Distribution of elongation factor 2 between particulate and soluble fractions of the brine shrimp *Artemia* during early development[1]

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The ADP-ribosylation of elongation factor 2 (EF-2) *in vitro* was used to quantitate EF-2 and to determine its subcellular distribution in extracts of *Artemia* embryos at different stages of development. In extracts from dormant cysts of *Artemia* 40–45% of EF-2 is complexed to macromolecules smaller than ribosomes, whereas the remainder is soluble or free in the cytosol. During early development the amount of "complexed" EF-2 decreases markedly concomitant with an increase in the pool of soluble EF-2. Complexed EF-2 was found to be associated with macromolecules which sediment at 16S–20S and 40S–50S and not with monoribosomes or polyribosomes as reported for mammalian systems. The data show that the decrease in complexed EF-2 is associated with the resumption of development in *Artemia*.

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Nous avons utilisé l'ADP ribosylation du facteur 2 d'élongation (EF-2) *in vitro* pour mesurer quantitativement l'EF-2 et pour déterminer sa distribution subcellulaire dans des extraits embryonnaires d'*Artemia* à différents stades du développement. Dans des extraits de kystes dormants d'*Artemia*, 40–45% du EF-2 forme un complexe avec des macromolécules plus petites que les ribosomes, tandis que le reste est soluble ou libre dans le cytosol. Au début du développement, la quantité d'EF-2 sous "forme de complexe" diminue de façon marquée en même temps que le pool du EF-2 sous forme soluble augmente. L'EF-2 est associé à des macromolécules qui sédimentent à 16S–20S et 40S–50S et non à des monoribosomes ou des polyribosomes comme c'est le cas dans les systèmes mammaliens. Les résultats montrent que la diminution du EF-2 sous forme de complexe est associée à la reprise du développement chez *Artemia*.

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Introduction

Dormant encysted embryos in the brine shrimp *Artemia* are inactive in protein synthesis and cell-free extracts from these embryos are unable to translate endogenous mRNA (1, 2, 3). However, various cell-free systems from dormant cysts are active in poly(U)-directed protein synthesis (2, 4, 5, 6). These latter studies have indicated that EF-1 and EF-2 are present and active in extracts from dormant cysts. Nevertheless, cell-free systems derived from dormant cysts are less active in poly(U) translation than systems derived from developed embryos (4). Slobin and Moller have demonstrated that EF-1 undergoes a size transformation from a heavy to light form during early development of *Artemia* and they suggested that this mechanism may provide a means by which the rate of protein synthesis is altered during embryogenesis (5, 7). 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) translation *in vitro* (6, 8). Hence, EF-2 might play a central role in the regulation of protein synthesis during early development of *Artemia*.

This study was initiated to determine the quantity and the subcellular distribution of EF-2 in *Artemia* embryos at different stages of development.

Materials and methods

Materials

Encysted embryos of *Artemia* were from the salterns in Utah and supplied by Longlife Fishfood Products (Harrison, NJ). About 65% of the cysts gave rise to swimming nauplii after 48 h of incubation in hatch medium (9). Diphtheria toxin (lot No. D-298, 1800 L_f/mL, 13.2 mg/mL) was obtained from Connaught Laboratories (Toronto, Ont.). Dithiothreitol and NAD^+ were from Sigma (St. Louis, MO), while [*adenine*-2,8-^{3}H]NAD^+ (sp.act. 109.5 GBq/mmol) was from New England Nuclear (Boston, MA). Creatine phosphate, phosphocreatine kinase, GTP, ATP, and poly(U) were from P-L Biochemicals (Milwaukee, WI). DEAE-cellulose (Whatman, DE-32) and phosphocellulose (Whatman, P-11) were from Mandel Scientific (Montreal, P.Q.). Ultrogel AcA34 (LKB Instruments) and

ABBREVIATIONS: EF-1, and EF-2, elongation factors 1 and 2; poly(U), polyuridylic acid; L_f, flocculating unit; NAD^+, nicotinamide adenine dinucleotide; sp.act., specific activity; DTT, dithiothreitol; UV, ultraviolet; SDS, sodium dodecyl sulfate; BSA, bovine serum albumin; ADP-Rib, adenosine 5′-diphosphoribose.

