MORPHOLOGICAL ALTERATIONS AND GANGLIOSIDE SIALYLTRANSFERASE ACTIVITY INDUCED BY SMALL FATTY ACIDS IN HELA CELLS

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ABSTRACT

Incubation of HeLa cells in the presence of millimolar concentrations of propionate, butyrate, or pentanoate increases the specific activity of CMP-sialic acid:lactosylceramide sialyltransferase 7–20-fold within 24 h. Longer-chain saturated fatty acids or acetate are much less effective, decanoate showing no induction. Unsaturated fatty acid analogs of butyrate and other compounds are ineffective. Only the three most effective compounds also produce characteristic smooth extended cell processes in HeLa cells. Butyrate (5 mM) induces the sialyltransferase after a 4-h lag, producing maximum specific activity by 24 h. The amount of sialyl-lactosylceramide, the glycolipid product of the enzyme, increases during that time 3.5 times more than in control cultures. No other glycosphingolipid enzyme is significantly altered by butyrate exposure. The cellular shape changes occur 2–3 h later than the increase of sialyltransferase activity, and both processes require the continuous presence of inducer and the synthesis of RNA and protein but not the synthesis of DNA or the presence of serum.

Transformation of mammalian fibroblasts, which occurs spontaneously or after exposure to viruses or carcinogens, gives rise to cells that are more rounded and compact, have shorter processes, and grow without contact inhibition in tissue cultures (30, 27). Transformed cells are also unable to synthesize the more complex glycosphingolipids found in normal cells, because the activities of the corresponding glycosyltransferases are decreased (2, 5, 8, 21). Revertants of transformed cells have been isolated which exhibit a return of many normal phenotypic properties including cell growth and shape (24, 7). Exposure of transformed fibroblasts to dibutyryl cyclic adenosine-monophosphate (cAMP) or other N^6-adenine derivatives produces a more normal phenotype, including more extended cell processes (16, 18, 19). Recently, it has been shown that short-chain fatty acids, such as butyrate, can alone produce a drastic extension of cell processes in epithelioid cells such as the (transformed) HeLa cell line (14).[1] It was furthermore found that butyrate causes in HeLa cells the specific increase of one glycosphin-

[1] C. W. Sheu, D. Salomon, J. L. Simmons, T. Sreevalsan, and E. Freese, manuscript in preparation.

golipid, the ganglioside *N*-acetylneuraminylgalactosylglucosylceramide (G_{M3}), and induces the corresponding enzyme activity CMP-sialic acid: lactosylceramide sialyltransferase (13). This effect provides the unique opportunity to study the temporal relationship between enzyme increase and morphological alteration. This relationship has been examined, with inconclusive results, after reversion of transformed cells (22, 8, 6) or quantitative transformation of normal cells with RNA sarcoma viruses (23, 15, 28). In this paper we report on the specificity and kinetics of sialyltransferase induction and relate it to the change in morphology investigated in the following paper.[2]

MATERIALS AND METHODS

Cell Culture

HeLa cells, strain 'R (Grand Island Biological Company, Grand Island, N. Y.), were cultured in minimal essential Eagle's medium supplemented with nonessential amino acids (MEM) (11) and 10% calf serum (Flow Laboratories, Inc., Rockville, Md.); this will be called "medium." Cells were grown in plastic Petri dishes of 150 mm diameter (Falcon Plastics, Oxnard, Calif.) in a humidified incubator gassed with 5% CO_2 in air at 37°C. In a typical experiment, dishes containing about 10^7 cells were washed three times in Dulbecco's phosphate-buffered saline (10) without Ca^{++} and Mg^{++} (PBS) and incubated for 10 min in 2.5 ml (per dish) 0.05% trypsin in PBS at 37°C. The cells were suspended in medium and a portion, diluted in physiological saline, was counted in a Cellozone particle counter (Particle Data, Inc., Elmhurst, Ill.). Cells were then seeded in 150-mm plastic Petri dishes at a density of 2×10^4 cells per cm^2 and incubated at 37°C for 24–36 h. To initiate the experiment, the growth medium was aspirated from the dish and replaced with medium containing the desired experimental compound. The cells were then incubated for different periods of time, usually 12 h. At the end of that time, the degree of morphological alteration was photographically recorded with a Leitz inverted microscope at a magnification of 200. The cells were harvested for enzyme assay and glycolipid analysis as follows. The experimental medium was aspirated and the cell sheet washed three times with warm (37°C) PBS. The cells were detached as stated above for enzyme assays, or by mechanical scraping for glycolipid analysis, immediately suspended in 10 vol of ice-cold PBS, and centrifuged at 200 *g* for 3 min at 4°C. The cells were suspended in (10 ml per original dish) ice-cold PBS and centrifuged again. The cell pellet was maintained on ice for early glycolipid extraction and enzyme assay.

