The Intracellular Accumulation of UDP-N-acetylhexosamines Is Concomitant with the Inability of Human Colon Cancer Cells to Differentiate*

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The relationship between the intracellular concentration of various nucleotides as measured by high-performance liquid chromatography analysis, and the differentiation of 2 human colon cancer cell lines was studied. HT-29 cells were induced to undergo both structural and functional enterocytic differentiation after they reached confluency when they were confluent. The significance of the accumulation of these compounds is not yet understood; however, alterations in cell membranes, single-stranded nicks in DNA, changes in DNA structure, and decreased membrane fluidity and permeability have all been implicated (reviewed in Ref. 9).

In order to study the differentiation of cells in culture, polar organic solvents such as dimethyl sulfoxide (1, 2) N,N-dimethylformamide (3, 4), hexamethylen bisacetamide (5), and sodium butyrate (6–8) have been used to induce differentiation in a wide variety of cell types including a murine virus-transformed erythroblast cell line (1, 6), human colon cancer cells (2, 8), Syrian hamster cells (4), glioblastoma multiforme (6), and mouse rhabdomyosarcoma cells (7). The mechanism of action of these compounds is not yet understood; however, alterations in cell membranes, single-stranded nicks in DNA, changes in DNA structure, and decreased membrane fluidity and permeability have all been implicated (reviewed in Ref. 9). Recently it was shown that the human colon cancer cell line HT-29, which was adapted to grow on galactose (10) or in the complete absence of sugar (11) (e.g. reduced rates of glycolysis), underwent both structural and functional enterocytic differentiation after the cells reached stationary phase. The same cells repassaged in the presence of 25 mM glucose did not differentiate. The ability to regulate differentiation in these cells by simply changing the carbon source in the media, therefore, presents a valuable approach for defining the biochemical changes associated with this cell process. This would be difficult using only organic solvents as the inducing agent, since it is not obvious which metabolic pathways would be affected.

The purpose of the present study is to correlate specific biochemical changes with the regulation of differentiation of human colon cancer cells cultured in media with various carbon sources. Preliminary results indicated that the rate of glycolysis (and presumably the levels of glycolytic intermediates), although reduced in cells that undergo differentiation, did not necessarily change as a function of growth phase and differentiation of HT-29 cells. Therefore, we chose to investigate whether modifications in the intracellular nucleotide levels could be associated with the regulation of differentiation. In this paper, we show that the accumulation of UDP-N-acetylhexosamines is concomitant with the inability of these cells to differentiate.

MATERIALS AND METHODS

Cells and Culture Conditions—The human colon tumor cell lines HT-29 (12) and Caco-2 (13) were obtained from J. Fogh (Sloan-Kettering Institute, Rye, NY). HT-29 (Glc*) and HT-29 (Gal) were adapted to grow in the absence of sugar (without the addition of nucleosides) (11) or on 5 mM galactose (10), respectively. HT-29 (Glc*) (55 passages without sugar) and HT-29 (Gal) (30 passages on galactose) were also repassaged on 25 mM glucose for at least 6
passages before being used in an experiment to serve as their respective glucose, nondifferentiated controls. These cells will be referred to as (Glc/"Glc") and (Gal/Glc). HT-29 (std) refers to HT-29 cells that have been cultured in 25 mM glucose since the strain was originated. Cells were maintained as monolayers in 25 cm² T-flasks (Corning) at 37°C, 5% CO₂-balanced air, 100% humidity, atmosphere. Dulbecco's minimum essential medium was prepared from powder lacking glucose and sucrose (Eurobio). Glutamine (4 mM, final concentration), sugar and/or nucleoside (as indicated), fetal calf serum (Boehringer Mannheim) (10% for HT-29 and 20% for Caco-2, and 1 x minimal essential medium nonessential amino acids (for Caco-2 only) were added just before media were used. Unless specifically indicated, media do not contain any sugar or nucleoside. All experiments and maintenance of cells were done in the presence of dialyzed fetal calf serum which was dialyzed in our laboratory. Complete media were not stored for more than 1 week and were changed daily (5 ml/25 cm² flasks). At confluency, cells were treated with 0.25% trypsin (EC 3.4.21.4) plus 0.53 mM EDTA in phosphate-buffered saline (NaCl, 0.14 M; KCl, 2.7 mM; Na₂HPO₄, 8.1 mM; and KH₂PO₄, 1.5 mM; pH 7.3) and seeded at 1 x 10⁶ cells/cm². Caco-2 cells were seeded at 3 x 10⁶ cells/cm².