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filter paper disks (Whatman 3, 2.3 cm) for the carbon-14 incorporation experiments were from Fisher Scientific (Toronto, Ont.). Liquid scintillation fluors and [^{14}C]phenylalanine (18 GBq/mmol) were from Amersham (Oakville, Ont.). Sephadex G-25 (medium) and Sepharose 6B were from Pharmacia (Montreal, P.Q.) and microporous filters (2.5 cm, 0.45 μm) were from Amicon (Lexington, MA). All other chemicals were reagent grade.

Preparation of Artemia *embryos for EF-2 studies*

Encysted dormant embryos were hydrated and washed before use as described by Warner *et al.* avoiding antiformin treatment (9). The washed cysts (0-h embryos) were rinsed with homogenization buffer and ground as described later. When developing embryos were required, hydrated and washed cysts were rinsed with hatch medium and 12 g of the fully hydrated cysts were incubated at 30°C with 500 mL of hatch medium in a 2800 mL Fernbach culture flask. The incubation medium and conditions were as described before, except that the concentration of penicillin and streptomycin was reduced to 500 U/mL and 25 μg/mL, respectively (9). Developing embryos were collected on nylon filters, washed with homogenization buffer, and processed as described for the 0-h embryos.

Preparation of particulate and soluble fractions from Artemia *embryos by differential centrifugation*

All embryo fractions were prepared at 0–4°C and stored at −70°C until needed. Embryos from different stages of development were ground to a thick paste with buffer A (50 m*M* Tris–HCl (pH 7.9, 4°C), 5 m*M* KCl, 10 m*M* $MgCl_2$, 1 m*M* DTT) using an electric mortar (6, 9). Additional buffer A was added to a final volume of eight times the initial volume of starting cysts, and the preparation was stirred for 10 min. The homogenate was centrifuged at 12 000 × *g* for 10 min and the supernatant fluid was passed through a cheesecloth – glass wool – cheesecloth filter. The filtrate was centrifuged at 30 000 × *g* for 45 min and the supernatant fraction was removed with a syringe avoiding the floating lipid layer. This supernatant fraction was centrifuged further at 150 000 × *g* (Beckman rotor 60Ti) for 150 min, and the pellet obtained (P-150 fraction) was suspended in buffer C (50 Tris–HCl (pH 7.9; 4°C), 100 m*M* KCl, 10 m*M* $MgCl_2$, 1 m*M* DTT, 10% glycerol). The fluid above the P-150 fraction was adjusted to 70% saturation with solid $(NH_4)_2SO_4$ and the preparation was allowed to stand for 2.5 h. The precipitate that formed was collected by centrifugation, suspended in buffer D (50 m*M* Tris–HCl (pH 7.9; 4°C), 50 m*M* KCl, 0.1 m*M* EDTA, 1 m*M* DTT, 10% glycerol), and dialyzed for 16 h against two changes of buffer D. This preparation is referred to as the S-150 fraction or soluble fraction prepared by differential centrifugation.

Fractionation of Artemia *embryo extracts on columns of Sepharose 6B*

The 30 000 × *g* supernatant fraction was prepared from 12 g wet weight starting cysts as previously described, except that buffer C was used as the homogenization buffer and the volume of the homogenate was 10 times the volume of the starting cysts. The 30 000 × *g* fraction was concentrated to about 8 mL by vacuum dialysis and clarified by centrifugation (10 000 × *g*; 15 min), and about 1000 A_{260} units was applied to a column of Sepharose 6B (2.5 × 91 cm) previously equilibrated with buffer C. The column was developed with buffer C at 30 mL/h and fractions of 7.5 mL were collected. All column fractions were assayed for UV absorbance and selected fractions were tested for the presence of EF-2 using the diphtheria-toxin-dependent ADP-ribosylation assay described later. The column fractions (see Fig. 1) that were positive for EF-2 (when assayed without any pretreatments) were pooled and treated with solid $(NH_4)_2SO_4$ to obtain the protein which is insoluble between 30 and 70% saturation with respect to $(NH_4)_2SO_4$. The precipitate was dissolved in buffer D and dialyzed for 8 h against five changes of buffer D and any insoluble material that formed was removed by centrifugation. This preparation is referred to as the soluble fraction prepared by chromatography on Sepharose 6B. All UV-absorbing fractions which eluted from Sepharose 6B ahead of the free EF-2 fractions (about 800 A_{260} units) were pooled, adjusted to contain 0.5 *M* NH_4Cl, and saved for the isolation of "complexed" EF-2 as described later.

