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EFFECT OF SODIUM BUTYRATE ON PRIMARY CULTURES OF ADULT RAT HEPATOCYTES

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SUMMARY

Sodium butyrate, at millimolar concentrations, seems to mediate or initiate multiple effects on many mammalian cells in culture. Although many transformed cell lines respond to butyrate treatment with acquisition of normal cellular characteristics, the effect of butyrate on a normal cell type, the parenchymal hepatocyte, has not been studied. Serum-free primary cultures of adult rat hepatocytes maintain many adult characteristics, yet after several days in culture a loss of adult characteristics occurs while fetal characteristics are often reexpressed. Therefore, we investigated whether butyrate treatment would improve the morphologic and biochemical characteristics of cultured hepatocytes. Exposure to 5 m*M* butyrate for 3 d did not affect hepatocyte viability or morphology but retarded the progressive decline in cytochrome P-450 levels and 5′-nucleotidase activity. The spontaneous increase in alkaline phosphatase activity was reduced and the induction of tyrosine aminotransferase was inhibited after 3 d in culture. The fetal liver characteristic, gamma glutamyltranspeptidase, was not affected by butyrate treatment. Results of this study suggest that butyrate represents a nontoxic compound capable of improving the maintenance of cell culture characteristics of adult rat hepatocytes.

Key words: cultured hepatocytes; butyrate; P-450; differentiated functions.

INTRODUCTION

Sodium butyrate, a 4-carbon fatty acid, produces a wide variety of effects on cells in culture (21,31,32). The effect of sodium butyrate on mammalian cells in culture markedly varies depending on the cell type investigated (31,32). The most consistent effect of butyrate on cultured cells is the differentiation of transformed cells in culture into more mature or "normal" cells after butyrate treatment (21,31,32). Most studies on the effect of butyrate on cultured cells have utilized malignant cell lines or embryonic cells in culture (25). Studies on the effects of sodium butyrate on nontransformed cells have been limited to fibroblasts and lymphocytes. Primary cultures of adult rat hepatocytes represent a normal, differentiated cell type that resembles, in many respects, the in vivo metabolic and morphologic characteristics of liver parenchymal cells (15,18,27,30). However, some characteristics of cultured liver parenchymal cells (hepatocytes) begin to deviate from what is observed in vivo. Fetal isozymes become apparent under the appropriate culture conditions (35). Inasmuch as the treatment of some transformed cells with butyrate results in the differentiation of several normal cellular characteristics, we were interested in determining if butyrate treatment would preserve more of the in vivo-like characteristics of cultured hepatocytes.

MATERIALS AND METHODS

Hepatocyte isolation and culture. Hepatocytes were isolated from adult (10 to 12 mo. of age) female F-344 (Harla Sprague Dawley, Inc., Madison, WI) rats by collagenase perfusion and cultured on collagen coated tissue culture dishes as previously described (9,12). All incubations were performed at 35° C in a humidified atmosphere of air.

Enzymatic analysis and protein and DNA determinations. After a 4-h attachment period, attached hepatocytes were washed once, fresh medium was added, and cultured continuously with 5 m*M* sodium butyrate (J. T. Baker Chemical Co., Phillipsburg, NJ). Both culture medium and sodium butyrate were added fresh each day. The hepatocytes were removed from culture at the times indicated, homogenized, sampled for DNA content (23), and a sample removed for enzyme analysis. Freshly isolated hepatocytes (representing the zero-hour values) were processed similarly. The following enzyme assays were performed on the cultured hepatocyte homogenate:gamma glutamyltranspeptidase (GGT), alanine aminotransferase (ALT), alkaline phosphatase (AP), lactate dehydrogenase (LDH), and tyrosine aminotransferase (TAT) as previously described (11,12). In addition, 5′-nucleotidase (5′-ND) was assayed according to the method of Arkesteijn (2). The remaining homogenate was

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TABLE 1

EFFECT OF BUTYRATE ON HISTONE H4 ACETYLATION BY CULTURED RAT HEPATOCYTES[a]

