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Aspects of nucleic acid metabolism during development of the brine shrimp Artemia salina.

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ASPECTS OF NUCLEIC ACID METABOLISM DURING DEVELOPMENT OF THE BRINE SHRIMP *ARTEMIA SALINA*

BY

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ABSTRACT

Nucleic acid metabolism of the brine shrimp, Artemia salina, has been the subject of recent investigation at the Oak Ridge National Laboratories (U.S.A.) to determine the source of nucleic acid purines relative to the cellular nucleotide pools during development. As a sequel to this work, and to further investigate the biochemical events following hatching of Artemia, newly hatched larvae (nauplii) are maintained for 6 hour periods in Pi^{32} and adenosine-H^{3} and the nucleic acids analyzed for radioactive incorporation. From this study it is apparent that virtually all RNA synthesis is terminated by 36 hours of development, whereas DNA synthesis continues for 18-20 additional hours. The relationship between RNA and DNA metabolism, nucleic acid purine content, and the presence of an unusually large amount of a high molecular-weight RNA species in Artemia is discussed.

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INTRODUCTION

Embryos of the brine shrimp, Artemia salina, develop either directly into nauplius larvae in the maternal ovisac or become encysted as dormant gastrulae (Fautrez-Firlefyn, 1951) which undergo nearly complete dessication and subsequently cessation of metabolism in the embryo (Clegg, 1967). In the latter case, cryptobiosis can be terminated and development resumed by rehydration. Although hatching of nauplii is accompanied by a marked increase in mitotic activity, extensive morphogenesis occurs prior to hatching and without DNA[1] synthesis or cell division (Emerson, 1963; Nakanishi et. al., 1962, 1963). Unfortunately the brine shrimp is almost completely impervious to RNA precursors during this period of high morphogenetic activity. Consequently, this thesis will be concerned with nucleic acid metabolism occurring shortly after hatching of nauplii.

A recent investigation of Finamore and Clegg (1969) has shown that nucleic acid purines are drawn from separate cellular pools, in particular DNA adenine from diguanosine tetraphosphate. As a sequel to this work, Artemia nauplii were "pulse-labelled" in an attempt to ascertain when RNA and DNA syntheses are turned on and/or off. Analysis of isotope incorporation into the nucleic acids, the acid-soluble

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[1] The following abbreviations have been used throughout this thesis: RNA, ribonucleic acid; DNA, deoxyribonucleic acid; EDTA, ethylenediaminetetraacetic acid; MAK, methylated albumin on kieselguhr; DEAE, diethylaminoethyl; AMP, ADP, and ATP, adenosine 5'-mono-, di-, and triphosphates respectively; GMP, GDP, and GTP, guanosine 5'-mono-, di-, and triphosphates respectively; diguanosine tetraphosphate or Gp_4G, P^1, P^4-diguanosine 5'-tetraphosphate; UMP, uridine 5'-monophophosphate; UDP-X, carbohydrate derivative of uridine 5'-diphosphate; and Pi, orthophosphate.

nucleotide precursors, and total nucleic acid purine concentrations indicate that maximum synthetic activity of RNA is complete by 36 hours of development, whereas DNA synthesis is not complete until 54 hours. Also, five species of nucleic acids from Artemia have been separated on columns of methylated albumin on kieselguhr and their importance and/or function in Artemia development are discussed. These biochemical observations, as presented in this thesis, are in agreement with the cytological evidence for resumption of cell division and morphogenesis (Bellini, 1960; Nakanishi et. al., 1962, 1963) in Artemia.

It is hoped that this thesis will further elucidate the role of the nucleic acids during the development of Artemia.

EXPERIMENTAL PROCEDURE

Preparation of Artemia Embryos

All experiments were carried out on the brine shrimp, Artemia salina. Dried encysted gastrulae(cysts) of the Great Salt Lake variety were obtained from Sanders Brine Shrimp Company, Ogden, Utah, and stored at 5°C.

Prior to use the embryos were sterilized by immersion in 7 % antiformin solution for 15 minutes at 5°C as previously described (Nakanishi et. al., 1963). Following removal of floating cysts and debris by suction, the remaining cysts were collected and washed several times with cold distilled water on a fritted glass filter. This treatment removes the outer granular part of the shell without damaging the embryos, and renders the preparation free of cryptobiotic microorganisms and debris which always accompany commercially available Artemia cysts.

