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EFFECTS OF SODIUM BUTYRATE ON MOUSE NEUROBLASTOMA CELLS IN CULTURE

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Abstract—Uncloned and cloned mouse neuroblastoma cells *in vitro* developed neurite-like processes upon treatment for 48 hr with 10^{-6} to 3×10^{-4} M sodium butyrate. Although treatment with 5×10^{-4} M sodium butyrate caused neurite formation after 18 hr, the per cent of the cells having neurities returned to control level by 48 hr of treatment. Cell division decreased and acetylcholinesterase activity increased with 5×10^{-4} M sodium butyrate; these parameters were unaltered in the presence of 10^{-6} or 10^{-5} M sodium butyrate. Sodium butyrate was more effective than sodium propionate or sodium valerate in causing a decrease in cell division and an increase in acetylcholinesterase activity, and sodium isobutyrate or gamma aminobutyric acid was ineffective. The effects of sodium butyrate on cell division and acetylcholinesterase activity were reversible after treatment was discontinued. It is concluded that the ability of butyrate to stimulate neurite formation at low concentrations is opposed by additional actions on the cell as its concentration is increased.

Exposure *in vitro* of mouse neuroblastoma cells to dibutyryl adenosine 3′, 5′ cyclic monophosphoric acid (B_2cAMP) or to phosphodiesterase inhibitors, such as papaverine or 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (R020-1724), stimulates the generation of features similar to those of mature nerve cells. These features include a reduction in cell division [1, 2], formation of axon-like processes or neurites (referred to generally as morphologic differentiation) [2, 3], and increases in the activities of tyrosine hydroxylase [4–6] and acetylcholinesterase [2, 7]. Sodium butyrate, used as a control for B_2cAMP since it can be generated within the cell from B_2cAMP [8], also causes some of these changes. For example, 5×10^{-4} or 10^{-3} M sodium butyrate causes a decrease in cell division [1, 2, 4, 6] and an increase in the activities of tyrosine hydroxylase [4, 7], cathechol-*O*-methyl transferase [9] and acetylcholinesterase [7]. Sodium butyrate in these concentrations also causes an increase in neuroblastoma cyclic AMP [10], RNA and protein, with an accompanying decrease in cellular DNA [11]. Although many of the agents which cause similar changes in neuroblastoma cells also stimulate "morphologic differentiation," 5×10^{-4} or 10^{-3} M sodium butyrate has been reported to lack this effect [1, 2, 4, 6]. However, Glazer and Schneider [12] showed that 10^{-6} to 3×10^{-4} M sodium butyrate is capable of stimulating process formation, and at 3×10^{-4} M also increases acetylcholinesterase activity and decreases cell division. Higher concentration did not stimulate neurite formation. Sodium butyrate has also been reported to alter the morphology and growth rate of Chinese hamster cells in culture [13].

In view of the varied effects of sodium butyrate on neuroblastoma cells in culture, and of the apparent concentration dependency of the effects, a more thorough study of this agent was undertaken, the results of which are reported here.

MATERIALS AND METHODS

The uncloned population of mouse C1300 neuroblastoma cells designated T59 and the clone of C1300 neuroblastoma cells designated NBA_2 used in this study were both generously provided by Dr. K. N. Prasad (Department of Radiology, University of Colorado School of Medicine) and have been described previously [3]. Cell culture conditions used in this laboratory have been reported elsewhere [12]. The NCTC clone 929 of mouse strain L connective tissue cells was obtained from the Center for Disease Control, Atlanta, Ga., and was grown under conditions identical to those for growth of neuroblastoma cells. Generation times for cells in exponential growth were between 19 and 24 hr.

The formation of neurite-like processes greater in length than the average cell diameters of 25–50 μm was considered indicative of morphological differentiation, and was expressed as the per cent of the total number of cells counted (250–350 cells) that had processes fitting this criterion. The number of cells sloughed into the medium were counted separately from those harvested from the flask with Viokase (0.25%, v/v), and a total flask cell count represents the cells in the medium plus those harvested. Per cent viability of the cells was measured with 0.4% trypan blue in PBS (8.0 g NaCl, 2.0 g KCl, 1.15 g Na_2PO_4 and 2.0 g KH_2PO_4 in distilled water to yield 1 liter) for 2 min at 36°. The values for the number of experiments (N) in the statistical data presented in the

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Results section represent the number of separate flasks used for analysis.

Media were routinely changed every other day unless a drug was present, in which case media were changed every day unless stated otherwise. The drugs used in this study were dissolved in PBS and added to the cultures in volumes of 0.1 ml or less. The organic acids were neutralized to pH 7.2 to 7.4 with sodium hydroxide prior to use. The drugs used in this study were obtained from commercial sources, with the exception of 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (R020-1724), which was generously supplied by Dr. W. G. Scott of Hoffmann-LaRoche Inc.

Enzyme activity of 1 to 10×10^6 cells was measured after their lysis by freezing and thawing in 50 μl of 10% (v/v) Trition X-100. The lysed sample was diluted to 0.5 ml with water and used for analysis of acetylcholinesterase activity by the spectrophotometric method of Ellman *et al.* [14]. Enzyme activity of extracts of cells treated with 5×10^{-4} M sodium butyrate for 30–48 hr using 5×10^{-4} or 5×10^{-3} M butyrylthiocholine as substrate was only 4 and 8 per cent, respectively, of the activity measured with 5×10^{-4} M acetylthiocholine as substrate, indicating that the activity of pseudo-cholinesterase is very low relative to that of acetylcholinesterase [15]. Glucose 6-phosphate dehydrogenase activity of the diluted Trition X-100 extracts was measured by the spectrophotometric procedure of Zinkham *et al.* [16] as modified by O'Brien *et al.* [17], and lactate dehydrogenase activity of the extracts was measured by the spectrophotometric procedure of Wroblewski and LaDue [18]. Enzyme activity was linear with the amount of extract and with time up to at least 15 min for each of these assays. There was no detectable loss of enzyme activity upon storage of the Trition X-100 extracts in the freezer (−20°) for up to 7 days. Aliquots (100 μl) of the dilute lysate were added to 5 ml of 5% trichloroacetic acid for precipitation of protein which was subsequently assayed by the microbiuret method [19].

