THE METABOLISM OF BASIC PROTEINS IN HELA CELL NUCLEI*

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It has been widely suggested that histones, the basic proteins of the nucleus, are integral units of mammalian chromosomes and that they assist in the regulation of genetic expression.[1-3] If so, the metabolism of these proteins might be expected to show a specific temporal relationship to the replication of the chromosomal DNA. In the present study, synchronized cultures of HeLa cells have been used to look for such a relationship. For this purpose three types of experiments were carried out: (1) Direct labeling studies, in which the specific activities of isolated basic nuclear proteins were compared after incorporation of leucine-C^{14} during different intervals in the synchronized cell cycle. (2) Accumulation studies, in which the amounts of the individual basic proteins were determined at different intervals in the cell cycle. (3) Dilution studies, in which cultures were prelabeled with leucine-C^{14} and the changes in specific activities of the isolated bands were measured during subsequent synchronized or logarithmic growth in nonradioactive media.

The three types of data supplement each other and permit four fundamental conclusions: (1) A fraction of the basic proteins of the nucleus is metabolically unstable and can be renewed in the absence of DNA synthesis. (2) Another fraction of the basic proteins is conserved in a form that was not isolatable during interphase; this form contributes to the bands of extractable, metabolically labile proteins during nuclear replication. (3) The accumulation of basic nuclear proteins is closely coupled to the synthesis of DNA and coincides with the accumulation of the latter. (4) Individual basic nuclear proteins exhibit independent metabolic patterns during the cell cycle. The implications of these findings for the regulation of genetic expression are discussed.

Materials and Methods.—Growth of HeLa cells: HeLa cells were grown in suspension cultures using media, methods, and a cell strain described earlier.[4] Aliquots of logarithmically growing stock cultures ($2.0 \pm 0.2 \times 10^5$ cells/ml) were incubated at 37° in 500-ml Florence flasks on a rotary shaker. The cells were counted in a Coulter counter.

DNA synthesis was synchronized by inducing a thymidineless state with 10^{-6} *M* amethopterin and 5×10^{-5} *M* adenosine[5] or by using thymidine (TdR, 10^{-3} *M*) which interferes with deoxycytidine metabolism.[6-8] After 16 hr of interphase the amethopterin block was reversed by adding TdR (5 μg/10^6 cells) or the block with 10^{-3} *M* TdR was reversed by changing the medium. Either of these procedures produced a synchronous wave of DNA synthesis. In some experiments puromycin (20 μg/ml) was added to inhibit protein synthesis, or DNA synthesis was prevented or interrupted with high concentrations of TdR (10^{-3} *M*) or N-hydroxyurea (HU 5×10^{-3} *M*).

At intervals during logarithmic growth, during inhibition of DNA synthesis, or during the period of synchronous DNA synthesis, DL-leucine-C^{14} (28.6 mc/mmole, New England Nuclear Corp.) was added to measure the rate of accumulation of isotope into proteins.

Extraction of basic nuclear protein: The cells were centrifuged at 400 *g* for 10 min, washed once with 0.154 *M* NaCl-0.25 *M* sucrose, and were resedimented at 600 *g* for 10 min. The cell pellet was suspended in twice-distilled water (2.0×10^7 cells/ml) and the suspension was immediately centrifuged for 5 min at 1300 *g*. After being suspended in water, the cells (3.0×10^7/ml) were lysed with 12-15 strokes in a Dounce-type homogenizer. Microscopic examination of the homogenate showed that all cells had been lysed. Within 30 min after their first exposure to distilled water, the lysate was centrifuged for 20 min at 1300 *g*, the soluble phase was decanted, and the

nuclear pellet was washed twice with 0.154 M NaCl. The final nuclear preparations were free of microscopically visible debris, and the nuclear membranes were intact and free from particles.

The isolated nuclei were suspended in 0.02 N H_2SO_4 ($2.5 \pm 0.5 \times 10^7$ nuclei/ml) for 60 min. The suspension was centrifuged for 60 min at 65,000 g in a Spinco model L preparative centrifuge, and the supernatant, which contained the nuclear proteins, was designated as the acid-soluble extract. In some experiments similar extractions were made with 0.25 N HCl. In these instances the acid-soluble fractions were dialyzed against 0.02 N H_2SO_4 before electrophoresis.