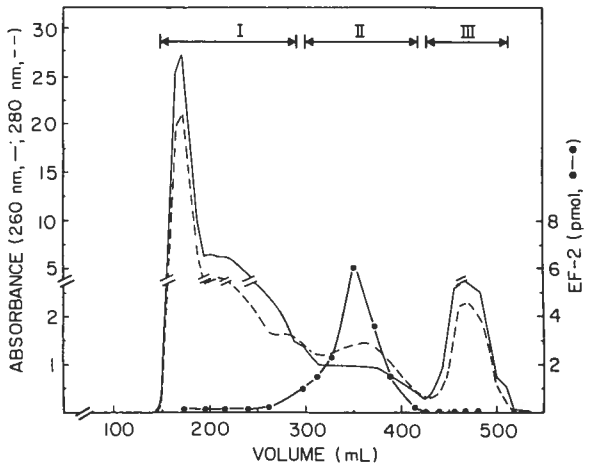

FIG. 1. Chromatography of *Artemia* cyst S-30 fraction on a column of Sepharose 6B. The 30 000 × *g* supernatant fraction from 12 g hydrated 0-h *Artemia* cysts was applied to a column of Sepharose 6B (2.5 × 91 cm) previously equilibrated with buffer C. The column was developed with buffer C at a flow 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 cm (--) and for EF-2 (●—●) by the ADP-ribosylation assay. The EF-2 content is in picomoles per 0.05-mL column fractions. Fraction I (140 mL) containing complexed EF-2 and fraction II (120 mL) containing free EF-2 were pooled separately and saved for further studies.

Fractionation of Artemia *embryo extracts on sucrose density gradients*

Sucrose density gradient analyses were performed on the 30 000 × *g* supernatant fluid prepared with buffer C as previously described (2), except that prior to sample application 0.4 mL of glycerol-free buffer C was layered on the 15–50% sucrose gradient and the sample to be analyzed (10–11 A_{260} units) was layered under the buffer.

