CHAPTER 3

Sodium Butyrate Induced Differentiation of SW620 Human Malignant Colorectal Cells

3.1 INTRODUCTION ........................................................................................................ 67

3.2 RESULTS ..................................................................................................................... 70

3.2.1 Effect of NaBT on growth characteristics of SW620 cells .................................... 70

3.2.1.1 Growth rate ........................................................................................................ 70

3.2.1.2 Cell cycle phases ............................................................................................... 74

3.2.1.3 Cell morphology ............................................................................................... 75

3.2.1.4 Viability and adherence ..................................................................................... 78

3.2.2 Effect of NaBT on marker enzyme activities ......................................................... 79

3.2.3 Effects of NaBT not necessarily related to cellular differentiation ................. 82

3.2.3.1 pH of cell culture medium ............................................................................... 82

3.2.3.2 Glucose consumption ....................................................................................... 83

3.3 DISCUSSION ............................................................................................................... 85

3.4 CONCLUSION ............................................................................................................. 93
3.1 INTRODUCTION

Butyrate, a four carbon chain fatty acid, is one of the main volatile fatty acids formed by microbial fermentation of dietary fibre carbohydrates in the colon, specifically in the caecum. Together with acetate and propionate, butyrate plays an important role in maintaining an acidic intracolonic pH, and in the regulation of the absorption of water and sodium. Further, butyrate, rather than glucose is the preferred energy source of colon cells (Roediger, 1982; Rodriguez et al., 1995).

In vitro, butyrate is a known anti-neoplastic agent. The effects of butyrate on transformed cultured cells include:

- increased cellular duplication time by arresting cells in the G1 phase (Boffa et al., 1981; Kruh et al., 1992)
- increased activity of plasma membrane-associated enzymes which are specific to brush border (Pinto et al., 1982)
- stimulated antiviral and cell growth inhibition properties of interferon (Rodriguez et al. 1995; Goto et al., 1996)
- modified cell morphology via alterations in elements of the cytoskeleton [induction of vimentin and cytokeratin, formation of microfilaments, microtubules and actin filaments (Miyashita et al., 1994)], formation of chromatin, the nucleoli and Golgi
apparatus (Rodriguez et al. 1995). These changes lead to a reversal of the transformation characteristics of cancerous cells.

- induced expression of carcinoembryonic antigen (CEA) (Tsao et al., 1982).

- inhibition of a nuclear enzyme, histone deacetylase activity (Miyashita et al., 1994), resulting in hyperacetylation of histones and expression of various genes.

- increased expression of c-fos and inhibits c-myc oncogenes in all phases of the cell cycle (Miyashita et al. 1994).

- changes in external matrix and cell surface glycoproteins and gangliosides consistent with the loss of cell transformation characteristics (Miyashita et al. 1994).

In human colonic tumor cell lines, including SW620, sodium butyrate has been reported to induce apoptosis (Hague et al., 1993, Heerdt et al., 1994). However, it has also been postulated (Paraskeva et al., 1990) that NaBT acts as a tumor promoter of colorectal carcinogenesis in vivo: under physiological concentrations (about 28 mM NaBT in the faecal water according to Cummings, 1981) NaBT may select for epithelial cells with a reduced response to terminal differentiation signals.

The in vitro differentiation-inducing capacity of sodium butyrate has been demonstrated (Pinto et al., 1982; Augeron and Laboisse, 1984; Whitehead et al., 1986). Some of the butyrate effects listed above, namely:

- growth delay

- appearance of brush border and activation of specific marker enzymes
- changes in morphology
- increased expression of CEA

are regarded as markers of differentiation in colorectal cells. Moreover, apoptosis (programmed cell death) was also associated with the process of cellular differentiation (Franceschi, 1989).

The objectives of this chapter were to determine:

a) the efficiency of sodium butyrate as a differentiation inducing agent in a poorly differentiated colorectal carcinoma cell line SW620 by examining its effect on:
   ! growth characteristics of cells in the culture
   ! brush-border marker enzyme activities

b) the optimal concentration of NaBT required to induce differentiation (while maintaining cell viability) in SW620 cells.

c) other effects of NaBT-treatment, which being unrelated to induction of differentiation, may help with the interpretation of MRS data.
3.2 RESULTS

3.2.1 Effect of NaBT on growth characteristics of SW620 cells

3.2.1.1 Growth rate

In order to determine optimal concentration of sodium butyrate comparative growth curves for SW620 cells cultured in different concentrations (0-5 mM) of NaBT were undertaken in duplicates by a research assistant and are shown in Figure 3.2.

![Graph showing short-term growth curve of SW620 cells](image)

**FIGURE 3.2.** Short-term growth curve of human malignant colorectal cells (SW620) exposed to sodium butyrate at different concentrations: 0 - 5mM, plotted as number of monolayer-growing cells per cm² of flask area vs. time (in hours). Cells were not passaged during the experiment.

