CHAPTER 5

Effects of NaBT-induced culture conditions on MR spectra of cells

5.1 INTRODUCTION ............................................................................................................ 151

5.2 RESULTS ...................................................................................................................... 153
  5.2.1 Growth characteristics ............................................................................................ 153
    5.2.1.1 Growth phase ................................................................................................ 153
    5.2.1.2 Cell viability .................................................................................................. 156
  5.2.2 Extracellular pH ..................................................................................................... 163
  5.2.3 Glucose concentration .......................................................................................... 165

5.3 DISCUSSION ................................................................................................................. 172

5.4 CONCLUSION .............................................................................................................. 179
5.1 INTRODUCTION

Sodium butyrate (NaBT) was shown in chapter 3 to be a powerful inducer of differentiation for the colorectal SW620 cell line. The increased differentiation caused by NaBT in SW620 cells was associated with a number of changes to the MR spectra. These spectral changes indicated increase in lipid and decrease in carbohydrate moieties (chapter 4).

Sodium butyrate treatment also caused a number of changes to the cell culture conditions (section 3.2.3) including: altered growth phases, higher extracellular pH, reduced cell viability, and a higher availability of energy sources such as glucose. Others have shown that such changes in cell culture conditions such result in significant changes to the MRS profile of cultured cells:

! Delikatny et al. (1996) found a correlation between an increase in extracellular pH, as occurs with lower cell density, and a decrease in the intensity of MRS signals from mobile lipid.

! Shedd et al. (1993) and Franks et al. (1996) reported that both changes in culture medium pH and concentration of glucose were responsible for the levels of MR-visible UDP-hexoses and other metabolites in human colorectal cells.

! Kuesel et al. (1990) showed that phosphocholine level was reduced and glycerophosphocholine level in CX-1 colorectal cells increased with the acidification of cell culture medium down to pH=6.1-6.3, without effects on cell viability.

! A high proportion of dead cells in excised tumor tissues also caused changes in lipid signal intensity. Some reported an increase (Kuesel et al., 1994; Blankenberg et al., 1996), while others reported a decrease (Nakai et al., 1996).
From the practical point of view, modelling of some effects of NaBT-treatment cannot be done by precisely reproducing the culture conditions. This is because the kinetics of the changes in these parameters are not the same in control and butyrate-treated SW620 cells. The data shown in chapter 3 indicate that the parameters of cell cultures which underwent changes in time depending on NaBT concentration were extracellular pH and glucose concentration. Nevertheless, correlating the direction of these changes in culture conditions with the MR spectral alterations can provide valuable information for or against the use of some MRS changes as markers of cellular differentiation.

The purpose of this chapter is to investigate MR changes caused by those effects of sodium butyrate on SW620 cultured cells which might not be the direct causes of cellular differentiation. Later they will be referred to as ‘side-effects’. In particular, the influence of: altered growth phases, decrease in viability, increased extracellular pH, and higher availability of glycolysis substrate on the proton MR spectra was studied.
5.2 RESULTS

5.2.1 Growth characteristics

5.2.1.1 Growth phase

Sodium butyrate caused alterations in growth of SW620 (as described in Chapter 3), which include shorter and flatter line of exponential growth phase, as well as earlier plateau’ing of the growth curve of cultured cells. Figure 5.1 is a schematic representation showing comparison between growth curves for control and differentiated cells. Whencells destined to be treated with NaBT were split at the same density as control cells for an MRS experiment (described in section 2.4.1.1), the cells would never be able to reach confluency post treatment, as their saturation density was lower.

FIGURE 5.1. Schematic comparison of growth curves of control SW620 and treated with butyric acid. Control cells in exponential phase of growth are sub-confluent and enter plateau phase in a state of post-confluent multilayer. NaBT-treated cells (thick line) in their plateau phase form a sub-confluent or early confluent monolayer.
Thus, in order to have a sufficient cell number and comparable density, cells to be treated
with NaBT and examined later by MRS, were split at a twice higher density than control and
exposed to butyrate just prior to reaching confluency.

Despite a lower density of the monolayer, due to the increase in their doubling time,
the butyrate-treated cells exhibited growth inhibition. This resulted in early plateau’ing of
their growth curve compared to control cells. Therefore, unlike control cells, those treated
with NaBT when harvested for MRS show characteristics of over-confluent cells.

The effect of growth phase on proton MR spectra was studied by comparing data
obtained from sub-confluent (exponential phase), confluent, and post-confluent (plateau
phase) cell cultures. MRS samples of untreated control cells were obtained in the way
described in chapter 2.4.1.1, by initial seeding different concentrations of cells in the cell
culture flasks. The final cell densities (number of cells per cm$^2$) at harvest were: $5.4 \times 10^5 \pm$
$1.3 \times 10^5$ 1D (sub-confluent), $1.1 \times 10^6 \pm 1.7 \times 10^5$ (confluent) and $1.9 \times 10^6 \pm 2.8 \times 10^5$
(post-confluent). Viability of these cells was 93-99%.

1D spectral data are presented in the Table 5.1. Cross-peak volume ratios are in
Table 5.2. The ‘p’ values were calculated between sub-confluent vs. confluent and post-
confluent vs. confluent groups using the two-tail, unpaired Student t-test, assuming equal
variances, ” =5%. In each case, 1D peak intensity and 2D cross-peak volume ratios,
showed no significant differences (p>0.05) in the carbohydrate or lipid profile of the cells.
### 5. Effects of NaBT-induced culture conditions...

