Histone Synthesis during Early Embryogenesis in *Xenopus laevis* (South African Clawed Toad)†

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ABSTRACT: Electrophoretic and chromatographic experiments indicate that histone synthesis takes place during the cleavage and swimming embryo stages of development in *X. laevis*. Furthermore, the major classes of histones (I, II, III, and IV) made during these different stages appear to be qualitatively the same, as shown by electrophoresis on polyacrylamide gels and by Amberlite chromatography, and include histones similar to those found in adult somatic tissues of this amphibian. Experiments utilizing polyacrylamide gels show that these proteins coelectrophorese with known histone markers I, IIb1, and III from trout testes and histone IV from peas. Individual basic proteins were purified from 8 M urea–2 M LiCl extracts of total embryo protein by carboxymethylcellulose chromatography and separated by Amberlite chromatography. The amino acid composition of the Amberlite peaks from swimming embryos indicates that these peaks contain histones comparable to those of other vertebrate species. Rechromatography of the first lysine-rich peak on Amberlite with a very shallow guanidine hydrochloride gradient resolved this peak into a ribosomal contaminant and at least three lysine-rich fractions in swimming embryos, suggesting that the microheterogeneity in *X. laevis* lysine-rich histone I is similar to that demonstrated by Bustin and Cole (Bustin, M., and Cole, R. D. (1968), *J. Biol. Chem. 243*, 4500) for other vertebrate tissues.

Are histones synthesized prior to gastrulation during the period of rapid cellular division in the early development of the amphibian embryo? If so, are these newly synthesized histones qualitatively the same or different at progressive stages of development?

We have investigated these questions by looking at the pattern of basic protein synthesis in early embryos of *Xenopus laevis* using a combination of chromatographic and electrophoretic techniques. In particular, we have focused on the synthesis of basic proteins during the cleavage and swimming embryo stages when the patterns of RNA synthesis are known to be markedly different.

In the course of a study on ribosomal protein synthesis in *X. laevis* embryos, Hallberg and Brown (1969) noted that about 8% of the total protein synthesized by cleaving embryos pulsed with $^{14}CO_2$ eluted as a single peak from a carboxymethylcellulose column. Kasinsky (1969) observed that electrophoresis of this radioactive peak on acrylamide gels showed the presence of two radioactive bands which were distinct from known ribosomal proteins and which migrated similarly to histone fractions isolated from adult somatic tissues. The experiments discussed in this paper now demonstrate that these two radioactive bands present in extracts both of cleaving and swimming *X. laevis* embryos represent the synthesis of the major classes of histones. A preliminary communication of this work has been published (Kasinsky and Byrd, 1972).

Methods and Materials

Mating and Labeling of X. laevis Embryos. X. laevis males were injected with 0.2 ml of Antuitrin "S" (Parke-Davis, 1000 I.U./ml) and females with 0.25 ml 1 week prior to mating. On the day of mating, males were injected with 0.25 ml of hormone and females with 0.75 ml. The mating pairs were placed in dechlorinated water in breeding tanks overnight at 19°. Eggs were shed and fertilized overnight. The embryos were collected the next day and dejellied for 4 min in a 2% cysteine–hydrochloride solution, pH 7.8, but omitting papain (Dawid, 1965).

Batches of 500 cleaving embryos were labeled for 6 hr after fertilization (stages 1–9) in small disposable tissue culture flasks (50 ml) containing 100 μCi (0.5 ml) of $NaH^{14}CO_3$ (specific activity *ca.* 50 mCi/mmol, Schwarz/Mann, No. 0023-92) in 10 ml of $^1/_{10}$ Holtfreter's solution modified to remove excess CO_2 by deletion of $NaHCO_3$ (Brown and Caston, 1962). (*X. laevis* embryos were labeled with this $^{14}CO_2$ precursor as they are impermeable to radioactive amino acids (Friedberg and Eakin, 1949).) Bromocresol Purple (0.01%) was added as a pH indicator and 0.1 N HCl was used to adjust the pH to 6.4. Labeling was stopped by cooling the embryos to 4° when they reached late blastula (stage 9) (Nieuwkoop and Faber, 1956) and they were stored at −20°.

Swimming tadpoles were first labeled at stage 37–38 with 50 μCi of $NaH^{14}CO_3$ for 8 hr as described for cleaving embryos. The radioactivity was removed and the embryos permitted to develop overnight to stage 40–41. They were then pulsed for 6 hr with 50 μCi of $NaH^{14}CO_3$, the isotope was removed, and the embryos were allowed to develop to stage 42, collected, and stored at −20°.

Isolation of Basic Proteins from Cleaving and Swimming X. laevis Embryos. Basic proteins were isolated following the procedure of Hallberg and Brown (1969). Six thousand labeled embryos were homogenized in ultra pure 8 M urea and 2 M LiCl to solubilize the bulk of the proteins and precipitate RNA. After centrifugation, the supernate was incubated with

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10% trichloroacetic acid (Cl_3CCOOH) for 24 hr at 37°, a method which hydrolyzes RNA and DNA without affecting the protein. The solution was then centrifuged at 27,000*g* for 20 min. The precipitate was stirred into 10% Cl_3CCOOH and centrifuged again. The insoluble precipitate was then collected and extracted with ether and ethanol–ether to remove lipid contamination. The protein precipitate was air-dried, dissolved in 0.1 N HCl, and dialyzed against several liters of 0.1 N HCl to solubilize the basic proteins. After high-speed centrifugation, the supernatant was treated with 9 M urea and 0.05 M dithiothreitol and then dialyzed against starting buffer (8 M urea–0.001 M dithiothreitol–0.05 M sodium acetate, pH 5.5) for chromatography. The procedure of Hallberg and Brown (1969) was followed throughout except that it was scaled up so that protein from 6000 to 12,000 radioactive embryos was extracted during the procedure, instead of 1000 embryos.

