

Biochemistry of Artemia Development

Joseph C. Bagshaw
Alden H. Warner



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Cover photos from top: pre-nauplius larva fully emerged from its shell, but still inside the hatching membrane; newly hatched nauplius larva; second instar nauplius larva, about 24 hours after hatching; same.

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by
Joseph C. Bagshaw
and
Alden H. Warner

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Dedicated to Frank J. Vinamore, our teacher and
colleague, for his encouragement and guidance during
our formative years as "Artemiologists"

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INTRODUCTION

In recent years the brine shrimp, *Artemia salina*, has become increasingly important as a useful model system for biochemical studies of development. This volume contains the proceedings of a symposium on *Artemia* biochemistry - the first of its kind - held July 7, 1979 in Toronto. The speakers came from fourteen different laboratories in seven countries to present their work and to exchange ideas and information. All the participants were, of course, familiar with the developmental biology of *Artemia*; however, for the benefit of readers who may be less familiar, I include here a brief summary of the salient features of *Artemia* development.

Artemia salina is a small anostracan crustacean found in hypersaline bodies of water throughout the world. Fertilization occurs internally, and embryos develop in the ovisac of the female up to the early gastrula stage. At this point two pathways are possible. The embryos may continue to develop inside the ovisac, giving rise to free-swimming nauplius larvae, which are released. Alternatively, the embryos may enter a dormant state (cryptobiosis) and become encysted in a hard, chitinous shell. The dormant cysts are expelled from the ovisac and are dessicated first osmotically, then by wind and sun after being washed ashore. The encysted embryos, commonly but erroneously called brine shrimp "eggs", are collected and sold to aquarium suppliers and hatcheries; *Artemia* larvae are the best known food source for the fry of many commercially valuable species. Availability at any time in large quantity is only one of the practical advantages to the biochemist. The dormant embryos are completely ametabolic, yet they will resume development immediately upon rehydration at a suitable temperature. About 16 hours after immersion of cysts in sea water at 30°, nauplius larvae hatch out. The larvae will continue to develop synchronously for two days without feeding, and even longer if fed. Thus it is easy to obtain large quantities of embryos or larvae at any stage within this period. Many interesting biochemical phenomena can be observed and studied during this period of embryonic and larval development in *Artemia* - and therein lies the subject of this book.

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CHARACTERIZATION OF POLYPEPTIDE CHAIN INITIATION FACTORS DURING ARTEMIA SALINA DEVELOPMENT

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ABSTRACT

Dormant and developing embryos of Artemia salina contain equivalent amounts of eIF-2, the eukaryotic chain initiation factor which forms a ternary complex with GTP and Met-tRNA_f. After purification, preparations of eIF-2 from both types of embryos have similar specific activities and are immunologically identical, and each stimulates amino acid incorporation in a cell-free system of protein synthesis.

The binding of [³⁵S]Met-tRNA_f to 40S ribosomal subunits is catalyzed by eIF-2 isolated from dormant or developing embryos, and is dependent upon GTP and AUG. Another protein, cofactor 1, present in the 0.5 M NH₄Cl ribosomal wash, stimulates 2-3-fold the binding of [³⁵S]Met-tRNA_f to 40S ribosomal subunits, as well as the formation of the eIF-2 ternary complex. This factor does not exhibit GTP-dependent binding of [³⁵S]Met-tRNA_f.

The eukaryotic chain initiation factor eIF-3, consisting of numerous polypeptides, was isolated by chromatography on heparin-Sepharose 4B, DEAE-cellulose, hydroxylapatite and Ultrogel AcA-34, and by centrifugation on sucrose density gradients. As assayed by its ability to stimulate [³⁵S]Met-tRNA_f and (¹²⁵I)-labeled mRNA binding to 40S ribosomal subunits in the presence of eIF-2, the concentration of this factor is at least 5-fold greater in developing than in dormant embryos. Experiments are in progress to determine the significance of this difference in eIF-3 activity.

INTRODUCTION

During embryogenesis of A. salina, gastrulae may develop to adults, or may encyst, enter a dormant state and be released into the environment where they may become dessicated (1-3). Cell-free extracts from dormant embryos contain active mRNA (4-7), large quantities of 80S ribosomes (8,9), elongation factors (10,11), termination factors (12)

and aminoacyl-tRNA synthetases (13); however, they are unable to translate natural mRNA (14). The absence of protein synthesis in cell-free extracts has been attributed partially to a deficiency of chain initiation factors (15,16). The GTP-dependent Met-tRNA_f binding protein, initiation factor eIF-2, was reported to increase over 20-fold following resumption of embryo development (16). According to those results, embryo development subsequent to encystment would depend upon the replacement of depleted or inactive initiation factors. Furthermore, the absence or inactivation of chain initiation factors in cell-free extracts of dormant embryos, may reflect intracellular conditions prior to encystment under which initiation factor deficiencies may trigger cessation of protein synthesis and eventual cyst formation.

To test some of these ideas, we began to purify and characterize chain initiation factors from dormant and developing embryos of A. salina. We have shown that eIF-2 is present in equivalent amounts at all stages of embryo development. In addition, a protein which stimulates eIF-2 activity was found in the 0.5 M NH₄Cl ribosomal washes (17). Embryo extracts were assayed for eIF-3, the eukaryotic chain initiation factor which is required for translation of natural mRNA and facilitates the binding of the eIF-2 ternary complex to 40S ribosomes (18-21). The quantity of eIF-3 in dormant embryos was only 20% of the amount in developing embryos. The results presented here indicate that eIF-3 and not eIF-2, as previously reported (15,16), may limit protein synthesis and resumption of embryo development subsequent to cryptobiosis.

MATERIALS AND METHODS

Preparations

Aminoacyl-tRNA - [³⁵S]Met-tRNA_f was prepared by charging unfractionated yeast tRNA with [³⁵S]methionine in the presence of E. coli synthetase (22,23).

(¹²⁵I)-Labeled mRNA - Iodinated poly A-rich hemoglobin mRNA was prepared by the procedure of Getz et al. (24). The specific activity of the (¹²⁵I)-labeled mRNA was 12.3 x 10⁶ cpm/A₂₆₀ unit when measured in a toluene-based scintillation fluid in a Beckman LS-9000 counter.

Antisera to eIF-2 - Antisera to eIF-2 from developing embryos were obtained from four-month old mice which had been injected intramuscularly with the phosphocellulose fraction of eIF-2 (17).

Embryo development - Dormant embryos of *Artemia salina* were sterilized in Clorox diluted with an equal volume of water. Sterilized embryos were washed 6 times with water and collected by suction. For development, 500 g wet weight of sterilized embryos were suspended in 5 l of artificial sea water, and incubated 11 h at 30° with vigorous aeration (25). For the preparation of chain initiation factors from dormant embryos, cysts were not sterilized, but hydrated for 3 h at 4° in 0.5 M NaCl.

Preparation of ribosomes and 40S ribosomal subunits - Hydrated embryos were ground in a motorized mortar and pestle (25). The ground embryos were suspended in buffer A (KCl, 5 mM; magnesium acetate, 10 mM; 2-mercaptoethanol, 10 mM; Tris·Cl, pH 7.4, 50 mM), stirred for 15 min and centrifuged at 20,000 x g for 20 min. The supernatant was filtered through cheese cloth and glass wool, centrifuged at 37,000 x g for 30 min and the resulting supernatant was centrifuged at 150,000 x g for 2 h. The ribosomal pellets thus obtained were resuspended overnight in buffer B (NH₄Cl, 500 mM; magnesium acetate, 5 mM; dithiothreitol, 1 mM; Tris·Cl, pH 7.4, 50 mM), and this suspension was then centrifuged at 150,000 x g for 2 h. 40S ribosomal subunits were prepared from the pellets and the high salt wash was stored at -70° for the preparation of eIF-2, eIF-3 and cofactor 1 (17,25).

Assays

Ternary complex formation with eIF-2, GTP, and [³⁵S]Met-tRNA_f, 40S ribosomal binding of [³⁵S]Met-tRNA_f, and translation of natural mRNA were performed as previously described (17). eIF-3 was assayed by its ability to stimulate eIF-2-dependent binding of [³⁵S]Met-tRNA_f to 40S ribosomal subunits in the presence of AUG or (¹²⁵I)-labeled mRNA.

Isolation of Chain Initiation Factors

Purification of eIF-2 and cofactor 1. These proteins were purified from the 0.5 M NH₄Cl ribosomal wash of *A. salina*. eIF-2 and cofactor 1 activities were found in the 35-60% (NH₄)₂SO₄ fraction of the ribosomal wash. After dialysis, this fraction was chromatographed on heparin-Sepharose 4B and DEAE-cellulose. Cofactor 1 was not retained on DEAE-cellulose, whereas eIF-2 was eluted with a linear gradient from 50-350 mM KCl. eIF-2 was further purified by chromatography on hydroxylapatite and phosphocellulose (17).

Purification of eIF-3. This factor was found in the

0-40% (NH₄)₂SO₄ fraction of the 0.5 M NH₄Cl wash of *A. salina* ribosomes. This fraction, after dialysis against buffer C (Tris·Cl, pH 7.4, 50 mM; KCl, 50 mM; magnesium acetate 0.2 mM; 2-mercaptoethanol, 10 mM; and glycerol, 10%) was adsorbed and eluted stepwise from heparin-Sepharose 4B with buffer C containing 0.5 M KCl. Further purification was achieved by chromatography on DEAE-cellulose in buffer C. eIF-3 was eluted with a linear gradient from 50-400 mM KCl. The active fractions, eluting between 170 and 300 mM KCl, were dialyzed, adsorbed and eluted stepwise from hydroxylapatite with increasing concentrations of phosphate. The active fractions of eIF-3, which eluted with 500 mM potassium phosphate, were concentrated by addition of ammonium sulfate, dialyzed, and purified further by filtration on Ultrogel AcA-34 and by sucrose density gradient centrifugation.

RESULTS

The total [³⁵S]Met-tRNA_f bound by the ribosomal salt washes and the post-ribosomal supernatants from dormant and developing embryos was identical (Table 1). Variations in the length of embryo hydration and development did not alter the specific activity of eIF-2 as measured by ternary complex formation (Table 2).

Table 1. eIF-2 activity in *A. salina* embryos

Embryo description	[³⁵ S]Met-tRNA _f bound per 100 g wet weight of embryos		
	Ribosomal salt wash ^a	Post-ribosomal supernatant ^b	Total
	pmol	pmol	pmol
Dormant	61	55	116
Developing (11 h)	71	39	110

^a35-60% (NH₄)₂SO₄ fractions of NH₄Cl washes of ribosomes were used.

^bPost-ribosomal supernatants were chromatographed on heparin-Sepharose 4B.

As previously observed (26,27), eIF-2 activity in $(\text{NH}_4)_2\text{SO}_4$ fractions was found to adsorb to heparin-Sepharose 4B. The elution pattern of eIF-2 from DEAE-cellulose is shown in Fig. 1. The column was initially washed with buffer containing 50 mM KCl and the fraction not adsorbed to DEAE-cellulose contained a factor, termed here cofactor 1, which stimulated 2-3-fold ternary complex formation with eIF-2. Therefore, in this and subsequent purification steps, 40 μg of cofactor 1 were added in the assays for eIF-2 activity.

Table 2. eIF-2 activity in dormant and developing embryos of *A. salina*^a

Embryo Description	[³⁵ S]Met-tRNA bound
	pmol/mg protein
Dormant (1 h hydration)	3.0
Dormant (3 h hydration)	4.0
Dormant (24 h hydration)	4.2
Developing (11 h)	3.5
Emerged (nauplii)	3.1

^aThe specific activity of the eIF-2 fractions in formation of a ternary complex was determined with the 35-60% $(\text{NH}_4)_2\text{SO}_4$ fractions of ribosomal washes.

At each stage of purification, the specific activity of eIF-2 from dormant and developing embryos was similar (Tables 3,4). A 123-150-fold purification of eIF-2, with a final specific activity of approximately 300 units/mg of protein and an overall yield of 5-7% was obtained. As shown by SDS gel electrophoresis (Fig. 2), phosphocellulose purified eIF-2 consisted of 3 polypeptides with molecular weights of 52,000, 38,000 and 36,000. Based on densitometer tracings of the gels, eIF-2 from either dormant or developing embryos was about 70% pure with the polypeptides present in a ratio of 1.00:1.24:1.20.

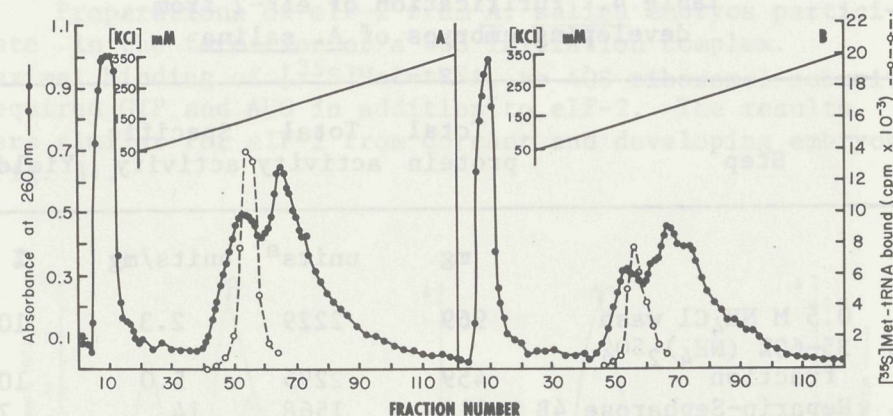


Fig. 1. Elution of eIF-2 from DEAE-cellulose. Chromatography of (A) 127 mg of step 3 eIF-2 from 5064 g wet weight of dormant embryos, and (B) 112 mg of step 3 eIF-2 from 3213 g wet weight of developing embryos was as described in Materials and Methods. Aliquots of each fraction (20 μl) were assayed for GTP-dependent [³⁵S]Met-tRNA_f binding. [³⁵S]Met-tRNA_f binding, ○-----○; absorbance at 280 nm, ●-----●. From MacRae *et al.* (17).

Table 3. Purification of eIF-2 from dormant embryos of *A. salina*

Step	Total protein mg	Total activity units ^a	Specific activity units/mg	Yield %
1. 0.5 M NH_4Cl wash	1649	3298	2.0	100
2. 35-60% $(\text{NH}_4)_2\text{SO}_4$ fraction	671	3489	5.2	106
3. Heparin-Sepharose 4B	127	2286	18	69
4. DEAE-cellulose	15.7	1036	66	31
5. Hydroxylapatite	4.9	495	101	15
6. Phosphocellulose	0.8	240	300	7

^aOne unit = 1 pmol of [³⁵S]Met-tRNA_f bound in a ternary complex under standard assay conditions (10 min, 30°). From MacRae *et al.* (17)

Table 4. Purification of eIF-2 from developing embryos of *A. salina*

Step	Total protein mg	Total activity units ^a	Specific activity units/mg	Yield %
1. 0.5 M NH ₄ Cl wash	969	2229	2.3	100
2. 35-60% (NH ₄) ₂ SO ₄ fraction	459	2295	5.0	103
3. Heparin-Sepharose 4B	112	1568	14	70
4. DEAE-cellulose	10.2	459	45	21
5. Hydroxylapatite	1.9	266	140	12
6. Phosphocellulose	0.4	113	283	5

^aOne unit = 1 pmol of [³⁵S]Met-tRNA_f bound in a ternary complex under standard assay conditions (10 min, 30°). From MacRae *et al.* (17).

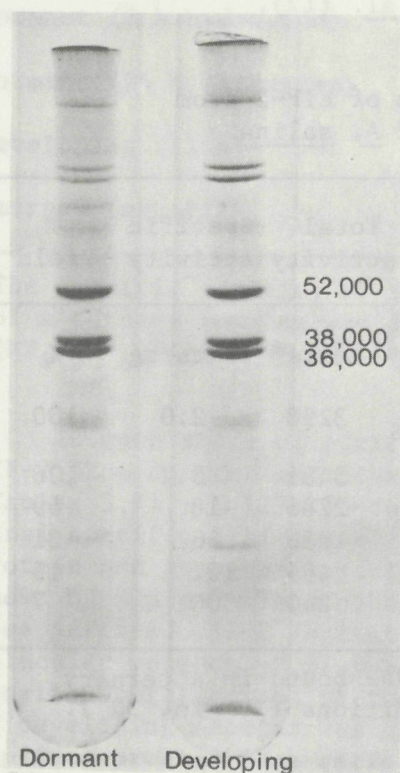


Fig. 2. SDS-polyacrylamide gel electrophoresis of the phosphocellulose fraction of eIF-2. From MacRae *et al.* (17).

Preparations of eIF-2 from *A. salina* embryos participate in the formation of a 40S initiation complex. Maximal binding of [³⁵S]Met-tRNA_f to 40S ribosomal subunits required GTP and AUG in addition to eIF-2. The results were similar for eIF-2 from dormant and developing embryos (Fig. 3).

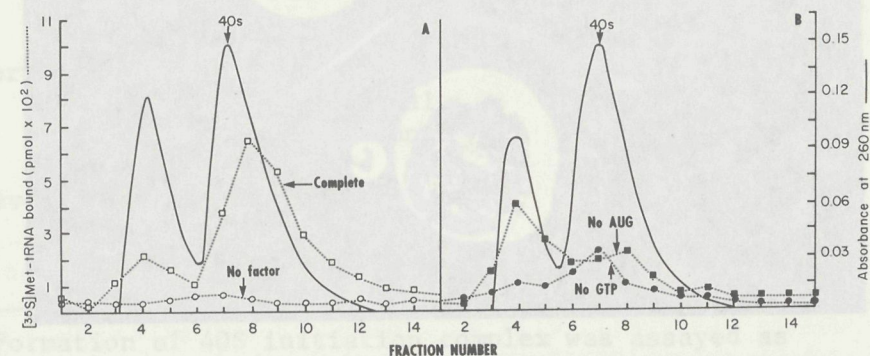


Fig. 3. 40S initiation complex formation with eIF-2 from dormant embryos. Binding of [³⁵S]Met-tRNA_f to 40S ribosomal subunits with 5.7 µg of step 6 eIF-2 from dormant embryos. (A) Complete reaction mixture, □.....□; no factor added, ○.....○; 260 nm absorbance —. (B) No AUG added, ■.....■; no GTP added, ●.....●; 260 nm absorbance —.

Assay and centrifugation was as described in Materials and Methods, and sedimentation was from left to right. From MacRae *et al.* (17).

Antibodies prepared against phosphocellulose-purified eIF-2 from developing embryos cross-reacted with eIF-2 from dormant embryos in Ouchterlony preparations (Fig. 4). Furthermore, this antiserum inhibited the binding of [³⁵S]Met-tRNA_f to 40S subunits with eIF-2 from either dormant or developing embryos. The results are given in Table 5. The observed inhibition in the presence of immune and normal serum was 90% and 7%, respectively.

Purified eIF-2 stimulated protein synthesis when it was tested in a cell-free protein synthesizing system containing limiting amounts of initiation factors from rabbit reticulocytes (Table 6). The stimulation with 3.8 µg of eIF-2 from dormant and developing embryos was 72 and 108%, respectively.

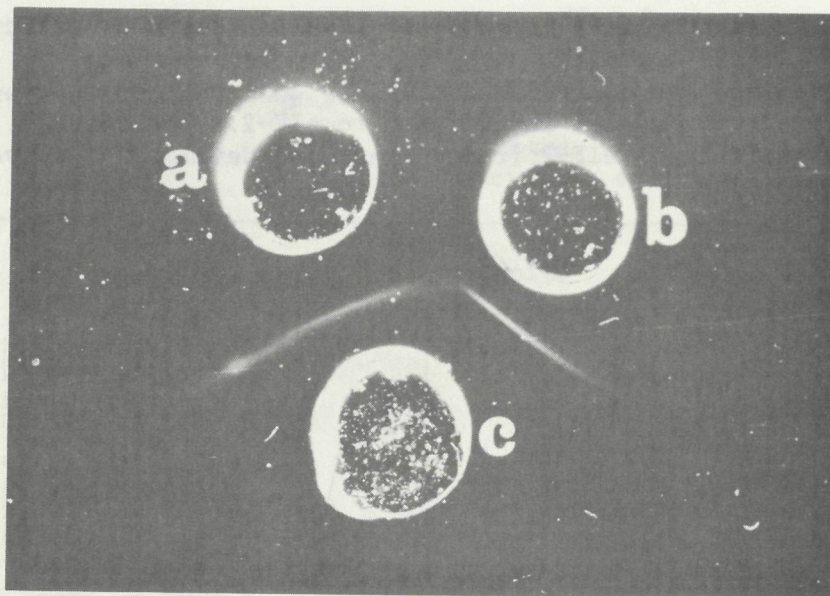


Fig. 4. Immunological cross-reactivity of eIF-2 from dormant and developing embryos. Mouse antibodies were prepared against step 6 eIF-2 from developing embryos as described in Materials and Methods. (a) contained 13 μ g of step 6 eIF-2 from developing embryos; (b) 6 μ g of step 6 eIF-2 from dormant embryos; (c) 25 μ l of antiserum. From MacRae *et al.* (17).

Cofactor 1 may be involved in eIF-2 activity. This protein does not exhibit GTP-dependent binding of [35 S]Met-tRNA_f; however, with 4-6 μ g of phosphocellulose-purified eIF-2 from either dormant or developing embryos, it stimulated 2-3-fold ternary complex formation (Fig. 5). Cofactor 1, from either dormant or developing embryos, also stimulated 2-3-fold 40S ribosomal binding of [35 S]Met-tRNA_f (Fig. 6).

The eukaryotic polypeptide chain initiation factor, eIF-3, was purified from 0.5 M NH₄Cl ribosomal washes of *A. salina* embryos. eIF-3 was found in the 0-40% (NH₄)₂SO₄ fraction from ribosomal washes and was adsorbed to heparin-Sepharose 4B. Chromatography on DEAE-cellulose partially separated eIF-3 from an inhibitor of cell-free protein synthesis which eluted at 50 mM KCl. Further purification of eIF-3 was achieved by chromatography on hydroxylapatite and gel filtration. The factor eluted at

Table 5. Inhibition of 40S initiation complex by antiserum against eIF-2^a

Source of eIF-2	Serum	[35 S]Met-tRNA _f bound	Inhibition
		pmol	%
Dormant embryos	None	0.245	0
	Normal	0.225	8
	Immune	0.031	87
Developing embryos	None	0.180	0
	Normal	0.169	7
	Immune	0.014	92

^aFormation of 40S initiation complex was assayed as described (17). Each reaction mixture contained 6 μ g of step 6 eIF-2 and, where indicated, 20 μ l of serum. Cofactor 1 was not added. A blank value in the absence of eIF-2 (0.033 pmol) was subtracted from each value.

the void volume of Ultrogel AcA-34, indicating that it had a high molecular weight (Fig. 7). This was confirmed by sucrose density gradient centrifugation of the protein from developing embryos which sedimented to the bottom of the gradient during centrifugation (Fig. 8). The factor from dormant embryos does not produce a sharp peak on sucrose gradients, but sediments as a high molecular weight aggregate.

Sodium dodecylsulfate polyacrylamide gel electrophoresis of Ultrogel purified eIF-3 from dormant and developing embryos shows that this factor from *A. salina* is a complex of many subunits (Fig. 9). At the present time, no detectable difference between both preparations of eIF-3 may be observed. Preliminary analysis of eIF-3 isolated after sucrose density gradient centrifugation indicates that several polypeptides may be removed and that a detailed comparison of the subunit composition of eIF-3 from dormant and developing embryos will be possible.

Table 6. Stimulation of polypeptide synthesis by eIF-2^a

Source of eIF-2	eIF-2 added	[¹⁴ C] Leucine incorporated	Stimulation
	μg	pmol	%
		70	0
Dormant embryos	0.76	89	27
	1.90	105	50
	3.80	121	72
Developing embryos	0.76	86	23
	1.90	116	66
	3.80	146	108

^aConditions for amino acid incorporation were as described (17). Each reaction mixture contained 0.5 A₂₆₀ unit of *A. salina* ribosomes, 0.15 A₂₆₀ unit of poly(A)+ mRNA, and 300 μg of reticulocyte unfractionated ribosomal salt wash. Incubation was for 90 min at 30°. Blanks, in the absence of reticulocyte initiation factors or in the presence of eIF-2 from dormant or developing embryos, averaged 1.6 pmol, and were subtracted from each value.

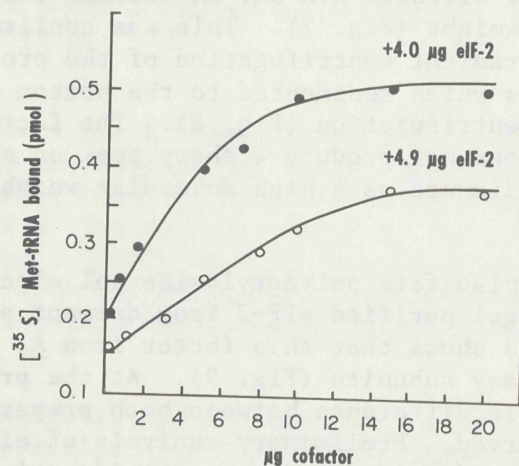


Fig. 5. Effect of cofactor 1 on ternary complex formation. Conditions were as described (17). 4.9 μg of step 6 eIF-2 (stored in an ice bath) from dormant embryos, ○—○; 4.0 μg of step 6 eIF-2 (stored at -70°) from developing embryos, ●—●. From MacRae *et al.* (17).

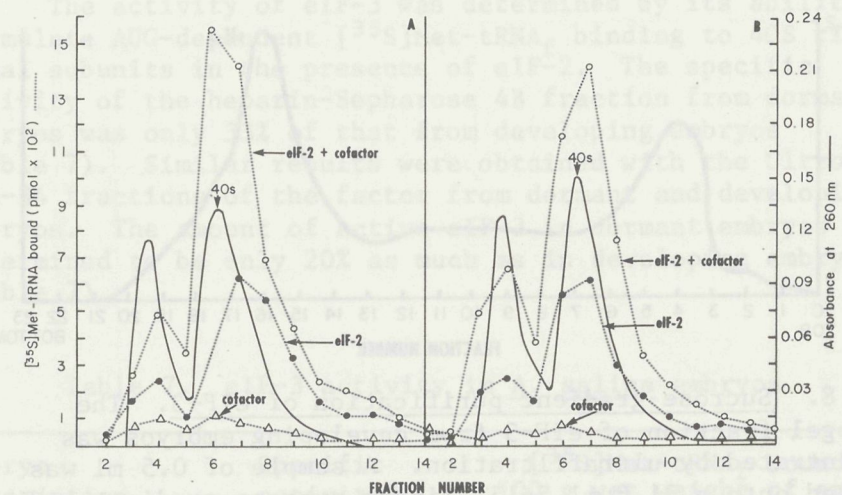


Fig. 6. Effect of cofactor 1 on eIF-2-dependent formation of 40S initiation complex. [³⁵S]Met-tRNA_f binding to 40S ribosomal subunits was assayed as in (17). (A) 40S initiation complex with 5.7 μg of step 6 eIF-2 from dormant embryos. (B) 40S initiation complex with 4.4 μg of step 6 eIF-2 from developing embryos. For panels A and B: 260 nm absorbance, —; eIF-2 added, ●—●; eIF-2 and cofactor 1 added, ○—○; cofactor 1 added, but no eIF-2, △—△. Where indicated, 38 μg of cofactor were used per reaction mixture. Sedimentation was from left to right. From MacRae *et al.* (17).

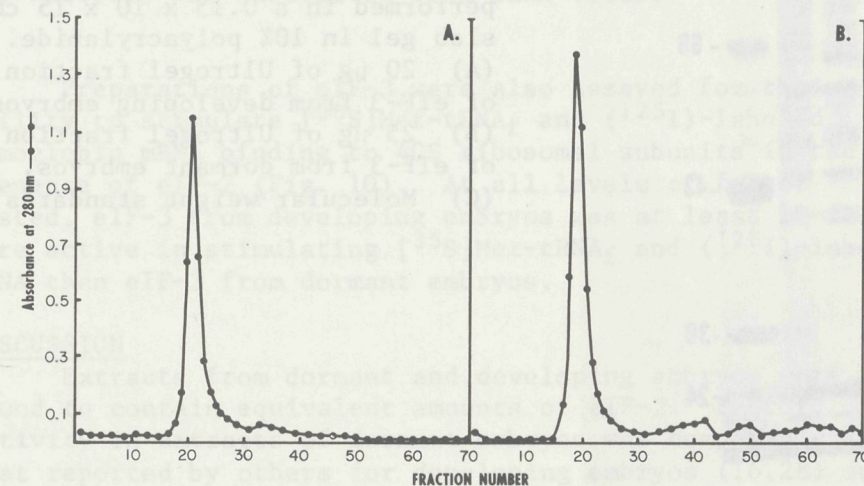


Fig. 7. Elution of eIF-3 from Ultrogel AcA-34. Chromatography of (A) 9.8 mg of hydroxylapatite-purified eIF-3 from dormant embryos and (B) 18.3 mg of hydroxylapatite-purified eIF-3 from developing embryos.

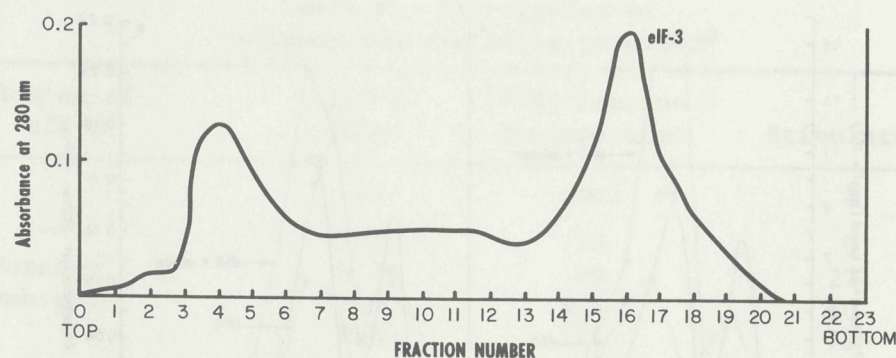


Fig. 8. Sucrose gradient purification of eIF-3. The Ultrogel fraction of eIF-3 from developing embryos was concentrated by ultrafiltration. A sample of 0.5 ml was layered onto a 11.6 ml, 5-15% linear sucrose gradient and centrifuged 20 h at 40,000 rpm and 4° in a Beckman SW41 rotor.

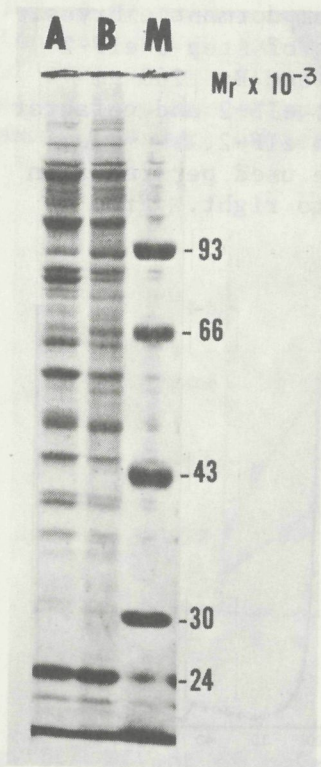


Fig. 9. SDS-polyacrylamide gel electrophoresis of *A. salina* eIF-3. Electrophoresis was performed in a 0.15 x 10 x 15 cm slab gel in 10% polyacrylamide. (A) 20 µg of Ultrogel fraction of eIF-3 from developing embryos. (B) 25 µg of Ultrogel fraction of eIF-3 from dormant embryos. (C) Molecular weight standards.

The activity of eIF-3 was determined by its ability to stimulate AUG-dependent [³⁵S]Met-tRNA_f binding to 40S ribosomal subunits in the presence of eIF-2. The specific activity of the heparin-Sepharose 4B fraction from dormant embryos was only 33% of that from developing embryos (Table 7). Similar results were obtained with the Ultrogel AcA-34 fractions of the factor from dormant and developing embryos. The amount of active eIF-3 in dormant embryos was determined to be only 20% as much as in developing embryos (Table 7).

Table 7. eIF-3 activity in *A. salina* embryos

Embryo description	Specific activity ^a	[³⁵ S]Met-tRNA _f bound per 100 g wet weight of embryos
	units/mg	pmol
Developing	6	39
Dormant	2	8

^aOne unit = 1 pmol of [³⁵S]Met-tRNA_f bound to 40S ribosomal subunits under standard assay conditions for AUG-directed binding. For each determination a blank, in the absence of factors, was subtracted from each value.

Preparations of eIF-3 were also assayed for their ability to stimulate [³⁵S]Met-tRNA_f and (¹²⁵I)-labeled hemoglobin mRNA binding to 40S ribosomal subunits in the presence of eIF-2 (Fig. 10). At all levels of factor tested, eIF-3 from developing embryos was at least 10-fold more active in stimulating [³⁵S]Met-tRNA_f and (¹²⁵I)-labeled mRNA than eIF-3 from dormant embryos.

DISCUSSION

Extracts from dormant and developing embryos were found to contain equivalent amounts of eIF-2. Factor activity in extracts of dormant embryos was comparable to that reported by others for developing embryos (16,28) and much greater than what was reported for dormant embryos (16). Neither did the length of embryo hydration, nor the resumption of cell division which occurs upon emergence of developing embryos (3), modify eIF-2 activity. The factor from either dormant or developing embryos was purified

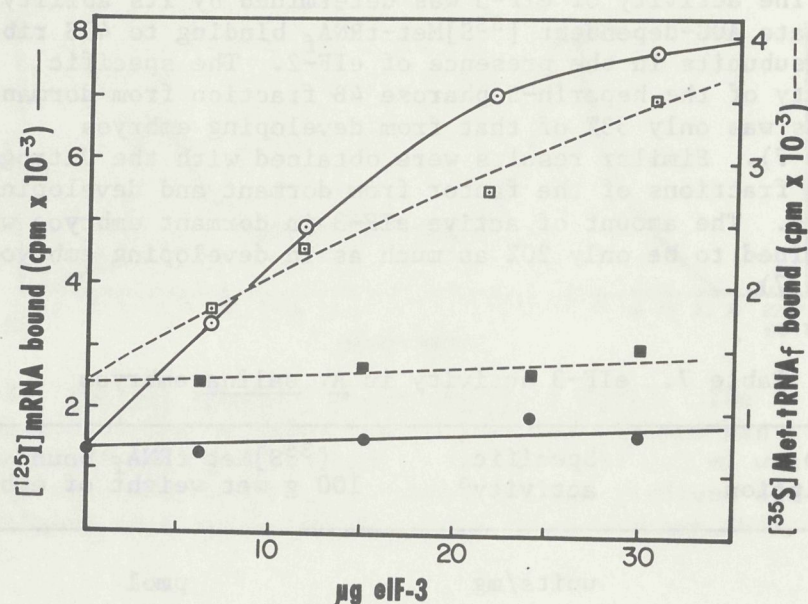


Fig. 10. Effect of eIF-3 on binding $[^{35}\text{S}]\text{Met-tRNA}_f$ and (^{125}I) -labeled mRNA to 40S ribosomal subunits. Reaction conditions were as described for mRNA translation (Table 6), except no reticulocyte initiation factors or S100 fractions were added. Each 75 μl reaction contained: 40S ribosomal subunits, 0.45 A_{260} unit; $[^{35}\text{S}]\text{Met-tRNA}_f$, 3 pmols; (^{125}I) -labeled mRNA, 3 pmols (153,000 cpm); and phosphocellulose fraction of eIF-2, 3 μg . The Ultrogel AcA-34 fractions of eIF-3 were used. Incubation and analysis of tRNA and mRNA binding were as described in Fig. 3. ●, ■, dormant embryos; ○, □, developing embryos.

125-fold, yielding a preparation with a specific activity of about 300 units/mg of protein. When analyzed by dodecylsulfate polyacrylamide gel electrophoresis, eIF-2 from either type of embryo exhibited three polypeptides with molecular weights of 52,000, 38,000 and 36,000. Preparations of eIF-2 from other sources, such as wheat germ (29), rabbit reticulocytes (30,31), ascites cells (32) and pig liver (33) are also composed of 3 subunits, but the middle polypeptide tends to have a molecular weight of 50,000.

Binding of $[^{35}\text{S}]\text{Met-tRNA}_f$ to 40S ribosomal subunits with eIF-2 from dormant or developing embryos required GTP and AUG, indicating the eIF-2 from either type of embryo functions similarly in 40S initiation complex formation.

The GTP requirements are common to eIF-2 from all sources examined, whereas the AUG or mRNA requirements are less well defined. In some cases an absolute requirement for mRNA has been reported (34-36), while in others mRNA exhibits a stabilizing effect (33,37) or is not required at all (16,18-20,38,39).

Immunological identity between eIF-2 from dormant and developing embryos was shown. Antibodies raised against eIF-2 from developing embryos cross reacted with the protein prepared from dormant embryos in Ouchterlony preparations and 40S initiation complexes. Immunological cross-reactivity, in concert with the stimulation of polypeptide synthesis by eIF-2 from both types of embryos, provide further evidence that the factor is not modified during development of *A. salina*.

Cofactor 1, which stimulated ternary complex formation, as well as $[^{35}\text{S}]\text{Met-tRNA}_f$ binding to 40S ribosomal subunits, was found in dormant and developing embryos. Factors with similar activities, such as Co-eIF-1 from rabbit reticulocytes (40-42) and developing *A. salina* embryos (43) have been isolated. The relationship of cofactor 1 to the other eIF-2 stimulatory proteins and its role in protein synthesis remain to be determined.

The eukaryotic initiation factor eIF-3, was observed to stimulate or stabilize Met-tRNA_f and mRNA binding to 40S ribosomal subunits in the presence of eIF-2 (18,21). The factor from developing embryos of *A. salina* was also found to possess these activities. The increase in $[^{35}\text{S}]\text{Met-tRNA}_f$ and (^{125}I) -labeled hemoglobin mRNA binding to 40S ribosomal subunits was 3-fold and 7-fold, respectively (Fig. 10).

Precipitation in the 0-40% $(\text{NH}_4)_2\text{SO}_4$ fraction of ribosomal washes, the large size as indicated by Ultrogel AcA-34 chromatography and sucrose density gradient centrifugation, as well as the complex banding pattern on dodecylsulfate gel electrophoresis, strongly suggest that the *A. salina* factor which stimulates Met-tRNA_f and mRNA binding to 40S subunits is similar to eIF-3 from other sources (30,32,44-48).

The specific activity of eIF-3 is at least 3-fold greater and its concentration is 5-fold more in developing than in dormant embryos. In addition, eIF-3 from each type of embryo behaves differently in its ability to stimulate

binding of [^{35}S]Met-tRNA_f to 40S subunits in the absence of message, a reported eIF-3 activity (18-20). Only the factor from developing embryos was found to stimulate eIF-2 mediated binding of [^{35}S]Met-tRNA_f to 40S subunits in the absence of message (data not presented).

Our results, in contrast to Filipowicz *et al.* (16), demonstrate that dormant and developing embryos of *A. salina* contain equivalent amounts of eIF-2. Both types of embryos also contain a cofactor which was shown to stimulate eIF-2 activity. On the other hand, the quantity of eIF-3 is much less in dormant than in developing embryos. These data suggest that a modification of eIF-3 may occur during early embryo development, which could lead to cessation of protein synthesis and eventual encystment of embryos. Furthermore, eIF-3, and not eIF-2 as previously suggested, may limit the resumption of embryo development in *A. salina*. The modified eIF-3 may permit selective translation of various classes of mRNA, a function suggested for eIF-3 from other sources (48,49). Such interesting possibilities are currently under investigation.

ACKNOWLEDGMENTS

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CHARACTERIZATION OF ELONGATION FACTOR 2
IN DORMANT CYSTS AND DEVELOPING
EMBRYOS OF ARTEMIA SALINA

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ABSTRACT

The ADP ribosylation of elongation factor 2 (EF-2) catalyzed by diphtheria toxin was used in vitro to determine the subcellular location and molecular weight of EF-2 in extracts from Artemia embryos at different stages of development. We have found that part of the EF-2 is soluble in the embryo and the remainder is bound to the particulate fraction which sediments at 150,000 x g. During early development, the amount of EF-2 in the particulate fraction decreases markedly concomitant with an increase in the amount of soluble EF-2. Our data suggest that most of the EF-2 in the particulate fraction is bound to a macromolecule which is different from ribosomes. No differences could be detected in the molecular weight of the free and bound EF-2 after the release of the latter from its complex. However, the molecular weight studies have revealed that extracts from hatched embryos exhibit smaller ADP ribose accepting polypeptide(s) than extracts from pre-hatched and dormant embryos. Our data indicate that the smaller polypeptides are specific degradation products of the native EF-2 and that the enzyme which catalyzes the production of these fragments is present in a sequestered form in crude extracts of dormant cysts. The characterization of the degradation process of EF-2 from Artemia raises the possibility that this event may be a general phenomenon in the metabolism of EF-2 in eukaryotes.

INTRODUCTION

Dormant embryos of Artemia salina are inactive in protein synthesis and cell-free extracts from these embryos are unable to translate exogenous mRNA (1,2,3). However, cell-free systems prepared from dormant cysts by various methods are active in poly(U)-directed protein synthesis (2,4,5). These studies indicate that elongation factors 1 and 2 (EF-1 and EF-2) are present and active in extracts from dormant cysts. Nevertheless, studies by Moller and Slobin have suggested that EF-1 is regulated during early development of the brine shrimp. These workers demonstrated that EF-1 undergoes a size transformation from a heavy to a light form during early development of Artemia, and they

suggested that the size transformation of EF-1 may provide a mechanism by which the rate of protein synthesis can be altered during embryogenesis (5,6). In addition, studies by Warner et al. showed that a protein from dormant cysts of Artemia which resembles EF-2 acts at the level of elongation to inhibit poly(U)-directed in vitro protein synthesis (7, these proceedings). Hence, EF-2 might play a role in the regulation of protein synthesis during early development of Artemia.

The purpose of the present study was to determine whether EF-2 is regulated during early development of Artemia and the subcellular localization and molecular size of EF-2 at different stages of development. Most of the results were obtained using the [³H] ADP-ribosylation assay for EF-2 (8), and these findings should contribute to our understanding of the role and metabolism of EF-2 in eukaryotes and during development.

MATERIALS AND METHODS

Encysted dormant embryos of Artemia salina were from the Utah salterns and they were hydrated and washed before use as described by Warner et al. (9). Unless noted otherwise antiformin treatment was avoided. The incubation medium was as described previously (9) except that the concentration of penicillin and streptomycin were reduced to 500 units/ml and 25 µg/ml, respectively. Two different incubation procedures were used. In some experiments 12 grams of fully hydrated cysts were incubated with 500 ml hatch medium in a 2800 ml Fernbach culture flask. In other experiments 1 gram of fully hydrated cysts was incubated with 75 ml of hatch medium in 100 x 80 cm glass storage dishes. In the latter case emergence started at about 9-h incubation and about 62-65% of the embryos hatched by 48-h incubation. When 12 grams of fully hydrated cysts were incubated emergence and hatching began later and the extent of hatching was lower due to a higher density of embryos in the culture flask (see Results).

The particulate and soluble fractions of Artemia embryos were prepared from the post-mitochondrial supernatant fluids of embryo extracts in an attempt to determine the distribution of EF-2 within the embryo. The post-mitochondrial fraction was prepared as previously described (7) except that buffer C (50 mM Tris-HCl, pH 7.9 (4°C), 100 mM KCl, 10 mM MgCl₂, 1 mM DTT, 10% glycerol) was used, and this fraction was applied to a column of Sepharose 6B. Column fractions containing particulate (I) and soluble proteins (II) were pooled as described in Fig. 1. In some experiments particulate and soluble fractions were prepared from the post-mitochondrial fraction by centrifugation at

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150,000 x g as described previously using buffer A (50 mM Tris-HCl, pH 7.9 (4°C), 5 mM KCl, 10 mM MgCl₂, 1 mM DTT) for the extraction (7). When the latter method was used the pellet (P-150) obtained by the high speed centrifugation was suspended in buffer C. The particulate fractions obtained by the above two methods were then treated with 0.5 M NH₄Cl (75 min, 0°C) to release EF-2 and the NH₄Cl soluble material was collected by centrifugation at 150,000 x g for 4.5 h and concentrated by vacuum dialysis. The soluble fractions from above were fractionated with ammonium sulfate (30-70% saturation), equilibrated with buffer D (50 mM Tris-HCl, pH 7.9 (4°C), 50 mM KCl, 0.6 mM EDTA, 1 mM DTT and 10% glycerol) then stored at -70°C until needed. The protein fraction obtained by ammonium sulfate fractionation of fraction II above is referred to as the free protein fraction and it contains free EF-2. The protein released from the particulate fraction by the NH₄Cl treatment is referred to as the bound protein fraction and the EF-2 in this fraction is designated as bound EF-2.

Soluble embryo extracts were prepared from *Artemia* at different stages of development to study the molecular size of EF-2, alkaline protease activity and the capacity of embryo extracts to support poly(U)-directed protein synthesis. Embryos used to prepare these soluble extracts were treated with antiformin during the washing procedure (9). The embryos were extracted with buffer C using a Dual type ground-glass homogenizer and the 30,000 x g supernatant (S-30) was prepared as described above for the post-mitochondrial fraction. The S-30 fraction was then treated with 0.5 M NH₄Cl to release EF-2 from particles and the salt soluble material (which in these experiments includes free and bound EF-2) was collected as described above. All fractions were equilibrated with buffer D and kept at -70°C until needed.

In some experiments embryo extracts were fractionated on sucrose density gradients to determine the quantity of EF-2 actually bound to monoribosomes and polyribosomes. Post-mitochondrial supernatant fluids were prepared as described above then 10 absorbance units (A₂₆₀) were fractionated on each of six 15-50% sucrose gradients as described previously (2). The cytosol, monoribosome and polyribosome fractions were pooled separately (see Fig. 2) and the latter two fractions were adjusted to contain 0.5 M NH₄Cl to release EF-2 from the ribosomes as described above. All fractions were equilibrated with buffer D then stored at -70°C until needed.

The assay for the quantitation of EF-2 was as described by Maxwell *et al.* (8,10,11) using [adenine-2, 8-³H] NAD

(122 pmole/assay) and diphtheria toxin (1.65 µg/assay) for the ADP ribosylation of EF-2. The functional capacity of EF-2 and soluble extracts from various stages of development were tested in an *in vitro* system as described by Warner *et al.* (7, these proceedings) except that salt washed *Artemia* cyst ribosomes were used. When the activity of EF-2 was tested in this assay the post-ribosomal fraction from 12-h *Artemia* embryos was added as a source of additional factors.

General protease activity in soluble extracts was determined by measuring the amino groups liberated during proteolysis as described by Nagainis and Warner (12) except that the final pH was 8.0 (23°C) and N,N dimethyl BSA was used as substrate.

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis of *Artemia* proteins was according to Laemmli using 10% polyacrylamide gel slabs (13). When [³H] ADP-ribosylated proteins were analyzed the gels were sliced into 2 mm slices, swollen in 90% tissue solubilizer (NCS) and counted in a scintillation counter as described by Basch (14).

Protein was determined according to the method of Lowry *et al.* using BSA as a standard (15).

The source of all chemicals and media was as described by Warner *et al.* (7, these proceedings).

RESULTS AND DISCUSSION

Distribution of EF-2 between the soluble and particulate fractions in *Artemia* embryos. The quantity and distribution of EF-2 between the soluble and particulate fractions of the post-mitochondrial supernatant were determined in extracts of dormant and developing *Artemia* embryos. When the post-mitochondrial fluid from 0-h embryos was chromatographed on a column of Sepharose 6B the results shown in Fig. 1 were obtained. The column fractions were tested for the presence of EF-2 by the toxin-NAD assay and virtually all of the EF-2 was found in region II which contains the soluble proteins. However, when the contents of region I were treated with 0.5 M NH₄Cl then centrifuged at 150,000 x g considerable EF-2 was found in the NH₄Cl soluble fraction as measured by the ADP-ribosylation assay. Since only free EF-2 can be assayed quantitatively by the ADP-ribosylation reaction the NH₄Cl treatment must have released EF-2 from the particulate material in fraction I. The difference in the ADPR acceptance activity of the particulate and soluble fractions suggests that the particulate fraction contains bound EF-2, whereas the soluble fraction contains free EF-2.

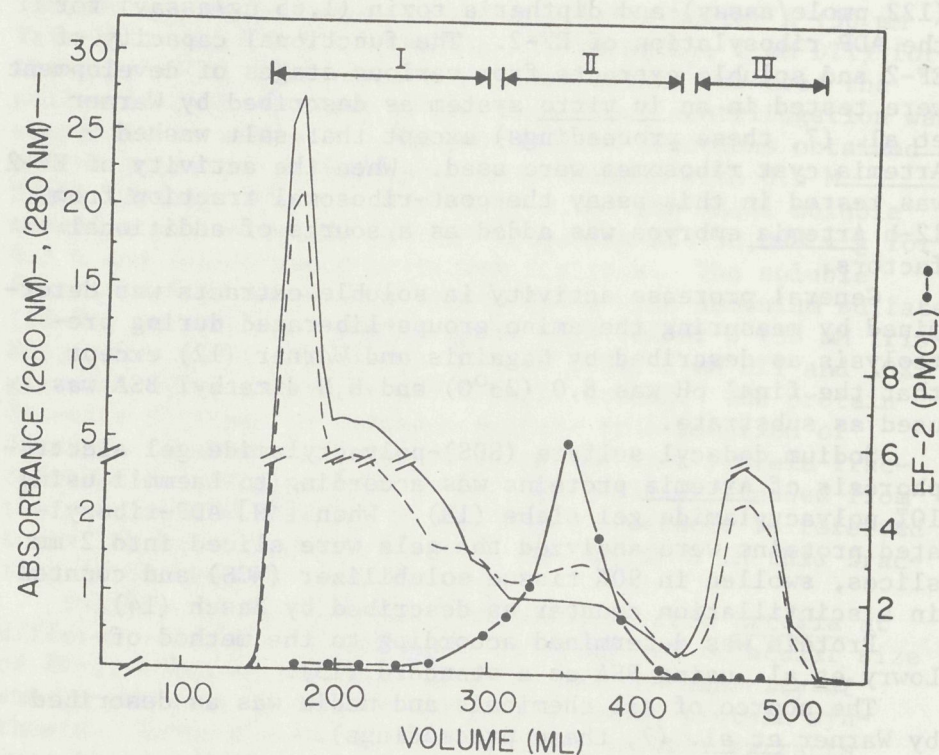


Fig. 1. Chromatography of *Artemia* cyst S-30 fraction on a column of Sepharose 6B. The 30,000 x g supernatant fraction from 12 grams hydrated 0-h *Artemia* cysts was applied to a column of Sepharose 6B (2.5 x 91 cm) previously equilibrated with buffer C. The column was developed with buffer C at a flow rate of approximately 30 ml/h and fractions of 7.5 ml were collected. Column fractions were assayed for absorbance at 260 nm (—) and 280 nm (---) and for EF-2 (●—●) as described in Materials and Methods.

The quantity and distribution of EF-2 from the bound and free fractions prepared from embryos at different stages of development is shown in Table 1. The data show that a gradual decrease occurs in total EF-2 during the first 31 h development followed by a marked increase during the next 2 h. More striking, however, is the observation that bound EF-2 (and other bound proteins) decreases by almost 50% during the first 31 h development in association with a 17% increase in free EF-2. During this period the level of free proteins does not change appreciably. Between 31 and 33 h development an increase occurs in both free EF-2 (42%) and free proteins (21%), while the content of bound EF-2 and

TABLE 1. EF-2 and protein content of bound and free protein fractions from *Artemia* embryos at different stages of development^a

Incubation time ^b	h	Embryos Emerged Hatched		Protein		EF-2		EF-2	
		z		Bound	Free	Bound	Free	Bound	Free
				mg		nmol		z	
	0	0	0	4.2 (100)	16.2 (100)	1.14 (100)	1.41 (100)	45	55
	9	0	0	4.4 (105)	16.1 (99)	0.80 (70)	1.49 (106)	35	65
	23	21	3	3.4 (81)	16.0 (99)	0.60 (53)	1.56 (111)	28	72
	31	10	14	2.3 (55)	15.7 (97)	0.52 (46)	1.65 (117)	24	76
	33	4	29	2.3 (55)	19.2 (118)	0.56 (49)	2.25 (159)	20	80

^aThe bound and free protein fractions were prepared by chromatography on columns of Sepharose 6B of extracts from 12 grams hydrated cysts and developing embryos as described in Methods. The quantities of protein and EF-2 are given per gram dry weight cysts initially incubated. Bound EF-2 and proteins were measured after release from associated macromolecular components with 0.5 M NH_4Cl as described in Methods. The numbers in parenthesis are percentages of the amount found in each fraction compared to that found in 0-h embryos. The values for mg and nmol are averages of 3 and 8 determinations, respectively.

^bHatching started at about 23-h incubation.

bound proteins remains relatively constant. The importance of the increase in the free proteins and free EF-2 is not clear.

In other experiments, the particulate and soluble fractions were prepared by high speed centrifugation as described in Materials and Methods. The results of these experiments (not shown here) demonstrate that the S-150 fraction contains free EF-2 while the P-150 fraction contains EF-2 which is sedimentable and must be released from the particulate material in the sediment in order to accept the ADPR moiety of NAD in the diphtheria toxin catalyzed reaction. These results are analogous to those obtained by gel filtration and they support the idea that considerable EF-2 is released from the bound state during development.

It has been established that the rate of protein synthesis increases throughout development of *Artemia* (1,16). Hence the decrease in bound EF-2 and the increase in free EF-2 during early development of *Artemia* are related to a general increase in the rate of protein synthesis in the intact embryo. These findings suggest that the change in subcellular location of EF-2 might be part of a regulatory mechanism controlling the overall rate of protein synthesis. Other investigators have also noted changes in the distribution of EF-2 in animal cells associated with changes in protein synthesis activity (17,18).

The proportion of bound and free EF-2 in *Artemia* after hatching (about 20 and 80%, respectively) is very similar to the proportion of EF-2 in HeLa cells (17), rabbit reticulocytes (19) and myogenic cells (16). In contrast to the above findings, the distribution of EF-2 in several rat organs favors the bound form (70-90% bound versus 10-30% free) (20); thus the distribution of EF-2 in the above tissues is similar to that found in dormant *Artemia* embryos. It appears that the distribution of EF-2 in rat organs and dormant cysts, compared to the distribution of EF-2 in hatched embryos and mammalian cells, is related to the protein synthetic activity in these systems. Thus, the possibility exists that the release of EF-2 from its bound state activates or enhances protein synthesis in animal cells.

The content of EF-2 bound to ribosomes and the nature of the EF-2 containing particles. We have studied the amount of EF-2 bound to ribosomes by fractionating the post-mitochondrial supernatants of 0 and 20-h embryo extracts on sucrose density gradients as described in Materials and Methods. As expected from previous studies 0-h embryos were found to be deficient in polyribosomes compared to developed embryos (2). The cytosol, monoribosome and

polyribosome fractions were pooled as described in Fig. 2 and the EF-2 was released from the particulate material in each fraction as described in Materials and Methods. Only 1-3% of the total EF-2 in the post-mitochondrial extracts was found to be associated with the ribosomes and no significant differences were found in the EF-2 content of monoribosomes and polyribosomes from different stages of development. A similar proportion of ribosome-bound EF-2 (1-3%) was found when ribosomes were prepared from the P-150 fraction by sedimentation of this fraction through a sucrose cushion. Hence, it appears that most of the bound EF-2 that is recovered in region I from Sepharose 6B (Fig. 1) or sedimentable in the absence of sucrose remains in the cytosol fraction after sucrose gradient fractionation.

To determine whether the sucrose gradient cytosol (fraction A, Fig. 2) contains bound EF-2 which is sedimentable, the 0 and 20-h untreated cytosols (in buffer C) were subjected to high speed centrifugation under conditions similar to those used to prepare the P-150 fraction. This treatment sedimented a red-colored pellet whose polypeptide pattern (0-h cytosol only) is shown in Fig. 3. When the latter sediment was tested in the diphtheria toxin-NAD assay it exhibited ADPR acceptance activity but only after the EF-2 was released from the sedimentable material by dissociating reagents such as EDTA.

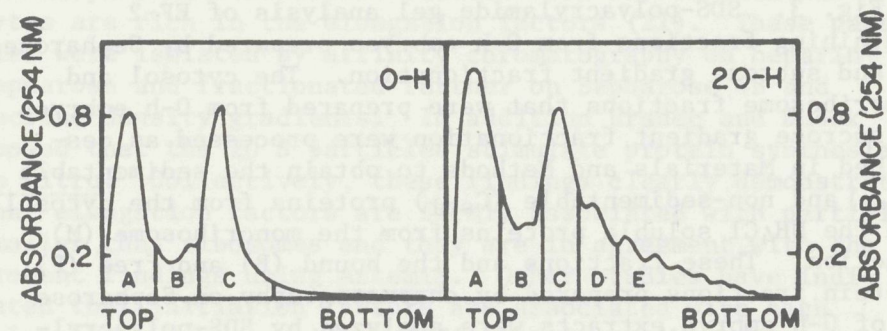


Fig. 2. Sucrose density gradient profiles of the post-mitochondrial supernatant fractions from 0 and 20-h *Artemia* embryos. The post-mitochondrial supernatant fractions were obtained from 0 and 20-h *Artemia* embryos described in Materials and Methods and 10 A₂₆₀ units of these fractions were layered onto separate 12 ml sucrose gradients (15-50%). The preparations were centrifuged for 80 min at 39,000 rpm (SW 41 rotor) then fractionated with an Isco gradient-fractionator. Cytosol fractions (A), monoribosome fractions (C) and polyribosome fractions (E) were pooled separately as illustrated and concentrated by vacuum dialysis.

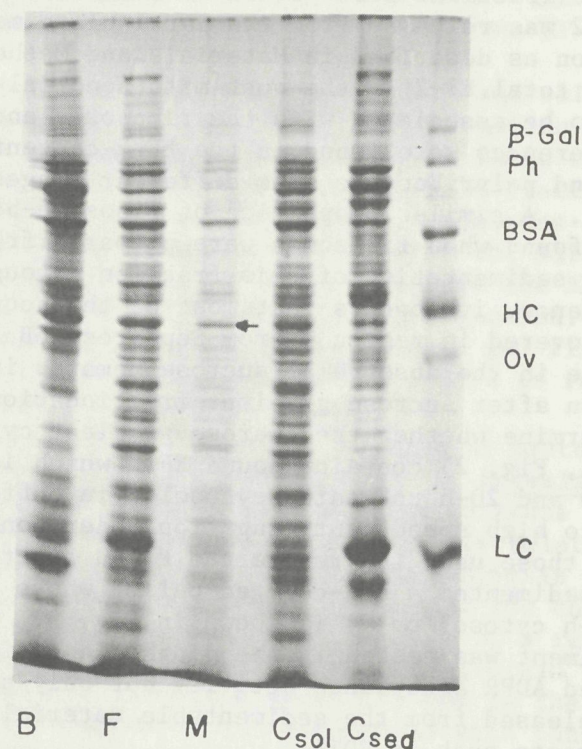


Fig. 3. SDS-polyacrylamide gel analysis of EF-2 containing fractions from 0-h embryos prepared by Sepharose 6B and sucrose gradient fractionation. The cytosol and monoribosome fractions that were prepared from 0-h embryos by sucrose gradient fractionation were processed as described in Materials and Methods to obtain the sedimentable (C_{sed}) and non-sedimentable (C_{sol}) proteins from the cytosol and the NH_4Cl soluble proteins from the monoribosome (M) fraction. These fractions and the bound (B) and free (F) protein fractions prepared by chromatography on Sepharose 6B of 0-h embryo extracts were analyzed by SDS-polyacrylamide gel electrophoresis. About 3 μg protein from M and 20 μg protein from all other samples were applied to the gel slab. The migration position of standard molecular weight markers is shown at the right B-Gal, B-galactosidase, 130,000; Ph, phosphorylase a, 94,000; BSA, bovine serum albumin, 67,000; HC, heavy chain of gamma globulin, 50,000; OV, ovalbumin, 43,000; LC, light chain of gamma globulin, 23,500.

