*CHANGING PATTERNS OF HISTONE ACETYLATION AND RNA SYNTHESIS IN REGENERATION OF THE LIVER**

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Recent studies of histone metabolism in the cells of higher organisms have indicated that the basic proteins of the nucleus are modified after synthesis by the attachment of acetyl,[1-7] methyl,[8-12] and, possibly, phosphoryl groups.[13-16] The biological significance of these structural modifications of proteins associated with DNA in the chromatin[17] remains an unsolved problem, but there is growing evidence that acetylation of the histones represents one of the earliest chemical events in the process of gene activation for ribonucleic acid synthesis.

The experiments now to be described are concerned with histone acetylation in regenerating liver, a system in which the regenerative response is accompanied by heightened nuclear activity in ribonucleic acid synthesis.[18] Gene activation is known to be a relatively early event in the first cycle of cell division after partial hepatectomy; it has been characterized in terms of the production of "new" species of RNA (demonstrable by DNA-RNA hybridization experiments[19] and by increases in the DNA-template activity of isolated nuclei[20] and chromatin fractions[21-24]). For this reason we have studied acetate incorporation and "turnover" in different histone fractions of normal and regenerating liver, comparing rates of histone acetylation and RNA synthesis at different times. It has been found that the patterns of histone acetylation are strikingly altered soon after partial hepatectomy, and that a peak of acetylation of the "arginine-rich" histones occurs before maximal rates of RNA synthesis are achieved.

Materials and Methods.—Male albino rats of the Sprague-Dawley strain, weighing 250–280 gm and maintained on Purina Chow *ad libitum*, were used throughout the experiments. Partial hepatectomy was performed by excising the left lateral and median lobes as described by Higgins and Anderson.[25] Sham-operated animals, injected with isotopic precursors and killed at corresponding times after the operation, were used as controls.

Acetylation of histones was followed by the intraperitoneal injection of acetic acid (methyl-H^3) (1.5 mc per 100 gm rat weight; sp. act. 377 mc/mmole and 588 mc/mmole). For measurements of histone synthesis, L-lysine-UL-C^{14} (5 μc per 100 gm; sp. act. 208 mc/mmole) was administered. In tests for contamination of histone fractions by acetylated mucopolysaccharides, rats were injected with D-galactose-1-H^3 (60 μc per 100 gm; sp. act. 70 mc/mmole). All isotopic precursors were dissolved in 0.14 *M* NaCl.

At 15 and 60 min (and sometimes 30 min) after the administration of the appropriate isotope, nuclei were isolated from normal and regenerating liver as described elsewhere.[20] The purified nuclear fraction was resuspended in 0.25 *M* sucrose–4 mM $MgCl_2$–0.01 *M* tris–HCl buffer (pH 8.3) (TMS) and centrifuged. The nuclei were washed twice with TMS, once with 0.01 *M* citric acid, and once with 88% ethanol–0.01 *N* HCl. The histones were then extracted from the washed nuclei by two procedures. Total liver histone was prepared by extraction in 0.2 *N* HCl followed by precipitation in acetone. Histones in the precipitate were further purified and fractionated by electrophoresis on cellulose polyacetate membranes as described elsewhere.[4] A fractionation of liver histones was also performed by the method of Johns.[26] The F2a fraction so obtained was further fractionated by the method of Phillips and Johns[27] to yield the F2a1 and F2a2 histones.

These fractions were further purified by electrophoresis on cellulose polyacetate at pH 9.[4] The specific activities of the histones in the electrophoretic bands were determined as described earlier.[4]

The specific activities of the proteins remaining in the nuclei after extraction of the histones were also determined following removal of the nucleic acids in 0.5 *N* perchloric acid (PCA) at 70°C for 20 min and extraction of the lipids with 3:1 ethanol–ether, and ether. Radioactivity was also measured in cytoplasmic ribosomal proteins prepared from the supernatant remaining after sedimentation of the nuclei. The suspension was centrifuged at 14,000 rpm (20,000 × g_{av}) for 10 min to remove mitochondria and other large particulates. Sodium deoxycholate was added to the supernatant to a final concentration of 1.0%, and the ribosomal fraction was then collected by centrifugation at 64,000 rpm (270,000 × g_{av}) for 2 hr. The pellet was resuspended in cold 0.5 *N* PCA, centrifuged, and washed twice with 0.5 *N* PCA, with ethanol, ethanol–ether (3:1), and ether. Nucleic acids were removed in 0.5 *N* PCA at 70°C for 20 min. The remaining protein was measured by biuret analysis[28] and its radioactivity determined before and after acid hydrolysis and steam distillation (to remove volatile H^3-acetate).