Measurement of Differentiation of Human Colon Adenocarcinoma Cells—Cells were examined for structural differentiation (the presence of brush borders and tight junctions) by transmission electron microscopy and scanning electron microscopy as described (10, 14). For the measurement of brush-border associated enzymes, cells were trypsinized and then centrifuged in the presence of 10% serum. The cells were then resuspended in and centrifuged 3 times in physiological saline and the final pellet was stored at −80°C until assayed. The enzymes sucrase α-D-glucohydrolase (sucrase) (EC 3.2.1.48), alkaline phosphatase (EC 3.1.3.1), and aminopeptidase (microsomal) (EC 3.4.11.2) were assayed as described (Refs. 15, 16, and 17, respectively) on whole cell homogenates as well as the "P2" fraction of partially purified brush-border membranes (18). In some experiments, indirect immunofluorescence was performed on paraformaldehyde fixed cells with anti-sucrase-isomaltase antibodies as described (14) except cells attached to a T-flask were used after excising a section of the bottom of the flask with a scribing gun.

Measurement of Intracellular Metabolite Levels—For the measurement of intracellular metabolites, the medium was aspirated from a 25-cm² T-flask and the cells were rinsed 1 time with physiological saline at 0°C. The cells were then covered with 1 ml of 0.5 N perchloric acid at 0°C. Less than 10 s elapsed between the time the cells were at 37°C in culture media and the addition of perchloric acid. The cells were then scraped into the acid with a silicone rubber policeman and transferred to a centrifuge tube. The flask was then rinsed with 1 ml of perchloric acid and added to the first ml of acid plus cells. The precipitate was pelleted by centrifugation (10 min at 2,500 g) and the supernatant was transferred to another centrifuge tube. The acid precipitate was stored at −20°C until assayed for protein as described (19). The perchloric acid was then extracted from the supernatant with 2 ml of trioctylamine (Merck) in 1,1,2-trichlorotrifluoroethane (Merck) as described (20) and the aqueous phase containing the nucleotides was stored at −80°C until analysis. The extracts were prepared 1x, adjusted for cell volume/flask, and the difference was calculated in the T-flasks. All nucleotides and nucleotide sugars discussed in this paper were found to be stable to this extraction method, and their recoveries were ≈96%.

The acid extracts were analyzed for their nucleotide contents using a Waters HPLC® system consisting of 2 model 510 pumps, a model 440 absorbance detector, a U6K injector, model 680 gradient controller, a model 780 integrator/plotted, and a Partisil 10-SAX analytical anion-exchange column (Whatman). A Seleno precolumn (Whatman) was installed before the injector to saturate the solvent with silica, and a guard column with pellicular anion exchanger (Whatman) was installed between the injector and analytical column. A modification of the solvent system of Pogolotti and Santi (21) was used which consisted of a 50-min programmed gradient (No. 7; concave) from 15 mM H₂PO₄ (pH 3.8 with NH₄OH) to 1 M H₂PO₄ (pH 2.8 with NH₄OH) at a flow rate of 1 ml/min. Buffers were not degassed before use. To re-equilibrate the column, the 1 M buffer was continued for 10 min followed by the starting buffer for 10 min at a flow rate of 2 ml/min. Generally, 100–200 µl of acid extract (representing up to 1 mg of cell protein) was injected. Typical chromatograms of standards and cell extracts are shown in Fig. 1.

Peaks were identified by comparing their retention times with standards, co-injection of standards and samples, and the difference in absorbance at 254 and 280 nm. The identities of the UDP-N-acetyhexosamines were also confirmed by chemical and enzymatic methods as described below. In order to calculate the intracellular concentrations of metabolites, the peak areas of samples were converted to nanomoles by comparison with standard curves derived after the injection of known quantities of each compound. Cell volumes were determined as described (22) for each cell type and culture condition. Knowing the nanomoles of each compound/flask and the cell volume/flask, one can calculate the intracellular concentration of each metabolite.

