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EFFECT OF SODIUM BUTYRATE ON CHROMATIN STRUCTURE

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Summary : Sodium butyrate is able to modify gene expression. It was then interesting to study its effect on chromatin structure. Butyrate modified the accessibility of chromatin from HTC cells to micrococcal nuclease : chromatin was digested to a higher rate until 1.5 % of the DNA was rendered acid soluble, in longer digestion it was attacked to a lesser extent than chromatin from untreated cells. When digestion was prolonged, both chromatins were equally digested. The released fragments were submitted to sucrose gradient analysis. The results confirmed the previous observations, in addition it showed that the mono- and dimers of nucleosomes preferentially originated from butyrate-treated cells, while the larger fragments were released from untreated cells. The increase in accessibility of a small part of chromatin induced by butyrate could be correlated with the effect of this compound on gene expression.

Butyrate is able to induce differentiated function in various cell types (1, 2). It is likely that it modifies the structure of chromatin. It has been suggested that butyrate acts by inhibiting histone deacetylase, since in its presence histone H3 and H4 were found hyperacetylated (3-5). When chromatin was attacked by DNase I under conditions in which active genes were preferentially digested, the released histone molecules were found enriched in acetylated forms (4, 6).

In order to establish whether butyrate is able to modify chromatin structure, we have studied the effect of this compound on the accessibility of HTC cell chromatin to micrococcal nuclease under very mild attack conditions since it has been shown that the active parts of chromatin are preferentially digested by this enzyme (7, 8). We have also determined the size of the fragments released by the nuclease attack.

MATERIALS AND METHODS

Cell culture and labelling : HTC cells were grown at 37° in suspension in SWIM's 77 medium supplemented with 10 % newborn calf serum as described by Hershko and Tomkins (9). When indicated, cells were cultured for 24 hrs in the presence of 5 mM sodium butyrate. The hyperacetylation of histones was

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controlled by polyacrylamide gels performed according to Panyim and Chalkley (10) as modified by Shaw and Huang (11). The labelling of DNA was obtained by culture of the cells in the presence of 10 µCi/ml of ^{3}H thymidine (S.A. 25 Ci/mmole, CEA, Saclay, France) before the addition of sodium butyrate, the labelling of control cells was obtained with 0.1 µCi/ml of ^{14}C thymidine (S.A. 50 µCi/mmole, CEA, Saclay, France). An equal number, 6.10^{8}, of ^{3}H butyrate-treated and ^{14}C control cells were mixed and the nuclei prepared as previously described (12).

Micrococcal nuclease digestion : Chromatin from 100 A_{260}/ml nuclei was digested with 100-200 U/ml of micrococcal nuclease (Worthington, N.Y., USA) according to Shaw et al. (13). After addition of EDTA to a final 2 mM concentration, the percentage of DNA rendered acid soluble was measured at 260 nm after addition of perchloric acid and removal of the precipitate by centrifugation of 3,000 g for 10 min. The material released by nuclease treatment was studied after removal of undigested chromatin and membranes by 10 min of centrifugation at 3,000 g. The radioactivity was measured on aliquots of the total supernatant or in the supernatant material precipitate by 20 % trichloroacetic acid.

Sucrose gradient fractionation : The released chromatin fragments in 0.3 ml volume were layered on a 5-20 % sucrose linear gradient in 10 mM Tris cacodylate buffer pH 7.3, 0.7 mM EDTA and 1 mM β mercaptoethanol and centrifuged at 21,000 rpm for 16 hrs in a SW 41 Beckman rotor. Fractions of 0.3 ml were collected. The ^{3}H and ^{14}C radioactivities were measured in each sample. Absorbance at 260 nm was automatically recorded.

In all cases an additional experiment was performed at zero time, before the addition of the nuclease, and the results obtained were substracted from those obtained at the indicated times.