The presence of radioactive acetyl groups in the histones was verified by acid hydrolysis and steam distillation of volatile H^3-acetic acid as described earlier.[2, 4] Similar tests of residual nuclear proteins and ribosomal protein fractions indicated that virtually all of their radioactivity was present as amino acids derived from acetate by metabolic conversions in the liver.

In tests for contamination of protein fractions by radioactive polysaccharides, rats were injected with D-galactose-1-H^3 and sacrificed 15 min later. The livers were homogenized and nuclei isolated as described.[20] A portion of the homogenate was precipitated in 10 vol of ethanol and the precipitate washed twice with ethanol, ethanol–ether (3:1), ether, and dried. The specific activities of this fraction, of the residual nuclear proteins, and of the purified histones were determined and expressed in cpm/mg dry weight.

Estimation of acetate "pool" sizes: A rough estimate of acetate "pool" sizes in norma and regenerating liver was made by comparing the specific activities of acetate-labeled lipids following the administration of two levels of the isotopic precursor, H^3-acetate. Assuming that the specific activity of the lipid fraction accurately reflects the average specific activity of the acetate (or acetyl-coenzyme A) "pools" at the time of synthesis, the ratio of the specific activity of the lipid after labeling with 6.0 mc/100 gm to that obtained using 1.5 mc/100 gm can be used to estimate precursor "pool" sizes (by solving the simple simultaneous equations for dilution of administered isotope by unlabeled acetate already in the "pool").

Twelve rats were partially hepatectomized and 12 were sham-operated. Matched pairs were inoculated with H^3-acetate 3–4 hr later (1.5 mc/100 gm rat weight and 6.0 mc/100 gm), and sacrificed at 15, 30, and 60 min. The livers were homogenized in 0.32 *M* sucrose–3 mM $MgCl_2$ and the lipids were extracted as described below for measurement of their specific activities. Livers were homogenized in an Omni-Mixer at full speed for 2 min and the suspension was filtered through flannellette. Aliquots (5.0-ml) were adjusted to 0.5 *N* with PCA and the resulting precipitates were washed twice with cold 0.5 *N* PCA. The lipids were extracted in 10 vol of ethanol at room temperature followed by 10 vol of ethanol–chloroform–ether (2:2:1) for 5 min at 60°C. The extracts were combined, left in the cold overnight, and centrifuged at 3000 × *g* for 5 min to remove a white biuret-positive precipitate. Equal aliquots (1.0-ml) of the lipid extract were delivered to stainless-steel planchets for drying and weighing and to 15 ml of Bray's scintillation mixture[29] for measurement of radioactivity. All samples were counted in triplicate in a Nuclear-Chicago scintillation spectrometer with an efficiency for tritium of 13%. From the observed ratios of lipid activity after labeling with 6.0 mc and 1.5 mc H^3-acetate (2.41 in normal liver and 3.17 in regenerating liver), the "pool" sizes were estimated as 9.0 μmoles acetate/100 gm rat wt in sham-operated animals and 27 μmole/100 gm in regenerating liver at 3–4 hr after partial hepatectomy.

Results.—Acetate incorporation and "turnover" in histones of normal and regenerating liver: The kinetics of histone acetylation have been followed by tracer techniques, measuring the specific activities of different histone fractions at different times following a single injection of acetic acid(methyl-H^3). Normal, sham-operated, and partially hepatectomized animals were inoculated intraperitoneally with the tritiated precursor and killed after 15 and 60 minutes. The liver nuclei were isolated and washed prior to extraction of the histones in 0.2 *N* HCl. Histones in the extract were precipitated and then purified by electrophoresis on cellulose polyacetate membranes at pH 9. This simple procedure separates a leading band of "lysine-rich" histones from a trailing band that contains the "arginine-rich" histone fractions.[4, 12]

The specific activity of the "arginine-rich" band in normal liver is plotted as a function of time in Figure 1. The peak specific activity is observed at 15 minutes;