Confluency in control cells was achieved 6-7 days after a 1:32 split with $7.8 \times 10^5$ cells/cm². Because there are no points in the linear log phase for 0-1 mM NaBT it was not possible to calculate doubling times of these cells from short-time growth curves. Culture
of SW620 cells in the presence of 0.5 mM NaBT had no appreciable effect on growth rate. Exposure to 1 mM sodium butyrate initially reduced the growth rate of the cells (about 16% increase in doubling time for the first 3 days), delaying the time point of confluence to 9-10 days. The eventual cell density was the same as for control cells. Growth in 3 mM NaBT further slowed the cell growth. In the plateau phase these cells had a density of $4.9 \times 10^5$ cells/cm$^2$. At no time did the cells reach the same density as confluent control, even though the experiment with 3 mM NaBT was carried out five days longer. The cell growth following addition of 5 mM NaBT was initially even more reduced and stopped completely after 200 hours of culture. The maximal cell density (on day 9) was only 16% of control (post-confluent on that day).

Cells were also cultured in the presence of NaBT for an extended time period (800±30 hours) and their growth is shown in Figure 3.3.

![Graph A](image1.png)  ![Graph B](image2.png)

**FIGURE 3.3.** Long-term growth curves of adhered SW620 cells grown in medium containing **A)** 3 mM and **B)** 5 mM sodium butyrate.
During this time cell division continued when the cells were exposed to 3 mM NaBT, but stopped after only 200 hours when exposed to 5 mM NaBT. The total cell number present at 200 h in cultures exposed to 5 mM NaBT remained steady over a six week period indicating that at this concentration of butyrate:
- either the culture was senescent, or
- cells were proliferating and dying/detaching at the same rate.

Cells treated for 72 hours with 3 and 5 mM NaBT resumed normal growth within 3-4 days after the inducer was removed from the culture.

Doubling times of SW620 cells, calculated only from points in log phases from two separate ‘long term’ growth curves were: control - 31.1±3.2 h; 3 mM NaBT - 53.2±3.7 h (statistically significant difference, p=0.007; n=10 for control, 5 for 3 mM NaBT; unequal variances). Cells treated with 5 mM NaBT did not grow steadily during the time of experiment, but their doubling time increased progressively from 55.3 to 585.5h (average 243.8 ± 234.6 h).

**Contribution of cell death and detachment to the decrease in growth rate.**

Whilst Figures 3.2 and 3.3 only include the number of monolayer cells, both viable and dead, detached cells present in the medium were also counted for each time point. As will be shown in section 3.2.1.4, NaBT affected cell detachment and viability. It could therefore be established whether a decrease in growth rate of a culture was caused by cell death and/or detachment from the monolayer, rather than by the actual decrease in proliferation rate.
In order to evaluate the contribution of death&detachment rate on doubling time of the culture after treatment of cells with butyrate, the proliferation rate independent of cell death and escape from monolayer to suspension phase \( (t_d) \) was calculated from extended-time growth curves data of control SW620 and treated with 3 mM NaBT (see section 2.2.1.1). The method of successive approximations was applied as described in Appendix I. As the \( 0^{th} \) approximation of doubling time, \( t_d^0 \), the value of control (31.144 h) was taken, forming a hypothesis that proliferation rate of NaBT-treated cells and control cells are equal when we neglect higher death rate and increased ratio of ‘non-adherent : adhered’ cells after treatment. Table 3.1. presents doubling times calculated for subsequent successive approximation steps of control SW620 cells and 3 mM NaBT-treated assuming that there is no cell death or detachment from the flask bottom.

<table>
<thead>
<tr>
<th>Doubling Time</th>
<th>Control</th>
<th>NaBT 3 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>( t_d^0 )</td>
<td>31.100 h</td>
<td>31.100 h</td>
</tr>
<tr>
<td>( t_d^1 )</td>
<td>30.869 h</td>
<td>51.872 h</td>
</tr>
<tr>
<td>( t_d^2 )</td>
<td>30.869 h</td>
<td>52.075 h</td>
</tr>
<tr>
<td>( t_d^3 )</td>
<td></td>
<td>52.081 h</td>
</tr>
<tr>
<td>( t_d^4 )</td>
<td></td>
<td>52.082 h</td>
</tr>
<tr>
<td>Real</td>
<td>31.144±3.168 h</td>
<td>53.190±3.664 h</td>
</tr>
</tbody>
</table>

TABLE 3.1. Doubling times calculated for subsequent successive approximation steps of control SW620 cells and 3 mM NaBT-treated based on assumption that all cells proliferate at a constant rate (equal \( t_d^{n-1} \), i.e. doubling time value resulting from each previous step), therefore eliminating effect of mortality and detachment from monolayer.

