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*HISTONE-BOUND RNA, A COMPONENT OF NATIVE NUCLEOHISTONE**

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Communicated July 27, 1965

We have found that native histone molecules as they occur in the nucleohistone component of pea bud chromatin contain RNA molecules chemically linked to them. The histone-bound RNA constitutes a new class of RNA, differing from other classes in base composition and in chain length. That the RNA content of native histone has not been discovered heretofore is due to the fact that histones are commonly extracted from chromatin with acid, a condition under which the RNA-histone bond is cleaved. Since the nucleohistone component of chromatin is inactive in the support of DNA-dependent RNA synthesis and since the histones are one class of agents responsible for such repression, their association with a specific class of RNA is relevant to considerations of the control of genetic activity.

Materials and Methods.—P^{32}-labeled nucleohistone: We have used P^{32}-labeled histone-RNA complex for the major part of this work. For each preparation *ca.* 5 kg of pea seeds (var. Alaska) were soaked in such a volume of P^{32}-solution (50 mc carrier-free $H_3P^{32}O_4$ adjusted to pH 7.5 with 1 *M* tris) that all was imbibed by the seeds. These were then planted in vermiculite and germinated in the dark for 6 days at 25°C. The apical buds were then removed, each preparation yielding *ca.* 600 gm fresh weight of buds. From these, chromatin was prepared by the methods of Huang and Bonner.[1] The buds were ground (Waring Blendor) in grinding medium consisting of sucrose 0.25 *M*, tris buffer, pH 8, 0.05 *M*, and $MgCl_2$, 0.001 *M*, the homogenate was filtered successively through cheesecloth and Miracloth, and pelleted for 30 min at 4000 × *g*. The crude chromatin was scraped from the underlying starch, resuspended in grinding medium, and repelleted for 10 min at 10,000 × *g*. The crude chromatin was then repelleted 4 further times from 0.05 *M* tris pH 8. The 5 × repelleting of the crude chromatin removes much nonchromosomal protein and contributes importantly to the purity of the final product. The thus purified crude chromatin was next layered on 1.7 *M* sucrose, and the upper third of the tube stirred to form a rough gradient. It was then centrifuged for 105 min at 22 krpm in the SW25 Spinco head. The chromatin pellets as a clear gelatinous material. At this stage we recover *ca.* 600 OD_{260} (approximately 30 mg) of chromosomal DNA. The purified chromatin was next dialyzed for 1 hr against 4 × diluted dilute saline citrate (DSC, 0.015 *M* NaCl, 0.001 *M* Na citrate) and then sheared to produce soluble nucleohistone by the method of Bonner and Huang.[2] The chromatin, at a concentration of 0.5 mg DNA/ml, was sheared for 90 sec in a Waring Blendor at 80 v. The suspension was then centrifuged for 30 min at 10 krpm. The supernatant contains the soluble nucleohistone. Approximately 70% of the chromosomal DNA is recovered as soluble nucleohistone. In general, the soluble nucleohistone was concentrated, as well as freed of contamination by small molecules, by pelleting for 16 hr at 40 krpm in the no. 40 Spinco head.

CsCl density gradient centrifugation: Materials to be fractionated by CsCl density gradient centrifugation were made up to the required concentration of CsCl (Harshaw, optical grade) and in 0.01 *M* tris, pH 8.0. For the separation of histone-RNA from nucleohistone, *ca.* 35 OD_{260} of nucleohistone dissolved in 5 ml of 2.09 *M* CsCl was placed in each bucket of the SW39 Spinco rotor. Concentrations of nucleohistone over the range 3.5–35 OD_{260} per 5 ml exhibit similar banding behavior, but higher concentrations lead to aggregation of the histone as it bands. In some runs, the sample was dissolved in 3 ml of CsCl of the required concentration (and in 0.01 *M* tris, pH 8.0). The sample was then overlayered by 2 ml of paraffin oil. Centrifugation was at 39 krpm for 48 hr, and at a rotor temperature of 20°C. At the end of this time the samples were removed and dripped from the bottom with a Buchler dripping device. The hypodermic needle was driven sufficiently far into the tube to assure that the DNA, which pellets in 2.09 *M* CsCl, was not collected. Ten-drop samples were collected. To each sample 0.6 ml of 0.2 *N* H_2SO_4 was added and protein then determined by OD at 230 mμ. P^{32}-labeled samples were dried on planchets and counted on a Nuclear-Chicago D-47 counting system. In each run one tube containing CsCl but lacking sample was included for determination of the final gradient. This tube was dripped, and the refractive index of each sample determined in a temperature-controlled Zeiss refractometer. From the index of refraction, density was calculated from the relation $\rho = 10.2402\ n_D^{25.0} - 12.6483$. Note that this is the relation appropriate for CsCl concentrations yielding values of $\rho < 1.37$.[3]