Glycolipid Isolation and Analysis

Glycosphingolipids were isolated from washed cell pellets and partitioned into neutral and sialic acid-containing classes as previously described (9). The neutral glycosphingolipids were further purified by saponification (9) and separated by thin-layer chromatography on silica gel G plates (E. Merck, Darmstadt, West Germany) with the solvent system chloroform-methanol-water (65:25:4, vol/vol/vol). The glycosphingolipids were detected by spraying the plates with 50% ammonium bisulfate and heating at 180°C for 15 min. They were identified by comparing their migration to authentic standards and quantified by densitometric scanning. The ganglioside fraction was separated by thin-layer chromatography (23), detected with resorcinol reagent, and quantified by densitometric scanning as previously described (22).

Incorporation of radioactively labeled precursors was measured in cells grown in either [1-^{14}C]galactose (1.25 μCi/ml, 28 mCi/mmol, International Chemical and Nuclear Corp., Irvine, Calif.) or *N*-[acetyl-^{3}H]-D-mannosamine (62.5 μCi/ml, 400 μCi/mmol, Tracerlab, Waltham, Mass.) for 24 h. The radioactive glycosphingolipids were extracted and separated by thin-layer chromatography as above, and the radioactivity in individual glycolipids was detected and quantitated with a Berthold chromatogram radioscanner (Varian Associates, Walnut Creek, Calif.).

Assay of CMP-Sialic Acid: Lactosylceramide Sialyltransferase

The glycolipid acceptor, lactosylceramide, was obtained from Miles-Yeda (Rehovoth, Israel) and the sugar nucleotide donor, CMP-[4-^{14}C]sialic acid (6.5–9.0 mCi/mmol), from New England Nuclear, Boston, Mass. The washed cell pellets were suspended in 2.5 vol of 0.25 M sucrose containing 0.1% 2-mercaptoethanol and disrupted by freezing and thawing four times (12).

The assay of sialyltransferase activity was similar to that described by Fishman et al. (13) but modified for optimum results with HeLa cell extracts. The reaction mixture contained the following: 5 nmol of lactosylceramide, 30 nmol of CMP-[^{14}C]-NAN, 25 μg of bovine cardiolipin (Applied Science Labs, Inc., State College, Pa.), 1.25 μmol of sodium-cacodylate buffer (pH 6.8), 375 μg of Cutscum (Fisher Scientific Co., Fairlawn, N.J.), and 300 μg of cell protein in a final volume of 25 μl. For each assay the same reaction mixture minus lactosylceramide was incubated as a control. The reactions were terminated after 3 h, at which time the activity still increased linearly.

The reaction products were isolated by either of two procedures. The first procedure, using the separation of

[2] J. L. Simmons and A. Breuer, manuscript in preparation.

the labeled glycolipid from the precursors on small Sephadex columns, has been described (12). The second procedure took advantage of the lipophilic nature of gangliosides in acidic calcium solutions (3). To each incubation tube, 1.0 ml of chloroform, 0.5 ml methanol, 0.3 ml 2% $CaCl_2$, and 0.15 ml 1.0 M formic acid were added. After vigorous mixing on a Vortex mixer (Science Industries, Inc., Springfield, Mass.) and centrifugation, the upper phase was discarded. The lower phases were washed twice with 1.0 ml of upper phase solvent and dried in counting vials. Recovery of G_{M3} was over 90%. Radioactivity was determined by liquid scintillation counting. Both procedures produced comparable results but the second partitioning method was simpler and faster. Activities are reported as picomoles of product formed from the exogenous acceptor per milligram protein per hour.

The reaction product G_{M3} was identified by its migration on thin-layer silica gel and its degradability by purified neuraminidase from *Clostridium perfringens* (Sigma Chemical Co., St. Louis, Mo.). After complete digestion (16 h with 0.1 U of enzyme in 0.1 ml of 0.05 M sodium-acetate buffer, pH 5.0), all of the radioactivity was recovered as sialic acid.

Chemicals

Propionic, butyric, and pentanoic acids (obtained from Sigma Chemical Co.) were prepared as 0.5 M stock solutions in H_2O titrated to pH 7.45 with sodium hydroxide. All other test compounds were added directly to MEM plus 10% calf serum, titrated to pH 7.45 with sodium hydroxide, and sterilized by filtration with a 0.45 μm Nalgene filter unit (Sybron Corp., Rochester, N. Y.). Other compounds tested were acetic, hexanoic, octanoic, decanoic, crotonic, α-aminoisobutyric, L-α-amino-*n*-butyric, γ-amino-*n*-butyric, and salicylic acids obtained from Sigma. Also tested (from Sigma) were *n*-butyric acid methyl ester, *n*-butanol, methylparabens, propyl parabens, and dimethylsulfoxide. Isobutyric acid was obtained from Fisher Scientific Co., and DL-2-aminobutric acid, phenethyl alcohol, and phenylacetic acid were obtained from Eastman Kodak Co., Rochester, N. Y. Tetrolic acid was obtained from Farchan Research Laboratories, Cleveland, Ohio. Actinomycin D (Calbiochem, San Diego, Calif.) was also utilized.

