The Effect of Inhibition of Deoxyribonucleic Acid Synthesis on Histone Phosphorylation†

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ABSTRACT: The effect on histone phosphorylation of the inhibition of DNA synthesis has been studied under conditions which allow for cell survival upon removal of the metabolic inhibitors. DNA synthesis was rapidly inhibited by using either hydroxyurea or cycloheximide. Histone synthesis continues relatively efficiently in the presence of concentrations of hydroxyurea which almost completely inhibit DNA synthesis, while both DNA and histone synthesis are inhibited effectively by cycloheximide. Hydroxyurea treatment leads to a slow reduction in the rate of phosphorylation of the lysine-rich histone to a value 45% of control after about 5 hr of treatment. The rate of histone phosphate turnover is reduced slightly from the normal 4.5 hr half-life to 6 hr in the presence of hydroxyurea. On the other hand, cycloheximide leads to a more rapid reduction in the rate of phosphorylation and it has attained its final level of 40 of control within 1 hr. The turnover of histone phosphate is dramatically increased in the presence of cycloheximide. The data indicate that DNA synthesis, histone synthesis, and histone phosphorylation are not tightly coupled events.

There is now a considerable body of evidence which supports the notion that histone phosphorylation and cell division are closely correlated (Balhorn *et al.*, 1971, 1972a,b,d). More specifically, we have shown that the bulk of histone phosphorylation in HTC cells occurs during the S phase of the cell cycle, prompting the suggestion that chromosome replication and histone phosphorylation are linked in some way (Balhorn *et al.*, 1972c). However, the rate of histone phosphate hydrolysis in these cells is quite slow (Balhorn *et al.*, 1972e), and it therefore seems likely that the low rate of phosphorylation in phases of the cell cycle other than S phase might be due to unavailability of appropriate sites as they are already phosphorylated. The extensive phosphorylation observed in S phase might well be reflecting the large increase of new sites as a direct consequence of histone synthesis which occurs at this time (Borun *et al.*, 1967; Robbins and Borun, 1967). This conclusion is supported by our recent finding that phosphorylation of newly synthesized histone occurs some 20–30 min after deposition on the chromosome (Oliver *et al.*, 1972).

In order to further assess the interdependence (or otherwise) of DNA replication and histone phosphorylation we have sought to block DNA synthesis using two metabolic inhibitors which function in different ways. On the one hand we have blocked DNA synthesis directly using hydroxyurea, and on the other hand we have utilized the ability of cycloheximide to rapidly and effectively block DNA synthesis secondary to its known property of inhibiting protein synthesis.

A variety of results have been obtained concerning the effect of inhibiting DNA synthesis on the continuation of histone synthesis. In HeLa cells, Young and Hodas (1964) found that the inhibition of DNA synthesis with hydroxyurea did not affect the incorporation of leucine into histone. Similar results were obtained by Bases and Mendez (1970) using X-irradiation, while Spalding *et al.* (1966) found that histone synthesis was not affected immediately but that synthesis began to decrease approximately 2 hr after cessation of DNA synthesis. Sadgopal and Bonner (1969), on the other hand, found that histone synthesis was totally inhibited. In Ehrlich ascites tumor cells Chae *et al.* (1970) showed that cytosine arabinoside resulted in a depression of histone synthesis within 30 min after the inhibition of DNA synthesis, whereas general cytoplasmic protein synthesis continued. Ontko and Moorehead (1964) found that inhibition of DNA synthesis by X-irradiation in these cells *in vivo* did not inhibit histone synthesis. Inhibition of DNA synthesis in tobacco cells with fluorodeoxyuridine was also reported to have no effect upon histone synthesis (Flamm and Birnstiel, 1964). We will argue that in HTC cells, 1 mM hydroxyurea causes a rapid and efficient cessation of DNA synthesis while exerting little effect on histone synthesis, though high dosage (5–10 mM) and extended time periods of treatment do reduce the capacity of cells to make histones.