Real doubling time (for comparison) - calculated from unmodified experimental data (including live as well as dead cells in the monolayer) expressed as mean ± standard deviation.

For control cells already the 2nd approximation of hypothetical time \( t_d \) gave the
exact value of doubling time equal 30.869 h. This is shorter by 1% than the ‘real doubling
time’ for control cells when the total number of cells is included in the growth curve. For cells
treated with 3 mM NaBT, after four approximation steps \( t_d = 52.082 \) h was obtained, which
is shorter by 2.2% than the ‘real doubling time’. These results are in agreement with the fact
that after treatment with butyrate a higher percentage of dead cells and cells in suspension
was found control cells (see section 3.2.1.4).

3.2.1.2 Cell cycle phases

In order to further investigate causes of lower proliferation rate of SW620 cells after
treatment with sodium butyrate, the cell cycle profile was measured as described in section
2.2.1.4, \( n=2 \). Figure 3.4 shows percentage of cell cycle phases in the control cells and 3
mM butyrate treated at 48, 72 and 96 hours of culture. The results show that in the
beginning phase of treatment with 3 mM sodium butyrate (after 48 h) most of cells i.e.
75.6±4.2% (but only 53±10.2% of untreated) were blocked in the \( G_1/G_0 \) phase (Figure
3.4), which is correlated with slowed proliferation.

![Figure 3.4. Percentage of cell cycle phases in SW620 after treatment with 3 mM sodium butyrate; n=2](image-url)
The percentage of cells in the S phase correspondingly decreased. However, after 72 hours they partially recovered from the “stressful effect” of this drug (and presumably resumed proliferation). For cells grown in the presence of sodium butyrate after 72 hours of treatment there was no significant difference in percentage of cells in G₁/G₀ phase between NaBT-treated and control. However, at n=2 it is difficult to estimate other possible variations in the percentage of cell cycle phases.

3.2.1.3 Cell morphology

Morphologically differentiated appearance of cells indicates differentiated state. During culture of cells their growth and morphology in vitro were observed. There were visible differences between cells depending on the concentration of sodium butyrate in their medium. Control SW620 cells (no NaBT) are spherical or bipolar in appearance and do not form a dense monolayer (see Figures 2.1A, 3.5A). Treatment with 0.5 mM resulted in a less dense monolayer than control. The same was recorded at 1 mM NaBT, but still no morphological changes were apparent. In 3 mM NaBT many cells had formed enlarged flattened star-shapes (Figure 3.5B). Extending the culture beyond 72 hours resulted in the majority of cells having long membranous processes though some retained the appearance of untreated SW620 cells. At 5 mM NaBT, similar effects were observed though the changes were more rapid and severe (Fig. 3.5C).

Following treatment with 3 or 5 mM sodium butyrate SW620 malignant colorectal cells underwent changes in morphology. The changes began as early as day 2 and by day 5 they included an increase in cell diameter by 68% of control size (measurement taken after trypsinization, when the cell shape was spherical; increase in cell volume to 474%).
FIGURE 3.5. Sodium butyrate - treated SW620 cells: Contrast phase micrographs: **A**) control; **B**) 3 mM NaBT - treated (72 hours); **C**) 5 mM NaBT - treated (72 hours); **D**) 3 mM NaBT - treated (60 days). Photographed at 500× magnification.
and as a result of changing shape from spherical to star-like appearance cells became more tightly attached to the flask.

After 14 days in 3 mM NaBT visible clumps (or domes) of SW620 cells were apparent, these consisted of spherical cells closely attached forming 3-dimensional structures. Morphologically differentiated cells were still present spread on the flask surface, but grouped in flattened net-like structures rather than uniformly attached across the surface (Figure 3.5D). Since the appearance of morphological changes correlated with an increase in the doubling time of cell cultures, it was expected that the presence of morphologically undifferentiated cells after 14-day treatment will result in faster growth. However, despite of the presence of morphologically undifferentiated cells in dome structures, the doubling time of the culture wasn’t shorter than in early stages of treatment. Cells grown in 5 mM NaBT arrived at the stage of domes even earlier (after 11 days) if initial cell density at the time of beginning of treatment was relatively high $50,000$ cells per cm$^2$.

It was observed that passaging of cells growing in the medium containing NaBT resulted in their expression of more differentiated phenotype. This was most visible when passaging flasks containing dome structures: the previously spherical cells after split assumed separate star-shapes.