RESULTS

Effect of Butyrate on the Glycosphingolipid Composition

Fig. 1 shows the ganglioside pattern of HeLa cells grown for 24 h in normal medium and in medium supplemented with butyrate (5 mM). Only two resorcinol-positive areas were present on the chromatogram. The doublet corresponding to G_{M3} was more intensely stained in the glycosphingolipid extract of cells exposed to butyrate than in that of normal cells. The level of the slower-migrating ganglioside, which probably is G_{D3}, was unaffected by butyrate. The two gangliosides were further identified by their sensitivity to bacterial neuraminidase. The ganglioside extract isolated from cells grown in the presence of both butyrate (5 mM) and small amounts of *N*-[*acetyl*-^{3}H]-D-mannosamine was incubated with purified neuraminidase and the products were separated on thin-layer plates (Fig. 2). The resorcinol-positive material and the radioactivity migrated with the sialic acid standard. Only terminal sialic acids are hydrolyzed under these conditions. The other hydrolysis product of G_{M3}, the lactosylceramide Gl-2, was also observed upon charring the chromatogram. The gangliosides of untreated cells were similarly identified.

The glycolipid content of normal and butyrate-treated HeLa cells is given in Table I. Three neutral glycosphingolipids (the mono-, di-, and trihexosylceramide) and two gangliosides (G_{M3} and G_{D3}) were observed in this cell line. When the cells were exposed for 24 h to 5 mM butyrate in growth medium, the G_{M3} content increased 3.5-fold, the Gl-1 content increased about 70%, while the slight changes of the other glycosphingolipids were insignificant. Although *N*-acetylgalactosaminylgalactosylgalactosylglucosylceramide (globoside) is present in many human tissues, none was detected in HeLa cells. The cell extracts contained some lipid material that migrated on the chromatogram between the globoside and G_{M3} standards, but this material was not labeled with [^{14}C]-galactose, in contrast to the other glycosphingolipids.

The incorporation of radioactive sugars into different glycosphingolipids in the presence and absence of butyrate is shown in Table II. When HeLa cells were grown for 24 h in medium containing *N*-[*acetyl*-^{3}H]-D-mannosamine, a specific precursor of sialic acid, tritium incorporation into G_{M3} was 6.5 times greater in the presence of butyrate than in the controls and the amount of radioactive material per nanomole of G_{M3} was twice as large. Tritium incorporation into G_{D3} was similar for both treated and untreated cells. Butyrate-treated cells also showed an almost twofold increase in incorporation of [1-^{14}C]galactose into G_{M3} but in this case the amount of radioactive material per nanomole of glycosphingolipid was approximately half that of control cells (Table II).

Induction of Sialyltransferase Activity

The increased amount of G_{M3} has been correlated with an increase in the specific activity of CMP-sialic acid:lactosyl ceramide sialyltransferase (briefly called sialyltransferase), as a result of HeLa cell exposure to 5 mM butyrate (13). Fig. 3 shows that the specific activity increased upon butyrate exposure after a 4-h lag, reaching within 12 h a level 14 times greater than that of untreated cells. The possibility of sialyltransferase activation or inhibition by some other cell component was excluded by a mixing experiment (Table III). Addition of butyrate (5 mM) to the assay mixture also did not affect the enzyme activity.

The effect of different butyrate concentrations on enzyme induction, measured 24 h after butyrate addition to HeLa, is shown in Fig. 4. Morphologi-