Incubation Procedures

Dormancy was terminated by incubating 2-gm portions of sterile gastrulae in large glass petri dishes (2 x 14 cm) containing 100 ml of sterile artifical sea water (see Appendix A) and maintained at 30°C with constant agitation to ensure an adequate supply of oxygen. Emergence of pre-naupliar larvæ begins in these populations after about 7-8 hours of incubation and approximately 50 % of these embryos produce swimming nauplii after 20 hours incubation. When desired, pre-naupliar larvæ were collected in the following way. The total population of 12 hour embryos was transferred to a 500-ml Erlenmeyer flask and rocked gently at a 45° angle. With this treatment the emerged embryos, usually still attached to the empty cysts, collect at the surface and can be

collected easily by suction. This procedure was repeated until the population contained at least 60 % pre-naupliar larvae. Nauplii were harvested at 19 or 30 hours by transferring the contents of the petri dishes into 500-ml separatory funnels and collecting the nauplii that gathered at the stopcock. This procedure was repeated until the nauplii were free of cysts and debris. Nauplii (about 50,000) were pipetted into large glass petri dishes (2 x 14 cm) and covered with 100 ml of artificial sea water fortified with 5 gm NaCl, 100,000 I.U. penicillin, and 10 mg streptomycin sulfate. Nauplii treated this way can be maintained aseptically and without feeding for about four days at 25°C.

Isotope Incorporation Studies

At the desired times, Pi^{32} and adenosine-H^{3}, either separately or in combination, were added to the petri dishes containing nauplii, and maintained for 6 additional hours at 25°C. The Pi^{32} (carrier-free orthophosphate) and adenosine-H^{3} (S.A. 500 mC/mmole) were employed at a final concentration of 10 µC/ml and 5 µC/ml of medium, respectively. After 6 hours incubation in the radioisotope(s) the nauplii were collected on a nylon screen and washed well with distilled water containing 100-fold excess of the unlabelled compound. The nauplii were quick frozen, and when all samples were collected, used either for isolation of acid-soluble nucleotides or nucleic acids as described below.

Extraction and Purification of RNA

The frozen nauplii preparations were homogenized in 0.05 M Tris-HCl containing 0.10 M KCl, 0.005 M $MgCl_2$, and 1 % sodium dodecyl sulfate, pH 7.5. Hereafter this buffered solution will be referred to as TKMS buffer. To the homogenate was added an equal volume of 90 % phenol

containing 0.10 M 8-hydroxyquinoline and the mixture agitated vigorously at 60°C for 1 hour. The aqueous phase was separated by centrifugation (12,000 g; 10 minutes) then re-extracted once with a half-volume of the phenol solution at 60°C for 30 minutes. The nucleic acids were precipitated from the aqueous phase by addition of 3 volumes of ethanol containing 0.10 M NaCl at 0°C. The alcohol precipitate was then dissolved in 0.05 M Tris-HCl containing 0.10 M KCl and 0.001 M EDTA, pH 7.5 (this buffered solution will be designated hereafter as TKE buffer), and shaken with an equal volume of the phenol solution at 0°C for 10 minutes. The nucleic acids were precipitated from the aqueous phase as previously described, dissolved in 0.05 M Tris-HCl, pH 7.5, and treated with pronase[2] (250 µg/ml) at 37°C for 45 minutes. The nucleic acids were collected by ethanol precipitation and centrifugation, dissolved in 0.05 M sodium phosphate, pH 6.7, and passed through a column of G-25 Sephadex (1.5 x 75 cm) equilibriated with the same buffer. This step removes residual phenol and separates the nucleic acids from the large amount of diguanosine tetraphosphate present in these embryos (Finamore and Warner, 1963). The nucleic acid fraction was then stored at -15°C until needed.

Separation of RNA Fractions by Methylated Albumin on Kieselguhr Column Chromatography

Columns of methylated albumin absorbed on kieselguhr (MAK) were prepared essentially according to Mandell and Hershey (1960, and see Appendix B). The Sephadex-treated preparation was adjusted to 0.2 M NaCl in 0.05 M sodium phosphate, pH 6.7, and approximately 50 O.D. units (260 mµ) were applied to the column. After washing with the starting

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[2] A mixture of proteases free of nuclease activity.

buffer, the column was developed with a linear gradient of NaCl in the phosphate buffer (see Appendix C). Elution of the nucleic acids was followed spectrophotometrically at 260 and 280 mμ. When radioactivity determinations were to be made, 0.5 ml was taken from each column fraction, added to 15 ml of scintillation fluid (see Appendix D), and analyzed in a Nuclear-Chicago scintillation system (Model 6850).

Preparation and Fractionation of Acid-soluble Fraction

In addition to the analysis of nucleic acids during post-hatch development of Artemia, nauplii from similar developmental stages were homogenized in ice-cold N $HClO_4$ and the acid-soluble material collected by centrifugation at 12,000 g for 10 minutes. The acid-soluble fraction was neutralized with Alamine (Warner and Finamore, 1967), then stored at -15°C until needed. In addition, the acid-insoluble fraction was neutralized with M CH_3COONH_4 and stored for analysis of nucleic acid purines as described below.

Isolation and Separation of Nucleic Acid Purines

To achieve isolation of total nucleic acid purines, the acid-insoluble precipitate remaining from each developmental stage analyzed for free nucleotides was delipidated with ethanol-ether (3:1) at 45°C then extracted with hot (90°C) 2 M NaCl according to Tyner et. al., (1953). The nucleic acids were recovered by ethanol precipitation of the soluble fraction and the RNA nucleotides obtained by hydrolysis in 0.2 M NaOH at 37°C for 12 hours. The DNA was recovered from the alkaline hydrolyzates as the acid-insoluble precipitate. The RNA and DNA fractions were adjusted to 2 N with respect to HCl and heated at 100°C for 1 hour. After cooling, the fractions were applied to separate

Dowex 50-H^+ columns (1 x 10 cm) and the purine bases and pyrimidine nucleotides separated from each other by elution with 2 N HCl (Cohn, 1955). The amount of each purine fraction was determined using the proper extinction coefficients (Sober, 1968) and all values are expressed for 100,000 nauplii.

