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Growth Inhibition and Morphological Changes Caused by Lipophilic Acids in Mammalian Cells

(food additives/short-chain fatty acids)

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ABSTRACT **Human (HeLa, Chang liver, L-132, and Intestine 407) and other mammalian (XC, SV3T3, and chick-embryo) cells in tissue culture are at least as sensitive to inhibition by lipophilic acids and nitrite as bacteria. Some of these compounds are the most frequently used antimicrobial food additives. Short-chain fatty acids (up to hexanoate) and parabens induce, at partially inhibitory concentrations, a jagged cell shape in continuous epithelial-like cell lines, such as HeLa, Chang liver, L-132, and Intestine 407. This morphological effect is not mediated or enhanced by butyryl cyclic AMP, which specifically affects fibroblasts.**

Fatty acids and other lipophilic acids, some of which are used as antimicrobial food additives, inhibit the growth of *Bacillus subtilis* and the uptake of amino and keto acids into bacterial membrane vesicles (1-3). Since these results indicated that lipophilic acids alter the properties of cell membranes, it appeared likely that they would also affect mammalian cells. This report demonstrates that they inhibit the growth of mammalian cells at least as effectively as that of bacteria. In addition, it shows that short-chain lipophilic acids induce filamentous protrusions in epithelial-like cells but not in cells with fibroblast morphology. These morphological changes are not mediated or enhanced by butyryl cyclic AMP (Bu-cAMP), which alters the morphology of fibroblasts but not of epithelial cells (4, 5).

MATERIALS AND METHODS

Cell Cultures. HeLa cells, strain R, were obtained from Grand Island Biological Co. (GIBCO), Grand Island, N.Y. and propagated in our laboratory. Cells of Chang liver (normal human epithelial cells), L-132 (originating from human embryonic lung), and Intestine 407 (epithelial cells originally derived from human embryonic jejunum and ileum) were bought from Flow Laboratories, Rockville, Md. XC cells (a cell line derived from a rat sarcoma induced by Rous sarcoma virus) were a gift from Dr. Janet Hartley, National Cancer Institute, Bethesda, Md. Chick-embryo cells were prepared from 9-day-old chick embryo (6). All the above cells were cultivated in 60-mm plastic petri dishes or 75-cm^2 T flasks (Falcon Plastics, Los Angeles, Calif.) in Eagle's minimal essential medium (pH 7.4) containing 10% fetal-calf serum (GIBCO), 2 mM L-glutamine, 100 units/ml of penicillin, and 100 μg/ml of kanamycin. The medium containing all these additions will in the following be called "minimal essential medium." The cultures were grown at 37° in a humidified incubator provided with 4% CO_2. Unless specified otherwise, HeLa cell cultures in 60-mm petri dishes were inoculated with 2.5×10^5 cells per culture and used after incubation at 37° for 20 hr. SV3T3 cells (mouse fibroblasts transformed by simian virus 40) were kindly provided by Dr. Aaronson of the National Cancer Institute, Bethesda, Md. They were grown at 37° in Dulbecco's modified minimal essential medium (GIBCO), including the above additions to Eagle's minimal essential medium in a humidified incubator provided with 10% CO_2.

Chemicals. Stock solutions of 100 mM Na propionate and Na butyrate were prepared in minimal essential medium, while 2.5 M Na hexanoate (pH 7.0) was prepared in distilled water. 1 M solution of octanoic and decanoic acid, methyl- and propyl-paraben (paraben = *p*-hydroxybenzoic acid ester) were prepared in 99% dimethyl sulfoxide (Matheson, Coleman & Bell, spectral grade) and diluted to the desired concentrations in minimal essential medium; the medium was then adjusted to pH 7 with NaOH and equilibrated for 2 hr in the incubator with 4% CO_2. The sodium salts of cyclic AMP (cAMP) and *N*-monobutyryl cyclic AMP (Bu-cAMP) were dissolved in minimal essential medium (at 10 mM). All of the compounds were purchased from Sigma Chemical Co., St. Louis, Mo. The solutions were sterilized by filtration through membrane filters (pore size 0.45 μm) (Millipore Corp., Milwaukee, Wis.). 1 mg/ml stock solutions of actinomycin D (a gift from Merck Sharp & Dohme, Pa.) were prepared in 95% ethanol.