From this experiment it was concluded that EF-2 is present in the red-colored pellet in a bound form. In contrast, the supernatant fluid above the red pellet contained EF-2 which could be ADP ribosylated without prior treatment with dissociating reagents. The SDS-polyacrylamide gel analysis of the cytosol proteins (soluble and sedimentable) from 0-h embryos along with the 0.5 M NH_4Cl soluble proteins of the monoribosomes of the 0-h embryos is shown in Fig. 3 and indicates that the three fractions differ markedly in polypeptide composition. This observation indicates that cross-contamination between these fractions is minimal. In addition many of the polypeptides which disappear from extracts of developed embryos are present in the sedimentable material of the cytosol of the 0-h embryos prepared by sucrose gradient fractionation (data not shown). Also, a polypeptide which is present in the NH_4Cl soluble fraction from 0-h monoribosomes (Fig. 3, sample M, indicated by arrow) is absent from the NH_4Cl soluble fraction of either polyribosomes or monoribosomes from 20-h embryos.

The results represented above suggest that *Artemia* embryos contain EF-2 that is free in the cytosol, EF-2 which is bound to monoribosomes and polyribosomes, and EF-2 which is bound to a macromolecular component(s) in the cytosol that is different from ribosomes.

Recently, Hradec and Dusek found that 20 S particles in the post-mitochondrial fraction from rabbit reticulocytes are rich in the elongation factors (21). These particles were isolated by affinity chromatography on heparin-Sepharose and fractionated further on Sepharose 4B and sucrose density gradients. In addition Hradec and Dusek showed that the 20 S particles stimulate protein synthesis *in vitro*. Collectively, these findings clearly demonstrate that elongation factors are firmly associated with particles smaller than ribosomes and they are in agreement with the present findings using *Artemia*. Other studies have indicated that initiation factors are associated with high molecular weight particles different from ribosomes or ribosomal subunits (22). Hence many components of the protein synthesis apparatus appear to exist as part of large aggregates or macromolecular complexes.

Changes in the molecular weight of the ADPR accepting polypeptides in extracts from *Artemia* embryos and the possible involvement of a specific hydrolase(s) in the process. A study of the molecular weight of EF-2 was conducted to determine the molecular size of EF-2 and to ascertain whether differences exist in the molecular weight of free and bound EF-2 at different stages of development. To obviate

the need for purified EF-2, all EF-2 containing fractions were [^3H] ADP-ribosylated in the diptheria toxin-dependent reaction and analyzed by electrophoresis on SDS-polyacrylamide gel slabs. The protein fractions used for these analyses are identical to those described in Table 1 and the results are summarized in Fig. 4.

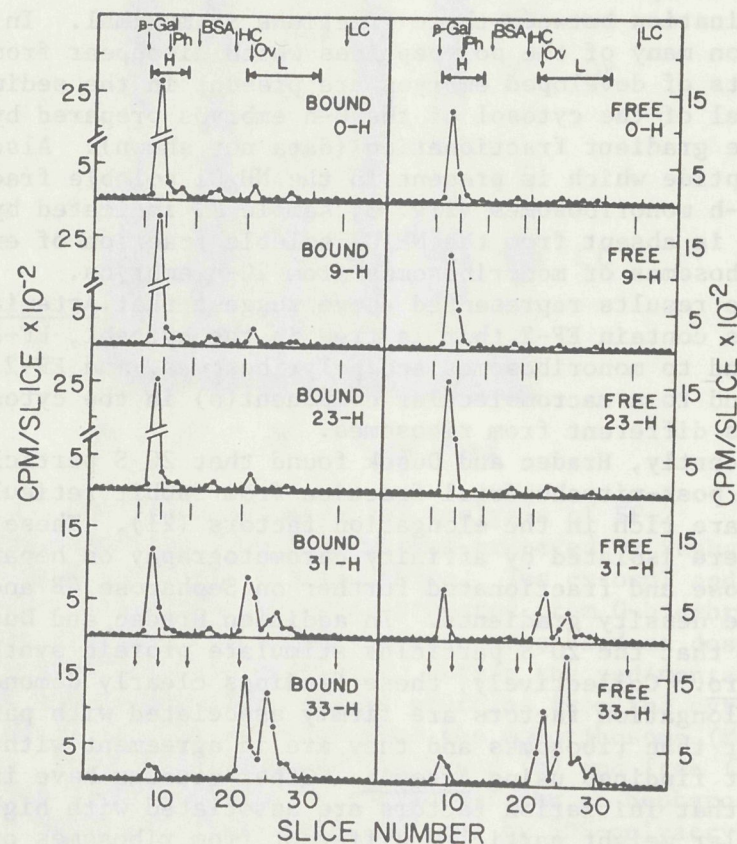


Fig. 4. SDS-polyacrylamide gel analysis of bound and free [^3H] ADP-ribosylated proteins from *Artemia* embryos at different stages of development. Protein preparations containing bound and free EF-2 were radiolabeled in a standard toxin-NAD assay and then analyzed by SDS gel electrophoresis as described in Materials and Methods. H and L indicate the regions where heavy and light forms of EF-2 migrate, respectively. The migration of molecular weight markers is indicated at the top of the figure and their description is given in the legend of Fig. 3.

The results indicate that several radioactive proteins are resolved by SDS-polyacrylamide gel electrophoresis and that a polypeptide of 95,000 molecular weight is the dominant radiolabeled protein in all fractions from pre-hatched and newly hatched embryos. Beyond 23 h development at least two low molecular weight (41,000 and 36,000) polypeptides appear in extracts and partially purified protein preparations which can be [^3H] ADP-ribosylated. The appearance of the latter forms occurs concomitant with the disappearance of the heavier form. The ADPR accepting proteins are considered to be EF-2 or fragments thereof since it has been established that only this protein can be ADP ribosylated in the diptheria toxin-dependent reaction (23). Regardless of their activity or function the 95,000 MW species is referred to as heavy EF-2 and the low molecular weight ADPR accepting proteins are referred to as light EF-2's. The appearance of the light EF-2 proteins can be detected in embryo extracts within 2 h after the onset of hatching but the exact time of appearance of the light EF-2 proteins is dependent on the incubation conditions (16). The disappearance of the heavy EF-2 concomitant with the appearance of the light EF-2's was also observed when embryo extracts were fractionated on analytical columns of Sephadex G-150 prior to the toxin - NAD treatment (16). The latter results indicate that the low molecular weight EF-2 proteins are not generated from the 95,000 MW form of EF-2 during the incubation period required for [^3H] ADP ribosylation of EF-2 or analysis of the resultant derivative on SDS-polyacrylamide gels.

The heavy form of *Artemia* EF-2 has a molecular weight similar to that of EF-2 in other eukaryotes (24). In addition, the heavy EF-2 from *Artemia* embryos catalyzes ribosome-dependent GTP binding (a property of functional EF-2), whereas the light EF-2 species regardless of their origin are inactive in this respect (16). Thus, the appearance of the light forms of EF-2 cannot account for the increase in rate of protein synthesis observed in the intact embryo.

The activity of the post-ribosomal fraction (prepared in the presence of 0.5 M NH_4Cl) in protein synthesis was studied *in vitro* using preparations from embryos at various stages of development and poly(U) as messenger. The results of this study are summarized in Table 2. The data indicate that the extract from 12-h embryos is the most active in polyphenylalanine synthesis. Moreover, the ability of extracts from embryos beyond 12-h development to support polyphenylalanine synthesis drops markedly, and no activity could be detected in extracts prepared from embryos beyond 24-h development. The addition of purified EF-2 to the

TABLE 2. Activity of embryo extracts from various stages of *Artemia* development in polyphenylalanine synthesis^a

Embryo age	Experiment 1 ^b			Experiment 2		
	In extract Protein	EF-2	Phenylalanine incorporated	In extract Protein	EF-2	Phenylalanine incorporated Extract plus added EF-2
h	μg	pmol	pmol/30 min	μg	pmol	pmol/30 min
0	120	7	0.32	60	3.5	0.14
5	120	7	1.84	60	3.5	0.90
12	120	7	4.30	60	3.5	2.90
20	120	13	0.80	60	6.5	0.42
24	120	14	0.41	60	7.0	0.23
30	60	8	0.00			0.43
						%
						42
						78
						23
						149
						87

^aOne gram of cysts was incubated as in Table 3 and the developmental profile was as in Table 3.
^bTo each reaction vessel was added 1 A260 unit of salt washed ribosomes from 0-h embryos in a final volume of 150 μl extent of ¹⁴C₃ polyphenylalanine synthesis is given for 0.05 ml of the reaction mixture. Background values of 220-250 cpm were subtracted for ribosomes assayed alone, for extract alone or for ribosomes to which purified EF-2 only was added. In experiment 2, 10 μg of partially purified EF-2 (36 pmol) in buffer D or the buffer alone were added as indicated.

reaction mixtures stimulated polyphenylalanine synthesis in all cases, but added EF-2 was most effective when added to extracts prepared from 20 and 24-h embryos. These data suggest that the light EF-2 proteins in the 20 and 24-h preparations are probably inactive in protein synthesis.

In addition to the change in molecular size of the ADPR accepting proteins and reduction in the capacity of soluble extracts to support polyphenylalanine synthesis, extracts from hatched embryos exhibit fewer polypeptides on SDS-polyacrylamide gels than extracts from pre-hatched embryos. All of these changes occur in extracts from hatched embryos prior to the rise in general alkaline protease activity which has been reported to occur at about 10 h after the onset of hatching (24). When we tested soluble embryo extracts for the presence of alkaline protease activity using the method described by Nagainis and Warner (12) we confirmed the previous findings. These results are shown in Table 3.

TABLE 3. General protease activity in soluble extracts of *Artemia* at different stages of development^a

Embryo age ^b	Embryos		Protease activity ^c		
	Emerg	Hatched	5°C	28°C	40°C
h	%		units/gram dry cysts		
0	0	0	0	0	0
5	0	0	0	0	0
12	16	0	0	0	0
20	14	15	0	0	0
24	8	28	trace	trace	trace
30	7	39	0.06	0.9	12.3
35.5	6	42	0.10	2.6	52.9
48	0	62	0.14	3.1	76.0

^aThe soluble extracts were identical to the ones assayed in Table 2. For the protease assay 350 and 120 μg protein were used for 10 to 24 and 30 to 48-h embryo extracts, respectively.

^bHatching started at about 18-h incubation.

^cOne activity unit is defined as the amount of enzyme that produces a change of 0.01 A₄₂₀ unit per minute. Protease activity is given as units per gram dry weight starting cysts. Since a lag in protease activity was observed occasionally, the units of protease activity were calculated from the linear part of the curve.

Moreover, under enzyme extraction and purification conditions (5°C) general protease activity is detectable in extracts from post-hatched embryos although it is suppressed substantially. When the extracts in Table 3 were assayed for the appearance of light EF-2 proteins, we observed that these EF-2 fragments are present within 2-h after the commencement of hatching. In another experiment we incubated purified EF-2 with selected soluble preparations as described in Tables 2 and 3) for 40 h at 5°C. These reaction mixtures were then analyzed by SDS-polyacrylamide gel electrophoresis and the results are shown in Fig. 5.

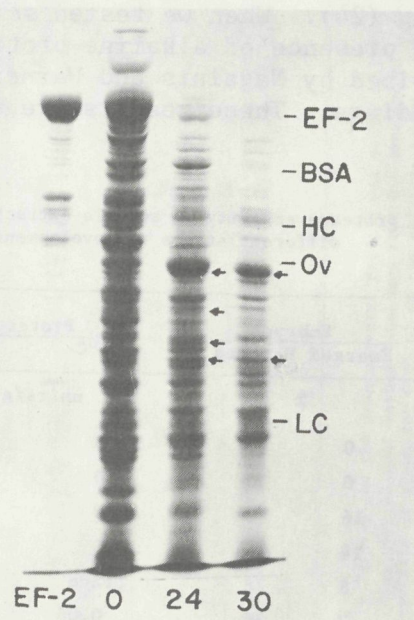


Fig. 5. SDS-polyacrylamide gel analysis of EF-2 hydrolysis by soluble extracts from *Artemia* embryos at different stages of development. Aliquots from 0, 24 and 30-h embryo extracts were mixed separately with 10 µg of partially purified EF-2, incubated at 5°C for 40 hours, then analyzed on an SDS-polyacrylamide gel slab. The partially purified EF-2 was also incubated under the same conditions. The migration position of EF-2 and the standard protein markers is indicated at the right and the arrows indicate the position of bands as described in the text.

These results show that no breakdown of EF-2 occurs in the reaction vessel containing the 0-h extract; however, in the presence of the 24-h extract only trace amounts of the intact EF-2 polypeptide remain. In the mixture containing the 30-h extract the existence of trace amounts of intact EF-2 is questionable. Concomitant with the proteolysis of EF-2 by extracts from 24 and 30-h embryos is the appearance of several new protein bands which are indicated by arrows in Fig. 5. Of the new bands the dominant one migrates slightly faster than ovalbumin (Ov) and these new bands were not observed in control reactions lacking EF-2. Furthermore, incubation of purified EF-2 alone had no effect on its electrophoretic profile (data not shown). This analysis shows clearly that considerable EF-2 hydrolase activity exists in the 24 and 30-h extracts although general protease activity was barely detectable. Hence, by the use of the latter method to monitor protease activity, alkaline protease activity could be detected within 6 h after hatching and prior to the increase in the general alkaline protease activity as measured by other methods. Moreover, this 'early' protease activity hydrolyzes EF-2 giving rise to several polypeptides of which two resemble (in molecular weight) the light forms of EF-2 identified in extracts from hatched embryos. Since the hydrolysis of added EF-2 occurs under extraction conditions (5°C), it appears that the low molecular weight EF-2 proteins in extracts from hatched embryos are generated, at least partially, during homogenization and sample preparation. The enzyme that hydrolyzes EF-2 appears to be specific for EF-2. However, since partially purified EF-2 only was tested by the SDS gel assay as a substrate for the 'early' alkaline protease in extracts from hatched *Artemia* embryos, no conclusion can be drawn at this time about the specificity of the EF-2 hydrolase activity in these extracts. The presence of EF-2 hydrolase and/or other proteolytic enzymes in extracts from early hatched embryos could also explain the reduction in activity of these extracts to support *in vitro* polyphenylalanine synthesis (see Table 2).

As demonstrated in Fig. 4 trace amounts of the light forms of EF-2 are present in extracts from early stages of development and prior to the large increase in these forms. Furthermore, since we observed extensive degradation of the 95,000 MW EF-2 species during attempts to purify EF-2 from dormant cysts (see Ref. 16), we examined extracts and preparations from dormant cysts for the existence of EF-2 hydrolase activity. When the S-150 fraction from dormant cysts was applied to a column of DEAE-cellulose and eluted with a buffered KCl gradient, only one peak of EF-2 eluted from the

column (16). When the EF-2 fraction from the DEAE-cellulose column was pooled, [^3H] ADP ribosylated and analyzed by SDS-polyacrylamide gel electrophoresis compared to unfractionated S-150, the results illustrated in Fig. 6 were obtained. These results show that during (or subsequent to) DEAE-cellulose fractionation new polypeptides appear which accept ADPR in the diphtheria toxin-dependent reaction. These polypeptides have molecular weights of 41,000 and 36,000

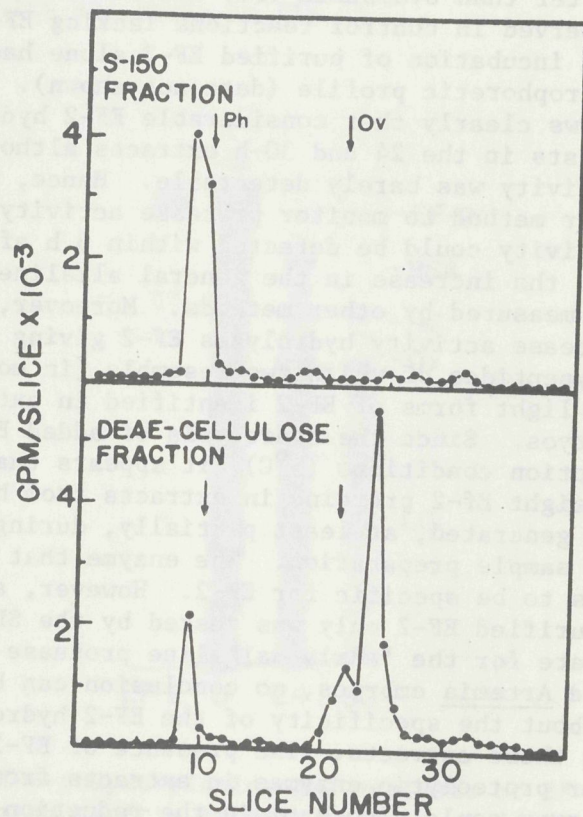


Fig. 6. SDS-polyacrylamide gel analysis of [^3H] ADP-ribosylated proteins from S-150 fraction before and after fractionation on DEAE-cellulose. The S-150 fraction from dormant cysts and the EF-2 fraction from the DEAE-cellulose column were radiolabeled in the toxin-NAD assay then analyzed on SDS-polyacrylamide gel slab. The gel was sliced and analyzed for radiolabeled polypeptides as described in Materials and Methods. The migration position of phosphorylase (Ph) and ovalbumin (Ov) is indicated.

compared to the untreated S-150 fraction which contains mainly the 95,000 MW species. Chromatography of the EF-2 fraction prepared by DEAE-cellulose on an analytical column of Sephadex G-150 also showed the presence of heavy and light EF-2 fractions in the DEAE-cellulose purified EF-2 peak supporting the idea that the DEAE-cellulose treatment and not incubation with the toxin-NAD mixture or preparation of the sample for electrophoresis leads to the formation of ADPR accepting EF-2 fragments. The light EF-2 proteins generated during DEAE-cellulose fractionation were found to be inactive in protein synthesis and in ribosome-dependent GTP binding (16). Thus, the polypeptides derived from EF-2 which are generated during DEAE-cellulose fractionation appear to be identical to the EF-2 fragments found in embryo extracts after hatching. These experiments suggest that EF-2 hydrolase activity is present in crude extracts of dormant cysts but sequestered by some type of regulatory mechanism which controls the metabolism of EF-2 during development.

From the *in vitro* and *in vivo* data it appears that the light forms of EF-2 found in extracts from hatched *Artemia* embryos may not represent the physiological situation in the intact embryo. If we assume that this idea is correct, what process permits the generation of EF-2 fragments to the extent observed in these extracts? Goldberg and Dice have suggested that proteins may accumulate breaks along their polypeptide chain which do not alter the protein structure or biological activity in the intact tissue (25). The existence of such a state in respect to EF-2 from hatched *Artemia* embryos could lead to the fragmentation of EF-2 upon extraction in the presence of a reducing agent such as DTT. However, since the breakdown of EF-2 to specific fragments was achieved during chromatography of protein fractions from dormant cysts on DEAE-cellulose, the possibility should not be overlooked that the homogenization process and/or extraction conditions enhance the breakdown of EF-2 in extracts from hatched embryos. Moreover, if the protease responsible for the hydrolysis of EF-2 is more active in *Artemia* nauplii than in cysts, the control of its activity may be lost during extraction of nauplii. Conversely, if EF-2 is more susceptible to proteolysis after hatching, the control of its degradation might be lost in extracts of nauplii. The existence of specific hydrolases which degrade protein once its conformation is changed has been reported (26). The latter mechanism occurs due to the interaction of various ligands with the protein which changes the sensitivity of the protein to proteases. For a similar reason EF-2 in extracts of hatched embryos or

after DEAE-cellulose fractionation might be susceptible to EF-2 hydrolase.

Further studies will be needed to elucidate the importance, if any, of the sudden increase in the light forms of EF-2 in extracts of hatched *Artemia* embryos. However, the striking similarities between the proteolysis products of EF-2 which appear during development of *Artemia* and during aging of rat liver EF-2 in vitro (11) suggest that there may be a general process of EF-2 metabolism in animal cells. In the study of rat liver EF-2 Collins et al. observed the breakdown of EF-2 into 64,000, 42,000 and 37,000 MW fragments following aging of highly purified EF-2 for one month at 0-4°C (11). Although none of the fragments of rat liver EF-2 were active in protein synthesis, the two low molecular weight fragments accepted ADPR in the diphtheria toxin-dependent reaction similar to that observed by us with *Artemia* EF-2. These results together with others not reported here support the view that the degradation process of EF-2 may be conserved during evolution.

The importance of a protein-degrading process in the control of development has been generally acknowledged (25). However, the significance of the degradation process in the control of protein synthesis has been largely ignored by most workers. It is clear that further studies are needed in this area to ascertain whether control of protein synthesis occurs at the elongation stage and whether this control involves degradative activities.

ACKNOWLEDGEMENTS

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A RELEASE FACTOR FROM THE CYST OF *ARTEMIA SALINA*: CHARACTERISATION AND ACTIVITY ON STAGE SPECIFIC RIBOSOMES

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ABSTRACT

A release factor has been isolated and partially purified from the cyst of *Artemia salina*. The factor recognises all three termination codons, its activity is stimulated by GTP and it exhibits a ribosome-dependent GTPase activity. The factor functions significantly better on salt washed ribosomes from later stages of development. Both cyst and prenauplius ribosomes increase their activity for *in vitro* termination by further salt washing although cyst ribosomes are much more sensitive to denaturation by high salt. Ribosomes from each stage of development show no differences in their peptidyltransferase activities, capacity to bind a model peptidyl-tRNA at the P site or to bind artificial mRNA; three known requirements for release factor-mediated *in vitro* termination.

INTRODUCTION

To date the rabbit reticulocyte is the only eukaryotic cell from which a polypeptide release factor (RF) has been at all well characterised (1). Activities have been partially purified from guinea pig liver, Chinese hamster liver (2) rabbit liver (3) and *Tenebrio molitor* (4). In addition a preliminary finding of a stimulatory factor in rabbit reticulocytes has been reported (1). The reticulocyte release factor is markedly different from the corresponding bacterial factors (RF-1 or RF-2). For example the bacterial factors are single polypeptides and each recognises two of the three possible termination codons (RF-1, UAA, or UAG; RF-2, UAA or UGA) (5) whereas the reticulocyte factor is a dimeric protein and all three termination codons are recognised at the same site (6). While there is evidence that GTP affects the interaction of the bacterial factors with the ribosome indirectly through the stimulatory factor, RF-3 (7), the reticulocyte factor itself exhibits a ribosome independent GTPase activity (2). In this case, GTP and its hydrolysis appear to be involved directly in the interaction of RF with the ribosome (6).

Although the rabbit reticulocyte is a good source for the purification of the release factor, low yields are obtained and relatively large numbers of animals need to be processed for each preparation. We therefore felt that the brine shrimp because of its abundance might be a good alternative source to study the overall mechanism of peptide chain termination, and the various factors in higher organisms which influence the event.

MATERIALS

Artemia salina dehydrated cysts were marketed as San Francisco Bay Brand, a division of Metaframe Corporation, Newark, Calif 94560. The isotopes were purchased from Amersham/Searle, purified tRNA^{Met} from Boehringer Mannheim, UAA and AUG from Miles Laboratories, and PolyU and Poly(U,G,A) and micrococcal nuclease from Sigma. The *E. coli* release factors RF1, and RF-2, and the reticulocyte release factor were purified as previously described (8, 1).

METHODS

Development of *Artemia salina* embryos. Dry cysts were pretreated with 1%(v/v) NaOCl and after extensive washing incubated in 4%(w/v) NaCl (500 ml/10g cysts) with vigorous aeration at 27°C. Prenauplius embryos appeared after about 16h and free swimming larvae at 22h.

Cell-free Extracts and Ribosomes of *Artemia salina*. The cell free fractions were prepared from various stages of development as described (9). Essentially, washed cysts or developing embryos were ground with sand in a buffer of 20mM Hepes, pH 7.6, 70mM KCl, 9mM Mg(OAc)₂, 0.1mM EDTA, 1mM DTT, 5%(v/v) glycerol. An S30 was obtained after filtration through cheese cloth, centrifugation of the filtrate at 15,000xg for 20 min, filtration of the supernatant through glass wool and further centrifugation of this extract at 30,000xg for 30 min. The ribosomes were recovered by sedimentation at 100,000xg for 3.5h. From the resulting supernatant the 0-75% (NH₄)₂SO₄ fraction was obtained and resuspended in and dialysed extensively against 20mM Hepes, pH 7.6, 50mM KCl, 0.1mM EDTA, 1mM DTT, 5%(v/v) glycerol. This is routinely referred to as S-100. The crude ribosome pellet was suspended with gentle stirring at 4° in a small volume of a buffer, 20mM Hepes, pH 7.6, 0.5M KCl, 5mM Mg(OAc)₂, 0.1mM EDTA, 2mM DTT, 5%(v/v) glycerol, pretreated with 0.1% diethylpyrocarbonate. The suspension was layered over the same buffer but containing 30%(w/v) sucrose and 0.1M KCl and centrifuged at 86,000xg for 12h. The clear

colourless pellet was suspended in the above buffer (0.1M KCl) to a concentration of about 200-300A₂₆₀. Any insoluble denatured material was removed by centrifuging at 12,000xg for 20 minutes. In some cases the ribosome wash solution (0.5M KCl or 1.0M KCl or 1.5M KCl) was recovered after sedimenting the ribosomes at 100,000xg for 3.5h. In other cases it was recovered as the solution above the sucrose layer after sedimenting the ribosomes through sucrose as described above.

Synthesis of Globin on Reticulocyte Polysomes with Cyst Extracts. Each 0.1ml of reaction mixture contained in addition to the components necessary for protein synthesis 1A₂₆₀ of reticulocyte polysomes, 1086 μ mol of [³H]leucine (2026 cpm/ μ mol) and 400 μ g of S-100 from *Artemia* cyst extracts. After 45min incubation at 30° the products were separated from the polysomal material by sedimentation through 10% sucrose. The released proteins from the top of the tube were precipitated with 10% TCA containing 20 μ M leucine and washed with 70% ethanol. The sample was analysed by electrophoresis in 20% SDS-polyacrylamide gels (2mA/gel). The gels were cut into 1mm sections digested with 30%(w/v) hydrogen peroxide at 50° for 3h. The radioactivity released was determined.

Purification of the *Artemia salina* Release Factor. The purification is as described (10). Cysts were broken in a Manton Gaulin Laboratory Homogenizer in 10mM Tris-HCl pH 7.6, 50mM KCl, 9mM MgCl₂, 1mM DTT, 5%(v/v) glycerol, debris removed by centrifugation, and a 30-75% ammonium sulphate fraction of the 100,000xg supernatant prepared. This fraction was applied to a column of DEAE Sephadex A50 (50 x 2.5cm), and eluted with 500ml of the extraction buffer, containing a KCl gradient (50-600mM). The fractions containing the major GTP binding activity were applied to phosphocellulose (30 x 1.5cm) and eluted with 150ml of the same buffer containing a KCl gradient (50-400mM).

Assay for the Release Factor. The RF activity was measured on a f[³H]Met-tRNA^{Met}/AUG/ribosome substrate as described (15). The salt washed (0.5M KCl) ribosomes were either derived from cyst or 18h prenauplius embryos of *Artemia*. A typical reaction would contain within 0.05ml: 2-4 μ mol of substrate, polynucleotide or oligonucleotide as indicated at a concentration of 2.0A₂₆₀, 0.1mM GTP and a buffer, 20mM Tris HCl, pH 7.5, 50mM KCl and a fraction containing release factor. The product of the reaction, f[³H]Met was extracted at pH 1 into ethyl

acetate. The reticulocyte release factor was assayed either with *Artemia* substrate or that prepared with reticulocyte ribosomes. *E. coli* release factor was assayed in the absence of GTP under conditions previously described (8).

Assay for "Interfering" Activity "A". About 5-10 μ mol of f[³H]Met-tRNA (~3000 cpm/ μ mol) was hydrolysed by heating at 90° for 20 minutes. The products were incubated at 30° for 10 minutes with a fraction containing "A" activity in a buffer, 20mM Tris HCl, pH 7.5, 50mM KCl, or buffer alone. The f[³H]Met remaining was extracted into ethylacetate at pH 1. When the products of the incubation of f[³H]Met with "A" were to be further analysed they were applied to a Sephadex G-25 column (8cm x 0.5cm) and eluted in water. The fractions corresponding to the free amino acid were pooled and lyophilised. Samples in deionised water were applied to Whatman 3MM in the centre of a strip and electrophoresed for 3h at 2000v in 3% pyridine, 3% acetic acid, pH 3.5 (11).

The areas containing radioactivity were located with a strip scanner and then cut into 10mm pieces and the radioactivity analysed in a toluene based scintillant.

In vitro Translation of PolyU with cell-free components. The reaction mixture contained typically within 0.1ml: 20mM Hepes, pH 7.6, 1.75 μ g spermidine, 2.4mM DTT, 1mM ATP, 0.3mM GTP, 100mM KCl, 7.5mM Mg(OAc)₂, 9.5mM phosphocreatine, 6.0 μ g creatine kinase, 380 μ moles [³H]phenylalanine (1970 cpm/ μ mole), 0.02mM of each of the remaining amino acids, polyU at a concentration of 0.4A₂₆₀, *Artemia salina* ribosomes at a concentration between 1-10 A₂₆₀ and S-100 (generally about 200-250 μ g protein). The reaction mixture containing all the above components except [³H]phenylalanine and poly(U) was preincubated for 20 minutes at 30°C to "run off" ribosomes from endogenous mRNA. After the addition of the isotope and Poly(U), incubation was for a further 30 minutes at 30°C and the radioactivity insoluble in 10%(w/v) TCA (heated for 20 minutes at 90°) was determined.

Assay of [³H]PolyU binding capacity of *Artemia salina* ribosomes. The method used was that of Moore (1966) (12). Ribosome samples (0.2A₂₆₀) were diluted to 3.0ml in 10mM MgCl₂, 5mM Tris-HCl, pH 7.4, and transferred onto Millipore filters under gentle suction. Filters were then washed successively with 5mg of BSA and [³H] PolyU

(102 pmoles, 297 cpm/pmole) both in the same buffer. The dried filters were counted in a toluene based scintillant.

Binding of f[³H]Met-tRNA to ribosomes and Assay of Peptidyl transferase Activities. The binding of f[³H]Met-tRNA to *Artemia* ribosomes and peptidyltransferase activities were determined essentially as described for reticulocyte ribosomes (13). However, salt conditions giving maximum binding to *Artemia* ribosomes, were markedly different. 13 pmoles of f[³H]Met-tRNA, (2460cpm/pmol) approximately 2.0A₂₆₀ of ribosomes, 0.02A₂₆₀AUG, and a buffer, 50mM Tris-HCl, pH 7.0, 20mM MgCl₂, 50mM NH₄Cl in a volume of 25μl were incubated 30° for 30 minutes. The bound f[³H]Met-tRNA/AUG/ribosome complex was determined on Millipore filters.

To test for peptidyl transferase activity samples of the above reaction mixture containing 2-3 pmoles of f[³H]Met-tRNA/AUG/ribosome were incubated in 50μl with 12μM puromycin, and the same buffer, at 30° for 30 minutes (or 5-10 minutes if rate measurements were required).

Treatment of Inhibitor fractions with Micrococcal Nuclease. Components required to translate PolyU, with the exception of the artificial mRNA, and an amount of an inhibitor fraction giving about 50% inhibition were preincubated with 1-10μg of micrococcal nuclease (0.2 units/μg) and 1mM CaCl₂ at 30° for 20 minutes. At the end of the preincubation 2mM EGTA was added along with PolyU and the reaction mixture incubated for a further 30 minutes at 30°C. This treatment with micrococcal nuclease was essentially as described by Pehlam and Jackson (14). When the inhibitor fraction was to be tested in termination reactions it was pretreated with micrococcal nuclease and CaCl₂ as described before the mixture was added along with EGTA to the *in vitro* termination assay.

RESULTS AND DISCUSSION

A natural mRNA cannot be translated by extracts of the cyst of *Artemia salina* because the cysts are deficient in at least one factor essential for the initiation of synthesis of proteins (16). Consequently it was not evident whether cyst extracts contained the components necessary to correctly terminate the synthesis of proteins. We tested this by following the synthesis and release of rabbit globin from isolated reticulocyte

polysomes which had been washed free of factors. As indicated in Fig. 1 the cyst extract catalysed the release of a product of Mr identical with that of rabbit globin isolated from reticulocyte lysates. The rate of synthesis and release of globin was similar if the *Artemia* extract was from the cyst or prenauplius. These data suggested that not only was the termination components non-limiting in the cyst compared with later stages of development, but also that the *Artemia* release factor was likely to be similar to the reticulocyte factor since it could function efficiently on reticulocyte ribosomes (9).

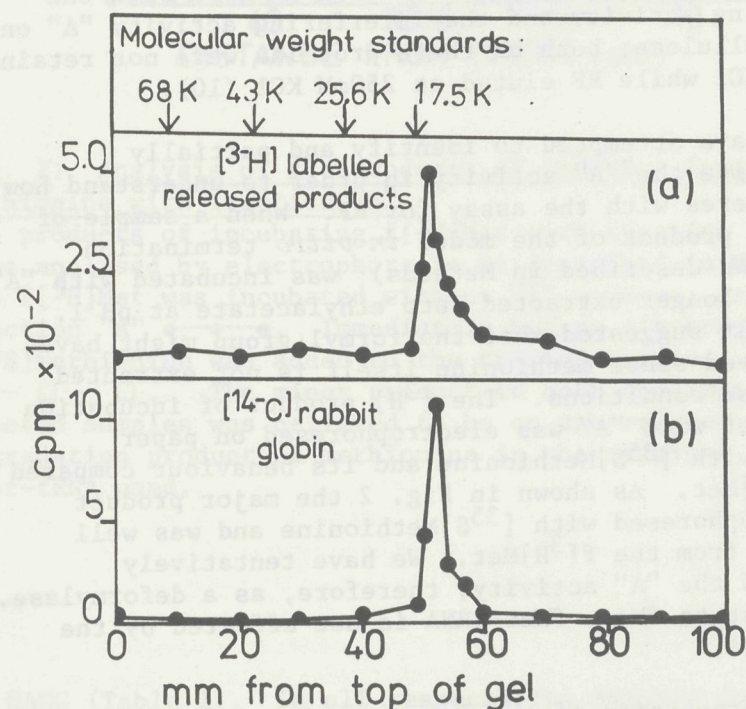


Fig. 1. Analysis of the globin products synthesised on reticulocyte polysomes with *Artemia* cyst extract. The synthesis of globin and its isolation are described in Methods. This sample, ¹⁴C-labelled rabbit globin isolated from a reticulocyte lysate, and a standard protein solution containing 5μg of each of bovine serum albumin (M_r 68,000), ovalbumin (M_r 43,000) chymotrypsinogen (M_r 25,700) and myoglobin (M_r 17,500) were electrophoresed on 20% SDS-polyacrylamide gels. Analysis of the gels is described in Methods.

It was not possible to assay a release factor in extracts of the *Artemia salina* cysts initially for two reasons. Firstly cyst ribosomes support *in vitro* termination poorly as described below, and secondly an activity ("A") which interferes with the analysis of the product of the release assay copurifies with the factor until a late state of the purification. Attempts to rearrange the purification protocol were unsuccessful because the substrate was destroyed non-specifically by other contaminating proteins. For this reason the major GTP binding protein, presumed to be EF-1, was used as a marker protein until the release factor could be detected. In the purification of the rabbit reticulocyte factor, EF-1 cochromatographs with RF until a late step. The *Artemia* release factor was resolved from both the GTP binding activity and the interfering activity "A" on phosphocellulose; both of these proteins were not retained at 50mM KCl while RF eluted at 250mM KCl (10).

We have attempted to identify and partially characterise the "A" activity in order to understand how it interferes with the assay for RF. When a sample of fMet (the product of the model *in vitro* termination reaction as described in Methods) was incubated with "A" it was no longer extracted into ethylacetate at pH 1. This result suggested that the formyl group might have been removed since methionine itself is not extracted under these conditions. The [^3H] product of incubation of f[^3H]Met with "A" was electrophoresed on paper together with [^{35}S]Methionine and its behaviour compared with f[^3H]Met. As shown in Fig. 2 the major product co-electrophoresed with [^{35}S]Methionine and was well separated from the f[^3H]Met. We have tentatively identified the "A" activity, therefore, as a deformylase. In contrast to fMet, fMet-tRNA is not affected by the deformylase.

The properties of the *Artemia* release factor have been investigated and compared with those of the reticulocyte and *E. coli* factors (Table 1). The *Artemia* RF (Column A) requires an oligonucleotide or random copolymer containing possible terminator codons for its activity and will not respond to an artificial mRNA from which it is not possible to generate such codons. However, it requires a minimum of tetranucleotide in the *in vitro* termination assay. All three termination codons are recognised by the factor. At several concentrations UAAA or UGAA support *in vitro* termination better than UAGA

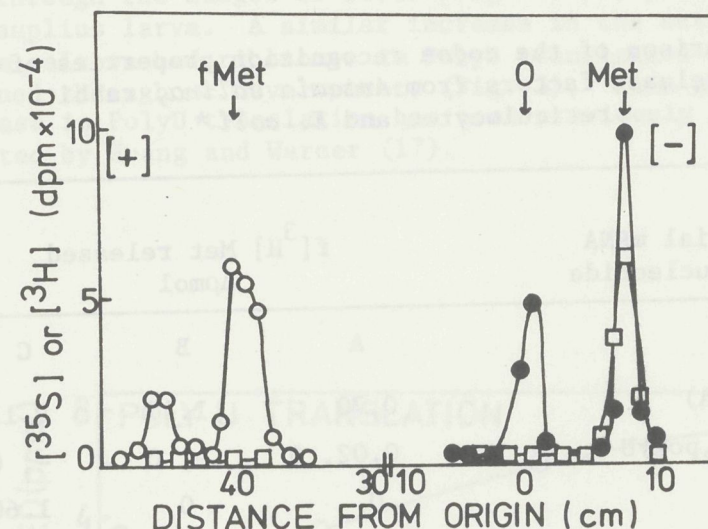


Fig. 2. Analysis of the products of treating formyl methionine with fraction "A".

The products of incubating f[^3H]Met with fraction "A" were analysed by electrophoresis as described in Methods. The f[^3H]Met was incubated without "A" $\circ-\circ-\circ$, or with fraction "A" $\bullet-\bullet-\bullet$. Immediately before electrophoresis [^{35}S]methionine was added to the treated sample $\square-\square-\square$. The minor product in both the fMet and treated samples was believed to be an age-related degradation product of methionine in the original fMet-tRNA used.

or UAGG (Table 1). In all respects the *Artemia* factor resembles the reticulocyte factor (Column B) and differs in codon recognition properties from the *E. coli* component RF-2 (Column C).

The *Artemia* factor is stimulated 4-fold by GTP and it exhibits a ribosome dependent GTPase activity which is enhanced by artificial mRNA. Although Poly(U,G,A) was most effective, artificial mRNAs not containing terminator codons also raised the RF dependent GTPase activity. These properties indicate that the *Artemia* RF closely resembles the codon recognition factor from rabbit reticulocytes.

Table 1

A comparison of the codon recognition properties of the release factors from *Artemia salina*, rabbit reticulocytes and *E. coli**

Artificial mRNA or oligonucleotide	f[³ H] Met released Δpmol		
	A	B	C
Poly(U,G,A)	0.70	1.47	0.12
Poly(U,G),polyU	0.02, 0	-, 0	-, 0
UAA	0	0	1.60
UAAA	0.55	1.93	1.42
UGAA	0.64	2.21	2.19
UAGA	0.29	0.84	0
UAGG	0.15	0.59	0

*Each reaction contained within 0.05ml: 3.6pmol f[³H]Met-tRNA/AUG/ribosome substrate, the polynucleotides at a concentration of 2A₂₆₀, the oligonucleotides at a concentration of 1.2A₂₆₀, and amounts of either *Artemia* RF (Column A), reticulocyte RF (Column B) or *E. coli* RF-2 (Column C). Incubations were for 5-20 minutes as appropriate to measure the release of f[³H]Met at the initial rate. The f[³H]Met extracted in the absence of termination codon was subtracted in each case.

We used cyst salt-washed ribosomes in our initial unsuccessful attempts to assay for the release factor. However, when ribosomes from the prenauplius stage were used to prepare the f[³H]Met-tRNA-AUG-ribosome substrate an increase in the release factor activity of up to ten-fold was detected over a range of concentrations of the factor. This result suggested that the factor involvement in the termination reaction was limited by a property of the cyst ribosomes, and that the termination activity at least *in vitro* might be stage specific. We have investigated the activity of ribosomes throughout development with the *Artemia* release factor. As shown in Fig. 3 there is a gradual increase in activity from the

cyst through the stages of developing embryo, prenauplius and nauplius larva. A similar increase in the activity of the salt-washed ribosomes in PolyU translation is obtained throughout development, (Fig. 3). Such an increase in PolyU translation has been previously reported by Huang and Warner (17).

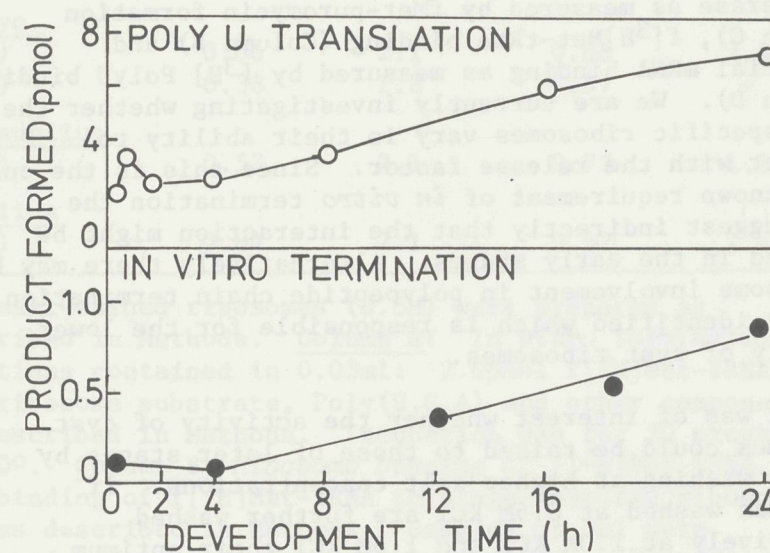


Fig. 3. Activity of stage-specific ribosomes in PolyU translation and *in vitro* termination.

Salt-washed ribosomes (0.5M KCl) were isolated from various stages of development of *Artemia salina*. Their relative activities were determined in PolyU translation by the procedure described in Methods. Ribosomal substrate for *in vitro* termination, f[³H]Met-tRNA/AUG/ribosome was prepared with 2A₂₆₀ ribosomes from each stage. *In vitro* termination was assayed with Poly(U,G,A) as described in Methods.

The known requirements of polypeptide chain termination *in vitro* include an active ribosomal peptidyl transferase, correct positioning of the model peptidyl-tRNA at the ribosome "P" site, ribosome binding of the artificial mRNA or oligonucleotide containing the terminator codon and a functional interaction between the release factor and the ribosome. As shown in Table 2 while there are significant differences in the overall *in vitro* termination event (Fig. 3 and Table 2, Column A) there seems to be no significant differences in the activities of ribosomes isolated throughout development for the first three requirements, that is, peptidyl-transferase as measured by fMet-puromycin formation (Column C), f[³H]Met-tRNA binding (Column B) and artificial mRNA binding as measured by [³H] PolyU binding (Column D). We are currently investigating whether the stage specific ribosomes vary in their ability to interact with the release factor. Since this is the only other known requirement of *in vitro* termination the data suggest indirectly that the interaction might be impaired in the early stages. Alternatively there may be a ribosome involvement in polypeptide chain termination not yet identified which is responsible for the lower activity of cyst ribosomes.

It was of interest whether the activity of cyst ribosomes could be raised to those of later stages by further washing at higher salt concentrations. If ribosomes washed at 0.5M KCl are further washed successively at 1.0M KCl and 1.5M KCl their optimum activity in PolyU translation increases 2-fold with each treatment. However, this increase in activity is not restricted to cyst ribosomes but is also found with ribosomes isolated at other stages throughout development. The rate of *in vitro* termination is also raised with the further washing of the ribosomes at the high salt concentrations. However, ribosomes treated with 1.5M KCl in particular bind fMet-tRNA with lower efficiency and as a result of the poor substrate a less dramatic increase in release factor mediated termination is observed.

We have tested the effects of the wash fractions on PolyU translation and on *in vitro* termination. Both partial events are significantly inhibited to a similar degree by the fraction removed from the ribosome at 0.5M KCl or 1.0M KCl. Moreover the wash fractions obtained from ribosomes throughout development also inhibit PolyU

Table 2.

In vitro termination reactions with *Artemia salina*
Stage-Specific Ribosomes

Stage	A (Δpmol)	B (Δpmol)	C (Δpmol)	D (cpm×10 ⁻³)
<u>Cyst</u> (0h)	0.12	1.8	0.45	1.8
<u>Embryo</u> (4h)	0.06	2.1	0.68	2.4
(12h)	0.38	1.8	0.57	-
<u>Prenauplius</u> (18h)	0.53	2.1	0.73	1.6
<u>Nauplius</u> (24h)	0.84	2.1	0.52	1.4

The salt-washed ribosomes (0.5M) were prepared as described in Methods. Column A: *In vitro* termination. Reactions contained in 0.05ml: 2.0pmol f[³H]Met-tRNA/AUG/ribosome substrate, Poly(U,G,A) and other components as described in Methods. Incubation was for 20 minutes at 30°. Column B: Ribosome binding of f[³H]Met-tRNA. The binding of f[³H]Met-tRNA to stage-specific ribosomes was as described in Methods. Samples of 5μl were analysed. Column C: Peptidyl transferase. Peptidyl-transferase activity was measured by the formation of fMet-puromycin as described in Methods. Incubations were for 10 minutes at 30°. Column D: Binding of artificial mRNA. The binding of PolyU to stage-specific ribosomes was determined with [³H]Poly U as described in Methods. The [³H]Poly U bound in the absence of ribosomes, 400 cpm, was subtracted in each case.

translation and *in vitro* termination although those obtained from equivalent amounts of ribosomes at the later stages (pre-nauplius, nauplius) are somewhat less potent. It is of interest that the inhibitory fraction does not affect the ribosomal peptidyltransferase, the ribosomal binding of PolyU or the binding of fMet-tRNA at the ribosome "P" site, three of the requirements for *in vitro* termination (see Table 2).

The nature of the inhibitor or inhibitors in the wash fraction have been investigated. To test for the possibility of an inhibitory RNA we have used the approach of Pehlam and Jackson (14) who utilised micrococcal nuclease to prepare a mRNA dependent reticulocyte lysate. In our case, the components required to translate PolyU and the inhibitor fraction were together preincubated with micrococcal nuclease, the Ca^{2+} dependent nuclease was then inactivated with the chelating agent EGTA, and PolyU was added for *in vitro* translation. The general conclusion is that the micrococcal nuclease could destroy most of the inhibitory activity of the ribosome wash fraction. As shown in Table 3, the nuclease was active since it destroyed PolyU if it were included in the preincubation. However, the nuclease was inactivated by addition of EGTA at the end of the preincubation; addition of PolyU together with EGTA gave a level of incorporation of [^3H] Phe comparable with that obtained in the absence of

Table 3.

Sensitivity of Inhibitor to Micrococcal Nuclease		
Preincubation	Incubations	[^3H]Phe Incorporated cpm
S-100, Ribosomes	-	450
S-100, Ribosomes	PolyU	4850
S-100, Ribosomes Nuclease, CaCl_2 , PolyU	EGTA	450
S-100, Ribosomes Nuclease, CaCl_2 , PolyU	EGTA, PolyU	4750
S-100, Ribosomes Inhibitor	PolyU	2600
S-100, Ribosomes Inhibitor Nuclease CaCl_2	EGTA, PolyU	4100

Each reaction contained the components in the preincubation mixture as described in Methods, and in addition where indicated, micrococcal nuclease (2 μg), CaCl_2 (1mM) and an amount of inhibitor fraction sufficient to give about 50% inhibition. PolyU and EGTA (2mM), where indicated, were added during the subsequent incubation. Preincubation was for 20 minutes at 30° and the incubation 30 minutes at 30°.

nuclease. The inhibition of PolyU translation by the cyst ribosome wash fraction (0-1.2M KCl) was significantly relieved by the nuclease treatment in the preincubation (Table 3). These data suggest that at least one inhibitory component in the wash fraction is an RNA. Moreover, this conclusion is reinforced since the inhibitor fraction was stable to heating at 80°C for 20 minutes. The inhibitory activity was lost on dialysis although it could not be reconstituted from the dialysis fluid.

The nature of the inhibitors of *in vitro* termination has proved more difficult to define. Preliminary experiments with the concentrated 0.5M KCl ribosome wash fraction indicate that one of the activities in this fraction is the "A" activity described above which was tentatively identified as a deformylase (Fig. 2). This clearly gives an apparent inhibition of *in vitro* termination by altering the product of the reaction. The presence of this deformylase indicates that it will be necessary to fractionate the ribosome wash components for further characterisation of other possible inhibitors.

Lee-Huang *et al.*, (1977) have reported the isolation of oligoribonucleotides affecting mRNA translation from extracts of *Artemia*. An inhibitor oligonucleotide was characterised as affecting EF-1 dependent aminoacyl-tRNA binding whereas another oligonucleotide found in developing embryos neutralised this inhibitor. These authors raise the interesting question whether these "oligonucleotide pairs" might act as a translational repressor-derepressor and be responsible for the onset of protein synthesis after hydration (18). Warner *et al.*, (1977) have partially characterised a heat labile protein associated with the large subunits of ribosomes from the cyst but not later stages which inhibits PolyU translation. This is of particular interest since it binds to GTP (19). Such an inhibitor might affect the function of the release factor whose ribosome associated activity probably involves an interaction with GTP. For example, both inhibitor and release factor might interact with a similar site associated with a GTP site on the ribosome.

During our studies on the effects of successive high salt washing (0.5M to 1.0M KCl and 1.0M KCl to 1.5M KCl) on the activity of ribosomes we discovered that the recovery of ribosomes varied dramatically with the stage from which the ribosomes were isolated. Similar amounts of ribosomes were recovered from each stage after 0.5M KCl treatment. After 1.0M KCl treatment ribosomes from

the cyst or early developing embryo were extensively denatured and recovered in low amounts (Fig. 4). In contrast those ribosomes from the late developing embryo and prenauplius (12h, 18h) suffered little denaturation and were almost completely recovered (Fig. 4). Ribosomes, 1.0M KCl washed, from all stages were extensively denatured when exposed to 1.5M KCl but those from the later stages were recovered in higher amounts. These studies suggest that in addition to the inhibitors which may be associated with the ribosomes in the cyst, there may be differences in structure or conformation between these cyst ribosomes and those at later stages of development. We are continuing studies to investigate this possibility further.

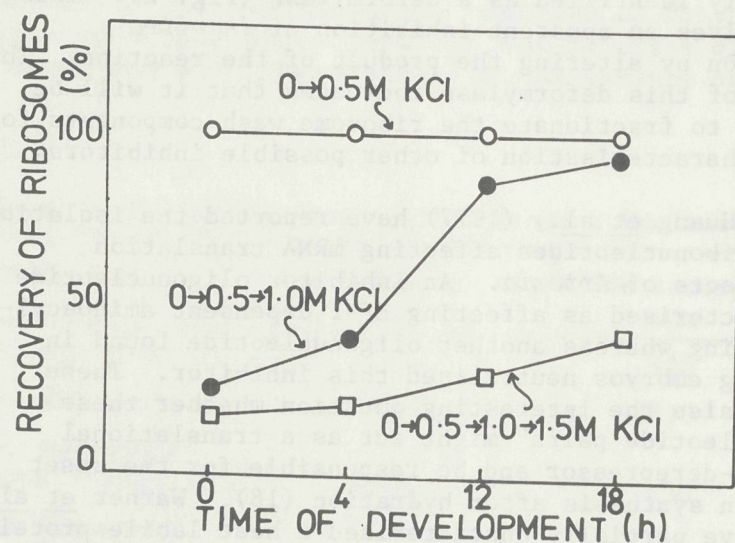


Fig. 4. Recovery of stage-specific ribosomes after high-salt washing.

Ribosomes were isolated from 50g of cysts, developing embryos (4h and 12h) and prenauplius larva (18h). One third of each preparation was washed with buffer containing 0.5M KCl, another third successively washed with 0.5M KCl and then 1.0M KCl and the remaining washed successively with 0.5M KCl, 1.0M KCl and 1.5M KCl. The recoveries of the ribosomes treated with two higher salt concentrations were compared with those of the 0.5M KCl washed ribosomes in each case. The amount of these ribosomes recovered were very similar at each stage of development.

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PARTIAL CHARACTERIZATION OF A PROTEIN SYNTHESIS INHIBITOR FROM ARTEMIA CYSTS

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ABSTRACT

In a previous study we described a procedure for the partial purification of a protein synthesis inhibitor from both the cytosol and ribosome fractions of Artemia cysts. In the present study we have attempted to characterize the inhibitor in the ribosome-rich P-150 fraction, and we found that Artemia cysts contain at least two populations of elongation factor 2 (EF-2), one which is inhibitory and one which is fully functional. The functional EF-2 is "tightly bound" to the P-150 fraction while the inhibitory EF-2 is "loosely bound" to the P-150 fraction. Based on similar EF-2 content loosely bound EF-2 is 15-20 times more inhibitory than tightly bound EF-2 and this ratio remains nearly constant following chromatography of these fractions on hydroxyapatite and Sephadex G-150. The inhibitor effect is not modified by ADP-ribosylation of EF-2, but it is abolished by treatment with NEM and pCMB. Our results suggest that the protein synthesis inhibitor in Artemia cysts is a modified form of EF-2.

INTRODUCTION

During the past few years two studies have shown that cell-free systems derived from undeveloped cysts of the brine shrimp, Artemia salina, are unable to translate natural mRNA's (1,2). Moreover, cell-free systems derived from cysts are much less active than their developed counterpart in poly(U) translation (3). The exact reasons for the depressed translation capacity of extracts of the undeveloped system are unknown but we have considered the possibility that the low translational capacity of the undeveloped system is due to the presence of an inhibitor(s) of protein synthesis in the cysts (3,4). Previously, we purified an inhibitor of poly(U) translation about 20-fold from both the cytosol and ribosome-rich fractions of Artemia cysts, and we identified the inhibitor to be a heat-labile, trypsin-sensitive substance with properties similar to elongation factor 2 (4). In the results reported here we present additional data to support the view that the protein synthesis inhibitor protein in Artemia cysts is a modified form of elongation factor 2.

MATERIALS AND METHODS

The encysted embryos of the brine shrimp, Artemia salina, used in this study were from the Utah salterns. They were purchased from Longlife Fishfood Products, Harrison, N.J. in 1970 and stored since that time at -15°C. The cysts give rise to about 62% swimming nauplii after incubation in hatching sea water at 30°C (5).

The in vitro assay for the inhibition of polyphenylalanine synthesis was similar to that reported previously with slight modification (4). Except for assays of column fractions, all reaction mixtures contained the following in 0.3 ml final volume: Tris-HCl, pH 7.3 (23°C), 50 mM; KCl, 100 mM; MgCl₂, 10 mM; EDTA, 0.1 mM; 2.5% glycerol; DTT, 1 mM; ATP, 2 mM; GTP, 0.8 mM; creatine phosphate, 2 mM; creatine phosphokinase, 25 µg; deacylated Artemia cyst tRNA, 1 A₂₆₀ unit; poly(U), 25 µg; [¹⁴C] phenylalanine, 0.25 µCi (specific activity 486 Ci/mol); crude cyst ribosomes (P-150 fraction), 1 A₂₆₀ and inhibitor or EF-2 fractions (or buffer in controls) at various concentrations as indicated in the figures and tables. The assays of column fractions were conducted using 0.15 ml reaction mixtures at the same concentration of components as described above. All assays were performed at 30°C and the reactions were initiated by the addition of the crude ribosome preparation from cysts which contains sufficient ribosomes, phenylalanyl-tRNA synthetase and elongation factors to support a linear rate of [¹⁴C] phenylalanine incorporation into polypeptide for at least 60 minutes. It should be noted, however, that the activity of the crude ribosome preparation is depressed somewhat due to the presence of the inhibitor in this fraction. At the desired incubation times 0.05 ml was removed from each assay vessel and processed for protein synthesis as described by Mans and Novelli (6). The extent of [¹⁴C] polyphenylalanine synthesis in the presence of various protein fractions was compared to controls containing buffer in place of the column fractions and the results are expressed as percent inhibition.

The crude ribosome or P-150 fraction from Artemia cysts was prepared as described previously using buffer A as the homogenizing medium (4). Buffer A contained 50 mM Tris-HCl, pH 7.3 (23°C), 5 mM KCl, 10 mM MgCl₂ and 1 mM DTT. The ribosome-rich P-150 pellet obtained by ultracentrifugation was suspended in buffer B (similar to buffer A except that it contains 50 mM KCl and 25% glycerol) and either stored at -20°C or used immediately for the isolation of the protein synthesis inhibitor. The protein

synthesis inhibitor was released from the P-150 fraction by adjusting this fraction (in buffer B) to contain 0.75 M NH_4Cl and the soluble fraction obtained after centrifugation at 50,000 rpm (Beckman 60 Ti) was chromatographed on a column of Ultrogel AcA34 as described previously (4).

The EF-2 content of all preparations and column fractions was determined using the diphtheria toxin catalyzed [^{14}C] ADP ribosylation (of EF-2) assay of Goor and Maxwell (7). The diphtheria toxin was obtained from Cannaught Laboratories (Toronto) as a purified preparation (1800 Lf/ml) and [adenine-2,8- ^3H] NAD (specific activity 3.2 Ci/mmol) was purchased from New England Nuclear (Boston).

The electrophoretic analyses were carried out on 10% polyacrylamide gel slabs containing 0.1% SDS as described by Laemmli (8). All other chromatographic media and reagents were as described previously (4).

RESULTS

Chromatography of Protein Synthesis Inhibitor of P-150 Fraction on Columns of Ultrogel AcA 34 and Agarose-GTP. In earlier experiments we reported that the protein synthesis inhibitor in *Artemia* is distributed almost equally between the post-ribosomal fraction (S-150) and the ribosomal-rich sediment (P-150). Moreover, we demonstrated that the inhibitor in the ribosome-rich P-150 fraction could be solubilized by 0.75 M NH_4Cl and purified from the bulk protein by chromatography on Ultrogel AcA 34. In this study, the P-150 associated inhibitor from the first Ultrogel AcA 34 column was rechromatographed on an analytical column of AcA 34 in the presence of buffer C (50 mM Tris-HCl, pH7.3, 50 mM KCl, 0.6 mM EDTA and 10% glycerol) and the column fractions were analyzed for UV absorbance at 280 nm, inhibitor activity and EF-2 content. The results of this experiment are shown in Fig. 1 and indicate that the inhibitor and EF-2 co-elute from Ultrogel AcA 34. The inhibitor EF-2 mixture was concentrated by vacuum dialysis then chromatographed on an Agarose-hexane-GTP column. The results shown in Fig. 2 indicate that most of the EF-2 and inhibitor co-elute with the last UV-absorbing peak to elute from the column. The small amounts of EF-2 and inhibitor which elute early as heterogeneous fractions from the column are variable from experiment to experiment and may represent degradation products of EF-2. SDS-polyacrylamide gel analysis of the column fractions containing both EF-2 and inhibitor revealed only one major band of molecular weight about 95,000. Collectively, these results indicate that EF-2 from the P-150 fraction of *Artemia* cysts is inhibitory in an *in vitro* system optimized for polyphenylalanine

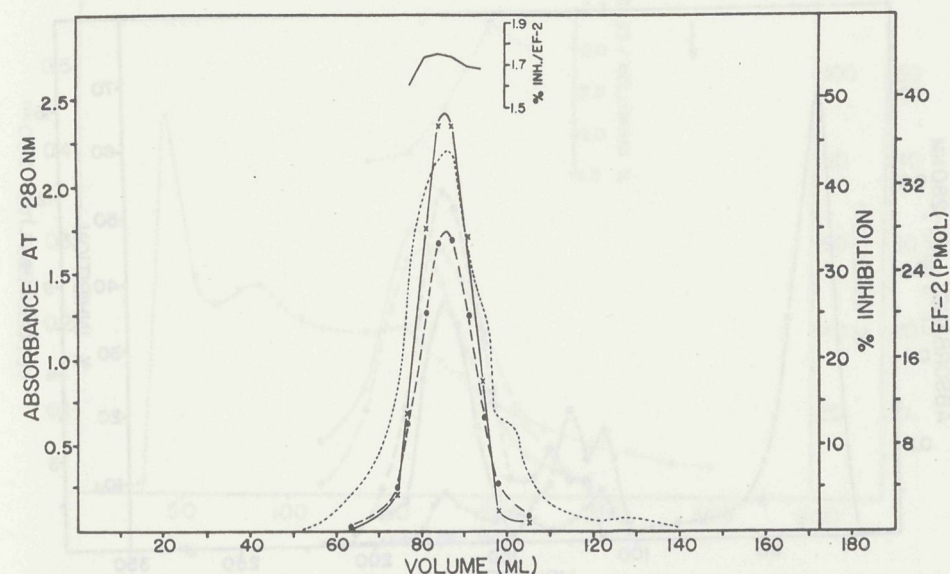


Fig. 1. Chromatography of inhibitor/EF-2 preparation on Ultrogel AcA 34. The total inhibitor/EF-2 fraction from the preparative AcA 34 column described in Materials and Methods was applied to an analytical column of Ultrogel AcA 34 (1.5 x 87 cm) equilibrated with buffer C (without glycerol) and eluted with this buffer. Selected column fractions were then analyzed for absorbance at 280 nm (-----), inhibitor activity (x — x) and EF-2 content (●----●) as described in Materials and Methods.

synthesis.