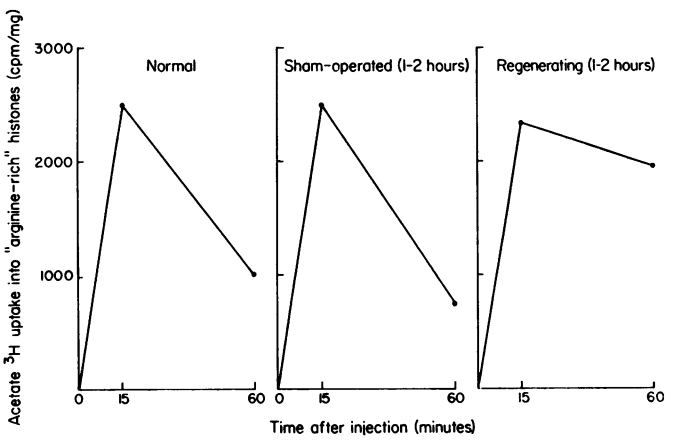

FIG. 1.—A comparison of acetate-H^3 incorporation and "turnover" in the "arginine-rich" histones of normal, sham-operated, and regenerating rat liver (1–2 hr after partial hepatectomy). Note the decreased rate of histone deacetylation in regenerating liver. The uptake data have not been corrected for the threefold increase in the acetate "pool-size" in regenerating liver (see text), a factor which masks the increased acetylation of the histones by dilution of the administered isotopic precursor.

it falls rapidly after that time, so that at 60 minutes only about one third of the acetyl groups originally incorporated in the histones remain. This rapid "turnover" of histone acetyl groups is also observed in sham-operated animals (Fig. 1).

The pattern of acetylation is dramatically altered in regenerating liver. Changes are evident even at one hour after partial hepatectomy (Fig. 1). One of the most striking differences between normal and regenerating liver is the decreased rate of acetyl group "turnover" in regeneration, since most of the acetate incorporated into the histones of the regenerating tissue is retained over the time period studied.

It should be stressed that there is no appreciable labeling of histones with radio-

active amino acids at these early times, so the differences observed are not likely to be due to differential rates of histone synthesis and degradation. The question arises as to whether the differences in acetyl group "turnover" might reflect differences in acetate "pool" sizes rather than altered rates of acetylation and deacetylation of particular histones. If such fluctuations exist, they should also be evident in simultaneous studies of lipid biosynthesis and "turnover," since both lipids and histones would incorporate acetate from the common precursor, acetyl-coenzyme A. To test this, the acetate "pool" sizes were measured by the isotope dilution techniques described under *Methods*. It was found that the "pool" of unlabeled acetate in regenerating liver (3–4 hr after partial hepatectomy) is about three times greater than that observed in sham-operated animals. If one corrects for the dilution of the isotopic precursor in the larger "pool" of the regenerating liver, the true extent of histone acetylation is about three times that shown in Figure 1, and much higher than that in the corresponding controls.

Studies of lipid synthesis using radioactive acetate as a precursor indicate that "turnover" occurs in both normal and regenerating liver. In experiments carried out 3–4 hours after partial hepatectomy, it was found that about half of the radioactivity is lost from the lipid fraction between 15 and 60 minutes, under conditions in which no loss of acetate is observed in the "arginine-rich" histones (Table 1.) Since the "turnover" of acetate-labeled lipids persists during regenera-

TABLE 1. *Acetate-H³ incorporation into components of normal and regenerating liver.*

Conditions of experiment	Time (min)	Acetate Uptake in: Lipids ($\mu\mu$moles/mg)	Arginine-rich histones ($\mu\mu$moles/mg)	Ribosomal proteins ($\mu\mu$moles/mg)
Regenerating liver*	15	27.4	55.0	—
	60	14.0	61.0	0.21
Normal liver†	15	23.4	50.0	—
	60	15.5	27.0	0.18

* Liver at 3–4 hr after partial hepatectomy.
† Liver at 3–4 hr after sham operation.

tion, it seems most likely that the failure to detect a "turnover" of histone acetyl groups indicates lower rates of deacetylation rather than fluctuations in the acetate "pool." This conclusion is supported by other experiments involving cortisol stimulation of histone acetylation and RNA synthesis in rat liver. In hormone-stimulated animals, acetylation of the "arginine-rich" histones is increased and acetyl "turnover" ceases, while no obvious changes in acetate "pool" sizes are observed.[30]

Table 1 also lists data for the incorporation of H^3-acetate into proteins of the ribosomal fraction. It can be seen that acetate uptake into the basic proteins of the ribosome is negligible compared to that occurring in basic proteins of the chromatin. That the incorporation of tritiated acetate into histones truly represents acetylation has been verified in several ways. Apart from the presence of H^3-acetate in purified histone fractions (see below), the acetyl groups have been recovered as H^3-acetic acid by steam distillation of 6 *N*

H_3PO_4 hydrolysates of purified histones. Other tests indicate the absence of contaminating acetylated polysaccharides, an important point because polysaccharides associated with some proteins can be acetylated after synthesis (e.g. ref. 31). Treatment with 16 per cent trichloroacetic acid at 90° for 15 minutes extracts polysaccharides and nucleic acids but does not remove radioactivity from the histone fractions. Moreover, tests for contamination by radioactive polysaccharides proved negative. Rats were injected with D-galactose-1-H^3 and sacrificed 15 minutes later. No counts were detectable in the histones after electrophoresis, although the liver homogenate had incorporated 67.2–73.5 $\mu\mu$moles/mg dry weight, and the nuclear residues after histone extraction contained 0.60–0.80 $\mu\mu$mole/mg dry weight. Though the latter test is perhaps not altogether satisfactory, it does indicate the absence of newly synthesized polysaccharides (containing galactose) in the electrophoretically purified histones.