Disk electrophoresis of histones: Disk electrophoresis of histones was carried out by the method of Reisfeld *et al.*[4] Acid-extracted histones were dissolved in 2 parts of β-alanine-acetic acid buffer (3.2% β-alanine and 0.8% glacial acetic acid) pH 4, and one part of 1.7 *M* sucrose. A sample of 0.05 ml containing 20–40 μg protein was applied to each gel. Acrylamide gel was made from 0.5% of tetraethylenediamine, 15% acrylamide, 0.1% methylene bis acrylamide with 0.14% ammonium persulfate as catalyst. Electrophoresis was carried out at constant current, 6 mA per tube, for 1 hr at room temperature. A solution 0.6% in buffalo black (naphthol blue-black) in 7.5% acetic acid was used for staining. After 18 hr of staining, the gel was destained by electrophoresis in 7.5% acetic acid solution. The histone bands of each gel were then traced with a Canalco model E microdensitometer.

Analytical methods: Analyses of DNA and RNA were done by the methods standard to our laboratory.[1, 2] Histone was determined by the method of Lowry *et al.*,[5] appropriately calibrated with pure histone or by OD at 230 mμ using the relation 1.0 mg pea bud histone = 3.5 OD_{230}.

Experimental Results.—We first demonstrate that histone dissociated from native nucleohistone by high ionic strength contains associated RNA. For this purpose native nucleohistone was made up in 2.09 *M* CsCl and centrifuged at 39 krpm in the no. 39 Spinco head, until equilibrium had been attained (48 hr). The tubes were then punctured at the bottom and 10-drop fractions collected. The data of Figure 1 show that protein and nucleic acid band together at a main peak density of 1.286. The protein of this band is all soluble in 0.2 *N* H_2SO_4 and hence histone; the nucleic acid is all hydrolyzed by 0.3 *N* KOH or 0.1 *N* NaOH (18 hr, 37°C) and hence RNA. The identities of the two components are further confirmed below by disk electrophoresis and column chromatography of the histone and by nucleotide analysis of the RNA. The banding behavior of the histone dissociated from nucleohistone by high ionic strength is different from that of the same histone after ethanol precipitation from 0.2 *N* H_2SO_4, the classical method for histone preparation (Rasmussen *et al.*[6]). Such acid-treated histone, banded in CsCl pH 8.0, exhibits, as shown in Figure 2, two incompletely resolved peaks at densities of 1.240 and 1.218, respectively. Histone associated with RNA is therefore denser than histone itself. The data of Figure 1 show that the RNA-associated histone bands as two incompletely resolved peaks of different densities. These two components may be quantitatively separated from one another. Nucleohistone is dispersed in 0.4 *M* $NaClO_4$ and pelleted for 16 hr at 40 krpm (Spinco no. 40 head). The pellet,