Chromatography of Artemia Cysts Protein Synthesis Inhibitor on Hydroxyapatite. Although the protein synthesis inhibitor from the P-150 fraction of *Artemia* cysts co-chromatographs with EF-2 on gel filtration and GTP affinity columns, partial resolution of the inhibitor activity and EF-2 protein has been achieved by chromatography on hydroxyapatite. The results shown in Fig. 3 indicate that the protein synthesis inhibitor activity in the partially purified salt wash of the P-150 fraction elutes slightly behind the EF-2 protein. These results contrast with those obtained by gel filtration and affinity chromatography and indicate that the inhibitor may not be EF-2 but some other closely related protein. However, an analysis of the column fractions throughout the EF-2/inhibitor region by SDS-polyacrylamide gel electrophoresis shows that the trailing edge of

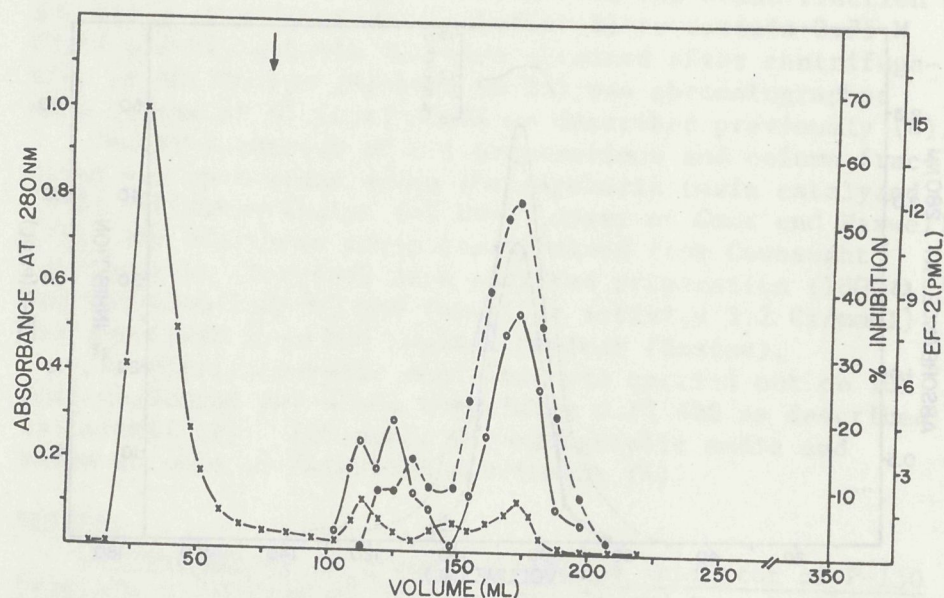


Fig. 2. Chromatography of inhibitor/EF-2 preparation on Agarose-GTP. The inhibitor/EF-2 fraction in Figure 1 was applied to a column (5 ml) of Agarose-GTP equilibrated with buffer C. The column was washed with 65 ml additional buffer C then developed with a 250 ml gradient of KCl (to 1 M) in buffer C. The arrow indicates the beginning of the KCl gradient. Selected column fractions were assayed for absorbance at 280 nm (x — x), inhibitor activity (o — o) and EF-2 content (● — ●).

of the EF-2 peak gives a more intensely staining EF-2 band with coomassie brilliant blue than could be measured by the diphtheria toxin assay for EF-2 (results not shown). These data together with those obtained previously suggest that perhaps a population of EF-2 exists which cannot be ADP-ribosylated, and that this population of EF-2 may be the form of EF-2 that is inhibitory in extracts of *Artemia* embryos. Alternatively, another protein with chromatographic properties very similar to EF-2 may be present which possesses the properties of the protein synthesis inhibitor.

Fractionation of Loosely Bound and Tightly Bound EF-2 on Sepharose 6B and the Inhibitor Activity of each EF-2 Preparation. In an attempt to ascertain whether subpopulations of EF-2 exist in the P-150 fraction of *Artemia* cyst,

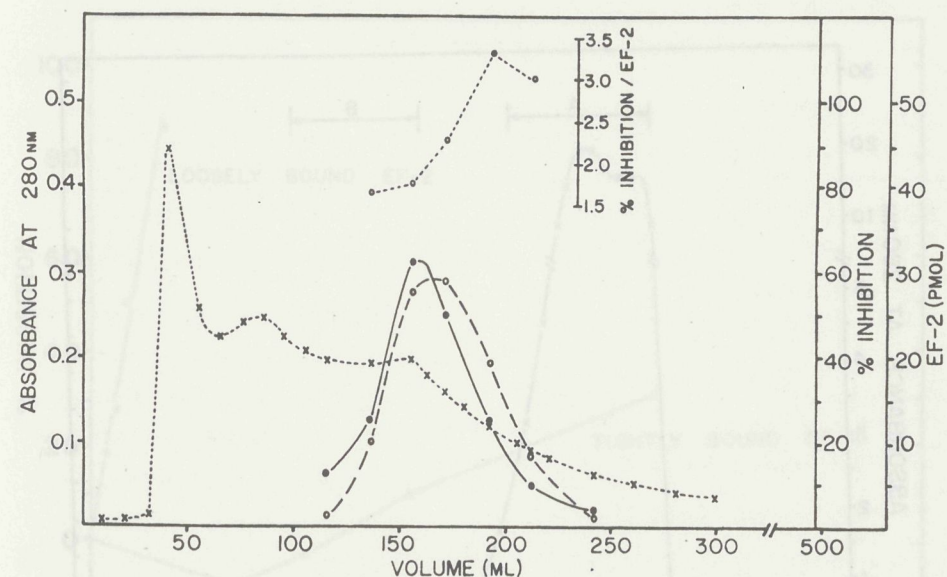


Fig. 3. Chromatography of inhibitor/EF-2 preparation on hydroxyapatite. The total inhibitor/EF-2 fraction from a preparative AcA 34 column was applied to a hydroxyapatite column (1 x 17 cm) equilibrated with 50 mM Tris-HCl, pH7.3 (23°C), containing 50 mM KCl and the protein was washed on to the column with 75 ml of this buffer. The column was developed with a 480 ml gradient of potassium phosphate, pH6.8, to a final concentration of 0.5 M phosphate. Selected column fractions were analyzed for absorbance at 280 nm (x — x), percent inhibition (o — o) and EF-2 content (● — ●) as described in Materials and Methods.

the crude P-150 fraction was suspended in buffer D (same as buffer A except that the buffer contains 100 mM KCl and 10% glycerol) and chromatographed on a column of Sepharose 6B using this buffer. The results of this experiment are shown in Fig. 4. Fraction A is rich in ribosomes and contains about 90% of the EF-2 and 40% of the inhibitor units of the crude P-150 fraction. However, the EF-2 content and inhibitor activity can be determined only after their release from the macromolecular fraction by washing with 0.75 M NH_4Cl . In contrast to the above, fraction B represents the loosely bound proteins in the P-150 fraction and contains 10% of the EF-2 and 60% of the inhibitor units of the crude P-150 fraction. The proteins released from fraction A by the NH_4Cl wash (tightly bound) and those

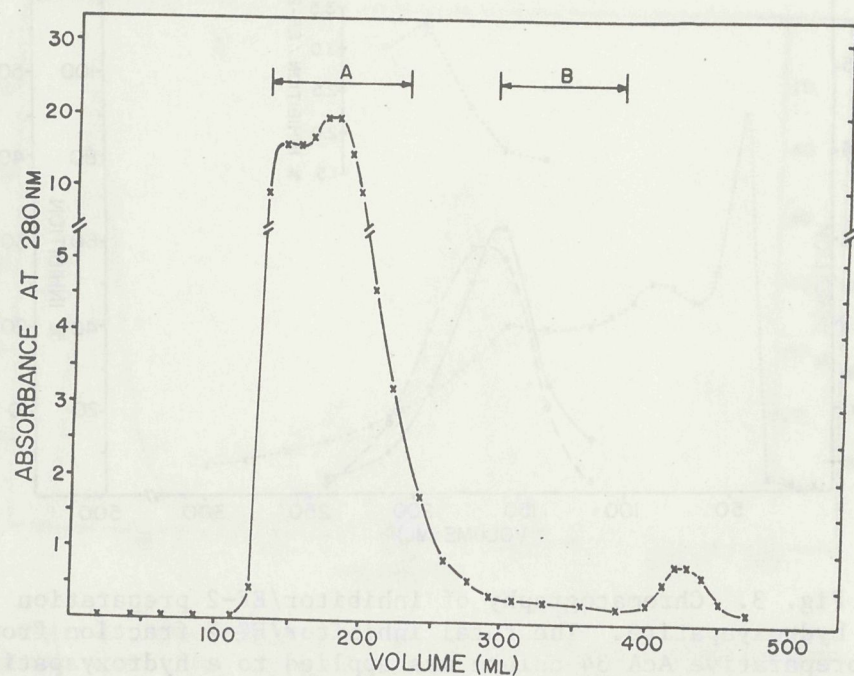


Fig. 4. Chromatography of P-150 fraction on Sepharose 6B. The crude P-150 fraction from 50 grams of cysts was suspended in buffer D and fractionated on a column of Sepharose 6B (2.6 x 90 cm) using the same buffer.

in fraction B (loosely bound) were concentrated separately by ammonium sulfate precipitation at 0.75 saturation and the EF-2/inhibitor purified by gel filtration as shown in Fig. 1. The inhibitor activity in each EF-2 preparation was determined as a function of the EF-2 content and these results are shown in Fig. 5. These results clearly indicate that the loosely bound EF-2 is 15-20 times more inhibitory per unit EF-2 than the EF-2 released from the macromolecular fraction (tightly bound EF-2). The tightly bound EF-2 is inhibitory in protein synthesis only at concentrations in excess of 100 pmol per ml.

If we purify the inhibitor/EF-2 proteins further on hydroxyapatite then on Sephadex G-150 the differences in inhibitor activity persist between the loosely bound and tightly bound protein fractions. These results are summarized in Table 1 and compared to the inhibitor activity of the total EF-2 in the salt wash fraction of the crude P-150 preparation. These experiments support the view that

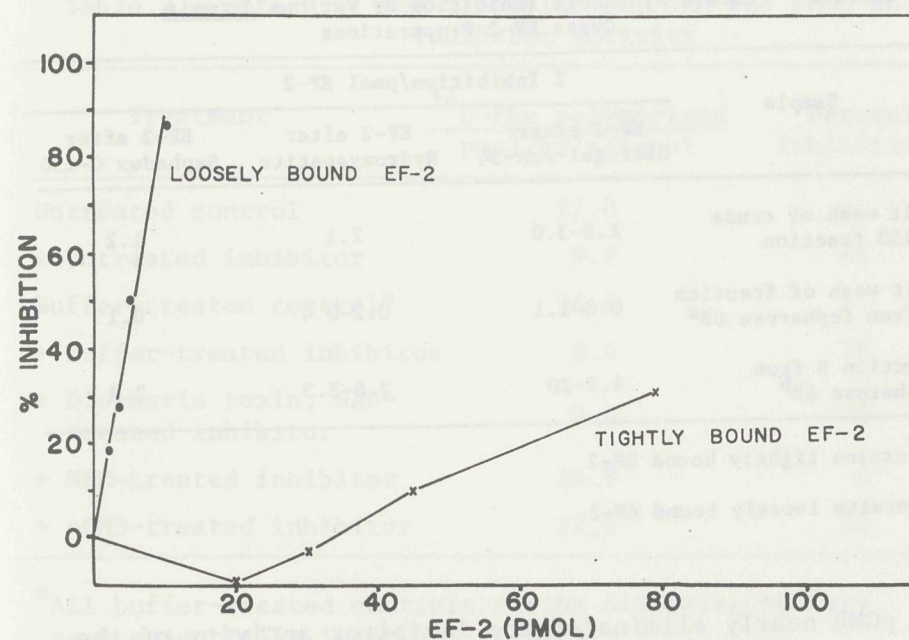


Fig. 5. Effect of EF-2 from loosely bound and tightly bound protein fractions on poly(U) translation. The EF-2 from fractions A (tightly bound) and B (loosely bound) shown in Fig. 4 were purified by gel filtration as shown in Fig. 1 then tested for inhibitor activity in the standard protein synthesis inhibitor assay.

there may be two populations of EF-2 in *Artemia* cysts, one that is functional and another that is inhibitory for as yet unknown reasons.

Effect of Inhibitors of EF-2 and Storage on the Activity of the Protein Synthesis Inhibitor. In an effort to elucidate some of the properties of the protein synthesis inhibitor, we treated an Ultrogel Aca 34 purified inhibitor/EF-2 preparation from the crude P-150 fraction with three reagents known to inactivate EF-2 in protein synthesis. When we assayed each preparation for its ability to inhibit poly(U) translation compared to control preparations treated with buffer only, we obtained the results shown in Table 2. It is clear that ADP-ribosylation of the EF-2 in the inhibitor preparation has very little effect on the inhibitor activity, whereas treatment of the inhibitor/EF-2 preparation with the sulfhydryl blocking reagents NEM

Table 1. Protein Synthesis Inhibition by Various Artemia Cysts EF-2 Preparations

Sample	% Inhibition/pmol EF-2		
	EF-2 after Ultrogel AcA-34	EF-2 after Hydroxyapatite	EF-2 after Sephadex G-150
Salt wash of crude P-150 fraction	1.8-3.0	2.1	1.2
Salt wash of fraction A from Sepharose 6B ^a	0.3-1.1	0.2-0.4	0.1
Fraction B from Sepharose 6B ^b	4.2-20	2.8-3.3	2.3

^acontains tightly bound EF-2

^bcontains loosely bound EF-2

and pCMB nearly eliminated the inhibitor activity of the preparation. Therefore, whatever the nature of the inhibitor, its function appears to require free sulfhydryl group(s).

In another series of experiments we studied the stability of the loosely bound and tightly bound inhibitor/EF-2 preparations under various conditions following purification of these fractions on Ultrogel AcA 34. In buffer C both preparations are stable for several months at -70°C, but at 0°C the inhibitor/EF-2 fraction prepared from the loosely bound preparation (fraction B, Fig. 4) becomes progressively more inhibitory with storage compared to EF-2 purified from the tightly bound protein fraction. The results in Fig. 6 support this observation and show that the inhibitor activity of loosely bound EF-2 increases nearly 3-fold during the first week of storage in buffer C at 0°C, whereas the tightly bound EF-2 remains relatively unaffected. The increase in inhibitor activity of the loosely bound inhibitor/EF-2 preparation is not altered by storage in buffer C containing 5 mM GSH or GSSG although storage in the presence of 5 mM DTT stabilizes the inhibitor activity during storage at 0°C. In view of the fact that EF-2 is rich in SH-groups, these results suggest that the inhibitor activity may be due to conformationally altered EF-2.

Table 2. Effect of ADP Ribosylation, NEM and pCMB on Inhibitor Activity

Treatment	¹⁴ C-Phe polymerized pmol/hr/aliquot	Percent Inhibition
Untreated control	27.0	-
+ Untreated inhibitor	9.7	64
Buffer-treated control ^a	26.4	-
+ Buffer-treated inhibitor	6.4	76
+ Diphtheria toxin, NAD- treated inhibitor	8.0	70
+ NEM-treated inhibitor	24.9	6
+ pCMB-treated inhibitor	22.6	14

^aAll buffer-treated controls (using dialyzing buffer) gave essentially identical results.

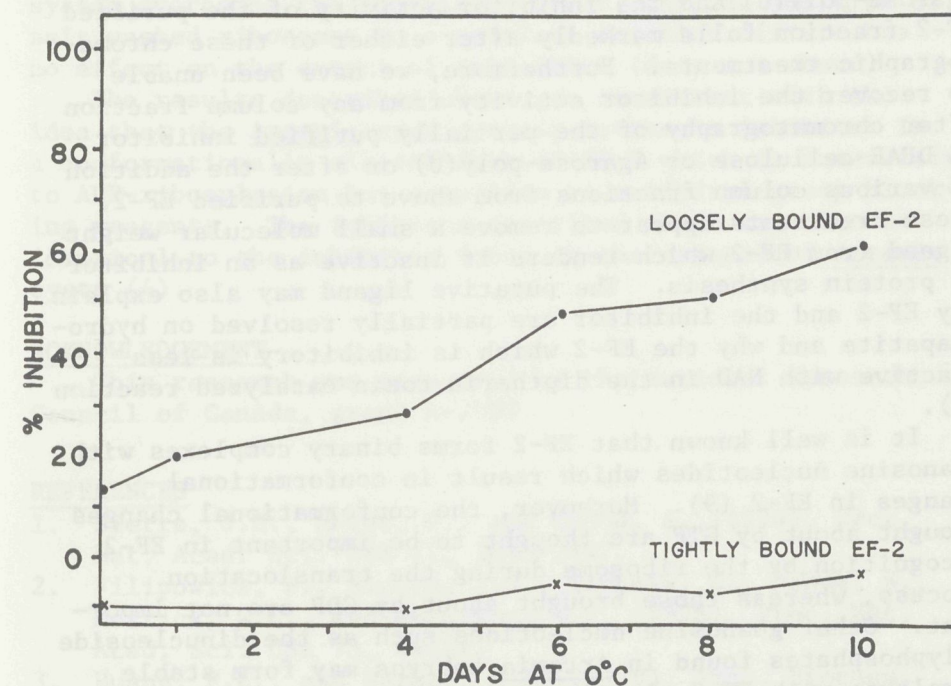


Fig. 6. Effect of storage of different/EF-2 preparations on inhibitor activity. The inhibitor/EF-2 preparations used in Fig. 5 were stored in buffer C in an ice bath and assayed at various times for inhibitor activity in the standard protein synthesis inhibitor assay.

DISCUSSION

Protein synthesis in Artemia embryos appears to be regulated by a complex translational control mechanism which modulates the rate of protein synthesis during early development. In our study of this regulatory process we have isolated and partially purified a protein from Artemia cysts which inhibits poly(U) translation. The inhibitor appears to act at the level of elongation and it has physical properties remarkably similar to elongation factor 2 (EF-2) which is required in the movement of ribosomes along the mRNA. However, the inhibitor does not promote elongation (of polyphenylalanine) in a reaction vessel lacking only elongation factor 2 despite the fact that all inhibitor preparations contain EF-2 that is reactive with NAD in a diphtheria toxin catalyzed reaction.

All attempts by us to separate the inhibitor from EF-2 without extensive loss of inhibitor activity have been unsuccessful and only on hydroxyapatite have we been able to partially resolve these two activities without a marked loss of inhibitor activity. The inhibitor is also sensitive to chromatography on columns of DEAE-cellulose and Agarose-poly(U) and the inhibitor activity of the purified EF-2 fraction falls markedly after either of these chromatographic treatments. Furthermore, we have been unable to recover the inhibitor activity from any column fraction after chromatography of the partially purified inhibitor on DEAE-cellulose or Agarose-poly(U) or after the addition of various column fractions from above to purified EF-2. These treatments appear to remove a small molecular weight ligand from EF-2 which renders it inactive as an inhibitor of protein synthesis. The putative ligand may also explain why EF-2 and the inhibitor are partially resolved on hydroxyapatite and why the EF-2 which is inhibitory is less reactive with NAD in the diphtheria toxin catalyzed reaction (7).

It is well known that EF-2 forms binary complexes with guanosine nucleotides which result in conformational changes in EF-2 (9). Moreover, the conformational changes brought about by GTP are thought to be important in EF-2 recognition by the ribosome during the translocation process, whereas those brought about by GDP are not important. Other guanosine nucleotides such as the dinucleoside polyphosphates found in Artemia embryos may form stable complexes with EF-2 altering its conformation (10). Despite the abundance of nucleotides in Artemia embryos the identification of the inhibitor as an EF-2•nucleotide complex has not yet been demonstrated.

During this study we observed that most of the inhibitor protein (60%) in the crude P-150 fraction can be separated from the macromolecular components and bound EF-2 by filtration of the P-150 fraction on Sepharose 6B in the presence of 100 mM KCl. We also observed that the inhibitor activity of loosely bound EF-2 increases markedly upon storage compared to EF-2 isolated from the macromolecular fraction which is more stable. The activity of the former during storage appears to be due to the production of polypeptide fragments of EF-2 since inhibition of EF-2 hydrolysis by a cyst protease inhibitor reduces the rate of inhibitor formation during storage (unpublished observation). However, the initial inhibitor activity is not due to fragments of EF-2 although we have not yet ruled out the possibility that "nicked" EF-2 is inhibitory in protein synthesis.

The mechanism of action of the Artemia cyst inhibitor on elongation is not known but it appears to act through a factor(s) loosely associated with the ribosome. This view is supported by the observation that addition of Sepharose 6B purified ribosomes to the standard protein synthesis system overcomes the inhibitor effect, whereas addition of salt washed ribosomes to a complete reaction mixture has no effect on the extent of inhibition (data not shown).

The results described above are consistent with the idea that the Artemia cyst protein synthesis inhibitor is a conformationally altered form of EF-2 which is insensitive to ADP-ribosylation but sensitive to sulfhydryl-group blocking reagents. The inhibitor described above may also be identical to the inhibitor found in the cytosol of Artemia cysts (4).

ACKNOWLEDGEMENT

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BIOCHEMICAL ANALYSIS OF DEVELOPMENTAL FAILURE IN GREAT SALT LAKE EMBRYOS

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ABSTRACT

The viability (percent hatching) of encysted embryos from the Great Salt Lake in Utah, the major commercial source for many years, has declined dramatically in the past decade. We sought to determine the extent of pre-emergence development in these defective embryos by analyzing biochemical parameters characteristic of normal development. Polyribosome assembly in vivo and nuclear RNA polymerase activity measured in vitro were examined. Defective embryos contain apparently normal amounts of monomeric ribosomes but do not assemble polyribosomes, even after prolonged incubation at 30°. Defective embryos do not exhibit the increase in nuclear RNA polymerase activity that occurs during normal pre-emergence development. Analysis of DNA content shows that the defective embryos are not merely uncleaved eggs, although they may have entered dormancy at a premature stage. We conclude that the defective embryos do not develop beyond the stage at which they were arrested.

INTRODUCTION

The developmental biochemistry of Artemia salina has been extensively studied in laboratories around the world, due in part to the commercial availability of large quantities of dormant encysted gastrulae (cysts). For many years, the major source of brine shrimp "eggs" was Utah's Great Salt Lake. Normally, most of these embryos would develop and hatch within about 24 hours of incubation in artificial sea water, yielding a nearly synchronous population of free-swimming nauplius larvae. However, the viability of these embryos, as judged by percent hatching has declined dramatically over the past decade. We first encountered this problem in 1975, when a new batch of Utah cysts failed to yield significant numbers of nauplii. We subsequently learned from the distributor (Long Life Fish Food Products) that the viability of all cysts from the Great Salt Lake began to decrease around 1970. As of this writing, our information is that cysts are no longer harvested around the Great Salt Lake. The reason for this decline in viability is unknown.

A fundamental question regarding the defective embryos is whether they are irreversibly arrested at gastrulation or develop partially, only to be arrested again prior to emergence. The thick shell makes direct observation of morphogenesis nearly impossible, and even after chemical removal of the opaque chorion, the remaining cuticle is impermeable to most organic molecules. The use of radio-labeled precursors of specific macromolecular synthesis (e.g. amino acids and nucleotides) or the use of inhibitors of translation and transcription is prevented (reviewed in ref. 1). $^{14}\text{CO}_2$ is absorbed and metabolized by developing embryos, and is eventually incorporated into both protein and RNA (2, 3). Alternative means of studying protein and RNA synthesis involve the use of cell-free systems. Polyribosomes are not found in dormant cysts, but are rapidly formed upon resumption of development (4). Nuclear RNA polymerase activity also increases several-fold during normal pre-emergence development (5). These parameters, then, can be considered diagnostic indicators of normal development, and we have used them to ask whether the defective embryos are at all viable. Our results indicate that these embryos never develop beyond the stage at which they were arrested.

MATERIALS AND METHODS

Dormant encysted embryos were obtained from two commercial sources through a local supplier. Four large cans of cysts from the Great Salt Lake (Long Life Aquarium Products, Lot No. 568) were purchased in the Spring of 1975; these are referred to herein as "recent Great Salt Lake" (RGSL) embryos. When the hatching problem was discovered, we were able to obtain a small supply of "old Great Salt Lake" (OGSL) cysts. These cysts (Longlife, Lot No. 414) were harvested at some unspecified date prior to 1970. We now routinely use cysts harvested from salterns near San Francisco (Metaframe Corporation); these are referred to as "San Francisco" (SF) cysts. The lot used in these experiments (No. 1737) was purchased in the Summer of 1977. All cysts were stored at -20° , and sterilized before use by treatment with dilute antiformin solution (6). Washed cysts were stored at -20° in foil packets containing 5 g each.

Cysts were incubated by placing a 5 g packet of washed cysts in a 2 liter Erlenmyer flask containing 250 ml of the following sterile sea water medium: NaCl, 423 mM; MgSO_4 , 25.5 mM; MgCl_2 , 22.6 mM; KCl, 7.19 mM; $\text{Na}_2\text{B}_4\text{O}_7$, 5.00 mM; CaCl_2 , 1.33 mM; penicillin, 50 I.U./ml; and streptomycin, 50 $\mu\text{g}/\text{ml}$. Flasks were kept in an incubator-shaker set at 75 revolutions per minute and at 30° . Following incubation,

cysts were collected in Miracloth filters and washed with distilled water. Excess water was removed by blotting with paper toweling. The cysts were weighed and stored in foil packets at -20° .

Viability of the three cyst populations (SF, OGSL, RGSL) was determined according to Warner *et al.* (7). One packet of washed cysts was incubated as described above. After 28 hours, three 1 ml samples of the culture were removed while the culture was swirled to mix the contents, and each sample was quickly diluted to 5 ml with culture medium. Each of the three five-fold dilutions was counted in triplicate by withdrawing 1 ml with a pipet, and spreading this aliquot along a 57 cm by 2.5 cm strip of Whatman #1 chromatography paper ruled at 1 cm intervals. The material was then counted using a dissecting microscope. Unhatched cysts and empty shells were counted together since they are difficult to distinguish. Pre-nauplii and nauplii were scored separately.

Nuclei were prepared from cysts that had been hydrated in artificial sea water for 3 hours, then collected by filtration. In some cases, cysts were incubated 8 hours at 30° before collection. Cysts (1 g) were ground in a cold mortar and pestle until breakage was virtually complete, as determined with a dissecting microscope. A 2 ml aliquot of cold homogenizing medium (Tricine, pH 8, 20 mM; EDTA, 0.5 mM; MgCl_2 , 5 mM; sucrose, 0.25 M; phenylmethyl-sulfonylfluoride (PMSF), 0.5 mM; dithiothreitol (DTT), 0.5 mM; soybean trypsin inhibitor (STI), 10 $\mu\text{g}/\text{ml}$) was added to the mortar and mixed. The homogenate was filtered through Miracloth; the mortar was rinsed with 2 ml of homogenizing medium, and the rinse also filtered into the same centrifuge tube. The combined (4 ml) filtrate was centrifuged at 3,000 rpm (1000 x g) for ten minutes. The pellet was resuspended in an equal volume of homogenizing medium, and centrifuged at 2,500 rpm (755 x g) for 10 minutes to sediment nuclei. The pellet was resuspended in 0.4 ml of homogenizing medium, and frozen -70° until needed. RNA polymerase activity in intact isolated nuclei was assayed by measuring incorporation of [^3H] UTP into RNA as previously described (8). Assay mixtures were incubated 30 minutes at 20° . Controls contained aliquots of nuclear suspensions that had been placed in boiling water for one minute. The DNA content of nuclear preparations was determined by the diphenylamine method (9).

For preparation and analysis of polyribosomes, washed cysts were incubated as described above or thawed and used without incubation. Cysts were ground in a cold mortar and

pestle until breakage was complete. Cold TEMN buffer (Tris, pH 8.9 at 0°C, 20 mM; EGTA, 25 mM; MgCl₂, 50 mM; NaCl, 250 mM; DTT, 1 mM; Triton X-100, 2%) was added to the mortar to a volume of 2 ml buffer per g wet weight of cysts. The homogenate was filtered through Miracloth to remove shell fragments, and centrifuged at 15,000 x g for 10 min. to sediment small shell fragments, nuclei, yolk platelets, and mitochondria. The absorbance of the supernate (S-15) was determined at 260 nm. Because suspensions of ribosomes tend to scatter light, net absorbance at 260 nm was taken to be

$$A_{260} - (2.29)A_{320}$$

This corrected absorbance was considered a measure of RNA concentration; one A₂₆₀ unit in 1 ml gives a corrected absorbance of 1.0 in a 1 cm light path at 260 nm.

Samples of the S-15 supernates were analyzed for polyribosomes by sucrose density gradients centrifugation. Linear sucrose density gradients, 15-50%, were made with a 30 ml gradient generator. The gradients contained 10 mM Tris (pH 8.9 at 0°C), 10 mM MgCl₂, and 50 mM NaCl in addition to sucrose. The gradients were formed in cellulose nitrate centrifuge tubes for the Spinco SW41 rotor. Five to ten A₂₆₀ units of S-15 (ca. 100 µl) were layered onto the sucrose gradients, which were centrifuged for 120 min. at 250,000 x g (39,000 rpm) and at 2°C. Gradients were analyzed for absorbance at 254 nm with an ISCO automatic gradient fractionating system.

RESULTS

The decline in the hatch rate of cysts from the Great Salt Lake has been noticed in several different laboratories in recent years. The magnitude of this problem is illustrated by the data in Table 1. In this experiment, cysts of the three populations were incubated under identical conditions of temperature and aeration. Free-swimming nauplius larvae were scored as hatched, and pre-nauplius larvae in any stage of emergence were scored as emerged. The sum of unhatched cysts and empty shells was taken as 100%; we have found virtually no empty shells prior to incubation. The results show that cysts of the RGSL population are almost completely non-viable in terms of emergence and hatching.

Table 1. Determination of cyst viability

Cyst Population	Percent Hatched ^a	Percent Emerged ^a
SF	76.2 ± 4.7	0.6 ± 0.3
OGSL	16.7 ± 2.3	5.6 ± 1.2
RGSL	0.6 ± 0.3	0.7 ± 0.1

^aMean ± standard deviation of three separate determinations, 28 hours of incubation.

To assess the ability of these encysted embryos to form polysomes, we prepared post-mitochondrial (15,000 x g) supernates from incubated and unincubated embryos, and fractionated these on linear sucrose gradients. Each gradient was displaced upward through an absorbance monitor, and the resulting scans are presented in Figures 1-4. Each gradient contained a "landmark" of 80 S monoribosomes, as well as soluble material and small amounts of ribosomal subunits. That the material sedimenting at > 80 S is polyribosomes has already been demonstrated (4).

The polyribosome profiles show that the 15,000 x g supernates from SF and OGSL cysts incubated for one hour or more contained discrete sedimentation classes of polyribosomes. The amount of heavy (> 80 S) material absorbing at 254 nm increased with incubation time, indicating that the number of large polyribosomes increased as development continued. The 15,000 x g supernates from RGSL cysts, however, contained no detectable polyribosomes, even after eight hours of incubation. One trivial cause of this result would be a unique characteristic of RGSL embryos, such as unusually high ribonuclease activity, which could destroy existing polyribosomes during the extraction procedure. We tested for the possibility of such a hypothetical artifact by performing a mixing experiment. The 15,000 x g supernates from 8 hr. SF and RGSL cysts were mixed in a 1:1 and a 1:9 ratio. Figure 5 shows that even a nine-fold excess of RGSL supernate failed to destroy the polyribosomes contributed by the small amount of SF supernate.

RNA polymerase activity in isolated nuclei was measured as an independent criterion for pre-emergence development (5), and the results are shown in Figure 6. These data are from a single experiment, and thus do not permit statistical evaluation. Nevertheless, a striking qualitative difference can be seen. During the first 8 hours of pre-emergence development, the level of RNA polymerase activity increased in SF and, to a lesser extent, in OGSL cysts, but no significant increase was seen in RGSL cyst. There was also a

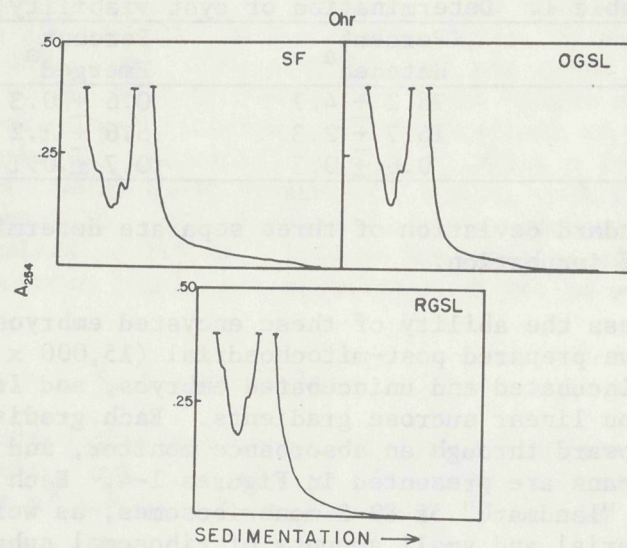


Figure 1. Sucrose gradient centrifugation of S-15 supernates from unincubated embryos. S-15 supernates from each of the three populations of encysted embryos were analyzed for polysome content as described in Methods.

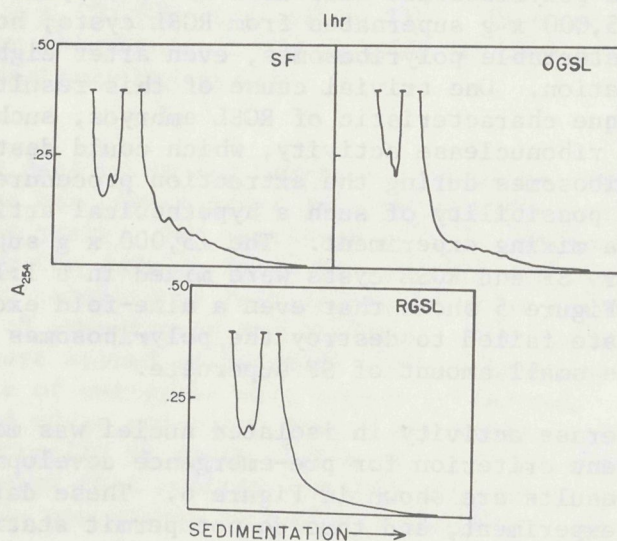


Figure 2. Sucrose gradient analysis of S-15 from embryos incubated 1 hr at 30°.

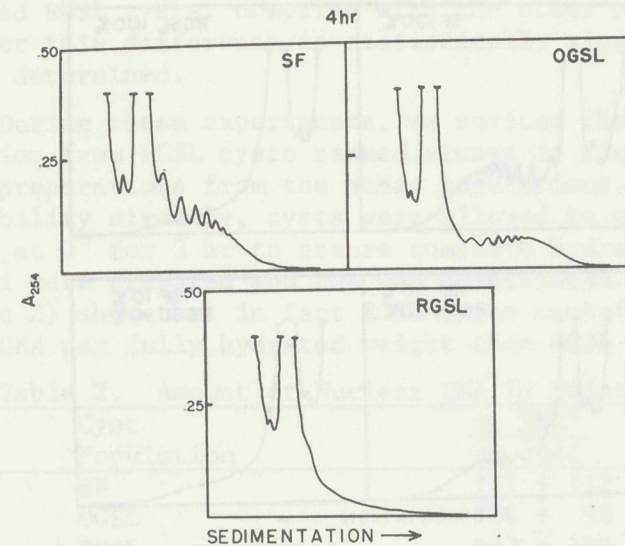


Figure 3. Sucrose gradient analysis of S-15 from embryos incubated 4 hr at 30°.

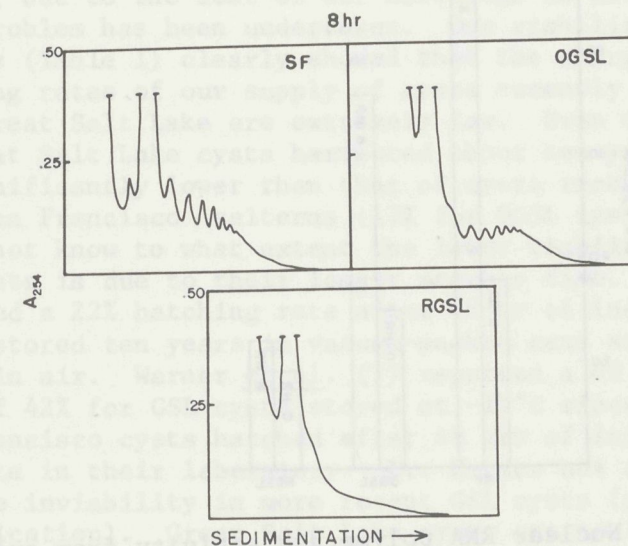


Figure 4. Sucrose gradient analysis of S-15 from embryos incubated 8 hr at 30°.

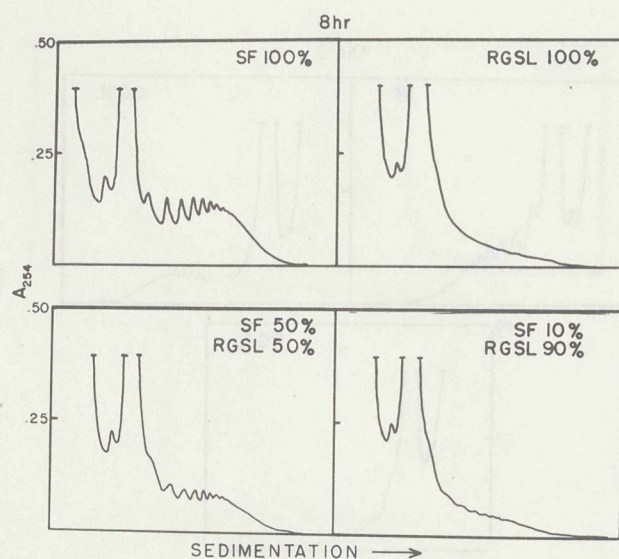


Figure 5. Sucrose gradient analysis of mixed S-15 supernates. S-15 fractions from SF and OGSL embryos incubated 8 hr were analyzed separately and in mixtures.

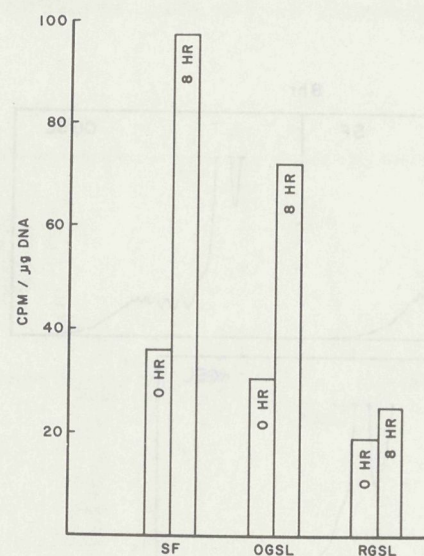


Figure 6. Nuclear RNA polymerase activity from cysts. Nuclei were prepared from embryos of each population, either unincubated (0 hr) or incubated 8 hr at 30°, and assayed for RNA polymerase activity *in vitro* as previously described (8).

definite trend toward lower RNA polymerase activity in unincubated RGSL cysts, compared with the other populations, but whether this difference is statistically significant remains to be determined.

During these experiments, we noticed that nuclear preparation from RGSL cysts seemed always to contain less DNA than preparations from the other populations. To test this possibility directly, cysts were allowed to stand in sea water at 0° for 3 hr to assure complete hydration, and then nuclei were prepared and DNA was quantitated. The results (Table 2) show that in fact RGSL cysts contain substantially less DNA per fully hydrated weight than OGSL and SF cysts.

Table 2. Amount of Nuclear DNA in Unincubated Cysts.

Cyst Population	ng DNA ^a mg cyst
SF	771 ± 112
OGSL	744 ± 98
RGSL	397 ± 102

^aMean ± standard deviation of 3 determinations.

DISCUSSION

Poor yields of nauplii from recent batches of Great Salt Lake cysts have been observed in a number of laboratories, but to the best of our knowledge no direct study of this problem has been undertaken. Our viability determinations (Table 1) clearly showed that the emergence and hatching rates of our supply of cysts recently harvested from Great Salt Lake are extremely low. Even the viability of Great Salt Lake cysts harvested about ten years ago (OGSL) is significantly lower than that of cysts recently harvested from San Francisco salterns (17% for OGSL cysts, 76% for SF). We do not know to what extent the lower viability of our old GSL cysts is due to their longer storage time. Clegg (10) reported a 22% hatching rate after 48 hr of incubation for cysts stored ten years in vacuum-packed cans and then five years in air. Warner *et al.* (7) reported a 48 hr hatching rate of 42% for GSL cysts stored at -15°C since before 1970. San Francisco cysts hatched after 48 hrs of incubation at a 72% rate in their laboratory. Dr. Warner has also observed extreme inviability in more recent GSL cysts (personal communication). Great Salt Lake cysts are no longer commercially available.

We have not yet identified the nature of the defect in RGSL cysts. A severe problem in any number of metabolic systems would eventually affect other life processes and halt development. An inability to initiate respiration (carbo-

hydrate catabolism) would starve the embryo of ATP needed for macromolecular synthesis, although *Artemia* embryos have a large pool of diguanosine tetraphosphate as a probable alternative source of phosphate bond energy for short period of anoxia (11). A lack of transcription would obviously prevent gene expression; the embryo could possibly survive briefly if pre-formed RNAs could be utilized. A defective translation system would soon be lethal, since all metabolically active cells need new proteins.

It would be at least interesting, and possibly useful, to know whether the defective embryos are irreversibly arrested in the dormant state, or at some later point during pre-emergence development. This is not a trivial point, because the underlying mechanisms could be fundamentally different. If the defective embryos do not develop, then the defect must be somehow related to the maintenance and termination of the cryptobiotic state. On the other hand, if these embryos develop but stop short of emergence, the defect must involve the interruption of one or more vital processes. Because of the opaque shell, observation of pre-emergence embryos is extremely difficult. As an alternative we investigated two parameters--polysome formation and increasing RNA polymerase activity--that are characteristic of normal development.

Golub and Clegg (4) showed that the abundant supply of monomeric ribosomes in dormant encysted embryos is quickly recruited to assemble polyribosomes when dormancy is broken. Results presented here (Figures 1-4) show that SF and OGSL embryos assemble polyribosomes as expected, but RGSL embryos do not. No polyribosomes could be found in the defective embryos even after 8 hr of incubation. This could not be attributed to an artifactual disassembly of polysomes in vitro, as shown by mixing experiments (Figure 5). We conclude that the translation apparatus in the defective embryos fails to function on schedule. This may reflect a lack of a necessary component (mRNA?) or of an essential regulatory signal. The apparently complete failure to synthesize proteins would in itself be lethal, but may also be symptomatic of a more general malaise.

RNA polymerase assays in isolated nuclei were performed to compare the transcriptional capability of each cyst population. In nauplius larvae, the rate of RNA synthesis in vivo is quantitatively paralleled by the RNA polymerase activity of isolated nuclei (8). Dormant embryos yield very little soluble RNA polymerase, but this yield increases during pre-emergence development (5). The results presented

in Figure 6 show that the RNA polymerase activity of isolated nuclei increases during incubation of SF and OGSL embryos, but no such increase was found in RGSL embryos. These assays were designed to measure transcription of the endogenous chromatin template under conditions that maintained the structural integrity of the nuclei. It would be incorrect to conclude that the defective embryos are transcriptionally "dead", because the level of endogenous nuclear transcription is nearly normal. It is the scheduled increase in RNA polymerase activity that is defective. Recent experiments in this laboratory by Dr. Judith Helder have shown that RNA polymerase II is synthesized de novo during preemergence development, and the same is presumably true for polymerase I. Given the inability of defective embryos to synthesize proteins, the failure to increase RNA polymerase levels is not surprising. By both these criteria--polysome formation and RNA polymerase levels--the defective embryos are stuck in the cryptobiotic state and do not develop beyond it.

DNA assays on nuclear preparations produced interesting data in themselves, though the assays were performed simply to normalize RNA polymerase activities. We noticed that the DNA content per wet weight of cysts was lower in the RGSL population than in the SF or OGSL populations. One possible cause of this result would be a significantly greater wet weight per cyst in the RGSL populations, perhaps reflecting a thicker shell. Since we know that a fully hydrated cyst from any of the three populations weighs about the same (unpublished data of J. Helder), the lower DNA content per wet weight cysts implies a lower DNA content per individual cyst. The RGSL embryos may be somewhat underdeveloped at the onset of dormancy. Relevant to this hypothesis are earlier observations of variations in developmental stage among batches of commercially available Great Salt Lake cysts (12). Our determinations of DNA content permit us to exclude the possibility that the defective cysts are merely eggs which never began cleavage. If the embryos were still at the one-cell stage, then we would expect only 2.5×10^{-4} as much DNA per wet weight cysts as in normal cysts, for normal dormant embryos contain about 4,000 cells (13).

Dramatic ecological changes have occurred in the Great Salt Lake since the Southern Pacific Railroad Company's Lucin Cutoff route was completed in 1959. The railroad embankment is a 32 km semipermeable quarry-run rock causeway that essentially divides the lake in two. Ninety-five percent of the lake's total inflow volume enters the south

arm, precipitation is slightly higher in the south arm and evaporation occurs slightly faster in the north arm. These forces have combined to give the south arm an elevation almost one meter greater than that of the north arm, causing a net flow of saltwater from the south to the north arm (14). Water flows by seepage through the embankment, or through two 4.3 by 6.1 meter culverts. Prior to construction of the causeway, tides and winds mixed the lake's water to give a relatively homogeneous solids load throughout. Since 1959, however, water flow has caused northward salt migration, increasing the salinity of the north arm at the expense of the south arm. By 1963, just five summers after the railroad embankment was complete, about one quarter of the salt formerly in the south arm had migrated to the north arm (14). Prior to 1955 the solids content of the lake was roughly 200 g per liter. In 1975 the north arm carried 330 to 350 g per liter, and the south arm only 120 to 130 g per liter (15). Attempts to hatch brine shrimp cysts in north arm water have failed, probably because the embryos could not raise their internal osmotic pressure enough to absorb water, swell, and excyst (15, 16). Brine shrimp still reproduce in the south arm of the lake, but cyst production has declined significantly. The relationship between decreasing numbers of *Artemia* cysts and decreasing cyst viability is not known.

We have no evidence directly linking the salt migration in Great Salt Lake to the inviability of RGSL cysts. Recent industrialization in the area may also be a factor. Regardless of the reasons for defective cysts, their presence must be recognized by biochemists and others using brine shrimp in laboratory research. Large amounts of defective cysts have been shipped to laboratories around the world. Some of these cysts may have been used in experiments wherein their inviability would go unrecognized, and give rise to inappropriately interpreted results. We know of at least one laboratory which performed several experiments with defective cysts before recognizing their inability to hatch. All literature published since 1970 describing pre-emergence development in Great Salt Lake cysts should be carefully scrutinized. Particularly suspect would be results indicating little difference in biochemical parameters between incubated and unincubated cysts. Even reported changes in biochemical parameters, such as increased enzyme activities, could be due to effects of incubation other than embryonic development. For example, extracts of unincubated cysts may contain residual NaOCl from an antiformin wash, whereas incubation may remove residual NaOCl from cyst shells and result in uncontaminated extracts and higher enzyme activities (A. H. Warner, personal communication).

A better understanding of defective Great Salt Lake cysts may lead to a better understanding of normal brine shrimp development, and of cryptobiosis and its termination. Characterization of the cysts' condition would also greatly facilitate identification of environmental cause(s) of the defect(s).

ACKNOWLEDGEMENTS

The work reported here was supported by a Faculty Research Award from Wayne State University. We would like to thank Drs. Roger Acey, Judith Helder and Alden Warner for many helpful suggestions and stimulating conversations.

NOTE ADDED IN PROOF

We have recently been informed that Salt Lake area cysts are again commercially available, and that the reported hatch rate is > 50%. This improved viability follows an increase in salinity in the south arm, resulting from several successive dry summers. We have not yet tested the hatch rate or biochemical parameters of these cysts.

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CHARACTERIZATION OF REPRESSED POLY(A)-CONTAINING RIBONUCLEOPROTEINS IN CRYTOBIOTIC GASTRULAE OF ARTEMIA SALINA

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ABSTRACT

In the free cytoplasmic fraction of cryptobiotic embryos the main active particle in protein synthesis is a 22S poly(A)-lacking RNP containing 9S RNA which codes for a basic protein with a Mr of 26,000.

Contrary to this particle, the poly(A)⁺mRNP exists in a functionally repressed form by interaction with a translational inhibitor RNA. The latter RNP was isolated by affinity chromatography on oligo(dT)-cellulose and purified by sucrose isopycnic centrifugation. The purified particles sedimented between 5-30S and banded at a density of 1.38-1.40 g/cm³ and 1.27-1.28 g/cm³ in CsCl and sucrose isopycnic centrifugation, respectively. Analysis by SDS gel electrophoresis of the poly(A) mRNP protein components revealed polypeptides with Mr of 38,000, 45,000, 50,000, 52,000, 65,000, 76,000 and 94,000. The 38,000-protein constitutes the main fraction among these proteins and has a high affinity for poly(A)- and poly(U)-sequences.

INTRODUCTION

Free ribonucleoprotein particles were first detected in the cytoplasm of fish embryos in 1964 (1). Since then analogous informational RNP has been observed in a great number of eukaryotic organisms and in different cellular localizations, indicating their universal nature. Independent of their origin these particles have common properties s.a. a RNA/protein ratio of 1/3, resistance to EDTA treatment etc. (2-3). The protein moiety of these RNPs differs depending on their cellular localization. In fact, transport of mRNA from nucleus to cytoplasm and the transfer into polyribosomes seems to be related with changes in the protein composition. Recently, Spirin (4) has even postulated that mRNA carries on itself the proteins necessary for its transport from the nucleus, transformation into a temporarily inactive form and reactivation into an active template.

The functional meaning of informational RNP can only be elucidated by the study of the relationship among the different types of RNP. The regulatory function of these RNPs in protein synthesis remains unresolved as a consequence of

the failure to isolate pure and intact RNP. *Artemia salina* is very suitable for the study of the different functional states of mRNP. In certain conditions the development of the embryos of the brine shrimp is interrupted at the gastrula stage followed by a dormant period. Since no polysomal structures have been found in these dormant embryos, the rapid formation of polysomes observed after redevelopment is due to the presence of stored mRNP (5-7). Furthermore, besides the functional state of mRNA, the repression of ribosome activity and the availability of initiation factors seem to have an important regulatory function on the development of the encysted gastrulae (8-10).

In this communication we describe our observations on the regulation of protein synthesis by temporary repression of mRNA activity. We present evidence that only the free cytoplasmic poly(A)⁺RNP of the cryptobiotic embryos are in a repressed form. The latter particles have been isolated and purified. The protein composition has been determined. The relationship between the protein moiety and the functional state of the RNP is discussed.

MATERIALS AND METHODS

Cryptobiotic embryos of *A. salina* were obtained from Metaframe (San Francisco Bay Brand, Newark, CA, U.S.A.). The postnuclear supernatant (PNS) of gastrulae (100-200 g, dry weight) was prepared in buffer C (10 mM sodium phosphate pH 6.8, 5 mM MgCl₂, 50 mM NaCl) and the postmitochondrial supernatant (PMS) in buffer C, buffer G (10 mM Tris-HCl pH 7.6, 100 mM NaCl), buffer J (10 mM Hepes pH 7.2, 5 mM MgCl₂, 50 mM NaCl), buffer K (10 mM Hepes pH 7.2, 1 mM EDTA, 50 mM NaCl) and buffer M (10 mM Tris-HCl pH 7.5, 1 mM EDTA, 50 mM NaCl) as described previously (11-12). The PNS was fractionated by centrifugation at 18,000 g for 15 min and the membrane bound particles released by dissolving the 18,000 g sediment in buffer C containing 1% Triton X-100. The postribosomal supernatant (PRS) was obtained from PMS by differential centrifugation in a R60 rotor at 255,800 g for 50 min and at 4°C. PMS RNA was prepared as described (13).

Affinity chromatography of PRS or PMS on oligo(dT)-cellulose (type T2, Collaborative Research Inc., Waltham, MA, U.S.A.) was in buffer C, buffer J or buffer L (10 mM Hepes pH 7.2, 1 mM EDTA, 250 mM NaCl) as indicated in the figure legends. The bound poly(A)⁺RNP was eluted in buffer without salts by temperature elevation. Affinity chromatography of PMS RNA on poly(U)-Sephacrose (Pharmacia Fine Chemicals, Uppsala, Sweden) was in buffer H (10 mM Tris-HCl pH 7.5, 10 mM EDTA, 500 mM NaCl). The poly(A)⁺RNP and poly(A) RNA were concentrated by Amicon Ultrafiltration (Amicon, Oosterhout, The Netherlands) using a PM10 or PM30

membrane pretreated with 5% formaldehyde. Poly(A)-sequences were assayed by hybridisation with (^3H)-poly(U) (Miles Laboratories Inc., Kankakee, IL, U.S.A.) (11).

Poly(A)⁺RNP was analyzed by sucrose and CsCl isopycnic centrifugation. For isopycnic centrifugation in sucrose, samples were placed on three layers (9 ml each) of sucrose solutions (30, 50 and 70% w/w) in buffer C or buffer K and centrifuged in a Beckman R60 rotor at 250,000 g for 90h and at 4°C. Poly(A)⁺RNP was fixed by dialysis for 12h and at 4°C against 5% neutralized formaldehyde in buffer K and a subsequent dialysis for 12h against buffer K. The fixed RNP was placed on three layers (1.3 ml each) of CsCl (50, 35 and 20% w/w). Centrifugation was in a Beckman SW65 rotor at 301,000 g for 25h and at 4°C. The protein content of each fraction was determined by the method of Ovchinnikov et al. (14). Linear 10-30% (w/v) or 5-20% (w/v) sucrose gradient centrifugation was in a Beckman SW27 or SW41 rotor. Conditions are described in the figure legends.

Proteins used in affinity chromatography on poly(A)-, poly(U)- and rRNA-Sepharose were extracted from poly(A)⁺RNP by LiCl (15) and dialysed against buffer N (10 mM Hepes pH 7.2, 10 mM KCl, 3 mM MgCl₂, 7 mM 2-mercaptoethanol). rRNA-Sepharose was prepared according to Ovchinnikov et al. (14). Poly(A)-, poly(U)- and cyanogen bromide activated Sepharose 4B were from Pharmacia Fine Chemicals. Bound proteins were eluted in buffer N with a KCl gradient from 10 mM up to 2 M.

For SDS gel electrophoresis, proteins were precipitated with 10% trichloroacetic acid and dissolved in sample buffer (10 mM Tris-HCl pH 8.3, 2% SDS, 2% 2-mercaptoethanol, 20% glycerol). Samples were denatured by heating at 95°C for 3 min and analyzed on 10% polyacrylamide gels (0.6x10 cm) containing 0.21% N-N'-methylenebisacrylamide. The following proteins were used as standards for molecular weight estimation: RNA-polymerase (165,000, 155,000, 39,000), phosphorylase b (94,000), serum albumin (68,000), ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (21,500) and α -lactalbumin (14,000).

In vitro proteins synthesis in a wheat embryo system and in micrococcal nuclease treated rabbit reticulocyte lysate was as described (16-17). A reaction mixture (90 μl) was diluted with redistilled water to 700 $\mu\text{g}/\text{ml}$, digested with pancreatic RNAase (5 $\mu\text{g}/\text{ml}$, Sigma Chemical Co., St. Louis, U.S.A.) at 37°C for 15 min. The proteins were precipitated with 20% TCA and dissolved in sample buffer for SDS gel electrophoresis. Gels were frozen at -20°C, sliced and the (^{35}S)-methionine (Radiochemical Centre, Amersham, U.K.) extracted with 1 ml of 0.1% SDS by shaking overnight at 37°C. The radioactivity was determined in 9 ml of a toluene based cocktail (1 L toluene, 0.5 L Triton X-100, 6 g

PPO, 150 mg POPOP). Protein products were analyzed by acid urea gel electrophoresis (18) and SDS gel electrophoresis as above. Calf thymus histones (Sigma Chemical Co.) were used as markers.

RESULTS AND DISCUSSION

Apparent contradictory results have been reported on the nature of the translatable mRNA present in undeveloped embryos. On the one hand, according to Sierra et al. (19), the free cytoplasmic poly(A) mRNA was not translatable while the poly(A) mRNA of the membrane fraction and the poly(A) mRNA of both fractions were readily translatable; on the other hand, Grosfeld and Littauer (20) and Felicetti et al. (21) reported evidence that poly(A) mRNA of both fractions was active in translation. The apparent contradiction concerning the translatability of poly(A) mRNA of the free cytoplasmic fraction prompted us to reinvestigate the translation of stored mRNA.

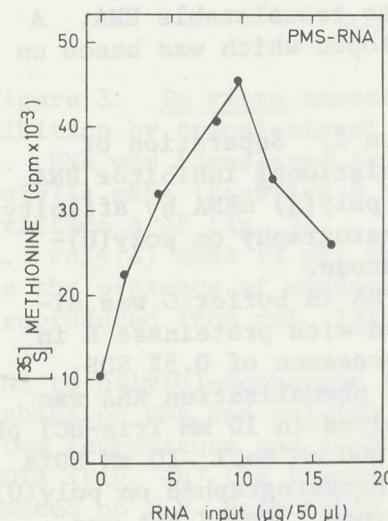


Figure 1. *In vitro* translation of total unfractionated PMS RNA of the free cytoplasmic fraction. RNA from PMS in buffer C was prepared by phenolization. RNA was dissolved in distilled water and used in a wheat embryo protein synthesizing system (reproduced from ref. 13).

Total unfractionated PMS RNA prepared in the absence of EDTA stimulated protein synthesis in a wheat germ extract (Fig. 1). Poly(A)⁺RNP prepared from PMS in the absence of EDTA and purified by affinity chromatography on oligo(dT)-cellulose as well as the extracted poly(A)⁺RNA were totally inactive in a wheat germ cell free system (Table I) in agreement with the results of Sierra et al. (19).

Table I. Translation of poly(A)⁺RNA in a wheat embryo protein synthesizing system.

Template	Amount (μg)	(³⁵ S)-Methionine incorporation (cpm) ^a
poly(A) ⁺ RNA	0.52	770
poly(A) ⁺ RNA	1.04	250
poly(A) ⁺ RNA	1.56	990
TMV RNA	9.00	112,870

^aAverage of duplicate experiments; background has been subtracted

Poly(A)⁺RNA isolated by affinity chromatography on oligo(dT)-cellulose from PMS RNA prepared in the absence of EDTA but purified by linear sucrose gradient centrifugation in the presence of EDTA showed an increase in template activity in the 7-14S region. This observation suggested that EDTA converted the inactive poly(A)⁺RNA into translatable RNA. A separation procedure has been developed which was based on the annealing of poly(A)⁺-

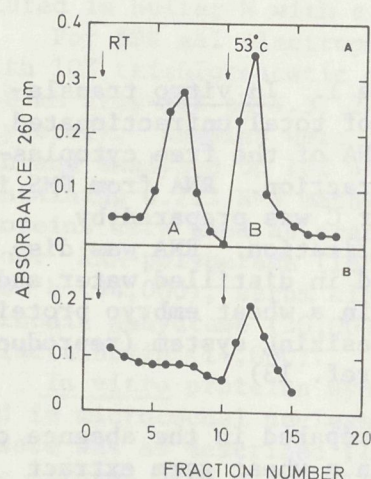


Figure 2. Separation of translational inhibitor RNA from poly(A)⁺mRNA by affinity chromatography on poly(U)-Sephadex.