In one set of experiments (see Figs. 2*a* and 2*b*) the

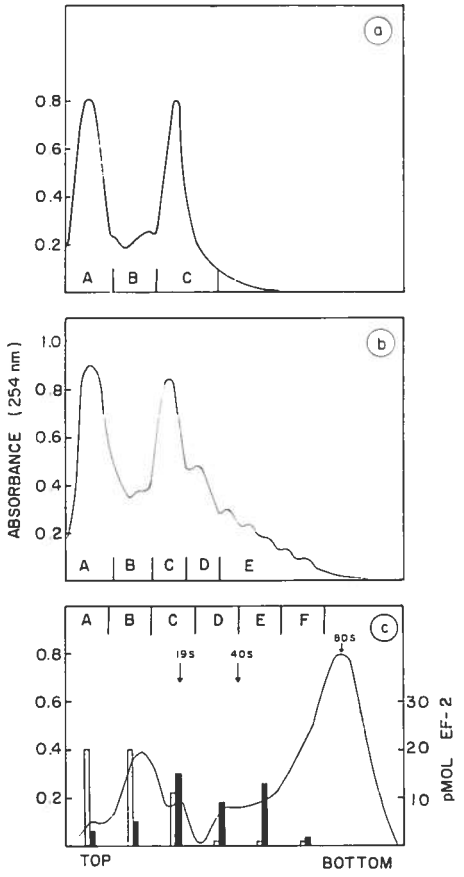

FIG. 2. Sucrose density gradient profiles of the postmitochondrial supernatant fractions from 0- and 20-h *Artemia* embryos. In *a* and *b* 10 A_{260} units of the postmitochondrial supernatant fractions from 0- and 20-h *Artemia* embryos, respectively, were layered onto separate 12-mL sucrose gradients (15–50%, w/v). The preparations were centrifuged for 80 min at 39 000 rpm (SW 41 rotor) then fractionated at a flow rate of 0.5 mL/min as described in Materials and methods. The cytosol (A), monoribosome (C), and polyribosome (E) fractions were pooled separately as illustrated and concentrated by vacuum dialysis. In *c* 11 A_{260} units of the postmitochondrial fraction from 0-h embryos were layered onto a sucrose gradient as above and then centrifuged for 5 h at 39 000 rpm. At the end of the run the sucrose gradient was fractionated as described in Materials and methods and fractions were pooled as indicated (A to F). In *a*, *b*, and *c*, the solid line represents the absorption measured at 254 nm. In *c*, the open bars represent the free or soluble EF-2 in each pooled fraction and the solid bars indicate the amount of complexed or sedimentable EF-2 in each pooled fraction.

monoribosome and polyribosome fractions from 15 gradients were obtained using an ISCO gradient fractionator (model 640), pooled, concentrated by vacuum dialysis, and treated with NH_4Cl as described later to liberate any complexed EF-2. The cytosol fractions that remained on top of the 15 sucrose gradients were also pooled, concentrated by vacuum dialysis, equilbrated with buffer D by dialysis, and then stored at −20°C until needed.

In a second set of experiments the 30 000 × *g* supernatant fluid was centrifuged for 5 h on a 15–50% sucrose gradient and 0.35-mL fractions were collected as previously described. Similar fractions from nine gradients were pooled (see Fig. 2*c*), diluted with buffer C containing 15% sucrose (in place of glycerol) and then centrifuged at 100 000 × *g* for 18 h. The supernatant fluids from this step were concentrated separately by vacuum dialysis and saved for the measurement of "free" EF-2, while the sediments were resuspended in buffer C containing 15% sucrose and saved for measurements of complexed EF-2 activity and content as described later.

The sedimentation constants of the sucrose density fractions were determined according to the method of Martin and Ames (10) using previously established density markers in *Artemia* extracts (11).

Release of elongation factor 2 from particulate and macromolecular fractions

The particulate and macromolecular fractions obtained by centrifugation and Sepharose 6B fractionation were treated with NH_4Cl to release complexed EF-2. Solid NH_4Cl was added to a final concentration of 0.5 *M* and the preparation was stirred gently for 75 min. The insoluble material and ribosomes were removed by centrifugation at 150 000 × *g* for 4.5 h and the supernatant fluid was concentrated by vacuum dialysis. The concentrate was dialyzed for 12 h against four changes of buffer D, clarified by centrifugation, and stored at −70°C. Protein and EF-2 fractions prepared in this way are referred to as complexed fractions.

Purification of elongation factor 2

EF-2 from *Artemia* cysts was purified from the P-150 fraction rather than from the total embryo extract, to avoid problems with protease and EF-2 inhibitor activity in the S-150 fraction (8, 12). The P-150 fraction from 112 g of fully hydrated cysts (in buffer C) containing 7320 A_{260} units was applied to a Sepharose 6B column (2.5 × 93 cm) previously equilibrated with buffer C. The column was developed with buffer C and the contents of the first UV-absorbing peak (A_{260}) to elute from the column were concentrated by vacuum dialysis. EF-2 was "released" from this fraction with 0.5 *M* NH_4Cl as described in the preceding section, concentrated by vacuum dialysis, and then dialyzed against five changes of buffer D for 10 h. The precipitate that formed during dialysis was removed by centrifugation and then the protein preparation (89 mg in 8 mL) was chromatographed on a column of Ultrogel AcA34 (2.5 × 85.5 cm) previously equilibrated with buffer D. Fractions of about 7 mL were collected (flow rate 30 mL/h) and EF-2 was detected by the ADP-ribosylation assay described later. The fractions containing EF-2 were pooled and then applied to a DEAE-cellulose column (DE-32, 1.5 × 17.8 cm) previously equilibrated with buffer D. Following the application and washing of protein on the column, EF-2 was eluted with a 400-mL linear gradient of KCl from 50 to 350 m*M* in buffer D at a flow rate of 30 mL/h. The EF-2 fractions which eluted between 90 and 120 m*M* KCl were pooled, concentrated to about 4 mL by vacuum dialysis, and then dialyzed against