Cells were grown on glass coverslips for photomicroscopy. Coverslips on which cells were growing were washed for 1 min in serum-free media, 1 min in PBS and approximately 1 sec in distilled water and then placed in methanol for 5 min for cell fixation. The cells were stained with the nonspecific protein stain, amido-Schwartz dye, in order to visualize the processes maximally. The fixed cells were dipped in a solution of 0.03% dye in 7.5% acetic acid for 15 sec. The slips were then washed in three changes of acetone for 1 min each and finally dehydrated by two successive treatments with acetone–xylene (1:1) for 1 min and with xylene for 5 min. All these steps were carried out at room temperature. Cells growing in flasks were also photographed through an inverted stage microscope equipped with phase contrast optics using a Polaroid camera.

RESULTS

Neurite formation. Previous studies have shown that 5×10^{-4} or 10^{-3} M sodium butyrate fails to stimulate neurite formation in mouse neuroblastoma cells growing in culture, even though it inhibits cell division. However, exposure of neuroblastoma cells to sodium butyrate in concentrations between 10^{-6} and 3×10^{-4} M did cause formation of neurite-like processes (Fig. 1), although lower or higher concentrations were ineffective. The neurites formed after 48 hr in the presence of the lower concentrations of sodium butyrate (10^{-6} and 5×10^{-5} M, in Fig. 2 B–D) are similar to those which develop in the pres-

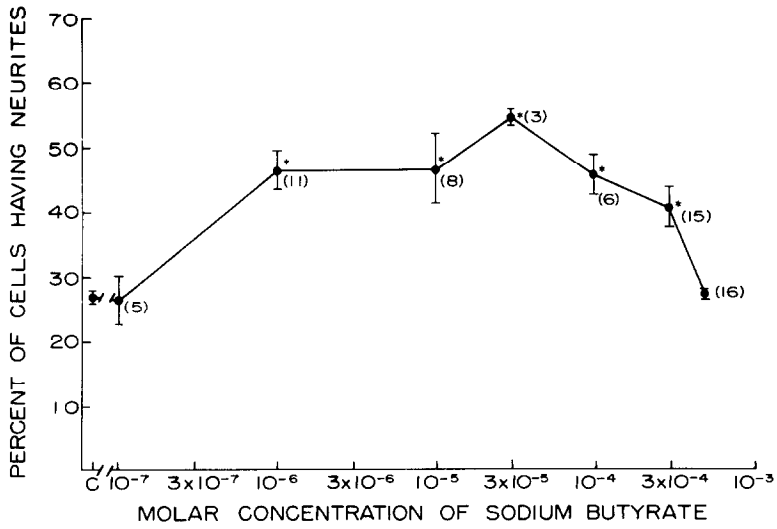

Fig. 1. Neurite formation in T59 neuroblastoma cells after 48 hr treatment with sodium butyrate. Cells were plated at 0.025×10^6/ml in 25 cm^2 Falcon plastic flasks containing 5 ml media, and allowed to grow for 3 days. On day 3, the media were changed and sodium butyrate was added to give the concentrations indicated on the abscissa. Media and drug were changed 24 hr later and the cells were harvested for analysis the following day. The numbers in parentheses by each point indicate the number of experiments and the asterisks indicate values which are statistically different from control at a $P < 0.05$ level or better. Neurite formation was measured by means of an inverted stage microscope and reflects the per cent of the cells having processes longer than the diameter of the cell body.