In some experiments, the acid extracts were treated with alkaline phosphatase (23) before injection to confirm the identity of and to quantitate some of the nucleotide sugars since all nucleotides were not resolved (e.g. UDP-GlcUA was not well resolved from NADP or UDP). After alkaline phosphatase treatment, up to 750 µl of sample

![HPLC analysis of cellular nucleotides. Panel a, a mixture of known nucleotides in water was analyzed in the phosphate buffer system (see "Materials and Methods"). Similar results were obtained when standards were extracted with perchloric acid before injection. Each peak represents ≈2 nmol of nucleotide. Panel b, HT-29 (Glc-) cells were cultured for 20 days in the absence of glucose and nucleoside. The cells were then extracted with perchloric acid (see "Materials and Methods") and 100 µl of extract (representing ≈500 µg of cell protein) were analyzed as in panel a. Peaks were identified as 1, NAD plus; 2, AMP plus UMP; 3, CMP-NeuAc; 4, UDP-GlcNAc; 5, UDP-GalNAc; 6, UDP-Glc plus UDP-Gal; 7, GDP-Man; 8, UDP-GlcUA; 12, ADP; 13, GDP; 14, UTP; 15, CTP; 16, ATP, and 17, GTP. Panel c, HT-29 (Glc/Glc+) cells which had been cultured on 25 mM glucose for 20 days were extracted with perchloric acid. The extract was then treated with alkaline phosphate to remove all terminal phosphates (see "Materials and Methods") and 750 µl was then injected in the HPLC as in panel b. The presumed UDP-HexNAc peaks (represented by peaks 4 and 5 in panel b) were collected and 100 µl of this fraction (without further treatment) was reanalyzed using a borate-containing buffer system designed to separate nucleotide sugars (24).
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were injected (representing up to 460 mg of protein) and the peaks could be collected for reanalysis in a second solvent system designed to separate nucleotide sugars based on their sugar moiety (e.g. UDP-Glc from UDP-Gal) (Fig. 1c) (24).

Purification of UDP-N-acetylhexosamines—in order to confirm the identity of the presumed UDP-N-acetylhexosamines, UDP-N-acetyl-[U-14C]glucosamine (54 mCi/mmol) [U-14C]acetylgalactosamine (40 mCi/mmol) (0.1 μCi of each) were added to 2 ml of perchloric acid extracts, prepared from HT-29 (std) cells harvested on day 15. This volume of cell extract represents 179 nmol of peak 4 and 69 nmol of peak 5 (assuming that these peaks are indeed UDP-N-acetylhexosamines). The [14C]compounds contribute less than 1% of the chemical quantity of peaks 4 and 5 derived from the cell extract. 250-μl aliquots were injected 8 times in the HPLC using the phosphate buffer system, and peaks 4 and 5 were collected in 0.1-min fractions. Fractions (from all 8 injections) representing peak 4 were pooled and the same was done with the fractions for peak 5. The fractions representing the valley between peaks 4 and 5 (the 2 peaks are not base-line resolved) were not added to either of the pooled peaks. Analysis of an aliquot of each pooled peak in the HPLC using the borate buffer system indicated that peak 4 was virtually 100% pure and peak 5 was ≤50% pure with respect to absorbance at 254 nm. In order to remove the phosphate buffer and any sugar-P that might have co-migrated with peak 4 and/or 5, the samples were treated with activated carbon. The activated carbon was washed 3 times with 0.3 N perchloric acid and then stored as a 5% (v/w) suspension in 0.3 N perchloric acid. Just before use, 1 ml (for each sample) of carbon suspension was pelleted by centrifugation and then washed 4 times with 3 ml of water. Each pooled peak was then added to the carbon pellet in less than 3 ml, (total volume) and incubated for 15 min at 0°C with gentle shaking. This resulted in >95% adsorption of the UDP-N-acetyl-[14C]hexosamines, whereas less than 20% of [14C]Glc-1-P was adsorbed. The samples were then centrifuged and the supernatants were discarded. The pellets were suspended/centrifuged 3 times with 3 ml of water and then 3 times with 1 ml of 50% ethanol. This ethanol wash eluted the remaining [14C]Glc-1-P but did not elute the nucleotide sugars. Finally, the nucleotide sugars were eluted with 50% ethanol, 0.2% NH4OH (3 x 1 ml). The ethanol/ NH4OH fractions were combined, filtered (0.2-μm pore size filters) to remove residual carbon, evaporated to dryness, resuspended in 50 μl of water, and then stored at −80°C. The ethanol/NH4OH elution resulted in a 65% recovery of the adsorbed nucleotide sugars. The remaining 35% appears to be irreversibly bound to the carbon. No degradation of the UDP-N-acetylhexosamines was observed with this procedure.

