[9] Dubnau, D., and J. Marmur, unpublished observations (1964).
[10] Cox, E. C., J. R. White, and J. G. Flaks, these PROCEEDINGS, 51, 703 (1964).
[11] Davies, J. E., these PROCEEDINGS, 51, 659 (1964).
[12] Schildkraut, C. L., K. L. Wierzchowski, J. Marmur, D. M. Green, and P. Doty, *Virology*, 18, 43 (1962).
[13] Marmur, J., E. Seaman, and J. Levine, *J. Bacteriol.*, 85, 461 (1963).
[14] Catlin, B. W., *J. Gen. Microbiol.*, 37, 369 (1964).
[15] Pakula, R., *Acta Microbiol. Polon.*, 10, 249 (1961).
[16] Schaeffer, P., thesis, Faculté des Sciences de L'Université de Paris (1961).
[17] Nickel, L., and S. H. Goodgal, *J. Bacteriol.*, 88, 1538 (1964).
[18] Ravin, A., and J. D. H. De Sa, *J. Bacteriol.*, 87, 86 (1964).
[19] Brown, G. L., *Progr. Nucleic Acid Res.*, 2, 259 (1963).
[20] Hecht, L. I., M. L. Stephenson, and P. C. Zamecnik, *Biochim. Biophys. Acta*, 29, 460 (1958).
[21] Singer, M. F., and G. L. Cantoni, *Biochim. Biophys. Acta*, 39, 182 (1960).
[22] Crick, F. H. C., *Progr. Nucleic Acid Res.*, 1, 163 (1963).
[23] Zamir, A., R. W. Holley, and M. Marquisse, *J. Biol. Chem.*, 240, 1267 (1965).
[24] Peterman, M. L., *The Physical and Chemical Properties of Ribosomes* (New York: Elsevier, 1964).
[25] Schaeffer, P., *Symp. Soc. Exptl. Biol.*, 12, 60 (1958).
[26] Arber, W., and D. Dussoix, *J. Mol. Biol.*, 5, 18 (1962).
[27] Arber, W., and M. L. Morse, *Genetics*, 51, 137 (1965).
[28] Takahashi, I., *J. Bacteriol.*, 89, 1065 (1965).
[29] Dubnau, D., I. Smith, and J. Marmur, these PROCEEDINGS, in press.
[30] Yoshikawa, H., and N. Sueoka, these PROCEEDINGS, 49, 559 (1963); personal communication.
[31] Taubman, S. B., A. G. So, F. E. Young, E. W. Davie, and J. W. Corcoran, *Antimicrobial Agents and Chemotherapy* (American Society for Microbiology, 1963), p. 395.
[32] Taubman, S. B., F. E. Young, and J. W. Corcoran, these PROCEEDINGS, 50, 955 (1963).
[33] Wolfe, A. D., and F. E. Hahn, *Biochim. Biophys. Acta*, 95, 146 (1965).
[34] Roberts, R. B., *J. Theoret. Biol.*, 8, 49 (1965).
[35] Nakada, D., *J. Mol. Biol.*, in press.

---

*TURNOVER OF TEMPLATE-BOUND HISTONE**

BY G. ROGER CHALKLEY AND H. RAINER MAURER

DIVISION OF BIOLOGY, CALIFORNIA INSTITUTE OF TECHNOLOGY

Communicated by James Bonner, June 14, 1965

The histone with which DNA is complexed in the genetic material of higher organisms has as one of its roles the regulation of DNA-dependent RNA synthesis.[1-3] DNA fully complexed with an appropriate histone is not transcribed by RNA polymerase. For the deeper understanding of this regulation it is necessary to know whether it is reversible; whether in life a histone molecule once complexed to the DNA template can dissociate again. The present paper is addressed to this point. The experimental strategy consists in determining whether histone molecules of the chromosomal nucleohistone complex are replaced by freshly synthesized ones in cells in which there is no replication of DNA and hence no requirement for net *de novo* synthesis of histone. It will be shown that histones may be divided into two classes, those which are replaced (turnover) in cells in which DNA is not replicating, and those of the alternative class which do not turn over at all. The latter are synthesized only during, and in fact are dependent upon, DNA replication.