Chromatography

A. Carboxymethylcellulose Chromatography. Proteins extracted by the above method were fractioned at room temperature on microgranular carboxymethylcellulose (Whatman CM-32) as indicated in Figure 1. The protein content was measured by absorbance at 278 nm. Radioactive assays were performed by the filter paper disk technique of Bollum (1968) using 50-μl samples taken from alternate tubes. The samples were counted in Bray's scintillation fluid in a Nuclear-Chicago liquid scintillation counter (Unilux II-A).

Fractions C and D (Figure 1) were isolated from the 0.175–0.25 M portion of the NaCl gradient on CM-cellulose.[1] We took a larger fraction than did Hallberg and Brown (1969) in order to obtain a better yield of the proteins we presumed were histones that were present in this region.

B. Amberlite (IRC-50) Chromatography. Amberlite resin was prepared by the method of Luck *et al.* (1958). After equilibration, the resin was suspended in 8% (w/v) guanidine hydrochloride–0.1 M sodium phosphate buffer, pH 6.8. A 0.6 × 55 cm column was packed with resin under air pressure. Lyophylized samples (8–10 mg) of C and D proteins were brought up in a few milliliters of 8% guanidine hydrochloride and applied to the column. A linear gradient of 8–14% guanidine hydrochloride was used to separate histones from each other and from ribosomal protein in the run-off peak, according to the procedure of Luck *et al.* (1958). After the gradient was completed, 25 ml of 40% guanidine hydrochloride were added to elute the final adhering protein. The eluted protein was collected in 0.3-ml fractions during a 3–5 day run at room temperature and ^{14}C-labeled proteins were assayed as described for CM-cellulose chromatography.

Acrylamide gel electrophoresis (disc) was performed using 15% polyacrylamide gels made 6 M with urea. Proteins were run 90 min at room temperature according to the procedure of Bonner *et al.* (1968). No starter gel was utilized. All samples were reduced with 0.05 M dithiothreitol to prevent histone aggregation (Fambrough and Bonner, 1969). Eight gels at a time were prerun for 90 min with the electrodes reversed to remove persulfate. The samples were run and the gels were stained with 1% (w/v) Amido Black in 7% (v/v) acetic acid and destained in a diffusion destaining apparatus (Hoefer Scientific No. DE 105) utilizing charcoal and 7% acetic acid. Densitometric tracings of the stained gels were obtained either on the Joyce-Loebl microdensitometer (Mark III CS) or on the Gilford linear scanner Model 2400 at 600 nm. For autoradiography, gels were sliced longitudinally, dried onto filter paper, and subjected to autoradiography (7–30 days) using Kodak No-Screen Medical X-ray film (No. NS-54T) according to the method of Fairbanks *et al.* (1965). Tracings of the autoradiograms were obtained as above for stained gels.

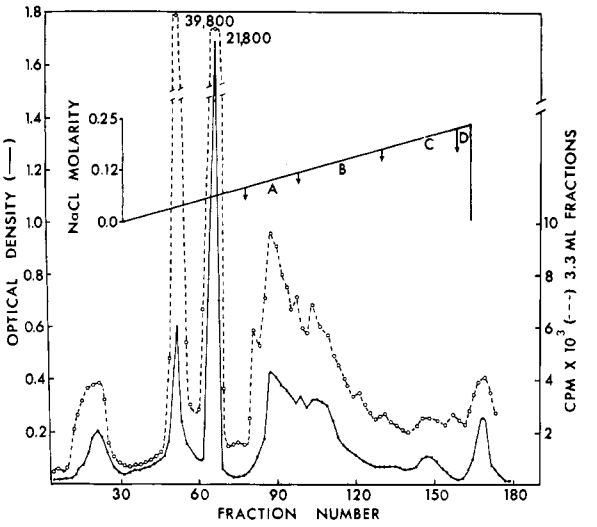

FIGURE 1: Carboxymethylcellulose chromatography of basic proteins synthesized by swimming embryos of *X. laevis*. The basic protein fraction from 3000 embryos was washed into a 2 × 25 cm column with 100 ml of starting buffer and eluted with a 400-ml linear gradient of NaCl (0–0.25 M) in starting buffer. Final adhering protein was eluted with 200 ml of 8 M urea, pH 2. Fractions of 3.3 ml were collected at a flow rate of 0.5 ml/min. Radioactive assays were performed by the filter paper disk technique of Bollum (1968) using 50-μl samples from alternate tubes.

Radioactive assays were performed by making 0.5-mm slices of frozen gels on a gel slicer (Mickle Laboratory Engineering Co., Comshal, England). The gel slices were dissolved by incubating them in 0.1 ml of 30% hydrogen peroxide in scintillation vials for 60 min at 50°. Aquasol (New England Nuclear) was added to the scintillation vials, the vials were shaken and then counted on the liquid scintillation counter.

Preparation of Histones from X. laevis

A. X. laevis Erythrocyte Histones. Nucleated erythrocytes were chosen as a source of adult somatic tissue because of the ease of isolation of the nuclei and because the cells were not synthesizing proteins, so that contamination with ribosomal proteins would be minimal. Adult *X. laevis* females were chilled on ice, sacrificed, and the exposed heart bled into standard saline citrate (SSC, 0.15 M NaCl–0.015 M sodium citrate), about 30 ml/frog. The intact erythrocytes were filtered through four layers of cheesecloth to remove debris and precipitated by centrifugation at 2000 rpm for 15 min in glass bottles in the International refrigerated centrifuge, Model PR2. The red precipitate was washed twice in 0.15 M NaCl. After washing with physiological saline, the erythrocytes were lysed in distilled water by osmotic shock to obtain the nuclei. Nuclei showed a slight cytoplasmic ghost by phase contrast microscopy. The residual cytoplasmic contents were lyophilized in order to obtain a supply of concentrated erythrocyte hemoglobin.