Counting of Viable Cells. Cells were detached from monolayer cultures in 60-mm petri dishes by incubation at 37° for 5 min with 3 ml of a mixture of 0.1% trypsin and 0.05% ethylenediamine tetraacetic acid (EDTA), pH 7.0. At the end of incubation, calf serum (0.5 ml) was added to the cultures to neutralize the trypsin. The cell suspension was incubated for 5 min with 0.2% trypan blue, and the titer of viable and nonviable (stainable) cells was determined in duplicate with a hemocytometer.

Osmolarity of Solutions Was Determined with an automatic osmometer of Precision Systems, Inc., Mass.

Assay of Growth Inhibition. Unless specified otherwise, inhibition of cellular growth was determined as follows. Cell suspensions were prepared by trypsinization of confluent monolayers of cells grown in 120-cm^2 glass bottles. Plastic petri dishes (60 mm) were each seeded with 2.5×10^5 cells in 5 ml of minimal essential medium and incubated at 37° for 20 hr. The culture fluid was then removed and replaced with 5 ml of minimal essential medium and different concentrations of the compound to be tested. Duplicate cultures

Abbreviations: Bu-cAMP, *N*-monobutyryl cyclic AMP; paraben, *p*-hydroxybenzoic acid ester.

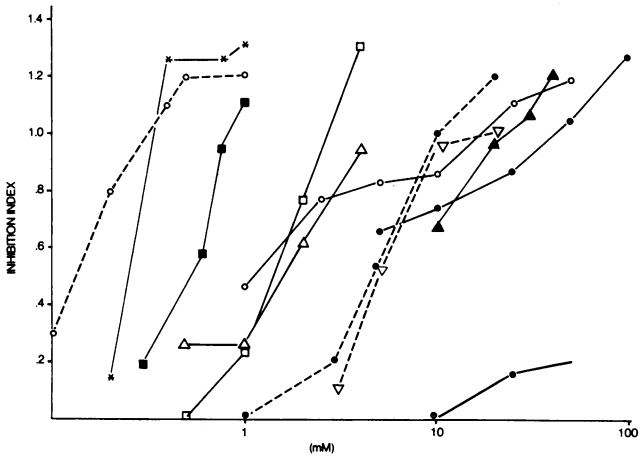

FIG. 1. Growth inhibition of cells. We prepared monolayer cultures of HeLa cells in 60-mm petri dishes by plating 2.5×10^5 cells per culture and incubating them for 20 hr at 37° in minimal essential medium. The medium was replaced with fresh medium containing one of the stated compounds. The cultures contained about 5×10^5 cells per flask. After further incubation for 48 hr, the number of cells was assayed. The growth inhibition index was calculated. (●—●) propionate (C_3); (▲—▲) hexanoate (C_6); (■—■) decanoate (C_{10}); (×—×) propyl-paraben; (○- - -○) 2,4-dinitrophenol; (▽- - -▽) sorbate; (○——○) butyrate (C_4); (△—△) octanoate (C_8); (□—□) methyl-paraben; (⊙—⊙) NaCl; (●- - -●) $NaNO_2$.

were used for each concentration, and the arithmetic average of the results was determined. The cultures were incubated at 37°, examined every 24 hr under a phase-contrast microscope for morphological changes, and then provided with new medium. After the desired time (48 hr) they were briefly washed with 0.1% trypsin + 0.05% EDTA to remove any floating cells (no attached cells were liberated thereby), and the number of viable cells was assayed as described. An inhibition index was defined as

$$1 - \frac{E_{(48)} - E_{(0)}}{C_{(48)} - C_{(0)}} = \frac{C_{(48)} - E_{(48)}}{C_{(48)} - C_{(0)}} \text{ (since } E_{(0)} = C_{(0)}).$$

E and C are the numbers of viable cells in the experimental and control cultures, respectively. The subscripts refer to the time of exposure (0 or 48 hr).

