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CHARACTERIZATION OF ELONGATION FACTOR 2
DURING EARLY DEVELOPMENT OF THE
BRINE SHRIMP *ARTEMIA SALINA*

by

Zipora Yablonka-Reuveni

A Dissertation
submitted to the Faculty of Graduate Studies
through the Department of Biology
in Partial Fulfillment of the requirements
for the Degree of Doctor of Philosophy
at The University of Windsor

Windsor, Ontario, Canada

1979

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DEDICATION

To my family and colleagues whose
understanding and encouragement
have led me through many
years of learning

ABSTRACT

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

by

Zipora Yablonka-Reuveni

The $[^3H]$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. In *Artemia* embryos part of the EF-2 is soluble (free) while 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. Very little EF-2 was found associated with either monoribosomes or polyribosomes of *Artemia* embryos and the data suggest that most of the EF-2 in the particulate fraction is bound to a macromolecule or complex other than ribosomes. The molecular weight of *Artemia* embryo EF-2 was found to be 95,000 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and no differences could be detected in the molecular weight or GTP binding activity of the free and bound EF-2 after release of the latter from its complex. However, the molecular weight studies revealed that extracts from hatched embryos contain more low molecular weight polypeptides which accept

ADP ribose than extracts from pre-hatched and dormant embryos. Furthermore, the low molecular weight polypeptides did not exhibit the functional properties of EF-2. In the intact embryo the rate of protein synthesis was found to increase throughout development, whereas the capacity of soluble extracts from newly hatched or post-hatched embryos to support poly(U)-directed protein synthesis was less than that for extracts of prenauplii. The data indicate that the small polypeptides which accept ADP ribose are specific degradation products of intact 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. This degradation process in Artemia embryos raises the possibility that this event may be a general phenomenon in the metabolism of EF-2 in eukaryotes. The changes in distribution of EF-2 and subsequent proteolytic cleavage of this protein during early development may function as a means of regulating the amount of EF-2 at the site of protein synthesis. A method for the purification of EF-2 from the particulate fraction of Artemia cysts has also been developed. The purified EF-2 preparation exhibits one major protein of 95,000 molecular weight which binds the ADP-ribose moiety of NAD in the diphtheria toxin-dependent reaction.

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I. INTRODUCTION

The unfertilized egg is generally considered to be metabolically repressed and fertilization results in metabolic derepression or activation. This is often true for eggs in which fertilization activates completion of meiosis and in eggs which complete meiosis before ovulation (e.g surf clam and sea urchin, see refs. 1 and 2 for summary of this work). This derepression is especially apparent in altering the rate of protein synthesis which may increase 2-30 times depending on the organism (1-5). The most studied organism in this area of research is the sea urchin. Protein synthesis in unfertilized eggs of the sea urchin proceeds at a low rate. Following fertilization the zygotes synthesize the same spectrum of polypeptides at a rate that is 10-30 times greater than in the unfertilized eggs (4-6). By the gastrula stage a rate of protein synthesis is reached which is 100 times higher than that in the mature egg (7). This increase in rate of protein synthesis is related to an increase in the total number of ribosomes but it is independent of ribosomal or messenger RNA synthesis (5, 8-10). Evidence has accumulated that unmasking of maternal mRNA results in a large stimulation of protein synthesis after fertilization of the sea urchin egg (11). In addition to the unmasking process, the translational efficiency (elongation plus termination) in the sea urchin zygotes increases 2 to 3-fold shortly after fertilization (12, 13).

In contrast to the sea urchins, oocytes of amphibia complete meiosis upon hormonal induction of ovulation and the rate of protein synthesis increases at this stage rather than after fertilization (1, 14, 15). Ecker and Smith have shown that eggs and embryos of _Rana_ _pipiens_ synthesize proteins at the same rate at least through the first 4-6 hours of cleavage (14). Studies by Woodland have shown that oocytes of _Xenopus_ _laevis_ retain only 1-2% of the total ribosomes in polyribosomes. Upon conversion to an egg the polyribosome content rises about 3-fold and after fertilization a further 2-fold increase in polyribosome content occurs. Only at the onset of organogenesis in Xenopus does the polyribosome content increase markedly (16). New synthesis of RNA is not required for the first stages of amphibian embryogenesis and the ribosomes that are entering polyribosomes during early development of amphibia are those stored in the oocytes (16-18). It has been postulated that ribosomes are not rate limiting of protein synthesis in amphibian oocytes since cell-free systems from Xenopus oocytes support poly(U)-directed protein synthesis (19). This idea is also supported by the findings that oocytes and eggs of Xenopus translate exogenous mRNA efficiently when it is introduced by microinjection (20). Results obtained using the latter technique also support the view that the initiation step in protein synthesis is not rate limiting in Xenopus oocytes (21).

Some molecular aspects of mammalian embryogenesis have been studied (see refs. 22 and 23 for the most recent publications in this field). Early development in mammals resembles the amphibian development in respect to hormonal induction of egg ovulation and egg activation (1, 23). Studies by Epstein and Smith on mouse embryos have shown that a slight increase in protein synthesis occurs in preimplantation embryos; only in the early blastocyst stage was a 3 to 9-fold increase in protein synthesis observed (24). Studies on rabbit embryos have revealed a similar pattern of protein synthesis during early development (25).

Activation of protein synthesis may be evident in a completely different context during embryogenesis in the brine shrimp, Artemia salina. Gastrulae of Artemia follow one of two developmental routes; they may give rise to swimming nauplius larvae directly, or they may encyst and become dormant. Development will resume if the dormant cysts are hydrated and incubated under aerobic conditions at 15-30°C. At 30°C prenauplii will emerge from the cysts after 10 to 12-h incubation in sea water. After further incubation the prenauplii break free of their extra-embryonic membrane giving rise to free swimming nauplii by a process called hatching (26). Early studies by Clegg showed that protein synthesis resumes upon termination of dormancy (27). Cytological studies have also indicated that no new nuclear synthesis occurs between the dormant cyst and prenauplius stages but resumes after hatching (28, 29). However, during this time considerable differentiation occurs. The separation of morphogenesis from cell division in Artemia makes this organism an ideal system to study the program for protein synthesis during early development since changes in macromolecular synthesis clearly reflect differentiation of pre-existing cells. Additional studies by Golub and Clegg showed that dormant cysts contain a large store of monoribosomes but very few polyribosomes (30, 31). However, upon resumption of development at 30°C polyribosome formation is initiated and the activity of the protein synthesis apparatus increases markedly (26, 27, 31). In contrast to the above findings, cell-free systems from dormant embryos display no endogenous protein synthesis and they are unable to translate exogenous natural mRNA (31-33). However, cell-free extracts of developing embryos are active in protein synthesis (31-33). Thus, although brine shrimp cysts are not at the same developmental stage as sea urchin zygotes, many of the biochemical events associated with the resumption of protein synthesis in Artemia embryos are similar to those of newly fertilized sea urchins. For this reason Artemia salina embryos have become a useful developmental system for the study of protein synthesis.

The above findings raise two questions of fundamental importance regarding protein synthesis in Artemia. Firstly, what mechanism operates to control protein synthesis as the embryos enter dormancy? Secondly, how is protein synthesis reactivated and regulated following the termination of dormancy?

Protein synthesis may be regulated by the availability of certain tRNA's or their ability to be acylated. Variations in isoaccepting tRNA populations have been shown to occur during growth and development of many animal tissues (34-37). Similarily, differences have been demonstrated in the aminoacyl-tRNA synthetases from different developmental stages (38, 39). Studies using eggs and embryos of the sea urchin, Paracentrotus lividus, have demonstrated that differences exist in the leucyl- and lysyl-tRNA patterns and in the appearance of a new seryl-tRNA during early development (40, 41). Spadafora et al. have suggested that the alteration in leucyl-tRNA is due to changes in the synthetase level and not the tRNA level (41). In other studies, Yang and Comb observed changes in the distribution of lysyl-tRNA's between the soluble and particulate cell fractions of young embryos of Lytechinus variegatus (42). These data suggest that certain tRNA's might be important in protein synthesis regulation during development.

In an attempt to test the role of tRNA in controlling protein synthesis in Artemia embryos, Bagshaw et al. compared the tRNA populations of cysts and nauplii (43). Their findings showed that quantitative differences exist in nine isoaccepting tRNA species of dormant cysts and nauplius larvae but that qualitatively the tRNA's are identical. They also found that tRNA's from different stages can be acylated using enzymes from both dormant embryos and nauplii indicating that aminoacylation is independent of the developmental stage used for the enzyme preparation. It is also known that tRNA's from cysts and nauplii are interchangable and that they support protein synthesis in vitro to the same extent. Although quantitative differences in tRNA might reflect a control mechanism operating in vivo, it appears most likely that protein synthesis during development of Artemia is not controlled by changes in the tRNA populations.

The origin and intracellular distribution of mRNA's which direct protein synthesis in young embryos have been investigated as possible clues to the lack of endogenous protein synthesis in extracts from dormant Artemia cysts. Nilsson and Hultin showed that protein synthesis in young embryos utilizes pre-existing mRNA (44, 45). They demonstrated that dormant Artemia embryos contain membrane-associated poly(A)-containing heterogeneous RNA which stimulates protein synthesis in a cell-free system. In other studies Grosfeld and Littauer showed that dormant cysts contain mRNA associated with proteins (ribonucleoprotein particles) which when deproteinized stimulate protein synthesis in a heterologous cell-free system (46). A similar observation was reported by Felicetti et al. (47), Sierra et al. (48) and by Slegers and Kondo (49). Thus, the criteria for establishing the existence of a pool of mRNA in dormant Artemia cysts have been fulfilled and this mRNA appears to exist in an inactive or masked form in the dormant cysts. The analogy to the sea urchin system is clear since masked mRNA also appears to be stored in these embryos (11).

In the sea urchin, activation of masked mRNA may occur through polyadenylation (50). A similar activation by polyadenylation of mRNA has been suggested by Sierra et al. (48) and can be concluded from the studies of Simons et al. (51) in early development of Artemia. Slegers et al. have demonstrated that an RNA which inhibits translation is complexed with mRNA from dormant cysts and that removal of this RNA from mRNA restores the translatability of the mRNA in vitro (52). The inhibitory RNA may be responsible for the inactivity of extracts from dormant Artemia embryos in protein synthesis. The possibility that masked mRNA lacks the cap structure at the 5' terminus has also been tested but this does not appear to be the case. In Artemia most mRNA from dormant cysts is capped and dormant embryos contain measurable amounts of mRNA methylase activity which can add methyl groups to the 7-position of the terminal guanosine group of capped mRNA (53, 54). These results, however, do not exclude the possibility that compartments exist within the embryos which allow selective capping of groups of masked messengers with their subsequent activation. Whatever the mechanism for activating masked messengers, it seems probable that the translation of a particular population of mRNA is dependent on the developmental state of the embryo since ribonucleoprotein particles containing mRNA are present even after extensive development has occurred (46). Moreover, in vitro translation products synthesized on Artemia polyribosomes (55) and using Artemia mRNA (56) show distinct developmental specificity.

Studies by Ochoa's group led to the suggestion that dry cysts are markedly deficient in certain initiation factors and that this deficiency is removed as development proceeds (33, 57). They reported that a 20-fold increase occurs in eIF-2 activity in developed embryos compared to dormant cysts (58). In contrast, MacRae and Wahba found equivalent amounts of eIF-2 in encysted and developing embryos and suggested that eIF-2 and at least one other initiation factor do not limit protein synthesis in encysted Artemia embryos (59). Based on these results, it appears doubtfull that a deficiency of initiation factors is the reason why dormant embryos are unable to translate endogenous or exogenous mRNA. The idea that specific initiation factors are required for muscle differentiation (60) and during insect development (61) has not been confirmed or firmly established.

Extracts from both dormant and developed embryos of Artemia are able to translate poly(U) under conditions which circumvent the requirement for the normal initiation mechanism (31). These results suggest that the elongation factors from the dormant embryos are active in protein synthesis. However, Huang and Warner demonstrated that ribosomes from developing embryos are more efficient in poly(U) translation than ribosomes from undeveloped cysts (62). The latter investigators also showed that the binding efficiency of phenylalanyl-tRNA to ribosomes increases as development proceeds. The cyst ribosomes appear to be repressed by an inhibitor on the large ribosomal subunit which is released or inactivated during development (62). In contrast to the above, purified ribosomes from cysts or developed embryos do not show any apparent differences in structure (63) and activity (62).

The idea of repressed ribosomes has also been suggested in sea urchin eggs but this view has not been adopted by most workers. Monroy _et_ _al_. showed that trypsin treatment of ribosomes from unfertilized sea urchin eggs increases the activity of these ribosomes in poly(U) translation (64). They suggested that similar derepression of ribosomes can occur _in_ _vivo_ following fertilization. In other studies, Metafora and co-workers have isolated an inhibitor of protein synthesis from ribosomes of unfertilized eggs of _Paracentrotus_ _lividus_ (65, 66). The inhibitor is not a ribonuclease but a protein that decreases the binding of poly(U) and of phenylalanyl-tRNA to ribosomes. Although Gambino _et_ _al_. reported that the sea urchin protein synthesis inhibitor is absent from ribosomes of blastulae (66), Hille suggested that the inhibitor is still present in developing embryos of _Strongylocentrotus_ _purpuratus_ particularly in the monoribosome fraction (67).

Other mechanisms than those mentioned above may inhibit protein synthesis in dormant cysts and explain the inactivity of cell-free extracts from dormant cysts in the translation of natural mRNA's. In this respect Warner _et_ _al_. found a protein which resembles elongation factor 2 (EF-2) in both supernatant and ribosome-rich fractions from dormant cysts (68, 69). This protein acts at the level of elongation to inhibit poly(U)-directed protein synthesis. A different type of elongation inhibitor was detected by Lee-Huang _et al_. in the supernatant and ribosome fractions from dormant and developing _Artemia_ embryos (70). The inhibitor has been identified as an oligonucleotide which inhibits aminoacyl-tRNA binding to ribosomes. High concentrations of this compound also inhibit initiation of protein synthesis. This inhibitor can be inactivated by another oligonucleotide which has been found only in developing embryos. The latter oligonucleotide appears to function as an activator by base pairing to the inhibitor. The oligonucleotide inhibitor is similar in size and base composition to Heywood's chick embryo muscle translational control RNA (71), but it differs in that it has no specificity for mRNA. Inhibitors of the elongation step of protein synthesis have been reported in other systems including wheat germ (72) and muscle tissues (73) and probably such inhibitors have a general role in the regulation of protein synthesis.

In contrast to the vast studies on elongation factors in respect to their general catalytic activity in protein synthesis, studies of these factors during embryogenesis are few in number. In many eukaryotes elongation factor 1 (EF-1, the enzyme responsible for the binding of aminoacyl-tRNA to ribosomes) occurs in high (EF-1$_{H}$) and low (EF-1$_{L}$) molecular weight forms (74-80). During development the relative proportion of EF-1$_{L}$ increases in the nematode, _Turbatrix aceti_ (75) and in the brine shrimp (76). Slobin and Möller have shown that EF-1$_{H}$ is the main form of EF-1 in the dormant cyst of _Artemia_ and at hatching it is transformed to EF-1$_{L}$ (76). More recently Slobin and Möller have demonstrated that the EF-1$_{L}$ from developing embryos is similar to the bacterial factor EF-Tu and that EF-1$_{H}$ contains protein factors analogous to the bacterial EF-Tu and EF-Ts (77, 79). Moreover, they were able to purify a low molecular weight protein from developing embryos of *Artemia* which is analogous to the bacterial EF-Ts (79). Similar observation was reported for other eukaryotic systems (78, 80). From these studies Slobin and Möller have suggested that the EF-1_H is the storage form of eukaryotic EF-Tu and EF-Ts, whereas the active forms of the factors *in vivo* are the individual polypeptides. Thus, the size transformation of EF-1 may provide a mechanism by which the rate of protein synthesis can be altered during early development of *Artemia* (79).

The general role of eukaryotic elongation factor 2 (EF-2) and its prokaryotic counterpart (EF-G) in protein biosynthesis has been elucidated (81-83). The protein factor EF-2 is essential for the translocation process that appears to involve three coordinated reactions: ejection of a deacylated tRNA from the ribosome, movement of newly elongated peptidyl-tRNA from the aminoacyl site to the peptidyl site and the movement of the mRNA so that a new codon can be translated. All of the above events of translocation are coupled to the hydrolysis of GTP to GDP and inorganic phosphate. A unique feature of EF-2 is its inactivation by diphtheria toxin (84, 85). Diphtheria toxin catalyzes the transfer of the adenosine diphosphate ribose (ADPR) moiety of nicotinamide adenine dinucleotide (NAD) to the eukaryotic elonation factor 2 (EF-2) but not to the prokaryotic elongation factor (bacterial and organelle EF-G) or any other protein (86-89). Since one molecule of ADPR binds to one molecule of EF-2 in the presence of excess NAD and diphtheria toxin (90), the ADP-ribosylation reaction has provided a measure of the amount of EF-2 in extracts when isotopically labeled NAD is used (90-95).

Thus far no attempt has been made to study the role of EF-2 in the regulation of protein synthesis during early development. Studies using sea urchins showed that the activity of EF-2 in the post-ribosomal supernatant fraction increased by about 50% within 2 min after fertilization (96). Studies by Warner et al. have shown 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 (68, 69). Although these studies and the EF-1 studies are incomplete, they suggest that the elongation factors are regulated during development. Hence, changes in the content and/or activity of elongation factors may account for the observed increase in activity of the protein synthesizing apparatus following fertilization of the sea urchin (12, 13), following early embryogenesis in amphibia and upon resumption of development in Artemia.

In order to assess the regulatory role (if any) of EF-2 in protein synthesis during development, a detailed study of EF-2 was initiated using embryos of the brine shrimp, Artemia salina. In this dissertation research the content and distribution of EF-2 between the soluble and particulate fractions have been studied together with changes in the molecular size and functional properties of this elongation factor. Moreover, a purification procedure for EF-2 from Artemia embryos has been developed. The results of this study should contribute to our understanding of the role and metabolism of EF-2 during development.

II. MATERIALS AND METHODS

A. Materials

Encysted embryos of Artemia salina were from the Utah salterns and were supplied by Longlife Fishfood Products (Harrison, NJ and St. Thomas, Ont.). Diphtheria toxin (lot number D-298, 1800 lf/ml, about 13.2 mg/ml) was purchased from Connaught (Toronto, Ont.). Creatine phosphate, phosphocreatine kinase, GTP, ATP and poly(U) were from P-L Biochemicals (Milwaukee, Wis.). NAD, dithiothreitol (DTT), B-mercaptoethanol and 2,4,6-trinitrobenzenesulfonic acid (TNBS) were from Sigma (St. Louis, Mo.). Ribonuclease-free sucrose, soybean trypsin inhibitor and streptomycin sulfate were from Schwarz/Mann (Orangeburg, NY). Penicillin G (sodium) was from Ayerst (Montreal, Que.). Liquid scintillation fluors, 2,5-diphenyloxazol (PPO) and 1,4-bis[2-(5-phenyloxazolyl)]-benzene (POPOP), and tissue solubilizer NCS were from Amersham (Oakville, Ont.). All other reagents used were A.C.S grade.

The proteins used as molecular weight standards for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and gel filtration were as follows: E. coli B-galactosidase (β-Gal) (P-L Biochemicals); rabbit muscle phosphorylase a (Ph) and bovine serum albumin (BSA) (Sigma); human gamma globulin, ovalbumin (Ov), bovine pancreatic chymotrypsinogen and bovine pancreatic ribonuclease (RNase) (Mann Research Laboratories, New york, NY).

SDS-polyacrylamide gel electrophoresis reagents were from Bio-Rad (Richmond, Ca.). Sephadex G-25 (medium), Sephadex G-150 (superfine) and Sepharose 6B were from Pharamacia (Montreal, Que.). Ultrogel AcA 34 is a product of LKB and it was purchased from Fisher Scientific (Toronto, Ont.). DEAE-cellulose (Whatman, DE-32) and phosphocellulose (Whatman, P-11) were from Mandel Scientific (Montreal, Que.). Microporous filters (2.5 cm, 0.45 μm) for the

ADP-ribosylation assay were from Amicon (Lexington, Ma.); nitrocellulose filters (B-6, 2.4 cm, 0.45 µm) for the GTP binding assay were from Schleicher and Schuell (Keene, NH); filter paper disks (type 3, 2.3 cm) for all carbon-14 incorporation measurements were from Whatman (Fischer Scientific).

[8-^{3}H]GTP (specific activity 13.1 Ci/mmol), [adenine-2,8-^{3}H]NAD (specific activity 2.96 Ci/mmol) and [^{14}C]NaHCO$_3$ (specific activity 7.7 mCi/mmol) were from New England Nuclear (Boston Ma.); [^{14}C-U]phenylalanine (specific activity 486 mCi/mmol) was from Amersham.

B. Media, buffers and scintillation fluids

Hatch medium: 422 mM NaCl, 9.4 mM KCl, 25.4 mM $MgSO_4$, 22.7 mM $MgCl_2$, 1.4 mM $CaCl_2$, 0.5 mM $NaHCO_3$, 500 units/ml of penicillin G (Na), 25 µg/ml streptomycin sulfate.
For isotope incorporation studies $NaHCO_3$ was replaced with 10 mM Tris-HCl, pH 8.2 (23°C).

Buffer A: 50 mM Tris-HCl, pH 7.9 (4°C), 5 mM KCl, 10 mM $MgCl_2$, 1 mM DTT

Buffer B: 50 mM Tris-HCl, pH 7.9 (4°C), 50 mM KCl, 10 mM $MgCl_2$, 1 mM DTT

Buffer C: 50 mM Tris-HCl, pH 7.9 (4°C), 100 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, 10% glycerol

Buffer D: 50 mM Tris-HCl, pH 7.9 (4°C), 50 mM KCl, 0.6 mM EDTA, 1 mM DTT, 10% glycerol

Acrylamide gel sample buffer: 125 mM Tris-HCl, pH 6.8 (23°C), 10% SDS, 25% β-mercaptoethanol, 50% glycerol, 0.004% bromophenol blue

Scintillation fluid A: 0.4% PPO, 0.015% POPOP, in toluene

Scintillation fluid B: 58.8% scintillation fluid A, 41.2% ethylene glycol monomethyl ether

C. Methods

1. Preparation of Artemia embryos and incubation procedures

Embryos of Artemia at different stages of development were prepared to study the concentration and activity of EF-2 and other properties of embryo extracts. Encysted dormant embryos were hydrated in ice-cold 0.5 M NaCl then washed with ice-cold distilled water to remove floating cysts, debris and sand (97). The washed cysts were rinsed with homogenization buffer and either stored at -70°C or extracted directly (0-h embryos). When developing embryos were required for analysis, the cysts were rinsed with hatch medium and incubated in this medium at 30°C. The embryos were collected after various incubation periods, washed with homogenization buffer and either processed directly or stored at -70°C.

Two different incubation procedures were used. In some experiments 12-12.5 grams of fully hydrated cysts were incubated with 500 ml sterile hatch medium in a 2800 ml Fernbach culture flask. In other experiments 1 gram of fully hydrated cysts was incubated with 75 ml of sterile hatch medium in 100 x 80 cm glass storage dishes. (1 gram of fully hydrated cysts equals 0.4 gram dry cysts). In the latter case emergence started at about 9-h incubation and about 62-65% of the embryos hatched by 48-h incubation. When 12-12.5 grams of fully hydrated cysts were incubated, emergence and hatching began later and the extent of hatching was lower than when 1 gram of cysts was incubated (see Results). The decreased level of emergence and hatching observed using the first procedure above does not appear to be due to a loss of cyst viability and may be the result of an higher population density of embryos.

For isotope incorporation experiments cysts were treated with antiformin prior to incubation to remove bacterial spores attached to the cyst chorion (28, 97) and all glassware and reagents were sterilized before use. In early experiments the cysts were also washed with 0.1% benzalkonium chloride to remove fungi (97) but this treatment was omitted in this study since it was found that no fungi appeared in the embryos cultures after 5 days of incubation.

Unless indicated otherwise, the same commercial batch of Artemia cysts was used throughout the study and the methods for handling and counting the embryos were as described by Warner et al. (97).