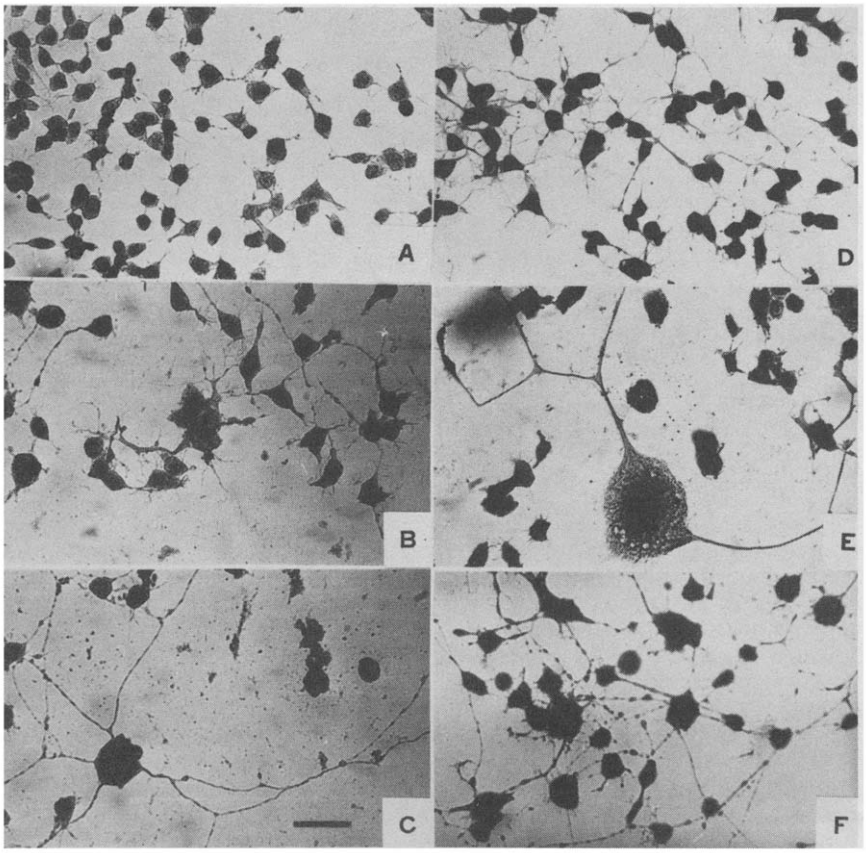

Fig. 2. Photomicrographs of T59 neuroblastoma cells growing on glass coverslips contained in plastic petri dishes. The cells were fixed in methanol and stained with 0.03% amido Schwartz dye in 7.5% acetic acid (see Methods for details). Cell inoculations were 0.025×10^6/ml in 5 ml. Fresh media and sodium butyrate were added after 3 days; a media change and drug addition were made 24 hr later. The cells were prepared for microscopy 24 hr after the second drug treatment. The magnification of the photographs is 273. Panel A, control; panels B and C, 10^{-6} M sodium butyrate; panel D, 5×10^{-5} M sodium butyrate; panel E, 5×10^{-4} M sodium butyrate; and panel F, 1.0×10^{-5} M 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (R020-1724).

ence of other agents known to induce neurite formation in neuroblastoma cells; the processes, which can be quite long, often have a beaded appearance and enlarged endings. In many instances, the processes formed after treatment with sodium butyrate are wider than those formed in the presence of other inducing agents, an effect which is more pronounced at the higher concentrations. The number of cells with abnormal morphology generally increases as the concentration of butyrate increases, evidenced by the giant cell shown in Fig. 2E. Concentrations of sodium butyrate above 3×10^{-4} M produce enlargement and vacuolization of the cells, fewer of the cells having neurites than at the lower concentrations. Process formation in cells treated with R020-1724 for 48 hr is shown in Fig. 2F for comparison. This concentration of R020-1724 routinely stimulates process formation in 80–85 per cent of T59 cells.

The possibility that sodium butyrate in concentrations of 5×10^{-5} M or higher produces cytotoxic effects which oppose neurite formation or cause retraction of formed neurites was tested by examining process formation in T59 cells at short time intervals during a 48-hr 5×10^{-4} M sodium butyrate treatment period (Table 1). Neurite formation increased 18 hr after treatment, remained high up to 24 hr, then declined to pretreatment level by 48 hr. The processes formed by 18 hr were similar in appearance to those formed after 48 hr exposure to the lower sodium butyrate concentrations, reaching a length up to 1 mm. Untreated cells remained at control levels over the 48-hr period. The ability of 5×10^{-4} M sodium butyrate to reduce or prevent neurite formation was further tested by examining its effects on processes induced by 10^{-5} M papaverine. Table 2 shows that neurite formation stimulated by exposure of the cells to papaverine for 48 hr was slightly less if 5×10^{-4} M sodium butyrate was present (67 per cent for papaverine vs 54 per cent for papaverine + sodium butyrate; $P < 0.001$). Addition of sodium butyrate after treatment of the cells with papaverine for 48 hr did not reduce the per cent of cells having neurites over the following 48 hr if papaverine was also present, although it did if papaverine treatment was not continued over this time (25 per cent for sodium butyrate alone in the second 48-hr period vs 49 per cent for sodium butyrate + papaverine present in the second 48-hr period; $P < 0.001$). Treatment with sodium

Table 1. Effects of 5×10^{-4} M sodium butyrate on T59 neuroblastoma cells after various times of treatment*

	Length of treatment (hr)				
	0	6	18	24	48
Neurite formation	31 ± 2 (6)	37 ± 3 (5)	41 ± 3† (7)	42 ± 1† (5)	29 ± 1 (5)
Acetylcholinesterase	0.43 ± 0.07 (7)	0.38 ± 0.07 (5)	0.68 ± 0.11 (5)	2.12 ± 0.69† (4)	5.29 ± 0.44† (7)
Protein	0.26 ± 0.02 (12)	0.23 ± 0.02 (8)	0.26 ± 0.03 (8)	0.28 ± 0.03 (8)	0.37 ± 0.02† (8)

* T59 cells (0.25×10^6) were plated in 25 cm^2 Falcon plastic flasks containing 5 ml media. Fresh media were added on day 3 after plating. The addition of sodium butyrate was staggered so that all flasks were harvested at the same time. Results are expressed as mean ± standard error and the figures in parentheses represent the number of separate flasks analyzed. Neurite formation is expressed as the per cent of the cells having processes longer than the diameter of the cell body; acetylcholinesterase activity is expressed as nmoles substrate converted/min/10^6 cells; protein is expressed as mg/10^6 cells.

† Indicates statistical significance at $P < 0.05$ or greater.

butyrate for either 48 or 96 hr did not prevent induction of neurite formation by the addition of papaverine in the second 48-hr period (34 per cent for sodium butyrate only for 96 hr, 64 per cent for sodium butyrate for 48 hr followed by papaverine for 48 hr, and 71 per cent for sodium butyrate for 96 hr with papaverine present during the second 48-hr period).

