AN ABSTRACT OF THE THESIS OF

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Title: ISOLATION AND CHARACTERIZATION OF THE NUCLEAR HISTONES FROM DEVELOPING EMBRYOS OF THE BRINE SHRIMP, ARTEMIA SALINA

Abstract approved: Redacted for privacy
John E. Morris

Techniques were developed for extracting histones from isolated chromatin of encysted embryos of Artemia salina. Histone extracts from embryos collected at eight time intervals after rehydration and initiation of development were analyzed by electrophoresis for qualitative and quantitative changes in composition. Histone to DNA ratios were determined for the same eight times.

Proteins were extracted at 17 time intervals from 0 hr up to 60 hr after rehydration. The extracts were subjected to visual analysis by polyacrylamide gel electrophoresis in an effort to determine when new proteins first appear, as an index of differentiation. An attempt was then made to correlate changes in the differentiated state with changes in the nuclear histones.

Changes in total protein were not evident until after 16 hr of hydration, when naupliar structures are well-formed. Two changes in histone pattern were observed. The first was the disappearance of a slow-moving band between 0 and 4 hr of development (band 2). The second was the increase in the proportion of the slightly lysine-rich fraction II (band 6) histones between 36 and 48 hr of development, and a decrease in band 3, a lysine-rich histone.

The evidence supports the conclusion that genes are activated in order to direct differentiation only several hours after hatching of the nauplius larva has occurred. New proteins which appeared shortly before emergence of the nauplius may be synthesized under the direction of pre-existing mRNA.

Isolation and Characterization of the Nuclear Histones from Developing Embryos of the Brine Shrimp, *Artemia salina*

by

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INTRODUCTION

The work of Stedman and Stedman (1950) first brought attention to the possibility that the main function of histones might be the control of gene expression. Their simple experiments demonstrated that salmon erythrocyte histone, liver histone and sperm histone varied greatly in arginine content.

Since that time, periodic bursts of research activity have made available sensitive techniques for the analysis of individual histone components and for the analysis of differential DNA[1] transcription activity (an indicator of gene expression) of isolated chromatin preparations. Bonner et al. (1968b) showed that deproteinized rat liver chromatin (stripped of histone with 0.2 N HCl or trypsin) used as a template for in vitro exogeneous RNA polymerase activity assay generated over four times the mRNA (as judged by incorporation of labeled AMP into the system) as pure chromatin or stripped chromatin to which whole histone had been restored. Addition of histone fractions to in vitro polymerase assay systems using naked calf

[1]Abbreviations used in this paper: DNA, deoxyribonucleic acid; RNA, ribonucleic acid; mRNA messenger ribonucleic acid; AMP, adenosine monophosphate; rRNA, ribosomal ribonucleic acid; hRNA, heavy ribonucleic acid.

thymus DNA as a template resulted in a differentially depressed template activity. Histone IV (arginine-rich) fractions restricted template activity to a greater degree than the histone II (slightly lysine-rich) fractions and histone I (lysine-rich) fractions depressed template activity the least (Allfrey and Mirsky, 1964; Spelsberg et al., 1971).

Marushige and Ozaki (1967) dealt with the role of nuclear proteins in a developmental analysis of sea urchin chromatin. Their experiments showed that the template activity of pluteus chromatin was less than that of blastula chromatin. Total removal of proteins associated with DNA in the chromatin greatly increased template activity and abolished the difference in template activity between blastula and pluteus chromatin. Thus, removal of histone protein or acidic nuclear proteins or both resulted in the disruption of a delicate control system of gene expression operative in the two stages. Furthermore, the histone to DNA ratio was higher in the blastula stages than in the pluteus stages. Purified extracts of histones in the pluteus exhibited a histone band upon electrophoresis that was not present in the blastula. Even without evidence for an exact function, their work clearly implies that histones have an important developmental role.

Numerous papers have appeared in recent years which compare histones from several developmental stages and different tissues of species of plants and of animals. The customary means of analyses have been cytochemical staining with fast green (Das et al., 1964;

Yanagi and Kusanagi, 1970), column chromatography using Amberlite CG-50 (Asao, 1970; Srivastava, 1971), starch gel electrophoresis (Lindsay, 1964; Hnilica et al., 1966) and polyacrylamide gel electrophoresis (Fambrough et al., 1968; Mohberg and Rusch, 1970; Easton and Chalkley, 1972). The last technique has become accepted as the most sensitive for qualitative and quantitative determination of histones.

Most reports indicate that there are differences in histone properties during development. Although the possible significance of these differences for gene control has been emphasized repeatedly, the mechanism by which histones might act is still a matter of intense debate (Hearst and Botchan, 1971). It is worth noting that some workers found histone to DNA ratios and histone properties to be similar in tissues which differ strongly with respect to synthetic activity. Kischer and Hnilica (1967) concluded that adult and embryonic chicken brain, liver and dorsal skin histones were practically identical by starch gel electrophoresis as did Beeson and Triplett, (1970) using acrylamide gel electrophoresis. No changes in histones between heterochromatin vs. euchromatin, metaphase cells vs. interphase cells or mature vs. phytohemagglutinin stimulated lymphocytes was reported by Comings (1967). Histones prepared from heterochromatin and euchromatin in mealy bugs were found to be identical (Pallotta et al., 1970).

One of the most informative reports of histone changes during development was that of Mohberg and Rusch (1970), who indicated a relatively high percentage of moderately lysine-rich histones in non-growing cultures of spherules and of starving and sporulating plasmodia of Physarum polycephalum, the slime mold. However, histone to DNA ratios were quite similar for growing, starving and dormant cultures.

Excellent work by Fambrough et al. (1968) showed that as pea seedlings develop to maturity, a steady increase in the amount of very lysine-rich histones (fraction I) occurred relative to total histone. This is consistent with the view of many authors that lysine-rich histones are important in the formation of heterochromatin. Further, the low histone I content of the immature cotyledons (7.8%) was paralleled by a low histone to DNA ratio (0.76) and high template activity supporting mRNA synthesis (32%). Dormant buds, which are high in histone I (15.8%) had a high histone to DNA ratio (1.30) and low template activity (6%).

Histones from different regions of the newt, Triturus pyrrhogaster, embryo in the course of development were analyzed qualitatively and quantitatively by Asao (1970) using an unusually sensitive procedure. He found that lysine-rich histone (histone I) continuously increases during early development, more rapidly in ectodermal than in mesodermal or endodermal tissue. Arginine-rich histones, a

major component of the blastula, tended to decrease in every region as development proceeded, and lingered longest in the endoderm throughout embryogenesis.

Histones were isolated from various developmental stages of the sea urchin Strongylocentrotus drobachiensis and the loach Misgurnus fossilis by Vorobyev et al. (1969). In both cases many small, less mobile, electrophoretic fractions disappeared during the course of development and later stages of development contained a relatively smaller amount of arginine-rich histones. The greatest changes in histone proportions occurred at gastrulation, when a large quantity of new mRNA appeared. Thus, as in the previously described experiments, there appeared to be a correlation between changes in the population of cells synthesizing mRNA and changes in the composition of histones in those cells during development.

In the literature on histones in development, two persistent and important technical problems have created inconsistencies in interpretation. First, the various methods of extraction and various extents of purification of histone molecules yield different results. For example, embryonic tissue from developmental stages of the sea urchin have been reported to contain as few as three histones (Ozaki, 1971) and as many as 32 histones (Spiegel et al., 1970). Thaler et al. (1970) reported eight sea urchin histones, Vorobyev et al. (1969) recovered ten, Hill et al. (1971) reported 11 and Repsis (1967) found

13 histone fractions. Thus, many contaminants, breakdown products and artifacts appear to be reported in the literature as histone. Second, most changes in histone composition during development were based on extraction from two or at most three separate (sometimes arbitrary) stages. I believe that a dynamic picture of histone composition can only be obtained by sampling developing embryos at closer intervals.

Some interesting work by Neelin (1964) deserves brief mention. He found a new (previously uncharacterized) major histone component in mature chicken erythrocytes which could not be detected in other avian tissues by starch gel electrophoresis. The presence of this new histone, as well as the high histone to DNA ratio (1.30) of this tissue (Bonner, 1967), are correlated with the following peculiar properties of avian erythrocytes: (1) simple cytoplasm, terminally differentiated, (2) small, condensed nuclei, (3) absence of mitosis, and (4) no mRNA synthesis. This certainly suggests that histones play some role in the regulation of gene expression.

The brine shrimp, Artemia salina (Order Anostraca, Subclass Branchiopoda), appears to have excellent potential for further investigation of histone developmental behavior. Dormant, desiccated, encysted gastrulae are completely ametabolic and developmentally inert (Clegg, 1967). Upon hydration in dilute salt solutions they are capable of considerable differentiation without cell division (Nakanishi

et al., (1962). Since histones usually are synthesized coordinately with DNA synthesis, there is probably no increase in histone content during at least the first ten hr of development under conditions where emergence occurs at about 12 hr. Any changes in amount of histone probably would reflect developmental events that are not a result of mitosis itself as described by Mohberg and Rusch (1970).

Cell division is resumed in the embryo after it emerges from the cyst and increases considerably during the formation of the nauplius larva (12 to 24 hr). A detailed review of reproduction and development in Artemia has been made by Finamore and Clegg (1969).

Polyribosomes capable of support of in vitro protein synthesis are present during pre-emergence development (Hultin and Morris, 1969; Finamore and Clegg, 1969). Also, $NaH^{14}CO_3$ was shown to be incorporated mainly into ribosomal RNA (rRNA) during the first two hr of incubation by Clegg and Golub (1969). McClean and Warner (1970) have isolated what they call "heavy" RNA (hRNA) which is synthesized intensely between 20 and 36 hr of naupliar development (i.e., after hatching). Much of the hRNA is present already in the encysted gastrula. The exact function of hRNA or whether it represents mRNA is not yet known. It may be stored or masked informational RNA (mmRNA) (McClean and Warner, 1970). Incorporation of $^{14}CO_2$ (^{14}C amino acids will not enter the cysts) in encysted Artemia embryos indicated that some protein synthesis was taking place as

early as three hr of development, but at a much lower rate than after emergence of the embryo from the cyst (Clegg, 1966).

Obviously the details of the molecular events in early Artemia development are far from clear, particularly with regard to the synthesis of RNA. However, this system appears to be a unique one for examining the relationship between protein synthesis, its regulation and the process of differentiation. Because of the extreme environmental pressures to which these embryos are subjected (exposure to heat, freezing, etc.) highly stable mechanisms probably evolved to protect the protein synthesizing machinery and the regulatory molecules for gene control in early development. The studies I have described in this Introduction indicate that histones might, indeed, play an important role in the control of transcription in Artemia.

In order to draw conclusions about the probable role of histones in maintaining the integrity of genes and in directing their expression in development, the present work was designed and executed with the following specific goals in mind:

1. To design a procedure for the extraction of histones in Artemia salina which gives reproducible results when assayed by polyacrylamide gel electrophoresis and which can be characterized by electrophoretic comparison to the two best known histone types, pea and calf thymus.

2. To determine whether there are changes in the electrophoretic character of histones and in histone to DNA ratio during development from gastrula to nauplius stages. Qualitative changes in histone fractions and histone to DNA ratios were noted at eight separate stages: 0, 4, 8, 16, 24, 30, 36 and 48 hr after rehydration of the desiccated cysts.

3. To determine changes in the electrophoretic character in the total protein make-up of the entire embryo for use as an index of actual degree of differentiation attained at each stage. Qualitative and quantitative protein determinations were prepared every two hr from 0 to 26 hr plus 36, 48 and 60 hr.

METHODS AND MATERIALS

Artemia salina Culture

Encysted gastrulae of the brine shrimp, Artemia salina, were obtained in 12-1/4 oz evacuated containers from Maury Rakowicz Biologicals (1655 West Winton Avenue, Haywood, California 94505) and once opened were stored under vacuum at room temperature. The cysts were incubated at 1.28 g aliquants in 600 ml of Millipore^{R}-filtered 0.5 strength Instant Ocean^{R} (Aquarium Systems, Inc., 1450 East 289 Street, Wickliffe, Ohio 44092) in sterilized 29 x 19 cm enamel pans. Each incubation procedure was carried out at room temperature (approximately 23^{o}C) and evaporation was kept to a minimum by covering the pans with Saran Wrap^{R}.

Collection of the cultures was accomplished immediately after each timed interval by pouring the incubating embryos over filter paper in a Büchner funnel and concentrating them by suction filtration onto the surface of the paper. After six washes in the funnel with ice cold distilled water, the embryos were transferred to a cold mortar and pestle for extraction of either histone or total protein.

Periodic assay of the viability of the cyst batches and the determination of the timing of developmental stages at 23^{o}C was accomplished by incubating about 20 cysts per drop in 16 hanging drop suspensions under the lid of a polystyrene petri dish. The lid was

inverted over 20 ml of the incubation medium to retard evaporation of the drops. Hourly counts of emerged prenauplii and swimming nauplii were made with the use of a dissecting microscope.

Microscopy

Scanning electron micrographs of cysts were made in an attempt to detect any unusual surface features which might aid in the maintenance of the cup-shaped contour of the cyst shell during dehydration. Examination of the cyst was performed using a Cambridge Stereoscan Mark II^{R} A scanning electron microscope at Materials Analysis Cost Center, Department of Mining, Metallurgical and Ceramic Engineering, University of Washington, Seattle, Washington. The specimen was coated with 50 Å of carbon followed by a 200 Å layer of gold under vacuum.

Light microscope photographs of the developmental stages were taken under a Zeiss^{R} Standard R A Microscope with the aid of a Nikon^{R} Microflex Model AFM automatic photomicrographic attachment. Individual embryos were mounted in drops of incubation medium without stain.

