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Accessibility of Newly Synthesized Chromatin to Histone Acetylase*

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Pulse-chase experiments with [^{3}H]lysine-labeled tissue culture cells reveal that newly synthesized nucleosomal histones H2B, H3, H4 (and possibly H2A) in chromatin are more accessible to histone acetylase *in vivo* than are older, pre-existing histones. Thus, when rat hepatoma cells are first pulse-labeled and then incubated in medium containing *n*-butyrate which blocks histone deacetylation, these newly synthesized histones become acetylated to a far greater extent than do their older homologues. As judged by its increased susceptibility to acetylation, the new chromatin matures at a surprisingly slow rate, the estimated half-time for maturation being about 35 min. Based on this data, we suggest that newly synthesized chromatin is in a relatively extended, accessible conformation, and that it slowly returns to a more compact conformation as it matures.

One important difference between prokaryotic and eukaryotic DNA replication (Hand, 1978; DePamphilis and Wassarman, 1980) is that eukaryotic DNA is highly compacted by histones to form nucleosomes, which are then packed together to form higher order chromatin structures (Paulson, 1981; McGhee and Felsenfeld, 1980; Rattner and Hamkalo, 1978; Sedat and Manuelidis, 1978; DuPraw, 1970). Separation of the parental DNA template strands topologically requires that the prereplicative chromatin structure be at least partially decondensed. How these higher order chromatin structures are altered during replication is not known. However, there is some evidence that the predicted chromatin decondensation actually occurs, since newly synthesized DNA has been reported to be unusually susceptible to nuclease digestion (Seale, 1975; Hildebrand and Walters, 1976; Hewish, 1977; Worcel *et al.*, 1978, Levy and Jakob, 1978).

It appears that the histone octamer which forms the core of the nucleosome remains intact throughout DNA replication (Leffak *et al.*, 1977). Moreover, adjacent parental histone octamers are thought to remain as neighbors as the fork passes (Leffak *et al.*, 1977; Riley and Weintraub, 1979), being inherited by one of the two daughter DNA helices. The newly synthesized histones have been reported to be deposited as octamers on the daughter DNA helix whose new strand has been synthesized on the "lagging" side of the replication fork (Seidman *et al.*, 1979). These new histones are probably deposited very soon after the replication fork passes, since electron microscopy of replicating SV40 and Drosophila chromatin reveals nucleosome-like particles on both newly replicated daughter chromatin strands within 500 base pairs of a replication fork (McKnight and Miller, 1977; Cremisi *et al.*, 1978).

In this report, we show that the newly synthesized chromosomal histones made in normally growing tissue culture cells are more accessible to acetylation *in vivo* than are older histones, and suggest that the new histones are in a more open chromatin conformation. Our results indicate that this unusual conformation begins to mature to the normal state immediately, but that the transition is not complete for about 2 h.

EXPERIMENTAL PROCEDURES

A tissue culture cell line derived from a rat hepatoma (HTC cells) was grown on the surface of a 100-mm petri dish to about half-confluence (~5 × 10^6 cells/plate) in Dulbecco's modified Eagle's medium containing 10% calf serum, plus penicillin and streptomycin. The attached cells were rinsed briefly with 2 ml of the same medium without lysine. The cells were then labeled for the desired time in 2 ml of this medium containing 100 to 200 μCi [^{3}H]lysine (~80 Ci/mmol, Amersham). Cessation of labeling and "chase" incubations were carried out as previously described (Cousens *et al.*, 1979), as were all other methods.

