[CANCER RESEARCH 57, 3697–3707, September 1, 1997]

Induction of Caspase-3 Protease Activity and Apoptosis by Butyrate and Trichostatin A (Inhibitors of Histone Deacetylase): Dependence on Protein Synthesis and Synergy with a Mitochondrial/ Cytochrome *c*-dependent Pathway[1]

Vicente Medina, Belinda Edmonds, Graeme P. Young, Robert James, Sarah Appleton, and Peter D. Zalewski[2]

Department of Medicine, University of Adelaide, The Queen Elizabeth Hospital, Woodville, South Australia 5011

ABSTRACT

The induction of apoptosis of tumor cells by the colonic fermentation product butyrate is thought to be an important mechanism in protection against colorectal cancer. Because a major action of butyrate is to inhibit histone deacetylase (leading to chromatin relaxation and altered gene expression), butyrate may induce apoptosis by derepression of specific cell death genes. Here we show that butyrate and trichostatin A (a more selective inhibitor of histone deacetylase) induce the same program of apoptosis in Jurkat lymphoid and LIM 1215 colorectal cancer cell lines that is strictly dependent on new protein synthesis (within 10 h) and that leads to the conversion of the proenzyme form of caspase-3 to the catalytically active effector protease (within 16 h) and apoptotic death (within 24 h). Cells primed with a low concentration of butyrate that itself did not induce activation of caspase-3 or apoptosis were, nevertheless, rendered highly susceptible to induction of apoptosis by staurosporine (an agent that has recently been shown to act by causing mitochondrial release of cytochrome *c*). Synergy between butyrate and staurosporine was due to the presence of a factor in the cytosol of butyrate-primed cells which enhanced over 7-fold the activation of caspase-3 induced by the addition of cytochrome *c* and dATP to isolated cytosol. We propose that changes at the level of chromatin structure, induced by a physiological substance butyrate, lead to the expression of a protein that facilitates the pathway by which mitochondria activate caspase-3 and trigger apoptotic death of lymphoid and colorectal cancer cells.

INTRODUCTION

The protective effect of high fiber diets in colon cancer has been attributed, in large part, to the production of butyrate by anaerobic fermentation of insoluble fiber in the colon (1, 2); an inverse relationship between tumor mass and fecal butyrate levels is found in a rat model of colon cancer (2). One hypothesis to explain the tumor suppressor activity of butyrate is that this short-chain fatty acid is a potent inducer of apoptosis (gene-directed cell death) in cancer cells (3–6). Apoptosis, a normal mechanism for deleting unwanted or moderately damaged cells (7), has important implications for cancer; suppression of apoptosis promotes tumor growth and, many anticancer agents induce it (8, 9). Because, butyrate may also trigger cell cycle arrest and terminal differentiation of tumor cells (1, 10), growth arrest may be followed sequentially by terminal differentiation and then apoptosis, as has been proposed to occur in the normal colonic crypt (11), or it may lead to separate pathways of differentiation and apoptosis. A greater understanding of how butyrate induces apoptosis should have implications for models of the coupling between differentiation and apoptosis.

In recent years, there have been major insights into the mechanisms by which apoptosis is triggered in cells. Although some signal transduction pathways are coupled directly to the apoptotic pathway (*e.g.*, via activation of membrane Fas/APO-1), other pathways require the expression of one or more cell death genes and are blocked by inhibitors of RNA and protein synthesis. Several putative cell death genes have been identified (12), but little is known about their mechanism of action. It is now generally believed that healthy cells constitutively express the molecules required for their own destruction, albeit in a latent form, and that the function of cell death genes is to render these molecules active (13–16). Principle among these effector molecules are the family of cytoplasmic ICE[3]-like proteases/ caspases (17–21), especially CPP-32 (18), now designated caspase-3 (21), which normally exists as an inactive proenzyme but which, immediately prior to apoptotic cell death, is cleaved to a heterodimer of M_r 17,000 and M_r 12,000 subunits that constitute the active protease (18). Roles for other ICE-like proteases in apoptosis are being defined presently (19, 20).

Intact and cell-free models of apoptosis have shown that activation of caspase-3 and induction of apoptosis by the protein kinase inhibitor staurosporine, anti-Fas, and etoposide involve increased mitochondrial membrane permeability and release of mitochondrial constituents including cytochrome *c* (16, 22–24). Members of the pro-apoptotic Bax and antiapoptotic Bcl-2 families, which are concentrated in the outer mitochondrial membrane (25), may serve to regulate cytochrome *c* release (23, 24). On the basis of these studies, a cell-free model of apoptosis has been developed (16) in which the cytoplasm of healthy cells is made pro-apoptotic by the addition of cytochrome *c* and dATP. The combination of these reagents with cytosol triggers, by unknown mechanisms, both proteolytic processing of caspase-3 and apoptosis of exogenously added nuclei.

Butyrate may be a useful tool for elucidating the mechanism by which products of cell death genes interact with the cytoplasmic pathway of apoptosis, because it induces apoptosis (3–6) and alters gene expression (10, 26), at similar concentrations and in diverse types of cell. Effects of butyrate on gene expression are attributed to the noncompetitive inhibition of histone deacetylase, increasing the half-life for deacetylation from 3 to 900 min (10, 27–29). Histone hyperacetylation leads to a more relaxed chromatin structure with increased availability of the promoter regions of certain genes to transcription factors. Although a number of butyrate-inducible genes have been identified (10), it is not known whether the expression of cell death genes is also regulated at the level of histone acetylation/ deacetylation. Recently, a highly specific and sensitive inhibitor of histone deacetylase, TSA has become available (30), allowing further investigation of the involvement of histone acetylation in butyrate-mediated responses. TSA has already been shown to share with

Received 1/29/97; accepted 7/3/97.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This study was supported by grants from the Australian Research Council and the University of Adelaide. V. M. was supported by a scholarship from the Government of the Canary Islands.

[2] To whom requests for reprints should be addressed. Phone: 61 8 8222 7344; Fax: 61 8 8222 6042.

[3] The abbreviations used are: ICE, interleukin 1B converting enzyme; TSA, trichostatin A; H-7, 1-[5-isoquinolinesulfonyl]-2-methyl-piperazine dihydrochloride; zDEVD-AFC, *z*-asp-glu-val-asp-7-amino-4-trifluoromethyl coumarin; zDEVD-fmk, *z*-asp-glu-val-asp-fluoro-methylketone; zVAD-fmk, *z*-val-ala-asp-fluoro-methylketone.

butyrate the properties of inducing new gene expression, cell cycle arrest (in both G_1 and G_2), differentiation, replicative senescence, and reversion of transformed phenotype in a number of cell lines (30–32). We have, therefore, compared the effects and sensitivity to protein synthesis blocking of butyrate and TSA on caspase-3 activation and apoptosis in malignant human T lymphoma/leukemia Jurkat cells and colorectal cancer LIM 1215 cells. The effects of priming cells with low concentrations of butyrate on caspase-3 activation in a cell-free model dependent on the addition of cytochrome *c* and dATP is then described.

MATERIALS AND METHODS

Materials. Major materials and their suppliers were: cycloheximide, EDTA, EGTA, herring sperm DNA, NP40, acridine orange, isobutyric acid, DTT, staurosporine, horse heart type III cytochrome *c*, sucrose, phosphocreatinine, creatine kinase, HEPES (Sigma Chemical Co., St. Louis, MO); sodium butyrate (BDH, Poole, England); H-7 (Seikagaku Kogyo, Tokyo, Japan); TSA (Wako Pure Chemicals Industry, Hiroshima, Japan); penicillin/streptomycin, EDTA/trypsin, glutamine, 3-[(3-cholamidopropyl)dimethylamino]-1-propanesulfonate (ICN, Aurora, OH); gentamicin (David Bull Labs, Melbourne, Victoria, Australia); dATP (Promega, Sydney, Australia); diphenylamine (Ajax, Auburn, New South Wales, Australia); zDEVD-AFC, zDEVD-fmk, Et-18-O-CH_3 (Kamiya Biomedical Co., Tukwila, Wa); and zVAD-fmk (Enzyme Systems, Portland, OR). All other reagents not listed were reagent grade.

Cell Cultures. Cell lines were grown from frozen stocks and cultured in 25-cm^2 vented tissue culture flasks (Sarstedt, Newton, NC) in a humidified atmosphere containing 5% CO_2. Medium was RPMI 1640, HEPES buffered, pH 7.4 (ICN, Aurora, OH), supplemented with 2 mM glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, 160 μg/ml gentamicin, and 10% fetal bovine serum (Biosciences, Sydney, Australia). Adherent LIM 1215 cells were allowed to attach and grow for 2–3 days in tissue culture flasks prior to exposure to test reagents.

For cultures with butyrate or isobutyrate, stock solutions (400 mM) in RPMI were made fresh each day and diluted into the cell suspension to give the desired final concentration (usually a 1 mM suboptimal concentration was used for synergy experiments and for priming cytoplasm for activation by cytochrome *c* and dATP, whereas a 4 mM optimal concentration was used for induction of apoptosis and related events). The pH of the isobutyric acid stock solution was adjusted to 7.4 with NaOH prior to the addition to cells. TSA was dissolved in DMSO as a 10 mM stock solution and stored in aliquots at −20°C. Before use, the stock solution was diluted 1:50 in DMSO, and one volume was added to 200 volumes of cell suspension to give the desired final concentration (usually 1 μM).

Assays for Apoptosis. Apoptosis was assessed by morphological criteria and, in some experiments, by DNA fragmentation as described previously (33). For morphological assessment, Jurkat and other suspension culture cells were examined by phase contrast microscopy after the addition of an equal volume of 0.2% trypan blue in HBSS, pH 7.4. For LIM 1215 and other adherent cell populations, the culture supernatant containing nonadherent cells was first decanted into a tube, and the adherent layer was washed twice with 1 ml of HBSS. The adherent cells were removed by trypsinization (as for subculturing). Apoptotic cells, which were almost entirely within the nonadherent fraction, were easily distinguished from normal cells by their characteristic morphology (see "Results"). At least 300 cells from replicate tubes were scored. In some experiments, acridine orange was added to a final concentration of 10 μM, and cells were analyzed by fluorescence microscopy for chromatin segregation.