Extraction of Histones

The procedure for exacting histone was basically that of Bonner et al. (1968a), designed for pea tissue. Peculiarities of embryonic

Artemia cells dictated several important procedural deviations.

Quantitative extraction of nucleohistones was accomplished by the following steps, each carried out at 0-3°C in an ice bath:

1. 1.280 g of cold, washed cysts were ground to a thick slurry with a cold mortar and pestle in 75 ml of ice cold grinding medium which consisted of 0.25 M sucrose (Mallinckrodt Chemical Works), 0.01 M tris (hydroxymethyl) aminomethane HCl pH 7.2, 0.1% (w/v) Triton X-100 (Sigma) and 0.002 M $CaCl_2 \cdot 2H_2O$. The slurry was then transferred to a Ten Broeck type tissue grinder (Bellco Glass Co.) for more complete pulverization.
2. The mixture was passed twice through four layers of cheese cloth to remove cyst shells and then passed twice through one layer of Miracloth^R (Calbiochem) to eliminate smaller shell fragments.
3. The slurry was centrifuged at 4,000 x g (5,750 rpm) for 30 min on a Sorvall^R RC-2B refrigerated centrifuge at 0°C. The resulting pellets were carefully resuspended in 40 ml of grinding medium with a tissue grinder. Centrifugation was repeated at 10,000 x g (9,250 rpm) for ten min.
4. The 10,000 x g pellets were suspended, again with the aid of a tissue grinder, in 40 ml washing medium (0.01 M tris-HCl pH 7.2, 0.1% (w/v) Triton X-100, 0.002 M $CaCl_2 \cdot 2H_2O$) and subjected to centrifugation at 10,000 x g (9,250 rpm) for ten

min. This washing procedure was repeated four times.

5. Washed pellets of crude chromatin were then suspended in 5.0 ml of washing medium with the tissue grinder and pipetted onto the surface of 23 ml of chromatin centrifuging solution in a cellulose acetate ultracentrifuge tube (7.5 x 1.5 cm). The chromatin centrifuging solution was made up of 2.0 M sucrose, 0.01 M tris HCl pH 7.2, 0.1% (w/v) Triton X-100 and 0.002 M $CaCl_2 \cdot 2H_2O$. After stirring the crude chromatin sample 1/3 to 1/2 of the way into the dense centrifuging medium with a glass rod, the sample was spun at 58,000 x g (24,000 rpm) in a SW 25-1 rotor on a Spinco^R ultracentrifuge for 90 min. This step was performed to remove ribosomal material which contains basic proteins electrophoretically similar to histones.

6. The supernatant fraction was pipetted off the dense "purified chromatin" pellet. The dense residue was then extracted with either 30 ml 0.4 M H_2SO_4 or 30 ml 0.1 N HCl by grinding in a tissue grinder and stirred for 30 min over ice. After centrifugation at 17,750 x g (12,000 rpm) for two hr the pellet was again extracted with 20 ml acid.

7. Both supernatant fractions were pooled and added to five volumes (250 ml) of cold absolute ethanol and allowed to stand at -20°C in a freezer for 72 hr. One-tenth normal HCl extracts did not form a precipitate at the extraction pH (pH 1.5) and required

adjustment to pH 10.1 with 1.0 N NaOH after addition to the ethanol. The flocculent white precipitate which formed was collected by centrifugation at 10,000 x g (9,250 rpm) for 20 min.

8. The precipitate, containing both histone and glycogen, was air dried at room temperature in an evaporating dish and ground to a white powder and then suspended in 2.0 ml 0.9 N acetic acid and stirred overnight at 4°C. This thick suspension was finally centrifuged at 17,750 x g (12,000 rpm) for 30 min. The supernatant fraction was analyzed for protein content and then subjected to qualitative assay for histones by electrophoresis. The precipitate (i.e., the glycogen pellet) was extracted three times to fully recover the histone for quantitative analysis.

See Figure 1 for a summary of the above procedure.

Alternative Methods for the Extraction of Histones

The extraction of histones using "crude chromatin" as prepared by the method of Lindsay (1964) was also attempted. Briefly, this procedure consisted of grinding and filtering the cells in 2.2 M sucrose and repeating the procedure on a 40,000 x g (18,250 rpm) pellet. The second pellet was extracted directly with 0.1 N HCl and centrifuged for 30 min at 40,000 x g (18,250 rpm). The basic proteins from the acid extract, those in the 18,000 rpm supernatant fraction, were allowed to precipitate in five volumes absolute ethanol when

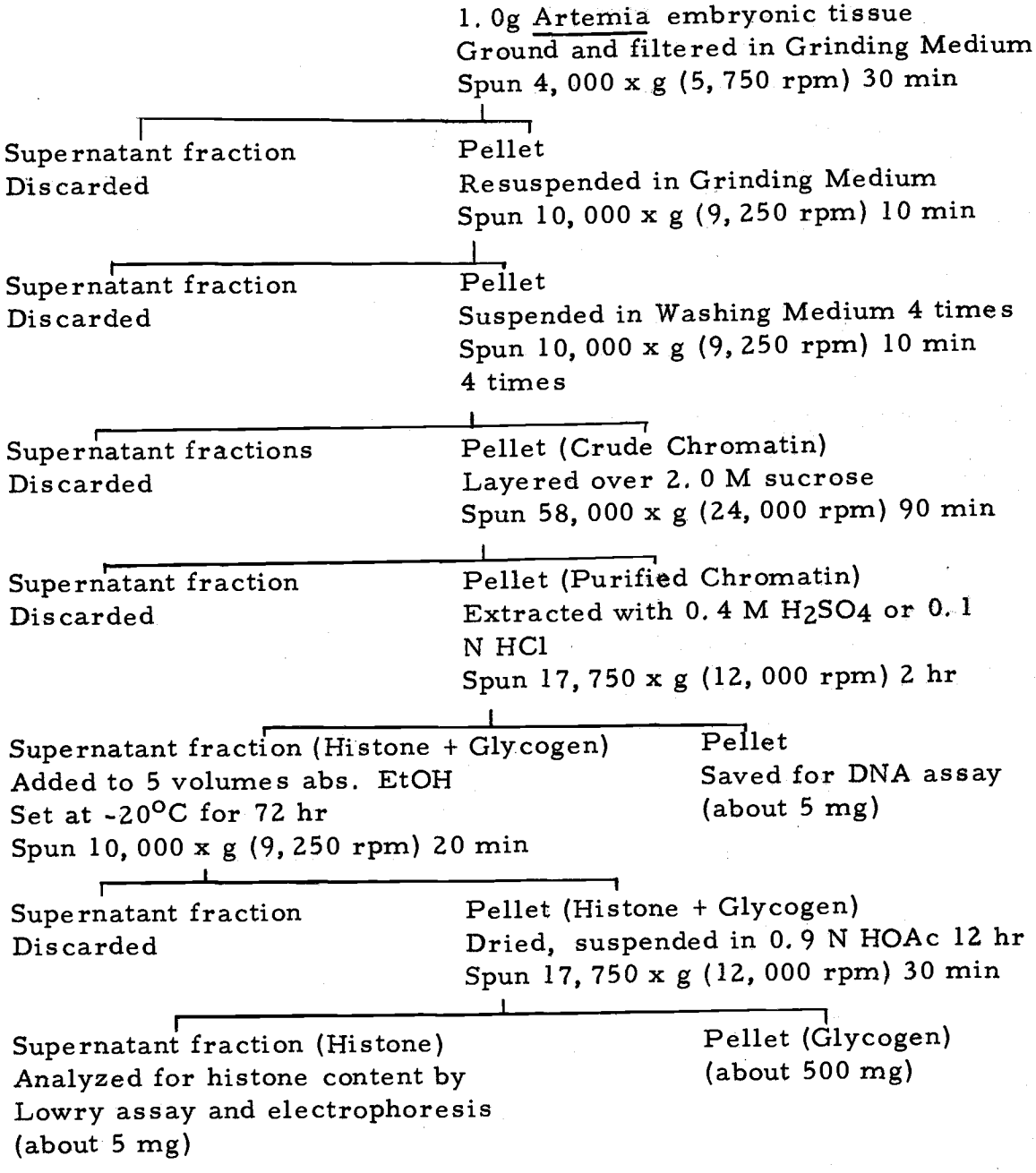


Figure 1. Basic procedure for histone extraction.

brought to pH 10.1 and were recovered from the precipitate as described in the previous detailed procedure.

Recovery of histones by the method of Mohberg and Rusch (1970) was also performed. In this case the purified chromatin was extracted with 1.0 M $CaCl_2$ overnight at 4°C. The 40,000 x g (18,250 rpm) supernatant fraction from the extract was brought to 25% trichloroacetic acid for histone precipitation. The pellet from a second 40,000 x g (18,250 rpm) centrifugation of the trichloroacetic acid precipitation was suspended, in this case, in 2.0 ml 0.02 M H_2SO_4, transferred to a dialysis tube and dialyzed against 0.02 M H_2SO_4 for 24 hr. The contents of the tube were drained and spun at 500 x g for five min in a clinical centrifuge, and the supernatant fractions were analyzed for protein by the method of Lowry et al. (1951) and for histone character by electrophoresis.

Electrophoresis of Histones

Histones were analyzed by subjecting purified extracts to electrophoresis according to the method of Panyium and Chalkley (1969) using a conventional disc electrophoresis apparatus as described by Davis (1964). The running gel (i.e., actual separating medium) consisted of 15% acrylamide (w/v) (Eastman Kodak Co., Rochester, N. Y.), 0.1% N, N'-Methylenebisacrylamide (w/v) (Eastman Kodak), 5.4% glacial acetic acid (v/v) and 0.5% N,N, N, N'-Tetramethylethylenediamine

(w/v) (Eastman Kodak). Sixty-five mm of the gel was polymerized catalytically in glass tubes (5 mm inner diameter by 7 mm outer diameter by 75 mm) by bringing the acrylamide mixture to 0.94% ammonium persulfate (w/v) and 5.0 M urea. Bubble formation at the time of polymerization was prevented by subjecting each stock mixture to reduced pressure from a laboratory faucet aspirator before use. This appeared to remove all dissolved gases accumulated during mixing and storage. The pH of the buffer reservoirs was 4.3 (0.9 N acetic acid). Neither a stacking gel (a purifying spacer) nor a sample gel (a gel which actually contains the sample) were used. A pre-electrophoretic charge of 2 ma per tube was applied to the system for three hr at room temperature to establish a continuous ionic character between the reservoir buffer and the gel.

Histone samples which had been dissolved previously in 0.9 N acetic acid were brought to 15% sucrose or 8 M urea and an appropriate amount (50-100 μg, as judged by Lowry protein assay) was pipetted on top of the gel. The density of the sample solution was sufficient to displace the reservoir buffer above the gel as well as restrict the basic proteins to the originally pipetted volume just above the gel. A constant current of 2 ma per gel was applied to the chamber with a Buchler^{R} Model 3-1014 DC power supply. Because the cathode was placed in the lower buffer reservoir basic proteins were pulled down into the gel. Separation in this system occurs on the basis of charge

character and molecular size. A 0.1% solution of methylene blue in 0.9 N acetic acid and 15% sucrose was layered on top of one of the samples as a tracking dye. When the tracking dye moved into the lower buffer chamber after about 90 min of electrophoresis, the power supply was switched off and the tubes were removed. The gels were teased free of the tubing with the aid of a fine jet of water forced through a blunted needle. The gels were then fixed and stained for general protein analysis with 0.7% naphthol blue black (Eastman Kodak) in 7% acetic acid for four hr. Destaining by exhaustive changes of 7% acetic acid over 48 hr revealed the presence of the basic proteins as sharp blue or black bands. The gels were stored in 5% glycerol (v/v) in a refrigerator and appeared to maintain their band patterns even after prolonged storage.

DNA Assay

Quantitative determinations of DNA content were made using the method of Burton (1956). A standard curve for the colorometric reaction was prepared using calf thymus DNA. The 17,750 x g pellet residues from the acid extraction for histone analysis (Figure 1) were extracted three times in a total of 20 ml 1.0 N $HClO_4$ (perchloric acid or PCA). Suspension of the pellet with a tissue grinder in PCA was followed by 25 min of stirring at 70°C and centrifugation on a clinical centrifuge at 1,000 x g for ten min. The supernatant fractions were

pooled and 1.0 ml of the extract was added to 2.0 ml of the following mixture: 1.5% diphenylamine (w/v), 1.5% H_2SO_4 (v/v) and 0.08% (w/v) aqueous acetaldehyde in glacial acetic acid. After 16 hr of incubation at 30°C in tubes covered by Parafilm^R, the color reaction was read at 600 mμ on a Coleman Junior II^R spectrophotometer.

Cytoplasmic Protein Extraction

Soluble cytoplasmic proteins were extracted from 1.0g of embryonic Artemia cells in 6.0 ml of the following grinding medium: 0.05 M tris HCl (pH 7.5), 1.0×10^{-4} M EDTA and 40% sucrose. Identical incubation, grinding and filtering procedures were used for protein extraction as in the case of the previously described histone extraction. The resulting slurry was allowed to stand for 60 min in an ice bucket, a procedure which allowed the proteins to be drawn slowly from the mascerated bits of tissue into the dense sucrose solution by osmotic pressure. After centrifugation at 9,250 x g (10,000 rpm) the supernatant fraction was carefully pipetted from beneath the orange lipid surface film and subjected immediately to electrophoresis without freezing. Two 1.0 ml aliquots were reserved for protein assay by a method similar to that of Lowry et al. (1951). One sample was added to 1.0 ml of 10% trichloroacetic acid, allowed to stand for 15 min and centrifuged at 1,000 rpm on a clinical centrifuge. The precipitated proteins were then washed with 3.0 ml 5%

trichloroacetic acid, centrifuged at 1,000 rpm and suspended in 5.0 ml 0.5 M NaOH until the white pellet dissolved. This sample, as well as a 1:4 dilution of the remaining 1.0 ml extract with 0.05 M tris HCl pH 7.5 were measured for protein content as described below.