*The reversibility of the morphological changes* caused by sodium butyrate was investigated by monitoring growth of cells pretreated with 3 mM NaBT for 72 hours, on the day 2, 3, 6, 10 and 17 after removal of NaBT from the culture medium. The 2-day period
of NaBT-withdrawal was insufficient to show any progress in dedifferentiation characteristics of the cell culture. On the day 3 after media change cells were growing visibly faster than cells still kept in 3 mM NaBT, however their appearance was still fully differentiated. On day 6 the cell density in flasks was visibly higher than in 9 day-NaBT-treated culture, and was almost as high as in flasks containing control medium. The morphology of some cells also resembled untreated SW620, however differentiated cells were also present to a level of about 50%. The complete recovery of cells occurred by the day 10, when the density of monolayer as well as cell morphology were indistinguishable from control cultures (passaged meanwhile in the same way) and cells maintained this state till day 17.

3.2.1.4 Viability and adherence

Apoptotic death of cells was associated with the process of differentiation (Franceschi, 1989). It was observed that sodium butyrate stimulated release of cells from monolayer to the suspension phase to a greater degree than occurred in control cultures. The viability of these detached cells was only 38±17% for 3 mM NaBT, n=12 (control: 66±19%, n=8). The percentage of total adhered and adhered viable cells was measured during cell counting for the growth curves as described in section 2.2.1.1. Growth characteristics, including percentage of adhered cells and % of adhered viable SW620 cells grown in 0-5 mM sodium butyrate after 4, 9 and 15 days of treatment (one day after the last supply of fresh medium) are presented in Table 3.2.

On day 4 after a 1:32 split, cells were subconfluent in their exponential growth phase with 95% of the total cell population firmly attached. The viability of these cells was 89%.
On day 9 control cells were in their plateau phase. The percentage of cells growing in monolayer was still high at 95% and their viability at 94%. In post-confluent cultures (day 15) a higher cell detachment (14% floating cells) and some (but not significant) decrease in viability of adhered cells (to 86%) were observed.

Addition of 0.5 and 1 mM NaBT had no significant effect on cell adherence.

Treatment of cells with 3 mM NaBT slightly reduced cell viability in the cell culture i.e. to 82-86% for adhered cells. The percentage of adhered cells was also slightly lower than control on days 4 and 9.

<table>
<thead>
<tr>
<th>NaBT Treatment</th>
<th>Day 4</th>
<th></th>
<th>Day 9</th>
<th></th>
<th>Day 15</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% adhered</td>
<td>% viable adhered</td>
<td>% adhered</td>
<td>% viable adhered</td>
<td>% adhered</td>
</tr>
<tr>
<td>0 mM</td>
<td>95</td>
<td>89</td>
<td>95</td>
<td>94</td>
<td>86</td>
</tr>
<tr>
<td>3 mM</td>
<td>92</td>
<td>82</td>
<td>87</td>
<td>86</td>
<td>96</td>
</tr>
<tr>
<td>5 mM</td>
<td>90</td>
<td>81</td>
<td>80</td>
<td>78</td>
<td>88</td>
</tr>
</tbody>
</table>

TABLE 3.2. Percentage of adhered (monolayer) SW620 cells and their viability after treatment with 0-5mM NaBT, measured on days 4, 9, 15 of treatment, one day after adding fresh media to cell culture flasks. Data are means of 2 measurements.

Treatment with 5 mM NaBT to SW620 cells initially reduced the adhered cells’ viability to 81% on day 4 and this decreased down to 66% on day 15. The proportion of adhered vs. floating cells was lower than in control and 3 mM NaBT-treated cells on days 4 and 9.
3.2.2 Effect of NaBT on marker enzyme activities

Brush borders are characteristic of differentiated colonocytes, therefore increased activity of their marker enzymes is also indicative of differentiation. Activities of four enzymes specifically associated with brush border, namely alkaline phosphatase (EC 3.1.3.1), amino-oligopeptidase (EC 3.4.11.2) and sucrase (EC 3.2.1.48) were assayed as described in section 2.2.2.

To establish the effect of sodium butyrate on brush-border marker enzymes of SW620 cells the enzyme activities were measured in the homogenates of cells treated with 3 mM and 5 mM NaBT for 72 hours (Table 3.3).

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Treatment</th>
<th>Alkaline phosphatase</th>
<th>Sucrase</th>
<th>Amino-oligopeptidase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>control</td>
<td>3.10 ± 0.17 (n=5)</td>
<td>3.09 ± 0.16 (n=5)</td>
<td>2.51 ± 0.57 (n=6)</td>
</tr>
<tr>
<td></td>
<td>3 mM NaBT</td>
<td>7.19 ± 0.08 (n=5)</td>
<td>18.37 ± 3.98 (n=8)</td>
<td>9.82 ± 1.25 (n=6)</td>
</tr>
<tr>
<td></td>
<td>5 mM NaBT</td>
<td>8.28 ± 0.54 (n=11)</td>
<td>74.32 ± 21.82 (n=6)</td>
<td>16.16 ± 1.21 (n=8)</td>
</tr>
</tbody>
</table>

Table 3.3. Marker enzyme activities in sodium butyrate treated SW620 cells expressed as nanomoles of product formed per minute by milligram of total protein. n - number of experiments; † - statistical significance (p<0.05). Shaded area - change indicative of cellular differentiation.

