Biochem. J. (1984) **217**, 701–708
Printed in Great Britain

D-Glucosamine-induced changes in nucleotide metabolism and growth of colon-carcinoma cells in culture

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(*Received 1 August 1983/Accepted 12 October 1983*)

Human colon-carcinoma cells were exposed to D-glucosamine at 2.5, 5 and 10mM, concentrations that were growth-inhibitory but not cytocidal in the presence of a physiological glucose concentration. Labelling of these HT-29 cells with D-[^{14}C]-glucosamine, followed by nucleotide analyses, demonstrated that UDP-*N*-acetylhexosamines represented the major intracellular nucleotide pool and the predominant metabolite of the amino sugar. D-[^{14}C]Glucosamine was not a precursor of UDP-glucosamine. After 4h exposure to D-glucosamine (2.5mM), the pool of UDP-*N*-acetylhexosamines was increased more than 6-fold, whereas UTP and CTP were markedly decreased. UDP-glucuronate content increased by more than 2-fold, whereas purine nucleotide content was little altered. Uridine (0.1 mM) largely reversed the decrease in UTP, CTP, UDP-glucose and UDP-galactose, while intensifying the expansion of the UDP-*N*-acetylhexosamine pool. Uridine did not reverse the D-glucosamine-induced retardation of growth in culture. A 50% decrease in growth also persisted when uridine and cytidine, cytidine alone, or UDP, were added together with D-glucosamine. The growth-inhibitory effect of the amino sugar could therefore be best correlated with the quantitative change in the pattern of sugar nucleotides, and, in particular, with the many-fold increase in UDP-*N*-acetylglucosamine and UDP-*N*-acetylgalactosamine.

The glucose analogue D-glucosamine was recognized as an inhibitor of tumour growth by Quastel & Cantero (1953). The mechanisms proposed for this action of the amino sugar include (i) decrease in the rate of glycolysis (Yushok, 1958; Sukeno *et al.*, 1971), (ii) alterations in uracil and adenine nucleotide contents (Bekesi & Winzler, 1969; Bosmann, 1971) and (iii) interference with glycosylation reactions (Koch *et al.*, 1979; Morin *et al.*, 1983).

Exogenous D-glucosamine is converted into physiological intermediates of amino sugar metabolism (Kornfeld *et al.*, 1964; Winterburn & Phelps, 1971). There is no evidence for UDP-glucosamine biosynthesis from D-glucosamine, even though this sugar nucleotide can be formed from UTP and glucosamine 1-phosphate *in vitro* in a reaction catalysed by UDP-glucose pyrophosphorylase (Maley *et al.*, 1956; Weckbecker & Keppler, 1982). This non-physiological UDP-glucosamine was identified as an inhibitor of lipid-dependent glycosylation reactions in the micromolar concentration range (McDowell *et al.*, 1983). It seemed relevant therefore to rule out the possibility that it can be formed in amounts sufficient to inhibit glycoconjugate synthesis when cells are exposed to D-glucosamine.

D-Glucosamine diverts uridylate from UTP into UDP-*N*-acetylglucosamine (Kornfeld *et al.*, 1964; Bekesi & Winzler, 1969). This uridylate-trapping action can lead to a depletion of UTP and other pyrimidine nucleotides, with subsequent inhibition of pyrimidine-nucleotide-dependent biosyntheses (Decker & Keppler, 1974). The expansion of the UDP-*N*-acetylglucosamine and UDP-*N*-acetyl-

Abbreviation used; h.p.l.c., high-performance liquid chromatography.

galactosamine pools in turn creates an imbalance in the sugar nucleotide pattern (Keppler *et al.*, 1970). Exogenous uridine provides a means of restoring the lowered UTP content, and can therefore be used to investigate a possible relation between pyrimidine nucleotide contents and cell growth in culture. In order to investigate this correlation, cultures of a human colon-carcinoma cell line, HT-29 (Fogh & Trempe, 1975), were exposed to low concentrations of D-glucosamine and/or uridine in the presence of physiological concentrations of glucose. In this system, the enlarged UDP-*N*-acetylhexosamine pool, rather than the decreased pyrimidine nucleotide content, was related to the inhibition of cell growth. This conclusion is in line with a study on the action of D-glucosamine in L1210 cells (Morin *et al.*, 1983).