Variable patterns of histone acetylation at different times during regeneration: Figure 2 summarizes the results of experiments measuring the incorporation

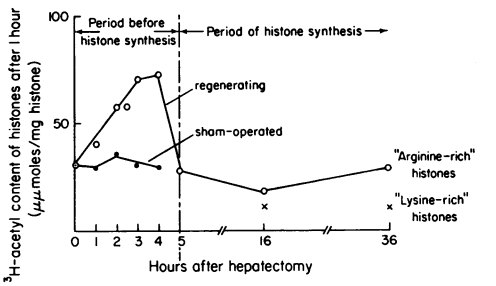

FIG. 2.—A comparison of acetate-H^3 incorporation into the histones of sham-operated and regenerating animals at different intervals after partial hepatectomy. The number of $\mu\mu$moles of acetate incorporated/mg histone in a 60-min labeling period is plotted against the time of regeneration. Note the peak of acetylation of the "arginine-rich" histones at 3–4 hr.

of H^3-acetate into the "arginine-rich" histone band at different intervals after partial hepatectomy. The number of $\mu\mu$moles of acetate incorporated per milligram histone in a 60-minute labeling period is plotted against the time of regeneration. It is clear that an increase in the acetyl content of the "arginine-rich" histones occurs within one hour, and a peak of acetylation occurs between three and four hours. The extent of acetylation then drops abruptly. No such changes are seen in sham-operated animals.

In contrast, the acetylation of the "lysine-rich" histones appears to be a relatively late event in regeneration. Very little uptake is detected in the early stages, but at 16 hours posthepatectomy labeling of the "lysine-rich" fraction becomes appreciable (Fig. 2).

Comparative timing of changes in histone acetylation and DNA-template activity: Studies of the RNA synthetic capacities of nuclei isolated from the liver at different times after partial hepatectomy indicate a great increase in DNA-template activity during the regenerative response.[20] The RNA polymerase assays show that gene activation begins at one to two hours after partial hepatectomy and reaches a plateau at about six hours. Similar findings have recently been reported in liver chromatin fractions.[24]

The timing of gene activation is to be compared with the present results on histone acetylation. Acetylation of the "arginine-rich" fraction reaches its

peak in three to four hours and then declines; this is two hours before maximal rates of RNA synthesis are achieved. Thus, as in the case of human lymphocytes stimulated by phytohemagglutinin,[4] the acetylation of the "arginine-rich" histones appears to precede the increase in nuclear capacity for RNA synthesis.

The results strongly suggest that increased rates of histone acetylation and greater stability of the incorporated acetyl groups are involved in the mechanism of gene activation. It has been proposed that acetylation may modify interactions between histones and DNA templates,[2] thus altering the state and function of the chromatin. It should be stressed, however, that this is an hypothesis, and that direct evidence for acetylation as a control mechanism in RNA synthesis has yet to be obtained.

Comparative acetylation of different histone fractions: The experiments described above have indicated that most of the newly incorporated acetyl groups appear in "arginine-rich" histone fractions prepared by electrophoresis. To study which of the histones becomes labeled and to compare relative rates of acetylation in different histone fractions, the total liver histone was fractionated by the methods of Johns[26] and Phillips and Johns.[27] Rats were injected with H^3-acetic acid as before, and the histones were prepared from nuclei isolated 15 and 60 minutes later. All fractions were further purified by electrophoresis. Acetyl groups were recovered from the fractions after acid hydrolysis and steam distillation.[2, 4]

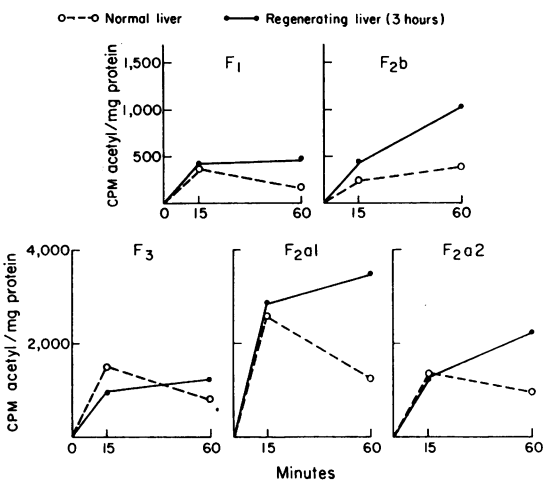

FIG. 3.—Comparative kinetics of acetate-H^3 uptake and "turnover" in different histone fractions of normal and regenerating liver. Note the high level of acetate incorporation into the F2a1 fraction, and the apparent cessation of acetyl group "turnover" in regenerating liver.