Electrophoretic separation of basic nuclear proteins: The polyacrylamide gel system, a modification of that of Reisfeld *et al.*,[9] used a 10% gel and a pH 4.5 β-alanine-acetic acid buffer. Aliquots of the acid-soluble extract containing 100 ± 10 μg of protein in 5% sucrose were applied directly to the top of the gel. Electrophoresis was carried out for 3 hr at 300 ± 10 v and 59 ± 2 ma in a Canalco model 12 apparatus with a model 1400 power supply. The gels were placed for 10–15 min in tetrabromophenolphthalein ethyl ester (1 mg/ml in 50% ethanol, in pH 4.5 buffer) to stain the protein-containing zones. After removing the excess dye in distilled water, the three most basic, front-running bands were cut from the 12 gels, pooled, and forced through a fine-meshed, stainless steel screen fitted into a 10-ml syringe. The protein was eluted by suspending the homogenized gel in a small volume of 0.02 N H_2SO_4 overnight at room temperature. After dialysis against 0.02 N H_2SO_4 in the cold for 12 hr, the extracts were analyzed for protein[10] and radioactivity in a Packard liquid scintillation spectrometer.

To examine the incorporation of leucine-C^{14} into the acid-insoluble proteins of the total cell, cytoplasm, or nuclei, the appropriate fractions were precipitated in 10% trichloroacetic acid; extracted successively with 80% ethanol, 100% ethanol, and ethyl ether; dissolved in formic acid; and the protein and radioactivity in each were determined.

Results.—Synthesis of basic nuclear proteins in the absence of DNA synthesis: About 30 per cent of the cells in a logarithmically growing culture of HeLa cells are replicating DNA at any one time. Amethopterin (10^{-6} M) or TdR (10^{-3} M), which act by different means to induce deoxyribotide deficiencies,[4, 7] quickly interrupt this process. The remainder of the cell population, which continues to make and accumulate RNA and protein at nearly 80 per cent of the normal rates,[5] progresses through the interphase events of the cell cycle, and reaches a state of readiness for DNA synthesis within 16 hr. Although cells in such cultures were unable to synthesize DNA because of the block in deoxyribotide metabolism, leucine-C^{14} *was* incorporated

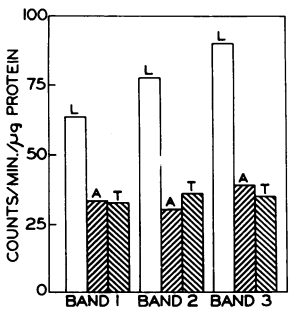

FIG. 1.—Effect of 10^{-6} M amethopterin (A) and 10^{-3} M TdR (T) on incorporation of DL-leucine-C^{14} (25 μc/300 ml) over 16 hr into three basic proteins isolated from HeLa nuclei. Comparison made with logarithmically growing (L) cultures labeled simultaneously.

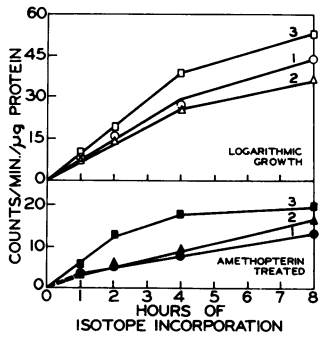

FIG. 2.—Incorporation of DL-leucine-C^{14} (25 μc/300 ml) into three electrophoretically isolated bands of basic proteins from HeLa nuclei from logarithmically growing cultures or cultures prevented from synthesizing DNA with 10^{-6} M amethopterin (separate expts. shown).

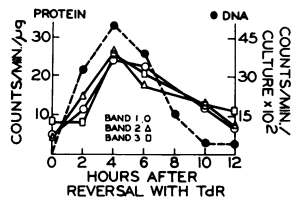

FIG. 3.—Incorporation of DL-leucine-C^{14} (25 μc/300 ml) into three basic proteins from HeLa nuclei during successive 2-hr intervals following initiation of DNA synthesis. Synchronization produced by 16 hr of treatment with 10^{-6} M amethopterin. The data giving the kinetics of DNA synthesis (*closed circles*) were taken from a separate experiment; such data are highly reproducible.

TABLE 1

INCORPORATION OF LEUCINE-C^{14} INTO BASIC NUCLEOPROTEINS DURING DIFFERENT GROWTH STATES OF HELA CELLS

Stage of cell cycle	Bands of Basic Nuclear Proteins 1	2	3
	(cpm/μg protein*)		
Logarithmic growth†	34	32	27
DNA synthesis‡	65	55	63
Mitosis and cell division§	23	21	25

* Incorporation of leucine-C^{14} (25 μc/300 ml culture) over 6 hr.
† Culture of an age comparable to that in mitosis and cell division.
‡ Six-hr period following reversal of amethopterin block.
§ Period from 6 to 12 hr after reversal of amethopterin block.