Materials and methods

Cell culture

The human epithelial colon adenocarcinoma-cell line HT-29, obtained from Dr. J. Fogh, Sloan Kettering Institute for Cancer Research, Rye, NY, U.S.A., was grown in Dulbecco's modified Eagle's minimum essential medium supplemented with 10% foetal bovine serum (Rousset *et al.*, 1979). For the present study, cells were seeded in medium containing 5mM-glucose (0.8×10^6–2.0×10^6 cells in 5ml of medium per 25cm^2 Corning plastic flask), with a change of medium every 24h. They were used 4 days later when still in the constant-growth phase; cell doubling time was of the order of 24h; 10^6 cells were equivalent to 250μg of protein; cells were checked to be mycoplasma-free. Experiments were initiated by replacing the medium.

Chemicals, radiochemicals and enzymes

D-Glucosamine hydrochloride, uridine and cytidine were Sigma products. D-[U-^{14}C]Glucosamine (Amersham International) was used at a specific radioactivity of 0.2 or 2Ci/mol. Alkaline phosphatase from calf intestine (1500 units/mg of protein as defined by the supplier) and all nucleotides were from Boehringer Mannheim.

Cell collection and preparation of neutral cell extracts

Cells were rapidly rinsed twice with ice-cold 0.9% NaCl, and the cell film was immediately snap-frozen by floating the flasks on liquid N_2. Nucleotide extraction was performed by laying the flasks on an ice bed and covering the cell film with 400μl of cold $HClO_4$ (0.9M) for 15min. Cells were detached with a Teflon-tipped spatula; the extract was transferred into a preweighed tube, which was reweighed and then centrifuged for 2min at 8000*g*. The supernatant was neutralized with about 35μl of KOH (8M)/K_3PO_4 (1M); the $KClO_4$ precipitate was again centrifuged and the supernatant reserved for h.p.l.c. All the experimental conditions studied included two further flasks for determinations of cell number as described previously (Krug *et al.*, 1983).

High-performance liquid chromatography of nucleotides

Acid-soluble nucleotides were separated by anion-exchange h.p.l.c. on a Partisil-10 SAX (Whatman, Clifton, NJ, U.S.A.) column by using buffer and flow gradients as described previously (Holstege *et al.*, 1982). ^{14}C-labelled metabolites were collected every 20s and counted for radioactivity. For a more detailed analysis of sugar nucleotides, the acid-soluble cell extracts were pretreated for 3h at 30°C with alkaline phosphatase at a final concentration of 15 units/ml in Tris/HCl buffer (50mM, pH9.0) containing magnesium acetate (0.3mM). This pretreatment eliminated interference by nucleoside phosphates with the subsequent h.p.l.c. analysis of sugar nucleotides. Synthetic UDP-glucosamine (Weckbecker & Keppler, 1982) served as a standard.

Ion-pair reverse-phase h.p.l.c. (Hoffman & Liao, 1977; Walseth *et al.*, 1980) was used for further analysis of sugar nucleotides with a 5μm-particle ODS-Hypersil RP 18 column (Shandon) and isocratic elution at a flow rate of 1.5ml/min. The aqueous eluent was composed of KH_2PO_4 (20mM), tetrabutylammonium hydroxide (5mM) and 5% (v/v) methanol at pH5.6. For analysis of radioactively labelled metabolites, fractions were collected every 30s.