A. PMS in buffer G was digested with proteinase K in the presence of 0.5% SDS. After phenolization RNA was dissolved in 10 mM Tris-HCl pH 7.6, 500 mM NaCl, 10 mM EDTA and chromatographed on poly(U)-Sephadex. Bound RNA was eluted at 20°C and at 53°C. B. Poly(A)⁺RNP of the free cytoplasmic fraction was isolated by chromatography of PMS in buffer C on oligo(dT)-cellulose.

The extracted poly(A)⁺RNA dissolved in buffer C was fractionated on poly(U)-Sephadex as in A. (reproduced from ref. 13).

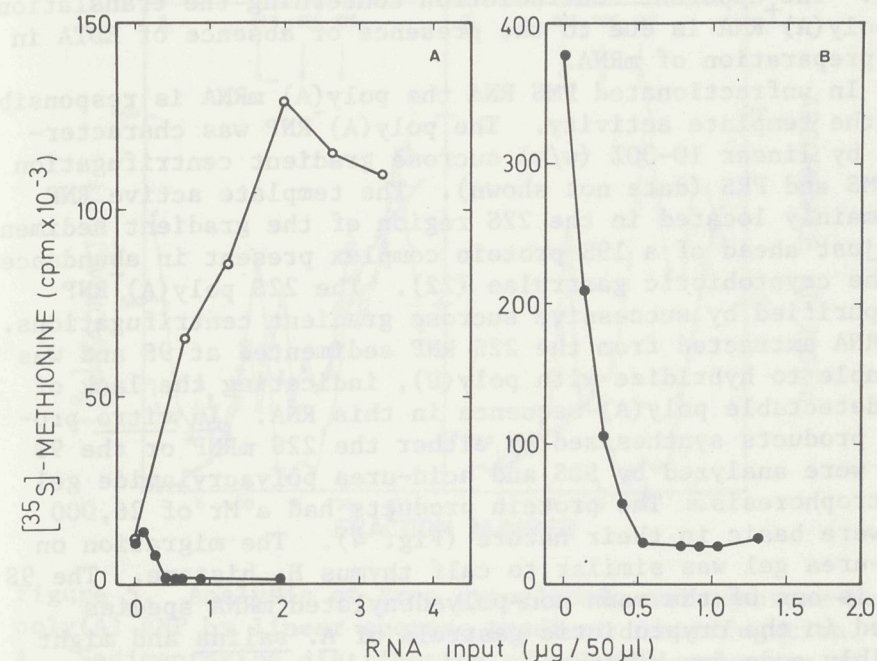


Figure 3. *In vitro* translation of poly(A)⁺mRNA and its inhibition by translational inhibitor RNA. A. RNA was translated in a wheat embryo system: (o-o) poly(A)⁺mRNA (fraction B of fig. 2A) and (●-●) RNA eluted in fraction A of fig. 2A. B. Poly(A)⁺mRNA (2 μg) translated in a wheat embryo system in the presence of various amounts of fraction A RNA. (reproduced from ref. 13).

RNA to poly(U)-Sephadex in the presence of 10 mM EDTA. The inhibitor RNA and poly(A)⁺RNA were separated by elution at room temperature and at 53°C (Fig. 2). In the absence of EDTA (Fig. 2b) the fraction eluted at room temperature contained no U.V. detectable material and the RNA eluted at 53°C was not translatable in a wheat germ cell free system. Contrary the poly(A)⁺RNA eluted from poly(U)-Sephadex at 53°C after EDTA treatment (Fig. 2a) was an excellent template in the same translational system. Moreover the RNA eluted at room temperature in the presence of EDTA not only repressed residual endogeneous activity but also exerted a strong inhibition on the translation of active poly(A)⁺RNA (Fig. 3). These experiments establish the presence of a translational inhibitor RNA which is stably bound to the free cytoplasmic poly(A)⁺-mRNP in the presence of divalent

ions. The apparent contradiction concerning the translation of poly(A)⁺ RNA is due to the presence or absence of EDTA in the preparation of mRNA.

In unfractionated PMS RNA the poly(A)⁻ mRNA is responsible for the template activity. The poly(A) RNP was characterized by linear 10-30% (w/v) sucrose gradient centrifugation of PMS and PRS (data not shown). The template active RNP was mainly located in the 22S region of the gradient sedimenting just ahead of a 19S protein complex present in abundance in the cryptobiotic gastrulae (22). The 22S poly(A) RNP was purified by successive sucrose gradient centrifugations. The RNA extracted from the 22S RNP sedimented at 9S and was not able to hybridize with poly(U), indicating the lack of any detectable poly(A)-sequence in this RNA. *In vitro* protein products synthesized by either the 22S mRNP or the 9S mRNA were analyzed by SDS and acid-urea polyacrylamide gel electrophoresis. The protein products had a Mr of 26,000 and were basic in their nature (Fig. 4). The migration on acid-urea gel was similar to calf thymus H₁ histone. The 9S mRNA is one of the main non-polyadenylated mRNA species stored in the cryptobiotic gastrula of *A. salina* and might possibly code for histones.

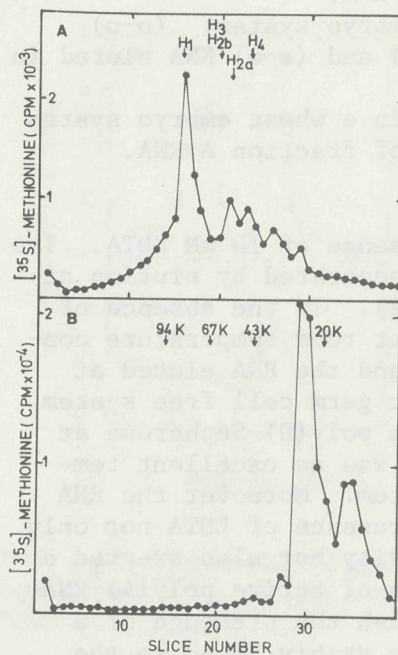


Figure 4. Analysis of *in vitro* protein products synthesized by the 9S RNA extracted from 22S poly(A) RNP. Proteins synthesized in a rabbit reticulocyte lysate were analyzed by acid-urea gel electrophoresis (A) and SDS polyacrylamide gel electrophoresis (B). Protein markers were phosphorylase b (94,000), serum albumin (68,000), ovalbumin (43,000) and trypsin inhibitor (20,100) for SDS polyacrylamide gel electrophoresis and calf thymus for the acid-urea gel electrophoresis.

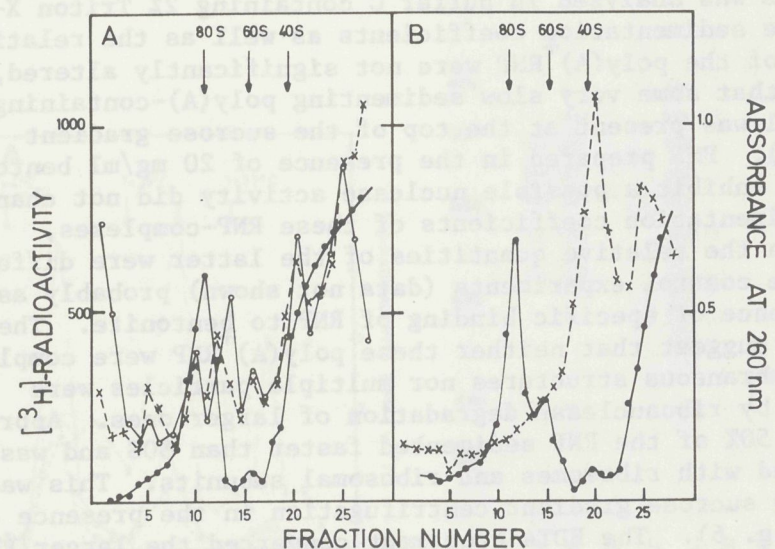


Figure 5. Analysis of free cytoplasmic and membrane-bound poly(A) RNP by linear sucrose gradient centrifugation. A. Sedimentation distribution of poly(A)-sequences of the free cytoplasmic fraction before (o-o) and after (x-x) treatment with 1% Triton X-100. A₂₆₀ (o-o). B. Sedimentation distribution of poly(A) RNP released from the membrane fraction by 1% Triton X-100 (x-x). A₂₆₀ (o-o). Centrifugation was in the Beckman SW41 rotor at 105,000g for 15h and at 4°C. (reproduced from ref. 12).

The repressed poly(A)⁺ RNP was characterized by linear 10-30% (w/v) sucrose gradient centrifugation of PMS and subsequent hybridization of RNA extracted from each gradient fraction with (³H)-poly(U). The poly(A) RNP exhibited a heterogeneous distribution in which at least six distinct RNP-complexes have been identified (Fig. 5a). The estimated sedimentation coefficients are presented in Table II together with those determined in different conditions.

Table II. Sedimentation coefficients of poly(A)⁺ RNP

PMS prepared in the presence of	S _{20,w} (±5S)					
none	15S	40S	60S	70S	85S	110S
Triton X-100(2%)	15S	35S	60S	75S	85S	105S
bentonite(20 mg/ml)	10S	40S	55S	70S	80S	100S

When PMS was analyzed in buffer C containing 2% Triton X-100, the sedimentation coefficients as well as the relative amount of the poly(A)⁺RNP were not significantly altered, except that some very slow sedimenting poly(A)-containing material was present at the top of the sucrose gradient (Fig. 5). PMS prepared in the presence of 20 mg/ml bentonite to inhibit a possible nuclease activity did not change the sedimentation coefficients of these RNP-complexes, although the relative quantities of the latter were different from the control experiments (data not shown) probably as a consequence of specific binding of RNP to bentonite. These results suggest that neither these poly(A)⁺RNP were complexed with membranous structures nor multiple particles were created by ribonuclease degradation of larger ones. Approximately 50% of the RNP sedimented faster than 50S and was complexed with ribosomes and ribosomal subunits. This was shown by sucrose gradient centrifugation in the presence of EDTA (Fig. 6). The EDTA treatment converted the larger RNP-complexes into free RNP sedimenting at 20-35S. Contrary to the free cytoplasmic poly(A)⁺RNP the distribution of particles released from the membrane fraction by treatment with 1% Triton X-100 consisted of only two peaks sedimenting at 16S and 36S, containing no RNP-ribosome complexes (Fig. 5b). The latter particles are not associated with a translational inhibitor RNA and have been translated in an *in vitro* protein synthesizing system (19). At the moment there is not sufficient experimental evidence to correlate the observed differences in template activity and the differences in sedimentation distribution of poly(A)⁺RNP of the free cytoplasmic fraction and the membrane fraction.

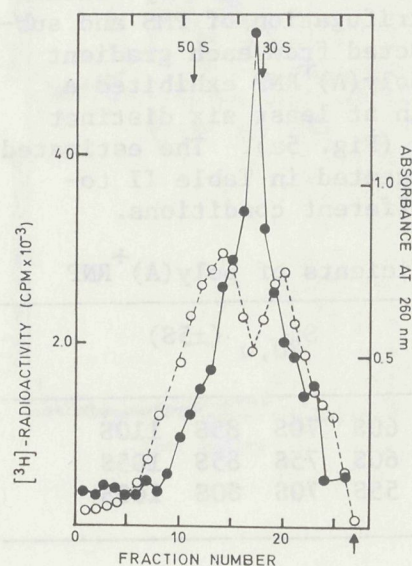


Figure 6. Effect of EDTA on the sedimentation behaviour of poly(A)⁺RNP complexes. RNP was analyzed on 5-20% sucrose gradients in buffer C containing 25 mM EDTA. Centrifugation was in the Beckman SW41 rotor at 286,000g for 120 min and at 4°C. (o-o) (3H)-poly(U) radioactivity, (o-o) absorbance at 260 nm. (reproduced from ref. 11).

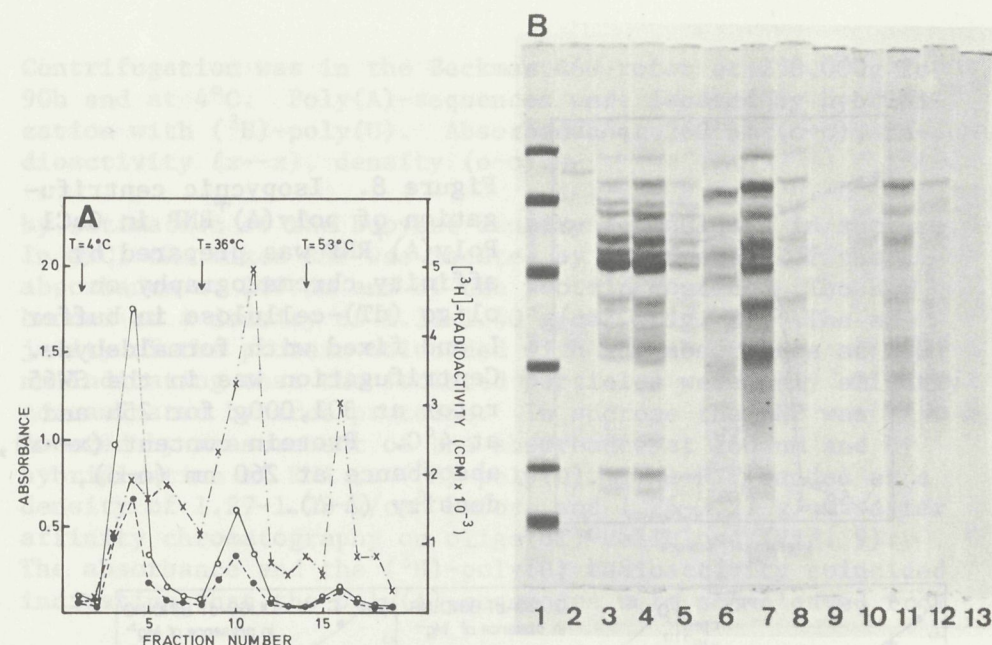


Figure 7. Affinity chromatography of poly(A)⁺RNP on oligo(dT)-cellulose.

A. PMS was prepared in buffer K. Poly(A)⁺RNP was bound to oligo(dT)-cellulose in buffer L and eluted in buffer without salts at 4°C, 36°C and 53°C. Each fraction was assayed for poly(A)-sequences by hybridization with (3H)-poly(U) (x'-x). Absorbance at 260 nm (o-o) and at 280 nm (o---o).

B. Analysis of poly(A)⁺RNP proteins present in oligo(dT)-cellulose eluted fractions.

gel 1: markers, phosphorylase b (94,000), serum albumin (68,000), ovalbumin (43,000), trypsin inhibitor (20,000) and α-lactalbumin (14,000); gel 2-5: RNP proteins eluted at 4°C; gel 6-9: RNP proteins eluted at 36°C; gel 10-13: RNP proteins eluted at 53°C.

Poly(A)⁺mRNP has been purified by affinity chromatography on oligo(dT)-cellulose and sucrose isopycnic centrifugation. The RNP was bound to oligo(dT)-cellulose in buffer L and eluted in 10 mM Hepes pH 7.2 at 4°C, 36°C and 53°C (Fig. 7a). Poly(A)⁺RNP was localized by hybridization with (3H)-poly(U). The majority was recovered in the fraction eluted at 36°C. The RNP protein components were analyzed by SDS gel electrophoresis. The main proteins have Mr of 38,000, 45,000, 50,000, 52,000, 65,000, 76,000 and 94,000 (Fig. 7b). Binding of RNP in Mg²⁺-containing buffers (buffer C, buffer J) resulted in more non-specific protein binding and also in a cleavage of the majority of poly(A)⁺mRNP in the vicinity of the 3' poly(A)-sequence (23). The intact nature of particles isolated in buffer L was confirmed

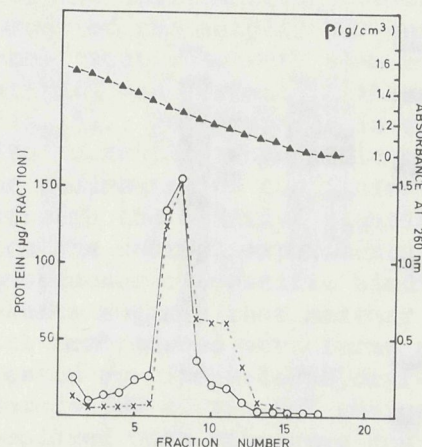


Figure 8. Isopycnic centrifugation of poly(A)⁺RNP in CsCl. Poly(A)⁺RNP was prepared by affinity chromatography on oligo (dT)-cellulose in buffer L and fixed with formaldehyde. Centrifugation was in the SW65 rotor at 301,000g for 25h and at 4°C. Protein content (x--x), absorbance at 260 nm (o-o), density (Δ-Δ).

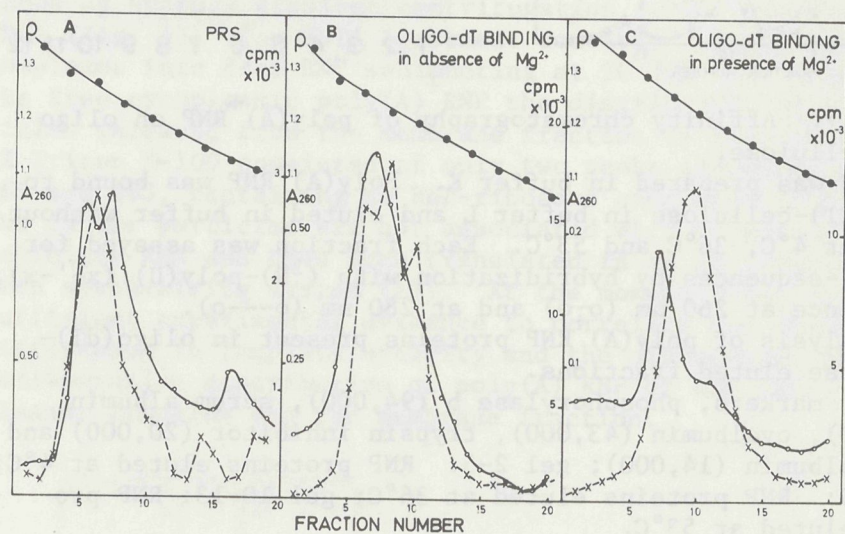


Figure 9. Isopycnic centrifugation of PRS and poly(A)⁺RNP in sucrose.

PRS was prepared in buffer C and in buffer K. Poly(A)⁺RNP was prepared from PRS by affinity chromatography on oligo(dT)-cellulose in buffer C or in buffer L. RNP was eluted in buffer without salts at 36°C.

A. Centrifugation of PRS in buffer K before affinity chromatography on oligo(dT)-cellulose.

B. Centrifugation of poly(A)⁺RNP prepared by affinity chromatography on oligo(dT)-cellulose in buffer L.

C. Centrifugation of poly(A)⁺RNP prepared by affinity chromatography on oligo(dT)-cellulose in buffer C.

Centrifugation was in the Beckman R60 rotor at 250,000g for 90h and at 4°C. Poly(A)-sequences were located by hybridization with (³H)-poly(U). Absorbance at 260 nm (o-o), radioactivity (x--x), density (Δ-Δ).

by estimation of the buoyant density in CsCl and in sucrose. In CsCl the fixed RNP was located by measurement of the absorbance at 260 nm and of the protein content. The RNP banded at a density of 1.38-1.40 g/cm³ (Fig. 8). The majority of the protein coincided with the absorbance at 260 nm indicating that the isolated particles were only slightly contaminated by free proteins. In sucrose the RNP was located by measurement of the absorbance at 260 nm and by hybridization of RNA to (³H)-poly(U). The RNP banded at a density of 1.27-1.28 g/cm³ before and 1.26-1.27 g/cm³ after affinity chromatography on oligo(dT)-cellulose (Fig. 9). The absorbance and the (³H)-poly(U) radioactivity coincided indicating that the poly(A)-sequences were not cleaved from

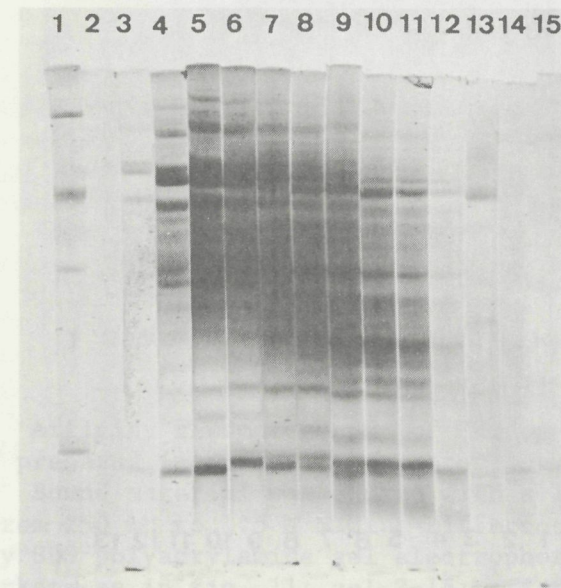


Figure 10. Analysis by SDS polyacrylamide gel electrophoresis of the protein composition of the poly(A)-containing and poly(A)-lacking RNP parts separated by isopycnic centrifugation in sucrose.

gel 1: markers, RNA-polymerase (165,000, 155,000, 39,000), serum albumin (68,000), trypsin inhibitor (20,000); gel 2-15: fractions 4-17 of the isopycnic centrifugation of fig. 9C.

the isolated ribonucleoproteins. Sucrose isopycnic centrifugation was also used to separate the poly(A)-containing and -lacking parts in the case of Mg^{2+} -activated ribonuclease cleavage (Fig. 9c). The poly(A)-lacking part has a density of 1.28-1.29 g/cm³, while the poly(A)-containing part banded at a density of 1.22-1.23 g/cm³. Analysis of the proteins of both fractions revealed that the proteins with Mr of 45,000, 50,000 and 65,000 were present in the poly(A)-lacking part while 38,000 and 76,000 were located in the poly(A)-containing part of the RNP (Fig. 10). The poly(A)⁺ mRNP isolated in buffer L sedimented between 5-30S in sucrose gradient centrifugation. The absorbance at 260 nm, (³H)-poly(U) radioactivity and the protein analysis indicate that different types of poly(A)⁺ RNP were present (23). Proteins with Mr of 45,000 and 65,000, 76,000 and 94,000, 45,000, 50,000 and 52,000 sedimented at 20-25S, 10-20S and 5-10S respectively.

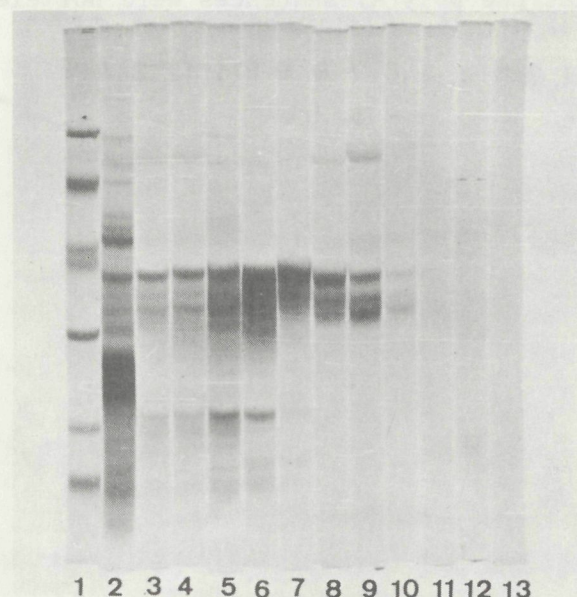


Figure 11. Affinity chromatography of poly(A)⁺RNP proteins on poly(A)-Sepharose.

Proteins were extracted from poly(A)⁺RNP by LiCl and bound to poly(A)-Sepharose in buffer N. Bound proteins were eluted from the column by a linear KCl gradient from 10 mM up to 2 M. Proteins eluted in different fractions were analyzed by SDS polyacrylamide gel electrophoresis.

gel 1: markers, phosphorylase b (94,000), serum albumin (68,000), ovalbumin (43,000), trypsin inhibitor (20,100) and α -lactalbumin (14,000); gel 2: unretained material; gel 3-13: fractions eluted with a linear salt gradient from 10 mM to 900 mM.

The protein with Mr of 38,000 was observed in each of the sedimenting regions. It is generally accepted that a 76,000-protein is attached to the 3' poly(A)-segment of polysomal mRNP (25-28). Our results indicate that some of the stored mRNP are devoid of the latter protein. The absence of a 78,000-protein is also reported for free mRNP of ascites tumor cells and rabbit reticulocytes (28). Contrary to these reports Jain and Sarkar (29) only observed differences in the proteins of the non-poly(A) part of free and polysomal mRNP.

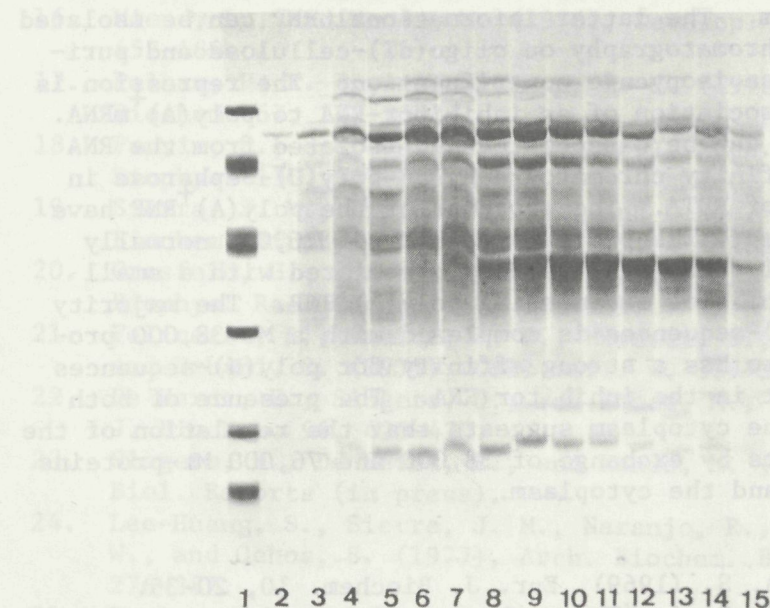


Figure 12. Affinity chromatography of PRS on poly(A)-Sepharose. PRS prepared in buffer L was chromatographed on poly(A)-Sepharose. Bound material was eluted with a linear NaCl gradient from 250 mM to 1.5 M NaCl. Different fractions were analyzed by SDS polyacrylamide gel electrophoresis.

gel 1: markers as in fig. 11; gel 2-15: fractions eluted with a linear salt gradient from 250 mM to 1.5 M NaCl.

The proteins of poly(A)⁺RNP with affinity for the poly(A)-sequence were isolated by affinity chromatography on poly(A)-Sepharose. Only proteins with Mr of 38,000 and 76,000 were retained on this column (Fig. 11). The Mr-38,000 protein constitutes the main fraction. Affinity chromatography of PRS on poly(A)-Sepharose resulted in the binding of Mr-38,000 and Mr-76,000 proteins in approximately the same amount indicating that these proteins also exist free in the

cytoplasm of cryptobiotic embryos (Fig. 12). These results suggest that the repression is regulated by exchanges of the Mr-38,000 and Mr-76,000 between the poly(A)⁺ RNP and the cytoplasm. The relation between the repression and the presence of the Mr-38,000 protein is strengthened by the fact that this protein is also bound to poly(U)-Sephadex but not to rRNA-Sephadex. Furthermore, the inhibitor oligonucleotide characterized by Lee-Huang et al. (24) contained as much as 47% U.

We may conclude that only the free cytoplasmic fraction of poly(A)⁺ mRNP of the cryptobiotic embryos exists in a repressed form. The latter informational RNP can be isolated by affinity chromatography on oligo(dT)-cellulose and purified by sucrose isopycnic centrifugation. The repression is due to the association of an inhibitor RNA to poly(A)⁺ mRNA. The inhibitor can be dissociated and isolated from the RNA complex by affinity chromatography on poly(U)-Sephadex in the presence of EDTA. The proteins of the poly(A)⁺ RNP have been identified. The proteins with Mr of 76,000 normally present in polysomal mRNP is only associated with a small fraction of the slow sedimenting poly(A)⁺ RNP. The majority of the poly(A)-sequences is complexed with a Mr-38,000 protein which also has a strong affinity for poly(U)-sequences known to exist in the inhibitor RNA. The presence of both proteins in the cytoplasm suggests that the regulation of the repression acts by exchange of 38,000 and 76,000 Mr-proteins between mRNP and the cytoplasm.

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THE NAUPLIAR HEMOGLOBINS OF THREE POPULATIONS OF ARTEMIA

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ABSTRACT

In *Artemia* adults, we have found four hemoglobins: Hb-1, Hb-2, Hb-X, and Hb-3. Other investigators have characterized Hb-N, which has the same electrophoretic mobility as Hb-2, but which is found only in the nauplius. We find no evidence for a unique naupliar hemoglobin. On polyacrylamide gels, naupliar Hb-2 and adult Hb-2 had the same mobility in three populations where Hb-2 relative mobilities were 0.99, 1.00, and 1.04. When logarithm of relative mobility was plotted against concentration of acrylamide, three of the hemoglobins (Hb-1, Hb-2, and Hb-X) gave a parallel family of lines. They make up a family of charge isomers with the same shape and size ($MW: 3.1 \times 10^5$). Because the plot of Hb-3 has a greater negative slope, Hb-3 may overlap Hb-X when hemoglobins from some populations are run on gels with little molecular sieving effect.

INTRODUCTION

When brine shrimps are reared under an atmosphere which is 100% oxygen, no hemoglobin is detectable in adult hemolymph. When adults are cultured under an atmosphere of 6% oxygen, four hemoglobins are present: Hb-1, Hb-2, Hb-X, and Hb-3, listed in descending order of electrophoretic mobility on 6% polyacrylamide gels. When adults are reared under air (20% oxygen), Hb-X is usually not detected. When Hb-X was first seen, it was thought to be a genetic variant of one of the other hemoglobins. Therefore, it was designated by letter rather than number (1). We have now shown that if shrimps are reared under 6% oxygen, the four hemoglobins are present in most adults of the 30 populations we have studied (2). Although many populations are monomorphic, genetic variants have been found for each of the four hemoglobins. The relative amounts of the hemoglobins change during ontogenesis (3,4). Hb-3 cannot be detected in adults older than six weeks of age (1,4) when reared under air.

Hb-1 is an alpha homopolymer, $(\alpha\alpha)_n$. Hb-2 is a heteropolymer, $(\alpha\beta)_n$. Hb-X is a beta homopolymer, $(\beta\beta)_n$. Hb-3 contains neither alpha nor beta polypeptides (5,6). The ratio of concentrations of alpha/beta polypeptides decreases as oxygen partial pressure over the culture medium decreases (5), as shown in Fig. 1. When two populations have

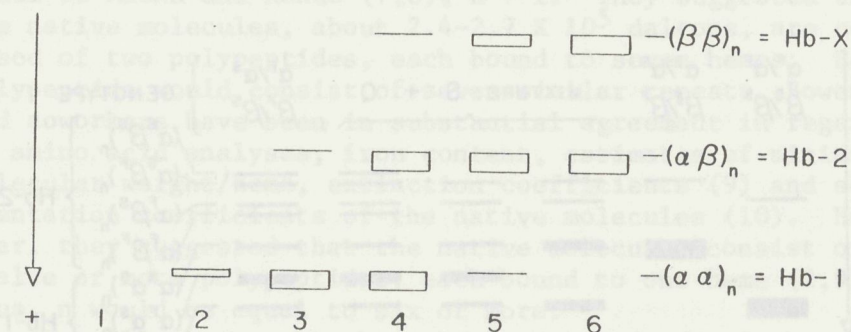


Figure 1. Electrophoretic patterns of hemoglobins of six adult females with the same genotype (homozygous for the alleles most common in the San Francisco population). Phenotypes differ because shrimps were cultured under decreasing oxygen partial pressure (from left to right). Shrimp #1 was maintained under 80%, #3 under 20% and #6 under 3% oxygen atmosphere. Concentration of total hemoglobin in shrimps #1, #3, #4, and #6 is 0, 6, 9, and 20 mg/ml hemolymph, respectively. Hb-3 is not present in these eight week old adults. Technique described earlier (5). Anode at bottom of figure; 6% polyacrylamide slab gel.

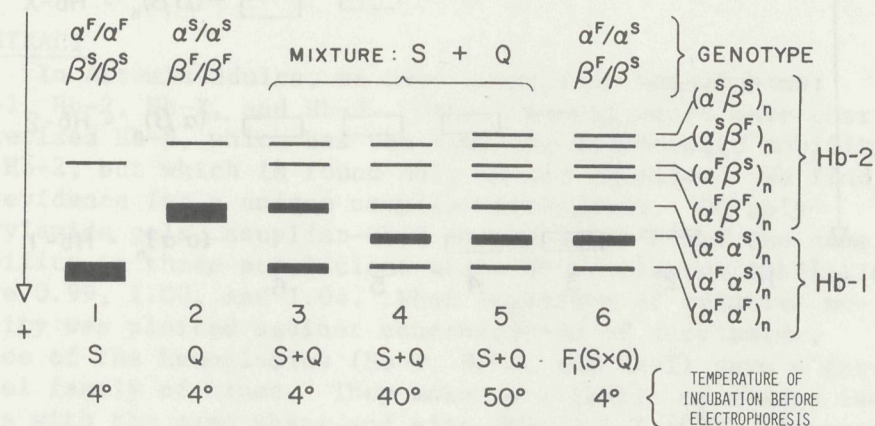


Figure 2. Electrophoretic patterns of six samples of hemolymph on a 6% polyacrylamide slab gel. Hb-X and Hb-3 are not illustrated. Hemolymph samples from inbred lines S and Q are in slots #1 and #2, respectively. A mixture of equal amounts of the two is run in slots #3-5. Before electrophoresis, aliquots were incubated for two hours at 4°, 40°, or 50° (slots #3, 4, and 5, respectively). Generation of a new $\alpha^F\alpha^S$ dimer occurs in aliquot #4 but not in #3. Although Hb-1 and Hb-2 native molecules do not dissociate into monomers during electrophoresis in the refrigerated cell, Hb-1 dissociates at 40° and Hb-2 dissociates to monomers at 50°. In slot #5, the mixture of two hemolymphs incubated at 50° generates a four-banded Hb-2, identical to that seen in F_1 shrimps obtained by crossing parents S and Q (slot #6). Stock S is homozygous for the prevalent α^{100} allele and rare β^S allele in the San Francisco population. Stock Q is homozygous for the α^{96} and β^F alleles which are the alleles with highest frequency in the Quemado population (2). Hybridization method described earlier (5).

different alleles at alpha and beta loci, hybridization *in vivo* or *in vitro* (5,6) results in a three-banded Hb-1 and a four-banded Hb-2 (Fig. 2).

The value of n , the number of dimers in the native hemoglobins, is a matter of controversy. According to the model of Moens and Kondo (7,8), $n = 1$. They suggested that the native molecules, about $2.4-2.7 \times 10^5$ daltons, are composed of two polypeptides, each bound to seven hemes. Each polypeptide would consist of seven similar repeats. Bowen and coworkers have been in substantial agreement in regard to amino acid analyses, iron content, estimates of minimum molecular weight/heme, extinction coefficients (9) and sedimentation coefficients of the native molecules (10). However, they suggested that the native molecules consist of twelve or more polypeptides, each bound to one heme (2,9). Thus, n would be equal to six or more.

We will attempt to show the correspondence of the four hemoglobins observed by Moens and Kondo and coworkers on cellulose acetate electrophoresis with the four hemoglobins we have observed on polyacrylamide electrophoresis.

MATERIALS AND METHODS

Cysts from San Francisco Bay (SFR), the Gulf of Kutch, India (KUT), and Great Salt Lake, Utah (GSL) were stored at -20°. They were hatched and maintained without feeding in artificial sea water (1 cm depth) at 24°. Nauplii were harvested at 6, 24, and 48 hours after hatching, washed in Tris-chloride buffer (0.05 M Tris) at 4°, homogenized in a chilled glass tissue grinder (drained nauplii: buffer, 10:1, v/v), and centrifuged. The supernate was concentrated 15-fold in a Minicon B-15 macrosolute concentrator (Amicon Corp., Lexington, Mass.) for four hours at 4°.

Adults were females, either 2-3 or 8-10 weeks of age, reared either under air or under 6% oxygen atmosphere by our standard method (2). Hb-3 was present in the hemolymph of young adults but not detectable in older adults. Hb-X was not detectable in the hemolymph of adults reared under air but was present in those reared under the reduced oxygen regimen. Adults were washed in Tris-Cl buffer, the hemolymph removed (6), and placed on the electrophoresis slab gel within 2h. For comparison of relative mobilities of adult and naupliar hemoglobins, hemolymph samples from four adults (of the same age and from the same regimen) were pooled, then divided into four aliquots. One aliquot was run (beside the naupliar extract from the same population) on each of the four slab gels (4, 5, 6, and 7% acrylamide concentrations). For calculation of retardation coefficients, both pooled and individual adult hemolymphs,

homogenized adults and naupliar extracts were run on the same slabs with reference proteins. Samples were mixed with an equal volume of 20% sucrose in Tris-Cl buffer containing bromphenol blue. All samples were made up to the same volume (11 μ l). Ovalbumin, bovine serum albumin, horse apoferritin (Mann Research) and bovine liver catalase (Sigma C-100) were run separately and together as molecular weight standards. Slab gels (0.75 X 160 X 105 mm) were cooled by circulating water (4°C). The Tris-glycinate discontinuous buffer system and apparatus have been described (6,10). All gels were made by dilution of one 40% acrylamide stock solution. T, the percent acrylamide concentration, was defined as g% acrylamide (11), rather than the sum of Bis and acrylamide. The ratio of Bis to acrylamide was 1:37.5. No spacer gel was used.

For demonstration of peroxidase activity, gels were stained overnight in 0.2% ortho-dianisidine dissolved in 5% glacial acetic acid - 0.06% H₂O₂ (w/v/v). If gels were stored in darkness, the color was stable for three months. For relative mobility measurements, gels were stained in Coomassie Blue. Distances from the origin to the bands of dye or protein were measured with calipers. R_m , the relative mobility, was defined as the distance of migration of protein/distance of migration of marker dye. In "Ferguson plots", the common logarithm (100 X R_m) was plotted against T (11,12). The slope of this plot (K_R , the retardation coefficient), was obtained by unweighted linear regression analysis (13). Molecular weights of reference proteins were taken from two sources (11,13).

RESULTS AND DISCUSSION

1. Protein stains. When adults are maintained under certain conditions(2), their hemolymph contains "slow proteins" which migrate more slowly than the hemoglobins. They are sometimes red or green and may stain with Oil Red O or be peroxidase positive (1,2,4,14). However, in the present study, none of the hemolymph proteins were green and none stained with Oil Red O. Only the four hemoglobins were red and peroxidase positive. When detected by peroxidase activity, the four hemoglobins were also demonstrable by Coomassie Blue and vice versa.

2. Molecular weight estimates. Ferguson plots of four hemoglobins from each of two *Artemia* populations are shown in Fig. 3. In all three populations, Hb-1, Hb-2, and Hb-X give a parallel family of lines with $K_R = 18.1$, evidence that the three proteins have the same molecular size and shape (11). In each population, the three lines are equidistant. Fig. 4 is a plot of the K_R values of

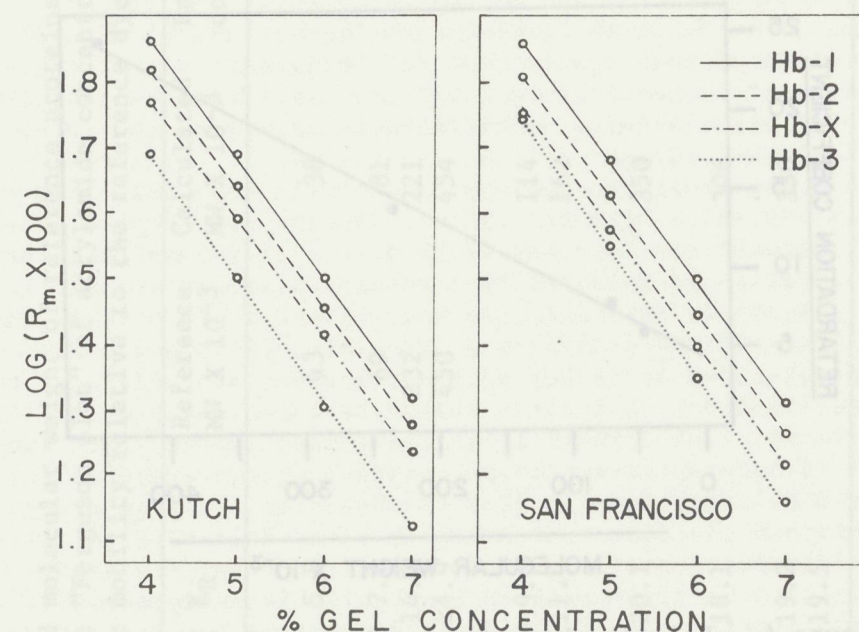


Figure 3. Comparison of Ferguson plots of four hemoglobins from shrimps of two populations: Kutch and San Francisco. In order of descending electrophoretic mobility, the hemoglobins are: Hb-1, Hb-2, Hb-X, and Hb-3. Values for adult and naupliar hemoglobins were pooled because there was no significant difference between them. K_R , the retardation coefficient of each protein is defined as the negative slope of the equation, $\log(R_m \times 100) = K_R T + b$, where R_m is the mobility relative to marker dye, T the percent acrylamide, and b a value characteristic of each protein. K_R values are given in Table 1.

Figure 4. Relationship of retardation coefficient and molecular weight in reference proteins (identified in Table 1). $K_R = 4.08 + (MW \times 4.6 \times 10^{-5})$.

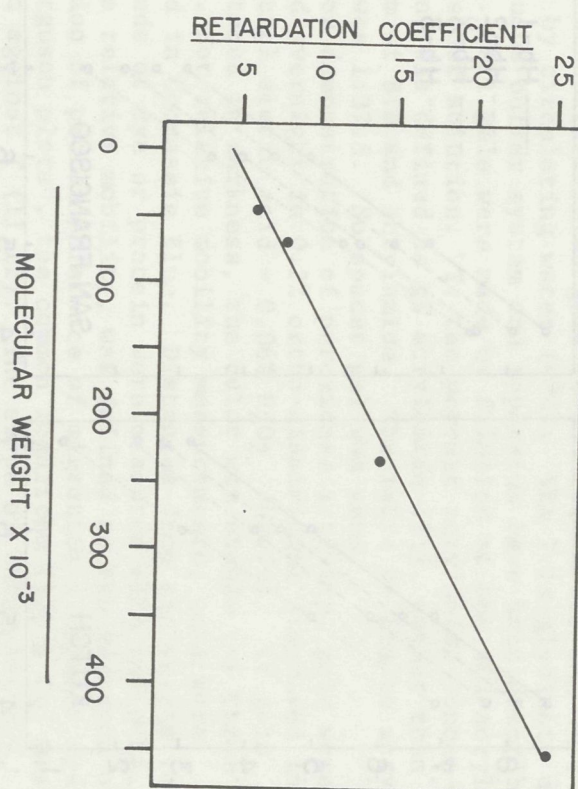


Table 1. Retardation coefficient (K_R) and molecular weight of reference proteins and Artemia proteins. K_R is the negative slope of the "Ferguson plot" of acrylamide concentration against $\log(100 \times R_m)$, where R_m is electrophoretic mobility relative to the reference dye.

Protein	K_R	Reference MW X 10 ⁻³	Calculated MW X 10 ⁻³	Range of gel concentration
Reference proteins				
1. ovalbumin	5.8	43	38	5 -10%
2. bovine serum albumin monomer	7.8	69	81	5 -10%
3. bovine liver catalase	14.2	232	221	4 - 8%
4. horse apoferritin	24.9	450	454	4 - 8%
Artemia isozymes				
esterase (EST-1)	9.3		114	8 -10%
alkaline phosphatase (ALP-1)	11.5		162	8 -10%
Artemia hemolymph "slow proteins" (SFR population) S-2 and S-4				
	20.1		350	4 - 7%
Artemia hemoglobins				
Hb-1, Hb-2, Hb-X in three populations	18.1		306	4 - 7%
Hb-3 in GSL and KUT populations	19.2		330	4 - 7%
Hb-3 in SFR population	19.5		336	4 - 7%

standard proteins against molecular weight. Table 1 lists estimates of molecular weight of *Artemia* proteins. Earlier estimates for the hemoglobins were $2.3 - 2.7 \times 10^5$ daltons (7,8,9,10) based upon sedimentation coefficients. We now revise our estimate to 3.1×10^5 for Hb-1, Hb-2, and Hb-X and 3.3×10^5 for Hb-3. Two other hemolymph proteins, S-2 and S-4, are estimated to be 3.5×10^5 daltons.

3. Possible overlap of hemoglobins. In the 30 wild populations, Hb-1, Hb-2, Hb-X, and Hb-3 retain this same rank order of mobility on 5-7% polyacrylamide gels (2,5). However, it is possible to construct inbred stocks carrying slow alpha alleles and fast beta alleles so that Hb-2 overlaps Hb-1 (2).

In San Francisco shrimps, Hb-3 has a high value and Hb-X a low value of mobility relative to other populations (2). Because the K_R value of Hb-3 is higher than that for Hb-X, overlap of Hb-3 and Hb-X occurs on support media with low molecular sieving capacity, such as 4% acrylamide gels (Fig. 3) and cellulose polyacetate. If polyacetate strips are used for study of Hb-X, shrimps from Kiatuthlanna, Tallaboa, or Tunisia should be chosen because of the low values of Hb-3 mobility in those populations (2). Two other factors which sometimes contribute to overlap is the genetic polymorphism (in Hb-X and Hb-3) in San Francisco shrimps and the fact that mobility of Hb-3 in PAGE is inversely related to its concentration. However, in regard to Hb-1 and Hb-2, protein concentration is independent of electrophoretic mobility, provided distance is estimated from the trailing edge of the protein (6).

4. Number of hemoglobins in nauplii. No hemoglobins were detected in nauplii of the three populations within 6h after hatching. At 24 and 48h posthatching, Kutch nauplii contained Hb-2 and Hb-X (relative concentrations of 2:1, respectively, by Coomassie Blue staining). In the two batches of cysts from pond A18 in San Francisco Bay, different patterns were seen. Nauplii hatched from SFR cysts collected in 1963 contained Hb-2 and Hb-X (concentration ratio of 2:1) at 24h and 48h posthatching. Nauplii hatched under the same conditions from 1972 cysts showed no detectable hemoglobin at 24h. Similarly, cysts collected from Great Salt Lake at three different times showed different patterns 48h after hatching. Although all contained approximately equal amounts of Hb-2, one batch contained only Hb-2, another Hb-2 and Hb-X (2:1 ratio), and the third Hb-1, Hb-2, and Hb-X (concentration ratio of 1:10:5). Whereas there is a predictable shift in relative amounts of hemoglobins in ontogeny (4), the absolute amount of each hemoglobin in the nauplius may be influenced by

conditions of cyst storage and hatching, nauplius culture, or environment of the females which produced the cysts.

5. Search for Hb-N, a naupliar hemoglobin. Moens and Kondo (7) reported that although adult Hb-II and naupliar Hb-N had the same isoelectric point and the same mobility on cellulose acetate electrophoresis, the sedimentation coefficients differed (11.3 for Hb-II; 11.8 for Hb-N). The two proteins had somewhat different patterns on SDS gels (7,8). We compared naupliar and adult hemoglobins on 4-7% polyacrylamide slab gels. An analysis of variance revealed more variance attributed to discrepancies between repeated measurements of the same sample (due to gel stretching or interpretation of the location of the edge or midpoint of the band) than to differences between relative mobilities of adult and naupliar hemoglobins. In Fig. 5, it is seen that relative mobilities of adult Hb-2 and naupliar Hb-2 (putative Hb-N) are identical in three populations where mobilities of Hb-2 are 1.04, 1.00, and 0.99. We failed to demonstrate differences in K_R values for Hb-2 in nauplius and adult, as would have been expected if they had different sedimentation coefficients. We find no evidence for a hemoglobin unique to the nauplius.

6. Relationship of symbols used by two groups of investigators. The probable correspondence of Hb-I, Hb-II, Hb-III, and Hb-N of Moens and Kondo to our Hb-1, Hb-2, Hb-X and Hb-3 is shown in Table 2. We perceive their Hb-III as two proteins because of the improved resolution of 6% polyacrylamide gels compared with cellulose polyacetate electrophoresis. In Table 2, the correspondence of the two systems is based on the following observations. In adults of the same genotype reared under the same conditions, the "Hb-1/Hb-2 ratio" and "Hb-I/Hb-II ratio" are higher in females than in males (1,4,6). ("Hb-1/Hb-2 ratio" denotes the ratio of amount of Hb-1 to amount of Hb-2, determined by Coomassie Blue staining.) In adults of the same sex and genotype, as the oxygen content of the culture medium decreases, Hb-X and Hb-III increase (3,5). The alpha polypeptide is detectable at a higher oxygen partial pressure than is the beta polypeptide (5). This is in accord with the observation that Hb-1 has a lower oxygen affinity than Hb-2 (15). In the present study, adults of the same sex and genotype, reared on the same regimen, Hb-3 was present at three weeks of age but undetectable at eight weeks of age. This confirms observations that concentration of Hb-3 and of Hb-III decline with ageing of the adult (1,4).

7. Identification of hemoglobins. In addition to the correlation of concentration with age, Hb-3 from parthenogenetic or SFR populations can be identified by the fact

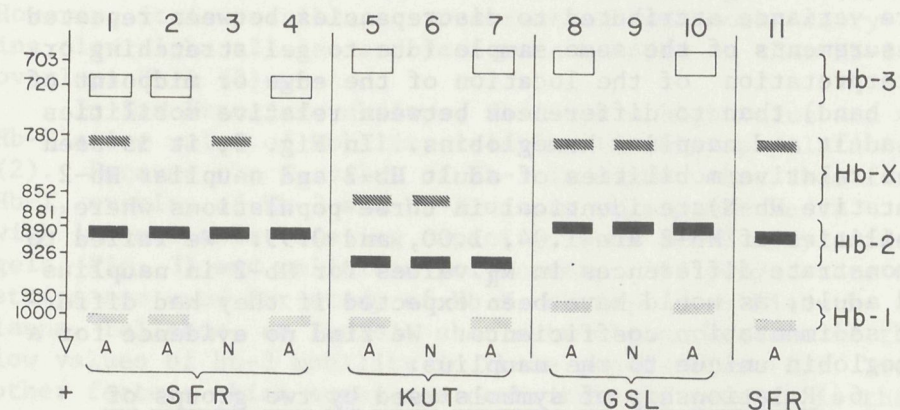


Figure 5. Comparison of relative electrophoretic mobilities of four hemoglobins in adults (A) and in nauplii (N) of three populations (SFR, San Francisco; KUT, Kutch; GSL, Great Salt Lake). Hb-1, stippled; Hb-2, solid; Hb-X, hatched; and Hb-3, open bar. Mobilities are measured from the origin to the middle of the Hb-3 band and to the trailing edge of the other hemoglobins. Mobility values are normalized, with 1000 representing the Kutch Hb-1 relative mobility. No genetic variation has been found in hemoglobins of the parthenogenetic Kutch shrimps. In zygogenetic SFR and GSL populations, mobility values are given for the most common alleles (2). Hb-X is present in adults #1, 5, 8, and 11 which were reared under 6% oxygen. Other adults were reared under air. Hb-3 is absent in adult #11 (eight weeks old) and present in the other adults (three weeks of age). Note that the distance between Hb-1 and Hb-2 is equal to the distance between Hb-2 and Hb-X. Tris-glycinate discontinuous buffer system, 6% acrylamide, anode at bottom of figure.

Table 2. Probable correspondence of hemoglobins described by two groups of investigators

Investigators (Support medium for electrophoresis)	Hemoglobins				
Moens, Kondo, Heip (Cellulose polyacetate electrophoresis)	Hb-I	Hb-II	Hb-III	Hb-III	Hb-N
Bowen, Sterling, Bucks (Cellulose polyacetate and 4-7% polyacrylamide gel electrophoresis)	Hb-1	Hb-2	Hb-X	Hb-3	Hb-2

that at high concentrations, it generates an additional band (or bands) which migrate faster than Hb-1 on 6-7.5% polyacrylamide gel electrophoresis at 24° (10). Because rank order of the four hemoglobins may change with genetic variation (section #3 above), the best guide to identification of the proteins is the ratio S/R, where S is the distance between Hb-2 and Hb-X and R is the distance between Hb-2 and Hb-1 (5). The mean value of S/R is 1.15 on cellulose polyacetate and 1.05 on 4-7% polyacrylamide gel electrophoresis.

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STUDY OF EXTRACELLULAR HEMOGLOBIN, FROM ARTEMIA SALINA, BY SCATTERING TECHNIQUES

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ABSTRACT

Hemoglobin II from Artemia salina has been studied with classical light scattering and with photon-correlation spectroscopy combined with sedimentation measurements. A molecular weight of $260,000 \pm 10,000$ can be concluded. Also some hydrodynamic parameters have been calculated.

INTRODUCTION

During the development of Artemia salina, several proteins are translated, and it is very important to have a well characterized marker protein, which could be followed during the whole developing period, to get more information about the different mechanisms of the regulation of the protein synthesis. Extracellular hemoglobin was chosen for this purpose (1) and a physical-chemical characterization of the structure has been started because classical biochemical studies haven't revealed a unique structure until now (2,3,4). The hemoglobin has been investigated using light scattering and hydrodynamical techniques. These methods give us the molecular weight and some macroscopic parameters such as the maximal asymmetry, maximal hydration and the most probable asymmetry assuming a mean value for the hydration. Based on the data of these methods, X-ray scattering measurements have been performed at small and larger angles in cooperation with Dr. G. Damaschun. The interpretation of these experiments will give us a detailed picture of the quaternary structure.

MATERIALS AND METHODS

We have followed the isolation procedure of Moens and Kondo (3). Special care has been paid to avoid degradation of the material and during chromatographic separation, only the innermost peak fractions have been used. All measurements have been performed on hemoglobin II (3). The quality of our material, even after several days of light scattering measurements at room temperature for several hours, was still very good as could be concluded from polyacrylamide gradient gel electrophoresis. The ratio $A_{415 \text{ nm}}/A_{280 \text{ nm}}$ has been measured as another criterion for the quality of the material: only a ratio of 3.2 and larger for oxygenated solutions has been accepted as good. The absorbance of the hemoglobin

solution has been used as a method for the concentration determination. The Kjeldahl method for nitrogen determination has been slightly modified so that it is possible to determine the relation absorbance/nitrogen content, starting with 10 mg of protein material, with a reliability and accuracy better than 2%. Taking into account the known amino-acid composition (3), the absorbance $A_{1\text{cm}, 280\text{nm}}^{1\%}$ can be calculated (5).

The use of light scattering for the study of hemoglobin is hampered by the high absorbance of these solutions at the two wavelengths normally used for light scattering, namely 436 nm and 546 nm. The hemoglobin was investigated at 645 nm in a Brice Phoenix Photometer, using small conical cells (minimum content 3 ml). The light scattering instrument has been calibrated in two different ways, using tungstosilicic acid as a molecular weight standard (6): the scattered intensities were measured in comparison to the scattered intensity at 90° of benzene and in comparison to the primary intensity using a diffusor in front of the detector (7). The reliability of the method has been controlled by the measurements of highly purified lysozyme solutions. The molecular weight M has been calculated from the formula

$$\frac{1}{M} = \frac{K_c}{R_\theta} + 2Bc$$

in which: K : optical constant, depending on the wavelength, refractive index of the solution and refractive index increment of the polymer (dn/dc).

c : concentration

$2B$: second virial coefficient

R_θ : absolute Rayleigh ratio

In order to avoid a high second virial coefficient, the measurements have been made in a phosphate buffer (0.01 M, pH 7) containing 0.1 M NaCl. Due to the small size of the particle, the scattered intensity, corrected for the volume effect, is independent of the angle of measurement. So, only measurements at 90° have been made.

The refractive index increment (dn/dc) has been measured using a Rayleigh-Haber-Löwe interferometer. Due to the fact that the refractive index difference is measured on the basis of interferometry, dilute solutions can be measured (from 0.5 to 2 mg/ml).

The analysis of the fluctuations of the light of a laser beam of high intensity, scattered by the hemoglobin solution

can yield the translational diffusion coefficient D . In photoncount autocorrelation spectroscopy, the fluctuations in the time domain are analyzed using an autocorrelator. The diffusion coefficient can be calculated from the second order correlation function:

$$g^2(iT) = 1 + A \exp(-2DK^2 iT)$$

where i = channel number

T = sample time

A = constant

K = scattering vector

The autocorrelation function was determined using a Malvern autocorrelator with 24 channels. The use of an argon laser, at 488 nm, limits the concentration range of the hemoglobin solutions which can be studied (< 2 mg/ml) due to absorption at this wavelength. Although it has been shown that this absorption does not influence the correlation function (8), it limits the scattered intensity and in this way the accuracy. The autocorrelation functions have been analyzed according to a procedure proposed by Nieuwenhuysen (9). This procedure involves different curve fitting methods and takes account of the disturbances in the correlation functions, due to dust particles and others, in an optimal way.

The sedimentation coefficient has been determined with a MSE analytical ultracentrifuge equipped with a monochromator and automatic scanner. The sedimentation has been determined by following the movement of the boundary with the absorption method using light of 415 nm wavelength. The high absorbance at this wavelength, allows the use of low concentrated solutions and makes the concentration corrections for the S value unnecessary. The use of the Svedberg relation for the calculation of the molecular weight from the sedimentation and diffusion coefficient requires the knowledge of the partial specific volume $\bar{v}$ of the protein, in the same conditions in which the S and D values have been measured. The quantity $\bar{v}$ can be calculated from the density increment dp/dc of the hemoglobin solutions, according to the relation

$$1 - \bar{v}\rho_0 = \frac{dp}{dc}$$

where ρ_0 is the solvent density, to which the hemoglobin solution is in thermodynamical equilibrium after extensive dialysis. The density of the solutions have been measured using the mechanical oscillator technique (10). The frequency f of the U shaped oscillator depends on the density

ρ of the solution according to the relation

$$f = \frac{1}{2\pi} \frac{\epsilon}{\rho V + M_0}^{1/2} \quad \text{where}$$

M_0 : mass of the empty oscillator

V_0 : internal volume of the oscillator

ϵ : instrumental constant

Different hemoglobin solutions have been measured up to concentrations of 10 mg/ml.

RESULTS AND DISCUSSION

Absorbance, Sedimentation, Diffusion and Density measurements

The relation absorbance/nitrogen content of hemoglobin solutions has been determined using the Kjeldahl method. Accepting a nitrogen content of 16.35% for hemoglobin II (2,3) an absorbance $A_{1\text{ cm}, 280\text{ nm}}^{1\%}$ of 17.8 ± 0.3 can be calculated. This value is somewhat higher than published data (2,11) which were based on dry weight determinations. Because it is known that dry weight determinations of the absorbance can underestimate the real value (12), a somewhat higher value looks reasonable. Carbohydrates, which can be present as glucosamines in a small proportion, probably do not influence the result to a great extent.

Sedimentation measurements have been performed in a great range of ionic strengths on different hemoglobin samples. A mean value for $S_{20,w}$ of 11.0 ± 0.2 S has been obtained. From measurements at different pH values, it can be concluded that the protein keeps its native macromolecular structure up to a pH value of 11.0. Due to the absorption of hemoglobin at 488 nm, the wavelength at which the photoncorrelation spectra have been taken, only low concentrations of protein can be used. Also the small size of the protein increases the measurement times. This increases the requirements for a very stable laser output and the absolute absence of dust. Although both conditions were not always perfectly met, the analysis of the autocorrelation functions with different values of the quality factor Q or a mismatching factor δ yields consistent values of the diffusion coefficient, when the calculated values of D were extrapolated to a quality factor Q or a mismatching factor δ equal to zero. In that way, measurements at different angles (45° to 135°) yield the same value for the diffusion coefficient, reduced to standard conditions, $D_{20,w}$ of $(3.7 \pm 0.1) \times 10^{-7}$ cm²/sec. From

density measurements of different hemoglobin solutions, as compared to the solvent, a $\bar{v}$ value of (0.737 ± 0.005) cm³/g has been calculated. This rather high value fits to a peptide which contains a haem group. From the Svedberg relation

$$M = \frac{S}{D} \cdot \frac{RT}{(1 - \bar{v}\rho_0)}$$

a molecular weight of $275,000 \pm 15,000$ can be deduced.