There is more general agreement concerning the effect of the inhibition of protein synthesis upon the continuation of DNA synthesis (Robbins and Borun, 1967; Mueller *et al.*, 1962; Weiss, 1969). Neither the inhibitor (puromycin, cycloheximide, acetoxycycloheximide, pactomycin, emetine, or pederine) nor the cell type (mouse L cells, HeLa cells, regenerating rat liver, rabbit kidney cortex, mouse intestinal villi, and others) appears to affect the results. In every case, total inhibition of protein synthesis resulted in an almost immediate (<15 min) inhibition in DNA synthesis, although recently Weintraub reported that this amounted to only a 50–60% inhibition in primitive chick erythroblasts (Weintraub, 1972).

Accordingly, the extent of the coupling between DNA replication and histone synthesis and phosphorylation has been examined by inhibiting either DNA synthesis or protein (histone) synthesis and observing the subsequent effects upon histone synthesis, phosphorylation, and histone phosphate turnover. The results suggest that these events, although interrelated, are not so tightly coupled as to be totally interdependent.

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Materials and Methods

Cell Culture. Suspension cultures of HTC cells double approximately every 24 hr at 37° and stay in exponential growth at cell densities between 2×10^5 and 8×10^5 cells/ml. Up to 600 ml of cells were grown in 1-l. erlenmeyer flasks, in modified Swim's medium S77 as previously described (Balhorn *et al.*, 1972c).

Assay of DNA, RNA, and Total Protein Synthesis. Replicate 100-ml cultures of HTC cells in exponential growth at a cell density of $\sim 4.5 \times 10^5$ cells/ml were set up containing hydroxyurea (Sigma Chemical Co.) or an equivalent volume of fresh medium; 10-ml aliquots were pulsed for 15-min intervals with 5 μCi/mol of [^{3}H]thymidine (BNC), 1 μCi/mol of [^{3}H]-uridine (New England Nuclear), or 1 μCi/mol of [^{3}H]-L-amino acid mixture (New England Nuclear). After the pulse, the cells were poured into a prechilled tube containing carrier HTC cells ($\sim 10^6$ cells) and either sufficient unlabeled thymidine to give a final concentration of 10^{-2} M (DNA synthesis) or 10X Medium 199 concentrate (protein synthesis) was added. The cells were sedimented (800*g* for 2 min) and resuspended in 1 ml of redistilled water. The cell suspension was sonicated for 15 sec using a Sonifier Cell Disruptor Model W140; 1 ml of 40% trichloroacetic acid was added, the suspension was again sonicated and the precipitate collected by centrifugation. After resuspending the pellets in 20% trichloroacetic acid by sonication, the suspension was again centrifuged. The pellets were dissolved in sodium hydroxide and 200-μl aliquots were counted in 10 ml of BioSolve.

Total protein synthesis was inhibited under similar culture conditions with 10 μg/ml of cycloheximide (Sigma Chemical Co.); 10-ml aliquots of cells were removed at appropriate intervals, and pulsed with [^{3}H]thymidine or [^{3}H]lysine. DNA and total protein were isolated and counted as described above.

Preparation of ^{3}H- and ^{32}P-Labeled Histone. Radiolabel incorporation was studied variously in the presence of hydroxyurea or cycloheximide or in control cultures. The details of the system used are reported in the text wherever appropriate. Cells were in the concentration range of 4–5 $\times 10^5$ cells/ml. [^{3}H]Lysine labeled histones were isolated from cultures containing 150–200 ml of medium. The cells were labeled with [^{3}H]lysine (1 μCi/ml) for the required time period. After a subsequent centrifugation the cells were frozen. In short term pulses (~20 min) with [^{32}P]phosphate we used 5 μCi/ml and for longer term experiments (2 hr) we have used 3 μCi/ml.

Histone was isolated from the frozen cell pellets as previously described (Balhorn *et al.*, 1972c). The extent of radiolabel incorporation was determined following electrophoretic analysis and counting as described below.

Turnover of F_1 Phosphate in the Absence of DNA or Protein Synthesis. Exponentially growing HTC cells (1800 ml) were labeled with 4 mCi of [^{32}P]phosphate for 3 hr. The cells were then sedimented by centrifugation at 700*g* for 15 min and resuspended in fresh medium without [^{32}P]phosphate. After 30 min, 1 M hydroxyurea was added to a final concentration of 5 mM, and during the following 24 hr 250-ml samples were removed at defined intervals. The samples were chilled in a Dry Ice–acetone bath and centrifuged and the cell pellets rinsed with 0.1 M phosphate buffer (pH 7.4), collected, and immediately frozen.