**TABLE 5.1. 1D peak height ratios in the spectra of untreated SW620 cells at 3 different stages of culture.** † p< 0.05 - significant difference (absent), calculated using the two-tail, unpaired Student t-test, equal variances, * = 5%. NA - non applicable. Chemical shifts of 1D peaks are: CH$_2$ (1.33 ppm), CH$_3$ (0.89 ppm), cholines (3.2 ppm), UDP-hexose (5.99 ppm), N-Ac (2.05 ppm)

<table>
<thead>
<tr>
<th>Peak height ratios</th>
<th>sub-confi. (n=10)</th>
<th>confluent (n=8)</th>
<th>post-confi. (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$&gt;\text{CH}_2 : 1.72$ppm</td>
<td>6.14 ± 1.55</td>
<td>7.01 ± 1.28</td>
<td>6.21 ± 0.84</td>
</tr>
<tr>
<td>$p^†$</td>
<td>0.2204</td>
<td></td>
<td>0.3491</td>
</tr>
<tr>
<td>$&gt;\text{CH}_2 : -\text{CH}_3$</td>
<td>2.68 ± 0.42</td>
<td>3.03 ± 0.40</td>
<td>2.68 ± 0.21</td>
</tr>
<tr>
<td>$p$</td>
<td>0.0918</td>
<td></td>
<td>0.1919</td>
</tr>
<tr>
<td>Cholines : 1.72ppm</td>
<td>2.38 ± 0.87</td>
<td>2.46 ± 1.47</td>
<td>3.51 ± 1.74</td>
</tr>
<tr>
<td>$p$</td>
<td>0.8873</td>
<td></td>
<td>0.3384</td>
</tr>
<tr>
<td>UDP-hexose:1.72ppm</td>
<td>0.28 ± 0.11</td>
<td>0.26 ± 0.06</td>
<td>0.23 ± 0.09</td>
</tr>
<tr>
<td>$p$</td>
<td>0.6513</td>
<td></td>
<td>0.5299</td>
</tr>
<tr>
<td>N-Ac : 1.72ppm</td>
<td>1.71 ± 0.26</td>
<td>1.79 ± 0.17</td>
<td>1.69 ± 0.33</td>
</tr>
<tr>
<td>$p$</td>
<td>0.4646</td>
<td></td>
<td>0.5114</td>
</tr>
</tbody>
</table>

**TABLE 5.2. Cross-peak volume ratios in the spectra of untreated SW620 cells at 3 different stages of culture.** † p< 0.05 - significant difference (absent), calculated using the two-tail, unpaired Student t-test, ‡ unequal variances, * = 5%. NA - non applicable. Cross-peak coordinates are listed in section 4.2.1.1.

<table>
<thead>
<tr>
<th>Cross-peak volume ratios</th>
<th>sub-confi. (n=10)</th>
<th>confluent (n=8)</th>
<th>post-confi. (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B : Lys</td>
<td>avg ± S.D.</td>
<td>$p^†$</td>
<td>avg ± S.D.</td>
</tr>
<tr>
<td>0.98 ± 0.60</td>
<td>0.4443</td>
<td>1.20 ± 0.58</td>
<td>1.83 ± 0.97</td>
</tr>
<tr>
<td>B : A</td>
<td>0.77 ± 0.08</td>
<td>1.0000</td>
<td>0.77 ± 0.09</td>
</tr>
<tr>
<td>Cho / Ins : Lys</td>
<td>0.54 ± 0.25</td>
<td>0.4130</td>
<td>0.45 ± 0.19</td>
</tr>
<tr>
<td>Fuc I / Thr : Lys</td>
<td>0.56 ± 0.12</td>
<td>0.8627</td>
<td>0.55 ± 0.12</td>
</tr>
<tr>
<td>Fuc II : Lys</td>
<td>0.14 ± 0.11</td>
<td>0.6029</td>
<td>0.17 ± 0.13</td>
</tr>
<tr>
<td>Fuc III : Lys</td>
<td>0.07 ± 0.01</td>
<td>1.0000‡</td>
<td>0.07 ± 0.03</td>
</tr>
<tr>
<td>Fucose block : Lys</td>
<td>0.78 ± 0.16</td>
<td>1.0000</td>
<td>0.78 ± 0.19</td>
</tr>
<tr>
<td>UDP-hexose : Lys</td>
<td>0.23 ± 0.10</td>
<td>0.6522</td>
<td>0.25 ± 0.08</td>
</tr>
</tbody>
</table>
5.2.1.2 Cell viability

Cellular viability was significantly lower in MRS samples of butyrate-treated cells than in the samples of control SW620. Although NaBT caused a noticeable increase in cell death during culturing [this phenomenon is probably due to apoptosis (Hague et al., 1993) and therefore related to cellular differentiation], it was mechanical injury during harvest for MRS examination that resulted in greatest differences in cell viability in the samples (see section 4.2.2.1, Table 4.3). Freezing and thawing of the MRS sample of untreated cells was selected as a model for induction of mechanical injury involving disintegration of plasma membranes (Kressel and Groscurth, 1994), which occurs in NaBT-treated cells. Therefore, experiments investigating the role of cellular viability in MR spectroscopy were undertaken as previously described in section 2.4.1.2. The preparation of MRS samples was the same as the standard procedure, except that after the final centrifuging, the pellet was resuspended in 0.2 ml PBS/D$_2$O and frozen at −20°C. After thawing, the cells were not washed, but another 0.2 ml PBS/D$_2$O was added.

