470.
Gattner, H., & Fahr, E. (1963) *Justus Liebigs Ann. Chem. 670,* 84.
Grossman, L. (1968) *Photochem. Photobiol. 7,* 727.
Grossman, L., & Rogers, E. (1968) *Biochem. Biophys. Res. Commun. 33,* 975.
Hayatsu, H., Wataya, Y., & Kai, K. (1970) *J. Am. Chem. Soc. 92,* 724.
Hollis, D. P. (1976) in *Photochemistry and Photobiology of Nucleic Acids* (Wang, S. Y., Ed.) Vol. 1, p 466, Academic Press, New York, N.Y.
Howard, B. D., & Tessman, I. (1964) *J. Mol. Biol. 9,* 372.
Johns, H. E., LeBlanc, J. C., & Freeman, K. B. (1965) *J. Mol. Biol. 13,* 849.
Kleber, R., Fahr, E., & Boebinger, E. (1965) *Naturwissenschaften 52,* 513.
Liu, F.-T., & Yang, N. C. (1978) *Biochemistry, 17* (preceding paper in this issue).
Lomart, A. J., & Fresco, J. R. (1972) *J. Mol. Biol. 66,* 49.
Miller, N., & Cerutti, P. (1968) *Proc. Natl. Acad. Sci. U.S.A. 59,* 34.
Moore, A. M. (1958) *Can. J. Chem. 36,* 281.
Moore, A. M., & Thomson, C. H. (1957) *Can. J. Chem. 35,* 163.
Ono, J., Wilson, R. G., & Grossman, L. (1965) *J. Mol. Biol. 11,* 600.
Patrick, M. H., & Rahn, R. O. (1976) in *Photochemistry and Photobiology of Nucleic Acids* (Wang, S. Y., Ed.) Vol. 2, p 35, Academic Press, New York, N.Y.
Remsen, J. F., Miller, N., & Cerutti, P. A. (1970) *Proc. Natl. Acad. Sci. U.S.A. 65,* 460.
Rohrer, D. C., & Sundaralingam, M. (1970) *Acta Crystallogr., Sect. B 26,* 546.
Rouiller, P., Delmau, J., & Nofre, C. (1966a) *Bull. Soc. Chim. Fr.,* 3515.
Rouiller, P., Delmau, J., Duplan, J., & Nofre, C. (1966b) *Tetrahedron Lett.,* 4189.
Shapiro, R., Servis, R. E., & Welcher, M. (1970) *J. Am. Chem. Soc. 92,* 422.
Singer, B., & Fraenkel-Conrat, H. (1970) *Biochemistry 9,* 3694.
Sinsheimer, R. L. (1957) *Radiat. Res. 6,* 121.
Skaric, V., Gaspert, B., Hohnjec, M., & Lecan, G. (1974) *J. Chem. Soc., Perkin Trans. 1,* 267.
Summer, W. A., Jr., Enwall, C., Burr, J. G., & Letsinger, R. L. (1973) *Photochem. Photobiol. 17,* 295.
Vandehoek, J. Y., & Cerutti, P. A. (1973) *Biochem. Biophys. Res. Commun. 52,* 1156.
Wang, S. Y., Apicella, M., & Stone, B. R. (1956) *J. Am. Chem. Soc. 78,* 4180.
Wechter, W. J., & Smith, K. C. (1968) *Biochemistry 7,* 4064.
Wierzchowski, K. L., & Shugar, D. (1961) *Acta Biochim. Polon. 8,* 219.
Yang, N. C., & Liu, F.-T. (1976) *Abstracts,* 4th Annual Meeting of the American Society of Photobiology, p 104,

Effect of Inhibition of DNA Synthesis on Histone Synthesis and Deposition†

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ABSTRACT: We have reinvestigated the degree of coupling between DNA and histone synthesis in mammalian cells. In at least one cell line (HTC cells), the coupling is not nearly as tight as had previously been inferred from experiments with HeLa cells. The site of deposition of such histones which continue to be made in the presence of sufficient hydroxyurea to depress DNA synthesis almost totally has been studied. Deposition seems to be on material which absorbs at 260 nm. This material is not a part of the bulk chromatin and binds histone in a relatively tight manner. The possible role of such a material in histone synthesis and deposition is discussed.

There is a notion that histone synthesis is very tightly coupled to DNA synthesis (Spalding et al., 1966). This idea has been most exhaustively demonstrated in HeLa cells (Robbins and Borun, 1967; Gallwitz and Mueller, 1969). The origin of this coupling appears to lie in the availability of cytoplasmic histone mRNA, since it seems that this RNA is made throughout the cell cycle (Jacobs-Lorena, et al., 1972; Thompson et al., 1976; Stein et al., 1977; Melli et al. 1977) but appears in polyribosomes primarily during the S phase. Furthermore, the addition of inhibitors of DNA synthesis to S-phase cells causes a loss of cytoplasmic histone mRNA even though its nuclear synthesis continues.

However, the tight coupling of DNA and histone synthesis is not always observed. For example, in developing frog oocytes, Adamson and Woodland (1974) have noted a vast excess of histone synthesis which appears to function as a reservoir for histones during the very rapid phase of DNA synthesis following fertilization. Furthermore, a lack of coupling has also been reported in mammalian systems during SV 40 (Kay and Singer, 1977) and HTC replication (Balhorn et al. 1973). Thus, it seems that the tight coupling observed in HeLa cells might prove to be an extreme case. Since we have previously (Balhorn et al., 1973) observed only partial coupling between

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histone and DNA synthesis in HTC cells, we have further studied this phenomenon. It was also hoped that a better understanding of the deposition of histones synthesized in the absence of DNA synthesis might help to understand the phenomenon in the uninhibited cell.

We will argue that the coupling between histone and DNA synthesis is very leaky in HTC cells, that much of the histone synthesized in the absence of DNA synthesis is associated with nuclear macromolecules, and that it is in a form which can be recognized as distinct from the bulk chromatin.

Experimental Procedure

Determination of Synthesis Rates of Nuclear DNA and of Cellular RNA and Proteins. Hydroxyurea (Calbiochem) was added to HTC cells in Swim's-S77 (Gibco) medium (5×10^5 cells/mL) to a final concentration of 2.5 mM. Aliquots were taken out after 0, 10, 30, 60, 90, 140, 240 min and the cells were pulsed for 15 min with 0.5 μCi/mL [^{3}H]thymidine, 1 μCi/mL [^{3}H]uridine, or 1 μCi/mL [^{3}H]lysine. Cells were then rapidly cooled, collected by centrifugation (500g, 10 min), and kept frozen at −20 °C until used.

Preparation of [^{3}H]Thymidine-Labeled Samples. Cells were broken by 12 strokes of a hand homogenizer in washing medium (Jackson and Chalkley, 1974), and the nuclei were pelleted (1000g, 5 min) and washed twice under the same conditions. The nuclei were then washed once in 5% perchloric acid and heated at 90 °C for 20 min in 5% perchloric acid. After centrifugation (3000g, 5 min), the supernatants were neutralized and counted in Bray's (1960) scintillation cocktail.