Other effects. The effects of three concentrations (10^{-6}, 10^{-5} and 5×10^{-4} M) of sodium butyrate on cell growth, protein content, process formation and acetylcholinesterase activity for uncloned neuroblastoma cells are shown in Table 3. The most pronounced effects of sodium butyrate occurred at the highest concentration, and consisted of decreased cell growth and increased acetylcholinesterase activity. Protein content of the cells increased almost 100 per cent after exposure of the cells to the highest concentration of sodium butyrate. Table 1 shows that acetylcholinesterase activity increased with increasing length of exposure to 5×10^{-4} M sodium butyrate over a 48-hr treatment period, although an increase in cell protein was not evident until 48 hr of treatment. Process formation, cell protein content and acetylcholinesterase activity were unaltered over the 48-hr period in untreated cells. There was no increase in sloughing of cells from the flask surface at any of the concentrations tested.

Inclusion of 1.1×10^{-5} M cycloheximide during a 24-hr treatment period markedly reduced the sodium butyrate (5×10^{-4} M)-induced increase in acetylcholinesterase activity; the reduction in two separate experiments was 96 and 100 per cent respectively. Cycloheximide at this concentration also completely inhibited cell division and increased cell sloughing from 4.2 ± 0.3 per cent (N = 12) to 42.6 ± 2.8 per cent (N = 12). Neurites were absent in cells treated with 1.1×10^{-5} M cycloheximide for 24 hr. However, viability of cells remaining attached to the flask, and which were used for analysis of acetylcholinesterase activity, remained high, with a value of 79.3 ± 7.2 per cent (N = 4), compared to 91.3 ± 3.2 per cent (N = 4) for untreated cells. In the presence of 1.1×10^{-5} M cycloheximide and 5×10^{-4} M sodium butyrate, cell sloughing was 39.3 ± 1.8 per cent (N = 4), and viability of harvested cells was 88.3 ± 2.2 per cent (N = 4). Treatment of the cells

Table 2. Effects of sodium butyrate and papaverine on neurite formation in T59 neuroblastoma cells*

48-hr Treatment		96-hr Treatment		
0–48 hr	Neurite formation	0–48 hr	48–96 hr	Neurite formation
Regular media	29 ± 1	Regular media	Regular media	Too dense†
Papaverine	67 ± 2	Papaverine	Regular media	62 ± 2
Sodium butyrate	30 ± 1	Papaverine	Papaverine + sodium butyrate	62 ± 2
Papaverine + sodium butyrate	54 ± 2	Paperverine	Regular media	49 ± 2
		Papaverine	Sodium butyrate	25 ± 1
		Sodium butyrate	Sodium butyrate	34 ± 2
		Sodium butyrate	Papaverine	64 ± 2
		Sodium butyrate	Papaverine + sodium butyrate	71 ± 4

* Papaverine and sodium butyrate concentrations were 10^{-5} and 5×10^{-4} M respectively. Neurite formation is expressed as the per cent of cells having processes longer than the diameter of the cell body. Cells (0.075×10^6) were plated in 25 cm Falcon flasks containing 5 ml media and allowed to grow 24 hr before treatment was initiated at 0 time. Fresh media and drugs were added after the first 48-hr treatment period; N = 5 in each case. See text for statistically significant differences.

† Cells were at confluent density.

Table 3. Effects of short-chain fatty acids and GABA on uncloned neuroblastoma cells in culture*

Agent and molar concn	Cell No. (as 10^6 cells)	Neurite formation (% of cells with neurites)	Protein (mg/10^6 cells)	Acetylcholinesterase (nmoles/min/10^6 cells)
Control	6.8 ± 0.7 (24)	31 ± 1 (20)	0.33 ± 0.03 (20)	0.56 ± 0.07 (29)
Propionate				
10^{-6}	7.5 ± 1.3 (5)	46 ± 7† (6)	0.25 ± 0.04 (4)	0.50 ± 0.08 (12)
10^{-5}	6.8 ± 1.4 (5)	39 ± 5 (6)	0.27 ± 0.04 (4)	0.57 ± 0.12 (8)
5×10^{-4}	5.6 ± 1.0 (5)	35 ± 5 (6)	0.39 ± 0.09 (4)	1.97 ± 0.29† (12)
Butyrate				
10^{-6}	5.6 ± 0.7 (10)	47 ± 3† (11)	0.30 ± 0.02 (10)	0.47 ± 0.04 (16)
10^{-5}	4.8 ± 0.8 (10)	47 ± 5† (8)	0.37 ± 0.04 (10)	0.81 ± 0.17 (14)
5×10^{-4}	3.4 ± 0.4† (21)	27 ± 1 (16)	0.57 ± 0.06† (16)	6.00 ± 0.60† (26)
Valerate				
10^{-6}	6.4 ± 1.9 (4)	58 ± 6† (5)	0.39 ± 0.08 (3)	0.55 ± 0.13 (6)
10^{-5}	5.8 ± 1.6 (5)	47 ± 4† (5)	0.60 ± 0.18 (3)	0.58 ± 0.21 (8)
5×10^{-4}	4.5 ± 1.4 (5)	32 ± 2 (5)	0.49 ± 0.12 (3)	2.70 ± 0.71† (18)
Isobutyrate				
10^{-6}	7.2 ± 1.3 (5)	47 ± 8† (5)	0.17 ± 0.01† (4)	0.41 ± 0.07 (8)
10^{-5}	7.0 ± 1.5 (5)	39 ± 4 (5)	0.24 ± 0.01† (4)	0.49 ± 0.07 (8)
5×10^{-4}	7.1 ± 1.1 (5)	38 ± 5 (5)	0.31 ± 0.07 (4)	0.55 ± 0.10 (8)
GABA				
10^{-6}	6.6 ± 1.5 (5)	45 ± 4† (5)	0.28 ± 0.08 (6)	0.91 ± 0.19 (6)
10^{-5}	6.7 ± 1.5 (5)	48 ± 3 (3)	0.30 ± 0.08 (4)	1.00 ± 0.22 (4)
5×10^{-4}	7.0 ± 2.1 (5)	34 ± 2 (5)	0.28 ± 0.09 (6)	0.80 ± 0.16 (6)

* Cells (0.25×10^6) were plated in 25 cm^2 Falcon plastic flasks containing 5 ml media. Fresh media and the agents were added on day 3 after plating. The flasks contained between 1.7 and 2.1 $\times 10^6$ cells on the initial day (day 3) of drug treatment. Fresh drugs and media were added 24 hr later and the cells were harvested 24 hr after the second treatment. Total exposure to the agents was 48 hr and the cells were in culture for a total of 5 days. The parameters listed in the table were measured by methods described in Materials and Methods. All acids were used as the sodium salts. Results are expressed as mean ± standard error. The numbers in parentheses represent the number of experiments.

