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# EFFECT OF SODIUM BUTYRATE ON MYOBLAST GROWTH AND DIFFERENTIATION

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Summary : Myoblasts from $L_6$ line, after a period of cell division, undergo differentiation into large multinucleated syncitia in 8-9 days. Butyrate was added for 24 hours at various times of culture. In all samples growth was strongly inhibited. After removal of butyrate, growth continued at the same rate for 2 days, afterwards the growth rate became the same as in control cells. Morphological and biochemical differentiations, estimated by creatine phosphokinase assay, occur with a 1-3 day delay according to the time of addition of butyrate, when compared to untreated cells. Only the M form of creatine phosphokinase was present in butyrate-treated cells as in untreated myoblasts.

Sodium butyrate has been reported to have a wide variety of morphological and physiological effects on mammalian cells in culture. It reversibly inhibits cell growth and DNA synthesis (1, 2) possibly by forming a replication inhibitor (2). Butyrate does not prevent cell differentiation, in some cases it even induces some differentiated functions : it increases the specific activity of several characteristic enzymes in neuroblastoma cells (1), it also induces hemoglobin in erythroleukemic cells (3).

The molecular mechanism of action of butyrate has been shown to involve a hyperacetylation of histones H3 and H4 by inhibition of histone deacetylase (4, 5, 6). Since the regions of chromatin preferentially digested by DNase I are rich in acetylated histones (5, 7, 8), and since acetylated histone H4 is preferentially associated with template active parts of chromatin (9), it has been suggested that butyrate could be able to activate genes. However it has not been proven that the effect of butyrate on DNA synthesis

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and on cell morphology is a direct consequence of its action on histone acetylation.

It is interesting to study the effect of butyrate on muscle cells cultivated *in vitro*, since these cells, after a period of division, undergo specific differentiation characterized by cell fusion, formation of myotubes and of multinucleated syncitia (10), by a strong increase in the synthesis of muscle specific proteins including creatine phosphokinase (11-13) and by a change in the RNA pattern (14-16). This could be related to specific changes in the pattern of chromatin phosphorylation which have been observed during myoblast differentiation (17). Dimethyl sulfoxide, which induces hemoglobin synthesis in erythroleukemic cells, when applied to proliferating $L_8$ myoblasts, completely prevented cell fusion and differentiation, but when applied at a later stage, it did not prevent cell differentiation, but induced a phenotypic conversion from myoblasts to fibroblast-like cells (18).

## MATERIALS AND METHODS

### *Culture conditions*

Cells from $L_6$ line were cultured in 100 mm diameter Corning dishes at 37° in 10 % $CO_2$ in a mixture of minimum essential medium (19) and of Dulbecco modified Eagle medium supplemented with 10 % fetal calf serum and 0.5 % chick embryo extract, at a density of $2.10^5$ cells/dish. After three days of culture, this medium was replaced by the same medium supplemented with 5 % horse serum and this medium was changed every 48 hours. Under these conditions, 90-100 % of cells are fused in multinucleated syncitia after 8-9 days.

Sodium butyrate was added to the culture medium at a 7 mM final concentration. After 24 hours butyrate was removed, the cells were washed and replaced in a normal medium.

### *Creatine phosphokinase determination*

Cells were treated with trypsin, washed three times by centrifugation with phosphate buffered saline, PBS (132 mM NaCl, 2.6 mM KCl, 6.5 mM $Na_2HPO_4$, 1.5 mM $KH_2PO_4$) (20). Cells were extracted after freezing and thawing with the PBS solution.

Creatine phosphokinase assays were performed with the use of the reagent supplied by Calbiochem. The reaction was performed at 30° and the formation of NADPH was followed spectrophotometrically at 340 nm.

*Electrophoresis and enzyme revelation*

Electrophoreses of cell extracts were performed for 50 min at 4° C with a 60 mM veronal buffer (pH 8.6) containing 0.06 % (v/v) 2-mercaptoethanol on cellulose polyacetate strips according to Penhoet et al. (19). The samples were placed towards the cathodic end of the strips.

Strips were treated for creatine phosphokinase activity as described by Cohen et al. (20), using 15 mg/ml agar (Bacto Agar, Difco) to reduce diffusion of the bands. Myoblast extracts contain a high level of myokinase which migrates like the M form of creatine phosphokinase and which is stained with the same reagent. It led us to add to the gel an inhibitor of myokinase which does not interfere with the creatine phosphokinase reaction : $P^1$-$P^5$ diadenosine 5' pentaphosphate (20).