TABLE 2

EFFECT OF STAGE IN CELL CYCLE ON SPECIFIC ACTIVITIES OF HELA CELL PROTEINS

Stage of cycle	Cell Fraction: Whole cells	Cyto-plasm	Whole nuclei	Nuclear residue*	Bands of nuclear basic proteins† 1	2	3
	(cpm/μg protein‡)						
Interphase§	91	100	62	68	18	16	24
DNA synthesis**	96	100	92	75	91	99	97

* Residue left after three extractions with 0.25 *N* HCl.
† Isolated from 0.25 *N* HCl extract.
‡ Radioactivity incorporated during 6 hr with leucine-C^{14} (50 μc/300 ml culture).
§ Measured during 16th to 22nd hr with 10^{-6} *M* amethopterin; simultaneous with **.
** Synchronized by reversal of amethopterin block.

into the electrophoretically separated bands of basic nuclear proteins. During a 16-hr labeling interval, cultures held in interphase incorporated 40–50 per cent as much leucine into the basic protein as did the logarithmically growing control cultures (Fig. 1). When the ability to incorporate label was measured over shorter intervals, the rate of incorporation into proteins of the inhibited cultures was also 40–50 per cent of the control rate. In both amethopterin-treated and control cultures the incorporation into the isolated proteins was essentially linear during the first 4 hr of exposure to isotope (Fig. 2). The differences in the specific activities and labeling patterns of the three isolated proteins suggested that each was synthesized at a different rate. Similar results were obtained using lysine-H^3 as a precursor, or when 0.25 *N* HCl was used for the extractions.

Accelerated labeling of basic proteins during DNA replication: The addition of 4×10^{-6} *M* TdR to cultures treated with amethopterin for 16 hr initiates DNA synthesis in nearly all cells of the population. The synchronous wave of DNA synthesis lasts about 6 hr and nearly doubles the DNA content of the culture.[4] During this period leucine-C^{14} incorporation into the electrophoretically isolated, basic proteins was greatly stimulated (Table 1). The specific activity of all three bands labeled during the 6-hr interval of DNA synthesis approximately doubled; then, during the 6 hr immediately after DNA synthesis, it fell below that of the logarithmically growing cultures. The relationship between the rate of DNA synthesis and the rate of labeling of basic proteins was demonstrated even more clearly when leucine-C^{14}-incorporation was measured in successive 2-hr intervals during the period of DNA replication (Fig. 3). The maximal rate of incorporation of labeled amino acid into all three bands occurred in the interval from 2 to 4 hr after reversal of the thymidineless state. This corresponds to the interval of most rapid DNA synthesis. During the period of cell division, the rate declined toward the basal level of the amethopterin-blocked cells. While grossly similar, each of the

three bands of protein again displayed individual differences. Studies using lysine-H^3 also showed stimulated incorporation at the time of DNA synthesis.

A comparison of the specific activities of whole cell, cytoplasmic, and nuclear protein labeled during this interval illustrates the selectivity of the effect of DNA synthesis on the labeling of basic nuclear proteins (Table 2). No significant difference was observed between the specific activities of whole cell and cytoplasmic proteins from cells synthesizing and not synthesizing DNA. The increased labeling seen in the total nuclear protein reflects the accelerated incorporation into the basic nuclear proteins.

Labeling of basic proteins after interrupting DNA synthesis: Although the maximal rate of basic protein labeling coincided with the period of most rapid synthesis of DNA in synchronized cultures (Fig. 3), it was not clear whether these processes were metabolically coupled. To gain further insight into this relationship, synchronized cultures that were making DNA at a maximal rate (3 hr after reversal of the thymidineless state) were treated with 10^{-3} *M* TdR or 5×10^{-3} *M* HU. These agents immediately and selectively block the synthesis of DNA, without affecting measurably the general synthesis of RNA and protein.[11, 12] As Figure 4 shows, stopping DNA synthesis with these agents rapidly reduces leucine incorporation into the basic proteins; however, the fall was measurably slower than the immediate effect on DNA synthesis. These results support the view that the accelerated labeling of basic proteins during DNA synthesis depends on the presence of recently replicated DNA, rather than on some reaction preparing the nucleus for DNA synthesis, or on the process of DNA synthesis itself.

