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Butyrate Inhibits Mouse Fibroblasts at a Control Point in the G1 Phase

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Butyrate block 3T6 cells in the G1 phase of the cell cycle approximately 5–6 h prior to the start of the S phase. Serum factors are required before as well as after the butyrate-sensitive steps in G1 in order to allow cells to start DNA synthesis. 3T6 cells infected with SV40 or with polyoma virus are also blocked at the same stage in G1 in the presence of the fatty acid. However, events before as well as after the butyrate-sensitive step do not require serum in virus-infected cells. The sensitivity of the initiation of cellular DNA synthesis to increasing concentrations of butyrate is the same for serum-stimulated or for virus-infected cells. A similar and parallel effect on DNA synthesis is observed if cells are incubated in the presence of very small amounts of cycloheximide. After release of the cycloheximide-induced G1 arrest about 4–6 h have to pass before cells enter the S phase. Cells stably transformed by SV40 are considerably more resistant to low cycloheximide concentrations and to butyrate. These data are discussed in the light of the hypothesis that both low concentrations of cycloheximide and sodium butyrate block cells at a control point in G1 by interference with the synthesis of one or more rapidly turning over, cell cycle-specific proteins.

Key words: serum stimulation, SV40, polyoma virus, DNA synthesis, 3T6 mouse fibroblasts, cell cycle

Various effects of sodium butyrate on animal cells, seemingly dependent on the cell type, have been observed. The substance was shown to induce terminal differentiation in several types of cultured cells [1–4] as well as to inhibit DNA synthesis [5–10] and to cause cells to accumulate in the G1 phase of the cell cycle [10, 11]. On the other hand, McKnight et al [12] reported that the fatty acid inhibits the estrogen-mediated induction of ovalbumin and transferrin by blocking the production of mRNA for these proteins. These effects of butyrate contrast with the observation that the substance appears not to influence the rate of overall RNA and protein synthesis [13, 14]. The biochemical reaction following most closely the addition of butyrate in all cell systems so far tested is an inhibition of a histone deacetylase which results in the accumulation of acetylated forms of histones [11, 15–20]. It is not known, however,

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how far this effect of sodium butyrate is related to the above mentioned actions of the substance.

Our main interest is the regulation of DNA replication, especially that of its initation. We are therefore studying the sequence of events to be passed through by serum-stimulated or papovavirus-infected mouse cells in order to proceed from the G1 to the S phase of the cell cycle. Here we describe the effect of sodium butyrate on the initation of DNA synthesis in serum-starved, resting mouse 3T6 fibroblasts following the addition of serum or an infection with polyoma virus or SV40.

METHODS

Cell Culture

Swiss 3T6 mouse fibroblasts were grown in Dulbecco's modified Eagle's medium containing 5% calf serum. For growth arrest, medium was removed from semiconfluent Petri dishes (55 mm diameter) and replaced by medium containing 0.5% serum. After 2 days in low serum, cells had become confluent and less than 1% of them synthesized DNA. Upon addition of serum to a concentration of 5%, DNA synthesis started after a lag period of approximately 12 h. Infection of resting cells with wild-type strains of SV40 or polyoma virus (at a multiplicity of infection of about 20) was carried out as described by Wawra et al [21]. Butyrate or cycloheximide was added to cultures that were just confluent but not dense (around 2.5–2.8 $\times$ 10^4 cells/cm^2) and was left there for 24 h. Cells were released from the butyrate block by removing the medium, washing the cells once, and then adding fresh medium containing the desired amount of serum (as indicated in the figure legends) but no butyrate. Each experiment was carried out at least twice.

Measurement of DNA, RNA, and Protein Synthesis

These were measured at 37°C by 20 min incorporation of radioactive precursors into cells grown in 55-mm Petri dishes (DNA: [^{3}H]-thymidine; RNA: 5-[^{3}H]-uridine; protein: [^{3}H]-leucine, final radioactivity was in all cases 1 μCi/ml medium). Cells were processed as described [21]. Radioactively labeled macromolecules were precipitated with 10% TCA. In case of leucine incorporation the precipitate was heated for 10 min at 90°C before filtration. DNA-sythesizing capacity of cells was also determined by autoradiography as described earlier [21].