1D and COSY spectra of cells killed by freezing in PBS/D$_2$O at −20°C, subsequently referred to as “frozen”, are shown in Figures 5.2. and 5.3B, respectively. Tables 5.3 and 5.4 present numerical comparison of lipid and carbohydrate resonances in frozen and control cells. Peak intensities in 1D spectra were normalised to the resonance at 1.72 ppm. Cross-peak volume ratios relative to lysine cross-peak (1.72, 3.05 ppm) volume were calculated for COSY spectra.
FIGURE 5.2. 0% viable (frozen) SW620 cells: 360 MHz $^1$H MR 1D spectra of SW620 cells suspended in PBS/D$_2$O: A) control; B) frozen cells. NS = 128, SR = 4915. Data were collected at 37°C with sample spinning at 20 Hz. NS = 128, SR = 4915. A line broadening of 1 Hz has been applied to the spectra. Data were collected, processed and plotted as described in section 2.3.
1D spectra

The 1D spectral ratios must be interpreted with caution due to partially overlapping signals (see section 4.2.2.2). The 1D spectra of both control and “frozen” SW620 cells contain resonances from the fatty acid chains of lipids. The resonance from methyl groups (-CH₃) at 0.9 ppm did not change with differences in cell viability. Also methylene (-CH₂-) peaks at 1.33, 1.6, 2.01 and 2.3 ppm were comparable in the spectra of frozen and control SW620 cells. The peak ratios CH₂ : 1.72 and CH₂ : CH₃ calculated from 1D data are shown in Table 5.3. Signals from olefinic groups (-CH=CH-) at 5.3 ppm also remained unchanged by freezing of the cells.

<table>
<thead>
<tr>
<th>Peak height ratios</th>
<th>Control (n=12)</th>
<th>Frozen (n=3)</th>
<th>p †</th>
</tr>
</thead>
<tbody>
<tr>
<td>UDP : 1.72ppm</td>
<td>0.25 ± 0.07</td>
<td>0.23 ± 0.05</td>
<td>0.6529</td>
</tr>
<tr>
<td>N-Ac. : 1.72ppm</td>
<td>1.75 ± 0.21</td>
<td>2.05 ± 0.46</td>
<td>0.3856 §</td>
</tr>
<tr>
<td>&gt;CH₂ : 1.72ppm</td>
<td>6.67 ± 1.42</td>
<td>6.88 ± 1.86</td>
<td>0.8312</td>
</tr>
<tr>
<td>&gt;CH₂ : -CH₃</td>
<td>2.89 ± 0.42</td>
<td>2.44 ± 0.26</td>
<td>0.1046</td>
</tr>
<tr>
<td>Cholines : 1.72ppm</td>
<td>2.39 ± 1.27</td>
<td>2.32 ± 0.22</td>
<td>0.8596 §</td>
</tr>
</tbody>
</table>

**TABLE 5.3.** 1D - spectral changes occurring in cells after freezing. Values expressed as an average of n experiments ± standard deviation. † p< 0.05 - significant difference, calculated using the two-tail, unpaired Student t-test, § - unequal variances, **=5%. Shaded areas indicate statistical significance. Chemical shifts of 1D spectra and cross-peak coordinates are listed in Tables 4.1-2

The resonance at 2.05 ppm assigned to N-acetyl groups of various compounds is well resolved and comparable in both spectra of viable and dead cells (Table 5.3). The resonance at 3.0 ppm, which is a composite of methyl group of creatine, phosphocreatine and N-CH₂ - group of lysine, is also resolved and constant in intensity in the spectra of
control and frozen cells. However, in the case of frozen cells, this peak is split into two lines: lower at 2.98 ppm and higher at 3.03 ppm. The same concerns another narrow resonance at 3.20 ppm from the -N+(CH₃)₃ group of choline and related species (phosphocholine, glycerophosphocholine), which reveals contribution of two peaks separated by 0.02 ppm. Intensity of the choline resonance decreased in the spectra of “frozen” cells, however this change was not statistically significant (p>0.05).

In the region of the spectrum 3.5 to 4.5 ppm there are significant contributions from the resonances of carbohydrates, amino acids and phospholipid precursors (previously assigned in section 4.2.1.1). Resonances in this spectral range increased in resolution following freezing of SW620 cells. The ribose resonance of UDP at 5.99 ppm does not appear to change in 1D MR spectra with the degree of cellular viability.

2D spectra

The dominant triglyceride cross-peaks denoted A-F in Figure 5.3B are present in control and “frozen” cells. As shown in Table 5.4 there is no statistically significant difference in neither B : A nor B : Lys cross-peak volume ratios in the spectra of frozen cells vs. control.

Cross-peaks for the following compounds (assigned in Table 4.2) are visible in spectra of control and frozen cells: cholesterol, choline, phosphocholine, phosphoethanolamine and glycerophosphocholine; the amino acids - alanine, aspartate, glutamate, glutamine, leucine, lysine, proline, threonine and valine, and other molecules including myo-inositol, taurine, and the ribose and glucosamine moieties of uridine.
FIGURE 5.3A. SW620 cells (control): 360 MHz $^1$H COSY (NS=32, NE=200, $F_1 = 0.0 - 6.5$ ppm, $F_2 = 0.0 - 6.5$ ppm; symmetrized) spectra of poorly differentiated SW620 cells suspended in PBS/D$_2$O. Data were collected at 37°C with sample spinning at 20 Hz. SR = 4915. A sinebell window function in $t_1$ domain, and a Lorentzian-Gaussian window function (LB = -40, GB = 0.15) in the $t_2$ domain have been applied to the spectrum. Data were plotted as described in section 2.3.5.
FIGURE 5.3B. 0% viable (frozen) SW620 cells: 360 MHz $^1$H MR COSY (NS=32, NE=200, $F_1 = 0.0 - 6.5$ ppm, $F_2 = 0.0 - 6.5$ ppm; symmetrized) spectrum of poorly differentiated SW620 cells suspended in PBS/D$_2$O. Data were collected at 37°C with sample spinning at 20 Hz. SR = 4915. A sinebell window function in $t_1$ domain, and a Lorentzian-Gaussian window function (LB = -40, GB = 0.15) in the $t_2$ domain have been applied to COSY.
diphospho-\(N\)-acetylglucosamine. The most significant change concerning the above resonances is a decrease in the choline / inositol cross-peak at 3.50, 4.07 ppm, in the spectra of frozen cells (Table 5.4). The UDP-hexose (ribose H1',H2') cross-peak at 5.99,4.38 ppm did not change after cell death.