Protein Assay

Both the histone preparations and the total soluble protein extracts were measured for protein content using a variation of the method described by Lowry et al. (1951) which I found to be very sensitive for Artemia proteins. Calf thymus histone, type IIa (Sigma) was used as a standard for histone samples, and bovine serum albumin (Sigma) was used for preparation of the soluble protein standard curve.

Prior to each assay the following solution was prepared from individual stock solutions: 0.01% $CuSO_4 \cdot 5H_2O$, 0.02% sodium tartrate, 2% Na_2CO_3, 0.1 N NaOH. Five ml of this solution were added to 0.5 ml of the sample. After mixing and allowing the solution to stand at room temperature for ten min, 0.5 ml of a 1:2 dilution of Folin phenol reagent with double distilled water was added with rapid mixing. Optical density at 600 mμ was read on a Coleman Junior II^{R} spectrophotometer after a second ten min at room temperature.

Total Protein Electrophoresis

Protein separation was accomplished by means of a standard disc electrophoresis technique for serum proteins which was described by Davis (1964). In essence, proteins are separated on the basis of their anionic behavior (migratory tendencies towards a positive electrode) in the electrophoretic system as well as separated by size due to the sieving effect of a polymerized acrylamide gel lattice.

The "running gel" or small pore gel was made up of 7% acrylamide (w/v) at pH 8.9. "Stacking" and "sample" gels (large pore gels) consisted of 2.5% acrylamide at pH 6.7. The soluble protein extract from embryonic Artemia cells was diluted 1:9 with the large pore gel. An appropriate amount of this "sample gel" was layered on top of the "stacking" gel and photopolymerized in the same manner as the "stacking" gel. After electrophoresis at 4 ma per tube for 75 min, the gels were removed from the glass tubes, and each was fixed and stained in 10.0 ml 1% naphthol blue black in 7% acetic acid one hr. They were destained over 24 hr with eight changes of 7% acetic acid. The destained gels were stored in a refrigerator in 5% glycerol.

Densitometric Analysis

Fixed and stained polyacrylamide gels were scanned with a Schoeffel^{R} Model 3000 spectrodensitometer. The resulting protein

band profiles plus data from the automatic integrating device were used to judge qualitative and quantitative differences in histone content and total protein content at various stages of Artemia development. The stained histone gels were scanned at 625 mμ and the stained total protein gels scanned at 650 mμ.

A gel containing 50 μg of electrophoretically separated calf thymus histone, type IIa, was scanned as a standard for determination of protein content in the other histone samples. For total protein samples, an identical determination was made with 40 μg of bovine serum albumin.

RESULTS

Scanning Electron Microscopy

Scanning EM micrographs of cysts reveal a smoothly textured surface (Figure 2) which bears very little resemblance to the highly sculptured exterior of many desiccated insect eggs which have evolved complex structures to allow the exchange of gases without loss of water (Hinton, 1970). I noted, however, an extension of the cyst wall which is not evident under the light microscope (Figure 2B). It is possibly a former point of attachment to the maternal ovisac.

Postgastrular Developmental Sequence

Completely synchronous cultures of Artemia embryos were never obtained. As reported by McClean and Warner (1971), the embryos are capable of surviving desiccation either in the blastular or gastrular state; and for this reason (and possibly others) they do not all emerge from the cysts at the same time. However, within a range of two-three hr predictable levels of development are attained in the majority of cysts when they are incubated in 0.5 strength Instant Ocean^{R} at 23°C.

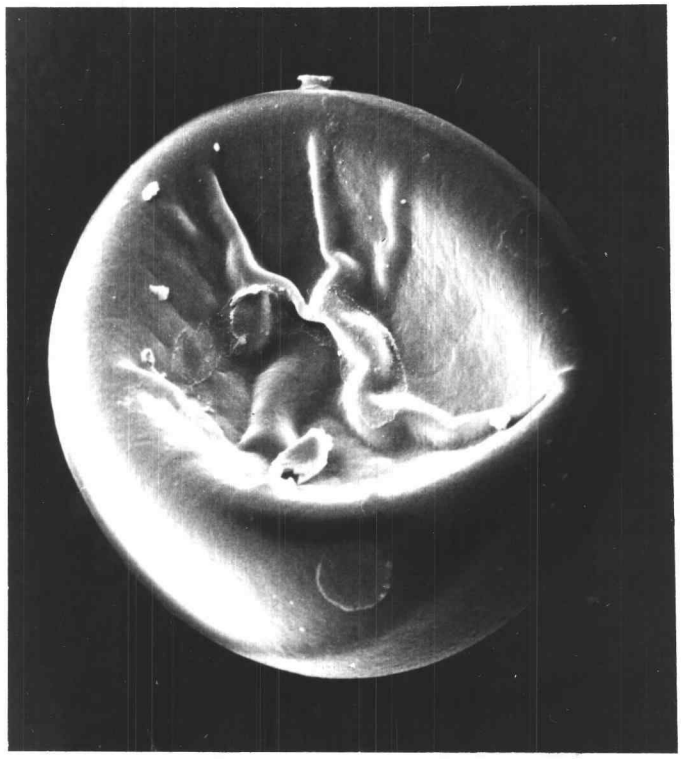
Upon hydration in the incubation medium, the cupped cyst swells to a sphere (Figure 3A, B) and appears to be completely hydrated within one hr (Morris, 1971). Between one and ten hr the spherical

Figure 2. Scanning electron micrographs of a desiccated encysted gastrula of Artemia.

(A) Whole cyst (437x).

(B) Detail of (A) showing probable attachment point to maternal ovisac (1990 x).

A



B

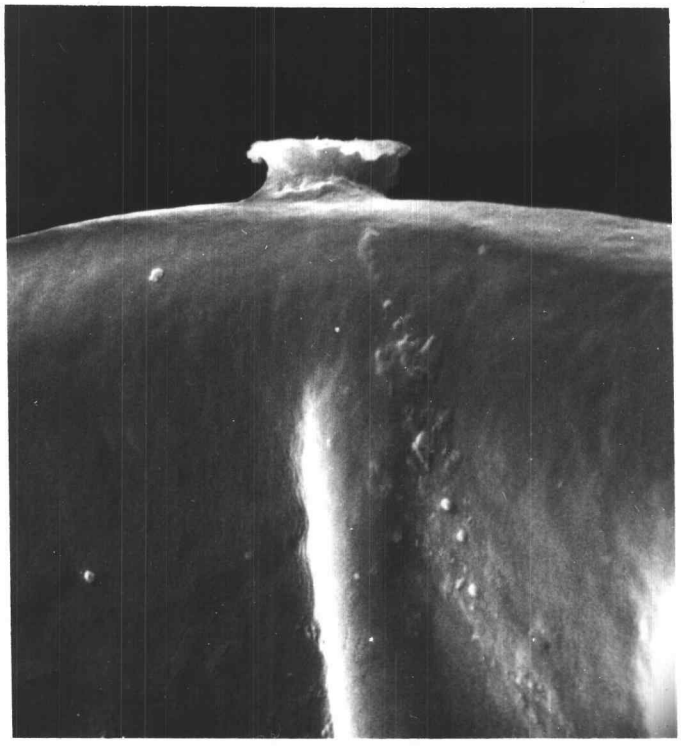


Figure 3. Developmental sequence of Artemia salina encysted gastrulae incubated in shallow pans at 23°C in 0.5 strength Instant Ocean®. All magnifications are 110 x.

(A) Desiccated encysted gastrulae.

(B) 1-10-hr post-incubated embryo.

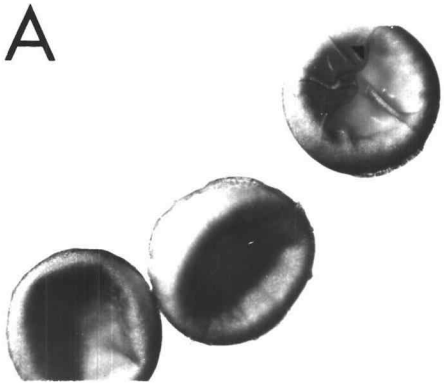
(C) 12-hr embryo, newly emerged.

(D) 16-hr early nauplius, showing signs of rapid tissue differentiation.

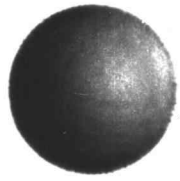
(E) 20-24-hr nauplius, fully emerged, arrow indicates intact hatching membrane.

(F) 24-28-hr swimming nauplius.

A

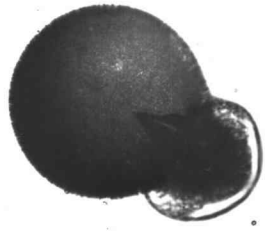


B

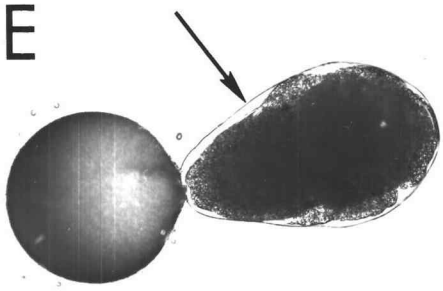


C

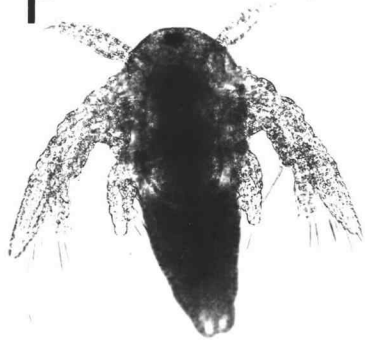
D



E



F



shell remains intact. Little evidence of tissue differentiation is noted during this time when the proteinaceous outer cyst wall is dissolved by 15 min of soaking in cold 5% $NaHClO_4$ (Chlorox^R). A bright red eyespot is seen in the otherwise homogeneous appearing, encysted embryo at about ten hr.

By 12 hr (Figure 3C) osmotic pressure ruptures the cyst wall (Clegg, 1964) and the rapidly developing early nauplius larva emerges. The differentiation of the gut and exoskeletal limb structures is obvious in the 16-hr post-hydration larva (Figure 3D). Between 20 and 24 hr (Figure 3E) the nauplius emerges fully and can be seen moving inside a hatching membrane. At this time the naupliar structures appear to be fully formed. Most of the nauplii have escaped from the hatching membrane and are swimming by 24 hr (Figure 3F). The nauplius continues to swim actively, utilizing glycogen and other carbohydrate reserves, and doubles in size by 30 hr. At least one molt and the addition of several abdominal segments has occurred by 48 hr.

Viability, as determined by percentage hatch, was found to be about 60-75% by 24-28 hr. Delayed hatching cysts, described by Morris (1971), which hatch only when subjected to hydration a second time may account for some of the apparently undeveloped embryos. Only about 5% were determined to be dead. These could not be effectively separated from the viable cysts.

Figure 4. Fifteen percent polyacrylamide gels through which basic, nuclear proteins (mostly histones) from Artemia salina have been separated electrophoretically, using various techniques of histone extraction. With the exception of (F) all samples represent extracts from desiccated gastrulae or 0-hr samples. Electrophoresis was carried out for 90 min at room temperature at 2 ma per gel. The pH of the system was 4.3.

(A) Calf thymus histone, type IIa, 25 μg, extracted with 1.0 N NaCl, then acid.

(B) Whole pea histone, 50 μg, method of Bonner et al. (1968a).

(C) Artemia histone, 10 μg, method of Bonner et al. (1968a), using 0.4 M H_2SO_4 for extraction, with suspension of the final pellet in water.

(D) Artemia histone, 100 μg, method of Mohberg and Rusch (1970), using 1.0 M $CaCl_2$ for extraction.

(E) Artemia histone, 75 μg, method of Lindsay (1964), using crude chromatin and 0.1 N HCl for extraction.