† Indicates statistical significance at $P < 0.05$ or greater.

with this concentration of cycloheximide for 24 hr reduced the incorporation of [^{3}H]leucine into protein during a subsequent 4-hr incubation period by 97.4 per cent.

The lowest and intermediate concentrations of sodium butyrate did not produce a change in acetylcholinesterase activity, although both concentrations caused an increase in neurite formation. Sodium valerate at 10^{-6} M was more effective than butyrate in causing neurite formation. Cell viability, as measured by trypan blue exclusion, was unaltered over the 48-hr time period by the two lowest concentrations tested, but was less than controls after exposure to 5×10^{-4} M (control, 87 ± 2, N = 18; sodium butyrate, 70 ± 4, N = 15: $P < 0.001$).

The reversibility of the increase in acetylcholinesterase activity was tested by treating T59 cells in duplicate flasks for 4 days with 5×10^{-4} M sodium butyrate, resulting in an enzyme level of 5.30 nmoles/min/10^6 cells. These cells were then harvested and replated in regular media at a density of 0.036×10^6 cells/ml in 25 cm^2 Falcon plastic flasks containing 5 ml media. Within 24 hr of replating, the acetylcholinesterase activity, as nmoles/min/10^6 cells, was 0.80 and 24 hr later was 0.10. The cells grew to confluent density by day 9, at which time the activity had increased to 1.00 nmoles/min/10^6 cells. Addition of sodium butyrate directly to the acetylcholinesterase assay mixture to give a concentration of 5×10^{-4} M had no effect on the enzyme activity.

Cells treated for 48 hr with 5×10^{-4} M sodium butyrate did not show an increase in specific activities (O.D. units/min/mg of protein) of lactate dehydrogenase (LDH) or glucose 6-phosphate dehydrogenase (G-6-PD), even though acetylcholinesterase was markedly elevated at this time (LDH, control, 1.80 ± 0.15, N = 5; LDH, sodium butyrate-treated, 1.23 ± 0.16, N = 4; G-6-PD, control, 0.16 ± 0.02, N = 7; G-6-PD, sodium butyrate-treated, 0.10 ± 0.01, N = 5).

Structure–activity relationships. The effects of other short-chain fatty acids on T59 neuroblastoma cells are shown in Table 3. Sodium butyrate was the only agent which caused a statistically significant decrease in cell growth, reflected in absolute cell number at the time of harvest, even though the mean values for cell numbers tended to be lower for the highest concentration of propionate and valerate. However, analysis of the data expressed as per cent of control for each experiment showed a statistically significant decrease in cell number with propionate, butyrate and valerate (81 ± 7 per cent control for propionate, $P < 0.025$; 48 ± 3 per cent of control for butyrate, $P < 0.001$; 76 ± 6 per cent of control for valerate, $P < 0.001$). There was no reduction in cell division with sodium isobutyrate or gamma aminobutyric acid (GABA). Acetylcholinesterase activity was increased by treatment with both propionate and valerate, although the increases were less than that for sodium butyrate. Acetylcholinesterase activity was unaltered by isobutyrate or GABA. The specific activity of acetylcholinesterase (nmoles substrate converted/min/mg of protein) was also elevated after 48 hr of treatment with 5×10^{-4} M propionate, butyrate and valerate

Table 4. Effect of 5×10^{-4} sodium butyrate on the NBA_2 clone of neuroblastoma and the L929 mouse fibroblast cell line*

Cell line and molarity of sodium butyrate	Cell No. (as 10^6 cells)	Process formation (% of cells with neurites)	Sloughing $\frac{\text{cells in media}}{\text{total cells}} \times 100$	Protein (mg/10^6 cells)	Acetylcholinesterase (nmoles/min/10^6 cells)
NBA_2 clone					
0	7.4 ± 1.7 (8)	35 ± 1 (7)	8 ± 1 (8)	0.25 ± 0.04 (8)	0.26 ± 0.07 (10)
10^{-6}	8.2 ± 1.5 (8)	33 ± 6 (6)	7 ± 1 (8)	0.23 ± 0.10 (4)	0.52 ± 0.07† (9)
10^{-5}	6.0 ± 1.4 (8)	36 ± 5 (7)	8 ± 1 (7)	0.27 ± 0.04 (6)	0.44 ± 0.09 (10)
5×10^{-4}	3.4 ± 0.6† (9)	23 ± 5† (7)	10 ± 2 (9)	0.54 ± 0.06† (8)	5.68 ± 0.80† (11)
L929					
0	2.6 ± 0.1 (10)		4 ± 0 (10)	0.38 ± 0.06 (10)	0.21 ± 0.08 (10)
10^{-6}	2.6 ± 0.2 (8)		3 ± 0 (8)	0.34 ± 0.06 (7)	0.27 ± 0.09 (8)
10^{-5}	2.6 ± 0.1 (8)		3 ± 0 (8)	0.35 ± 0.05 (8)	0.38 ± 0.15 (8)
5×10^{-4}	1.9 ± 0.2† (8)		4 ± 0 (8)	0.36 ± 0.08 (8)	0.21 ± 0.06 (8)

* Cells were plated and treated as described for Table 3. The parameters listed in the table were measured by methods described in Materials and Methods. Results are expressed as mean ± standard error. The numbers in parentheses represent the number of experiments.