RESULTS

Extraction of Nucleic Acids by the Three-Step Phenol Procedure

From several previous reports it has become apparent that RNA extractions from embryonic tissues using phenol is highly variable and often depends upon temperature, pH, and the amount of phenol employed (Wilt, 1964; Cherry and Chroboczek, 1966; Clegg and Golub, 1969). Although Cherry and Chroboczek (1966) state that hot phenol causes "widespread degradation" in peanut cotyledons, Landesman and Gross (1969) used hot phenol to "minimize aggregation and degradation" by ribonuclease while ensuring high extraction of nuclear RNA from X. laevis ovarian tissue. Clegg and Golub (1969) have used phenol at 0°C but were not concerned with quantitative extraction of RNA.

When 48 hour Artemia nauplii were extracted with the phenol procedure described above at 5, 37, and 60°C the data shown in Table 1 were obtained. Although extraction at 37°C yielded the most RNA, the RNA was highly degraded as evidenced by chromatography on MAK columns. This is shown in Figure 1. Comparison of the RNA profile of 5 and 60°C (Table 1) shows little difference, but 60°C provides 10 % more RNA and greater amounts of the 'heavy' RNA (hRNA) and was therefore used in subsequent extractions at various developmental stages.

Separation of Nucleic Acids by MAK Column Chromatography

Employment of MAK column chromatography for fractionation of nucleic acids has been used extensively. Several laboratories have demonstrated that at least five distinct species of nucleic acid may be separated using this fractionating system (Sueoka and Cheng, 1962; Ishihama et. al., 1962; Ewing and Cherry, 1967). Preliminary to

TABLE 1

Extraction of Nucleic Acids from *Artemia* Nauplii at Different Temperatures

Nucleic acid preparation and MAK chromatography were preformed as described under Experimental Procedure. The extraction temperatures employed are indicated. Nauplii 2 days after the onset of development (48 hour nauplii) were used for these determinations