To assay DNA fragmentation, cells (5×10^6-10^7 in total) were lysed at 4°C in 1 ml of NP40 lysis buffer (5 mM Tris-HCl, 5 mM EDTA, and 0.5% NP40, pH 7.5) and centrifuged at 13,000 × *g* for 10 min at 4°C. For LIM 1215 cells, similar results were obtained if the adherent cell fraction was lysed either after removal from plates by trypsinization or lysed within the plates. Supernatants containing low molecular weight DNA fragments were assayed for DNA by a colorometric technique (33). To calculate total DNA, the whole lysate was sonicated for 5 min in a Branson ultrasonifier on setting 5. DNA fragmentation was calculated as the percentage ratio of DNA in supernatant to DNA in total cell lysate.

Western Blotting. Aliquots of cells were lysed in SDS-loading buffer and electrophoresed on 10% minigels (Bio-Rad Lab, Hercules, CA) at 180 V for 45 min, according to the method of Hague *et al.* (34). Proteins were blotted onto nitrocellulose (Hybond, Amersham Life Sciences, Sydney, Australia). Membranes were preblocked with 2.5% powdered milk in PBS/Tween 20 for 60 min at room temperature and then incubated sequentially with mouse monoclonal antihuman Bcl-2 (1:60 dilution, clone 124; Boehringer Mannheim, Mannheim, Germany) and sheep antimouse IgG peroxidase conjugate (1:1000 dilution, Fab fragment; Boehringer Mannheim) for Bcl-2, polyclonal rabbit anti-Bax p-19 (1:60 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), and sheep anti-rabbit immunoglobulin peroxidase conjugate (1:1000 dilution, whole antibody; Amersham Life Sciences, Sydney, Australia) for Bax and mouse monoclonal antihuman CPP-32 (1:1000 dilution; Transduction Laboratories, Lexington, KY) and sheep anti-mouse IgG peroxidase conjugate (1:1000 dilution, Fab fragment) for CPP-32. Primary antibodies were added for 60 min and secondary antibodies for 45 min at room temperature. Membranes were then soaked in ECL Western Blotting reagent (Amersham Life Sciences, Sydney, Australia), and bands were detected by autoradiography. Rainbow high molecular weight markers (Amersham Life Sciences, Sydney, Australia) were used for determination of molecular weight. For negative controls in Bax experiments, membranes were exposed to primary antibody in the presence of excess immunizing peptide P-19 (Santa Cruz Biotechnology). For Western blotting of Bcl-2, recombinant human Bcl-2 baculovirus lysate (PharMingen, San Diego, CA) was used to confirm the identity of the Bcl-2 band.

Measurement of CPP-32/Caspase-3 Protease Activity. Caspase-3 was assayed by the cleavage of DEVD-AFC, a fluorogenic substrate based on the peptide sequence at the caspase-3 cleavage site of poly(ADP-ribose) polymerase (18). Cells (5×10^6–10^7 per flask) were cultured overnight in the absence or presence of 4 mM butyrate, washed 1 time with 5 ml of HBSS, and resuspended in 1 ml of NP40 lysis buffer (as for DNA fragmentation). LIM 1215 cells were first separated into nonadherent or adherent cell populations as for assay of DNA fragmentation. After 2 h in lysis buffer at 4°C, insoluble material was pelleted at 15,000 × *g*, and an aliquot of the lysate was tested for protease activity. In some experiments, lysates were prepared by repeated freeze-thawing as described by Enari *et al.* (20). For the assay, to each tube containing 8 μM of substrate in 1 ml of protease buffer [50 mM HEPES, 10% sucrose, 10 mM DTT, and 0.1% 3-[(3-cholamidopropyl)dimethylamino]-1-propanesulfonate, pH 7.4] was added 20–40 μl of cell lysate. After 45 min at room temperature, yellow-green fluorescence at 505 nm (excitation 400 nm), due to release of AFC, was quantified in a Perkin-Elmer LS50 spectrofluorimeter. Optimal amounts of added lysate and duration of assay were determined in preliminary experiments.

For the assay of caspase-3 in cytoplasmic *versus* nuclear fractions of cells, cells (10^7 in total) were gently lysed in a buffer containing 25 mM Tris-HCl (pH 7.4), 25 mM KCl, 7.5 mM $MgCl_2$, 30% (w/v) sucrose, and 0.1% NP40. After 10 min on ice, lysates were centrifuged at 1400 × *g* for 10 min. Nuclei were pelleted from the cytoplasmic fraction by centrifugation at 800 × *g* for 10 min through a sucrose gradient and then homogenized by ultrasonication.

Cell-free Model of Apoptosis. Jurkat cells were cultured for 13–18 h in the presence or absence of 1 mM butyrate. The cells were lysed, and cytosols were tested for cytochrome *c*- and dATP-dependent activation of endogenous caspase-3 proenzyme (15, 16). Cells (15–40 × 10^6 in total) were washed two times with HBSS, one time with cell extract buffer [50 mM Pipes (pH 7.4), 50 mM KCl, 5 mM EGTA, 2 mM $MgCl_2$, 1 mM DTT, 10 μM cytochalasin B, and 1 mM phenylmethylsulfonyl fluoride] and then allowed to swell in an equal volume of cell extract buffer for 15 min on ice. In some experiments, extract buffer contained 250 mM sucrose to minimize leakage of cytochrome *c* from mitochondria during cell disruption. However, the presence or absence of sucrose did not affect the results. Cells were disrupted by at least 30 passages through a 22-gauge needle attached to a 1-ml syringe, maintained in a tube of ice. Cell disruption was monitored by testing 5-μl aliquots for trypan blue uptake. Lysate was centrifuged at 15,000 × *g*, and the clear supernatant was removed and used as cytosol. Cytosols were stored at −20°C or used immediately. For the assay, 10 μl of cytosol were incubated in disposable fluorimeter cuvettes in a total volume of 50 μl of the following buffer [10 mM HEPES (pH 7.4), 50 mM NaCl, 5 mM EGTA, 2 mM $MgCl_2$, 1 mM DTT, 2 mM ATP, 10 mM phosphocreatinine, and 50 μg/ml creatine kinase], supplemented with or without 1 mM dATP and 300 μg/ml horse heart cytochrome *c*. After 90 min at 37°C, 1 ml of caspase-3 protease assay buffer containing DEVD-AFC was

added to each cuvette, and the caspase-3 protease assay was performed as described earlier.

Experimental Design. All experiments were repeated a minimum of three times. Typical experiments are described, or data were pooled, as indicated in the text. Apoptosis was assessed by scoring at least 300 cells from three separate culture flasks for each treatment. Statistical significance was determined by Student's *t* test.

RESULTS

Induction of Apoptosis by Butyrate. At physiologically relevant concentrations (2–4 mM), sodium butyrate induced morphological changes of apoptosis and DNA fragmentation in a variety of cancer cell lines (lymphoblastoid, promyelocytic, and colorectal cancer). Thus, in typical experiments, overnight culture of Jurkat lymphoid cells with butyrate increased the percentage of apoptotic cells from 2.2 ± 1.5 to 37.7 ± 5.0 ($P < 0.005$), whereas the percentage of DNA fragmentation increased from 0.9 ± 1.8 to 60.8 ± 9.3 ($P < 0.005$); in LIM 1215 colorectal cancer cells, the percentage of apoptotic cells increased from 4.1 ± 0.7 to 26.5 ± 5.2 ($P < 0.005$), whereas the percentage of DNA fragmentation increased from 11.5 ± 6.4 to 29.5 ± 9.2 ($P < 0.05$). With the adherent LIM 1215 cell population, apoptotic cells were greatly enriched in the population of detached (nonadherent) cells, where 93% of the cells were apoptotic, compared with 21% apoptotic cells in the adherent fraction. Typical morphological features in the butyrate-treated cells included the presence of one or more intracellular apoptotic bodies, a granularity that was often localized to one pole of the cell, exclusion of the vital dye trypan blue, membrane blebbing, and in some cells, reduction in volume. Labeling of the DNA with acridine orange revealed segmentation of the chromatin into discrete masses in the butyrate-treated cells, compared with the uniform mass of chromatin in untreated cells.

Apoptosis with butyrate was characterized by a lag phase, lasting 12–14 h in Jurkat cells (Fig. 1*A*) and 8–12 h in LIM 1215 (Fig. 1*B*). Thereafter, cells progressively underwent apoptosis, and the time course curve plateaued by 24 h.