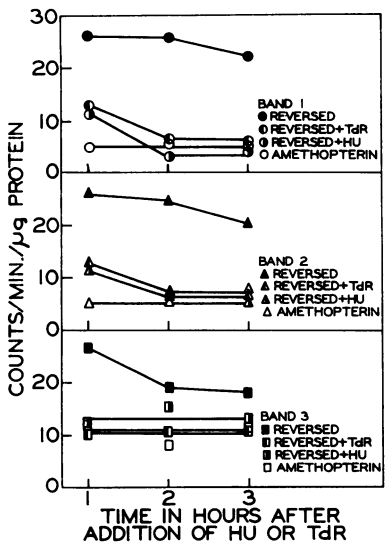

FIG. 4.—Effect of hydroxyurea (HU, 5×10^{-3} *M*) on incorporation of DL-leucine-C^{14} (50 µc/300 ml) into three basic proteins from HeLa nuclei. The inhibitors of DNA synthesis were added 3 hr after initiation of DNA synthesis; incorporation was measured during subsequent 1-hr intervals. DNA synthesis was synchronized by 16 hr of treatment with 10^{-6} *M* amethopterin.

Effect of puromycin on the labeling of nuclear basic proteins: Puromycin, at a level which blocks 90 per cent of the protein synthesis in the cell, also interferes with DNA synthesis. This action depends on adding puromycin early in the period of DNA synthesis. If added at the time of reversal of the thymidineless state, it strikingly limits the acceleration of DNA synthesis associated with the duplication of the late-replicating DNA; addition 2 hr later has much less effect on the process of DNA synthesis.[4] To determine whether puromycin affected the accelerated labeling of basic proteins which concurs with the synthesis of DNA, synchronized cultures were treated with 20 µg/ml of the agent 2 hr after the initiation of DNA synthesis. After exposure to puromycin for 20 min to ensure maximal inhibition of protein synthesis, leucine-C^{14} was added and the incorporated radioactivity measured 2.5 hr later. Table 3 shows that puromycin inhibited the incorporation of leucine-C^{14} into the three isolated bands of basic protein in both the

amethopterin-treated and released cultures. The effect, however, was less than that on the proteins of the whole cells and other cell fractions, suggesting that some of the incorporation of leucine into basic protein may occur by a puromycin-insensitive process, or that the site of synthesis of these proteins may be less accessible to puromycin.

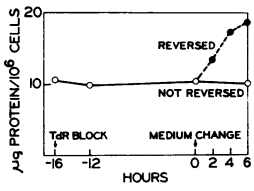

FIG. 5.—Amount of basic nuclear protein in bands 1, 2, and 3 (combined) during a cell cycle synchronized by 16 hr of treatment with TdR (10^{-3} *M*). Reversed cultures transferred to medium free of TdR at 0 hr; nonreversed cultures were kept in the medium containing 10^{-3} *M* TdR. Protein was determined by the method of Oyama and Eagle.[10]

Accumulation of basic proteins in synchronized cultures: While the incorporation of labeled amino acids into the bands of basic proteins implied synthesis of these entities, it seemed important to measure directly their accumulation relative to the replication of DNA. For this purpose the amount of protein in the electrophoretically separated bands was eluted quantitatively and determined at several different points in the cell cycle. In apparent contrast to the incorporation data, cultures in which DNA synthesis was blocked by 10^{-3} *M* TdR maintained a constant level of the three basic proteins during a 24-hr period (Fig. 5). During the 6-hr period after DNA synthesis was initiated, the basic proteins approximately doubled in amount. The accumulation curves for the individual bands show that these isolated proteins responded independently during this interval (Fig. 6). Thus it is evident that the levels of these three basic nuclear proteins in the cell remain constant except for the period of DNA synthesis. At that time they accumulate in much the same way as the DNA. Possible fluctua-

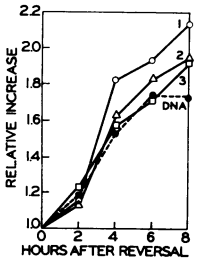

FIG. 6.—Amotun of three electrophoretically isolated basic proteins from HeLa nuclei during the period of DNA synthesis. DNA synthesis was synchronized by 16 hr of treatment with 10^{-6} *M* amethopterin. DNA content of cells was measured by the method of Kissane and Robins;[15] protein measured by the method of Oyama and Eagle.[10]

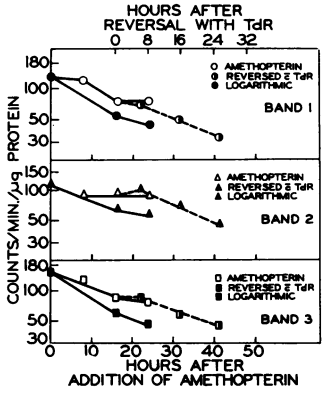

FIG. 7.—Changes of specific activity of three bands of basic protein isolated from HeLa nuclei during logarithmic growth, during inhibition of DNA synthesis by 10^{-6} *M* amethopterin, and during synchronous DNA synthesis. Cultures were labeled with DL-leucine-C^{14} (50 μc/300 ml) for 24 hr (one-generation time), and at zero time were resuspended in fresh, isotope-free medium.