† Indicates statistical significance at $P < 0.05$ or greater.

(control, 1.86 ± 16, N = 19; propionate, 8.30 ± 1.36, N = 6, $P < 0.001$; butyrate, 12.86 ± 2.20, N = 18, $P < 0.001$; valerate, 9.20 ± 2.00, N = 4, $P < 0.001$).

There was no effect of the agents on cell sloughing, with the exception of 10^{-5} M valerate, which increased sloughing from 11 ± 2 per cent (N = 19) to 19 ± 3 per cent (N = 5). Each of the agents tested produced moderate neurite formation at 10^{-6} M, with decreasing effects as the concentrations were increased to 10^{-5} and 5×10^{-4} M. A lack of general cell toxicity of the agents listed in Table 3 is reflected in the lack of extensive rounding of the cells, even at the highest concentration tested. Sodium acetate at 10^{-5}, 5×10^{-4} and 10^{-3} M (N = 3 to 6) produced no change in cell growth, process formation, sloughing of cells or acetylcholinesterase activity. The T59 neuroblastoma cells were actually capable of more extensive neurite formation as shown by a 48-hr exposure to 3×10^{-5} M papaverine, which caused 79 ± 3 per cent (N = 4) of the cells to form processes.

NBA_2 clone and L929 fibroblasts. Sodium butyrate also decreased growth and increased acetylcholinesterase activity of the NBA_2 clone of neuroblastoma (Table 4). This agent had no effect on sloughing or viability of this clone, but at 5×10^{-4} M did cause a slight decrease in the number of cells having neurities; it also increased cell protein content at this concentration. Cell growth was inhibited at the high concentration only, whereas acetylcholinesterase was approximately double at 10^{-6} M and increased approximately 20-fold at 5×10^{-4} M. This clone followed a time course for neurite formation similar to that for the T59 cells upon treatment with 5×10^{-4} M sodium butyrate; morphologic differentiation was 50 per cent above control after 8 hr, but back to pretreatment levels at 24 hr of treatment and actually lower at 48 hr. Process formation was unchanged over this time period in NBA_2 cells maintained in regular media.

Growth of L929 fibroblasts was inhibited by 5×10^{-4} M sodium butyrate, but not by 10^{-5} or 10^{-6} M. Sloughing, cell protein content and acetylcholinesterase activity were unchanged by any of the concentrations of sodium butyrate.

Reversibility of the effect of sodium butyrate on growth. The reversibility of the effect of sodium butyrate on the growth of neuroblastoma cells was also tested. Two types of experiments were carried out for this purpose: (1) the medium was changed from that containing sodium butyrate to normal media, and (2) the cells were harvested from media containing sodium butyrate and replated at a lower density in regular media. In both procedures, cell number, morphological differentiation, cell sloughing, cell viability and responses to addition of sodium butyrate were measured. When either the uncloned or cloned cells were allowed to remain in media containing 5×10^{-4} M sodium butyrate for longer than 3–4 days, their rate of cell division, which either slows or ceases completely, gradually began to increase again, although generation times were still greater than those for untreated cells. The growth curves shown in Fig. 3 show the effect of a 4-day and of a 14-day exposure to 5×10^{-4} M sodium butyrate for the NBA_2 clone. The short exposure produces essentially complete inhibition of cell division, yielding 0.39×10^6 cells on day 5. Cell division occurred rapidly (generation time = 16 hr) upon return to normal media. However, upon continued treatment with sodium butyrate, the cells grew to 2.7×10^6 by day 15. The average generation time between days 5 and 9 in the presence of sodium butyrate was 45 hr; generation time for untreated cells in log growth was 24 hr in this experiment. The cells which had been treated with sodium butyrate for 4 days and the untreated cells were both harvested on day 15 and replated in normal media at 0.015×10^6 cells/ml. After a period of slow growth for 4 days both cell populations grew to confluency by day 24, with generation times of 27 hr for controls and 29 hr for the cells previously treated with sodium butyrate.

Results similar to those described above for the NBA_2 clone were obtained with uncloned T59 cells (Fig. 4). Panel a in Fig. 4 shows that treatment of