After incubation of the strips at 37° the colored bands were fixed in a 70 % (v/v) ethanol, 5 % (v/v) acetic acid solution containing a few drops of glycerol, dried and stored.

## RESULTS

*Effect of sodium butyrate on cell growth*

Cells were plated at a concentration of $2.10^5$ cells per dish and the standard medium replaced for 24 hours on the indicated day by the same medium containing 7 mM butyrate or by standard medium in control experiments. This concentration of butyrate was used because preliminary experiments have shown that it delayed differentiation without inhibiting completely cell growth.

After 24 hours the cells were washed and cultured in standard medium (fig. 1). Samples were harvested every day for counting, creatine phosphokinase assay and Giemsa coloration.

When butyrate was added during the phase of cell division, between the 1st and the 4th day of culture, growth was inhibited to approximately 35 % of the growth rate of the control cells. After removal of butyrate, cells grew at the same reduced rate for approximately 2 days, afterwards they were submitted to the same growth rate as the control cells. If the cells were cultured in the presence of butyrate for 48 hours, growth was completely inhibited but it resumed after removal of the drug (*data not shown*).

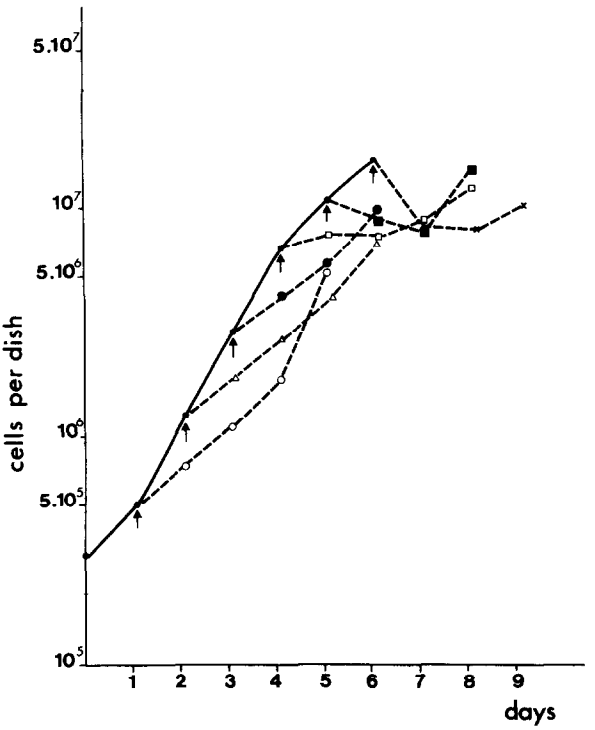


Figure 1 : Effect of butyrate on myoblast growth. Myoblasts are grown in a standard medium. Every day samples were supplemented with 7 mM butyrate for 24 hrs (arrows). The number of cells was counted in each sample (average of 2-3 dishes per experiment). Sodium butyrate added after 1 day (o---o), after 2 days (Δ---Δ), after 3 days (●---●), after 4 days (□---□), after 5 days (■---■), after 6 days (✱---✱). Control growth ( •——• ).

When butyrate was added on the 5th or 6th day of culture, growth was completely inhibited. Some cells died, probably the cells which were engaged in the fusion process. Approximately 2 days after removal of butyrate, growth resumed, cells fused and formed myotubes.

Optical microscopy showed that treated myoblasts had larger and clearer soma and nucleus than control cells. They were very well displayed on their support with some spindle shapes. This morphological aspect of the cells could be due to the appearances of actin filaments, as it has been observed in other transformed

Table I : Effect of sodium butyrate on creatine phosphokinase (CPK) specific activity.

| Day of culture when butyrate was added (CPK I mU in control) | Number of days required to obtain the highest CPK specific activity | CPK specific activity in differentiated cells I.mU. |
|---|---|---|
| (Control) | 9 | 80 |
| 1 day | 10 | 74.5 |
| 2 | 11 | 80 |
| 3 (23 U) | 11 | 73.5 |
| 4 | 12 | 63.5 |
| 5 (32 U) | 12 | 76.5 |
| 6 | 12 | 65 |
| 7 (52 U) | | |
| 8 | | |
| 9 (80 U) | | |

Butyrate was added to a 7 mM final concentration for 24 hours to cells cultured for 1-6 days. Creatine phosphokinase was assayed every day. Its specific activity progressively increased. The number of days of culture required to obtain the highest specific activity is indicated in column 2, this specific activity is indicated in column 3.

cells (21). The cells did not seem to suffer really from the butyrate treatment.