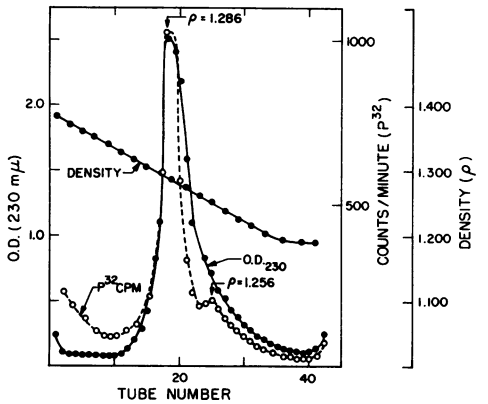


FIG. 1.—CsCl density gradient centrifugation of P^{32}-labeled pea bud nucleohistone. The nucleohistone was dissolved in 2.09 *M* CsCl, 0.01 *M* tris pH 8.0, and centrifuged at 39 krpm to equilibrium. The DNA of the nucleohistone is pelleted at the bottom and is not recovered. Histone and associated RNA bands with the peak densities indicated.

dialyzed, dissolved, and banded in 2.09 *M* CsCl, yields the result shown in Figure 3, namely, it contains only that portion of histone and RNA which bands at a density of 1.286. The supernatant, dialyzed, dissolved, and banded in 2.09 *M* CsCl, contains the material which bands at a density of 1.256, identical to the density at the second peak of Figure 1. It appears then that there are two classes of RNA-histone complex and these can be separated by treatment of nucleohistone with 0.4 *M* $NaClO_4$.[7] Both contain RNA, but in different proportions, as is shown in Table 1. The RNA-histone complex which is not extracted from nucleohistone by 0.4 *M* $NaClO_4$ possesses a mass ratio of RNA to histone of 0.1, that which is extracted of approximately 0.5. The buoyant densities found for the two classes, 1.286 and 1.256, are those predicted on the basis of their RNA contents and the densities of RNA (1.900) and RNA-free histone (mean density = 1.229, Fig. 2), respectively.[8] It is clear, therefore, that histone which has been dissociated from nucleohistone by 2.09 *M* CsCl contains RNA. In addition, all of the histone bands at the densities characteristic of histone-RNA and none at the densities characteristic of RNA-free histone. It is also clear therefore that all of the histone of native nucleohistone is involved in RNA-histone complex formation.

It may be stated parenthetically that the two histone-RNA complexes of different densities (Fig. 1) described above differ in their histone composition. For the study of this matter the histones of the extract and pellet fractions from 0.4 *M* $NaClO_4$-treated nucleohistone were prepared by alcohol precipitation from 0.2 *N* H_2SO_4 and analyzed by disk electrophoresis and by chromatography on the Amberlite IRC-50 analytical column of Fambrough.[9] Both methods show, as demonstrated for one of them in Figure 4, that 0.4 *M* $NaClO_4$ extracts from nucleohistone

TABLE 1

RNA AND HISTONE COMPOSITIONS OF PEA BUD NUCLEOHISTONE AND HISTONE-RNA BANDS*

Component	Mass Ratios RNA/DNA	Mass Ratios RNA/histone
Pea bud nucleohistone†	0.115	0.086
Bands of histone-RNA in 2.09 *M* CsCl:		
From nucleohistone	0.109‡	0.083§
From 0.4 *M* $NaClO_4$ extracted nucleohistone	0.106‡	0.101§
From 0.4 *M* $NaClO_4$ extract of nucleohistone	—	0.056§

* Materials derived from nucleohistone by banding in 2.09 *M* CsCl.
† The mass ratio of histone/DNA is approximately 1.33 in pea bud nucleohistone. Approximately 25% of the total histone is removed by extraction with 0.4 *M* $NaClO_4$.
‡ Ratio of RNA in band to DNA contained in sample from which band was derived by centrifugation.
§ Ratio of RNA/histone in band.

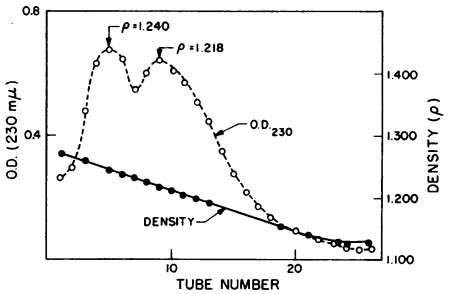


FIG. 2.—CsCl density gradient centrifugation of acid-extracted pea bud histone. The native histone was dissolved in 0.2 N H_2SO_4, precipitated with 2 vol of EtOH at −20°C, redissolved, reprecipitated, and the final precipitate lyophilized. The histone mixture was then made up in 1.60 M CsCl, 0.01 M tris pH 8.0, and centrifuged at 39 krpm to equilibrium.