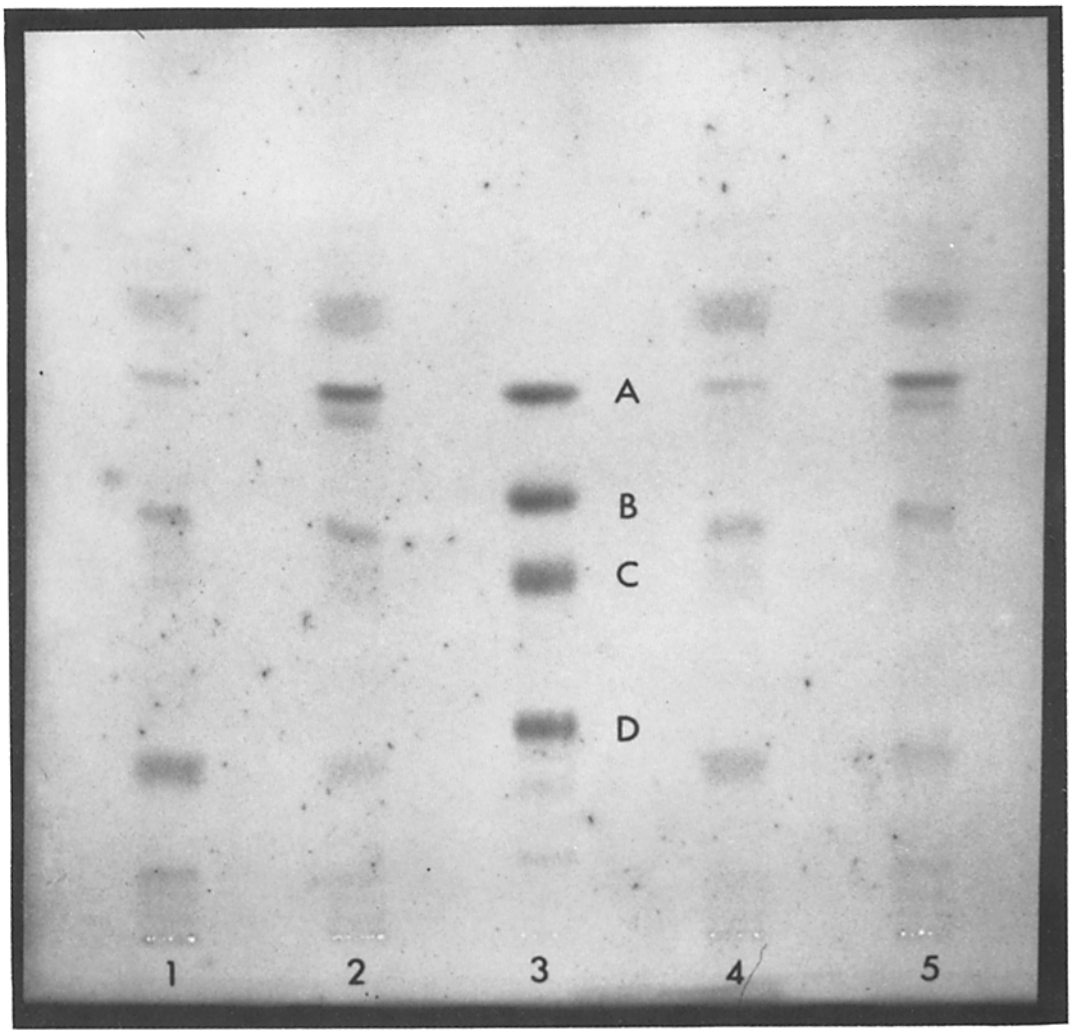

FIGURE 1 Thin-layer chromatogram of gangliosides extracted from control and butyrate-treated HeLa cells. 24 h after growth of HeLa cells in normal medium or medium containing 5 mM sodium butyrate, gangliosides were extracted from an amount of cells representing 5 mg protein, isolated, chromatographed, and stained with resorcinol reagent as described in Materials and Methods. Gangliosides are visualized as resorcinol-positive purple bands. Lanes 1 and 4: gangliosides from control cells; lanes 2 and 5: gangliosides from butyrate-treated cells; lane 3: ganglioside standards, (A) G_{M3}, (B) G_{M2} (*N*-acetylgalactosaminyl-[*N*-acetylneuraminyl]-galactosylglucosylceramide), (C) G_{M1} (galactosyl-*N*-acetylgalactosaminyl-[*N*-acetylneuraminyl]-galactosylglucosylceramide), (D) G_{D1a} (*N*-acetylneuraminylgalactosyl-*N*-acetylgalactosaminyl-[*N*-acetylneuraminyl]-galactosylglucosylceramide).

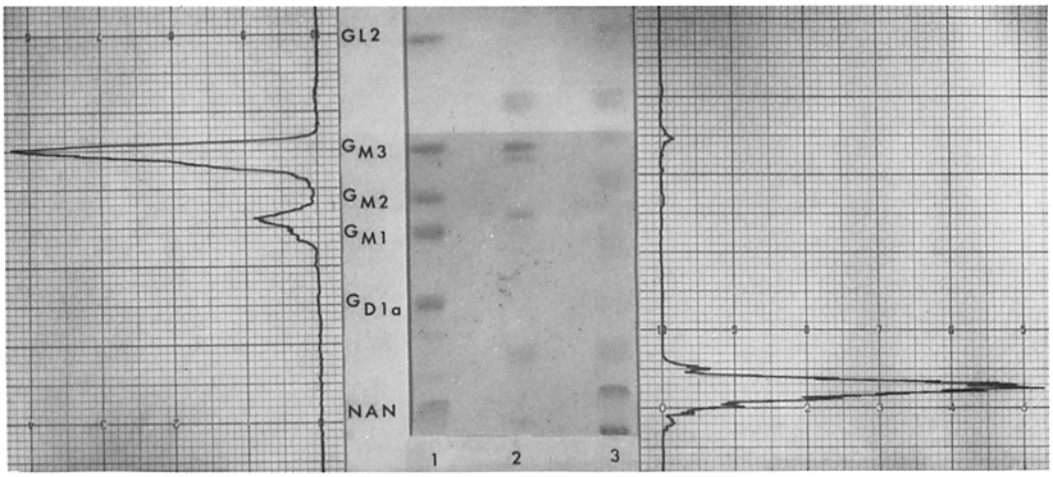

FIGURE 2 Radioscan of thin-layer chromatogram of HeLa-derived gangliosides before and after neuraminidase treatment. Gangliosides were extracted and isolated from HeLa cells grown for 24 h in medium containing sodium butyrate (5 mM) and *N*-[acetyl-^{3}H]-D-mannosamine (156 μM, 62.5 μCi/ml). One aliquot was untreated (lane 2, in middle) while another aliquot was incubated overnight with 0.1 U neuraminidase (in 0.2 ml of 0.05 M sodium acetate and 0.154 M NaCl, pH 5.0) before chromatography (lane 3). Lane 1 contains the designated standards. The chromatogram was then scanned for radioactivity. The left panel is the scan of lane 2 and the right panel that of lane 3. After the radioactive scan, the lower portion of the chromatogram was stained with the resorcinol reagent, and then the upper portion was charred as described.

cal changes were seen in cells exposed to as little as 0.5 mM butyrate but they were more apparent at 1 mM butyrate or higher. To establish whether the decreased induction of sialyltranferase activity and cell shape changes was simply a matter of inadequate exposure time, cells were incubated in media containing 0.5 mM or 5 mM butyrate for up to 3 days. No further morphological changes or increase in enzyme activity were seen beyond the effects apparent at 20 h.