A similar experiment was performed in which protein synthesis was inhibited with 10 μg/ml of cycloheximide. Samples were removed from cycloheximide-treated cultures (prelabeled with [^{32}P]phosphate in the absence of cycloheximide) and the cells were washed, pelleted, and stored at −20° prior to histone isolation.

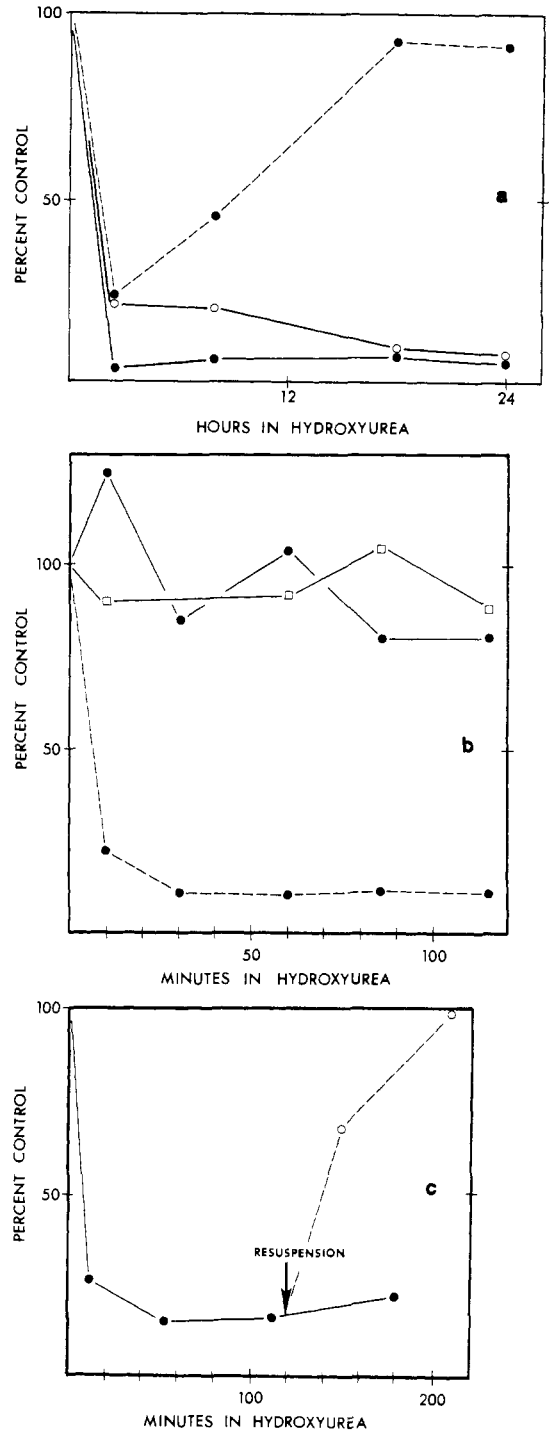

FIGURE 1: Effect of hydroxyurea on DNA, RNA, and protein synthesis in HTC cells. (A) DNA synthesis was assayed by [^{3}H]-thymidine incorporation in the presence of 1 mM (●- - - -●), 5 mM (○—○), and 10 mM (●—●) hydroxyurea. (B) RNA (●—●), DNA (●- - -●), and protein synthesis (□—□) were monitored by pulse labeling with [^{3}H]uridine, [^{3}H]thymidine, or [^{3}H]lysine, respectively, in the presence of 1 mM hydroxyurea. (C) The reversibility of hydroxyurea treatment was determined by pulse labeling cell aliquots with [^{3}H]thymidine before (●—●) and after (○- - -○) resuspension in medium without hydroxyurea.