The activities of all three brush-border marker enzymes (i.e. alkaline phosphatase, amino-oligo peptidase and sucrase) increased significantly in SW620 cells treated with 3
or 5 mM sodium butyrate compared to control cells.

**Alkaline phosphatase** was 2.32 times after 3 mM and 2.67 times higher after 5 mM NaBT treatment. **Amino-oligo peptidase** increased in activity even more: 3.91 and 6.44 times. The highest increase was observed in **sucrase** level: 5.94 and 24.05 times for 3 and 5 mM NaBT respectively.

The degree of increased activity, always higher at the higher concentration of sodium butyrate applied, indicates that the degree of cellular differentiation achieved was higher in the presence of 5 mM compared to 3 mM NaBT.

**Changes in protein concentration.**

Because the previously performed chemical analysis of colorectal cell lines (Mackinnon *et al.*, 1994) showed changes in protein content in cell lines at different levels of cellular differentiation, a possibility exists that protein content is also altered in butyrate-treated cells. Since an increase in enzyme activity per cell, rather than per mg of total protein, is indicative of differentiation, the increase in activity of brush border marker enzymes after NaBT-treatment was investigated in the view of possible changes in total protein content in the cells. Table 3.4 shows total protein content per cell before and after NaBT treatment.
### TABLE 3.4. Total protein concentration (ng/cell) in control cells and treated with 3 mM and 5 mM sodium butyrate. n=3, adherent cells viable+dead.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Protein concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>10.51 ± 0.96 ng/cell</td>
</tr>
<tr>
<td>3 mM NaBT</td>
<td>11.55 ± 0.72 ng/cell</td>
</tr>
<tr>
<td>5 mM NaBT</td>
<td>9.80 ± 0.35 ng/cell</td>
</tr>
</tbody>
</table>

3 mM butyrate treatment resulted in a 9% increase in protein content in SW620 cells, 5 mM NaBT caused only 7% reduction compared to control. These changes were not statistically significant. The increase in marker enzyme activities was high at both NaBT concentrations relative to the detected differences in total protein, therefore they are unlikely to be due directly to the alterations in total protein content in cells.

#### 3.2.3 Effects of NaBT not necessarily related to cellular differentiation

Differentiation as manifested by slower growth and metabolism rate produced changes in the pH and glucose concentration of the medium. These observations reflect changes in cellular differentiation, however are not directly related to it. It must also be considered that they may be a consequence of such parameters of cell culture as buffer capacity, time in the culture, frequency of feeding, or medium formulation (although they were kept comparable in experiments). It is therefore important to distinguish between primary (specific) and secondary (unspecific) symptoms of differentiation.
3.2.3.1 pH of cell culture medium

It has been noticed during cell culture maintenance that medium in the flasks with butyrate-treated cells becomes acidic at a lower rate than in flasks with control cells, as indicated by phenol red, one of the components of DMEM. The pH value of culture medium in the flasks containing cells in their exponential phase of growth was measured. pH was significantly higher in the flasks containing sodium butyrate and the dependence was linear with the concentration of NaBT used (Figure 3.6). The pH was recorded 48-hours after beginning of treatment, as this time point was considered to be most representative of average culture conditions over 3 days.

![Figure 3.6](image)

**FIGURE 3.6.** Extracellular pH changes vs NaBT concentration. Data are expressed as a mean of 8 measurements ± standard deviation

3.2.3.2 Glucose consumption

In addition to pH, the second parameter for evaluating metabolic rate of cells is the intensity of glucose consumption. Since both growth curves and lactate release intensity
indicate that sodium butyrate may slow down the cellular metabolism, the concentration of glucose was measured in the culture medium of SW620 cells after 48 hour-treatment with 3 mM and 5 mM NaBT as described in section 2.2.1.5. Table 3.5 presents final values of glucose concentration in the media containing 0, 3 and 5 mM NaBT.

Sodium butyrate treatment resulted in a slightly higher glucose concentration in the cell culture medium compared to control cells. The initial concentration of glucose in DMEM medium (containing 0, 3 and 5 mM NaBT) was 25 mM, as defined by the supplier. After 48 hours of experiment the glucose content was 15.18 ± 2.31 mM in control cells, 17.49 ± 0.89 mM in 3 mM and 19.37 ± 0.65 mM in 5 mM butyrate. Therefore, the glucose consumption by cells is about 15% and 23% lower after 48 hour-treatment with 3 mM and 5 mM NaBT respectively than in untreated SW620 colorectal carcinoma cells.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Glucose concentration (mM)</th>
<th>0 hours</th>
<th>48 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>25</td>
<td></td>
<td>15.18 ± 2.31</td>
</tr>
<tr>
<td>3 mM NaBT</td>
<td>25</td>
<td></td>
<td>17.49 ± 0.89</td>
</tr>
<tr>
<td>5 mM NaBT</td>
<td>25</td>
<td></td>
<td>19.37 ± 0.65</td>
</tr>
</tbody>
</table>

**TABLE 3.5. Concentration of glucose in the media** collected from cell cultures after 48 hours of treatment with sodium butyrate. Number of samples was n=2 for each treatment.