2. Preparation of particulate and soluble fractions from Artemia embryos

Particulate and soluble fractions were prepared from post-mitochondrial supernatant fluids of Artemia embryos to determine the distribution of EF-2 within the embryo. Two different methods were used to obtain these fractions and each method is described below.

a. Fractionation of Artemia embryo extracts by differential centrifugation. Embryos from different stages of development were ground to a thick paste using an electric mortar (Torsion, model MG-2) with buffer A at 5°C. (Unless otherwise mentioned, all fractionation steps were performed at 0-5°C). Additional buffer A was added to a final volume of 8 times the initial volume of cysts and the preparation was stirred for 10 additional minutes. The homogenate was then centrifuged at 12,000 x g for 10 min and the supernatant fluid passed through a cheesecloth-glass wool-cheesecloth filter. The filtrate was centrifuged at 30,000 x g for 45 min and the supernatant fraction was removed avoiding the floating orange lipid layer. This fraction was centrifuged further at 150,000 x g (Beckman rotor 60 Ti) for 150 min. The supernatant fluid from this step was fractionated with $(NH_4)_2SO_4$ and the precipitate that formed between 0-70% saturation was collected by centrifugation, suspended in buffer D, and dialyzed against two changes of the same buffer for 16 h. Following dialysis the preparation was clarified by centrifugation. This fraction will be referred to as the S-150 fraction or free fraction prepared by differential centrifugation. This fraction contains the soluble or free EF-2. The pellet from the high speed centrifugation designated as P-150 fraction was suspended in buffer C and treated with NH_4Cl as described below. All fractions prepared as above and below were stored at -70°C.

b. Fractionation of Artemia embryo extracts on columns of Sepharose 6B. The 30,000 x g fraction was prepared as described above except that buffer C was used as the homogenization medium and the volume of the homogenate was 10 times the volume of the starting dry cysts. The 30,000 x g supernatant fluid was concentrated by vacuum dialysis to about 8 ml, clarified by centrifugation and applied immediately to a column of Sepharose 6B (2.5 x 91 cm) previously equilibrated with buffer C. The column was developed with buffer C at approximately 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 as described below. The column fractions that displayed EF-2 (in the absence of other treatments; see Fig. 1, peak II) were pooled and fractionated by $(NH_4)_2SO_4$. The precipitate that formed between 30-70% saturation was collected by centrifugation, dissolved in buffer D and dialyzed against 5 changes of this buffer over an 8-h period. Any insoluble material that formed was removed by centrifugation. This preparation will be referred to as free fraction prepared by chromatography on Sepharose 6B; it contains soluble or free EF-2. The contents of those fractions that showed trace amounts of EF-2 activity (see Fig. 1, peak I) were pooled and treated immediately with NH_4Cl as described below. The material that eluted last from the column (see Fig. 1, peak III) was not saved.

3. Fractionation of Artemia embryo extracts on sucrose density gradients

Sucrose density gradient analyses were performed to ascertain the amount of EF-2 associated with monoribosomes and polyribosomes of Artemia embryos. Embryos from different stages of development were ground to a thick paste using buffer C as described above except that the final volume of the homogenate was 7 times the volume of the starting dry cysts. The 30,000 x g supernatant fluid was obtained as described above and fractionated immediately on sucrose gradients or frozen in liquid nitrogen. Ten A_{260} units were fractionated on 12 ml of a 15-50% sucrose gradient in buffer C lacking glycerol. Prior to sample application, 0.4 ml of glycerol-free buffer C was layered on the sucrose gradient and the sample to be analyzed was layered under this buffer. Centrifugation was then carried out at 39,000 rpm (Beckman rotor SW 41) for 80 min (31). After the centrifugation fractions of about 0.3 ml were collected using an Isco gradient fractionator with continuous recording of absorbancy at 254 nm at a flow rate of 0.5 ml/min. The monoribosome and polyribosome fractions were pooled separately (see Fig. 5), concentrated by vacuum dialysis, dialyzed against 5 changes of buffer C over a 10-h period and treated with NH_4Cl as described below. The cytosol fraction that remained on top of the sucrose gradient (Fig. 5, fraction A) was divided into two parts. One half was concentrated by vacuum dialysis then passed through a column of Sephadex G-25 (1.5 x 18 cm) previously equilibrated with buffer D and the protein which eluted in the void volume was concentrated by vacuum dialysis. (This column filtration was carried out to remove small molecules from the cytosol fraction; in other experiments this was achieved by ammonium sulfate fractionation and dialysis). The second half was centrifuged at 140,000 x g for 7 h. The resultant supernatant fluid was concentrated and processed as described above for the first half of the sucrose gradient cytosol. The 140,000 x g sediment obtained above was suspended in buffer D unless indicated otherwise.

4. Preparation of EF-2 from the particulate fractions

To enable us to measure EF-2 in the particulate fractions (P-150 suspended in buffer C, fraction I from Sepharose 6B, monoribosome and polyribosome fractions from sucrose gradients) EF-2 was released from the particulate fraction by a treatment with 0.5 M NH_4Cl. Such a treatment was previously used to release EF-2 and other proteins from particulate fractions (95, 98, 99). 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 was removed by centrifugation at 150,000 x g for 4.5 h and the supernatant fluid was concentrated by vacuum dialysis. Finally, the sample was dialyzed against 4 changes of buffer D for 12 h, clarified by centrifugation and stored at -70°C. These fractions will be referred to as bound fractions unless specified otherwise; they contain bound EF-2. Also crude EF-2 released from partially purified P-150 fraction by the NH_4Cl treatment as described here was used as starting material in developing the EF-2 purification procedure described under Results.

5. Preparation of soluble extracts from Artemia embryos

Soluble extracts from *Artemia* embryos were prepared to determine the total quantity of EF-2 (free and bound) in the post-mitochondrial supernatant fraction and to test the ability of embryo extracts to support protein synthesis *in vitro*. One gram of fully hydrated cysts or incubated embryos was homogenized with a Duall type ground-glass homogenizer in 6 ml of buffer C. The homogenate was centrifuged at 12,000 x g for 10 min and the pellet obtained was washed with 2 ml of buffer C followed by centrifugation as above. The 12,000 x g supernatant fluids were pooled then centrifuged at 30,000 x g for 45 min. The entire supernatant fraction was collected, adjusted to contain 0.5 M NH_4Cl, then stirred gently at 0°C for 75 min. The insoluble macromolecules (including ribosomes) were removed by centrifugation at 140,000 x g for 7 h and the 0.5 M NH_4Cl soluble fraction was dialyzed for 16 h against 4 changes of buffer D. The preparation was clarified by centrifugation and part of the soluble material was concentrated by vacuum dialysis. All fractions were stored at -70°C. These preparations will be referred to as soluble embryo extracts.

6. Preparation of salt washed ribosomes from dormant cysts of Artemia

Salt washed ribosomes were prepared to study the biological activity of EF-2 and the capacity of soluble extracts to support protein synthesis *in vitro*. The 150,000 x g pellet (P-150) from hydrated cysts was prepared as outlined above. The pellet was suspended in buffer C and treated with 0.5 M NH_4Cl as described above. The NH_4Cl treated preparation was layered over a cushion of 26% sucrose in glycerol-free buffer C and centrifuged at 150,000 x g for 4.5 h. The ribosome-rich pellet was suspended in the glycerol-free buffer C and the above centrifugation step was repeated. The transparent ribosome pellet was suspended in buffer B containing 25% glycerol to a final concentration of 200 A_{260} units per ml and stored in small aliquots at -70°C until needed. Ribosomes prepared in this way were found to be free of most non-ribosomal proteins and active in polyphenylalanine synthesis in the presence of soluble factors.

7. Uptake of $[^{14}C]$bicarbonate into Artemia embryo fractions

In order to measure the rate of protein synthesis at different stages of development, embryos (1 gram sterile hydrated cysts or 1 gram embryos incubated in 75 ml of hatch medium) at different stages of development were incubated for 2 h with $[^{14}C]$ $NaHCO_3$ at a concentration of 2.38 uCi per ml of hatch medium. At the end of the 2-h period, unlabeled $NaHCO_3$ was added to the medium to a final concentration of 0.02 M and the embryos were collected on miracloth filters (2.5 cm). The embryos were washed with 4x50 ml of 0.5 M NaCl containing 0.02 M $NaHCO_3$ and finally with 4x5 ml of buffer C. The embryos were stored at -70°C until needed. Embryos from different stages of development were homogenized with a Duall type ground-glass homogenizer in 7.5 ml of buffer C and the homogenates were centrifuged at 12,000 x g for 10 min. The supernatant fractions were saved (separately) and the 12,000 x g pellets were washed with 2.5 ml of buffer C. The washes were pooled with the original supernatant fractions and the combined soluble fractions were saved for radioactivity measurements. The buffer C-insoluble fractions were suspended in 2.5 ml of 0.1 N NaOH and incubated for 30 min at 30°C with occasional mixing. The alkali soluble material was collected by centrifugation at 12,000 x g for 10 min and the insoluble material (containing mainly shell fragments) was re-extracted with 1 ml of 0.1 N NaOH. The alkali soluble washes were pooled and the combined fractions were saved for radioactivity measurements. The amount of carbon-14 incorporated into the embryo fractions was determined as follows.

a. Total radioactivity incorporated. Samples of 25, 50 and 100 µl of the buffer C-soluble and the alkali-soluble fractions were transferred to filter paper disks, dried by circulating warm air and counted with 10 ml of scintillation fluid A using a Beckman LS 3150P liquid scintillation spectrophotometer.

b. Radioactivity incorporated into the fraction soluble in perchloric acid (PCA). To 1 ml of buffer C-soluble fraction 0.2 ml of 6N $HClO_4$ was added. The mixture was allowed to stand for 15 min at 0°C then centrifuged at 12,000 x g for 10 min. The supernatant fluid was removed and aliquots of 20, 25 and 50 µl were transferred to filter paper disks for the determination of radioactivity as described above. The alkali-soluble fraction was processed in the same way except that 0.3 ml of 6 N $HClO_4$ was added to 1.2 ml of alkali-soluble material.

C. Radioactivity incorporated into fractions insoluble in trichloroacetic acid (TCA). Aliquots of 25, 50 and 75 µl from the buffer C-soluble and alkali-soluble fractions were transferred to filter paper disks, dried by circulating warm air and processed according to Mans and Novelli (100) in order to determine the radioactivity in the protein fraction (hot TCA insoluble fraction). To determine the radioactivity in the combined protein-nucleic acid fraction, the hot TCA treatment was omitted (ethanol-ether insoluble fraction).

The radioactivity data from the buffer C-soluble and alkali-soluble fractions were combined for presentation under Results.

8. Diphtheria toxin-catalyzed ADP ribosylation of EF-2

The assay for the diphtheria toxin-dependent ADP ribosylation of EF-2 was according to the method of Maxwell *et al*. (86, 90, 91) as modified by A.H. Warner. The reaction mixture contained the following components in 0.1 ml: 1.65 µg diphtheria toxin, 6.25 µg BSA, 122 pmol [^{3}H]NAD (specific activity 820 Ci/mol), 0.25 µmol $MgCl_2$ and various quantities of EF-2 containing preparations or column fractions. The reaction mixture was incubated at 28°C for 45 min then the reaction was stopped by adding 2 ml of 5% ice-cold TCA containing 0.1 mM unlabeled NAD and 0.05 ml of BSA (10 mg/ml) was added as carrier protein. The protein-[^{3}H]ADPR complex was collected on microporous filters (0.45 µm) and washed with 3x2 ml of 5% ice-cold TCA. The filters were oven-dried at 125°C for 10 min and counted in 5 ml of scintillation fluid A as described above. The concentration of EF-2 was determined from the extent of $[^3H]$ADP ribosylation assuming 1 molecule of ADPR is bound to 1 molecule of EF-2 in the presence of diphtheria toxin and excess of NAD (90). The amount of EF-2 was determined by assaying at least 3 different protein concentrations in the linear range of the ADPR binding curve. In those experiments where $[^3H]$ADP-ribosylated protein preparations were required for analysis on SDS-polyacrylamide gels, the reactions were stopped by addition of one fourth volume of the polyacrylamide gel sample buffer.

9. Ribosome-dependent GTP binding activity of EF-2 preparations

The ribosome-dependent GTP binding assay employed here was a modification of previous methods (101, 102). The different EF-2 fractions were incubated in 0.2 ml final volume of the following mixture: 50 mM Tris-HCl, pH 7.0 (28°C), 50 mM KCl, 10 mM $MgCl_2$, 0.15 mM EDTA, 3.1% glycerol, 1 mM DTT, 576 pmol $[^3H]$GTP (specific activity 6050 Ci/mol) and 1 A_{260} unit salt washed Artemia cyst ribosomes. The reaction mixture was incubated for 10 min at 28°C and then diluted with 3 ml of ice-cold buffer B' (same as buffer B but at pH 7.3 (4°C)). The bound $[^3H]$GTP was collected on nitrocellulose filters (B-6, 0.45 µm) previously soaked in buffer B' and washed with 3x2 ml of this buffer. The filters were then dried at 125°C for 10 min, dissolved in 10 ml of scintillation fluid B and counted as described above.

10. Activity of EF-2 and soluble embryo extracts in polyphenylalanine synthesis

The synthesis of polyphenylalanine was determined by a modification of the method of Huang and Warner (62). To measure the activity of soluble extracts in supporting polyphenylalanine synthesis, aliquots of these extracts were incubated in 0.15 ml final volume of the following mixture:

50 mM Tris-HCl, pH 7.0 (30°C), 100 mM KCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 2.5% glycerol, 2 mM ATP, 0.8 mM GTP, 75 µg cratine phosphate, 12.5 µg phosphocreatine kinase, 12.5 µg poly(U), 0.5 A_{260} units of deacylated Artemia cyst tRNA (97), 257 pmol [^{14}C]phenylalanine (specific activity 486 Ci/mol) and 1 A_{260} unit of salt washed Artemia cyst ribosomes. The activity of partially purified EF-2 from Artemia in polyphenylalnine synthesis was tested in the mixture described above to which was added 60 µg protein from the 12-h soluble embryo extract as a source of additional factors. The reaction vessels were incubated at 30°C and after various times, 0.05 ml samples were removed from each vessel, transferred to filter paper disks and processed for polypeptide synthesis according to Mans and Novelli (100). The washed paper disks were suspended in 5 ml of scintillation fluid A and the radioactivity determined as described above. The extent of [^{14}C]polyphenylalanine formation was expressed as pmol phenylalanine polymerized per 30 min per 0.05 ml of reaction mixture.

11. General protease activity in extracts of Artemia embryos

The standard assay for protease activity measured the amino groups liberated during proteolysis as described by Nagainis and Warner (103) with slight modification. Aliquots of embryo extracts were incubated with 1.6 mg N,N-dimethyl BSA (104) and 0.1 M potassium phosphate, pH 8.0 (23°C) in a final volume of 0.4 ml. The incubation was performed at 5°, 28° and 40°C as indicated and at various times 0.05 ml aliquots were removed from each reaction vessel and processed for proteolysis using the TNBS assay described by Nagainis and Warner (103). One unit of protease activity was defined as the amount of enzyme that produces a change of 0.01 A_{420} unit in 1 min (as judged by the TNBS reaction). Occasionally, a lag in the protease activity was observed; in such assays the activity was calculated from the linear part of the absorbance curve (103).

12. Determination of protein content

Protein content was determined according to the method of Lowry et al. using BSA as a standard (105).

13. SDS-polyacrylamide gel electrophoresis of Artemia proteins

All protein preparations including the [^{3}H]ADP-ribosylated proteins were analyzed on 10% polyacrylamide gel slabs (1.5 x 140 x 80 mm) containing 0.1% SDS (sodium dodecyl sulfate) as described by Laemmli (106). To all protein samples to be analyzed by SDS gel electrophoresis, one fourth volume of polyacrylamide gel sample buffer was added and the mixture was heated at 90°C for 5 min. Electrophoresis was carried out at 120 volts until the marker dye (bromophenol blue) moved to the bottom of the gel (about 3 h). The gels were stained in a solution containing 0.1% coomassie brilliant blue, 50% methanol and 7.5% acetic acid (for about 1 h) and then destained in a solution containing 5% methanol and 7.5% acetic acid. When [^{3}H]ADP-ribosylated proteins were analyzed on SDS gels that portion of the gel representing a protein sample was sliced into 2 mm slices using a Bio-Rad® gel slicer (model 190). The slices were swollen in 0.5 ml of 90% tissue solubilizer NCS at 50°C for about 3 h and the radioactivity in each slice was measured after the addition of 10 ml of scintillation fluid A (107).

14. Molecular weight measurements of Artemia proteins

Molecular weight measurements of specific protein bands and [^{3}H]ADP-ribosylated polypeptides following electrophoresis on SDS-polyacrylamide gels were computed from a calibration curve obtained by plotting the electrophoretic mobilities of the standard polypeptides against the logarithm of their known polypeptide molecular weight (108).

The size of ADPR accepting proteins in different EF-2 preparations was also estimated from the elution position of these proteins following fractionation on columns of Sephadex G-150 (superfine, 0.9 x 41 cm). A sample of 0.3 ml from various EF-2 preparations was applied to the column and eluted with buffer D as described in the figure legends. The molecular size of the ADPR accepting proteins was estimated by comparison to the elution position of standard proteins.

III. RESULTS

A. Determination of elongation factor 2 content in Artemia during early development

1. Free and bound EF-2 at different stages of development

The quantity and distribution of EF-2 between the free and particulate fractions of *Artemia* post-mitochondrial supernatant fluid were determined in dormant and developing embryos. First, a method was devised to separate the soluble and particulate EF-2 and to determine the quantity of EF-2 in each fraction. When the 30,000 x 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 content using the diphtheria toxin-dependent ADP-ribosylation assay. Under these conditions virtually all of the EF-2 was found in fraction II while trace amounts were detected in fraction I. When the contents of fraction I were treated with 0.5 M NH_4Cl (75 min, 0°C) then centrifuged at 150,000 x g, considerable EF-2 was found in the salt soluble fraction as measured by the ADP-ribosylation assay. Since only free EF-2 can be assayed quantitatively by the ADP-ribosylation reaction (95, 99), the NH_4Cl treatment must have released EF-2 from the particulate substances in fraction I. No additional EF-2 was released by higher NH_4Cl concentrations. The difference in ADPR acceptance ability of Sepharose 6B column fractions I and II suggests that fraction I contains bound EF-2, whereas fraction II contains free EF-2. Fraction III was found to be devoid of EF-2.

When extracts from developing *Artemia* embryos 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 bound and free EF-2 were determined. These results are summarized in Table 1. The data show that a gradual decrease occurs in

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 Methods. Fraction I (140 ml) containing bound EF-2 and fraction II (120 ml) containing free EF-2 were pooled separately and saved for further studies.

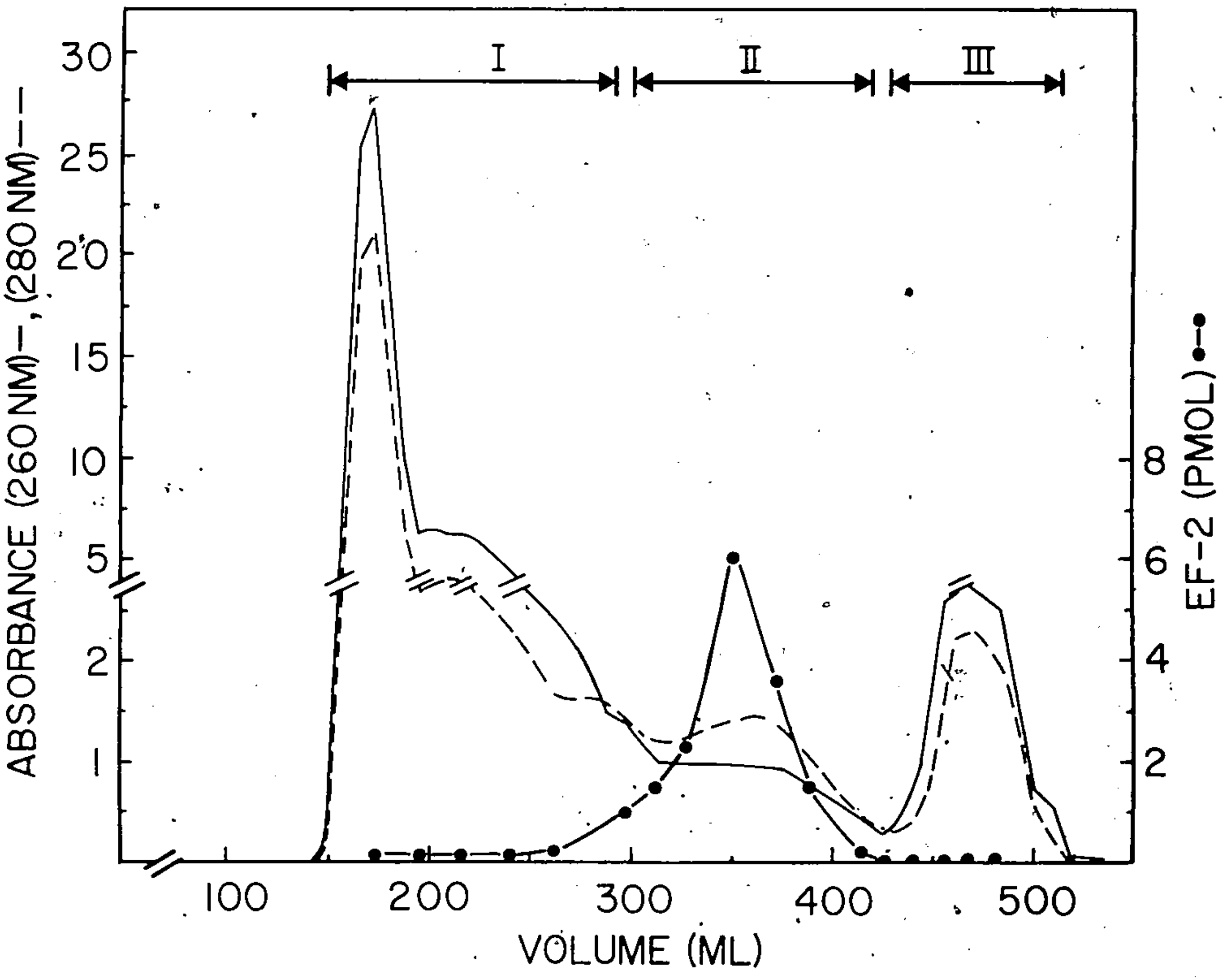

I
II
III
30
25
20
15
10
5
2
1
ABSORBANCE (260 NM)—, (280 NM)--
8
6
4
2
EF-2 (PMOL) •—•
100
200
300
400
500
VOLUME (ML)

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]	Embryos		Protein		EF-2			EF-2	
	Emerged	Hatched	Bound	Free	Bound	Free	Total	Bound	Free
h	%		mg		nmol			%	
0	0	0	4.2 (100)	16.2 (100)	1.14 (100)	1.41 (100)	2.55 (100)	45	55
9	0	0	4.4 (105)	16.1 (99)	0.80 (70)	1.49 (106)	2.29 (90)	35	65
23	21	3	3.4 (81)	16.0 (99)	0.60 (53)	1.56 (111)	2.16 (85)	28	72
31	10	14	2.3 (55)	15.7 (97)	0.52 (46)	1.65 (117)	2.17 (85)	24	76
33	4	29	2.3 (55)	19.2 (118)	0.56 (49)	2.25 (159)	2.81 (110)	20	80

[a] The 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.