RESULTS

Addition of sodium butyrate (final concentration 7 mmol/liter) to logarithmically growing 3T6 mouse fibroblasts resulted in a decline in the rate of DNA synthesis after a lag period of about 5 h (Table I). The minimum was reached after 20–24 h when cells accumulated in the G1 phase from which they could be released by removal of the drug (see below). Figure 1 summarizes the effect of the addition of increasing amounts of butyrate to cells that were stimulated to synthesize DNA by serum or by infection with polyoma virus or SV40. When the drug was added 2 h after serum addition or after infection a dose-dependent inhibition of DNA replication could be measured 24 h after setting the mitogenic stimuli. The fatty acid has no immediate effect on DNA synthesis of cells that have already reached the beginning of the S phase (Fig. 2). Taken together these data indicate that butyrate arrests the

TABLE I. Inhibition of Asynchronously Growing 3T6 Cells by 7 mM Butyrate

Hours after addition of butyrate	Percent incorporation of [^{3}H]-thymidine
0	100
5	100
10	63
15	22
20	5
25	2
30	2

Two sets of dishes were used for each time point. One received butyrate, the other one not. The data given correspond to the percentage of DNA synthesis in the butyrate-treated sample relative to the untreated control.
100% (at 0 time) was 83,750 cpm incorporated/dish.

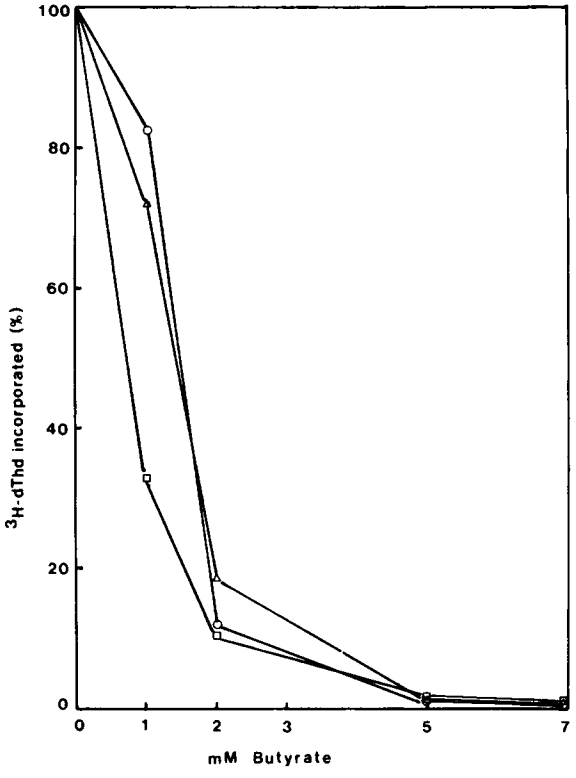


Fig. 1. Effect of increasing concentrations of sodium butyrate on DNA synthesis in serum-stimulated or virus-infected 3T6 cells. Cells, arrested by 48 h culture in 0.5% serum, were stimulated either by addition of serum to 5% (○), by infection with SV40 (□) or polyomavirus (△) (about 20 pfu/cell) in the absence of serum. Sodium butyrate was added 2 h after serum addition or virus infection to give the final concentration indicated in the abscissa. DNA synthesis was measured 24 h after growth stimulation. 100% equals 108,086 cpm/dish for serum-stimulated cells, 43,583 cpm/dish for SV40, and 71,119 cpm/dish for polyomavirus-infected cells.

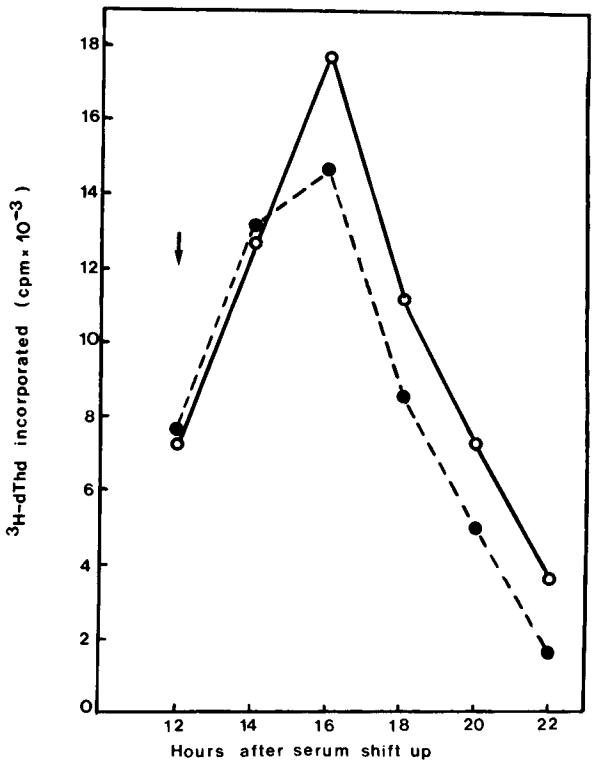


Fig. 2. Influence of butyrate added to 3T6 cells 12 h after serum stimulation. Cells were arrested by keeping them for 2 days in medium containing 0.5% serum. Serum was then added to a final concentration of 5%; 12 h later (arrow) half the plates received butyrate (final concentration 7 mM). DNA-synthesis rates were measured at intervals thereafter in the absence (○) or presence (●) of butyrate as described in Methods.