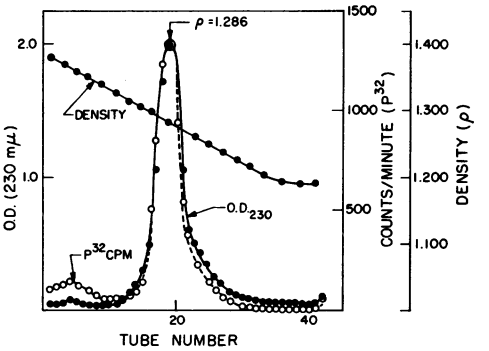


FIG. 3.—CsCl density gradient centrifugation of nucleohistone previously pelleted from 0.4 M $NaClO_4$. The pellet was redissolved in 2.09 M CsCl, 0.01 M tris pH 8.0, and centrifuged at 39 krpm to equilibrium.

nearly all of histone I together with some histone II (nomenclature of Murray[10]) and that the pellet from such extraction contains histones of classes II, III, and IV, but is essentially free of histone I.

We now consider the properties of the histone-associated RNA. P^{32}-labeled histone-RNA, separated from DNA by banding in 2.09 M CsCl, was subjected to hydrolysis in 0.1 N NaOH and the histone precipitated with 1.65 M TCA. The TCA was extracted from the supernatant with ether and the resultant mixture of nucleotides analyzed on a Dowex 1 × 8 column according to Cohn and Volkin[11] as shown in Figure 5. It may be noted first that the histone-associated RNA contains a high proportion of a nucleotide which elutes with the solvent front in 0.01 N

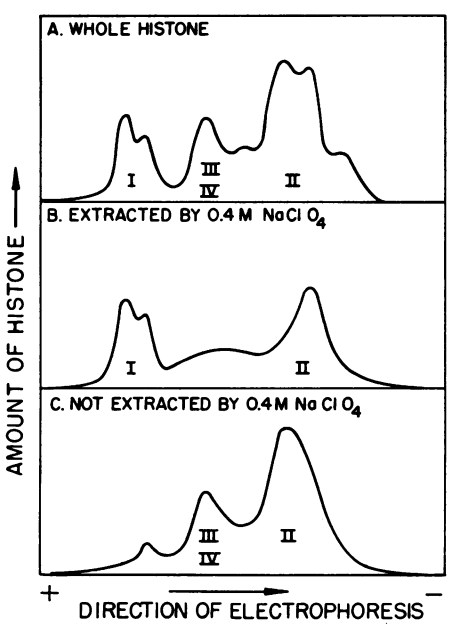


FIG. 4.—Profiles of acid-extracted histones of pea bud nucleohistones after electrophoresis on polyacrylamide gel. (*A*) All histones of pea bud nucleohistone. (*B*) Histones extracted from pea bud nucleohistone by 0.4 M $NaClO_4$. (*C*) Histones which remain attached to DNA during extraction of nucleohistone with 0.4 M $NaClO_4$. The numerals indicate to which class of histone each peak belongs (nomenclature of Murray[10]). Such identification was established by electrophoresis of individual histone fractions previously separated on the Amberlite IRC-50 column and subjected to amino acid analyses.[9]

formic acid-0.05 *M* ammonium formate, that is, ahead (Fig. 5, tubes 28–30) of 2′, 3′-uridylic acid. This nucleotide has been identified as 5,6-dihydrouridylic acid on the basis of spectral properties[12] (OD_{230}/OD_{260} = > 10 in neutral solution), positive ureido group test (yellow color with paradimethylaminobenzaldehyde, in ethanolic HCl, carried out according to Fink *et al.*[13]), and chromatographic similarity to alkali- and 1.65 *M* TCA-treated, authentic dihydrouridylic acid[14] in two solvent systems.[15]

