Effect of Growth Conditions on the Content of the Major Groups of Carbohydrates in Chick Embryo Fibroblasts

David E. Roll, Michael J. Weber, and H. Edward Conrad

Departments of Biochemistry [D. E. R., H. E. C.] and Microbiology [M. J. W.], University of Illinois, Urbana, Illinois 61801

ABSTRACT

The levels of glycogen, hyaluronic acid, chondroitin sulfates, N-acetylneuraminic acid, all of the monosaccharide components of the glycoprotein and glycolipid fractions, and the monosaccharide phosphate pools were measured in cultured chick embryo fibroblasts. Under all growth conditions, the glycogen plus the glucose phosphate pool contained approximately 50% of total monosaccharide content of the cells. However, marked qualitative and quantitative alterations were found in the glycoprotein, glycolipid, and mucopolysaccharide fractions when growing cells reached confluence, when the growth temperature was shifted from 36 to 41°C, or when the cells were transformed with Rous sarcoma virus. From 65 to 95% of the total monosaccharide residues in these complex carbohydrates were found in the glycoprotein fraction, while the glycolipids contained only 5 to 10% of the residues, and the mucopolysaccharides contained 5 to 25%. Changes in the complex carbohydrates in normal cells following changes in cell density or growth temperature were so great that they obscured any transformation-dependent changes that might have occurred consistently in the virus-infected cells under different growth conditions.

INTRODUCTION

The major groups of carbohydrates found in animal cells include glycogen, the mucopolysaccharides, the glycoproteins, the glycolipids, and those phosphorylated sugars which make up the metabolic pools (17, 21, 22). Within most of these groups, there are multiple molecular species, and each structure may contain several different monosaccharides in glycosidic linkage. Special attention has been devoted to changes in complex carbohydrates following viral transformation of cells (1, 12, 32, 33). Most of these studies have dealt with a single oligosaccharide precursor which is metabolically processed after transfer of the preassembled oligosaccharide from its dolichol phosphate carrier to a spectrum of different growth conditions. Special attention has been devoted to changes observed by 50% of total monosaccharide content of the cells. There is also some suggestion that the processing is more extensive in transformed cells than in normal cells (2, 29), but the differences are less pronounced.

The present study was undertaken to determine whether transformation-dependent carbohydrate changes could be observed in chick embryo fibroblasts infected with a mutant of Rous sarcoma virus that is temperature sensitive for transformation (25). Sensitive procedures, developed earlier (37) for assay of glycogen, hyaluronic acid, chondroitin sulfates, N-acetylneuraminic acid, all of the monosaccharide components of the glycoprotein and glycolipid fractions, and the monosaccharide phosphate pools, were applied here to determine changes that occur in the cellular carbohydrates when growing cells reach confluence when the growth temperature is shifted from 36 to 41°C and when cells are virally transformed. Marked changes in the quantitative distribution of the monosaccharides in the major groups of carbohydrates resulted when growth conditions were altered. The data show that, if significant changes in the complex carbohydrates occur as a result of viral transformation of the cells, such changes are largely obscured by changes that are normally observed in cellular carbohydrates following alterations in cell density or growth temperature.

MATERIALS AND METHODS

Cell Culture. Chick embryo fibroblast cultures were prepared from 10-day White Leghorn chick embryos (SPAFAS, Roanoke, Ill.), as described previously (47). Some of the cultures were infected with Rous sarcoma virus at a multiplicity of one (44) 12 hr after plating the primary cultures. Two strains of the virus were used, the Schmidt-Ruppin strain, subgroup A, and Martin’s T-5 mutant, which is temperature sensitive for transformation (25). All primary cultures were grown at 39°C in an atmosphere containing 5% CO2:95% air. After 4 days, all cultures were subcultured at 0.3 to 2 x 106 cells/100-mm culture dish in 12.5 ml Dulbecco’s medium (Grand Island Biological Co., Grand Island, N. Y.) containing 10% tryptose phosphate broth, 4% calf serum, and 1% chick serum. The secondary cultures were grown for 2 to 3 days at 36 or 41°C in the 5% CO2:95% air atmosphere before harvesting the cells for characterization and carbohydrate analysis.