A major obstacle to earlier studies of this kind has been the difficulty of clear-cut histone fractionation.[4] The development by Rasmussen, Murray, and Luck[5] of a cation-exchange chromatographic method has made reproducible histone fractionation possible. We have applied this method to the study of the genetic material of calf endometrium and other nonreplicating cells, as well as to that of cells multiplying exponentially in tissue culture.

Materials and Methods.—Preparation of calf endometrium: Calf endometrium was prepared by cutting and scraping off the inner lining of the opened uterine horns of freshly killed 15–20-week-old calves. Uteri from 70 calves yielded approximately 84 gm endometrial tissue. The size of the slices and particles was 0.5–2 mm.

Incubation of calf endometrium: Incubations were carried out for 6 hr at 38°C under a stream of 95% CO_2-5% O_2 with shaking (90 cycles/min), using the following incubation medium: 75 mM NaCl, 5 mM KCl, 10 mM Na_2HPO_4, 6 mM KH_2PO_4, 25 mM glucose, 55 mM sucrose, 1.5 mM $CaCl_2 \cdot 2H_2O$, 3 mM $MgCl_2 \cdot 6H_2O$, pH 6.8. In each experiment, 10 gm of fresh tissue were incubated with 35 ml of medium containing 50 μc C^{14}-algal hydrolysate (uniformly labeled, 125 μc/mg). During the incubation, the pH of the suspension was continuously adjusted to 7.1–7.5 by the dropwise addition of 1 *N* NaOH (phenol red as indicator).

Isolation of endometrial nuclei and nucleohistone: All procedures were performed at approximately 2°C. Following incubation, the tissues were washed 3 × with 0.14 *M* NaCl and homogenized in a medium consisting of 0.25 *M* sucrose, 3 mM $CaCl_2 \cdot 2H_2O$, and 5 mM Tris, pH 7.35 (Waring Blendor). The homogenates were filtered twice through 3 layers of cheesecloth and once through 2 layers of Miracloth, and centrifuged at 1200 × *g* for 20 min. The pellets, washed twice with homogenization medium, consisted of 90–94% pure nuclei as determined by phase-contrast microscopy and staining procedures. The pellets were resuspended (Potter Teflon homogenizer) in 0.15 *N* H_2SO_4; the resulting suspension was stirred for 30 min and centrifuged at 30,000 × *g* for 30 min. Five volumes of cold ethanol were added to the clear supernatant, and the mixture was stored at −20°C for at least 24 hr to complete precipitation of histones. The histones were then separated by centrifugation (1800 × *g* for 20 min), washed twice with cold ethanol and ether, and dried under reduced pressure in a desiccator for 2 hr.

Incubation of endometrial nuclei: The preparation of the nuclei intended for the nuclei incubation experiments followed essentially the same procedure. Fifty gm of fresh tissue yielded about 4.5 gm nuclei (wet weight). In each experiment, 2.7 ml nuclear suspension (*ca.* 2 × 10^9 nuclei/ml) were added to 25 ml incubation medium containing 50 μc C^{14}-algal hydrolysate. No clumping of the nuclei was visible after 1 hr incubation (38°C, CO_2–O_2 stream). Phase-contrast microscopy revealed the formation of some clusters of nuclei, but no lysis or morphological deformations were observed.

Preparation of endometrial chromatin: For the preparation of the chromatin the nuclei were freed of excess label by exhaustively washing with homogenization medium. They were then washed once with 75 mM NaCl, 24 mM Na_2EDTA, pH 8, and lysed in 10 mM Tris, pH 8. After 2 hr of stirring, lysis was completed by additional homogenization. The resulting crude chromatin suspension was layered on 1.7 *M* sucrose and centrifuged in the SW25 swinging bucket Spinco rotor at 22,000 rpm for 2 hr. The gelatinous, faintly turbid pellet was dialyzed overnight against 50 mM Tris pH 8, and centrifuged at 105,000 × *g* for 6 hr. The packed chromatin pellet was vigorously homogenized in 0.15 *N* H_2SO_4, until a fine milky suspension was achieved, and centrifuged at 30,000 × *g* for 20 min. From the resulting supernatant the histones were isolated as above.

Preparation of labeled rat liver histones: Two Sprague-Dawley rats, 180–200 gm, were injected intraperitoneally, each with 50 μc L-C^{14}-leucine (200 μc/μmole). After 18 hr the rats were anesthetized and the livers perfused with cold 0.9% saline and excised. Histones were isolated from the liver chromatin as described for the endometrium tissue.