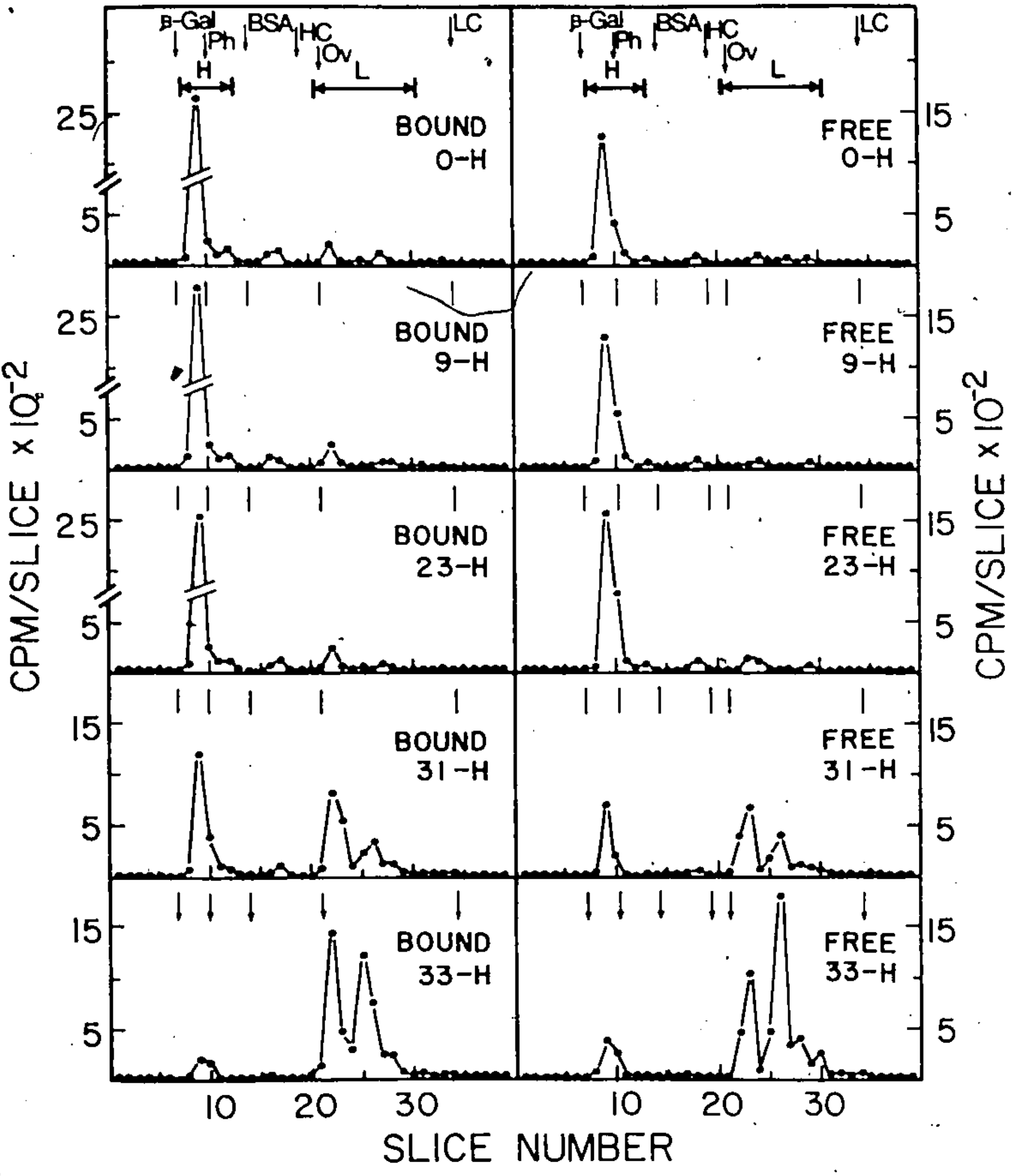
[b] Hatching started at about 23-h incubation.

total EF-2 during the first 31-h development followed by a marked increase during the next two hours. More striking however is the observation that bound EF-2 and other bound proteins decrease by almost 50% during the first 31-h development. In contrast, the level of free proteins does not change appreciably during the first 31-h development and the content of free EF-2 increases by only 17%. 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 bound proteins remains relatively constant.

2. Molecular weight of free and bound EF-2 from Artemia embryos at different stages of development

A study of the molecular weight of EF-2 was conducted to determine the molecular size of EF-2 and to access whether differences in molecular weight exist between free and bound EF-2 at different stages of development. In order to obviate the necessity for purified EF-2, all EF-2 containing fractions were $[^3H]$ADP ribosylated in the diphtheria toxin-dependent reaction and analyzed by electrophoresis on SDS-polyacrylamide gel slab. A similar analysis of EF-2 was described previously by Collins et al. (91) and it was also reported that the ADPR·EF-2 complex is not effected by denaturing reagents (109, 110). Gel electrophoretic analyses of ADP-ribosylated polypeptides from the free and bound protein fractions are shown in Fig. 2. The protein fractions used for these analyses are identical to those described in Table 1. The results indicate that several radioactive peaks are resolved by SDS gel electrophoresis. Moreover, a radiolabeled polypeptide of 95,000 molecular weight (MW) is dominant in all protein fractions from pre-hatched and newly-hatched embryos (0 to 23-h incubation), but beyond 23-h development at least two low molecular weight-radiolabeled polypeptides appear with molecular weights of 41,000 and 36,000. The appearance of the latter forms occurs concomitant with the disappearance

Fig. 2. SDS-polyacrylamide gel analysis of bound and free [^{3}H]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 under 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. 4. Control gels gave a background radioactivity of 20-25 cpm/gel slice.


β-Gal
Ph
BSA
HC
Ov
LC
H
L
BOUND 0-H
BOUND 9-H
BOUND 23-H
BOUND 31-H
BOUND 33-H
FREE 0-H
FREE 9-H
FREE 23-H
FREE 31-H
FREE 33-H
25
5
15
CPM/SLICE × 10^{-2}
10
20
30
SLICE NUMBER

of the heavier form. 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 distribution of EF-2 between the heavy and light forms in the bound and free protein preparations as they appear during development is given in Table 2. The ratio of light/heavy EF-2 has been used to measure the relative increase in the light EF-2's after hatching and it permits an estimation of trace amounts of light EF-2.

In order to determine the origin of the light EF-2 polypeptides, the bound and free protein preparations from 0 and 31-h *Artemia* embryos described in Table 1 were fractionated on columns of Sephadex G-150 and the elution position of ADPR accepting proteins was determined. The results in Fig. 3 indicate that both free and bound preparations from 31-h embryos contain the high and low molecular weight EF-2 proteins. Since chromatography on Sephadex G-150 will not resolve the two similar light EF-2 proteins observed on SDS-polyacrylamide gels, the second ADPR accepting peak to elute from the column probably contains the 41,000 and 36,000 MW forms of EF-2. These results indicate that the low molecular weight EF-2 proteins are not generated from the 95,000 MW form during the incubation period required for [^{3}H]ADP ribosylation of EF-2 or analysis of the resultant derivative on SDS-polyacrylamide gels.

3. SDS-polyacrylamide gel electrophoresis of free and bound protein preparations from Artemia embryos

To determine whether the change in the size of EF-2 and its distribution between free and bound subcellular fractions are associated with changes in the size of other soluble proteins from *Artemia* embryos, free and bound protein fractions from different developmetal stages were analyzed by SDS-polyacrylamide gel electrophoresis. This analysis is

TABLE 2

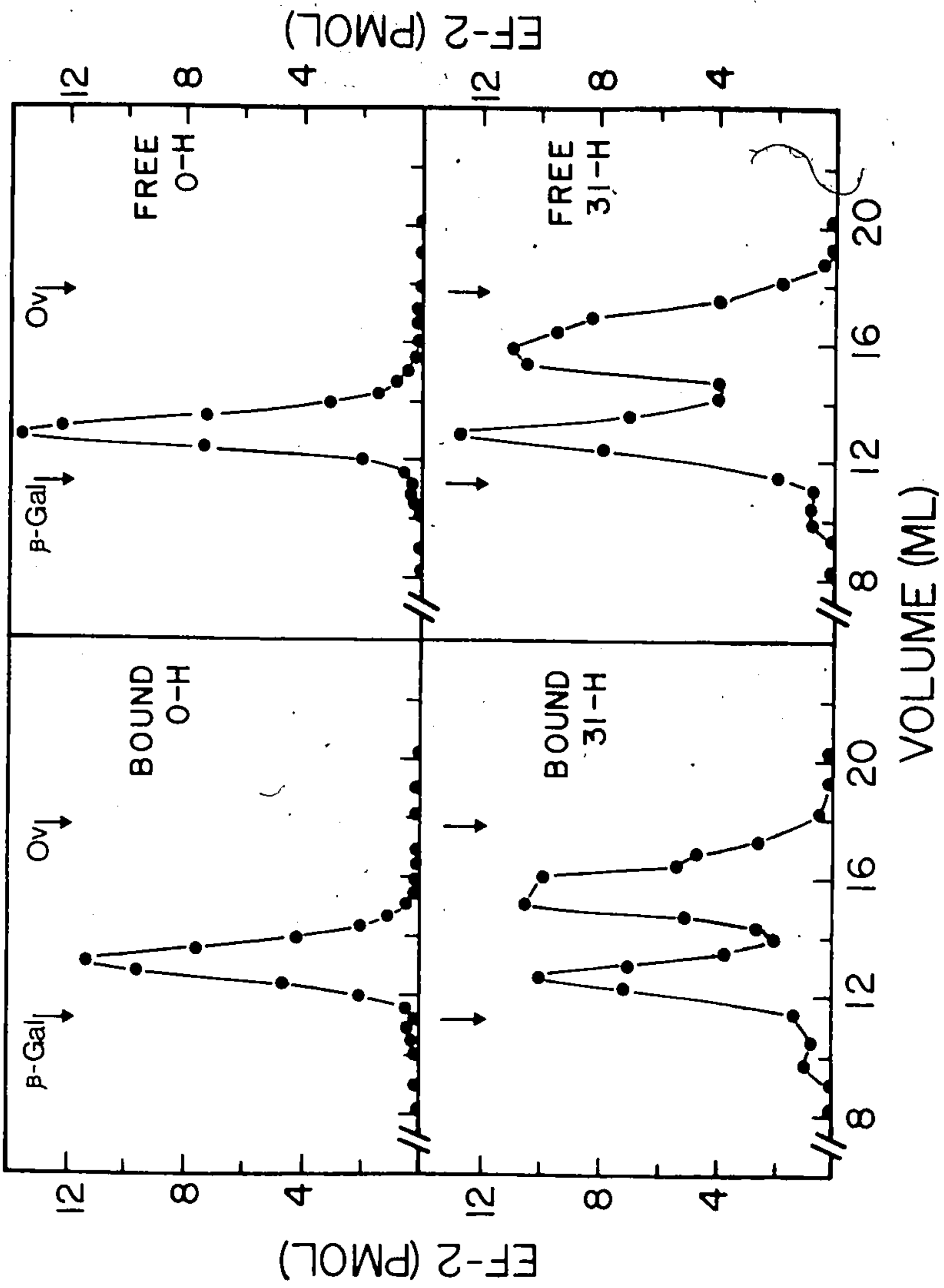
Distribution of light and heavy EF-2 in bound and free protein preparations during development[a]

Incubation time[b]	Embryos		EF-2	
	Emerged	Hatched	Bound	Free
h	%		light/heavy	
0	0	0	0.16	0.16
9	0	0	0.16	0.12
23	21	3	0.18	0.17
31	10	14	1.30	1.97
33	4	22	12.65	7.18

[a] The bound and free protein fractions were prepared by chromatography on columns of Sepharose 6B and are the same as those described in Table 1. The radioactivity in the heavy (H) and light (L) regions in Fig. 3 was quantitated following subtraction of background counts of 25 cpm/gel slice, and the data are expressed as ratios (L/H) of EF-2 in the different molecular weight regions.

[b] Hatching started at about 23-h incubation.

Fig. 3. Chromatography of bound and free protein preparations on a column of Sephadex G-150. Protein fractions containing bound and free EF-2 were prepared by chromatography of embryo extracts from 0 and 31-h incubated embryos on Sepharose 6B (see Table 1). In separate experiments aliquots (0.3 ml) of these preparations were then chromatographed on a column of Sephadex G-150 (superfine, 0.9 x 41 cm) previously equilibrated with buffer D. Proteins were eluted with buffer D at a flow rate of 1.3 ml/h. Fractions of 0.35-0.40 ml were collected and 0.05 ml of selected fractions was assayed for EF-2 as described in Methods. The elution position of β-galactosidase (β-Gal) and ovalbumin (Ov) chromatographed separately but under identical conditions is indicated at the top of the figure.


BOUND
0-H
FREE
0-H
BOUND
31-H
FREE
31-H
β-Gal
Ov
EF-2 (PMOL)
12
8
4
VOLUME (ML)
8
12
16
20

shown in Fig. 4. Free and bound protein preparations from extracts of embryos beyond 23-h development exhibit less polypeptides than the earlier stages. The decrease in intensity of a protein band with a mobility similar to phosphorylase in the latter preparations might reflect the decrease in the 95,000 MW form of EF-2 observed by the [^{3}H]ADP-ribosylation analysis. In addition, the appearance of new protein bands in the BSA and under the Ov (ovalbumin) regions of the gel slab was observed in bound protein preparations from 31 and 33-h embryos. Thus, this analysis has demonstrated that the polypeptide pattern changes in embryo extracts beyond 23-h development; these changes include the disappearance of the 95,000 molecular weight EF-2.

4. Fractionation of Artemia embryo extracts by differential centrifugation

The quantity, distribution and molecular size of Artemia embryo EF-2 were studied in embryo extracts prepared by differential centrifugation and the results were compared to data obtained by chromatography on Sepharose 6B. In this study the 30,000 x g supernatant fraction from 0 and 22-h embryos was subjected to centrifugation at 150,000 x g and the bound and free EF-2 fractions were prepared from the P-150 and S-150 fractions as described under Methods. A summary of the distribution of EF-2 between the bound and free fractions is given in Table 3. These results are similar to those shown in Table 1 and they support the idea that considerable EF-2 is released from the bound state during development. In other experiments the molecular size of the [^{3}H]ADPR accepting polypeptides from the P-150 and S-150 fractions of 0 and 22-h Artemia embryos was studied by SDS-polyacrylamide gel electrophoresis. The distribution of radioactivity was similar to that described in Fig. 2. The ratios between the light and heavy forms of EF-2 are given in Table 4. These results confirm those

Fig. 4. SDS-polyacrylamide gel analysis of free and bound protein fractions from Artemia embryos at different stages of development. The free and bound protein fractions were prepared by chromatography of embryo extracts on Sepharose 6B as shown in Fig. 1. Approximately 20 µg of free and bound proteins from the developmental times (h) shown at the bottom of the gel slab were electrophoresed. Free and Bound refer to free and bound protein fractions, respectively. The migration of known molecular weight markers is shown at the right. β-Gal, β-galactosidase, 130,000 MW; Ph, phosphorylase a, 94,000 MW; BSA, bovine serum albumin, 67,000 MW; HC, heavy chain of gamma globulin, 50,000 MW; Ov, ovalbumin, 43,000 MW; LC, light chain of gamma globulin, 23,500 MW; RNase, ribonuclease, 13,700 MW.

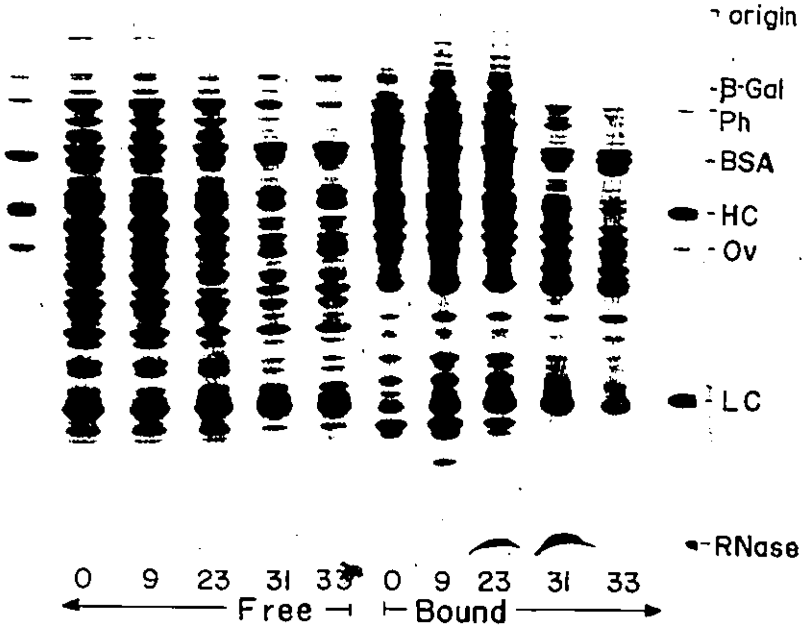

origin
β-Gal
Ph
BSA
HC
Ov
LC
RNase
0
9
23
31
33
0
9
23
31
33
Free
Bound

TABLE 3

Distribution of EF-2 between bound and free protein fractions prepared by differential centrifugation[a]

Incubation time[b]	Embryos		EF-2		
	Emerged	Hatched	Total	Bound	Free
h	%		nmol	%	
0	0	0	2.25	42	58
22	28	9	2.06	19	81

[a] The quantities of EF-2 are given per gram dry weight cysts initially incubated and the values are averages of 5 determinations.

[b] A different batch of *Artemia* cysts from the one described in Table 1 was used in this experiment and the incubation conditions were slightly different. In this study 12.5 grams fully hydrated cysts were incubated in 500 ml hatch medium. Hatching started at about 20-h incubation.

TABLE 4

Distribution of light and heavy forms of EF-2 in *Artemia* extracts prepared by differential centrifugation[a]

Incubation time	Embryos		EF-2	
	Emerged	Hatched	Bound	Free
h	%		light/heavy	
0	0	0	0.10	0.13
22	28	9	1.21	1.73

---

[a] Calculated from SDS-polyacrylamide gel slices as described for Table 2. The embryo incubation conditions are as described in Table 3.

observed using extracts fractionated by Sepharose 6B and indicate that the heavy molecular weigt EF-2 (95,000) is replaced by light forms of EF-2 following hatching of the embryos. In the present study the appearance of the light EF-2 proteins was observed earlier (22-h embryos) than in a previous experiment (see Table 2). This discrepancy might be due to the different commercial batches of cysts used in the two experiments.

5. Distribution of EF-2 between monoribosome, polyribosome and cytosol fractions at different stages of development

An analysis of polyribosome and monoribosome associated EF-2 as well as cytosol EF-2 was conducted to determine the amount of EF-2 bound to ribosomes and to ascertain whether the size of EF-2 is identical in the different fraction. For this part of the study, post-mitochondrial fractions of embryo extracts were fractionated on sucrose density gradients to obtain monoribosomes and/or polyribosomes free of particulate material which chromatographs with ribosomes on columns of Sepharose 6B or which sediments with ribosomes in the P-150 fraction prepared using the conditions described in Methods. Post-mitochondrial supernatant fractions were prepared from dormant cysts and developed embryos that were just beginning to hatch (20-h embryos, 20% emerged, 0.5% hatched). These supernatant fractions were then fractionated on sucrose density gradients and the monoribosome, polyribosome and cytosol fractions were pooled as described in Fig. 5. As expected from previous studies (31, 55) 0-h embryos were found to be deficient in polyribosomes compared to developed embryos. The monoribosome (C) and polyribosome (E) fractions were treated separately with 0.5 M NH_4Cl and the salt soluble fractions were collected by centrifugation at 150,000 x g. These salt soluble fractions and one-half of the cytosol fraction (A) were equilibrated with buffer D, $[^3H]$ADP ribosylated and the amount of EF-2 in each fraction was determined as shown in Table 5. The radiolabeled protein

Fig. 5. Sucrose density gradient profiles of the post-mitochondrial supernatant fractions from 0 and 20-h Artemia embryos. Twelve grams of hydrated cysts and an equivalent amount of 20-h embryos (20% emerged, 0.5% hatched) were homogenized separately with buffer C and the post-mitochondrial supernatant fractions were obtained as described in Methods. Ten A_{260} units of these fractions were layered onto separate 12 ml sucrose gradients (15-50%) and the preparations were centrifuged for 80 min at 39,000 rpm using SW 41 rotor. The gradients were fractionated with an Isco gradient-fractionator with continuous recording of absorbancy at 254 nm. Cytosol fractions (A), monoribosome fractions (C) and polyribosome fractions (E) were pooled separately as illustrated to avoid cross-contamination with adjacent components and concentrated by vacuum dialysis. Regions B and D were not saved for further analysis.

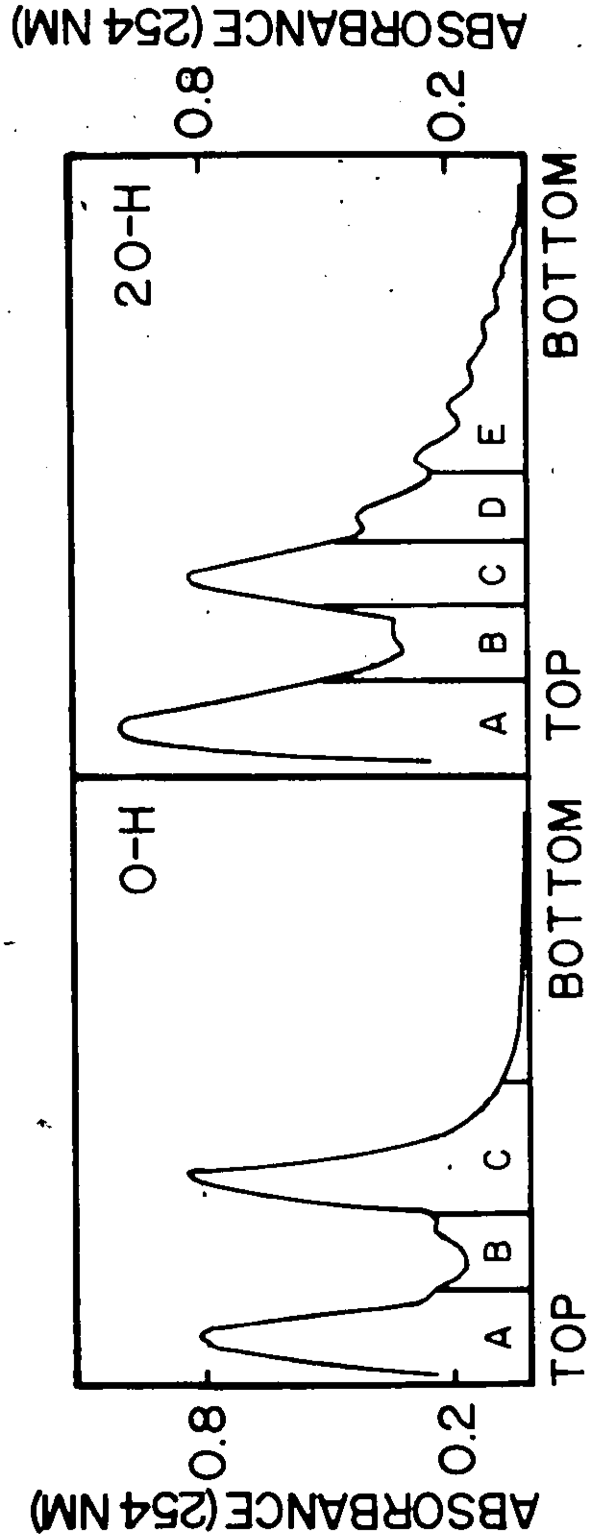

ABSORBANCE (254 NM)
0.8
0.2
20-H
A
B
C
D
E
TOP
BOTTOM
0-H
A
B
C
TOP
BOTTOM
ABSORBANCE (254 NM)
0.8
0.2

TABLE 5

Distribution of EF-2 between cytosol, monoribosomes and polyribosomes prepared by sucrose density gradient centrifugation from 0 and 20-h embryos[a]

Incubation time	Cytosol EF-2	Monoribosomes			Polyribosomes			Total EF-2/Total Rib
		EF-2	Rib[b]	EF-2/Rib	EF-2	Rib	EF-2/Rib	
h	pmol	pmol			pmol			
0	740	11.2	468	0.02	-	-	-	1.6
20	845	8.6	252	0.03	15.3	348	0.04	1.4

[a] Embryo incubation conditions were as described in Table 1. In the 20-h sample 20% of the embryos had emerged and 0.5% had hatched; about 2% hatching was observed after 22-h incubation of parallel cultures. All values are the averages of 5 measurements on samples pooled from 15 sucrose gradients (about 10 A_{260} units were applied per gradient). The cytosol, monoribosome and polyribosome fractions were pooled as described in Fig. 5.

[b] The amount of ribosomes (Rib) was calculated from their absorbance at 260 nm assuming the molecular weight of 3.8×10^6 and $E_{260}^{1mg/ml}$ equal to 12.0 for *Artemia* ribosomes (111).

fractions were also fractionated by SDS-polyacrylamide gel electrophoresis and the ratios between the light and heavy forms of EF-2 (L/H) were determined. These results are shown in Fig. 6. The dominant EF-2 in the cytosol and monoribosome fractions of 0 and 20-h embryos is the 95,000 MW species and the L/H values for the cytosol and the monoribosome fractions of 20-h embryos (0.23 and 0.51, respectively) are similar to their counterparts in 0-h embryos (0.11 and 0.46, respectively). In contrast to the above, the EF-2 in the polyribosome fraction of the 20-h embryos contains mostly the 41,000 MW form (L/H equals 3.11). Thus, the polyribosome EF-2 differs in size from the cytosol and monoribosome EF-2 of 20 or 0-h embryos and it is similar in size to the EF-2 that appears in extracts from post-hatched embryos.

The distribution of EF-2 among the various embryo fractions is shown in Table 5 and indicates that only 1-3% of the total embryo EF-2 is bound to the ribosomes in both 0 and 20-h embryo extracts. However, since some of the sucrose gradient fractions were discarded to avoid cross-contamination (Fig. 5 fractions B and D) this value is probably somewhat conservative. Nevertheless, it appears that most of the bound EF-2 recovered by Sepharose 6B and differential centrifugation remains in the cytosol fraction during sucrose gradient fractionation. To test whether the sucrose gradient cytosol contains bound EF-2 which is sedimentable, the remaining one-half of the 0 and 20-h cytosol fractions from the sucrose gradients described above (untreated cytosol) was subjected to high speed centrifugation (140,000 x g). This treatment sedimented a red-coloured pellet whose polypeptide pattern (for 0-h cytosol only) is shown in Fig. 7. When the latter sediment was tested in the diphtheria toxin-NAD assay it exhibited ADPR acceptence activity confirming the presence of EF-2 in this sediment. However, the EF-2 was detectable by ADP ribosylation only after suspension of the pellet in buffer D.