Results

Separation of D-glucosamine metabolites

The pattern of intermediates from D-[^{14}C]-glucosamine after 4h indicated that UDP-*N*-acetylhexosamines, which are a mixture of UDP-*N*-acetylglucosamine and UDP-*N*-acetylgalactosamine, are the major metabolites in colon-carcinoma cells, accounting for 47% of the intracellular radioactivity (Fig. 1). A minor fraction, corresponding to 3% of the metabolite radioactivity, was eluted between 4 and 5min (Fig. 1), the retention time of synthetic UDP-glucosamine in this h.p.l.c. system (Weckbecker & Keppler, 1983). This radioactive and u.v.-absorbing peak was eluted at the same position after alkaline-phosphatase treatment (Fig. 2). As these properties would have been consistent with UDP-glucosamine, we collected this material and rechromatographed it by ion-pair reverse-phase h.p.l.c. (Fig. 3). Standard UDP-glucosamine was clearly separated from the radioactive metabolites of D-[^{14}C]-glucosamine. This approach provided unam-

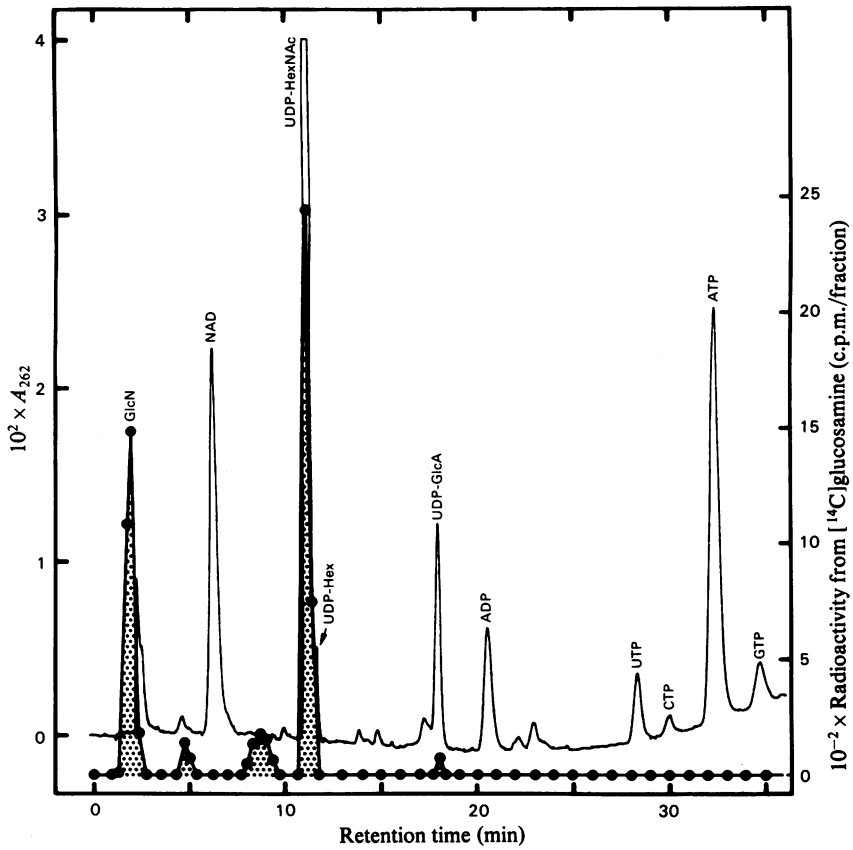

Fig. 1. *Separation of acid-soluble nucleotides by anion-exchange h.p.l.c. after labelling with* D-[^{14}C]*glucosamine (2.5 mM; 0.2 Ci/mol) for 4 h*

The cell extract (50 μl) applied to the column corresponded to 10^6 cells. Nucleotide absorbance was recorded at 262 nm. Stippled areas represent the radioactivity from D-[^{14}C]glucosamine measured at the times indicated (●). Abbreviations: UDP-Hex, UDP-glucose and UDP-galactose; UDP-HexNAc, UDP-*N*-acetylglucosamine and UDP-*N*-acetylgalactosamine; UDP-GlcA, UDP-glucuronic acid; GlcN, D-glucosamine.