Electrophoresis and Radioactivity Determination. Electrophoresis of histones on short (9 cm) gels was performed as described previously (Balhorn *et al.*, 1972d). The gels were scanned on a Gilford microdensitometer and the ^{32}P-labeled

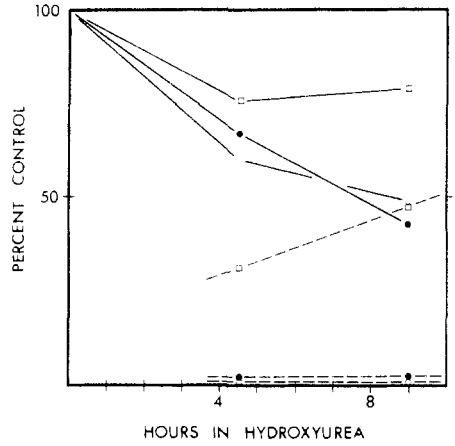

FIGURE 2: Effect of hydroxyurea on histone and DNA synthesis. Cells were incubated in 1 mM (□), 5 mM (●), or 10 mM (○) hydroxyurea and assayed for DNA synthesis (- - - -) or histone synthesis (—) as described in the Materials and Methods.

TABLE I: Histone Synthesis in the Presence of Hydroxyurea.[a]

Hours in HU	HU Concentration (mM)	DNA Synthesis (% control)	% Control Synthesis				
			F_1	F_3	F_{2b}	F_{2a2}	F_{2a1}
4	1	31	54	68	45	52	33
	5	2	32	50	21	27	29
	10	1	38	63	20	24	41
8	1	49	55	84	47	44	64
	5	3	19	42	20	20	12
	10	1	17	24	8	10	9

[a] Cells were pulse labeled for 30 min with 2 μCi/ml of [^{3}H]-lysine at the end of each incubation period with hydroxyurea. Control histone samples at 4 and 8 hr had specific activities of 49,000 and 41,000 cpm/mg, respectively.

lysine-rich histone bands sliced out, dried, and counted on a Biospan planchet counter. Tritium-labeled bands were sliced, digested in 0.3 ml of 30% hydrogen peroxide at 60° for 1 hr, and mixed with 10 ml of Bray's solution (Bray, 1960).

Results

Hydroxyurea Inhibition of DNA Synthesis. The most desirable inhibitor of DNA synthesis would be one that rapidly and specifically inhibited DNA synthesis while allowing RNA and total protein synthesis to continue during the period under study. In addition, the effects of the inhibitor should be reversible; it would be very difficult to interpret the results obtained using an inhibitor that simply killed the cells.

Hydroxyurea appears to meet these qualifications. The level of DNA synthesis in log-phase HTC cells in 1 or 5 mM hydroxyurea is decreased to 12 or 2%, respectively, of the level observed in control cultures (Figure 1a). The inhibition of DNA synthesis is rapid and is complete in <20 min (Figure 1c). Cells in 1 mM hydroxyurea slowly recover the ability to synthesize DNA after about 2 hr, while those in 5 mM hydroxyurea remain inhibited for 24 hr (Figure 1a). Neither RNA synthesis nor total cell protein synthesis is affected to any significant extent (Figure 1b). Hydroxyurea acts reversibly since DNA synthesis returns to normal within 90 min after washing the inhibitor from the cells and resuspending them in fresh medium (Figure 1c). Cytotoxicity is observed only after the cells have remained in the presence of hydroxyurea for longer than 24 hr and accordingly no experiments were continued beyond this point.

Effect of Hydroxyurea on Histone Synthesis. Previous studies have suggested that a fairly close relationship might exist between the continuation of DNA synthesis and histone synthesis. Within the first 2 hr after the addition of 1 mM hydroxyurea, however, [^{3}H]lysine incorporation into F_1 histone continues relatively unaffected. It is only after 9 hr of treatment at higher levels of hydroxyurea that incorporation of [^{3}H]lysine has decreased to 40% of control (Figure 2).

In order to test whether all histone fractions continue to be synthesized, whole histone was isolated after a pulse with [^{3}H]lysine in the presence of 5 mM hydroxyurea. After electrophoretic separation, bands were cut from the gel and counted as described previously. The data presented in Table I show that all histones continue to be synthesized at roughly comparable rates in the presence of hydroxyurea, and that the level of synthesis after 8 hr in the presence of 5 mM hydroxyurea ranges from 42% of control for F_3 to 12% for F_{2a1}. The level of DNA synthesis in 5 and 10 mM hydroxyurea at both time periods was 2% of that of control.