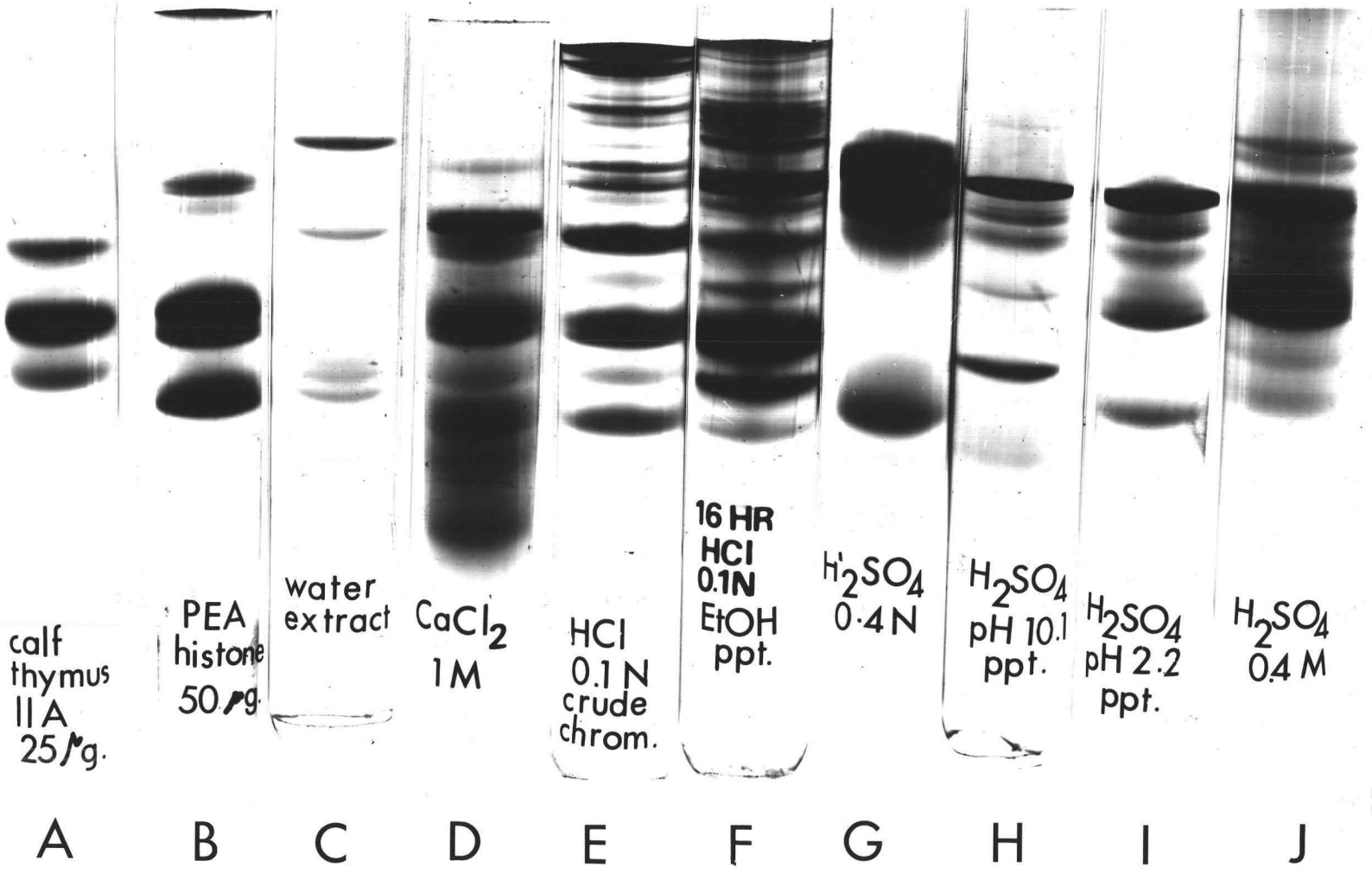
(F) Artemia histone, 16-hr extract, 118 μg, 0.1 N HCl extract of purified chromatin.

(G) Artemia histone, 100 μg, method of Bonner et al. (1968a), using a 0.4 M H_2SO_4 extract of purified chromatin, with final suspension of the pellet in 0.9 N acetic acid.

(H) Artemia histone, 50 μg, same as (G) except histone was precipitated in ethanol at pH 10.1.

(I) Artemia histone, 50 μg, same as (G).

(J) Artemia histone, 81 μg, using procedure outlined in Methods and Materials (Extraction of Histones).


calf thymus II A 25 µg.
PEA histone 50 µg.
water extract
CaCl2 1M
HCl 0.1 N crude chrom.
16 HR HCl 0.1N EtOH ppt.
H2SO4 0.4 N
H2SO4 pH 10.1 ppt.
H2SO4 pH 2.2 ppt.
H2SO4 0.4 M
A
B
C
D
E
F
G
H
I
J

The method of Mohberg and Rusch (1970), which employed a 1.0 M $CaCl_2$ extract, produced four major histone bands without contaminants. The yield was only 12.7% of the expected value and the bands were fuzzy (Figure 4D).

The technique of Lindsay (1964), which involved isolation of only crude chromatin with 0.1 N HCl, demonstrated at least 16 clear bands (Figure 4E). By comparing these band patterns with those of whole pea histone, the four widest, faster moving bands are probably histone. The identity of the slower moving, narrow bands in this case is not clear. A comparison with highly purified chromatin 0.1 N HCl extracts (that is, washed crude chromatin that had been centrifuged through 2.0 M sucrose) in Figure 4F shows that the low molecular weight and/or only slightly charged, slow moving molecules must be broken histones, other basic, nuclear proteins or cytoplasmic contaminants (such as ribosomal proteins) which are irreversibly bound to the chromatin preparations (Hearst and Botchan, 1971). Both techniques shown in Figures 4E and 4F gave high yields, slightly over the expected 5 mg per g of dry weight of tissue.

Figures 4G, 4H and 4I represent basic proteins extracted by the method of Bonner et al. (1968a) for pea histone, using the following grinding medium: 0.25 M sucrose, 0.05 M tris HCl pH 8.0, 1.0×10^{-3} M $MgCl_2$. Care was taken to extract the 10,000 x g pellet (histone plus glycogen) from the 83% ethanol precipitation process (see Figure 1)

with 0.9 N acetic acid. The histone yield was adequate for developmental comparisons (30-60%); the resolution was good and the lack of slow moving, small bands was aesthetically gratifying. As is seen by comparison of the three 0-hr samples (Figures 4G, 4H and 4I), this technique never allowed me to achieve electrophoretically reproducible results from one experiment to the next. The slowest moving component appears to represent a clumping of all of the histones which, to varying degrees, are resolved in their proper positions only under unknown circumstances of the isolation procedure.

The method of choice for histone extraction in embryonic Artemia cells is represented by the sample in Figure 4J. The procedure is outlined in detail in the Methods and Materials section (Extraction of Histones). Although the extracts obviously cannot be well-resolved, the technique has the advantages of: (1) highly reproducible results showing eight major bands, (2) band patterns electrophoretically comparable in migration distance to whole pea histone, (3) reproducible histone yields of 40-70% of expected values, and (4) lack of slow-moving contaminants.

The Results of Sulfate versus Chloride Extraction

Electrophoretic data from Figures 5 and 6 clearly demonstrate that the form of basic, nuclear protein molecules of Artemia embryos is extremely sensitive to manipulation in acid solutions and to

Figure 5. Electrophoretic separation of basic, nuclear proteins from desiccated encysted gastrulae of Artemia salina. All three samples represent equal volumes of extracted protein based on the weight of the cysts before extraction. Electrophoresis was carried out in 15% polyacrylamide gels at pH 4.3 for 90 min. The origin is at the top of the gel.

(A) Basic proteins which exhibit a clumped pattern at the origin due to a change in pH from 1.5 to 10.1 before ethanol precipitation of a 0.1 N HCl extract. 31 μg, 8.7 μg enter gel fully.

(B) Basic protein pattern which results when (A) is re-extracted with 0.4 M H_2SO_4. 76 μg, 56 μg enter gel fully.

(C) Basic protein pattern which results when (A) is re-extracted with 0.1 N HCl and adjusted to pH 10.1 after addition to cold ethanol. 191 μg, 124 μg of which enter gel.

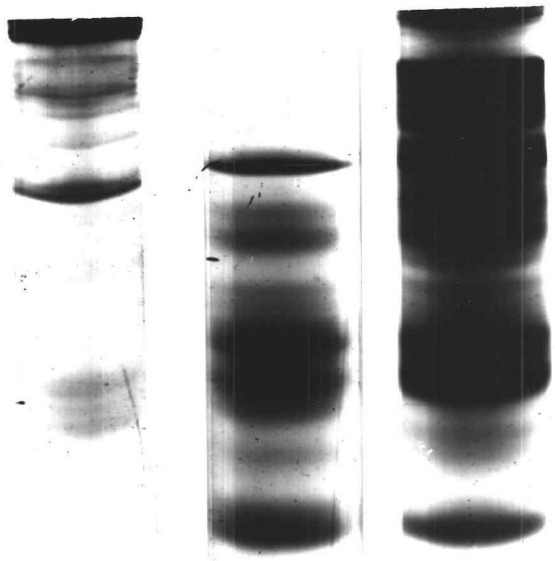
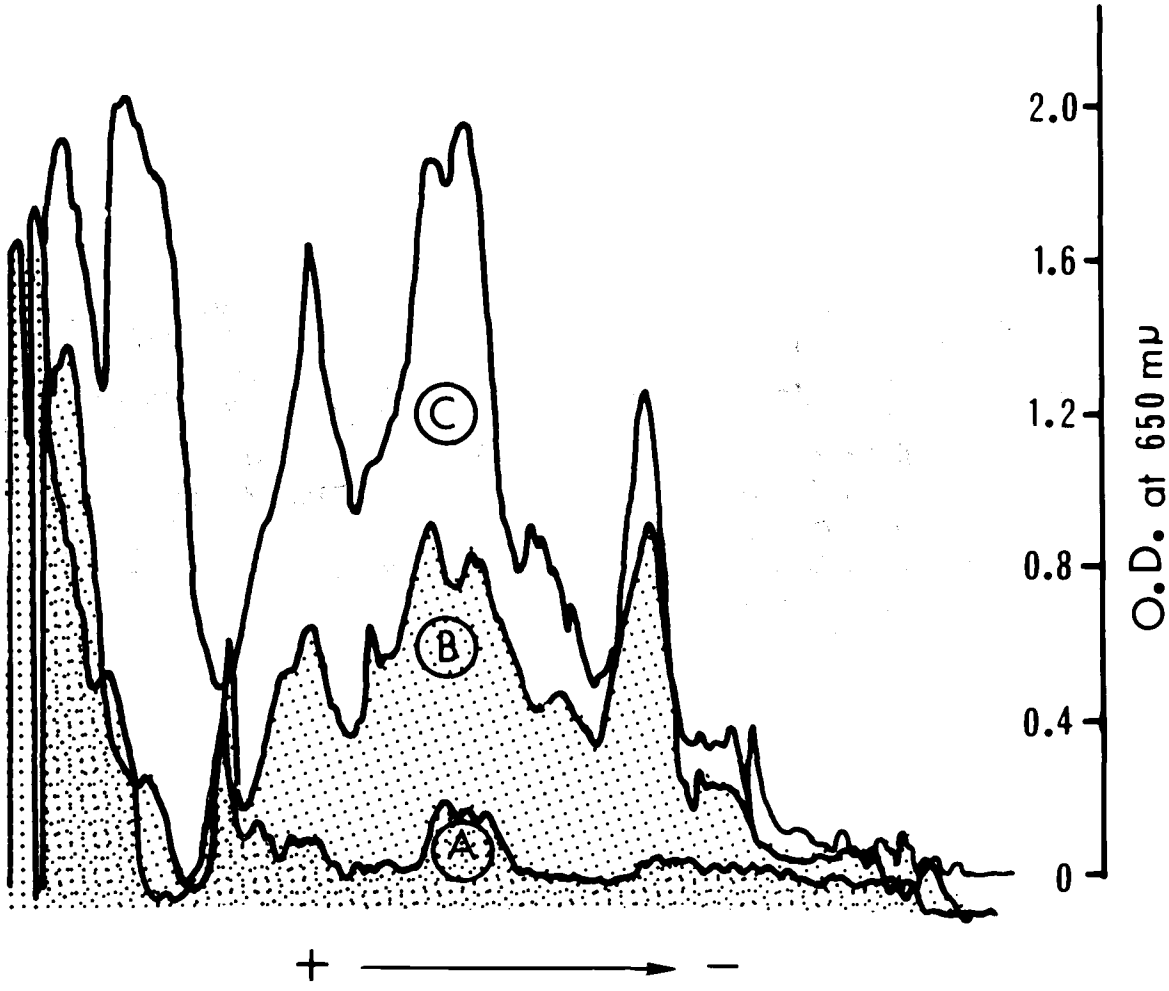

0 HR
0.1 N
HCl
A
0 HR
0.4 M
H_2SO_4
B
0 HR
0.1 N
HCl
C

Figure 6. Superimposed densitometric tracings of scanned electrophoretic gel separations of histones as illustrated in Figures 5A, 5B and 5C. Evidence of differential extraction capabilities between (B) 0.4 M H_2SO_4 and (C) 0.1 N HCl is indicated. The area under the curve in (A) demonstrates the limited ability of histone molecules to migrate in an electrophoretic field when precipitated in ethanol after adjustment to pH 10.1. The top of the gel is at the left (anode).


2.0
1.6
1.2
0.8
0.4
0
O.D. at 650 mμ
C
B
A
+
−

conditions of precipitation in ethanol. Prior to electrophoresis the proteins in Figure 5A were extracted with 0.1 N HCl (pH 1.5) from the purified chromatin pellet as outlined in Figure 1. Before addition of this extract to the five volumes ethanol, the pH was adjusted to 10.1 with 1.0 N NaOH. The heavy, white ethanol precipitate was then prepared as usual for histone electrophoresis. As can be seen from the photograph (Figure 5A) the molecules were clumped in a form which did not migrate in the electrophoretic field. This same extract was recombined with the glycogen pellet and divided in half. To one of the halves was added 15 ml of 0.4 M H_2SO_4. The slurry was stirred for 30 min, spun at 10,000 x g and the resulting supernatant fractions precipitated in five volumes of cold ethanol. The second half was treated with 15 ml of 0.1 N HCl in an identical manner and added to five volumes of cold ethanol. As usual, no precipitate formed at pH 1.5, but a copious flocculation appeared when the acid-ethanol mixture was brought to pH 10.1 with 1.0 N NaOH. Figures 5B and 5C represent preparations that had been extracted in equal volumes of H_2SO_4 and HCl.

Figure 6 exhibits three superimposed densitometric tracings of the electrophoretic gels pictured in Figure 5. The two illustrations demonstrate that: (1) basic proteins are clumped if subjected to a high pH before precipitation in ethanol, (2) the clumping is reversible upon further acid extraction, (3) 0.1 N HCl extracts 2.5 times as much

basic protein from a chromatin sample as does an equal volume of 0.4 M H_2SO_4 (191 μg compared to 76 μg), (4) of the two ions, Cl^- appears either to extract many more impurities or the histones fragment and clump together and show up as slow moving bands, and (5) HCl and H_2SO_4 extract different histone fractions. As can be seen in the tracing in Figures 6B and 6C, 0.4 M H_2SO_4 more completely extracts the faster moving proteins compared to 0.1 N HCl than it does the more slowly migrating bands.