RESULTS

Newly synthesized, chromosomally located histones are readily labeled with a brief pulse of [^{3}H]lysine. Due to the small pool of free histones, newly synthesized histones are rapidly incorporated into replicating chromatin (Oliver *et al.*, 1974; Groppi and Coffino, 1980). When the nuclear histones from HTC cells are labeled for 10 min in this way and separated on a Triton-containing acid-urea gel, the pattern of radioactive histones shown in the *leftmost lane* of Fig. 1*B* is obtained. As indicated on the margins, the fastest migrating set of protein bands is histone H4, with various modified H4 forms running slower than the unmodified H4 band (these are the mono-, di-, tri- and tetra-acetylated H4 species). Each of the other three nucleosomal histones similarly migrates as a set of variously modified bands. Newly synthesized histone H4 has been reported to be unusual in being either monoacetylated and monophosphorylated (Ruiz-Carillo *et al.*, 1975) or diacetylated (Jackson *et al.*, 1976). In either case, most of the newly synthesized histone H4 migrates approximately 2 charges slower than unmodified H4. (Labeling times shorter than 10 min result in virtually all of the new H4 appearing as this specifically modified form; data not shown.) Examination of the other histones reveals that new H1, new H2A, and new H2B are apparently unmodified. The same conclusion is reached by analysis on acid-urea gels with or without Triton (see also Ruiz-Carillo *et al.*, 1975; Sealy and Chalkley, 1979).

Although new histone H3 is frequently seen to migrate as a doublet of bands after both types of gel electrophoresis, this doublet is not always observed. Morever, unlike new H4, it does not reproducibly mature with age. Thus, it seems unlikely that the new chromosomal H3 is synthesized in a specially modified form.

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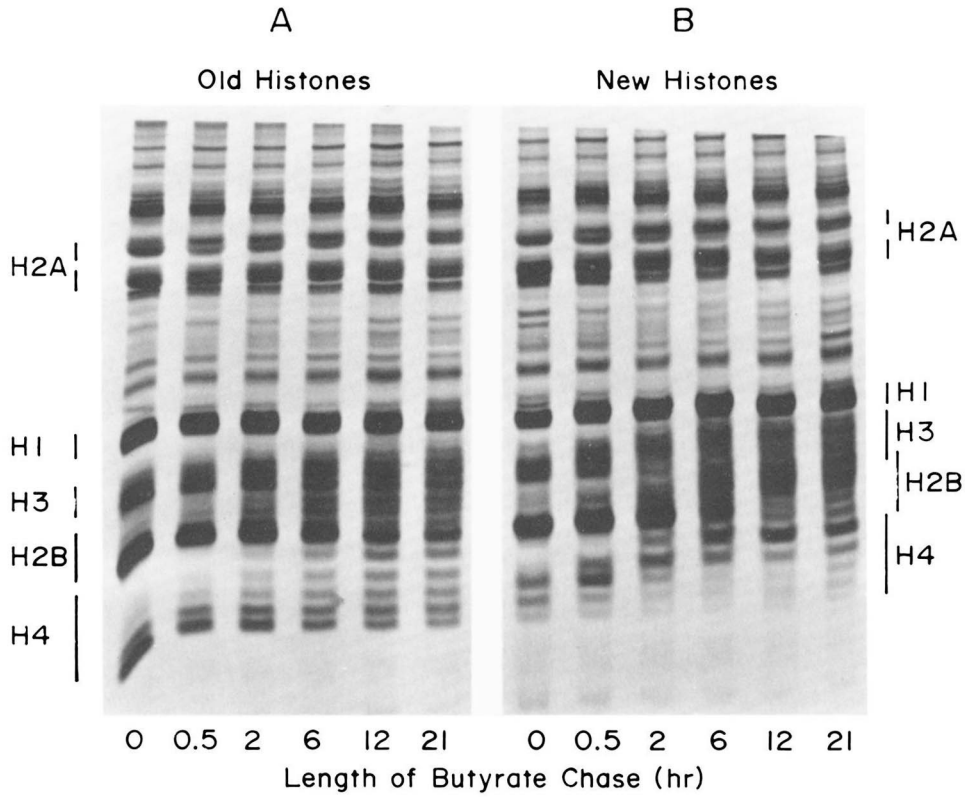