Butyrate Concentration (m*M*)	Percent Histone H4 Acetylation			
	Parental	Mono	Di-	Tri-
0	50.3 ± 0.3	35.7 ± 0.9	8.7 ± 0.3	5.3 ± 0.3
5	32.7 ± 2.2[b]	39.3 ± 3.0	17.7 ± 1.7[b]	10.0 ± 2.1[b]
10	31.6 ± 1.5[b]	39.8 ± 1.7	17.4 ± 1.0[b]	10.8 ± 1.4[b]

[a]Data represent the mean ± SEM obtained from three to five animals cultured for 24 h in the presence or absence of butyrate. Parental: Nonacetylated H4; mono: mono-acetylated H4; di: Di-acetylated H4; tri: Tri-acetylated H4.
[b]$P < 0.05$ compared to untreated cultures.

used for microsomal isolation (43) and determination of tryptophan pyrrolase (TP) enzyme activity (45). The extinction coefficients of 91 mM^{-1} and 185 mM^{-1} were used for the quantification of cytochrome P-450 and cytochrome b_5, respectively (28). Protein determinations were performed by the method of Bradford (4). One unit of enzyme activity equals that amount of enzyme catalyzing the formation of a product or a decrease in a substrate of 1 μmol/min at the defined conditions for each assay. All statistical analyses were performed using a one-way ANOVA (36).

Nuclei isolation, histone extraction, and electrophoretic separation. Nuclei were prepared from the cultured hepatocytes after 24-h incubation in the presence or absence of butyrate by the method described by Sealey and Chalkley (34). Histones were extracted from the isolated nuclei with 0.25 *N* HCl at 4° C for 3 h, with mixing every 30 min. The nuclei were collected by centrifugation (5000 × *g* for 10 min), supernatant carefully removed, and histones precipitated by the addition of 10 vol of acetone. The mixture was maintained at −20° C until analyzed. Histones were separated by electrophoresis according to the method of Alfageme et al. (1), stained with Coomassie blue, and scanned at 550 nm in a Beckman DU-8 spectrophotometer.

RESULTS

To determine the optimum concentration of butyrate that results in a readily detected effect, the viability of cultured hepatocytes to butyrate treatment was examined. It was determined that butyrate concentrations exceeding 20 m*M* resulted in cellular detachment and corresponding vital dye uptake (data not shown). A unique biochemical characteristic of butyrate treatment, histone hyperacetylation, was also analyzed as a criterion of response to various concentrations of butyrate (Table 1). When cultured hepatocytes were treated with either 5 or 10 m*M* butyrate, a significant increase ($P < 0.05$) in the percentage of di- and triacetylated histone H4 was detected. Histone H4 acetylation was analyzed because it is well resolved from the other histones electrophoretically. Because similar acetylation profiles were observed with either 5 or 10 m*M* butyrate, 5 m*M* butyrate was used for all further studies. No attempt was made to utilize any lower concentrations of butyrate in this study.

TABLE 2

EFFECT OF SODIUM BUTYRATE ON CELLULAR ENZYME LEVELS[a]

Culture Time (h)	Culture Medium Additions	Cellular Enzyme Activities (mU/mg protein)		
		LDH[b]	ALT[c]	GGTP[d]
0	—	3142 ± 217	177.60 ± 15.4	2.73 ± 0.33
24	Control	3174 ± 114	107.10 ± 20.6[e]	1.92 ± 0.27
24	5 m*M* Butyrate	3172 ± 137	109.30 ± 21.6[e]	2.13 ± 0.51
48	Control	3919 ± 214[f]	92.77 ± 14.9[e]	2.15 ± 0.25
48	5 m*M* Butyrate	3824 ± 227[f]	90.77 ± 12.2[e]	1.76 ± 0.18
72	Control	3767 ± 168[f]	78.84 ± 2.7[e]	2.39 ± 0.24
72	5 m*M* Butyrate	4035 ± 340[f]	75.24 ± 9.5[e]	2.25 ± 0.20

[a]Data represent the mean ± SEM obtained from four to nine animals.
[b]Lactate dehydrogenase.
[c]Alanine aminotransferase.
[d]Gamma glutamyl transpeptidase.
[e]$P < 0.001$ compared to zero-hour value.
[f]$P < 0.05$ compared to zero-hour value.