*Effect of sodium butyrate on cell differentiation*

Addition of butyrate to the culture for 24 hours between the 2nd and the 6th day of culture delayed myoblast fusion for 2-3 days, when compared to control cells, but in all cases apparently normal morphological differentiation occurred.

Biochemical differentiation may be characterized by creatine phosphokinase whose specific activity increases 3-4 times between the 3rd and the 9th day of culture in control cells (table I). Addition of butyrate delayed this increase, the highest specific activity was obtained with a delay of 1-3 days according to the time of butyrate addition, the latest the addition, the longer the delay.

No differentiation occurred when butyrate was present. After a lag period following butyrate removal, creatine phosphokinase specific activity increased progressively and reached the highest value as indicated in table I.

Several creatine phosphokinase isozymes have been described. The form B found in brain is also present in undifferentiated cells, the form M is specific of muscle cells (13). Only form M was found in rat myoblasts from the 3rd to the 9th day of culture, in agreement with Cohen et al. (20) (fig. 2).

Butyrate treatment did not modify the isozymic pattern. The increase in creatine phosphokinase specific activity during differentiation may be seen in electrophoretic gels in control as well as in butyrate-treated cells since the same amount of material was used in each gel.

## DISCUSSION

Sodium butyrate at the used concentration inhibits strongly cell growth but not completely as in several other cell types (1). After its removal the reduced cell growth rate is maintained for approximately 2 days. This could be explained by the persistence of some butyrate in the cells although cells were washed. Nevertheless it is unlikely since butyrate removal is quickly followed by a reactivation of histone deacetylase, but growth inhibition could be due to a different mechanism. Butyrate could cause a persistent abnormality in the cell, it could induce the formation of a replication inhibitor (2). A 1-3 day delay was also observed before fully differentiated cells were observed : multinucleated syncitia and highest creatine phosphokinase specific activity. This observation is consistent with the concept that a certain number of cell divisions are required before the cells can reach the fully differentiated state

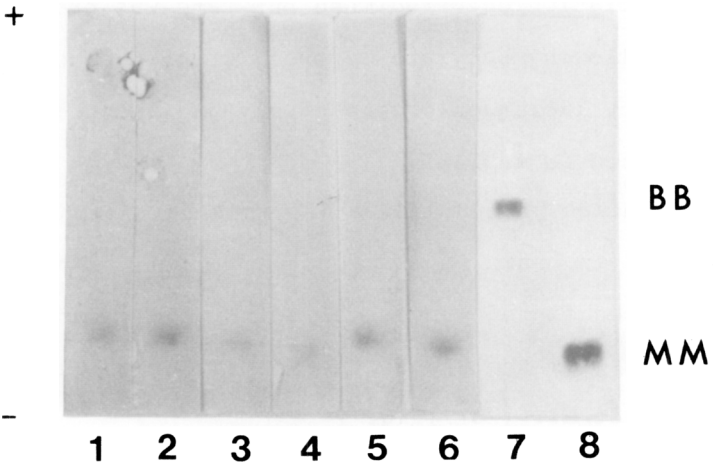


Figure 2 : Electrophoretic pattern of creatine phosphokinase. Electrophoreses were performed according to Cohen et al. (20) using a myokinase inhibitor. The same amount of material (2 µg of protein) was placed in each cellulose polyacetate strip.
(1) and (2) : control myoblasts culture - (1) 3rd day, (2) 9th day of culture. (3) - (6) : myoblasts treated with 7 mM sodium butyrate for 24 hours after 5 days of culture - (3) 7th day ; (4) 9th day ; (5) 10th day ; (6) 12th day of culture; (7) rat brain extract ; (8) rat muscle extract.

(10). The divisions may have occurred before or after butyrate treatment.

From these observations and observations by others, it appears that the effect of butyrate strongly varies from one type of cell to another. As opposed to what was observed in neuroblastoma cells (1) and in Friend cells (3), butyrate does not induce differentiated functions in myoblasts, it does not induce filamentous protrusion as in HeLa cells (22).

The inhibitory effect of butyrate on $L_6$ myogenic cells growth and differentiation is completely reversible, as opposed to dimethylsulfoxide (18).

Butyrate seems to be a very useful tool for the study of cell differentiation and of its relationship with cell growth.

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