<table>
<thead>
<tr>
<th>Cross-peak volume ratios</th>
<th>Control (n=12)</th>
<th>Frozen (n=3)</th>
<th>(p)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>UDP : Lys</td>
<td>0.21 ± 0.06</td>
<td>0.22 ± 0.03</td>
<td>0.788</td>
</tr>
<tr>
<td>Fucose block: Lys</td>
<td>0.68 ± 0.14</td>
<td>0.54 ± 0.07</td>
<td>0.1235</td>
</tr>
<tr>
<td>Fuc I/Thr : Lys</td>
<td>0.52 ± 0.16</td>
<td>0.36 ± 0.05</td>
<td>0.1189</td>
</tr>
<tr>
<td>Fuc II : Lys</td>
<td>0.08 ± 0.01</td>
<td>0.11 ± 0.06</td>
<td>0.4791§</td>
</tr>
<tr>
<td>Fuc III : Lys</td>
<td>0.07 ± 0.01</td>
<td>0.05 ± 0.02</td>
<td>0.0236</td>
</tr>
<tr>
<td>B : Lys</td>
<td>1.81 ± 0.54</td>
<td>1.28 ± 0.16</td>
<td>0.125</td>
</tr>
<tr>
<td>B : A</td>
<td>0.82 ± 0.09</td>
<td>0.88 ± 0.10</td>
<td>0.3288</td>
</tr>
<tr>
<td>Cho / Ins : Lys</td>
<td>0.49 ± 0.18</td>
<td>0.24 ± 0.08</td>
<td>0.0388</td>
</tr>
</tbody>
</table>

TABLE 5.4. 2D - spectral changes occurring in cells after freezing. Values are expressed as an average of \(n\) experiments ± standard deviation. † \(p<0.05\) - significant difference, calculated using the two-tail, unpaired Student t-test, equal variances, § - unequal variances, \("\) =5%. Shaded areas indicate statistical significance. Cross-peak coordinates are listed in Table 4.2

" -Fucose couplings (-CH(OH)-CH\(_3\)) result in a group of cross-peaks at around 4.2, 1.3 ppm (see Table 4.2 for assignment). Their volumes were measured as a group and separately. The methyl-methine coupling regions of COSY spectra of control and frozen cells are plotted in the Figure 5.4. Table 5.4 lists ratios of three individual fucose cross-peaks (denoted Fuc I, Fuc II, Fuc III) and total fucose block (containing region 4.20-4.40, 1.20-1.50 ppm) to lysine. The only significant change in fucose cross-peaks in cells killed
by freezing concerned Fuc III, which decreased in frozen cells. Fuc II and Fuc I/Thr remained unchanged by cell freezing. The methyl - methine cross-peak of lactate anion (1.33, 4.12 ppm) is present and comparable in the spectra of both control and frozen cells.

**FIGURE 5.4.** 0% viable (frozen) SW620 cells: 360 MHz $^1$H MR symmetrized COSY spectra showing methyl - methine coupling region ($F_1 = 4.05 - 4.3$ ppm, $F_2 = 1.1 - 1.5$ ppm) of SW620 cells suspended in PBS/D$_2$O: A) control; B) frozen cells. Data were collected at 37°C with sample spinning at 20 Hz (NS=32, NE=200). A sinebell window function in $t_1$ domain, and a Lorentzian-Gaussian window function (LB = -40, GB = 0.15) in the $t_2$ domain have been applied.

In the COSY spectra of frozen cells there is also a significant increase in the volume of an unassigned cross-peak at 3.1, 3.85 ppm (Figure 5.3).

### 5.2.2 Extracellular pH

Because NaBT caused less acidification of cell culture medium (section 3.2.3.1), the effect of altered extracellular pH on MR spectra of cells was studied by modifying the pH of medium subsequently added to control cells, as described previously in section 2.4.2. However a pH-stabilizing buffer wasn’t used, and it was observed that the cultured
cells could adjust their metabolism rate under the imposed pH to their own physiological requirements. The final pH’s after 24 hours were as follows:

<table>
<thead>
<tr>
<th>initial pH</th>
<th>final pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.20 ± 0.01</td>
<td>6.59 ± 0.09</td>
</tr>
<tr>
<td>7.20 ± 0.01</td>
<td>6.77 ± 0.07</td>
</tr>
<tr>
<td>8.20 ± 0.01</td>
<td>6.94 ± 0.18</td>
</tr>
</tbody>
</table>

it was therefore difficult to design an experiment to simulate a process of natural cellular response to a drug treatment. Nevertheless, these experiments provide an information on which resonances were affected by a change in pH and the direction of these changes. However, no quantitative comparison to butyrate treatment is appropriate.