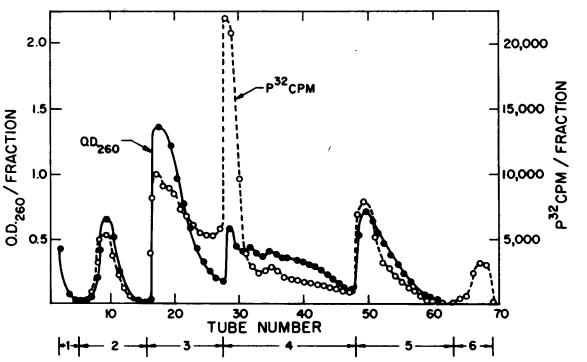


FIG. 5.—Separation on Dowex 1 × 8 of the products of alkaline digestion of histone-bound RNA. The nucleoside-nucleotide mixture prepared as described in the text was eluted successively with 3 × 10 ml of 0.005 *N* formic acid for elution of nucleosides (region *1*); 12 × 5 ml of 0.025 *N* formic acid for elution of cytidylic acids (region *2*); 12 × 5 ml of 0.2 *N* formic acid for elution of adenylic acids (region *3*); 20 × 5 ml of 0.01 *N* formic acid, 0.05 *N* NH_4COOH for elution of uridylic acids (region *4*); 17 × 5 ml of 0.1 *N* formic acid, 0.2 *N* NH_4COOH for elution of guanylic acids (region *5*), and then with successive portions of 0.1, 0.2, 0.45, 0.55, 0.75, and 2.5 *M* NH_4COOH for elution of nucleotide diphosphates (region *6*). The analysis illustrated above is carrier-free, but all steps were first calibrated with authentic nucleotide mono- and diphosphates as well as with authentic dihydrouridylic acid. Dihydrouridylic acid as determined by the color reaction of Fink[13] is contained in the last tube of region *3* and the first 2 tubes of region *4*. Separate experiments with P^{32}-orthophosphate have shown that less than 1.5% of any P^{32}-orthophosphate, if present, would elute in these 3 tubes.

The data for nucleotide composition derived from a typical analysis (that of Fig. 5) of histone-associated RNA is given in Table 2. Histone-associated RNA is characteristically rich in dihydrouridylic acid and in adenylic acid and poor in guanylic and cytidylic acids, in this respect different from transfer RNA and

TABLE 2

NUCLEOTIDE COMPOSITION OF THE HISTONE-ASSOCIATED RNA*

Component	P^{32}, cpm	Mole %
Cytidylic acid	24,440	10.4
Adenylic acid	72,920	31.6
Dihydrouridylic acid	63,100	27.5
Uridylic acid	34,940	15.2
Guanylic acid	35,160	15.3
Total nucleoside diphosphates	11,900	

Ratio of total nucleoside monophosphates to total nucleoside diphosphates: 230,560 ÷ 11,900/2 = 38.

* Removed from nucleohistone by banding in 2.09 *M* CsCl. Experiment of Fig. 5.

ribosomal RNA from the same pea bud material whose compositions are summarized in Table 3. From the ratio of terminal nucleotide diphosphates to total nucleotide monophosphates, the average chain length can be estimated as 38 (Table 2). The same value is yielded by the ratio of nucleoside to total nucleotide mono-

TABLE 3

COMPARISON OF THE NUCLEOTIDE COMPOSITIONS OF VARIED SPECIES OF PEA BUD RNA

Species of RNA	Mole % C	A	U	DiHU	U + DiHU	G
RNA of whole nucleohistone*	14.0	30.6	—	—	38.6	16.7
RNA assoc. with histone* recovered by 2.09 *M* CsCl banding	10.4	31.6	15.2	27.5	42.7	15.3
Transfer RNA*, †	18.5	23.5	—	—	30.6	27.4
Ribosomal RNA‡	22.3	24.3	22.0	—	—	31.4

* The authentic 2′,3′-nucleotide monophosphates, AMP, CMP, UMP, DiHUMP, and GMP, were used as carriers for the analysis of the P^{32}-labeled ribonucleotide mixtures of the several species of RNA.
† Transfer RNA separated from whole phenol-extracted pea bud cytoplasmic RNA by sucrose density gradient centrifugation.
‡ Data of Wallace and Ts'o.[16]

phosphate in the hydrolysate. The data of Table 3 include the base composition of the RNA of whole nucleohistone as well as of the histone-associated RNA as removed by CsCl banding. The two are evidently quite similar. This is to be expected, since CsCl banding removes essentially all RNA as well as all histone from nucleohistone.