Characterization of Cell Cultures. Tris-saline used in the cell characterization work contained 8 g NaCl solution: 0.38 g KCl, 3 g Tris, and 1 g Na2HPO4. These salts were dissolved in water, and the solution was adjusted to pH 7.5 with concentrated HCl and then diluted to 1 liter with water. Phosphate-buffered saline contained 0.5 g KCl, 1.07 g Na2HPO4·7H2O, 0.26 g CaCl2·2H2O, 0.2 g MgSO4·7H2O, and 0.138 g NaH2PO4 in 1 liter of water.

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2 Present address: Department of Chemistry, Roberts Wesleyan College, Rochester, N. Y. 14624.
3 Recipient of NIH Research Career Development Award CA00092.
4 To whom requests for reprints should be addressed.

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were used for each analysis. At the time of harvesting the cells for carbohydrate analysis, one of these dishes was taken for measurement of cell density, a second was used to assay the rates of 2-deoxy-D-glucose transport and DNA synthesis, and the cells on the remaining dishes were combined for carbohydrate analyses. For cell counts, the cells were removed from the culture dish in 0.05% trypsin (twice crystallized, type X: Sigma Chemical Co., St. Louis, Mo.) in Tris-saline and counted in a Coulter counter. For measurement of the metabolic activities, the culture medium was removed from a second dish, and the cells were washed once with phosphate-buffered saline. The cells were then incubated at 37° with 5 ml of phosphate-buffered saline containing 2.5 µCi each of [14C]-2-deoxy-D-glucose (500 µCi/mmole) and [3H]thymidine (500 mCi/mmole), both from New England Nuclear, Boston, Mass. After 15 min, the labeling medium was removed, and the cells were washed 4 times with 5 ml of cold phosphate-buffered saline. Five ml of 5% aqueous trichloroacetic acid were added to the washed cells to solubilize the intracellular pools of [14C]-2-deoxy-D-glucose and [3H]thymidine, leaving the insoluble material attached to the dish. After 10 min at 0°, the trichloroacetic acid solution was removed from the dish, and an aliquot was counted to determine the amount of intracellular [14C]-2-deoxy-D-glucose. The cell debris remaining on the dish was washed twice with cold 5% trichloroacetic acid, and then 5 ml of 5% trichloroacetic acid were added. The dish was covered and heated at 70° for 2 hr to hydrolyze the DNA, and an aliquot of the hydrolysate was counted to determine the amount of [3H]thymidine released from the DNA. The protein, which remained precipitated on the dish, was dissolved in 1 N NaOH and assayed using the procedure of Lowry et al. (24).

**Carbohydrate Analyses.** The procedures used to prepare cultured cells for analysis and to assay for each of the major groups of carbohydrates have been described in detail (6, 37). Briefly, the cells were removed from the culture dish and lysed by treatment with hypotonic trypsin. Separate aliquots were removed from the lysed cell suspension for each of the analyses. Glycogen was assayed by measuring the amount of D-glucose released by amyloglucosidase digestion. Chondroitin 4-sulfate, chondroitin 6-sulfate, and hyaluronic acid were quantitated by measuring the amounts of their respective disaccharides released by digesting cell samples with bacterial chondroitinases. Glycolipids were extracted from the cell samples with chloroform:methanol, and the monosaccharides released by acid hydrolysis of the extracts were measured. The residual aqueous sample from the glycolipid extraction was hydrolyzed to release monosaccharides from the glycoproteins. N-Acetylgalactosaminic acid was selectively released by hydrolysis of cell samples with 0.2 N HCl at 80° for 1 hr. The phosphorylated derivatives of monosaccharides in the metabolic pools were converted to free sugars by treatment of an aliquot of the cell sample with alkaline phosphatase. In all cases, the reducing sugars that were selectively released from different aliquots of the cell sample were analyzed by radiochromatography (6). In the glycoprotein assay, appropriate corrections were made for contributions of glycogen and the monosaccharide pools to the sugars released by acid hydrolysis.

**RESULTS**

**Physiological Characterization of Cell Cultures.** Cells were grown under a variety of culture conditions, and the effects of variations in growth states on the carbohydrate composition were determined. Cells were grown at 36 or 41° and were harvested either at low cell densities (2 to 6 x 10⁶ cells/100-mm dish) or when the cells had reached confluence (approximately 10⁷ cells/dish). Some of the cultures were infected with the Schmidt-Ruppin strain of Rous sarcoma virus, and some with T5-RSV. Table 1 shows the characteristics of each cell culture analyzed in the present study. Cultures in which the cells had not reached confluence are referred to as sparse cultures. As expected, these cells incorporate labeled thymidine into DNA at rates that are higher than those of the corresponding confluent (dense) cultures grown under the same conditions. In the uninfected cultures and in the T5-RSV culture which was grown at the nonpermissive temperature, the cells exhibited a fibroblastic morphology, while the cells in all of the Rous sarcoma virus-infected cultures were rounded and refractile and were only loosely attached to the dish.

