We have examined the effects of the "differentiating agent," sodium butyrate, on the induction of alkaline phosphatase in human colonic tumor cell line LS174T. Culture of these cells in the presence of 2 mM butyrate caused this activity to increase from $<0.0001$ unit/mg of protein to $>0.7$ unit/mg of protein over an 8-day period. This induction proceeded in a nonlinear fashion with a lag time of 2–3 days occurring before enzymatic activity began to rise. These increases in activity were accompanied by elevations in the content of a placental-like isozyme of alkaline phosphatase as demonstrated by "Western" immunoblots. Dome formation, indicative of differentiation in cultured cells, also required 3 days treatment with butyrate before becoming evident. The rate of biosynthesis of the enzyme, examined using metabolic labeling with $\text{L-}^{[35\text{S}]}\text{methionine}$ and immunoprecipitation, was found to increase continuously between days 2 and 6 of butyrate treatment. "Northern" blot analysis indicated that treatment of these cells with butyrate caused $>20$-fold induction of a $2700$-base mRNA that hybridized to a cDNA probe for placental alkaline phosphatase. The mRNA for alkaline phosphatase produced by these cells upon butyrate treatment was approximately $300$–$400$ bases smaller than the mRNA for alkaline phosphatase found in placenta. Human small intestine also contained two mRNAs that hybridized relatively weakly with the placental alkaline phosphatase probe. These results indicate that a placental alkaline phosphatase-like protein and mRNA are induced by butyrate in LS174T cells with a time course consistent with cellular differentiation preceding induction.

Human alkaline phosphatases comprise a family of isozymes encoded in at least three distinct genetic loci. These are the tissue unspecific or liver-bone-kidney alkaline phosphatase gene, the intestinal alkaline phosphatase gene, and the placental alkaline phosphatase gene (1–3). Although the physiological functions of alkaline phosphatases have yet to be established, this family of enzymes has been extensively studied due to its usefulness in clinical diagnosis. These three isozymes of alkaline phosphatase are known to differ in their molecular weights, thermal stabilities, immunological properties, and sensitivity to inhibitors (1–5). The placental isozyme of alkaline phosphatase is of particular interest because it is sometimes present in tumors that arise from tissues that do not normally contain this enzyme (6–9).

Butyrate has been shown to increase the levels of alkaline phosphatase in several cell lines including those derived from human colorectal cancers (10–14). In addition, this compound induces the expression of a number of glycoproteins other than alkaline phosphatase and causes alterations in cellular differentiation (15–19). These alterations include the inhibition of growth and changes in cell morphology and polarization (16–19). Butyrate has recently been shown to induce the differentiation of colon cancer cell line HT-29 into a layer of polarized epithelial cells exhibiting tight junctions and well-defined basolateral and brush border membranes (19).

In previous work, we found that alkaline phosphatase was present in each of 14 human colorectal cancer cell lines examined and, moreover, that this activity was inducible by butyrate to at least some extent in most of these lines (14). In the present study, we have examined in greater detail the induction of alkaline phosphatase by butyrate in one of these, human colon cancer cell line LS174T. Here we report that butyrate induces a placental-like form of alkaline phosphatase in these cells by increasing the level of the mRNA that codes for this enzyme. This mRNA induction begins only after a 2–3 day lag time and then proceeds to increase rapidly over the next several days. Dome formation, a well-characterized marker of the differentiation of cultured cells into a polarized epithelial monolayer (20), is also induced in LS174T cells by treatment with butyrate for 3 days. Hence, the lag time exhibited in alkaline phosphatase mRNA induction by butyrate may reflect a necessity for cellular differentiation to occur prior to induction.