HCl Hydrolysis of UDP-N-acetylhexosamines—1 μl of each purified peak was added to 100 μl of 10 mM HCl and then boiled for 15 min. These conditions liberate virtually 100% of the N-acetylglucosamine from UDP-N-acetylglucosamine (25) with the concomitant production of UDP and UTP. Reaction products were analyzed by HPLC in the phosphate buffer system.

Snake Venom Phosphodiesterase Digestion of Nucleotide Sugars—Snake venom phosphodiesterase I (EC 3.1.4.1) hydrolysis of nucleotide sugars produces nucleoside-5’-P plus sugar-1-P. Digestion was performed essentially as described (26) by adding 4 μl of 100 mM Tris (pH 8.0) and 2 μg of snake venom phosphodiesterase to 20 μl of purified peak 4 or 5. Samples were then incubated for 1 h at 37°C. An aliquot of each sample (≤1 μl) was added to an equal volume of glacial acetic acid and then analyzed by HPLC in the phosphate buffer system.

TLC system 1 is modified from Ref. 27. Samples were spotted on 20-cm² plastic-backed PEI-cellulose layers (Merck) (up to 20 μl of snake venom phosphodiesterase mix could be spotted per lane). 4% boric acid was run to the origin followed by (without intermittent drying) 4% boric acid, 4 ml of LiCl (92.5/7.5) to the top of the layer. The layer was then dried and autoradiographed. After autoradiography, the thin layer was treated with ammonium molybdate reagent (28) to detect phosphate-containing compounds.

TLC system 2 is modified from Ref. 29. PEI-cellulose thin layers were spotted in 4% boric acid (pH 9.0) with NH4OH and the excess buffer was removed by blotting the thin layer with Whatman paper. The layer was dried at 50°C for 1 h before loading samples. The thin layer was then developed 2 times in the same direction (with intermittent drying of layers) to 10 cm from the origin in 95% ethanol, 4% boric acid (pH 9 with NH4OH) (60/40). Spot visualized as with system 1. The Rf values of sugar-1-Ps, nucleoside-5’-Ps, and P, are given in Table 1. All standards (≥20 nmol) were spotted in the same volume of snake venom phosphodiesterase assay mix as the samples. Less than 4 nmol of GlcNAc-1-P were detectable with the ammonium molybdate reagent.

UDPGlucose Labeling of Intracellular UDP-N-acetylhexosamines—After 14 days in culture, one 25-cm² T-flask of HT-29 (std) cells was reseeded with 7 ml of media. 12 hours later, 3.5 ml of media were discarded and 5 μCi of [14C]glucosamine (278 mCi/mmol) (25-μl volume) were added to the flask. After 3 h, cells were extracted with perchloric acid. 500 μl of extract (of 2 ml total) were then subjected to the same purification and analysis procedures as described for the UDP-N-acetylhexosamines.

Chemical and Biochemicals—All chemicals were reagent grade or better. AMP, ADP, ATP, AMP, CMP, CDP, CTP, GDP, GTP, UDP, UTP, NAD, NADP, alkaline phosphatase, and snake venom phosphodiesterase were purchased from Boehringer Mannheim. GMP, UDP-GlcUA, UDP-GlcNAc, UDP-GalNAc, ADP-Glc, GDP-Man, CMP-NeuAc, and all sugar phosphates were purchased from Sigma. All radioactive compounds were purchased from Amerham.

RESULTS

Metabolite Levels in Differentiated and Nondifferentiated HT-29 Cells in Culture—Since HT-29 (Glc−) and HT-29 (Gal−) cells underwent both structural and functional differentiation (10, 11), and this differentiation is a growth phase-related event (10, 11), we measured the intracellular nucleotide levels as a function of these parameters.