Light Scattering

Light scattering measurements of solutions up to concentrations of 3.5 mg/ml, using natural and vertically polarized light of 645 nm, resulted in a molecular weight of $250,000 \pm 6000$. In order to obtain this result, the experimentally determined value of 0.186 cm³/g for the refractive index increment has been used.

Hydrodynamic behavior

From the diffusion coefficient and the partial specific volume, the following hydrodynamic parameters can be calculated:

$$f = \frac{kT}{D}; \quad f : \text{frictional coefficient}$$

$$f_{\min} = 6\pi\eta \frac{3\bar{M}\bar{v}}{4\pi N_a}^{1/3}; \quad f_{\min} : \text{minimal frictional coefficient}$$

$$f_0 = 6\pi\eta R_0$$

The minimal frictional coefficient $f_{\min}$ corresponds to a spherical particle with a volume equal to that of the dry particle; the frictional coefficient f_0 corresponds to a hypothetical spherical particle with radius R_0 and having the same volume as the real hydrodynamic macromolecule. The relation

$$\frac{f}{f_{\min}} = \frac{f}{f_0} \frac{\bar{v} + \delta v_0}{\bar{v}}^{1/3}$$

in which: f/f_0 : form factor

δ : solvation in (g of water/g of protein)

v_0 : specific volume of the solvent (water)

takes into account that the real particle deviates from a spherical particle (form factor f/f_0) and also that the real particle is hydrated (δ).

From this, the maximal solvation (accepting a spherical particle) or the maximal asymmetry (accepting no solvation) or the most probable asymmetry accepting some mediate solvation, can be calculated (13). A maximal solvation of 1.0 g water/g protein, a maximal asymmetry a/b of 6 for an unsolvated ellipsoid, or an axial ratio of 1.7 for an oblate ellipsoid can be calculated using the Perrin functions accepting a solvation of 0.25 (14).

Dissociation

Sedimentation measurements of hemoglobin solutions at different alkaline pH values have shown that hemoglobin II dissociates mainly to a smaller unit, which has a sedimentation coefficient of 6.3 S and some other minor small fragments. The dissociation cannot be reversed and quick alkalization (by adding KOH) or slow treatment (by dialysis against a alkaline buffer) and slow or quick neutralization afterwards yield the same components. These data agree with the results of Bowen et al (2). Equilibrium sedimentation of native hemoglobin and the 6.3 S component in identical experimental conditions have shown that the molecular weight of the native molecule is two times as large as the molecular weight of the 6.3 S component if we accept the same partial specific volume for the two molecules. This result perfectly fits the idea of a dimeric structure of the hemoglobin as proposed by Moens and Kondo (3).

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BIOGENESIS OF THE (Na+K)-ACTIVATED ADENOSINE TRIPHOSPHATASE DURING NAUPLIAR DEVELOPMENT IN ARTEMIA

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ABSTRACT

Artemia (Na+K)-activated adenosine triphosphatase, Na,K-ATPase, has been purified 200-fold over the homogenate to a purity of about 60%. The specific activity of the partially purified enzyme is about 600 $\mu\text{mol P}_i/\text{hr/mg}$ protein, and the yield is about 10 mg/100 g dry cysts. By SDS-polyacrylamide gel electrophoresis, two large subunits ($\alpha 1$ and $\alpha 2$) and a small subunit (β) are identified with molecular weights 100,400, 94,900 and 41,100, respectively, of which the large ($\alpha 1 + \alpha 2$) and small subunits are found in a molar ratio of 1:1. In the newly hatched nauplius, $\alpha 1$ and $\alpha 2$ are nearly equal in concentration, and later in development, $\alpha 2$ is progressively lost. Na,K-ATPase activity first appears 8 hr after hydration and initiation of development and is maximum by 32 hr. Preliminary results of pulse-chase experiments with $\text{NaH}^{14}\text{CO}_3$ indicate that synthesis of Na,K-ATPase parallels that of other membrane proteins and occurs about 2-4 hr in advance of enzyme activity.

INTRODUCTION

The developing brine shrimp nauplius is being pursued as a model for the study of the biogenesis of the (Na+K)-activated adenosine triphosphatase (Na,K-ATPase). This enzyme is responsible for the active coupled transports of Na^+ and K^+ across biological membranes (1). The enzyme is an integral plasma membrane-lipoprotein complex consisting of two different protein subunits, a large subunit of molecular weight 95-100,000 and a small subunit of molecular weight 40-60,000, and both are glycoproteins (2,3). The large subunit, which is often referred to as the catalytic subunit since it is specifically phosphorylated from ATP (4), is a transmembrane protein (5). The small subunit is exposed to the external surface (6), but it is not known if it spans the bilayer. To date, its function remains unknown. Biogenesis of this enzyme presents special problems with respect to translation of the two highly lipophilic subunit proteins, unique glycosylation of each, transport of the subunits to the plasma membrane and insertion into it, and at some point the coordinated assembly of the subunits into the holoenzyme. Understanding these

interesting biochemical problems is fundamental to understanding the turnover and regulation of this metabolically important enzyme.

Earlier work on the Great Salt Lake variety of Artemia demonstrated that the preemergent brine shrimp cysts do not contain any detectable Na,K-ATPase activity. Enzyme activity first appeared in the emergent stages (E1 and E2) (6) and developed to high levels in the nauplius (7), which was maximum by 40 hr after initiation of development of the encysted embryo (6,8). This large increase in enzyme activity was found to be due to de novo synthesis (8).

In continuing with these studies, we have switched to the San Francisco variety of brine shrimp. Efforts were made to improve the purification of the brine shrimp Na,K-ATPase. The previous partial purification procedure (2) routinely gave yields of about 10 mg protein per 200 g dry cysts at a specific activity of about 300 $\mu\text{mol P}_i$ released per hr per mg protein, which was about 25-30% pure on analysis by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). We have developed a new partial purification procedure which gives a two-fold increase in yield and purity. We have also determined the stoichiometry of the two brine shrimp Na,K-ATPase subunits by very careful molecular weight and mass ratio analysis. This information is important in order to relate the coordinated synthesis and assembly of the two subunits. In agreement with previous findings (2), the large subunit consists of two bands. The relationship to these two bands or isozymes to development are examined. Pulse-chase experiments with $\text{NaH}^{14}\text{CO}_3$ have been performed to accurately stage the synthesis of the Na,K-ATPase subunits in relation to the appearance of functional enzyme.

MATERIALS AND METHODS

Brine shrimp eggs were obtained from San Francisco Bay Brand (Newark, California) and stored at -20°C . The dried cysts were prehydrated for a minimum of 4 hr at 4°C , washed, and then incubated in one-half strength sea water (Instant Ocean Sea Salts) with aeration at 28°C for up to 48 hr.

Four steps were involved in purification of the brine shrimp Na,K-ATPase. 1. Crude membranes -- The harvested nauplii were washed with distilled water and homogenized in a Waring blender containing 100-150 ml homogenizing medium (250 mM sucrose, 50 mM imidazole-HCl, 2 mM Na_2EDTA , 2 mM β -mercaptoethanol, pH 7.2) per 25 g original dry cysts. The homogenate was centrifuged for 2 hr at 12,000 rpm in a Sorvall GSA rotor and the top membrane layer of the pellet recovered. The remaining pellet was rehomogenized and

centrifuged, and the combined membranes were resuspended in homogenizing medium to a final volume of 60 ml per 25 g original dry cysts and stored at -90°C . 2. Gradient membranes -- The crude membranes were centrifuged through a step gradient consisting of 20% and 40% sucrose using either a Beckman SW 27 swinging bucket rotor (small scale) or a Beckman Ti-15 zonal rotor (large scale) for 1 hr at 27,000 rpm. The membranes banding at the 20-40% interface were collected, the sucrose was removed by diaflo filtration and centrifugation, and the final membranes were resuspended in buffer (25 mM imidazole-HCl, 2 mM Na_2EDTA , pH 7.2) to a final protein concentration of 6 mg/ml. 3. Lubrol pellet -- The gradient membranes were made 0.15 mM in ATP and 0.16% in Lubrol WX and centrifuged at 100,000 x g for 2 hr, and the pellet was resuspended in homogenizing medium to a final protein concentration of 3.0 mg/ml. 4. SDS enzyme -- The Lubrol pellet was treated with 0.4 volumes of 15 mM ATP and 0.6 volumes of 1.67 mg/ml SDS, and the mixture was incubated for 30 min at 30°C . This material was then centrifuged through a step gradient consisting of 15% and 35% sucrose using either a Beckman SW 27 swinging bucket rotor (small scale) or a Beckman Ti-15 zonal rotor (large scale). The 15-35% sucrose interface contained the final partially purified brine shrimp Na,K-ATPase. Further details of this procedure will be published elsewhere (9).

Molecular weight determinations were made by SDS-PAGE at ten different individual acrylamide concentrations using the buffer system of Laemmli (10) and by 3-30% acrylamide gradient SDS-PAGE as described by Lambin *et al.* (11) and Lambin (12). The individual acrylamide concentration gels were run on 0.6 x 12 cm cylindrical tubes, and the gradient gels were run on 0.3 x 14 x 12 cm slab gels. Samples were solubilized in 2.5% SDS for 2-3 min at 100°C , reduced with β -mercaptoethanol, and dialyzed overnight against 0.2% SDS and 0.75% β -mercaptoethanol. The gels were calibrated against 16 proteins whose molecular weight (M_r) and number of amino acid residues (N) were known from sequence data. Three different estimates of the apparent molecular weight of the brine shrimp Na,K-ATPase subunits were obtained by calibration of $\log M_r$ or $\log N$ vs R_f for the ten different acrylamide concentrations, of $\log M_r$ or $\log N$ vs $\log K_R$ from the retardation coefficient determined from Ferguson plots, and of $\log M_r$ or $\log N$ vs $\log \%T$ from the acrylamide gradient gels.

Determination of the mass ratio of the brine shrimp Na,K-ATPase subunits was done by gel scanning and dye elution methods (13), for Coomassie brilliant blue (R-250)

stained gels. The linear region for quantitation by these methods was verified by examining in quadruplicate 12 different amounts of purified electric eel Na,K-ATPase varying from 0-100 μg protein per gel. Disc gels (0.6 x 12 cm) were used and staining was for 16-18 hr in 0.05% Coomassie blue. Brine shrimp enzyme at 10 μg and 50 μg protein per gel was found to be within the linear regions for the gel scanning and dye elution methods, respectively. Further details of the molecular weight determinations and subunit mass ratio analysis will be published elsewhere (14), together with similar studies on the dog kidney, eel electroplax and dogfish rectal gland Na,K-ATPase preparations.

The brine shrimp Na,K-ATPase subunits were labeled during early development in 1.0-1.2 mCi $\text{NaH}^{14}\text{CO}_3$ (about 50 mCi/mmol) per 25 g dry cysts. The animals were pulsed for 2 hr periods up to 24 hr after hydration and the Na,K-ATPase purified as described above after a total of 32 hr of development. Approximately 1 g wet weight of animals were sampled at the end of the 2 hr radioactive pulse period for determination of the specific radioactivity of the free amino acids. These samples were homogenized in TCA, and the TCA-soluble fraction was extracted with ethyl ether and lyophilized. The specific radioactivity of the free amino acids were determined with the aid of a Beckman Spinco Model 120 amino acid analyzer.

RESULTS

Partial purification of the brine shrimp Na,K-ATPase by either small scale (25 g dry cysts) or large scale (300-1,000 g dry cysts) procedures results in comparable yields and purity. An example of a representative large scale purification from 48 hr animals reared from 900 g dry cysts is shown in Table 1. The homogenate, which was not assayed in this preparation, is usually less than 3 $\mu\text{mol P}_i/\text{hr/mg}$ protein for 48 hr animals. Thus the overall purification is about 200-fold. The gradient membrane fraction is about four-fold enriched in the Na,K-ATPase over that in the crude membranes, with a 60-70% recovery of enzyme. The increase in specific activity of the Lubrol pellet fraction is due in part to activation (about one-third) and in part to loss of non-enzyme protein (about two-thirds). Recovery of the Na,K-ATPase at this step is near 100%. The Lubrol treatment is necessary in preparation for the SDS step. Without prior Lubrol treatment, the SDS step results in little purification. The SDS step results in a 4-5-fold purification with a one-third recovery of the amount of enzyme found in the Lubrol pellet. In 48 hr animals, the specific activity of the final SDS

TABLE 1

Large Scale Partial Purification of the Brine Shrimp Na,K-ATPase from 48 hr Animals Reared from 900 g Dry Cysts

Purification step	Total activity ($\frac{\mu\text{mol P}_i}{\text{hr}}$)	Total protein (mg)	Specific activity ($\frac{\mu\text{mol P}_i}{\text{hr}}$)	Enzyme yield (%)
Crude membranes	168,000	11,100	15.2	100
Gradient membranes	106,000	1,800	58.6	63
Lubrol pellet	153,000	968	158	91
SDS enzyme	57,200	96.2	594	34

enzyme ranges from 500–600 $\mu\text{mol P}_i/\text{hr}/\text{mg}$ protein. At the time that enzyme activity is at its maximum level (approximately 32 hr; see Fig. 3), specific activities somewhat greater than 600 are obtained. For animals 20 hr old, the specific activity is about 400.

The SDS treatment results in loss of enzyme activity to an extent dependent on protein and SDS concentration. At 0.5 mg/ml SDS and 1.5 mg/ml protein, about one-third of the enzyme activity is immediately and irreversibly lost. Subsequent addition of bovine serum albumin or removal of the SDS by the sucrose gradient results in recovery of about 120% of the activity, indicating that a low level of reversible inactivation also occurs. The conditions worked out for the SDS step were arrived at by using the lowest concentration of SDS compatible with maximum purification. Above 0.5 mg/ml SDS, the yield of purified enzyme drops dramatically. Protein concentrations greater than 1.5 mg/ml result in slightly greater yields due to less inactivation but at the expense of decreased specific activities. The Lubrol pellet prepared from younger animals (near 20 hr) is more sensitive to SDS inactivation; however, in these cases, the protein concentration can be increased to balance the inactivation without apparent loss in specific activity of the SDS enzyme. Thus for the younger animals, 2 mg/ml protein can be used with 0.5 mg/ml SDS.

The large and small subunits of the Na,K-ATPase partially purified by the above method show properties similar to those previously described for the less pure enzyme isolated from the Great Salt Lake brine shrimp (2). The large subunit consists of two bands on SDS-PAGE, and the small subunit consists of a broad diffuse band. The two subunits account for about 60% of the total protein.

The molecular weights of the brine shrimp Na,K-ATPase subunits determined by SDS-PAGE are shown in Table 2. The

TABLE 2

Molecular Weights of the Brine Shrimp Na,K-ATPase Subunits Determined by SDS-PAGE

Method of estimation	Large subunit		Small subunit
	$\alpha 1$	$\alpha 2$	β
Individual acrylamide	101,100	96,100	40,200–41,800
Ferguson data	101,200	89,500	41,000–42,500
Acrylamide gradient	99,000		40,500
Average of above	100,400	94,900	40,600–41,600

molecular weights in Table 2 were determined by calibration against N (number of amino acid residues) and converted to molecular weight using an average molecular weight per 100 amino acid residues calculated from the amino acid compositions of the corresponding subunits from the dogfish and eel (15) and dog (16) Na,K-ATPases. These values were 11,273 for the large subunit and 11,676 for the small subunit. Calibration against N, which is theoretically more precise, was found to produce less variability in the estimates. The plots of $\log N$ (or $\log M_r$) vs R_f for the individual gels of different acrylamide concentrations were sigmoid and fitted by an iterative least squares method to the sigmoid function

$$R_f = \frac{a}{(1 + \frac{X}{c})^m},$$

where a , c and m are constants and $X = N$ or M_r . The coefficient of determination, r^2 , for all these regression lines was 0.99 or better. The Ferguson plots demonstrated that the brine shrimp Na,K-ATPase subunits behaved normally on SDS-PAGE. The Ferguson parameters were determined by weighted least squares linear regression for which r^2 was greater than 0.99 for each protein examined. The plot of $\log N$ vs $\log K_R$ was linear with $r^2 = 0.995$. The plot of $\log N$ vs $\log \%T$ from the acrylamide gradient gel was linear with $r^2 = 0.963$.

The measured large:small mass ratios for the brine shrimp Na,K-ATPase are 2.60 as determined by gel scanning and 2.20 as determined by dye elution of the Coomassie blue stained gels. The ratio calculated from the molecular

weight data ranges from 2.28 to 2.56. The mole ratio of the large:small subunits is thus 1:1.

The double banding pattern of the large subunit from the brine shrimp Na,K-ATPase is not an apparent artifact due to oxidation or proteolysis occurring during the purification procedure. Addition of the potent lipid antioxidant and free radical scavenger 2,6-di-*tert*-butyl-4-hydroxy-methylphenol (17) or the serine protease inhibitors phenyl-methylsulfonyl fluoride and L-1-tosylamide-phenylethyl-chloromethyl ketone during purification resulted in no change in the purification or of the appearance of the double bands. Incubating the homogenate or gradient membrane fractions for 2 hr at 37°C without lipid antioxidant or exogenous protease inhibitors also had no effect on the appearance of the doublets, and there was no apparent loss of enzymatic activity.

Isolation of the brine shrimp Na,K-ATPase at various times during development indicated that the upper band of the doublet (α_1) was approximately equal in amount to the lower band (α_2) in the younger animals (14 hr). In the older animals (48 hr), the upper band was predominant. As shown in Figure 1, the ratio of α_1 to α_2 increased progressively with developmental age.

Figure 2 shows the results of $\text{NaH}^{14}\text{CO}_3$ incorporation into total TCA precipitable material. At the end of the 2 hr pulse periods, the radioactivity is about half that observed after the animals have been allowed to continue development up to 32 hr. Apparently the radioactive precursors of macromolecules (proteins, nucleic acids, etc.) that have accumulated during $^{14}\text{CO}_2$ -fixation account for the remaining incorporation, which takes place after the $\text{NaH}^{14}\text{CO}_3$ is removed. Since the end points of the pulse periods represent approximately the 50% incorporation level, they are used as the point at which the incorporation data are plotted in relation to development of enzyme activity.

Preliminary findings on $\text{NaH}^{14}\text{CO}_3$ incorporation into the Na,K-ATPase and other membrane proteins is shown in Figure 3. Membrane proteins represented by the gradient membrane fraction obtained during partial purification of the Na,K-ATPase consists primarily of plasma membranes and endoplasmic reticulum membranes according to previous marker enzyme activity measurements (8). Incorporation into the SDS enzyme fraction roughly parallels that of the other membrane proteins. The radioactivity of the SDS enzyme appears about 2-4 hr in advance of the appearance of enzyme activity.

At this writing, analysis of the specific radioactivities of the amino acid pools have been examined for the 8-10 and 14-16 hr pulse periods; the results are shown in Table 3.

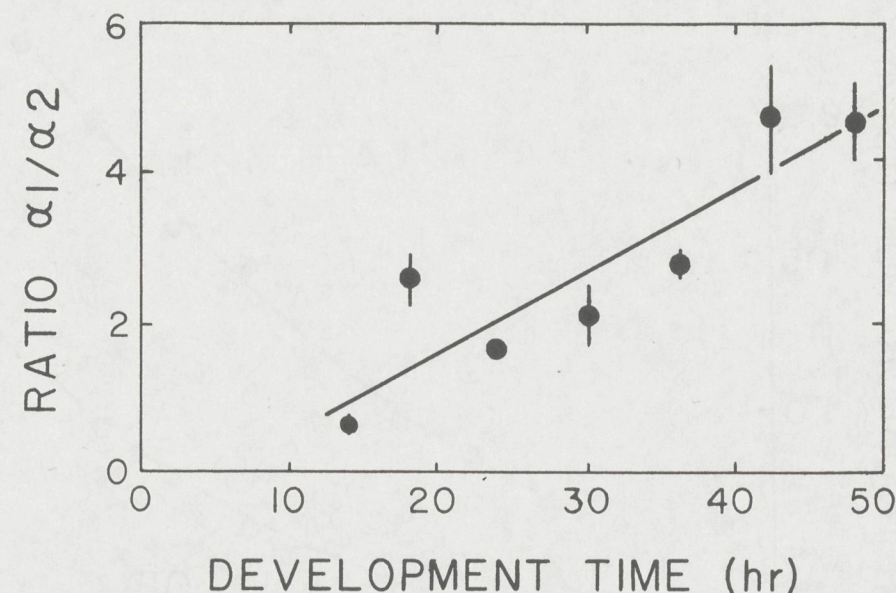


Figure 1. Ratio of the two large subunit bands (α_1/α_2) of the brine shrimp Na,K-ATPase isolated from animals at different times during development. Quantitation of the bands was done by dye elution methods (13) of Coomassie blue stained gels containing 50 μg total enzyme protein. The linear regression line was calculated by the method of least squares. Vertical bars represent one standard deviation above.

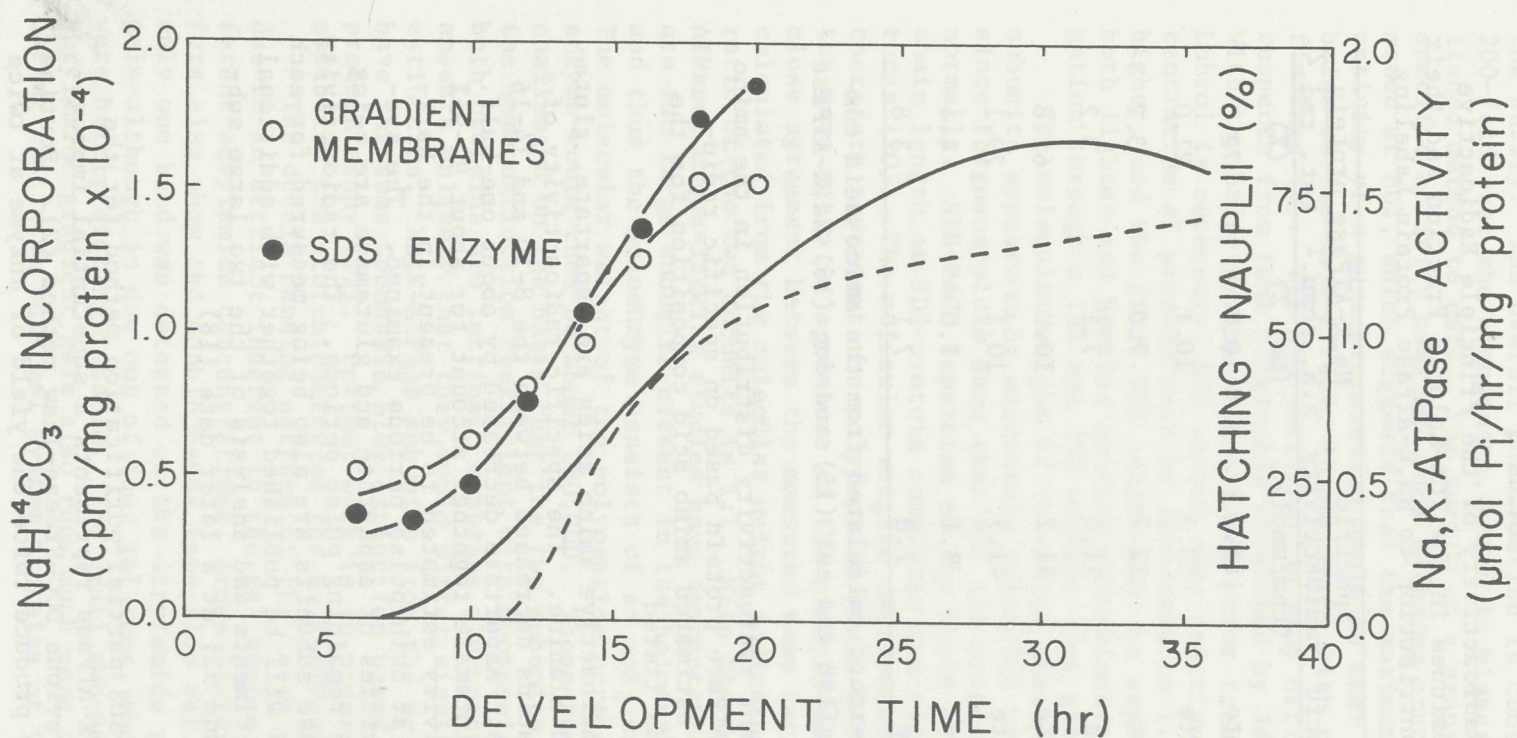
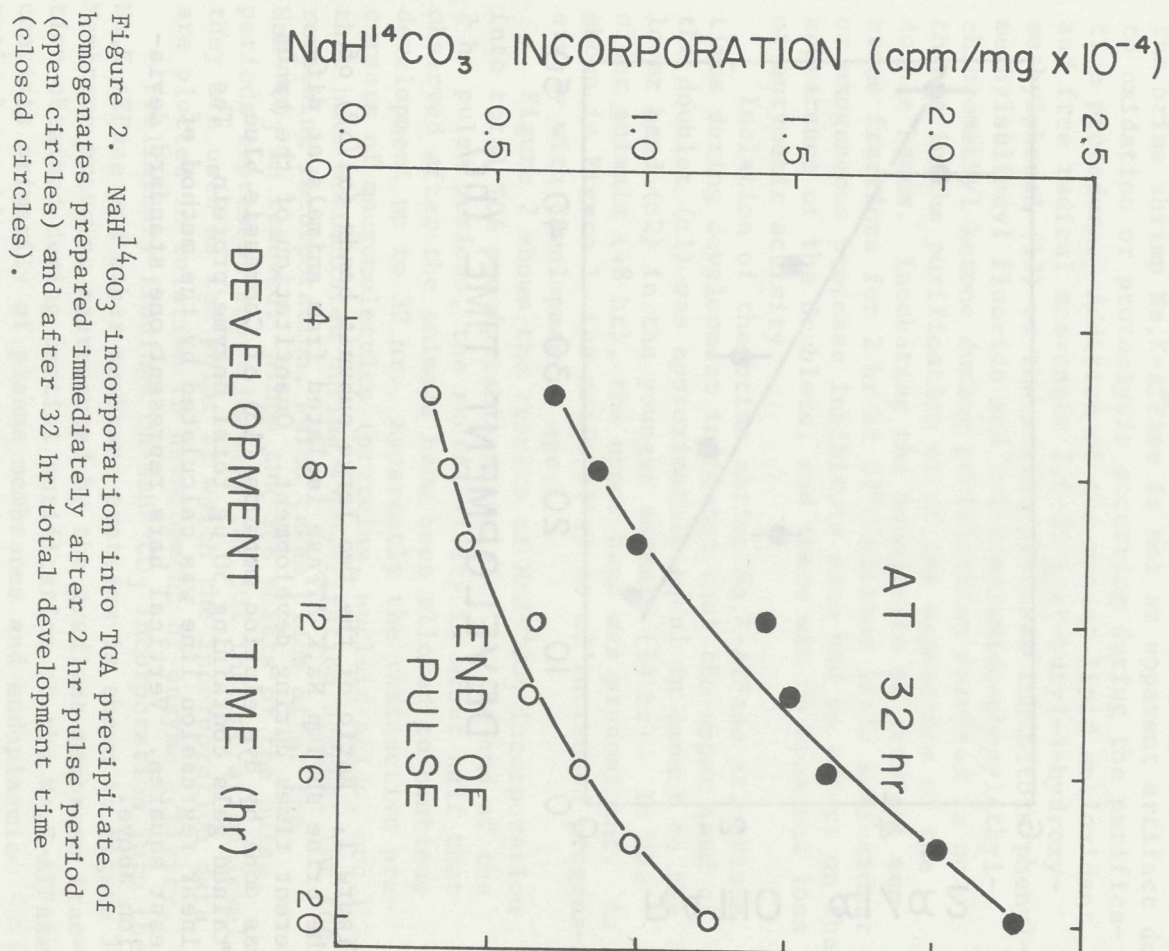


TABLE 3

Specific Radioactivity of the Principle Radioactive Amino Acid Residues in the TCA-Soluble Fraction and their Estimated Contribution to Na,K-ATPase Protein Labeling

Pulse period	Amino acid	Specific radioactivity (cpm/nmol)	Na,K-ATPase protein	
			a.a. comp. ¹ (mol %)	est. rad. ² (%)
8-10	aspartate	185.2	10.0	72.5
	glutamate	53.3	10.1	21.0
	serine	11.7	7.0	3.2
	alanine	11.8	7.1	3.2
14-16	aspartate	91.2	10.0	62.8
	glutamate	41.9	10.1	29.1
	serine	9.1	7.0	4.4
	alanine	7.8	7.1	3.8

¹ average mole percent calculated from the amino acid composition of dogfish and eel (15) and dog (16) Na,K-ATPase subunits

² estimated percent radioactivity distribution in the amino acids of Na,K-ATPase protein based on specific radioactivities and estimated amino acid composition for the four amino acids listed

The principle radioactive amino acids are aspartate, glutamate, serine and alanine. The specific radioactivity of the four amino acids decreased between the 8-10 and 14-16 hr pulse periods. Aspartate decreased by about one-half. Aspartate and glutamate together account for about 92-94% of the radioactivity estimated to be present in the Na,K-ATPase subunits at both pulse periods examined. The specific radioactivities of aspartate and glutamate are being examined for the remaining pulse periods. The radioactivity of the Na,K-ATPase subunits are also being measured for each pulse period and will be published together with additional pulse-chase experiments and analysis of the two large subunit bands ($\alpha 1$ and $\alpha 2$) at a later date (18).

DISCUSSION

The Lubrol-SDS partial purification method for the brine shrimp Na,K-ATPase represents a substantial improvement over the previous DOC-Freezing-Thaw method (2). Routine preparations now produce twice the yield of enzyme at twice

the purity. The overall purification is consistently about 200-fold over the homogenate, which is higher than any published method to date. The purity of a preparation with a specific activity near 600 $\mu\text{mol P}_i/\text{hr/mg}$ protein is estimated at 60%, which suggests that the maximum specific activity of a pure preparation would be near 1,000 for the brine shrimp Na,K-ATPase, in agreement with the previous findings (2). The SDS purification step differs in several respects from that originally developed by Jørgensen (19). With the brine shrimp Na,K-ATPase, prior treatment with Lubrol is necessary, SDS causes only inactivation, the concentration of protein must be increased to 1.5 mg/ml or higher, and the final SDS enzyme must be separated from both lighter and heavier density contaminants by centrifugation through a 15% and 35% sucrose step gradient.

The molecular weight of the brine shrimp Na,K-ATPase subunits appears to be accurately estimated by SDS-PAGE since Ferguson plots show that all the protein bands behave normally. SDS-PAGE separates on the basis of polypeptide chain length as SDS-protein complexes are rod-like structures (20). The molecular weights determined from SDS-PAGE therefore represent the polypeptide chain portion only. As the mass ratio is a measure of the protein mass ratio, the close agreement between the measured mass ratio and that calculated from the molecular weight indicates that the mole ratio of the two subunits is 1:1. With the dog kidney Na,K-ATPase, cross-linking studies have demonstrated that there are two large subunits present in the holoenzyme (21,22), and thus the holoenzyme consists of an $\alpha_2\beta_2$ conformation. The molecular weight of the polypeptide portion of the holoenzyme would then be about 280,000. These results may be challenged on the basis that the two subunits do not show the same extinction coefficient with Coomassie blue. Since both proteins are intrinsic membrane glycoproteins, it appears unlikely that they would differ significantly in extinction. Na,K-ATPase subunits from the eel and dogfish have the same Coomassie blue extinction based on the Lowry protein assay (15). Further work is in progress to firmly establish the stoichiometry of the Na,K-ATPase subunits.

The double banding pattern of the large subunit is not due to an artifact inherent in the gel system since different acrylamide concentrations, cross-linkers, and buffers also show the double bands (see also ref. 2). Usually only one band was detected on the acrylamide gradient SDS gels although in one out of four gel runs, the two bands were barely distinguishable. The small subunit on the acrylamide gradient gels also runs much more compact, indicating that with this gel system proteins of very similar

molecular weight often are not resolved. The joint 95% confidence envelopes for the Ferguson parameters, free electrophoretic mobility (Y_0) and retardation coefficient (K_R), demonstrated that the two large subunit bands were significantly different with $P = 0.0025$ (data not shown). The double banding is also apparently not due to an artifact resulting from the purification procedures. Two different purification procedures (Lubrol-SDS and DOC-Freezing-Thaw) show the same double banding pattern. The use of lipid antioxidant, serine protease inhibitors, or intentional promotion of conditions favoring oxidation or proteolysis do not alter the enzyme activity, purification or double banding pattern of the large subunit.

Since the lower band (α_2) of the large subunit doublet is most prominent in the young nauplius and disappears (or nearly so) in the older nauplius, it is suggested that this protein is either a precursor of the upper band (α_1) or that it is in some way a "juvenile" isozyme of the Na,K-ATPase found only in the young nauplius. Demonstration that α_2 is large subunit protein rests principally on evidence that it is specifically phosphorylated from [γ - 32 P] ATP in the presence of Na^+ and Mg^{++} and dephosphorylated in the presence of K^+ (ref. 2 and experiments with the present preparation, data not shown). The labeling of α_2 reproducibly appears as a shoulder or a peak separated by a single gel slice of lower radioactivity from the radioactive peak corresponding to α_1 . Attempts to improve the resolution of the two bands by prolonged electrophoresis have not been successful due to the lability of the phosphorylated intermediate. Copper phenanthroline cross-linking of the brine shrimp SDS enzyme causes formation of multimers with both α_1 and α_2 , without affecting any of the other proteins in the preparation, the small subunit or four other contaminating protein bands (data not shown). This would suggest that α_1 and α_2 are very similar in nature. Further experiments are in progress to identify the relationship, if any, between α_1 and α_2 by peptide mapping and $\text{NaH}^{14}\text{CO}_3$ pulse-chase studies.

Preliminary results of the $\text{NaH}^{14}\text{CO}_3$ incorporation studies suggest that the Na,K-ATPase is synthesized roughly at the same time as other proteins of the plasma and endoplasmic reticulum membranes. This pattern is expected in systems such as the developing brine shrimp which are undergoing extensive differentiation. The Na,K-ATPase in the gradient membrane fraction comprises about 5% of the total protein as estimated from the relative purity of the Na,K-ATPase at this stage in the purification. Since most of these proteins are probably integral membrane proteins,

which are probably synthesized on membrane-bound polyribosomes (23), somewhere near 5% of the membrane-bound polyribosomes might then be expected to be involved in synthesis of the Na,K-ATPase subunits. If this is the case, it should be possible to isolate the specific subunit-mRNA polyribosome complexes for use in cell-free protein translation studies.

The $\text{NaH}^{14}\text{CO}_3$ incorporation into the Na,K-ATPase subunits was previously found to be similar to that present in the partially purified enzyme (8). In the current experiments, the level of specific radioactivity incorporated into the partially purified enzyme is about ten-fold higher than that obtained by the previous method (8), and the pulse periods have been reduced from 6 hr to 2 hr to permit more accurate staging of Na,K-ATPase synthesis in relation to the appearance of enzyme activity. The appearance of radioactivity into the SDS enzyme occurs about 2-4 hr in advance of enzyme activity. This measurement would be a maximum value since incorporation is continuing after removal of the $\text{NaH}^{14}\text{CO}_3$. The rate of radioactive tracer incorporation into protein, however, depends on changes in the amino acid pool. Preliminary evidence indicates that in the 8-16 hr developmental period, the specific radioactivity of the precursor amino acids decreases during development. If this holds up in further analysis, the slope of the $\text{NaH}^{14}\text{CO}_3$ incorporation curves in Figure 3 would be steeper than shown. At least it is clear that the increase in protein specific radioactivity is due to an increase in protein synthesis and not merely to an increase in the specific radioactivity of precursor amino acids. A lag period of 2 hr has been observed between polypeptide synthesis of the electric eel Na,K-ATPase subunits and assembly of the holoenzyme in the plasma membrane (24). The lag probably involves glycosylation and transport to the plasma membrane.

In brine shrimp, $\text{NaH}^{14}\text{CO}_3$ is fixed primarily into certain amino acids but also into pyrimidines and certain organic acids (25). Clegg (25) observed that the principle amino acids labeled within the first 1-2 hr of development were aspartate > threonine > alanine > glutamate. Our data showed that at 8-10 hr and at 14-16 hr after development, the principle amino acids labeled were glutamate > aspartate > alanine > serine. Threonine was not labeled. However, in terms of the specific radioactivity of the amino acids, the order was aspartate > glutamate > serine $\approx$ alanine. Aspartate and glutamate accounted for 90% or more of the radioactivity expected in the Na,K-ATPase protein. Thus measurement of the specific radioactivity of both aspartate

and glutamate will serve as a reliable index of changes in the precursor amino acid pools at different pulse periods.

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STAGE-SPECIFIC GENE EXPRESSION IN *ARTEMIA*

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ABSTRACT

During embryonic and larval development of *Artemia*, certain genes are expressed in a stage-specific manner. We have used *in vivo* radiolabeling to identify proteins that are synthesized in hatched nauplii but not in developing encysted embryos. Histones are synthesized coordinately with DNA replication in nauplii. Hybridization of *Artemia* RNA to cloned sea urchin histone genes suggests that the onset of histone gene expression involves synthesis of histone mRNAs. Another protein, initially named UP1, was found by electrophoretic fractionation of soluble proteins. UP1, which is not found in pre-emergence embryos, is rapidly synthesized in hatched larvae. We present evidence that UP1 is *Artemia* hemoglobin.

INTRODUCTION

Understanding the mechanisms that regulate gene expression has long been a major objective of developmental and molecular biology, and embryonic and larval development of *Artemia* provide some excellent opportunities for studies of gene expression. The availability of dormant encysted embryos and facile culture in the laboratory provide quantities of synchronously developing embryos and larvae equalled by few other eukaryotic organisms. In this laboratory, the emphasis has been on transcriptional regulation in *Artemia*. RNA synthesis *in vivo* increases during development of encysted embryos and declines in nauplius larvae over a period of 24-48 hr (1,2). Since RNA polymerases must, by definition, be involved in all transcriptional regulation, we have examined the levels of RNA polymerases from the resumption of development in dormant embryos through the first 56 hr of post-hatching larval development. We have found that RNA polymerases are themselves quantitatively stage-specific enzymes, increasing and decreasing in concert with the rate of RNA synthesis *in vivo* (3-5).

More recently we have begun to explore situations in which we can study the expression of specific genes in *Artemia*. Within the last decade, methodological and conceptual advances have made possible new approaches to studies of developmentally regulated genes. Reverse transcription of purified mRNAs provides specific reagents ("probes") for

detection and quantitation of those mRNA sequences even in the presence of overwhelming quantities of other RNA molecules. Recombinant DNA technology permits the amplification and identification of individual genes, as well as the cloning of entire genomes. DNA sequencing techniques can in short order define the primary structure of the gene. However, the developmental biochemist must still deal with the biology of the organism at hand. Full exploitation of this technological windfall requires identification of genes that are expressed in a stage-specific manner. We have identified two sets of genes in *Artemia* that are expressed in nauplius larvae but not in developing encysted embryos. DNA synthesis does not occur in developing encysted embryos and is confined to a period of about 36 hr in nauplius larvae (ref. 2 and our unpublished observations). It seemed likely that histone synthesis should be coordinated with DNA replication, and results presented here show that indeed histones are synthesized in nauplius larvae but not in pre-emergence embryos. Another stage-specific protein was found by acrylamide gel electrophoresis of soluble proteins. This protein, initially designated UP1, appears during development of nauplius larvae. We now have evidence that UP1 is *Artemia* hemoglobin.

MATERIALS AND METHODS

Dormant encysted embryos of *Artemia* (Metaframe brand, San Francisco) were purchased from a local supplier and stored at -20° . Cysts were sterilized by treatment with dilute antiformin (6), and 5 g batches were incubated at 30° in 250 ml sterile artificial sea water with 25,000 units penicillin, 25 mg streptomycin sulfate and 0.48 g sodium tetraborate. Under these conditions, 90% of the embryos produced hatched larvae within 24 hr. Newly hatched nauplius larvae were harvested after 16 or 24 hr of incubation and separated from unhatched cysts and empty shells in a separatory funnel (7). Older larvae were obtained by returning newly hatched larvae to sea water and continuing incubation at 30° . Nauplii were harvested by filtration through Miracloth and either used immediately or stored at -80° . To detect synthesis of proteins *in vivo*, encysted embryos or larvae (1 g) were incubated in 50 ml sea water containing 2.5 mCi $\text{NaH}^{14}\text{CO}_3$ (50-60 mCi/mmol). Unless otherwise specified, all subsequent procedures were carried out at 4° .

Nauplii were homogenized in all-glass Dounce homogenizers; cysts were first ground with a chilled mortar and pestle. Homogenates were filtered through Miracloth, and nuclei were isolated by repeated low speed centrifugation as described previously (3,4) except that the homogenizing medium was supplemented with 1 mM phenylmethane sulfonylfluoride and

10 µg/ml soybean trypsin inhibitor. Soluble proteins were prepared by centrifugation of the low speed supernate at 15,000 x g for 10 min, and centrifugation of the resulting supernate at 150,000 x g for 90 min. The high speed supernate was stored at -80°. Crude histones were obtained by overnight extraction of isolated nuclei in 0.4 N H₂SO₄ containing 50 mM sodium sulfite. Acid-soluble proteins were recovered by precipitation with acetone overnight at -20°; precipitates were collected by centrifugation, dried under vacuum, and stored in a dessicator at -20°.

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) was performed in slab gels at 25°, using the buffer system of Wycoff *et al.* (8). Gels contained linear gradients of total monomer (2.5% methylene bisacrylamide crosslinker), 8-16% T for histones and 6-12% T for soluble proteins. Protein samples were dissolved in sample buffer at 1-5 mg/ml and heated at 95-100° for 2 min before being applied to the gel. Non-denaturing PAGE was performed in 6% T gels at 0° using buffer system 2860 of Jovin and Chrambach (9). Gels were stained with 0.2 % Coomassie blue in 10% acetic acid + 50% methanol and destained first in 10% acetic acid + 25% methanol, then in 7.5% acetic acid + 5% methanol. Gels were photographed on Kodak high contrast copy film using a yellow filter, then prepared for fluorography as described by Bonner and Laskey (10). Dried gels were exposed to pre-flashed Kodak X-Omat R X-ray film at -80° (11).

To prepare total RNA for hybridization, cysts or nauplii were homogenized in a buffer containing 50 mM Na acetate, pH 5.5, 0.10 M NaCl, 1 mM EDTA, 0.5% SDS. RNA was isolated by phenol extraction at 60° and recovered by ethanol precipitation. Residual DNA was removed by chromatography on hydroxyapatite in Na phosphate buffer, pH 6.8.

E. coli strain HB101 carrying the recombinant plasmid pCh22 was a generous gift of Dr. Max Birnstiel. Bacteria were grown in L broth and plasmid DNA was amplified by chloramphenicol treatment and isolated as described by Tanaka and Weissblum (13). All procedures were conducted under P2 containment conditions in accordance with the NIH Guidelines for Recombinant DNA Research. Plasmid DNA was radiolabeled (specific activity ~4 x 10⁷ cpm/µg) by nick translation (14) using [α-³²P]dCTP as substrate. Labeled pCh22 DNA was hybridized with a 50,000-fold mass excess of RNA in 50 µl sealed glass capillaries. Hybridization mixtures containing 0.5 mg RNA/ml in 0.12 M PB (potassium phosphate, pH 6.8) + 0.2% SDS were placed in a boiling water bath for 10 min, then incubated at 50° to a final R₀t (ribonucleotide mole/l x sec) of ~100 (20 hr). Mixtures were applied to columns of hydroxyapatite (0.2 ml) equilibrated with 0.12 M PB +

0.2% SDS at 50°, and the columns were washed with 2 ml 0.12 M PB + 0.2% SDS. Hybrids were eluted with 1 ml 0.5 M PB + 0.2% SDS, and radioactivity in all fractions was determined by Cherenkov counting.

RESULTS

During pre-emergence development of *Artemia* embryos, the mass of DNA and number of nuclei per embryo remain constant (2,6). Thus there is no DNA replication during this period. DNA synthesis begins just after hatching of nauplius larvae, and ends about 36 hr later; by this time the mass of DNA per embryo has doubled (2). Synthesis of histones is in most cases coordinated with DNA replication. The known exceptions to this rule occur during cleavage (15,16), and the encysted embryos are well past this stage. Thus it seemed likely that histone synthesis would be coordinated with DNA replication during this period of *Artemia* development. To test this hypothesis, we examined the synthesis of histones *in vivo* by incubating embryos or larvae with NaH¹⁴CO₃. The use of this precursor is necessitated by the impermeability of cyst shells to amino acids. Fortunately, a substantial amount of the ¹⁴C taken up is incorporated into amino acids (17). We have found that even in newly hatched larvae prior to the first molt, NaH¹⁴CO₃ is a more effective precursor than [4,5-³H]leucine (unpublished observations of J. Helder). The specific radioactivities we obtained (4-20 x 10⁵ dpm/mg protein) are quite adequate for detection of labeled proteins by fluorography.

Nuclei were isolated from embryos or larvae labeled for periods of 8 hr, and histones were prepared by acid extraction of nuclei. Extraction of histones from chromatin resulted only in lower yields, with no increase in purity (unpublished observation of L. Illuminati). Moreover, core histones (H2A, H2B, H3 and H4) were easily recognized as major components of crude preparations, especially in older larvae. Histones were separated by SDS-PAGE on 8-16% acrylamide gels; the gels were stained, photographed, and subjected to fluorography. The results of such an experiment are shown in Figure 1. In this case the labeling periods were 0-8 hr (encysted embryos), 16-24 hr (newly hatched larvae), 40-48 hr (peak of DNA synthesis), and 64-72 hr (past the peak of DNA synthesis). In each panel in Figure 1, the left half is the stained gel and the right half is the fluorogram of the same sample. The positions of calf thymus histones run in the same gel are indicated on the left. The electrophoretic mobility of *Artemia* and calf core histones are identical on these gels, but we are not certain that the band that co-migrates with calf thymus

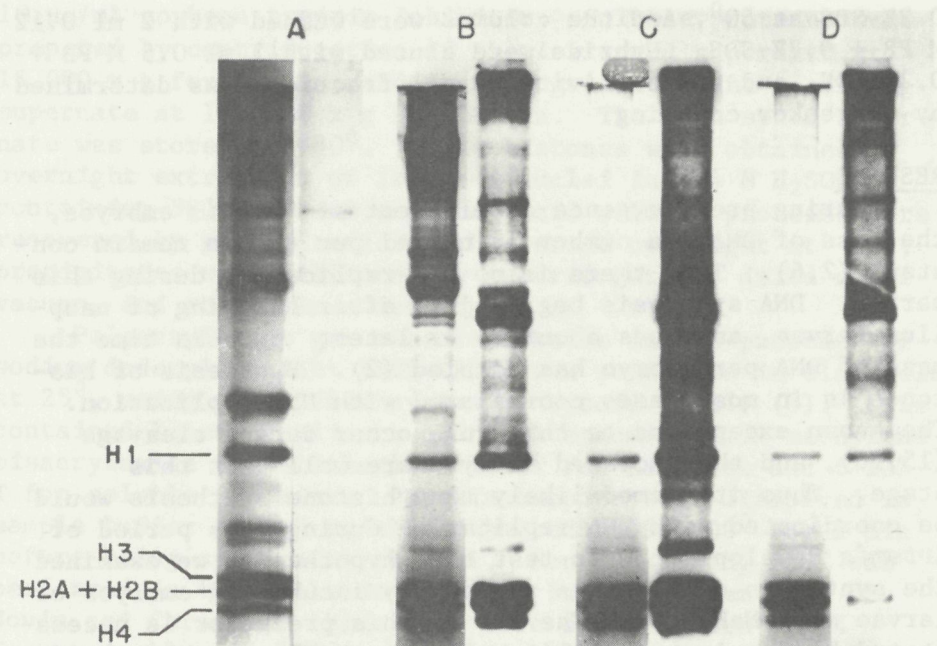


Figure 1. SDS-PAGE analysis of radiolabeled histones. Embryos or larvae were incubated with $\text{NaH}^{14}\text{CO}_3$ for 8 hr beginning at 0 hr (A), 16 hr (B), 40 hr (C), and 64 hr (D). Acid-soluble nuclear proteins were fractionated by SDS-PAGE on an 8-16% acrylamide gradient gel. The gel was stained with Coomassie blue, photographed, and then subjected to fluorography for 30 days. In each panel, the left side is the stained gel and the right is the fluorogram. The positions of calf thymus histone markers are shown at left.

H1 is *Artemia* H1 (see below). The only radiolabeled protein band that can be seen in the sample labeled at 0-8 hr is near the top of the gel; no labeling of histones was detected, even though the X-ray film was exposed for 30 days. In contrast, histones were clearly radiolabeled in nauplii. The entire period from 16 hr to 72 hr was examined, and only the "highlights" are shown in Figure 1. The period of maximal histone synthesis was 40-48 hr, corresponding to the period of maximal DNA synthesis.

An important consideration in these experiments is whether we could have seen histone synthesis in encysted embryos if it occurred. To clarify this point, we subjected soluble proteins from the same labeled preparations to SDS-PAGE in a 6-12% gel, followed by staining and fluorography.

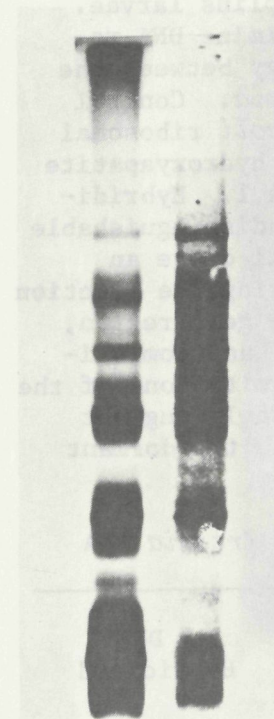


Figure 2. SDS-PAGE analysis of radiolabeled soluble proteins. Soluble ($150,000 \times g$ supernate) proteins from the same preparation shown in Figure 1, panel A, were fractionated by SDS-PAGE on a 6-12% acrylamide gel. The gel was treated as described in the legend to Figure 1 except that fluorography was for 5 days. The stained gel is on the left, the fluorogram on the right.

The result for the preparation labeled 0-8 hr is shown in Figure 2. Labeling of even very minor bands was easily detectable, even though this film was exposed for only 5 days. If histones had been labeled in these embryos to only a few percent of the specific radioactivity of soluble proteins, this labeling would have been readily detectable. From the complete absence of detectable label in histones from encysted embryos, we conclude that histone synthesis does not occur during at least the first 8 hr of pre-emergence development, and that histone synthesis is coordinated with DNA replication.

Further exploitation of histone synthesis as a model for transcriptional regulation in *Artemia* will require proof that histone gene expression is regulated at the level of transcription. This in turn will require purification of histone mRNAs and preparation of cDNA probes, and these experiments are now in progress. In the meantime, we have conducted a preliminary search for histone mRNA sequences by using the recombinant plasmid pCh22. This recombinant carries the histone genes of the sea urchin *Psammechinus miliaris* (18). Plasmid DNA was isolated and radiolabeled by nick translation in this laboratory by Dr. Michael Hudspeth. We then performed RNA-driven liquid hybridization of pCh22 DNA to a 50,000-fold mass excess of total RNA isolated from

dormant encysted embryos and from 48 hr nauplius larvae. Hybridization conditions were chosen to minimize DNA re-association and to tolerate some non-homology between the genes of a sea urchin and the mRNAs of *Artemia*. Control hybridization mixtures contained either *E. coli* ribosomal RNA or no RNA, and hybrids were measured by hydroxyapatite binding. The results are presented in Table 1. Hybridization of pCh22 DNA to RNA from cysts was indistinguishable from controls; in contrast, RNA from nauplii drove an additional ~7% of DNA into hybrid. Considering the fraction of pCh22 that is codogenic DNA strand in the gene region, about 1/12 of the counts (not corrected for base composition) should be hybridizable. Within the limitations of the heterologous hybridization, the results strongly suggest the histone mRNA sequences are not present in the dormant encysted embryo.

Table 1. Hybridization of pCh22 DNA to *Artemia* RNA

RNA source	% DNA Hybridized
Encysted embryos	3.8
48 hr larvae	11.3
<i>E. coli</i> rRNA	4.0
None	3.9

A second stage-specific protein was identified by inspection of soluble proteins of nauplii of different ages. The homogenizing medium (3,4) contained only buffered sucrose without salt, thus selecting for readily soluble proteins and excluding structural proteins such as actin and myosin. After removal of particulate material by high speed centrifugation, the soluble proteins were subjected directly to SDS-PAGE without further fractionation. The objective of this approach was to look for one or more proteins that are produced in sufficient amount to be seen as new stained protein bands. The results of such an experiment are shown in Figure 3. In this case, the time span was from 24 hr to 72 hr of development, and nauplii hatched at 16 hr. The pattern of soluble proteins is strikingly similar over this period of development; the only

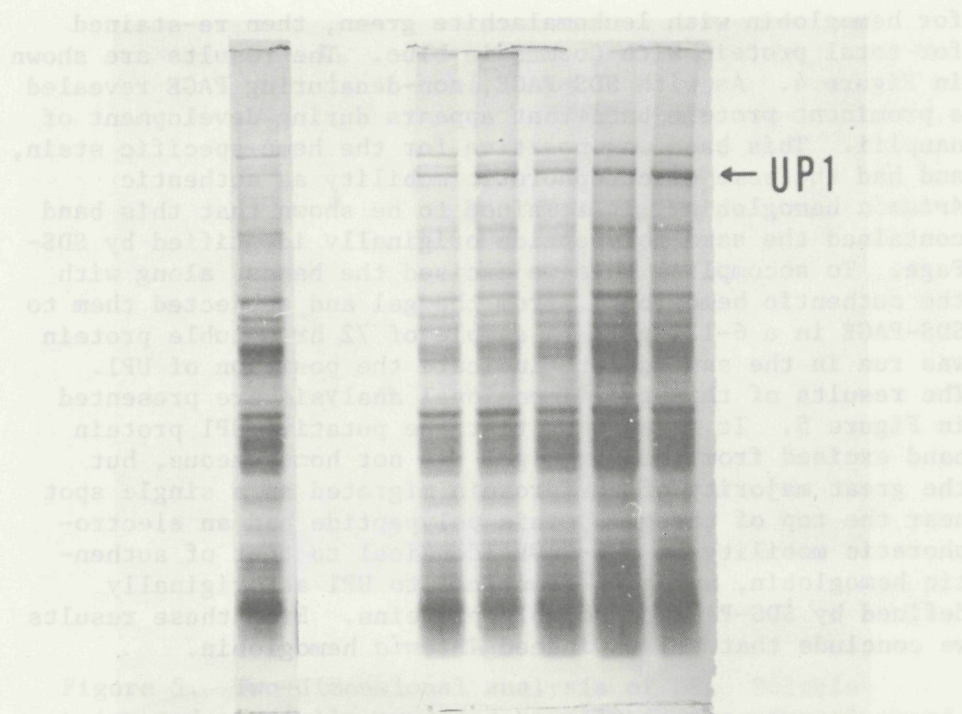


Figure 3. SDS-PAGE analysis of soluble proteins from nauplius larvae. Soluble protein were prepared from nauplii harvested at (left to right) 24, 32, 40, 48, 56 and 72 hr, and fractionated by SDS-PAGE on a 6-12% acrylamide gel. The gel was stained with Coomassie blue

major change is the appearance of a single polypeptide indicated in Figure 3 and designated UP1 (for "unknown protein 1"). UP1 is barely detectable in 24 hr nauplii, and undetectable in 16 hr nauplii and pre-emergence embryos (not shown). It becomes prominent by 48 hr, and by 72 hr UP1 is the most abundant single protein in the preparation.

Several facts suggested that UP1 might be *Artemia* hemoglobin(s). Hemoglobins are usually very soluble, and the appearance of hemoglobins during development of nauplii has been reported (19,20). The molecular weight of UP1 is about 130,000, similar to the molecular weight reported by Moens and Kondo for subunits of *Artemia* hemoglobins (21). To test this hypothesis, we fractionated soluble proteins from different stages by non-denaturing PAGE. The gel was stained

for hemoglobin with leukomalachite green, then re-stained for total protein with Coomassie blue. The results are shown in Figure 4. As with SDS-PAGE, non-denaturing PAGE revealed a prominent protein band that appears during development of nauplii. This band was positive for the heme-specific stain, and had the same electrophoretic mobility as authentic *Artemia* hemoglobin. It remained to be shown that this band contained the same polypeptide originally identified by SDS-PAGE. To accomplish this we excised the bands, along with the authentic hemoglobin, from the gel and subjected them to SDS-PAGE in a 6-12% gel. A sample of 72 hr soluble protein was run in the same gel to indicate the position of UP1. The results of this two-dimensional analysis are presented in Figure 5. It is evident that the putative UP1 protein band excised from the first gel was not homogeneous, but the great majority of the protein migrated as a single spot near the top of the gel. This polypeptide had an electrophoretic mobility in SDS-PAGE identical to that of authentic hemoglobin, and also identical to UP1 as originally defined by SDS-PAGE of soluble proteins. From these results we conclude that UP1 is indeed *Artemia* hemoglobin.

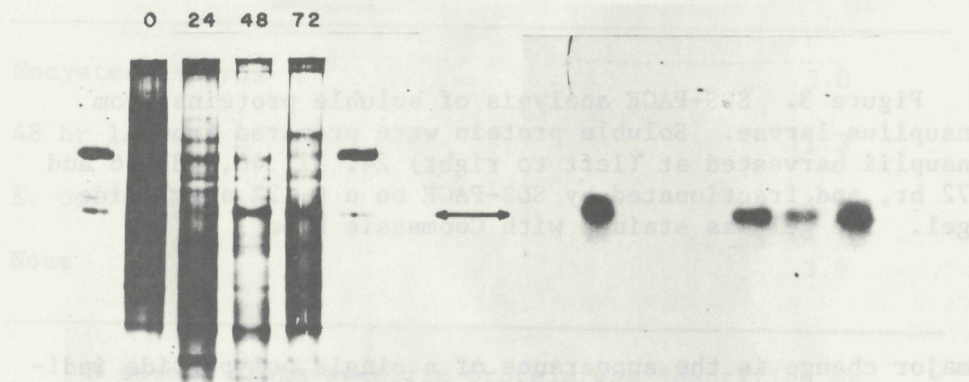


Figure 4. Analysis of soluble proteins by non-denaturing PAGE. Soluble proteins from unincubated embryos (0 hr) and from nauplius larvae harvested at 24, 48 and 72 hr, were fractionated by PAGE in an 8% acrylamide gel under non-denaturing conditions. The gel was stained first with leukomalachite green, then with Coomassie blue. Samples of partially purified *Artemia* hemoglobin were also run. The total protein pattern is shown on the left and the hemoglobin staining on the right. The arrow indicates the position of authentic hemoglobin.

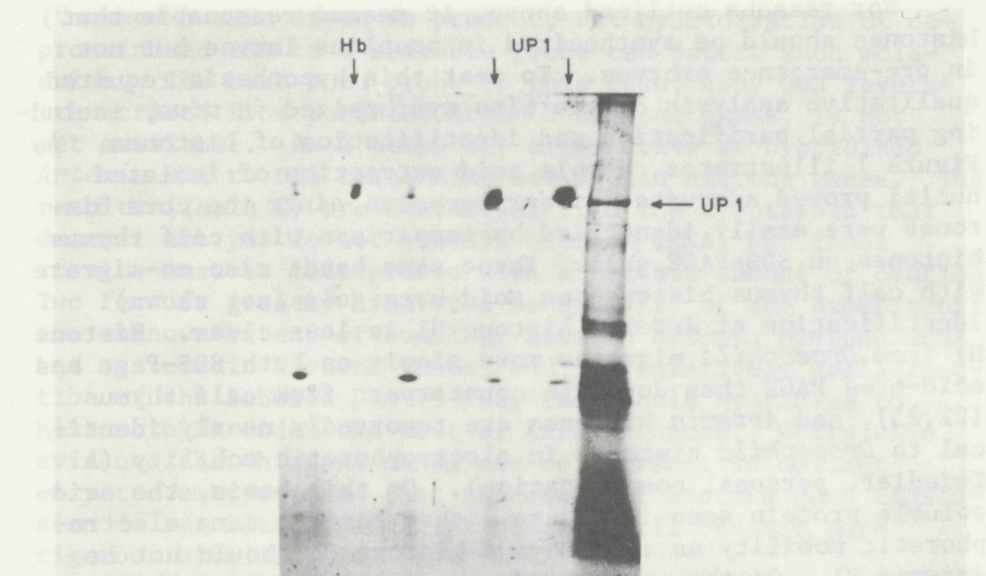


Figure 5. Two-dimensional analysis of UP1 Soluble proteins and partially purified hemoglobin were fractionated by PAGE on a 6% acrylamide gel, and the putative and authentic hemoglobin bands were cut out of the gel and run on an SDS-PAGE gel (6-12% acrylamide along with a sample of soluble protein. Lanes 1 and 3, putative hemoglobin from 48 hr larvae; lanes 4 and 5, putative hemoglobin from 72 hr nauplii; lane 2, authentic hemoglobin; lane 6, total soluble protein from 72 hr nauplii.