FIG. 1. **Comparison of the kinetics of acetylation of newly synthesized and old histones in HTC cells.** HTC cells growing on the surface of a 100-mm petri dish were pulse-labeled with [^{3}H]lysine for 10 min to label newly synthesized histones (analyzed in the *leftmost lane* of *B*). Similarly labeled cells were further incubated in nonradioactive medium for 2 h to shift the label into "old histones" (analyzed in the *leftmost lane* of *A*). The two types of cells were then incubated, for the various times indicated, in medium which contained 50 mM *n*-butyrate but without radioactive lysine. The histones were isolated and separated by electrophoresis on a Triton acid-urea gel, in which the differently acetylated species of each of the histones can be identified as bands migrating slower than the parental unmodified form. Note also that the two primary sequence variants of H2A (Franklin and Zweidler, 1977) migrate as two separate sets of bands. The fluorograph shown reveals the positions of the ^{3}H-lysine-labeled radioactive bands. The response of the old histones to the butyrate incubation is shown in *A*, and that of the new histones is shown in *B*.

If briefly pulse-labeled cells (those for which the histone pattern has been displayed in the leftmost lane of Fig. 1*B*) are incubated in nonradioactive medium for an additional 2 h, the histone pattern shown in the leftmost lane of Fig. 1*A* is generated. The labeled H4 histone now migrates mostly as unmodified H4, with about 20% of the molecules being monoacetylated, as is commonly found in these cells (Cousens *et al.*, 1979). Similar pulse-chase experiments reveal that the half-time for the disappearance of the characteristically modified, new H4 is about 15 min (see Fig. 4*A*), in agreement with previous results (Ruiz-Carillo *et al.*, 1975; Jackson *et al.*, 1976). As judged by the loss of the special H4 modification, the [^{3}H] lysine-labeled chromatin is fully mature after the 2-h chase used in Fig. 1, and therefore we shall refer to these 2-h-old, radioactively-labeled histones as being in "old" chromatin.

Even though newly synthesized histone H2B (and seemingly new H3) do not migrate differently than the corresponding mature histones on a polyacrylamide gel, both these histones and histone H4 appear to be in a specially accessible form shortly after their deposition onto DNA. This can be seen when the susceptability to *in vivo* acetylation of the [^{3}H] lysine labeled histones in "old" and "new" chromatin is examined by incubating each type of labeled cell with 50 mM *n*-butyrate. At this concentration, butyrate completely blocks the histone deacetylation reaction that otherwise rapidly removes acetyl groups from histone lysines, and thereby allows any acetylated histones being formed to accumulate to a high level (Cousens *et al.*, 1979; Riggs *et al.*, 1977; Sealy and Chalkley, 1978a; Boffa *et al.*, 1978; Candido *et al.*, 1978).

Fig. 1*A* shows the response of the old histones to this butyrate treatment, and Fig. 1*B* shows the response of the new histones. The hours of incubation in *n*-butyrate are indicated beneath each gel lane. An obvious difference between the old and new histones can be seen after 0.5 h in *n*-butyrate, when very little of the old H4 has been acetylated, while much of the new H4 has been. By 2 h, this difference is even more pronounced. The eventual extent of acetylation is much greater for the new histones than for the old histones. For example, in Fig. 2 we plot the percentage of H4 as the tetra-acetylated species *versus* the time in *n*-butyrate. The new histones are seen to contain about 2.7-fold more of the tetra-acetylated species after a prolonged period in butyrate. The fact that the half-time for maximal acetylation (~5 h) is the same for new and old histones suggests that the fraction of accessible histone is much greater in the former group, but that the rate at which each accessible molecule becomes acetylated is about the same. A related observation was made by Sealy and Chalkley (1979), who reported an extensive acetylation of new H4 molecules when cells grown in butyrate were pulse-labeled with lysine.

The above difference in the *in vivo* reactivity to histone acetylase is not due merely to the specially modified nature of the new histone H4, since it is also seen for several other histones. Thus, new histone H2B can be seen to be more readily acetylated than old, preexisting H2B (Fig. 1). After 21 h in *n*-butyrate, for example, the major form of old H2B is the unacetylated species, while the major forms of new H2B are the tri- and tetra-acetylated species. Similarly, after 21 h in *n*-

butyrate, the old histone H3 accumulates little of the tri-acetylated form, whereas this becomes the major species for new H3.