Preparation of Uridine- and Lysine-Labeled Samples. Cell pellets were suspended in distilled water by sonication, and the suspension was made 20% in trichloroacetic acid. After centrifugation (3000g, 5 min), the pellets were washed twice with 20% trichloroacetic acid and finally solubilized in 1 N sodium hydroxide. Samples were neutralized and counted in Bray's (1960) solution.

Determination of the Rate of Synthesis of Histones. After incubation of HTC cells in presence of hydroxyurea (2.5 mM) for 0, 30, 60, 90, 150, and 240 min, aliquots were labeled for 30 min with [^{3}H]lysine (2.5 μCi/mL). Cells were collected immediately after the pulse and frozen. Chromatin was prepared and histones were obtained as previously described by Jackson and Chalkley (1974). Histone electrophoresis was performed on 15% acrylamide–1 M urea gels (25 cm) (Panyim and Chalkley, 1969) which were stained in Amido Black, scanned on a Beckman Acta III spectrophotometer, and sliced. The slices were solubilized at 60 °C in 30% H_2O_2 and counted in Bray's (1960) solution, and the specific activity (cpm/unit value of absorbance at 600 nm of the histone fractions) was determined.

Measure of Turnover Rates. Control HTC cells (log phase) were labeled for 60 min with [^{3}H]lysine (0.5 μCi/mL); after the incubation, cells were collected by centrifugation (300g, 10 min) and resuspended in fresh unlabeled medium. Treated cells were first incubated in the presence of 2.5 mM hydroxyurea for 30 min, and [^{3}H]lysine was added for the next 60 min. These cells were then collected by centrifugation and resuspended in fresh unlabeled medium containing 2.5 mM hydroxyurea. Samples were taken out after 0, 2, 4, 5, and 8 h of chase. Histones were extracted and their specific activity was determined for each time point.

pH Washes of Chromatin. Control and hydroxyurea-treated cells were labeled with [^{3}H]lysine for 4 h. Chromatin was isolated, solubilized in water, and brought to pH 3.2. After centrifugation (5000g, 5 min), the specific activity of histones in the supernatant was determined and the pellet resuspended in water. The same procedure was repeated for washes at pH 3.0 and 2.8.

Mitochondrial DNA Synthesis during Hydroxyurea Treatment. HTC cells were treated simultaneously with hydroxyurea (2.5 mM) and ethidium bromide (1 μg/mL) (Lord, 1974). Aliquots were taken out after 0, 10, 30, 90, and 180 min and pulsed with 0.5 μCi/mL [^{3}H]thymidine for 15 min. After centrifugation and freezing, cells were homogenized in washing medium without Triton X-100 and nuclear material was prepared as previously described (see section "Preparation of Thymidine-Labeled Samples" under Experimental Procedure). Mitochondria were obtained by a high-speed centrifugation (12 000g, 10 min) of the homogenate supernatant. The pellets were then suspended in water and counted in Bray's (1960) solution.

Fixation. Chromatin was fixed at 0 °C, in 5×10^{-4} M triethanolamine (pH 7.4), at a final concentration of 2% formaldehyde (pH 7.2) for 4 h. Sulfuric acid extraction and gel electrophoresis show complete fixation of material. Formaldehyde was removed by dialysis against 100 volumes of triethanolamine buffer.

Gradients. Chromatin was analyzed on "standard" (1.7 M) or "light" (1.5 M) cesium chloride gradients containing 4.0 M guanidine hydrochloride, 0.1 M Tris[1] (pH 7.4). Seven-milliliter gradients were run on a Beckman Type 50 rotor at 40 000 rpm for 72 h. Fractions were collected, read at 260 nm, and counted in Bray's (1960) solution.

Electrophoresis. Column electrophoresis was performed in a sucrose gradient according to a technique (Schwimmer and Olivera, 1966) modified in this laboratory. The buffer used in the system is 0.01 M Tris (pH 7.4); this system consists in the superposition of a heavy electrode solution (40% sucrose, 2 M NaCl, 10 mL), a heavy cushion (30% sucrose, 3 mL), a gradient (22%/12% sucrose, 32 mL), a sample layer (10% sucrose, 0.5–1 mL), a light cushion (8% sucrose, 3 mL), and a light electrode solution (0.5 M NaCl, 15 mL). The column used was 15 mm in diameter. Migration was for 3 h at 250 V at 0 °C. Fractions were then collected, read at 260 nm, and counted in Bray's (1960) solution.

Effect of Chase on Histones Synthesized during a Hydroxyurea Treatment. HTC cells were treated with hydroxyurea (2.5 mM) for 30 min before the addition of [^{3}H]lysine (2.5 μCi/mL) for 60 min. Cells were then centrifuged and the pellets resuspended in unlabeled medium in the absence of hydroxyurea and chased for 24 h.

Reversal of Fixation and Analysis of Proteins. Standard cesium chloride gradients were divided into three aliquots, and chromatin fixation was reversed by making the samples 0.35 M in glycine, 1% in $NaDodSO_4$ and dialyzing them against 500 volumes of 0.1% $NaDodSO_4$, 0.01 M Tris (pH 7.4) at 37 °C for 2 days. Electrophoresis was then performed according to Weber and Osborn (1969). Gels were stained with Coomassie brilliant blue and scanned on a Beckman Acta III spectrophotometer. They were next sliced, solubilized in 30% H_2O_2 at 60 °C, and counted in Bray's (1960) solution.

Results

Continued Synthesis of Histone Despite Cessation of DNA Synthesis. DNA synthesis is effectively inhibited in HTC cells by hydroxyurea concentrations in the range of 1–5 mM. The liver-derived tumor cells can metabolize hydroxyurea, and the inhibitory effect at low concentrations (less than 1 mM) is

[1] Abbreviation used: Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.