The glucose consumption rate of entire cell cultures was linearly dependent on the concentration of sodium butyrate in the medium, as shown in Figure 3.7. Because of a relatively long duration (48 h) of the experiment the number of cells changed by proliferation.
Therefore, another factor, the cell number, may contribute to the obtained results. Because of this, the values are not expressed as glucose consumption per cell and should be used only as an indicator of the concentration of glucose in the media.

Since NaBT-treated cells were seeded at twice higher concentration than control, the final numbers of cells were not significantly different between cultures [control - \((9.594 \pm 0.03) \times 10^5\) cells/ml; NaBT 5 mM \((8.259 \pm 0.13) \times 10^5\) cells/ml].

![Graph showing glucose consumption vs concentration of NaBT](image)

**FIGURE 3.7.** Glucose consumption vs concentration of NaBT, expressed as mM of glucose consumed in 48 hours in the experimental cell cultures. Correlation coefficient \(R^2 = 0.9970\), \(n=2\).

### 3.3 DISCUSSION

The objective was to determine the efficiency of sodium butyrate (at different concentrations) as a differentiation inducing agent in a poorly differentiated human malignant colorectal cell line SW620, and to establish what concentration of NaBT and duration of treatment resulted in sufficiently increased differentiation with a minimal cell
death (a factor important for the subsequent MRS analysis).

NaBT has been reported by others (Tsao et al. 1982; Kim et al., 1984; Augeron and Laboisse, 1984; Whitehead et al., 1986) to affect the differentiation status of many colorectal cell lines. Changes in cell characteristics described included altered cell morphology, reduced growth rate in culture, dome formation, mucus secretion and increased activity of brush-border marker enzymes. Some of these features, namely: growth rate, morphology and brushborder marker enzyme activity, were characterized here for the poorly differentiated SW620 colorectal cell line treated with 0.5, 1, 3, 5 mM NaBT.

**Growth rate**

Lower concentrations of NaBT tested had minimal effect on cell growth rate with up to 16% increase in doubling time for the first 3 days in cells exposed to culture containing 1 mM sodium butyrate. However, 3 mM sodium butyrate increased doubling time by 71% which was a significant change representing a more differentiated state of the cells.

Higher concentration (5 mM) gradually slowed cell growth until it stopped completely at 200 h of treatment. It is likely that these cells were still proliferating (at least a fraction of the population), but the rate of proliferation was not higher than the death rate. The existence of possible heterogeneity of cells (some proliferate, some do not) in the culture is supported by morphological evidence described below (domes). It cannot be excluded, however, that proliferation was completely blocked at this stage because the number of cells was steady or slowly decreasing with time and no morphological characteristics of dividing cells were observed. This would mean that at this stage the proliferation is stopped and the culture becomes senescent.
A possibility that increased cell death and detachment from monolayer, observed usually for NaBT-treated cultures, was the primary cause of the increase in doubling time was excluded for 3 mM butyrate treatment, since it was established that these factors account only for 1% (control) and 2.2% (NaBT) of decrease in the growth rate. Differences in parameters of culture medium of NaBT treated cells, namely increased pH and glucose concentration, are likely to stimulate rather than inhibit cell proliferation. Therefore, it can be said that side-effects of NaBT- treatment had minor effect on doubling time, and assumed that the most important factors contributing to the increase in doubling time of NaBT-treated culture are changes in cell cycle and contact inhibition of proliferation, both reviewed below.