The response of new histone H2A has been variable. In some experiments (for example, the 12-h data in Fig. 1) new H2A reaches a higher acetylation level in *n*-butyrate than does old H2A. Yet, in other experiments (for instance, in Fig. 3*B*) the age of H2A seems to have little or no effect on its extent of acetylation.

For how long does the new chromatin retain its increased susceptibility to acetylation? To determine this lifetime, HTC cells were pulsed with [^{3}H]lysine for 5 min and then further incubated in nonradioactive medium for periods of 0, 10, 20, 30 or 65 min. The histones from these various cells are analyzed by electrophoresis in Fig. 3A. As expected, the newly synthesized form of H4 is predominant initially, but it changes to the unmodified form with time. Each of the above "differently-aged" preparations of labeled cells was then incubated in medium containing 50 mM *n*-butyrate for 16 h. The radioactive histones from each of these butyrate-treated cell populations are displayed in Fig. 3*B*. The data reveal that chromatin maturation begins within 10 min, since 10 min of aging causes increased numbers of the histone molecules to remain at low levels of acetylation after an incubation in *n*-butyrate. But this chromatin maturation is rather slow. Even after 65 min of aging the pulse-labeled histones show an enchanced response to butyrate compared to old histones. In other experiments, we tested for an enhanced susceptibility of these histones to acetylation after longer times and found that this susceptibility could no longer be detected by 2 h (data not shown).

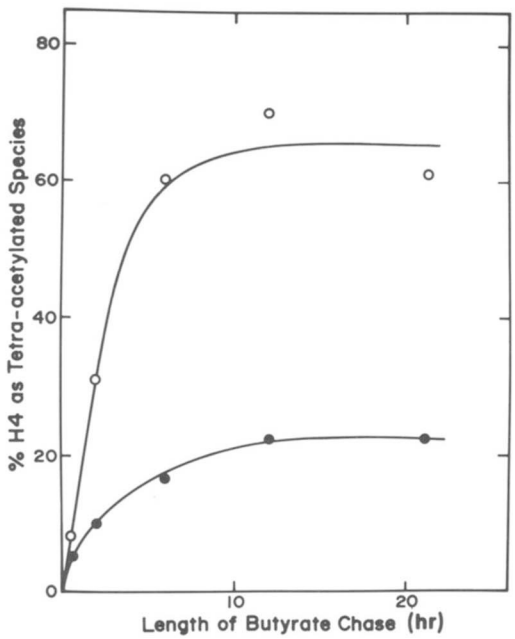

FIG. 2. **Comparison of the rate and extent of acetylation of old and new histones.** The H4 region of the fluorograph in Fig. 1 was scanned on a Joyce-Loebel densitometer. Peak height measurements were used to estimate the amount of label in a given acetylated species. The percentage of total labeled H4 as the tetra-acetylated species for both old (●——●) and new (○——○) histones is plotted *versus* the time the cells were incubated in *n*-butyrate.

Three different maturation rates have been determined by densitometry of the autoradiograph shown in Fig. 3. Two of these are measures for the rate of loss of an enhanced accessibility to acetylation. First, in Fig. 4*A*, the percentage of H4 which becomes tetra-acetylated in *n*-butyrate is plotted *versus* its increase in age. As shown, the ability of H4 to become tetra-acetylated in *n*-butyrate decays with a half-life of at least 38 min. Secondly, in Fig. 4*B*, the rate of formation of the "inaccessible" class of H4 (molecules that are not acetylated in butyrate—see Cousens *et al.*, 1979) is shown. Here, the percent H4 remaining unacetylated after a prolonged butyrate treatment is plotted *versus* the increase in age of the histones. By 65 min, the normally observed level of 26% unacetylated H4 is nearly reached, and the half-time for formation of this inaccessible H4 histone class is at least 32 min. For comparison with these two similar maturation rates, the per cent H4 remaining as the new, di-modified form is also plotted *versus* its increase in age. The new form of H4 is seen to decay more quickly, with a half-life of about 15 min (Fig. 4*A*, and Ruiz-Carillo *et al.*, 1975; Jackson *et al.*, 1976).