Effects of Butyrate Are Mimicked by TSA. To determine whether histone acetylation is important in initiating butyrate-induced apoptosis, TSA, a highly specific inhibitor of histone deacetylase (31), was tested for its capacity to induce apoptosis in Jurkat and LIM 1215 cells. TSA not only induced extensive apoptosis (*e.g.*, in LIM 1215 cells, the percentage of apoptosis increased from 4.1 ± 0.7 to 42.8 ± 6.1, $P < 0.005$, and the percentage of DNA fragmentation increased from 11.5 ± 6.4 to 54.4 ± 6.9, $P < 0.005$), but also the kinetics were identical to those seen with butyrate (Fig. 1). Whereas in Jurkat cells the maximal responses of butyrate and TSA were similar, in LIM 1215 cells butyrate induced a significantly lower level of apoptosis and DNA fragmentation than did TSA (Fig. 1*B*). This was a consistent finding and might reflect significant metabolism of butyrate by mitochondrial β-oxidation in the colon cancer cells (35), thereby lowering the intracellular concentration of butyrate. To inves-

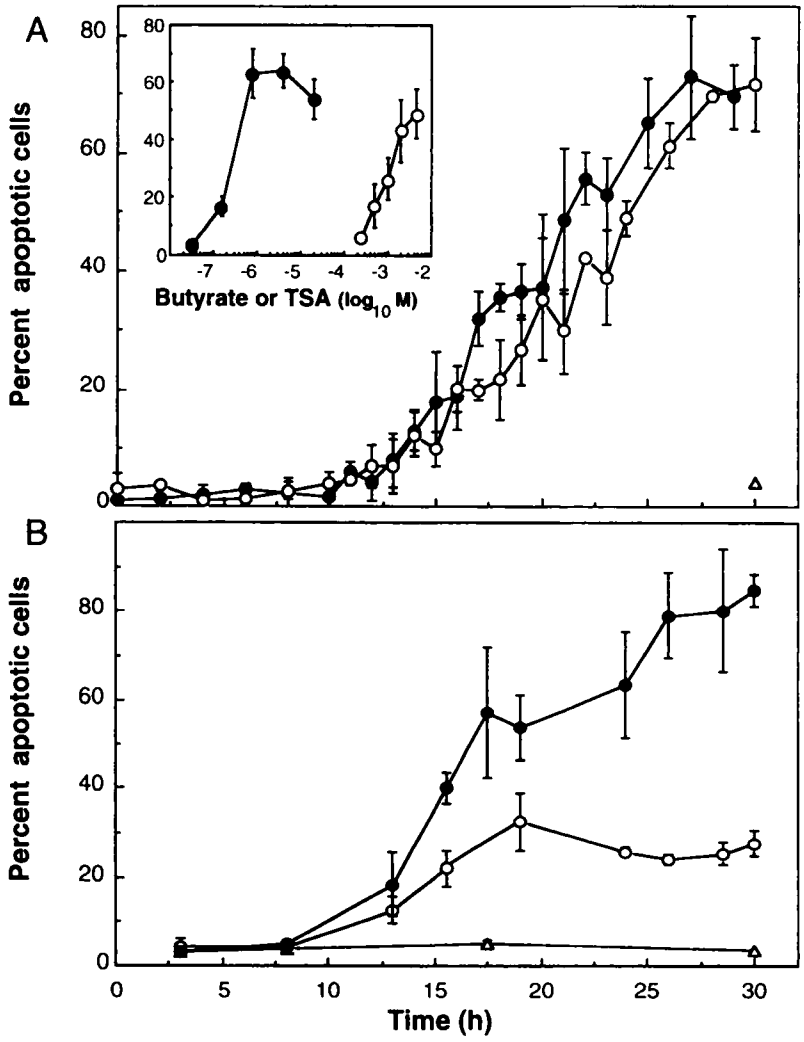


Fig. 1. Concentration-dependence and kinetics of induction of apoptosis by butyrate and TSA. Jurkat cells (*A*) or LIM 1215 cells (*B*) were cultured alone (△), with 4 mM butyrate (○), or 1 μM TSA (●), and at the indicated times, apoptosis was quantified by determining the percentage of cells with morphological features of apoptosis under phase contrast microscopy (see the text). The graph shows similar time courses for butyrate and TSA following a lag phase of 14 h. *A, inset,* Jurkat cells were cultured for 22 h in the presence of varying concentrations of butyrate (○) or TSA (●), and apoptosis was quantified. TSA was at least three orders of magnitude more effective than butyrate in inducing apoptosis. Each point represents a mean of triplicates (different culture flasks); *bars,* SD.

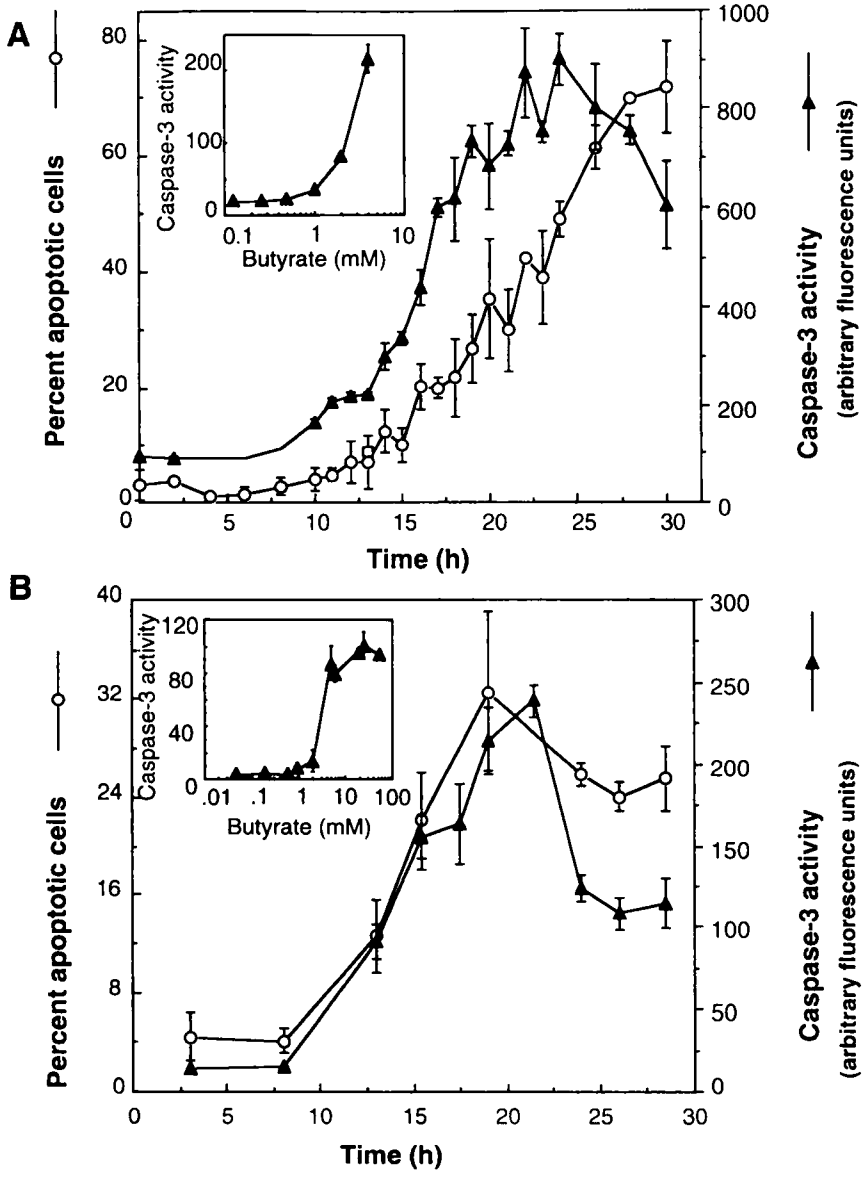


Fig. 2. Increase in caspase-3 protease activity during butyrate-induced apoptosis. Jurkat cells (*A*) or LIM 1215 cells (*B*) were cultured with 4 mM butyrate, and at the indicated times, apoptosis (○) or caspase-3 protease activity (▲) was quantified. The graphs show a peak of caspase-3 protease activity preceding the morphological cell death in Jurkat cells and occurring simultaneously with apoptosis in LIM 1215 cells. The *inset* in each panel shows concentration-dependence of butyrate induction of caspase-3 protease activity in the two types of cell. Each point represents a mean of triplicates (different culture flasks); *bars*, SD.

tigate this further, LIM 1215 cells were cultured for 22 h in the presence of butyrate at concentrations up to 160 mM (the highest concentration tested that did not lyse the cells). The apoptotic response to butyrate did not increase at concentrations beyond 4 mM (data not shown). This finding tentatively argues against the hypothesis that the metabolism of butyrate in LIM 1215 cells limits its intracellular concentration required for triggering apoptosis, although the possibility exists that carrier-mediated mechanisms for transport of butyrate across either plasma or nuclear membranes were saturated at 4 mM butyrate. The effective concentrations of butyrate (2–4 mM) and TSA (0.5–1 μM) in inducing apoptosis of Jurkat (Fig. 1*A*, *inset*) and LIM 1215 (Fig. 1*B*, *inset*) cells corresponded to those reported to inhibit histone deacetylase in other types of cells (27–31). TSA has been reported previously to kill SV40-transformed 3Y1 cells by an undefined mechanism (30) and, more recently, to induce apoptosis in thymocytes (36).

A structural analogue of butyrate, isobutyrate, which is much less effective in inducing histone acetylation (29), did not induce either morphological changes or DNA fragmentation in Jurkat and LIM 1215 cells over a 24-h period. Thus, where Jurkat cells were treated for 24 h with 10 mM isobutyric acid, there were only 2.3 ± 2.1% apoptotic cells compared to 1.3 ± 1.5% in control cultures.

Activation of Caspase-3. Both butyrate and TSA induced the appearance of caspase-3 protease activity in NP40-soluble extracts of Jurkat and LIM 1215 cells, as determined by its capacity to cleave a fluorogenic substrate, zDEVD-AFC. There was a modest amount of caspase-3 activity at 0 h, which may reflect the presence of a small proportion (up to 5%) of apoptotic cells in the untreated cell cultures. The increase in caspase-3 protease activity (Fig. 2) began about 8 h after the addition of butyrate and peaked at 18–24 h in both types of cells, reaching levels more than 10-fold those of control populations. The induction of caspase-3 activity in cells treated with butyrate or TSA was also observed when extracts were prepared by repeated freeze-thawing instead of by detergent (data not shown). When cytoplasm and nuclei of butyrate-treated Jurkat cells were compared, 92% of the caspase-3 protease activity was found to be cytoplasmic.