The bonds by which histone is bound to its associated RNA are not ionic ones, since the two are not dissociated by concentrations of CsCl as high as 4 *M*. They are, however, cleaved by acid, as is shown in Table 4. For this experiment, P^{32}-

TABLE 4

CLEAVAGE OF RNA FROM HISTONE BY ACID*

Acid treatment	RNA as P^{32} Cpm in: Pellet	Supernatant	% of Total in pellet
1.65 *M* TCA†	1680	68	97
0.2 *N* H_2SO_4	515	275	65

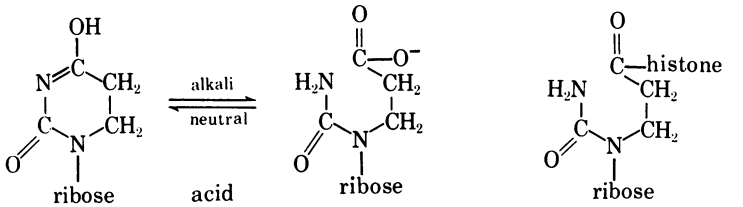
* P^{32}-labeled histone-RNA, prepared by banding in 2.09 *M* CsCl as in Fig. 1 was treated with acid at 0°C. The acid-treated material was rebanded in 1.85 *M* CsCl containing 0.2 *N* H_2SO_4. The distribution of the P^{32}-labeled RNA was determined after attainment of equilibrium.
† After treatment the TCA was removed with ether and the sample made up to 1.85 *M* CsCl in 0.2 *N* H_2SO_4.

labeled histone-RNA isolated by banding in 2.09 *M* CsCl was treated with 1.65 *M* TCA at 0°C for 48 hr. The TCA was then removed by ether extraction and the material rebanded in 1.85 *M* CsCl, pH 1. The data of Table 4 show that the RNA now pellets essentially completely while the histone remains in the supernatant. Treatment with 0.2 *N* H_2SO_4 for 48 hr cleaves 65 per cent or more of the RNA from the histone as judged by pelletability of the RNA in 1.85 *M* CsCl, pH 1.

Discussion.—Histone molecules as prepared by the usual extraction with acid possess molecular weights of the order of 10,000 (histone I) to 20,000 (histone II).[10] The histone-RNA complex as isolated by CsCl banding contains, by mass, 92 per cent histone and 8 per cent RNA. On this basis there are available approximately 3–6 nucleotides per histone molecule. It has been shown above, however, that the RNA molecules associated with histone are approximately 10 times this large. We find no native histone which bands in CsCl at the density characteristic of RNA-free histone. It follows, therefore, that in the native state several histone molecules must be associated into a larger unit with which an RNA molecule is in turn associated. This conclusion follows also from comparison of the sharp CsCl gradient banding profiles of histone-RNA (Figs. 1 and 3) with the diffuse profiles of acid-extracted, RNA-free histone (Fig. 2) studied under similar conditions of histone concentration. We conclude that the histones studied by classical histone chemis-

try are subunits formed by dissociation of larger structures characteristic of native nucleohistone.

With regard to the union between histone and RNA we know only that it is cleaved by strong acid. It is possible that the dihydrouridylic component of the RNA may play a role in the bond to histone. The dihydrouracil ring which is closed at neutral pH possesses the property of opening in base to form ureidoproprionic acid[12] which is the form which gives the yellow color reaction with p-



Dihydrouridine ring form | Dihydrouridine open form | Possible histone derivative of dihydrouridine

dimethylaminobenzaldehyde. The histone-RNA complex gives the yellow color reaction typical of the ureido group of ureidoproprionic acid without alkaline treatment. This indicates that the dihydrouracil of the histone-RNA complex is held in the open form. Binding to histone may be responsible for preventing closure of the dihydrouracil ring. It is of interest that transfer RNA which must also interact with protein also contains dihydrouridylic acid.[17]