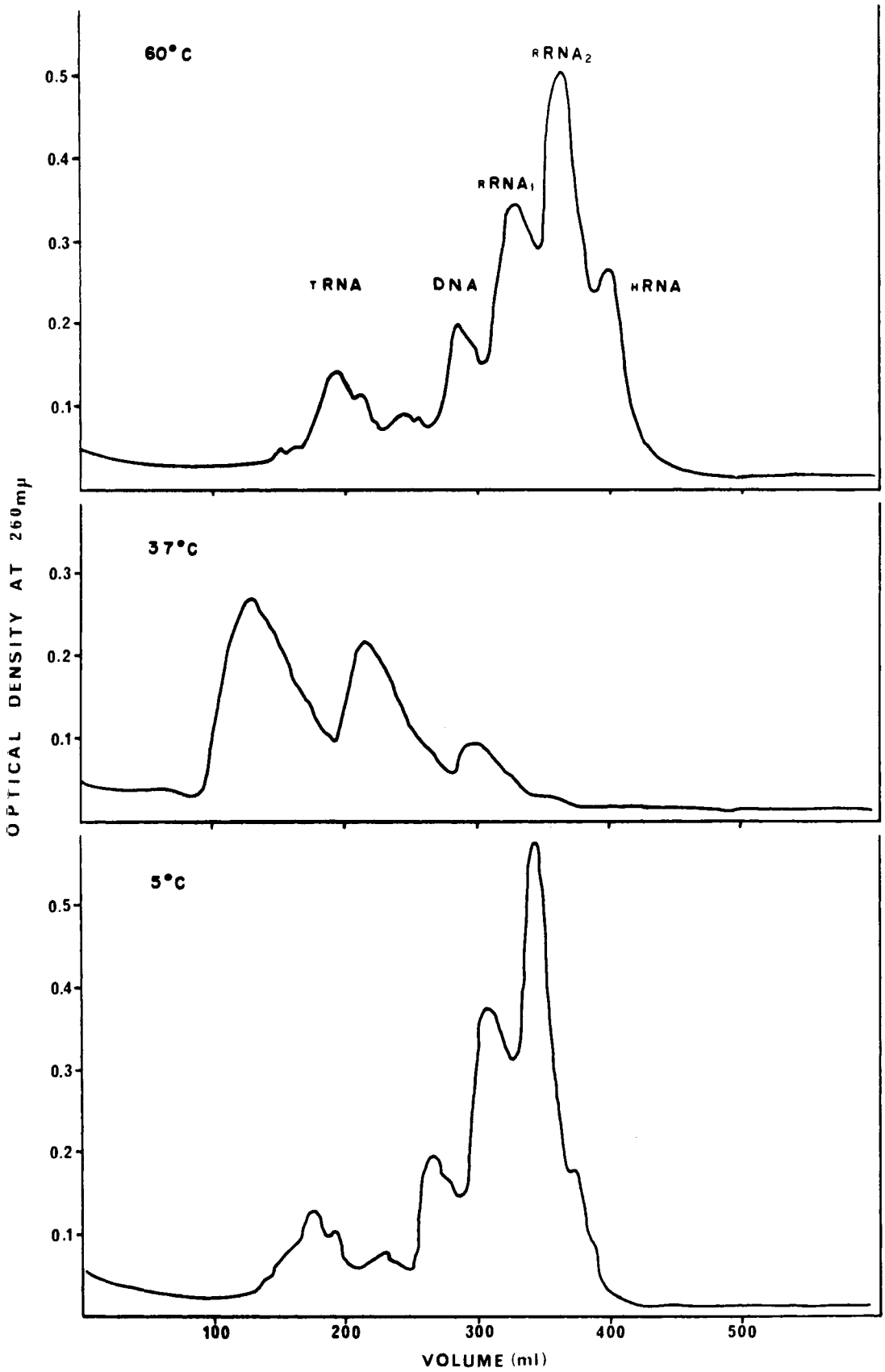

Fig. 1. Fractionation on MAK Columns of Artemia Nucleic Acids Extracted with Phenol at 5, 37, and 60°C.

FIGURE 1

Fractionation on MAK Columns of *Artemia* Nucleic Acids Extracted with Phenol at 5, 37, and 60°C

Nucleic acid extraction and MAK chromatography were preformed as described under Experimental Procedure. The extraction temperatures employed are indicated. Nauplii (48 hour) were used for all determinations. Nucleic acid samples of 50 O.D. (260 mμ) were placed on each MAK column and 4-ml fractions were collected at a rate of 2.2 ml/min.

TABLE 1

Extraction of Nucleic Acids from Artemia Nauplii at Different Temperatures.

TEMPERATURE (°C)	% OF TOTAL NUCLEIC ACID EXTRACTED					TOTAL NUCLEIC ACID EXTRACTED
	tRNA	DNA	$rRNA_1$	$rRNA_2$	hRNA	O.D.(260mμ)/gm Nauplii
5	12	14	29	37	8	29.9
37	NR[a]	NR	NR	NR	NR	38.2
60	13	12	26	38	11	33.3

[a] NR refers to no MAK chromatographic resolution.

analysis of total nucleic acids from Artemia embryos, it was decided to standardize the MAK columns and elution system using individual species of purified nucleic acids[3]. When purified tRNA, DNA, and rRNA from Artemia cysts are chromatographed separately on MAK columns, it is found that the rRNA preparation is resolvable on MAK columns into three major fractions. In this thesis these fractions have been designated, in order of elution, as $rRNA_1$, $rRNA_2$, and hRNA, and they elute at 0.86, 0.92, and 1.0 M NaCl, respectively. In addition, tRNA elutes at 0.58 M and DNA at 0.77 M NaCl. Although separation of the ribosomal RNA's is not always optimal, the NaCl concentration needed to elute the individual nucleic acid fractions is highly reproducible.

Estimation of Various Nucleic Acid Fractions from Artemia Embryos at Different Stages of Development

Using the phenol procedure at 60°C, the nucleic acids at several stages of Artemia development were prepared and fractionated on MAK columns as outlined (see Appendices B and C). The results of this experiment appear in Table 2. From these data, it is evident that only slight fluctuations occur in all nucleic acid fractions from the onset of emergence of pre-naupliar larvae through 108 hours of development. Included with these data is the analysis of RNA isolated from cyst ribosomes. It is apparent that the hRNA fraction constitutes a significant portion of the ribosomal preparation. The importance of this RNA moiety in Artemia development is of considerable interest and remains to be elucidated.

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[3] tRNA isolated by the three-step phenol procedure was supplied by A.H. Warner. DNA was isolated from Artemia cysts according to A.H. Warner (unpublished procedure). rRNA was isolated by the method of Keller and Zamecnek (1956).

TABLE 2

Distribution of Nucleic Acids in *Artemia* Embryos

Nucleic acid preparation and MAK chromatography were preformed as described under Experimental Procedure. The ribosome preparation was prepared according to Keller and Zamecnek (1956).

TABLE 2

Distribution of Nucleic Acids in *Artemia* Embryos.

TISSUE PREPARATION	% OF TOTAL NUCLEIC ACID EXTRACTED			
	tRNA	DNA	rRNA[a]	hRNA
12 Hour Prenauplius Larvae	17	7	61	15
26 Hour Nauplii	14	6	59	21
36 Hour Nauplii	14	5	63	15
60 Hour Nauplii	15	7	63	15
84 Hour Nauplii	14	7	62	17
108 Hour Nauplii	16	9	56	19
Ribosome Dessicated Cyst	6	-	54	40

[a] This fraction represents total rRNA ($rRNA_1$ + $rRNA_2$).