The induction of sialyltransferase activity and of process formation is fully reversible. The specific activity of the enzyme decreases to the level found in control cells within 16–24 h when the butyrate is removed from the medium (Fig. 5); the cells return to the normal epithelioid morphology within 18–24 h. The half-life of the induced enzyme is around 6–8 h.

Actinomycin D (as well as cycloheximide) has been shown to prevent both enzyme induction (13) and morphological alteration (14); Table IV demonstrates that it does so at very low concentrations at which RNA synthesis can just be inhibited whereas the other toxic effects observed at higher actinomycin concentrations (17) are not yet effective. Note that an actinomycin D concentration of 0.25 μg/ml allowed the production of some enzyme while no morphological alteration was observed.

Other glycosyltransferases involved in glycolipid biosynthesis (12) did not increase in the presence of butyrate (Table V).

TABLE I

Glycolipid Composition of HeLa Cells Grown in Normal and Butyrate-Supplemented Media

Glycolipid	Structure	Control	Butyrate
		nmol/mg cell protein	
Gl-1	cer-glc*	0.55	0.95
Gl-2	cer-glc-gal	0.41	0.45
Gl-3	cer-glc-gal-gal	1.85	2.21
G_{M3}	cer-glc-gal-NAN	0.49	1.72
G_{D3}	cer-glc-gal-NAN-NAN	0.23	0.19

HeLa cells were cultured for 24 h at 37°C in normal and sodium butyrate (5 mM)-supplemented media. Glycolipids were extracted, isolated, separated, and quantitated as described in Materials and Methods. Results are the average of two experiments.

* Abbreviations: cer, ceramide; glc, glucose; gal, galactose; NAN, *N*-acetylneuraminic acid.

TABLE II

Incorporation of [^{14}C]Galactose and N-[Acetyl-^{3}H]-D-Mannosamine into Glycosphingolipids of Normal and Butyrate-Treated HeLa Cells

Glycosphingolipid	Control Cells				Butyrate-Treated Cells			
	^{14}C		^{3}H		^{14}C		^{3}H	
	cpm/mg protein	*cpm/nmol*	*cpm/mg protein*	*cpm/nmol*	*cpm/mg protein*	*cpm/nmol*	*cpm/mg protein*	*cpm/nmol*
Gl-1	4,465	8,118	—*		2,165	2,279	—	
Gl-2	2,938	7,166	—		1,698	3,773	—	
Gl-3	17,128	9,258	—		9,375	4,422	—	
G_{M3}	3,033	6,190	7,249	14,794	5,681	3,303	47,421	27,570
G_{D3}	6,957	15,124	14,983	32,572	3,274	8,616	11,646	30,647

HeLa cells were cultured at 37°C in normal and sodium butyrate (5 mM)-supplemented medium which also contained [1-^{14}C]galactose (45 μM, 1.25 μCi/ml) or *N*-[*acetyl*-^{3}H]-D-mannosamine (156 μM, 62.5 μCi/ml). After 24 h, the cells were harvested and the radioactive glycosphingolipids were extracted, separated by thin-layer chromatography, and quantitated by radioscanning as described in Materials and Methods.

* No radioactivity was observed.

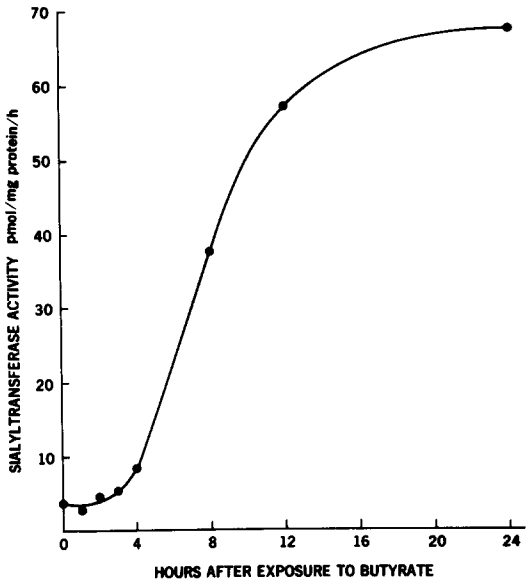

FIGURE 3 Increase of sialyltransferase activity in HeLa cells after exposure to butyrate. At time 0, the medium of HeLa cell cultures was replaced with medium containing 5 mM sodium butyrate. At the designated times, some cultures were harvested and assayed for sialyltransferase activity as described in Materials and Methods.