**EXPERIMENTAL PROCEDURES**

Cell Culture, Labeling, and Assays—Human colonic adenocarcinoma cell line LS174T (21) was a gift of Dr. Barry D. Kahan of the University of Texas Medical School at Houston. Cells were maintained as described earlier in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal bovine serum, 100 units/ml penicillin, and 100 $\mu$g/ml streptomycin (14). Confluent monolayers of cells were used in the present study. These were produced by seeding $6-25 \times 10^4$ cells/cm$^2$ of culture area in 35- or 100-mm tissue culture dishes (Lux or Falcon) and allowing 3–4 days growth prior to use. The medium was replenished daily. To metabolically label cellular protein, confluent cells in 35-mm dishes were cultured for 3 h with 1 ml of medium containing 100 $\mu$Ci of $\text{L-}^{[35\text{S}]}\text{methionine}$. In these experiments, supplemented medium prepared using Dulbecco’s modified Eagle’s medium with 20% of the normal amount of $\text{L-methionine}$ was used to facilitate label incorporation. Labeled (or unlabeled) cells were harvested and lysates were prepared as described previously and stored at $-20^\circ C$ (14). Protein concentration (22) and trichloroacetic

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**Effects of Sodium Butyrate on Human Colonic Adenocarcinoma Cells**

**INDUCTION OF PLACENTAL-LIKE ALKALINE PHOSPHATASE**

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acid-insoluble radioactivity (29) were determined as described. One unit of alkaline phosphatase activity is defined as 1 pmol of p-nitrophenylphosphate hydrolyzed per min at 37 °C using previously described assay conditions (13).

**RNA Isolation and In Vitro Translation**—Total cellular RNA was isolated using the guanidinium thiocyanate-chloride procedure of Chirgwin et al. (24). Typical yield of total RNA from LS174T cells was 100 μg, and the RNA was used in RNA blot reactions using the same procedure except that a quantity of poly(A)⁺ RNA was prepared using one cycle of oligo(dT)-cellulose chromatography (25), except as noted. In vitro translation was conducted using rabbit reticulocyte lysates and the rabbit reticulocyte system of Maniatis et al. (25). Either 20 μg of total RNA or 2.5 μg of poly(A)⁺ RNA, amounts found to stimulate maximal incorporation of radioactivity into trichloroacetic acid-insoluble peptides, was used with 100 μCi of [35S]methionine in 52-μl reaction volumes.

**Immunoprecipitations, SDS-Polyacrylamide Gel Electrophoresis, and “Western Blots”**—For immunoprecipitation of alkaline phosphatase, lysates containing 1 × 10⁶ cpm of trichloroacetic acid-insoluble L-[35S]methionine were thawed, sonicated for 10 s, and dissolved in buffer A (1% Nonidet P-40, 0.50 mM NaCl, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 1 mg/ml bovine serum albumin, and 10 mM Tris-HCl, pH 8.9). A sufficient quantity of nonradioactive carrier lysate, prepared from cells treated 6 days with 2 mM butyrate, was added as necessary to obtain a level of 0.11 unit/ml alkaline phosphatase activity. A 1-μl aliquot of normal rabbit serum was added to a final volume of 1 ml, and the mixtures were incubated for 20 min at 4 °C. After centrifugation at 14,000 g for 20 min, the supernatants were added to 1 ml of a 1:500 dilution of anti-alkaline phosphatase (prepared in the laboratory) or to 1 ml of a 1:500 dilution of nonimmune rabbit serum. The samples were treated for 1 h with occasional shaking at 4 °C with 100 μl of a 10% (w/v) solution of formalin-treated Staphylococcus aureus cells and centrifuged for 2 rain at 12,000 × g in a microcentrifuge. The supernatant was then treated with 5 μl of anti-alkaline phosphatase IgG for 30 min at 30 °C, for 15 min at 4 °C, and then for 1 h with S. aureus cells as above. The cell pellet was then washed and prepared for electrophoresis as described (26). Electrophoresis was conducted using 7% polyacrylamide gels in the presence of sodium dodecyl sulfate (27). Proteins were transferred electrophoretically from the gel to nitrocellulose filters using the procedure of Towbin et al. (28), the filters soaked 1 h in Autofluor (National Diagnostica), and fluorography conducted at -70 °C using Kodak XAR-2 film presensitized to achieve maximum linearity as described by Laskey and Mills (29). Alkaline phosphatase was immunoprecipitated from in vitro translation reactions using the same procedure except that a quantity of carrier lysate containing 0.01 unit/ml alkaline phosphatase was used with 2 μl of normal rabbit serum and anti-alkaline phosphatase IgG being used in the preabsorption and immunoprecipitation steps, respectively. Assay of alkaline phosphatase activity indicated that none of the enzyme was lost in the preabsorption step and that greater than 97% and 99% of enzyme activity was immunoprecipitated at alkaline phosphatase levels of 0.01 and 0.11 unit/ml, respectively. “Western” blots were conducted as described (28), using a 1:500 dilution of anti-alkaline phosphatase IgG followed by 100 cpm/ml 125I-labeled protein A.