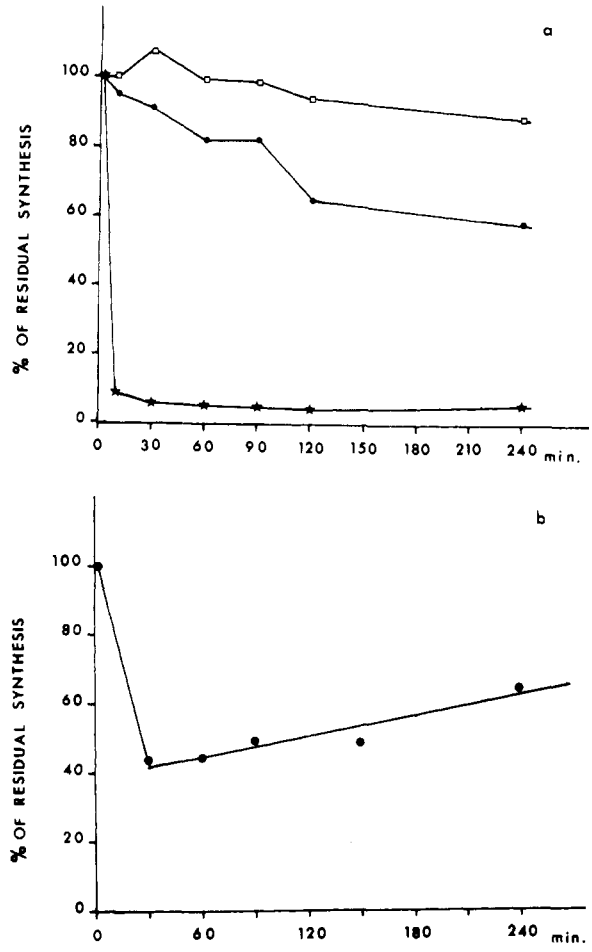

FIGURE 1: (a) Effect of hydroxyurea (2.5 mM) on the synthesis of nuclear DNA (★) and cellular RNA (●) and proteins (□). Hydroxyurea was added to a midlog phase population of HTC cells. Samples were taken after various time intervals and pulsed for 15 min with [^{3}H]thymidine, [^{3}H]-uridine, or [^{3}H]lysine, and the level of incorporation was compared with the respective controls. 100% was defined as the incorporation at $t = 0$. (b) Effect of hydroxyurea (2.5 mM) on the synthesis of total histones. After incubating in hydroxyurea for various time intervals, HTC cells were pulsed with [^{3}H]lysine for 30 min. Histones were extracted and run on acrylamide gels, and their specific activity was compared to the control (100%, $t = 0$). All the histone bands were pooled together for the determination of the specific activity.

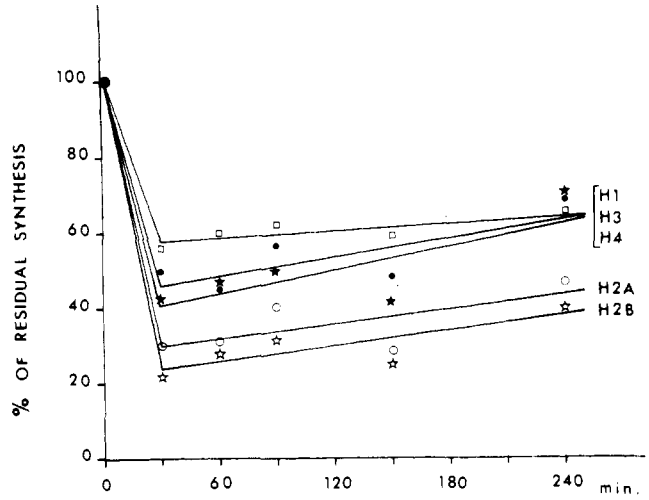

FIGURE 2: Effect of hydroxyurea (2.5 mM) on the synthesis of the individual histone fractions. After incubation for various time periods, HTC cells were pulsed for 30 min with [^{3}H]lysine. Histones were extracted and run on long (25 cm) 15% acrylamide–1 M urea gels. The specific activity of the individual fractions was determined and compared to the control (100%, $t = 0$). The lines drawn represent the best fitting lines obtained by a least-squares analysis: H1 (□), H3 (●), H4 (★), H2A (○), H2B (☆).

reversed after 60–120 min (Balhorn, 1974). Accordingly, we have utilized a concentration of 2.5 mM hydroxyurea for these studies and extended the time of analysis to 240 min. DNA synthesis is reduced to 3–4% of control during the entire course of such experiments, as shown in Figure 1a. Autoradiographic experiments (data not shown) have indicated that this represents a very low rate of synthesis in about 50% of the population of previously randomly growing cells, and it seems likely that we are indeed seeing primarily a gross reduction in the overall rate of synthesis rather than merely repair synthesis. The effect of this treatment upon RNA and protein synthesis in these cells is also shown in Figure 1a. There is a 40% reduction in the rate of RNA synthesis during the time course of these experiments, whereas no significant decrease was noted on total protein synthesis. We note that there was no increase in cell death during the course of these exposures to hydroxyurea. It is only upon exposure to hydroxyurea for periods longer than 12 h that serious disturbances to the health of the population were noted, although we cannot exclude the very likely possibility that cells exposed to hydroxyurea for 4 h may have suffered irreversible changes which lead to cell death after an extended time period.

Changes in the apparent rate of DNA synthesis are sometimes due to changes in the pool size of thymidine. However, Bjursell and Reichard (1973) have shown that hydroxyurea has essentially no effect upon the thymidine pool size in CHO cells. Moreover, the nearly constant level of total protein synthesis suggests strongly that hydroxyurea is not influencing the size of the amino acid pools.

In addition, we have determined that residual DNA synthesis observed in the presence of hydroxyurea was not the effect of mitochondrial contamination. HTC cells were treated with hydroxyurea (2.5 mM) and sufficient ethidium bromide to inhibit completely mitochondrial DNA synthesis (1 μg/mL) (Lord, 1974). No differences in the residual level of DNA synthesis were observed between this double inhibition and the level obtained from a hydroxyurea treatment alone.

Although there is little overall decrease in total protein synthesis as a result of exposure to hydroxyurea, histones behave in a different manner to bulk proteins. As shown in Figure 1b, histone synthesis drops rapidly to about 40% of control, after which time there is a slow but gradual increase in histone synthesis to 65% of control. In no experiment was a recovery in DNA synthesis noted. Separation of histones on long gels permitted us to ask whether all the major histone subfractions behave in the same way. Histones H2a and H2b appear to be the most extensively inhibited and their synthesis is reduced to almost 20% of the control value, though it recovers to 40% of control after 4 h, as shown in Figure 2. Histone H1 appears to be the least affected and is reduced to only 60% of the control synthesis level. These variable levels of inhibition cannot be explained by selective proteolysis as (1) no degradation products are observable on the gels; (2) even if H1 and H3 are the histones which are the most sensitive to degradation, their synthesis was the less extensively inhibited.

The histones which continue to be made were isolated from purified nuclei and, therefore, in a sense, can be said to have been deposited. Since newly deposited histone is extensively modified (Jackson et al., 1975), we have measured the extent of modification of histones both after a short period (15 min) and after a larger time period (4 h) of synthesis in the presence of hydroxyurea. As shown in Table I and in accord with Figure

TABLE I: Relative Incorporation of [^{3}H]Lysine in HTC Histone Fractions and Their Modified Forms.[a]

	15-min incorp (%)		4-h incorp (%)	
	control	hydroxyurea-treated	control	hydroxyurea-treated
H1-1	17.1	15.3	12.8	16.0
H1-2	23.2	22.9	16.8	22.5
H3-1	4.4	12.7	5.0	14.9
H3-2	6.5	9.5	5.5	6.3
H3-3*	12.4	9.9	10.7	8.9
H2B	15.9	13.0	22.9	13.0
H2A	10.4	4.3	12.3	6.0
H4-1	3.3	3.2	1.8	4.0
H4-2	3.0	3.1	5.4	2.6
H4-3*	3.7	6.2	6.8	4.8

[a] After a pulse of [^{3}H]lysine for 15 min or 4 h in the absence or presence of hydroxyurea (2.5 mM), histones were extracted and run on long (25 cm) 15% polyacrylamide–1 M urea gels. Bands were cut and counted for radioactivity incorporation. 100% is defined as the total number of counts incorporated in all the histone fractions. The modified forms were identified after staining, referring to relative positions given by Jackson et al. (1975). Histones with an asterisk designate parental forms.