cell cycle of 3T6 cells in the G1 phase, confirming earlier reports [10, 11] on different cell systems. They furthermore indicate that this block is obtained regardless of whether cells are growth-stimulated by addition of serum or by infection with papovaviruses. Also in agreement with data on other cells [13,14] is our observation that concentrations of butyrate which cause a complete block of DNA replication have no effect on overall RNA and protein synthesis (Table II). The difference in the rates of RNA and protein synthesis observed in serum-treated or virus-infected compared to starved control cells, however, is significant.

For the study of processes occurring after a release of cells from the butyrate-induced block, cells were treated with 7 mmol/liter of butyrate for 24 h at 37°C, the drug containing medium was then removed, and the cells were washed and then covered with fresh medium as indicated in the legend to Figure 3, which summarizes the following experiments: resting 3T6 cells, treated for 24 h with butyrate under low (0.5%) serum conditions and then placed into medium containing 5% serum but no butyrate started DNA synthesis only 12 h thereafter (Fig. 3, curve 5). This lag period is the same as that observed if starved 3T6 cells, not treated with butyrate, are stimulated by serum shift up [22] (unpublished observation). Cells stimulated by

TABLE II. RNA and Protein Synthesis in 3T6 Cells Stimulated by Serum or by Infection With Polyoma Virus and Incubated in the Absence or Presence of 7 mM Butyrate

Conditions	RNA synthesis: [^{3}H]-uridine-incorporated (cpm/10^6 cells)	Protein synthesis: [^{3}H]-leucine-incorporated (cpm/10^6 cells)
0.5% serum without bytyrate	6,848	719
with butyrate	5,940	661
5% serum without butyrate	10,919	954
with butyrate	9,485	1,043
Polyomavirus-infected cells		
without butyrate	9,906	953
with butyrate	9,254	895

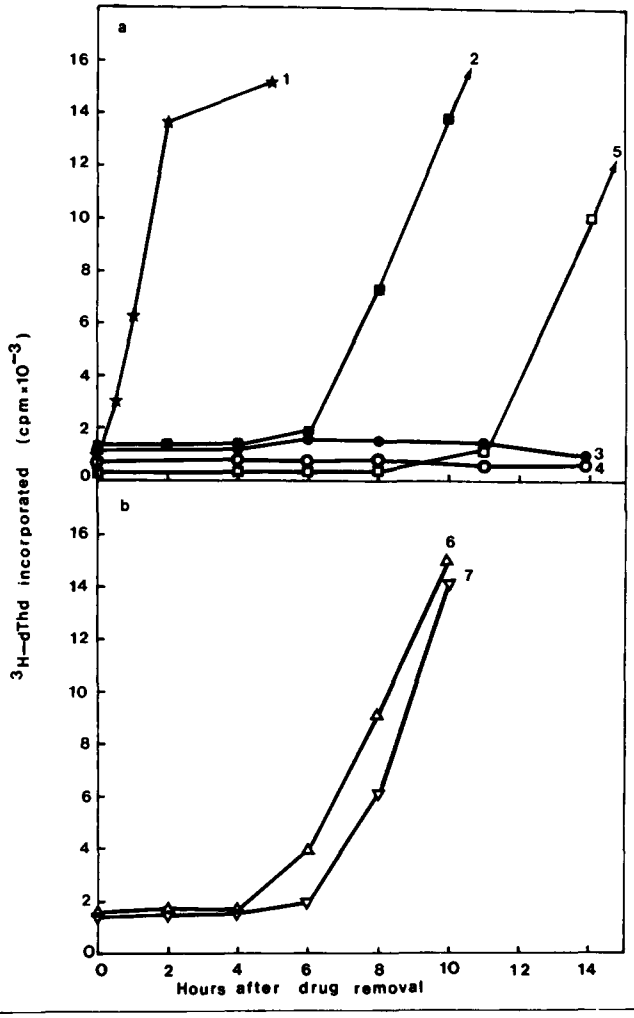
RNA and protein synthesis were determined 20 h after addition of butyrate (22 h after growth stimulation).