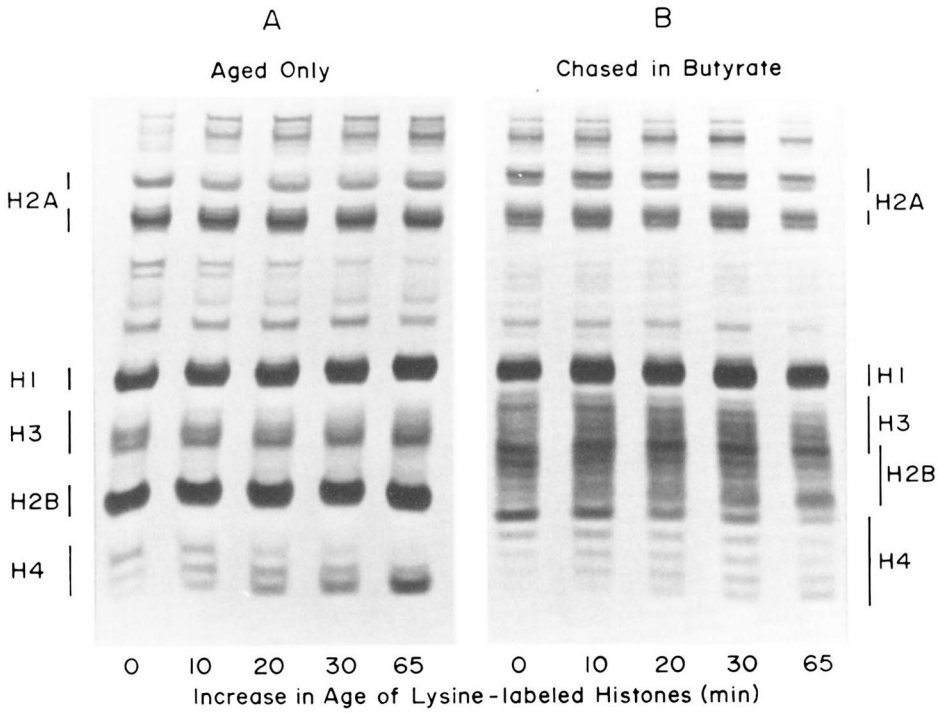

FIG. 3. **The rate of maturation of newly synthesized histones based on enhanced susceptibility to acetylation.** HTC cells were pulse-labeled with [^{3}H]lysine for 5 min, then incubated for the indicated times in unlabeled medium to increase their age, after which histones were isolated and analyzed by electrophoresis on a Triton acid-urea gel (*A*). For each time point, an identical petri dish of aged cells was treated for 16 h in unlabeled medium containing 50 mM *n*-butyrate, prior to an identical histone isolation and electrophoresis (*B*).