2, the accumulation of lysine into H2a and H2b in both instances was lower than in the other fractions. Furthermore, both H3 and H4 show an increased degree of incorporation of [^{3}H]lysine into highly modified forms (most likely acetate modifications) relative to the control histones. This is particularly evident after 4 h of synthesis in hydroxyurea. These observations are consistant with a depositional process which was never completed. For instance, there is a rapid deacetylation of the highly modified forms of histones seen relatively soon after entrance to the nucleus (Jackson et al., 1975).

The following experiment was performed in order to test whether the histones synthesized in the presence of hydroxyurea are degraded very rapidly to maintain correct histone to DNA ratios. HTC cells were incubated with [^{3}H]lysine for 30 min after exposure of half the culture to hydroxyurea. After the pulse, the cells were collected and resuspended in fresh medium lacking radiolabel, though containing hydroxyurea whenever appropriate. At various time points in the chase, aliquots of the cells were collected and frozen. Histones were isolated and analyzed electrophoretically so that their specific activity could be determined. The results are shown in Figure 3. Clearly, there is no dramatic, selective turnover of histones. Both control and hydroxyurea-treated cells show a degree of turnover (as judged by the deviation of the two lines from the horizontal), but no significant differences between the two lines were noted. In 8 h, the control culture should have increased in histone content by approximately 25% (cell generation time $\simeq$28 h) and even if there were no turnover of histone per se there should be an isotope dilution effect, so that the final specific activity of the control should be 75% of the initial value. Similarly, the histone from the hydroxyurea-treated cells should have a specific activity approximately 85% of zero time. Thus, within the limits of precision of this experiment, we conclude that there has been no selective turnover of the histones labeled in the presence of hydroxyurea. Some of the specific activities which are shown in Figure 3 have standard deviations amounting to as much as 20% of their values. Despite massive efforts, the lack of reproducibility of the staining appears to be an unavoidable consequence of the use of long gels. We cannot determine the cause of this large variation nor

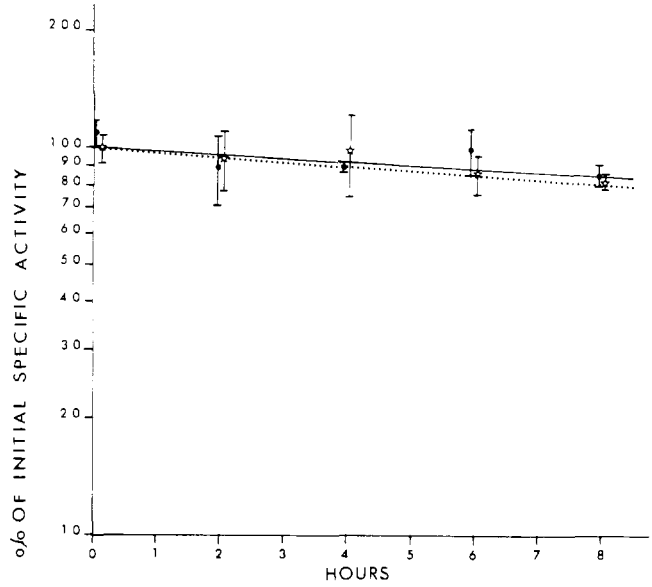

FIGURE 3: Effect of hydroxyurea (2.5 mM) on the turnover of histones. After 30 min in the absence (control) or presence (treated) of hydroxyurea, cells were labeled with [^{3}H]lysine for 60 min. Cells were then resuspended in unlabeled medium without (control) or with hydroxyurea and chased during 8 h. Samples were taken out at every 2 h, and the specific activity of the histones was determined. The graph presents the best fitting lines obtained from a least-squares analysis: control (●), hydroxyurea treated (☆).

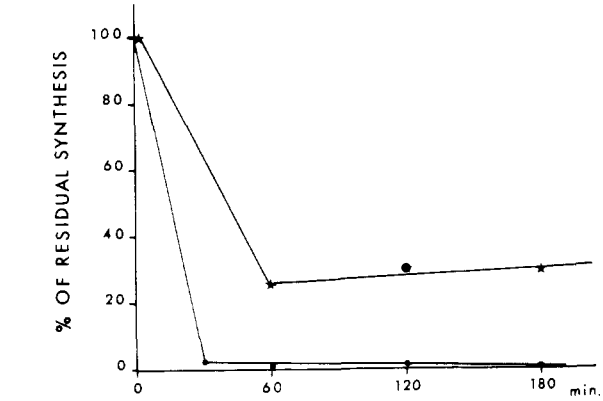

FIGURE 4: Effect of hydroxyurea on the synthesis of DNA (●) and histones (★) in HeLa cells. After incubating in hydroxyurea (5 mM) for various time periods, samples were pulsed with [^{3}H]thymidine or [^{3}H]-lysine, and the level of incorporation in DNA and histone was compared to the control values (100%, t = 0). All the histone bands were pooled together for the determination of the specific activity.

can we avoid it. Nonetheless, this does not detract from the major conclusion of this experiment, namely, that there was no major selective destruction of histone synthesized in the presence of hydroxyurea.

Since these results differ from the generally accepted picture, we have repeated the study of continued histone synthesis in HeLa S-3 cells, the system from which the earlier conclusions were drawn. As shown in Figure 4, HeLa cells respond more extensively than HTC cells in an analysis of the coupling of histone and DNA synthesis. Nonetheless, when HeLa DNA synthesis is reduced to 3% of the control, we observed approximately 20–30% rate of histone synthesis relative to control. It would appear that the conclusion of a very tight coupling between DNA and histone synthesis may not have been totally justified.