addition of 5% serum in the presence of butyrate followed by a release from the butyrate block in medium lacking serum did not initiate DNA synthesis (Fig. 3, curve 3); neither did those cells start DNA synthesis which were kept under low serum concentration during the incubation with butyrate (Fig. 3, curve 4). Serum-starved cells treated for 24 h with 5% serum in the presence of the fatty acid and released from the butyrate block with medium again containing high serum concentration started DNA synthesis after a lag period of 5–6 h (Fig. 3, curve 2). The same result was obtained if cells, not previously starved but randomly grown in 5% serum, were treated with sodium butyrate for 24 h and then released from the butyrate-induced block (not shown). These observations indicate that serum is required both before and after the butyrate-sensitive step in the G1 period. Proof for the significance of the thymidine incorporation experiments presented in Figure 3 was obtained by an autoradiographic study in which the percentage of labeled nuclei was determined at different times after the release of cells from a butyrate-induced block (Table III). The results are in good agreement with the data of Figure 3 (curve 2). They furthermore indicate that the butyrate block is indeed reversible as about 50% of the cells synthesize DNA 12 h after release from the butyrate block.

According to our results, the butyrate-sensitive step can be placed approximately 5 h prior to the beginning of the S phase. This makes butyrate an inhibitor of DNA synthesis the effect of which differs distinctly from that of others, such as hydroxyurea or fluordeoxyuridine, which cause cells to accumulate in early S or at the G1/S border. After release from a hydroxyurea–induced block, cells immediately and rather synchronously enter DNA synthesis (Fig. 3, curve 1).

Resting cells infected with polyoma virus or SV40 were also inhibited by butyrate (Fig. 1) and did not enter the S phase as long as the drug was present. After the fatty acid was removed, cells started DNA synthesis *in the absence of serum* after a lag period of 4–6 h (Fig. 3, curves 6 and 7).

All the results described so far indicate that butyrate inhibits cells in mid G1 at a point that may execute a critical function in the cell cycle. Such a point was postulated several years ago by Pardee and was termed "restriction point" [23]. One characteristic of the "restriction point" is that cells tend to accumulate there when



Curve no.	Symbol	Conditions prior to and during treatment	Conditions after removal of the inhibitor
1	★	5% serum; hydroxyurea	5% serum
2	■	5% serum; butyrate	5% serum
3	●	5% serum; butyrate	0% serum
4	○	0.5% serum; butyrate	0% serum
5	□	0.5% serum; butyrate	5% serum
6	△	0.5% serum; polyoma-infected; butyrate	0% serum
7	▽	0.5% serum; SV40-infected; butyrate	0% serum

Fig. 3. Release of 3T6 cells from a block of DNA synthesis caused by butyrate or by hydroxyurea. Cells were grown to near confluence and then arrested by 48 h growth in 0.5% serum. Further treatment was as outlined below for the various experiments. Growth stimulation was by addition of serum or by virus infection for 24 h, and butyrate (final concentration 7 mM) or hydroxyurea (final concentration 5 mM) was added at the time of serum addition or 2 h after virus infection. Release from the drug-induced block was by removal of the medium. Cells were washed once and covered with medium (time 0) as indicated in the table below. DNA synthesis was measured by pulse-labeling with [^{3}H]-thymidine as described in Methods. a) Growth stimulation by serum; b) growth stimulation by infection with polyoma virus or SV40.

TABLE III. Onset of DNA Synthesis After Release of Cells From a Butyrate-caused Block Measured by Autoradiography.

Hours after removal of butyrate	Percent labeled nuclei
0	< 2
4	2
6	5
8	10
10	24
12	53

The experiment was carried out like that shown in Figure 3, curve 2. Autoradiography was done as described [21].

protein synthesis is inhibited by very small amounts of cycloheximide [24], a condition that is assumed to affect the synthesis of a rapidly turning over, cell cycle–specific protein. In fact, if experiments analogous to those shown in Figure 1 were carried out with varying concentrations of cycloheximide in place of butyrate, the outcome was surprisingly similar. A small amount (0.1–1 μg/ml) of cycloheximide led to an inhibition of DNA replication in cells stimulated by either serum or by papovavirus infection. Fifty percent inhibition was obtained with 0.1–0.15 μg/ml cycloheximide. Even more important, a release of cells from a block induced by 0.2 μg/ml of cycloheximide (about 80% inhibition of DNA synthesis) resulted in an initiation of DNA synthesis after a lag period of 4–5 h which is only slightly shorter than the lag period after release from a butyrate-induced block (see Fig. 3).