TABLE III

Sialyltransferase Activity in Cell Homogenates of Normal and Butyrate-Treated HeLa Cells Assayed Separately and Admixed

Homogenate		Sialyltransferase activity		Difference
		Endogenous	+Acceptor	
	μg	*cpm/2 h incubation*		
Control	297	46	106	60
Butyrate-treated	305	153	639	486
Control	212	76	308	232
Butyrate-treated	127			
		Expected if no inhibition		245

HeLa cells were grown for 12 h in normal or butyrate (5 mM)-supplemented media. Cell homogenates were prepared and assayed separately and admixed for sialyltransferase activity by the standard assay except that the reaction mixtures were incubated for 2 h.

Effect of Cell Cycle Inhibitors on Enzyme Induction

In order to establish whether the increase of sialyltransferase was simply a result of cellular arrest at a particular stage of the cell cycle, the effects of thymidine and Colcemid were examined. Cells incubated for 24 h in medium containing 3 mM thymidine, and thus all arrested in S phase, showed no altered morphology and no enzyme increase. But when their incubation was continued for another 12 h in the presence of thymidine and 5 mM butyrate, their morphology was altered and the enzyme had reached typical elevated levels (Table VI). When Colcemid (0.6 μg/ml) and butyrate were added simultaneously to normally

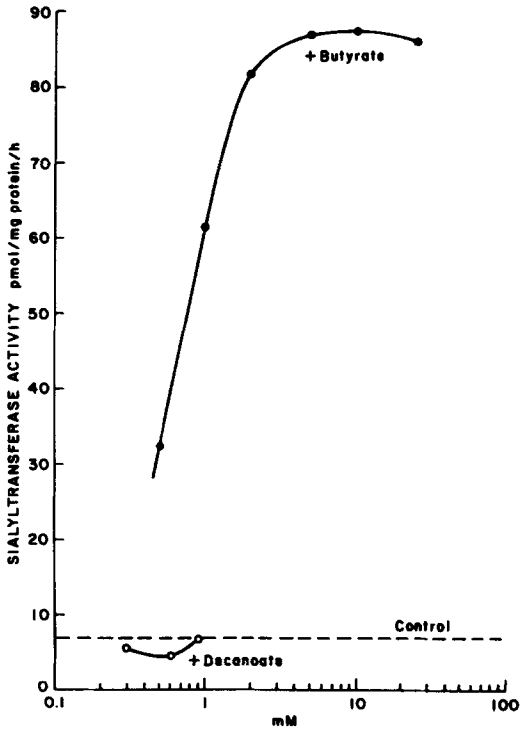

FIGURE 4 Effect of butyrate and decanoate on sialyltransferase activity in HeLa cells. Cells were grown for 12 h in media containing different concentrations of sodium butyrate (●) or decanoate (O), harvested, and assayed for sialyltransferase activity. Activity in untreated cells was 7 pmol/mg protein/h (-----).

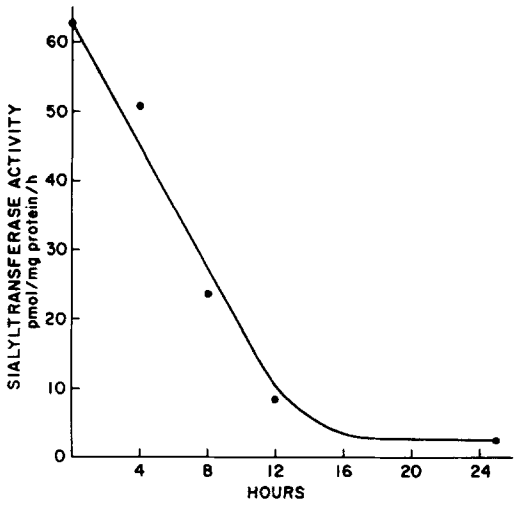

FIGURE 5 Effect of butyrate removal on sialyltransferase activity in HeLa cells. Cells were grown for 12 h in medium containing 5 mM sodium butyrate. At time 0, the medium of the cultures was replaced with normal medium. At the designated times, cells were harvested and assayed for sialyltransferase activity. Enzyme activity in untreated cells was 5.6 pmol/mg protein/h.

TABLE IV

Actinomycin D Inhibition of Butyrate-Induced Sialyltransferase Increase and Cell Shape Changes

Actinomycin D	Sialyltransferase Activity		Morphology (+Butyrate)
	Control	+ Butyrate	
μg/ml	*pmol/h/mg protein*		
0	5	46	+
0.03	—	61	+
0.06	—	56	+
0.25	8	28	—
0.50	—	3	—

HeLa cells were grown for 12 h in normal and butyrate (5 mM)-supplemented media containing different amounts of actinomycin D. The cells were then analyzed for process formation (+) and sialyltransferase activity.

growing HeLa cells, sialyltransferase activity increased but no process formation was seen.

Since serum withdrawal causes process formation in neuroblastoma cells (27), its effect on HeLa cells was investigated. When these cells were incubated for 12 h in serum-free medium, no alteration in morphology or enzyme level was seen (Table VI). However, when the cells were incubated without serum but with 5 mM butyrate, the typical cell processes and the characteristic enzyme increase were observed.