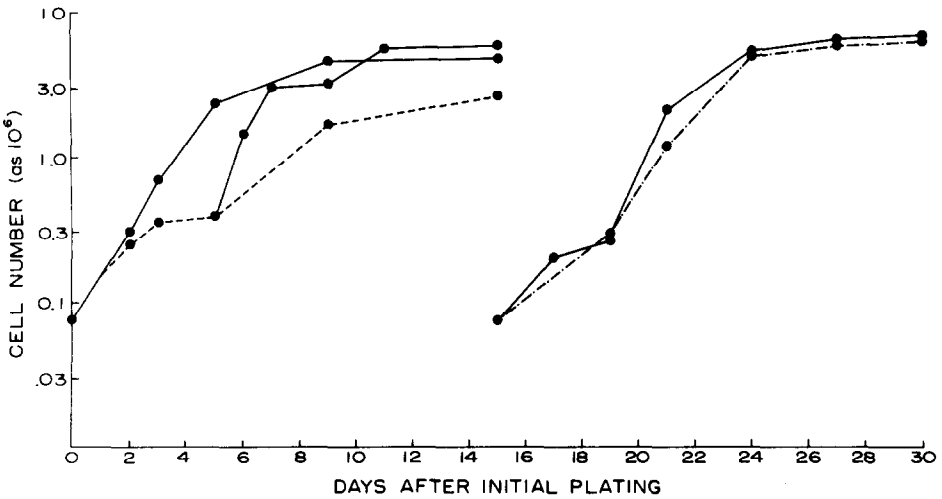

Fig. 3. Effects of treatment for 4 or 14 days with 5×10^{-4} M sodium butyrate on the growth of NBA_2 neuroblastoma cells. The cells were plated initially at a density of 0.015×10^6/ml in 25 cm^2 Falcon plastic tissue culture flasks containing 5 ml media. On day 1 after plating, one group of flasks was treated with 5×10^{-4} M sodium butyrate, depicted by the dashed line (– – – –), and another group was maintained in regular media, shown by the solid line (——). On day 5, a portion of the cells being treated with sodium butyrate was returned to regular media (solid line) and another portion was maintained in sodium butyrate-containing media (dashed line) for an additional 10 days. On day 15, the control and sodium butyrate-treated cells were harvested and replated at a density of 0.015×10^6/ml. The growth of these cells is shown in the two curves on the right. Cells previously treated with sodium butyrate, but replated in regular media, are shown by a broken line (– · – · –); cells growing in regular media are shown by a solid line (——).

the cells with 5×10^{-4} M sodium butyrate results in inhibition of cell division after treatment for 4 days. Cell division occurred at a normal rate upon changing the cells to sodium butyrate-free media. In another experiment, shown in panel b of Fig. 4, the cells were harvested after treatment with 5×10^{-4} M sodium butyrate for 4 days and replated at a lower density. Both the control cells and the cells previously treated grew to a confluent level by day 11 of the experiment. Generation times between days 6 and 9 were 21 hr for the previously untreated cells and 24 hr for the cells previously treated with sodium butyrate.

DISCUSSION

Sodium butyrate produces a variety of changes in cells growing in culture. For example, Wright [13] showed that the addition of 5×10^{-4} M sodium

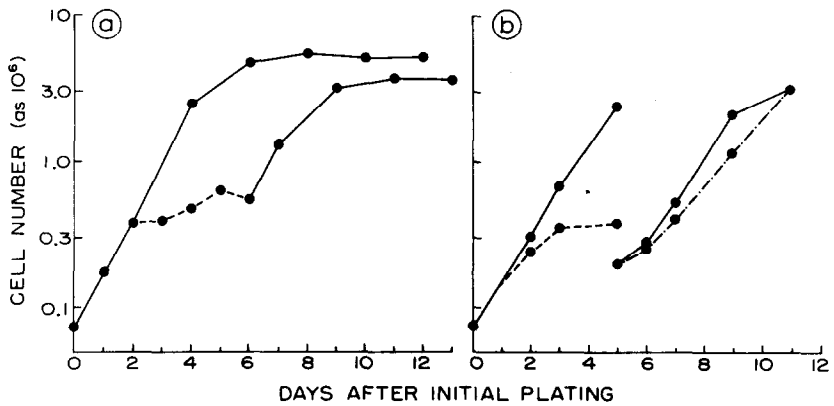

Fig. 4. Growth curves of T59 neuroblastoma cells during and after treatment with 5×10^{-4} M sodium butyrate. Cells were plated at a density of 0.015×10^6/ml in 25 cm^2 Falcon plastic tissue culture flasks containing 5 ml media. Sodium butyrate (5×10^{-4} M) was present in the media during the times indicated by the dashed line (– – – – – –), in panels a and b. Total cell counts were obtained only on the days for which points are included, and each value represents the average of duplicate flasks. In panel a, the cells treated with sodium butyrate were returned to regular media on day 6. In panel b, the cells treated with sodium butyrate and those growing in regular media were harvested on day 5 and replated in regular media at a density of 0.04×10^6/ml. The broken line (– · – · – · –) in panel b represents growth of the cells which had been previously treated with sodium butyrate. In each experiment (panels a and b), sodium butyrate, with fresh media, was added on days 1 and 3 of treatment.

butyrate, but not sodium isobutyrate, to Chinese hamster cells increased their length, enhanced their tendency to grow in monolayer and decreased their growth rate. Sodium butyrate (5×10^{-4} to 10^{-3} M) also causes a decrease in cell division and altered morphology, but without increased neurite formation, of mouse neuroblastoma cells in culture [1, 2, 4, 6]. However, quite different results were obtained in the study reported here when sodium butyrate was used in concentrations lower than those used in previous investigations. Neurite formation, both as the per cent of cells having neurite and in the length of the processes, was observed with concentrations of 3×10^{-4} M or below. The per cent of cells having processes in the presence of low sodium butyrate was not as high as that seen with some other agents. This does not reflect an inherent limitation of these cells, since the phosphodiesterase inhibitors R020-1724 and papaverine, which are known to induce process formation [2, 4, 6], both caused extensive neurite formation. Also interesting is the difference observed between the uncloned T59 neuroblastoma cells and the NBA_2 clone; in contrast to the uncloned cells, the cloned line did not respond to the lower concentrations of butyrate with neurite formation. However, neurite formation was seen 8 hr after treatment with 5×10^{-4} M butyrate, but then returned to lower values. The effect on cell growth was not specific for neuroblastoma cells, since division of mouse L929 fibroblasts was also reduced in the presence of this acid.

This work also showed that neurite formation can occur without a corresponding decrease in cell division. The per cent of cells having neurites in the presence of 10^{-6} M sodium butyrate was almost double that of untreated cells, although the total cell number after 48 hr of treatment with the acid was no different than for cells maintained in drug-free media. Furthermore, the neurites in the treated cells were greater in length, and did not represent only an increase in the number of short processes normally present in untreated cultures. Increased neurite formation in growing cells has also been observed upon treatment of T59 neuroblastoma cells for 48 hr with 3×10^{-4} M cyclic AMP, which stimulated cell growth and increased neurite formation 64 per cent [12]. These findings support previous observations [7, 20, 21] that decreased cell division is not a requisite for neurite formation, although there are many instances where neurite formation occurs after a reduction in cell division [1, 4].