RESULTS

Inhibition of Growth and the killing of cells by different lipophilic acids were measured in HeLa cells growing in minimal essential medium containing 10% fetal-calf serum. The reduction of the increase in the viable cell number is expressed by an inhibition index that is 0 for no inhibition, between 0 and 1 for inhibition, and >1 for cell killing. Killing could also be ascertained by stainability with trypan blue. We have not always determined to what extent cell killing contributes to an inhibition index between 0 and 1, but the lethal effects must be small, as will be shown below for butyrate. For values above 1, the loss of viable cells was well correlated with the appearance of cells floating in the culture medium. The results presented in Fig. 1 show that the degree of growth inhibition and/or cell death caused by fatty acids and parabens increased with concentration of the compounds. Propionate (C_3) and butyrate (C_4) showed an exceptionally broad and concave concentration dependence, suggesting the existence of two different inhibitory effects of which one operates only at high concentrations. The latter effect was exerted by all fatty acids, and the concentration needed decreased with increasing length of the carbon chain. 2,4-Dinitrophenol, which also uncouples amino-acid transport from the electron-transport system (3), also inhibited growth effectively (Fig. 1). Since many of the above compounds are used as antimicrobial food additives, another additive, Na nitrite, was also tested. Its inhibitory effectiveness was between that of hexanoate and octanoate. At high concentrations all compounds were lethal.

Conceivably, inhibition of cell growth might result from the increased osmolarity of the medium containing various additions. This possibility can be ruled out because 20 mM propionate, butyrate, or hexanoate increased the osmolarity of the medium about as much as did 10 mM sodium chloride, which caused no significant growth inhibition (Fig. 1).

Morphological Alteration. At high concentrations of the inhibitory compounds, HeLa cells appeared under a phase-contrast microscope unusually opaque and with ragged outlines. At medium growth-inhibitory concentrations, some of the compounds produced an even more pronounced morphological alteration, as is shown for butyrate in Fig. 2. HeLa cells normally are round or polygonal, with few cells showing elongations (Fig. 2*a*). When these cells were incubated in the presence of 5 mM butyrate, filamentous protrusions began to appear after 7–8 hr, and by 14 hr had developed into long spikes. 2.5 mM butyrate caused the maximal morphological response in all cells, while 1 mM affected only 25% of the cells (Fig. 2). The morphological changes did not result from a salt

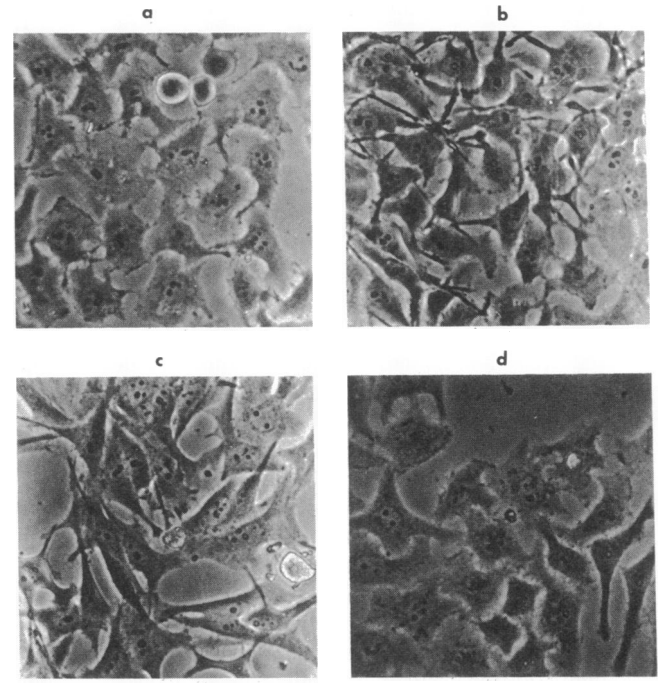

FIG. 2. Morphological alterations of HeLa cells induced by *n*-butyrate. HeLa cell cultures were prepared as in Fig. 1 and grown for 24 hr in minimal essential medium containing the following additions. Then the photographs were taken; magnification ×330. (*a*) control (no addition); (*b*) 2.5 mM butyrate; (*c*) 1.0 mM butyrate; (*d*) 25 mM NaCl.

or osmotic effect, because 25 mM sodium chloride did not produce similar changes. Propionate (10 mM), hexanoate (5 mM), methyl-paraben (1.0 mM), or propyl-paraben (0.5 mM) produced the same morphological changes as 2.5 mM butyrate. In contrast, octanoate, decanoate, 2,4-dinitrophenol, or nitrite did not cause morphological alterations at any growth-inhibitory concentrations used.

To determine whether 2.5 mM butyrate significantly affected viability, we grew cells as described in the legend to Fig. 3. In the presence of butyrate, the number of cells increased within 24 hr only slightly less than in control cultures, but then decreased slightly during the next 72 hr.