In Jurkat cells, caspase-3 protease activity preceded, by about 4–6 h, the onset of morphological changes of apoptosis (Fig. 2*A*) or DNA fragmentation (data not shown), whereas in LIM 1215 cells, there was less delay between the rise in caspase-3 and either morphological

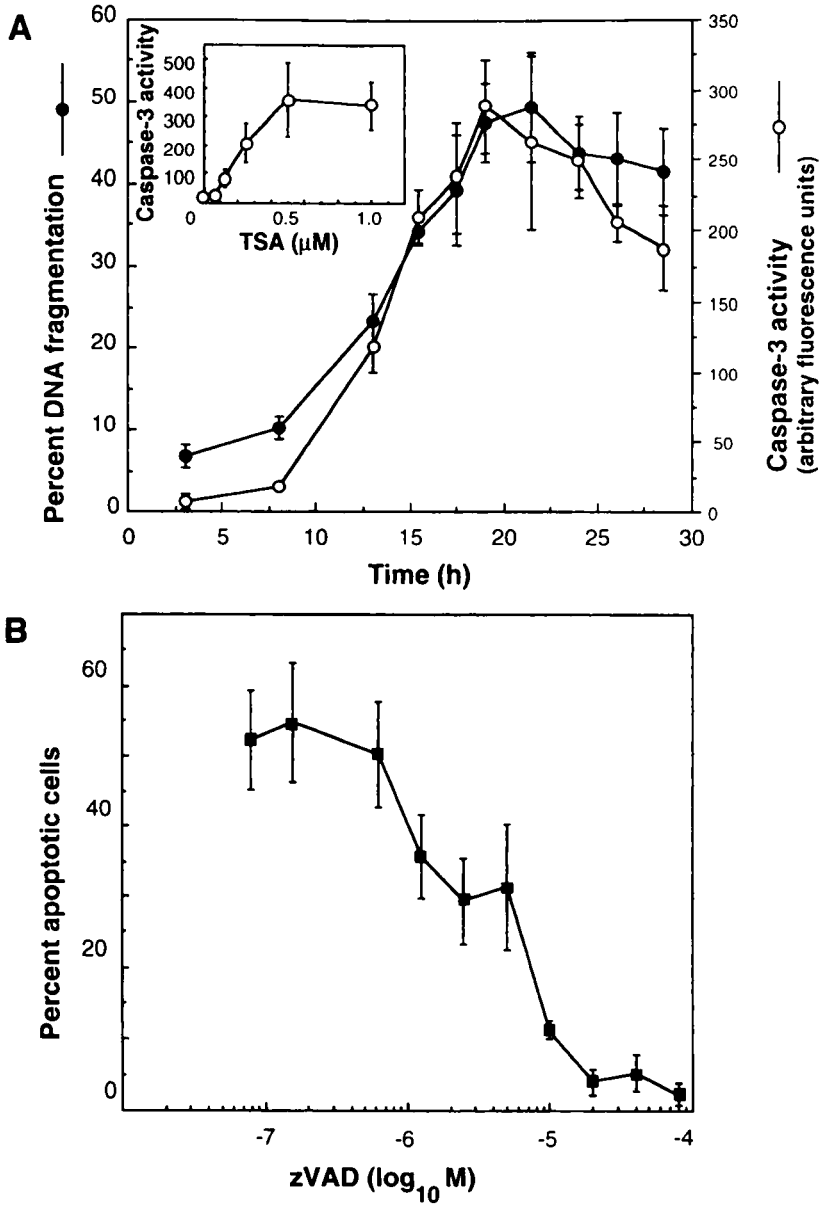


Fig. 3. Involvement of caspase-3 in apoptosis of cancer cells. In *A*, LIM 1215 cells were cultured in the presence of 1 μM TSA, and at the indicated times, DNA fragmentation (●) or caspase-3 protease activity (○) was determined. The graph shows good correlation between the induction of DNA fragmentation and caspase-3 activity. The *inset* shows a plateau in the induction of caspase-3 activity at concentrations of TSA above 0.5 μM. In *B*, Jurkat cells were cultured with 4 mM butyrate for 22 h in the presence of the indicated concentrations of peptide inhibitor zVAD-fmk. The graphs show inhibition of apoptosis by zVAD-fmk. Each point represents a mean of triplicates (different culture flasks); *bars*, SD.

changes or DNA fragmentation in response to butyrate (Fig. 2*B*) or TSA (Fig. 3*A*). Concentrations of butyrate effective in activating caspase-3 in Jurkat and LIM 1215 cells were similar to those inducing morphological changes and DNA fragmentation (*insets* in Figs. 2 and 3*A*). As with the induction of morphological changes, the increase in caspase-3 activity in butyrate-treated LIM 1215 cells plateaued at 4 mM (Fig. 2*B*, *inset*).

Effect of Membrane-permeable Inhibitors of ICE-like Proteases. To determine whether the rise in caspase-3 protease activity was essential for the induction of apoptosis by butyrate and TSA, two membrane-permeable inhibitors of caspases were used. zVAD-fmk is based on the peptide sequence at the cleavage site of interleukin 1β (mediated by ICE/caspase-1), whereas zDEVD-fmk corresponds to the sequence at the cleavage site of poly-(ADP-ribose) polymerase mediated by caspase-3 (18). Although the peptide inhibitors exhibit selective inhibition of purified caspase-1 and caspase-3 at nanomolar concentrations, their specificities toward different members of the caspase family in intact cells at the higher (micromolar) concentrations required to inhibit apoptosis in most cellular systems have not been determined.

In Jurkat cells incubated with either zVAD-fmk (Fig. 3*B*) or zDEVD-fmk (data not shown), there was suppression of both caspase-3 activation and the induction of apoptosis. Thus, the butyrate-induced formation of apoptotic bodies (Fig. 4*A*) and DNA fragmentation (data not shown) were completely blocked in the presence of zVAD-fmk (Fig. 4*B*). However, zVAD-fmk did not prevent all of the apoptosis-associated morphological change, because in about 30% of the cells, there was a variable reduction in cell volume and a more refractile appearance of the cytoplasm, consistent with condensation of the cytoplasm (arrowed cell in Fig. 4*B*). zVAD-fmk was fully active as an inhibitor, even when added 7 h after butyrate; thus, in cultures treated with butyrate alone for 21 h, there were 35.7 (± 6.0) percentage of apoptotic cells, whereas cultures treated with butyrate and zVAD-fmk (added 7 h after butyrate) contained only 3.3 (± 0.6) percentage of apoptotic cells. The effectiveness of zVAD-fmk added late in culture is consistent with the onset of caspase-3 activity as a relatively late event during lag phase. Half-maximal inhibition of apoptosis by zVAD-fmk in butyrate-treated Jurkat cells occurred at 2.5 μM, but complete inhibition occurred only at concentrations of 20

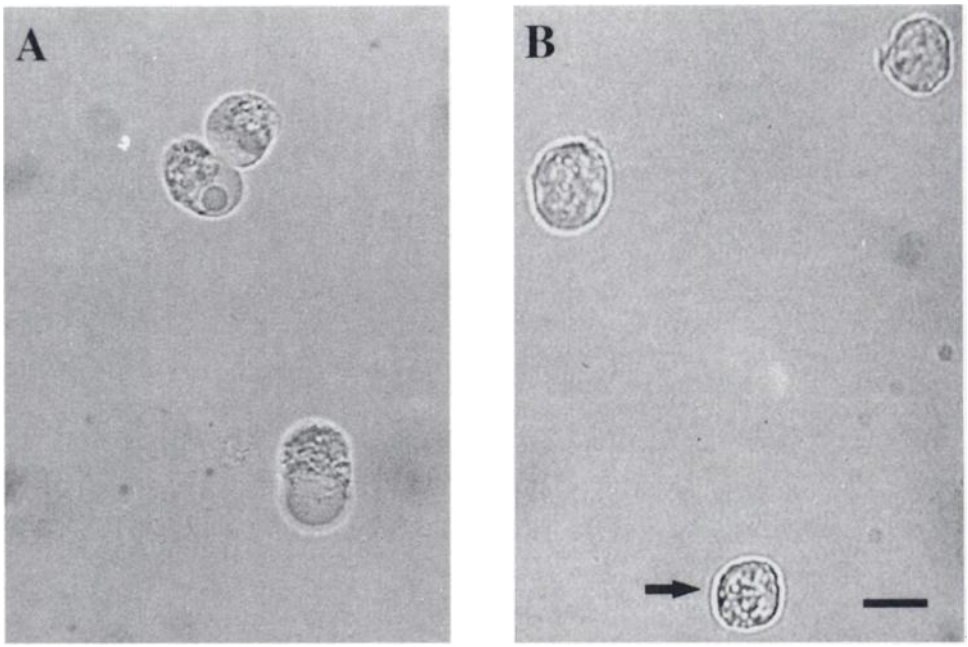


Fig. 4. Morphology of cells after zVAD-fmk inhibition. Jurkat cells were treated with 4 mM butyrate in the absence (*A*) or presence (*B*) of 10 μM zVAD-fmk. *B* shows a lack of apoptotic bodies in zVAD-treated cells, compared with numerous bodies in cells in *A*. The *arrow* in *B* indicates a cell with a more refractile appearance and reduced volume, typical of about 30% of the cells, suggesting that zVAD blocks only some of the morphological changes. *Bar*, 10 μm.

μM and greater (Fig. 3*B*). Because induction of caspase-3 activity in Jurkat cells was completely suppressed by zVAD-fmk at 2.5 μM, it is likely that apoptosis of Jurkat cells requires, in addition, the action of another caspase that is only inhibited at much higher zVAD concentrations.

Role of Protein Synthesis in Commitment to Apoptosis. To determine whether protein synthesis was required for induction of apoptosis and caspase-3 protease activity by inhibitors of histone deacetylase, the protein synthesis inhibitor cycloheximide was used. Concentrations of cycloheximide in the range 3–6 μM almost completely prevented the formation of apoptotic bodies and the nuclear fragmentation in Jurkat cells treated with either butyrate or TSA (Fig. 5*A*). Half-maximal effects of cycloheximide were 0.35 and 0.4 μM, respectively, typical of concentrations of cycloheximide known to inhibit protein synthesis. It was not possible to confirm these results in LIM 1215 cells because these cells were killed by exposure to concentrations of cycloheximide required to block protein synthesis. The results with Jurkat cells suggest that *de novo* protein synthesis is required for apoptosis initiated by a decrease in histone deacetylase activity. To further investigate this phenomenon, the time of the addition of cycloheximide was delayed, relative to that of butyrate (time, 0 h), and apoptosis was assayed after an additional overnight incubation. If cycloheximide was added within 3 h of butyrate, there was complete inhibition of apoptosis (Fig. 5*A*, *inset*). However, when the addition of cycloheximide was delayed 5 h or more, there was progressively less protection, culminating in no protection 10 h after butyrate. This suggests that the resulting cascade of gene activation is completed within 10 h of the addition of butyrate.