HT-29 cells were seeded into 25-cm² T-flasks in the appropriate media and 2 flasks of each cell line were harvested on days 5 (exponential phase), 11 (asynchronous), 20, and 30, for the measurement of intracellular metabolites, and 1 flask of each series for the measurement of brush-border-associated enzymes (see "Materials and Methods"). As can be seen in Fig. 2, several different patterns in the nucleotide levels were observed. The levels of UTP and CTP (Fig. 2, b and f) varied as a function of growth rate, but not as a function of the state of differentiation of the cells, since HT-29 (Gal) and HT-29 (Gal/Glc+) cells exhibited identical levels of these metabolites versus time. UDP-Hex levels (Fig. 2e) were lower in differentiated versus nondifferentiated cells; however, the levels did not change as a function of growth phase with HT-29 (Glc−) versus HT-29 (Glc−/Glc+). In short, the levels of ATP, GTP, CTP, UTP, ADP, GDP, and UDP-Hex (Fig. 2, b, h and i) and NAD, NADP, UDP, UDP-GlcUA, UDP-Man, and CMP-NeuAc (not shown) did not change in a fashion which could be correlated with both the carbon source and growth phase dependent differentiation of these cells. However, two metabolites which were tentatively identified as UDP-GlcNAc and UDP-GalNAc (peaks 4 and 5 of Fig. 1b) varied in a unique fashion (Fig. 2a). During the exponential phase of growth (day 5), cells in the presence or absence of glucose had similar intracellular concentrations of these metabolites. However, as the cells approached stationary phase the levels of these compounds rose 10-fold only when glucose was present in the culture media.

The levels of UDP-HexNAc (Fig. 2a) exhibited an inverse relationship to the differentiation of HT-29 cells. This differentiation, as exemplified by the specific activities of sucrase, aminopeptidase, and alkaline phosphatase in brush-border enriched membrane fractions, occurred after day 11 with HT-29 (Glc−) (Fig. 3) and HT-29 (Gal) (not shown) and was not associated with changes in the UDP-HexNAc levels which remained low and constant. In contrast, HT-29 (Glc−/Glc+) (Fig. 3) and HT-29 (Gal/Glc−) (not shown), which did not differentiate, had high and constant levels of UDP-HexNAc after confluence (Fig. 2a).

Identification of UDP-N-acetylglucosamine and UDP-N-acetylgalactosamine—Since the only metabolites (of those we
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**Fig. 2.** Nucleotide levels in HT-29 cells as a function of culture media and time in culture. HT-29 (Glc-) (O), HT-29 (Glc-/Glc+) (C), HT-29 (Gal) (●) and HT-29 (Gal/Glc+) (■) were cultured in the complete absence of sugar and nucleotide, 25 mM glucose, 5 mM galactose, or 25 mM glucose, respectively, and extracted with perchloric acid on the indicated days. 100-200 μl of cell extract were then analyzed by HPLC using the phosphate buffer system, and the intracellular concentrations of metabolites were calculated (see "Materials and Methods"). Each point represents the average of 2 flasks which usually varied less than 10%. UDP-HexNAc represents the sum of peaks 4 and 5 in panel b of Fig. 1.