Incorporation of Pi^{32} and Adenosine-H^{3} into Nucleic Acids

At various times after the onset of emergence, Artemia larvae were given a mixture of Pi^{32} and adenosine-H^{3} for 6 hours and then the nucleic acids analyzed for isotope incorporation. The data appearing in Figure 2 and Table 3 were obtained. It is seen that maximum specific activities are attained during the 30 - 36 hour incubation period for all fractions and with both nucleic acid precursors. In addition, the specific activity of DNA is about double that of the RNA's when Pi^{32} is used, whereas it is only half that of the RNA when adenosine-H^{3} incorporation is determined. Of the four RNA species fractionated on MAK columns, tRNA and hRNA show the greatest incorporation when Pi^{32} is used and hRNA is highest when adenosine-H^{3} incorporation is analyzed. Beyond 36 hours development the rate of Pi^{32} and adenosine-H^{3} incorporation into the nucleic acids declines markedly.

Incorporation of Pi^{32} into Acid-soluble Nucleotides

In order to more fully understand nucleic acid metabolism in Artemia, metabolism of the free nucleotide pool was investigated using Pi^{32}. When nauplii were given Pi^{32} (10 μC/ml) for 6 hours, extensive incorporation into the acid-soluble nucleic acid precursors occurs at all stages except 20 - 26 hours. These data are shown in Table 4. Whereas incorporation of Pi^{32} into nucleic acid decreases several fold between the 36 and 60 hour stages, incorporation of this isotope into the nucleic acid precursors increases at least 2-fold during this same period of development. Also, the ∝-phosphates of GTP and ATP account for approximately 70 to 85 % of all radioactivity in these nucleic acid precursors.

FIGURE 2

Fractionation on MAK Columns of Artemia Nucleic Acids Incubated 30 - 36 Hours with Pi^{32}

Incubation, isotope incorporation, nucleic acid extraction, and MAK chromatography were preformed as described under Experimental Procedure. Incorporation was determined by counting aliquants of each column fraction. No quench corrections were made as the degree of quenching changed little throughout the elution. Nucleic acid samples of 50 O.D. (260 mμ) were placed on each MAK column and 4-ml fractions were collected at a rate of 2.2 ml/min. M refers to NaCl concentration in Moles.