Effects of Lipophilic Agents on Sialyltransferase Induction and Morphological Alterations

To determine the specificity of sialyltransferase induction and the correlation between this induction and the morphological changes, the effect of several other compounds was measured. As Table VII shows, all fatty acids that produced the typical process formation[1] also caused enzyme induction. The absence of enzyme induction with decanoate is shown for different decanoate concentrations in Fig. 4. The tight grouping of the C_3, C_4, and C_5 saturated fatty acids as the only compounds producing these effects is particularly noteworthy. Acetate and octanoate, ineffective morphologically, produced only small increases in enzyme

TABLE V

Activities of Different Glycosyltransferases in HeLa Cells Grown in Normal and Butyrate-Supplemented Media

Glycosyltransferase	Reaction	Control	Butyrate
		pmol/mg	*protein h*
Glucosyltransferase*	Cer + UDP-Glc → Gl-1 + UDP	107‡	104‡
Galactosyltransferase§	Gl-2 + UDP-Gal → Gl-3 + UDP	154	193
Sialyltransferase	Gl-2 + CMP-NAN → G_{M3} + CMP	5	69‖

Cells were grown for 24 h in normal or sodium-butyrate (5 mM)-supplemented medium, harvested, and the enzymes were assayed in cell homogenates. It has been shown previously that UDP-Gal:Gl-1 galactosyltransferase activity is not increased in butyrate-treated cells (13).

* Glucosyltransferase activity was assayed by the method of Basu et al (1).

‡ Glucosyltransferase activities are given in cpm/1.5 h/mg protein.

§ Galactosyltransferase activity was assayed by the method of Kijimoto and Hakomori (21).

‖ With saturating concentrations of CMP-NAN, sialyltransferase activity was 220 pmol/mg protein/h in butyrate-treated cells.

TABLE VI

Effect of Thymidine, Colcemid, and Serum-Deprivation on the Induction of Sialyltransferase Activity by Butyrate

Inhibitor	Sialyltransferase activity	
	Control	Butyrate
	pmol/mg protein/h	
Experiment 1		
None	8.2 ± 1.6	67.2 ± 7.8
Thymidine	6.5 ± 0.9	93.8 ± 7.7
Colcemid	7.5 ± 0.8	90.5 ± 8.2
Experiment 2		
None	3.0	62
Serum-Deprivation	2.0	52

In Experiment 1, HeLa cells were grown for 24 h in normal or 3 mM thymidine-containing media. Normal medium was then replaced with normal or 5 mM butyrate-supplemented medium containing either 0.6 μg/ml Colcemid or no further addition. Cells grown with thymidine were maintained in thymidine-containing medium with and without added butyrate (5 mM). After 12 h the cells were harvested and the enzyme was assayed. Stated activities are averages of two separate determinations. In Experiment 2, HeLa cells were grown for 12 h in normal and serum-free media with and without butyrate (5 mM).

TABLE VII

Induction of Sialyltransferase by Chain Fatty Acids

Fatty acid (sodium salt)	Sialyltransferase activity	Morphology
	pmol/mg protein/h	
None	7	−
Acetate (20 mM)	18	−
Propionate (10 mM)	63	+
Butyrate (1 mM)	46	+
Butyrate (5 mM)	65	+
Crotonate (2 mM)	0	−
Tetrolate (2 mM)	3	−
Pentanoate (5 mM)	48	+
Hexanoate (5 mM)	7	−
Octanoate (2 mM)	15	−
Decanoate (0.9 mM)	7	−

HeLa cells were grown for 24 h in medium supplemented with the above fatty acids (sodium salts). Morphological changes were determined microscopically: (+) indicates process formation, (−) no process formation.

activity whereas other fatty acids, including the unsaturated analogs of butyrate, were ineffective. Additional analogs of butyrate (α-aminoisobutyric, L-α-amino-*n*-butyric, γ-amino-*n*-butyric, DL-2-aminobutyric, and isobutyric acids as well as butyric acid methylester and *n*-butanol) were tested (at concentrations up to 5 mM) for their effect on morphology alone and were found ineffective.

Other compounds, some of which were reported to cause some morphological alterations (14)[1] were also tested for enzyme induction (Table VIII). However, they did not produce the characteristic smooth, elongated processes caused by propionate,

TABLE VIII

Absence of Sialyltransferase Induction by Other Compounds

Compound	Sialyltransferase activity	Morphology
	pmol/mg protein/h	
None	9	–
5 mM butyrate	63	+
1% DMSO	0	–
5 mM methylparabens	5	*
1 mM propylparabens	14	*
5 mM 2-phenylethanol	2	–
5 mM salicylate	0	–
5 mM phenylacetate	7	*

Conditions as in Table VII.

* Cell processes found which are distinctly shorter, more branched, and less smooth than those produced by butyrate.

butyrate, and pentanoate, nor did they induce sialyltransferase activity.

DISCUSSION

The alteration of glycosphingolipid biosynthesis by butyrate observed earlier (13) was investigated in detail. The cellular content of G_{M3} is increased 3.5-fold without significant increase in the other membrane glycosphingolipids. G_{M3} has been identified by its characteristic chromatographic migration, its susceptibility to neuraminidase treatment, and by the incorporation of a labeled sialic acid precursor, *N*-acetyl-mannosamine, which was clearly higher, per nanomole of G_{M3}, than the incorporation into control cells. Interestingly, the incorporation of labeled galactose per nanomole of G_{M3}, as well as the other glycosphingolipids, was less in the presence of butyrate than in its absence. This effect could have resulted from the more extended growth of the control than the butyrate exposed cells (14), or from a decreased sugar transport in the presence of butyrate. Increased sugar transport has been reported after viral transformation of chick embryo (31) and baby hamster kidney (20) cells. Sugar transport is also higher in growing compared to nongrowing cells.