**“Northern” Analysis of Alkaline Phosphatase mRNA—**Poly(A)⁺ RNA was incubated at 65 °C for 5 min in a solution containing 50% deionized formamide and 6.6% formaldehyde and subjected to electrophoresis in 1.5% agarose gels containing 6.6% formaldehyde. A buffer system consisting of 10 mM sodium phosphate (pH 7.0), 1 mM EDTA, and 5 mM sodium acetate was used for electrophoresis. Following capillary transfer of the RNA to Biotrans A nylon membranes using 20 × standard saline citrate and baking at 80 °C in a vacuum oven, prehybridization was conducted at 42 °C for 18-22 h in 5 × Denhardt's solution, 5 × standard saline citrate, 50 mM sodium phosphate (pH 6.5), 0.1% SDS, 50 μg/ml salmon sperm DNA, and 50% deionized formamide (25). The 1.9-kilobase KpnI fragment of λAP27 that contains the entire coding sequence of placental alkaline phosphatase (30) was nick translated, and hybridization was conducted as prehybridization except that 1-5 × 10⁶ cpm/ml of labeled probe and 10% dextran sulfate was used. The membranes were then washed with 2 × standard saline citrate containing 0.1% SDS for 1.5 h at room temperature followed by 1-h washes at 25 °C and then at 55 °C in 0.1 × standard saline citrate, 0.1% SDS. After drying in air, the membranes were autoradiographed.

**Northern Procedures—**Fetal bovine serum and Dulbecco's modified Eagle's medium were obtained from Irvine Scientific. Medium without methionine was obtained from the University of California at San Francisco cell culture facility and combined with normal medium to obtain the desired level of L-methionine. Medium containing butyrate was prepared by adding butyric acid (Matheson, Coleman and Bell) to supplemented medium and titrating to pH 7.0 with NaOH. L-[35S]Methionine (1200 Ci/mmol) and deoxy-cytidine 5'-triphosphate (3000 Ci/mmol) were from Amer sham Corp. Carrier-free Na⁺ and Biotrans A nylon membranes were from ICN. Protein A and formalin-fixed S. aureus were from Zymed Laboratories. The latter was washed 3 times by centrifugation in buffer A immediately before use. Protein A was labeled with 125I using chloramine T (31), obtained from Sigma. Rabbit polyclonal anti-alkaline phosphatase IgG was obtained from the Dako Corp. Nick translations were performed as described (25) using enzymes and reagents purchased as a kit from Worthington.

**RESULTS**

**Effect of Butyrate on Alkaline Phosphatase Levels in LS174T Cells**—The addition of 2 mM sodium butyrate to cultures of LS174T cells initially has little effect on alkaline phosphatase activity (Fig. 1). Only after 2-3 days of incubation with butyrate does this activity begin to rise. After this lag time, alkaline phosphatase activity begins a sustained increase continuing for at least 5 additional days until levels exceed 0.7 unit/ml. The extent of this induction varies somewhat between experiments (see Fig. 4); however, the lag time is always observed. Since purified mammalian alkaline phosphatases have specific activities ranging between 500 and 2000 units/mg of protein (32-34), the alkaline phosphatase levels in the most highly induced lysates observed in these experiments are approximately 0.05% of total cellular protein. This effect is reversible as indicated by the decrease in activity observed following butyrate removal (Fig. 1).