Isolation of labeled pea cotyledon histones: Individual pea cotyledons (300), from which the growing embryonic axes had been removed with a razor blade, were obtained from pea seeds (var. *Alaska*), previously soaked in water for 24 hr at 25°C. These were incubated for 6 hr in Petri dishes, each containing 15 ml of a medium consisting of 0.1 μc/ml of L-C^{14}-leucine, 16 μg/ml penicillin, and 0.01 *M* Tris, pH 7.2. After the incubation, the cotyledons were washed with 0.01 *M* Tris pH 7.2.

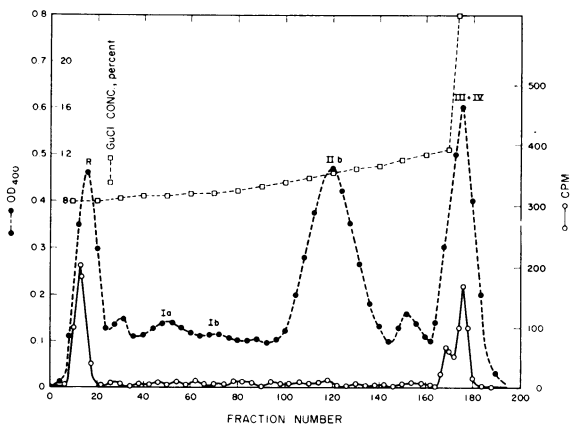

FIG. 1.—Chromatographic fractionation of calf endometrium histones isolated from chromatin of incubated nuclei, on Amberlite IRC-50 (column: 60 × 0.5-cm diameter) with a concentration gradient of guanidinium chloride (GuCl); 0.3-ml fractions were collected. Protein concentration of the fractions was determined by optical density at 400 mμ of the turbid solutions resulting when 0.2 ml of the effluent sample were mixed with 1.1 *M* trichloroacetic acid in a total volume of 1.2 ml. For radioactivity determinations, 0.3-ml fractions were treated with 15 ml of liquid scintillation mixture.

Untreated cotyledons (200 gm) were then added as carrier, and chromatin isolated following the methods of Huang and Bonner,[1] except that the concentration of Tris buffer was maintained at 0.01 *M* throughout. Histones were obtained from the purified chromatin as described above.

Preparation of labeled tobacco histones: A tobacco (*Nicotiana tabacum* var. *Xanthi*) pith-cell line, which grows exponentially in a chemically defined liquid medium was used.[6] The cells were used on the fifth day after subculture into fresh medium and at a concentration of 4–6 gm/500 ml. Usually, 5 liters of medium in 10 flasks were used for each experiment. Treatment with 10^{-6} *M* 5-fluorodeoxyuridine (5-FDU) followed the procedure of Flamm and Birnstiel.[7] This concentration of 5-FDU essentially stopped DNA synthesis within 2 hr. Sterile L-C^{14}-leucine (5 μc) was added for each 500 ml of medium. Incubation was normally for 3 hr, with the exception that the replicating cells (5-FDU absent) were incubated for 12 hr.

The cells (40–60 gm) were collected by filtration through Miracloth and washed with cold culture medium. Chromatin was isolated as described for pea cotyledons, but with two differences: (1) the initial grinding procedure was more vigorous and prolonged to disrupt the tobacco cells, and (2) cysteine (1 mM) was added to prevent extensive discoloration.

Chromatography: Cation-exchange column chromatography was performed according to the methods of Rasmussen *et al.*[4] using Amberlite IRC-50 (column size 0.6 ID × 60 cm) with a gradient of guanidinium chloride (GuCl). Two to six mg of material were ordinarily applied to the column. This column permits the resolution of acid-soluble nucleoproteins into 6 major fractions; the material carried along with the solvent front is known as the run-off peak, the other fractions in order of appearance in the column (see Fig. 1) are known as Ia, Ib, IIb, III, and IV, respectively. The molar ratios lysine:arginine for these histone fractions are: Ia, 8.4; Ib, 10.0; IIb, 1.7; III and IV, both 0.7.

