that of the embryos. The total amount of acid-soluble nucleotides increased strikingly during oogenesis, as the ribonucleotides accumulated (Tables IV and V, line 2). Increase in deoxynucleoside monophosphates is also obvious at this stage. When the approximately tenfold difference in weight of adults and embryo clusters is taken into account it is evident that the amounts of ribodinucleotides and deoxyribonucleoside 5′-phosphates which accumulate during oogenesis corresponds quantitatively to the amounts found in the embryo clusters. Consequently the function of the accumulation must be to provide nucleotides for the mature oocytes.

Some embryo clusters were cultured, and the acid-soluble nucleotides of the newly hatched nauplii (0–2 days after hatching) were examined in the same way (Tables IV and V, line 4). The dinucleotides decrease at this stage although the rate is different for various dinucleotides. It is conceivable that the dinucleotides are used in the rapid development at this stage. The big pool of deoxynucleoside monophosphates in the prehatching embryos decreases on hatching, with the production of an equivalent amount of DNA.

Incorporation of Radioactivity. Females undergoing oogenesis and the embryos were placed in petri dishes with sea water containing [^{3}H]adenosine and [^{14}C]cytidine for 24 hr, and acid-soluble nucleotides and RNA were examined for the incorporation study. In the embryos both [^{3}H]adenosine and [^{14}C]-cytidine did not show appreciable incorporation into the dinucleotides in spite of their large quantities. The other mononucleotides and RNA in the embryos were labeled. On the other hand, the dinucleotides in the females at the oogenesis stage incorporated both [^{3}H]adenosine and [^{14}C]cytidine. The intact embryo clusters attached to the parent females were maintained in sea water containing [^{3}H]adenosine for 24 hr. None of the dinucleotides in the embryos incorporated labeled adenosine. These results showed that the dinucleotides in the embryos were not newly synthesized materials, nor were they transported from the parent females to the developing embryos. Table VI summarizes the results of the incorporation of [^{3}H]adenosine.

The accumulation of dinucleotides during oogenesis was next investigated. Females with developing ovaries were placed in sea water containing [^{3}H]adenosine for 8 hr, then in sea water containing unlabeled adenosine, at 100 times the previous [^{3}H]adenosine concentration, for 40 hr. As may be seen in Figure 3A, radioactivity in mononucleotides decreased after 8 hr; that in dinucleotides, however, increased linearly. This implies that radioactivity in mononucleotides has transferred into the dinucleotides. Radioactivity in RNA was very low and decreased during the chase period. Comparing change of specific activity (Figure 3B) and that of total radioactivity incorporated (Figure 3C) in the major components, it is seen that pApA has a slow increase in specific activity. This supports the concept that pApA is accumulated without rapid turnover. ATP, which has a big change in specific activity, but a small amount of total radioactivity, shows its rapid turnover.

Discussion

The presence of nucleotides with unusual structures in eggs or embryos has been reported in several species; acid-soluble RNA in amphibian eggs and embryos (Finamore and Volkin, 1961 and Finamore, 1964), acid-soluble RNA in fish eggs (Lu and Finamore, 1963) and P^1,P^4-diguanosine 5′-tetraphosphate and P^1,P^3-diguanosine 5′-triphosphate in embryos of several species of the crustacean subclass Branchiopoda (Finamore and Warner, 1963; Warner and Finamore, 1965; Oikawa and Smith, 1966; Warner and McClean, 1968).

Because the branchiopods contain nucleotides which were both unusual and readily characterizable, we decided to examine the nucleotides of a second crustacean subclass. The copepod *E. japonica* was chosen because of its availability in

TABLE V: Amounts of Nucleotides and Nucleic Acid at Different Stages of Development (μmoles/g).

Compound	Parent Female	Female at Oogenesis	Embryo	Hatched Nauplius
Deoxyribonucleoside 5′-phosphates	0.04	0.82	9.24	1.62
Ribonucleoside 5′-phosphates	1.59	1.37	3.43	1.55
Ribonucleoside 5′-diphosphates	0.68	0.88	0.07	0.50
Ribonucleoside 5′-triphosphates	1.01	1.00	1.05	1.04
pAp, pGp		0.05	2.87	0.92
UDPX	0.06	0.14	0.28	0.33
Mononucleotides	3.38	4.26	16.94	5.96
Dinucleotides	0.19	3.00	30.54	13.27
Nucleotides	3.57	7.26	47.48	19.23
RNA	0.36	0.30	0.49	0.48
DNA	1.00	0.97	1.36	10.51

the seas adjacent to Vancouver. It also has the advantages of breeding on a year round basis rather than in a specific season, and the embryos and ovaries are readily identified because of their bright blue coloration. In this investigation of *E. japonica*, observations have been made on three groups of acid-soluble nucleotide, ribodinucleotides, purine ribonucleoside 3′,5′-bisphosphates, and deoxyribonucleoside 5′-phosphates.