The induction of caspase-3 protease activity by either butyrate or TSA in Jurkat cells was also prevented by cycloheximide (Fig. 5*B*). It is unlikely that caspase-3 is the essential protein requiring synthesis, because the level of M_r 32,000 caspase-3 proenzyme, assessed by Western blotting using an antibody that reacts with an epitope contained within both the precursor and the M_r 17,000 domain, did not appear to change with time after the addition of butyrate. However, there was a marked increase in the M_r 17,000 fragment with time, especially after 12 h of exposure to butyrate (Fig. 6*A*), consistent with cleavage of the proenzyme to the active M_r 17,000 and M_r 12,000 subunits, shown previously to recombine to form a catalytically active site (18).

Lack of Effects on Expression of Bcl-2 and Bax. Some apoptotic stimuli appear to induce apoptosis by up-regulating the pro-apoptotic Bax-like subfamily of proteins and/or down-regulating the antiapoptotic Bcl-2-like proteins (25). To determine the effects of butyrate and TSA on Bcl-2 and Bax, Jurkat and LIM 1215 cells were treated with 4 mM butyrate, and at varying intervals, the amounts of Bax and Bcl-2 proteins were quantified in cell lysates by Western blotting (see "Materials and Methods"). Fig. 6*B* shows the blot of a typical experiment in which there was no difference in levels of Bax at several time points following the addition of butyrate to Jurkat cells; similar blots were obtained with LIM 1215 cells (data not shown). Also, there were no changes in Bcl-2 expression in Jurkat or LIM 1215 cells treated for up to 7 h with butyrate (four separate experiments; data not shown).

Synergy between Butyrate and Staurosporine or Other Protein Kinase Inhibitors. The protein kinase inhibitor staurosporine triggers apoptosis and caspase-3 activation by direct effects on the cell cytoplasm that are: (*a*) independent of new gene expression; (*b*) regulated by Bcl-2; and (*c*) likely to be mediated by changes in mitochondrial membrane potential (14, 16, 22). If butyrate acts by a distinct pathway (dependent on new gene expression and independent of Bcl-2), butyrate and staurosporine should act synergistically rather than additively in inducing apoptosis.

When suboptimal concentrations of butyrate (1 mM) and staurosporine (1 μM) were added to Jurkat (Fig. 7) or LIM 1215 cells (data not shown), a potent synergy was evident both for the activation of caspase-3 and induction of apoptosis. Synergy occurred regardless of whether butyrate and staurosporine were added simultaneously (data not shown) or whether cells were treated overnight with butyrate and then staurosporine added for the final 6.5 h (Fig. 7). Thus, the treatment of Jurkat cells for up to 24.5 h with 1 mM butyrate did not enhance caspase-3 protease activity or the percentage of cells with the morphological features of apoptosis over that in control cultured cells (Fig. 7). If staurosporine was added to control cultures at 18 h, only a little or no increase in caspase-3 activity was evident over the next 6.5 h, and only a slight increase in the percentage of cell apoptosis occurred. However, when staurosporine was added to cultures pretreated for 18 h with 1 mM butyrate, there was a 5-fold increase in caspase-3 protease activity, which peaked about 2 h after the addition of staurosporine and then decreased thereafter to about a 2–3-fold increase 6.5 h after the addition of staurosporine (Fig. 7*A*). Synergy

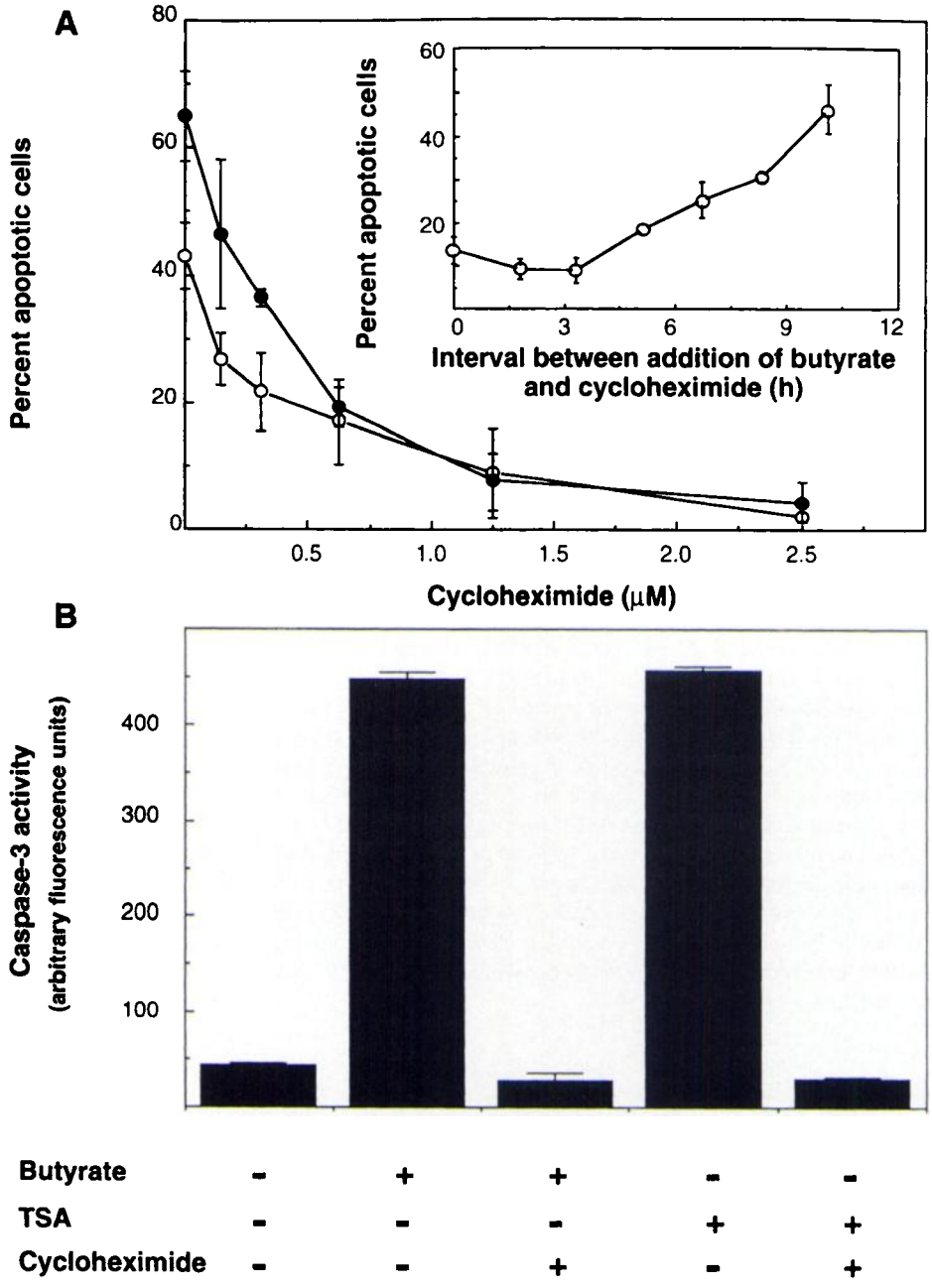


Fig. 5. Effects of cycloheximide on butyrate and TSA-induced caspase-3 protease activation and apoptosis. In *A*, Jurkat cells were cultured for 22 h with 4 mM butyrate (○) or 1 μM TSA (●) in the presence of the indicated concentrations of cycloheximide (added simultaneously with butyrate or TSA). The graph shows similar inhibition of apoptosis by cycloheximide in cells induced to apoptose by treatment with butyrate or TSA. *Inset*, cycloheximide (3 μM) was added at indicated times (h) after butyrate. After 22 h, apoptosis was determined. The graph shows complete suppression of apoptosis by cycloheximide, when added within 3 h after butyrate and partial suppression, when added between 3 and 10 h. Each point represents a mean of triplicates (different culture flasks); *bars*, SD. In *B*, Jurkat cells were cultured for 24 h with (+) or without (−) 4 mM butyrate, 1 μM TSA, and 3 μM cycloheximide. Cells were lysed in NP40 lysis buffer. Lysates were centrifuged, and supernatants were assayed for caspase-3 protease activity as in "Materials and Methods." The graph shows induction of protease activity by both butyrate and TSA and complete suppression of induction by cycloheximide. Each point represents a mean of triplicates (different culture flasks); *bars*, SD. For cells treated with butyrate or TSA, columns in the absence of cycloheximide were significantly higher ($P < 0.005$) from those in the presence of cycloheximide.

was also evident with other protein kinase inhibitors, although the kinetics of increase differed. Caspase-3 protease activity peaked at levels 5–6-fold and 2–3-fold those of controls by about 5 h after the addition of H-7 or Et-18-O-CH_3, respectively, to cultures pretreated overnight with 1 mM butyrate; however, there were no increases if H-7 or Et-18-O-CH_3 were added to control cultures. In agreement with these findings, H-7 and Et-18-O-CH_3 synergized with butyrate in the induction of apoptosis (Fig. 7*B*).

