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STIMULATION OF MINOR FETAL HEMOGLOBIN SYNTHESIS IN CORD BLOOD RETICULOCYTES BY BUTYRATE[1]

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Summary: As it had been shown that sodium butyrate promoted hyperacetylation of nuclear histones the effect of 5, 10, and 50 mM sodium butyrate on the in vitro synthesis of Hb F_{Ic} (an acetylated component of Hb F) and Hb F in cord blood reticulocytes was studied. The presence of 5 mM butyrate showed a significant increase in the production of Hb F_{Ic}, whereas Hb F synthesis was unaffected. Higher concentrations of butyrate, however, had no stimulatory effect on Hb F_{Ic} synthesis. Results of pulse-chase studies indicated that sodium butyrate inhibited the turnover of preformed Hb F_{Ic}, probably by inhibiting deacetylase.

In addition to the major fetal hemoglobin component (Hb F_o), minor components, namely Hb F_{Ia}, Hb F_{Ib}, and Hb F_{Ic} are present in the red cell lysates of newborn infants (1-4). Hb F_{Ia} is formed by the reaction of Hb F with phosphorylated glycolytic intermediates (3,4). Hb F_{Ib} is a glycosylated minor component, however, the mechanism of its formation is not known (4). On the other hand, Hb F_{Ic} contains γ chains mostly acetylated at the amino terminal (1-4). A previous report has supported the concept that the synthesis of Hb F_{Ic} is dependent on Hb F synthesis and the acetylation of γ chains may occur at an early stage in Hb F synthesis, while nascent γ chains are ribosome bound (3).
Using transformed cell cultures several investigators have shown that sodium butyrate inhibits nuclear histone deacetylation resulting in hyperacetylation of histones in HeLa cells, Friend erythroleukemia cells, and in hepatoma cell cultures (5-7). In the present communication we report that in the presence of 5 mM sodium butyrate the in vitro synthesis of Hb F_{Ic} is increased in the reticulocytes from cord blood and the turnover of preformed Hb F_{Ic} is inhibited.

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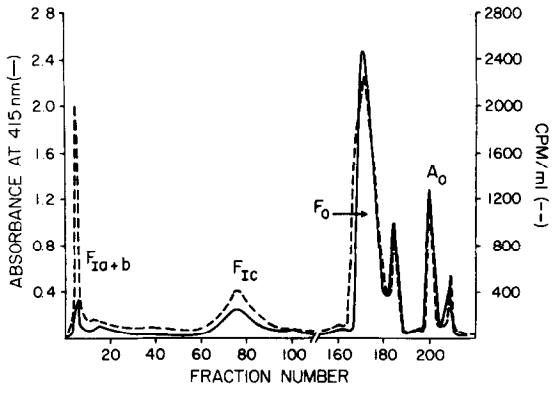
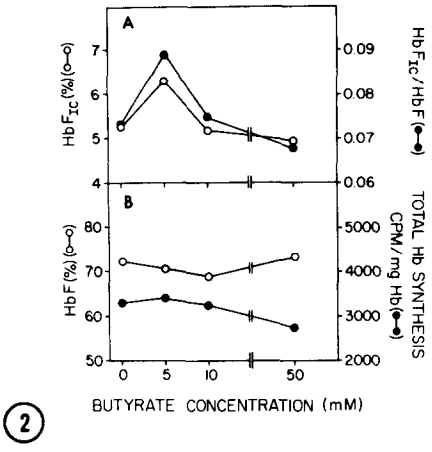


Figure 1. Biorex 70 chromatographic separation of hemoglobins present in a red cell lysate after incubation of cord blood reticulocytes in a medium supporting hemoglobin synthesis and containing [^{14}C]leucine. See text for method.