biguous evidence that these cells do not synthesize significant amounts of UDP-glucosamine (< 30 pmol/10^6 cells after 4 h exposure to 2.5 mM-D-glucosamine). As the radioactive metabolites detected in Fig. 3 had the same retention times as radioactive material recovered in the corresponding extracellular medium, they might represent catabolic products of D-[^{14}C]glucosamine, including lactate (Sukeno *et al.*, 1971).

D-Glucosamine-induced changes in nucleotide contents of HT-29 cells

The studies were initiated at the different D-glucosamine concentrations indicated in Figs. 4 and 5 and at a glucose concentration of 5 mM. These amino sugar concentrations did not affect cell viability (Krug *et al.*, 1983).

The most pronounced changes caused by D-glucosamine concern pyrimidine nucleotide metabolism. The accumulation of UDP-*N*-acetylhexosamines (Figs. 1, 2, 4 and 5) was associated with a fall in the contents of UTP, CTP and UDP-hexoses, the latter comprising UDP-glucose and UDP-galactose. UTP decreased to 36% of the control value after 2 h exposure to 2.5 mM-D-glucosamine; this fall was not significantly intensified by increasing the concentration of the hexosamine up to 10 mM (Fig. 4). After 4 h, UTP content had recovered partially, which reflects a compensation by enhanced formation of uracil nucleotides. This was indicated by the enlargement of the pool of total acid-soluble uracil nucleotides, which was dependent both on the time of exposure and on the D-glucosamine concentration (Fig. 5). With 10 mM-hexosamine, total uracil nucleotides increased by factors of 1.9 and 3.7 after 2 and 4 h respectively. The major components of the acid-soluble uracil nucleotides were the UDP-*N*-acetylhexosamines,