Distribution of Histone Synthesized in the Presence of Hydroxyurea. Initially, we wished to test whether the newly

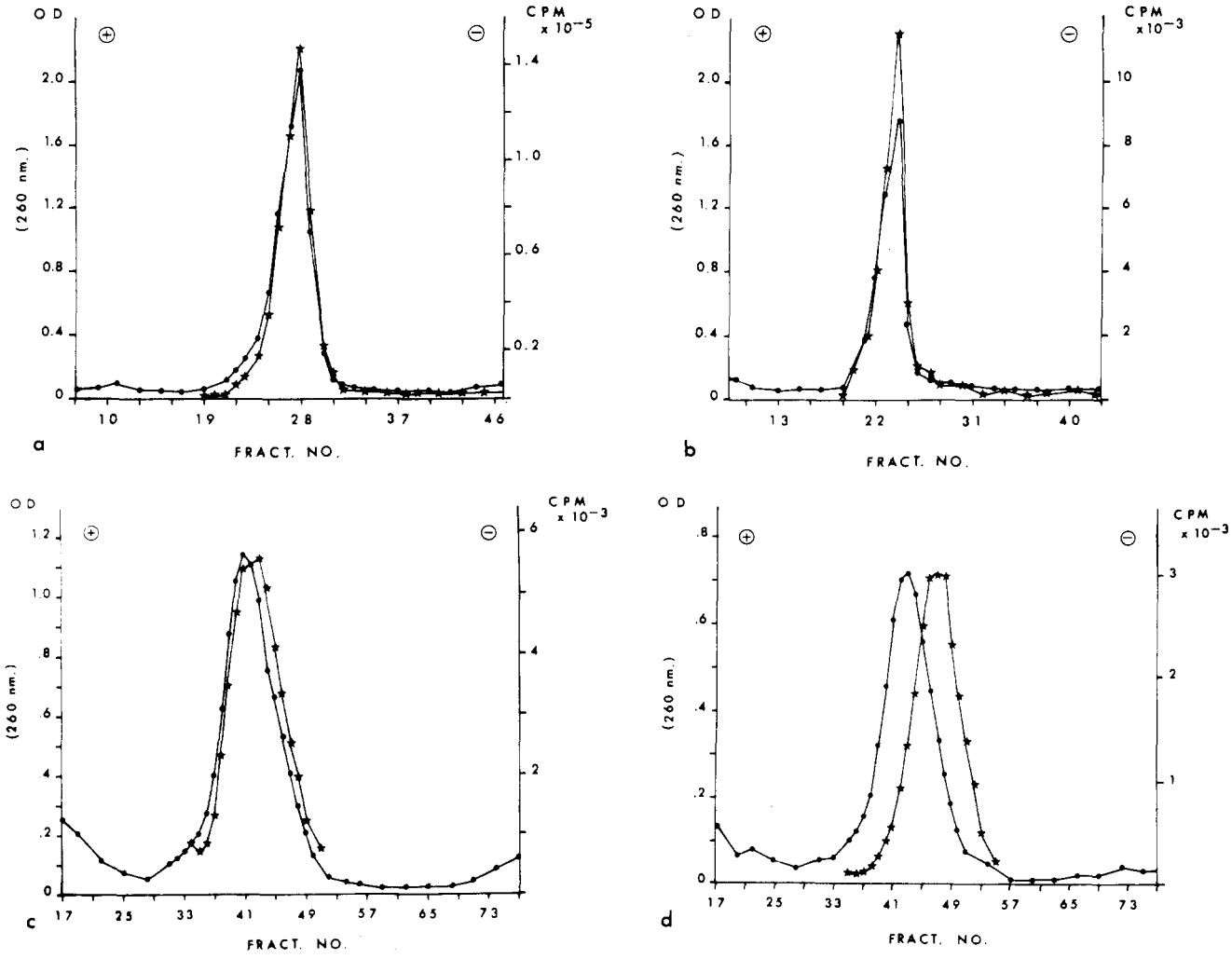

FIGURE 5: Effect of hydroxyurea (2.5 mM) on the electrophoretic behavior of newly synthesized chromatin. HTC cells were incubated for 4 h either in [^{3}H]thymidine or [^{3}H]lysine, in the absence or presence of hydroxyurea. After purification of the chromatin, column electrophoresis was performed on a sucrose gradient. After fractionation, the optical density and distribution of the radioactivity were measured: Optical density (●), cpm (★); (a) [^{3}H]thymidine-labeled control chromatin; (b) [^{3}H]thymidine-labeled, hydroxyurea-treated chromatin; (c) [^{3}H]lysine labeled control chromatin; (d) [^{3}H]lysine-labeled, hydroxyurea-treated chromatin.

synthesized histones were really intranuclear and not merely bound to the nuclear membrane, perhaps as the result of an artifact of preparation. To investigate this possibility, we added lysine-labeled histones to purified nuclei (prepared in the absence of detergents) and observed that a fivefold excess of DNA (relative to the intranuclear DNA in the suspension) was able to remove a substantial proportion (54%) of the added counts. We then subjected lysine-labeled nuclei and lysine-labeled hydroxyurea-treated nuclei to a fivefold excess of DNA. In these two experiments, a small proportion of the counts (20%) was extracted from the nuclei, and no observable difference was seen between control and hydroxyurea-treated material. These results show clearly that the bulk of newly synthesized histones (in the presence of hydroxyurea) are intranuclear and are not adsorbed on the exterior of the nuclear membrane.

The continued histone synthesis and lack of turnover noted above should lead to an accumulation of approximately 10% extra histone during an 8-h time period. Given the intrinsic difficulties of quantitating small increases of histone in HTC cells, we have been unable to demonstrate a significant increase in the histone to DNA ratio. Accordingly, we have utilized indirect means to assay for chromatin containing extra histone.

The accumulation of extra histone on the chromatin should lead to a progressive decrease in the overall negative charge density of the complex. This should be detected upon electrophoresis of the intact complex. Both control and hydroxyurea-treated cultures were labeled in parallel experiments with either [^{3}H]lysine or [^{3}H]thymidine. Chromatin was isolated and analyzed by column electrophoresis using a modification of the method of Schwimmer and Olivera (1966). The results of such an analysis are shown in Figure 5. The distribution of [^{3}H]lysine and [^{3}H]thymidine in chromatin from control cells follows the distribution of A_{260}, as might be expected (Figure 5a,c). However, chromatin from hydroxyurea-treated cells shows a radical departure in distribution. Although [^{3}H]thymidine (admittedly incorporated at a low level but most probably into DNA in a normal manner, see above) is distributed normally, the [^{3}H]lysine, representing an incorporation which is 60% into histone, is present in material which migrates with a substantially lower mobility, consistent with extra, positively charged histones being present. That the bulk of the radioactivity migrates differently from the A_{260} indicates that the extra histone is not randomly distributed over the entire chromatin but is selectively deposited at a site which is distinct from the site of the freshly incorporated [^{3}H]thymidine.