measured) that changed in a fashion consistent with their involvement in the inhibition of differentiation were tentatively identified as UDP-HexNAc, it seemed important to confirm the identity of these compounds. Several criteria were used to do this. First, the cell extracts were reinjected in the same HPLC system with added UDP-N-acetyl-[14C]hexosamines: UDP-N-acetyl-[U-14C]glucosamine co-migrated with peak 4 of Fig. 1; and UDP-N-acetyl-[1-14C]galactosamine co-migrated with peak 5 (data not shown). Secondly, the acid extract was treated with alkaline phosphatase under conditions that removed all terminal phosphates (see "Materials and Methods"), and the sample was then reanalyzed by HPLC. One hundred per cent of the peaks in question remained in the extract after the phosphatase treatment as well as other compounds, some of which were identified as NAD, UDP-Glu + UDP-Gal, UDP-GlcrUA, GDP-Man, and CMP-NeuAc (not shown). This result means that the compounds in question have no terminal phosphate groups. Thirdly, cells were labeled with [14C]glucosamine, a compound known to be incorporated into UDP-N-acetylhexosamines (24, 30). HPLC analysis of the perchloric acid extracts of these cells indicated that both peaks 4 and 5 were the predominant labeled compounds (not shown). Fourthly, after alkaline phosphatase treatment, acid extracts from HT-29 (Gal) and HT-29 (Gal/Glc+) from days 5 and 20 were injected in the HPLC with the phosphate buffer system, and the compounds in question (peaks 4 plus 5 of Fig. 1b) were collected. An aliquot was then reinjected in a second HPLC solvent system containing borate which separates nucleotide sugars based on their sugar moiety (24). Two well-resolved peaks appeared in this system, the first co-migrated with UDP-GlcNAc and the second with UDP-GalNAc (e.g. Fig. 1c). Integration of the peak areas indicated that these 2 peaks account for all of the peak area in the first HPLC system. Furthermore, a ratio of UDP-GlcNAc to UDP-GalNAc of 2.6 was observed and this ratio did not change as a function of culture media or growth phase of the cells. This ratio is similar to the ratio of 3.8 observed in liver after perfusion with glucosamine (24).

Although 4 independent lines of evidence suggest that peaks 4 and 5 are indeed UDP-N-acetylhexosamines, final confirmation of their identities was obtained by purification and chemical/enzymatic characterization of these peaks (see "Materials and Methods"). Both UDP-N-acetyl-[14C]glucosamine and UDP-N-acetyl-[14C]galactosamine were added to a perchloric acid extract prepared on day 15 from HT-29 (std) cells. The extract was then injected in the HPLC with the phosphate buffer system and fractions were collected. The fractions representing peak 4 plus UDP-N-acetyl-[14C]glucosamine were pooled as were those representing peak 5 plus UDP-N-acetyl-[14C]galactosamine. Reanalysis of an aliquot of each peak with the second HPLC system (with borate) indicated that each peak was >95% pure with regard to absorbance at 254 nm (not shown). The samples were then adsorbed to and eluted from activated carbon (to remove nonnucleotide material), evaporated to dryness, and then resuspended in H2O (see "Materials and Methods"). Recov-
The purified compounds were next subjected to mild acid hydrolysis (see “Materials and Methods”), and the reaction products were analyzed by HPLC in the phosphate buffer system. As can be seen in Fig. 4, HCl hydrolysis resulted in a 98% hydrolysis of both UDP-N-acetyl-[14C]glucosamine and peak 4. Furthermore, the production of UDP and UMP as the 254 nm reaction products confirms that peak 4 is indeed a uridine-containing nucleotide sugar. Identical results were obtained for peak 5 and UDP-N-acetyl-[14C]galactosamine (not shown).

Snake venom phosphodiesterase digestion of the purified compounds was also performed, followed by HPLC as well as TLC analysis of reaction products (see “Materials and Methods”). As can be seen in Fig. 4, snake venom phosphodiesterase treatment of peak 4 resulted in a 90% destruction of both peak 4 and UDP-N-acetyl-[14C]glucosamine with the concomitant production of UMP and N-acetyl-[14C]glucosamine-1-P. Analysis of peak 5 gave virtually identical results except N-acetyl-[14C]galactosamine-1-P was the labeled degradation product. Furthermore, the snake venom phosphodiesterase products were analyzed in 2 different TLC systems designed to identify all possible reaction products (i.e. nucleoside-5'-P and sugar-1-P) (see Table 1). Autoradiography revealed the migration of the N-acetyl-[14C]hexosamines-1-Ps (from the UDP-N-acetyl-[14C]hexosamines added at the beginning of the purification), and treatment with ammonium molybdate reagent revealed the phosphate-containing compounds. UMP was produced in both samples, reconfirming the identity of the nucleoside moiety. The peak 4 sample also contained a compound that exactly co-migrated with the N-acetyl-[14C]glucosamine-1-P (not shown) whereas the peak 5 sample revealed a second product which exactly co-migrated with the N-acetyl-[14C]galactosamine-1-P (not shown).