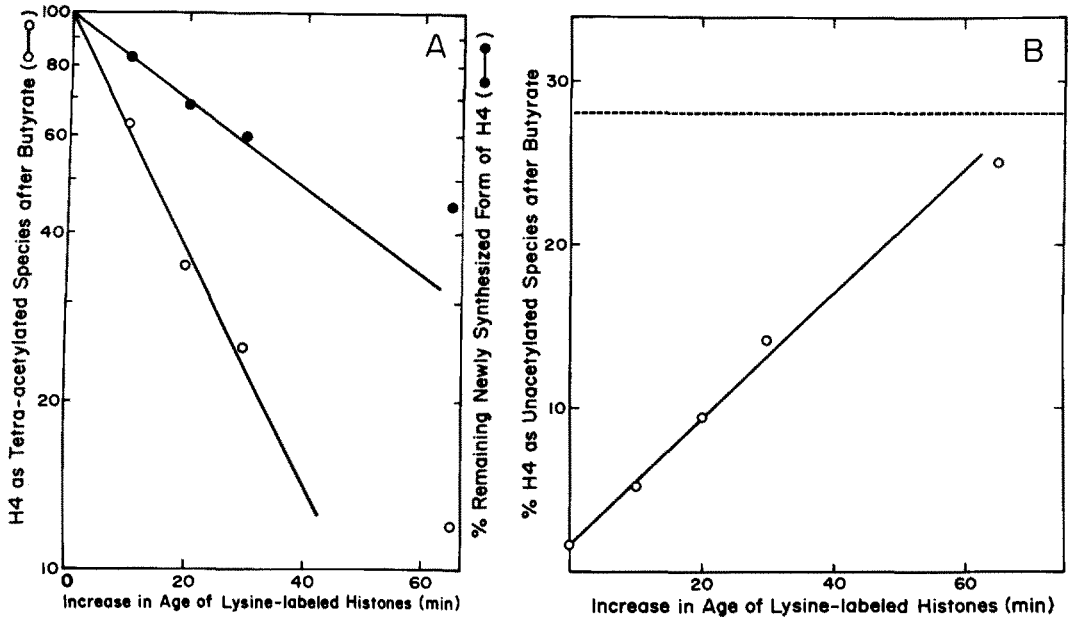

FIG. 4. **The rate of maturation of new histones.** The H4 region of the fluorograph in Fig. 3 was scanned on a Joyce-Loebel densitometer, and the peak heights used to estimate the tritium content of each band. *A*, the relative amount of H4 that becomes tetra-acetylated after 16 h in *n*-butyrate is plotted on a semi-log plot *versus* the increase in age (●——●). Note that after butyrate ~65% of the new H4 (0 min point) is tetra-acetylated, and that this value is normalized to 100 to facilitate these comparisons. Also shown for comparison is the rate of maturation of the new dimodified H4 (see Fig. 3*A*) during the incubation in unlabeled medium without *n*-butyrate (○——○). Here, the percentage of the remaining dimodified form of newly synthesized H4 is plotted *versus* the length of the incubation in unlabeled medium without *n*-butyrate. *B*, the percentage of H4 that remains unacetylated after 16 h in *n*-butyrate is plotted *versus* the length of the "aging" incubation. The *dashed line* indicates the expected total complement of unacetylated H4 after 16 h in butyrate for fully mature histones, derived from our data in Cousens *et al.*, 1979.

DISCUSSION

The increased accessibility of new histones H2B, H3, H4 (and possibly H2A) to acetylation persists over twice as long as does the uniquely modified form in which newly made histone H4 is deposited onto chromatin (Fig. 4). Since most of this form of H4 is gone when the chromatin is still unusually accessible to acetylation, it would seem not to be directly responsible for this effect. However, because each nucleosome contains two molecules of histones H2A, H2B, H3 and H4, a newly assembled nucleosome is expected to contain two H4 molecules in the uniquely modified form. This form loses one or more of its two modifications with a half-life of 15 min. However, half of the nucleosomes will lose modifications on *both* of their H4 molecules in about 27 min (assuming a two hit kinetic process). This latter rate is similar to the rate of nucleosome maturation measured by acetylase accessibility. However, our limited data suggest that this maturation follows single hit rather than two hit kinetics (see Fig. 4*A*). Thus, while the loss of modifications on *both* H4 molecules could be required for the chromatin maturation observed, something else would also appear to be involved. We suggest that the attainment of full chromatin condensation is necessary, a process that may entail a complex series of different molecular changes.

The rate of chromatin maturation measured in our studies is significantly slower than the rate of maturation measured by studies of nuclease sensitivity. By the latter criterion, the new chromatin is fully mature within 10–20 min (Seale, 1975; Hildebrand and Walters, 1976; Hewish, 1977; Worcel *et al.*, 1978; Levy and Jakob, 1978). This suggests that chromatin maturation is a multistep process: the step involving loss of nuclease sensitivity may occur simultaneously with the loss of some of the H4 modifications, but it occurs before the loss of an enhanced accessibility to histone acetylase.

Because nearly all of the newly synthesized chromatin can be readily acetylated if butyrate is added shortly after chromatin synthesis, even the normally "inaccessible" class of nucleosomes (Cousens *et al.*, 1979; Garcea and Alberts, 1980) must be temporarily accessible to the histone acetylase(s). Thus the factor(s) responsible for maintaining the inaccessible state are apparently disrupted during replication. During chromatin maturation, the appropriate structure and controls on acetylation are then presumably re-established differently for different regions of the chromatin. This includes the re-establishment of the special conformation of chromatin on active genes that is responsible for its abnormally DNase I-sensitive state (Weintraub and Groudine, 1976; Garel and Axel, 1976). Since this process has been reported to require only 3 min (Weintraub, 1979), it must operate on the new, immature chromatin studied in this report.