[1] Abbreviations used are: CM-cellulose, carboxymethylcellulose; MEM, minimum essential medium; RSB, reticulocyte standard buffer (0.01 M Tris–0.01 M NaCl–0.005 M $MgCl_2$); SSC, standard saline citrate (0.15 M NaCl–0.015 M sodium citrate); TES buffer, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.

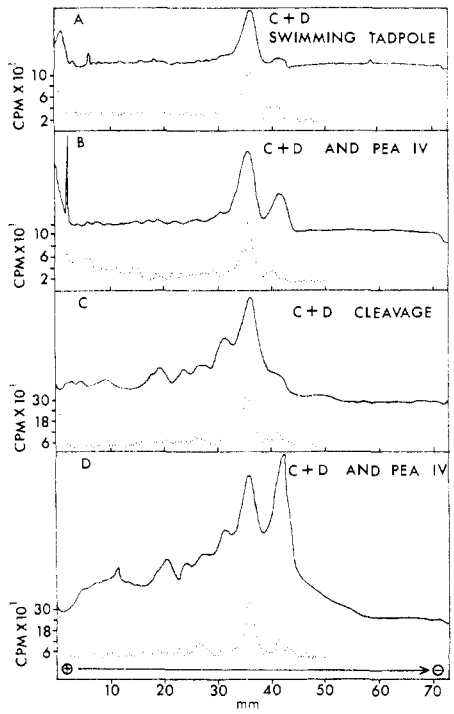

FIGURE 2: Tracings of acrylamide gels from fractions C and D for stage 42 swimming embryos and cleavage embryos: solid lines, optical density at 660 nm; dotted profiles, radioactivity. Disc electrophoresis was performed using 15% polyacrylamide gels made 6 M with urea. Proteins were run for 90 min at room temperature according to the procedure of Bonner *et al.* (1968). All samples were reduced with 0.05 M dithiothreitol to prevent histone aggregation. Gels were preelectrophoresed for 90 min to remove persulfate. After the final run, gels were stained with 1% Amido Black in 7% acetic acid and destained in 7% acetic acid in a diffusion destainer. Tracings were taken using the Gilford linear scanner (Model 2400) at 660 nm. Radioactive assays were performed on 0.5-mm gel slices after peroxidation and solubilization with Aquasol for scintillation counting: (A) radicactive fractions C + D, swimming embryos; (B) fractions C + D, swimming embryos electrophoresed on the same gel with unlabeled pea histone IV; (C) radioactive fractions C + D, cleavage embryos; (D) fractions C + D cleavage embryos electrophoresed on the same gel with unlabeled pea histone IV.

Chromatin and histones were obtained from isolated nuclei according to the method of Bonner *et al.* (1968). The erythrocyte preparations exhibited an A_{320}/A_{260} ratio less than 0.1, indicating that the preparations were not contaminated.

Histones were extracted from purified chromatin containing less than 0.5 mg/ml of DNA, as measured by ultraviolet absorbancy, by stirring with 0.2 N H_2SO_4 (final concentration) for 30 min on ice. Following the method of Bonner *et al.* (1968), we centrifuged the solution for 20 min at 12,000*g* and stirred the residual precipitate as before but with a final concentration of 0.4 N H_2SO_4. The supernatant fractions were combined, dialyzed against distilled water, and lyophilized to yield *X. laevis* erythrocyte histone. The yield was about 1 mg of total histone per adult frog.

B. X. laevis Kidney Cells in Culture. In order to obtain radioactive histones from somatic tissues, we grew tissue culture cells originally derived from adult kidneys of *X. laevis* using the nutrient medium of Brown and Weber (1968). The cells were pulsed for 4 days with 50 μCi of [^{14}C]leucine (uniformly labeled, specific activity of 333 mCi/mmol, New England Nuclear, No. 279) in 100 ml of MEM with Earle's salts but without L-leucine (Grand Island Biological Co., No. 189G), made 0.01 M in TES buffer, pH 7.4. The cells were then chased for 1 day with 2 mg of cold L-leucine in 0.01 M TES buffer, pH 7.4, added to the same medium. This was about one-third the usual concentration of leucine in MEM. After centrifugation the nuclei were full of cytoplasmic tabs as seen by phase contrast microscopy. Preparation of chromatin and histones followed the same precedures used in the isolation of erythrocyte histones. The kidney cell chromatin preparations exhibited an A_{320}/A_{260} ratio greater than 0.1, the turbidity perhaps being due to aggregation of the chromatin.

Amino Acid Analyses

Samples of the various histones were hydrolyzed *in vacuo* at 110° for 24 hr in 6 N HCl. After evaporation to dryness, the hydrolysates were dissolved in distilled H_2O and their amino acid composition determined on a Technicon amino acid analyzer. The presence of tryptophan was measured by the method of Opienska-Blauth *et al.* (1963).

Results

When subjected to chromatography on carboxymethylcellulose, the basic proteins synthesized either in cleaving or swimming embryos of *X. laevis* were fractionated as shown in Figure 1. The region of particular interest consisted of fractions C and D in which Kasinsky (1969) observed proteins similar to adult somatic histones of *X. laevis* in their electrophoretic properties. This fraction eluted between 0.175 and 0.25 M NaCl in the gradient.