Radioactivity measurements: For liquid scintillation counting, the following mixture was used: PPO, 4 gm; POPOP, 50 mg; naphthalene, 120 gm; Cabosil, 20 gm; and dioxane to 1 liter. Counting efficiency was 54%. No quenching effect was observed as the GuCl gradient rose from 8% to 40%.

Disk electrophoresis: The disk electrophoresis techniques employed were essentially those of Reisfeld *et al.*[8] (15% polyacrylamide gel, pH 4.3). Instead of using sample and spacer gels, the protein was applied directly upon the running gel in 1 *M* sucrose solution, containing the electrophoresis buffer.[9] Pyronine B (1 μl of a 0.1% aqueous solution) was used as a buffer front dye marker.

Experimental Results.—Endometrium experiments: That the cells of the endo-

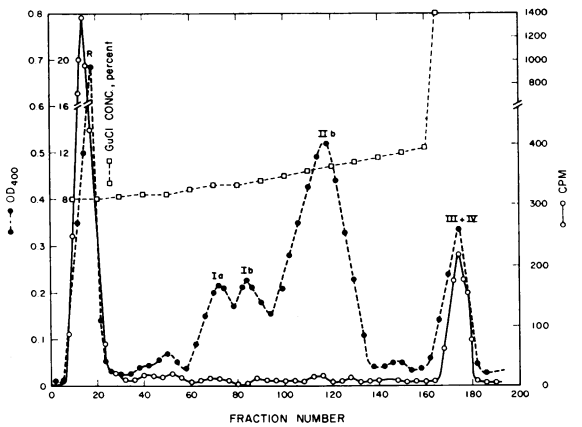

FIG. 2.—Chromatographic fractionation of calf endometrium histones isolated from nuclei of incubated tissue slices. Analytical details as for Fig. 1.

metria of immature calves are not engaged in DNA replication is shown by the following facts: (1) Endometrial cells cultured in the presence of H^3-thymidine incorporate a negligibly small amount of the material into DNA. The low level incorporation (60 cpm/mg) found is similar to that found in other nonreplicating tissues under similar conditions.[10] (2) Endometrial cells from immature calves cultured in Eagle's medium exhibit essentially no mitoses when examined by time-lapse microphotography[11] over a period of 44 hr.

The data of Figure 1 concern the histones from the isolated chromatin of endometrial nuclei which had been previously incubated with C^{14}-labeled amino acid as described under *Materials and Methods*. It is apparent that the histones of such chromatin include, as do the histones of other chromatins, the runoff peak material, histones Ia and Ib, histone IIb, and histones III and IV, which are unresolved. It is also clear that only two groups of these template-bound histones become labeled under the conditions of the experiment, namely, the runoff peak (fractions 10-20)

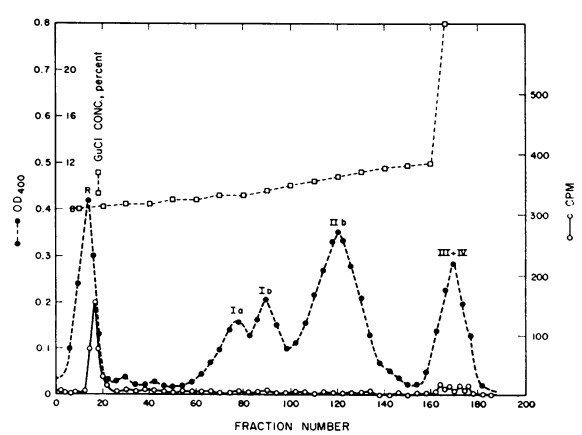

FIG. 3.—Chromatographic fractionation of calf endometrium histones isolated from nuclei of tissue slices incubated in the presence of puromycin (120 μg/ml). Analytical details as for Fig. 1.