Eight ribodinucleotides, pApA, pApC, pCpA, pApU, pUpA, pGpA, pGpC, and pCpC have been isolated from *Euchaeta* embryos. The dinucleotides amount to approximately 30 μmoles of nucleotides/g, *i.e.*, 15 μmoles of dinucleotides/g or 23 nmoles of nucleotides/cluster of approximately 15 embryos. This extraordinarily large amount of ribonucleotides can be compared with the small amounts, about 1 μmole/kg, of dinucleotides derived from cytidine and uridine which have been isolated from hen oviduct (Suzuki *et al.*, 1964). The amount of ribonucleotides in *Euchaeta* embryos is 60 times the amount of RNA present. The present investigation, both by isolation at different stages of development and from studies on incorporation of radioactive nucleosides, shows that in *Euchaeta* the ribodinucleotides accumulate during oogenesis. On the evidence of the studies of incorporation of radioactive

TABLE VI: Incorporation of [^{3}H]Adenosine into Adenosine-Containing Nucleotides and RNA.[a] cpm/Female or Embryo Cluster (specific activity, cpm/nmole).

Compound	Female at Oogenesis	Embryo Cluster
AMP	1,904 (390)	154 (227)
ADP	834 (503)	11 (219)
ATP	208 (517)	82 (171)
pApA	1,275 (166)	4 (0.3)
pApC	94 (58)	1 (0.4)
pCpA	77 (55)	1 (0.4)
pApU + pUpA	21 (27)	0.4 (0.3)
pGpA	89 (59)	
pAp		19 (29)
RNA	148 (104)	13 (34)

[a] Females with developing ovaries or with embryo clusters attached were incubated in [^{3}H]adenosine (25 μCi/25 ml) for 24 hr. Embryo clusters were detached from the parent females after the incubation. The acid-soluble fraction was separated by DEAE-cellulose and by following paper chromatography. Aliquots from each nucleotide or RNA were counted, and the total activity and the specific activity were determined. Quench corrections were made for the nucleotides which were eluted from paper chromatogram in 0.1 M sodium phosphate–$(NH_4)_2SO_4$–1-propanol (100:60:2).

TABLE VII: Amounts of Dinucleotides in Females with Developing Ovaries and in Embryo Clusters (nmoles as nucleotide/animal or embryo cluster).

Compound	Female at Oogenesis	Embryo Cluster
pApA	5.87	7.13
pCpA	1.71	1.51
pApC	1.19	1.41
pApU	0.30	0.27
pUpA	0.30	0.27
pGpA	1.12	0.15
pGpC	0.60	0.05
pCpC	0.15	0.16