The increase in acetylcholinesterase activity stimulated by sodium butyrate is similar to that reported previously for this agent [7, 15], as well as for others [7, 22, 23]. Acetylcholinesterase induction and neurite formation did not appear to be causally related since process formation with 5×10^{-4} M sodium butyrate occurred within 18 hr after initiation of treatment, but then fell off to control values by 48 hr, whereas enzyme activity increased gradually over the 48-hr period. Likewise, process formation for a 48-hr treatment period was most pronounced at 10^{-6} to 5×10^{-5} M sodium butyrate, while the increase in enzyme activity is greater at 5×10^{-4} M than at the lower concentrations. It appears that the increase in acetylcholinesterase activity stimulated by sodium butyrate is related more closely to cell growth, and in the present study the magnitude of the increase in acetylcholinesterase activity caused by the three straight-chain acids appeared to be related to the degree of inhibition of cell growth [7, 22, 23]. Yet there are exceptions to this hypothesis also. For example, acetylcholine causes an induction of neuroblastoma acetylcholinesterase activity without an effect on cell growth [21]. Several workers [2, 23, 24] have shown that the increases in acetylcholinesterase activity seen after treatment with various agents are prevented by inhibitors of protein synthesis, and Lanks *et al.* [24] reported that the increased specific activity of acetylcholinesterase in neuroblastoma cells maintained in serum-free media reflects an increased rate of synthesis of the enzyme. Since the increase in acetylcholinesterase activity seen upon treatment with sodium butyrate (1) reflects an increase in specific activity, (2) occurred over a period of time similar to that required for induction of activity by other conditions, and (3) was prevented by cycloheximide, it may represent true enzyme induction. However, further work is required before this point can be established with certainty.

Both the decrease in cell division and the increase in acetylcholinesterase activity stimulated by 5×10^{-4} M sodium butyrate were reversible upon removal of the agent. The reversibility was seen even after 14 days of treatment, and was demonstrated in cells which were harvested and replated at a lower density and on cells which were not harvested but changed into regular media from sodium butyrate-containing media. Another interesting point is that cells gradually emerged which were able to grow in the presence of 5×10^{-4} M sodium butyrate (Fig. 3). It is not possible to say at this time whether this reflects selection of resistant mutants or drug-induced alterations in the cells.

The reversibility of changes in neuroblastoma cells which are thought to reflect neuronal differentiation is controversial. Prasad [25] has reported that dibutyryl cyclic AMP, prostaglandin E_1 and inhibitors of cyclic nucleotide phosphodiesterase induce irreversible morphological differentiation. On the other hand, others have reported that the neurites which were formed in serum-free media or in the presence of dibutyryl cyclic AMP are promptly retracted upon return of the cells to regular media [2, 26]. Schubert *et al.* [26] also showed that cell growth returned after changing cells from serum-free media to media containing 10% fetal-calf serum, although the onset of growth was delayed with longer times (up to 5 days) of maintenance in serum-free media. It is likely that the reversibility of the "differentiation" in neuroblastoma in culture is dependent upon the mechanism of induction, length of exposure and characteristics of the clone or population of cells being studied.

The importance of the specific characteristics of given clones in determining responses to pharmacological agents is apparent in the marked biochemical differences among specific neuroblastoma clones [3, 27, 28]. It follows that, when assessing the pharmacological effects of a chemical agent, uncloned as well as cloned lines can be used profitably. The uncloned cells are probably more representative of the actual tumor tissue, and might, therefore, give a better indi-

cation of the effect of the agent on neuroblastoma tumor cells *in vivo*. The generality of effects observed on uncloned cells can be tested by employing individual clones. This approach was used in this study to show that sodium butyrate causes reduced cell growth and elevated acetylcholinesterase activity in uncloned cells and in the clone NBA_2, whereas at a lower concentration it causes neurite formation in the uncloned cells but not in this particular clone.

The complexity of the action of sodium butyrate on neuroblastoma cells may reflect the ability of pharmacological agents to produce multiple effects on cells, and also that these effects exhibit differing concentration–response relationships. Thus, the neurite formation stimulated by the lower concentrations of sodium butyrate may be opposed by an additional effect seen at higher concentrations, such as, for example, a nonspecific toxicity which results in rounding of the cells with retraction of processes. However, if a toxic action is responsible for this effect, it is a rather mild one, since detachment of cells from the flask surface is not increased and cell viability is only slightly reduced in the short-term experiments. A low degree of toxicity is also reflected in the observation that papaverine is capable of inducing neurite formation in the presence of 5×10^{-5} M sodium butyrate. Furthermore, cell growth is rapid in onset upon removal of the cells from contact with sodium butyrate.

Little can be said about the mechanism by which the short-chain fatty acids produce these effects. It is clear that the effect is optimal for a straight chain, four carbons in length, since isobutyrate, a branched-chain four-carbon acid, was ineffective, and propionic acid and valeric acid were less effective. It is unlikely that sodium butyrate is acting by mimicking GABA, a putative neurotransmitter, since this agent itself had no effect on the cells. Acetate was also ineffective, suggesting that butyrate is not acting by providing acetate as a substrate for cell metabolism. These data suggest that sodium butyrate is acting in some specific manner to alter cell function. Further work is required in order to clarify the mechanisms by which these effects are produced. This work also further strengthens the need to test butyrate as a control for studies in which dibutyryl cyclic AMP is used.

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