RESULTS AND DISCUSSION

Effect of sodium butyrate on chromatin digestion by micrococcal nuclease

In order to study the effect of butyrate on the accessibility of chromatin to nuclease attack, we have compared the kinetics of digestion by 100 U/ml of micrococcal nuclease at 37° of nuclei from HTC cells cultured for 24 hrs in the presence of 5 mM sodium butyrate with the kinetics of digestion of control cell nuclei. In butyrate-treated cells a larger amount of DNA was rendered acid soluble after 1-2 min of digestion, it corresponded to approximately 1.5 % of the total DNA (fig. 1). Afterwards DNA from untreated cells seemed to be attacked to a greater extent. After longer digestion, the effect of the nuclease was not significantly different in treated and control cells. Since butyrate alters DNA synthesis, the question arises whether the observed effect would not simply be due to the arrest of cell proliferation. It led us to culture HTC cells in the absence of serum which prevents cell growth. Fig. 1 shows that the most accessible parts of chromatin

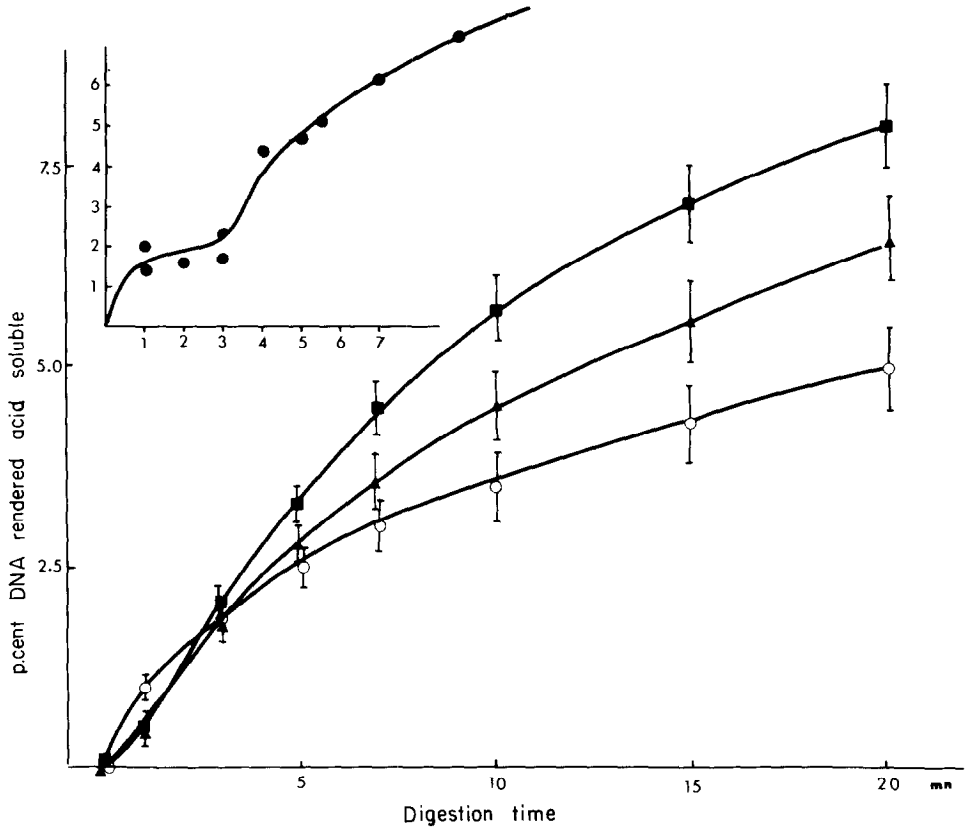

Figure 1. Kinetics of digestion by micrococcal nuclease of DNA from butyrate-treated and control HTC cells. Nuclei from butyrate-treated (o—o), from control growing cells (■—■) and from control cells cultured in the absence of serum (▲—▲), at 100 A_{260} concentrations were separately digested by 100 U/ml of micrococcal nuclease at 37° for various times. Perchloric acid to a final 4 % concentration was added. Insoluble material was discarded by centrifugation and the soluble material estimated at 260 nm. The same experiment was performed on nuclei from a mixture of equal amounts of butyrate-treated and control cells (●—●).

were digested to the same extent as in growing control cells. It is then likely that the increase in nuclease accessibility in butyrate treated cells is not due to the effect of this compound on DNA synthesis. The lower rate of digestion observed in the less accessible parts of chromatin in butyrate-treated cells may be partly due to the arrest of cell growth. However the mechanism by which butyrate inhibits cell proliferation is not presently understood.