Previous studies have shown that hydroxyurea produces only small changes in the pool size of TTP (Skoog and Nordenskjold, 1971) and thus [^{3}H]thymidine incorporation is in all likelihood a valid measure for the rate of DNA synthesis in both control and hydroxyurea-treated cells.

These results suggest that either a considerable level of additional histone deposition onto the interphase chromosome occurs in the absence of DNA replication or that turnover of histone, an event not normally observed in these cells (Oliver *et al.*, 1972), is initiated.

Cycloheximide Inhibition of Histone and DNA Synthesis. The following experiments utilized cycloheximide, an inhibitor of protein synthesis (and thus indirectly of DNA synthesis). The maximum length of treatment with cycloheximide at the concentration we have employed, which does not effect the viability of the cells upon its subsequent removal, is 8 hr. All the experiments to be described were conducted within this time constraint. At a concentration of 10 μg/ml both total protein synthesis and histone synthesis are decreased to less than 10% of the level observed in control HTC cell cultures within a few minutes after cycloheximide addition (Figure 3). DNA synthesis, as monitored by [^{3}H]thymidine incorporation, likewise decreases to very low levels (less than 10% of the control level) in the same time period. This decrease occurs coincidentally with the cessation of histone (and general protein) synthesis (Figure 3).

Histone Phosphorylation. The continuation of histone synthesis in the presence of hydroxyurea suggests that histone phosphorylation could continue if substrate availability were a limiting factor. On the other hand, if histone phosphorylation requires the actual movement of the chromosome through the replication point (*i.e.*, DNA replication), we might expect a rapid inhibition of histone phosphorylation in both hydroxyurea and cycloheximide-treated cells, since DNA replication is rapidly inhibited by both drugs.

The effects of hydroxyurea or cycloheximide upon the continuation of F_1 phosphorylation appear to be quite similar although significant differences were noted, as is discussed below. Histone phosphorylation, in the absence of DNA replication, continued at significant levels throughout the

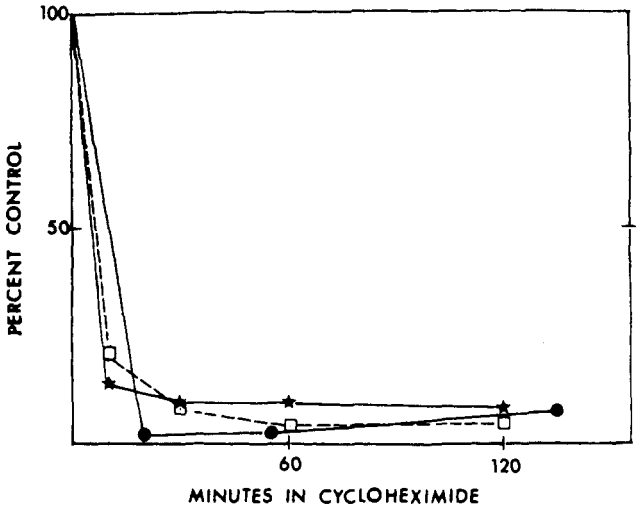

FIGURE 3: Inhibition of histone and DNA synthesis by cycloheximide. HTC cells in exponential growth were treated with 10 μg/ml of cycloheximide and aliquots pulsed with [^{3}H]thymidine or [^{3}H]lysine at appropriate times after introduction of the inhibitor. Histone synthesis was measured by isolation and electrophoresis: (★) overall protein synthesis, (●) histone synthesis, and (□) DNA synthesis.