Fig. 6. SDS-polyacrylamide gel analysis of [^{3}H]ADP-ribosylated proteins from cytosol, monoribosome and polyribosome fractions of 0 and 20-h Artemia embryos. The cytosol, monoribosome and polyribosome fractions were prepared from 15 sucrose gradients as described in Fig. 5. The monoribosome and polyribosome fractions were treated with 0.5 M NH_4Cl as described in Methods and the NH_4Cl soluble fractions and the cytosol fraction were equilibrated with buffer D. Samples of these fractions were radiolabeled in a standard toxin-NAD assay and then analyzed by SDS gel electrophoresis as described in Methods and the ratios between the light and heavy forms of EF-2 (L/H) were calculated. C, M and P refer to cytosol, monoribosome and polyribosome fractions, respectively. The migration of phosphorylase (Ph) and ovalbumin (Ov) is indicated at the top of the figure.

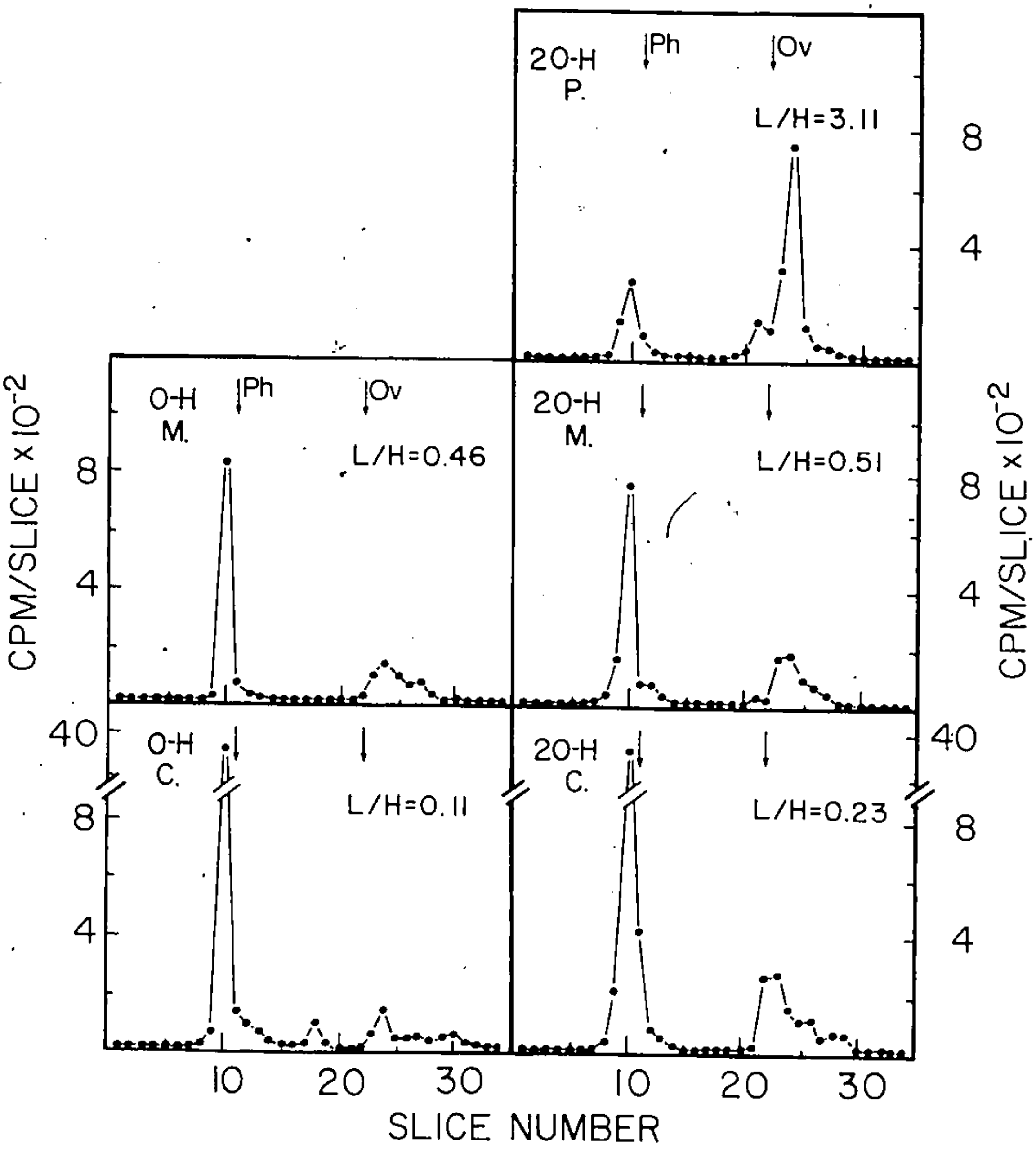
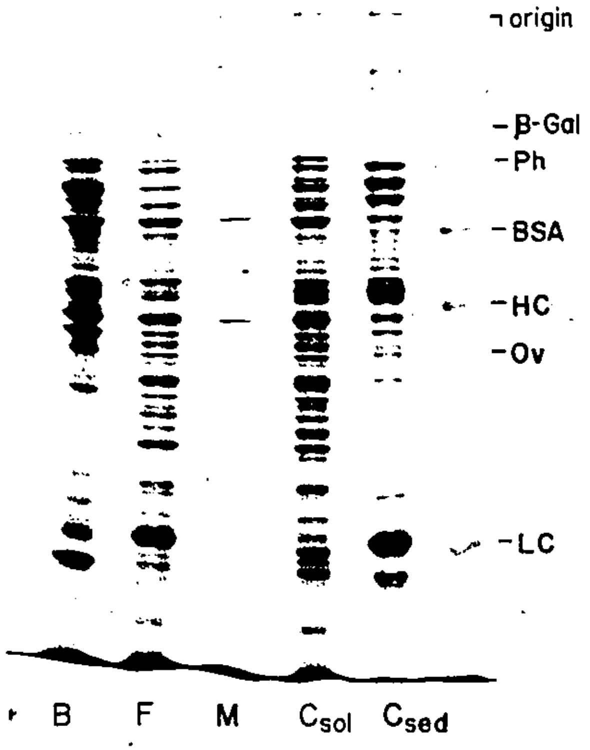

20-H
P.
Ph
Ov
L/H=3.11
0-H
M.
Ph
Ov
L/H=0.46
20-H
M.
L/H=0.51
0-H
C.
L/H=0.11
20-H
C.
L/H=0.23
CPM/SLICE x 10^-2
8
4
40
10
20
30
SLICE NUMBER

Fig. 7. SDS-polyacrylamide gel analysis of EF-2 containing fractions prepared from 0-h embryos 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 Methods to obtain the sedimentable and non-sedimentable proteins from the cytosol and the NH_4Cl soluble proteins from the monoribosome fraction. These fractions were analyzed by SDS gel electrophoresis and are indicated in the figure as C_{sed}, C_{sol} and M, respectively. For comparison, the bound and free protein fractions from 0-h embryos prepared by chromatography on Sepharose 6B were analyzed on the same gel slab. These samples are indicated in the figure as B and F, respectively. About 3 µg protein from M and 20 µg protein from all other samples were applied to the gel slab. The symbols 1-4 indicate polypeptide bands discussed in the text. The migration position of the 95,000 MW EF-2 and standard molecular weight markers is also indicated.


origin
β-Gal
Ph
BSA
HC
Ov
LC
B
F
M
C_{sol}
C_{sed}

Very little activity could be detected when the pellet was suspended in buffer C. Since buffer D contains EDTA which can release EF-2 from ribosomes (93, 94), it was concluded that EF-2 is present in the red-coloured pellet in a bound form. By comparison, the supernatant fluid above the red pellet contains EF-2 which can be ADP ribosylated by the toxin without prior treatment with dissociation reagents such as NH_4Cl or EDTA. The soluble and the sedimentable EF-2 prepared from the sucrose gradient cytosol fraction show the same pattern of $[^3H]$ADP ribosylation on SDS-polyacrylamide gel (data not shown). Collectively, the above results suggest that Artemia embryos contain EF-2 that is free in the cytosol and active in ADPR acceptance, EF-2 which is bound to monoribosomes and polyribosomes and EF-2 which is bound to some macromolecular component(s) in the cytosol with a particulate nature different from that of ribosomes.

The cytosol proteins from 0-h embryos which were prepared by sucrose gradient fractionation followed by high speed centrifugation were analyzed by SDS-polyacrylamide gel electrophoresis along with the 0.5 M NH_4Cl soluble proteins of the monoribosome fraction of 0-h embryos. The SDS gel patterns shown in Fig. 7 indicate that although the protein fractions have some polypeptides in common, they differ markedly in polypeptide composition suggesting that cross-contamination between these fractions is minimal. Furthermore, when the bound and free protein fractions prepared from 0-h embryos by Sepharose 6B fractionation were also analyzed on the same gel slab for comparison (Fig. 7, samples B and F), the band patterns show that several polypeptides in the sedimentable cytosol fraction (C_{sed}) are partitioned between the bound and free Sepharose 6B fractions (e.g polypeptides labeled 2, 3 and 4 in Fig. 7). The latter findings are probably a reflection of the inability of the Sepharose 6B fractionation procedure to resolve completely the particulate and non-particulate proteins from Artemia extracts. This conclusion is supported by the UV-absorbance profile shown in Fig. 1.

It is noteworthy that many of the polypeptides which disappear from developed embryo extracts (see Fig. 4) could be found in the cytosol sedimentable fraction from 0-h embryo extracts prepared by sucrose gradient fractionation (e.g band 4 in C_{sed}, Fig. 7). When sucrose gradient fractions from the 20-h embryos were analyzed on SDS-polyacrylamide gel it was observed that polypeptide 1 (Fig. 7, sample M) is not present in the salt soluble fraction of either polyribosomes or monoribosomes (data not shown). A similar finding has been reported to occur during development of sea urchin (66). In addition, a polypeptide which migrates to the position of BSA (67,000 MW) was found in the salt soluble fraction from 20-h embryo polyribosomes but was barely detectable in the salt soluble fractions from monoribosomes of 0 and 20-h embryos. The latter polypeptide might be a fragment of EF-2 and this possibility will be discussed later.

B. Biological activity of different EF-2 preparations compared to the protein synthesis activity of intact embryos

1. Effect of heavy and light EF-2 on ribosome-dependent GTP binding activity

Biologically active EF-2 in most eukaryotic organisms has a molecular weight of about 95,000 when studied by SDS-polyacrylamide gel electrophoresis and eukaryotic EF-2 reacts covalently with the ADPR moiety of NAD in the presence of diphtheria toxin (90, 112-114). Smaller polypeptides can be generated from native EF-2 and some of them bind ADPR but these fragments are usually devoid of EF-2 function (91). Therefore, it was important to determine whether the low molecular weight EF-2 proteins that appear in hatched embryos of Artemia have biological properties similar to high molecular weight EF-2 or whether they are inactive. In this study the ability of low molecular weight EF-2 proteins (which bind ADPR) to stimulate ribosome-dependent GTP binding was tested. Free and bound EF-2 preparations were prepared by the Sepharose 6B method and purified further by chromatography on a column of Sephadex G-150 as described in Fig. 4. The heavy and light EF-2 fractions were pooled and tested for ribosome-dependent GTP binding. The results of this experiment are shown in Table 6. As expected from previous studies (101, 102), the addition of ribosomes to a mixture of GTP and heavy EF-2 enhances GTP binding activity by about 3-fold. In contrast, ribosome addition to a mixture of light EF-2 and GTP inhibits or destabilizes GTP binding despite the fact that the light EF-2 preparations alone bind about twice the amount of GTP than heavy EF-2. Thus, the low molecular weight EF-2 fraction does not show the characteristic GTP binding property of native EF-2. The inactivity of the light EF-2 compared to that of the heavy EF-2 will be shown later using more purified preparations (see Table 14).

2. Protein synthesis in intact embryos compared to the appearance of light forms of EF-2 in embryo extracts

Since the light forms of EF-2 did not show the GTP binding characteristics of native EF-2, an experiment was designed to test the possibility that the appearance of the low molecular weight EF-2 proteins during development may reflect a shut off in protein synthesis in *Artemia* embryos. Since encysted embryos of *Artemia* are impermeable to exogenous amino acids but permeable to bicarbonate (27), *Artemia* embryos were radiolabeled at various times of development using 2-h pulses of $[^{14}C]NaHCO_3$ and the radioactivity incorporated into various embryo fractions was determined as described in Methods. In a parallel experiment non-radiolabeled embryos were used to determine the extent of emergence and hatching, the amount of EF-2 at each stage and the time of appearance of the light forms of

TABLE 6

The effect of different EF-2 preparations on ribosome-dependent GTP binding activity[a]

EF-2			GTP bound[b]	
Source	Form	Amount	-Ribosomes	+Ribosomes
		pmol	pmol	
buffer	-	-	0.0	1.0
bound	heavy	40	8.3	22.2
bound	light	40	17.9	3.3
free	heavy	35	6.0	19.0
free	light	35	10.9	4.3

[a] Bound and free EF-2 were prepared from 31-h Artemia embryos by the Sepharose 6B method and the heavy and light EF-2 were purified by Sephadex G-150 (see Fig. 4). Since the two light forms of EF-2 (36,000 and 41,000 MW) can not be separated by column fractionation, they were tested together.

[b] The GTP binding assay was carried out as described in Methods. One A_{260} unit of salt washed ribosomes from 0-h embryos was added where indicated.

EF-2. The results of these studies are summarized in Tables 7 and 8 and in Fig 8. The carbon-14 incorporation data (Table 7, A and A') show that the rate of protein synthesis in Artemia embryos, calculated as percentage of the total recovered radioactivity or as percentage of the perchloric acid soluble radioactivity, increases with the age of the embryos. Thus, protein synthesis occurs at an increasing rate throughout early development. By comparison the rate of nucleic acid synthesis (Table 7, A-B and A'-B') has a different pattern; total nucleic acid synthesis is constant in pre-hatched embryos then increases over 2-fold after hatching. A peak in the rate of nucleic acid synthesis is seen in the 24-h embryos followed by a gradual decrease in more developed embryos. The pattern of protein and nucleic acid syntheses is in agreement with the previous studies of Clegg (27) and McClean and Warner (115), respectively.

In Table 8 the amount of EF-2 in soluble extracts from embryos at different stages of development is illustrated. In this experiment no attempt was made to separate the free and bound EF-2. The results show that in encysted and pre-hatched embryos the total amount of EF-2 is constant but increases 2 to 3-fold following hatching. The protein content of the 0 to 24-h extracts is similar to the amount recovered by the Sepharose 6B method (Table 1, free plus bound protein columns); however in the present experiment less EF-2 was recovered from the pre-hatched embryos than in the Sepharose 6B studies (Table 1). The differences may be due to the crude nature of the unfractionated preparations in the present experiment which might contain factors that interfere with the ADP-ribosylation assay in extracts from pre-hatched embryos.

The appearance of light forms of EF-2 in the soluble extracts from developing Artemia embryos is shown in Fig. 8. The light forms of EF-2 were first observed in extracts from 20-h embryos and the transition from heavy to light forms of EF-2 is more pronounced in the 24-h embryo extract. In the

TABLE 7

Incorporation of [^{14}C]NaHCO$_3$ into various fractions of *Artemia* embryos during development[a]

Exposure time to [^{14}C]NaHCO$_3$ during development[b]	Embryos		Radio-activity per embryo	Fraction of total recovered radioactivity				Fraction of cold PCA soluble radioactivity		
	Emerged	Hatched		Cold PCA Soluble	Ethanol-ether Insoluble A	Hot TCA Insoluble B	A-B	Ethanol-ether Insoluble A'	Hot TCA Insoluble B'	A'-B'
h	%		cpm			%			%	
3-5	0	0	7.8	73.9	10.3	7.6	2.7	13.9	10.3	3.6
10-12	16	0	11.2	69.8	16.3	13.8	2.5	23.3	19.8	3.5
22-24	8	28	13.1	65.0	20.6	14.5	6.1	31.7	22.3	9.4
28-30	7	39	10.3	64.3	22.4	16.8	5.6	34.8	26.1	8.7
33.5-35.5	6	42	9.9	62.0	22.9	18.4	4.5	36.9	29.6	7.3

[a] In general terms the cold PCA (perchloric acid) soluble fraction contains small molecules including amino acids and nucleotides. The insoluble fractions A and A' measure mostly nucleic acids and proteins; it was obtained by ethanol-ether treatment after the cold TCA wash. The insoluble fractions B and B' measure mostly proteins; it was obtained by hot TCA treatment after the cold TCA wash and further treated with ethanol-ether. The difference between radioactivity in the ethanol-ether insoluble and hot TCA insoluble fractions (A-B and A'-B') represents radioactivity in the nucleic acids.

[b] One gram of fully hydrated cysts (90,000 embryos) was incubated for each time point. At the end of the incubation period the extent of development was determined by counting aliquots of the embryos from non-radiolabeled dishes. Hatching started at about 16-h incubation.

TABLE 8

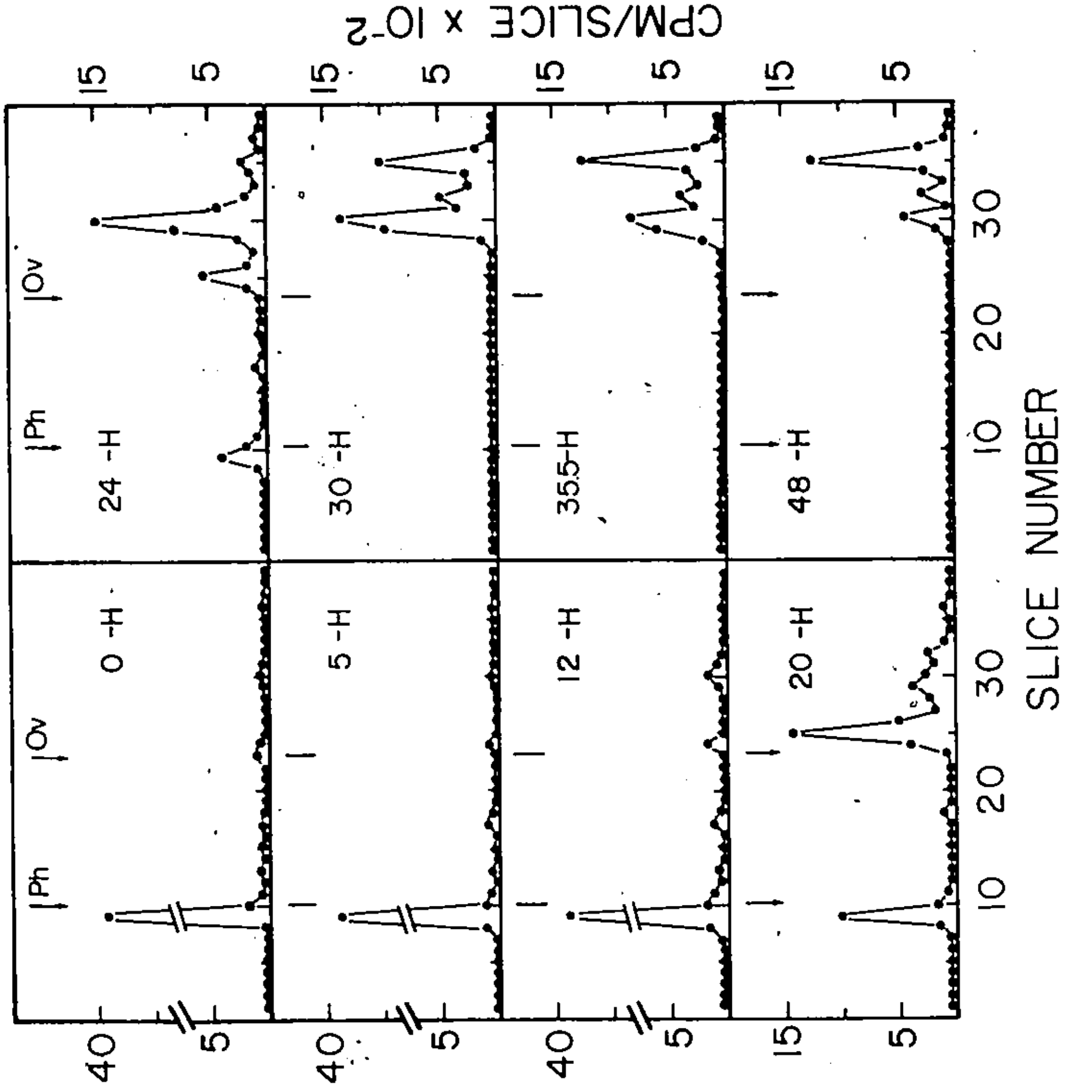
EF-2 and soluble protein content of *Artemia* embryos at different stages of development[a]

Incubation time[b]	Embryos		Protein	EF-2
	Emerged	Hatched		
h	%		mg	nmol
0	0	0	20.8	1.2
5	0	0	21.0	1.1
12	16	0	20.4	1.2
20	14	15	19.9	2.2
24	8	28	19.7	2.3
30	7	39	7.9	3.4
35.5	6	42	6.8	2.7
48	0	62	6.4	2.0

[a] One gram of fully hydrated cysts was incubated for each time point and the content of embryo EF-2 and protein was determined in the soluble extract (post ribosomal fraction prepared in the presence of 0.5 M NH_4Cl) after extensive dialysis. The values for protein and EF-2 are given for 1 gram dry weight of starting cysts and are averages of 5 measurements.

[b] Hatching started at about 18-h incubation.

Fig. 8. SDS-polyacrylamide gel analysis of [^{3}H]ADP-ribosylated proteins from soluble extracts of Artemia embryos at different stages of development. The soluble extracts described in Table 8 were treated with [^{3}H]NAD and diphtheria toxin and prepared for SDS gel electrophoresis as described in Methods. Following electrophoresis the migration position of the [^{3}H]ADP-ribosylated polypeptides was determined as described under Methods. The migration position of phosphorylase (Ph) and ovalbumin (Ov) is indicated at the top of the figure.


0 -H
5 -H
12 -H
20 -H
24 -H
30 -H
35.5-H
48 -H
Ph
Ov
40
5
15
10
20
30
CPM/SLICE x 10-2
SLICE NUMBER

latter extract there is also a preponderance of the 36,000 MW species. A further decrease in the molecular weight of ADPR accepting polypeptides is exhibited by soluble extracts from 30, 35.5 and 48-h embryos. The latter observation is probably due to protease activity that appears several hours after hatching. This protease activity may also account for the decrease in protein contents observed in extracts from 30 to 48-h embryos. This idea will be discussed later.

3. Activity of embryo extracts from various stages of Artemia development in polyphenylalanine synthesis

As described above, the light EF-2 proteins prepared from 31-h embryos are inactive in ribosome-dependent GTP binding (Table 6). This observation has suggested to us that the light forms of EF-2 in extracts from post-hatched embryos are probably inactive in protein synthesis. However, protein synthesis takes place in the intact embryos thoroughout the period studied (Table 7). Therefore, the activity of the post-ribosomal fraction of embryos at various stages of development (soluble extract prepared in the presence of 0.5 M NH_4Cl) in support of polyphenylalanine synthesis in vitro was studied. Also, the capacity of the soluble embryo extracts to synthesize polyphenylalanine was determined following the addition of purified EF-2 to these extracts. A summary of this study is given in Table 9. These results indicate that of those samples assayed the extract from 12-h embryos is the most active in polyphenylalanine synthesis. 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. These latter results are probably due to protease activity in the extracts. Data in support of this idea are presented below. The addition of purified EF-2 to the reaction mixtures stimulated polyphenylalanine synthesis in all cases,

TABLE 9

Activity of embryo extracts from various stages of *Artemia* development in polyphenylalanine synthesis[a]

Embryo age	Experiment 1[b]			Experiment 2				Increase by added EF-2
	In extract		Phenylalanine incorporated	In extract		Phenylalanine incorporated		
	Protein	EF-2		Protein	EF-2	Extract only	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	0.20	42
5	120	7	1.84	60	3.5	0.90	1.60	78
12	120	7	4.30	60	3.5	2.90	3.57	23
20	120	13	0.80	60	6.5	0.42	1.17	149
24	120	14	0.41	60	7.0	0.23	0.43	87
30	60	8	0.00					

[a] Embryo incubation conditions and the developmental profile were as in Table 8. The soluble extracts were prepared as described in Table 8 and concentrated by vacuum dialysis.