four changes of buffer D for 6 h. Finally, the dialyzed fraction was applied to a column of phosphocellulose (P-11, 1.5 × 10 cm) previously equilibrated with buffer D, washed with 50 mL of buffer D, and then eluted with a 400-mL linear gradient of KCl from 50 to 350 m*M* in buffer D. The fractions which contained EF-2 (as determined by the ADP-ribosylation assay) were pooled, concentrated by vacuum dialysis, and dialyzed against four changes of buffer D for 6 h. The yield was about 1 mg EF-2. Aliquots of about 0.1 mL at 0.8 mg/mL protein were stored at −70°C until needed.

Artemia cyst EF-2 prepared in this way was about 93% pure as judged by SDS–polyacrylamide gel electrophoresis. It had a molecular weight of 95 000 by SDS–polyacrylamide gel electrophoresis and gel filtration.

Assay for elongation factor 2

The content of EF-2 in various fractions was assayed using the diphtheria-toxin-dependent ADP-ribosylation method of Maxwell and colleagues slightly modified (13, 14). The amount of EF-2 was determined in a 0.1-mL reaction containing 12.5 m*M* Tris–HCl (pH 7.3; 28°C), 1.25 m*M* KCl, 0.15 m*M* EDTA, 0.25 m*M* DDT, 2.5 m*M* $MgCl_2$, 2.5% glycerol, 6.25 μg BSA, 1.65 μg diphtheria toxin, 122 pmol [^{3}H]NAD^+ (sp.act., 30 GBq/mol), and various quantities of EF-2 containing preparations. The reaction mixture was incubated at 28°C for 45 min, and then the reaction was stopped by the addition of 0.05 mL of 10 mg/mL BSA (as carrier protein) followed by 2 mL of 5% ice-cold trichloroacetic acid containing 0.1 m*M* unlabeled NAD^+. The precipitated proteins were collected on microporous filters (0.45 μm) and washed three times with 2 mL of 5% ice-cold trichloroacetic acid. The filters were oven dried at 125°C for 10 min and the radioactivity on the filters was measured after placing the filters in 5 mL of toluene base scintillation fluid containing 0.4% PPO and 0.015% POPOP. The concentration of EF-2 was determined from the extent of [^{3}H]ADP-ribosylation assuming that one molecule of ADP-Rib is bound to one molecule of EF-2 in the presence of diphtheria toxin and excess NAD^+ (14). The EF-2 content of each sample was determined at three different protein concentrations in the linear range of the ADP-Rib binding curve.

The activity of *Artemia* cyst EF-2 in the translation of poly(U) was measured *in vitro* by a procedure similar to that described previously (8), except that the reaction mixture (0.3 mL) contained 2.5 A_{260} units of salt-washed ribosomes (9), 45 μg of a postribosomal protein fraction from *Artemia* lacking EF-2 but containing nonlimiting amounts of EF-1 and phenylanyl-tRNA synthetase (second peak in Fig. 1 of Ref. 15), and varying amounts of purified *Artemia* EF-2. Prior to the addition of EF-2 and ribosomes, the reaction mixture was incubated for 30 min at 30°C to amino acylate fully $tRNA^{Phe}$. Thus at the onset of the polymerization phase of the assay, the reaction mixture contained 44.4 pmol [^{14}C]Phe-tRNA in an EF-1·GTP·Phe-tRNA complex. [^{14}C]Polyphenylalanine synthesis was determined according to the method of Mans and Novelli (16).

Protein content of various embryo fractions

The protein content was determined according to the method of Lowry *et al.* using BSA as a standard (17).

Results

Quantity and distribution of EF-2 between particulate and soluble fractions

When extracts from dormant cysts were prepared in buffer A and the 30 000 × *g* supernatant fraction was subjected to ultracentrifugation, we observed that about 40% of the embryo EF-2 sedimented at 150 000 × *g* (P-150 fraction), while the remainder was found in the supernatant fluid (S-150 fraction). The EF-2 in the P-150 fraction could be quantified by the ADP-ribosylation assay only after the EF-2 was released from the particles in this fraction. This was accomplished by treatment of the P-150 fraction with 0.5 *M* NH_4Cl, followed by centrifugation at 150 000 × *g* to remove ribosomes and insoluble material. Previously bound or complexed EF-2 is thus released into the salt-soluble fraction. In analogy to ribosome-bound EF-2 in other systems which reacts very slowly with NAD^+ in the presence of diphtheria toxin (18), the EF-2 in the P-150 fraction from *Artemia* cysts was designated complexed EF-2. The EF-2 which is soluble in the cysts (the S-150 fraction) was designated free EF-2.