Examples exist of cultured human tumor cell lines that express each of the three known isozymes of alkaline phosphatase (35, 36). As a preliminary experiment to determine the isozymic composition of LS174T cells, we examined the resistance of the activity present in this line to thermal denaturation. We found that the alkaline phosphatase activity present in lysates of both control and butyrate-treated LS174T cells is completely stable to incubation at 65 °C for 5 min, indicating that these cells express a form of the enzyme similar to the form found in placenta (data not shown). This

![Figure 1](https://example.com/fig1.jpg)

**FIG. 1.** The effect of butyrate on LS174T cell alkaline phosphatase activity. Confluent cultures in 35-mm tissue culture dishes were incubated in supplemented medium with (Ī——ī) or without (□□□□□□□□) 2 mM butyrate. At the indicated times, the cultures were harvested, and the lysates were prepared and assayed for alkaline phosphatase activity and protein content. The arrow indicates a time at which some cultures (■——■) were returned to butyrate-free medium and subsequently harvested and assayed.
was further examined by Western analysis using anti-placental alkaline phosphatase IgG. In these experiments, butyrate-treated LS174T cell lysates exhibited a single band at 68,500 (Fig. 2), the subunit molecular weight of placental alkaline phosphatase (37). As shown in Fig. 2, a band of indistinguishable molecular weight was also observed when placental homogenates were analyzed using this technique. The intensity of this band correlated well \( (r^2 = 0.978) \) with the alkaline phosphatase activity present in the lysate (Fig. 3), indicating that the increases in activity observed upon butyrate treatment are due to increases in placental-like alkaline phosphatase protein levels rather than activation of pre-existing inactive protein.

**Enhancement of Alkaline Phosphatase Labeling with \( L-[1^3S] \) Methionine in Intact Cells by Butyrate**—The changes in alkaline phosphatase activity and protein levels that are induced by butyrate in these cells require 2-3 days before becoming manifest. As an initial experiment to examine the mechanisms responsible for this lag time we treated cells with 2 mM butyrate for various lengths of time, incubated with \( L-[1^3S] \) methionine-containing medium for 3 h, and determined the relative amounts of alkaline phosphatase labeled by immunoprecipitation and fluorography. After several days treatment with butyrate, significant amounts of labeled alkaline phosphatase \( (M_r = 68,500) \) were immunoprecipitated by this procedure (Fig. 4). No detectable amount of alkaline phosphatase was labeled until the 2nd day of butyrate treatment, however, and then levels underwent a steady rise until the 6th day. Given the usual turnover times for membrane proteins (38-40), the amount of alkaline phosphatase labeled in a 3-h period should primarily reflect the relative biosynthetic rate of the enzyme at a particular stage of butyrate induction. Hence, the normalized increases in the labeled alkaline phosphatase observed in Fig. 4, which precede normalized increases in alkaline phosphatase activity, imply that continuously increasing biosynthetic rates are responsible for the observed increases in enzymatic activity.

**Fig. 2.** Western immunoblot of LS174T alkaline phosphatase. 50 \( \mu \)g of lysate from LS174T cells treated 4 days with 2 mM butyrate \((A)\) or 50 \( \mu \)g of term human placenta homogenate \((B)\) was subjected to Western analysis using anti-alkaline phosphatase IgG. Autoradiography was conducted for 18 h at \(-70^\circ\) C using Dupont Cronex intensifying screens and presensitized (29) Kodak XAR-2 film.

**Fig. 3.** Correlation between immunoreactive alkaline phosphatase and enzymatic activity. Top, lysates (75 \( \mu \)g) prepared from LS174T cells incubated the indicated number of days with 2 mM butyrate were subjected to Western analysis. The region of the gel containing the alkaline phosphatase band is shown. Bottom, lanes 3-8 of the above gel were scanned using an E-C Apparatus Corporation model EC910 densitometer and the relative areas under the peaks determined. Densitometric units were assigned by dividing these areas by a normalization constant and plotted as a function of the alkaline phosphatase activity in the lysate applied to the gel.