[b] To each reaction vessel was added 1 A_{260} unit of salt washed ribosomes from 0-h embryos in a final volume of 150 µl. extent of [^{14}C]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.

but added EF-2 was most effective in 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.

C. Protease activity in Artemia embryos in relation to the appearance of light forms of EF-2 during development

1. Protease activity in soluble extracts from developing embryos

Several properties of soluble extracts from post-hatched embryos were described above such as size transformation of EF-2 (Fig. 8), decrease in protein content in extracts from embryos beyond 24-h development (Table 8) and loss of polyphenylalanine synthesis capacity (Table 9). In addition, when the soluble extracts were analyzed on an SDS-polyacrylamide gel (Fig. 9a), a slight decrease in the number of polypeptide bands was detected in the 24-h embryo extract; this was followed by a sharp decrease in the number of polypeptide bands in extracts from embryos beyond 24-h development compared to extracts from earlier embryos. Since Nagainis (116) and Osuna _et_ _al_. (117, 118) observed an increase in protease activity (at pH 7.5-8.0) in _Artemia_ embryos about 10 h after the onset of hatching, it appears that protease activity might be involved in the various changes in extracts from post-hatched embryos.

In order to test the importance of proteases in the present study, assays were carried out at 5^{o}, 28^{o} and $40^{o}C$ (at pH 8.0) for general protease activity in soluble extracts from dormant and developing embryos using dimethyl BSA as substrate as described in Methods. The results of this study are shown in Table 10. The results show that general protease activity in the 24-h extract is barely detectable but that it increases markedly in older embryos. At enzyme extraction and purification conditions ($5^{o}C$) the general protease activity is suppressed substaintially but still detectable.

Fig. 9a. SDS-polyacrylamide gel analysis of proteins in the soluble extracts from Artemia embryos at different stages of development. The soluble extracts were obtained as described in Table 8 and concentrated by vacuum dialysis. These extracts were analyzed either directly or after incubation for 45 min at 28°C by SDS gel electrophoresis. The numbers at the bottom of the figure indicate the age (h) of the embryos extracted and samples marked with the letter "i" were incubated prior to electrophoresis. Since less protein (µg) was found in extracts from post-hatched embryos than from earlier embryos (see Table 8), different amounts of protein were applied to the gel. The amounts of protein applied directly to the gel or following incubation were as follows: 20 µg from 0, 5 and 12-h extracts, 18 µg from the 24-h extract and about 12 µg from the 30, 35.5 and 48-h extracts. The migration position of standard proteins as described in Fig. 4 is indicated at the right of the figure. The letters "A" and "B" and the arrow indicate polypeptides that are discussed in the text.

Fig. 9b. SDS-polyacrylamide gel analysis of EF-2 hydrolysis by soluble extracts from Artemia embryos at different stages of development. Aliquots from selected soluble extracts described in Fig. 9a (twice the protein amounts) were mixed separately with 10 ug of partially purified EF-2. The mixtures were incubated at 5°C for 40 h then analyzed on an SDS-polyacrylamide gel slab. The numbers at the bottom of the figure indicate the age (h) of the embryos from which the different extracts were prepared. The partially purified EF-2 preparation was also incubated under the same conditions as the mixtures; the minor bands in this preparation were present in the non-incubated sample and they are due to protein impurities in the EF-2 preparation. The migration position of EF-2 (minimum amount) and standard proteins is indicated. The letters "A" and "B" and the arrows indicate polypeptides that are discussed in the text.

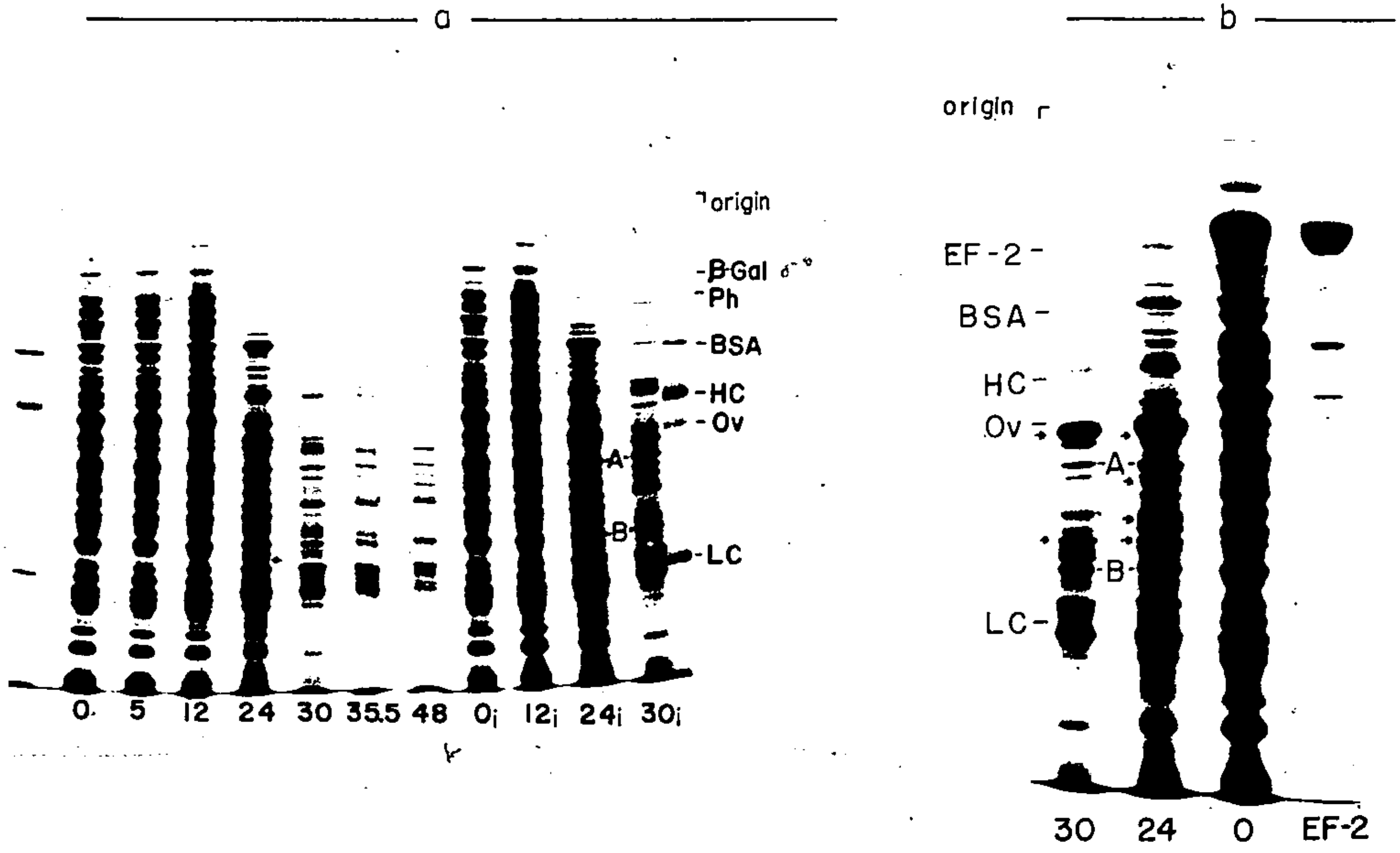

a
origin
-βGal
-Ph
-BSA
-HC
-Ov
-A-
-B-
-LC
0
5
12
24
30
35.5
48
0i
12i
24i
30i
b
origin
EF-2-
BSA-
HC-
Ov-
-A-
-B-
LC-
30
24
0
EF-2

TABLE 10

General protease activity in soluble extracts of *Artemia* at different stages of development[a]

Embryo age[b]	Embryos		Protease activity[c]		
	Emerged	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

[a] The soluble extracts were identical to the ones assayed in Table 9. For the protease assay 350 and 120 μg protein were used for 10 to 24 and 30 to 48-h embryo extracts, respectively.

[b] Hatching started at about 18-h incubation.

[c] One activity unit is defined as the amount of enzyme that produces a change of 0.01 A_{420} 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 (103).

In order to ascertain whether _Artemia_ proteins are substrates for the proteases detected above, soluble extracts were incubated at 28°C for 45 min (conditions that are similar to those required for ADP ribosylation and polyphenylalanine synthesis) then analyzed by SDS gel electrophoresis. The results of this experiment are given in Fig. 9a. These data show that the band profile of the 30-h extract was only slightly changed upon incubation but after this treatment the profile was similar to that of the control extract from 35.5-h embryos. No change in the protein band profile was observed after incubation of the 24-h embryo extract except for a reduction in one band (indicated by the arrow in Fig 9a). The protease activity in various soluble extracts was also tested under enzyme isolation conditions (5°C) using _Artemia_ EF-2 as substrate. In this experiment mixtures of embryo extracts and partially purified EF-2 were incubated for 40 h and then analyzed on SDS-polyacrylamide gel as shown in Fig. 9b. No breakdown of EF-2 occurs in the mixture containing the 0-h extract; however, in the presence of the 24-h extract only trace amounts of the EF-2 polypeptide remain intact. In the mixture containing the 30-h extract the existence of even 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. 9b. 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. Although such control samples are not shown, one can use the results shown in Fig. 9a (0_i-30_i) as controls since extracts incubated at 0°C for 40-h without EF-2 exhibit the same polypeptide pattern as those incubated at 28°C for 45 minutes. In addition, incubation of purified EF-2 alone has no effect on its band pattern (data not shown). By the use of internal markers such as band A and B and the molecular weight markers, a comparison can be made between the samples in

Figs. 9a and 9b. 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 even in the 30-h extract after incubation at 5°C.

2. Protease activity in free and bound protein preparations

To ascertain whether a correlation exist between the time of appearance of the light forms of EF-2 and the increase in protease activity during development, the time of appearance of general proteolytic activity (at pH 8.0) was determined in free and bound protein preparations whose origins have been described earlier (see Tables 1 and 2). As mentioned before, a slower rate of development occurs when 5 grams of hydrated cysts are incubated (in 500 ml of hatch medium) than when 1 gram is incubated (in 75 ml of hatch medium). Therefore, it was expected that the rise in general protease activity observed in the previous experiment should occur later in terms of incubation hours when large amounts of cysts are incubated. In the present experiment the protease activity in the Sepharose 6B fractions was tested in the standard assay at 40°C and the results are given in Table 11. These data show that general protease activity can be detected in extracts from embryos prior to the hatching process. This protease activity in the free preparations is fairly constant up to 10 h after hatching (20% hatching), whereas the protease activity in the bound fraction from 10-h hatched embryos decreases by about 4-fold. Since the free preparations contain 30-100 times more protease activity than the bound preparations, the protease activity in the latter fractions might be a contaminant of the free protein fractions. Since no increase in general protease activity could be detected after hatching in this experiment, in contrast to the previous experiment (Table 10), the free preparation from an extract of 46-h embryos (containing 30% nauplii) was studied for the presence of general protease activity. In this sample a 3-fold increase in protease units

TABLE 11

General protease activity in free and bound protein fractions of *Artemia* at different stages of development[a]

Embryo age[b]	Embryos		Protease activity[c]		EF-2	
	Emerged	Hatched	Free	Bound	Free	Bound
h	%		units/gram dry cysts		light/heavy[d]	
0	0	0	18.5	0.55	0.16	0.16
9	0	0	18.7	0.65	0.12	0.16
23	21	3	18.9	0.43	0.17	0.18
31	10	14	17.2	0.19	1.97	1.30
33	4	22	17.6	0.15	7.8	12.65
46	0.4	30	53.4	ND[e]	ND	ND

[a] The free and bound proteins were prepared using the Sepharose 6B method and the fractions used are identical to those described in Table 1. All general protease activity assays were conducted at 40°C. Approximately 320 and 200 μg protein from free and bound fractions, respectively, were added to the reaction vessels.

[b] Hatching started at about 23-h incubation.

[c] The definition of protease activity units is given in Table 10. All samples except for the 46-h one exhibit a 20 min lag in the onset of protease activity.

[d] calculated as described in Table 2.

[e] ND = Not determined.

was observed compared to the 33-h sample. Thus, protease activity in the two different experiments (Tables 10 and 11) in which different incubation procedures and different extraction methods were employed should be compared in terms of the degree of hatching rather than hours after starting the incubation. Moreover, the appearance of the light forms of EF-2 in the Sepharose 6B fractions occurs prior to any apparent change in the general protease activity which increases several hours after hatching. Hence, the different experiments clearly show that the appearance of the light forms of EF-2 after hatching is unrelated to the increase in the general protease activity, and that a specific protease which becomes active at the onset of hatching accounts for the appearance of the low molecular weight EF-2 proteins.

3. Evidence for the presence of EF-2 hydrolase activity in dormant cysts of Artemia

During initial attempts to purify EF-2 it became apparent that partially purified EF-2 is unstable and breaks down to smaller components during its isolation. When the S-150 fraction was applied to a DEAE-cellulose column and eluted with a buffered KCl gradient, the results shown in Fig. 10 were obtained. These results show that only one peak of EF-2 eluted from the column. When the EF-2 fraction was pooled (as shown in Fig. 10), $[^3H]$ADP ribosylated and analyzed by SDS-polyacrylamide gel electrophoresis compared to unfractionated S-150 fraction, the results illustrated in Fig. 11 were obtained. The results show that following DEAE-cellulose treatment new polypeptides appear which accept ADPR in the diphtheria toxin-dependent reaction. These polypeptides have molecular weights of 41,000 and 36,000 compared to the S-150 fraction which contains the 95,000 MW species only. When the EF-2 fraction from the DEAE-cellulose step was chromatographed on a Sephadex G-150 column then tested for ADPR acceptance the results illustrated in Fig. 12 were obtained. These results show that the DEAE-cellulose

Fig. 10. Chromatography of S-150 fraction from Artemia cysts on DEAE-cellulose. The 150,000 x g supernatant fluid (S-150) was fractionated with $(NH_4)_2SO_4$ and dialysed as described under Methods. About 75 A_{280} units of this fraction were applied to a column of DEAE-cellulose (DE-32, 1.5 x 18 cm) previously equilibrated with buffer D. The column was washed with the same buffer until no UV-absorbing material eluted from the column. The absorbed proteins were eluted using a 250 ml linear gradient of KCl from 50 to 350 mM in buffer D at a flow rate of 20 ml/h and 6 ml fractions were collected. Column fractions were assayed for absorbance at 280 nm (--) and EF-2 (●-●) as indicated using 0.05 ml aliquots in the toxin-NAD assay. Fractions containing EF-2 were pooled as shown, concentrated by vacuum dialysis, dialyzed for 10 h against 5 changes of buffer D and saved for further studies.

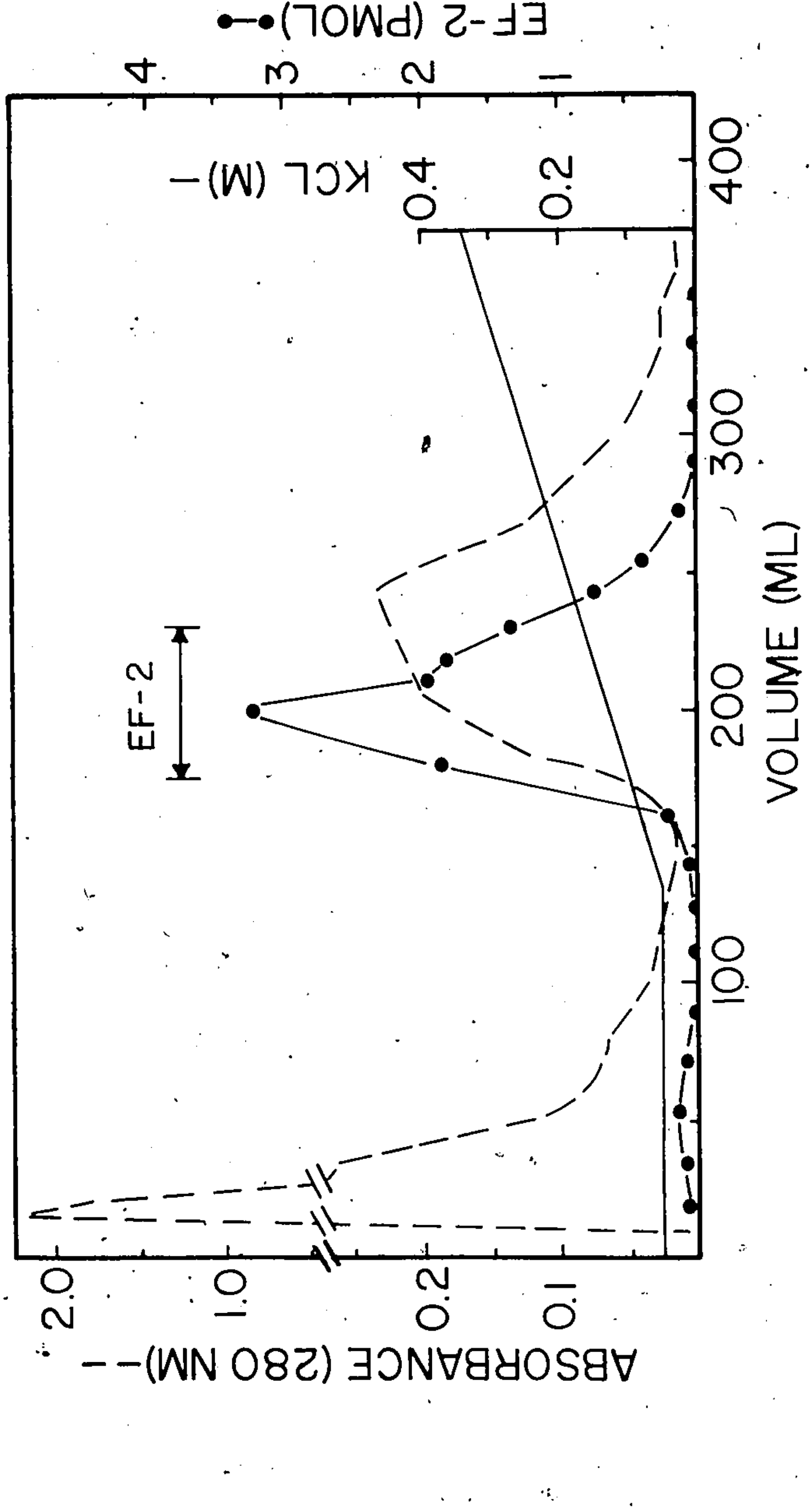

EF-2 (PMOL) ●—●
4
3
2
1
KCL (M) —
0.4
0.2
EF-2
ABSORBANCE (280 NM) – – –
2.0
1.0
0.2
0.1
100
200
300
400
VOLUME (ML)

Fig. 11. SDS-polyacrylamide gel analysis of [^{3}H]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 (see Fig. 10) 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 Methods. The migration position of phosphorylase (Ph) and ovalbumin (Ov) is indicated.

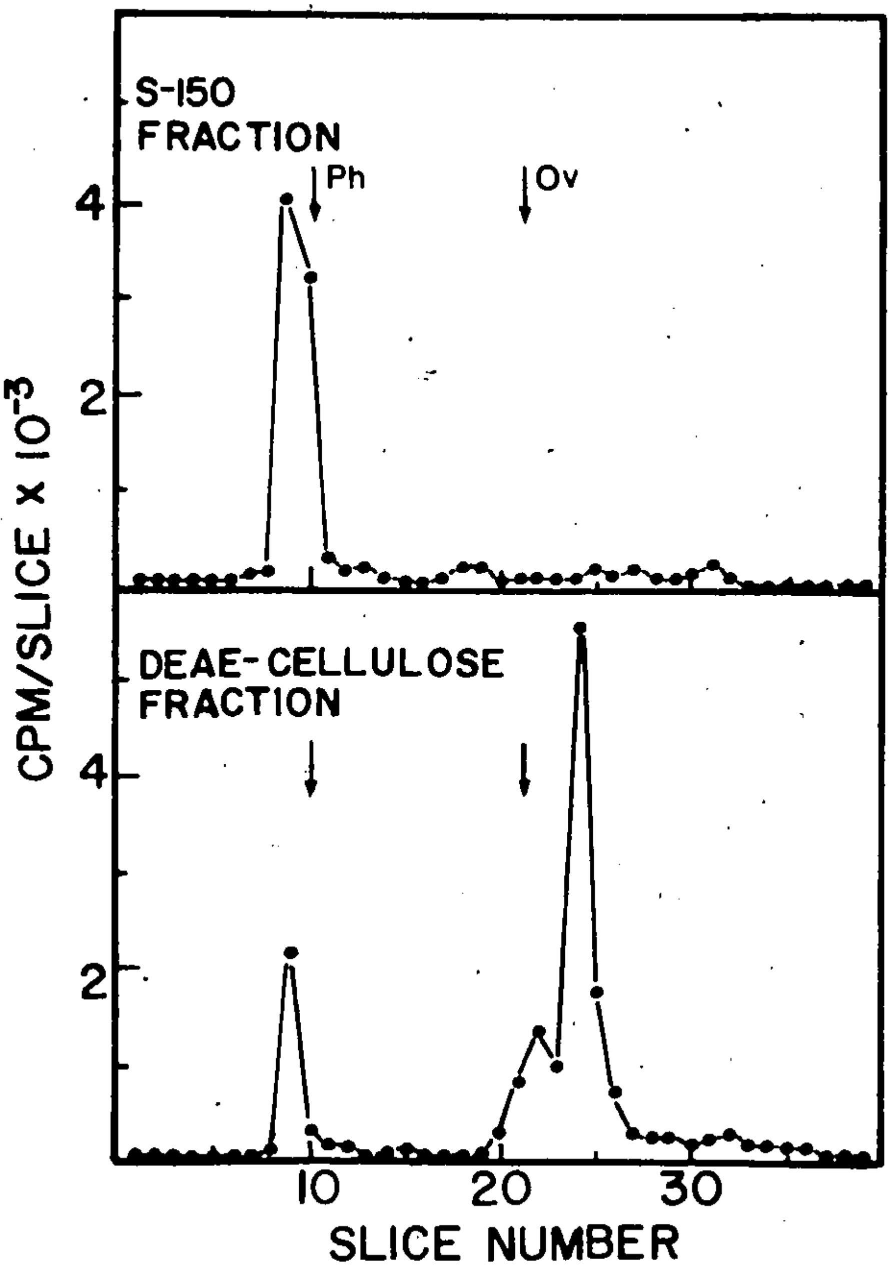
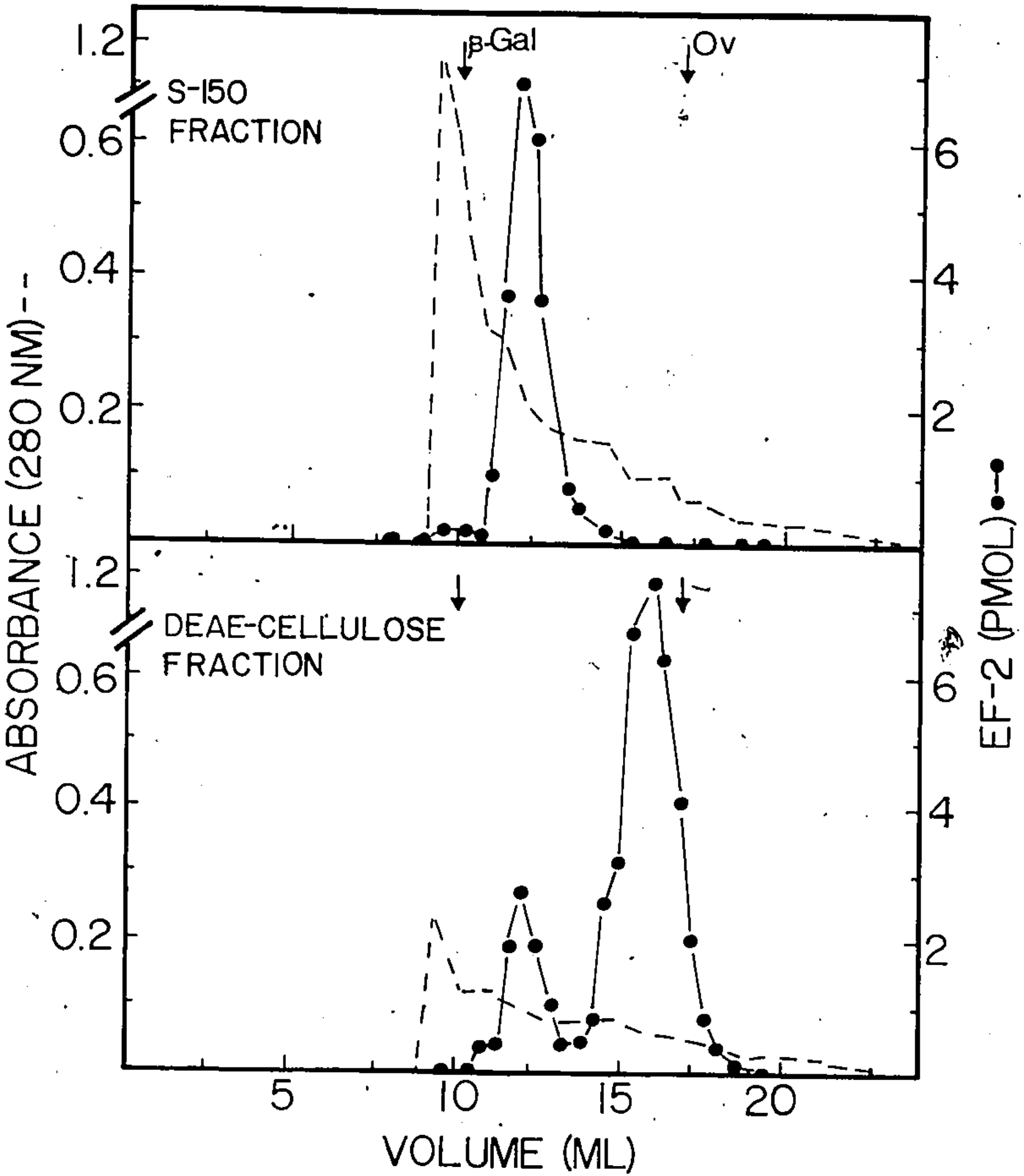

S-150
FRACTION
Ph
Ov
4
2
CPM/SLICE x 10^{-3}
DEAE-CELLULOSE
FRACTION
4
2
10
20
30
SLICE NUMBER

Fig. 12. Sephadex G-150 column analysis of [^{3}H]ADPR accepting proteins from cyst S-150 fraction before and after chromatography on DEAE-cellulose. The S-150 fraction from dormant cysts (containing 160 pmol of EF-2) and the EF-2 fraction from the DEAE-cellulose column (see Fig. 10, containing 470 pmol of EF-2) were chromatographed separately on a column of Sephadex G-150 (superfine, 0.9 x 41 cm) previously equilibrated with buffer D. The column was developed with buffer D at a flow rate of approximately 1.3 ml/h and fractions of 0.35-0.40 ml were collected. Aliquots of 0.05 ml were removed for the diphtheria toxin-dependent assay of EF-2 (●-●) and absorbance at 280 nm (--) was determined after adding 1 ml of buffer D to the remainder of each column fraction. The elution position of standard proteins β-galactosidase (β-Gal) and ovalbumin (Ov) chromatographed separately but under identical conditions is indicated.