Butyrate-enhanced Induction of Caspase-3 Protease Activity in the Cytochrome *c*- and dATP-dependent Cell-Free Model. Staurosporine (and some other inducers of apoptosis) have been shown recently to cause a release of cytochrome *c* from mitochondria into cytoplasm that occurs early in apoptosis and is blocked by addition of exogenous Bcl-2 (16, 23, 24). The importance of this release was shown by the induction of caspase-3 activity in control cell cytoplasms following the addition of cytochrome *c* (16). Because pretreatment with 1 mM butyrate greatly enhanced staurosporine-induced increase in caspase-3 activity in intact Jurkat cells (Fig. 7*A*), we asked whether the pretreatment of cells with butyrate would also enhance cytochrome *c*- and dATP-dependent activation of caspase-3 in the cell-free model.

We confirmed that the addition of both cytochrome *c* and dATP to cytoplasm of control culture Jurkat cells induced activation of cytosolic caspase-3. When cytosol from 5×10^7 control-treated Jurkat cells was incubated with both 300 μg/ml horse heart cytochrome *c* and 1 mM dATP for 90 min at 37°C, 31.4 (± 19.8) units of caspase-3 activity were induced. No induction of active caspase-3 occurred when only dATP or cytochrome *c*, alone, were added to cytosol (see the typical experiment in Fig. 8*A*). When cytosol was prepared from cells pretreated overnight with 1 mM butyrate, 229.8 (+ 8.1) units of caspase-3 activity were induced by dATP and cytochrome *c*, 7.3-fold higher than obtained with cytosol from control-treated cells. These figures represent the means of four separate experiments, each in triplicate ($P < 0.005$). Optimal induction of caspase-3 activity in

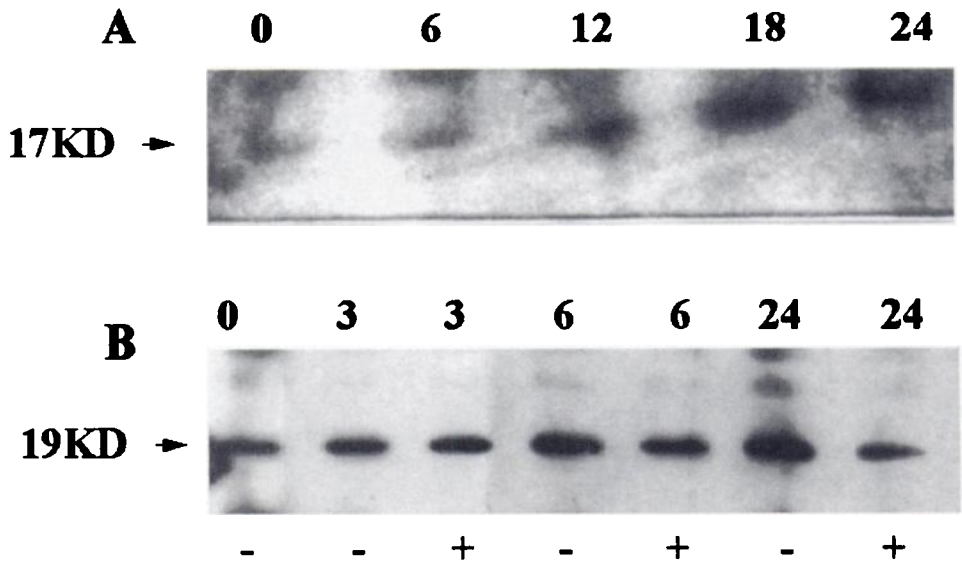


Fig. 6. Western blots of effects of butyrate on levels of M_r 17,000 caspase-3 and Bax. In *A*, Jurkat cells were cultured with 4 mM butyrate for 0, 6, 12, 18, or 24 h. Cell lysates were subjected to Western blotting with antibody to an epitope contained in the M_r 17,000 active subunit of caspase-3. The figure shows an increase in the active form of caspase-3 with increasing time after exposure to butyrate, particularly at 18 and 24 h. In *B*, Jurkat cells were cultured for 0, 3, 6, or 24 h in the absence (−) or presence (+) of 4 mM butyrate. Cell lysates were subjected to Western blotting with antibody to Bax. No change in the level of Bax was evident. Typical blots are shown.

butyrate-pretreated Jurkat cell cytosol required the addition of both cytochrome *c* and dATP; however, there was a much greater dependence on the addition of cytochrome *c* than dATP (Fig. 8*A*). The reason for this is unclear but may reflect differences in endogenous concentrations of dATP between cytosols from control and butyrate-treated cells. Optimal activation with cytochrome *c* occurred at 120 μg/ml, with partial activation at 24 μg/ml (Fig. 8*A*, *inset*).

When an optimal concentration of butyrate (4 mM) was used, there was a marked increase in the capacity of cytosolic caspase-3 to be activated by cytochrome *c* and dATP at a time point 11 h after addition of butyrate: following essential protein synthesis (complete by 10 h) and preceding the activation of caspase-3 (onset at 13–14 h) in intact Jurkat cells (Fig. 8*B*).

DISCUSSION

The main conclusions of this study are that butyrate induces a novel pathway of CPP-32/caspase-3 activation and apoptosis in cancer cell lines that is dependent on the inhibition of histone deacetylase and new protein synthesis, apparently independent of changes in levels of mitochondrial Bcl-2 and Bax and highly synergistic with the pathway induced by the staurosporine-mediated release of mitochondrial cytochrome *c*.

It is likely that the earliest effect of butyrate on cells is to cause hyperacetylation of histones (especially H4) due to the inhibition of histone deacetylase. This is known to be a direct effect of butyrate on the enzyme and, in various cell lines, occurs within 30 min and peaks

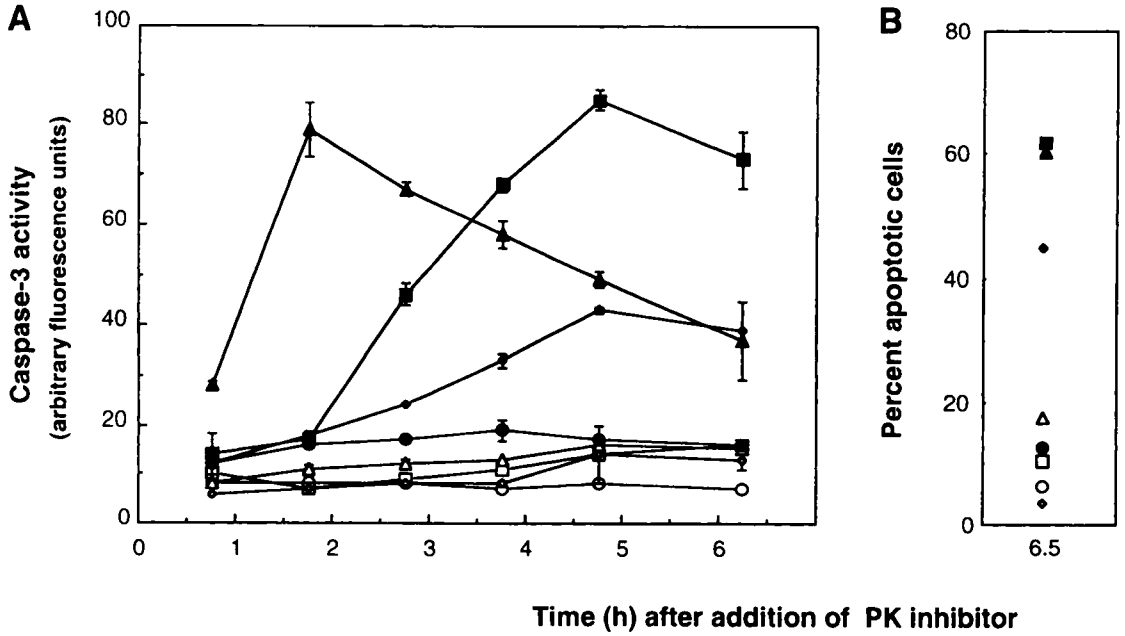
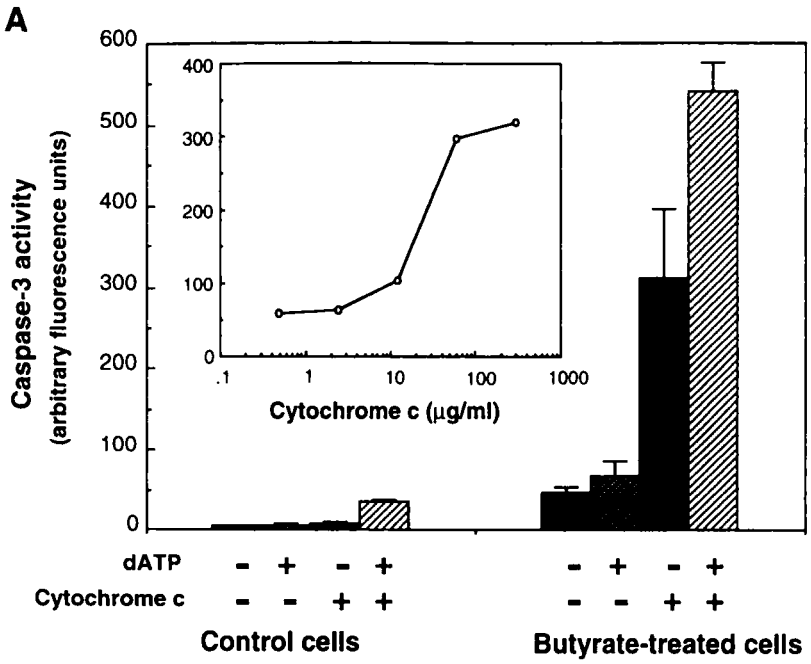


Fig. 7. Synergy between suboptimal concentration of butyrate and protein kinase inhibitors. Jurkat cells were cultured overnight in the presence or absence of 1 mM butyrate. The cells were then incubated for an additional 6.5 h in the absence or presence of protein kinase inhibitors: ○, no addition; ●, butyrate; △, 0.5 μM staurosporine; ▲, 0.5 μM staurosporine + butyrate; □, 25 μM H-7; ■, 25 μM H-7 + butyrate; ◇, 7 μM Et-18-O-CH_3; and ◆, 7 μM Et-18-O-CH_3 + butyrate. In *A*, at the indicated times, cells were washed, lysed, and assayed for caspase-3 protease. In *B*, after 6.5 h, cells were assayed for apoptotic morphology. The figure shows synergy between butyrate and the protein kinase inhibitors in activation of caspase-3 and induction of apoptosis. Each point represents a mean of triplicates (different culture flasks); *bars*, SD.