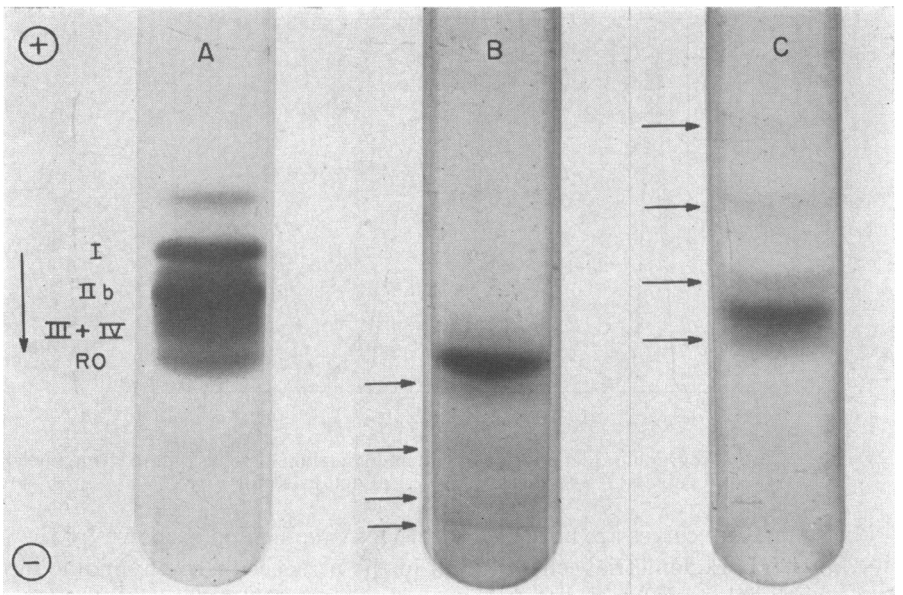

FIG. 4.—Disk electrophoresis patterns of calf endometrium histones isolated from nuclei of incubated tissue slices. (*A*) Total histone extract; (*B*) runoff peak; (*C*) III and IV jointly. (*B*) and (*C*) were overexposed to show weaker bands (arrowed). Thirty-five μg applied to the polyacrylamide gels. Electrophoresis at 3 mA/tube (0.5 cm ID) for 200 min. Amidoschwarz staining.

and the peak which contains the arginine-rich histones III and IV (fractions 165–185). Similar elution profiles are obtained for the histones extracted directly from whole endometrial nuclei, as is shown in Figure 2. These nuclei obtained from cultured endometrial cells show labeling only in the runoff peak and in histones III and IV, just as in the case of the chromosomally bound histones. That protein synthesis is involved in the labeling of these components is shown by the fact that such labeling is greatly reduced in the presence of puromycin (Fig. 3).

The chromatographic technique employed does not resolve and separate all

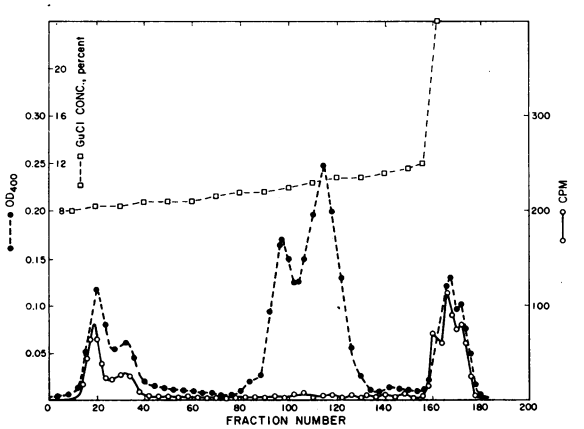

FIG. 5.—Chromatographic fractionation of histones isolated from rat liver chromatin. Analytical details as for Fig. 1.

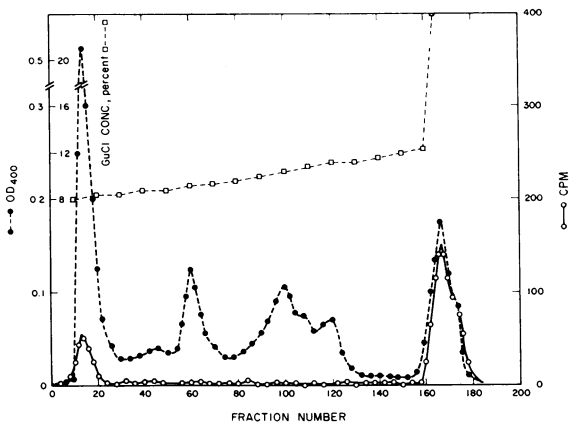

FIG. 6.—Chromatographic fractionation of histones isolated from pea cotyledon chromatin. Analytical details as for Fig. 1.

histone components which are present in either the chromosomal or nuclear extracts. The data of Figure 1 indicate that the radioactivity measurements have a higher resolving power than does the optical method, since histones III and IV are separated by the former and not by the latter. Moreover, the heterogeneity of both labeled fractions was confirmed by disk electrophoresis on polyacrylamide gels (Fig. 4). The runoff peak is split by this method into one major and four minor, faster moving, materials. Histones III and IV yield, on disk electrophoresis, one major band preceded by one minor and followed by three minor bands.