MATERIALS AND METHODS

In vitro Hemoglobin Synthesis: Umbilical cord blood samples (7-10 mls) were collected in vacutainer tubes containing EDTA as anticoagulant. The cell fractions enriched for reticulocytes by standard methods (8) were washed three times with reticulocyte saline and filtered through 2 layers of 3-ply cheesecloth to remove white cells. The washed cells were incubated at 37°C for one to three hours in an amino acid mixture containing [^{14}C]leucine according to the procedure described elsewhere (8). The incubation mixture in a total volume of 1.4 ml contained 0.22 ml cells, 0.95 ml amino acid mixture, 1.6 mg glucose, 0.06 ml transferin solution, and 0.02 ml [^{14}C]leucine (2 µci of 300 µci/µmol from Amersham). To study the effect of butyrate on the synthesis of various Hb components, sodium butyrate dissolved in reticulocyte saline was added to the incubation medium to give a final concentration of 5, 10, and 50 mM. In one experiment, the cells were preincubated for 30 minutes with 5 mM sodium butyrate, washed thoroughly with reticulocyte saline, and then the protein synthesis was initiated immediately thereafter. To study the effect of butyrate on the turnover of Hb F_{Ic}, reticulocytes were incubated with [^{14}C]leucine for 1 hour with sodium butyrate (5 mM), washed and resuspended in a medium in which [^{14}C]leucine was replaced with excess unlabeled leucine, and incubated for additional 2 hours in the presence or absence of sodium butyrate (5 mM). The rate of protein (hemoglobin) synthesis was evaluated by determining the acid-precipitable radioactivity. An aliquot containing about 0.5 mg Hb was precipitated with cold 10% trichloroacetic acid on a microfiber disc (2.4 cm, GF/A, from Whatman Inc.), washed with cold acid-acetone, boiling 5% trichloroacetic acid, cold 5% trichloroacetic acid, and finally with 95% ethanol. The discs were then dried at 70°C and counted for radioactivity. At the end of the incubation, hemolysates were prepared from washed cells by freezing - thawing and stroma removed by centrifugation at 20,000 x g for 30 minutes. The red cell lysates were dialyzed against the appropriate developer for Biorex 70 chromatographic separation of hemoglobins.

Biorex 70 Chromatographic Separation of Hemoglobins: Red cell lysates were chromatographed on 1 x 25 cm Biorex 70 columns using a steadily increasing pH gradient according to the procedure described elsewhere (3). About 25 mg of hemoglobin were applied to a column and developed at a constant flow rate of



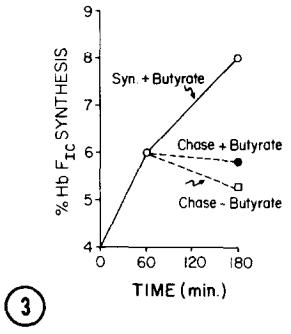


Figure 2. The sodium butyrate concentration dependence of in vitro Hb F_{Ic} synthesis in cord blood reticulocytes. (A) The percentage of Hb F_{Ic} synthesis and Hb F_{Ic}/Hb F synthesis ratios and (B) the percentages of total Hb F (Hb F_o + Hb F_{Ic}) synthesis and the total hemoglobin synthesis expressed as cpm of [^{14}C]leucine incorporated per mg of total hemoglobin present in the lysate.

Figure 3. The percentage synthesis (% radioactivity) of Hb F_{Ic} that was synthesized by reticulocytes from a cord blood sample in the presence of 5 mM sodium butyrate (O), and during a period of pulse-chase analysis in the presence (●) or absence (□) of 5 mM sodium butyrate.

12-15 ml/h. Twenty-minute fractions were collected and the absorbances of the fractions read at 415 nm. The radioactivity of 1 ml of each effluent fraction was determined after heating with 0.4 ml of 30% hydrogen peroxide and 0.2 ml of 70% perchloric acid for 1 hour at 70°C. The percentage synthesis of the various Hb components was calculated based on the total radioactivity of each Hb component. An example of the chromatographic separations is shown in Fig. 1.

RESULTS

Figure 2 shows the dependence of the synthesis of Hb F_{Ic}, Hb F (Hb F_{Ic} + Hb F_o), and total hemoglobin on the butyrate concentration. The percentage synthesis of Hb F_{Ic} and the Hb F_{Ic}/Hb F synthesis ratios showed a significant increase with 5 mM sodium butyrate. With 10 and 50 mM concentration either no effect or a moderate inhibition of the Hb F_{Ic} synthesis was observed. Hb F synthesis, on the other hand, was unaffected. The total hemoglobin synthesis (expressed as cpm of [^{14}C]leucine/mg Hb) remained the same between 0 and 10 mM butyrate and decreased about 20% with 50 mM butyrate.