**Alterations in Cellular Carbohydrates when Growing Cells Reach Confluence.** The data presented below show that the carbohydrate composition of chick embryo fibroblasts undergoes profound quantitative changes in response to changes in growth temperature, cell density, or viral transformation. Because the observed changes are so great and because they occur in response to all variations in culture conditions, the data are presented in bar graphs which show the changes that occur in response to a single variable in all of the cultures. Chart 1 compares sparse and dense cultures, and Chart 2 compares cultures grown at different temperatures. The amount of carbohydrate is plotted on 3 different scales, depending upon the carbohydrate levels in the different groups.

The data in Chart 1 show that cells in dense cultures contained lower D-glucose pools than did cells in sparse cultures, the drop in D-glucose being most pronounced in uninfected cells. With a single exception, the fall in the D-glucose pool was accompanied by a fall in cellular glycogen, a fall which again was much more pronounced in the uninfected cells. Decreased levels of mucopolysaccharides were also generally noted in the dense cell cultures, with the most striking changes observed in the hyaluronic acid content of transformed cells (see below). Changes in the monosaccharide compositions of the glycoproteins and glycolipid fractions were mixed, apparently reflecting a rise in some of the individual components of these mixtures and a fall in others as the cell density increased. The most pronounced change was the decreased D-mannose content of the glycoprotein fraction of the uninfected cells at both 36 and 41°. This was accompanied by a marked increase in the D-mannose pool. A final point of note is the sharp decline in the ribose pool (presumably 5-phosphoribosyl pyrophosphate) in all confluent cultures.

**Alterations in Cellular Carbohydrates when the Culture Temperature Was Raised from 36 to 41°.** The effects of temperature on the carbohydrate content of chick embryo fibroblasts are shown in Chart 2. In all cultures, the D-glucose pool and the glycogen content were reduced in the 41° cultures. These effects were most pronounced in the infected cultures. Although there were some very large changes in the carbohydrate compositions of the glycoproteins, the glycolipids, and the mucopolysaccharides, none of these changes...
Table 1

Physiological properties of the chick embryo fibroblast cultures analyzed for carbohydrate

All measurements were made on cells at the time of harvest.

<table>
<thead>
<tr>
<th>Culture</th>
<th>Infecting agent</th>
<th>Growth temperature</th>
<th>Cell density</th>
<th>Cell morphology</th>
<th>Cell density (cells/dish x 10^9)</th>
<th>Cell protein (mg glucose/mg, 15 DNA/mg, 15 culture agent)</th>
<th>Glucose transport (nmol 2-deoxy-D-glucose/mg, 15 min)</th>
<th>Thymidine incorporation (nmol DNA/mg, 15 min)</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>None</td>
<td>36</td>
<td>S*</td>
<td>F</td>
<td>1.8</td>
<td>2.4</td>
<td>65</td>
<td>9.0</td>
</tr>
<tr>
<td>2</td>
<td>None</td>
<td>41</td>
<td>S</td>
<td>F</td>
<td>2.5</td>
<td>2.4</td>
<td>81</td>
<td>7.4</td>
</tr>
<tr>
<td>3</td>
<td>None</td>
<td>36</td>
<td>D</td>
<td>F</td>
<td>12.5</td>
<td>1.9</td>
<td>17</td>
<td>4.0</td>
</tr>
<tr>
<td>4</td>
<td>None</td>
<td>41</td>
<td>D</td>
<td>F</td>
<td>9.1</td>
<td>2.4</td>
<td>10</td>
<td>1.1</td>
</tr>
<tr>
<td>5</td>
<td>SR-RSV</td>
<td>36</td>
<td>S</td>
<td>R</td>
<td>6.9</td>
<td>1.1</td>
<td>143</td>
<td>5.2</td>
</tr>
<tr>
<td>6</td>
<td>SR-RSV</td>
<td>41</td>
<td>S</td>
<td>R</td>
<td>24.3</td>
<td>1.6</td>
<td>90</td>
<td>1.3</td>
</tr>
<tr>
<td>7</td>
<td>SR-RSV</td>
<td>36</td>
<td>D</td>
<td>R</td>
<td>37.1</td>
<td>1.3</td>
<td>191</td>
<td>4.1</td>
</tr>
<tr>
<td>8</td>
<td>SR-RSV</td>
<td>41</td>
<td>D</td>
<td>R</td>
<td>44.4</td>
<td>1.4</td>
<td>70</td>
<td>1.3</td>
</tr>
<tr>
<td>9</td>
<td>TS-RSV</td>
<td>41</td>
<td>S</td>
<td>F</td>
<td>7.4</td>
<td>2.4</td>
<td>112</td>
<td>7.7</td>
</tr>
<tr>
<td>10</td>
<td>TS-RSV</td>
<td>36</td>
<td>S</td>
<td>R</td>
<td>8.3</td>
<td>3.1</td>
<td>277</td>
<td>3.3</td>
</tr>
</tbody>
</table>