Rat liver, pea cotyledon, and tobacco cell experiments: The differential histone-labeling pattern shown above for endometrial cells is not restricted to endometrial tissue not to our semi-*in vitro* incubation conditions. Rat liver histones from rats previously injected with C^{14}-amino acids (Fig. 5), histones from incubated pea cotyledons (Fig. 6), as well as histones from the chromatin of cultured tobacco cells, whose DNA synthesis had been inhibited by 5-fluorodeoxyuridine (Fig. 7), all exhibit similar labeling patterns in which only the runoff peak and histones III and IV become labeled.

Tobacco cells dividing exponentially in liquid medium provide a pattern of histone labeling which is interestingly different from that found for the cells and tissues described above, in which DNA synthesis is minimal or nonexistent. In such exponentially dividing cell cultures, all histone fractions become labeled (Fig. 8). That the synthesis of histones I and II is directly dependent upon DNA replication is indicated by the fact that the two tobacco experiments differed only in the presence or absence of 5-fluorodeoxyuridine.

Discussion.—Previous studies of histone synthesis in the absence of DNA replication have demonstrated a continuing synthesis of acid-soluble nuclear proteins.[7, 12, 13] Fractionation of such protein has been reported by Busch,[14] who found that arginine-rich histones are the more rapidly synthesized, although Busch noted some incorporation into lysine-rich histones. The present findings are therefore a refinement of Busch's observations and show in addition that histone actually associated with DNA undergoes such turnover. Incorporation into histones of the groups III and IV amounted to a specific activity under the conditions used of 1500–

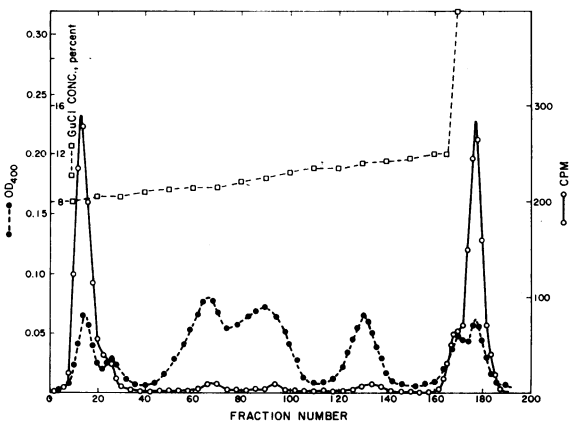

FIG. 7.—Chromatographic fractionation of histones isolated from tobacco chromatin of cells incubated in the presence of 5-fluorodeoxyuridine (10^{-6} *M*). Analytical details as for Fig. 1.

2000 cpm/mg of protein in separate experiments. This is of the same order of magnitude as the incorporation into total cytoplasmic protein (2500 cpm/mg). Thus, histones of groups III and IV turn over at a rate approximating that of whole cytoplasmic protein. That the incorporation represents *de novo* synthesis of histone was clearly demonstrated using puromycin.

Incorporation into the runoff peak is also substantially equal to incorporation into histones III and IV and into whole cytoplasmic protein. The nature of the proteins of the runoff peak is ambiguous. It is possible that a portion can be ascribed to acid-soluble protein components of nuclear ribosomes. We are able to say with certainty that acid-soluble protein from cytoplasmic ribosomes is found solely in the runoff peak. On the other hand, polyacrylamide gel electrophoresis shows that a further protein, different from acid-soluble cytoplasmic ribosomal protein, may be a major contributor to this material.