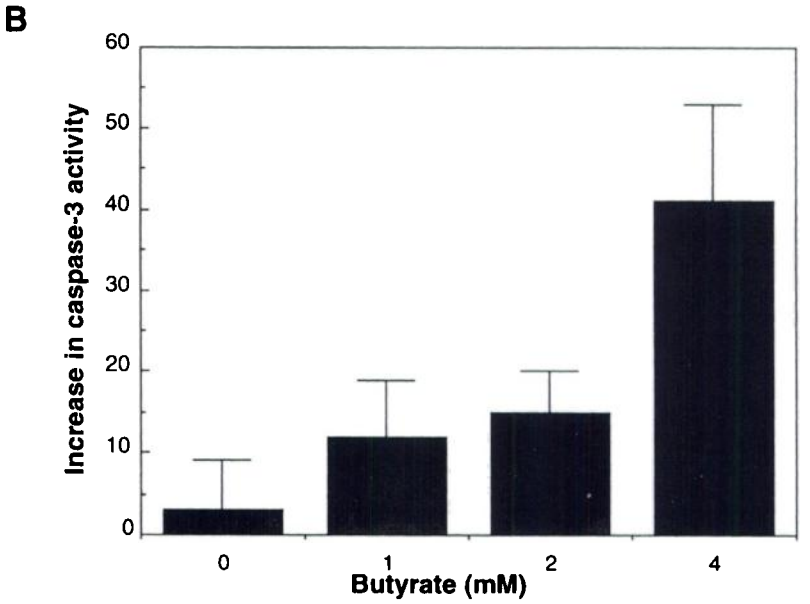


Fig. 8. Activation of caspase-3 in the cytoplasm of butyrate-treated cells assessed in a cell-free model. In *A*, Jurkat cells were cultured overnight in the presence or absence of 1 mM butyrate. Cells were then washed and lysed, and cytosols were prepared. Cytosols were incubated for 90 min at 37°C in the presence or absence of dATP and cytochrome *c*, before assaying caspase-3 protease activity. The figure shows a much greater cytochrome *c*- and dATP-induced activation of caspase-3 in cytosols from butyrate-treated cells. Activation was more dependent on exogenous cytochrome *c* than dATP, although both were required for optimal activation. *Inset*, concentration-dependence of cytochrome *c*-induced activation of caspase-3 in cytosols from butyrate-treated cells. All samples contained 1 mM dATP. In *B*, Jurkat cells were cultured for 11 h in the presence of the indicated concentrations of butyrate. Cytosols were prepared as in *A* and tested for caspase-3 activation by a mixture of dATP and cytochrome *c*. The figure shows a marked increase in the activity of caspase-3 in cells treated with a concentration of butyrate (4 mM) optimal for inducing apoptosis. *Bars*, SD.

by 6 h (27, 29). That histone acetylation triggers the induction of apoptosis is supported by the findings that: (*a*) the structural analogue, isobutyrate, which is much less effective than *n*-butyrate in inhibition of histone deacetylase (29) failed to induce apoptosis; and (*b*) TSA, a highly specific inhibitor of histone deacetylase, mimicked butyrate in the kinetics and cycloheximide sensitivity of induction of caspase-3 activation and apoptosis at nanomolar concentrations known to inhibit histone deacetylase. Future studies will directly correlate H4 hyperacetylation with the induction of morphological changes of apoptosis and caspase-3 activation in cells treated with inhibitors of histone deacetylase. In the absence of specific inhibitors of histone acetylases, final proof that butyrate induces apoptosis via histone deacetylase will have to await the isolation of cell lines with a butyrate-insensitive histone deacetylase as well as evidence that butyrate treatment exposes the promoter regions (and facilitates transcription) of putative cell death genes.

An alternative explanation for the effects of histone acetylation on apoptosis is that the more open chromatin structure renders the internucleosomal linker regions of DNA more susceptible to cleavage by apoptotic endonucleases. Enhanced sensitivity to nuclease involves about 20% of the DNA in butyrate-treated cells (37). However, the failure of butyrate to increase the sensitivity of DNA to micrococcal nuclease (38), which like the apoptotic endonuclease preferentially attacks internucleosomal sites, and the complete suppression of apoptosis by cycloheximide argues for the hypothesis that histone hyperacetylation facilitates apoptosis by increased expression of one or more cell death genes. Butyrate is known to affect the expression of various genes (reviewed in Ref. 10). It is conceivable that cell death genes are normally silenced by insertion in hypoacetylated, transcriptionally silent domains, where histone deacetylase activity is high, and that these are rendered more accessible to transcription factors by the suppression of deacetylation (see Ref. 32).

Although it has not yet been proved, it is believed that activation of caspases represents a terminal signaling event common to all of the different pathways by which cells can be induced to apoptose and which then trigger effector pathways resulting in morphological changes, DNA fragmentation, and cell death (17–20). The induction of caspase-3 activity in the cytoplasm of Jurkat and LIM 1215 cells preceding morphological changes, the suppression of this increase by cycloheximide, and the inhibition of butyrate-induced apoptosis by membrane-permeable inhibitors of ICE-like proteases suggest that butyrate-induced apoptosis requires involvement of caspase-3. Whether other members of the family act upstream or downstream of caspase-3, as shown recently for Fas-mediated apoptosis (20), or whether they act in parallel pathways, as described in a cell-free model of apoptosis (19), needs to be determined. The failure to inhibit all of the morphological changes of apoptosis by zVAD in butyrate-treated Jurkat cells, particularly at concentrations well above those required to block caspase-3 activity, raises questions as to whether zVAD-insensitive caspases or other effector pathways are also operative. Recently, McCarthy *et al.* (39) showed that zVAD effectively blocked nuclear changes but not membrane blebbing in cells undergoing apoptosis, arguing for more than one mechanism responsible for eliciting the cascade of morphological changes that characterize apoptotic cell death.

Much interest has focused on the role of mitochondria and mitochondria-derived factors (*e.g.*, cytochrome *c*) in the activation of caspase-3 and onset of apoptosis (16, 22–24). That we were unable to detect any change in the levels of the mitochondrial membrane proteins Bcl-2 and Bax, in response to butyrate, does not exclude the possibility that the butyrate-induced pathway is regulated by these proteins, but it does suggest that butyrate does not simply work by inducing a shift in the ratio of these proteins that might favor apoptosis. However, it must be emphasized that there are several other members of the Bcl-2/Bax family, both proapoptotic and antiapoptotic (25) and any of these may still influence butyrate-induced apoptosis. We are presently testing whether butyrate affects the levels of any of these proteins.

We believe, however, that the mechanism by which butyrate induces caspase-3 activation and apoptosis is independent of the mitochondrial pathway. The treatment of cells with a concentration of butyrate that was insufficient alone to activate caspase-3 or cause apoptosis primed cells for subsequent response to a suboptimal concentration of staurosporine. Staurosporine releases cytochrome *c* from mitochondria (16). It presumably acts to inhibit a protein kinase, because two other structurally different protein kinase inhibitors, the isoquinoline sulfonamide H-7 and the alkyl-lysophospholipid Et-18-O-CH_3, also synergized with butyrate in the activation of caspase-3. It is, therefore, likely that dephosphorylation of one or more proteins in the cascade leads to critical changes in the mitochondria; however, it needs to be determined whether these kinase inhibitors also induce mitochondria to release cytochrome *c*. Studies of the effects of selective protein kinase inhibitors may assist in the identification of the kinase that regulates events leading to the translocation of cytochrome *c* from mitochondria to cytosol.

In support of a synergy between the butyrate-induced and staurosporine-induced pathways, priming of cells with low concentrations of butyrate rendered the cytoplasm much more sensitive to cytochrome *c*-induced activation of caspase-3. Activation was strictly dependent on added cytochrome *c*, suggesting that butyrate alone, at least at the priming concentration, does not cause mitochondria to release cytochrome *c*. How butyrate renders the cytoplasm more sensitive to the action of cytochrome *c* is not yet known. Western blotting studies suggested no increase in the proenzyme form of caspase-3. Other possibilities include an increase in the intracellular concentration of dATP and alterations in levels or function of other cytoplasmic cofactors, including other caspases. Of interest, Orth *et al.* (40) have proposed that the physiological activation of caspase-3 is mediated by the ICE-like protease Mch2/caspase-6. If so, the relationship of caspase-6 to the butyrate-inducible death protein and the cytosolic factor that enhances caspase-3 activation in the cell-free model needs to be determined.

The millimolar concentrations of butyrate required to induce apoptosis are similar to those required to induce differentiation and growth arrest and are achieved *in vivo* in the lumen of the distal colon (1, 2, 28). The effective intracellular concentrations of butyrate are unknown, because butyrate is rapidly metabolized via β-oxidation in mitochondria (35). For this reason, cells that metabolize butyrate at a higher rate are likely to be less susceptible to its apoptosis-inducing effects. This may explain why normal colonocytes, which rely on butyrate as a major energy source (35), are unaffected by the very high levels of butyrate (reaching concentrations as high as 150 mM) in the distal colon. In the light of evidence that high fiber diets promote histone hyperacetylation in colonic epithelia of rats (28), the effects of these diets and colonic instillation of butyrate on histone acetylation, caspase-3 protease activation, and apoptosis in colorectal tumors now needs to be addressed in animal models. Of particular relevance to tumor prevention therapies are possible synergistic interactions between butyrate or TSA and protein kinase inhibitors.

The studies in this paper point to a critical effect of inhibitors of histone deacetylase on the apoptosis signaling pathway of cancer cells and have begun to identify some of the factors involved. Additional studies of the early interactions of butyrate with cells, the identity of the protein(s) that commit the cells to apoptose, and the possible involvement of histone hyperacetylation as a common early event induced by diverse apoptogens now require testing.