The lipid (cross-peak B), UDP-hexose, fucose (spectral region 4.2-4.4, 1.2-1.5 ppm) and choline profiles from COSY and N-acetyl resonance from 1D spectra obtained from cells, whose initial pH was adjusted to 6.2, 7.2 and 8.2 24 hours prior to experiment, were compared. The resonance intensities and cross-peak volumes are presented as a function of altered extracellular pH in Figure 5.5.
These data demonstrated that the triglyceride signal from methylene groups (1.33, 2.01 ppm) decreased with medium alkalization. Conversely, total fucose and N-acetyl resonances were higher in cells grown in more alkaline culture medium, although the 1D spectral ratios may not be very reliable (as described in section 4.2.2.2). Resonances of choline/inositol and UDP decreased with an increase in extracellular pH, although these changes were not statistically significant.

5.2.3 Glucose concentration

Glucose-free conditions were tested because of reduced consumption of glucose by butyrate-treated cells (see section 3.2.3.2). Butyrate can be used by colonocytes as a preferred energy source (as indicated by Roediger et al., 1982; Rodrigues et al., 1995), therefore it should be considered that the spectral changes observed in NaBT-treated cells resulted not from increased concentration, but rather from reduced glucose consumption. The influence of higher (40 mM) glucose concentration on cell metabolism is also important here because of slightly elevated glucose content in butyrate-treated cultures (see section 3.2.3.2). Although concentration of glucose in NaBT-treated cell cultures was never as high as 40 mM, butyrate itself was another available energy source. Also, larger experimental differences in glucose concentration would more clearly indicate possible MRS changes (especially because of fast glucose utilization by untreated cells, used in this model).

In order to test the effect of glucose concentration on the spectra of SW620, the cells were grown in the culture medium supplemented with 0, 25 or 40 mM glucose for 72 hours prior to the MRS experiment (as described previously in section 2.4.3). A concentration of 25 mM glucose represents standard culture conditions in DMEM.
The 1D and COSY (methyl-methine region) spectra of cells grown in 0, 25 and 40 mM glucose-containing medium are compared in Figures 5.6 and 5.7, respectively. Resonance intensities / cross-peak volumes relative to lysine are plotted vs. glucose concentration in Figure 5.8.

**Lipids**

The 1D proton MR spectra of cells cultured in 0-40 mM glucose contain all signals from the fatty acid chains of lipids. The resonance from methyl groups (-CH₃) appearing at 0.9 ppm is equal in intensity in 25 and 40 mM glucose-supplemented cell cultures, but significantly decreased in cells grown without glucose in the medium. Also methylene (-CH₂-) resonances at 1.33, 1.6, 2.01 and 2.3 ppm were significantly reduced in glucose-free SW620 cell cultures, and were slightly increased in 40 mM relative to 25 mM glucose grown cells. The signal from olefinic groups (-CH=CH-) at 5.3 ppm is significantly reduced by growth in the absence of glucose (0 mM), but remained unchanged when cells were cultured in increased, 40 mM glucose concentration (Figure 5.6). However, since often more than one chemical species resonates at a given discrete frequency in the 1D spectra, the resonance ratios should be verified by other (e.g. COSY) experiments.

All dominant lipid cross-peaks assigned to triglyceride decreased in the 2D COSY spectra of SW620 cells cultured without glucose. They were largest in the cells supplemented with 40 mM glucose, however the difference in the ratio of lipid B : Lys cross-peak volumes between cells grown in 25 and 40 mM glucose-containing cells was not as significant as this difference between cells cultured with 0 and 25 mM of glucose. The values of cross-peak volume ratios B : Lys are plotted as a function of glucose
Figure 5.6. Glucose concentration effect: 360 MHz $^1$H MR 1D spectra of SW620 cells suspended in PBS/D$_2$O grown for 72 hours in medium containing: 0 mM, 25 mM (control), or 40 mM glucose. NS = 128, SR = 4915. Data were collected at 37°C with sample spinning at 20 Hz. NS = 128, SR = 4915. A line broadening of 1 Hz has been applied to the spectra. Plots were scaled relative to the resonance at 1.72 ppm.
concentration in Figure 5.8.

**N-acetyl**

The resonance at 2.05 ppm assigned to N-acetyl groups of various compounds was significantly decreased in glucose-free (0 mM) cultures (Figures 5.6, 5.8). Excess of glucose in the medium (40 mM) did not significantly affect cellular N-acetylation. The intensity of this resonance is also influenced by the neighbouring lipid peak at 2.01 ppm.

**Fucose**

The enlarged methyl-methine (-CH(OH)-CH₃) coupling region of COSY spectra of cells grown in medium containing 0, 25 and 40 mM glucose is shown in Figure 5.7. The cross-peak of lactate anion was lowest in the spectra of cells grown without glucose, and was highest in those cultured at 40 mM glucose. The "-fucose couplings (-CH(OH)-CH₃) giving a group of cross-peaks at around 4.2, 1.3 ppm, denoted Fuc I/Thr, Fuc II and Fuc III (see Table 4.1 for assignment), were present in the spectra of SW620 cells grown in all three glucose concentrations studied (0, 25 and 40 mM). The dependence of the fucose pattern on the glucose concentration is complex. In the spectra of glucose-free (0 mM) cell cultures there is a significant increase only in the FucI/Thr cross-peak, however growth in 40 mM glucose caused a small reduction in Fuc II and Fuc III in comparison to 25 mM. The graph in Figure 5.8 presents changes in the volume of total cumulative fucose signal (containing region 4.20-4.40, 1.20-1.50 ppm) referenced to lysine.