In a previous study we observed that some proteins in the P-150 fraction are not tightly associated with the macromolecular components of this fraction and that they can be separated from the macromolecular fraction by chromatography on Sepharose 6B using a buffer containing 100 m*M* KCl (8). Thus we decided to use the filtration method on Sepharose 6B to study the distribution of EF-2 in the postmitochondrial fraction of *Artemia* embryos.

When the 30 000 × *g* supernatant fraction from 0-h embryos was chromatographed on a column of Sepharose 6B, the results shown in Fig. 1 were obtained. The column fractions were analyzed for absorbance at 260 and 280 nm and for EF-2 using the diphtheria-toxin-dependent ADP-ribosylation assay under conditions described in Fig. 1. Under these conditions virtually all of the detectable EF-2 was found in fraction II, while trace amounts were found in fraction I. When the contents of fraction I were treated with 0.5 *M* NH_4Cl and then centrifuged at 150 000 × *g*, considerable EF-2 was found in the salt-soluble fraction as measured by the ADP-ribosylation assay. Thus, as described for the P-150 fraction, the NH_4Cl treatment released EF-2 from the particulate or macromolecular substances in fraction I. No additional EF-2 was released by use of higher concentrations of NH_4Cl. The differences in ADP-Rib acceptance ability of Sepharose 6B column fractions I and II suggests that fraction I contains complexed EF-2, whereas fraction II contains free EF-2. Fraction III which contains mainly tRNA and other small molecules was found to be devoid of EF-2.

When extracts from developing *Artemia* embryos

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

Incubation time (h)	Embryos (%)		Protein (mg)		EF-2 (nmol)			EF-2 (%)	
	Emerged	Hatched	Complexed	Free	Complexed	Free	Total	Complexed	Free
0	0	0	4.2	16.2	1.14±0.05	1.41±0.08	2.55±0.13	45	55
9	0	0	4.4	16.1	0.80±0.04	1.49±0.07	2.29±0.11	35	65
23	21	3	3.4	16.0	0.60±0.04	1.56±0.09	2.16±0.13	28	72
31	10	14	2.3	15.7	0.52±0.04	1.65±0.09	2.17±0.13	24	76
33	4	29	2.3	19.2	0.56±0.04	2.25±0.20	2.81±0.24	20	80

NOTE: The complexed and free protein fractions were prepared by chromatography on columns of Sepharose 6B of extracts from 12 g hydrated cysts and developing embryos as described in Materials and methods. The quantities of protein and EF-2 are given per gram dry weight cysts initially incubated. Complexed EF-2 and proteins were measured after release from associated macromolecular components with 0.5 *M* NH_4Cl as described in Materials and methods. The values for milligrrams and nanomoles are averages of three and eight determinations, respectively. Hatching started at about 23 h of incubation.

were fractionated on columns of Sepharose 6B, elution profiles were obtained similar to that shown in Fig. 1. From these experiments the amount and distribution of complexed and free EF-2 were determined on samples from several stages of development. These results are summarized in Table 1. The data show that, while the amount of total EF-2 decreased by 15% during the first 31 h of development, the content of complexed EF-2 and other complexed proteins decreased by almost 50% during this period. During this same period of development the content of free EF-2 increased by 17%, whereas the level of free proteins did not change appreciably. Between 31 and 33 h of development a marked increase occurred in both free EF-2 (36%) and free proteins (22%), while the content of complexed EF-2 and proteins remained relatively constant. The data also showed that a gradual decrease occurred in total EF-2 during the first 31 h of development, followed by an increase during the next 2 h. It should be noted that the embryos used for the present study showed about 65% hatching when 1 g of fully hydrated cysts was incubated in glass storage dishes (9). The decreased level of hatching observed in the experiments summarized in Table 1 are probably the result of a high population density of embryos and not due to a loss of cyst viability (9).