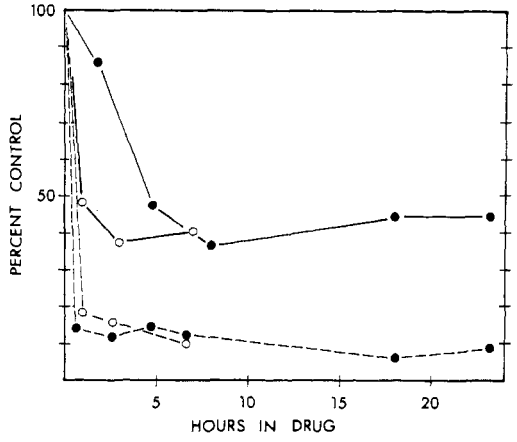

FIGURE 4: Phosphorylation of lysine-rich histone in the presence of inhibitors of DNA synthesis. Phosphorylation of the F_1 histone was studied in a series of short (20 min) pulses of [^{32}P]phosphate at appropriate times after adding the inhibitor: (●- - - - -●) DNA synthesis in 5 mM hydroxyurea; (●—●) histone phosphorylation in hydroxyurea; (○- - - -○) DNA synthesis in cycloheximide; and (○—○) histone phosphorylation in cycloheximide.

period of treatment. During the first 5 hr after the addition of hydroxyurea (Figure 4) or cycloheximide (Figure 4) the level of F_1 phosphorylation decreased until it reached 40–50% of control levels. The decrease in rate of phosphorylation in the presence of hydroxyurea occurs slowly, whereas cycloheximide exerts its effect much more rapidly and phosphorylation is reduced to its final level within about 1 hr after addition of the drug. Extended treatment with either drug failed to lower the rate of phosphorylation further.

Histone Phosphate Turnover. During exponential growth, HTC lysine-rich histone phosphate turns over with a half-life of 4–5 hr (Balhorn *et al.*, 1972e). In order to more accurately assess the effect of the inhibition of DNA synthesis upon F_1-phosphate metabolism, the rate of histone phosphate turnover was determined in hydroxyurea-treated cells (Figure 5). F_1-phosphate turnover during hydroxyurea treatment is slightly lower than in control cultures ($t_{1/2} \sim 6.5$ hr).

Addition of cycloheximide to HTC cells leads to a threefold increase in the rate of turnover of phosphate which became associated with histone during the 2 hr preceding the addition of the drug. This high rate of turnover continues for about 3 hr before returning to the half-life of about 5–6 hr, somewhat lower than that characteristic of untreated cells. Thus in the first 3 hr after addition of cycloheximide approximately 80% of the labeled phosphate is removed. Such an impressive removal of phosphate should be easily detected as a change in the intensity of the electrophoretic bands of phosphohistones. That this is indeed the case is shown in Figure 6a in which we see that a substantial proportion of the F_1-phosphate bands have been removed within 3 hr of cycloheximide treatment. In contrast, in the presence of hydroxyurea the phosphorylated bands decrease only slowly with time (Figure 6b), accurately reflecting the slightly reduced phosphorylation rate and rate of turnover of the phosphate group.

Discussion

The two inhibitors utilized in this study had dramatic, and at times surprising, effects on nucleoprotein metabolism in HTC cells. Cycloheximide rapidly and effectively inhibited histone and DNA synthesis. While this is consistent with several recent reports that continued histone synthesis is a prerequisite for DNA replication, the almost total inhibition of DNA synthesis is more complete than that reported by