* S, sparse; F, fibroblastic; D, dense; R, rounded; SR-RSV, Schmidt-Ruppin strain of Rous sarcoma virus.

Alterations in Cellular Carbohydrates When Cells Were Infected with Rous Sarcoma Virus. Chart 3 shows the carbohydrate changes that occurred when the cells were infected between cells grown at 36° and 41° were the changes in the D-mannose content of the glycoprotein fraction.
However, the normal T5-RSV cultures were virus-infected cells growing at 41 °, while the transformed T5-RSV cultures were cells at 41 ° (normal) or 36° (transformed); dense cultures at 36°, normal versus Rous sarcoma virus-infected; sparse cultures at 41 °, normal versus Schmidt-Ruppin strain of Rous sarcoma virus-infected; see Chart 1 for abbreviations.

The T5-RSV results showed few correlations that were consistent with the other data. These were low-density cultures that resulted from different growth temperatures alone (Chart 1) were superimposed on the transformation effect. The changes due to transformation seemed to prevail in some instances, especially in the virus-induced rise in hyaluronic acid and fall in glycoprotein L-fucose.

DISCUSSION

Reports on structural variations in animal cell carbohydrates following viral transformation have generally focused on a single group of the complex carbohydrates, namely, the glycolipids, the glycoproteins, or the mucopolysaccharides (1, 12, 17, 21, 32, 43, 45). Such studies have shown that plasma membranes contain much higher levels of glycolipids than do the intracellular membranes and that up to 80% of the carbohydrate in the cellular glycoproteins can be released from the cell surface by exposure of cells to levels of trypsin that do not alter cell viability (45). Thus, the complex carbohydrates found in animal cells are concentrated in the plasma membrane. Furthermore, it has been demonstrated that the same basic structural features are found in the carbohydrate moieties of intracellular and plasma membrane glycoproteins (3, 28), perhaps because much of the intracellular glycoprotein is in the process of being secreted. Thus, the structural features of cell surface carbohydrates appear to be a reflection of the composition of the total complex carbohydrate in the cell.

Identification of transformation-dependent changes in complex cellular carbohydrates is complicated by the finding (3, 28) that these structures may be altered in untransformed cells when culture conditions are varied or when growing cells reach confluence. For example, it has been reported that at confluence the carbohydrate chains of neutral glycolipids become extended (11, 18, 35, 38), and the proportions of those glycolipids which contain oligosaccharides with high oligomannosyl cores are decreased (3, 28). Furthermore, the composition of the carbohydrates of normal cells has been shown to vary, depending upon the monosaccharides supplied in the culture medium (31).

Attempts have been made to show differences between normal and transformed cells by measuring the incorporation of radioactive monosaccharides into glycoproteins. However, it has been shown (2) that the distribution of label in high- and low-molecular-weight glycopeptides released by proteolysis of prelabeled cells depends upon which labeled monosaccharide is administered to the cells. Clearly, differences in distribution of label may reflect differences in rates of carbohydrate metabolism rather than the usually inferred differences in carbohydrate composition. The distribution of label in the complex carbohydrates formed by a 24-hr incubation of chick embryo fibroblasts with [14C]glucosamine is significantly different from the actual carbohydrate composition of cells measured by the present procedures (36).