These results confirm that peaks 4 and 5 are indeed UDP-N-acetylgalactosamine and UDP-N-acetylglucosamine, respectively. Purification and analysis of [14C] in peaks 4 and 5 from cells labeled with [14C]glucosamine (see “Materials and Methods”) revealed the [14C] in these peaks was also UDP-N-acetylgalactosamine and UDP-N-acetylglucosamine, respectively (not shown).

**Differentiation of HT-29 Cells under Different Culture Conditions**—Since UDP-N-acetylglucosamine levels were greatly increased at confluence only in the presence of glucose (e.g. high rate of glycolysis), we looked at the process of differentiation under other culture conditions. Animal cells can be grown in the complete absence of sugar by the addition of uridine or inosine to standard culture media without cell mortality or the need to “adapt” the cells to these conditions (27, 31, 32). Also, in many tissue culture systems, fructose is considered a poor substrate for glycolysis (e.g. Refs. 22 and 33) and might be expected to have an effect similar to galactose. Many cells can also differentiate when Na butyrate is added to standard tissue culture media (6–8). This is of interest since glucose is still present in the culture media.

HT-29 (std) cells were seeded into 25-cm² T-flasks, and after 24 h the media were replaced with those containing: 25 mM fructose (since these cells utilize fructose at rate similar to glucose); 10 mM uridine ± 25 mM glucose; 2.5 mM inosine ± 25 mM glucose; or 25 mM glucose ± 2 mM Na butyrate. The cells were then cultured in the appropriate media for >4 passages before being used in an experiment. For an experiment, the cells were seeded into 25-cm² T-flasks in the appropriate media and were extracted with perchloric acid on days 4, 18, and 31 for the measurement of metabolites; also on day
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All samples were prepared in snake venom phosphodiesterase assay mix and spotted 2 cm from the bottom of the thin layer. Compounds were detected by pouring ammonium molybdate reagent (28) on the thin layer, drying the thin layer for 1 min, and then incubating it for 10 min at 80 °C between 2 glass plates. This yields light green spots on a white background. The spots are then rendered dark blue by a 5-min exposure to UV light (λ 254). $R_f$ is defined as the distance the compound migrated divided by the distance the solvent migrated from the origin.

| Compounds              | System I | System II |
|------------------------|----------|-----------|
| Rib-1-P                | 15       | 26        |
| Glc-1-P                | 40       | 45        |
| Gal-1-P                | 32       | 29        |
| Man-1-P                | 37       | 29        |
| GlcNAc-1-P             | 56       | 70        |
| GalNAc-1-P             | 50       | 54        |
| GlcN-1-P               | 72       | 40        |
| GaIN-1-P               | 68       | 24        |
| GlcUA-1-P              | 5.7      | 23        |
| GalUA-1-P              | 6.3      | 10        |
| P.                     | 23       | 29        |
| AMP                    | 9.5      | 22        |
| CMP                    | 12       | 17        |
| GMP                    | 1.9      | 8.6       |
| UMP                    | 18       | 23        |
| dTMP                   | 47       | 73        |
| UDP-GlcNAc             | 42       | 39        |
| UDP-GalNAc             | 35       | 21        |

* System I was performed with untreated 20 × 20-cm PEI-cellulose layers. 4% boric acid was run to the origin followed by 4% boric acid, 4 M LiCl (92.5/7.5) to the top of the layer. The layer was then dried before visualization of compounds.

* System II was performed with PEI-cellulose layers that were pretreated with 4% boric acid (pH 9.0 with NH₄OH) (see "Materials and Methods"). The layer was run 2 times with 95% ethanol, 4% boric acid (pH 9 with NH₄OH) (60/40) to 10 cm from the origin before spots were visualized as with system I.