In order to study the extent of the reduction in electrophoretic mobility due to the accumulation of extra histones, we

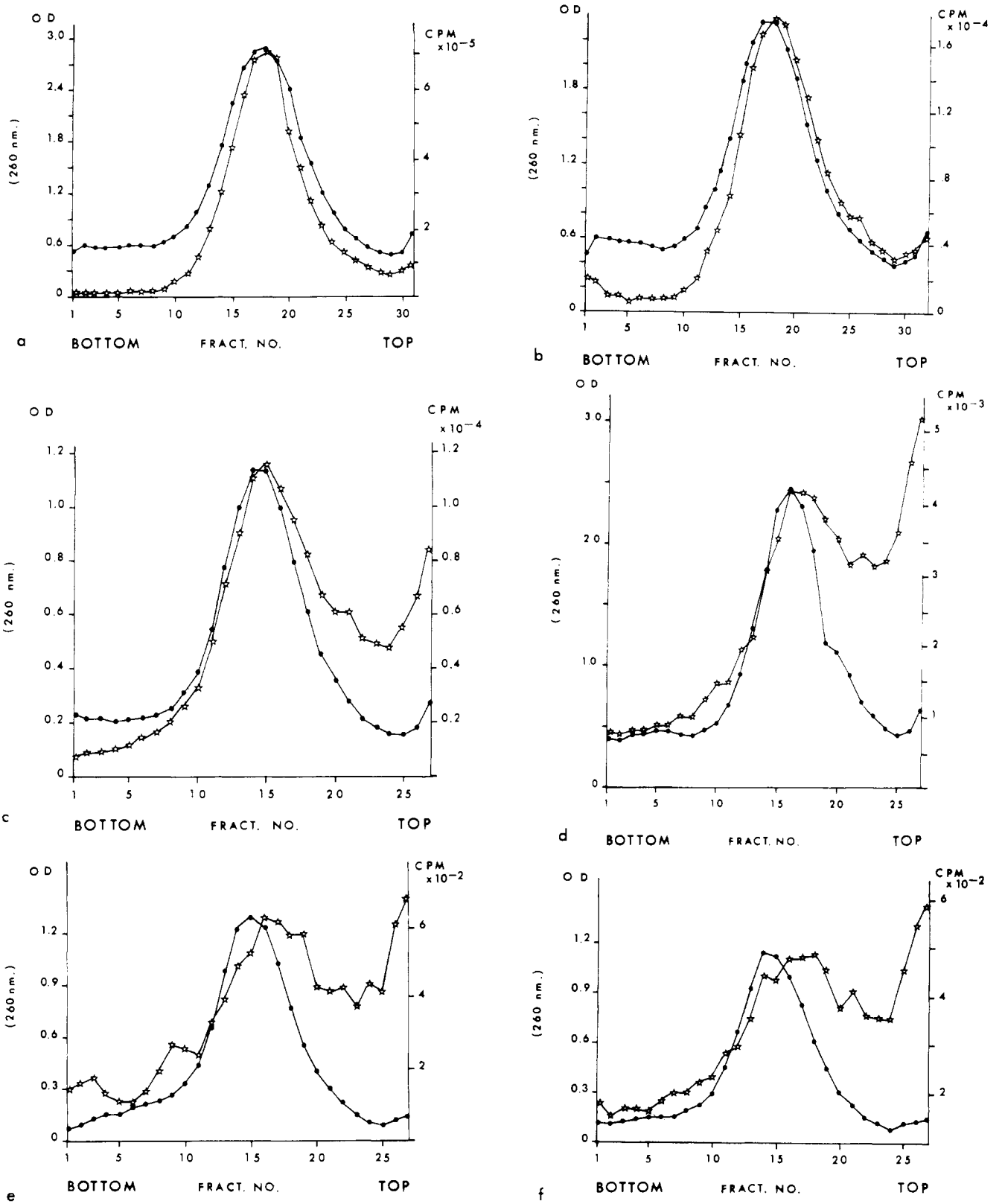

FIGURE 6: Effect of hydroxyurea on the density distribution of newly synthesized chromatin. HTC cells were incubated for 4 h in [^{3}H]thymidine, [^{3}H]lysine, or [^{3}H]tryptophan in the presence or absence of hydroxyurea (2.5 mM). Chromatin was then extracted, fixed with formaldehyde at very low ionic strength, and centrifuged in cesium chloride gradients. The optical density (●) and distribution of radioactivity (☆) were then measured through the gradient: (a) [^{3}H]thymidine-labeled control chromatin; (b) [^{3}H]thymidine-labeled hydroxyurea-treated chromatin; (c) [^{3}H]lysine-labeled control chromatin; (d) [^{3}H]lysine-labeled, hydroxyurea-treated chromatin; (e) [^{3}H]tryptophan-labeled control chromatin; (f) [^{3}H]tryptophan-labeled, hydroxyurea-treated chromatin.

enriched some control chromatin by adding [^{3}H]lysine-labeled histones (10%) and formaldehyde fixed this complex. Then, we mixed the enriched chromatin with an excess (tenfold) of untreated material. We noted that the mobility of the radioactivity peak, relative to the optical density peak, was very similar to the one observed for the distribution of radiolabel in protein from hydroxyurea treatment (data not shown). Thus, this observation shows that an increase of approximately 10%

TABLE II: Specific Activity of Histones from Regions of a Cesium Chloride Density Gradient.[a]

	control	hydroxyurea treated
Mid-peak	12.3	2.3
Light side	14.8	7.8

[a] The specific activity is expressed as the ratio of [^{3}H]lysine counts in histones to the absorbance values of the stained histones.

in the mass of histone, expected after 4 h of treatment with hydroxyurea, is easily detectable with the system used and should lead to a decrease in mobility similar to that observed.

If a part of the chromatin possesses extra histone, the buoyant density should be less than that of bulk chromatin. We have analyzed chromatin from hydroxyurea-treated and from control cells, following the distribution of [^{3}H]lysine or [^{3}H]thymidine on CsCl density gradients after formaldehyde fixation. We found that, under these conditions, fixation is more than 99% complete. The results of a 4-h pulse are shown in Figure 6. The distribution of [^{3}H]thymidine (Figure 6a,b) in the treated material (Figure 6b) is skewed slightly toward the light side on the main peak, a result analogous to that seen in very short pulses ($\simeq$3 min) of [^{3}H]thymidine into normal cells (Seale and Simpson, 1975). This indicates that the DNA synthesized in the presence of hydroxyurea is not able to shift to normal density, even during a 4-h period, and is possibly associated with proteins characteristic of newly replicated DNA. The distribution of [^{3}H]lysine (Figure 6c,d) in hydroxyurea-treated chromatin (Figure 6d) is skewed dramatically to the light end of the gradient, to a much greater degree than is seen in the control chromatin (Figure 6c). A parallel experiment using [^{3}H]tryptophan (Figure 6e,f) showed no difference between hydroxyurea-treated and control material, indicating that the extra material (at the top of the gradient) is due to the presence of extra histone after hydroxyurea treatment. This was also tested directly by isolating histones from the main peak and from the light region of the gradient. The specific activity of the histone was determined after electrophoresis on $NaDodSO_4$ gels. The specific activity in the light region of the gradient is only very slightly higher than in the main peak for the control material. However, the hydroxyurea-treated chromatin gave rise to a histone specific activity over three times greater in the light region than that in the main band, as shown in Table II.

Since the above experiment was conducted during a 4-h period and showed histone both in the main band and in the light region, we wondered if a shorter time period might only show a distribution into the light region, particularly if such an association were characteristic of newly deposited histone. We repeated this experiment during a 15-min pulse of [^{3}H]-lysine. The distribution of radiolabel (Figure 7a,b) was very similar to that seen after a 4-h pulse and there was no indication that the appearance in the light band is characteristic for recently deposited material and that it subsequently moves to the main band. Likewise, the 15-min control showed no extra radioactivity in the light-band region. On the other hand, histone transport to the nucleus is a very rapid process and may be essentially complete in 15 min in the absence of hydroxyurea.