DISCUSSION

The objective of the work reported here was to identify systems that will be useful for detailed studies of expression of specific genes in *Artemia*. The search for such systems was based on the following rationale. The genes in question should be expressed at one stage of development but not at another, thus enabling us to prepare large quantities of organisms that are or are not (our choice) expressing these genes. The gene products should be easily identified by simple fractionation procedures, and it would be advantageous if they were produced in relatively large amount. Ideally, since our basic interest is in transcriptional regulation, the stage-specific genes should be regulated at the level of transcription. Two approaches were used to identify such genes, one based on a logical guess and one on a "blind" search for a protein produced in quantity at a specific stage. Fortunately, both approaches were successful.

For reasons outlined above, it seemed reasonable that histones should be synthesized in nauplius larvae but not in pre-emergence embryos. To test this hypothesis required qualitative analysis of proteins synthesized *in vivo*, including partial purification and identification of histones. As Figure 1 illustrates, simple acid extraction of isolated nuclei proved adequate for our purposes, since the core histones were easily identified by comparison with calf thymus histones on SDS-PAGE gels. These same bands also co-migrate with calf thymus histones on acid-urea gels (not shown). Identification of *Artemia* histone H1 is less clear. Histone H1 from *Drosophila* migrates more slowly on both SDS-Page and acid-urea PAGE than does its counterpart from calf thymus (22,23), and *Artemia* histones are reportedly nearly identical to *Drosophila* histones in electrophoretic mobility (A. Zwiedler, personal communication). On this basis, the acid-soluble protein seen in Figure 1 that has the same electrophoretic mobility as calf thymus histone H1 should not be *Artemia* H1. On the other hand, there is no obvious alternative candidate for histone H1. It is entirely possible that histone H1 was lost, either by degradation during preparation or by elution from the gel into the staining and destaining solutions. This question will have to be resolved by selective extraction and/or chromatographic fractionation of *Artemia* histones.

From the results presented in Figure 1, it is clear that core histones were synthesized in nauplius larvae, and that no histone synthesis could be detected in encysted embryos. The argument that histone synthesis is stage-specific rests, then, on the question of whether the experiment could have detected histone synthesis in pre-emergence embryos. The results presented in Figure 2 show that synthesis of soluble proteins in pre-emergence embryos was easily detected. Moreover, labeling of a non-histone acid-soluble nuclear protein could also be seen. Even if radiolabeled amino acids were not equilibrated throughout the cell, and if histones were synthesized from a relatively underlabeled pool of amino acids (e.g., in the vicinity of the nucleus), histone synthesis should have been detectable. The most reasonable interpretation of the results is that histone synthesis is coordinated with DNA replication in hatched nauplius larvae. Since the period between 8 and 16 hr was not examined in these experiments, the precise time at which histone synthesis begins remains to be determined.

Amaldi *et al.* (24) have reported that poly (A)⁻ RNA from nauplii could be translated *in vitro* into histones, but found little or no histone synthesis with RNA from embryos. Translation *in vitro* will not detect repressed

("masked") mRNA if it is present, but hybridization of de-proteinized RNA to a suitable probe can detect such molecules. The best such probe in this case, copy DNA reverse transcribed from purified *Artemia* histone mRNAs, is not yet available. As a temporary alternative we hybridized *Artemia* RNA to DNA containing sea urchin histone genes. The results (Table 1) are consistent with the hypothesis that dormant embryos do not contain histone mRNA, but these results must be interpreted with a certain amount of caution. Two factors greatly limit the sensitivity of the experiment: the unknown extent of homology between *Artemia* histone mRNA and sea urchin histone genes, and the relatively small fraction of the labeled DNA (9-10%) that could hybridize to histone mRNA under the best of circumstances. Definitive evidence that histone mRNAs are not present in dormant encysted embryos, and that the onset of histone gene expression is regulated transcriptionally, will require more rigorous hybridization experiments.

A second stage-specific protein, initially named UPl, was identified as a change in the population of soluble proteins found by SDS-PAGE. The experiment was designed deliberately to search only for major changes. Only the most abundant proteins can be seen by electrophoresis of the total soluble protein fraction; dozens, perhaps hundreds, of quantitatively minor proteins could appear and disappear without being detected. For this reason, it is not surprising that so little change was seen in the soluble protein pattern. The results presented in Figure 3 do not indicate that UPl is the only protein to appear during early larval development, but that appearance of UPl is the most significant quantitative change.

The evidence that UPl identified by SDS-PAGE is *Artemia* hemoglobin can be summarized as follows. A protein with a similar developmental profile can be found by non-denaturing PAGE of soluble proteins. This protein is positive for heme staining, has the same electrophoretic mobility as authentic hemoglobin, and contains a polypeptide that co-migrates with both UPl and authentic hemoglobin on SDS-PAGE. These observations leave little reasonable doubt that UPl is hemoglobin, and we feel the point is made. This is a particularly exciting finding, because in addition to being stage-specific, hemoglobin is inducible by low oxygen (ref 25 and L. Moens, personal communication). This implies that there may be one or more regulatory genes for hemoglobin. Another particularly interesting aspect of this protein is the suggestion that *Artemia* hemoglobin may contain, within a polypeptide of 130,000 daltons, smaller domains of approximately equal amino acid composition (and possibly sequence). If this is true, then the gene may also comprise a collec-

tion of approximate repeat sequences. The structure and regulation of such a gene would be exceptionally interesting.

In summary, we have now identified two groups of proteins that are synthesized in nauplius larvae but not in pre-emergence embryos. Thus we can exploit the inherent biological advantages of *Artemia* to obtain substantial quantities of organisms in which these genes are either active or inactive. We are now in a position to shift from studies of gross changes in transcription patterns (e.g., RNA polymerase levels) to detailed studies of the structure and regulation of specific genes. Efforts to establish a complete library of cloned *Artemia* genes are now underway in this laboratory.

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DEVELOPMENTAL CHANGES OF ENZYME LEVELS DURING ARTEMIA SALINA DIFFERENTIATION

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ABSTRACT

The expression of the developmental program of *Artemia salina* involves changes in many biochemical parameters. We have studied the changes in enzymes involved in gene expression as well as others not directly related to this process. Histone acetylation shows a different pattern when studied in isolated nuclei or in cell free systems, suggesting the presence of regulatory mechanisms for the acetylation *in vivo*. RNA polymerases change independently during development. Poly(A) polymerase activity increases from cysts to nauplii and its distribution changes also in this period. Several hydrolytic enzymes (a ribonuclease, proteases and an aminoacyl-tRNA hydrolase) are induced at or after hatching, being this induction inhibited by cycloheximide. The mitochondrial carbamyl phosphate synthetase increases its activity, concomitant with the mitochondrial activation that occurs at hatching.

INTRODUCTION

The developmental pathway of an eukaryotic organism can be considered to be composed of three completely integrated, interacting, parallel processes: differential gene expression, cell proliferation and differentiation. The interactions of these processes give rise to a characteristic phenotype at any given time in development. Although far from definitively proved, differential gene expression is assumed to be a key process in development (1). The experimental approaches to the biochemical study of development can be divided *a priori* in two broad classes. One is an analytical, descriptive study in which one looks for quantitative as well as qualitative changes during development (different messengers, different proteins, different supramolecular organization, etc.); the other is to look for the mechanisms by which these

changes do occur, i.e., the regulation of the processes.

Our group has been centered for the past several years on the study of some biochemical events that take place during the embryonic and early larval development of *Artemia salina*. We have chosen *A. salina* cysts experimental system, among other reasons, because cell proliferation does not take place in the developmental period from dormant gastrulae to swimming nauplii (2, 3), thus allowing to simplify the developmental pathway stated above. In spite of the absence of cell division in this period, there is a very strong morphological reorganization of the embryo, and although the data is scarce, one can assume that differential gene expression may play a role in this reorganization (4-6). We have been mainly interested not only in the changes themselves, but in what happens with the enzymes involved in these changes and in their regulatory possibilities. The object of this communication is to present some of our results on these subjects.

MATERIALS AND METHODS

Artemia salina cysts were obtained from Longlife Fish Food Products, Division of Sternco Industries Inc., Harrison, N.J. 07029 and from San Francisco Bay Brand Inc., Division of Metaframe Co., Menlo Park, CA 94025. Cysts were hydrated overnight in ice-cold distilled water and floating cysts were discarded. The sedimented population was treated with 0.1% sodium hypochlorite for 5 min, washed twice with cold water and treated with 1% Merthiolate (Eli Lilly, Co.) for 5 min, followed by four washes with cold water. After filtering through a cloth, cysts were either homogenized or incubated (8-10 g aliquots) in 1.5 l of sterile Dutrieu's medium (7), supplemented with antibiotics, at 30° with agitation. Under these conditions hatching occurs in 16-18 hr. New born nauplii were isolated in separatory funnels, and if needed, incubated in Dutrieu's medium at 30° for subsequent growth. When needed, nauplii were fed with homogenates of the alga *Spirulina* (8). Samples were processed immediately or were frozen at -70° until use.

Homogenization and enzyme assays.

a) Histone acetyltransferase: procedures are detailed elsewhere (9, 10).

b) RNA polymerases: solubilization, assay and quantification of RNA polymerases was done as previously described (11, 12).

c) Poly(A) polymerase: solubilization was done with buffer containing 50 mM Tris-HCl, pH 8.5, 0.4 mM EDTA, 5 mM β -mercaptoethanol, 20% glycerol. When the subcellular distribution was studied, homogenization was done by the method of Marca *et al.* (13). Briefly, embryos at different stages of

development were homogenized in a manual glass homogenizer with 5 vol. of buffer containing 25 mM HEPES, pH 7.5, 5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.3 M sucrose and 15% Ficoll 400. The homogenate was centrifuged at 12,000xg for 20 min, obtaining the particulate fraction. The supernatant was further centrifuged at 27,000xg for 30 min and again at 105,000xg for 2 hr, to obtain the soluble fraction. The assay mixture contained (in a final volume of 0.15 ml), 50 mM Tris-HCl, pH 8.5, 2 mM $MnCl_2$, 0.12 mM (8- 3H)-ATP (55 mCi/mmol), 0.2 mg of RNA primer and enzyme preparation. After 30 min at 30°, the reaction was stopped with 5 ml of ice-cold 5% TCA-2% $Na_4P_2O_7$, measuring acid-insoluble counts. 1 unit of activity represents 1 nmol of AMP incorporated in 60 min at 30°.

d) Ribonuclease was assayed as described by Sebastián and Heredia (14).

e) Proteases were isolated and assayed as described by Osuna *et al.* (15) and Olalla *et al.* (16).

f) Aminoacyl-tRNA hydrolases were assayed as described by Coloma and Heredia (17) and Miralles *et al.* (18).

g) Carbamyl phosphate synthetase was obtained from frozen dry cysts or nauplii by homogenization in buffer containing 20 mM Tris-HCl, pH 7.5, 0.25 M sucrose, 1 mM EDTA, 2 mM ATP, 5 mM $MgCl_2$, 25 mM NaCl, 75 mM KCl, 15% glycerol. After filtering through a piece of cloth, this total homogenate was centrifuged at 500xg for 10 min, discarding the pellet. The mitochondrial fraction was obtained by centrifugation at 27,000xg for 20 min, being the supernatant the cytoplasmic fraction. The enzyme was assayed in 0.5 ml reactions containing 0.4 mg of bovine serum albumin, 100 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 5 mM reduced glutathione, 100 mM NH_4Cl , 5 mM L-glutamine, 5 mM N-acetyl-L-glutamate, 10 mM ATP and 10 mM (^{14}C)- $KHCO_3$ (3×10^5 cpm/ μ mol) and an appropriate amount of enzyme. The reaction was incubated 10 min at 37° and 0.1 ml of a mixture of 0.7 M NaOH and 2.7 M KOH were added and allowed to react 10 min at 37° to convert the (^{14}C)-carbamyl phosphate to (^{14}C)-cyanate. Cyanate was converted to (^{14}C)-urea by adding 0.2 ml of 4 M NH_4Cl , pH 8.5, and heating at 100° for 10 min. After adding 1 ml of water and 2-3 drops of n-octanol, the (^{14}C)- HCO_3^- was removed by bubbling CO_2 through the solution for 60 min at room temperature. After gassing, the whole volume was counted with a toluene-triton scintillation fluid. 1 unit of activity represents 1 μ mol of product formed in 10 min under the assay conditions.

RESULTS AND DISCUSSION

A. Histone acetylation. Among the different types of modification of histones, acetylation is now believed to be in-

volved in chromatin activation and in the regulation of gene expression (19). Most of the work done on this subject has largely dealt with the parallelism between hyperacetylated and active chromatin (20) and structural characteristics of acetylated chromatin (21).

We have focussed on the problem of the enzymatic regulation of histone acetylation during the early development of *A. salina*. We have developed an *in vitro* assay to study acetylation of endogenous histones in crude nuclear preparations from *A. salina* embryos and larvae at different stages of development (9, 10). The characteristics of the histone acetyltransferase are shown in Table 1.

Table 1. Characterization of Histone acetyltransferase activity in nuclear extracts from *A. salina* cysts.

Thermal stability	Low (80% inactivation in 2.5 min at 40°)
Optimum ionic strength	Low (0.05 M)
Optimum pH	8.5
Metal requirement	None (inhibition)
Sulfhydryl group requirement	High
Molecular weight	170,000 (aggregates at low ionic strength)
Substrate specificity	H1 > H3 >> H4
Histones acetylated in nuclear preparations	H3 and H4

data taken from (9, 10)

There are two interesting properties of this activity. One is the substrate specificity when individual histone fractions are used. H1 is a better acceptor than H3 and H4 (relative acetyl-group incorporation taken as 100 that of total histones, are 150, 80 and 40 for H1, H3 and H4 respectively). However, electrophoretic analysis of the endogenous acetylation shows that H3 and H4 are the only species acetylated, as it is also the case when the acetylation reaction is carried out *in vitro* with unfractionated histones. The second point to consider arise from experiments now in progress with purified preparations, which indicate that the histone acetyltransferase exhibits sigmoid kinetics at very low substrate concentrations and that H4 inhibits the acetylation

of H1. These data strongly suggest that the histone acetyltransferase is an allosteric enzyme, and therefore a potential site of regulation of the expression of genetic information.

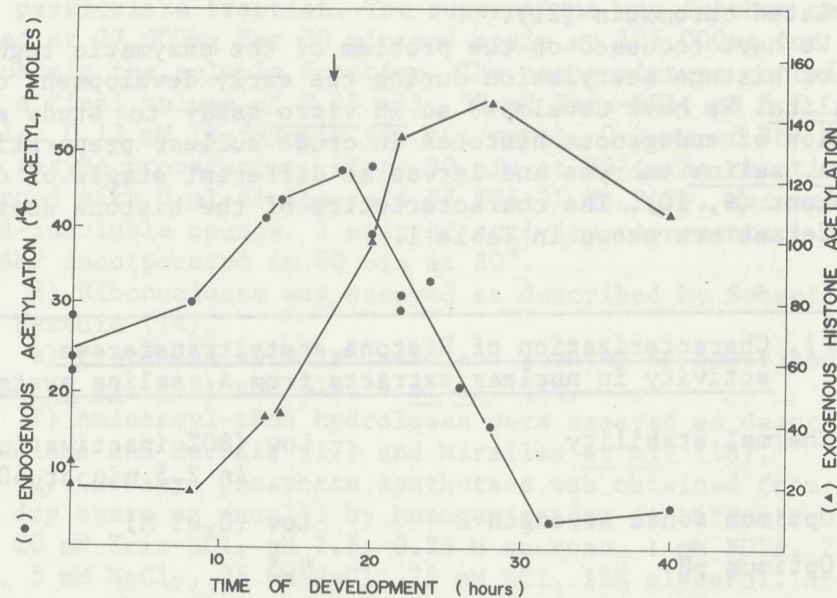


Figure 1. Endogenous and exogenous acetylation of histones in nuclear extracts of *A. salina* during development.

Details of extraction and quantification of the acetylation of histones are given in ref. 9. (●): endogenous acetylation; (▲): exogenous acetylation. The arrow signals hatching time in this experiment.

In order to obtain a complete description of the developmental changes in histone acetylation, nuclear fractions were prepared from *A. salina* embryos and larvae at different times from the onset of development. As shown in figure 1, there is a slow increase in the endogenous acetylation during the period of embryonic development which reaches a maximum after hatching, followed by a rapid fall along the early larval development. In contrast, the acetylation of exogenously supplied histones increases continuously through all the early larval development, reaching a maximum after 30 hr, when the endogenous acetylation was at the lowest level. Results similar to the exogenous acetylation were obtained with the histone acetyltransferase activity extracted from nuclei (data not shown).

The observed changes in endogenous acetylation in nuclei which were supposed to arise from the developmental appearance of an endogenous inhibitor (9), are now best explained by

changes in substrate availability or enzyme specificity as discussed in a forthcoming report (22).

As a result of this and previous reports (9, 10), we can conclude that the acetylation of histones in *A. salina* is under the control of enzyme levels and enzyme regulation.

B. RNA polymerases. The enzymatic machinery involved in RNA synthesis in eukaryotic systems is now well characterized; *A. salina* is not an exception and contains three isoenzymes of the RNA polymerases (23, 11, 12). We have studied the fluctuations of the levels of these enzymes to obtain some information on the possible role of RNA polymerases in the regulation of the synthesis of the different classes of RNA during the early development of *A. salina*.

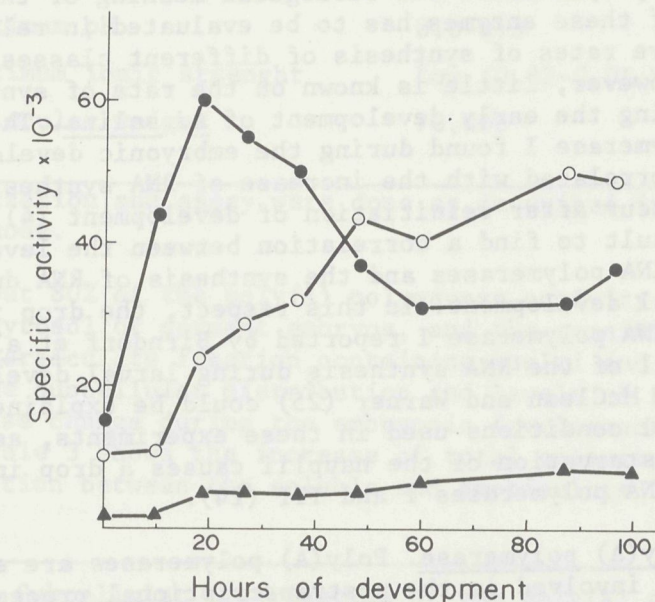


Figure 2. Levels of the RNA polymerases I, II and III during the early development of *A. salina*.

The growth medium for the nauplii was supplemented with extracts of the alga *Spirulina* (8). Extracts were made as described (11, 12) with 100 µg/ml of Soybean trypsin inhibitor in all the buffers used. (●): RNA polymerase I, (○): RNA polymerase II, (▲): RNA polymerase III.

The levels of the RNA polymerases are influenced by different experimental conditions, mainly after hatching, when the culturing conditions of the nauplii and the possibility of *in vitro* proteolytic degradation of RNA polymerases I and

III by protease B (see below) has to be considered (24). Figure 2 shows the levels of RNA polymerases I, II and III during the embryogenesis and early larval development of *A. salina*. The levels were determined in fed nauplii grown at 30° and the extracts were prepared in the presence of Soybean trypsin inhibitor, that inhibits protease B (15). The levels of RNA polymerase I increase during embryogenesis and decrease after hatching to reach a plateau that is maintained during the following 50 hr of larval development. The levels of RNA polymerase II increase steadily to reach, after 80 hr of development, a specific activity five times higher than in embryos. The levels of RNA polymerase III have a small increase during embryonic and early larval development. These results indicate the existence of independent changes in the levels of the RNA polymerases. The biological meaning of the fluctuations of these enzymes has to be evaluated in relation to the relative rates of synthesis of different classes of RNA *in vivo*. However, little is known on the rate of synthesis of RNA during the early development of *A. salina*. The increase of RNA polymerase I found during the embryonic development could be correlated with the increase of RNA synthesis that seems to occur after reinitiation of development (4). It is more difficult to find a correlation between the levels of the three RNA polymerases and the synthesis of RNA during the early larval development. In this respect, the drop in the levels of RNA polymerase I reported by Birndorf *et al.* (23) and the fall of the RNA synthesis during larval development reported by McClean and Warner (25) could be explained by the experimental conditions used in these experiments, as we have shown that starvation of the nauplii causes a drop in the levels of RNA polymerases I and III (24).

C. Poly(A) polymerase. Poly(A) polymerases are enzymes that can be involved in the postranscriptional processing of messenger RNAs (26), catalyzing the synthesis of a poly(A) chain covalently bound to the 3' end of a RNA primer. We have partially purified and characterized the poly(A) polymerase from dormant embryos of *A. salina* (27-29). Table 2 summarizes some of the properties of the enzyme. It requires for activity manganese, a RNA primer and ATP. dATP can replace ATP with 20% efficiency. Several natural and synthetic polynucleotides can be polyadenylated by the enzyme, but DNA is completely inactive as primer. The nature of the product has been characterized by different criteria, including binding to Millipore filters, sensitivity to ribonuclease and hybridization to poly(U). The average size of the poly(A) chain added by the enzyme is 10 nucleotides. The properties of the poly(A) polymerase purified from *A. salina* cysts are similar to the

properties described for the enzyme from other eukaryotic systems (26).

Table 2. Properties of the Poly adenylate polymerase purified from *A. salina* dormant embryos.

Substrate specificity	ATP > dATP >> CTP, GTP, UTP
Primer	RNA $\geq$ poly(A) > poly(AU) > poly(C) > poly(U) >> DNA
Metal requirement	Mn ²⁺ >> Mg ²⁺ $\approx$ Co ²⁺
Mean size of the poly(A) chain	10 nucleotides
Optimum pH	8.0-8.5
Optimum ionic strength	Low (0.02-0.05 M)
Molecular weight	70,000

Solubilization and assay were done as described in Materials and Methods.

About 80% of the poly(A) polymerase activity is located in the cytosol of dormant embryos, and the remaining 20% is in the particulate fraction containing nuclei and yolk platelets. The subcellular distribution and levels of the poly(A) polymerase change during the embryonic development of *A. salina*. Table 3 shows the increase of total activity and its distribution between the soluble and particulate fractions.

Table 3. Subcellular distribution of the poly(A) polymerase activity during the embryonic development of *A. salina*.

Hours of development	Enzyme activity (units/10 ⁵ embryos)		
	Total	Particulate	Soluble
0	63	13	50
10	71	33	38
18	119	62	57

Fractionation and assay were done as described in Materials and Methods.

In contrast to dormant embryos, more than 50% of the activity is located in the particulate fraction in new born nauplii. Experiments now in progress are consistent with the idea that there is only one molecular species throughout the developmental period studied.

These results are compatible with an initial polyadenylation of stored cytoplasmic mRNAs during the activation of the cysts and the reinitiation of development, followed by an increase in the polyadenylation and processing of newly synthesized mRNAs. Several laboratories have reported the existence of stored polyadenylated and non-polyadenylated mRNA in encysted embryos of *A. salina* (30-34). The stored mRNAs shift from subribosomal particles to polyribosomes and are translated during embryogenesis after resumption of development (33, 35, 36). The average size of the poly(A) chain found in the stored mRNAs is 45-65 nucleotides (37), which is lower than the size found in the mRNAs from most eukaryotic systems (26). Therefore, the polyadenylation of the stored mRNAs in the cysts could be a necessary step in their activation, being the cytoplasmic poly(A) polymerase the enzyme responsible for this activation. The active polyadenylation of stored maternal mRNAs has been reported after fertilization and early embryogenesis in different developing systems (38-41).

D. Larval ribonuclease. It has been known from the work done in the past several years that much more RNA is made in the nucleus than is exported to the cytoplasm to form mature messenger RNA (42). The understanding of this phenomenon is becoming clear with the finding of mRNA reorganization and splicing in almost all eukaryotic systems where this process has been looked for (43). Ribonucleases should be considered as potential enzymes in the regulation of the processing and turnover of structural and informational RNAs. We have studied and partially purified a ribonuclease induced during early larval development of *A. salina* (14). Table 4 shows the properties and substrate specificity of this ribonuclease. As can be seen, the enzyme is active on either transfer or ribosomal RNA, and has no detectable activity on DNA. Synthetic polyribonucleotides are hydrolyzed to different extent, but much more efficiently than natural RNAs. Poly(U) is hydrolyzed with the highest efficiency, followed by poly(A) and poly(C), while poly(G) is not hydrolyzed by the enzyme. The mode of action of the ribonuclease was examined by sucrose gradient analysis of the RNA products of the reaction, indicating an endonucleolytic attack of the RNA substrate by the enzyme. The ribonuclease requires a divalent cation for activity (Mg^{2+} or Mn^{2+}), is inactivated by treatment with EDTA and the inactive preparations can be reactivated by Mn^{2+} and Ca^{2+} ,

but not by Mg^{2+} , indicating that the cations play a role not only in the catalytic reaction but also in the maintenance of the active enzyme molecule (14).

Table 4. Properties of *A. salina* larval ribonuclease.

Optimum pH	7.5
Metal requirement	Mg^{2+} or Mn^{2+}
Heat stability	Low (50% inactivation in 3 min at 54°)
Substrate specificity (relative rates)	
rRNA (yeast)	100
rRNA (<i>A. salina</i>)	66
DNA (native or denatured)	undetectable
poly (U)	10^4
poly(A)	10^3
poly(C)	500
poly(G)	undetectable

data taken from (14)

When ribonuclease activity is measured at different stages of development (figure 3A) no activity is detected during embryonic development. Immediately after hatching the enzyme is induced, being the increase in activity maintained until 50 hr of development, when it reaches a plateau. The results shown in the figure are consistent with an induction of the activity, since treatment of the growing nauplii with cycloheximide completely prevents its expression.

Although the biological function of this larval ribonuclease is not known, its substrate specificity suggests a possible role in the processing of heterogeneous nuclear RNA, which has been reported to contain internal oligo(U) and oligo(A) sequences that have to be processed out during maturation (44). This activity could be effective also in the turnover of postranscriptionally added poly(A) tails present in most eukaryotic mRNAs (45). Alternatively, it could be one of the enzymes needed in the new tissues that nauplii have to develop (as the gastro intestinal tract, for instance).

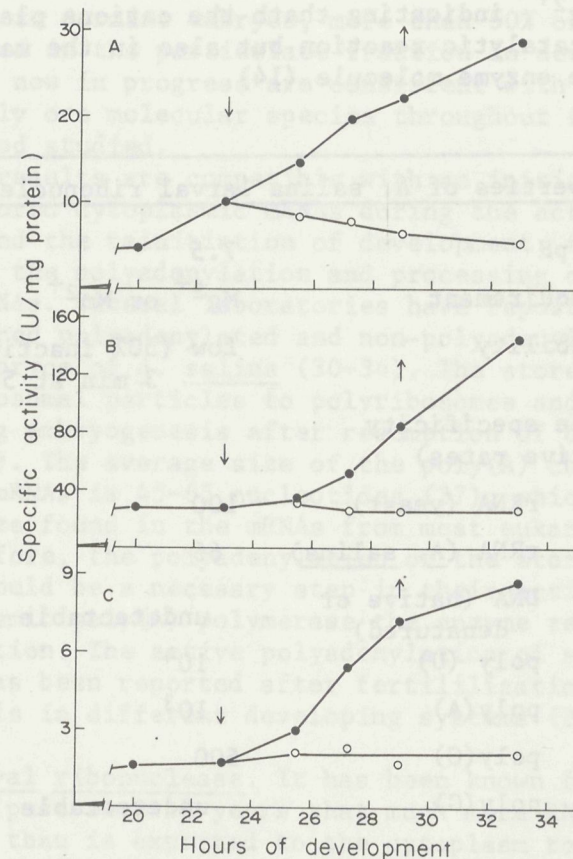


Figure 3. Induction of different enzymes during the early larval development of *A. salina*.

Growing of the embryos and nauplii was done as described (15). At the time indicated (↓), 50 µg/ml of cycloheximide were added to one culture flask, using other in identical conditions as control; 6 hr later (↑), nauplii were filtered off and resuspended in fresh medium without drug (in both flasks). Aliquots were taken at the indicated times and homogenized as described (15). A: larval ribonuclease; B: aminoacyl-tRNA hydrolase; C: protease A (similar results were obtained for proteases B and C, not shown for clarity). (●): no drug; (○): 50 µg/ml of cycloheximide added to the medium.

E. Aminoacyl-tRNA hydrolase. Enzymes which unspecifically hydrolyze both peptidyl and N-substituted aminoacyl-tRNAs to give free tRNA and the corresponding peptidyl or aminoacyl moiety are widely distributed among prokaryotic and

eukaryotic cells (46). Other more specific aminoacyl-tRNA hydrolases have also been reported (17, 47, 48). Encysted and developing embryos of *A. salina* contain almost undetectable levels of unspecific aminoacyl-tRNA hydrolases while they contain substantial amounts of a specific hydrolase active on N-acetylphenylalanyl-tRNA (18).

The specific hydrolase is present without appreciable changes of activity from dormant gastrulae to 40 hr old nauplii (18). The unspecific one, however, is induced during the early larval development as shown in figure 3B. This induction, as is the case for the ribonuclease and the proteases (see below), is inhibited by cycloheximide. The inducible hydrolase has a molecular weight of 55,000, is strictly dependent of a divalent metal for activity and shows a very broad specificity in hydrolyzing either free or N-substituted aminoacyl-tRNAs.

In spite of the wide distribution of the unspecific aminoacyl- or peptidyl-tRNA hydrolases, little is known about their physiological role, although it seems to be related with the process of protein biosynthesis (49). The independent expression of these two hydrolases at different stages of *A. salina* differentiation may facilitate the study of the role played by these enzymes in cell metabolism.

F. Proteases. The proteases can play an important role in the morphogenetic changes and structural reorganization that occur during development, as well as in the regulation of protein turnover (50). In the case of *A. salina*, a study of the proteases was also of interest to understand the metabolic fate of yolk platelets during development (51) and the modification of RNA polymerases (12).

Four different proteolytic activities, active at neutral or alkaline pH, have been detected in *A. salina* larval extracts which have been named A, B, C and D. Their characterization has been made after separation by chromatography on DEAE-cellulose, with the use of several substrates and inhibitors (15, 16). The levels of proteases A, B and C are undetectable in dormant cysts; only some activity on casein has been determined which corresponds most probably to protease D. Figure 3C shows that the induction of these proteolytic activities occurs shortly after hatching (15), and as happens with other enzymes (see above), the addition of cycloheximide to the medium stops the increase of the levels of proteases during development.

G. Carbamyl phosphate synthetase. During our studies on the general metabolism of nucleic acids during *A. salina* development, we have studied the synthetic pathway of pyrim-

idine nucleotides and more precisely the carbamyl phosphate synthetase (CPase), the regulatory enzyme in this pathway (52). *A. salina* dormant embryos contain a constitutive, cytosolic CPase involved in pyrimidine biosynthesis. However, shortly after hatching a new activity is detected (figure 4) clearly different from the constitutive one. The inducible CPase is mitochondrial, is activated by N-acetyl-L-glutamate and is not inhibited by uridine nucleotides, which are effectors of the CPase located in the cytosol. The induction of the mitochondrial CPase reported here correlates well with the mitochondrial activation that occurs after hatching (53, 54).

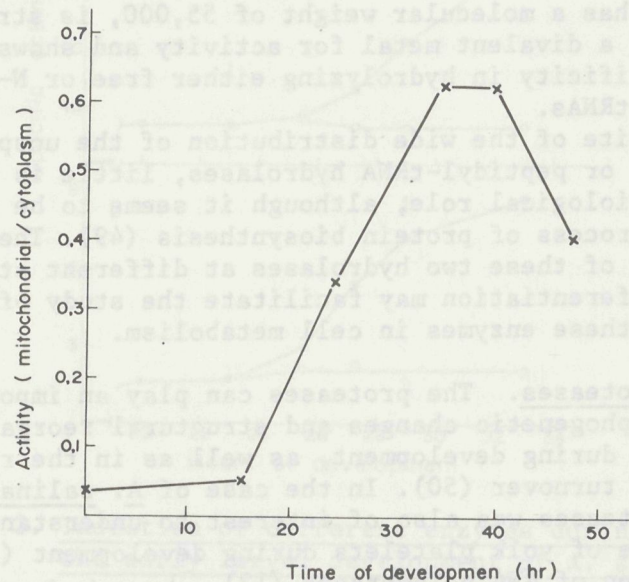


Figure 4. Ratio of mitochondrial and cytosolic carbamyl phosphate synthetase activities during early development of *A. salina*.

Extraction and assay of both activities was done as described in Materials and Methods.

The characteristics of the mitochondrial CPase are well known (55), and it is admitted that this synthetase is involved in the biosynthesis of arginine and urea. This fact raises the question of the functional role of this enzyme in *A. salina*, that, as all crustaceans, is reported to be ammoniotelic (56). Huggins et al. (57) have suggested that the synthesis of urea by organisms that do not excrete it could be a possible biochemical mechanism for osmotic regulation; according to Huggins' nomenclature, *A. salina* could be

considered as "ureosmotic", and the appearance of the mitochondrial CPase after hatching could be a response to the high-salinity habitat in which nauplii, and subsequent adult stages, do occur.

CONCLUSIONS

A general summary of events during *A. salina* early development is shown in figure 5. There are several processes, related with gene expression or with its regulation that are acting before hatching: histone acetylation, significant changes in the levels of RNA polymerases, and an increase and change in distribution of poly(A) polymerase. Histone acetylation and increase in RNA polymerase levels could be related with the activation of RNA synthesis; this point is clearly difficult to confirm as cysts are very impermeable to precursors; nevertheless, Clegg and Golub (17) have reported such an increase. Considering together these results and those from electronmicroscopic studies (R. Marco, personal communication), one can say, in a general way, that nuclear activation occurs soon after reinitiation of development. The significance of the changes in the localization of poly(A) polymerase was discussed previously. After hatching, it oc-

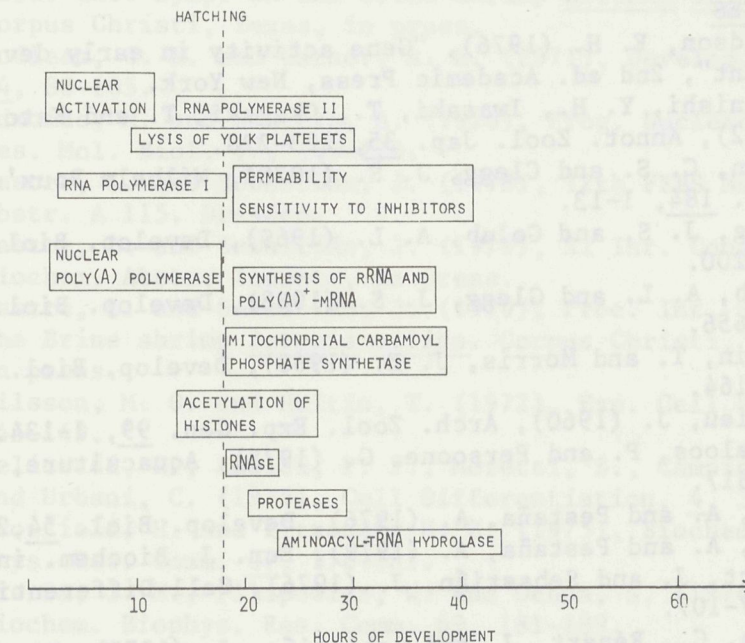


Figure 5. General summary of the developmental changes during *A. salina* embryonic and early larval development.

curs a new burst of biochemical processes. In fact, several degradative enzymes seem to be induced, as well as the mitochondrial carbamyl phosphate synthetase. One can notice also (25, 35 and experiments in progress) that there is an intense RNA synthesis, both of ribosomal and poly(A)⁺-messenger RNAs.

From all these data, and more not discussed in this communication, one is tempted to divide A. salina development in two main periods; the first one before hatching (embryonic development), where the main events are those related with the activation of the genetic apparatus and the morphological reorganization; the second one after hatching (early larval development), when the new hatched larvae prepares to be able to interact with the environment. In this period a wide variety of systems (eyes, gastro intestinal tract, etc.) are expressed, and therefore new enzymes are expressed not needed previously.

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GENE EXPRESSION DURING RESUMPTION
OF DEVELOPMENT OF ENCYSTED
ARTEMIA GASTRULAE

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ABSTRACT

We have analyzed during the first 24 hr of re-initiation of development of encysted Artemia gastrula: a) the efficiency of translation of polyadenylated and non-polyadenylated mRNA; b) the nature of translation products of messengers in vivo and in vitro; c) the sequence complexity of mRNA; d) the compartmentalization of RNA polymerases as template-engaged and free enzyme pools. Poly(A)-containing RNA from embryos at 5, 10 and 20 hr after reinitiation of development is translated more efficiently in a rabbit reticulocyte lysate cell-free protein synthesizing system than RNA from dormant gastrulae. Two major proteins coded for by dormant gastrular RNA disappeared by the hatched-out nauplius stage. Kinetics of hybridization with complementary DNA to polyadenylated RNA revealed that the sequence complexity of the three major abundance classes of mRNA increased markedly upon resumption of development following reactivation and then declined in the hatched-out nauplii, as well as a considerable divergence in messengers at these two developmental stages. These changes in RNA populations were accompanied by large increases in the fraction of template-engaged enzyme and smaller increases in the free enzyme molecules occurring at the pre-naupliar and naupliar stages. Thus, development of dormant Artemia gastrulae is characterized by a "reprogramming" seen as a simultaneous and rapid increase in (a) the absolute number of transcribing RNA polymerase molecules, (b) sequence complexity of polyadenylated RNA, and c) the amount of translatable messenger RNA.

INTRODUCTION

It is now known that dormant Artemia gastrulae contain a large store of "masked" messenger activity (1,2). The inactive stored mRNA contains polyadenylic acid [poly(A)] and is present as ribonucleoprotein particles which, when deproteinized, become active as templates in heterologous translation systems in vitro (2). Upon artificial induction of development, RNA and protein synthesis is resumed very actively but the mode of "masking" and

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"unmasking", or the reversal of inhibition of translation almost immediately upon reactivation, still remain unclear. Recent studies have also demonstrated that very substantial changes occur in transcription at the onset of resumption of development of encysted *Artemia* gastrulae. These include an overall increase in extractable polymerase activities (3,4) and labelling of RNA *in vivo* (5). However, virtually nothing is known about the nature of newly synthesized RNA at resumption of development relative to the stored pregastrular RNA, except that the majority of the RNA of dormant embryo codes for low molecular weight proteins, whereas high molecular weight proteins are synthesized on RNA obtained after resumption of development (6). In recent years, analysis of mRNA populations by molecular hybridization to complementary DNA as a means for deducing mRNA complexity has provided a fruitful approach to studying gene expression in eukaryotic cells (7). In most cases, a given mRNA population can be arbitrarily resolved into three classes depending on the approximate number of copies of each mRNA species present (8,9). From the kinetics of the hybridization reaction, some of the messenger species can be shown to be present in a few thousand copies per cell (high abundance class) while others occur as a few hundred (middle abundance class) or only 5-20 (low abundance class) copies per cell (10,11).

Regulation of transcription is often studied either at the level of the chromatin template or that of the isolated multiple eukaryotic RNA polymerases (12,13,14). However, studying chromatin and its endogenous enzymes separately would not allow one to detect possibly important functional compartmentation of the individual components as a normal regulatory process in developing cells. It is possible to examine isolated nuclei in which such functional compartmentation would be retained by following an assay procedure first described by Yu (15). It is based on the response of isolated nuclei to exogenously added synthetic DNA poly[d(A.T)] in the presence of high concentrations of actinomycin D. Thus it was possible for us to discriminate in nuclei of developing *Artemia* gastrulae between RNA polymerase molecules which are bound to the nuclear template, and are capable to elongating *in vitro* RNA chains initiated *in vivo* (referred to as engaged enzymes), and RNA polymerase molecules which have a loose or no association with the endogenous chromatin template and do not take part in RNA synthesis *in vitro* unless exogenously added DNA is supplied (referred to as free enzyme).

In this review we report our studies on analyzing changes in messenger RNA population upon resumption of development of encysted *Artemia* gastrulae and on the changes in compartmentation of transcribing and non-transcribing RNA polymerase

molecules. Towards this end, we have combined three different approaches. 1) Assay of cell-free protein synthesis in nuclease-treated rabbit reticulocyte lysates to assess the "translatability" and nature of translation products of RNA extracted from embryos at different developmental stages; 2) messenger RNA excess - cDNA hybridization to study the sequence complexity of polyadenylated RNA [poly(A)⁺-RNA] isolated from different stages of reactivated dormant gastrulae; 3) changes in the proportion of template-engaged and free RNA polymerases I and II in relation to alterations in total enzyme activities.

RESULTS AND DISCUSSION

Poly(A) Content of RNA

There is a gradual increase in the poly(A) content of the total RNA (as determined by hybridization to ³H-poly U) with increasing time of development following hydration of the dormant gastrulae (16). Total RNA from hatched-out nauplius larvae comprises approximately twice as much poly(A) when compared to a similar preparation from dormant embryos. Approximately 3 times more poly(A)⁺-RNA is obtained from total RNA preparation of nauplius larvae compared with that from dormant embryos. The poly(A) content/μg of poly(A)⁺-RNA did not however change with development. Thus, a *de novo* synthesis of polyadenylated RNAs must occur during reactivation of the dormant gastrulae. As will be shown below, the increase in the poly(A) content of the total RNA corresponds to greater efficiency of translation on the one hand, and a change in sequence complexity of the RNA on the other.

Translation of RNA from *Artemia salina* Embryos in a Heterologous Cell-Free System

As shown in Table 1, total RNA from dormant, desiccated embryos stimulated the incorporation into protein of detectable amounts of ³⁵S-methionine. When this RNA from dormant embryos was fractionated into poly(A)⁺ and poly(A)⁻ fractions, and the fractions assayed separately, the poly(A)⁻ fraction was more active in promoting the incorporation of ³⁵S-methionine than an equivalent amount of poly(A)⁺ fraction. However, this unusual situation was rapidly reversed when the dormant gastrulae were stimulated to develop further. Total RNA isolated from embryos 5, 10 or 20 hr after incubation was progressively more actively translated than preparations from dormant embryos, there being a 2-fold increase at 20 hr, i.e. with RNA from post-hatch nauplius larvae. This increase was considerably magnified when the total RNA was fractionated according to its poly(A) content, with a 10-fold increase in the amount of label

Table 1. Incorporation of ^{35}S -Methionine into Protein Directed by Various Fractions of RNA from *Artemia salina* Embryos in a Rabbit Reticulocyte Lysate Cell-Free System (16).

Developmental Stage (hours of incubation)	^{35}S -Methionine incorporated (cpm/100 μl lysate)		
	Total RNA	Poly(A) ⁺ -RNA	Poly(A) ⁻ -RNA
0 (dormant)	33,800	4,200	33,100
5	45,800	23,400	14,400
10	50,800	41,800	18,200
20	69,300	45,000	14,800

1 μg of poly(A)⁺-RNA or an equivalent amount of poly(A)⁻-RNA or total RNA were assayed for *in vitro* incorporation of ^{35}S -methionine into acid precipitable radioactivity in a rabbit reticulocyte lysate system.

incorporated by hatched-out larval poly(A)⁺-RNA preparation compared to that of preparations from dormant gastrulae. Whereas poly(A)⁻-RNA from dormant embryos was more efficiently translated than poly(A)⁺-RNA at the same developmental stage, poly(A)⁻-RNA from later stages was translated much less well. The very low messenger activity in RNA from dormant gastrulae could be due to: 1) the lack of functionally active messengers in the preparation, 2) the presence of some inhibitory RNA(s) or protein(s), or 3) some inherent inability of the messenger to be translated. The latter possibility may involve end modifications or lack of processing of RNA such that these would cause masking of initiation sites or introduction of premature termination sites for translation of the message. It also seemed possible that the RNA preparation from the dormant cysts is more susceptible to degradation due to endogenous nucleases, despite the fact that extraction of RNA from embryos at different developmental stages was carried out simultaneously and under identical conditions. This possibility was however ruled out by an experiment in which RNA from dormant cysts failed to decrease the translatability of the RNA from 10 hr postincubation embryos when the dormant and developing embryos were extracted together.

To test the possibility that the poly(A)⁺-RNA from dormant embryos might contain a fewer number of messenger sequences

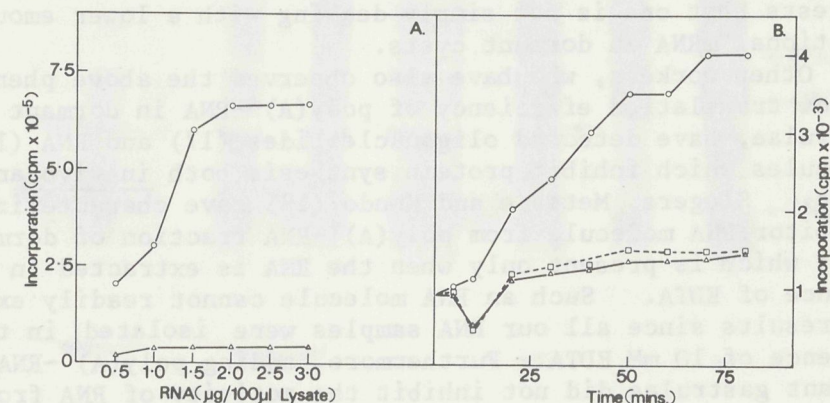


Figure 1. Kinetics of incorporation of ^{35}S -methionine into protein, directed by Poly(A)⁺-RNA from dormant *Artemia* gastrulae and from embryos 10 hr after activation in a rabbit reticulocyte lysate system

A: Effect of different amounts of RNA added.

B: Time course of incorporation with 1.0 μg RNA/100 μl lysate.

RNA from dormant gastrulae (Δ); RNA from 10 hr activated embryos ($\circ$); "heat-treated" dormant gastrulae RNA ($\square$).

From James and Tata (16).

per μg RNA, we studied the effect of varying the concentration of poly(A)⁺-RNA from the dormant and activated embryos (see Fig. 1A). There was no increase in the amount of ^{35}S -methionine incorporated into protein with increasing concentrations of poly(A)⁺-RNA from dormant embryos, but the incorporation increased with increasing concentrations of RNA from activated embryos up to 2 $\mu\text{g}/100\ \mu\text{l}$ of the reticulocyte lysate. The time-course of incorporation of ^{35}S -methionine with poly(A)⁺-RNA from

embryos at the 10 hr incubation stage (see Fig. 1B) also showed that this preparation was at least 2-5 times more effective in its messenger activity than that of a similar preparation from dormant embryos. The fact that increasing the amount of input RNA from dormant gastrulae did not enhance ^{35}S -methionine incorporation in a cell-free assay, whereas a linear relationship was observed with RNA from 10 hr postincubation embryos, further suggests that one is not simply dealing with a lower amount of functional mRNA in dormant cysts.

Other workers, who have also observed the above phenomenon of low translation efficiency of poly(A)⁺-RNA in dormant *Artemia* gastrulae, have detected oligonucleotides (17) and RNA (18) molecules which inhibit protein synthesis both *in vivo* and *in vitro*. Slegers, Mettrie and Kondo (18) have characterized an inhibitor RNA molecule from poly(A)⁺-RNA fraction of dormant cysts which is present only when the RNA is extracted in the absence of EDTA. Such an RNA molecule cannot readily explain our results since all our RNA samples were isolated in the presence of 10 mM EDTA. Furthermore, adding poly(A)⁺-RNA from dormant gastrulae did not inhibit the activity of RNA from 10 hr postincubated cysts so that dormant gastrular RNA preparations were unlikely to contain any translation inhibitor RNA or protein.

The low template activity of poly(A)⁺-RNA from dormant cysts may also possibly arise from some modifications of mRNA at the 3' and 5' termini. Preliminary studies from our laboratory on the 3' end analysis of dormant and nauplii mRNA using RNase A and T₁ digestion, showed that both dormant and nauplii poly(A)⁺-RNA have poly(A) tails of 50-200 nucleotides in length. Furthermore, cDNA transcription which begins at the 3' end proceeded with the same kinetics for poly(A)⁺-RNA isolated from dormant cysts or nauplii, thus further confirming that the 3' ends of these RNAs are similar. Groner, Grosfeld and Littauer (6) reported the presence of identical cap structures (19,20) at the 5' end of messenger RNA isolated from *Artemia* embryos 3 and 22 hr after incubation of dormant gastrulae. However, it does not rule out the possibility of an unprocessed or decapped 5' end of mRNA from dormant gastrulae. Gilmour and Warner (21) have detected the nucleotide guanosine 5' triphospho-5'-adenosine [G-(5')p3(5')A] in acid soluble extracts of dormant *Artemia* embryos, which they suggest could arise from hydrolysis of 'capped' messengers. It is possible that during desiccation and encystment, gastrular and pre-gastrular messenger RNAs were demethylated and 'decapped', thus resulting in cryptic or modified initiation triplet or possible recognition and binding sites for ribosomes and initiation factors on the noncoding region of mRNA, as suggested by Shine and Dalgarno (22) and Hagenbüchle *et al.* (23).

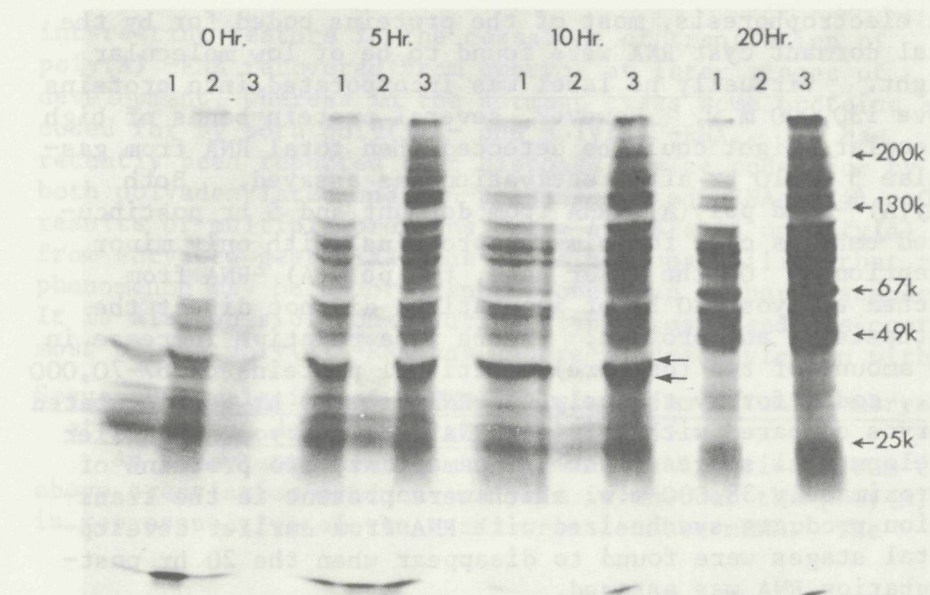


Figure 2. Electrophoretic analysis of translation products. Poly(A)⁺-RNA (track 1), poly(A)⁻-RNA (track 2) and total RNA (track 3) extracted from dormant (0 hr) and developing *Artemia* embryos at 5, 10 and 20 hr of incubation were translated in a rabbit reticulocyte lysate as described elsewhere (16). 20 μl of lysate were mixed with equal volume of sample buffer, electrophoresed for 5 hr at 130 volts on a 10-16% linear polyacrylamide gradient slab gel in 0.1% SDS at room temperature and the gels fluorographed. Protein bands indicated by arrows (bottom) are two protein bands disappearing at the nauplius stage. Note the overall similarity of the translation products from different stages. From James and Tata (16).

However, we found no detectable differences in the action of m⁷GDP on translation of RNA prepared both from dormant cysts and nauplii.

When the nature of labelled translation products in the rabbit reticulocyte lysate were analyzed by SDS-polyacrylamide

gel electrophoresis, most of the proteins coded for by the total dormant cyst RNA were found to be of low molecular weight. Virtually no label was incorporated into proteins above 130,000 m.w. However, several protein bands of high molecular weight could be detected when total RNA from gastrulae 5 or 10 hr after activation was assayed. Both poly(A)⁺- and poly(A)⁻-RNA from dormant and 5 hr postincubated embryos code for similar proteins, with only minor exceptions. On the other hand, the poly(A)⁻-RNA from hatched embryos (20 hr of incubation) did not direct the synthesis of any protein. There is a relative increase in the amount of two (or more) additional proteins of 67-70,000 m.w., coded for by the poly(A)⁺-RNA from 20 hr postincubated embryos compared with poly(A)⁺-RNA from embryos at earlier developmental stages. At the same time, two proteins of approximately 38,000 m.w. which were present in the translation products synthesized with RNA from earlier developmental stages were found to disappear when the 20 hr postincubation RNA was assayed.

In order to test the possibility that dormant cyst mRNAs coding for high molecular weight proteins were actually translated but difficult to detect by gel electrophoresis when total RNA preparation was used, we fractionated the RNA from dormant and hatched-out nauplii into 15 fractions according to size on a sucrose density gradient and assayed each fraction separately in a heterologous cell-free translation assay. The resultant fluorographs were deliberately over-exposed to show up any minor bands but still most of the proteins coded for by RNA extracted from dormant cysts fractionated on density gradients were of molecular weights below 90,000. On the other hand, the majority of proteins coded for by density gradient-fractionated RNA extracted from nauplii are in the range of molecular weights of 49,000 - 90,000 daltons, with a number of higher molecular weight proteins (m.w. >130,000 daltons). When each fraction obtained from the sucrose density gradients was assayed for its poly(A) content, a major shift was noticeable in the molecular weight of poly(A)-containing RNAs from <9S for the dormant cysts to 9-18S for RNA from nauplii. Furthermore, fractions from nauplii RNA had a higher poly(A) content compared with those from dormant cysts but in the case of RNA prepared from dormant embryos, the messenger activity did not exactly follow the poly(A) content, thus supporting the finding described in Table 1 that the poly(A)⁺-RNA from dormant cysts is translated less efficiently than the poly(A)⁻-RNA. Our data agree with the findings of Grosfeld and Littauer (6) who also observed the disappearance of two low molecular weight proteins coded for by gastrular RNA which are present at all other developmental stages. Another

interesting feature is the cessation of translation of poly(A)⁻-RNA recovered from embryos at later stages of development, whereas in the dormant cysts some proteins are coded for by both poly(A)⁺- and poly(A)⁻-RNA. It has recently been reported that histones may be coded for by both polyadenylated and non-polyadenylated mRNAs (24). Our results of multiple proteins being translated on poly(A)⁻-RNA from encysted gastrulae thus raise the possibility that the phenomenon may be a more general one than hitherto suspected. It is also possible that during cryptobiosis and desiccation, most of the poly(A)⁺-RNA has undergone deadenylation with time.

Sequence complexity of mRNAs isolated from *Artemia* embryos at different developmental stages

An important question that immediately arises from the above translation product analysis is whether the poly(A)⁺-RNA is representative of the total translatable mRNA. The

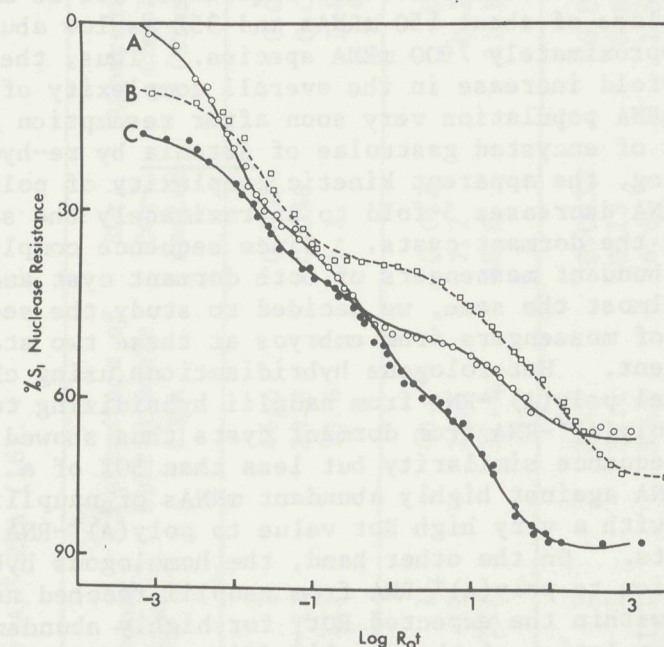


Figure 3. Sequence complexity of embryonic poly(A)⁺-RNA of *Artemia salina*. Hybridization of cDNA prepared against poly(A)⁺-RNA from A) dormant encysted gastrulae (○); B) embryos 10 hr after incubation (□) and C) hatched-out (20 hr postincubation) nauplii larvae (●) to homologous poly(A)⁺-RNA. All hybridization curves represent computer fits to the data and corrected for zero time values which were 2-4% of total. From James and Tata (16).

complexity of the polyadenylated mRNA sequences in a given cell or tissue can be better estimated by hybridizing an excess (usually 1000-fold) of mRNA with its complementary DNA (Fig. 3). In the dormant cysts, 46% of the total population of mRNA behaved as a highly abundant class (a few thousand copies/cell), 30% as a middle abundant class of about 85 different messengers present in a few hundred copies each, and about 24% as a low abundant class of 9700 mRNA species represented in a very few copies (<1-3 per cell). Poly(A)⁺-RNA isolated from 10 hr post-incubation embryos showed a different pattern of mRNA complexity than that seen for dormant gastrulae RNA (Table 2). 40% of the total RNA at this developmental stage contains a highly abundant class of 15 mRNA species, 22% forms a middle abundance class of about 1400 mRNA species, whilst 37% comprises a low abundance class of about 57000 different species of mRNA. The newly hatched-out larval (20 hr post-incubation) mRNA also falls into a 31% high abundance group of about four different messenger sequences, 33% as middle abundance class of about 150 mRNAs and 35% as low abundance class of approximately 7900 mRNA species. Thus, there occurs a 5-fold increase in the overall complexity of embryonic mRNA population very soon after resumption of development of encysted gastrulae of *Artemia* by re-hydration. Upon hatching, the apparent kinetic complexity of poly(A)⁺-messenger RNA decreases 5-fold to approximately the same level as in the dormant cysts. Since sequence complexities of highly abundant messengers of both dormant cyst and nauplii were almost the same, we decided to study the sequence similarity of messengers from embryos at these two stages of development. Heterologous hybridizations using cDNA against total poly(A)⁺-RNA from nauplii hybridizing to an excess of poly(A)⁺-RNA from dormant cysts thus showed considerable sequence similarity but less than 50% of a purified cDNA against highly abundant mRNAs of nauplii hybridized with a very high Rot value to poly(A)⁺-RNA from dormant cysts. On the other hand, the homologous hybridization reaction to poly(A)⁺-RNA from nauplii reached near completion within the expected Rot₁ for highly abundant messenger population of the nauplii (Fig. 4). Although this finding indicates a considerable degree of qualitative difference in the population of highly abundant messages at these two stages, it does not rule out the possibility of common sequences existing in other classes of mRNAs present in lower abundance.

The situation with developing *Artemia* gastrulae described above is different from that in *Xenopus* oocytes and eggs in which the stored messengers represent almost the

Table 2. Sequence complexity of mRNA populations present in *Artemia* embryos at different developmental stages as determined by kinetics of hybridization reaction between cDNA and excess of cytoplasmic poly(A)⁺-RNA (16).

Source of Cytoplasmic Poly(A) ⁺ -RNA	Abundance Class	Percentage of Hybridizable cDNA	Rot ₁ Corrected	Apparent Sequence Complexity (NT x 10 ⁻⁷)	Number of Messenger RNA Species	Number of Copies Per Cell
Dormant gastrular cysts (no incubation)	I	46.6	0.0016	0.00062	4	3580
	II	29.8	0.056	0.013	85	170
	III	23.6	6.1	1.466	9700	2
Embryos 10 hr postincubation	I	40.4	0.012	.003	15	1600
	II	22.4	1.2	0.289	1445	16
	III	37.2	48.0	11.52	57600	1
Hatched-out nauplius larvae 20 hr postincubation	I	31.1	0.0036	0.0008	4	8775
	II	33.1	0.13	0.031	155	226
	III	35.8	6.6	1.59	7950	5

Legend to Table 2.