Fig. 4. Snake venom phosphodiesterase digestion and 10 mM HCl hydrolysis of copurified UDP-N-acetyl-[U-¹⁴C]glucosamine and "peak 4". UDP-N-acetyl-[U-¹⁴C]glucosamine was added to perchloric acid extract prepared from HT-29 (std) cells cultured on 25 mM Glc for 15 days and then injected in the HPLC with the phosphate buffer system. Peak 4 plus UDP-N-acetyl-[¹⁴C] glucosamine was collected, treated with activated carbon, evaporated, and then resuspended in water (see "Materials and Methods"). Peaks are identified as follows: 1, UDP-GlcNAc; 2, UDP; 3, UMP; 4, N-acetylgalactosamine; 5, N-acetylgalactosamine-1-P; and 6, reagent. Panel a, HPLC analysis (phosphate system) of the purified peak 4 (solid line) and the UDP-N-acetyl-[¹⁴C]glucosamine (---) that coeluted with peak 4. Panel b, the purified sample was boiled for 15 min in 10 mM HCl before HPLC analysis as in panel a. Panel c, The purified sample was treated with snake venom phosphodiesterase (see "Materials and Methods") before HPLC analysis. The large peak at ≈4 min represents the reaction mixture plus glacial acetic acid since it is present in controls lacking extract.

31 cells were harvested for measurement of enzyme activities, and duplicate samples were fixed for TEM and SEM analysis. HT-29 (std) ± butyrate were harvested on additional days (as indicated in Fig. 5) in order to observe the kinetics of UDP-HexNAc accumulation versus growth phase of the cells. As indicated in Table II, cells cultured on uridine or inosine alone are well-differentiated with the presence of tight junctions, brush borders, and increased levels of sucrase in the brush-border enriched membrane fraction. This differentiation was not due to the presence of the nucleosides themselves, since nucleoside plus glucose prevented the differentiation of these cells. Cells cultured in 25 mM fructose were not differentiated as was the glucose control. Cells cultured in the presence of glucose plus 2 mM butyrate underwent a typical enterocytic
The effects of culture media on the expression of differentiation markers and UDP-N-acetyhexosamine levels in HT-29 cells

HT-29 (std) cells were trypsinized and seeded into 25-cm² T-flasks in the presence of 25 mM Glc. 24 hours later the cells were washed with phosphate-buffered saline, and media containing the indicated carbon source(s) were added. The cells were then cultured in the respective media for 4 passages before being seeded into T-flasks for this experiment. Each value represents 1 T-flask. + or − refers to the presence or absence of the indicated parameter.

### Table II

| Culture media | Intraacellular concentration of UDP-HexNAc | Presence of enterocytic differentiation markers |
|---------------|------------------------------------------|------------------------------------------------|
| 25 mM Glc     | Day 4: 0.32 3.62 3.22 | Functional: 0.44 − − − − − Structural: − − − − − |
| 25 mM Fru     | Day 18: 0.46 2.58 2.47 | − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − − -
After the core oligosaccharide is completed, it is attached to the protein (in the rough endoplasmic reticulum) before the glycoproteins move to the smooth endoplasmic reticulum-Golgi complex where additional sugars can be added to the sugar core. The initial step in the synthesis of the core oligosaccharide can be inhibited by the antibiotic tunicamycin (43, 44). When tunicamycin was added to L1210 leukemia cell cultures, incorporation of sugars into glycoproteins was greatly reduced (45, 46). However, when these cells were examined by electron microscopy (45), the endoplasmic reticulum showed extensive damage. Electron micrographs of HT-29 cells do not reveal any ultrastructural damage, regardless of the conditions under which they were cultured (not shown). This would suggest that the accumulation of UDP-HexNAc in HT-29 cells is not related to a total blockage of the N-glycosylation of proteins. However, it is also possible that tunicamycin has secondary effects which accompany this inhibition which do not occur when this process is blocked without the antibiotic. The O-glycosylation of proteins takes place without a lipid carrier after the direct attachment of GalNAc to serine or threonine residues of the protein (38, 39). Additional sugars can then be attached to the N-acetylgalactosamine by specific glycosyltransferases. Whether the levels of UDP-HexNAc can modify this process is not known. It is important to note that for the duration of our experiments (>40 days) the cells remained fully viable. Therefore, it would seem unlikely that there is a total blockage of either N- or O-glycosylation of proteins; rather there are specific modifications in certain glycoproteins. UDP-HexNAc levels could have a direct effect on (or result from) a specific(s) glycosyltransferase activity(s) which modifies the sugar content of glycoproteins. For example, UDP-GlcNAc has been classified in inhibition which do not occur when this process is blocked (47). The Biochemistry 1983, 25, 254, 98-112.

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