We tested if the histone synthesized during the hydroxyurea treatment might be in some way bound to intranuclear RNA. An RNase digestion (data not shown) of lysine-labeled nuclei (control and treated material) showed no modification of the

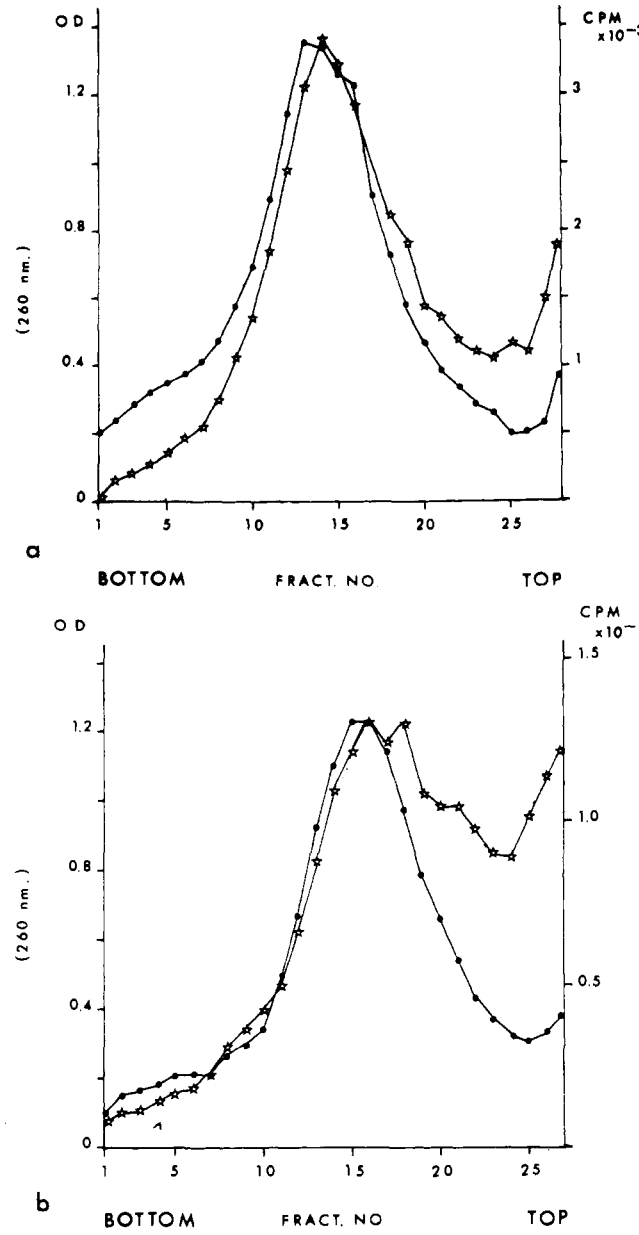

FIGURE 7: Effect of hydroxyurea on the density distribution of newly synthesized chromatin. The procedure described in Figure 6 was followed, except that the cells were labeled for 15 min: optical density (●); cpm (☆); (a) [^{3}H]lysine-labeled control chromatin; (b) [^{3}H]lysine labeled hydroxyurea-treated chromatin.

radioactivity profile on a density gradient, indicating that either the histone is not associated with RNA or that this RNA is protected against digestion by RNase. A similar experiment performed with uridine-labeled material showed a disappearance of the typical uridine incorporation profile, indicating that most of the RNA has been destroyed.

We wondered whether the histones associated with the light-banding chromatin would shift into the main band if hydroxyurea were to be removed. Cells were exposed to hydroxyurea for 30 min and then to [^{3}H]lysine for 60 min. Both radiolabel and hydroxyurea were then removed and the cells incubated under normal growing conditions for 24 h. The results of density gradient separation of fixed chromatin after the 24-h chase period are shown in Figure 9. By comparing to Figure 8b, where no chase was performed, no change is apparent during the chase period, and, indeed, the specific activity is likewise unchanged, indicating that no nucleic acid has been synthesized during this time. In addition, relatively little cell

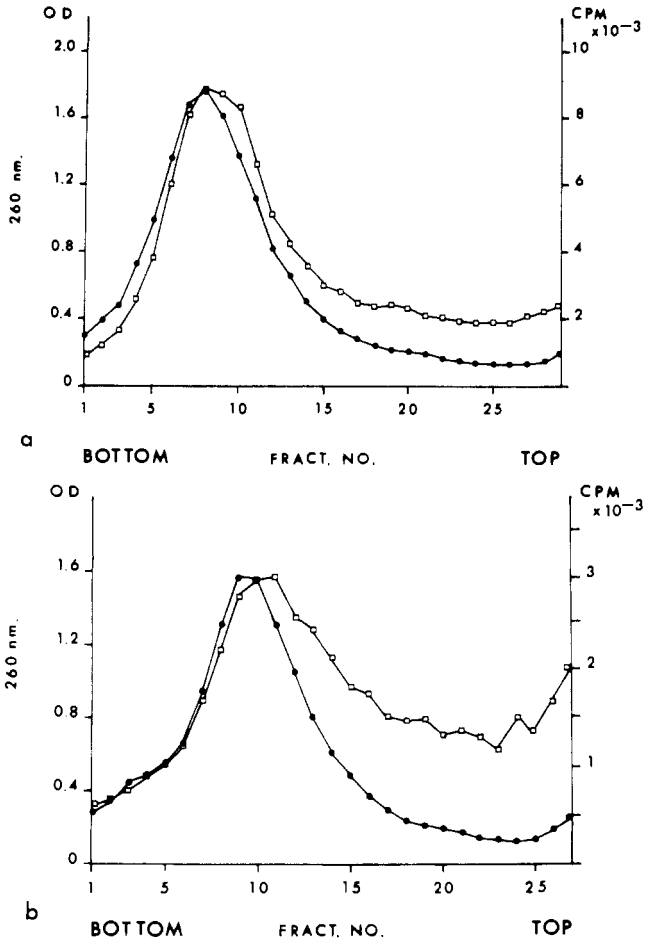

FIGURE 8: Effect of hydroxyurea on the density distribution of newly synthesized chromatin. The procedure described in Figure 6 was followed but "lighter" CsCl gradients were used: optical density (●); cpm (□); (a) [^{3}H]lysine-labeled control chromatin; (b) [^{3}H]lysine-labeled, hydroxyurea-treated chromatin.