**Fig. 4.** Labeling of alkaline phosphatase with \( L-[1^3S] \) methionine in cells at various times of butyrate induction. Confluent cultures in 35-mm tissue culture dishes were treated with 2 mM butyrate for various times and then labeled with \( L-[1^3S] \) methionine using 3-h incubations as described under “Materials and Methods.” Following the labeling period, the cells were harvested and assayed for alkaline phosphatase activity, protein concentration, and trichloroacetic acid-insoluble \( 35S \). Aliquots of lysates containing \( 10^6 \) cpm of acid-insoluble \( 35S \) were then subjected to immunoprecipitation, gel electrophoresis, and fluorography. Top, fluorograph of immunoprecipitated alkaline phosphatase band. Numbers indicate the approximate time (in days) of butyrate treatment prior to labeling. CL is control immunoprecipitation done using the 5-day butyrate-treated sample but without using anti-alkaline phosphatase IgG. Butyrate (2 mM) was included during each labeling except in the case of the "0 day" sample. Bottom, the lanes of the fluorograph were scanned densitometrically and the relative intensities of the alkaline phosphatase bands determined as described in the legend to Fig. 3 and plotted as a function of time of treatment with 2 mM butyrate (measured in hours to the midpoint of the labeling incubation). The specific activities of alkaline phosphatase in the lysates used for immunoprecipitation are also given.
Butyrate-induced Increases in Alkaline Phosphatase mRNA Levels—The results shown in Fig. 4 suggest that butyrate treatment induces alkaline phosphatase biosynthesis. To probe this further, total cellular and poly(A)+ RNA were isolated after various times of butyrate treatment and analyzed indirectly by in vitro translation and immunoprecipitation (Fig. 5) and also by hybridization analysis using placental alkaline phosphatase cDNA (Fig. 6). When RNA isolated following several days treatment with butyrate was used to program in vitro translation reactions and the products examined by immunoprecipitation and gel electrophoresis a product of 63,000 daltons was observed (Fig. 5). This is similar in size to the unglycosylated in vitro translation product obtained using RNA from choriocarcinoma cells (10) and in good agreement with the size of the placental alkaline phosphatase primary translation product predicted from the cDNA sequence (30). The amount of alkaline phosphatase produced by in vitro translation steadily increased with time of butyrate treatment whether total RNA or poly(A)+ RNA was used (Fig. 5). Northern analysis confirms that this increased level of product is due to increases in message levels (Fig. 6). Poly(A)+ RNA preparations prepared following increasing times of butyrate treatment exhibited increases in hybridization band intensities that paralleled results obtained by in vitro translation (<5, 22, 52, and 100% of maximum by densitometry at 1, 3, 5, and 7 days of butyrate treatment, respectively; analysis in Fig. 6). Using the 18 S and 28 S rRNA subunits as molecular size markers (41) this mRNA was determined to be 2700 bases in length.

Northern analysis of the alkaline phosphatase message found in butyrate-treated LS174T cells is compared to that present in human small intestine and term placenta in the experiment shown in Fig. 7. Poly(A)+ RNA isolated from human small intestine contains two species that hybridize relatively weakly to the placental alkaline phosphatase probe (Fig. 7, panel A). The major band found in small intestine...
poly(A)$^+$ RNA is approximately 80 bases larger than the band observed in butyrate-treated LS174T cell RNA. A second, minor, band is considerably larger (3700 bases). Human term placental poly(A)$^+$ RNA also contains two bands that hybridize to the placental alkaline phosphatase probe (Fig. 7, panel B). These are a very weak band that comigrates with the message from butyrate-treated LS174T cells and a very intense band 300–400 bases larger in size. The autoradiographs shown in Fig. 7 were performed without intensifying screens, a condition that is necessary to resolve the minor bands. The results indicate that the message for alkaline phosphatase expressed by butyrate-treated LS174T cells is different from the message expressed both by human small intestine and term placenta.

**Butyrate-induced Differentiation of LS174T Cells**—We observed in the course of this study that butyrate caused LS174T cells to become flattened and to extend processes in a manner that is generally similar to our previous results with HRT-18 cells (16). Further, the confluent cultures of cells used in the present study exhibited dome formation (Fig. 8). This phenomenon, originally described in Madin-Darby canine kidney cells, has been attributed to ion and water transport by a differentiated population of polarized epithelial cells that expresses tight junctions and well delineated brush border and basolateral membranes (20). This effect, which becomes apparent 3 days after treating newly confluent cultures with butyrate, also occurs with untreated cells. In this case, however, the time of onset is later and, although it is not apparent in Fig. 8, the size of the domes formed is much smaller. Hence, butyrate apparently causes LS174T cells to differentiate into a polarized cell type in much the same manner as it affects human colon tumor cell line HT-29 (19).