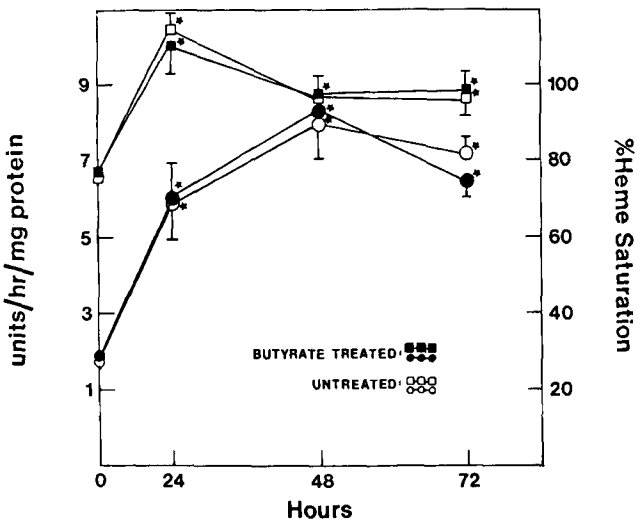

FIG. 1. Effect of 5 m*M* butyrate treatment on tryptophane pyrrolase activity (□,■) and corresponding percentage of heme saturation of tryptophane pyrrolase (○,●). Data represent the mean ± SEM from three to five cultures. Significantly different from zero-hour value, **P* <0.05.

A variety of cellular enzymes have been utilized as markers of normal, malignant, and fetal properties of adult rat hepatocytes in culture (15,18,30). In this regard, we have examined several "housekeeping" enzymes (LDH, ALT, 5'-ND, AP), two hormonally responsive enzymes (TAT, TP), and one fetal enzyme often associated with cultured hepatocytes (GGT) as to their response to butyrate treatment during hepatocyte cell culture.

The effect of 5 m*M* sodium butyrate treatment on three cellular enzymes is shown in Table 2. In response to the high pyruvate concentration of the culture medium (20 m*M*) (9,33), LDH activity showed a significant increase (*P* <0.05) in activity after 48 h of incubation in the presence or absence of butyrate. The specific activity of ALT declined significantly (*P* <0.001) during culture, with or without butyrate. The enzyme GGT, which in normal liver is expressed at highest levels in the fetus (39), demonstrated no significant changes in activity during the culture period, and butyrate treatment did not induce the expression of this fetal characteristic when hepatocytes were cultured on collagen-coated plastic.

The effect of butyrate treatment on TP induction and heme saturation is shown in Fig. 1. In response to the dexamethasone in the culture medium (10^{-7} *M*), TP activity showed a significant increase (*P* <0.05) in both enzyme-specific activity and the percentage of heme saturation. Butyrate treatment had no effect on TP induction or heme saturation.

When hepatocytes are cultured on collagen-coated plastic, significant increases in AP activity have been observed (11,12,18,29). When hepatocytes were cultured in the presence of 5 m*M* butyrate, a significant reduction (*P* <0.05) in the increase in AP activity was observed throughout the culture period (Fig. 2). The effect of butyrate treatment on the dexamethasone induction of TAT activity is shown in Fig. 3. When hepatocytes were treated with 5 m*M* butyrate for 3 d, a significant decrease (*P* <0.01) in TAT-specific activity was observed on the 3rd d when compared to the untreated cultures.

The cellular enzyme 5'-ND has been shown to decline in activity during the first several days of hepatocyte culture and then increase in activity after 5 d in culture (18). When hepatocytes were treated with 5 m*M* butyrate, no significant decline in activity below the zero-hour value was observed. The specific activity of 5'-ND was significantly higher (*P* <0.05) in the butyrate treated cultures throughout the culture period (Fig. 4).