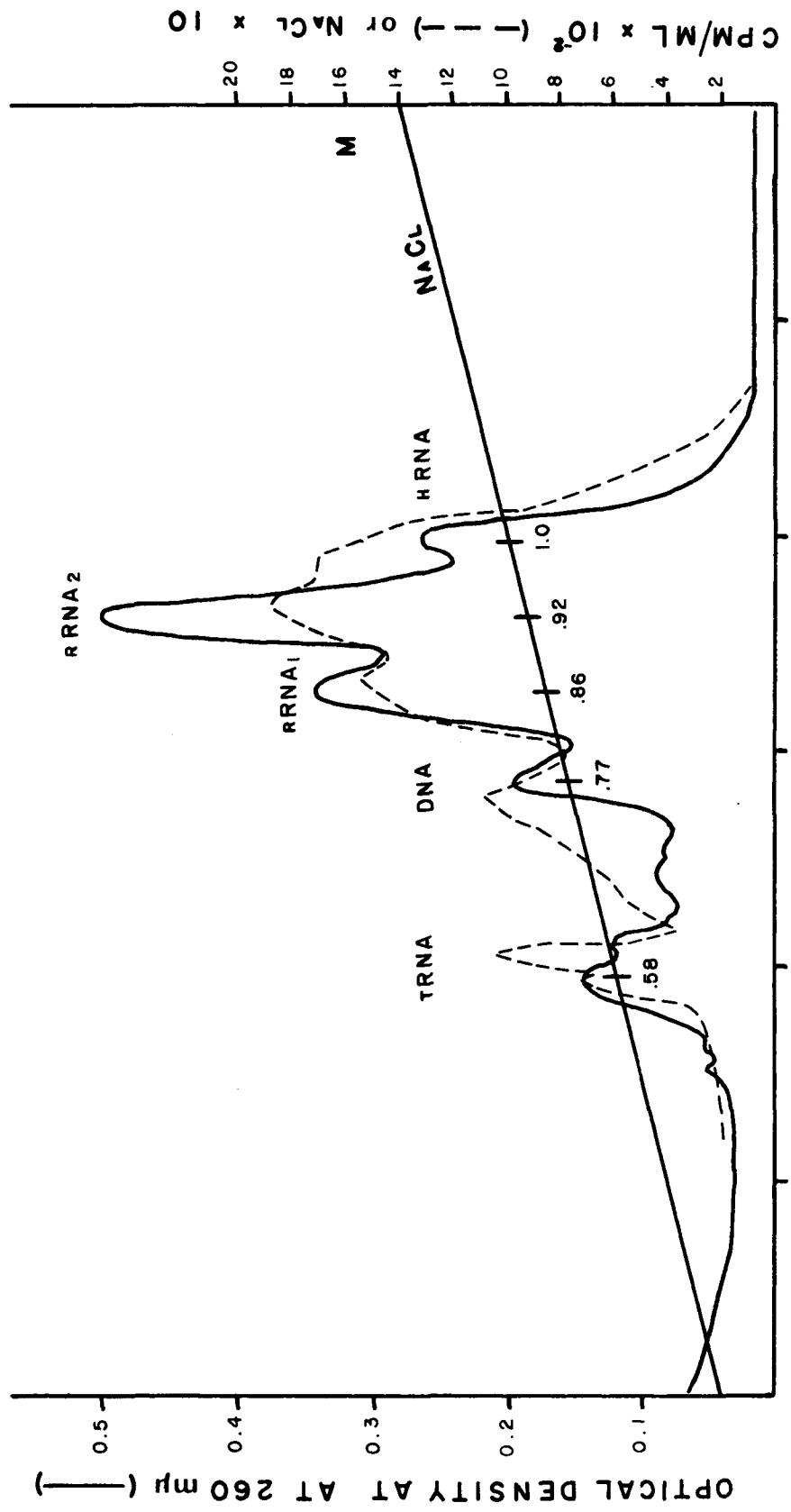

Fig. 2. Fractionation on MAK Column of Artemia Nucleic Acids Incubated 30 - 36 Hours with $P^{32}i$.

TABLE 3

Incorporation of Pi^{32} and Adenosine-H^{3} into Nucleic Acids of *Artemia* at Several Stages of Development

Incubation and isotope incorporation techniques as well as nucleic acid extraction and MAK chromatography were preformed as described under Experimental Procedure. The specific activities were determined by counting aliquants of each column fraction and the values for each UV absorbing peak averaged. No quench corrections were made, as the degree of quenching changed little throughout the elution. Each value represents at least two determinations.

TABLE 3

Incorporation of Pi^{32} and Adenosine - H^3 into Nucleic Acids of *Artemia* at Several Stages of Development

Specific Activity cpm/O.D.(260mμ)

PERIOD INCUBATION (Hours)	ISOTOPE	tRNA	DNA	$rRNA_1$	$rRNA_2$	hRNA
20 - 26	Pi^{32}	1947	2084	1120	1148	1302
30 - 36		15856	25452	9698	11935	15401
54 - 60		2042	2258	1678	1362	1699
76 - 84		1066	1027	803	938	1153
102 -108		348	291	237	204	278
20 - 26	Adenosine-H^3	82	116	147	154	182
30 - 36		1172	619	850	1134	1629
54 - 60		53	112	357	171	161
76 - 84		41	19	54	101	33
102 -108		2	4	13	22	9

TABLE 4

"Pulse-Labelling" of the Acid-Soluble Nucleotides in *Artemia* with Pi^{32} at Various Developmental Stages

The acid-soluble fraction was prepared and chromatographed on columns of DEAE-cellulose as described under Experimental Procedure. Aliquants from each column fraction were counted and the average specific activities under each UV-absorbing peak determined. No quench corrections were made as the degree of quenching changed little throughout the elution.

TABLE 4

"Pulse-Labelling" of the Acid-Soluble Nucleotides in *Artemia* with Pi^{32} at Various Developmental Stages

Specific Activity cpm/O.D.(260mμ)

DEVELOPMENTAL STAGE (Hours)	AMP[a]	GMP	ADP	ATP[b]	GTP	GP_4G
20 - 26	25,618	12,602	25,969	30,688	49,122	839
30 - 36	262,988	84,818	253,847	221,243	32,384	13,174
54 - 60	623,203	271,473	497,861	563,272	188,412	14,468
78 - 84	190,396	187,503	217,333	278,816	111,267	14,884

[a] Includes small quantities of UMP and UDP-x.

[b] Includes 5 - 10 % GDP.

Quantitative Changes in Nucleic Acid Purines in Artemia Nauplii

The specific activity data in Tables 3 and 4 indicate that most of the nucleic acid synthesis is terminated by 60 hours development. When the purine content of both RNA and DNA was determined at similar stages of development, it became apparent that RNA synthesis is completed by 36 hours, whereas DNA synthesis is completed by 60 hours. These data are shown in Table 5. It is noteworthy that the RNA content decreases markedly between 60 and 84 hours, whereas the DNA content per embryo remains constant.

TABLE 5

Changes in Purine Content of *Artemia* Nucleic Acids during Development

The acid-insoluble fractions were prepared for chromatography on Dowex-50-H^+ as described under Experimental Procedure. Micromoles were calculated from the optical density at 260 mμ and the appropriate extinction coefficients.

TABLE 5

Changes in Purine Content of *Artemia* Nucleic Acids during Development.