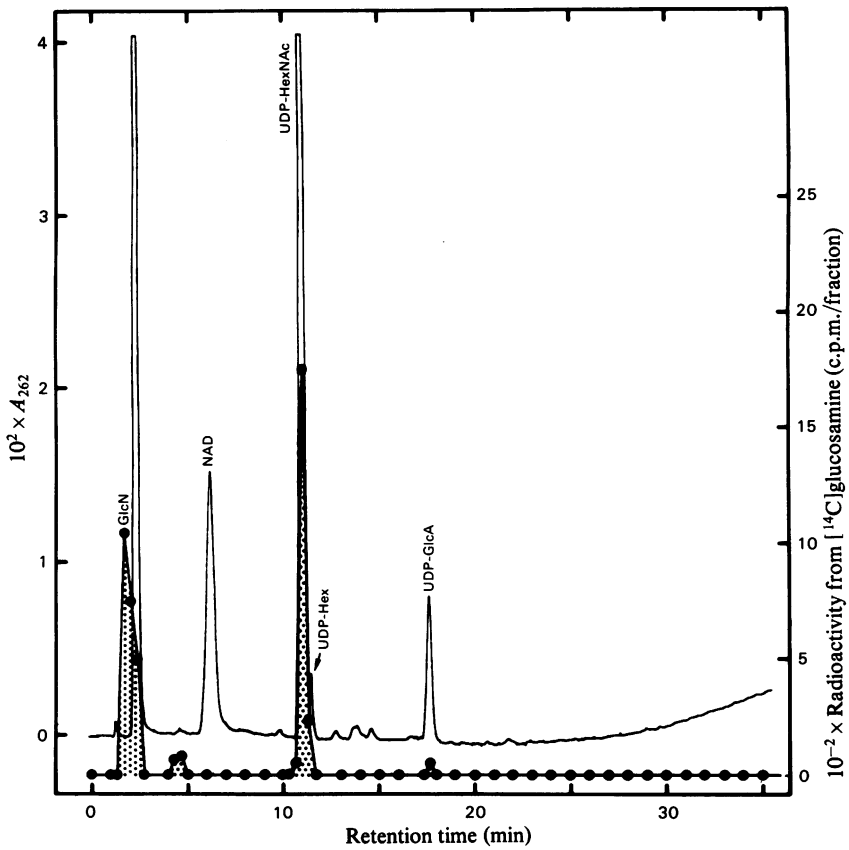

Fig. 2. *Separation by anion-exchange h.p.l.c. of acid-soluble nucleotides after treatment with alkaline phosphatase* The cells were labelled as described in the legend to Fig. 1. The $HClO_4$ extracts were incubated with alkaline phosphatase before chromatography as described in the Materials and methods section. For abbreviations see Fig. 1 legend.

which amounted to about 80% of the total acid-soluble uracil nucleotide pool.

Exposure of HT-29 cells to D-glucosamine had a marked effect on their UDP-glucuronate content, which was increased 2.7-fold after 4h with the highest concentration of the amino sugar (Fig. 5). This change required incubation times exceeding 2h (cf. Figs. 4 and 5).

Effect of uridine on D-*glucosamine-induced changes in nucleotide contents of HT-29 cells*

Uridine, at a concentration of 100 μM, enhanced uracil nucleotide as well as CTP contents (Table 1). This action of the nucleoside largely reversed the depression in UDP-hexoses, UTP and CTP caused by D-glucosamine. Uridine intensified the expansion of the UDP-*N*-acetylhexosamine pool and of total uracil nucleotides. Purine nucleotide contents as well as the ATP/ADP ratio in HT-29 cells were little affected by uridine or D-glucosamine in the concentration ranges studied (Table 1 and Fig. 5). Selective interference with pyrimidine nucleotide metabolism, together with the possibility for reversal by uridine, rendered the system useful for studying the consequences of these changes on cell growth.

Failure of pyrimidine nucleosides to reverse the D-*glucosamine-induced depression of cell growth*

To test whether the retardation of growth observed in the presence of D-glucosamine at the non-cytocidal concentration of 2.5 mM can be attributed to the described decrease in UTP, UDP-hexoses and CTP, HT-29 cells were cultured in the presence of various pyrimidines (Table 2). The pyrimidine concentrations used did not exceed 100 μM as higher values were themselves growth-inhibitory under our conditions of culture. Neither uridine nor cytidine, singly or in combination, counteracted the growth-retarding action of D-glu-

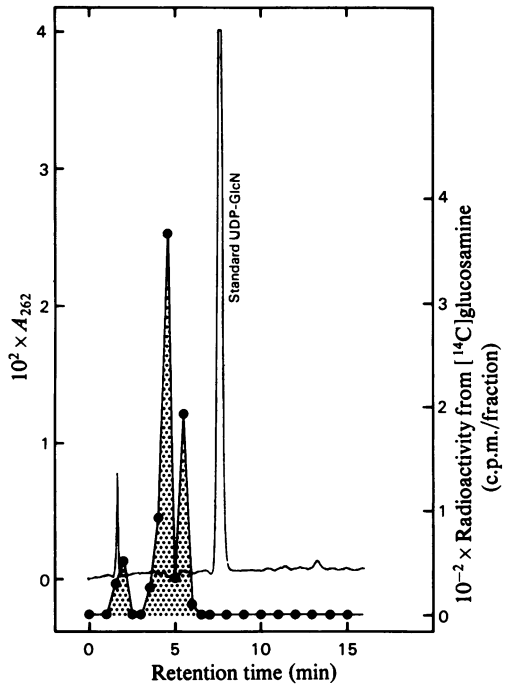

Fig. 3. *Rechromatography by ion-pair reverse-phase h.p.l.c. of material co-eluted on anion-exchange h.p.l.c. with standard UDP-glucosamine*

The specific radioactivity of D-[^{14}C]glucosamine in this experiment was raised to 2Ci/mol; the concentration of the amino sugar was 2.5mM. UDP-glucosamine (UDP-GlcN) is eluted on anion-exchange h.p.l.c. (Figs. 1 and 2) between 4 and 5min (Weckbecker & Keppler, 1983); this fraction was collected, freeze-dried and rechromatographed on a reverse-phase column as described in the Materials and methods section after addition of standard UDP-glucosamine. Note the separation of u.v.-absorbing standard UDP-glucosamine from the radioactivity indicated by the stippled areas.