It was reported that transformed cells are less sensitive to the inhibiting action of small amounts of cycloheximide than untransformed ones [25, 26]. This could be confirmed in our test system with 3T6 cells and SV40-transformed mouse cells. When we compared the same two cell lines in their sensitivity to butyrate, a surprisingly analogous result was obtained: SV40 transformed cells turned out to be remarkably resistant to the inhibitory action of the fatty acid. For instance, whereas 2 mM butyrate inhibited DNA synthesis in 3T6 cells by 90%, only 10% inhibition was observed with the SV40-transformed mouse cells.

DISCUSSION

According to current ideas there exist several points in the G1 phase of the cell cycle at which serum factors, hormones, or nutrients are required for further progress of growth-stimulated fibroblasts toward the initiation of the S phase [27–29].

Our data on the blockade of growth stimulated cells by butyrate and the raising of this blockade by removal of the drug allow several interesting conclusions: 1) Sodium butyrate blocks 3T6 cells in the G1 phase of the cell cycle around 5 h prior to the onset of the S phase. 2) Serum factors are required before as well as after the butyrate-sensitive step. 3) The start of DNA synthesis after release from the butyrate block is still asynchronous (compare gradient angles of curves 2 and 5 with that of curve 1 in Fig. 3). The results can be reconciled with the "restriction point" (or control point) hypothesis of cell-cycle regulation [23] by assuming that butyrate interferes with the synthesis of rapidly turning over protein(s) (possibly at the level of transcription) which are similar or identical to those proteins affected by low

concentrations of cycloheximide. Arrest of cells by butyrate might serve as valuable tool to analyze steps in the G0 to S transition of growth-stimulated cells. It should allow to separate in a rather specific way events occurring early after setting the mitogenic stimulus (prior to the butyrate-sensitive step) from those occurring shortly before entrance into the S phase (afer the butyrate-sensitive step) without interference with overall RNA or protein synthesis [see 21]. We have, for instance, successfully used sodium butyrate and hydroxyurea to measure the production of mRNAs for DNA-synthesis enzymes in late G1 between the cell-cycle events blocked by the two inhibitors (Müllner, Hofbauer, and Wintersberger in preparation).

Data in this paper, together with previously published data [21, 30], would favor the hypothesis that the primary action of polyoma virus or SV40 T-antigens on resting cells is the induction of cellular processes in G0/G1 which eventually lead to the initiation of the S phase. This hypothesis contrasts with another one in which T-antigens are assumed to have a more direct effect on the induction of cellular DNA synthesis [31, 32]. From a study on NIH 3T3 cells, published while this manuscript was in preparation, Kawasaki et al [32] concluded that SV40 can induce cellular DNA synthesis in the presence of butyrate, which is incompatible with our observation. There are several points which could explain the difference: 1)Kawasaki et al [32] based their conclusion and interpretation only on data obtained with one concentration of butyrate (3 mM), although they mentioned that at higher drug concentration they did observe an inhibitory effect on virus-infected cells. 2) Most important, the results obtained with virus-infected cells seem to depend strongly on the multiplicity of infection or on the amount of viral DNA introduced by transfection or injection. There is agreement between the results of Kawasaki et al, and our data on the point that SV40-transformed cells are much more resistant to butyrate than normal ones, and the same holds true for the sensitivity to low concentration of cycloheximide [24, 26]. We interpret this to mean that the resistance is caused by the relatively high concentrations of T-antigen present in transformed cells. A similarly high amount of T-antigen is probably synthesized in cells infected with SV40 at high multiplicity or in those receiving a large number of viral DNA copies (or at least the early region thereof) by transfection or by injection, conditons used by Kawasaki et al [32]. In our experiments cells were infected at a multiplicity of about 20, which is just sufficient to cause a stimulation of resting cells quite similar to that obtained by serum shift up. Under these conditions, SV40 or polyoma virus does not endow cells with resistance to butyrate. Thus, higher concentrations of T-antigen could play a role in stabilizing "restriction point" protein(s) as suggested [26]. One interesting candidate for such a protein is the 53K nonviral antigen found in transformed cells [33]. Amounts of T-antigen sufficient to cause cells to enter S phase, however, do not exhibit this effect. Our data do not, of course, exclude the possibility that the pleiotropic T-antigens of SV40 or polyoma virus function at several points in the G0/G1 to S transition of infected cells, but one of these points appears to lie quite early after mitogenic stimulation, prior to the butyrate-sensitive step.

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