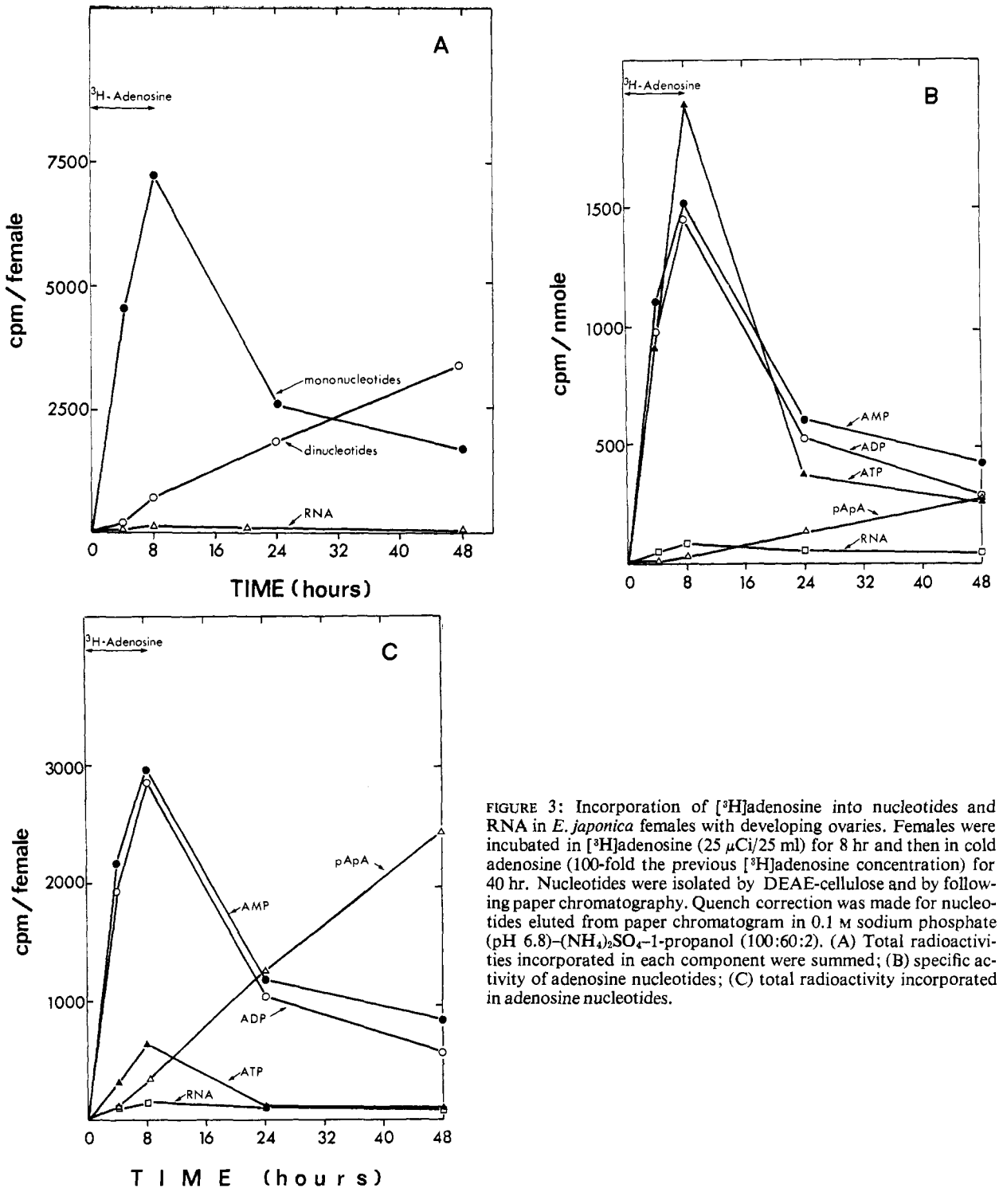

FIGURE 3: Incorporation of [^{3}H]adenosine into nucleotides and RNA in *E. japonica* females with developing ovaries. Females were incubated in [^{3}H]adenosine (25 μCi/25 ml) for 8 hr and then in cold adenosine (100-fold the previous [^{3}H]adenosine concentration) for 40 hr. Nucleotides were isolated by DEAE-cellulose and by following paper chromatography. Quench correction was made for nucleotides eluted from paper chromatogram in 0.1 M sodium phosphate (pH 6.8)–$(NH_4)_2SO_4$–1-propanol (100:60:2). (A) Total radioactivities incorporated in each component were summed; (B) specific activity of adenosine nucleotides; (C) total radioactivity incorporated in adenosine nucleotides.

nucleosides it appears that little or no synthesis of ribodinucleotides occurs after maturation of the ovary and that most of the dinucleotides are stored throughout the stages of embryogenesis prior to hatching. Subsequently, the stores of ribodinucleotides are depleted, the nucleotides being virtually absent from adult females with undeveloped ovaries. The data indicate that the depletion of the various ribodinucleotides may occur at different rates or at different stages of development. Thus, some of them diminish in amount in the embryos prior to hatching (Table VII; pGpA, 1.1 nmoles/female with developing ovaries; 0.15 nmole/embryo cluster; pGpC, 0.6 nmole/female with developing ovaries; 0.05 nmole/embryo cluster).

The mechanisms for the synthesis or the utilization of the ribonucleotides have not been determined nor has their function. It seems possible that they may be storage forms, a role similar to that suggested for the guanosine nucleotides in *Artemia* (Clegg *et al.*, 1967; Warner and McClean, 1968). In

this connection it is notable that AMP represents over 80% of the nucleotides present in the dinucleotides in line with the major roles of adenosine nucleotides.

The second class of ribonucleotides characteristic of *Euchaeta* is the purine ribonucleoside 3′,5′-bisphosphates. They accumulate either late in oogenesis or in the early stages of embryogenesis, *i.e.*, later than the ribonucleotides and their depletion parallels that of the major group of ribodinucleotides (Table IV). The studies on the incorporation of [^{3}H]-adenosine into nucleotides indicate that there is synthesis of pAp in embryos whereas little synthesis of ribodinucleotides is detectable (Table VI). This observation also suggests that nucleotides such as pApA are not the precursors of pAp. Quantitatively, there does not appear to be any relationship between the amounts of ribodinucleotides or RNA and the amounts of pAp and pGp.

The deoxyribonucleoside 5′-phosphates dCMP, dTMP, dGMP, and dIMP are notable because of the large amounts of these nucleotides which accumulate during oogenesis in *Euchaeta*. It is possible that these nucleotides are accumulated in anticipation of the extensive synthesis of DNA which occurs at the time of hatching when there is an approximately tenfold increase in the amount of DNA per unit weight of the organisms. The fact that dIMP and the other deoxyribonucleoside 5′-phosphates are the ones which are accumulated suggests possible roles for the amination of dIMP and the phosphorylation of deoxyribonucleoside 5′-phosphates in the control of DNA synthesis. It also may be possible that the synthesis of DNA is a useful prelude to the extensive series of metamorphic changes which occur after hatching in *E. japonica*.

A major problem with regard to the further exploration of the biochemistry of the nucleotides lies in the limited numbers in which *E. japonica* can be conveniently obtained. This almost certainly will prevent extensive studies of the enzymes of nucleotide metabolism in the organism. However, the discovery of the various classes of nucleotide in *E. japonica* increases the number of modes of nucleotide metabolism which may be anticipated in other forms.

Acknowledgments

This study would not have been possible without the help and advice on oceanographic matters from Dr. A. C. Lewis. The help of the officers and crews of the research vessels of the Canadian Department of Mines and Technical Services was invaluable as was that of the various friends and colleagues who assisted with the tedious task of collecting *E. japonica*.

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