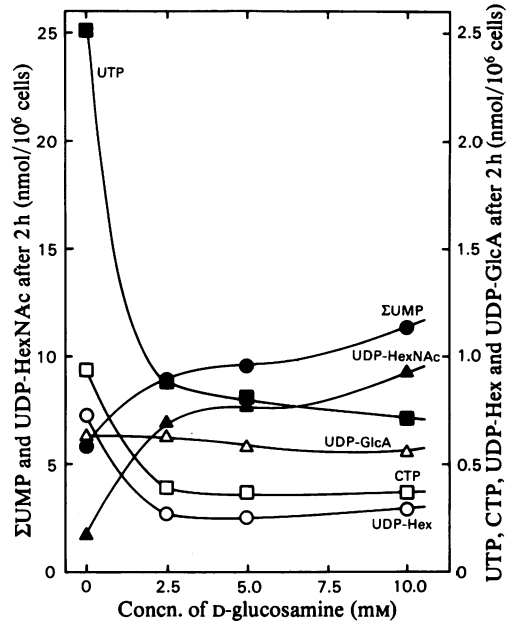

Fig. 4. *Changes in nucleotide contents of HT-29 cells after 2h of exposure to increasing concentrations of* D-*glucosamine*

Nucleotides were analysed by anion-exchange h.p.l.c. both directly and after alkaline-phosphatase treatment of the acid-soluble cell extracts. ATP values are not included, as they were essentially unchanged. Each point represents the mean from a minimum of two separate flasks, with average S.D. of 9%. Abbreviations: ΣUMP, sum of total acid-soluble uracil 5'-nucleotides; UDP-Hex, UDP-glucose and UDP-galactose; UDP-HexNAc, UDP-*N*-acetylglucosamine and UDP-*N*-acetylgalactosamine; UDP-GlcA, UDP-glucuronic acid.

cosamine. Comparable results were obtained when the medium was changed twice daily or when dialysed serum was used. UDP, which can provide uridine after its hydrolysis on the cell surface (Keppler & Holstege, 1982), was also unable to restore normal growth rates. D-Glucosamine may act as a phosphate-trapping agent (Plagemann & Erbe, 1973) in addition to trapping uridylate. However, doubling the phosphate concentration in the medium to 2mM failed to normalize growth in culture (results not shown).

Discussion

HT-29 human colon-carcinoma actively converted D-glucosamine into UDP-*N*-acetylhexosamines, which represented the major intracellular product, in a quantity exceeding all the other metabolites of the amino sugar. Accumulation of UDP derivatives can strain the ability of these cells to maintain contents of other uracil nucleotides; this is in agreement with previous studies in liver (Kornfeld *et al.*, 1964; Keppler *et al.*, 1970), sarcoma cells (Bekesi & Winzler, 1969), leukaemia cells (Bosmann, 1971) and fibroblasts (Scholtissek, 1971; Rapaport *et al.*, 1980). The decrease in UTP, UDP-hexoses and CTP in the colon-carcinoma cells was marked, but insufficient to cause irreversible cell damage, as indicated by the reversal of the depression of growth in D-glucosamine-free medium (Krug *et al.*, 1983). In hepatoma cells, lethal UTP deficiency was observed only when contents were maintained below 10% of control for several hours (Keppler, 1977). An inhibition of RNA polymerases by substrate deficiency would require this degree of UTP depletion (Decker &