The effect of butyrate treatment on the P-450 content of cultured hepatocytes is shown in Fig. 5. A significant decline in P-450 content (*P* <0.05) below the zero-hour value occurred in both the butyrate-treated and untreated hepatocyte cultures. However, after 72 h of butyrate treatment, the P-450 concentration was significantly higher (*P* <0.05) than the level detected in the untreated cultures. The effect of butyrate treatment on b_5 content was also examined (Fig. 6). A significant decline in b_5 content (*P* <0.05) below the zero-hour value also occurred in both butyrate-treated and untreated hepatocyte cultures. The significant decline in b_5 content was delayed for 24 h by butyrate treatment although the differences between butyrate-treated and untreated cultures failed to achieve statistical significance (*P* >0.05).

DISCUSSION

The pleiotrophic effects of butyrate on many cell types is well documented (21,31,32). The effect of butyrate on primary cultures of adult rat hepatocytes has not been reported. Primary cultures of adult rat hepatocytes have

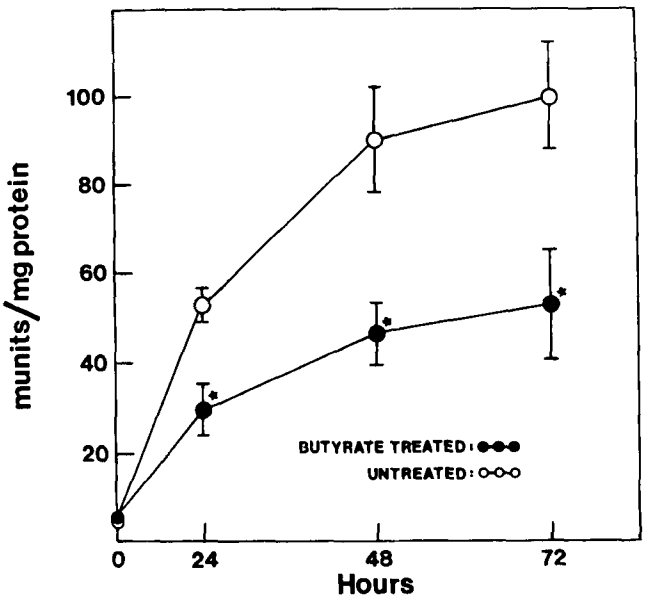

FIG. 2. Effect of 5 m*M* butyrate treatment on alkaline phosphatase activity in primary rat hepatocyte cultures. Data represent the mean ± SEM from three to five cultures. Significantly different from untreated cultures, **P* <0.05.

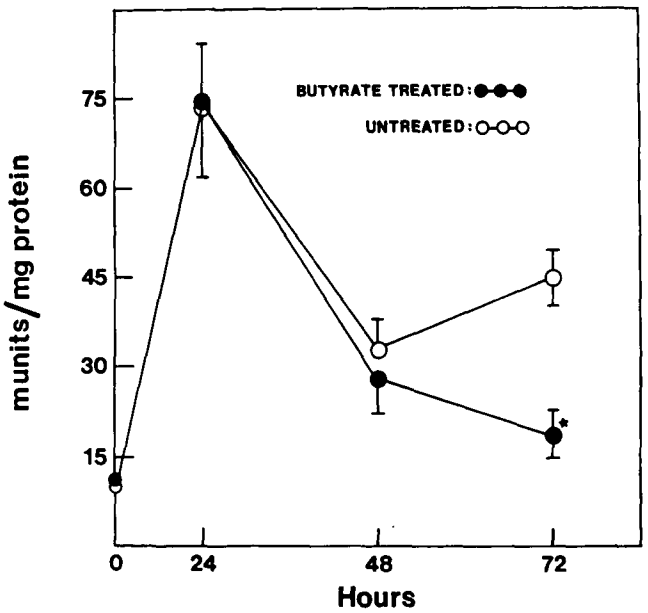

FIG. 3. Effect of 5 m*M* butyrate treatment on tyrosine aminotransferase activity in primary rat hepatocyte cultures. Data represent the mean ± SEM from three to five cultures. Significantly different from untreated cultures, $*P<0.05$.

been utilized as an in vitro biochemical and metabolic system that retains several in vivo liver characteristics (15,18,27,30). Although significant male-female variations exist in the specific activities of many rodent enzyme systems (42), the rate of change in vitro of several biotransformation enzymes of hepatocytes cultured from male or female rats has been reported to be generally similar (8). Therefore, the use of hepatocytes isolated from female rats in this study does not preclude generalizations to other studies utilizing male animals.