The $Rot_{1/2}$ values obtained in these experiments are compared with the $Rot_{1/2}$ of a control reaction between rabbit globin cDNA and an excess of globin mRNA which has a $Rot_{1/2}$ of 5×10^{-4} and a sequence complexity of 1200 nucleotides. Most of the poly(A)⁺-RNA from embryos at 10 and 20 hr after incubation sedimented at 9-20S, with a peak around 14S region on 0-30% isokinetic sucrose gradients (53). Poly(A)⁺-RNA from dormant cysts sedimented at 9-15S. The number average molecular weight for poly(A)⁺-RNA for 10 and 20 hr postincubation embryos is 660,000 daltons and that of dormant embryos 500,000 daltons. The observed $Rot_{1/2}$ value is corrected for the presence of other complexity components of the population. Abundance classes I, II and III refer to high, middle and low abundance mRNA species, respectively. 37, 80 and 117 μ g poly(A)⁺-RNA could be recovered from 3.3×10^{10} cells of dormant, 10 and 20 hr incubated embryos respectively. Number of copies per cell is obtained by dividing the total number of nucleotides per cell divided by sequence complexity (in nucleotides) in each class. Total number of nucleotides is given by Mass X Avogadro number/330 g (molecular weight of one nucleotide). Computer program was adapted by Dr. B. Young of The Beatson Institute for Cancer Research, Glasgow, from Monahan, Harris and O'Malley (54).

full potential of genes expressed in all tissues of the adult animal (25) and only partially resembles the situation in developing sea-urchin eggs soon after fertilization (26). In this context, it may be more relevant to compare our findings with those recently described by Farmer *et al.*, (27) on the substantial changes in the translatability of mRNA populations during reversible surface attachment of mouse fibroblasts. It could be argued that the higher sequence complexity of mRNA populations that we observe during early embryogenesis reflects the requirement of new protein synthesis for the resumption of metabolic activity of the dormant gastrula (28,29). It is also significant that the increase in sequence complexity upon reactivation is mainly reflected in the middle and low abundance classes of mRNA. Hastie and Bishop (30) have attributed "housekeeping" functions (those required for the growth and maintenance of all cells) to low abundance class mRNAs which raises the possibility that during the process of cryptobiosis *Artemia* genes coding for "housekeeping" proteins were selectively and reversibly "switched-off". In any event, it can be concluded that during early embryonic development of *Artemia salina*, there are both quantitative and qualitative changes in the sequence complexity of poly(A)⁺-messenger RNA molecules.

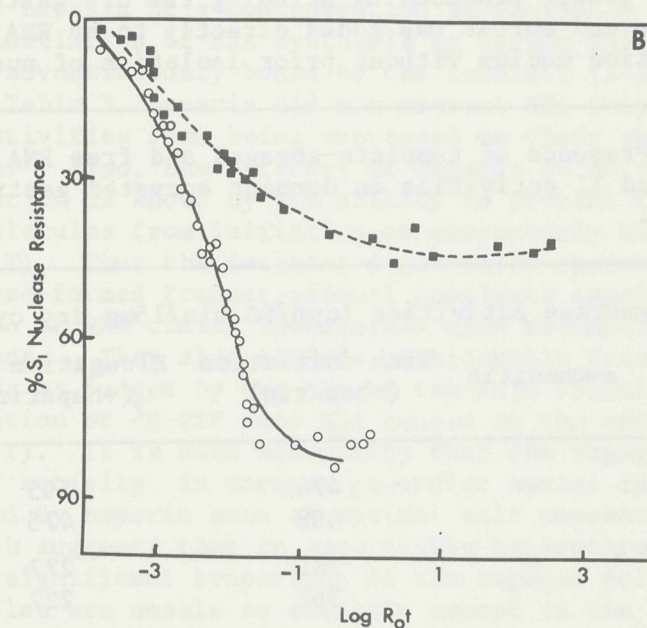


Figure 4. Comparison of hybridization kinetics of cDNA to total poly(A)⁺-RNA and isolated cDNA to abundant mRNA. The cDNA to abundant mRNA sequences was purified as described elsewhere (16) and cDNA to abundant nauplii RNA hybridized to poly(A)⁺-RNA from nauplii (O) and to dormant cyst poly(A)⁺-RNA (■). From James and Tata (16).

Nuclear RNA polymerase activities from dormant cysts

Clegg and Golub (31) found that RNA synthesis was detectable by isotopic labelling *in vivo* within 15 min of resumption of development of gastrulae. Hentschel and Tata (32) reported that nuclear preparations from the dormant gastrulae can synthesize RNA and that both RNA polymerase I and II were present as discriminated by their differential sensitivity to low concentrations of α -amanitin (33). Since RNA synthesis in isolated nuclei represents predominantly chain elongation (13) it seemed that the nuclei within the dormant gastrulae contained RNA polymerase I and II molecules that were engaged to their endogenous templates and which are capable of elongating RNA chains as soon as the cysts are hydrated and metabolic activity resumes. Alternatively, un-engaged polymerase molecules could have become attached to the template and initiated transcription during the isolation

of nuclei. To distinguish between these possibilities, a desiccated powder produced by grinding the dry gastrulae with pestle and mortar was added directly to an RNA synthesis incubation medium without prior isolation of nuclei in

Table 3. Presence of template-engaged and free RNA polymerase I and II activities in dormant encysted gastrulae of *Artemia*.

RNA polymerase activities (cpm/60 min/15mg dry cyst)			
Enzyme Activities	α -amanitin	With initiation (-heparin)	Elongation only (+heparin)
Engaged	-	474	595
		498	475
	+	281	277
		367	227
Free	-	3325	168
		3832	111
	+	2423	165
		2185	113

In order to establish that the presence of engaged polymerase molecules in the dormant material was not due to adventitious initiation caused by the procedure for isolation of nuclei in aqueous media, dry cysts were crushed in a pestle and mortar and 15mg of the powder was added to 100 μ l of RNA synthesizing medium containing 50 mM $(\text{NH}_4)_2\text{SO}_4$ and 2.5 μCi ^3H -UTP (49 Ci/mmol). The final UTP concentration was unknown. The incubation media also contained, where indicated, heparin (100 $\mu\text{g}/\text{ml}$), α -amanitin (1 $\mu\text{g}/\text{ml}$). Engaged enzyme activities were measured without any exogenous template added, whereas for the free enzyme assay poly d(A.T) (200 $\mu\text{g}/\text{ml}$) and actinomycin D (50 $\mu\text{g}/\text{ml}$) were added. All incubations were carried out at 30°C for 1 hr. Reactions were stopped by the addition of 25 μ l 2.5% SDS-0.1M EDTA and the tubes were heated for 30 sec in boiling water. Insoluble material was sedimented by centrifugation and the supernatant (50 μ l) was pipetted onto Whatman DEAE 81 discs and processed as described elsewhere (32).

an aqueous medium. Furthermore, the incubation medium contained heparin at a concentration sufficiently high to inhibit initiation of RNA synthesis by enzyme molecules becoming adventitiously bound to the template (34). As shown in Table 3, heparin did not prevent RNA polymerase I and II activities from being expressed on their endogenous templates. Also, the efficacy of heparin as an inhibitor of initiation is shown by its ability to prevent free polymerase molecules from initiating on exogenously added poly d(A.T). Thus the desiccated gastrulae appear to contain pre-formed transcriptional complexes capable of elongation of RNA chains immediately upon resumption of development. They also contain considerable free polymerase activities as judged by the almost ten-fold stimulation of incorporation of ^3H -UTP into RNA caused by the addition of poly d(A.T). It is also noteworthy that the engaged polymerase II activity in dormant gastrular nuclei is highly stimulated by heparin even at optimal salt concentrations (32) which suggests that in such highly heterochromatic nuclei a significant proportion of the engaged polymerase II molecules are unable to elongate except in the conditions of extreme chromatin decondensation produced by the combination of high ionic strength and heparin.

Reactivation of engaged and free RNA polymerases with development

The reactivation of RNA synthesis occurring during *Artemia* pre-hatch development was clearly reflected in template engaged polymerase I and II activities. Maximum enzyme activities were detected shortly after hatching which corresponds to the period originally found by Warner and McClean (35) to be the most active in RNA synthesis *in vivo*. As regards free RNA polymerase I and II, which were detected in nuclear preparations from the dormant gastrulae, the enzyme activity was found to be highly dependent on the concentration of the exogenous template (32). The free enzyme activities in different nuclear preparations from developing embryos had therefore to be standardized to constant chromatin DNA and exogenous DNA [poly d(A.T)] concentrations before comparisons could be made. Figure 5 compares the free and engaged polymerase I and II activities during this period and shows that in the nuclei derived from the dormant gastrulae a large proportion of both polymerase I and II activities is free. When development was reinitiated, both free and engaged polymerase I and II activities increased as a function of time but the kinetics of the activation are quite different, the free polymerase activity increasing earlier than that of the template-engaged fraction. Renart and Sebastian (36)

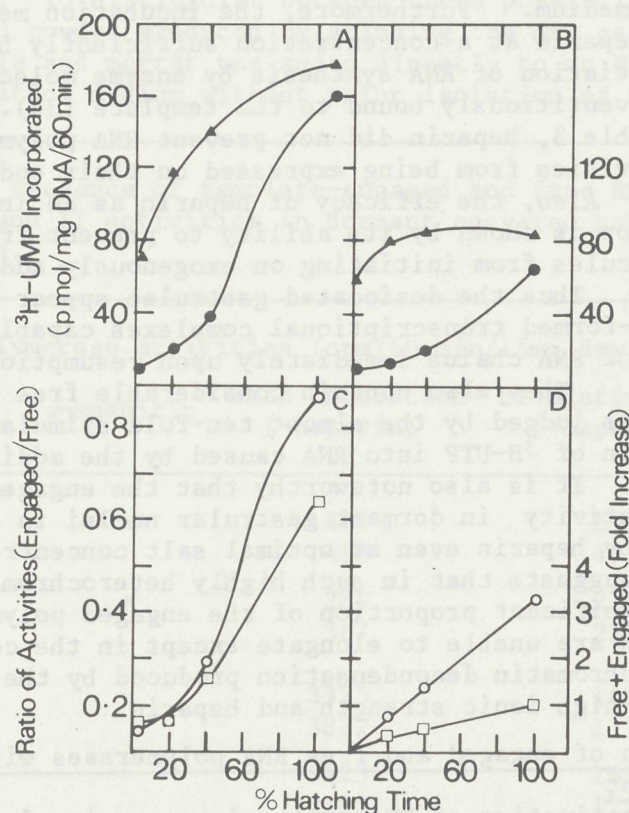


Figure 5. Panels A and B: Differential activation of free (▲) and engaged (●) RNA polymerase I and II activities respectively following the resumption of development of dormant gastrulae. Nuclear fractions were prepared from developing embryos at different time intervals following the resumption of development and free and engaged polymerase I and II activities were assayed as described elsewhere (32). Incubations were carried out for 60 min at 30°C with no added ammonium sulphate so that free and engaged activities could be assayed under identical ionic conditions. Under these conditions RNA polymerase I (α -amanitin insensitive) represented the major transcriptional activity. Panel C: Ratio of engaged to free RNA polymerase I (○) and II (□) activities during pre-hatch development. Panel D: Fold increase in total (free plus engaged) RNA polymerase I (○) and II (□) activities during pre-hatch development. From Hentschel and Tata (32).

also reported increases in the activities of solubilized polymerase I and II transcribing exogenous template similar to those described in our work for total (free + engaged)

polymerase activities (panel D) during the same developmental period. At a time when RNA synthesis is very active as in newly hatched nauplii the proportion of total enzyme as free polymerase is much reduced, particularly in the case of polymerase I. These results suggest that the activation of RNA synthesis which has occurred *in vivo* may, at least in part, be due to the engagement of free polymerase molecules already present in the nuclei of the desiccated gastrulae. Furthermore, there is also an increase in the total cellular levels of the polymerases during this period of development either due to new enzyme synthesis or activation of latent enzymes.

In order to test the possibility that the absolute number of engaged polymerase molecules increases during this period of development, the number of elongating RNA chains were quantitated in nuclei derived from dormant gastrulae and nauplii, using a method based on the fact that alkaline hydrolysis of RNA chains results in the release of internal nucleotide residues as nucleotide monophosphates whereas the 3' termini are released as free nucleosides which can be easily quantitated by thin layer chromatography. This method has been shown to be reliable for estimating the actual number of engaged polymerase molecules per cell, as well as to determine relative changes in initiation or elongation rates during a change in nuclear transcriptional activity (37,38). When such an analysis is carried out in nuclear preparations from dormant gastrulae and from nauplii, as shown in Table 4, it was found that change in incorporation of ³H-UTP into 3' termini of RNA product closely parallel the total engaged RNA polymerase activities thus demonstrating a large increase in the absolute number of transcribing polymerase I and II molecules during the period of development analyzed.

In other situations in which an increase in both free and engaged polymerase activities have been reported, such as after partial hepatectomy of rat liver (39,40) and after transformation of lymphocytes by mitogen (41), it was concluded that an increase in the absolute amount of polymerase I was responsible for the result. The possible regulation of RNA synthesis at the level of modulation of the proportion of transcribing (engaged) to non-transcribing (free) polymerase molecules is implied in a number of dormant early developmental situations where the polymerase may be in excess and acting as storage products (42,43). Thus, Colman (44) found that injection of poly d(A.T) into unfertilized *Xenopus* eggs, which are transcriptionally inactive, resulted in a very large stimulation of RNA synthesis as one would expect if there was a large pool of free polymerase. Oocytes which are active in RNA synthesis showed far less stimulation when similarly micro-injected. Earlier, Roeder (45) had failed to detect any

Table 4. A comparison of the relative number of elongating RNA chains in nuclei derived from dormant gastrulae and nauplii by quantitation of ^3H -UTP incorporated into 3' chain termini.

Develop- mental Stage	Total RNA synthesis (pmol UMP/mg- DNA/10 min)	Internal residues (pmol UMP/mg DNA)	3' Termini (fmol Uridine /mg DNA)
Dormant	24	13	112
Gastrulae	23	14	145
Nauplii	234	165	1086

RNA hydrolysis products from RNA chains elongated *in vitro* in nuclei derived from dormant gastrulae and from nauplii were quantitated as described elsewhere (32). Under the experimental conditions (^3H -UTP specific activity 49 Ci/mmol) 1 fmol of uridine recovered was measured as 40 cpm. Adapted from Hentschel and Tata (32).

alteration in the total polymerase activities during the maturation of oocytes to eggs. Similarly, while little change in the total polymerase activity of the fat body of the tobacco hornworm (*Manduca sexta*) could be detected during development from larval to pupal stages (46), it was noted that most of the larval polymerase was easily soluble and that during development there was a progressive shift from soluble to insoluble activity as would be expected if free enzymes were increasingly mobilized to become template-engaged during this period.

The nature of polymerase binding to chromatin during the initiation of transcription is poorly understood in eukaryotes but is probably a multi-step process whose final extent is dependent on the number of true initiation sites available on the active chromatin fraction (47). Recent results from this laboratory suggest that these active chromatin fractions are discrete structural units which may be selectively released from the nuclei by minimal micrococcal nuclease digestion (48). Other studies from various laboratories, including ours, suggest that functional compartmentation of the multiple RNA polymerase may play a significant regulatory role in controlling the dramatic switches in the synthesis of specific RNA

classes observed in a number of developmental systems (7,32,49). The mechanism governing any such compartmentation remains unclear but could be due either to specific modifications of the components of the transcriptional apparatus or to changes in the levels of specific regulatory factors required for initiation of the polymerases. What is evident is that investigation of these regulatory phenomena requires the conservation of the chromatin template as a functional unit with its endogenous bound RNA polymerase and regulatory factors.

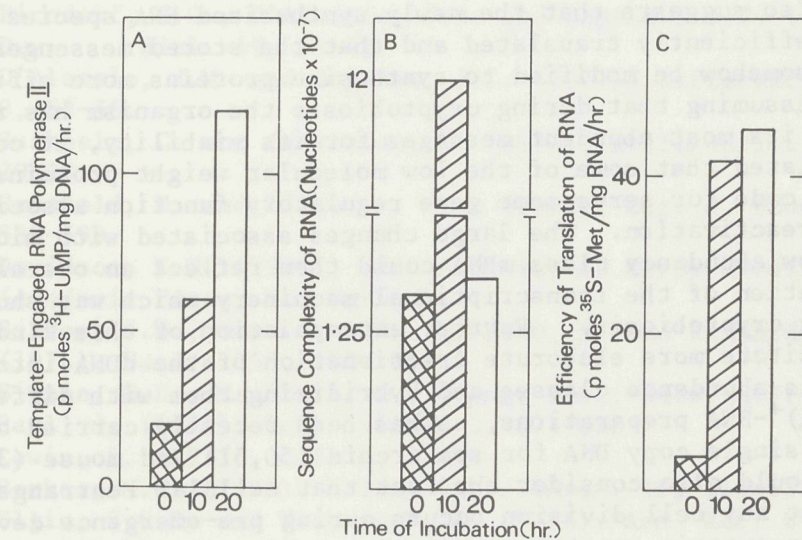


Figure 6. Histogram summarizing relative changes in: A) template-engaged RNA polymerase II activity; B) sequence complexity of poly(A)⁺-RNA and C) efficiency of translation (in 100 μl rabbit reticulocyte lysate) of cytoplasmic poly(A)⁺-RNA from dormant *Artemia* gastrulae and in embryos at 10 and 20 hr after activation. Based on data from Hentschel and Tata (32) and James and Tata (16).

General Implications

The mechanism underlying a shift in the nature of gene transcripts during developmental reactivation is hard to assess but it must involve the compartmentation of the transcriptional process within the nucleus. As depicted in Fig. 6, the redistribution of RNA polymerase II pool accompanies the 5-fold increase in sequence complexity as well as the increased efficiency of translation of cytoplasmic poly(A)⁺-RNA that we have observed upon reactivation of dormant gastrulae. It would be intriguing to determine if some of the RNA polymerase II molecules newly engaged on the template during reactivation of the gastrulae were initiating transcription at sites not previously transcribed. The question is, however, complicated by the fact that RNA prepared from the hatched-out nauplii larvae has almost the same overall messenger sequence complexity as that of the dormant cysts although the RNA of nauplii is translated with a much higher efficiency. Translational efficiency is therefore not directly linked to the apparent sequence complexity. The 10-fold increase in translational efficiency of RNA also suggests that the newly synthesized RNA species are more efficiently translated and that the stored messengers may also somehow be modified to synthesize proteins more efficiently.

Assuming that during cryptobiosis the organism has to conserve its most abundant messages for its viability, it could be speculated that some of the low molecular weight proteins that these code for serve some gene regulatory function associated with reactivation. The large changes associated with middle and low abundance class mRNA could then reflect an overall reactivation of the transcriptional machinery which was shut down during cryptobiosis. Further extrapolation of this kind would necessitate more elaborate fractionation of the cDNA into various abundance classes and hybridizing them with different poly(A)⁺-RNA preparations, as has been recently carried out using single copy DNA for sea urchin (50,51) and mouse (30). One should also consider the fact that cellular rearrangement without any cell division occurs during pre-emergence development in *Artemia* so that a compartmentalization of the different messenger sequences would have to occur.

The manner in which populations of messenger RNAs undergo alterations in their distribution and function is now central to the study of gene expression during early development (7,26,52). Our present studies were designed to investigate simultaneously alterations in mRNA complexity, the efficiency with which these messengers are translated and the redistribution of RNA polymerase pools, soon after resumption of development is induced in encysted *Artemia* gastrulae. Towards this end, we have established that not only are the stored

messengers in the dormant embryos unavailable for translation but that a major shift in the sequence complexity of mRNA accompanies their availability as templates for translation very rapidly as development resumes. We are now extending these studies to analyzing the different abundance classes of mRNAs separately to characterize some of the messengers in terms of proteins they code for. Whatever the outcome of these investigations, it is clear that the ease with which transcription is experimentally resumed in embryos in which it is totally arrested, in the absence of any cell division, once again confirms the enormous advantage offered by dormant *Artemia* gastrulae in designing experiments to unravel factors controlling gene expression during early development.

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DNA SEQUENCE ORGANIZATION
IN THE GENOME OF THE BRINE SHRIMP, ARTEMIA SALINA

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ABSTRACT

The arrangement of single copy and repetitive sequences has been determined in the chromosomal DNA of the brine shrimp, Artemia salina. The methods of investigation were: (1) analysis of reassociation kinetics of short (425 nucleotide) and long (2,450 nucleotide) DNA fragments; (2) hyperchromicity analysis of these reassociated DNAs; and (3) S1 nuclease digestion studies of these same fragments. It is concluded that a major fraction of the genome consists of single copy sequences less than 2,450 nucleotides in length, interspersed with short repetitive sequences. The length of the repetitive elements was estimated electrophoretically following S1 nuclease digestion of reassociated 2,450 nucleotide fragments, and found to contain a prominent short component whose average length is 275 nucleotides. Short inverted repeat sequences, which comprise 6% of the genome, are interspersed throughout a major part of the chromosomal DNA.

INTRODUCTION

Chromosomal DNA in eucaryotic organisms contains both repeated copy and single copy sequences (1). The organization of these sequences with respect to one another has been hypothesized to play an important role in genetic regulation (2, 3). Davidson et al. (4) first devised a method to demonstrate the general organization (i.e. average spacing and dimensions) of these two DNA classes in the frog, Xenopus laevis. Slightly over 50% of this genome was found to consist of closely interspersed non-repetitive and repetitive sequences, a pattern termed short period interspersion. These repetitive sequences were found to average 300 nucleotides in length, and the single copy sequences to average about 800-1,000 nucleotides. About 8% of the genome in this species was found to consist of long clustered repetitive sequences. The remainder of the DNA was found to consist of very long single copy elements averaging 4,000 to 8,000 nucleotides, also containing rare repetitive elements.

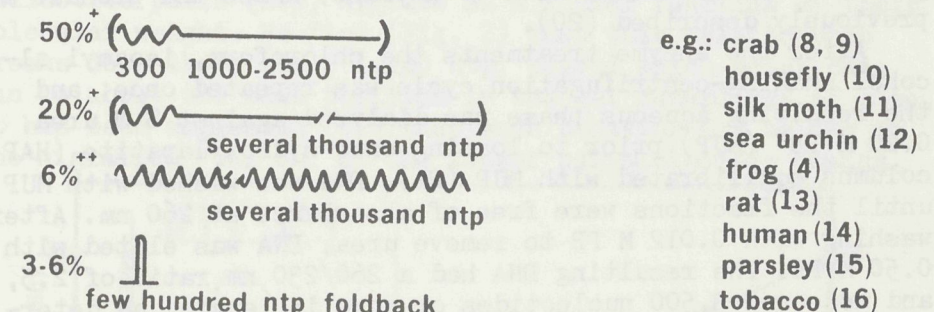
A different pattern of genome organization has been described in other species, including the fly Drosophila (5, 6). Here, repetitive sequences averaging 5,600 nucleotides in length are interspersed with single copy elements which average 13,000 nucleotides. This pattern is termed long period interspersion, and appears to be a feature of eucary-

otic species having a very small genome size.

Since the original report by Davidson's group, numerous other species have been investigated using their procedures, so that a general picture is now emerging, although there are exceptions (7). Although the percentage values of the DNA components differ from species to species, a qualitatively similar pattern has been reported for several species, ranging from lower and higher plants to invertebrates and vertebrates. These major patterns of DNA sequence organization are summarized in Figure 1.

The pattern of DNA sequence organization has now been determined for the brine shrimp Artemia salina: a member of one of the most primitive crustacean groups (Subclass Sarsostraca, Order Anostraca). A fraction of the repetitive sequences has been found to exhibit short period interspersion in this species. Knowledge of the pattern of sequence organization in Artemia, which is a particularly useful system for developmental studies, should now facilitate analysis of RNA/genome relationships during development. This in turn should contribute to our understanding of the possible role of the regular sequence organization which has been observed.

(A) Xenopus-type : short period interspersion



(B) Drosophila-type : long period interspersion

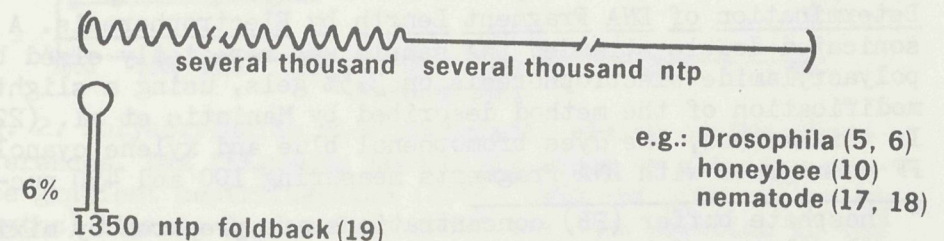


Fig. 1. Major patterns of DNA sequence organization reported for various representative eucaryotes. Except for foldback DNA, repeated sequences are represented by a wavy line.

MATERIALS AND METHODS

Dehydrated brine shrimp embryos, derived from salterns in San Francisco, Calif., were obtained from the Metaframe Corp., Newark, Calif. Nauplius larvae were hatched in salt water at room temperature with aeration. Calf thymus was obtained fresh at a local slaughterhouse.

DNA Isolation and Fragmentation. Small thymus fragments or fresh larvae hatched from 7-15 g of dehydrated cysts were homogenized in a stainless steel Waring vessel in 5-10 volumes of ice cold 0.15 M NaCl, 0.015 M Na-citrate, pH 7.0 (1 X SSC) for 1 min. Alternatively, materials were blended in 0.10 M NaCl, 0.20 M disodium EDTA, pH 8.0. The homogenate was at once made to 1% sodium dodecyl sulfate by adding a concentrate. An equal volume of freshly distilled SSC-saturated phenol was added, which had previously been mixed with a 1/4 volume of chloroform, and the slurry was stirred overnight at room temperature. Phases were separated by centrifugation at 10,000 rev/min for 10 min at room temperature. The aqueous phase was removed with a large bore pipet, an equal volume of chloroform, isoamyl alcohol (24:1, v/v) was added, the mixture was shaken on a wrist action shaker for 30 min, and centrifuged as before. The aqueous phase was recovered and dialyzed against two changes of 1 X SSC in preparation for treatment with α -amylase, RNase and pronase as previously described (20).

After the enzyme treatments the chloroform, isoamyl alcohol shaking-centrifugation cycle was repeated once, and the resulting aqueous phase was dialyzed against 8 M urea, 0.20 M PB* (MUP) prior to loading onto hydroxylapatite (HAP) columns equilibrated with MUP (21). DNA was washed with MUP until the fractions were free of absorbance at 260 nm. After washing with 0.012 M PB to remove urea, DNA was eluted with 0.50 M PB. The resulting DNA had a 260/230 nm ratio of 2.5, and measured 3,500 nucleotides or more in length, as determined by alkaline sucrose gradient centrifugation (below).

DNAs of various average fragment length were prepared by blending at different speeds with a Virtis homogenizer (4); blending speed was monitored with a tachometer. Small DNA fragments were obtained by sonication (20).

Determination of DNA Fragment Length by Electrophoresis. A sonicated double stranded DNA sample was repeatedly sized by polyacrylamide electrophoresis on 3.5% gels, using a slight modification of the method described by Maniatis et al. (22). In this method, the dyes bromophenol blue and xylene cyanol FF co-migrate with DNA fragments measuring 100 and 450 nuc-

* Phosphate buffer (PB) concentrations are prepared by mixing equal volumes of equimolar monobasic and dibasic sodium phosphate solutions. Thus, 0.12 M PB is 0.06 M monobasic sodium phosphate, 0.06 M dibasic sodium phosphate, pH 6.8.

leotide pairs, respectively, and serve as internal standards. Since these dyes were found to absorb somewhat at 260 nm, we either ran them in parallel gels or incubated freshly run gels in dist. water for 3-5 h to remove most of the dye. This treatment did not alter the absorption profile of DNAs, which will easily tolerate a much longer incubation, but did decrease the absorption of the dyes at 260 nm. It was found that prior treatment of DNAs with S1 nuclease, followed by recovery of resistant duplex DNA on HAP columns at 60°C, resulted in aberrant DNA migration. In these experiments, the dyes were always run in separate parallel gels. After electrophoresis, gels were scanned at 260 nm in the linear transport attachment of a Gilford spectrophotometer to locate the DNA peak(s), then at 600 nm to record the position of the dye reference peaks. DNA size was then read from the resulting standard curve.

The sonicated size reference was found to be about 340 nucleotide pairs long, as determined by the 50% of peak value in the distribution (Fig. 2, inset). As expected, the sample was heterogeneous, with a size range of about 100-900 nucleotide pairs. Both labeled and unlabeled DNA size references were calibrated in this way. Maniatis et al. (22) found that in this system, DNAs in the size range 100-1,000 nucleotide pairs (ntp) migrate as a linear function of their molecular weight. We find (Fig. 2) that this relationship breaks down above about 1,100 ntp, beyond which point DNAs run to about the same position in the gel regardless of size. It has been suggested (22) that such behavior may be due to the migration "end on" of the longer polynucleotide chains.

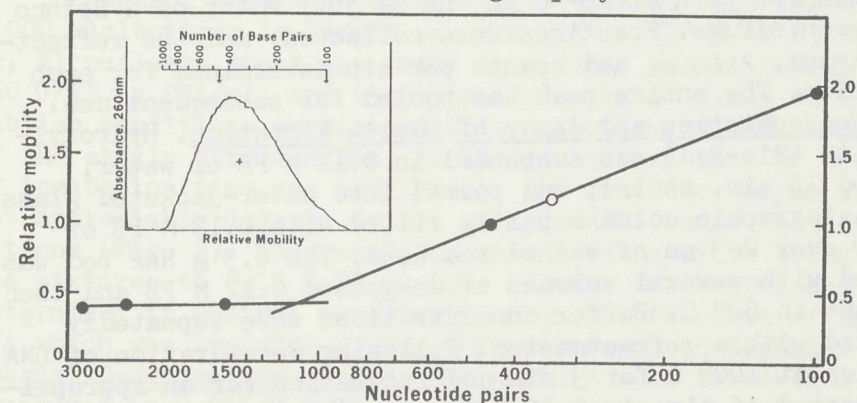


Fig. 2. Polyacrylamide electrophoretic sizing of DNA size references. DNA fragments previously sized by alkaline sucrose gradient centrifugation (1600, 2450 and 3000 nucleotide lengths) migrate "end on" to about the same position in the gels, while dyes which co-migrate with DNAs measuring 100 and 450 nucleotide pairs follow a linear relationship (22). The position of the sonicated DNA sample is shown by the open circle; its profile is shown in the inset.

Determination of Fragment Length on Sucrose Gradients. The sonicated 125-I labeled 340 nucleotide long reference DNA was utilized as an internal standard to determine the size of DNA fragments in 5-20% linear alkaline sucrose gradients prepared in 0.10 N NaOH (4). In each case, the sedimentation coefficient was determined based on the 50% of peak value in the distribution. We emphasize that size determinations are in each case the average of a heterogeneous population of DNA fragments.

Preparation of 125-I Labeled DNA. Impermeability of the encysted *Artemia* embryo reportedly makes it difficult to obtain high specific activity DNA (23). We therefore elected to label high molecular weight DNA *in vitro*, using a modification of the Commerford method (24). This routinely resulted in specific activities exceeding 1×10^6 counts/min/ μ g, but invariably resulted in some intrastrand breakage, so that labeled molecules were always sized after iodination. For example, in one typical experiment, a length reduction of 27% was observed following iodination to a specific activity of 1×10^6 counts/min/ μ g. Although iodinated DNAs were freed from reactant molecules by Sephadex G-50 chromatography, followed by washing on HAP columns in 0.03 M PB at 60° C, we were concerned that traces of labeled impurities may have followed the DNA through these steps. Therefore, as a final purification step, labeled DNAs were banded in neutral CsCl density gradients. DNA was dialyzed into 0.10 M Tris-HCl, pH 8.0, and CsCl was added until the initial refractive index was 1.4000 ($\rho = 1.707$ g/ml). Samples (3.75 ml) were overlaid with paraffin oil and centrifuged at 35,000 rev/min for 72 h at 25° C in the SW 50.1 rotor of a Spinco ultracentrifuge. Fractions were collected, and the refractive index, A₂₆₀ nm and counts per min determined for each fraction. The entire peak was pooled for subsequent use.

DNA Reassociation and Assay of Duplex Formation. Hydroxylapatite (Bio-Rad) was suspended in 0.12 M PB or water, boiled 15 min, cooled, and poured into water-jacketed glass chromatographic columns partly filled with 0.12 M PB or water over 2-3 mm of washed sea sand. The 0.3 g HAP bed was washed with several volumes of de-gassed 0.12 M PB and then brought to 60° C. Buffer concentrations were repeatedly checked with a refractometer. Following denaturation of DNA samples at 100° C for 3 min and incubation for an appropriate period of time in 0.12 M PB, DNA (25-75 μ g) was at once stirred into the HAP bed. After it had run into the bed, DNA was washed with several volumes of 0.12 M PB. Fractions 1 ml each were collected from the point of introduction of the sample to the bed. Duplex DNA was then eluted with 0.50 M PB. The absorbance of each fraction was read at 260 nm, the volume was measured and (where appropriate) each frac-

tion was counted in a gamma counter to determine the 125-I labeled DNA.

In order to facilitate comparison of cot values of incubated DNAs having different length, which do not reassociate at the same rate, we have expressed cots in such cases as "length equivalent cot," relative to the cot which would be developed by DNA having an average fragment length of 340 nucleotides. This is necessary because the rate of reassociation increases as the square root of the fragment length (21).

Determination of Zero-time Binding (Foldback) Component. In the zero-time binding determinations, 125-I labeled DNAs of various lengths having a concentration of $1.2-1.4 \times 10^{-2}$ μ g/ml were denatured at 100° C for 3 min and at once applied to 1 ml (0.3 g) HAP columns operating at 60° C. The columns were not stirred at this point. After waiting 20 sec for temperature equilibration, the samples were forced through the bed with a large syringe and washed with several volumes of 0.12 M PB, collecting 1 ml fractions. The maximum cot which can be calculated under these conditions is 5×10^{-6} . Duplex DNA was then either eluted by bringing the columns to 95° C, or with 0.50 M PB. The two alternative procedures gave identical results.

S1 Nuclease Digestion. S1 nuclease was purchased from Miles Lab. This was diluted with S1 buffer (0.025 M Na-acetate, 0.10 M NaCl, 1×10^{-4} M ZnSO₄, pH 4.5) to a stock concentration of 100 U/ μ l and stored at -20° C after addition of an equal volume of glycerol. The preparation was characterized before use.

In control experiments, heat-denatured *E. coli* DNA about 2,000 nucleotides in length was brought to 37° C in S1 buffer, S1 nuclease stock was added to a final concentration of 150 U/25 μ g DNA/ml, and incubated for various times to establish conditions consistent with digestion of 95% or more of the single stranded DNA. It was determined that 30 min of incubation time was sufficient, and that the enzyme did not appreciably degrade double stranded DNA under these conditions (Fig. 3). The reactions were terminated by bringing the mixtures to 0.12 M PB on ice. Percent DNA hydrolyzed was determined in control experiments from the fraction of the DNA which was included during chromatography on Sephadex G-50. Thermal Denaturation of DNA. DNA samples were automatically denatured in 0.015 M NaCl, 0.0015 M Na-citrate, pH 7.0 (0.1 X SSC), using a Gilford spectrophotometer equipped with a thermo-programmer and chart recorder. To calibrate the instrument, bovine DNA was always included in one of the four sample ports of the thermal cuvette. The %(G+C) for bovine DNA was taken to be 42% (25), corresponding to a T_m in 0.1 X SSC of 71.1° C, using the equation derived by Mandel and

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Marmur (26). Percent base pair mismatching was calculated from the T_m values, using the relationship described by Laird et al. (27).

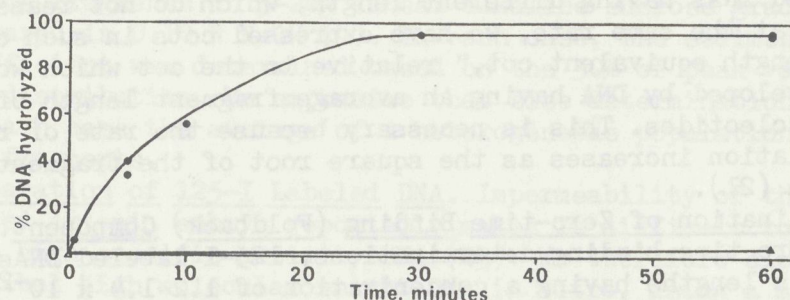


Fig. 3. Percent *E. coli* single stranded (circles) and double stranded (triangles) DNA hydrolyzed by S1 nuclease under standard conditions, as evidenced by loss of exclusion on Sephadex G-50.

RESULTS AND DISCUSSION

The arrangement of single copy and repetitive sequence elements in chromosomal DNA is at the present time determined by using a variety of techniques differing in principle from one another. Each of these techniques yields some limited further bit of information: the aggregate has resulted in a reasonably detailed understanding of sequence organization in *Artemia*.

DNA Reassociation Kinetic Analysis. In an earlier kinetic study utilizing short DNA fragments (28), we reported that the slowest reassociating *Artemia* DNA component ($\text{cot}_{1/2} = 1,900$ in whole DNA, uncorrected) renatures about twice as quickly as the slowest reassociating single copy bovine component ($\text{cot}_{1/2} = 3,900$ in whole DNA, uncorrected). Since the haploid bovine genome contains 3.2 pg DNA, it was concluded that the haploid *Artemia* genome contains 1.5 pg. That study emphasized the single copy DNA component.

A complete kinetic curve for *Artemia*, showing data derived from a number of experiments utilizing fragments with sizes ranging from 340-425 nucleotides, is given in Figure 4. A broad plateau separates single copy from repeated copy sequences in the vicinity of $\text{cot } 50$, greatly facilitating experiments in which it is desirable to renature only the repeated DNA. Incubation of short fragments to $\text{cot } 50$ results in the HAP binding of 55% of the fragments. This value represents an over-estimate of the percent repetitive DNA in this species, owing to the presence of single stranded, single copy "tails" on these fragments, as will be shown. Casual visual inspection of the data given in Figure 4 suggests that intermediately repetitive sequences appear in

roughly the cot range 10^0 to 10^{-2} and highly repetitive, rapidly reassociating sequences in roughly the cot range 10^{-2} to 10^{-4} .

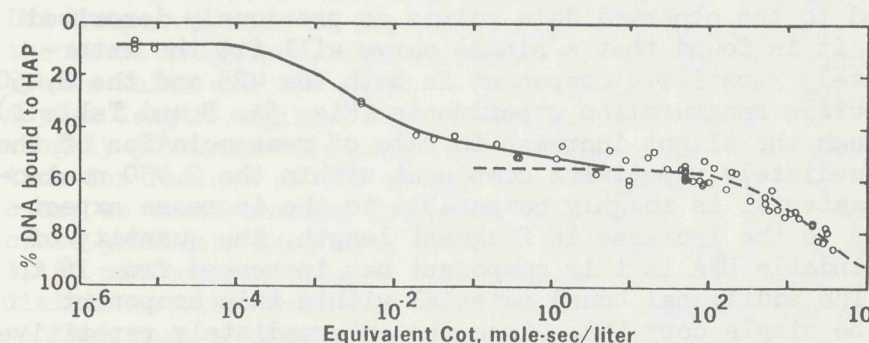


Fig. 4. Short fragment renaturation kinetic curve for the genome of *Artemia salina*, assayed by hydroxylapatite chromatography. The dashed line represents single component calculated theoretical second-order reaction kinetics which best fit the observed data points for the slowest reassociating component. The $\text{cot}_{1/2}$ for this component, which comprises 45% of the genome, is 1900.

Since the data shown in Figure 4 were derived from several experiments done at different times, utilizing somewhat different DNA fragment lengths, there is considerable scatter in the data points. In order to more critically characterize the repetition frequency and quantity of middle repetitive sequences, and also to determine the effect of fragment length on the kinetics and HAP binding properties of this DNA component, this work was repeated using fragments measuring 425 and 2,450 nucleotides in length. In an organism having primarily long period interspersion (Fig. 1) the longer fragments would be largely "pure" single copy or "pure" repetitive and the rate of reassociation of these long fragments would be expected to merely increase as the square root of the fragment length (21). The predicted effect on long fragment kinetics in a species having long period interspersion would then be a 2.40-fold shift to the left for each component in the kinetic curve shown in Figure 5A ($\sqrt{2,450} / \sqrt{425} = 2.40$). Since the $\text{cot}_{1/2}$ of the single copy component shown in Figure 5A is 2100, this would decrease to 875. This is known to be the case for the *Drosophila* genome (6), which has been shown to have the long period pattern (5).

Reassociation of the single copy component in 2,450 nucleotide *Artemia* DNA fragments is completed long before $\text{cot } 875$ is attained (Fig. 5B). The absence of any detectable

reassociation of single copy sequences in the expected range must be due to their virtually complete covalent linkage to repetitive elements which have previously reassociated. When calculated theoretical second-order kinetic curves (29) are fitted to the observed data points as previously described (20), it is found that a single curve will fit the intermediately repetitive component in both the 425 and the 2,450 nucleotide renaturation experiments (Fig. 5A, B and Table 1). Although the slight increase in rate of reassociation of the intermediately repetitive component within the 2,450 nucleotide material is roughly comparable to the increase expected due to the increase in fragment length, the quantity of HAP-bindable DNA in this component has increased from 16 to 32%. The additional bound material within this component must be single copy DNA, since the intermediately repetitive component does not comprise more than about 16% of the genome (Fig. 5A). This data constitutes our first evidence that a major fraction of the single copy sequences is intermingled with repetitive elements at an average distance of less than 2,450 nucleotides: a characteristic of a short period genome.

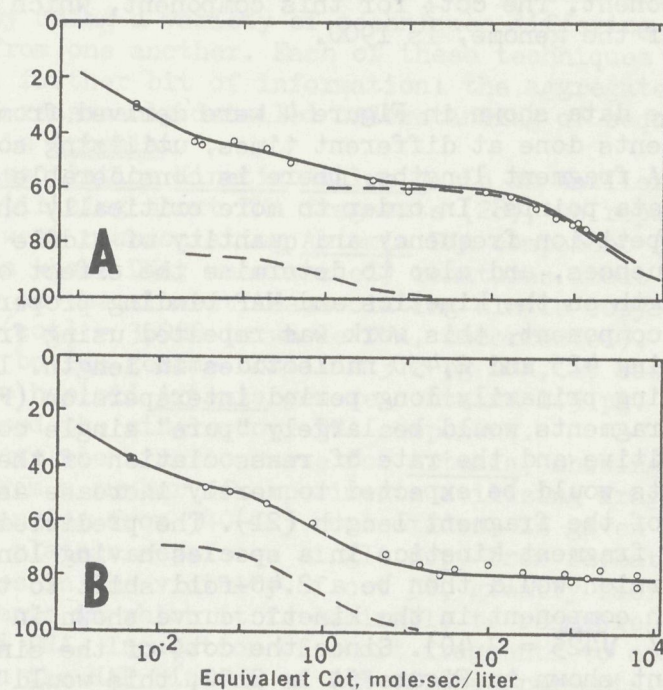


Fig. 5. Reassociation of *Artemia* DNA 425 nucleotides long (upper curve) and 2,450 nucleotides long (lower curve). The dashed lines represent the calculated second-order kinetic curves described in Table 1. The ordinate shows the percent reassociation, assayed by binding to hydroxylapatite.

Figure 5B shows that 20% of the 2,450 nucleotide long fragments have failed to reassociate. Using short fragments of the order of a few hundred (5) or a few thousand nucleotides long (10), it has been reported that up to 15% of the DNA fragments fail to reassociate in such experiments. For the short fragments, this material may simply represent duplexes which are too short to bind to HAP, as the minimal duplex length required for adsorption onto HAP at 60° C is roughly 50 nucleotide pairs (30). This is not the explanation for failure of 20% of our long fragments to bind, however, as these unbound fragments were sized on alkaline sucrose gradients following elution from HAP and found to be 1,150 nucleotides long on the average. Since fragment length diminishes with prolonged incubation at elevated temperature, we feel that this material could represent single copy DNA which was linked to reassociated repetitive elements early in the incubation and thus was prevented from hybridization by inclusion in hyperpolymers (31). During prolonged incubation at elevated temperature, the demonstrated decrease in average DNA fragment length resulted in the release of some of these sequences, which now fail to bind to HAP. If this is so, we have no information as to whether these liberated single copy sequences were originally linked to the intermediate or to the fast reassociating repetitive DNA, although it is perhaps significant that their length is about equal to that of the interspersed single copy elements described in other crustacean species (8).

Table 1
Kinetic components in *Artemia salina* DNA

Fragment length, nucleotides	Trivial terminology	Percent of DNA	Cot _{1/2}	Average repetition frequency
425	single copy	40	2.1 x 10 ³	1
	intermediate	16	1.1	2,000
	fast(a)	44	< 1.0 x 10 ⁻²	> 20,000
2,450	intermediate	32	7.4 x 10 ⁻¹	--
	fast(a)	47	--	--

(a) This includes zero-time binding component, and has not yet been completely characterized.

Hyperchromicity of Reassociated DNA Fragments. Thermal denaturation of high molecular weight Artemia DNA (Fig. 6) does not reveal any d(A+T)-rich satellite component, which is a striking feature of many other crustacean genomes (32-34). When Artemia DNA was denatured in 0.1 X SSC, it showed 29% hyperchromicity and its T_m was 68.0-68.5° C. This T_m value is somewhat higher than we have previously obtained using DNA isolated by a different method (28), but is consistent. From the T_m , we calculate that in the brine shrimp Artemia, the %(G+C) is 34.4-35.6% (26).

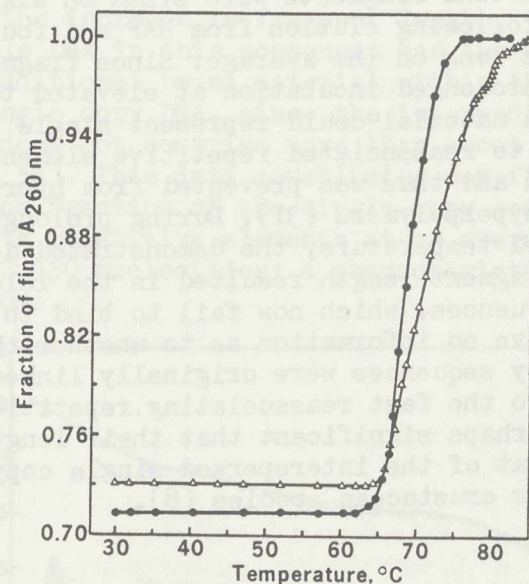


Fig. 6. Thermal denaturation profiles of high molecular weight DNA from Artemia (circles) and calf thymus (triangles) in 0.1 X SSC, denatured in different ports of the same thermal cuvette.

The hyperchromicity developed by reassociated DNA fragments can also be used to provide information on DNA sequence organization (4). In this approach, short (a few hundred nucleotide) and long (a few thousand nucleotide) DNA fragments are denatured and incubated to length equivalent cot 50, at which point all repeated sequences will have reassociated. Single copy sequences will not have reassociated, as shown in Figure 4. Fragments containing double stranded (i.e. repetitive) regions are then collected by HAP chromatography. The hyperchromicity of these fragments is then determined and compared to that developed by native DNA. Provided that a correction is made for the small contribution of single stranded "tails" to the hyperchromicity, the

fraction of repetitive duplex in such hybrids can then be determined by comparing the hyperchromicity of the hybrids to that of native DNA, or to that of S1-treated hybrids.

In a species having the long period interspersion pattern (Fig. 1), only a relatively small percent increase in linked single stranded single copy "tails" accompanies the increase in fragment length. This is because fragments of the order of a few thousand nucleotides long are more likely to be relatively "pure" repetitive or "pure" single copy. This results in a relatively small decrease in hyperchromicity upon denaturation of the reassociated long fragments. In Drosophila, for example, reassociated fragments 350 nucleotides long are reportedly 95% double stranded; those 3,200 nucleotides long are 79% double stranded (6). In a species containing the short period interspersion pattern, the reassociation to low cot of DNA fragments of the order of a few thousand nucleotides in length will involve hybrid formation between large numbers of fragments containing both repetitive and single copy sequences. Here, a relatively great percent increase in single stranded single copy "tails" linked to duplex repetitive regions accompanies the increase in fragment length. This results in a relatively large decrease in hyperchromicity upon denaturation of the reassociated long fragments. The two major interspersion patterns can therefore be distinguished from studying the hyperchromicity developed by the hybrids. Here and in the subsequent sections, we have used bovine DNA as a convenient control, as this species is known to contain the short period interspersion pattern (35 and D. Graham, personal communication).

The results of our hyperchromicity studies on reassociated Artemia DNA fragments are shown in Figure 7A; those for bovine DNA are shown in Figure 7B. These results are tabulated for further analysis in Table 2. Using 340 nucleotide Artemia fragments, 56.2% bind to HAP following denaturation and reassociation to cot 50: at which point only repetitive sequences could have renatured (Fig. 4). Our hyperchromicity and S1 nuclease digestion studies show that 72-75% of this bound material is actually duplex (i.e. purely repetitive); the remainder is attributable to linked single stranded single copy "tails." When 2,450 nucleotide Artemia fragments are reassociated to length equivalent cot 50, 71.1% bind to HAP and therefore contain repetitive regions. Our hyperchromicity and S1 nuclease digestion studies show that only 42-45% of this bound material is actually duplex. There is therefore a marked increase in the percentage of single copy material which is linked to repetitive DNA after reassociation of long fragments to low cot. These results are quite similar to those obtained for the bovine DNA fragments, and are the expected results for genomes in which a

major fraction of the DNA consists of short repeated sequences interspersed with single copy elements. This constitutes the second major evidence for short period interspersal in Artemia.

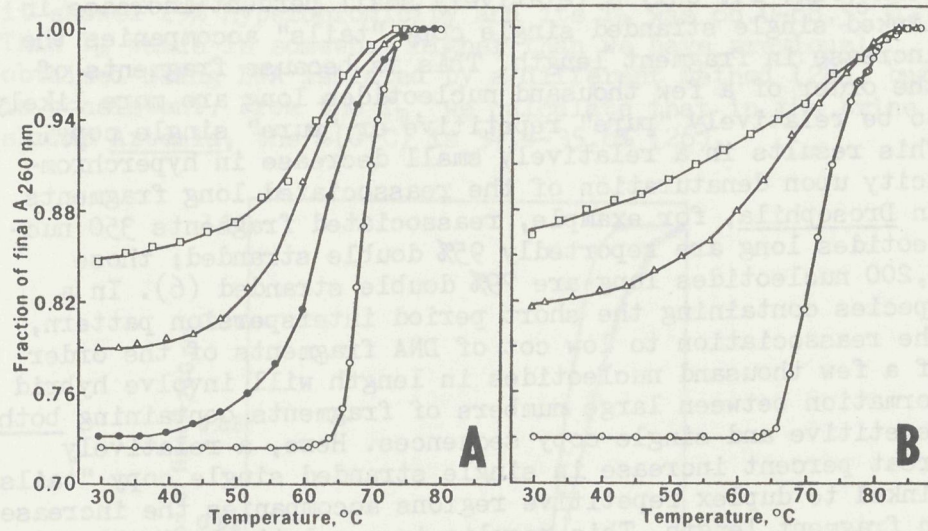


Fig. 7. Hyperchromicity of native and reassociated DNA from Artemia (left) and calf thymus (right). In Fig. 7A, native high molecular weight DNA is shown by open circles; reassociated 340 nucleotide fragments by open triangles; reassociated 2,450 nucleotide fragments by open squares; reassociated 2,450 nucleotide fragments treated with S1 nuclease by closed circles. In Fig. 7B, native high molecular weight DNA is shown by circles; reassociated 340 nucleotide fragments by triangles; reassociated 2,000 nucleotide fragments by squares.

Determination of Repetitive Sequence Length. Artemia DNA fragments 2,450 nucleotides long were denatured and reassociated to length equivalent cot 50, which is sufficient to just renature the repetitive sequences (Fig. 4). Fragments containing repetitive duplex were collected by HAP chromatography, dialyzed into S1 buffer, and treated with S1 nuclease. The standard S1 procedure described in Materials and Methods was used, although in some trials the concentration of S1 nuclease was increased several fold. Bovine DNA was processed in parallel, as a control, to assure that the S1 enzyme was working properly. Following enzyme hydrolysis for 1 hour, the S1 resistant duplex was collected on HAP, eluted, and sized by electrophoresis. Purified Artemia and bovine repetitive duplexes prepared in this way are qualitatively very similar (Fig. 8). Both preparations contain a long repetitive component which is greater than 1,150 nucleotide pairs

Table 2
Characteristics of length equivalent cot 50 reassociated DNAs

Characteristics of reagent equivalent col 30 reassociated DNAs							
Reassociated DNA sample	% bound to HAP	T _m in SSC/10	% absorbance increase upon melting	% in duplex (from hyperchromicity)	% in duplex (after S1 nuclease)	% repeated DNA in genome	
						From hyper-(a) chromicity	From S1 nuclease
<u>Artemia</u>							
340 ntp (b)	56.2	59.7	26.2	76.6 (72.0)	75.2	40.5	
340 ntp + S1	--	59.5	34.2	--	--		42.3
2450 ntp	71.1	60.2	18.0	47.0 (41.5)	45.1	29.5	
2450 ntp + S1	--	62.6	38.3	100	--		32.1
native DNA	--	68.5	38.3	--	--		
<u>Bovine</u>							
340 ntp (b)	53.2	64.5	22.3	63.4 (59.5)	57.0	31.7	
340 ntp + S1	--	64.1	35.2	--	--		30.3
2000 ntp	82.7	62.7	15.4	41.7 (36.7)	31.5	30.4	
2000 ntp + S1	--	--	--	--	--		26.1
native DNA	--	71.6	36.9	--	--		

(a) Corrected for effect of collapse hyperchromicity (parentheses).

(b) We find that the T_m of native 340 nucleotide pair long DNA is 2.0° C below that of native high molecular weight DNA, which is 68.0-68.5° C in 0.1 X SSC. This is close to the ΔT_m predicted from the "650/L" equation (Britten et al., 1974), which is 1.9° C. The ΔT_m for short reassociated Artemia DNA is therefore 66.5 - 59.7 = 6.8° C. Using the relationship derived by Laird et al. (1969), which is that 1.5% base pair mismatching is reflected by a lowering in T_m by 1.0° C, the short Artemia hybrids contain 10.2% mismatched bases. A similar situation is seen for the reassociated short bovine fragments.

in length. In addition, both preparations clearly contain a short repetitive component. The size of this short repetitive component in *Artemia* averages 275 nucleotides; in the bovine genome it is also 275 nucleotides. Our *Artemia* value compares closely with the 285 nucleotide average short repetitive length obtained by Holland and Skinner (8) for the crustacean *Gecarcinus*, who determined their repetitive duplex fragment length by agarose A-50 chromatography. Our bovine value agrees favorably with the ca. 300 nucleotide average short repetitive length obtained for other mammalian systems, as in rat (13) and human (14). We estimate that about 70% of the *Artemia* repetitive sequences isolated and characterized in this manner fall under the short fragment region shown by the dashed line in Figure 8A. This is similar to the result obtained for the bovine genome, in which 55% of the genome falls under the short fragment region shown by the dashed line in Figure 8B. These results confirm the prediction obtained from kinetic analysis and hyperchromicity of reassociated DNA fragments that short repetitive sequences are present for interspersions with non-repetitive single copy elements.

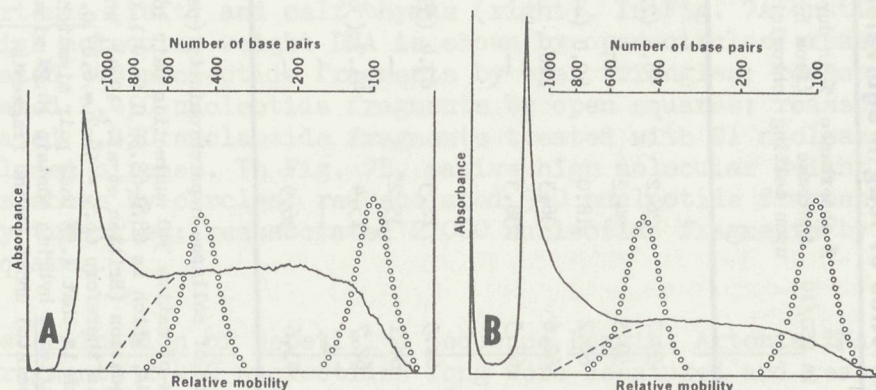


Fig. 8. Polyacrylamide electrophoretic sizing of repetitive DNA sequences isolated from *Artemia* (left) and calf thymus (right). DNAs were reassociated to length equivalent cot 50 and fragments containing repetitive duplex were collected by HAP chromatography. These fragments were then digested with S1 nuclease in S1 buffer and the resistant purified repetitive DNA duplexes collected on HAP prior to electrophoresis. The positions of dye markers which co-migrate with DNAs containing 100 and 450 nucleotide pairs are shown by the open circles. The dashed lines represent our estimate as to the extent of the short repetitive component in each case.

Zero-time Binding Component. The fraction of 125-I labeled DNA binding to HAP at cot 5×10^{-6} increases as the fragment length increases (Fig. 9). This zero-time binding component is due to the presence of foldback sequences in the *Artemia* genome. We believe that the percent bound DNA increases because single stranded DNA is linked to short reassociating foldback sequences. Extrapolation of the curve to the ordinate accomplishes something which we cannot do experimentally: it gives the percent foldback in the genome which would be obtained if fragments approaching one nucleotide in length were reassociated to this very low cot. This approach shows that the fraction of the *Artemia* genome containing foldback sequences is about 6%. The increase in percent DNA bound to HAP with moderate increase in fragment length demonstrates the interspersions of short foldback sequences amongst non-reassociated sequences whose average length is $>1,600$ nucleotides throughout at least 20% of the genome.

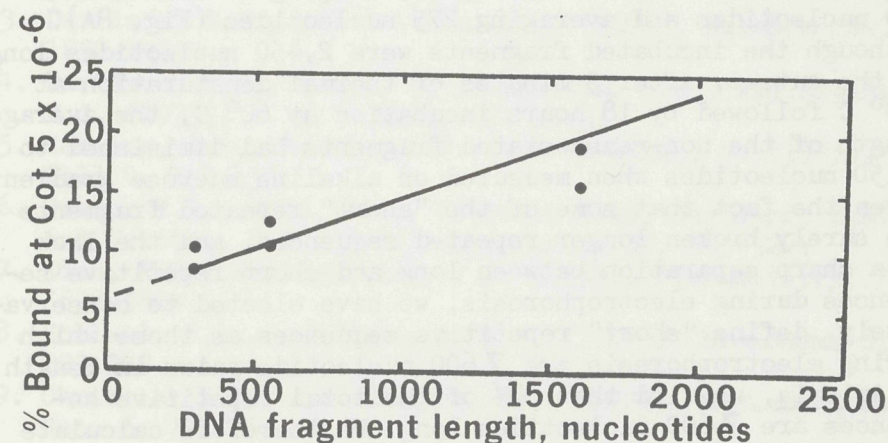


Fig. 9. Zero-time binding of 125-I labeled *Artemia* DNA fragments of known length to hydroxylapatite, carried out as described in Materials and Methods.

Estimation of DNA Sequence Organization. Our best estimate as to the total percent repetitive sequence DNA in *Artemia* is obtained from hyperchromicity measurements obtained from short fragments reassociated to cot 50. This value is 41%, in close agreement with the 42% value obtained upon S1 nuclease digestion of these same fragments (Table 2). It is clear that reassociation of 2,450 nucleotide long fragments to length equivalent cot 50 does not result in reassociation of all available repetitive elements in *Artemia*. This effect was first observed by Britten and Waring (36), who suggested that in reassociation of short fragments, sufficient freedom

will exist for complementary sequences to locate each other, whereas in the longer fragments fewer complementary sequences will locate partners because of the distribution of pairing regions in various fragments of DNA. Flavell and Smith (31) have also cautioned against the use of long fragment reassociation in the estimation of total repetitive content, pointing out that hyperpolymer formation can lead to underestimation of repetitive duplex. We are somewhat surprised that the use of long bovine DNA fragments yields the same value for repetitive sequence content as is obtained from short fragments (Table 2). Perhaps it is significant that in bovine DNA there is a smaller percent of short repeated elements, and that our long bovine fragments were 20% shorter than those used in the Artemia study.