The effect of butyrate was confirmed and extended by the use of the double labelling method on a mixture of butyrate-treated and control cells. The re-

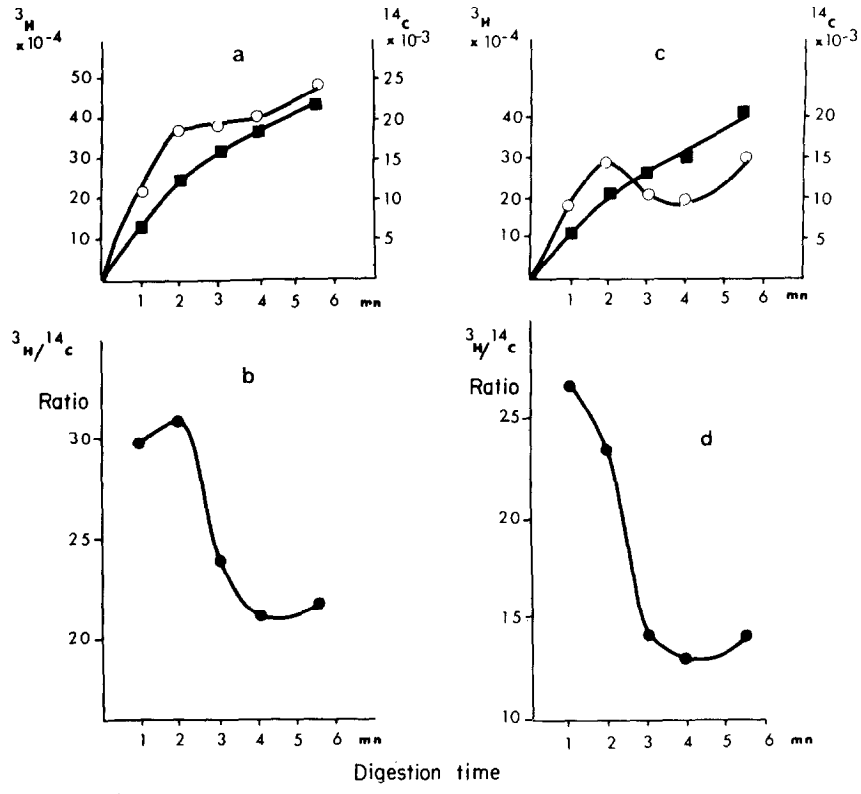

Figure 2. Kinetics of digestion by micrococcal nuclease of labelled DNA from a mixture of butyrate-treated and control cells. DNA from butyrate-treated cells was labelled with ^{3}H thymidine (o—o), DNA from control cells was labelled with ^{14}C thymidine (■—■). Samples of each cell type were mixed. The $^{3}H/^{14}C$ ratio was 20. The nuclei were prepared and submitted to 200 U/ml of micrococcal nuclease at 37° for various times. The ^{3}H and ^{14}C radioactivities were measured in the supernatant after removal of nuclear membrane and undigested chromatin (fig. 2a), the $^{3}H/^{14}C$ ratio was determined for each digestion time (fig. 2b). Trichloroacetic acid was added to the supernatant, the ^{3}H and ^{14}C radioactivities measured in the insoluble material (fig. 2c), the $^{3}H/^{14}C$ ratio determined for each digestion time (fig. 2d).

leased ^{14}C radioactivity (control cells) increased progressively for at least 6 min of digestion, while the released ^{3}H radioactivity (treated cells) increased at a faster rate during the 2 first min (fig. 2a). The $^{3}H/^{14}C$ ratio was measured under the same conditions (fig. 2b). The ratio was 20 in the mixture of cells. In the released fragments the ratio was very much higher during the 2 first min of digestion, indicating a preferential attack of the chromatin from treated cells, then it strongly decreased but remained higher than 20. It indicates that during that time, more chromatin from control cells was digested, although the majority of the released fragments originated from the butyrate-treated cells.