The extent of butyrate-induced acetylation varies according to the cell type studied (5). We measured the extent of histone H4 hyperacetylation (Table 1) after hepatocytes were cultured in the presence of 5 or 10 m*M* butyrate for 24 h. It is interesting to note that although butyrate treatment increased histone H4 acetylation, no tetraacetylated H4 histone species were detected. This contrasts with what has been observed in transformed cell types. For example, treatment of Chinese hamster ovary cells with 7 m*M* butyrate resulted in 14% of the histone H4 being tetraacetylated (3). It is possible that butyrate treatment of primary cultures of adult rat hepatocytes results in less histone acetylation because of the postmitotic characteristic of mature hepatocytes. When compared to other cell types treated with butyrate, the similar increase in histone H4 acetylation observed after 5 or 10 m*M* butyrate treatment in the cultured hepatocytes indicates that butyrate metabolism was not responsible for the decreased level of histone H4 acetylation detected.

As shown in Table 1, several enzyme systems of cultured hepatocytes adapt to the in vitro environment in different ways. The expression of these enzyme activities were unaffected by butyrate treatment and butyrate did not increase the expression of the fetal enzyme GGT when hepatocytes were cultured on collagen-coated plastic dishes. In contrast to transformed cell lines, LDH activity was not inhibited by butyrate treatment. This may be because primary cultures of rat hepatocytes utilize primarily oxidative metabolic processes whereas transformed cells utilize less oxidative dependent metabolic processes. Butyrate-induced cell lethality and growth inhibition of mouse neuroblastoma cells have been reported to be associated with an inhibition of anaerobic respiration via LDH (32). Because high concentrations of pyruvate have been shown to be necessary to satisfy the energy requirements of cultured hepatocytes (33), oxidative consumption of pyruvate by hepatocyte cultures remains unaffected by butyrate treatment. Butyrate treatment of cultured male rat hepatocytes that characteristically exhibit increased activity of the fetal enzyme GGT (30,35) has also been shown to inhibit the "fetalization" process and thereby maintain more normal cellular characteristics (10) and unpublished data. The effect of butyrate on GGT expression in hepatocytes cultured on collagen-coated nylon mesh is dependent on both dosage and continuous exposure to butyrate (Personal communication, Staecker and Pitot).

Butyrate has been shown to be a potent inducer of differentiation, enzyme activity, and gene expression (21,31,32). When hormonal induction of several hepatocyte enzyme systems was examined, butyrate either inhibited the increase in enzyme activity or had no effect. Figure 1 shows that the induction of TP activity was unaffected by butyrate treatment. Similar increases in the specific activity of TP in untreated hepatocyte cultures have also been reported (11,12,18).

Alkaline phosphatase has been shown to be induced by butyrate treatment in several cell lines (20,26). We have

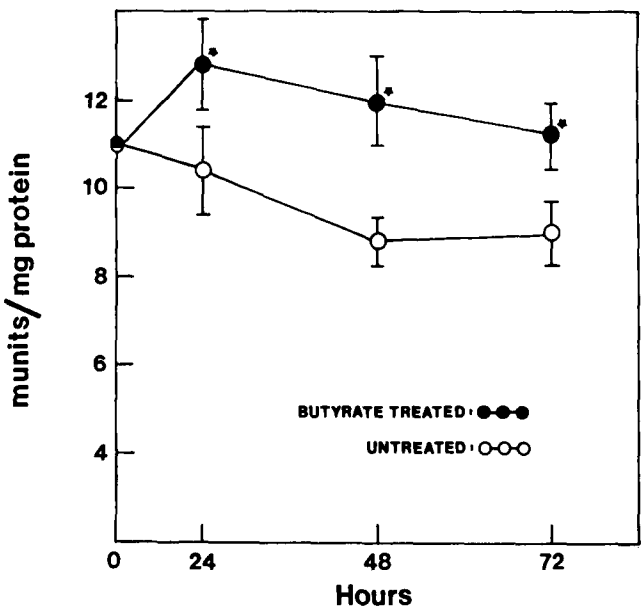

FIG. 4. Effect of 5 m*M* butyrate treatment on 5'-nucleotidase activity in primary rat hepatocyte cultures. Data represent the mean ± SEM from three to five cultures. Significantly different from untreated cultures, $*P<0.05$.