Fractions C and D were reduced with dithiothreitol and examined by electrophoresis in 15% acrylamide gels by the method of Bonner *et al.* (1968) (Figure 2). Tracings of these gels stained with Amido Black showed the presence of a fast moving doublet in both swimming tadpole and cleavage embryos that coincided with a radioactive doublet as determined by counting gel slices in a liquid scintillation counter. Electrophoresis on the same gel with a known histone marker, the arginine-rich histone IV from pea or calf thymus (a gift of Drs. D. M. Fambrough and J. Bonner), indicated that the fastest migrating band in both cleaving and swimming embryos and the pea IV histone migrated together. These data indicated that we were looking at a histone fraction. As we shall see from the subsequent data on Amberlite chromatography and gel electrophoresis, the first peak of the radioactive doublet represented the synthesis of the other major classes of histones in both the cleavage and swimming embryos.

The basic proteins of fractions C and D were also compared with preparations of histones isolated from chromatin of adult somatic tissues of *X. laevis* by electrophoresis on gels. Figure 3 indicates that two of the main bands of *X. laevis* erythrocyte histones electrophoresed on the same gel with the two main bands of fractions C and D from swimming embryos. The microdensitometer tracings of these gels revealed that the stained bands of erythrocyte histones migrated identically with the two radioactive bands of fraction C and D proteins as measured by autoradiography. Histones extracted from *X. laevis* kidney cells labeled with [^{14}C]leucine showed a two-banded pattern. In each case, the fastest moving band from the somatic tissue was similar in electrophoretic mobility to that of the fastest moving band from fractions C and D of stage 42 embryos. These electrophoretic data suggested that somatic and embryonic histones were qualitatively similar.

Further evidence to characterize the newly synthesized basic proteins present in fractions C and D as histones was obtained from Amberlite chromatography (Luck *et al.*, 1958)

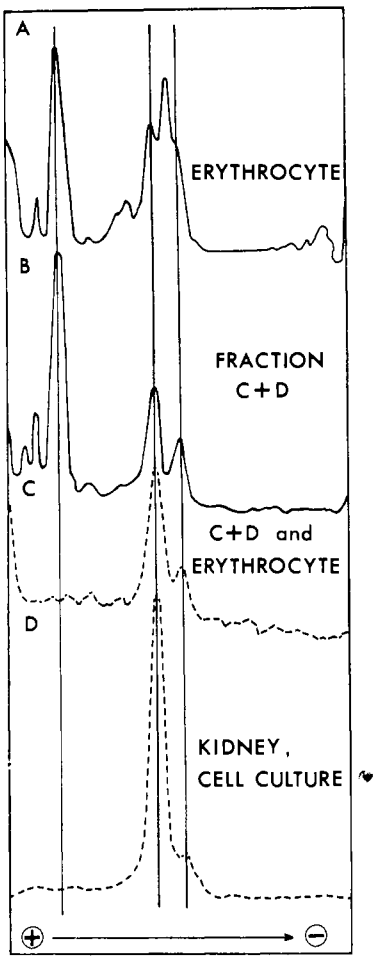

FIGURE 3: Tracings of acrylamide gels comparing adult somatic histones of *X. laevis* with swimming embryo fraction C + D. Conditions were the same as in Figure 2: solid lines, optical density at 660 nm; dashed lines, radioactivity of autoradiograms. (A) Erythrocyte histone fraction; (B) fractions C + D histone; (C) electrophoresis of unlabeled erythrocyte histone on the same gel with radioactive fractions C + D; only the labeled C + D is shown; (D) radioactive kidney cell histone fraction. The initial peaks near the anode in A and B were due to bovine serum albumin used as a marker.

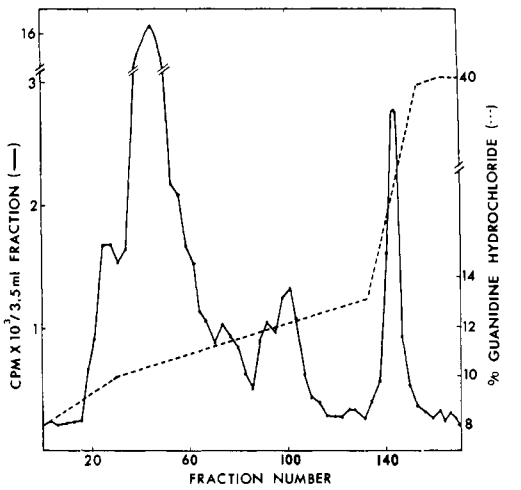

FIGURE 4: Amberlite chromatography of radioactive histones synthesized by cleavage embryos (stages 1–9) of *X. laevis*. Basic proteins (5–8 mg) were applied to a 0.6 × 55 cm column and eluted with a 50-ml linear gradient of 8–14% guanidine hydrochloride in 0.1 M sodium phosphate, pH 6.8, followed by a 40% guanidine hydrochloride wash (Luck *et al.*, 1958). Fractions of 0.35 ml were collected from a column over a 3-day period and radioactive assays were performed as described in Figure 1.

which separated most of the histone components from contaminating basic proteins. Figure 4, showing the fractionation of cleavage embryo C and D fractions on Amberlite, contained an initial broad peak of radioactivity (fractions 20–70) that included lysine-rich histone I and other contaminating basic proteins. The second radioactive peak (fractions 71–120) eluted by the guanidine hydrochloride gradient was due to the presence of the slightly lysine-rich histones IIb1 and IIb2. Finally, the last radioactive peak (fractions 121–160) eluted in the region where the arginine-rich histones III and IV were expected to appear. Amberlite chromatography of fractions C and D from stage 42 embryos yielded a similar elution pattern as seen in Figure 5. The similarity of the Amberlite fractionation profiles for both labeled cleavage and swimming tadpole histones on Amberlite suggested that there was no qualitative change in the pattern of histone synthesis throughout the early development of *X. laevis* embryos.