The morphological alteration induced by butyrate in the first 24 hr remained constant throughout the next 3 days. Throughout this time the cells remained as tightly attached to the flask as without butyrate, and during changes of medium only an insignificant fraction of cells floated. When butyrate was removed after 24 hr of incubation, cell division resumed, and 24 hr later at least 90% of the cells again displayed normal morphology. These results indicate that 2.5 mM butyrate reversibly inhibited cell division, affecting cell viability only slightly.

The morphological alterations of HeLa cells induced by butyrate were prevented by actinomycin-D (0.04 μg/ml) or cycloheximide (10 μg/ml). Apparently, synthesis of some RNA and protein is required for cells to undergo the morphological changes caused by butyrate. (This conclusion appeared justified although actinomycin or cycloheximide were partially lethal, as evidenced by rounding up and detachment of up to 50% of the cells from the monolayer.) Colchicine (1 μM) also prevented the morphological alterations, while it caused up to 50% of cell detachment in 24 hr.

Effect of Sodium Butyrate on Other Types of Cells. Based on their morphology and their ability to produce collagen, HeLa cells are regarded as epithelial cells. Morphological alterations similar to those observed with HeLa cells were induced by 2.5 mM sodium butyrate in other epithelial-like cells, such as Chang liver, L-132, and Intestine 407 (Fig. 4). However, primary epithelial cells (isolated from monkey or calf kidney) and fibroblastic cells (XC, SV3T3, or primary chick-embyro cells), which intrinsically produced a jagged cell shape, did not change their morphological appearance in the presence of different concentrations of butyrate (Fig. 5); nevertheless, the division of these cells was still inhibited by the same butyrate concentrations that inhibited HeLa cells.

TABLE 1. *Effects of butyrate and cAMP on the morphology of HeLa cells*

Additions to medium	No. of cells ($\times 10^{-6}$) at 24 hr	48 hr	Cellular morphology after 48 hr
None	1.65	3.20	Normal
1 mM butyrate	1.10	2.20	25% jagged
1 mM Bu-cAMP	1.65	2.10	Normal
5 mM cAMP	0.78	0.21	Normal
1 mM butyrate + 1 mM Bu-cAMP	1.10	1.20	25% jagged
1 mM butyrate + 5 mM cAMP	0.85	0.38	25% jagged

60-mm Petri dishes initially contained 5.5×10^5 HeLa cells per culture. The alteration in cellular morphology was observed under a phase-contrast microscope: *Normal* means no morphological alteration.