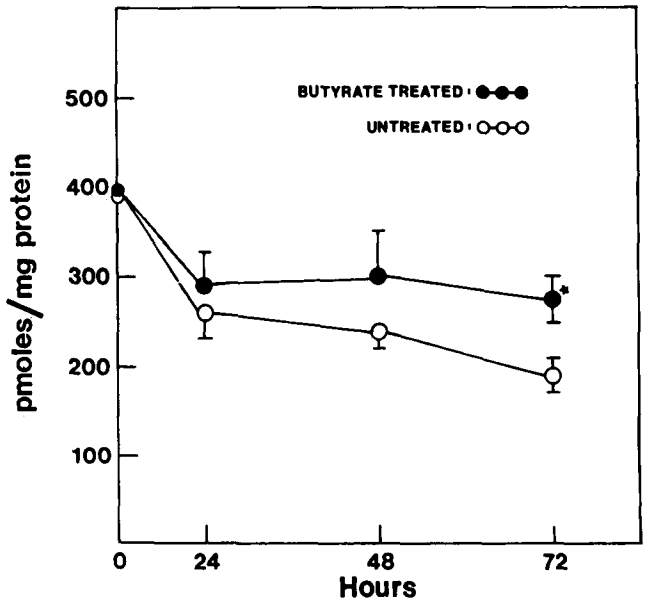

FIG. 5. Effect of 5 m*M* butyrate treatment on microsomal cytochrome P-450 content in primary rat hepatocyte cultures. Data represent the mean ± SEM from three to five cultures. Significantly different from untreated cultures $*P<0.05$.

previously shown that AP activity increased significantly in primary hepatocyte cultures due to hormonal stimulation and hyperosmolarity of the culture media (400 mOSM, 9,33) (11,12). Figure 2 shows that butyrate treatment of cultured hepatocytes reduces the increase in AP specific activity. This effect was seen by 24 h of treatment. Tsao et al. (41) have reported that both dimethyl sulfoxide (DMSO) and retinoic acid, additional differentiation agents, also decrease AP activity in HRT-18 adenocarcinoma cells. In contrast to the rapid inhibition of AP activity, the effect of butyrate on the induction of TAT activity required 72 h of treatment (Fig. 3). Tichonicky et al. (40) have reported that butyrate inhibited the induction of TAT in HTC cells after only several hours of treatment. Chalkley and Shires (7) have recently described an HTC variant that replicates in the presence of 6 m*M* butyrate yet TAT induction remains sensitive to butyrate inhibition. We have found that the inhibition of TAT induction in primary hepatocyte cultures requires 48 to 72 h of treatment and may represent fundamental differences in the two cell types. As shown for HTC cells (7,40), primary cultures of rat hepatocytes also maintain basal (zero-hour) TAT levels in the presence of butyrate. Weingarten et al. (44) have also reported that butyrate completely inhibits the glucocorticoid increase in glycerol phosphate dehydrogenase activity while having no effect on the induction of glutamine synthase in C6 cells. They reported that butyrate itself induces glutamine synthase and does not enhance the glucocorticoid induction of this enzyme.

The cell membrane enzyme 5′-ND has been shown to decline in activity during the first several days of hepatocyte culturing (18). We have shown that butyrate treatment eliminated the decline in 5′-ND activity (Fig. 4). The specific activity of 5′-ND was significantly greater in the butyrate-treated cultures throughout the culture period examined. Ichihara et al. (18) have reported that 5′-ND activity increases above the zero-hour value after 5 d in culture. We have observed similar findings when examining untreated cultures (data not shown). The stabilization of 5′-ND activity may represent a modification of the cell membrane of the butyrate-treated hepatocyte. A similar enhancement of membrane beta-adrenergic receptor activity has been reported in both HeLa cells (37) and fetal rat hepatocytes (24) after butyrate treatment. Butyrate treatment also enhances the formation of the external cytoskeletal fibronectin matrix on the cell surface (17,38).