β-Gal
Ov
S-150
FRACTION
DEAE-CELLULOSE
FRACTION
ABSORBANCE (280 NM) - -
EF-2 (PMOL) ●—●
1.2
0.6
0.4
0.2
6
4
2
5
10
15
20
VOLUME (ML)

treatment and not incubation with the toxin-NAD reaction mixture or preparation of the ADP-ribosylated sample for electrophoresis leads to the formation of ADPR accepting fragments.

The results obtained from the above SDS gel analysis (Fig. 11) reveals that the 36,000 MW fragment is the dominant ADPR accepting protein after the DEAE-cellulose fractionation. However, the elution profile of the ADPR accepting fragments following Sephadex G-150 fractionation suggests that the 36,000 MW fragment of EF-2 is not the result of proteolysis during the ADP-ribosylation assay. To test further the possibility that the 36,000 MW fragment is generated, at least to some extent, from the 41,000 MW protein during the ADP-ribosylation assay, the assay was carried out in the presence of soybean trypsin inhibitor (at 0.2 mg/ml). Although this inhibitor was reported to suppress the general alkaline protease activity in extracts from post-hatched Artemia embryos (116), it had no effect on the formation of the 36,000 MW fragment.

In a parallel study the 0.5 M NH_4Cl soluble proteins from the P-150 fraction of dormant cysts were fractionated on Ultrogel AcA 34 (see Fig. 14 for a similar fractionation experiment) then on DEAE-cellulose. The column fractions that contained EF-2 were pooled (as shown in Figs. 14 and 15) and the molecular weight of the ADPR accepting polypeptides was determined by SDS-polyacrylamide gel electrophoresis compared to the total 0.5 M NH_4Cl soluble fraction. The EF-2 fractions from DEAE-cellulose were also analyzed on Sephadex G-150 as described in Fig. 12 for the S-150 fraction.

The results of these experiments on the S-150 and P-150 fractions are summarized in Table 12. These data demonstrate that light forms of EF-2 are generated during or immediately after fractionation on DEAE-cellulose of extracts from dormant cysts and that the degradation activity is more pronounced in the S-150 preparation. These results suggest

TABLE 12

Distribution of light and heavy EF-2 in preparations from dormant cysts before and after DEAE-cellulose[a]

Source of preparation[b]	Treatment	EF-2 light/heavy
S-150	No treatment	0.130
	Stored at 5°C for 3 days	0.130
	After DEAE-cellulose[c]	4.630
P-150	0.5 M NH_4Cl soluble	0.075
	after Ultrogel AcA 34[d]	0.056
	after DEAE-cellulose (fraction A)[e]	0.007
	after DEAE-cellulose (fraction B)[e]	0.500
	after DEAE-cellulose (fraction A)[f]	0.007
	after DEAE-cellulose (fraction B)[f]	0.700

[a] The light/heavy values were calculated from SDS-polyacrylamide gel analysis of [^{3}H]ADP-ribosylated EF-2 as described in Table 2.

[b] The S-150 and P-150 fractions were prepared as described in Methods.

[c] Total EF-2 after column fractionation (see Fig. 10).

[d] From total EF-2 fraction which eluted from a column as shown in Fig. 14.

[e] From EF-2 fraction which eluted from a column as shown in Fig. 15.

[f] Stored at -70°C for 7 days after column fractionation.

the presence of EF-2 hydrolase(s) that is inactive in the unfractionated S-150 and P-150 fractions from dormant cysts.

4. Functional properties of the light forms of EF-2 generated by DEAE-cellulose fractionation

The effect of the light and heavy EF-2 on ribosome-dependent GTP binding and polyphenylalanine synthesis was studied using EF-2 preparations from the P-150 fraction of dormant cysts that had been chromatographed on Ultrogel AcA 34 and DEAE-cellulose (see Table 12). The light and the heavy forms of EF-2 were separated on a column of Sephadex G-150 as described in Fig 12 and the column fractions were assayed for ADPR acceptance, ribosome-dependent GTP binding and polyphenylalanine synthesis as shown in Table 13. All fractions containing heavy EF-2 stimulated ribosome-dependent GTP binding by about 3.5-fold, whereas fractions containing light forms of EF-2 did not stimulate this binding. Polyphenylalanine synthesis was stimulated slightly by column fractions containing heavy EF-2 and inhibited slightly by column fractions containing light EF-2's. Thus, the light EF-2 proteins generated during DEAE-cellulose fractionation were found to be inactive in protein synthesis and similar in some respects to the light EF-2 molecules which appear in embryo extracts after hatching.

D. Purification of EF-2 from dormant cysts of Artemia

1. Purification procedure

During initial attempts to purify EF-2 it became apparent that the EF-2 content in the bound protein preparations obtained by Sepharose 6B fractionation or differential centrifugation is 40-45% of the total EF-2 in dormant *Artemia* cysts (Tables 2 and 3). It was also observed that the specific activity of bound EF-2 (nmol EF-2/mg protein) after its separation from the particulate material is about 3 times higher than in the

TABLE 13

Effect of heavy(H) and light(L) forms of EF-2 on ribosome-dependent GTP binding and polyphenylalanine synthesis[a]

Fraction tested[b]	EF-2[c]	GTP bound[d]		Phenylalanine incorporation[e]	
		-Ribosomes	+Ribosomes		
	pmol	pmol		pmol	% of control
buffer	–	–	1.0	2.90	100
H_1	12.4	3.5	13.5	3.25	112
H_2	20.0	4.7	16.7	–	–
H_3	19.9	4.7	16.9	3.44	118
H_4	14.5	3.4	11.7	–	–
L_1	15.0	1.0	1.1	2.46	84
L_2	19.0	1.1	1.2	–	–
L_3	20.0	1.1	1.2	2.58	89
L_4	15.5	0.8	0.8	–	–

[a] EF-2 was prepared from the P-150 fraction of dormant cysts and further purified by chromatography on Ultrogel AcA 34 and DEAE-cellulose as described in Figs. 14 and 15. The EF-2 fractions which were eluted off DEAE-cellulose between 125-160 mM KCl (fraction B) were pooled and concentrated by vacuum dialysis. The preparation (3.3 A_{280} units and 4 nmol of EF-2) was chromatographed on a column of Sephadex G-150 as described in Fig. 12.

[b] All column fractions were tested for EF-2 by the toxin-NAD assay and the fractions with maximum EF-2 activity from the heavy(H) and light(L) peaks were selected for this study.

[c] Determined by the toxin-NAD assay.

[d] The GTP binding assay was carried out as described in Methods.

[e] The phenylalanine incorporation assay was done as described in Methods; aliquot of the soluble extract from 12-h embryos was added to each reaction vessel as the source of factors (60 µg protein including 3.5 pmol of EF-2). Background values of 70 cpm for ribosomes tested alone were subtracted.

free protein fractions (Table 1). Moreover, large quantities of EF-2 could be processed more easily using differential centrifugation to obtain bound proteins than by using Sepharose 6B fractionation. Thus, the 150,000 x g pellet (P-150) has been used as the starting material in developing a purification scheme for EF-2 from Artemia cysts.

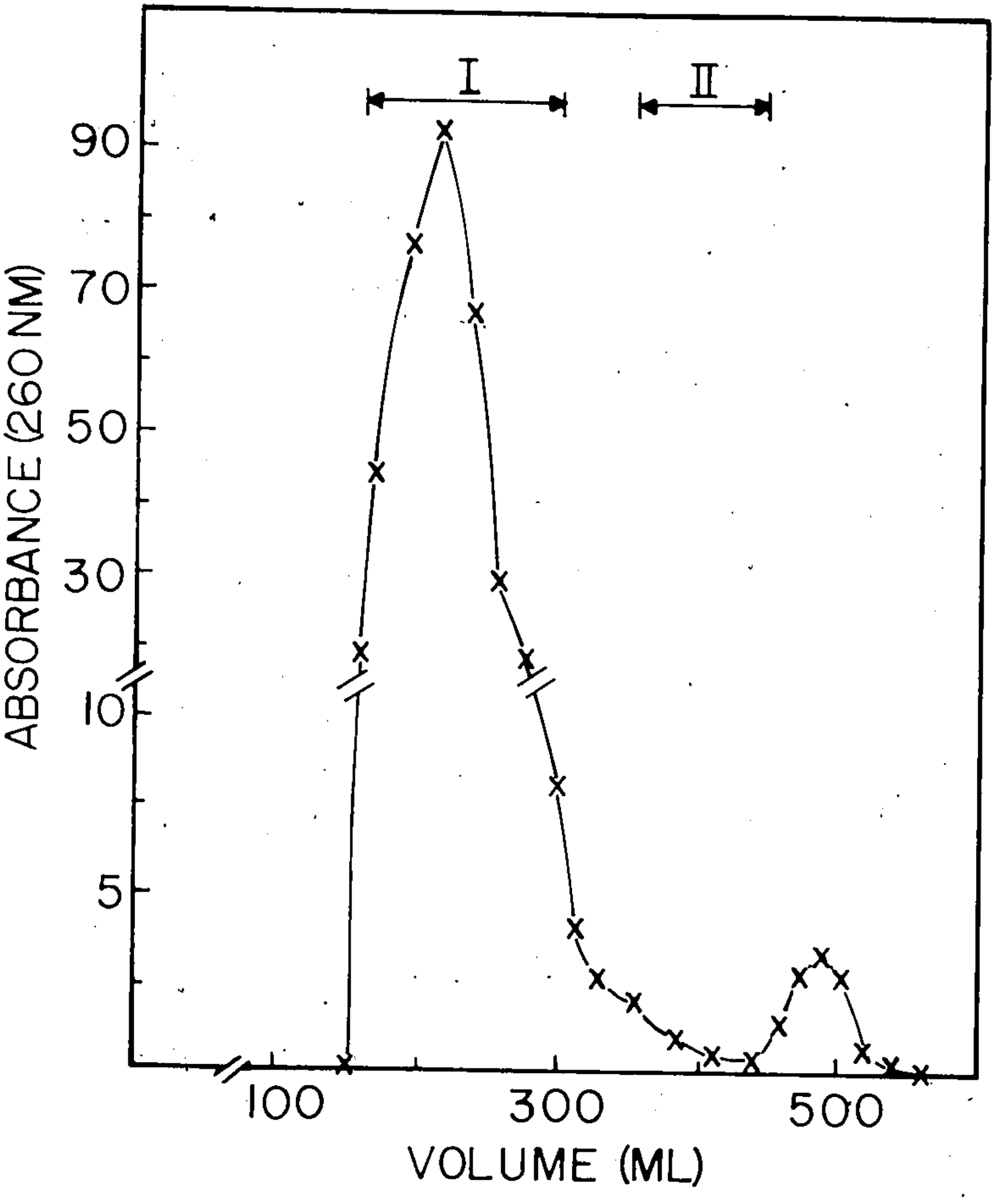
In initial attempts to purify EF-2 considerable degradation of EF-2 occurred as the result of hydrolase activity which contaminated all the EF-2 preparations. This contaminant caused very poor yields and unstable preparations. The purification steps described below have been developed to obtain EF-2 free of hydrolase activity and stable during storage at -70°C.

Step 1. Fractionation of the P-150 fraction on Sepharose 6B

The P-150 fraction was prepared from 112 grams of fully hydrated and washed cysts (equivalent to 45 grams dry weight) as described in Methods. The pellet was suspended in buffer C and the suspension was clarified by centrifugation at 7,000 x g for 10 minutes. The soluble material (7320 A_{260} units) was applied to a Sepharose 6B column (2.5 x 93 cm) previously equilibrated with buffer C and the column was developed with buffer C. The results of this experiment are shown in Fig. 13. The contents of all tubes in peak I were pooled and concentrated by vacuum dialysis. This peak contains ribosomes, glycogen, RNA, proteins, lipids and other undetermined high molecular weight components. If a smaller amount of material is applied to the column peak I can be partially resolved into two peaks. The EF-2 in peak I can be quantitated by the diphtheria toxin-dependent ADP-ribosylation assay only after EF-2 is released or solubilized as described in step 2.

The purpose of the Sepharose 6B step is to remove contaminating proteins that are loosely bound to the macromolecules of the P-150 fraction. Fraction II from the Sepharose 6B step contains about 10% of the total EF-2 and 50% of the A_{280} units found in the 0.5 M NH_4Cl soluble

Fig. 13. Chromatography of Artemia cyst P-150 fraction on Sepharose 6B. The P-150 fraction from dormant embryos (containing 7320 A_{260} units) was suspended in buffer C then applied to a Sepharose 6B column (2.5 x 93 cm) previously equilibrated with buffer C. The column was developed with buffer C at a flow rate of about 30 ml/h and 7.5 ml fractions were collected. Column fractions under peak I contain bound EF-2 and the contents of these fractions were pooled and concentrated by vacuum dialysis. Fractions under region II were not saved; these fractions contain residual amounts of free EF-2.


I
II
90
70
50
30
10
5
ABSORBANCE (260 NM)
100
300
500
VOLUME (ML)

fraction if the Sepharose 6B step is omitted.

Step 2. Preparation of 0.5 M NH_4Cl wash

Solid NH_4Cl was added to fraction I from Sepharose 6B column to a final concentration of 0.5 M in a final volume of 35 ml (in buffer C). The preparation was stirred gently at 0°C for 75 min then centrifuged at 150,000 x g for 4.5 hours. The clear supernatant fluid was collected, concentrated by vacuum dialysis and dialyzed against 5 changes of buffer D for 10 hours. The precipitate that formed upon concentration and dialysis was removed by centrifugation at 7,000 x g for 10 min and the soluble fraction was saved for the next step.

Step 3. Chromatography on Ultrogel AcA 34

The concentrated protein preparation from step 2 (8 ml in buffer D) was applied to a column of Ultrogel AcA 34 (2.5 x 85.5 cm) previously equilibrated with buffer D and the column was developed with this buffer. Fractions were collected and the EF-2 was detected by the ADP-ribosylation assay. The results of this experiment are shown in Fig. 14. The fractions containing EF-2 were pooled as shown and saved for the next step.

Step 4. Chromatography on DEAE-cellulose

The pooled fraction from step 3 (in buffer D) was applied to a DEAE-cellulose column (DE-32, 1.5 x 17.8 cm) previously equilibrated with buffer D. The column was washed with buffer D until no further protein could be washed from the column. The column was then developed with a buffered KCl gradient and column fractions were collected. The fractions containing EF-2 were detected by the ADP-ribosylation assay. The results of this experiment are shown in Fig. 15. The EF-2 fractions which eluted between 90 to 120 mM KCl (fraction A) were pooled, concentrated to about 4 ml by vacuum dialysis, dialyzed against 4 changes of buffer D for 6 h and saved for next step. The EF-2 fractions which eluted between 125 to 150 mM KCl (fraction B) were

Fig. 14. Chromatography of Sepharose 6B fraction I on Ultrogel AcA 34 after NH_4Cl treatment. The pooled fraction I from the Sepharose 6B column (see Fig. 13) was treated with 0.5 M NH_4Cl and the NH_4Cl soluble material was processed as described under Methods. The resultant soluble fraction (in buffer D) was chromatographed on a column of Ultrogel AcA 34 (2.5 x 85 cm) previously equilibrated with buffer D. The sample was eluted with buffer D at a flow rate of 30 ml/h and fractions of about 7 ml were collected. Fractions were assayed for absorbance at 280 nm (--) and for EF-2 (●-●) using 0.025 ml aliquots in the toxin-NAD assay. The active EF-2 fractions were pooled as shown.

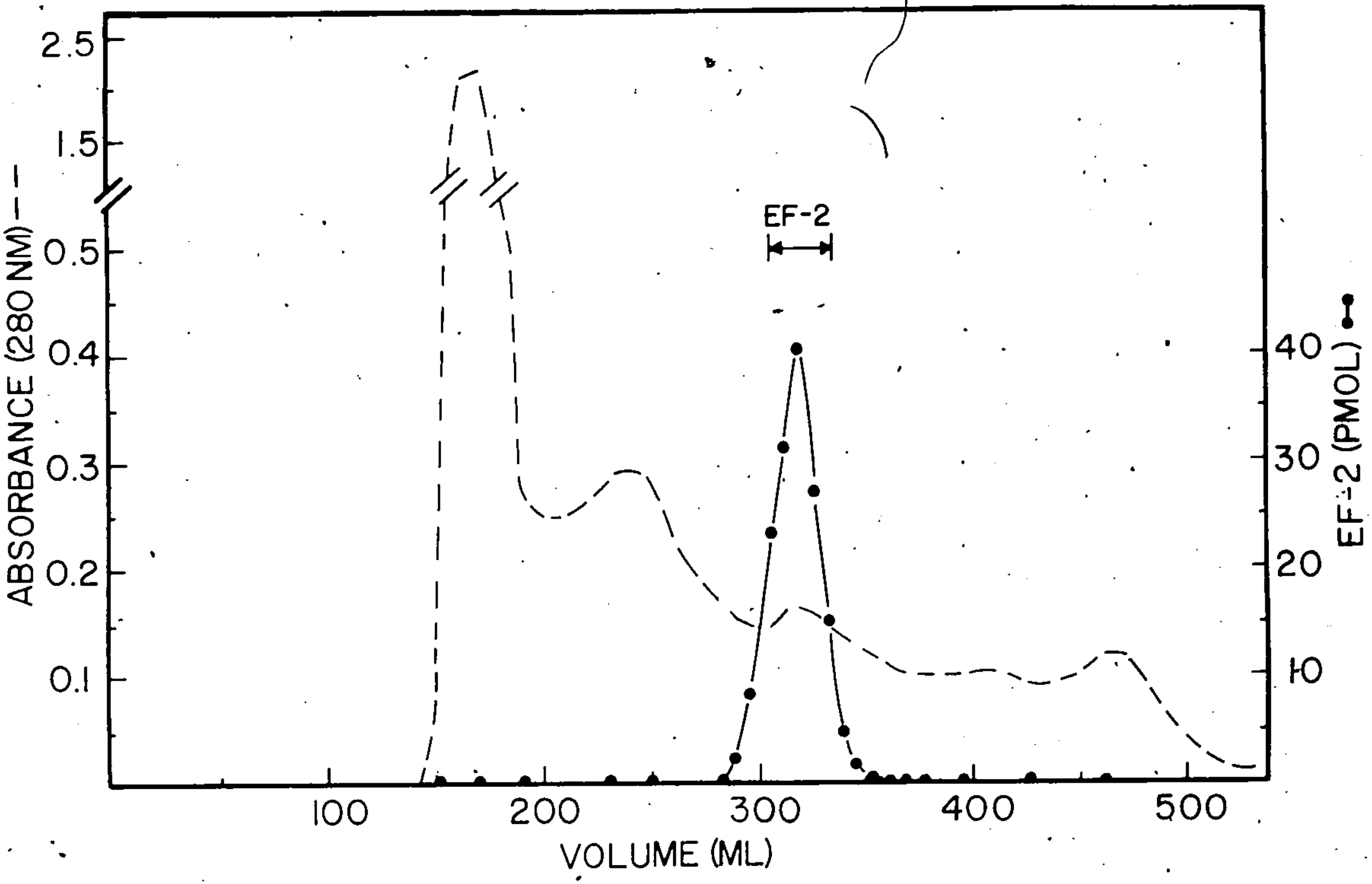
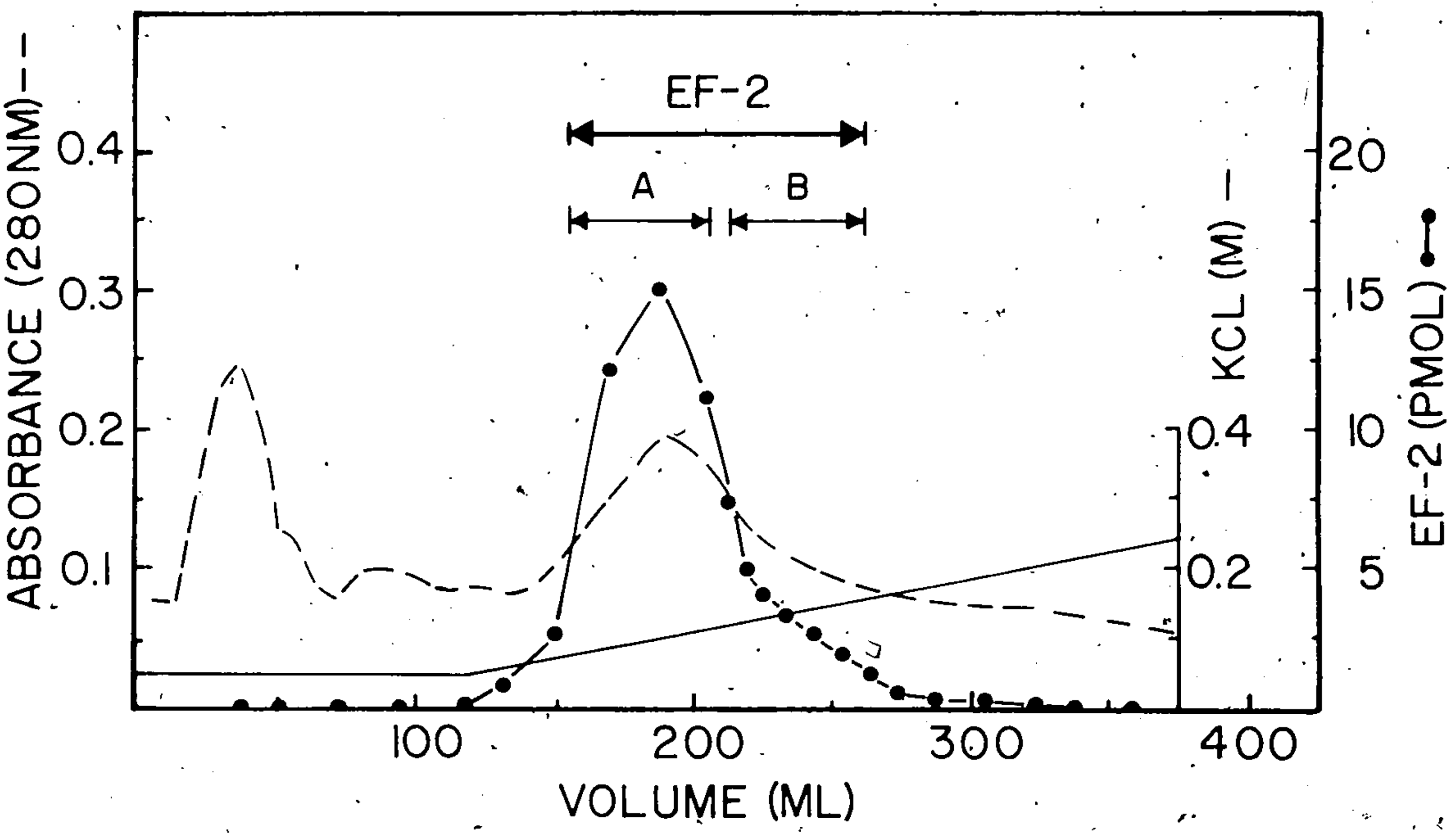

ABSORBANCE (280 NM) – –
2.5
1.5
0.5
0.4
0.3
0.2
0.1
EF-2
EF-2 (PMOL) •–•
40
30
20
10
100
200
300
400
500
VOLUME (ML)

Fig. 15. Chromatography of partially purified EF-2 on DEAE-cellulose. The EF-2 preparation from Ultrogel AcA 34 (see Fig. 14) was chromatographed on a column of DEAE-cellulose (DE-32, 1.5 x 17.8 cm) previously equilibrated with buffer D. The column was washed with buffer D and eluted with a 400 ml linear gradient of KCl from 50 to 350 mM in buffer D at a flow rate of 30 ml/h. Fractions of 6 ml were collected and each was analyzed for absorbance at 280 nm (--) and for EF-2 content (●-●) using 0.025 ml aliquots in the toxin-NAD assay. The EF-2 fractions A and B were pooled separately and concentrated by vacuum dialysis.