HOURS OF DEVELOPMENT	R N A µmoles/100,000 Nauplii			D N A µmoles/100,000 Nauplii		
	ADENINE	GUANINE	TOTAL	ADENINE	GUANINE	TOTAL
26	2.11	2.79	4.90	0.85	0.65	1.50
36	2.59	3.17	5.76	1.31	0.88	2.19
60	2.60	3.08	5.68	1.69	1.07	2.76
84	1.04	1.69	2.73	1.69	1.09	2.78

DISCUSSION

Before a study of nucleic acid metabolism in developing brine shrimp could be undertaken it was necessary to establish a reliable extraction and purification procedure which would yield undegraded nucleic acids in amounts proportional to their in vivo concentrations. After several studies employing extraction with cold buffered saline solutions, a hot phenol extraction procedure was tested and subsequently used because it provided superior profiles on MAK columns.

When the nucleic acids of Artemia embryos and nauplii are extracted with hot (60°C) phenol, chromatography on MAK columns resolves the extract into at least five distinct species. Their elution characteristics correspond satisfactorily to chromatograms of purified Artemia nucleic acids and are similar to the nucleic acids isolated from peanut cotyledons by Ewing and Cherry (1967). Extraction with phenol at lower temperatures (0 and 37°C) either lowers the yield or permits extensive degradation to occur. In addition, resolution of the hRNA fraction is always best when the extraction is carried out at 60°C. This is apparent in Figure 1. According to several investigators (Mandell and Hershey, 1960; Sueoka and Cheng, 1962), fractionation of nucleic acids on MAK columns is dependent on molecular size, hydrogen bonding, and guanine-cytosine composition. In this respect, Artemia nucleic acids lend themselves to further study using MAK chromatographic techniques.

When the chromatographic profile shown in Figure 2 is examined, it is clear that the tRNA fraction is bimodal with respect to both UV-absorption and Pi^{32} incorporation. Whether this pattern is due to

fractionation of tRNA (Sueoka and Yamane, 1962) or to the presence of a 5S ribosomal-type RNA species (Perry, 1962) has yet to be ascertained.

Analysis of the RNA extracted from both ribosomal and nuclear preparations indicates that the hRNA fraction is an important component of the ribosomal fraction and not of the nuclear fraction. That this fraction contributes heavily to the total rRNA fraction may indicate this fraction to be a ribosomal precursor species as proposed by Perry (1964, 1965), Girard (1965), and Gall (1966). However, precursors of rRNA are usually found in the nuclear fraction and not associated with ribosomes. In peanut cotyledons, Ewing and Cherry (1967) designate the fraction eluting after $rRNA_2$ as messenger RNA (mRNA) and have demonstrated its template activity in an in vitro amino acid incorporating system.(Jachymczyk and Cherry, 1968). Aggregation of rRNA has been reported to produce artifacts on MAK columns but the high specific activity of the hRNA fraction in Artemia in relation to the rRNA fractions does not support this contention. The exact function of hRNA in Artemia remains to be elucidated.

Finamore and Clegg (1969) in a recent report indicated that nucleic acid synthesis in Artemia, and in particular DNA synthesis, occurs in a novel fashion. In these experiments, however, the radiolabelled precursors of nucleic acids were introduced for 4 hours shortly after hatching, then chased for as long as 44 additional hours with unlabelled precursors. As a sequel to their work we decided to "pulse-label" Artemia nauplii in an attempt to ascertain the time at which RNA and DNA syntheses were turned on and off. The data in this thesis show that although little synthesis is apparent immediately after

hatching (20 - 26 hour period), the results are misleading and merely reflect the nucleotide pool specific activity in Artemia (see Tables 2 and 3). The nucleotide pool is very large in newly hatched Artemia embryos (Warner and Finamore, 1967) and may regulate or control entry of exogenously added precursors. Whether the nucleotide pool is rate limiting or a permeability barrier still exists remains to be studied. During the 30 - 36 hour period of development, a 10-fold increase in specific activity of the nucleic acids occurs concomitant with an 8-fold increase in the specific activity of the nucleotide fraction. This is a direct indication of nucleic acid synthesis throughout this period of development. Between 54 - 60 hours development there is a precipitous decrease in specific activity of all nucleic acid fractions yet the nucleotide pool specific activity increases almost 3-fold over the 30 - 36 hour period. These data indicate that both RNA and DNA synthesis are "turned off" by 54 hours. Beyond this time of development the embryos maintain only minimal nucleic acid synthetic activities whose rates appear to be similar for all nucleic acid fractions.

It is significant to note that the high specific activity of P^{32}-labelled DNA at 30 - 36 hours coincides with the resumption of cell division in Artemia. This datum, however, is not corroborated by adenosine-H^3 incorporation during this period of development. This observation lends support to the postulation of Finamore and Clegg (1969) that the source of DNA adenine in Artemia is primarily from diguanosine tetraphosphate and that RNA adenine comes from a separate cellular pool, probably ATP.

Examination of the count profile in Figure 2 shows that

considerable radioactivity elutes immediately in front of DNA and also contaminates the DNA fraction. According to Spiegleman (1963) and Chroboczek and Cherry (1966), DNA-like RNA (messenger-type RNA) elutes from MAK columns in this position but due to insufficient material this fraction has not been analyzed in Artemia.