ACKNOWLEDGMENTS

We acknowledge Dr. R. Whitehead for the supply of colorectal cancer cell lines and Ann Mcintyre for the donation of isobutyric acid.

REFERENCES

1. Young, G. P. Butyrate and the molecular biology of the large bowel. *In:* J. L. Rhombeau, J. H. Cummings, and T. Sakata (eds.), Short Chain Fatty Acids: Metabolism and Clinical Importance, pp. 39–44. Columbus, OH: Ross Laboratories, 1991.
2. McIntyre, A., Gibson, P. R., and Young, G. P. Butyrate production from dietary fibre and protection against large bowel cancer in a rat model. Gut, *34:* 386–391, 1993.
3. Hague, A., Manning, A. M., Hanlon, K. A., Huschtscha, L. I., Hart, D., and Paraskeva, C. Sodium butyrate induces apoptosis in human colonic tumour cell lines in a p53-independent pathway: implications for the possible role of dietary fibre in the prevention of large bowel cancer. Int. J. Cancer, *55:* 498–505, 1993.
4. Hague, A., Manning, A. M., van der Stappen, J. W. J., and Paraskeva, C. Escape from negative regulation of growth by transforming growth factor β and from the induction of apoptosis by the dietary agent sodium butyrate may be important in colorectal carcinogenesis. Cancer Metastasis Rev., *12:* 227–237, 1993.
5. Calabresse, C., Venturini, L., Ronco, G., Villa, P., Chomienne, C., and Belpomme, D. Butyric acid and its monosaccharide ester induce apoptosis in the HL-60 cell line. Biochem. Biophys. Res. Commun., *195:* 31–38, 1993.
6. Fillipovich, I., Sorokina, N., Khanna, K. K., and Lavin, M. F. Butyrate-induced apoptosis in lymphoid cells preceded by transient overexpression of HSP70 mRNA. Biochim. Biophys. Res. Commun., *198:* 257–265, 1994.
7. Kerr, J. F. R., Searle, J., Harmon, B. V., and Bishop, C. J. Apoptosis. *In:* C. S. Potten (ed.), Perspectives of Mammalian Cell Death, pp. 93–128. Oxford: Oxford University Press, 1987.
8. Wyllie, A. H. Apoptosis and the regulation of cell numbers in normal and neoplastic tissues: an overview. Cancer Metastasis Rev., *11:* 95–103, 1992.
9. Eastman, A. Activation of programmed cell death by anticancer agents: cisplatin as a model system. Cancer Cells, *2:* 275–280, 1990.
10. Kruh, J., Defer, N., and Tichonicky, L. Butyrate and the molecular biology of the large bowel. *In:* J. L. Rhombeau, J. H. Cummings, and T. Sakata (eds.), Short Chain Fatty Acids: Metabolism and Clinical Importance, pp. 45–50. Columbus, OH: Ross Laboratories, 1991.
11. Hall, P. A. Regulation of cell number in the mammalian gastrointestinal tract: the importance of apoptosis. J. Cell Sci., *107:* 3569–3577, 1994.

12. Schwartz, L. M. The role of cell death genes during development. Bioessays, *13:* 389–395, 1991.
13. Weil, M., Jacobson, M. D., Coles, H. S. R., Davies, T. J., Gardner, R. L., Raff, K. D., and Raff, M. C. Constitutive expression of the machinery for programmed cell death. J. Cell Biol., *133:* 1053–1059, 1996.
14. Jacobson, M. D., Burne, J. F., and Raff, M. C. Programmed cell death and Bcl-2 protection in the absence of a nucleus. EMBO J., *13:* 1899–1910, 1994.
15. Martin, S. J., and Green, D. R. Cell-free apoptosis. *In:* T. G. Cotter and S. J. Martin (eds.), Techniques in Apoptosis. A User's Guide, pp. 121–132. London: Portland Press Ltd., 1996.
16. Liu, X., Kim, C. N., Yang, J. Jemmerson, R., and Wang, X. Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. Cell, *86:* 147–157, 1996.
17. Kumar, S. ICE-like proteases in apoptosis. Trends Biochem. Sci., *20:* 198–202, 1995.
18. Nicholson, D. W., Ali, A., Thornberry, N. A., Vaillancourt, J. P., Ding, C. K., Gallant, M., Gareau, Y., Griffin, P. R., Labelle, M., Lazebnik, Y. A., Munday, N. A., Raju, S. M., Smulson, M. E., Yamin, T-T., Yu, V. L., and Miller, D. K. Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. Nature (Lond.), *376:* 37–43, 1995.
19. Lazebnik, Y. A., Takahashi, A., Moir, R. D., Goldman, R. D., Poirier, G. G., Kaufmann, S. H., and Earnshaw, W. C. Studies of the lamin proteinase reveal multiple parallel biochemical pathways during apoptotic execution. Proc. Natl. Acad. Sci. USA, *92:* 9042–9046, 1995.
20. Enari, M., Talanian, R. V., Wong, W. W., and Nagata, S. Sequential activation of ICE-like and CPP-32-like proteases during Fas-mediated apoptosis. Nature (Lond.), *380:* 723–726, 1996.
21. Alnemri, E. S., Livingston, D. J., Nicholson, D. W., Salvesen, G., Thornberry, N. A., Wong, W. W., and Yuan, J. Human ICE/CED-3 protease nomenclature. Cell, *87:* 171, 1996.
22. Zamzami, N., Susin, S. A., Marchetti, P., Hirsch, T., Gomez-Monterrey, I., Castedo, M., and Kroemer, G. Mitochondrial control of nuclear apoptosis. J. Exp. Med., *183:* 1533–1544, 1996.
23. Yang, J., Liu, X., Bhalla K., Kim, C. N., Ibrado, A. M., Cai, J., Peng, T., Jones, D. P., and Wang, X. Prevention of apoptosis by Bcl-2: release of cytochrome *c* from mitochondria blocked. Science (Washington DC), *275:* 1129–1132, 1997.
24. Kluck, R. M., Bossy-Wetzel, E., Green, D. R., and Newmeyer, D. D. The release of cytochrome *c* from mitochondria: a primary site for Bcl-2 regulation of apoptosis. Science (Washington DC), *275:* 1132–1136, 1997.
25. Nunez, G., and Clarke, M. F. The Bcl-2 family of proteins: regulators of cell death and survival. Trends Cell Biol., *4:* 399–403, 1994.
26. Arts, J., Lansink, M., Grimbergen, J., Toet, K. H., and Kooistra, T. Stimulation of tissue type plasminogen activator gene expression by sodium butyrate and TSA in human endothelial cells involves histone acetylation. Biochem. J., *310:* 171–176, 1995.
27. Boffa, L. C., Vidali, G., Mann, R. S., and Allfrey, V. G. Suppression of histone deacetylation *in vivo* and *in vitro* by sodium butyrate. J. Biol. Chem., *253:* 3364–3366, 1978.
28. Boffa, L. C., Lupton, J. R., Mariani, M. R., Ceppi, M., Newmark, H. L., Scalmati, A., and Lipkin, M. Modulation of colonic epithelial cell proliferation, histone acetylation, and luminal short chain fatty acids by variation of dietary fiber (wheat bran) in rats. Cancer Res., *52:* 5906–5912, 1992.
29. Sealy, L., and Chalkley, R. The effect of sodium butyrate on histone modification. Cell, *14:* 115–121, 1978.
30. Yoshida, M., Horinouchi, S., and Beppu, T. TSA and trapoxin: novel chemical probes for the role of histone acetylation in chromatin structure and function. Bioessays, *17:* 423–430, 1995.
31. Yoshida, M., Kijima, M., Akita, M., and Beppu, T. Potent and specific inhibition of mammalian histone deacetylase both *in vivo* and *in vitro* by TSA. J. Biol. Chem., *265:* 17174–17179, 1990.
32. Ogryzko, V. V., Hirai, T. H., Russanova, V. R., Barbie, D. A., and Howard, B. H. Human fibroblast commitment to a senescence-like state in response to histone deacetylase inhibitors is cell cycle dependent. Mol. Cell. Biol., *16:* 5210–5218, 1996.
33. Zalewski, P. D., Forbes, I. J., and Giannakis, C. Physiological role for zinc in prevention of apoptosis (gene-directed cell death). Biochem. Int., *24:* 1093–1101, 1991.
34. Hague, A., Moorghen, M., Hicks, M, D., Chapman, M., and Paraskeva, C. BCl-2 expression in human colorectal adenomas and carcinomas. Oncogene, *9:* 3367–3370, 1994.
35. Roediger, W. E. Utilization of nutrients by isolated epithelial cells of the rat colon. Gastroenterology, *83:* 424–429, 1982.
36. Lee, E., Furukubo, T., Miyabe, T., Yamauchi, A., and Kariya, K. Involvement of histone hyperacetylation in triggering DNA fragmentation of rat thymocytes undergoing apoptosis. FEBS Lett., *395:* 183–187, 1996.
37. Smith, P. J. *n*-Butyrate alters chromatin accessibility to DNA repair enzymes. Carcinogenesis (Lond.), *7:* 423–429, 1986.
38. Nelson, D. A., Perry, M., Sealy, L., and Chalkley, R. DNase I preferentially digests chromatin containing hyperacetylated histones. Biochim. Biophys. Res. Commun., *82:* 1346–1353, 1978.
39. McCarthy, N. J., Whyte, M. K. B., Gilbert, C. S., and Evan, G. I. Inhibition of Ced-3/ICE-related proteases does not prevent cell death induced by oncogenes, DNA damage, or the Bcl-2 homologue Bak. J. Cell Biol., *136:* 215–227, 1997.
40. Orth, K., O'Rourke, K., Salvesen, G. S., and Dixit, V. M. Molecular ordering of apoptotic mammalian ced-3/ICE-like proteases. J. Biol. Chem., *271:* 20977–20980, 1996.