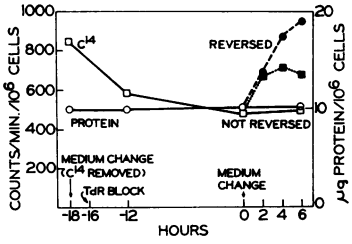

FIG. 8.—Total amount of C^{14} and protein present in basic protein bands 1, 2, and 3 (combined) from HeLa nuclei during a synchronized cell cycle. The cultures were synchronized by double blocking with 10^{-3} *M* TdR.[8] TdR was added for 16 hr, from −40 to −24 hr. The cultures were transferred at −24 hr to fresh medium containing DL-leucine-C^{14} (0.3 μc/ml) and no TdR. At −18 hr the isotope was removed by changing the medium, and at −16 hr 10^{-3} *M* TdR was again added for a 16-hr period. At zero time DNA synthesis was initiated by changing the medium (reversed cultures); part of the cultures (not reversed) remained in medium containing 10^{-3} *M* TdR.

TABLE 3

EFFECT OF PUROMYCIN ON SPECIFIC ACTIVITIES OF HeLa CELL PROTEINS

Stage of cycle	Treatment* ± amethopterin ± puromycin	Incorporation of Leucine (cpm/μg protein†) and Per Cent Inhibition					
		Whole cells	Cytoplasm	Whole nuclei	Bands of Basic Nuclear Proteins 1	2	3
Interphase	+a −p	81	49	17	7.2	6.5	11
	+a +p	8	7	3	3.2	2.9	3.5
		(90%)	(86%)	(82%)	(56%)	(55%)	(68%)
DNA synthesis‡	−a −p	73	48	21	42	33	38
	−a +p	7	4	3	10	10	13
		(90%)	(92%)	(86%)	(76%)	(68%)	(66%)

* 10^{-6} *M* amethopterin present for 21 hr; 20 μg/ml puromycin present for 3 hr.
† Radioactivity incorporated in 2½-hr period from leucine-C^{14} (25 μc/300 ml culture).
‡ Incorporation measured 2½–5 hr after reversal of amethopterin-induced interphase.

tions in levels of individual proteins during mitosis remain to be investigated.

Turnover and conservation of basic proteins during the cell cycle: The incorporation of labeled amino acids without increase in amount implied that the basic proteins were turning over during the period when DNA synthesis was restricted. To assess this possibility, logarithmically growing cultures were prelabeled by exposure to leucine-C^{14} for 24 hr (one growth cycle). The radioactive medium was then replaced with medium containing nonisotopic leucine. The decline in specific activity of the three bands of isolated basic protein was followed over 24 or 40 hr under three growth conditions: continued logarithmic growth, the amethopterin-induced thymidineless state (prevention of DNA synthesis), and the reversal of the thymidineless state (synchronization of DNA synthesis in all cells). The data have been plotted on a semilog scale (Fig. 7). During the first 16 hr of the amethopterin block, the specific activities of the isolated bands declined, and at a somewhat slower rate than in the logarithmically growing cells. This behavior could be expected from the observations illustrated in Figure 1. Surprisingly, during the 6-hr period of DNA synthesis when the proteins increase in amount, the specific activities did not drop precipitously but tended to remain constant. After DNA synthesis was complete and synchronous cell division had begun, specific activity decreased further.

The falling specific activities of the individual bands show more complicated patterns than can be explained by simple and continuous addition of newly made protein, especially in the plateau observed during DNA synthesis. One possible explanation for the relatively constant specific activity of the bands during the period of DNA synthesis is that the high rate of isotope incorporation characteristic of this period (Table 1 and Fig. 3) does not represent net synthesis of protein alone, but involves an additional metabolic process. A more likely explanation is that previously synthesized proteins are mobilized at the time of DNA synthesis, so that they appeared in these bands of basic nuclear protein.