Electrophoresis of length equivalent cot 50 reassociated 2,450 nucleotide long Artemia DNA following extensive S1 nuclease digestion shows that about 70% of the total repetitive sequences fall under a broad peak, ranging in size from 100-800 nucleotides and averaging 275 nucleotides (Fig. 8A). Although the incubated fragments were 2,450 nucleotides long at the outset, after 3 minutes of thermal denaturation at 100° C followed by 18 hours incubation at 60° C, the average length of the non-reassociated fragments had diminished to 1,150 nucleotides when measured on alkaline sucrose gradients. Given the fact that some of the "short" repeated fragments are surely broken longer repeated sequences, and the lack of a sharp separation between long and short repetitive sequences during electrophoresis, we have elected to conservatively define "short" repetitive sequences as those which during electrophoresis are ≈ 600 nucleotide pairs in length. In Artemia, we find that 64% of the total repetitive sequences are ≈ 600 nucleotides long. We therefore calculate that about $0.64 \times 41\% = 26\%$ of the Artemia genome consists of short repetitive sequences and that about $0.36 \times 41\% = 15\%$ consists of long, clustered repetitive sequences. The length of these long repetitious segments is not known, although they are greater than 1,150 nucleotides in length. Short foldback sequences, which comprise about 6% of the genome, are interspersed in Artemia (Fig. 9) and in many other eucaryotic species, including the crustacean Gecarcinus (8), where they comprise 5% of the genome. We therefore estimate that in Artemia, short repetitive sequences other than foldback comprise about $26 - 6 = 20\%$ of the total genome.

We have found that in Artemia interspersed single copy elements average less than 2,450 nucleotides in length. Although we do not yet have a reliable direct estimate as to the length of these sequences in Artemia, it is known that they average 800-900 nucleotides in two other crustacea (8, 9). Assuming this to also be the case in Artemia, since

there should be an equal number of short interspersed and single copy elements and the latter are about three times the average length of the former, which comprise about 20% of the genome, we would predict there are about $3 \times 20\% = 60\%$ interspersed single copy elements in Artemia.

In summary, we estimate from our studies that the brine shrimp genome consists of about 41% total repetitive DNA (6% foldback, 15% clustered repetitive, 20% short repetitive) and 59% single copy elements which are interspersed amongst the short repetitive elements. We are not able to detect the presence of appreciable quantities of very long single copy sequences in Artemia by our techniques.

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TRANSCRIPTION IN VITRO OF CHROMATIN BY HOMOLOGOUS DNA-DEPENDENT RNA POLYMERASES AND IN ISOLATED NUCLEI OF ARTEMIA SALINA LARVAE.

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ABSTRACT

Transcription in vitro of chromatin by homologous RNA polymerases I and II was studied using freshly hatched nauplii of Artemia salina. The enogeneous activity of isolated chromatin was predominantly exerted by RNA polymerase II (90%) and did not apparently involve the reinitiation of RNA synthesis as suggested by the lack of inhibition with high concentrations of aurintricarboxylic acid, as contrast to the transcription of chromatin by exogeneous RNA polymerase.

Two classes of transcription by RNA polymerase II were revealed by its sensitivity to actinomycin D; a minor highly sensitive transcription and a major less sensitive one having their 50% inhibition at 0.04 and 1.1 µg/ml, respectively; the latter inhibitor concentration being almost identical to that found for RNA polymerase I in the same in vitro system. Quite similar results were obtained by the titration of RNA synthesis in isolated nuclei with the inhibitor. In this system, several fold higher concentrations of actinomycin D were required to inhibit RNA polymerase II dependent transcription, whereas about three fold lower concentrations of the inhibitor were sufficient for the inhibition of RNA polymerase I dependent one.

RNA polymerase III was highly resistant to α-amanitin (50% inhibition at 650 µg/ml), but this enzyme was extremely sensitive to the in vitro inactivation by proteases specifically induced in hatching nauplii. Therefore, isolated nuclei of Artemia larvae contained a negligible amount, if at all, of active RNA polymerase III.

Diguanosin nucleotides, Gp₄G and Gp₃G, accumulated in the embryo at early developmental stages, were found to lack any effect on the in vitro transcription.

INTRODUCTION

Eukaryotic DNA-dependent RNA polymerase has been found essentially in three types of enzymatic activity (type I, II and III), each transcribing specific genes (1,2). These enzymes have been assumed to play a major role in the regulation of gene expression, hence also in the control of development and cell differentiation. The detailed mechanism, however, by which these regulations are exercised at the transcriptional level, is yet only poorly understood.

We have been studying the mode of induction or de-induction of three extracellular haemoglobins in the brine shrimp, *Artemia salina*, whose synthesis is found to be principally controlled by the ontogenesis and secondarily by environmental factors (3-5). Unlike vertebrate haemoglobins, *Artemia* haemoglobins are all in a dimeric form consisting of two large globin subunits of Mr 125,000 (6). Two of the three haemoglobins are induced only in freshly hatched nauplii for the first time in the development and a highly active biosynthesis of haemoglobins immediately takes place, accounting for 10-20% of the total *in vivo* radiolabelled proteins in these larvae (in the first instar stage) (7). Based on our acquired knowledge on the structure of messenger RNA and globin chains of *Artemia* haemoglobins, we intend to investigate the regulatory mechanism at the transcription level by which globin genes are specifically repressed or derepressed under well controlled conditions, e.g. developmental stages and oxygen concentrations.

This paper describes some characterization of the *in vitro* transcription by RNA polymerases I and II using a chromatin template and compares with the RNA synthesis in isolated nuclei of freshly hatched nauplii of *Artemia salina*.

EXPERIMENTAL PROCEDURES

Materials Encysted cryptobiotic gastrulae of *Artemia salina* were obtained from Metaframe, San Francisco Bay Brand (Newark, Ca., U.S.A.). α -Amanitin and actinomycin D were from Boehringer Mannheim (Mannheim, F.R.G.). Calf thymus DNA and nucleotides were purchased from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). Gp₃G and m Gp₃G were from P.L. Biochemicals, Inc. (Milwaukee, Wi., U.S.A.). Snake venom phosphodiesterase and *E. coli* alkaline phosphatase were obtained from Worthington Biochemical Co. (Freehold, N.J., U.S.A.). [³H]UTP (45 Ci/mmol) was purchased from Radiochemical Centre (Amersham, U.K.).

Buffers Buffer 1 (pH 7.5) contains 30 mM Tris-HCl/120 mM KCl/3mM Mg (OAc)₂/7 mM 2-mercaptoethanol. Buffer 2 (pH 7.6) contains 30 mM Tris-HCl/75 mM KCl/3 mM Mg (OAc)₂/2 mM 2-mercaptoethanol. Buffer 3 (pH 7.5) contains 50 mM Tris-HCl/ 5 mM 2-mercaptoethanol/0.2 mM EDTA.

Preparation of Artemia Larval Nuclei. Freshly hatched nauplii were harvested after culturing encysted cryptobiotic gastrulae as previously described (8). After washing they were suspended in buffer 1 supplemented with 250 mM sucrose and homogenized with one gentle stroke at 0-4°C in a Potter Elvehjem homogenizer at 100-150 rev./min. The homogenate was centrifuged at 4°C in a Beckman JS-13 rotor at 1,500 rev./min for 1.5 min to eliminate large cell debris and then at 2,000 rev./min for 2.5 min to pellet most of nuclei. The nuclear pellet was washed twice by centrifugation as in the last one with buffer 2 supplemented with 250 mM sucrose and finally suspended gently in the same buffer. After an appropriate dilution with this buffer, isolated nuclei were immediately used for an *in vitro* RNA polymerase assay. Although the nuclear preparation so prepared was contaminated with yolk granules, the latter particles were not eliminated further, because either isolated yolk granules themselves or exogenously added ones to a nuclear preparation in excess were totally inactive as template or effector in an *in vitro* RNA polymerase assay system (L.S. & M.K., unpublished observations). The number of nuclei and the content of DNA in isolated nuclei were estimated according to de Chaffoy and Kondo (9).

Preparation of Artemia Larval RNA Polymerases. Frozen nauplii (3-5g) were homogenized in a Virtis homogenizer at 30,000 rev./min for 15 min in the presence of buffer 3 supplemented with 600 mM ammonium sulfate and the homogenate was centrifuged at 4°C in a Beckman JA 20 rotor at 10,000 rev./min for 10 min, followed by second centrifugation in the same rotor at 15,000 rev./min for 30 min. The resulting supernatant was diluted with one volume of buffer 3 supplemented with 20% glycerol and centrifuged at 4°C in a Beckman R 50 rotor at 150,000 x g for 100 min. The supernatant was diluted again with the same buffer to the ammonium sulfate concentration of 75 mM and loaded on a DEAE-Sephadex A 25 column (50 ml) which had been equilibrated with buffer 3 supplemented with 20% glycerol and 75 mM ammonium sulfate. The column was developed by a linear gradient of ammonium sulfate (75-600 mM) in buffer 3 containing 20% glycerol (200 ml) with a flow rate of 10-15 ml/h. Fractions of 4 ml were collected and aliquots of 25 μ l were tested for the RNA polymerase activity in 100 μ l assay mixtures as described below. Pooled active fractions were made 40% glycerol and stored in aliquots

(300 μ l) at -70°C .

In vitro RNA Polymerase Assay. Isolated nuclei adjusted to final concentrations of $1-4 \times 10^7/\text{ml}$, were incubated at 30°C for 30 min, unless otherwise indicated, in a 50 μ l reaction mixture containing 30 mM Tris-HCl (pH 7.6), 3 mM Mg (OAc)₂, 2 mM MnCl₂, 90 mM KCl, 40 mM (NH₄)₂SO₄, 7 mM 2-mercaptoethanol, 12.5% glycerol, 1 mM ATP, 0.25 mM CTP and GTP, 5 μ M UTP and 1 μ Ci [³H] UTP as final concentrations. To terminate the reaction, 1 ml of 5% trichloroacetic acid/ 5% sodium pyrophosphate was added at 0°C and acid-insoluble material was processed to determine the [³H] radioactivity incorporated into RNA as previously described (8).

Isolated RNA polymerases were incubated at 30°C for 20 min in a 50 μ l reaction mixture containing 50 mM Tris-HCl (pH 7.5), 1 mM MnCl₂, 5 mM 2-mercaptoethanol, 60 (RNA polymerase I) or 105 mM (RNA polymerase II) (NH₄)₂SO₄, 0.25 mM ATP, CTP and GTP 0.01 mM UTP, 1.5 μ Ci [³H] UTP and template (as indicated in the figure legends) as final concentrations. The radioactivity was determined as above.

Preparation of Artemia Larval Chromatin. Freshly harvested nauplii in buffer 1 supplemented with 400 mM sucrose were homogenized with two and a half strokes at $0-4^{\circ}\text{C}$ in a Potter Elvehjem homogenizer at 1,000 rev./min and the homogenate was centrifuged at 4°C in a Beckman JS-13 rotor at 1,000 rev./min for 1 min, followed by a second centrifugation in the same rotor at 5,000 rev./min. The nuclear pellet was suspended in five volumes (of the original homogenate) of 75 mM NaCl/24 mM EDTA and centrifuged in the same rotor at 5,000 rev./min for 10 min. The pellet was washed successively with 10, 5 and 1 mM Tris-HCl (pH 8) by centrifugation in the same rotor at 10,000 rev./min for 10 min and the final pellet was resuspended in 5 mM Tris-HCl (pH 8). The chromatin suspension was layered over a 25 ml bed of 5 mM Tris-HCl (pH 8)/ 1.7 M sucrose and centrifuged at 4°C in a Beckman SW 27 rotor at 23,000 rev./min for 90 min. The chromatin pellet was homogenized with a Virtis homogenizer at 3,000 rev./min for 90 sec in the presence of 5 mM Tris-HCl (pH 8), followed by centrifugation in a Beckman JS 13 rotor at 10,000 rev./min for 30 min. The supernatant was stored under liquid nitrogen and used as a chromatin template.

Isolation of Gp₄G. Yolk granules were prepared from the cryptobiotic gastrulae and Gp₄G was isolated from the perchloric acid soluble fraction as described earlier (10). Identification of Gp₄G was carried out by paper electrophoresis and high pressure liquid chromatography (Spectro-Physics, Model 3500) using a column Partisil PXS 10/25 SAX

(Whatman) (11), after the digestion of samples with *E. coli* alkaline phosphatase or snake venom phosphodiesterase (12).

RESULTS

Absence of RNA Polymerase III Activity in isolated Larval Nuclei. It has been our experience that it is rather difficult to isolate *Artemia* RNA polymerase III from hatched nauplii, because of the presence of induced proteases in these animals which inactivate readily this enzyme in cell lysate. Furthermore, since reinitiation of RNA synthesis in isolated nuclei of erythroleukemic Friend cells (13) and in lysates of Ehrlich ascites cells (14) was predominantly, if not exclusively, carried out by RNA polymerase III, a large proportion of this enzyme in nuclei might possibly have a greater probability for a given time to be in the unbound (hence unprotected) state during *in vitro* experiments. If this were the case the contaminating proteases in the nuclear preparation would certainly destroy the activity of RNA polymerase III in isolated nuclei. The result shown in Figure 1a demonstrates that α -amanitin at 0.11 μ g/ml can cause 50% inhibition of RNA polymerase II activity but no further inhibition could be observed in isolated *Artemia* nuclei in the concentration range up to 6.3 mg/ml. Since the α -amanitin concentration required to achieve 50% inhibition of RNA polymerase II activity in nuclei was 10-15 fold higher than that (0.007 μ g/ml) determined with free enzyme (Fig. 1b), the concentration of 6.3 mg/ml of this toxin should have been sufficient to obtain nearly 50% inhibition of RNA polymerase III activity in nuclei, on the basis of the observed 50% inhibiting concentration (0.65 mg/ml) with free enzyme (Fig. 1b).

The negative result of Fig. 1a indicates the virtual absence of active RNA polymerase III in isolated larval nuclei of *Artemia*. The positive identification of RNA polymerase III activity in isolated nuclei in the similar manner using α -amanitin has been reported for HeLa cells (15) and *Drosophila* (16). Furthermore, solubilization of RNA polymerase from isolated *Artemia* nuclei, instead of whole cells employed for the standard procedure, was not successful to obtain RNA polymerase III upon DEAE-Sephadex chromatography (L.S. & M.K. unpublished observations).

Possible Existence of Two Classes of Transcription by RNA Polymerase II. Fig. 2 illustrates the actinomycin D sensitivity of α -amanitin resistant and sensitive RNA polymerase activities in isolated nuclei. The α -amanitin resistant activity exhibited essentially a monophasic inhibition curve by this antibiotic (Fig. 2a). Since

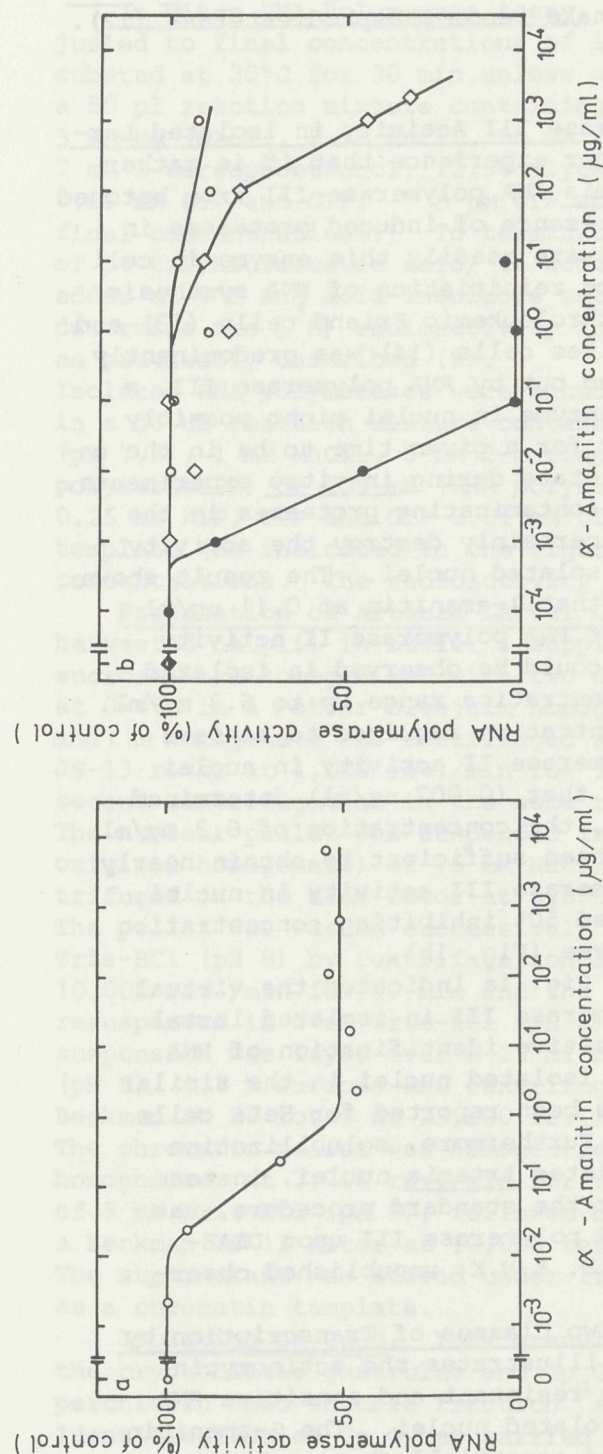


Fig. 1 Effect of α -amanitin on RNA synthesis in isolated nuclei and by isolated RNA polymerase.

(a) Nuclear RNA synthesis. Freshly isolated nuclei ($8 \times 10^5/50 \mu\text{l}$) were incubated at 30°C for 30 min in the standard reaction mixture (Experimental Procedures), in the presence or absence of α -amanitin as indicated in the figure. 100% incorporation of [^3H] UMP into RNA was 12,500 counts/min and background counts (470) obtained at 0°C in the presence of $2 \mu\text{g/ml}$ actinomycin D were subtracted from all values. (b) RNA synthesis by RNA polymerases using calf thymus DNA ($1.3 \mu\text{g}/50 \mu\text{l}$) as template. The standard reaction mixture (50 μl) was incubated at 30°C for 25 min in the presence or absence of α -amanitin as indicated. 100% incorporation values were for RNA polymerase I (2.0 μg protein) activity, no $(\text{NH}_4)_2\text{SO}_4$ was included. 100% incorporation values were 19,000, 10,000 and 4,000 counts/min for RNA polymerase I (O—O), II (●—●) or III (◇—◇), respectively. 850 counts/min was subtracted from all values as background obtained in the absence of added enzyme.

about 10 fold higher concentration of actinomycin D was required for 50% inhibition of free RNA polymerase III transcribing a chromatin template than those for RNA polymerase I and II (L.S. & M.K. unpublished results), the result of Fig. 2a indicates again that the α -amanitin resistant activity in isolated nuclei was principally exerted by RNA polymerase I, but not by RNA polymerase III.

In contrast to the α -amanitin resistant activity, the inhibition curve of RNA polymerase II activity was biphasic, having 50% inhibitions at 0.22 and 6.5 $\mu\text{g/ml}$ actinomycin D, respectively, (Fig. 2a). The biphasic inhibition curve of RNA polymerase II was also evident in the experiments using a chromatin template, where two 50% inhibitions were obtained at 0.04 and 1.1 $\mu\text{g/ml}$ actinomycin D (Fig. 2b). Although it is not clear at this moment whether or not these two classes of transcription by RNA polymerase II demonstrated in two systems were identical to each other, the actinomycin D concentrations required for 50% inhibition in isolated nuclei were respectively about 6 fold higher than those determined for the transcription of free chromatin (Fig. 2a and b). It is interesting to note that whereas a major transcription class by RNA polymerase II in isolated nuclei was nearly 20 fold more resistant to actinomycin D than that by RNA polymerase I, the identical sensitivity of the transcription using chromatin by two enzyme types was found as seen in Fig. 2. This suggests the possibility that the discriminatory transcription by specific RNA polymerases presumed in intact nuclei is prevented in the nucleus-free system, although the observation that the chromatin saturated with one of the two enzyme types is capable of acting as a template for the other favours the contrary (L.S. & M.K., unpublished results).

The precise nature of transcription by these enzymes using chromatin will remain to be elucidated. Furthermore, a minor highly actinomycin D sensitive transcription by RNA polymerase II was detectable only when the initiation of RNA synthesis on a chromatin template was allowed to proceed before the addition of actinomycin D and it is totally undetectable when the antibiotic was added simultaneously. These results indicate that the initiation of the minor class transcription by RNA polymerase II must be extraordinarily sensitive to actinomycin D and that this transcription class might be concerned with some regulatory functions. In fact, Lindell made similar observations using isolated nuclei of rat liver and suggested a hypothesis that the nucleolar transcription might be controlled extranucleolarly by the minor transcription class of RNA polymerase II which was as extremely sensitive to actinomycin D (17).

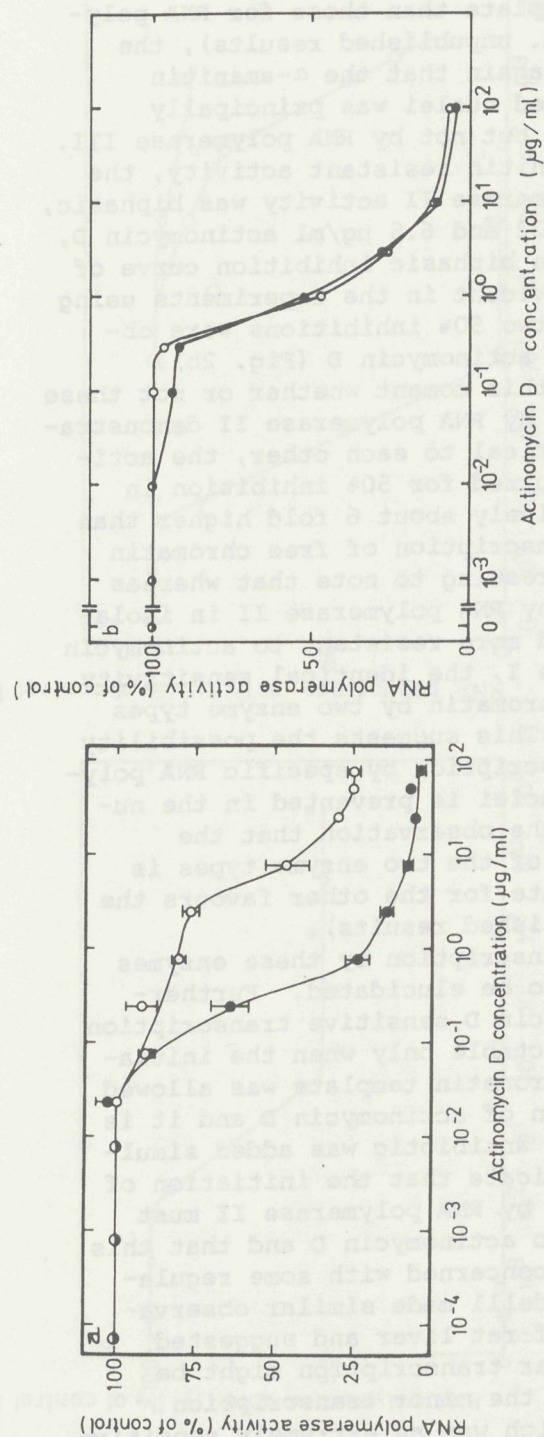


Fig. 2 Effect of actinomycin D on RNA synthesis in isolated nuclei and by isolated RNA polymerases.

(a) Nuclear RNA synthesis. RNA polymerase activity was determined with or without 10 µg/ml α -amanitin as in Fig. 1 a, using $7-11 \times 10^5$ nuclei/50 µl in the presence or absence of actinomycin D as indicated in the figure. Each value was expressed as the average of four to six determinations (each in duplicates) $\pm$ s.e.m., except the averaged two determinations without error bars. 100% value was between 10,000, 16,000 counts/min depending on the concentration of nuclei used. (O—O), α -amanitin sensitive or (●—●), resistant activity. (b) RNA synthesis by RNA polymerases using homologous chromatin DNA (10 µg/50 µl). RNA polymerase was preincubated with a chromatin template in the complete reaction mixture (50 µl) at 30°C for 5 min. After the preincubation, [3 H] UTP and actinomycin D as indicated in figure were added and RNA synthesis was continued for another 25 min at 30°C. 100% values were 16,500 and 21,500 counts/min for RNA polymerase I (O—O) and II (●—●), respectively. Appropriate background counts (300 and 1300) were subtracted from values for RNA polymerase I and II respectively.

As can be seen in Fig. 2a, about 25% of total RNA polymerase II-dependent transcription in nuclei was completely resistant to actinomycin D at nearly the concentration of 100 µg/ml. It was also found in isolated nuclei of the slime mold, *Dictiostelium discoideum*, that about 50% of α -amanitin sensitive transcription was resistant to even a concentration of 150 µg/ml of actinomycin D (18). In both cases, this resistant transcription should involve either a template having very low guanine/cytosine composition or no template at all, since no such transcription by RNA polymerase II could be detected using isolated chromatin (Fig. 2b). In the former case, it is still possible that a special class of template with low guanine/cytosine content was preferentially lost during the preparation of chromatin.

Effect of Diguanosine Nucleotides. Unusual diguanosine nucleotides, Gp₄G and Gp₃G, are synthesized during oogenesis and cleavage period, respectively, in *Artemia* and stored in yolk granules (19). Since it was demonstrated in the fungus, *Achlya*, that the rate of Gp₄G synthesis was inversely correlated to that of RNA synthesis *in vivo* (20) and that Gp₄G was a potent inhibitor to the *in vitro* transcription by isolated RNA polymerases I and II (21), it was tempting to speculate that Gp₄G and/or Gp₃G might play some role in the transcriptional control in *Artemia*, especially in connection with the cryptobiosis in the embryo. The results presented in Table 1 and Fig. 3 show conclusively that both diguanosine nucleotides exert any effect neither on initiation nor on elongation of RNA synthesis in *Artemia* using isolated nuclei as well as RNA polymerase on chromatin or free DNA template.

DISCUSSION

Actinomycin D distinguishes two classes of transcription by RNA polymerase II (Figs. 2a and b). A minor transcription class is extremely sensitive to this antibiotic and can be recognized only when the initiation of RNA synthesis on chromatin precedes the addition of this inhibitor or in isolated nuclei in which the elongation of RNA chains predominates. In isolated nuclei, several fold higher concentrations of the inhibitor are generally required to achieve the equivalent inhibition of RNA polymerase II obtained in the nucleus-free system since a similar effect on the inhibition by α -amanitin is observed (Figs. 1a and b), these differences are likely caused by lower effective intranuclear concentrations of the inhibitor under present conditions (without preincubation).

Lindell has already proposed the possibility of the extranucleolar regulation of nucleolar transcription (17).

Table 1 Effect of diguanosine nucleotides on RNA synthesis in isolated larval nuclei of *Artemia salina*.

Diguanosine nucleotides at concentrations indicated in the Table were tested on total nuclear RNA synthesis activity in the assay described in Experimental Procedures.

Nucleotide	Amount ($\mu\text{g/ml}$)	[^3H] UMP Incorporation (pmol/10 μg DNA)	%
Gp_4G	6	33.4	108
	60	33.3	107
	600	36.1	116
	none	31.0	100
Gp_3G	1.6	44.5	126
	8	42.8	121
	none	35.2	100
$7^m\text{Gp}_3\text{G}$	3	39.6	112
	30	37.9	107
	300	38.9	110
	none	35.2	100

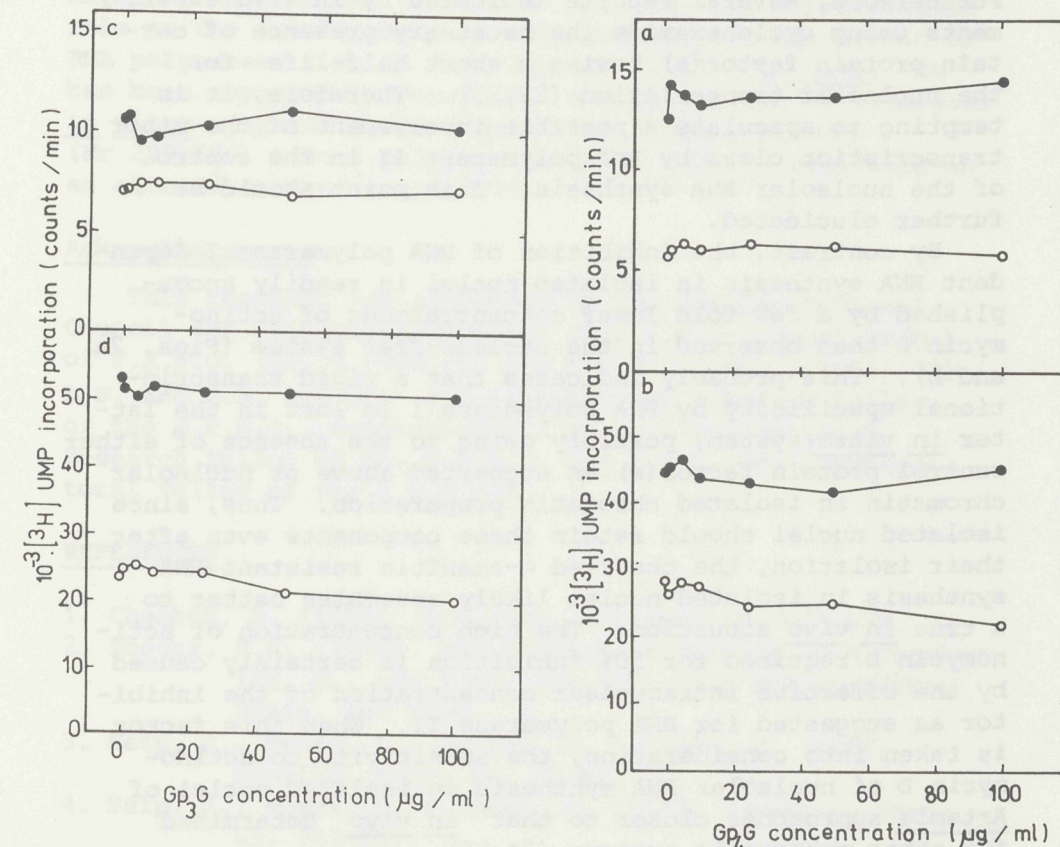


Fig.3 Effect of diguanosine nucleotides on RNA polymerase activity

The standard reaction mixture (50 μl) was incubated at 30°C for 30 min, using 5.4 or 10.5 μg homologous chromatin DNA, or 1.3 or 2.5 μg calf thymus DNA as template for RNA polymerase I (0.5 μg protein $\circ$ — $\circ$) or II (2.1 μg protein; $\bullet$ — $\bullet$), respectively, in the presence or absence of diguanosine nucleotides as indicated in the figure.

(a) and (c), chromatin DNA; (b) and (d), calf thymus DNA.

Furthermore, several reports indicated by in vivo experiments using cyclohexamide the necessary presence of certain protein factor(s) having a short half-life for the nucleolar transcription (22,23). Therefore, it is tempting to speculate a possible involvement of the minor transcription class by RNA polymerase II in the control of the nucleolar RNA synthesis. This point should be further elucidated.

By contrast, the inhibition of RNA polymerase I dependent RNA synthesis in isolated nuclei is readily accomplished by a few fold lower concentrations of actinomycin D than observed in the nucleus-free system (Figs. 2a and b). This probably indicates that a rigid transcriptional specificity by RNA polymerase I is lost in the latter in vitro system, possibly owing to the absence of either control protein factor(s) as suggested above or nucleolar chromatin in isolated chromatin preparation. Thus, since isolated nuclei should retain these components even after their isolation, the observed α -amanitin resistant RNA synthesis in isolated nuclei likely resembles better to a true in vivo situation. The high concentration of actinomycin D required for 50% inhibition is certainly caused by the effective intranuclear concentration of the inhibitor as suggested for RNA polymerase II. When this factor is taken into consideration, the sensitivity to actinomycin D of nucleolar RNA synthesis in isolated nuclei of Artemia approaches closer to that in vivo determined for other eukaryotic systems (24,25).

Isolated nuclei of Artemia larvae is found to lack RNA polymerase III activity. Since RNA polymerase III exhibits a higher resistance to actinomycin D in isolated nuclei (L.S. & M.K., unpublished results; 17) and in intact cells (25), our observation for the absence of such an activity demonstrated in Fig. 2 a further supports our conclusion on RNA polymerase III.

The inactivation of Artemia RNA polymerase III is exerted by the larva-specific proteases (26). This proteolytic attack affects not only RNA polymerase III but also RNA polymerase I. This observation might be correlated with antigenic similarities between these two enzymes, but not RNA polymerase II, demonstrated in calf thymus and HeLa cells (1, 27). However, Artemia RNA polymerase III is apparently a far better substrate for the proteases than RNA polymerase I and the postulated proteolysis of enzymes causes an immediate inactivation of the former enzyme but a mere conversion of the latter enzyme to an altered form retaining still its enzymatic activity. Although the altered enzyme Ia was not characterized in this study, it

might be interesting to examine its possible physiological role on the development, if this proteolytic conversion of RNA polymerase I to Ia were functional also in vivo. It has been reported in yeast that RNA polymerase II undergoes in vitro a specific cleavage of the largest subunit (Mr 220,000) into a polypeptide of Mr 180,000 resulting in an altered form of enzyme (28).

Acknowledgements

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THE HYDRATION DEPENDENCE OF METABOLISM IN ARTEMIA CYSTS

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ABSTRACT

Hydration levels of cysts can range from close to zero to a maximum of about 1.4 gH₂O/g dry wt. Previous work has shown that up to hydration levels of about 0.1 g/g no detectable metabolic activity occurs, and physical measurements (sorption isotherms, DSC, NMR, and microwave dielectrics) indicate that none of this water can provide a continuous dielectric for metabolic reactions. At hydrations of about 0.3 g/g a limited amount of metabolic activity begins, but the physical measurements still do not indicate the presence of "bulk water". However, at about 0.65 g/g, conventional metabolism and embryonic development are resumed, and the presence of "bulk water" is indicated for the first time in the hydration sequence. Evidence will be presented in this paper that suggests the rate of metabolism is strongly hydration-dependent between 0.65 and 0.8 g/g. In contrast, above 0.8 g/g (to maximum hydration) the metabolic rates are surprisingly very much less hydration-dependent. These results will be discussed in the context of previous work on the relationship between metabolic activity and the properties of intracellular water.

INTRODUCTION

Artemia cysts provide unique experimental opportunities for analysis of the relationships between intracellular water content, metabolism, and the physical properties of cell water (1,2). We have previously "mapped" the hydration dependence of the initiation of embryonic development (3), several parameters of carbohydrate metabolism (4), respiration (5), ¹⁴CO₂-fixation (6), changes in ATP concentration (7), the free amino acid pool (8), and the synthesis of RNA and protein (6,9). This work clearly indicates the occurrence of two critical hydrations (~0.3 g/g and ~0.65 g/g) at which specific metabolic pathways are initiated during the hydration sequence (10). Concurrent studies have been directed toward understanding the physical properties of cyst water, particularly at the hydration ranges of metabolic importance. This research has involved the use of sorption isotherm analysis (11), differential scanning calorimetry, or DSC (2,10), dielectric measurements (10), and nuclear

magnetic resonance (NMR) spectroscopy (12,13). Although most of the physical measurements are not yet complete, they provide a reasonably good indication that the presence of appreciable amounts of bulk water (i.e., that present in relatively dilute aqueous solutions) are not required for the initiation of much, or perhaps all of the metabolic parameters we have examined. As a result of this work an hypothesis has been proposed that deals with relationships between the physical properties of intracellular water and metabolism occurring in the aqueous compartments of cells - the cytosol, nucleoplasm, and interior compartments of membrane bounded cytoplasmic organelles (2,10). The present paper involves further examination of the hypothetical model, particularly with regard to the dependence of metabolic rate on cyst water concentrations.

MATERIALS AND METHODS

Cysts were purchased from San Francisco Bay Brand, Newark, California 94560, and were processed and stored at -20°C under 100% N_2 . Hydration was carried out from the vapor phase (using saturated solutions of various salts to generate known relative water vapor pressures (or a_w) and from the liquid phase (using NaCl solutions). All details of processing, storage, hydration and incubation have been given in detail elsewhere (2,3,10). In all cases the cysts were prehydrated at 0°C prior to incubation at 25°C (3).

Hydration levels were determined gravimetrically and are expressed in units of $\text{gH}_2\text{O/g}$ dry mass. These will be referred to as "g/g".

Shells were isolated by flotation methods (3) from populations of cysts that had been incubated for 72 hours in sea water (25°C) to allow maximum hatching of larvae. The cyst population used in these studies gave a hatch yield of about 86%. The shells constitute about 20% of the dry mass of cysts equilibrated over CaSO_4 and their hydration at different a_w 's is known (3,11).

Trehalose was isolated from the supernatant of 80% ethanol homogenates (15,000g for 15 min.) by paper chromatography and quantified by the anthrone procedure (14). Glycogen was extracted from the 80% ethanol insoluble fraction by KOH digestion followed by reprecipitation with ethanol (4) and quantified by the anthrone procedure. Most oxygen consumption measurements were performed with a Clark-type oxygen electrode attached to a YSI Biological Oxygen Monitor, Model 52 (Yellow Springs Instrument Co.)

with recorder output (5). Manometric measurements were made with a conventional Warburg apparatus (see ref. 5 for details). For studies of ^{14}C -incorporation, prehydrated cysts were incubated in the appropriate pre-hydrating NaCl solution at 25°C in the presence of 20 $\mu\text{curies/ml}$ $\text{NaH}^{14}\text{CO}_3$ (83 $\mu\text{curies/mg}$) obtained from New England Nuclear Corp. Following incubation, the cysts were assayed for incorporation into the total cold 5% TCA-soluble fraction, and the nucleic acid and protein fractions using a thin window planchet counter (~ 2 cpm background). In the latter two cases the cysts were pre-incubated at maximum hydration for 2 hours at 25°C in the presence of $^{14}\text{CO}_2$ to label the "precursor pool", then re-dried rapidly. This population was then used for rate studies at different hydration levels (9). Further details of these procedures have been published (6,9).

RESULTS

Figure 1 summarizes all of these measurements. Each point represents an individual rate curve obtained from at least 5 measurements over appropriate time intervals for the various parameters.

Figure 1A shows the hydration dependence of incorporation of $^{14}\text{CO}_2$ into the cold TCA-soluble fraction. We have previously shown the isotope to be contained in a variety of amino acids, Krebs' cycle-related organic acids and pyrimidine nucleotides (6). Note that the initiation of these metabolic pathways occurs in the hydration range of 0.3-0.35 g/g. This confirms the results of previous work (6,9). Above this hydration, the rate of incorporation is strongly dependent on hydration until a cyst water content in the range of 0.5-0.7 g/g is reached, at which the rate decreases with increased hydration. This decrease has been shown to be a result of the dilution of the precursor $^{14}\text{CO}_2$ by increases in the level of respiratory CO_2 (unlabelled) that occurs when respiration is initiated at hydrations of about 0.6 g/g (Fig. 1B; 5,6). Thus, the rate of apparent incorporation decreases only because the specific activity of the precursor decreases above hydration levels of about 0.6 g/g.

Figure 1B describes the hydration dependence of respiration. In this case, like that of $^{14}\text{CO}_2$ -incorporation, the rate is strongly dependent on hydration level between the point of initiation, in this case ~ 0.6 g/g, and up to a level of roughly 0.8 g/g. However, hydration in excess of the latter produces only a slight increase in rate, even

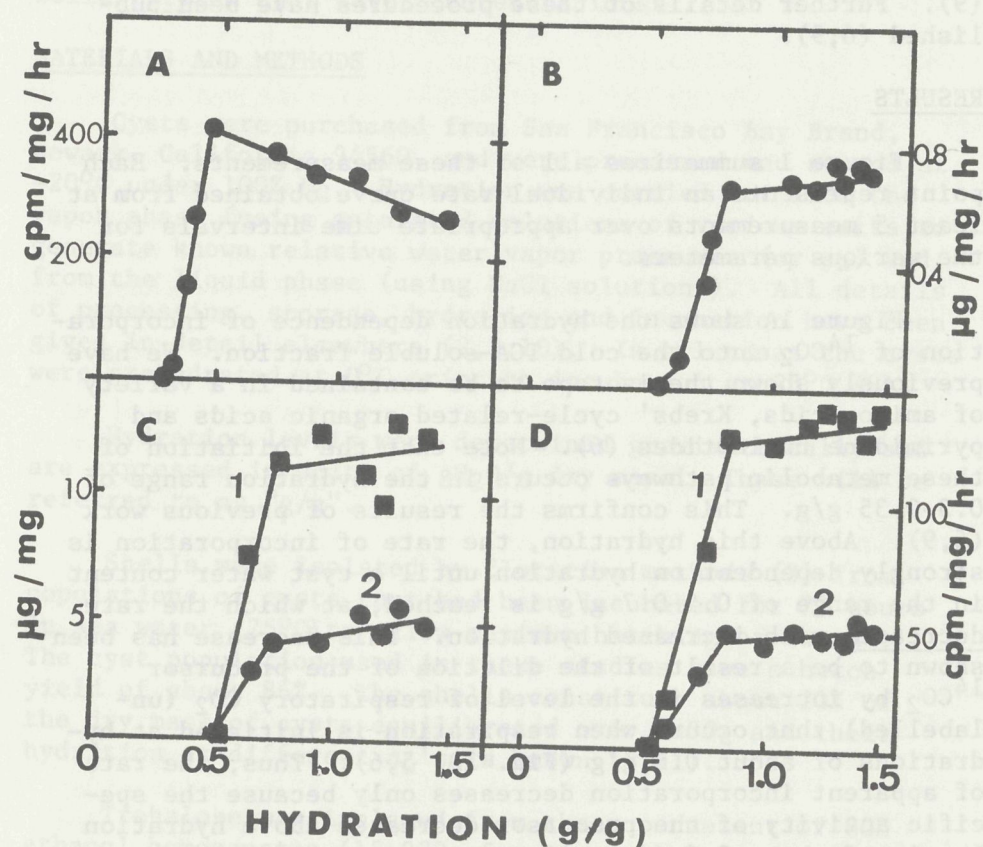


Figure 1. The hydration dependence of various metabolic rate processes in *Artemia* cysts. A, $^{14}\text{CO}_2$ incorporation into cold TCA-soluble material; B, respiration; C, 1. trehalose, 2. glycogen concentrations; D, incorporation of ^{14}C into 1. protein, and 2. RNA.

at maximum hydration. Essentially the same results are observed for the rate of incorporation of ^{14}C into protein and RNA (Fig. 1D) once these processes are initiated at cyst hydrations in the vicinity of 0.6 g/g.

The data for trehalose and glycogen concentration changes as a function of cyst hydration level show considerable scatter (Fig. 1C), in part because we are measuring relatively small changes in significant levels of these substances, particularly for trehalose. Nevertheless, it appears that a similar pattern emerges: the rate of trehalose decrease and glycogen increase show strong hydration dependence over the range of 0.6 to ~ 0.8 g/g, above which hydration level seems not to be a strong determinant of these rates.

I should stress that these measurements are somewhat preliminary. They do reveal, however, a uniform response of an array of metabolic processes to the level of cyst water, once such processes have been initiated within the cells.

On the basis of these findings, and the much more complete analysis of the initiation of metabolism (2,10) we can consider the hydration sequence to consist of three "metabolic domains" separated by "critical hydration levels" (Table 1).

Table 1. Summary of the Hydration Dependence of Metabolism in *Artemia* Cysts

Hydration Range (g/g)	Metabolic Domain	Critical Hydration for Initiation
0 - 0.3 ($\pm .06$)	Ametabolic	-
0.3 - 0.65 ($\pm .1$)	Restricted Metabolism	0.3
0.65 - 1.4	Conventional Metabolism	0.65
	a. strong rate dependence	0.65
	b. weak rate dependence	0.8

In the following section of this paper I will discuss several features of these metabolic results, summarize some of our previous work on the physical properties

of cyst water at the "critical hydration levels", and speculate on what relationships might exist between these sets of observations.

DISCUSSION

I believe the most surprising finding in this study is the weak metabolic rate dependence on hydration, above levels of about 0.8 g/g, up to the maximum of about 1.4 g/g. Our expectation was the opposite since it seemed reasonable to expect that such considerations as viscosity would play a strong role by retarding diffusive transfer of metabolites and so forth, within the cells. The latter could account for metabolic rate dependence observed between 0.6 and 0.8 g/g. But the question arises as to how such large changes in total water content, amounting to about 40% of the total, could have so little effect on metabolic rates over the range of 0.8 to 1.4 g/g. If one considers current views on the nature and disposition of metabolic pathways occurring in the "soluble" compartments of cells this result seems to be contrary to expectation. I believe it is useful to examine what such current views are in the context of the Artemia results.

Much of what we know about the organization and inter-relationships of metabolic processes within living cells, in general, has been derived from cell fractionation studies, combining methods of electron microscopy and biochemistry. Although this approach has been, and will continue to be fruitful, it is of very limited value for study of what might be called the "aqueous intracellular compartments" (in eucaryotic cells these would be the cytosol, nucleoplasm, and internal compartments of membrane-bounded cytoplasmic organelles). Such compartments cannot be "isolated", as such, and studied apart from the intact cell (or organelle) and we know very little about them as a result. Because even the gentle rupture of cells and membrane bounded organelles results in the release of enzymes from these compartments, it has generally been considered that such enzymes exist in free solution within the intact cell, frequently being referred to as "soluble enzymes". Based on compilations from many cell-fractionation studies (15,16) a large fraction of the total enzyme complement of cells consists of such enzymes which, along with coenzymes, substrates and the like, are often described as the "soluble pathways". An important implication of this view is that the operation and regulation of these pathways occur by means of random collisions of the participants and simple mass action mechanisms in a bulk water phase within the

various aqueous compartments. It is not obvious to me how the cells within Artemia cysts can manage to maintain control over their metabolism if the foregoing description of intracellular metabolism is considered to be correct. Thus, the cells would be faced with constant adjustments in all concentration-dependent parameters and, what is worse, the concentrations in the various parts of the cell are likely to be changing differentially since their total water contents are probably not the same (i.e., compare mitochondrial matrix with cytosol). Consequently, the occurrence in Artemia cysts of a simple mass-action based array of "soluble" metabolic pathways seems unlikely in view of our results. There is, in fact, very good reason to doubt such an occurrence for cells in general, based on a very large body of opposing evidence, collected from a wide variety of experiments, on different cell and tissue types. All of these matters have been the subject of very extensive recent reviews (10,17-28). These reviews convincingly demonstrate that much remains to be learned about the organization and operation of metabolic activity within the aqueous intracellular compartments. Furthermore, they strongly indicate that the view of "soluble" metabolic pathways being made up of randomly distributed collections of enzymes dissolved in bulk solution within the aqueous intracellular compartments requires re-examination.

In view of the metabolic results presented here (Fig. 1) and elsewhere (2,10) it seems appropriate to comment briefly on what is known about the physical properties of water in the cysts. The rationale of these studies has been to carry out physical measurements that provide kinetic as well as thermodynamic information, in the hope that the integration of these results will reveal properties and behavior of cellular water in the cysts that are not evident from use of a single methodology. Of course, the hazard in this approach is that it is exceedingly difficult to be technically expert in all of these areas, and virtually impossible to be on firm ground in terms of theory and interpretation. In an attempt to overcome these problems, I enlisted the aid of two research groups and a cooperative research program was initiated. The participating laboratories in this venture are those of Dr. Walter Drost-Hansen (Department of Chemistry, University of Miami) for differential scanning calorimetry (DSC), Dr. Carlton Hazlewood (Department of Physiology, Baylor Medical College and Department of Physics, Rice University) for nuclear magnetic resonance (NMR) spectroscopy, and Professor E. H. Grant (Physics Department, the Queen Elizabeth College of London University) for dielectric measurements in the microwave

and millimeter frequency range. These various programs have resulted in the collection of a large amount of information and it will not be possible to treat it all here. I will refer the reader to Seitz (13) for a detailed account of the NMR work (also see ref. 2) and details of the DSC and low frequency dielectric studies have been published (2). In the following (Table 2) I have summarized a few of our current estimates for several parameters relating to the properties of cyst water at the two hydrations of metabolic importance, and for the fully hydrated cysts. Values for fully hydrated cysts are compared with corresponding values for pure (or bulk) water in the far right column.

Table 2. Physical Parameters* at Hydrations of Metabolic Importance (A and B) and in Fully Hydrated Cysts (C)

Parameter Measured	Cyst Hydration (g/g)			Bulk Water Value	H ₂ O/C
	A 0.3	B 0.65	C 1.4		
Heat Capacity (DSC) (cal/g/deg)	1.47	1.11	0.98	1.00	1
T ₁ (msec) NMR	98	148	246	3000	12
T ₂ (msec) NMR	21	34	48	1750	37
D (10 ⁻⁶ cm ² /sec) NMR	0.6	1.5	3.5	25	7
τ (10 ⁻¹² sec.) dielectrics (for dispersion 0.8-70GHz)	23	16	9	8	1

*T₁ and T₂ are relaxation times; D is the self-diffusion coefficient; τ is the macroscopic dielectric relaxation time given by $2 \pi f_c^{-1}$, where f_c is the characteristic frequency estimated from the upper and lower frequency limits of the dispersion (ϵ'_∞ and ϵ'_s , respectively).

Inspection of these results will show that only at hydrations in excess of about 0.65 g/g (the critical hydration threshold for conventional metabolism) is there any reasonably good indication for the presence of appreciable amounts of water in cysts that exhibit the properties of

bulk water. Consequently, all of the metabolism that is initiated under these conditions is occurring in the absence of a bulk water phase and is likely to have an interfacial locus, in proximity to the various intracellular surfaces. Furthermore, a reasonably good argument can be advanced that a similar situation exists in fully hydrated Artemia cells. Based on the Artemia results (2,10) and on evidence in the literature from other systems (10) I have proposed that the "soluble" enzymes of the aqueous intracellular compartments may not be freely dissolved in bulk aqueous solutions, but instead are loosely associated with various intracellular surfaces (and the perturbed water in proximity to such surfaces) and that the enzymes of these metabolic pathways might also exist in a high degree of organization with respect to each other, operating more in a sequential 2-dimensional framework, than randomly in bulk solution.

Certainly, the rate dependence presented in this paper (Fig. 1) are consistent with this possibility since changes in cyst water content above hydrations of about 0.8 g/g would alter only the bulk phase and might not affect the operation of enzymes located at interfacial locations in the cells. Below 0.8 g/g, where rates of metabolism are hydration-dependent, it seems likely that such things as viscosity would be sufficiently great (in the relatively small bulk aqueous phase present at these cyst hydrations) as to retard diffusion-dependent processes such as metabolite transport, enzyme substrate collisions, and so on.

Of course, there are alternative interpretations that could be applied to these results, and the particular one I have presented here is considered tentative and hypothetical. However, I suggest that the experimental evidence in support of this hypothesis is considerable (2,10).

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INFLUENCE OF DIETARY PURINES AND PYRIMIDINES ON ARTEMIA POSTEMBRYONIC DEVELOPMENT

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ABSTRACT

During Artemia postembryonic development an abortive appendicular morphogenesis is induced on larvae reared in a medium lacking folic acid and thymidine. The appendicular morphogenesis is more abortive when the requirement for thymidine is increased by increasing the adenylic acid concentration provided the cytidylic supply is not limited. In a medium lacking thymidine there is no morphogenetic adaptation to a high adenylic/cytidylic acid ratio which results in a reduced survival percentage.

INTRODUCTION

Artemia postembryonic development is anamorphic, development progressing without any cataclysmic event through a number of larval stages successively adding new segments and appendages. Some degree of metamorphosis has been superimposed on these anamorphic stages resulting in the juveniles (1). The first visible sign of metamorphosis is the rotation of the antennae which takes place after the morphogenesis of eleven pairs of thoracic appendages (2).

Provasoli and d'Agostino using a complex artificial medium containing RNA and DNA hydrolysates reported that folic acid deletion resulted in the production of juveniles (3). In the refined medium they developed in 1969, these RNA and DNA hydrolysates were replaced by a purine and pyrimidine mixture (4). We have been using the refined medium with a different mixture than that formulated by Provasoli and d'Agostino (Table 1).

Using this medium we have shown that folic acid deletion resulted in the production of juveniles provided thymidine is supplied at an optimal level. In a medium lacking folic acid and thymidine, an abortive appendicular morphogenesis is induced with metamorphosis occurring after the morphogenesis of six instead of eleven pairs of thoracic appendages (5). The inhibition of thymidylate synthetase by 5-fluorodeoxyuridine (5FUdR) induces a teratogenic action on appendicular morphogenesis with numerous malformations. It was tentatively concluded that the abortive appendicular morphogenesis is not the result of a thymidine deficiency

Table 1. Purine and Pyrimidine Concentrations of Standard Media

Provasoli and d'Agostino		Hernandorena
1962	1969	
mg %	mg %	mg %
RNA 40	adenylic acid 60.0	60
	guanylic acid 2.5	—
DNA 10	cytidylic acid 5.0	10
	uridylic acid 5.0	10
	thymidine acid 2.5	5

but the result of a folic acid deficiency, thymidine acting as a folic acid sparer (6). In a medium lacking folic acid and thymidine, aminopterin, a folic acid reductase inhibitor, must be added in order to prevent any growth (7). This observation can be interpreted by an ovarian transfer of folic acid. One generation experiments are not suited to control this possible transfer and at that time the abortive appendicular morphogenesis was not further analyzed. We have been interested by a supernumerary gonopode morphogenesis induced by a reduced adenylic/cytidylic acid ratio. The induction of supernumerary gonopodes by a dietary cytidylic excess was supposed to be related to the production of a thymidine excess by opposition to the abortive appendicular morphogenesis (8). We have not yet obtained such evidence. The administration of a dietary thymidine excess induces a teratogenic action which is completely different from the induction of non-teratogenic abdominal gonopodes (9). Evidently the metabolic fate of an exogenously supplied thymidine excess is different from the endogenous production of thymidine at the level of folic acid metabolism.

We have shown that thymidine has no growth promoting action but increases the survival percentage in a medium containing folic acid and a high adenylic/cytidylic acid ratio (9). We thought it would be interesting to reexamine the effect of folic acid and thymidine deletion on appendicular morphogenesis in relation to the adenylic/cytidylic ratio of the diet.

MATERIALS AND METHODS

The method developed by Provasoli and d'Agostino for the Utah strain was used for axenic cultivation (4). The purine requirement was met by adenylic acid at 100 mg% con-

Table 2. Effect of Various Dietary Components on Growth and Mortality^a

Treatment	Growth Index 8th Day							Mortality 8th Day						
	CMP mg %							CMP mg %						
	0	10	20	40	60	80	100	0	10	20	40	60	80	100
+ folic acid	6.1	6.8	7.3	7.8	8.1	8.5	8.9	0	0	0	0	0	0	0
+ thymidine														
+ folic acid	6.1	6.4	7.1	7.9	8	8.6	8.7	50	30	20	0	0	0	0
- thymidine														
- folic acid	6.1	6.4	7.4	7.7	8	8.4	8.9	30	20	0	0	0	0	0
+ thymidine														
- folic acid	6	6	x	x	x	x	x	50	40	40	0	0	0	0
- thymidine														

^a AMP at 100 mg% was added to all growth media.

x Abortive appendicular morphogenesis.

centration and the pyrimidine requirement by cytidylic acid with folic acid omitted or supplied at the standard concentration (1400 µg%) and thymidine omitted or supplied at a 10 mg% concentration.

RESULTS

Growth Rate. In a medium containing 100 mg% adenylic acid the growth rate increases with increasing cytidylic acid concentration from 0 to 100 mg% in a medium containing folic acid and thymidine, in a medium containing folic acid and lacking thymidine and in a medium lacking folic acid but containing thymidine. In a medium lacking cytidylic acid or in a cytidylic acid deficient medium the growth rate is limited under every experimental condition (Table 2).

Survival. In a medium containing 100 mg% adenylic acid the mortality decreases with increasing cytidylic acid concentration. In a medium lacking cytidylic acid or in a cytidylic acid deficient medium, thymidine omission is detrimental. Thymidine addition reduces mortality not only in a medium lacking folic acid but also in a medium containing folic acid (Table 2).

Morphogenesis. In a medium containing folic acid or thymidine morphogenesis is normal under every experimental condition. The larvae reared in a medium containing thymidine but lacking folic acid do not reach sexual maturity.

In a medium lacking folic acid and thymidine an abortive appendicular morphogenesis is induced on larvae reared with cytidylic acid concentrations equal or superior to 20 mg%. The first signs of metamorphosis are evident by the 8th day of development. This morphogenesis shows an extreme abortive appendicular morphogenesis as compared to the abortive morphogenesis previously obtained in a medium lacking folic acid and thymidine but containing the standard adenylic, cytidylic and uridylic acids concentrations.

The appendicular morphogenesis of larvae surviving in a medium lacking cytidylic acid or in a cytidylic acid deficient medium is normal even in a medium lacking folic acid and thymidine, but these larvae are not going to reach the juvenile stage.

DISCUSSION

We have no comparative data to analyze the morphogenetic action of folic acid and thymidine deletion. Although folic acid is required by all but a few insects so far examined, folic acid deletion has no morphogenetic incidence (10).

However aminopterin treatment induces morphogenetic action on the wing of the vestigial heterozygote Drosophila (11) and aminopterin pulsed to late third instar of Drosophila wild type larvae caused various wing malformations and "crippled legs" (Sang's unpublished observation quoted in ref. 12). In Myzus persicae aminopterin depresses the wing formation and this effect is relieved by thymidine (13). A combination of folic acid and appropriate amino acids increases the production of brachypterous adults in Laodelphax striatellus (unpublished observation quoted in ref. 14).

Our observation may be particularly interesting when considering the general indication obtained in selected Drosophila lines that the faster the DNA synthesis, the shorter the developmental time and the smaller the eventual adults (15). In Artemia the problem of the relationships between DNA synthesis (that is thymidine requirement) the adenylic/cytidylic acid ratio (that is growth rate) and the timing of metamorphosis could be favourably approached since in Artemia folic acid is required for thymidine biosynthesis but not involved in purine biosynthesis. Artemia is incapable of synthesizing the purine ring de novo and can synthesize the pyrimidine ring (16, 17) but apparently at a limiting rate under our experimental conditions. Aspartic acid concentration is not the rate limiting factor (unpublished result). The positive effect of thymidine on survival in a medium containing folic acid and lacking cytidylic acid suggests that thymidine is sparing some vital folic acid function, possible by preventing the drain of methylenetetrahydrofolate to dihydrofolate.

As stressed by Costlow (1) metamorphosis in the Crustacea remains "a problem in developmental biology". In Artemia we can suggest that the onset of metamorphosis is a consequence of the metabolic conditions prevailing during early larval life at the level of folic acid metabolism as mediated by the adenylic and cytidylic acid balance of the diet.

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CONCLUDING REMARKS

Commercially available brine shrimp cysts are contaminated with a variety of microorganisms which many workers remove or kill by chemical sterilization using antiformin or NaOCl (1). Although these reagents rid the cysts of most microorganisms, enhance the synchrony and extent of hatching and increase the validity of radioisotope incorporation measurements, we have found that they are, in some cases, the cause of low measurements of enzyme activity in extracts and enzyme preparations from encysted embryos. In one series of experiments we observed that antiformin treatment of *Artemia* cysts caused a 50% reduction in elongation factor 2 values (from 2.4 nmol to 1.2 nmol/gm dry weight) despite extensive washing of the treated cysts with H₂O, 1% urea and homogenization buffer. Our data also showed that the residual antiformin affected primarily the elongation factor 2 associated with the crude ribosome fraction.

These findings appear to be related to those of Golub and Clegg who demonstrated that NaOCl causes inactivation of 80-S ribosomes (2). Abdul-Baki was able to neutralize residual NaOCl on plant seeds using 0.1N HCl in the wash medium (3), but we have found this reagent to be without effect on the residual NaOCl associated with *Artemia* cysts. Wash media containing 1 mM dithiothreitol were also ineffective on the *Artemia* problem.

Since *Artemia* cysts appear to retain antiformin or NaOCl in an active and non-exchangeable form, I'd like to suggest that all workers avoid the use of these sterilizing reagents unless their effects on cyst extracts have been given careful consideration.

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