The Effect of Ethanol Precipitation on Histones

Direct assay of acid extracts of chromatin for histone (without concentration by ethanol precipitation) have been partially successful using starch gel electrophoresis (Lindsay, 1964).

An experiment with 16-hr post-hydration Artemia embryos demonstrated clearly that direct electrophoresis of chromatin extracts resulted in weak histone bands (less than 10% of the expected yield). In addition, most of the basic proteins were clumped near the origin in direct extracts.

Figure 7 represents a comparison of four equal amounts of proteinaceous material extracted from a single purified chromatin pellet (see Figure 1) and subjected to four variations in technique: (1) direct electrophoresis of a 0.1 N HCl extract (Figure 6A), (2) electrophoresis of an ethanol precipitate of a 0.1 N HCl extract (Figure 6B),

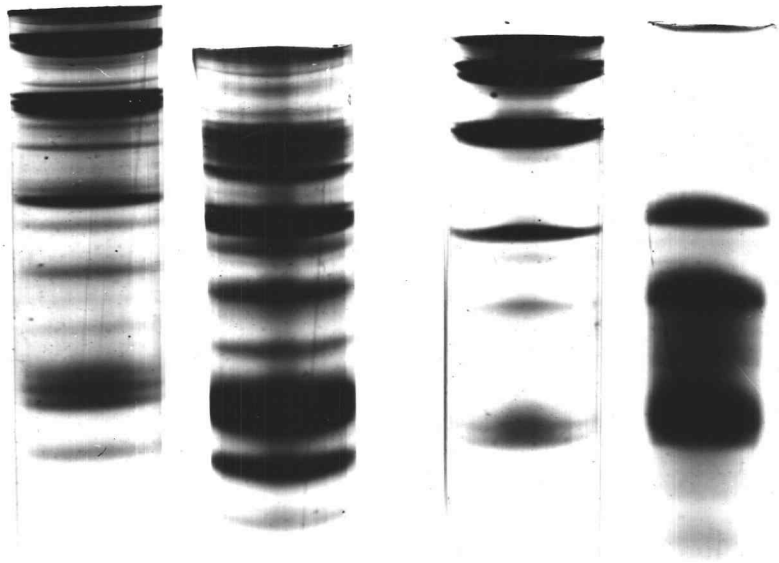
Figure 7. Patterns of basic, nuclear protein extracts of Artemia subjected to electrophoresis before and after precipitation with ethanol. Electrophoretic procedure was identical to that in Figure 5. All extracts were taken from 16-hr, post-hydration prenauplii and represent equal quantities of extracted material.

(A) Direct assay from a 0.1 N HCl extract. 39 μg.

(B) Assay after ethanol precipitation of a 0.1 N HCl extract. 118 μg.

(C) Direct assay of a 0.4 M H_2SO_4 extract. 21 μg.

(D) Assay after ethanol precipitation of a 0.4 M H_2SO_4 extract. 66.5 μg.


16HR
HCl
0.1N
direct
extract
A
16 HR
HCl
0.1N
EtOH
ppt.
B
16HR
H_2SO_4
0.4M
direct
extract
C
16 HR
H_2SO_4
0.4M
EtOH
ppt.
D

(3) direct electrophoresis of a 0.4 M H_2SO_4 extract (Figure 6C), and (4) electrophoresis of an ethanol precipitate of a 0.4 M H_2SO_4 extract (Figure 6D).

As with the desiccated cysts (Figure 5) there were marked quantitative differences between the chloride and sulfate extracts.

Developmental Changes in Histone Composition

No dramatic changes were seen in histone composition during the development of a simple Artemia gastrula to a highly differentiated nauplius when extracts were prepared by the technique outlined in Methods and Materials. However, Figures 8, 9 and 10 do demonstrate several interesting changes of histone constitution with time. The first is the immediate disappearance or marked reduction of band 2 from the 0-hr desiccated cyst upon hydration. This band is easily recognizable by its bright blue staining property with naphthol blue black (single arrow in Figure 8). Compare the desiccated 0-hr pattern with the 4-hr hydration pattern in Figure 8. Second, band 3, which corresponds in electrophoretic migration distance to fraction I of whole pea histone (Bonner et al., 1968b), decreases in content from 25% of the total histone protein in the gastrula to 11.8% in the highly differentiated nauplius. Third, the disappearance of band 3 is concurrent with a proportionate increase in band 6 (from 36.6% to 43.7%) which is a doublet corresponding to the IIa and IIb histone fractions of

Figure 8. Electrophoretic separation of nuclear histone preparations from eight developmental times (between gastrular and naupliar) in Artemia salina. A 15% polyacrylamide gel at pH 4.3 was used as the electrophoretic separating medium. The separations were performed at 2 ma per gel for 90 min.

Band numbers (see the 30-hr sample) have been assigned for convenience to identify Artemia nucleohistones only. They have not been correlated with other systems of nomenclature for histones because they have not been rigorously identified by amino acid analysis.

The single arrow designates band 2, a bright blue band conspicuous only in the 0-hr sample. The double arrow points out the increased quantity of the band 6 doublet in the 48-hr naupliar stage.

Whole pea and calf thymus IIa histone preparations are included as reference samples.

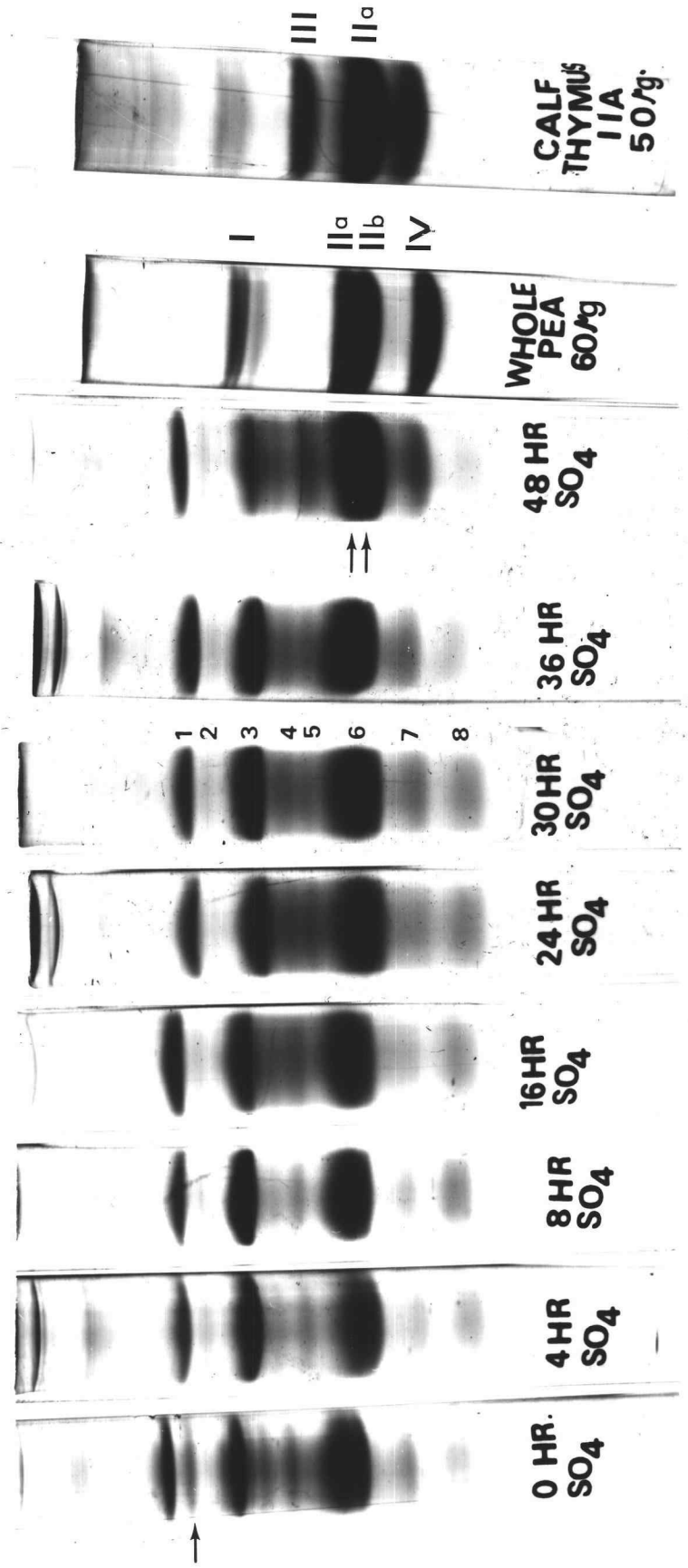

0 HR. SO4
4 HR SO4
8 HR SO4
16 HR SO4
24 HR SO4
30 HR SO4
36 HR SO4
48 HR SO4
WHOLE PEA 60 µg
CALF THYMUS IIA 50 µg.
I
IIa
IIb
III
IV
1
2
3
4
5
6
7
8

Figure 9. Densitometric tracings of electrophoretically separated histones from Figure 8. Note the increase in band 6 and decrease in band 3 between 36 and 48 hr.

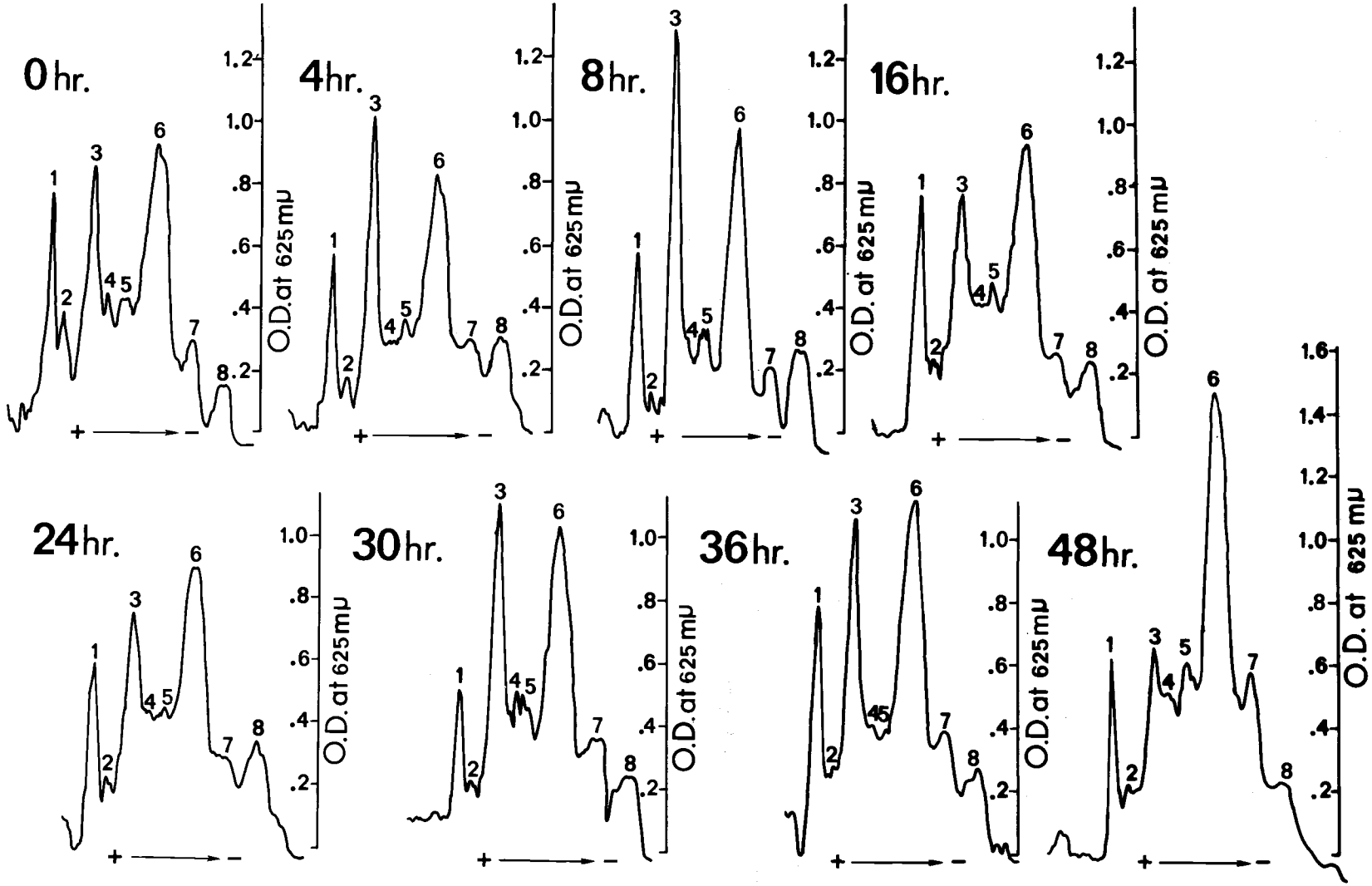
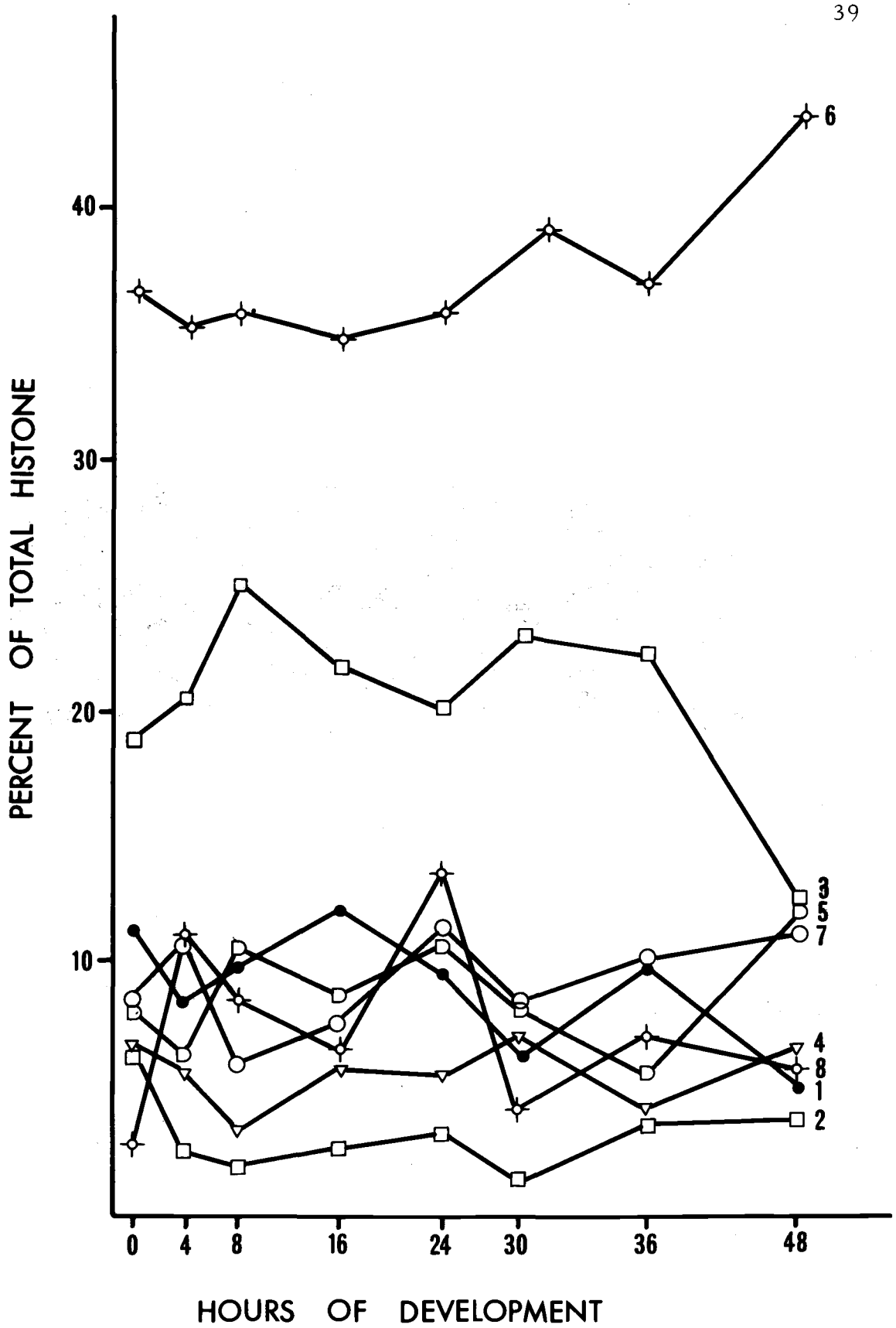