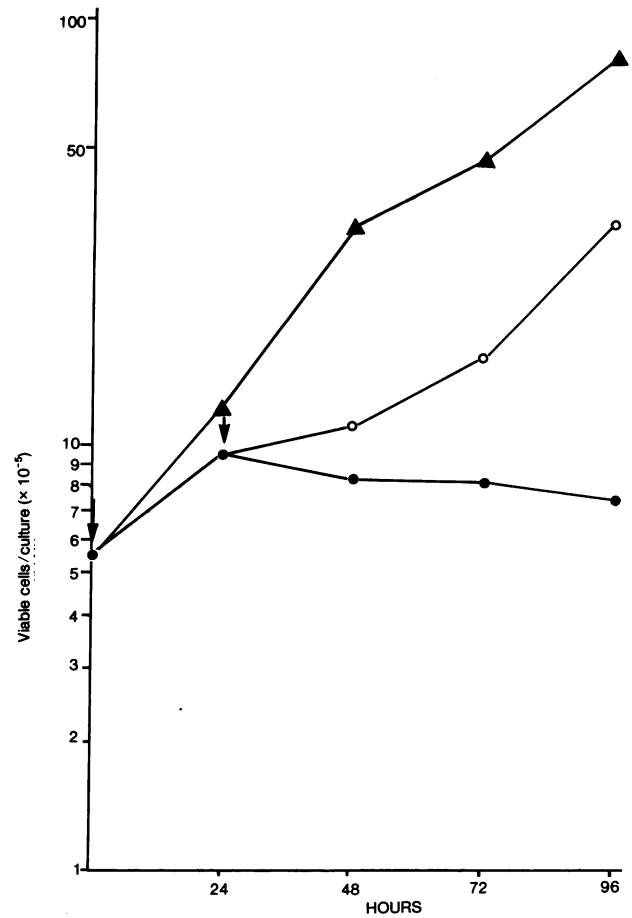

FIG. 3. Growth inhibition by butyrate and its reversibility. HeLa cell cultures were prepared as described in Fig. 1. One set of cultures was grown in minimal essential medium, while another set was grown in medium containing 2.5 mM butyrate. After 24 hr at 37°, all cultures were washed three times and then incubated with minimal essential medium. Half of the butyrate-treated cultures again received 2.5 mM butyrate. Each point represents the average number of cells in three cultures. *Arrows* indicate the time at which sodium butyrate was added or removed from the cultures. (▲) No butyrate; (●) butyrate throughout; (○) butyrate for the first 24 hr.

Effect of Cell Density on Growth Inhibition. Normal cells in tissue cultures usually cease to divide after a monolayer has been completed. This density-dependent or "contact" inhibition of growth is lost when cells undergo spontaneous or viral-induced transformation (4, 5, 7, 8). Since HeLa cells do not exhibit contact inhibition, the jagged cell appearance caused by butyrate conceivably might be correlated with a restitution of contact inhibition, which in turn might be responsible for the observed growth inhibition. To test such a possibility, the growth inhibitory effect of butyrate on HeLa cell cultures was examined at different cell densities (Fig. 6). After the usual increase in the first 24 hr, butyrate completely inhibited any subsequent cell division independent of the cell density; at low density, the cells were so far apart that they could not be subjected to contact inhibition. Therefore, contact inhibition does not seem to be the cause of growth inhibition.