Figure 3 summarizes the results in normal liver and in regenerating liver at three hours posthepatectomy. The "lysine-rich" fraction F1 is only slightly acetylated in either case, but acetylation of the moderately "lysine-rich" F2b picks up during regeneration. All the "arginine-rich" fractions have high levels of radioactivity. As before, there is a loss or "turnover" of acetyl groups in all (but F2b) fractions of normal liver, while all regenerating histones retain their activity or become more acetylated over the 60-minute time period.

Particularly high incorporations are seen in the F2a1 and F2a2 histone frac-

tions: 50–60 per cent of the total radioactivity incorporated commonly appears in the F2a1 fraction. The F2a1 contains high amounts of arginine and glycine and it is known to be acetylated on the nitrogen of the N-terminal residue (serine).[32, 33] In accord with this site of acetylation, we have found that all of the radioactivity is stable to treatment with 2 *M* neutral hydroxylamine for one hour, a procedure which would hydrolyze *O*-acetyl but not *N*-acetyl linkages.

In histone fraction F3, alanine is the terminal amino acid, but the sites of acetylation are less clear. After *in vivo* labeling experiments, hydroxylamine treatment removes 55 per cent of the radioactive acetyl groups, a result which indicates multiple sites of acetylation and agrees with the work of Nohara *et al.*[7] who reported *O*-acetylation of the F3 fraction *in vitro*.

Histone and "residual" protein synthesis during regeneration: Experiments using L-lysine-UL-C^{14} as a precursor show little or no histone synthesis during the first four hours after partial hepatectomy. A small amount of histone synthesis is detectable at five hours[12] and the uptake of amino acids proceeds rapidly thereafter.[12, 34–36] Acetylation of the "arginine-rich" fraction remains more or less constant during the period of most active histone synthesis.

Some of the administered H^3-acetate is converted to amino acids (asparate and glutamate) and tritium makes its appearance as a component of amino acids in newly synthesized proteins. (Unlike acetyl groups in histones, this radioactivity cannot be recovered as acetic acid by steam-distillation of acid hydrolysates.)

Studies of the "residual protein" remaining in the nucleus after washing and extraction of the histones indicate very rapid rates of synthesis and "turnover." The high metabolic activity of this fraction has been noted before (e.g., see refs. 37–39). A comparison of the rates of synthesis of the "residual protein" fraction in normal and regenerating liver nuclei is shown in Figure 4. An increased rate of synthesis is evident within one hour after partial hepatectomy, and after three hours a sharp rise occurs which is maintained over the entire time period studied. It is of interest that the synthesis of the nonhistone proteins in regenerating liver nuclei should increase during the period of gene activation, and long before histone synthesis is initiated. It remains to be seen whether the "residual protein" fraction includes nonhistone proteins concerned with the regulation of DNA-template activity at specific genetic loci.

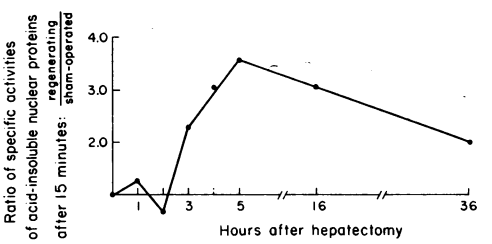

FIG. 4.—Evidence for increased synthesis of the nonhistone proteins of the nucleus following partial hepatectomy. The ratio of the specific activity of the "residual protein" fraction of regenerating liver nuclei to that of "sham-operated" nuclei is plotted as a function of the time after the operation.

Summary.—The acetylation of different histone fractions has been compared in normal and regenerating liver. The rate of incorporation of H^3-acetate into

several "arginine-rich" histones is increased during the early regenerative response after partial hepatectomy. The acetylation reaches a peak at three to four hours and then declines. This is a period of extensive gene activation in which DNA-template activity for RNA synthesis increases, reaching a plateau at about six hours. While rates of histone acetylation are increased, rates of deacetylation are apparently decreased. The results are consistent with the view that acetylation of histones modifies DNA-histone interactions, and the subsequent changes influence the template activity of the chromatin for RNA synthesis.

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