0hr.
4hr.
8hr.
16hr.
24hr.
30hr.
36hr.
48hr.
O.D. at 625 mμ
1.2
1.0
.8
.6
.4
.2
1.6
1.4
1
2
3
4
5
6
7
8
+
–

Figure 10. Graphic representation of changes in nuclear histone composition during development in Artemia embryos. Numerals 1-8 correspond to the assigned band designations in Figure 8 (30-hr sample) and peak designations in Figure 9. Histone quantity for each band is expressed as a percent of the total histone content of each gel. Data for these points were calculated with the aid of an automatic integrating device on the recording spectrodensitometer.


PERCENT OF TOTAL HISTONE
40
30
20
10
0
4
8
16
24
30
36
48
HOURS OF DEVELOPMENT
6
3
5
7
4
8
1
2

Bonner et al. (1968b). These two fractions (see double arrow in Figure 8) are the most conspicuous and persistent histone components of mature plant and animal tissue, usually comprising 50% of the histone complement. Fourth, there is an unusual slowly migrating "histone" fraction in Artemia (band 1) which has no counterpart in pea tissue or any other known tissue studied. It steadily decreases during the course of development from 0 to 48 hr by 11.3 to 5.5%.

In a general sense, the slower migrating histone bands which I have labeled bands 1 and 3 decrease in proportion to the whole during the course of development. Band 3, at least, is usually described as a "lysine rich" histone. A gradual increase is observed in one faster migrating component (Artemia band 6), usually labeled in electrophoretic analysis as "slightly lysine-rich" histone of pea and calf thymus tissue (Bonner et al., 1968b). This increase is more pronounced after 36 hr.

Artemia histone bands 4 and 5 appear to represent the equivalent of fraction III (cysteine-containing histone) in whole pea histone. The proportional make up of these two fractions each varies from 3 to 10%, but I believe no clear cut developmental trends in their relative quantities is indicated. The "arginine rich" or fraction IV histones (bands 7 and 8) do not change in content during development.

Changes in Histone to DNA Ratios

Figure 11 illustrates that histone to DNA ratios do change during the course of development in Artemia.

The choice of extracting ion was a critical factor in appraising the histone yield. One-tenth normal HCl extracts presented, I believe, an overestimation of the actual histone content by 30-40%, presumably due to non-histone proteins. Densitometric analysis of the gel separation shown in Figure 4F, for example, indicated that 30% of the protein was "non-histone" in migratory character. These contaminants varied considerably in content during development and interfered with attempts to establish definite trends in the histone to DNA ratio with the use of chloride ion.

Sulfuric acid extracts probably underestimate the histone yield (Figure 7B). However, definite trends in the histone to DNA ratio can be evaluated using 0.4 M H_2SO_4 for histone extraction. Ginzburg-Teitz et al. (1967) claim that the use of 0.1% Triton X-100 in the extraction medium results in a 30% loss of histone yield. If this is true, the histone quantities in Artemia extracts, which had been subjected to 0.1% Triton X-100 in the preliminary grinding processes and extracted with 0.4 M H_2SO_4, probably are too low. Taking this into account, the adjusted histone to DNA ratios (Figures 11C and 11D) would be 0.8 and 1.0. This range in histone to DNA ratio is

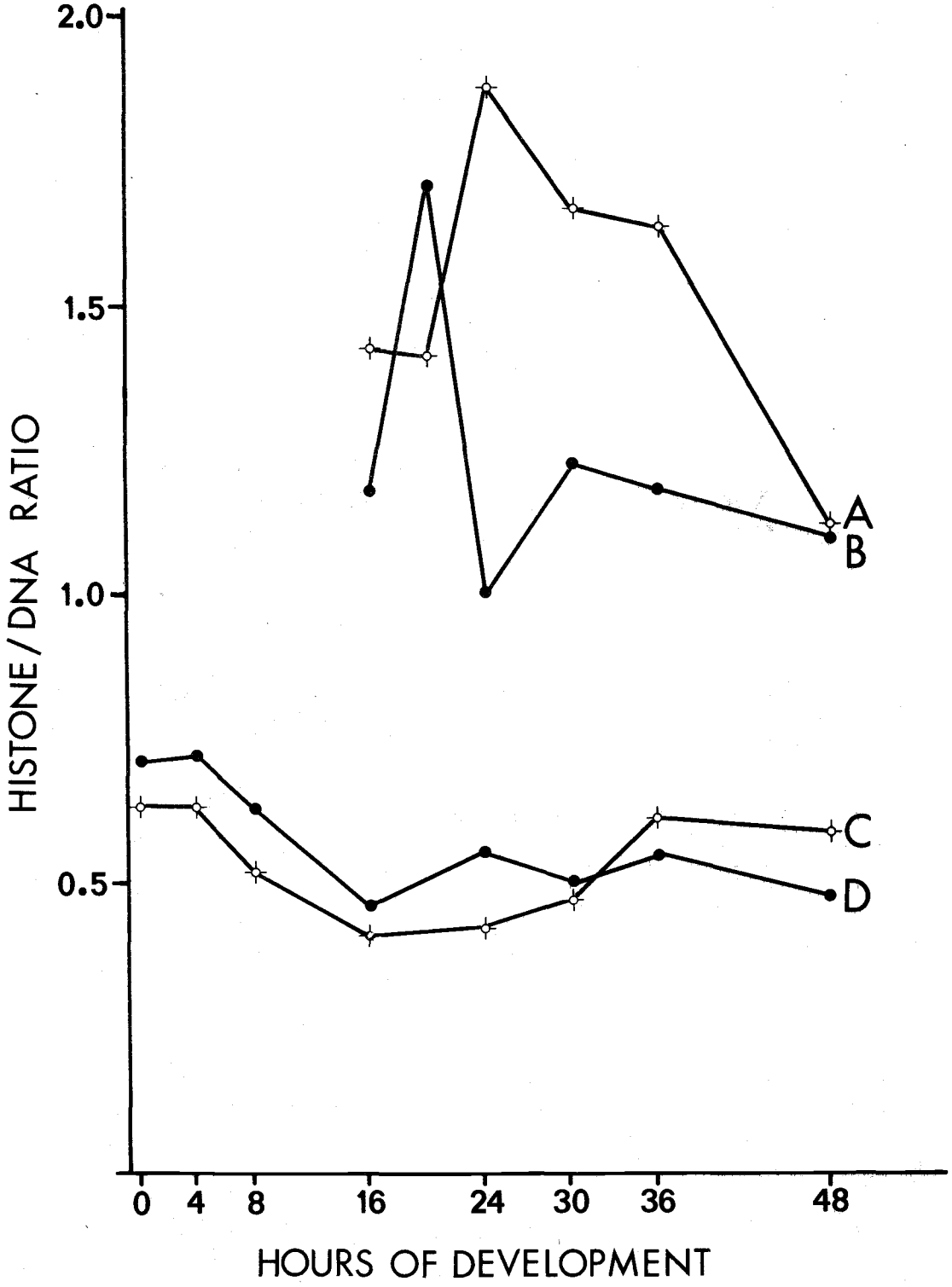
Figure 11. Nuclear histone to DNA ratios of Artemia salina during the course of development from an encysted gastrula to a 48-hr nauplius. Histone and DNA were obtained by the method outlined in Figure 1. DNA was determined by the method of Burton (1956). Histone was isolated and assayed using the following methods:

(A) 0.1 N HCl extraction, analyzed by densitometric quantification at 625 mμ of a polyacrylamide gel separation of the extract.

(B) 0.1 N HCl extraction, Lowry protein assay.

(C) 0.4 M H_2SO_4 extraction, densitometric determination of a gel separation.

(D) 0.4 M H_2SO_4 extraction, Lowry protein assay.


2.0
1.5
1.0
0.5
HISTONE / DNA RATIO
A
B
C
D
0
4
8
16
24
30
36
48
HOURS OF DEVELOPMENT

closer to that seen in most other tissues (Mohberg and Rusch, 1969; Bonner et al., 1968b).

Densitometric analysis at 625 mμ of the acrylamide gel samples of 0.4 M H_2SO_4 extracts (Figure 11C) revealed a depression of the histone to DNA ratio from 8 to 30 hr of development (see Figures 11C and 11D). This coincides with a period of intense morphogenic activity in the embryo. By 36 hr of development the histone to DNA ratio approximated the ratio in the metabolically inert gastrula.

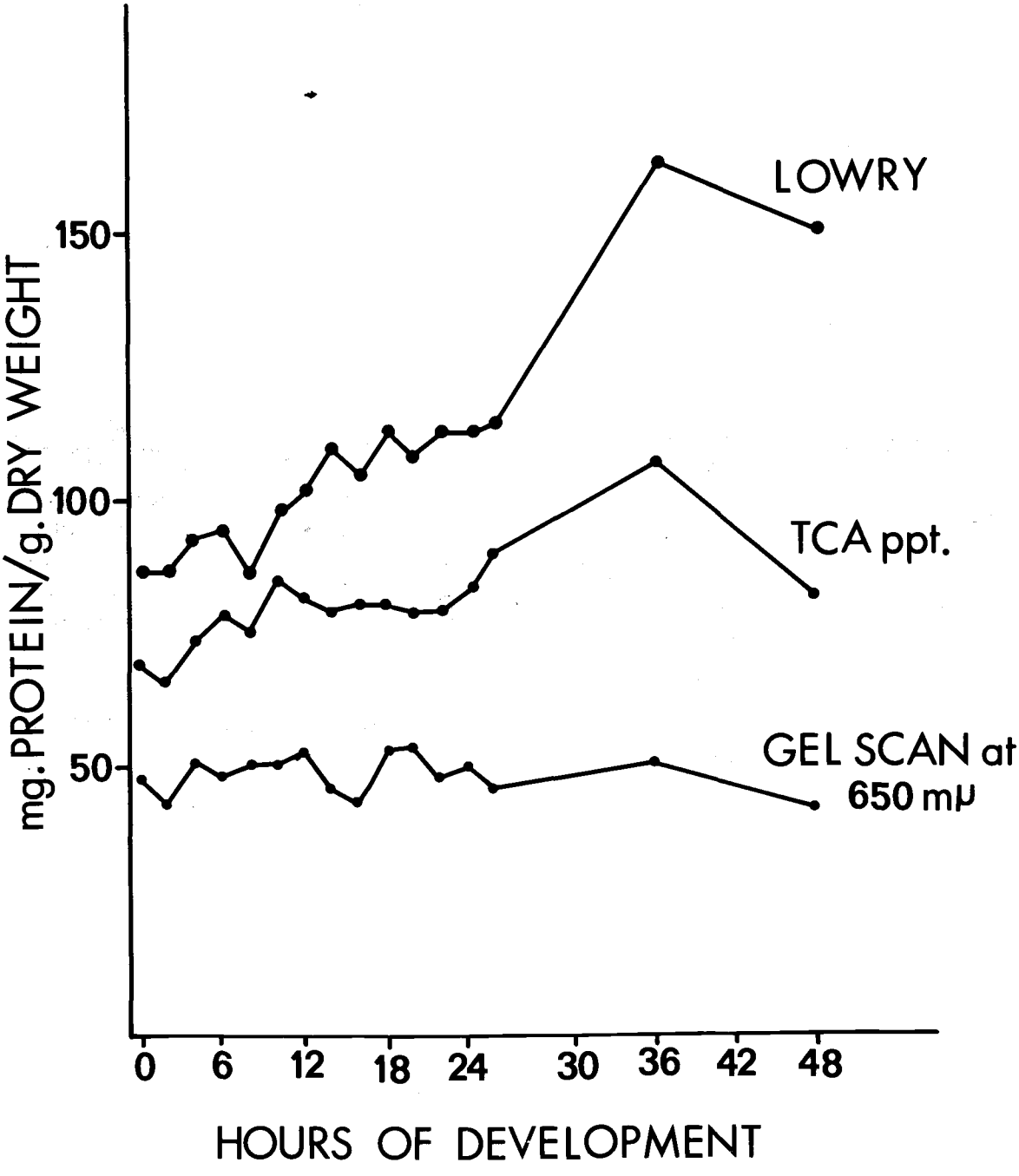
Changes in Cytoplasmic Proteins During Development

Total acidic cytoplasmic protein content appeared to be nearly constant during early development in Artemia. Lowry protein assays of direct 0.05 M tris HCl (pH 7.5) extracts did indicate a gradual increase in protein content, but this increase was less pronounced when samples were subjected to trichloroacetic acid precipitation before protein assay (Figure 12). Equal amounts of extract, when subjected to electrophoresis and scanned with a densitometer, did not reveal increased amounts of protein during development (see Figure 12). Urbani (1959) and Dutrieu (1960) also found little change in protein nitrogen during development in Artemia.