In order to have a further insight on the released fragments, we separated the particulate fragments by trichloroacetic precipitation. Fig. 2c shows that the amount of the fragments released from butyrate-treated cells increased during the 2 first min of digestion, then the radioactivity decreased for the 2 next min, which, by comparison with fig. 2a, indicates that between the 2nd and the 4th min of digestion, the nuclease has essentially digested the linker DNA from the fragments previously released from the butyrate-treated cells and rendered it acid soluble. The $^{3}H/^{14}C$ ratio strongly decreased during the 3 first min of digestion, and became lower than 20 (fig. 2d). It shows that after 2 min of digestion, micrococcal nuclease preferentially attacked the control cells, while it digested the linker DNA from the fragments released from the butyrate-treated cells.

Sucrose gradient fractionation of the chromatin fragments released by micrococcal nuclease digestion

Nuclei prepared from a mixture of double labelled butyrate-treated and control cells were digested for various times with 200 U/ml of micrococcal nuclease. Aliquots were fractionated on a sucrose linear gradient. Absorbance at 260 nm was automatically recorded. it produced the familiar pattern of DNA fragments seen in digestion from various types of chromatin (14). The $^{3}H/^{14}C$ ratio was measured in each fraction.

Fig. 3 shows : (a) when the digestion proceeded, the radioactivity ratio, which was high in the beginning, became progressively lower, which indicates that the nuclease has first digested chromatin from butyrate-treated cells and afterwards, more and more chromatin from control cells ; (b) in each sample, except the last, the ratio was higher in the small fragments than in the larger ones, it progressively decreased from the top to the bottom of the gradient, which means that the smallest fragments preferentially originated from the butyrate-treated cells, while the larger ones were essentially from the control cells. In order to determine the effect of each additional min of digestion, we substracted point by point the ^{14}C and the ^{3}H radio-