Distribution of EF-2 among various subcellular fractions

In an attempt to identify the macromolecular form of complexed EF-2, postmitochondrial fractions from 0-h *Artemia* embryos were fractionated on sucrose density gradients and the EF-2 content of the major fractions was measured. The results in Fig. 2 show representative sucrose density gradient profiles of postmitochondrial fractions from 0-h to 20-h *Artemia* embryos which are in agreement with previous studies (2). When the cytosol, monoribosome, and polyribosome fractions were pooled (avoiding overlapping regions) as shown in Figs. 2*a* and 2*b*, treated with 0.5 *M* NH_4Cl to release complexed EF-2 (if present), and assayed for EF-2 content, the results shown in Table 2 were obtained. These results indicate that only 1–3% of the total EF-2 of the postmitochondrial fraction is associated with monoribosomes or polyribosomes (when present) in both 0-h and 20-h embryos. Thus the complexed EF-2 must be associated with some other macromolecular fraction.

To test the idea that the cytosol from *Artemia* cysts contains both free and complexed EF-2, the postmitochondrial fraction was fractionated on sucrose density gradients as shown in Fig. 2*c* and then analyzed for the presence of free and complexed EF-2 as described in Materials and methods. The results of this experiment showed that *Artemia* cysts contain two fractions of EF-2 which sediment into the sucrose gradient and one that remains near the top of the gradient. On the basis of the sedimentation coefficient of known markers in the postmitochondrial fraction of *Artemia* embryos (11), it appears that the rapidly sedimenting EF-2 is associated with complexes having coefficients of 16S–20S and 40S–50S. The free or slow sedimenting EF-2 and complexed EF-2 released by NH_4Cl treatment had a molecular weight of 95 000 and sedimentation constant of 5.2S (unpublished observations).

Activity of complexed EF-2 in protein synthesis

The activity of complexed EF-2 purified by sucrose density gradient centrifugation compared with the purified EF-2 is shown in Table 3. These data indicate that complexed EF-2 is 25–75 times more active in promoting polypeptide chain elongation than EF-2 purified to near homogeneity from this EF-2 fraction. Reasons for these findings will be discussed.

Discussion

The diphtheria-toxin-catalyzed ADP-ribosylation reaction has provided a simple and rapid means to quantitate

TABLE 2. Distribution of EF-2 between cytosol, monoribosomes, and polyribosomes prepared by sucrose density gradient centrifugation from 0- to 20-h embryos

Incubation time (h)	Cytosol EF-2 (pmol)	Monoribosomes			Polyribosomes			Total EF-2/ total ribosomes
		EF-2 (pmol)	Ribosomes (pmol)	EF-2/ribosomes	EF-2 (pmol)	Ribosomes (pmol)	EF-2/ribosomes	
0	740	11.2	468	0.02	—	—	—	1.6
20	845	8.6	252	0.03	15.3	348	0.04	1.4

NOTE: Embryo incubation conditions were described in Table 1. In the 20-h sample, 20% of the embryos had emerged and 0.5% had hatched. All values are the averages of five measurements on samples pooled from 15 sucrose gradients (about 10 A_{260} units were applied per gradient). The cytosol (A), monoribosome (C), and polyribosome (E) fractions were pooled as described in Figs. 2*a* and 2*b*. The amount of ribosomes was calculated from their absorbance at 260 nm assuming the molecular weight of 3.8×10^6 and E_{260} 1 mg/mL equal to 12.0 for *Artemia* ribosomes (19).

TABLE 3. Polypeptide synthesizing activity of different EF-2 preparations from *Artemia* cysts[a]

Fraction	EF-2 added (pmol)	[^{14}C]Phenylalanine incorporation (pmol/h per microgram EF-2)
Control	Nil	2–3
Purified EF-2	1.0	42–50
Complexed EF-2	1.1	1088–3674

[a]The combined sucrose gradient pellets (in buffer C) shown in Fig. 2*c* were assayed for EF-2 activity in a cell-free protein synthesizing system as described in Materials and methods. The purified EF-2 was prepared from the P-150 fraction as described in Materials and methods. The EF-2 content of each fraction to be tested was determined using the [^{3}H]ADP-ribosylation method described herein and where 1 pmol EF-2 weighs 0.097 μg. The data shown represent the range of values obtained from three separate experiments.