EF-2
A
B
ABSORBANCE (280NM)--
0.4
0.3
0.2
0.1
KCL (M) —
0.4
0.2
EF-2 (PMOL) ●–●
20
15
10
5
100
200
300
400
VOLUME (ML)

pooled separately and treated as for fraction A above. EF-2 fractions A and B were kept separate since fraction B was shown previously to contain EF-2 hydrolase activity (see Table 12). In this experiment EF-2 fraction B contained very little EF-2 hydrolase activity as judged by the L/H ratio of [^{3}H]ADP-ribosylated EF-2. However, upon storage at -70°C for 6 days the L/H ratio increased from 0.05 to 0.13. In contrast, the concentrated EF-2 fraction A was not degraded at all after similar storage. The low EF-2 hydrolase activity in the present experiment compared to the hydrolase activity in the crude P-150 fraction (see Table 12) is probably due to the use of Sepharose 6B to remove material that is not firmly associated with the P-150 fraction. In order to avoid trace amounts of EF-2 hydrolase activity, fraction B was not pooled with fraction A even though the former fraction contains about 35% of the total EF-2 in the starting material.

Step 5. Chromatography on phosphocellulose

The pooled EF-2 (fraction A) obtained from the DEAE-cellulose step was diluted to 10 ml with buffer D and applied to a column of phosphocellulose (P-11, 1.5 x 10 cm) previously equilibrated with buffer D. The column was washed with buffer D until no further UV-absorbing material eluted from the column and the column was developed with a buffered KCl gradient as described in the legend to Fig. 16. The fractions containing EF-2 were detected by the ADP-ribosylation assay and the results of this experiment are shown in Fig. 16. The fractions which contained EF-2 were pooled as indicated, concentrated by vacuum dialysis and dialyzed against 4 changes of buffer D for 6 hours. The preparation was clarified by centrifugation and stored at -70°C in aliquots of about 0.1 ml at 0.8 mg protein per ml.

A summary of the purification procedure for EF-2 is outlined in Table 14.

Fig. 16. Chromatography of DEAE-cellulose purified EF-2 on phosphocellulose. The EF-2 fraction A from DEAE-cellulose (see Fig. 15) was chromatographed on a column of phosphocellulose (P-11, 1.5 x 10 cm) previously equilibrated with buffer D. The column was washed with buffer D and eluted with a 400 ml linear gradient of KCl from 50 to 350 mM in buffer D at a flow rate of 20 ml/h. Fractions of 7 ml were collected and analyzed for absorbance at 280 nm (--) and for EF-2 content (●-●) using 0.050 ml aliquots in the toxin-NAD assay. The column fractions containing EF-2 were pooled as shown and concentrated by vacuum dialysis.

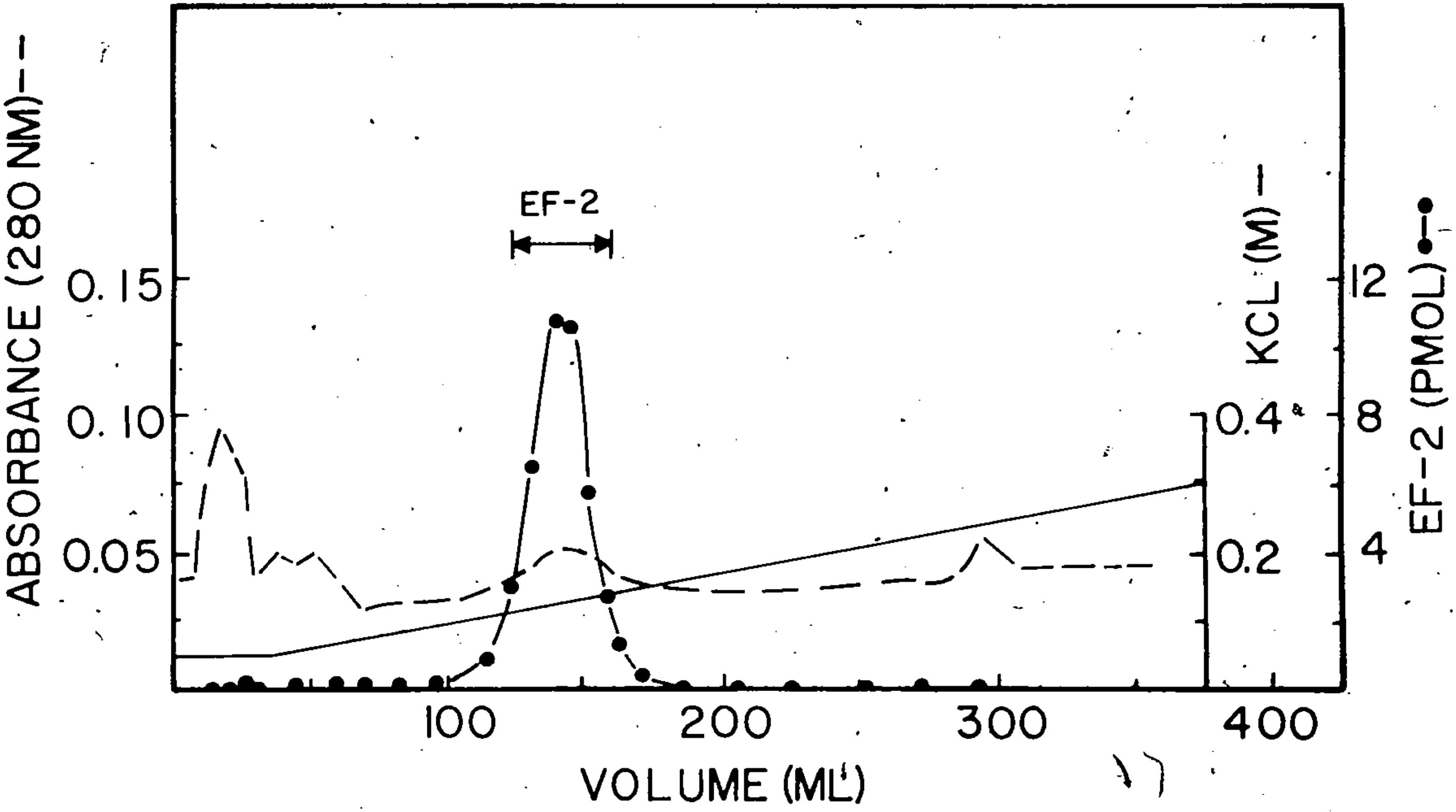

ABSORBANCE (280 NM)--
0.15
0.10
0.05
EF-2
KCL (M) —
0.4
0.2
EF-2 (PMOL) ●—●
12
8
4
100
200
300
400
VOLUME (ML)

TABLE 14

Summary of EF-2 purification procedure[a].

Step	Treatment	Total protein	Total EF-2	Specific activity[b]	Purification factor	Yield
		mg	nmol	nmol/mg		%
1	Sepharose 6B[c].	-	-	-	-	-
2	0.5 M NH_4Cl wash	89.0	41.2	0.46	1	100
3	Ultrogel AcA 34	6.6	35.6	5.40	11.7	86
4	DEAE-cellulose	2.4	19.7	8.20	17.8	48
5	Phosphocellulose	0.8	7.8	9.75	21.2	19

[a] From the 150,000 x g pellet (P-150) of 112 grams hydrated cysts.

[b] Assuming one equivalent of $[^3H]$ADPR bound per molecule of EF-2 (90).

[c] The EF-2 in the Sepharose 6B fraction is in a bound form and cannot be measured by the toxin-NAD assay. Therefore the EF-2 in step 2 was considered as the starting material for the determination of yield and extent of purification.

2. Molecular weight of EF-2 from the different purification steps

After each stage of purification the concentrated EF-2 fraction was $[^{3}H]$ADP ribosylated in the diphtheria toxin-dependent reaction and analyzed by SDS-polyacrylamide gel electrophoresis. The results of these analyses are shown in Fig. 17. In all cases the molecular weight of the major $[^{3}H]$ADP-ribosylated protein band is 95,000 which indicates that the integrity of the EF-2 molecule is preserved during purification. The final EF-2 preparation was stable after storage for at least two months at -70°C.

3. The degree of purity of the final EF-2 preparation

The specific activity of the final EF-2 preparation was 9.75 nmol per mg protein (see Table 14) and 93% pure assuming a molecular weight of 95,000 for *Artemia* cyst EF-2 and the binding of 1 molecule of ADPR per molecule of EF-2 (90). The degree of purity of the above EF-2 was confirmed by SDS-polyacrylamide gel electrophoresis as shown in Fig. 18. Some minor protein bands were observed in the final preparation of EF-2 which represent about 8% of the total band density as determined by scanning the stained gel at 560 nm in an Ortec 4310 densitometer and weighing the paper under the recorded peaks (data not shown). In addition, the major protein band (95,000 MW) accepted more than 99% of the $[^{3}H]$ADPR covalently bound to the EF-2 preparation in the diphtheria toxin-dependent reaction. Thus, the EF-2 appears to be about 92% pure after the five-step purification procedure described above.

Fig. 17. SDS-polyacrylamide gel analysis of [^3H]ADP-ribosylated proteins from EF-2 preparations at different stages of purification. The EF-2 preparations were as described in Table 14; step 2 (0.5 M NH_4Cl wash), step 3 (Ultrogel AcA 34), step 4 (DEAE-cellulose) and step 5 (phosphocellulose). In all cases the EF-2 preparation was radiolabeled in a standard toxin-NAD assay and analyzed on a 10% polyacrylamide gel slab containing 0.1% SDS. The same amount of radioactivity was applied from each EF-2 preparation and after electrophoresis the gels were sliced and the radioactivity was measured in gel slices as described in Methods. The migration position of phosphorylase (Ph) and ovalbumin (Ov) is indicated.

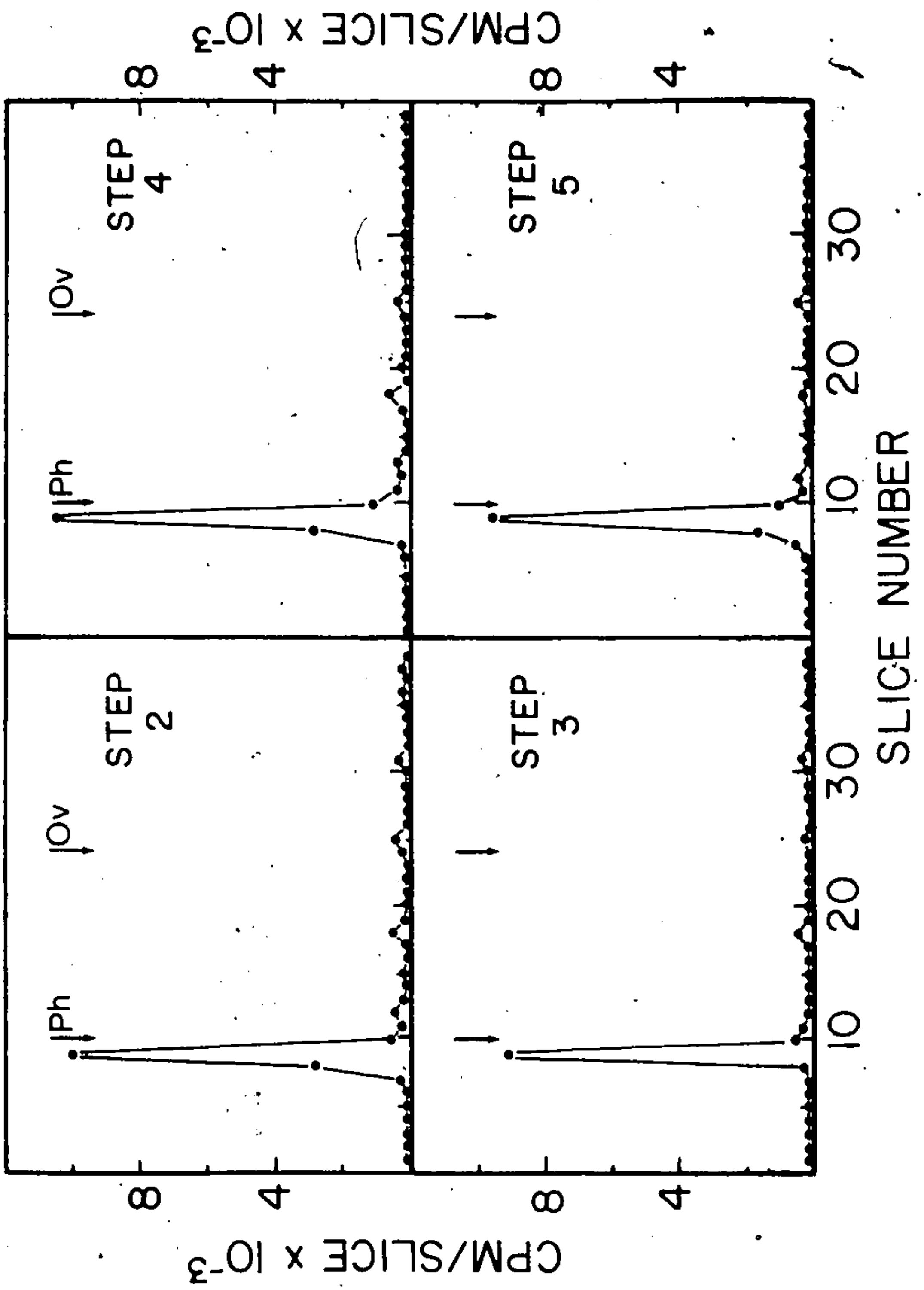
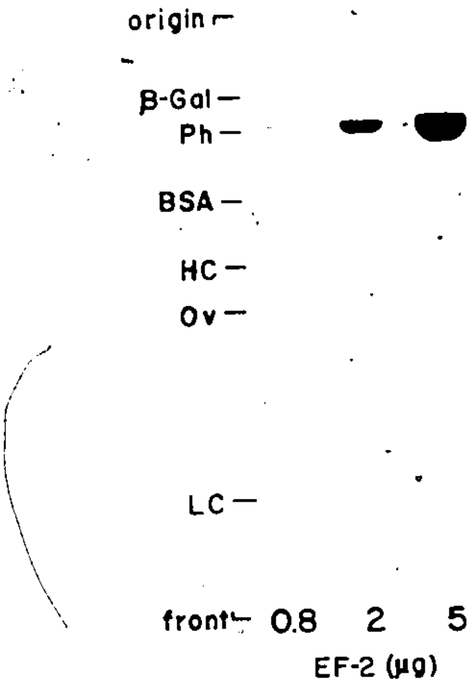

CPM/SLICE x 10-3
8
4
STEP 2
STEP 3
STEP 4
STEP 5
Ph
Ov
10
20
30
SLICE NUMBER

Fig. 18. SDS-polyacrylamide gel electrophoresis of EF-2 after chromatography on phosphocellulose. The EF-2 was analyzed on a 10% polyacrylamide gel slab containing 0.1% SDS. The amount of EF-2 (μg) applied to each slot is given at the bottom of the figure. The migration position of standard molecular weight proteins is indicated. The description of these proteins is given in the legend to Fig. 4.


origin
β-Gal
Ph
BSA
HC
Ov
LC
front
0.8
2
5
EF-2 (μg)

IV. DISCUSSION

It has been established that dormant embryos of _Artemia salina_ are inactive in protein synthesis and that cell-free extracts from these embryos are unable to translate exogenous mRNA (27, 31-33). However, cell-free systems prepared from dormant cysts by various methods are active in poly(U)-directed protein synthesis (31, 62, 77, 119). The latter studies have clearly suggested that elongation factors are present and active in extracts from dormant cysts. In addition, Slobin and Möller have demonstrated that EF-1 undergoes a size transformation during early development of _Artemia_ (76, 77), and studies by Warner _et al_. have suggested that EF-2 has a role in the regulation of protein synthesis in early development of _Artemia_ (68, 69). In order to ascertain whether EF-2 is regulated during early development of _Artemia_ and therefore important in the control of protein synthesis during embryogenesis, a detailed study of certain properties of this protein during development has been carried out using _Artemia_ embryos.

In this study the ADP-ribosylation reaction catalyzed by diphtheria toxin has provided a simple and rapid means to quantitate EF-2 and to determine its distribution and molecular weight during early development. This study has revealed two possible modes of regulation of EF-2 in _Artemia_ embryos: a) changes in the distribution of EF-2 between free and bound forms during early development and b) selective degradation of EF-2 in developing embryos. The latter process was observed mainly in extracts from hatched embryos, whereas changes in the distribution of EF-2 were found to occur prior to and after the commencement of hatching. Since these two mechanisms are distinctly different they will be discussed separately. In the last section of this dissertation a method for the purification of EF-2 from _Artemia_ embryos which was developed in this study will be discussed.

A. Free and bound EF-2 in developing embryos of Artemia

The present study has established the existence of EF-2 as free and bound forms in dormant cysts and developing embryos of Artemia salina. In addition, this study has shown that the distribution of EF-2 between the two forms changes during early development; in dormant cysts about 45% of the total EF-2 is in a bound form and this value decreases as development proceeds reaching the value of about 20% in hatched embryos (Tables 1 and 3). Several fractionation methods have been used to demonstrate the existence of bound EF-2 in extracts from Artemia embryos. Following fractionation of embryo extracts on columns of Sepharose 6B or by differential centrifugation, the bound EF-2 co-purifies with ribosomes. Moreover, the bound EF-2 resembles ribosome-bound EF-2 since it is unable to accept the ADPR moiety of NAD in the diphtheria toxin-NAD assay unless first treated with dissociation reagents such as NH_4Cl and EDTA (92-95, 99). However, sucrose density gradient fractionation has revealed that most of the bound EF-2 in Artemia embryos is not associated with ribosomes but with some other macromolecules. In fact only 1-3% of the total EF-2 from dormant cysts or developed embryos is bound to ribosomes (Table 5), whereas 20-45% of the total EF-2 is in a bound form. Despite the above data there is still some uncertainty about the exact amount of ribosome-bound EF-2 since some of the sucrose gradient fractions at the extreme ends of the monoribosome peak were discarded to avoid overlapping between the cystosol, monoribosome and polyribosome fractions. However, the discarded fractions could only account for less than 1% additional EF-2 bound to ribosomes. A similar proportion of ribosome-bound EF-2 (1-3%) was found when relatively pure ribosomes were prepared from the P-150 fraction by sedimentation of this fraction twice through a 26% sucrose cushion (data not shown).

It was reported previously that 20-30% of rabbit reticulocyte EF-2 is bound to ribosomes (94) and similar values were reported for ribosome-bound EF-2 from HeLa cells (93). Also, the reticulocyte study showed that the monoribosome and polyribosome fractions contain 0.65 and 0.3 molecules of EF-2 per ribosome, respectively. This contrasts with the present study using Artemia embryos which showed that monoribosome and polyribosome fractions contain 0.02-0.03 and 0.04 molecules of EF-2 per ribosome, respectively (Table 5). Thus only about 2-4% of Artemia ribosomes contain an average of one molecule of EF-2 per ribosome. The significance of the low EF-2 content of Artemia embryo monoribosomes and polyribosomes is not clear, but the differences observed between Artemia and reticulocytes and HeLa cells may be due to the low salt concentrations (0 to 10 mM) used in the latter two studies to isolate the ribosome fractions. Hence, the conditions employed in the present study using Artemia might have dissociated EF-2 or EF-2 containing particles from ribosomes.

Recently, Hradec and Dušek found that 20 S particles in the post-mitochondrial fraction from rabbit reticulocytes are rich in the elongation factors (120). These particles were isolated by affinity chromatography on heparin-Sepharose and fractionated further on Sepharose 4B and sucrose density gradients. In addition Hradec and Dušek 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. It is still possible, however, that the EF-2 containing macromolecular complex is loosely associated with ribosomes in the intact embryo. Other proteins may also exist as large complexes in vivo. Some evidence suggests that aminoacyl-tRNA synthetases from eukaryotes exist in multiple copies in relatively large complexes in the cytoplasm (120-122). Other studies have indicated that initiation factors are associated with high molecular weight particles different from ribosomes or ribosomal subunits (120, 123). Hence, many components of the protein synthesis apparatus appear to exist as part of large aggregates or macromolecular complexes.

The data presented in this dissertation show that a gradual decrease in the total amount of EF-2 occurs during prenauplier development. This event is associated with a substantial decrease in the amount of bound EF-2 and a slight increase in the content of free EF-2 (Table 1). At about 10 h after the onset of hatching, a marked increase was observed (35%) in the total amount of EF-2. This change occurred in the free EF-2 fraction while the content of bound EF-2 remained fairly constant compared to newly-hatched embryos. This increase in total EF-2 was also observed about 12 h after hatching in unfractionated embryo extracts (Table 8). However, in the latter experiment the values of EF-2 from pre-hatched embryo extracts were low compared to experiments where the extracts were fractionated to free and bound components (see Tables 1 and 3 versus Table 8). The low values of EF-2 observed in the latter experiment may be explained from the findings of Goor and Maxwell who reported that various nucleotides act as competitive inhibitors of ADP ribosylation of EF-2 (86). In the earlier part of the present study methods were chosen to avoid this problem since they ensured the removal of small molecules by dialysis, ammonium sulfate fractionation and filtration through Sephadex G-25. However, in the experiment where the recovery of EF-2 was low (in extracts from pre-hatched embryos, Table 8), the soluble embryo extracts were not fractionated into subcellular components nor treated with ammonium sulfate or Sephadex G-25 as the cytosol or free protein fractions in other experiments. Hence, some substances which are present in extracts of pre-hatched embryos even after dialysis may have interferred with the ADP-ribosylation assay of EF-2 in the experiment using unfractionated embryo extracts.

From the present methods it is also impossible to determine whether or not there is a further increase in the amount of total EF-2 in embryos beyond 30 to 35-h development since the protease activity in extracts from hatched embryos (discussed below) may hydrolyze the EF-2 polypeptides completely making it impossible to quantitate accurately EF-2 in extracts from these embryos.

The findings that bound EF-2 decreases in amount and that free EF-2 increases during early development of Artemia concomitant with a general increase in the rate of protein synthesis in the intact embryo (Table 7), suggests that the change in the 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. Smulson and Rideau showed that within 45 min after the release of HeLa cells from tyrosine deprivation the amount of EF-2 in the high speed supernatant fraction increased by about 50% with a concurrent decrease in the EF-2 content of the monoribosome fraction (93). The reverse change in distribution was observed by Smulson and Rideau when protein synthesis was inhibited by various conditions. These studies were conducted using a diphtheria toxin-NAD assay similar to that described in this dissertation. Using the sea urchin Felicetti et al. have shown that the activity of EF-2 in the supernatant fraction of zygotes increases by 50% within 2 min after fertilization but they didn't examine any particulate fraction for the presence of EF-2 (96). The latter study doesn't preclude the possibility that EF-2 activation occurs by some mechanism other than release of EF-2 from a macromolecular complex. The above findings as well as the results obtained in this study using Artemia support the idea that the release of EF-2 into the free pool is associated with an increase in the rate of protein synthesis. The release of bound EF-2 soon after the cessation of dormancy in Artemia might also include destruction of some EF-2 (bound, free or both) because the total amount of EF-2 decreases between the dormant cyst and 9-h development stages (see Table 1).