**UDP-hexoses**

The peak at 5.99 ppm, being a resonance from ribose (H1') and uracil (H5) of UDP-
FIGURE 5.7. Glucose concentration effect: 360 MHz $^1$H MR symmetrized COSY spectra (NS=32, NE=200) showing methyl - methine coupling region ($F_1 = 4.05 - 4.3$ ppm, $F_2 = 1.1 - 1.5$ ppm) of SW620 cells suspended in PBS/D$_2$O grown for 72 hours in medium containing: 0 mM, 25 mM (control), or 40 mM glucose. Data were collected at 37°C with sample spinning at 20 Hz. A sinebell window function in $t_1$ domain, and a Lorentzian-Gaussian window function ($LB = -40$, $GB = 0.15$) in the $t_2$ domain have been applied. Data were plotted as described in section 2.3.5.
hexoses, is significantly reduced in the spectra of cells grown without glucose but does not change significantly in 1D spectra of cells cultured within the range of 25 and 40 mM of glucose. However, in COSY spectra a significant decrease was observed in the volume of UDP-hexose cross-peak (ribose H1’,H2’) at 5.99,4.38 ppm in the spectra of glucose-free cell cultures relative to those grown in the presence of 25 mM glucose. This indicates that 1D spectra may not detect all changes in UDP-hexose levels. Similarly to the 1D data, this signal remained unchanged as a result of increased glucose concentration to 40 mM (Figure 5.8).

**FIGURE 5.8.** Glucose dependence of proton MRS signals: fucose region (4.2-4.4, 1.2-1.5 ppm), lipid B, UDP-hexose, lactate and choline 2D cross-peak volumes referenced to lysine, and N-acetyl 1D resonance intensity referenced to 1.72 ppm, in the SW620 cell line cultured in 0-40 mM glucose. MRS ratio values are averages over 3 separate experiments.
Other metabolites

The resonance at ca. 3.0 ppm, a composite of methyl groups of creatine, phosphocreatine and N-CH$_2$ - group of lysine, and other molecules, did not change with altering the concentration of glucose in the cell culture medium. The narrow 1D resonance at 3.20 ppm from the -N+(CH$_3$)$_3$ group of choline and related species (phosphocholine, glycero-phosphocholine) was found to decrease with increasing concentration of glucose in the medium, however all 1D peak ratios must be interpreted with caution (see section 4.2.2.2).

The most significant change concerning 2D resonances of lipid metabolites is a linear reduction in choline/inositol cross-peak at 3.50, 4.07 ppm with increasing extracellular concentration of glucose (Figure 5.8). Also the phosphorylcholine / threonine cross-peak at 3.61, 4.25 ppm was affected by the glucose content in the same direction as choline, however the glycerophosphocholine level was not reduced in cells grown in high-glucose media.

Resonances from carbohydrates, amino acids and phospholipid precursors in the 1D spectral region 3.5 - 4.5 ppm did not alter in cells grown in 25 and 40 mM glucose-supplemented media, however the spectral resolution was lower in cells grown without glucose. Of interest was the appearance of a peak at 3.55 ppm (possibly " -CH$_2$ of Gly) as a result of glucose withdrawal, not observed in any other culture conditions.

The cross-peak of lactate anion (1.33, 4.12 ppm) is present in the spectra of glucose containing cells (25 and 40 mM), but almost absent from spectra of glucose-free cell cultures. Although a tendency to the increase of lactate signal with the glucose
concentration can be seen in Figure 5.8, neither of these differences was statistically significant. This is probably due to large standard deviations when measuring this, mostly extracellular, compound. Other 1D resonances and cross-peaks (listed previously in Tables 4.1 and 4.2) were comparable in the spectra of cells grown in all tested glucose concentrations.

5.3 DISCUSSION

The purpose of this thesis is to find out which of the $^1$H MR spectral changes previously associated with tumor progression and development are due specifically to changes in cellular differentiation. In vitro effects of sodium butyrate on SW620 colorectal carcinoma cell line, including markers of differentiated phenotype, as well as side-effects of this treatment were described in Chapter 3. Chapter 4 of this thesis presented a range of spectral changes in NaBT- treated SW620 cells. The objective of this chapter was to investigate the influence of the side-effects (i.e. changes not specifically related to cellular differentiation) of NaBT treatment on MR spectra of SW620 cells.

Manipulation of the parameters (growth phase, viability, extracellular pH and glucose concentration) in the culture of untreated SW620 cells did not produce any phenotypical changes characteristic of the differentiated state.

Growth phase

The growth phase of cells in the culture had no significant effects on the $^1$H MR signals arising from lipids and carbohydrates, which have been previously investigated
(chapter 4) and were proposed as markers of cellular differentiation. This is contrary to the findings of others with different cell models. Methylene $^1$H MR signal was increased in the confluent state of human lung adenocarcinoma cells (Nakai et al., 1996). Although these differences may arise here from another factor, i.e. time in culture, which (unlike in the experiments of this chapter) varied in the study of Nakai et al., 1996, also Delikatny et al. (1996b) observed an increase in the intensity of $^1$H MR 1D peak ratio CH$_2$/CH$_3$ with the density of transformed murine fibroblasts seeded at a range of densities. In the study by Delikatny et al. (1996b), however, the variation range of cell concentration on the flask surface (seeding density $1.6\times10^3$ - $1.6\times10^4$ cells/cm$^2$) was much wider/broader than investigated in this chapter ($1.5\times10^4$ - $4.5\times10^4$ cells/cm$^2$). On the other hand, the same paper describes a strong dependence of the methylene signal intensity on extracellular pH, which can, at least partially, explain the behaviour of lipid peak, as pH supposedly varied with the degree of confluence in different growth phases. Since differences in proliferation rate between sub-confluent, confluent and overconfluent cultures are expected to change the rate of membrane turnover, it is most interesting that the choline resonance (normalised to lysine), indicative of membrane turnover, wasn’t found here to decrease with cell confluence; contrary to Nakai et al. (1996). This, again can be attributed to rather small differences in growth phases between compared cell cultures.