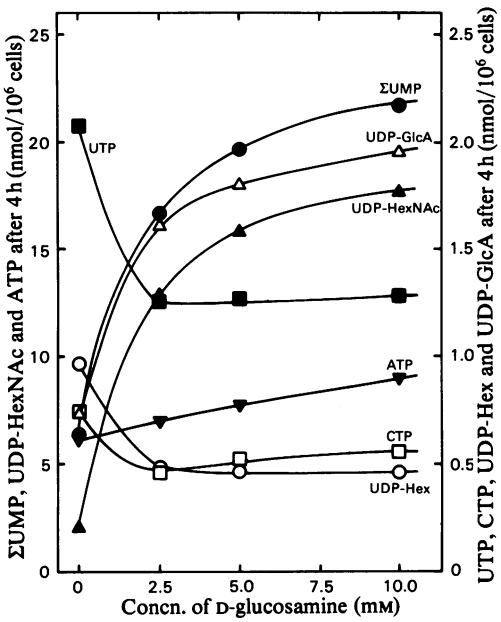

Fig. 5. *Changes in nucleotide contents of HT-29 cells after 4h of exposure to* D-*glucosamine*
Analyses and abbreviations were the same as described in the legend to Fig. 4; values are means from at least three different flasks, with average S.D. of 10%.

Keppler, 1974). The lowered content of UTP was associated with decreased content of its metabolic product CTP (Figs. 4 and 5). A severe depletion of UTP pools is prevented in the colon-carcinoma cells by additional formation of uracil nucleotides (Fig. 5). This compensatory expansion of the total uracil nucleotide pool was shown in other cell lines to be due to pyrimidine synthesis *de novo* (Holstege *et al.*, 1982). We expect that this is also the case in HT-29 cells, since extracellular uridine was not added as a source of uracil nucleotide synthesis on the salvage pathway.

As glucosamine 6-phosphate is an established metabolite of D-glucosamine (Sukeno *et al.*, 1971; Koch *et al.*, 1979), one could assume its conversion into glucosamine 1-phosphate and UDP-glucosamine. The latter can substitute for UDP-glucose in glycogen synthesis (Maley *et al.*, 1966; Romero *et al.*, 1980). A possible formation of UDP-glucosamine was also of interest to understand better the growth-inhibitory effect of D-glucosamine in HT-29 colon-carcinoma cells, which are characterized by a high glycogen content (Rousset *et al.*, 1979). The analytical techniques used in the present study (Figs. 1–3) clearly established that UDP-glucosamine was not an intermediate of D-glucosamine metabolism in HT-29 cells. Previous evidence for UDP-glucosamine formation in intact cells and

Table 1. *Nucleotide contents in colon carcinoma cells treated with* D-*glucosamine, uridine, or a combination of both*
HT-29 cells were cultured for 4h in the presence of glucose (5mM) with addition of D-glucosamine (2.5mM) and/or uridine (100 μM). Analyses were performed by anion-exchange h.p.l.c., with or without prior alkaline-phosphatase treatment of the cell extracts. The sum of NAD^+ and $NADP^+$ is given; ADPRP corresponds to NADPH which is degraded during $HClO_4$ deproteinization. ΣAMP and ΣUMP represent the respective sums of total acid-soluble adenine and uracil nucleotides. Mean values, differing on average by 13%, are from two separate flasks in controls and glucosamine-treated cells, and from four different flasks, ±S.D., in cells treated with uridine or uridine plus glucosamine. All extracts were obtained in the same experiment; corresponding results shown in Fig. 5 and derived from a separate experiment with a lower cell concentration are not included in the calculation.