Although we have only monitored the accessibility of chromatin to acetylation, it seems quite likely that similar differences in new chromatin accessibility apply generally to many other nuclear events as well. For example, the fact that the newly replicated chromatin does not reach a highly acetylated state in the absence of *n*-butyrate (see Fig. 3*A*) suggests that its temporarily increased rate of acetylation is balanced by an increased accessibility to the histone deacetylase enzymes(s).

An advantage of our experiments over other types of measurements that minotor the state of new chromatin is that our measurements are made *in vivo* by monitoring chromatin accessibility to an endogenous, nuclear enzyme. The *in vivo* nature of the experiments avoids potential artifacts that might be caused by the isolation of nuclei or chromatin. Although it could be argued that the use of a drug, *n*-butyrate, is creating distorting effects, this drug is added only *after* the histones have been allowed to age. Thus the differences between new and old chromatin are established under nonperturbed cellular conditions.

It is worth noting that the increased extent of acetylation of new chromatin is revealed only after a prolonged incubation in butyrate, reaching maximal acetylation after 6 h or more (Fig. 2). Yet, in the absence of butyrate, these new histones would have been mature by 2 h, and would have then responded to *n*-butyrate as old histones. One might expect, then, that both new and old histones would mostly respond to prolonged *n*-butyrate as do old histones. Since this is not the case, it appears that the new histones stop their maturation process very soon after the *n*-butyrate has been added. One possibility is that the acetylation induced during the early stage of *n*-butyrate treatment maintains the highly accessible state of the chromatin. This predicts that nucleosomes containing one or more acetylated histones will be unusually accessible to further acetylation in *n*-butyrate (Cousens *et al.*, 1979).

In summary, the results presented here strongly suggest that the nucleosomal histones H2B, H3, H4 (and possibly H2A) in newly synthesized chromatin are unusually accessible to histone acetylase *in vivo*. Significantly, the fact that this effect is seen on newly synthesized histone H2B, which has a newly synthesized form that is apparently identical to its mature form, strongly suggests that the difference in reactivity between old and new histones is due to a difference in some general feature of chromosome structure. The maturation process might involve either changes in the conformation of the new nucleosomes (Gordon *et al.*, 1978), a gain or a loss of the binding of some special non-histone protein(s) (Weisbrod and Weintraub, 1979), decreased involvement with the nuclear matrix (Pardoll *et al.*, 1980) or even delayed DNA (Razin and Riggs, 1980) or histone methylation (Thomas *et al.*, 1975). Alternatively, the decreased accessibility of the histones with time might result simply from a slow higher order folding of the chromatin into its mature conformation.

Our findings are relevant to several experiments which have shown that chromatin from *n*-butyrate-treated cells is more accessible to DNase I than normal chromatin (Simpson, 1978; Nelson *et al.*, 1978; Vidali *et al.*, 1978; Perry and Chalkley, 1981) and to a variety of other experiments involving *n*-butyrate (Whitlock *et al.*, 1980; Dobson and Ingram, 1980; Levy-Wilson, 1981). Histone and DNA synthesis continue for several hours in *n*-butyrate (Sealy and Chalkley, 1979; Hagopian *et al.*, 1977)[1] and from the data of Hagopian *et al.* (1977), we estimate that ~⅛ of the total histone will be newly synthesized during an extended period in *n*-butyrate (~20 h). Since these newly synthesized histones will preferentially reach high levels of acetylation, a substantial fraction of the highly acetylated histones obtained in the presence of *n*-butyrate will normally be derived from them. Therefore, as also noted by Sealy and Chalkley (1978b), it is possible that some of the differences between normal and hyperacetylated chromatin seen in the above studies may reflect the special structure of new chromatin, instead of a special structure caused by the acetylation of old nucleosomes.

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