**Viability**

Differences in a degree of cell viability did not affect 1-dimensional MR spectra, except for the narrowing of some signals, which was due to leakage of metabolites into the extracellular space and their degradation to simpler compounds after freezing and subsequent thawing of the cells. However, cell death caused a reduction in the COSY cross-peaks arising from fucose (cross-peak Fuc III) and choline. This indicates that the
COSY spectra showed differences which are not detected by 1D spectra due to contributions of many chemical species to 1D resonances, in particular the ‘total choline’ resonance at 3.2 ppm. No changes were observed in the mobile lipid signals. Cell injury causes rupture of cellular membranes and release of degradative enzymes, especially active in malignant cells (Kohn, 1991; Duffy, 1992), from intracellular compartments into the solution. During such procedures as cell harvest by scraping, or freezing, suspension volume is very small and the resultant concentration of these enzymes is quite high. This process can be responsible for degradation of glycocalyx components of other viable cells, including fucosylated species, clearly visible in the 2D spectra of colorectal carcinoma cells. In spite of the assumed neuraminidase action, the \( N \)-acetyl resonance was unaffected, which suggests that this group does not change conformation regardless on the localization of sialic acid molecules in the sample.

The total volume of the fucose region was not significantly different following cell death, which means that the reduction in this species is not associated with the loss of viability caused by NaBT. The analysis of the fucose region indicates that Fuc III cross-peak may be different in nature from the other two fucose signals, since it is reduced after cell freezing. In fact, the only case out of all studied in the thesis, it was cell death that caused a reduction in Fuc III. A possible explanation could be enzymatic cleavage of the core oligosaccharides from the cell surface by glucosidases, which are released from cells when their plasma membranes are disrupted during rapid freezing. Of interest are the other fucose species giving rise to Fuc I/Thr and Fuc II cross-peaks which do not change after freezing. These species may be protected from the enzymes in a shielded position within some other part of carbohydrate chains, or can be fucosylation precursors in the form of a nucleotide within the endoplasmic reticulum-Golgi complex inside the cell. Establishing the chemical basis of fucose resonances as observed in the spectra of colorectal cells is of great importance in understanding the role of aberrant glycosylation in the loss of cellular
differentiation and other processes associated with tumor progression, like invasiveness and metastatic potential. This can be achieved by studying glycosylated antigens associated with cell membranes and their complexes with monoclonal antibodies using $^1$H MRS.

The reduction in the choline resonance is consistent with ceased proliferation and membrane turnover in dead cells. It is also opposite to the results of sodium butyrate administration, where choline/inositol intensity increased despite reduced viability. This suggests that cell death caused by mechanical injury had little effect on choline/inositol level in the process of differentiation. That triglyceride resonances were unchanged by cell freezing is indicative of a difference between the processes of a rapid death and necrosis. Higher lipid resonances were obtained in necrotic regions of tumor tissue (Kuesel et al., 1994). On the contrary, a decrease in lipid signals, associated with the higher proportion of dead cells in the sample was reported by Nakai et al. (1996). This implies that not the viability alone, but rather physiological consequences of plasma membrane disintegration, are the cause for alterations in mobile lipid resonances.

**Extracellular pH**

Manipulations with extracellular pH caused changes in lipid, fucose and $N$-acetyl $^1$H resonances. There were also smaller (statistically insignificant) alterations in ribose of UDP-hexoses and choline/inositol signals. However, as was mentioned earlier (section 5.2.2), this experiment had the purpose of indicating the tendencies of MR changes with pH, but neither their significance or quantities can be compared to the case of NaBT-treated cells. Therefore all observed spectral changes are considered here, and lead to a conclusion that only the reduction in UDP-hexose (ribose) resonance in NaBT-treated cells was consistent with the pH effect.
Some of the data obtained are in agreement with previous publications. The same dependence of mobile lipid signals on extracellular pH (increase of CH$_2$/CH$_3$ 1D resonances in acidic environment) in L fibroblasts has been demonstrated by Delikatny et al. (1996). Composition and pH of cell culture medium was responsible for the levels of UDP-hexoses and other metabolites in human colorectal CX-1 cells measured by $^{31}$P MRS (Shedd et al., 1993). Kuesel et al. (1990) reported a decrease in Cho-P with medium acidification and an opposite effect on Gro-P-Cho, however no changes in free choline were measured. The trend of a reduction in free choline (and/or inositol) with the alkalization of medium also supports that the variations in extracellular pH alter phospholipid metabolism.

As butyrate treatment resulted in lower acidification of medium (see chapter 3.2.3.2), it can be concluded, that changes in mobile lipid, choline/inositol, N-acetyl and fucose signals (i.e. increase in lipid and reduction of fucose and N-acetyl residues) are pH-independent, because the direction of these changes is opposite to those induced by elevated pH of medium.

**Glucose concentration**

The concentration of glucose in cell culture medium had effects on the MR spectra of SW620 cells. The three glucose concentration values (0, 25 and 40 mM) were selected for experiments in order to find out directions of possible MRS changes and do not represent real values found in NaBT-treated cell cultures, as described in section 5.2.3. Since butyrate can be used by colonocytes as a preferred energy source (as indicated by Roediger et al., 1982; Rodrigues et al., 1995) it can be considered that the spectral changes observed in NaBT-treated cells resulted not from increased concentration, but rather from reduced glucose consumption. Also, because of this change in the glycolytic
substrate, the MRS alterations observed for glucose-enriched cells (but not treated with NaBT) could not be quantitatively compared to those of NaBT-treated SW620, because the kinetics of butyrate consumption weren’t monitored.