**Discussion**

Butyrate has been termed a “differentiating agent” because of the many effects it has on a variety of cultured cells (10–19). In human colonic cancer cell line LS174T, examined here, butyrate causes both morphological and biochemical alterations. After a lag time of 2–3 days this agent increases alkaline phosphatase activity in these cells. This activity increase is accompanied by elevated expression of a placental-like isozyme of alkaline phosphatase, as demonstrated by Western analysis. Increases in biosynthesis, as measured by $[^{35}S]$methionine incorporation into enzyme, preceded these increases in activity and enzyme content. The results of this study further demonstrate that butyrate affects this induction by increasing the level of alkaline phosphatase mRNA present. Northern analysis indicates that, again after a lag time, butyrate treatment sharply elevates the expression of a single 2700-base mRNA that hybridizes to placental alkaline phosphatase cDNA. This mRNA is different in size from both species of small intestine poly(A)$^+$ RNA that hybridize to the placental alkaline phosphatase probe and is also different from the major alkaline phosphatase mRNA found in term placentas. These results suggest a dissimilarity between the alkaline phosphatase induced in LS174T cells by butyrate and both small intestine and placental alkaline phosphatases.

Interestingly, choriocarcinoma cells have recently been shown to contain an alkaline phosphatase mRNA that is 400 bases smaller than the mRNA for this enzyme from placenta (42). The similarities between the LS174T enzyme and placental alkaline phosphatase are substantial, however, as both enzymes exhibit a near-identical molecular weight (by Western analysis) and resistance to thermal denaturation. Hence, the enzyme induced in LS174T cells appears to be closely related to the placental isozyme of alkaline phosphatase. Subtle differences in the two enzymes could be revealed by cloning and sequencing the cDNA for LS174T cell alkaline phosphatase.

Itto and Chou (10) have demonstrated that the level of placental alkaline phosphatase synthesized by choriocarcinoma cells is increased by incubation with butyrate for 3 days. Choriocarcinoma cells, however, exhibit a markedly different response to butyrate than the LS174T cells used in the present study. The relatively high basal levels of alkaline phosphatase activity in choriocarcinoma cells begin a linear increase immediately upon treatment with butyrate and reach a peak at 3 days before declining. LS174T cells, on the other hand, do not begin appreciable synthesis of this enzyme until 2–3 days following the initiation of butyrate treatment, and increases in biosynthetic levels then continue for several more days. These increases in biosynthetic (and message) levels in LS174T cells may be limited by a requirement for cellular differentiation to occur prior to induction. This hypothesis is consistent with the time course of dome formation in these cells.

Human colonic epithelial cells contain only low levels of alkaline phosphatase of uncertain isozymic composition (43). While it has been shown that the intestinal flora produces substantial levels of butyrate and other short chain fatty acids (44, 45) the role of this compound in the regulation of normal or cancerous colonic cell differentiation in vivo has not yet been established. Further, there is no evidence that butyrate induces colonic alkaline phosphatase under physiological conditions. It is known, however, that butyrate causes several types of alterations in the nucleus. It inhibits histone deacetylase which leads to increased levels of acetylated histones H3 and H4 in cultured cells (46–48). This causes changes in the structure of histone-associated DNA as shown by increased susceptibility to DNase I digestion (48). Butyrate also increases the phosphorylation of high mobility group proteins −14 and −17 associated with transcriptionally active chromatin (49). Moreover, this agent inhibits the phosphorylation of histones (50). Whether the effects of butyrate on alkaline phosphatase induction are the result of these or other relatively nonspecific effects on chromatin or due to a more specific interaction is currently unclear. Perhaps this, and the question as to why this enzyme is often ectopically expressed by tumors, can be addressed through detailed analysis.

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**Fig. 8.** Dome formation in LS174T cells. Confluent cultures in 35-mm dishes were incubated in the presence (—) or absence (—) of 2 mM butyrate for the times indicated, and the number of macroscopically visible domes formed per plate were counted. The error bars represent the range of values observed using duplicate plates. The merging of domes accounts for the decreasing number of domes observed after the 4th day in the presence of butyrate.
of the structure of the placental alkaline phosphatase gene in both tumor and normal cells.

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