That turnover of DNA-bound histones is confined to the runoff peak and to his-

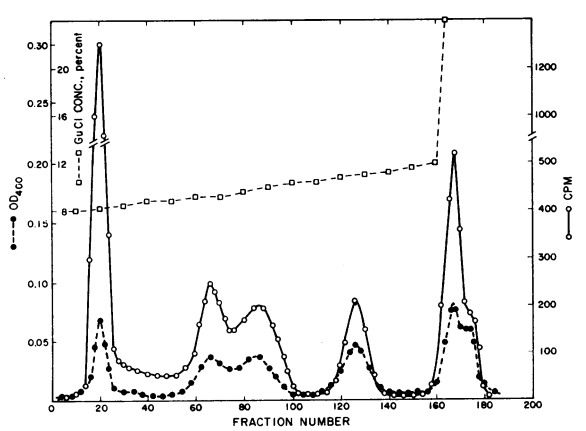

FIG. 8.—Chromatographic fractionation of histones isolated from tobacco cell chromatin of incubated cells. Analytical details as for Fig. 1.

tone fractions III and IV in cells in which DNA is not undergoing replication, appears to be a general characteristic of higher organisms, plants, and animals.

It further appears that the inhibition of DNA synthesis in cells in which such synthesis is occurring promptly stops the formation of histones of groups I and II. It is these histones which have been shown to be the most effective in inhibition of DNA-dependent RNA synthesis in reconstituted nucleohistones.[15] The tentative conclusion may be drawn that any portion of the genome which is associated with histones I and II is not available for transcription without replication of DNA.

Summary.—The use of an ion-exchange chromatographic technique for the fractionation of histones has shown that only two classes of histones turn over in cells in which DNA is not undergoing replication. These are histones of groups III and IV, and, in addition, the proteins of the so-called runoff peak which precedes the elution of the histones proper. The lysine-rich histones I and II on the contrary are only formed in cells in which DNA is undergoing replication.

The authors wish to acknowledge the advice of D. M. Fambrough on the use of the ion-exchange chromatography on an analytical scale. They are indebted to Dr. R. C. Huang and Professor J. Bonner for continuing counsel and support.

* Report of work supported by the U.S. Public Health Service, grants RG-03977 and A-3102-07; and by the Herman Frasch Foundation.

[1] Huang, R. C. C., and J. Bonner, these PROCEEDINGS, **48**, 1216 (1962).
[2] Allfrey, V. G., V. C. Littau, and A. E. Mirsky, these PROCEEDINGS, **49**, 414 (1963).
[3] Barr, G. C., and J. A. V. Butler, *Nature*, **199**, 1170 (1963).
[4] Busch, H., *Histones and Other Nuclear Proteins* (New York: Academic Press, 1965), p. 69.
[5] Rasmussen, P. S., K. Murray, and J. M. Luck, *Biochemistry*, **1**, 79 (1962).
[6] Filner, P., in press.
[7] Flamm, W. G., and M. L. Birnstiel, *Exptl. Cell Res.*, **33**, 616 (1964).
[8] Reisfeld, R. A., V. J. Lewis, and D. E. Williams, *Nature*, **195**, 281 (1962).
[9] McAllister, H. C., Jr., Y. C. Wan, and J. L. Irvin, *Anal. Biochem.*, **5**, 321 (1963).
[10] Bucher, N. L. R., *Intern. Rev. Cytol.*, **15**, 245 (1963).
[11] Maurer, H. R., and C. W. Raiborn, unpublished.
[12] Ontko, J. A., and W. R. Moorehead, *Biochim. Biophys. Acta*, **91**, 658 (1964).
[13] Starbuck, W. C., and H. Busch, *Cancer Res.*, **20**, 891 (1960).
[14] Davis, J. R., and H. Busch, *Cancer Res.*, **19**, 1157 (1959).
[15] Huang, R. C., J. Bonner, and K. Murray, *J. Mol. Biol.*, **8**, 54 (1964).

---

*EVIDENCE FOR VIRUS-SPECIFIC NONCAPSID PROTEINS IN POLIOVIRUS-INFECTED HELA CELLS**

BY DONALD F. SUMMERS,† JACOB V. MAIZEL, JR.,‡ AND JAMES E. DARNELL, JR.

DEPARTMENTS OF BIOCHEMISTRY AND CELL BIOLOGY,
ALBERT EINSTEIN COLLEGE OF MEDICINE, NEW YORK

Communicated by Harry Eagle, June 21, 1965

It is known that poliovirus RNA functions as messenger RNA in virus-specific cytoplasmic polyribosomes.[1, 2] If the entire viral RNA molecule specifies information for protein synthesis and the coding ratio is 3 nucleotides per amino acid,[3] the 6000 nucleotides of polio RNA[4] would encode peptide chains containing 2000