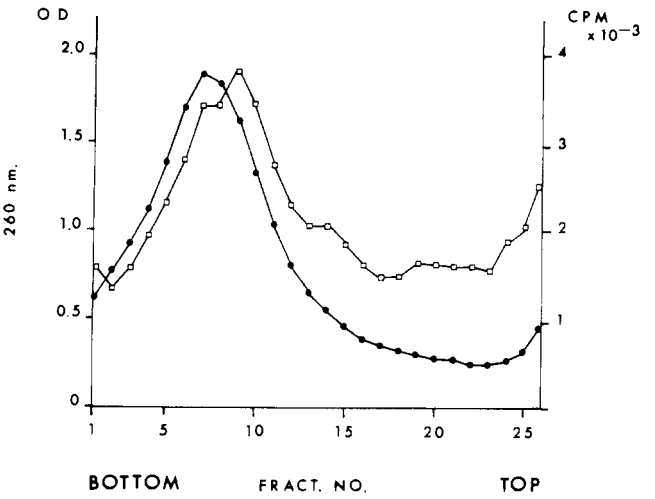

FIGURE 9: Effect of hydroxyurea on the density distribution of chromatin. After incubation for 30 min in hydroxyurea, cells were pulsed for 1 h with [^{3}H]lysine and chased in unlabeled medium without hydroxyurea for 24 h. Chromatin was prepared and "light" CsCl gradients were run. Figure 8b is representative of the situation before the chase: optical density (●); cpm (□).

TABLE III: Effect of pH Washes on the Release of Histone Counts from Chromatin.[a]

pH	control, % net	control, % cumulative	hydroxyurea treated, % net	hydroxyurea treated, % cumulative
3.2	6.7	6.7	4.6	4.6
3.0	1.8	8.5	3.1	7.7
2.8	4.1	12.6	2.1	9.8

[a] Percentages express the ratio of the histone counts extracted to the total histone counts in the samples.

lysis is observed and there is no cell division. The most likely interpretation is that the cells have suffered irreversible damage as a result of exposure to hydroxyurea. Whether or not the deposition of the extra histone is a cause or a result of this damage is a moot question.

Finally, we have studied the strength of the binding to the chromatin of histones synthesized in the presence of hydroxyurea. This assay is based upon observations that histones added to chromatin in vitro are more weakly bound so that they can be extracted at pH values not normally thought to dissociate histones from chromatin. Usually, the pH range 2.8–3.2 is used (Walker, 1965; Jackson et al., 1977). We have decreased the pH in three successive washes (see Table III). No significant difference in the release of histone counts is observable between the control and the hydroxyurea-treated material. This result indicates that the histones that appear to be in excess in the treated material are not interacting more loosely but, to the contrary, are indistinguishable in terms of their binding interaction with the complex.

Discussion

This study has addressed itself to two major points: (1) Can a significant measure of histone synthesis continue in a mammalian cell even though DNA synthesis has been effectively inhibited? (2) Where are such histones found within the nucleus?

It is clear that the answer to the first point is definitely in the affirmative, although it must be emphasized that there is a great deal of variability in different systems. Thus, in HTC cells, the degree of coupling between DNA and histone synthesis is limited. On the other hand, in HeLa cells, as seen both in this report and in earlier papers (Spalding et al., 1966; Robbins and Borun, 1967; Gallwitz and Mueller, 1969), the degree of coupling is quite strong. In all likelihood, we may expect that additional studies will reveal that the degree of coupling will be highly variable in different mammalian systems. The reason for the variability presumably lies in the nature of the partial coupling itself. Recent work has indicated in HeLa cells that the histone mRNA disappeared from the polyribosomes (Stein et al., 1977) or even from the cytoplasm (Melli et al., 1977) when it is not to be used, even though it is evidently synthesized throughout the cell cycle. Degradation or partial shift of the mRNA to the nucleus would appear to be the most likely explanations for the loss of cytoplasmic histone synthesizing capacity. Thus, in HTC cells we may evoke an increased cytoplasmic stability or a failure of histone mRNA to remain in the nucleus as causes for the decreased coupling between histone and DNA synthesis.

In view of the fact that histone synthesis does indeed continue quite vigorously in HTC cells even when DNA synthesis is inhibited, we have looked for the location of these histones within the nucleus (from whence they were extracted). The results presented in this paper indicate (1) they are not randomly distributed over the chromatin but rather reside at a specific site, (2) they are not associated with even the small amount of DNA which is made in hydroxyurea-blocked cells, and (3) they are not in the form of a complex of chromatin plus

extra histones, as they cannot be extracted under conditions of pH where extra histone (added in the test tube) can easily be removed. In other words, they are as tightly bound as histones normally are in their association with DNA. We have no evidence indicating when the histone becomes bound to this material. A very real possibility exists that the association of histone with a still unidentified material can be initiated in the cytoplasm as a device for the highly directed transport of these very positively charged proteins into the nucleus. These ideas would certainly be consistent with the observations reported in this paper and as such provide interesting areas for further study.

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References

Adamson, E. D., and Woodland, H. R. (1974), *J. Mol. Biol. 88,* 263–285.

Balhorn, R. (1974), Ph.D. Thesis, Department of Biochemistry, College of Medicine, The University of Iowa, Iowa City, Iowa.

Balhorn, R., Tanphaichitr, N., Chalkley, R., and Granner, D. K. (1973), *Biochemistry 12,* 5146–5150.

Bjursell, G., and Reichard, P. (1973), *J. Biol. Chem. 248,* 3904–3909.

Bray, G. A. (1960), *Anal. Biochem. 1,* 279–285.

Gallwitz, D., and Mueller, G. C. (1969), *Science 163,* 1351–1353.

Jackson, V., and Chalkley, R. (1974), *Biochemistry 13,* 3952–3956.

Jackson, V., Shires, A., Chalkley, R., and Granner, D. K. (1975), *J. Biol. Chem. 250,* 4856–4863.

Jackson, V., Hoffman, P., Hardison, R., Murphy, J., Eichner, M. E., and Chalkley, R. (1977), *Mol. Biol. Mamm. Genet. Appar. 1,* 281–300.

Jacobs-Lorena, M., Baglioni, C., and Borun, T. W. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 2095–2099.

Kay, A. C., and Singer, M. F. (1977), *Nucleic Acid Res. 4,* 3371–3386.

Lord, A. (1974), *J. Ultrastruct. Res. 46,* 117–130.

Melli, M., Spinelli, and Arnold, E. (1977), *Cell 12,* 167–174.

Panyim, S., and Chalkley, R. (1969), *Arch. Biochem. Biophys. 130,* 337–346.

Robbins, E., and Borun T. W. (1967), *Proc. Natl. Acad. Sci. U.S.A. 57,* 409–416.

Schwimmer, S., and Olivera, B. M. (1966), *J. Mol. Biol. 20,* 585–587.

Seale, R. L., and Simpson, R. T. (1975), *J. Mol. Biol. 94,* 479–501.

Spalding, J., Kajawara, K., and Mueller, G. C. (1966), *Proc. Natl. Acad. Sci. U.S.A. 56,* 1535–1542.

Stein, G., Stein, J., Shepard, E., Park, W., and Phillips, I. (1977), *Biochem. Biophys. Res. Commun. 77,* 245–252.

Thompson, J. A., Stein, J. L., Kleinsmith, L. J., and Stein, G. S. (1976), *Science 194,* 428–431.

Walker, I. O. (1965), *J. Mol. Biol. 14,* 381–398.

Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244,* 4406–4412.