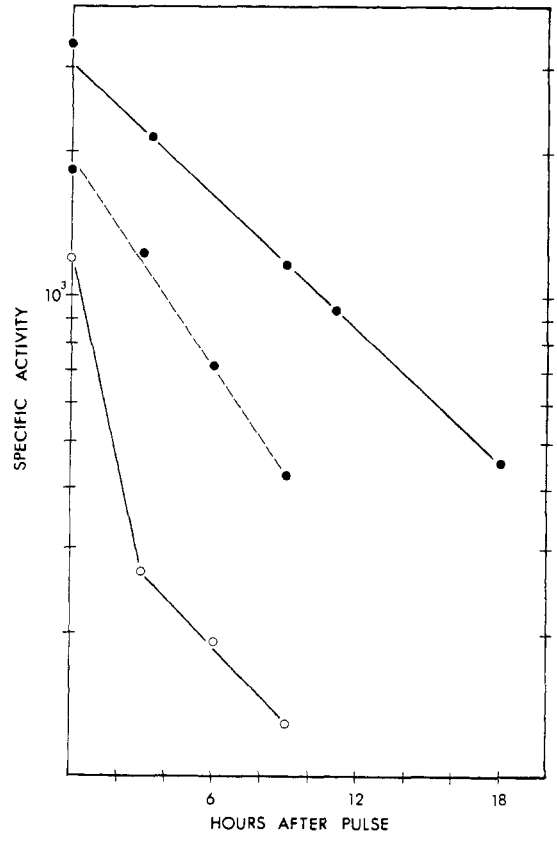

FIGURE 5: Turnover of F_1-associated phosphate. After a 3-hr pulse of [^{32}P]phosphate turnover was measured in (a) control cells (●- - - -●), (b) in the presence of hydroxyurea (5 mM) (●—●), and in the presence of cycloheximide (○—○). Each point is the result of several repeated electrophoretic analyses. The experiment was repeated four times and although absolute numbers vary depending upon amount of isotope used and cell densities, the relative values for the time course are unchanged. Because of slight different conditions between experiments the data were not normalized but an identical trend was noted in all cases.

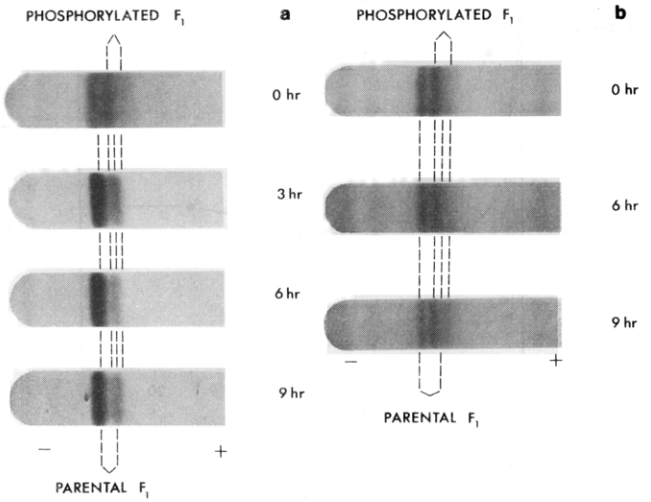

FIGURE 6: Bulk levels of phosphorylated lysine-rich histone. These were measured as a function of time of incubation in (a) cycloheximide (10 μg/ml) and (b) hydroxyurea (5 mM). Electrophoresis was for 80 hr at 4° so that only the lysine-rich histone remained in the gel with its microheterogeneity clearly visible.

other workers. Hydroxyurea effectively inhibits DNA replication and only partly reduces histone synthesis at the highest concentrations used (10 mM). This raises several points: (1) is this newly synthesized histone deposited on the chromosomal material, thus reducing the overall negative charge on the nucleoprotein, or (2) does this newly synthesized histone replace preexisting histone so that the overall charge density of the nucleoprotein complex remains unchanged? It seems likely that whichever of these proposals is correct may have considerable significance for the study of the mechanism of histone deposition in untreated cells. However, it is clear that although the processes of DNA and histone synthesis appear to be highly coupled in HeLa cells (Mueller *et al.*, 1962) this is not the case in HTC cells, where histone synthesis continues after the hydroxyurea-induced cessation of DNA synthesis.

There appears to be no direct dependence of histone phosphorylation on the act of DNA synthesis, though the converse is an attractive possibility and remains to be tested. This conclusion is derived primarily from the observations of phosphorylation in the presence of hydroxyurea where we found only a small decrease in phosphorylation rate even several hours after initiation of the block. Although the rate of phosphorylation does fall more rapidly in the presence of cycloheximide, occurring about 30–60 min after the cessation of DNA synthesis, it seems plausible that the cause of the decrease in rate of phosphorylation is due to the absence of a continuing supply of new substrates (*i.e.*, unphosphorylated lysine-rich histone). The observed rate of phosphorylation which continues at 30–40% of the control rate throughout extended treatment with cycloheximide must reflect continued phosphorylation of those sites on the F_1 histone made available through the action of the phosphatase. The increased rate of phosphate hydrolysis in the presence of cycloheximide may have a trivial origin in an activation of the phosphatase by the drug, may be due to inhibition of histone kinase synthesis, or may be due to inhibition of synthesis of a protein factor which controls the phosphatase. This is currently being studied.

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