Finally, we consider the nature of the association of histone-RNA with DNA in native nucleohistone. Although we know little about this matter, we have shown[18] that the RNA of the native nucleohistone is bound in the complex in such a manner as to be totally resistant to attack by RNase and that it is converted to a form susceptible to such attack either by heating the complex (at low ionic strength) to 60°C, a temperature below that at which any melting of DNA takes place, or alternatively, by treatment of the nucleohistone with DNase.

Summary.—Histones, as they occur in the native nucleohistone of pea bud chromatin, are bound to RNA. This RNA, of molecules approximately 40 nucleotides in length, constitutes a distinct class of RNA distinguished by its high content of dihydrouridylic acid. The histone molecules of classical histone chemistry appear to be subunits of much larger structures present in native nucleohistone. Each such large structure, made of several subunits, bears an RNA molecule of the class which we have here described.

We gratefully acknowledge the counsel and support of our colleagues Professors Norman Davidson and Jerome Vinograd, Drs. Roger Chalkley and Keiji Marushige, and Baldomero Olivera. We appreciate the counsel of Professor Robert Holley in connection with the identification of dihydrouridylic acid. We acknowledge with pleasure also the skillful assistance of Georgia Lau and Ludia Brown.

* Report of work supported in part by the Herman Frasch Foundation, and by the U.S. Public Health Service, grants GM-5143, GM-3977, and AM-3102.

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[7] The selective extraction of histone from native nucleohistone has been developed by our colleague Heiko Ohlenbusch.

[8] The densities expected for RNA-histone complexes of varied RNA-histone composition were calculated from the relation $\frac{1}{\rho} = \frac{W_1}{\rho_1} + \frac{W_2}{\rho_2}$, where W_1 and W_2 are the fractions of total weight of the sample contributed by each of the two components of densities, ρ_1 and ρ_2, respectively.

[9] We are indebted to our colleague Douglas Fambrough for instruction in the use of his analytical column procedures for histone separation. This method which involves elution of histones from Amberlite IRC-50 with a gradient of guanidinium chloride is a modification of that of Rasmussen, P. S., K. Murray, and J. M. Luck, *Biochemistry*, **1**, 79 (1962).

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[14] Authentic 2′,3′-dihydrouridylic acid was prepared for us by CalBiochem whose assistance we gratefully acknowledge. We have also found that the unknown is after treatment with phosphomonoesterase identical with authentic 5,6-dihydrouridine purchased from the Cyclo Corp., Los Angeles.

[15] The solvent systems used included t-butyl alcohol:methyl ethyl ketone:formic acid:H_2O (40:30:15:15) and n-butyl alcohol:water (85:15).

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THE REPRESSION AND INDUCTION BY THYROXIN OF HEMOGLOBIN SYNTHESIS DURING AMPHIBIAN METAMORPHOSIS

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Communicated by John T. Edsall, July 6, 1965

The tadpole and adult stages of the bullfrog (*Rana catesbeiana*) have different hemoglobins. Differences in oxygen binding between the larval and adult hemoglobins have been shown by McCutcheon[1] and Riggs.[2] Several laboratories have reported different electrophoretic patterns, chromatographic elution profiles, or sedimentation constants for hemoglobin from the tadpole and frog[3–8] and a change toward the adult pattern during metamorphosis.[4, 6, 7, 9] Baglioni and Sparks[6] and Sakai *et al.*[10] found differences in the primary structure of hemoglobins obtained from pre- and postmetamorphic animals. Although the hemoglobins have been shown to consist of subunits,[6] the number of different polypeptide chains has not been established. No studies, to our knowledge, have been reported on protein biosynthesis in tadpole or frog red blood cells.

The experiments to be reported here indicate that hemoglobin can be synthesized *in vitro* by circulating red blood cells of normal tadpoles. Soon after the administration of thyroxin, hemoglobin synthesis markedly declines. Following this, an