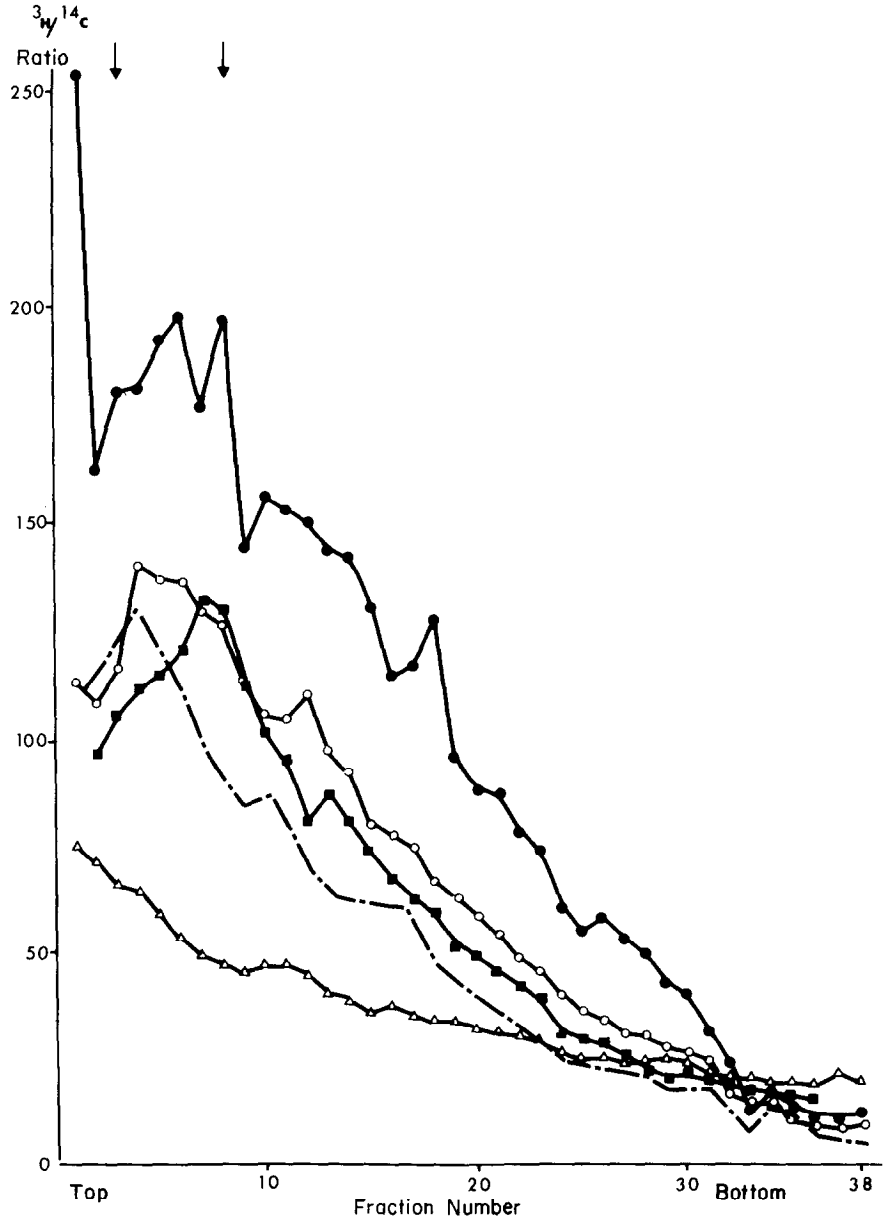

Figure 3. Sucrose gradient fractionation of chromatin fragments obtained by micrococcal nuclease digestion. Nuclei, 100 A_{260}/ml, were digested by 200 U/ml of micrococcal nuclease for various times at 37° : 1 min (●—●), 2 min (o—o), 3 min (■—■), 4 min (–.–.), 5.5 min (Δ—Δ). The released material was analysed by centrifugation on a 5-20 % sucrose linear gradient (centrifugation for 16 hrs at 21,000 rpm in a SW 41 Beckman rotor). The $^3H/^{14}C$ ratio was measured in each 0.3 ml collected fraction. Hemoglobin and catalase were used as sedimentation markers.

activities after a certain time of nuclease digestion from the radioactivities of the corresponding fraction after a longer time of digestion (fig. 4). The ratio was very high in the mono- and dimers of nucleosomes released during