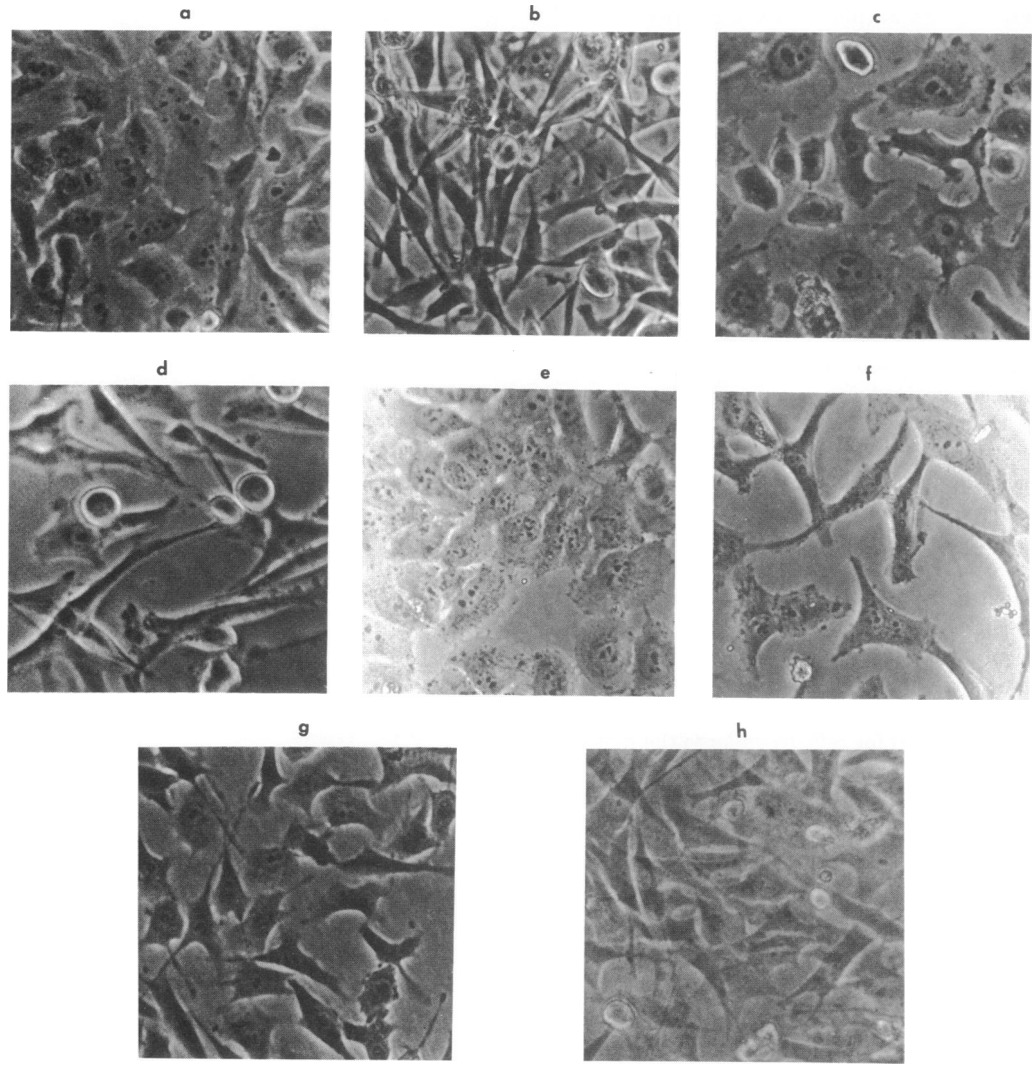

FIG. 4. Butyrate-induced morphological alterations in different cell lines. Cell cultures prepared as in Fig. 1 were incubated with minimal essential medium with or without 2.5 mM butyrate. The photographs were taken 24 hr later: magnification, ×330. Chang liver cells: (*a*) without and (*b*) with butyrate. L-132 cells: (*c*) without and (*d*) with butyrate. Intestine 407 cells: (*e*) without and (*f*) with butyrate. XC cells: (*g*) without and (*h*) with butyrate.

Effect of cAMP on Morphological Changes. Morphological changes and inhibition of cell division have been observed when fibroblastic XC or L cells were incubated with Bu-cAMP or diBu-cAMP, whereas HeLa cells were only inhibited (4, 5). cAMP or Bu-cAMP did not even enhance the morphological effects of butyrate on HeLa cells, as was demonstrated when these compounds were added to cultures incubated with 1 mM butyrate, a concentration that caused

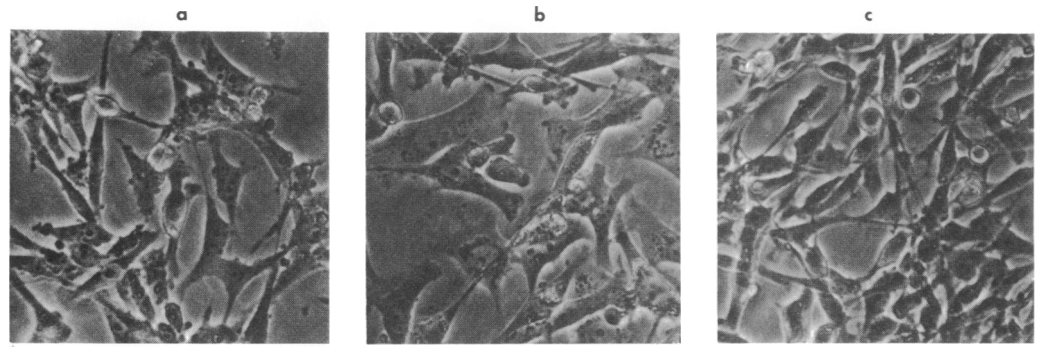

FIG. 5. Morphological effects of butyrate and Bu-cAMP on SV3T3 cells. SV3T3 cell cultures were prepared as in Fig. 1. The culture medium was supplemented with the stated compounds. The photographs were taken 24 hr later. Magnification, ×330. (*a*) No addition; (*b*) 2.5 mM butyrate; (*c*) 1 mM Bu-cAMP.

only partial growth inhibition and morphological alterations (Table 1). cAMP or Bu-cAMP alone did not produce any morphological changes in HeLa cells, while 1 mM Bu-cAMP did induce such changes in XC and SV3T3 cells (Fig. 5) in agreement with earlier reports (4, 5). When the HeLa cells exposed to 2.5 mM butyrate for 24 hr were washed and incubated with butyrate-free growth medium, addition of Bu-cAMP (1 mM) had no observable effect on resumption of the original morphology. Apparently, the morophological alterations induced by butyrate in HeLa cells were not mediated through or enhanced by Bu-cAMP.