**Cell cycle profile**

Another significant effect observed in 3 mM sodium butyrate-treated cells was their arrest in the G₁/G₀ phase. This is in agreement with previous findings by Kruh *et al.* (1992) and Boffa *et al.* (1981). This effect is most evident at 48 hours, after which some cells recovered from the stress of treatment and return to their cell cycle, while the rest of cells remained in the G₁ or G₀ phases. Although by 96 hours of culture, sodium butyrate did not have any effect on percentage of tetraploid cells (in mitotic and G₂ phases), the later decrease in the number of such cells is indicated by growth curves as permanent reduction in proliferation rate of butyrate-treated SW620. Therefore cells treated with 3 mM sodium butyrate show an important feature of a culture undergoing differentiation, namely became a “growing” (some cells remain latent in G₁/G₀ phase, while other are in their mitotic cycle) rather than “expanding” population (almost all cells proliferate) characteristic of untreated SW620. Except the arrest of cells in the G₁ phase, NaBT treatment resulted also in less
cells in the S phase compared to control, and this might be another factor influencing slower proliferation rate at least at this early stage of butyrate exposure. The growth curves of SW620 cells treated with 5 mM NaBT indicated that after 200 h the culture entered a steady state (cell number didn’t change). One possibility is that the culture became senescent, another that it assumed a type of renewing population (cells migrating, shed or dead are replaced by the same number of cells by proliferation) typical of differentiated colorectal tissue. It would be, therefore, interesting to measure a cell cycle profile at 200 h of treatment with 5 mM NaBT to further evidence this concept.

**Viability and adherence**

One of the first effects of exposure of cells to butyrate is a lower cell adherence. Cell adherence didn’t fall below 90% until day 15 of treatment in the cultures containing 0-1 mM NaBT. Higher concentrations affected cell adherence to a greater degree. Although a high significance of these changes cannot be claimed after analysis of data of 2 experiments, there is an indication that for 3 and 5 mM NaBT-treated cells adherence decreased compared to control. This observation was made on days 4 and 9 of the culture, but not on day 15, when adherence of control cells was lowest presumably due to overconfluence.

There was a general tendency for cell viability to decrease with time and butyrate concentration in the monolayer phase of cell culture. On the fourth day of treatment the viability of monolayer cells which had undergone differentiation as judged by other criteria (treated with 3 and 5 mM NaBT) was 82 and 81% (control 89%). This suggests that by day 4 both effective concentrations of butyrate are comparable in terms of induced toxicity. This effect on cellular viability may suggest that sodium butyrate, especially at higher concentrations and longer exposure (further decrease in viability was observed) can be
cytotoxic and cause necrotic changes in the cells.

On the other hand, Hague et al., (1993) and Heerdt et al., (1994) reported that sodium butyrate induced apoptosis (programmed cell death) in a number of colorectal cell lines, including SW620. Hague et al. (1993) observed a high percentage of apoptotic cells in the suspension phase of cultures treated with sodium butyrate. Also the viability of floating cells obtained here was lower in NaBT-treated cultures (for 3 mM - 58% of control) but did not depend on the duration of treatment. It is therefore likely that induced apoptotic cell death results in the detachment of cells from the surface of culture flasks. Since the process of apoptosis is thought to be associated with the differentiated state of cells and tissues (Franceschi, 1989), reduced cell viability and adherence to the flask surface, together can be regarded as indicators of cellular differentiation, when both these occur in given apoptotic cells.

**Morphology**

Sodium butyrate was able to induce morphological changes in the SW620 cells. The changes such as cell enlargement, flattening and assuming star-shape are characteristic of differentiated cells, as opposite to undifferentiated spherical or spindle shaped, and were achieved after 3 days of treatment with 3 or 5 mM sodium butyrate.

A decrease in the monolayer cell density also accompanied 3 and 5 mM butyrate treatment of SW620 cells. This was caused, at least in part, by a slowed growth rate but was also due to an earlier plateau’ing of the culture. This fact may suggest that a contact inhibition of cell growth was obtained after treatment with NaBT.

A dome formation after prolonged exposure to sodium butyrate has been observed for both 3 and 5 mM concentrations. This observation was unique to longer cultures of
NaBT-treated cells and was abolished by passaging. This indicates that longer time is required for the formation of these 3-dimensional structures. The nature of domes is unclear, they may be cell aggregates typical of a differentiated tissue. Certainly the cell aggregation was not a result of extreme cell density, as this was low in NaBT-treated cultures. Although the cell components of domes did not show any morphologically altered phenotype, dome structures are a normal growth pattern for a less metastatic cell line, SW1222, derived from a primary colorectal tumor. All these morphological and growth pattern characteristics are therefore associated with the increasing differentiation state of SW620 cells after 3 and 5 mM butyrate treatment.

**Marker enzymes**

Sodium butyrate treatment resulted in a marked (232 - 2405%) increase in the activities of all brush-border marker enzymes. The most significant was the increase in sucrase level, suggesting that this enzyme might have been almost absent from the membrane of untreated SW620. Because glucose oxidase only uses the $\alpha$-form of glucose, the non-enzymic anomerization of unphosphorylated glucose is a limiting step in the sucrase assay. Mutarotase was not used in the assay. Nevertheless, the rate of mutarotation of glucopyranose in the reaction mixture should be the same in all samples. If $K_m$ (the Michaelis-Menten constant) of glucose oxidase is not much higher than $K_m$ of sucrase then the rate of mutarotation should not obscure differences in sucrase activity between control and NaBT-treated cells. The data obtained indicate that this is the case and the sucrase assay can reflect the changes in cellular differentiation.