In order to identify the newly synthesized radioactive proteins in the Amberlite peaks of cleavage (Figure 4) and stage 42 (Figure 5) embryos as histones, we first electrophoresed them on one gel and then coelectrophoresed them with unabeled histone markers from trout testes (I, IIb1, and III, a gift of Dr. G. H. Dixon) and histone IV from pea. By coelectrophoresis we mean that each radioactive protein in the Amberlite peaks was run together with a nonradioactive marker on the same gel. Both the densitometric tracing of the Amido Black stained band and the radioactivity pattern of the gel were then determined. As seen in Figure 6, densitometric tracings indicated that the initial Amberlite radioactive peak from stage 42 embryos coelectrophoresed with the lysine-rich histone I from trout testes, although other contaminants were also present. In this gel and in each of the others in Figure 6, sufficient amounts of radioactive histones from the Amberlite peaks of swimming embryos were applied to the gels to obtain densitometric tracings of their Amido Black stained bands as well as their radioactivity profile. As the two patterns coincided, only the densitometric tracings at 660 nm for the stained bands are shown for both the radioactive proteins from the

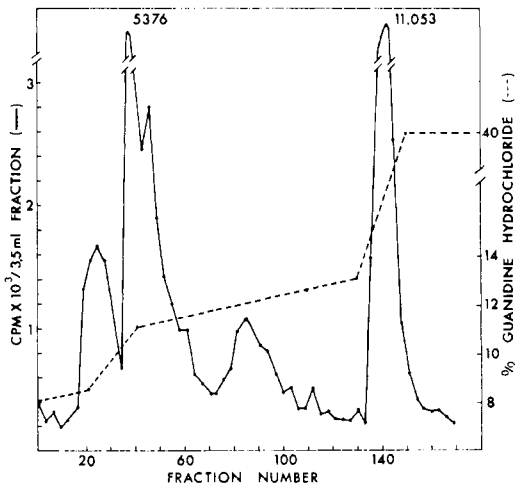

FIGURE 5: Amberlite chromatography of radioactive histones synthesized by swimming embryos (stage 42) of *X. laevis*. Conditions were the same as indicated in Figure 4.

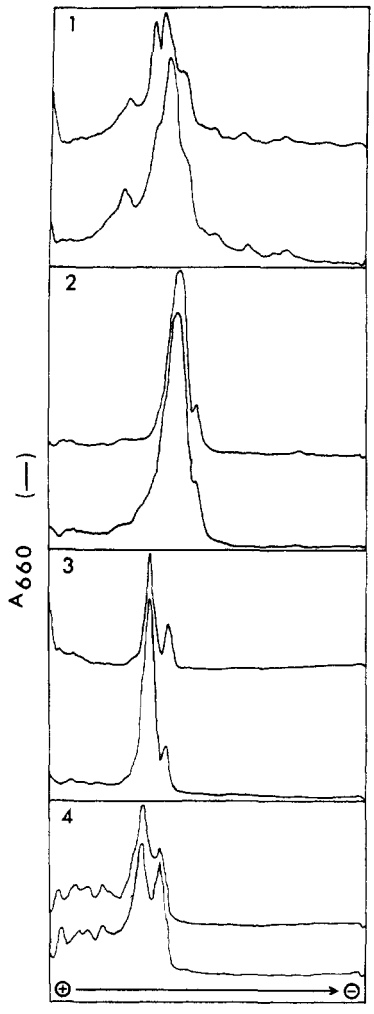

FIGURE 6: Tracings of acrylamide gels of Amberlite peaks from stage 42 embryos (Figure 5). Conditions were those indicated in Figure 2. Densitometric tracings at 660 nm both for proteins from the Amberlite peaks and histones used as markers are indicated by solid lines: (1) top gel, electrophoresis of the lysine-rich histone peak (fractions 20–70); bottom gel, coelectrophoresis of this peak with lysine-rich histone I from trout as a marker; (2) top gel, electrophoresis of the slightly lysine-rich histone peak (fractions 71–120); bottom gel, coelectrophoresis of this peak with slightly lysine-rich histone IIb1 from trout; (3) top gel, electrophoresis of the arginine-rich histone peak (fractions 121–160); bottom gel, coelectrophoresis of this peak with arginine-rich histone III from trout; (4) top gel, electrophoresis of the arginine-rich histone peak (fractions 121–160); bottom gel, coelectrophoresis of this peak with arginine-rich histone IV from pea.