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 (92, 93), rabbit reticulocytes (94) and myogenic cells (unpublished observation of the author). In the study using myogenic cells (cell line L8, see ref. 124) both proliferating cells and multinucleated fibers (differentiated myogenic cells) exhibited the above distribution. 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) (95); 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. In mature organs (and dormant cysts) the rate of protein synthesis is lower than in embryonic cells, cells in tissue cultures and reticulocytes where protein synthesis is very active. However, when cell division and growth are induced in mature liver by partial hepatectomy, the rate of protein synthesis is enhanced markedly; this increase in activity appears to be due to an increase in the efficiency of liver ribosomes (125). Henshaw et al. have suggested that factors which are not firmly bound to ribosomes might regulate protein synthesis in the rat under various physiological conditions and that this control is lost in malignant cells (126). Thus, the possibility exists that the release of EF-2 from its bound state activates or enhances protein synthesis in animal cells.

In addition to the above findings the sucrose gradient experiments revealed that the ratio of total EF-2 molecules (soluble plus particulate) per ribosome is 1.4-1.6 in cysts and developing embryos of Artemia (Table 5). In this respect the present study is in agreement with studies using rabbit reticulocytes (1.5-2.0 EF-2/ribosome (94)) and various organs from the rat (1.1-1.5 EF-2/ribosome (95)). In Escherichia coli (a prokaryote) a slightly lower ratio has been found (1 EF-G/ribosome (127)). However, the values for Artemia embryos may be somewhat conservative since some of the sucrose gradient fractions were not used for the study (see Fig. 5).

B. Changes in molecular weight of EF-2 in extracts from Artemia embryos and the possible involvement of specific hydrolase(s) in this process

Analysis of the [^{3}H]ADPR accepting proteins from extracts of Artemia embryos at different stages of development did not reveal any differences in the molecular weight of these proteins in the free and bound EF-2 preparations. However, the molecular weight studies revealed that the size distribution of the ADPR accepting proteins changes markedly after hatching.

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 diphtheria toxin-NAD dependent reaction (87-89). Thus, EF-2 in extracts from dormant cysts and pre-hatched embryos has a molecular weight of 95,000 (heavy form). Soon after the commencement of hatching EF-2 polypeptides of 41,000 and 36,000 MW (light forms) become the predominant species. Trace amounts of the light forms of EF-2 are detectable in extracts from pre-hatched embryos (see Figs. 2 and 6); therefore, it appears that light forms of EF-2 exist at all stages of Artemia embryogenesis but that the amount is enhanced dramatically in extracts from post-hatched embryos. The findings that both the heavy and light EF-2 proteins disappear sequentially in extracts as hatching proceeds suggest that the size-transformation process is due to hydrolytic activity and not synthesis of new ADPR accepting polypeptides.

The heavy form of Artemia EF-2 has a molecular weight of 95,000 which is similar to that of EF-2 in other eukaryotes (82, 83, 90, 112-114). In addition, the heavy EF-2 from Artemia embryos catalyzed ribosome-dependent GTP binding activity which is a property of functional EF-2 (81-83, 101, 102), whereas the light EF-2 species are inactive in this respect regardless of their origin (see Table 6). Thus, the appearance of the light forms of EF-2 can not account for the increase in rate of protein synthesis observed during Artemia development (see Table 7).

In addition to the disappearance of functional EF-2 from extracts of newly hatched embryos other polypeptides also disappear from extracts of newly hatched embryos and young nauplius larvae (Figs. 4 and 9a). In previous reports it was noted that some polypeptides are absent (or in decreased amounts) from in vitro-translation products when mRNA from developing embryos of Artemia is compared to that of dormant cysts (48, 56). This observation suggests that the disappearance (or lack of) certain polypeptides from extracts of hatched embryos occurs under physiological conditions and is not due to protease activity in the extracts. However, the observation that soluble extracts from newly hatched embryos exhibit less activity in polyphenylalanine synthesis than extracts from pre-hatched (12 h) embryos (see Table 9), and the findings that the rate of protein synthesis in vivo increases throughout the period of development studied (35.5 h) suggest that partial inactivation of the protein synthesizing machinary occurs during preparation of hatched embryo fractions for analysis. Moreover, the appearance of the light EF-2 polypeptides in extracts correlates well with the loss in protein synthetic capacity of embryo extracts and the onset of hatching (see Tables 8 and 9 and Fig. 8). (The mechanism of EF-2 degradation and subsequent loss of protein synthesis capacity will be discussed below). The findings of Moens and Kondo which showed that endogenous protein synthesis activity of extracts from newly hatched _Artemia_ embryos (20 h) decreases by over 2-fold compared to extracts from pre-hatched embryos (15 h) were probably due to the same problem observed in the present study (55).

The results of $[^{14}C]HCO_3^-$ incorporation into intact _Artemia_ embryos and the poly(U)-directed _in vitro_ protein synthesis experiment alluded to above deserve further consideration. Although the protein and the nucleic acid synthesis patterns differ markedly during _Artemia_ development (Table 7), both profiles are in good agreement with previous results of Clegg (27) and McClean and Warner (115). Also, the protein synthesis activity of pre-hatched embryo extracts is consistent with the _in vivo_ protein synthesis data. However, the low activity of soluble extracts from dormant cysts in poly(U)-directed protein synthesis is not consistent with the results of Clegg and Golub (31). The different results appear to be due to the extraction methods used in the two studies. In the present study the soluble embryo extract was prepared in the presence of 0.5 M NH_4Cl; therefore, any ribosome-associated elongation inhibitor in the dormant cyst extract would be solubilized by this treatment. In fact studies by Huang and Warner have shown that ribosomes from 12-h embryos are more active in poly(U)-directed protein synthesis compared to ribosomes from early stages of development due to the presence of a ribosome-associated inhibitor (62). The findings in the present study support those of Huang and Warner and suggest the presence of an inhibitor substance on the ribosomes.

In this study the alkaline protease activity has been detected in soluble extracts from hatched _Artemia_ embryos prior to the increase in general protease activity following hatching (Fig. 9b versus Table 10). Previously, other workers reported that various alkaline protease activities appear in Artemia embyos about 10 h after the onset of hatching (116-118). However, the assays that were employed in the above studies were not sensitive enough to detect limited proteolysis. In the present study using SDS-polyacrylamide gel electrophoresis to monitor protease activity, alkaline protease activity was detected in soluble extracts from 6-h hatched embryos or about 6 h 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 were generated, at least partially, during homogenization and sample preparation. The enzyme that hydrolyzes EF-2 appears to be specific for EF-2; nevertheless, it may hydrolyze other proteins in extracts from newly hatched embryos and account for the disappearance of polypeptides from these extracts as seen on SDS-polyacrylamide gels (Figs. 4 and 9a). 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 explain the reduction in activity of these extracts to support in vitro polyphenylalanine synthesis. The loss in protein synthesis capacity in vitro and the reduction in heavy EF-2 and other protein bands in extracts from older embryos (post-hatched) are probably not a reflection of the physiological state within the embryo but of uncontrolled protease activity in these extracts.

The present study has also shown that under certain conditions, alkaline protease activity can be detected in preparations from pre-hatched and newly hatched embryos. Considerable alkaline protease activity could be detected in the free protein fractions of these embryos if they were prepared by Sepharose 6B followed by $(NH_4)_2SO_4$ fractionation (see Table 11). Moreover, the EF-2 present in the above extracts was stable upon storage at -20° to -70°C for at least 3 months as judged by SDS gel analysis. In contrast, EF-2 in Sepharose 6B fractions (free and bound) from post-hatched embryos was not stable; upon storage as above the amount of heavy EF-2 decreased giving rise to additional light forms (data not shown). Also, specific proteolysis of heavy EF-2 to the light EF-2 proteins occurred after fractionation of the S-150 and NH_4Cl-soluble P-150 fractions from Artemia cysts on DEAE-cellulose (Table 12). The light forms of EF-2 generated by DEAE-cellulose fractionation resemble the light forms of EF-2 isolated from hatched embryos in their inability to catalyze ribosome-dependent GTP binding. In addition, the fragments generated by the DEAE-cellulose treatment inhibit slightly poly(U) translation in vitro. The significance of this finding is not clear but it might be related to the observation that a modified form of EF-2 is inhibitory in protein synthesis (68, 69).

The presence of sequestered EF-2 hydrolase activity in crude extracts of dormant cysts suggests that some type of regulatory mechanism operates in Artemia embryos to control the metabolism of EF-2 during development. In addition, the findings that light EF-2 (41,000 MW) is present on polyribosomes in substantial amounts, whereas it is present only in trace amounts in the other post-mitochondrial fractions (Fig. 6) suggest that the metabolism of EF-2 may be regulated at the polyribosome level.

During the present study evidence has accumulated in support of the idea that the light forms of EF-2 found in extracts from hatched embryos may not represent the physiological situation in the intact animal. If we assume that this idea is correct, what process permits the generation of EF-2 fragments to the extent observed in extracts from hatched embryos? 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 (128). 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.

Alteration of the rate of proteolysis is often controlled by activation-inhibition or translocation within the cell of the degrading enzyme (103, 129-132). A different mode of control of degradation may occur by altering the conformation of the protein substrate. The latter mechanism may occur as a result of the interaction of various ligands with the protein which changes the sensitivity of the protein to proteases (128, 133-135). If for various reasons (as discussed below) the protease which is 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. Goldberg and Dice have suggested that larger proteins are degraded more rapidly than smaller proteins and that the more rapid degradation of large proteins evolved because of special functions that large proteins may serve in the cell which may require rapid turnover (128). Hence, the degradation of EF-2 might be enhanced in Artemia embryos after hatching.

To determine whether heavy EF-2 in crude extracts from dormant Artemia embryos is susceptible to hydrolysis by EF-2 hydrolase, mixing experiments of crude extracts from dormant cysts and fractions that exhibit EF-2 hydrolase activity were carried out (data not shown). Incubation of the DEAE-cellulose fraction from the S-150 fraction of dormant cysts which contains EF-2 with the untreated S-150 fraction for 2 to 3 days at 5°C did not lead to the breakdown of the intact EF-2. A similar finding was observed when the soluble extract from 24-h embryos was incubated under these conditions with the soluble extract from 0-h embryos. However, these experiments did not resolve the question of whether EF-2 is insensitive to proteolysis in dormant cysts or whether an inhibitor of EF-2 hydrolase is present in these embryos. Perhaps both possibilities exist in the dormant cysts. In another experiment it was observed that no EF-2 degradation occurred when partially purified EF-2 was mixed with soluble extracts from 0-h embryos (Fig. 9b). These results support the conclusion that EF-2 hydrolase is not active in extracts from dormant cysts. However, if purified EF-2 regains its native conformation immediately upon mixing with an extract from 0-h embryos, it may be protected from hydrolysis even in the present of active EF-2 hydrolase. Consequently, this problem remains to be resolved.

Further studies will be needed to elucidate the importance (if any) of the sudden increase in light forms of EF-2 in Artemia nauplii as observed in extracts of hatched embryos. However, the striking similarity between the proteolysis products of EF-2 which appear during development of Artemia and during aging of rat liver EF-2 in vitro (91).

suggests that there may be a general process of EF-2 metabolism which is highly conserved in the animal kingdom. Unfortunately, the study of rat liver EF-2 in which the above process was found to occur has not been extended. Nevertheless, Collins et al. observed a specific breakdown of the 96,500 molecular weight EF-2 into components of 64,000, 42,000 and 37,000 MW following aging of highly purified EF-2 for one month at 0-4°C (91). None of the breakdown products was found to possess the enzymatic properties of EF-2, but the two smaller fragments accepted ADPR in the diphtheria toxin-dependent reaction. Using SDS-polyacrylamide gel electrophoresis the above workers demonstrated that the 64,000 MW fragment is the dominant breakdown product of rat liver EF-2. Careful examination of the SDS gel electrophoresis patterns of different Artemia embryo extracts (Fig. 4) revealed that several new protein bands appear in the region of the BSA marker concomitant with the disappearance of the 95,000 MW polypeptide (EF-2). One band appears in the same position or slightly under the BSA marker. This protein band (67,000) is very clear in the bound preparations from 31 and 33-h embryos and was observed in the NH_4Cl soluble fraction from polyribosomes of 20-h embryos along with the 41,000 MW fragment that can be ADP ribosylated. The 67,000 MW polypeptide was below the level of detection in the NH_4Cl washes of the monoribosome fraction from dormant cysts and 20-h embryos in which only trace amounts of the 41,000 MW fragment were found. In contrast to the above findings, the 67,000 MW protein was not observed when EF-2 was treated with a soluble extract from 24-h embryos under conditions where the 41,000 MW polypeptide was generated (Fig. 9b). Thus, it is not clear whether the same mechanism that hydrolyzes endogenous EF-2 in extracts from hatched embryos is responsible for the degradation of exogenous EF-2.

The present study with Artemia EF-2 suggests that the

41,000 MW fragment is the precursor of all other small ADPR accepting fragments including the 36,000 MW polypeptide. This idea is supported by the following observations. a) The 41,000 MW fragment was found on polyribosomes of 20-h embryos, whereas the 36,000 MW fragment was not detected on these organelles (Fig. 6). b) The various profiles of ADPR accepting proteins during development of Artemia indicate that the 41,000 MW fragment is the dominant one following initiation of the breakdown process of EF-2 (Figs. 2 and 8). c) In some experiments only the 41,000 MW fragment was found in the embryo extract immediately after hatching, whereas the 95,000 and 36,000 MW forms of EF-2 were barely detectable. Subsequent chromatography of this extract on DEAE-cellulose generated the 36,000 MW fragment. Collectively the data obtained in this study, in respect to the breakdown of EF-2, suggest that the hydrolytic activities responsible for the appearance of both the 41,000 and 36,000 MW fragments are sequestered in pre-hatched embryos and that their activation occurs sequentially during post-hatched development. Further degradation of the light EF-2 proteins may also involve other regulatory events since the pattern of appearance of the small ADPR accepting fragments is very distinct (see Fig. 8).

The assumption that fragments of 67,000 and 41,000 MW are the first degradation products of Artemia EF-2 was suggested from the findings of Collins et al. who studied the rat liver EF-2 (91). Summation of the molecular weights of these fragments gives a higher value than the molecular weight of the intact EF-2 as determined by SDS-polyacrylamide gel analysis (108,000 versus 95,000 MW). The fragments generated during aging of rat liver EF-2 also have an aggregate molecular weight greater than native EF-2 (91). Moreover, amino acid analysis data of rat liver EF-2 (native and ADP ribosylated) are consistent with a molecular weight of 112,000 for EF-2 (136). Therefore, it appears likely that native EF-2 behaves differently on SDS-polyacrylamide gels than standard proteins. Anomalous mobilities on SDS-polyacrylamide gels have been described for proteins that are complexed with non-protein material such as glycoproteins and lipoproteins. Similar problems have been described for proteins whose conformation is different from the standard molecular weight markers in the presence of SDS. Various aspects of the anomalous migration of certain types of proteins are discussed by Maddy (137).

In view of the chemical similarity between native EF-2 from mammals and aves (113, 114) and between the trypsin derived ADP-ribosylated peptide of rat liver EF-2 (109) and yeast (110), it appears that the degradation process of EF-2 might be conserved during evolution. Unfortunately, no literature values are available for comparison except those described above. However, Collier and Traugh have obtained some preliminary results related to this process (138). These workers observed the appearance of ADPR accepting fragments on ribosomes from rabbit reticulocytes aged for deveral days in an unfrozen state. They also stated that EF-2 from the supernatant fraction of yeast is smaller than the EF-2 prepared from yeast ribosomes, but these observations are hard to evaluate since no data were given by these workers.

During this dissertation research the presence of light forms of EF-2 was detected in the myogenic cell line L8 (for details about this cell line see ref. 124). Although these results were not presented above, it should be noted that trace amounts of $[^3H]$ADPR accepting fragments similar in molecular weight to the Artemia light forms of EF-2 were detected in the S-150 and P-150 fractions prepared from the mononucleated cells (proliferative state) and from the multinucleated fibers (differentiated state). The light forms of EF-2 represent only 1-4% of the total EF-2 in the myogenic cell line, whereas the light forms are the dominant ones in extracts from post-hatched Artemia embryos. Studies on other developmental systems such as the fertilized egg of the sea urchin should be conducted to elucidate further the relationship between embryogenesis and EF-2 metabolism.

This part of the discussion concludes with a very intriguing question. Is the observed degradation process of Artemia EF-2 a reflection of the catabolic process of the protein or is it related to the function of EF-2 in vivo? Moldave et al. found that the molecular weight of functional EF-2 from rat liver is 65,000 (139). Their study contrasts with the findings of Raeburn et al. who reported that EF-2 from rat liver has a molecular weight of 96,500 (90, 91). It should be noted, however, that Raeburn et al. employed different extraction conditions than Moldave et al. and they detected a 64,000 MW fragment of rat liver EF-2 after aging of the EF-2 at 0^{o}-4^{o}C. The 64,000 MW fragment which appeared during aging of rat liver EF-2 was not functional in protein synthesis (91). However, the sensitivity of EF-2 to hydrolysis (as described in this study and as a result of aging of rat liver preparations) might serve as a mechanism for the generation of active fragments of EF-2 from a high molecular weight precursor. Studies on wheat germ EF-2 which has a molecular weight of 70,000 support this idea (140).

In the present study the existence of EF-2 fragments active in protein synthesis was considered. However, when preparations containing the heavy and light EF-2 proteins were fractionated on Sephadex G-150 and the column fractions were tested for ribosome-dependent GTP binding or stimulation of polyphenylalanine synthesis only the 95,000 molecular weight EF-2 exhibited these properties. If active fragments of the 95,000 MW species of EF-2 exist in Artemia embryos, their detection in embryo extracts may be obscured by the presence of proteases which are activated during cell breakage.

Recently, Twardowski et al. have shown that extracts from nauplii of Artemia salina disaggregate EF-1_H without producing a significant change in the size of the EF-1 active polypeptide (about 50,000 MW) as determined by SDS-polyacrylamide gel analysis (141). They have also found that carboxypeptidase A (141) and elastase (142) disaggregate EF-1_H without loss in EF-1 activity. They concluded that the former enzyme removes a small fragment from the carboxyl end of the EF-1 active polypeptide that is required to maintain the EF-1_H aggregate. They also suggested that a protease in extracts from Artemia nauplii hydrolyzes the 50,000 MW polypeptide of EF-1 at a site similar to that hydrolyzed by carboxypeptidase, whereas elastase catalyzed the hydrolysis of this polypeptide at other sites along the chain. These findings are similar to those observed for EF-2 hydrolysis by extracts of Artemia embryos and both processes may be related to the release of protein synthesis during development.

The importance of a protein-degrading process in the control of development has been generally acknowledged (128, 131, 134). 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.

C. Purification of elongation factor 2 from dormant cysts of Artemia salina

A five-step procedure has been devised for the purification of EF-2 from dormant cysts of Artemia. This research was mainly concerned with establishing a procedure that avoids the breakdown of EF-2 which was observed during early attempts to purify this protein synthesis factor. The adoption of the diphtheria toxin-catalyzed ADP-ribosylation assay for EF-2 enabled us to understand the reasons for the instability of EF-2 in certain extracts of Artemia embryos. The choice of the P-150 fraction as the starting material for the purification of EF-2 has not been described previously. Starting with the P-150 fraction it was possible to obtain a crude fraction of EF-2 that had a specific activity several times higher than that of the S-150 fraction from Artemia. Moreover, using this procedure the enzyme (EF-2) was exposed to various fractionation conditions and media for a minimal time. Also, a large quantity of EF-2 from the DEAE-cellulose column was sacrificed to avoid contamination by a specific protease. The hydrolysis of EF-2 by an endogenous protease was also minimized by fractionating the crude P-150 fraction on Sepharose 6B as the initial step in the purification. It is noteworthy that the lability of EF-2 after chromatography on DEAE-cellulose was described previously by Hardesty and McKeehan in their report on the purification of rabbit reticulocyte EF-2 (143).

Using the procedure described herein an EF-2 preparation about 92% pure was obtained. The major protein in the preparation has a molecular weight of 95,000 as determined by SDS-polyacrylamide gel electrophoresis (Fig. 18), and it appears to be EF-2 because it exhibits most of the ADPR binding capacity in the preparation. The trace amounts of the light forms of EF-2 detected by [^{3}H]ADP-ribosylation represent less than 0.1% of the diphtheria toxin-catalyzed ADPR binding activity of the final EF-2 preparation. The quantity of small fragments in the final preparation did not increase after two months storage at -70°C. Therefore, it can be concluded that Artemia cyst EF-2 consists of a single polypeptide as previously described for other eukaryotes (82, 83) and for its prokaryotic counterpart EF-G (144, 145). The molecular weight of the Artemia cyst EF-2 (95,000) is similar to that reported previously for EF-2 from rat liver (90, 91), human tonsil (83), pig liver (112), rabbit reticulocytes (113) and hen oviduct (114) using SDS-polyacrylamide gel electrophoresis. By comparison, a molecular weight of 70,000 has been reported for wheat germ EF-2 (140) and early experiments by Moldave et al. suggested a molecular weight of 65,000 for rat liver EF-2 (139). (See discussion above in respect to the lower molecular weights of EF-2). Molecular weights of 84,000 and 78,000 were reported for the prokaryotic factor EF-G from Escherichia coli (144) and Thermus thermophilus (145), respectively.

In the present work some of the functional properties of the Artemia EF-2 were studied. Purified EF-2 was shown to support poly(U)-directed protein synthesis and ribosome-dependent GTP binding activity in vitro.

The yields and the specific activity of the Artemia EF-2 studied in this investigation were similar to values obtained by other investigators (90, 113, 114). An attempt to avoid protein impurities in the final EF-2 preparation produced lower yields of active enzyme. The purified EF-2 from Artemia was found to be sensitive to dilution and to adhere to glass surfaces. SDS-polyacrylamide gel analysis of the EF-2 containing fractions after chromatography on phosphocellulose showed that the elution profile of most contaminant proteins coincides with the elution profile of EF-2. Synthetic homoribopolymers might have some utility in the purification of EF-2 since Warner et al. (unpublished data) have found specific binding of EF-2 to poly(U)- and poly(G)-agarose. The binding of EF-2 to various homoribopolymers was previously reported by Traugh and Collier (146). Recently, Lan and Heinz described conditions in which EF-2 from reticulocytes was immobilized to a column of NAD-agarose in the presence of diphtheria toxin (147). Following binding to the column these workers were able to recover active EF-2 from the column by reversing the diphtheria toxin-dependent reaction. In future studies this technique might be useful for the purification of this protein from Artemia embryos at different stages of development since it removes contaminating proteins from solutions containing EF-2 in one step. Moreover, this latter purification method may achieve separation of EF-2 from its hydrolase more rapidly and avoid degradation of EF-2 such as that experienced in the present study.

Previously, Nombela and Ochoa reported that they had purified Artemia EF-2 to homogeneity using a three-step procedure and that the molecular weight of the purified EF-2 is 90,000 (148). More recently Slobin and Möller described a procedure for the purification of EF-2 from the post-ribosomal supernatant fraction of Artemia cysts without providing any information on the molecular weight of the protein (77). Although the latter report is more detailed than the former, neither study provided supporting data on the method of purification of EF-2. Thus, the present study is the first comprehensive study leading to the purification of EF-2 from Artemia salina.

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VITA AUCTORIS

Born: December 24, 1949, Tel-Aviv, Israel

Parents: Mr. Arie B. and Mrs. Bella Yablonka

Secondary Education: "Saul Tchernichovski" Municipal High School
Academic Stream - Biology
Netanya, Israel, 1963/64-1966/67

Military Service: (compulsory), 1968-1969

University Education: The Hebrew University of Jerusalem
Jerusalem, Israel, 1969/70-1971/72
Graduated with the degree of
Bachelor of Science in Biology

The Hebrew University of Jerusalem
Jerusalem, Israel, 1971/72
Rehovot , Israel, 1974/75
Secondary-Teacher Education
Graduated with Biology Teaching Diploma

The Feinberg Graduate School and the
Department of Cell Biology of
The Weizmann Institute of Science
Rehovot, Israel, 1972/73-1974/75
Graduated with the degree of
Master of Science in Life Sciences

The Feinberg Graduate School and the
Department of Cell Biology of
The Weizmann Institute of Science
Rehovot, Israel, 1975/76
Studies toward the degree of Ph.D.

On leave of absence from the Weizmann Institute
Working with Dr. A.H. Warner of the
Department of Biology, University of Windsor
Windsor, Ontario, 1976/77

The Faculty of Graduate Studies and the
Department of Biology of
The University of Windsor
Windsor, Ontario, 1977/78-1978/79
Studies toward the degree of Ph.D.