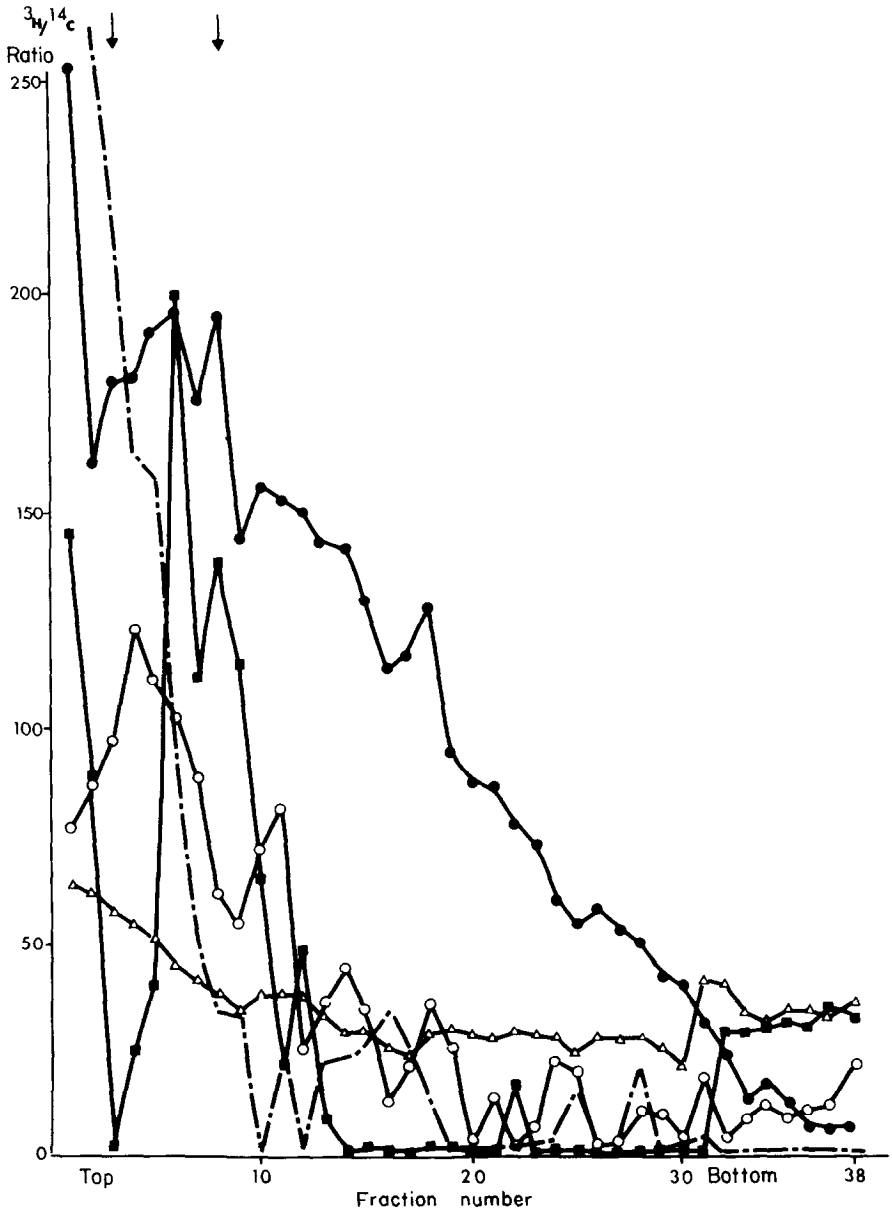

Figure 4. Sucrose gradient fractionation of chromatin fragments released during each interval of time of micrococcal nuclease digestion. The curves were drawn from the data shown in fig. 3 by substraction point by point in each fraction of the radioactivities measured after a certain time of nuclease digestion from the radioactivities of the corresponding fraction after a longer time of digestion : (1-0) min (●——●) ;(2-1) min (o——o) ; (3-2) min (■——■) ;(4-3) min (—.—.—) ;(5.5-4) min (Δ——Δ).

the 2 first min of digestion. During the 4th min of digestion, all the fragments, except those smaller than nucleosomes, originated from the control cells Between the 4th and the 5.5th min, both chromatins were digested at approxima-

tely similar rates (ratio equal to 20). These experiments clearly showed that the mono- and dimers from nucleosomes released during short nuclease attack originated from butyrate-treated cells.

This observation was confirmed by the analysis by electrophoresis performed according to Nichols et al. (15) of the chromatin fragments released after 2.5 min of digestion by micrococcal nuclease, the $^{3}H/^{14}C$ ratio of the mono- and dimers of nucleosomes was higher than 20, while the ratio in the larger fragments was much lower.

In this work we showed that butyrate treatment induces modifications in chromatin structure in such a way that a very small part of it becomes more accessible to nuclease digestion, while another part is less accessible than the chromatin of control cells. The major part of chromatin is equally digested in treated and control cells. This result is in apparent contradiction with the observation made by Mathis et al. (16) who did not find any effect of butyrate on the accessibility of HeLa cell chromatin to micrococcal nuclease. However these authors used much higher concentrations of nuclease and the digestion time was much longer. We also found that in these conditions no effect of butyrate can be detected.

The activation by butyrate treatment of the release of mono- and dinucleosomes after mild nuclease attack is consistent with the observation that butyrate is able to induce differentiated functions, since these fragments are very likely to originate at least partly from active genes (7, 8, 14).

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