To explore the latter hypothesis, synchronized cultures of HeLa were prelabeled with leucine-C^{14} during the 6-hr interval of DNA synthesis, and the medium was replaced with regular medium containing nonisotopic leucine. After allowing 2 hr for cell division, 10^{-3} *M* TdR was added to block DNA synthesis and resynchronize the cultures for a second nuclear replication cycle.[8] After 16 hr with 10^{-3} *M* TdR, the nucleotide was removed and a second, highly synchronous wave of DNA synthesis occurred. The absolute amount of C^{14} in the electrophoretically isolated basic pro-

teins was determined at different times during the 16-hr block by TdR and the 6-hr interval after its release. As supporting evidence of turnover in the absence of DNA synthesis, the amount of radioactivity fell rapidly during the first 6 hr, although the amount of basic protein remained constant (Fig. 8). The amount of radioactivity in the proteins did not change during the rest of the period of restricted DNA synthesis. A very interesting phenomenon took place when the thymidine block was reversed by changing the medium; in such cultures significantly greater amounts of radioactivity entered the pool of extractable basic proteins (Fig. 9). The amount of protein increased at the same time. Although the values given are for the combined bands, each of the electrophoretically isolated proteins showed these increases.

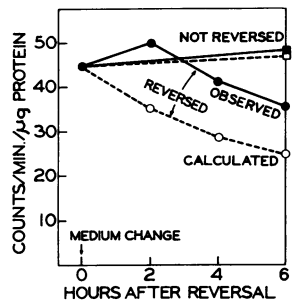

FIG. 9.—Changes in specific activity of basic protein bands 1, 2, and 3 (combined) from HeLa nuclei during DNA synthesis. Treatment and conditions of the culture are the same as in Fig. 8. Solid lines show observed specific activities. Broken lines show specific activities calculated from radioactivity at zero time and amounts of protein at 2, 4, and 6 hr.

The specific activities of the three basic proteins dropped slightly during this interval, but the decline was much less than that predicted from the measured increase in the amount of protein during the same interval (Fig. 9). This observation makes it highly likely that basic proteins of high specific activity were made and held in an unisolatable form during the previous labeling interval and contributed to the extractable bands during nuclear replication.

Discussion.—Any proposed role of nuclear histones in the regulation of genetic expression should account for their complex metabolism as revealed by these data. One facet to be considered is the relationship between the nuclear content of histones and their metabolic stability. Since they accumulate only during DNA replication, the level of acid-extractable histones appears to be coupled closely to the amount of DNA. Nevertheless, a fraction of these proteins turned over during interphase, an interval when the amount per nucleus remained constant. This behavior could be explained if basic proteins near active genes were involved in the metabolism of RNA formed at such sites and were renewed as the RNA turned over. As a corollary, the metabolically stable fraction of acid-extractable histones might well reflect the number of genes in a potentially active state, but not called into action by the prevailing environmental conditions. The reported isolation of histone fractions containing RNA[13, 14] is in accord with the concept that histones might be involved in RNA metabolism.

Of particular interest in our studies were the data implying that a significant portion of the histones was sequestered in an unavailable form during interphase. This fraction contributed to the isolated bands of acid-extractable histones only during nuclear replication. In accord with a concept of histone function related to RNA metabolism, a fraction such as this might be associated with genetically inactive chromatin. The possibility that the sequestered form of the basic proteins might be found in heterochromatin appears worthy of investigation.

Since each of the electrophoretically resolved bands of basic proteins exhibited these three metabolic subfractions, it appears unlikely that histones are specifically

concerned with the recognition of individual genes. It seems more probable that single genes are recognized by other means and that the metabolism of histones is only secondarily affected.

Summary.—Three basic protein bands (histones) were isolated from HeLa cell nuclei by extraction with 0.02 *N* H_2SO_4 and electrophoretic separation on polyacrylamide gel. The metabolism of these proteins was studied during different phases of the cell cycle, using cultures synchronized by treatment with amethopterin or high levels of thymidine. In the absence of DNA synthesis the amount of basic nuclear protein did not change, but isotopic studies showed that a certain fraction underwent turnover. During the period of synchronous DNA synthesis, incorporation into the protein bands increased greatly, and the amount of basic proteins doubled. At that time a form which had been synthesized previously, but was not extracted with the banded nuclear proteins during interphase, contributed to the extractable basic nuclear proteins. The three electrophoretically isolated proteins displayed generally similar metabolic patterns, but differences were observed which imply that they behave independently.

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