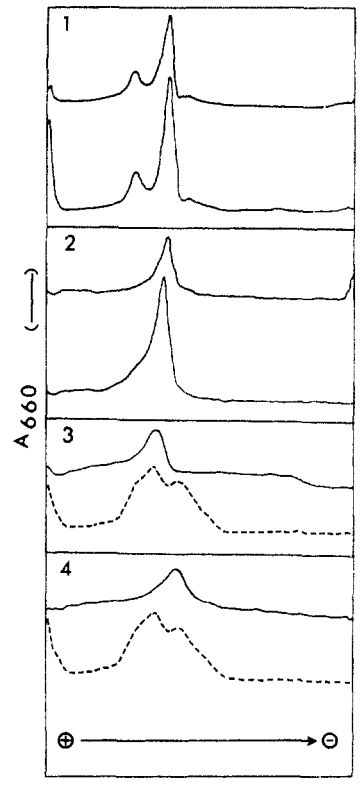

FIGURE 7: Tracings of acrylamide gels of Amberlite peaks from cleavage embryos (Figure 4). Conditions and fraction numbers were the same as indicated in Figures 2 and 6, respectively. Autoradiograms of the radioactive proteins from the Amberlite peaks are indicated by dashed lines in (3) and (4). Densitometric tracings at 660 nm for both the radioactive proteins and the nonradioactive histones used as markers are indicated by solid lines: (1) top gel, electrophoresis of the lysine-rich histone peak (fractions 20–70); bottom gel, coelectrophoresis of this peak with lysine-rich histone I from trout as a marker; (2) top gel, electrophoresis of the slightly lysine-rich histone peak (fractions 71–120); bottom gel, coelectrophoresis of this peak with slightly lysine-rich histone IIb1 from trout; (3) coelectrophoresis of the arginine-rich histone peak (fractions 121–160) with unlabeled histone II from trout; both tracings are from the same gel; (4) coelectrophoresis of the arginine-rich histone peak (fractions 121–160) with unlabeled histone IV from pea; both tracings are from the same gel.

Amberlite peaks and the same proteins coelectrophoresed with nonradioactive histone markers. The second peak coelectrophoresed with the slightly lysine-rich histone IIb1 from trout testes. We presume that the slightly lysine-rich histone IIb2 is also present in this fraction. Finally, in the 40% guanidine hydrochloride wash, electrophoresis showed two bands. The slower migrating band coelectrophoresed with arginine-rich histone III from trout and the faster moving band with histone IV from pea. There was still a low level of contaminating proteins in this region. They may represent acidic proteins liberated by the abrupt increase in guanidine hydrochloride as described by Stellwagen and Cole (1968).

Densitometric tracings were also obtained from acrylamide gels of the radioactive Amberlite peaks from cleavage embryos (Figure 4) coelectrophoresed with unlabeled histone markers in order to identify these newly synthesized proteins as histones. As seen in Figure 7, each of the major classes of histones present in these embryos was synthesized during this early period of development preceding gastrulation. As in Figure 6, sufficient amounts of the lysine-rich and slightly lysine-rich histones were obtained from the Amberlite peaks of cleavage embryos to obtain densitometric tracings of their Amido Black stained bands as well as their radioactivity profile. As the two patterns coincided we have shown only the densitometric tracings in part 1 and part 2 of Figure 7. However, dilution of the radioactive proteins in the arginine-rich histone peak from the Amberlite column onto several gels greatly decreased their staining with Amido Black. We therefore used autoradiography to establish the identity of these labeled histones by their coelectrophoresis with unlabeled arginine-rich histones III (trout) and IV (pea), respectively.

Histones prepared from stage 42 swimming embryos and separated on Amberlite chromatography were further identified by amino acid analysis. The amino acid compositions of the three peaks are shown in Table I where they are compared to newt embryo and calf thymus histones.

TABLE I: Amino Acid Composition of Histones from Stage 42 *X. laevis* Embryos Compared with Those of Stage 20 Newt Embryos and Calf Thymus Histone Fractions.

	X. laevis Histones (Mol %)			Newt Histones[a] (Mol %)			Calf Thymus Histones[c] (Mol %)		
Amino Acid	I	II	III + IV	FI	FIIa	FIII[b]	F1b	FIIb	FIII[b]
Lys	18.8	14.1	10.4	18.6	10.5	9.3	26.2	13.5	9.3
His	0.2	3.4	2.7	2.6	3.6	3.1	0.2	2.8	1.6
Arg	1.3	7.5	8.7	1.7	3.8	5.4	2.6	7.9	12.8
Asp	4.7	6.7	7.6	4.7	6.3	6.9	2.5	5.6	4.4
Thr	4.3	3.9	5.1	3.4	5.8	6.6	5.4	5.2	7.3
Ser	4.3	6.9	5.6	14.1	13.0	10.2	6.5	7.8	4.1
Glu	4.4	5.2	11.0	8.6	8.6	7.5	4.3	8.7	9.8
Pro	5.7	5.9	4.9	7.0	4.2	3.2	9.1	4.7	3.8
Gly	8.3	5.9	9.1	11.9	13.1	12.3	7.3	8.2	8.7
Ala	11.2	12.9	8.1	14.9	10.6	10.7	24.2	11.5	11.7
Cys/2	0	0	0.6	0	0	0	0	0	0
Val	6.2	6.9	6.5	5.1	5.8	6.7	4.0	6.7	5.8
Met	0.8	1.3	1.8	0.4	0.5	1.1	0.1	0.8	1.2
Ile	2.1	4.1	4.8	1.6	4.4	5.4	1.2	4.5	5.4
Leu	6.0	7.4	8.4	3.9	6.8	8.2	5.0	8.6	8.6
Tyr	0.3	3.5	3.0	0.3	0.9	0.7	0.7	3.0	2.4
Phe	1.2	3.5	3.9	1.3	2.0	2.9	0.6	1.3	2.5
B/A	2.2	2.1	1.2	1.7	1.9	1.2	4.3	1.7	1.6
Lys/Arg	10.5	1.9	1.2	10.8	2.8	1.7	10.1	1.7	0.8

[a] Asao, 1969. [b] Represents combined histones III + IV. [c] Rasmussen *et al.*, 1962.