The data presented here show the distribution of carbohydrates in all of the major groups of carbohydrates in chick embryo fibroblasts. The relative proportions of these different groups have not been emphasized in previous studies. Heparan sulfate is not included in this study, since methodology for its routine, multiple microanalysis is not available. Under most of the culture conditions described here, the glycogen and the glucose phosphate pools taken together contain approximately infected cultures grown at 36°. Consequently, the marked changes that resulted from different growth temperatures alone (Chart 1) were superimposed on the transformation effect. The changes due to transformation seemed to prevail in some instances, especially in the virus-induced rise in hyaluronic acid and fall in glycoprotein L-fucose.
50% of the total monosaccharide residues in these cells. It was shown earlier (19) that the glucose phosphate pool in cultured chondrocytes increases with increasing levels of D-glucose in the culture medium at the time of harvest. In a separate experiment (data not shown), we have found that the same relationship holds in chick embryo fibroblasts. The cellular glycogen levels, in turn, are directly proportional to the glucose phosphate pool levels. Consequently, variations in glycogen and in the glucose phosphate pools may reflect only the amount of D-glucose remaining in the culture medium at the time of cell harvest.

The complex carbohydrates in these cells include the glycoproteins, the glycolipids, and the mucopolysaccharides. From 65 to 95% of the total monosaccharide residues in the complex carbohydrates are in the glycoprotein fraction, while the glycolipids contain only 5 to 10% of the residues, and the mucopolysaccharides contain 5 to 25%. Thus, qualitatively at least, the glycoprotein fraction is of greatest significance. The total nmoles of each sugar per 10^7 cells or per mg cell protein in the metabolic pools and in the major groups of complex carbohydrates are comparable to those reported previously for a variety of cell types (7–9, 14, 15, 39, 49).

Previous studies have shown both qualitative and quantitative changes in cellular carbohydrates when normal cells become transformed (1, 12, 17, 21, 32, 43, 45), or when growing cells reach confluence (3, 11, 18, 28, 35, 38). The present study was initiated in the hope that, by using a temperature-sensitive viral mutant, the carbohydrate changes that are transformation dependent could be distinguished from other, transformation-independent changes that may result from the viral infection. However, the data show that the levels of carbohydrates in normal cells cultured at 36° (the permissive temperature) were so different from those in normal cells cultured at 41° (the nonpermissive temperature), that they obscured any differences that might be transformation dependent. The glycoprotein and glycolipid fractions each contain a large number of different molecular species, and the changes reported here represent the resultants of changes of all of the species in each fraction. The changes observed are similar to those reported previously. For example, the sharp drop in the D-mannose content of the glycoprotein fraction when normal cells reach confluence (Chart 1) is consistent with reports of Ceccarini et al. (3) and Muramatsu et al. (26), and the lowered levels of sialic acid and N-acetylgalactosamine when normal cells are transformed at 36° (Chart 3) have been reported by polyoma and SV40-transformed cells (10, 13, 20, 26, 30, 48). Also, the marked increase in hyaluronic acid in transformed cells has been seen previously (17), but the present study shows that the hyaluronic acid elevation in chick embryo fibroblasts occurs in growing but not confluent cells (27).

The striking feature of our data is the magnitude of the changes that are observed. For example, the total number of monosaccharide residues in the glycoprotein fraction ranges from 133 nmoles of monosaccharide per 10^7 cells (83 nmoi/mg protein) in Schmidt-Ruppin strain of Rous sarcoma virus-infected cells growing at 41° to 500 nmoles/10^7 cells (208 nmoi/mg protein) in normal cells growing at 41°. The most prominent monosaccharide in the glycoprotein fraction is D-mannose, and the content of this monosaccharide undergoes extreme variation from 4 nmoles/10^7 cells (2 nmoles/mg protein) in uninfected, confluent cultures at 36° to 253 nmoles/10^7 cells (155 nmoles/mg protein) in uninfected cells growing at 41°. The direction of this change might be anticipated from recent studies on the biosynthesis and processing of glycoproteins and the changes that occur when growing cells reach confluence (3, 28).

The transformation-dependent changes found under one set of culture conditions are quite different from those observed for a different set of conditions. The picture that emerges from these and other studies is that cells carry out a rapid, dynamic metabolism of all types of carbohydrates and that the mechanisms which regulate these metabolic pathways respond readily to alterations in the physiological state of cultured cells. The failure to observe consistent changes, even in the levels of hyaluronic acid or chondroitin sulfate, which are assayed as individual polymers, suggests a complex interaction between the metabolism of the complex carbohydrates and the other metabolic pathways that are also perturbed by changes in the conditions of culture.

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