The brush border membrane-associated hydrolases are indicative of differentiation
state of colorectal cells (Pinto et al., 1982; Chung et al., 1985). By comparing several cell lines showing different degree of tumor differentiation, it has been found that those classified as moderate to well differentiated have the highest alkaline phosphatase levels. The greatest increase in alkaline phosphatase activity by sodium butyrate was also found in cells derived from well-differentiated tumors, and this agent had lesser effect on poorly differentiated cell lines (Chung et al., 1985). The more differentiated cell lines have more brush borders which could allow a higher increase. However, it doesn’t explain the stimulatory role of butyrate on alkaline phosphatase within one cell line. The enzyme activities obtained by Chung et al. (1985) cannot be compared to those described in this thesis, because of different culture conditions and NaBT concentration used as well as other than SW620 colorectal cell lines studied. The effect of sodium butyrate on the level of DNA transcription was proposed as the reason for changes in brush-border hydrolase activities. Another possibility arises from the fact that more differentiated cells express lower levels of protease activity and secretion than less differentiated cells (Lokshina et al., 1993; Duffy, 1992). No protease inhibitors were used during or before measurements of enzyme activities, thus it might be assumed that the apparent ‘increase’ in the activity in NaBT-treated cells vs. control is in fact caused by less degradation of the enzymes measured. Whatever the reason, the increase in marker enzyme activities is related to the increase in cellular differentiation.

The increase in the activities of brush border marker enzymes (expressed as change in nanomoles of product formed per minute by milligram of total protein) was shown to be not due to a decrease in total protein concentration in control and butyrate-treated cells, as this was almost unchanged. It is possible that both changes in cell morphology (causing an
increase in the amount of plasma membrane per cell) and assumed appearance of brush borders (albeit not shown) was the cause of increased activity of these enzymes.

Other changes resulting from butyrate treatment which were not specifically associated with cellular differentiation were observed in treated SW620 colorectal cells.

**Extracellular pH**

The pH of cell culture medium decreased less rapidly with the increasing concentration of NaBT. In this light, the slower metabolism rate of butyrate-treated cells can reduce acidification of the environment, as the glycolysis product, pyruvate is totally used up in the citric acid cycle, therefore less lactic acid accumulates in the cells and their environment. The slower metabolism rate (and increased extracellular pH) can be a result (but not a cause) of cell contact inhibition (similarly to decreased proliferation rate), and therefore the effect of pH on MR spectra needs to be investigated.

**Glucose consumption**

Glucose consumption in SW620 cells exposed to butyrate was lower than in control cells. Two effects can explain this phenomenon. Slowed cell proliferation and metabolism rate may result in the concentration of glucose (and other nutrients) being higher in comparison with untreated cells, where the essential energy source is running out faster, despite of frequent supply of fresh media. On the other hand, butyrate is known to be preferred over glucose by colon cells, as a source of energy (Roediger, 1982; Rodriguez. et al., 1995). Glucose withdrawal and substitution of this sugar by another energy source is a popular method of inducing differentiation in malignant cells (Pinto et al., 1982; Zweibaum et al., 1985; Fantini et al., 1986 & 1989; Huet et al., 1987; Godefroy et al., 1988;
Magnusson *et al.*, 1990; Lu *et al.*, 1992), thus lowering glucose uptake by the SW620 cell culture may reflect in part the mechanism of sodium butyrate induced differentiation.

### 3.4 CONCLUSION

Sodium butyrate is able to induce cellular differentiation in the poorly differentiated SW620 colorectal adenocarcinoma cell line. 3 - 5 mM NaBT was found to be the optimal concentration range (cell viability is still acceptable within this range), and a 3-day treatment - sufficient to achieve differentiated phenotype.

To ensure sufficient viability of cells in MRS samples, it was decided that only monolayer cells be collected for these experiments.

The increased differentiation of 3-5 mM NaBT was manifested in:

- morphological changes
- markedly slower growth
- increased activity of enzyme markers of differentiation.

In the following chapter 4, MR spectra of cells grown for 72 hours in 3 and 5 mM sodium butyrate will be described.

An important remaining step, however, is to choose the proper control for MRS experiments, which would best match the growth phase of 3 day-butyrate-treated cells. The cell cycle profiles of both control and 72-hour-butyrate-treated cells are fairly similar. Taking slower proliferation into account, to ensure a sufficient number of cells in the sample,
butyrate should be added to almost confluent cells. This would result in cells reaching plateau phase after 3 days, however because of detachment, the resulting cell density is lower than in control cells in the plateau phase. Therefore the effect of growth phase and cell density on MR spectra will be investigated in chapter 5, together with other side-effects of NaBT like decreased viability, increased extracellular pH and lower glucose consumption.