The first peak, *X. laevis* histone I, is shown to have the highest lysine content of the separated histones. This high lysine to arginine ratio is characteristic of the lysine-rich histones. However, total mole percentages of alanine and proline are lower than would be expected for histone I as seen in calf thymus. This is probably due to contamination by other basic proteins that are eluted in the lysine-rich peak, as shown in Figure 8. The amino acid composition of the second peak, II, or the slightly lysine-rich histones IIb1 plus IIb2 is similar to known values for calf thymus. This fraction also compares to that of newt histone IIa except for the higher serine and glycine content and the greater lysine to arginine ratio shown in the newt. The third peak, eluted in the 40% gradient on Amberlite, appears to be characteristic for the arginine-rich histones III plus IV, having a low lysine to arginine ratio. Also, the presence of cysteine is noted in our arginine-rich fraction. Cysteine is absent in all histones except for histone III. The presence of tryptophan was measured in each peak by the method of Opienska-Blauth *et al.* (1963), which is a colorimetric assay for the determination of tryptophan. There was no tryptophan present in these peaks; thus the absence of tryptophan is another characteristic feature of histones.

As noted earlier, the lysine-rich histone peak from stage 42 embryos (Figure 5) was contaminated with other basic proteins after Amberlite chromatography. This may have been due to the synthesis of basic proteins, such as ribosomal proteins, which is known to occur during this period of development in *X. laevis* (Hallberg and Brown, 1969). To further characterize the lysine-rich histones in this region and to separate them from other basic proteins, we rechromatographed the initial Amberlite peak on another Amberlite column using a shallow gradient as described by Kinkade and Cole (1966) and Bustin and Cole (1968). We can see in Figure 8 that this gradient separated the ribosomal and other basic proteins from the lysine-rich histones, the latter emerging as three labeled peaks starting at 9.4% in the guanidine hydrochloride gradient. Bustin and Cole (1968) have shown that there is a similar microheterogeneity of lysine-rich histone I in rabbit and calf thymus as well as rabbit and chicken liver using this method. Rechromatography of the lysine-rich histone peak of cleavage embryos on a shallow gradient also showed a separation of histone I from basic protein contaminants.

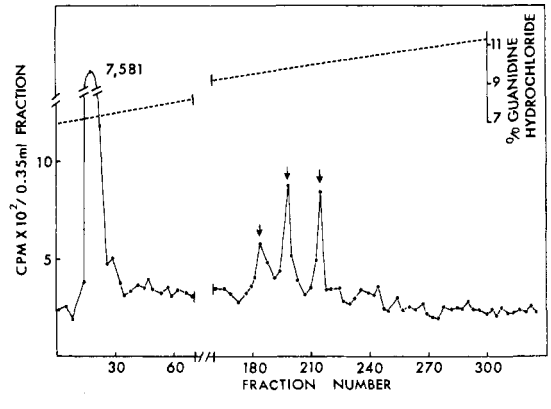

FIGURE 8: Chromatography of the lysine-rich histone peak from stage 42 embryos on Amberlite using a shallow gradient. Basic proteins (10 mg) were eluted with a 200-ml linear gradient of 7–14% guanidine hydrochloride in 0.1 M sodium phosphate, pH 6.8, according to the method of Bustin and Cole (1968). Fractions of 0.35 ml were collected over an 8-day period. Radioactive assays were done as described in Figure 1. The arrows point to the three lysine-rich histone peaks that began eluting at 9.4% in the guanidine hydrochloride gradient.

Discussion

Chromatographic and electrophoretic analyses indicate that the major classes of histones (I, II, III, and IV) are synthesized during cleavage (stages 1–9) and swimming tadpole (stages 37–42) periods of development in *X. laevis* embryos. Thus, *X. laevis* embryos appear to synthesize the full complement of histones throughout their early development and do not acquire qualitatively unique "cleavage histones" as had been suggested by some cytochemical experiments (Horn, 1962). In these experiments we have been looking at histone synthesis in total cell extracts. Since histones may be defined as "basic proteins that at some time are associated with DNA" (Murray, 1964), we must qualify our use of the term histone in this regard. We have been observing newly synthesized basic proteins in a total cell extract that appear to be histones by three different criteria: electrophoresis, Amberlite chromatography and amino acid analysis. The data presented here represent the first biochemical evidence for histone synthesis both before and after gastrulation in an amphibian.

We did not use nuclear preparations in this study for two reasons. First, nuclear preparations from early amphibian embryos are generally poor due to large, fragile nuclei and to extensive cytoplasmic yolk and pigment granule contamination. Second, in earlier experiments, Berlowitz and Birnstiel (1967) isolated a partially purified, $^{14}CO_2$-labeled nuclei preparation from swimming embryos of *X. laevis* and extracted histones directly from this preparation. Using Amberlite chromatography, they demonstrated peaks corresponding to the synthesis of major histone fractions in swimming tadpoles, although they did not characterize these fractions by gel electrophoresis or amino acid analysis. Berlowitz and Birnstiel found that the nuclei in the anucleolate mutant of *X. laevis* contained markedly different amounts of individual histones than did nuclei of tailbud embryos at comparable stages. This conclusion may be subject to doubt, however, as their nuclear preparations may have contained varying degrees of cytoplasmic contaminants. In fact, the data of Hallberg and Brown (1969) on the anucleolate mutant of *X. laevis* indicate that anucleolate embryos synthesized histones to almost the same extent (70%) as did the normal stage 42 embryos. Our procedure of isolating histones from a total cell homogenate rather than from partially purified nuclei and subsequently separating out contaminating basic proteins, particularly ribosomal proteins, attempted to overcome some of these difficulties.