	Amount (nmol/10^6 cells)			
Nucleotides	Control	D-Glucosamine	Uridine	D-Glucosamine + uridine
UDP-*N*-acetylhexosamines	1.43	9.40	1.77±0.11	11.50±1.05
UDP-glucose + UDP-galactose	0.62	0.39	0.83±0.06	0.60±0.07
UDP-glucuronate	0.53	1.02	0.60±0.04	1.36±0.20
UTP	1.52	0.66	2.15±0.16	1.21±0.14
UDP	0.17	0.21	0.27±0.04	0.22±0.04
ΣUMP	4.27	11.88	5.61±0.39	14.89±1.38
ATP	4.98	3.98	4.80±0.32	3.97±0.26
ADP	0.80	1.12	0.81±0.10	0.65±0.14
AMP	0.16	0.15	0.13±0.02	0.14±0.03
$NAD(P)^+$	1.63	1.53	1.46±0.02	1.33±0.20
ADPRP (NADPH)	0.16	0.22	0.14±0.02	0.10±0.002
ΣAMP	7.73	6.99	7.34±0.53	6.19±0.57
GTP	0.86	0.70	0.86±0.05	0.72±0.07
CTP	0.61	0.26	0.76±0.05	0.47±0.06

Table 2. *Effects of* D-*glucosamine, uridine, UDP and cytidine on the growth of human colon-carcinoma cells in culture*
Cells were seeded as described in the Materials and methods section 24h before replacement of the medium and addition of D-glucosamine (2.5mM) and/or pyrimidines. The initial glucose concentration was 5mM in all flasks. Changes of medium with the respective additions were made every 24h. Mean values from duplicate determinations, on average differing by 7%, or the means ± S.D. from six different flasks are given. Abbreviation: N.D., not determined.

Treatment	Cell number (% of control culture) Time in culture (days) ... 1	2	3
Controls	100	100 ± 9	100
+ Uridine (10 μM)	100	100	104
+ Uridine (100 μM)	N.D.	96 ± 10	N.D.
+ UDP (20 μM)	76	83	120
+ Cytidine (10 μM)	100	97	108
+ Cytidine (10 μM) + uridine (10 μM)	100	98	100
Glucosamine (2.5mM)	62	48 ± 10	43
+ Uridine (10 μM)	62	52	39
+ Uridine (100 μM)	N.D.	39 ± 12	N.D.
+ UDP (20 μM)	45	37	29
+ Cytidine (10 μM)	55	51	38
+ Cytidine (10 μM) + uridine (10 μM)	66	57	38

tissues has been obtained exclusively after addition of D-galactosamine (Maley *et al.*, 1968; Keppler, 1977), which follows a metabolic pathway different from that for D-glucosamine (Decker & Keppler, 1974).

The D-glucosamine-induced changes in metabolites should be related to the observed retardation of growth in culture. The uridine-reversal studies indicate that this effect can be attributed to the enhanced concentration of UDP-*N*-acetylhexosamines, and possibly also to the increase in UDP-glucuronate, rather than to decreases in UTP, UDP-hexoses and CTP in HT-29 cells. This imbalance in the pattern of UDP-sugars may well affect glycoconjugate synthesis. This latter change has been documented in other D-glucosamine-treated cells with subsequent alterations in membrane structure (Datema & Schwarz, 1979; Friedman & Skehan, 1980; Morin *et al.*, 1983; Chelibonova-Lorer *et al.*, 1983). It may be relevant that tunicamycin, an inhibitor of dolichol-dependent glycoconjugate synthesis, also enhanced UDP-*N*-acetylglucosamine pools several-fold, and that this inhibitor shared several of the effects of D-glucosamine in L1210 cells, including its antiproliferative action (Morin *et al.*, 1983).

We are indebted to Gisbert Weckbecker for this competent help in ion-pair reverse-phase h.p.l.c. This work was supported by grant CRL No. 81-70-21 from the Institut National de la Santé et de la Recherche Médicale (INSERM), Paris, France, and by the Deutsche Forschungsgemeinschaft through Forschergruppe Leber, Freiburg, Federal Republic of Germany.

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