DISCUSSION

Our results show that certain concentrations of fatty acids and parabens inhibit growth of mammalian cells while higher concentrations kill them. The inhibitory concentration dependence is similar to that observed for *B. subtilis*, which, in contrast to Gram-negative bacteria, is sensitive to all lipophilic acids (1, 3). Therefore, *B. subtilis* is particularly useful for determining the potential effect of lipophilic compounds on mammalian cells. It is not known whether the growth inhibition of mammalian cells is caused by the same mechanism as the antimicrobial activity; the latter results from uncoupling of the transport of amino and organic acids, phosphate, etc., as well as the oxidative phosporylation of ATP, from the electron transport system (2, 3).

Most antimicrobial food additives are lipophilic acids that have not exhibited any gross animal or human toxicity at the concentrations at which they are currently used (9). Since these compounds, as well as nitrite, inhibit all human cells tested in this study at least as effectively as they inhibit bacteria, the predominant consumption of food containing such compounds may potentially interfere with the function of some human cells. Similar considerations apply to extensively used drugs that are lipophilic acids, such as salicylates, e.g., aspirin. However, our studies were done in tissue cultures and did not involve whole tissues or animals.

The effective concentration of lipophilic acids in tissue surrounding the gut will be reduced by several mechanisms. Owing to the low pH in the stomach, the weak acids can be rapidly absorbed by ion trapping and then distributed throughout the body by blood circulation, decreasing their local concentration. Some of the absorbed compounds can be metabolized, e.g., long-chain fatty acids into glycerides. In addition, the mucous membrane may protect the underlying cells against the direct effect of insoluble free acids.

Short-chain fatty acids (up to hexanoate) and parabens induce jagged cell shapes in established epithelial-like cell lines. These morphological changes do not result merely from physical effects of the compounds on the cell, because they depend on RNA and protein synthesis. They are also not caused by the same mechanism by which mono- or dibutyryl cAMP induces morphological alterations in fibroblasts (4, 5), as is shown by the following observations: (*i*) Bu-cAMP does not produce significant morphological changes in HeLa cells, even when it is used at concentrations that inhibit growth, while such changes in XC fibroblasts are quite evident. In contrast, butyrate causes extensive morphological alterations in HeLa cells but not in fibroblasts. (*ii*) While the growth inhibition by Bu-cAMP and butyrate is additive, the morphological changes caused by suboptimal amounts of butyrate are not enhanced (or prevented) by Bu-cAMP, nor are they sustained by it when butyrate is removed. (*iii*) Preliminary experiments indicate that the replication of two enveloped viruses, i.e., Sindbis and vesicular stomatitis, is inhibited by 2.5 mM butyrate in HeLa cells, while it is not inhibited in fibroblasts, e.g., chick-embyro cells.

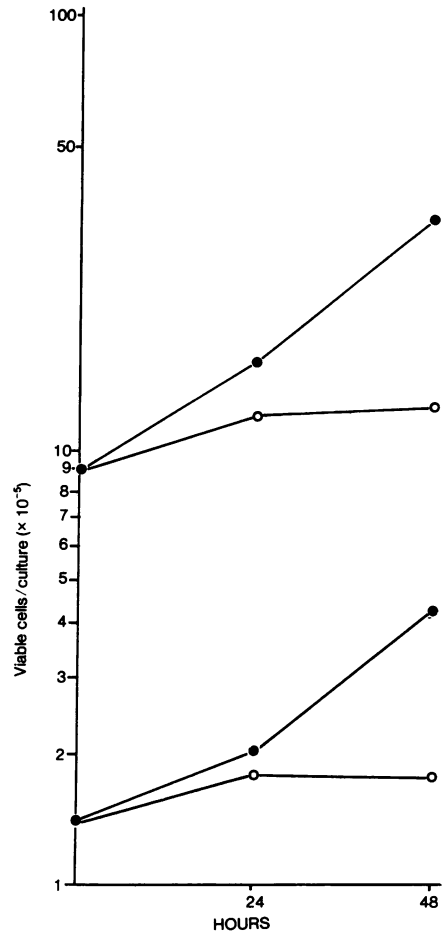

FIG. 6. Growth inhibition by butyrate at different cell densities. Cultures of HeLa cells were prepared by seeding 60-mm petri dishes with 0.5×10^5 or 5×10^5 cells per culture. After 20 hr of incubation at 37°, the medium was replaced with minimal essential medium plus 2.5 mM butyrate. Incubation was continued at 37°. The number of cells in the cultures was enumerated after 24 and 48 hr of incubation. (●) Without and (○) with butyrate.

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