The increased synthesis of G_{M3} results from the increase in the specific activity of CMP-sialic acid:lactosylceramide sialyltransferase activity in cells incubated in the presence of butyrate. Other glycosyltransferases involved in glycolipid biosynthesis are not elevated.[3] The increase in sialyltransferase activity appears to be due to induction of enzyme synthesis as it is blocked by inhibitors of RNA and protein synthesis. The induced sialyltransferase turns over within 16–24 h as indicated by the reduction in enzyme activity upon removal of butyrate; thus the continued presence of butyrate is required for enzyme induction. These results as well as those from experiments in which the mixture of cell homogenates from induced and uninduced cells is used seem to rule out the possibility that butyrate causes the increase of sialyltransferase by the activation of the enzyme or the removal of an enzyme inhibitor.

Butyrate induces the sialyltransferase activity and morphological changes in HeLa cells even in the absence of serum. In contrast, neuroblastoma cells rapidly form cell processes upon removal of serum from the medium by a cycloheximide-independent process (29). Interestingly, butyrate can slowly produce cell processes also in human neuroblastoma cells in the presence of serum (26).[1]

Butyrate appears to arrest HeLa cells in the G_1 phase of the cell cycle[2] and glycolipid biosynthesis appears to be maximal during G_1 (4). However, the arrest of the cell cycle by butyrate does not seem to be the sole source of elevated enzyme levels, because cells blocked by thymidine in the S phase do not show morphological or sialyltransferase alterations but they develop these characteristic alterations when butyrate is also added. Similarly, Colcemid-treated cells, blocked in the M phase, do not show any enzyme increase, but the enzyme is still induced by butyrate (although there is no evidence of process formation). This result suggests that microtubular assembly may play an essential part in process formation (18, 16, 29).

It is clear from this work that only the unmodified, straight-chain, saturated fatty acids (C_3, C_4, and C_5) are effective in producing both the type of cell shape alteration and the sialyltransferase induction described for butyrate. The shorter and longer fatty acids are much less effective or are ineffective in enzyme induction and they produce no shape changes. The mechanism by which the short-chain fatty acids cause these specific effects is unknown.

Simmons and Breuer[2] have shown that process elongation of HeLa cells starts 6–7 h after addition

[3] In addition, G_{M3}-sialidase activity is the same in control and butyrate-treated HeLa cells. (J. Tallman, P. H. Fishman, and R. C. Henneberry, unpublished observations.)

of butyrate. The increase in sialyltransferase activity demonstrated here begins earlier, 4 h after butyrate addition. It is therefore possible that the change of the glycosphingolipid pattern at the cell surface is necessary for the development of cell processes. For example, the surface change may be required to allow cell processes to attach to the surface of the culture dish, counteracting the tendency of the cell surface to reduce its surface area. This assumption agrees with the observation that, on butyrate removal, enzyme activity decreases well before morphological reversion occurs.

The effect of butyrate, and presumably of propionate and pentanoate, is not limited to HeLa cells. Butyrate-induced morphological alterations have been observed with CHO cells (32), with Chang liver, L-132, and Intestine 40 cells (14), and with neuroblastoma cells,[1] but not mouse neuroblastoma cells (26).

Morphological changes were also found associated with exposure to mono- or dibutyryl cyclic AMP and inhibitors of phosphodiesterase in Chinese hamster ovary (16), transformed fibroblast (19) and neuroblastoma (25) cell lines. In contrast, cAMP or mono- or dibutyryl cyclic AMP were ineffective in altering the morphology of HeLa cell lines (14) or inducing sialyltransferase activity.[4] When butyrate-treated and control HeLa cells were acid extracted at 4, 8, 12, and 24 h after butyrate (5 mM) addition, no difference in the cAMP content per milligram protein was found (as kindly measured by Dr. A. Peterkofsky). While work is being continued in this area, we have at this time no evidence that the butyrate effect is related to cAMP.

Finally, there is a direct temporal correlation between changes in cell morphology and glycolipid metabolism which may be relevant to the altered glycolipid metabolism observed in many, but not all transformed cells (for a recent review see reference 2). As indicated in the introductory paragraphs, previous studies with phenotypic revertants (22, 8, 6) or mass transformation of normal cells with RNA tumor viruses (23, 15, 27) have not clarified this association. Since reduced glycolipid glycosyltransferase activities are observed in transformed cells with altered glycolipid compositions (2, 5, 8, 21), the modulation of sialyltransferase activity (and thus G_{M3} levels) in HeLa cells by butyrate may provide a convenient model to explore the role of these complex carbohydrates in malignant processes.

Received for publication 15 October 1974, and in revised form 22 February 1975.

[4] P. H. Fishman, J. L. Simmons, and R. C. Henneberry, manuscript in preparation.

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