A major differentiated function of the liver in vivo is drug metabolism. This facet of liver physiology is not easily maintained in most hepatocyte culture systems (15,30). For example, a 70% decrease in P-450, the major component of the drug metabolizing system in the rodent hepatocyte, is often observed within 24 h of culturing (16). Although the microsomal protein P-450 represents the major component of the mixed-function-oxidase system (MFO), membrane lipid modifications can also influence P-450 structure and function (14). When hepatocytes are cultured in the presence of various hormones and the heme precursor delta aminolevulinic acid, the P-450 content can be maintained above 50% of the zero-hour level during the first 72 h of incubation (Fig. 5), (11,12). When cultured hepatocytes were additionally treated with butyrate, the concentration of P-450 was higher at all time periods and was significantly higher ($P<0.05$) after 72 h of incubation. Leshin (22) recently reported the induced expression of a P-450 enzyme (aromatase) in 5-azacytidine- and butyrate-treated fibroblasts. Additionally, Isom et al. (19) have reported

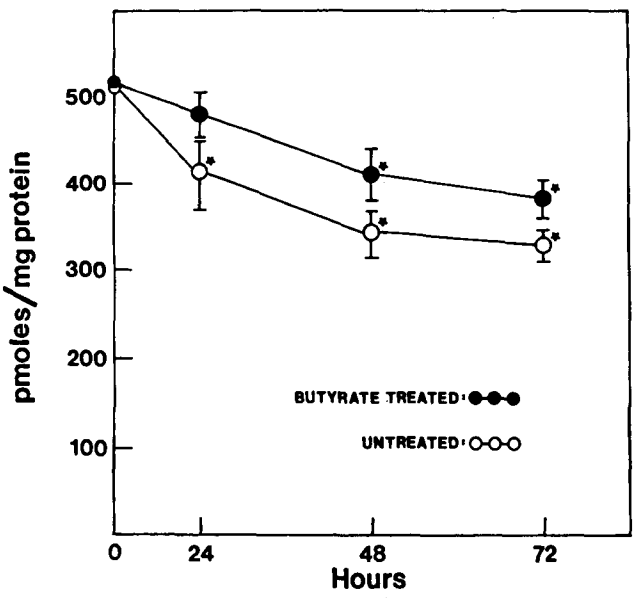

FIG. 6. Effect of 5 m*M* butyrate treatment on microsomal cytochrome b_5 content in primary rat hepatocyte cultures. Data represent the mean ± SEM from three to five cultures. Significantly different from zero-hour value $*P<0.05$.

that DMSO, a differentiation agent, improves the stability and maintenance of differentiated functions of hepatocytes in primary culture. Butyrate treatment also retarded the decline in b_5 content that was observed in untreated cultures (Fig. 6).

Because the main targets of butyrate treatment are cellular membranes and cytoskeleton (21), the butyrate-mediated enhancement of microsomal cytochrome content may represent modifications of these cellular components. Capuzzi et al. (6) have reported that butyrate is actively incorporated into free cholesterol and phospholipids of rat hepatocytes. At least half of the butyrate incorporated into lipids was found in the unesterified cholesterol fraction; about one fourth was identified in phospholipids and one eighth in triglycerides. Fiszman et al. (13) have also suggested that butyrate-mediated membrane modifications may enhance the normal program of biochemical differentiation of myoblasts. Because the lipid component of the MFO is required for both metabolic activity and P-450 stability (14), membrane lipid modifications may be related to the increased microsomal cytochrome levels of butyrate-treated cultured hepatocytes.

Treatment of liver parenchymal cells with 5 m*M* butyrate modified the appearance of atypical characteristics of hepatocytes cultured for brief periods of time. Butyrate treatment also modified the culture-related adaptive changes in several biochemical characteristics. In this regard, primary hepatocyte cell culture are similar to many transformed cell lines in that butyrate treatment resulted in the expression of more normal cellular characteristics.

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