The only other biochemical investigation of histone synthesis in the early development of an amphibian is that of Asao (1969, 1970). Asao injected labeled amino acid mixtures into female newts before mating and separated the labeled histones by Amberlite chromatography on a microscale. In contrast to our finding that synthesis of the major classes of histones in *X. laevis* did not change qualitatively throughout early development, Asao found that there was no accumulation of labeled histones in the nuclei of the Japanese newt, *Triturus pyrrhogaster*, before early gastrulation. He also observed that arginine-rich histones predominated over lysine-rich ones in late blastula and early gastrula and, quite surprisingly, that the dorsal lip region was relatively lacking in histones at late blastula. This suggested to Asao that the template activity of DNA was not repressed by these basic proteins during early development in the newt. One explanation for this difference in our observations and Asao's is that we may be looking at both cytoplasmic synthesis and nuclear accumulation of labeled histones. Asao (1969) did look at a fraction he called "cytoplasmic" basic proteins. However, this is a misnomer as this fraction consisted mostly of yolk granules and some other cytoplasmic components that remained as a surface layer after centrifugation of the nuclei, according to Asao. This would not correspond to the whole cell extract examined in this work. We are presently investigating possible differences between nuclear and cytoplasmic histones in *X. laevis* embryos.

Biochemical studies have shown that histones are also synthesized during early embryogenesis in the sea urchin (Johnson and Hnilica, 1971). Kedes *et al.* (1969) and Nemer and Lindsay (1969) used differential labeling with [^{14}C]lysine and [^{3}H]tryptophan to detect basic proteins which are deficient in tryptophan, hence are probably histones rather than ribosomal proteins (Borun *et al.*, 1967). Kedes *et al.* (1969) have estimated that about 25% of the total protein synthesized during cleavage in sea urchins may be histones. Further proof that histones are newly synthesized after fertilization in sea urchin has been obtained by Moav and Nemer (1971). These workers studied the function of the "s-polysomes" that make up 70% of the polysomes in the 200 cell blastula stage. Characterization of nascent proteins on these light polysomes by electrophoresis and by chromatography on Amberlite showed them to be histones.

Our observations on *X. laevis* embryos indicate that there are no qualitative changes in the synthesis of the major classes of histones during early development in this amphibian. These newly synthesized histones also appear to be the same as histones present in adult tissues of *X. laevis* as well as histones purified from other organisms, as shown by electrophoresis and amino acid analysis. Recently Claycomb and Villee (1972) have isolated proteins from *X. laevis* embryos that bind to a calf thymus DNA–cellulose column. They have shown that there is synthesis of proteins in the molecular weight range for histones during cleavage and blastula stages, a finding that agrees with our data.

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Subunit Composition of Haptoglobin 2-2 Polymers†

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ABSTRACT: Hp 2-2 forms a series of discrete polymers of increasing molecular weight. In the present study Hp 2-2 polymers have been isolated from each other by polyacrylamide gel electrophoresis. Quantitative amino-terminal analysis and amino acid analysis provide evidence that each polymer consists of $\alpha 2$ and β polypeptide chains in a 1:1 ratio. Molecular weight estimations by sodium dodecyl sulfate acrylamide gel electrophoresis and by ultracentrifugational analysis indicates that each polymer differs from the next member of the series by an average increment 54,500. This approximates a subunit consisting of an $\alpha 2$ and a β polypeptide chain. Experimental results indicate that the $\alpha 2/\beta$ subunits are linked together through disulfide bonds to form each polymer.

Haptoglobin (Hp)[1] is an α_2 globulin in human serum whose function is to bind free hemoglobin (Hb). Three common genetic types have been described on the basis of their electrophoretic migration patterns on vertical starch gel at alkaline pH (Smithies, 1955). More recently, polyacrylamide gel electrophoresis has demonstrated similar patterns (Raymond, 1962; Woodworth and Clark, 1967; McCombs and Bowman, 1970). An interesting feature of haptoglobin types 2-2 and 2-1 is their capacity to form a series of discrete stable polymers. During electrophoresis Hp 1-1 migrates as a single band, whereas Hp 2-2 and 2-1 each forms a series of polymeric bands of decreasing mobility and concentration.

The Hp molecule is comprised of two pairs of nonidentical polypeptide chains, designated α and β, which are held together by disulfide bonds (Smithies *et al.*, 1962). The α chain, which exists in two common phenotypes, is responsible for the genetic polymorphism observed in Hp. Hp 1-1 contains $\alpha 1$ chains, each of which has a molecular weight of 9000, while Hp 2-2 contains $\alpha 2$ chains, each of which has a molecular weight of 17,300 (Dixon, 1966). Both $\alpha 1$ and $\alpha 2$ chains are present in Hp 2-1. The β chain, 40,000 molecular weight, is identical in all three types of haptoglobins (Shim *et al.*, 1965).

There have been several different views concerning the number of α and β polypeptide chains which occur in the polymers of Hp 2-1 and Hp 2-2. By observing the number of bands on acrylamide gels of partially saturated Hp–Hb complexes, Sutton (1970) considered that each successively larger polymer was due to the addition of one β chain. The presence of α chains in proportion to β chains was suggested, but no experimental verification for this was given. An earlier hypothesis of Allison (1959) was that each successively larger polymer resulted from addition of one monomeric molecule. The number of chains of this monomer was unspecified. Parker and Bearn (1963) and Shim and Bearn (1964) proposed an addition of two α chains and two β chains as the increment between consecutive polymers. Marnay (1961) proposed that the difference between polymer size was due to the addition of half-molecules. Although the number of polypeptide chains was unspecified, this presumably had reference to α/β increments. Marinis and Ott (1965) proposed that each polymer is a dimer of the preceding smaller polymer.

In the present study the Hp 2-2 polymers (one through six) have been isolated by polyacrylamide gel electrophoresis

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[1] Abbreviations used are: Hp, haptoglobin; Hb, hemoglobin; β-ME, β-mercaptoethanol; Pth, phenylthiohydantoin derivative.