EF-2 and to determine its distribution in *Artemia* during development. The results of this study have established the existence of complexed and free forms of EF-2 in *Artemia* embryos and demonstrate that changes occur in the distribution of EF-2 (between complexed and free forms) during early development. In dormant cysts 40–45% of the total EF-2 is in macromolecular complexes and as development proceeds the amount of EF-2 present in this form decreases to about 20% in hatched embryos. Although the complexed EF-2 appears to contribute to the pool of free EF-2 in the embryo, the net effect is a 15% reduction in total EF-2 during prenaupliar development. Following hatching there is a marked increase (48%) in the pool of free EF-2 within the first 10 h which might be due to *de novo* synthesis. Additional studies on the EF-2 content and distribution in more advanced embryos have not been performed, owing to the high level of protease activity in hatched embryos (20). The decrease in complexed EF-2 and increase in free EF-2 during early development of *Artemia* coincides with a general increase in the rate of protein synthesis in the intact embryo (1, 12). A similar correlation has been observed by other workers using Hela cells (21, 22).

Although we have used three different fractionation procedures to demonstrate the presence of complexed EF-2 in extracts from *Artemia* embryos, the identity of the macromolecular complexes containing EF-2 has not yet been fully characterized. However, from the data in Fig. 2*c* and Table 2 and previous findings (12), it appears that complexed EF-2 in *Artemia* embryos is associated with two multiprotein complexes which sediment at 16S–20S and 40S–50S and not with ribosomes or polysomes. Also, preliminary analysis of these complexes by SDS–polyacrylamide gel electrophoresis suggests that these complexes are not merely aggregates of cytosol proteins (12); in fact, they may be similar to the elongation factor rich 20S particles found

in the postmitochondrial fraction of rabbit reticulocytes by Hradec and Dusek (23). It should be noted, however, that these results contrast with those of Smulson and co-workers who reported that between 20 and 30% of the EF-2 in Hela cells is ribosome bound (21, 22), with Traugh and Collier who observed similar results with rabbit reticulocytes (24), and with Gill and Dinius who reported that 70–90% of the EF-2 from various organs of the rat is sedimentable and probably associated with ribosomes (25). However, since most of the results by other investigators were obtained using ribosomes prepared by differential centrifugation or sucrose fractionation in low ionic strength buffers (conditions which do not permit the complete separation of ribosomes from other macromolecular complexes), the exact subcellular localization of bound or complexed EF-2 in these tissues remains an unanswered question. Our data indicate that neither ribosomes nor polyribosomes are storage sites of EF-2 in *Artemia* embryos. Despite the differences reported for the subcellular localization of bound or complexed EF-2, *Artemia* embryos contain approximately 1.4–1.6 molecules of EF-2 for each ribosome, compared with 1.5–2.0 EF-2 per ribosome in reticulocytes (24) and 1.1–1.5 EF-2 per ribosome in various organs of the rat (25).

The functional similarities (or differences) between complexed and free EF-2 from *Artemia* embryos have not been studied in detail owing to the presence of a protein synthesis inhibitor in the cytosol of *Artemia* embryos which resembles EF-2 and which interferes with the elongation reaction (8). Notwithstanding these difficulties, we have observed that the EF-2 rich complexes (16S–20S and 40S–50S) are severalfold more active in stimulating polyphenylalanine synthesis than purified EF-2 at similar concentrations (see Table 3). However, treatment of these complexes with 0.5 *M* NH_4Cl to solubilize the EF-2 lowers the polymerizing activity by over 80%, despite the fact that the solubilized EF-2 behaves like purified cyst EF-2 on sucrose gradients, gel filtration columns, and SDS–polyacrylamide gels (unpublished observations). Thus it appears that considerable EF-2 is stored in *Artemia* embryos in macromolecular complexes which are functionally active in protein synthesis. Whether the high level of polymerization activity of these complexes is due to EF-2 or some other protein such as EF-3 described in yeast extracts remains to be determined (26).

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