Thus, changes in protein composition, at least those visualized by electrophoresis, probably represent a reorganization of former proteins. Degradation of yolk protein into structural and enzyme

Figure 12. Protein content of developing embryos of Artemia salina expressed as mg of protein per g of dry weight of the embryos before incubation. Three methods were used in the determination: Lowry protein assay of a direct extract, Lowry protein assay of preparations precipitated with trichloroacetic acid and densitometric scan analysis of acidic proteins separated on polyacrylamide gels in Figure 13.

Bovine serum albumin was used as a standard for all protein determinations.


LOWRY
TCA ppt.
GEL SCAN at
650 mμ
mg. PROTEIN/g. DRY WEIGHT
150
100
50
0
6
12
18
24
30
36
42
48
HOURS OF DEVELOPMENT

proteins via a rather unstable amino acid pool in Artemia is discussed in detail by Emerson (1967).

Visual analysis of total protein changes by electrophoresis of proteins extracts was, I felt, of limited success. Resolution of individual bands was poor, the large number of proteins present could not be distinctly separated, and interaction of proteins with carbohydrates resulted in the blurred appearance of the slower moving components. These problems could not be remedied through protein purification by dialysis against buffer or urea, grinding in Sephadex G-25, Millipore^{R} filtration, phosphate buffer extraction, trichloroacetic acid precipitation, ammonium sulfate precipitation, ethanol or acetone precipitation, or any combination of these procedures.

Figure 13 compares electrophoretically separated acidic protein extracts of Artemia sampled every two hr of development up to 26 hr and also compares samples taken at 36, 48 and 60 hr. A reduction in the background blur due to metabolism of stored carbohydrates was apparent after 24 hr.

The ten protein profiles in Figure 14 and 15 are more easily interpreted than the gels themselves. Densitometric tracings are compared with regard to the R_f values of the most obvious protein peaks. I have assumed that differences in R_f values reflect differences in protein property and type. Arbitrary notation was assigned to each peak based on the time of its appearance in the scanned profile

Figure 13. Electrophoretic separations of acidic, soluble proteins of Artemia salina extracted at various stages of development in 0.05 M tris HCl buffer (pH 7.5).

A 7% polyacrylamide gel was used as a separating medium. Electrophoresis was carried out for 75 min at 4 ma per gel at 4°C. The origin (cathode) is at the top of the gel. The fastest moving component represents the tracking dye front.

New proteins appear at 8-10 hr, but do not completely replace the 0-hr proteins until 36-48 hr.

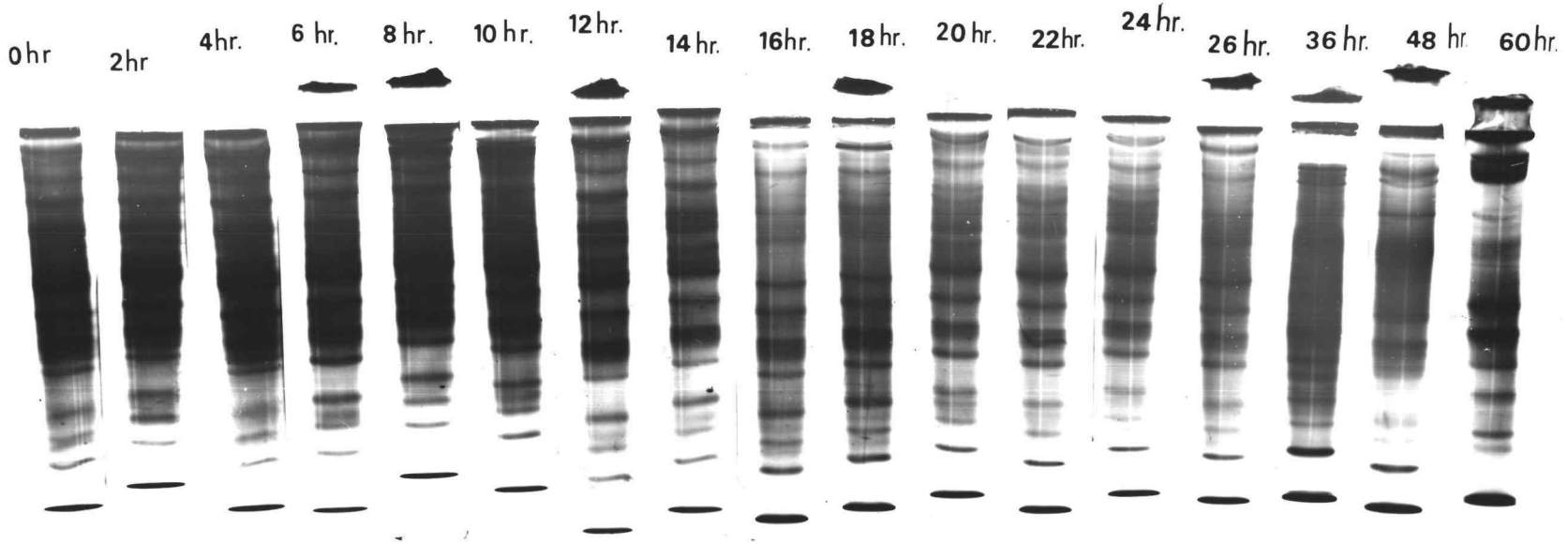
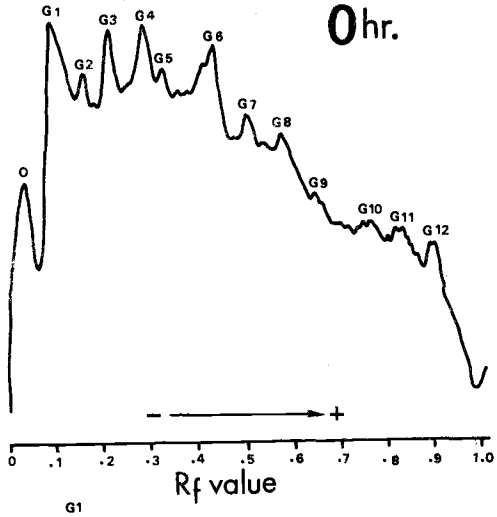
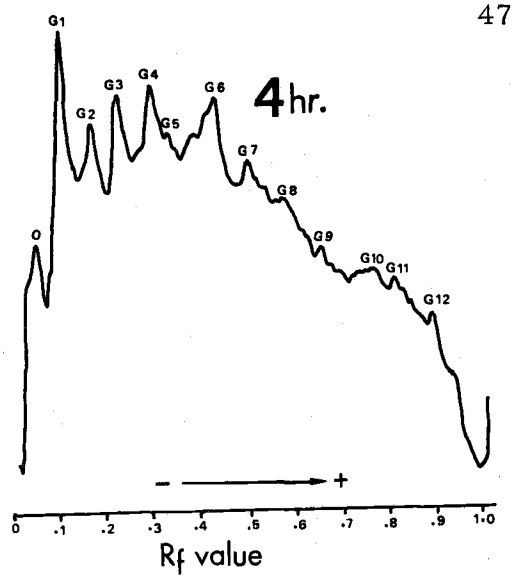

0 hr
2hr
4hr.
6 hr.
8 hr.
10 hr.
12 hr.
14 hr.
16hr.
18 hr.
20 hr.
22hr.
24 hr.
26 hr.
36 hr.
48 hr.
60 hr.

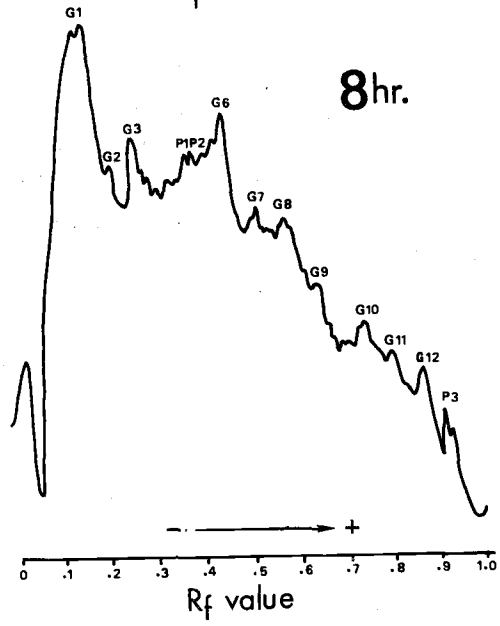
Figure 14. Densitometric tracings of selected polyacrylamide gels illustrated in Figure 13. Gels were scanned at 650 mμ. Protein peaks have been assigned arbitrary designations on the basis of their R_f values for identification purposes according to the following scheme:

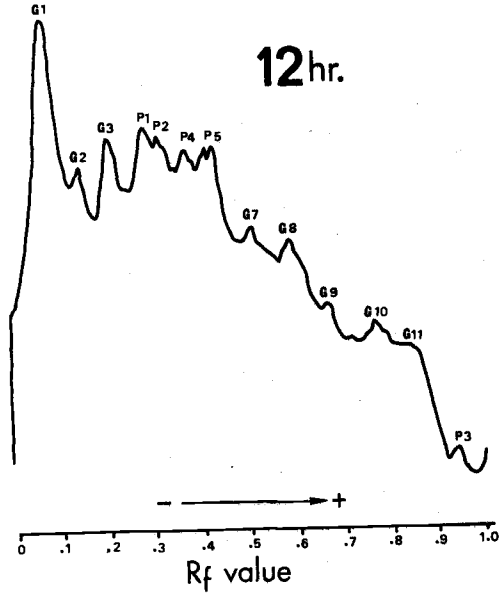
G = gastrular protein

P = prenaupliar protein


0hr.
O
G1
G2
G3
G4
G5
G6
G7
G8
G9
G10
G11
G12
−
+
0 .1 .2 .3 .4 .5 .6 .7 .8 .9 1.0
Rf value


4hr.
O
G1
G2
G3
G4
G5
G6
G7
G8
G9
G10
G11
G12
−
+
0 .1 .2 .3 .4 .5 .6 .7 .8 .9 1.0
Rf value


8hr.
G1
G2
G3
P1P2
G6
G7
G8
G9
G10
G11
G12
P3
−
+
0 .1 .2 .3 .4 .5 .6 .7 .8 .9 1.0
Rf value


12hr.
G1
G2
G3
P1
P2
P4
P5
G7
G8
G9
G10
G11
P3
−
+
0 .1 .2 .3 .4 .5 .6 .7 .8 .9 1.0
Rf value

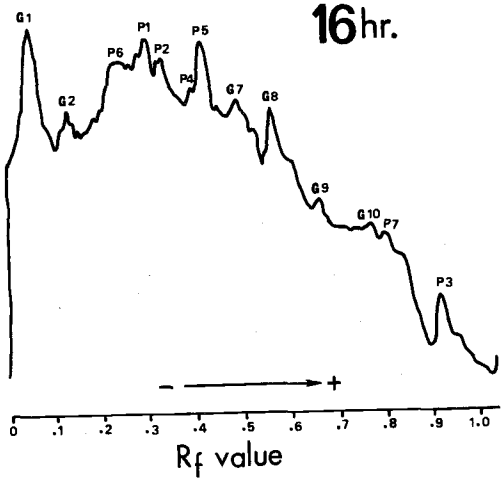

16hr.
G1
G2
P6
P1
P2
P4
P5
G7
G8
G9
G10
P7
P3
−
+
0 .1 .2 .3 .4 .5 .6 .7 .8 .9 1.0
Rf value

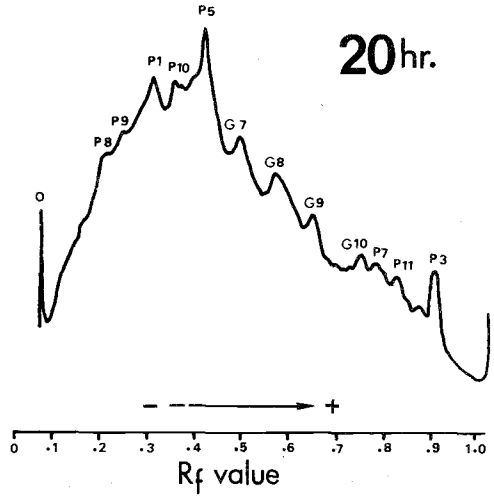
Figure 15. Densitometer tracings of selected polyacrylamide gels illustrated in Figure 13. Gels were scanned at 650 mμ. Protein peaks have been assigned arbitrary designations on the basis of their R_f values according to the following scheme:

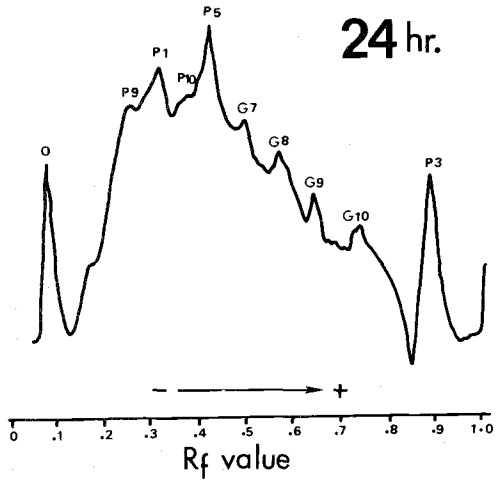
G = gastrular protein

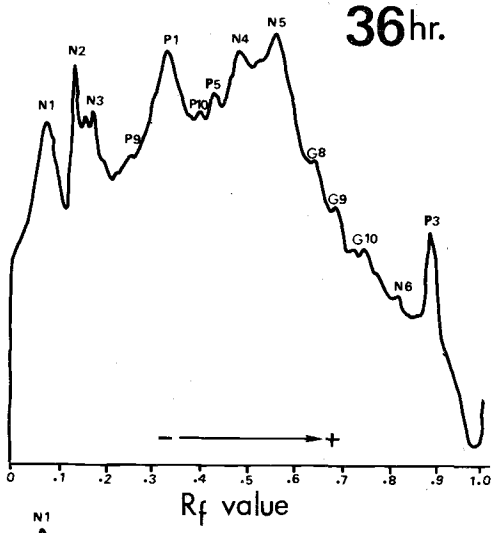
P = prenaupliar protein

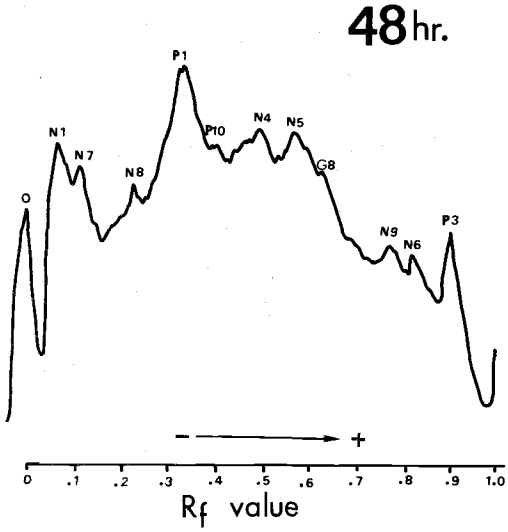
N = naupliar protein

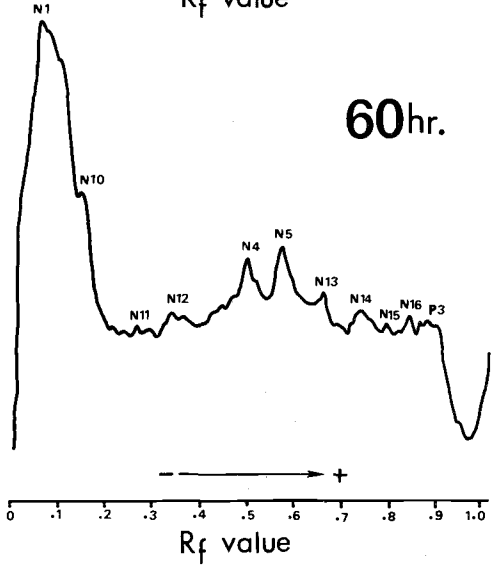
Figures 14 and 15 summarize the developmental changes in cytoplasmic, acidic proteins which can be visualized by electrophoresis in Artemia.


20hr.
O
P8
P9
P1
P10
P5
G 7
G8
G9
G10
P7
P11
P3
0 .1 .2 .3 .4 .5 .6 .7 .8 .9 1.0
Rf value


24 hr.
O
P9
P1
P10
P5
G7
G8
G9
G10
P3
0 .1 .2 .3 .4 .5 .6 .7 .8 .9 1.0
Rf value


36hr.
N1
N2
N3
P9
P1
P10
P5
N4
N5
G8
G9
G10
N6
P3
0 .1 .2 .3 .4 .5 .6 .7 .8 .9 1.0
Rf value


48hr.
O
N1
N7
N8
P1
P10
N4
N5
G8
N9
N6
P3
0 .1 .2 .3 .4 .5 .6 .7 .8 .9 1.0
Rf value


60hr.
N1
N10
N11
N12
N4
N5
N13
N14
N15
N16
P3
0 .1 .2 .3 .4 .5 .6 .7 .8 .9 1.0
Rf value

and its position in the gel (slowest moving bands were assigned numbers first). The letters designate the following protein classifications: G for gastrular protein (present at 0 hr), P for prenaupliar protein (appearing between 8 hr and 24 hr), and N for naupliar protein (appearing after 24 hr). Numbers were assigned simply on the order of appearance of a new band.

Although quantitative fluctuation in embryonic protein patterns was evident by four hr of development, new proteins did not appear until eight hr in this system. Between 16 and 24 hr the protein profiles had become distinct from the 0-8-hr profile. Zero hr "gastrular" proteins were still discernible in the 36 hr sample. During the period of most intense developmental activity (10-16 hr) half of the gastrular proteins (G3, G4, G5, G6, G11 and G12) were lost and were replaced by prenaupliar proteins (P1-P7). Maturation of the nauplius after 24 hr of development was accompanied by a substantial turnover of protein species.

These findings are consistent with the electrophoretic analysis of developmental changes in protein in other systems, both in clarity of protein separations and the limited interpretation possible from the experiments (Dennis, 1960; Steward et al., 1965; Jockusch et al., 1970; Spregel et al., 1965).

DISCUSSION

Histone Extraction

Due to the great diversity in structural and chemical composition of different eukaryotic cells, it is doubtful that there can be a single "best" method for the extraction of histone. If any non-histone material sedimenting with the chromatin is acid soluble, methods must be devised to separate it from the histone fraction. In the case of Artemia, the large amount of acid soluble storage glycogen appears to have the same density as chromatin. Because of this the glycogen remained with the histone until the final step (preparation for electrophoresis), when it could be separated from the histone on the basis of its greater insolubility in 0.9 N acetic acid. It is likely, however, that glycogen is somewhat soluble in the dilute acetic acid and, thus, may account for the blurred appearance of the basic protein bands after electrophoresis (Figure 4J).

The use of 0.1 N HCl (but not 0.4 M H_2SO_4) for acid extraction of histones resulted in the extraction of a mixture of non-histone proteins. Bonner et al. (1968b) concluded that the latter are acid soluble ribosomal proteins from ribosomes so closely associated with the chromosomes that they are not separable by centrifugation. They may also represent histone fragments broken due to proteolytic activity during the grinding procedure (Easton and Chalkley, 1972) or histones

that have clumped together.

Peculiarities of embryonic Artemia cells required that histones be precipitated with ethanol before they could be separated electrophoretically in the polyacrylamide gel (Figure 7). Furthermore, if 0.1 N HCl is used for histone extraction, the pH of the acid-ethanol mixture must be adjusted to 10.1 after the acid extract is added to the ethanol mixture. If it is adjusted before mixing with ethanol, 86% of the histones clump together and cannot be separated (Figures 5 and 6). In Artemia extracts ethanol probably does more than just precipitate histones. It appears to reduce either the binding of glycogen to histone (which otherwise would result in the clumping of material at the origin of the electrophoretic separating medium) or the clumping of the histones to each other. Or it may do both of these.

The histones isolated in this study have been identified only by comparison in electrophoretic migratory behavior to whole pea histone and calf thymus histone (Figure 4). Rigorous identification of the histone bands, however, would require purification of the extracts by column chromatography (Bonner et al., 1968b) or chemical separation of each histone component from whole histone (Johns, 1964) and amino acid analysis of each eluted fraction or each chemically separated fraction.

Developmental Changes in Histone and Total Protein

Encysted embryos of Artemia salina appear to have some unique histone properties compared with other known histone patterns from diverse organisms. During the course of development (at least from gastrula to nauplius) only slight changes in composition take place, but some of these changes appear rather abruptly at certain stages. Artemia bands 1 and 3 (the most lysine-rich, on the basis of comparison with pea and thymus histone) decrease during development. Band 6 (slightly lysine-rich histone) increases between 36 and 48 hr and bands 7 and 8 (arginine-rich) maintain essentially constant proportions (see Figures 8, 9 and 10). Between 36 and 48 hr of development, the electrophoretic histone pattern approximates that of whole, mature pea histone.

These findings are different from those of Fambrough et al. (1968), and Vorobyev et al. (1969) who found increases in lysine-rich histones during the development of sea urchins. Amino acid analyses of Artemia extracts would be necessary, however, in order to make strict comparison with these studies, which rely heavily on differential lysine and arginine contents for histone identification.

Band 2 appears to represent a unique histone fraction which is obvious only in the desiccated cyst (Figure 8). It comprises 6.5% of the total histone at this time. Its reduction upon hydration and

resumption of development suggests that it may play a role similar to that of the slow-moving avian erythrocyte histone described by Neelin (1964) in the maintenance of chromosomal condensation. It is also interesting to note, however, that there has been controversy in the literature about whether desiccated cysts contain blastulae or gastrulae (McClean and Warner, 1970). If both are present, it is possible that the shift in band 2 represents the development of the remaining blastulae to gastrulae.

Band 1 has no electrophoretic counterpart in pea tissue or calf thymus. It may be a lysine-rich fraction which is unique to Artemia, but probably is the cysteine-containing histone (fraction III) which has been oxidized during the isolation procedure to a slow-moving dimer form. Easton and Chalkley (1972) describe a slow-moving band similar to band 1 in this study which, upon reduction with mercaptoethanol, is converted from a slow-moving dimer form to a fast-moving monomeric form. The position of the faster moving monomer is identical with Artemia band 5. Thus, bands 1 and 5 may represent two forms of the same histone molecule. If this is the case, the total histone represented by bands 1 and 5 stays nearly constant during development.

In summary, the bands showing the greatest change were 2, 3 and 6. The histone in band 2 decreased rapidly soon after hydration and remained low. The histone content of bands 3 and 6, respectively,

increased and decreased slightly until 36 to 48 hr when these changes were greatly accelerated.

The acidic proteins were difficult to analyze because of persistent background material and the tendency for proteins to clump or fragment to an unknown extent. Nevertheless, there was little change in the pattern of gastrular proteins until about 16 hr when prenaupliar proteins began appearing. This was followed by a naupliar protein pattern beginning some time between 24 and 36 hr.

Developmental Considerations

Artemia has had an unusual history in the laboratory in that a large amount of physiological and biochemical data has accumulated in recent years, but there is almost no previous morphological background upon which to draw. This has been mainly due to the difficulties in preparing cysts for microscopy. No known fixatives penetrate the cyst wall, and large amounts of cytoplasmic yolk and lipid make sectioning difficult. These same difficulties in penetration have made it impossible to use the usual tools of the molecular biologist, labeled precursors and inhibitors, to study events within the intact embryo. Consequently, studies with Artemia have typically assumed that development resumed with rehydration of the cyst and that the highly differentiated nauplius that emerged some 16-24 hr later was a result of gradual and continual differentiation.

The present paper suggests that differentiation is discontinuous and ties together other recent evidence supporting this idea. On the basis of experiments showing that hydration and dehydration were completely reversible until shortly before the nauplius emerged, Morris (1971) proposed that approximately the first 60% of the time before hatching was preparatory and that differentiation did not begin until the end of this "latent" period. The time of appearance of new proteins in the present work correlates with the end of the latent period.

If the role of histones is gene suppression (Bonner et al., 1968b), the failure to find significant histone changes during the latent period or even during the following period of differentiation implies that the genes are not actively transcribing during this period. In support of this idea is the recent observation of Nilsson and Hultin (1972) that encysted embryos contain a store of latent mRNA which is fully capable of activity under the proper conditions with E. coli ribosomes. It is also known (Barros et al., 1966) that much of the mRNA of sea urchins produced during cleavage is stored for use after gastrulation.

The changes in acid proteins and histones between 36 and 48 hr, after most nauplii had hatched, correlate with the beginning of gut function, growth, onset of the first molt and osmoregulation. In this regard it is worth noting that the only detectable non-ribosomal RNA ("heavy" RNA) synthesis occurs between 20 and 36 hr (McClean and

Warner, 1970), but under temperature conditions that would correspond to roughly 36-48 hr in the present work.

If, as suggested by Hearst and Botchan (1971), stable histone populations are responsible for the maintenance of chromosomal condensation (and, thus, inaccessibility to RNA polymerase and other transcriptional activators) and if, indeed, periods of gross changes in histone composition indicate active transcriptional activity (Fambrough et al., 1968), then I suggest that the early post gastrular developmental activity in Artemia is carried out in the absence of gene activation until at least 36 hr of development. Histones probably act as passive repressors during the 36 hr after the gastrula has been initiated into the developmental regime. The presumably high lysine content indicates that the nucleus may not be in an "active" state at this time (Fambrough et al., 1968). It is concluded that the genetic information necessary for development to the nauplius stage is transcribed and stored in the cells before encystment in the gastrula stage.

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