Table I. Effect of Sodium Butyrate on the Synthesis of Hb F_{Ic} in Reticulocytes from Five Cord Blood Samples

Cord Blood	Treatment	Percentage Synthesis		Synthesis Ratio
		Hb F_{Ic}	Hb F_o	Hb F_{Ic}/Hb F
*1	none	2.6	51.4	0.048
	5 mM Butyrate	4.2	52.2	0.075
*2	none	5.3	67.0	0.073
	5 mM Butyrate	6.3	64.5	0.089
*3	none	3.6	72.5	0.047
	5 mM Butyrate	5.3	72.2	0.067
**4	none (1h)	1.7	51.3	0.032
	5 mM Butyrate (1h)	6.0	50.0	0.107
	none (3h)	2.3	54.7	0.040
	5 mM Butyrate (3h)	8.0	51.9	0.134
***5	none	4.2	51.5	0.075
	5 mM Butyrate pretreatment	4.5	51.4	0.081

* Incubations were done for 2 hours
** Incubations were done for 1 and 3 hours
*** Cells were pretreated with or without 5 mM butyrate for 30 minutes and incubated for an additional 2 hours after butyrate was washed out.

The synthesis of Hb F_{Ic} and Hb F was studied in five cord blood samples in the presence and absence of 5 mM sodium butyrate. The results are summarized in Tabel I. In the presence of 5 mM sodium butyrate the percentage synthesis of Hb F_{Ic} and the Hb F_{Ic}/Hb F synthesis ratios increased between 20 and 250% compared to the untreated samples. In one sample (cord blood #5), where the cells were pretreated with butyrate and subsequently butyrate washed off, no increase in Hb F_{Ic} synthesis was observed.

The results of pulse-chase studies are shown in Fig. 3. After a 1 hour pulse with [^{14}C]leucine, during a two hour chase period, the percentage of the preformed Hb F_{Ic} decreased faster in the absence of butyrate than in the presence of butyrate. Thus, butyrate apparently protected Hb F_{Ic} from degradation.

DISCUSSION

The presence of low levels of sodium butyrate enhanced the production of Hb F_{Ic} in cord blood reticulocytes. A similar effect of butyrate on histone H_3 and

H_4 acetylation in HeLa cells, Friend erythroleukemia cells, and in hepatoma cell cultures has been reported before and the maximum effect was observed with 5 to 7 mM sodium butyrate (5-7). The approach used in these studies was to determine the turnover of [^{14}C]acetate rather than to chromatographically separate the acetylated and nonacetylated proteins. The effect of butyrate seems to be reversible because pretreatment with sodium butyrate and subsequent removal of it from the incubation medium has no effect on Hb F_{Ic} synthesis. It has been shown that in HeLa cells sodium butyrate acts through suppression of histone deacetylation by inhibiting the enzyme deacetylase (6). Such an enzyme may be associated with the ribosomes to balance the acetylation of the histone and the nonhistone proteins by acetyltransferase (9). The results of pulse-chase studies show that, indeed, the presence of sodium butyrate protects the disappearance of Hb F_{Ic}, probably by inhibiting deacetylase. Thus, butyrate has an inhibitory effect on the turnover of previously formed Hb F_{Ic}.

It is apparent that sodium butyrate above 5 mM concentration has no stimulatory effect on the production of Hb F_{Ic}. At higher concentrations, it is possible that the metabolites of butyrate, such as propionate, accumulate and inhibit acetyltransferase. Evidence has been presented in reports from other laboratories to show that butyrate promotes increased formation of ε-N-acetyl-lysine rather than ε-N-butyryl-lysine (5). Moreover, if butyryl groups, rather than acetyl groups, were utilized for modification of the amino acid residues, a large increase in Hb F_{Ic} would have been observed with higher concentration of butyrate.

The substrate specificity of the acetyltransferase and the deacetylase is not known, however, it should be pointed that the acetylation of ribosomal proteins (9), including that of fetal hemoglobin (1-4), is mainly amino terminal. The balance between acetylation and deacetylation is subject to physiological control and several factors may be involved in the regulation of acetyltransferase - deacetylase system. It is suggested from these studies that sodium

butyrate shifts this balance towards acetylation, most probably by inhibiting deacetylase.

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