Finally, the purine content of both nucleic acids was determined in an attempt to corroborate the specific activity data. Examination of Table 5 indicates that RNA synthesis is complete by 36 hours, whereas DNA synthesis continues until 60 hours. DNA synthesis must be completed by at least 54 hours when one considers the specific activity data in Table 3. Apparently the period of most intense nucleic acid synthesis is over by 54 hours development. These observations are in general agreement with those of Bellini (1960) regarding nucleic acid content during development of Artemia. Total RNA adenine and guanine levels which drop significantly after 60 hours development are at variance with determinations of Clegg et. al., (1967) and must be investigated further.

During the period of post hatch development studied in Artemia, resumption of cell division and the onset of morphogenesis appear to be closely linked to enhanced RNA and DNA metabolism. It is hoped that this thesis provides additional information on the biochemical aspect of morphogenesis as related to development in Artemia salina.

SUMMARY

Analysis of the nucleic acid fraction during early development of the brine shrimp, Artemia salina, using a 3-step hot (60°C) phenol extraction and methylated albumin on kieselguhr column chromatography (MAK) resolves the fraction into at least five distinct species: tRNA, DNA, $rRNA_1$, $rRNA_2$, hRNA. Extraction with phenol at lower temperatures (0 and 37°C) either lowers the yield or permits extensive degradation to occur.

The last fraction to elute from the MAK column (hRNA) appears to be an important component of the ribosomal fraction and not of the nuclear fraction. This fraction, based on elution data only, may be similar to messenger RNA species observed in other developmental systems.

Incorporation of Pi^{32} and adenosine-H^3 into nucleic acids and Pi^{32} into free nucleotides demonstrates that maximum RNA synthetic activities are completed by 36 hours development, whereas DNA synthesis continues for an additional 18-20 hours.

Evidence is also presented which indicates that RNA and DNA synthetic mechanisms utilize different nucleotide pools.

APPENDIX A

The artificial sea water used contained the following salts (micromoles/liter): NaCl, 422; KCl, 9.4; $MgSO_4$, 25.4; $MgCl_2$, 22.7; $CaCl_2$, 1.4; $NaHCO_3$, 0.5. Sea water of this composition closely resembles that of the Great Salt Lakes region (Hutchinson, 1957).

APPENDIX B

Methylated albumin on kieselguhr (MAK) columns were prepared essentially according to Mandell and Hershey (1960). The kieselguhr used was of the grade available as "Celite" (Johns-Manville Products Corp.) and "Fraction V" bovine serum albumin powder as the source of albumin (Nutritional Biochemicals Corp.). The esterfied albumin and the washed, protein coated kieselguhr (used for the second layer in the column) were prepared exactly as reported by Mandell and Hershey (1960).

The column used (3.5 x 10 cm) for analysis of nucleic acids contained five layers and was prepared as follows. Suspensions of kieselguhr in three beakers (8 gm in 40 ml of 0.1 M NaCl; 6 gm in 40 ml of 0.4 M NaCl; 1 gm in 10 ml of 0.4 M NaCl) were brought to a boil then cooled. To the first beaker, 2 ml of 1 % esterfied albumin was added with stirring and then 15 ml additional salt solution added. With this suspension the bottom layer of the column was formed on 1 gm of cellulose powder, and the excess protein wiped off the column using Kleenex saturated with 0.10 M saline. The second layer was formed with the contents of the second beaker to which had been added 10 ml of protein-coated kieselguhr with brief, gentle stirring. Once again the excess protein was wiped clean of the column. Finally, the contents of the third beaker was added and overlaid with 1 gm of [illegible] cellulose powder. All packing operations were preformed under atmospheric pressure. The column was washed with 200 ml of 0.2 M NaCl containing 0.05 M sodium phosphate, pH 6.7. The bed volume of the finished column is about 55 ml, and the liquid displacement volume is about 53 ml.

APPENDIX C

The linear gradient system employed for MAK column elution consisted of two 1000-ml Pyrex aspirator jugs connected across the bottom spigots by a 20 cm length of Tygon tubing.

A 600 ml linear gradient of NaCl, 0.2 M to 1.4 M, containing 0.05 M sodium phosphate, pH 6.7, was employed under 110 cm hydrostatic pressure. The elution was run at room temperature and the flow rate was 100 ml/hr. Under these conditions no detectable chromatographic aberrations were apparent. The columns could be successfully reused but in practice they were discarded after each run.

APPENDIX D

The scintillation fluid used for counting aqueous samples was composed of toluene, ethylene glycol monomethyl ether, PPO, and POPOP in the following composition: 1000 ml, 700 ml, 4 g, and 50 mg, respectively.

APPENDIX E

The Pi^{32} (carrier-free orthophosphate) was purchased from Atomic Energy of Canada Ltd., and the adenosine-H^3 (S.A. 500 mC/mmole) from Amersham/searle Corp. Penicillin was purchased from Squibb and Sons Ltd., streptomycin sulfate from Nutritional Biochemicals Corp., pronase from Calbiochem, and sodium dodecyl sulfate from Fisher Scientific Co., Ltd.

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