The decrease in signals coming from triglycerides and some carbohydrates found in the cells grown in glucose-free medium are probably due to alterations in cellular metabolism: lipids can be used up as reserve energy source. The low level of glycosylation intermediates (N-acetyl-UDP-hexoses) as well as cell-surface fucose and N-acetyl sugars, indicates that glycosylation of cell surface is also blocked, because of free glucose shortage (compare chapter 6.4). Reduction in certain UDP-hexose levels in colorectal carcinoma cells grown in glucose-free conditions was also described by Wice et al. (1985) and Shedd et al. (1993). A significant increase in the fucose signal in glucose-free cell cultures is consistent with the same change observed in glucose-free galactose-enriched cells, presented in section 6.4, and shown there to be most likely due to the increase in threonine level, whose methyl-methine coupling contributes to the fucose Fuc I/Thr resonance.

Higher concentration of glucose did not affect UDP-hexoses, fucose and N-acetyl resonances. This suggests that an increasing glucose concentration in the culture of this colorectal cell line causes metabolic changes, but doesn’t influence processes of glycosylation and expression of cell surface carbohydrate antigens. Therefore, in butyrate-treated cells these species indicative of dedifferentiation and malignancy are independent on alterations in carbohydrate catabolism.

Only the change in triglyceride resonances, out of possible energy sources, can be regarded as due to increased glucose concentration. Elevation of glucose content of cell culture medium increased mobile lipid (albeit not statistically significant) and significantly reduced free choline/inositol content in the cells. There may be other effects on choline
metabolites, as Shedd et al. (1993) and Franks et al. (1996) showed and decrease in Cho-P signal with increased glucose concentration. The increase in cellular triglyceride signal may be indicative of reduced utilization of these lipids as a source of energy instead of glucose (now available in higher quantity). This fact could have practical implications in optimizing culture conditions for this cell line, i.e. concentration of glucose can be increased above standard 25 mM (DMEM medium) to minimize lipid catabolism. Considering the reduction in the cross-peak of choline, a precursor of membrane phospholipids and a marker of membrane turnover rate, it can be suggested that the two cellular triglyceride pools, located in plasma membrane and lipid droplets, are in dynamic exchange in SW620 cells.

Finally, the MRS changes related to different glucose concentration in the medium may be interpreted in conjunction with pH effects. It was observed that extracellular pH decreased as glucose content increased, it is also reflected by the trend showing accumulation of lactate. The pH-dependent changes in lipid (cross-peak B), fucose block, and UDP-hexose signals correlate with those observed for variations in the concentration of glucose. The correlation is particularly good for the case of pH 7.2 - 8.2 and [Glc] = 25 - 40 mM. Free choline / inositol and N-acetyl groups showed opposite trends to the pH effects in varied Glc concentrations. However, for a more conclusive analysis a linear dependence of signals on glucose concentration would be helpful. The lack of linearity in the data shown here may result from glucose-free conditions being rather extreme. Therefore, more data points are required for the range of concentrations between 0 and 25 mM Glc.
5.4 CONCLUSIONS

The objective here was to establish which of the $^1$H MRS changes in butyrate-treated SW620 colorectal carcinoma cells (namely: increase in lipid metabolites and reduction in carbohydrate levels) were a result of side-effects of NaBT treatment (changes in growth phase, increased cell death, pH and glucose content), and therefore should be excluded as specific markers of cellular differentiation.

These data indicated that there were two kinds of alterations to MR spectra resulting from modification of cell culture conditions:

1. Changes which can be easily compared with those caused by sodium butyrate with respect to their direction (increase or decrease) and extent (value by which the signal is altered).

2. Changes which can only be compared with those caused by NaBT with respect to their direction, because the determination of the time-averaged value of a given parameter of culture conditions wasn’t possible in the case of sodium butyrate treatment. Thus, quantitative distinction by MRS between NaBT-induced and artificially modified parameters couldn’t be done.

Considering direction and extent of MRS changes in SW620 cells, which were not treated with NaBT, but subjected to manipulations of cell density and viability, it was found that:

- Differences in cell density or growth phase do not affect measured resonances,
- Reduced viability caused different spectral changes than NaBT-treatment did.

Investigation of the role of changes in two parameters which cannot be quantitatively
compared with *in vitro* butyrate-treatment, i.e. extracellular pH and availability of glycolysis substrates, could provide information only about possible directions of MRS changes in NaBT-treated cells which are due to these parameters. It was established that:

- reduction in UDP-hexoses could be due to increased pH of culture medium,
- increase in unsaturated mobile lipid signal could be caused by higher concentration of glucose.

Because the direction of these two above changes caused by unspecific conditions is the same as for alterations observed in butyrate-treated differentiated cell cultures, it can be considered that MR-visible UDP-hexose and unsaturated lipid levels are not reliable markers of cellular differentiation. This is because these MRS changes could not be exclusively attributed to either altered differentiation or culture conditions. However, there was no sufficient quantitative evidence to exclude a possibility that these changes were due to cellular differentiation.

It can be concluded that the decrease in fucose and *N*-acetyl resonances, as well as the increase in free choline / inositol, are not caused by culture conditions, and are likely to be specific of cellular differentiation. Because of dependence of $^1$H MRS of cells on culture conditions and harvest techniques, a special caution is recommended in designing experiments involving use of differentiation inducers.