EFFECT OF D-GLUCOSAMINE ON GROWTH AND SEVERAL FUNCTIONS OF CULTURED MASTOCYTOMA P-815 CELLS*

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In order to elucidate the biochemical basis for the selective cytotoxicity of D-glucosamine to neoplastic cells, the effect of glucosamine on the growth and several functions of mastocytoma P-815 cells were examined. Incubation of mastocytoma cells with 5 mM glucosamine resulted in a marked inhibition of growth and a significant reduction of cellular uptake and oxidation of glucose and of cellular levels of adenosine triphosphatase (ATP). Glucosamine also reduced the uridine nucleotide pool sizes, and accumulated uridine diphosphate (UDP)-N-acetylglucosamine. However, growth inhibition by glucosamine, which was reversed by glucose, was not prevented by exogenous uridine. In addition, glucosamine suppressed the phosphorylation of thymidine and its incorporation into deoxyribonucleic acid (DNA). The suppression of cell division by glucosamine was accompanied by the elevation of several functions of mastocytoma cells, including the accumulation of adenosine-3', 5'-monophosphate (cAMP), histamine, and serotonin. The incorporation of [³⁵S]SO₄²⁻ into acidic glycosaminoglycan was also increased. Of these functional alterations, the elevation of cAMP levels was the earliest detectable change, indicating that growth and functions of mastocytoma cells are also regulated by cAMP. However, glucosamine did not affect the adenylate cyclase activity of plasma membrane *in vitro*, suggesting the necessity of intact membrane structure for the action of glucosamine.

Keywords—Mastocytoma P-815 cells; glucosamine; pyrimidine biosynthesis; cAMP; cell growth and functions

In 1953 Quastel and Cantero¹⁾ reported that the daily administration of D-glucosamine (GlcN) resulted in a marked inhibition of the rate of growth of the tumor sarcoma 37 in mice. Since their original observation, growth-inhibiting and cytotoxic effects of GlcN were demonstrated on several neoplastic cells *in vivo*^{2,3)} and *in vitro*.^{4,5)} Although the mechanism of GlcN action was not fully elucidated yet, GlcN was shown to compete with glucose at several sites of the metabolic pathway in normal animal tissues^{6–9)} and tumor cells.¹⁰⁾ In addition, GlcN was known to suppress the biosynthesis of deoxyribonucleic acid (DNA),

ribonucleic acid (RNA), and protein,^{3,11,12)} and alter the cellular pool size of uridine nucleotides in several neoplastic cells^{3,12,13)} as well as in the normal rat liver.¹⁴⁾ Since adenosine also inhibited the growth and uridine nucleotide biosynthesis of mastocytoma P-815 cells,¹⁵⁾ we examined the effect of GlcN on the growth and functions of this neoplastic cell line. As reported below, the growth inhibition induced by GlcN was not prevented by exogenous uridine, although GlcN significantly affected the synthesis of uridine nucleotides in mastocytoma P-815 cells. GlcN also elevated the synthesis of histamine, serotonin,

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acidic glycosaminoglycan, and adenosine-3', 5'-monophosphate (cAMP).

EXPERIMENTAL PROCEDURES

Cells — Mastocytoma P-815 cells were maintained and grown in suspension culture in Fischer — Sartorelli's medium supplemented with 5% fetal calf serum (standard medium) as previously described.¹⁵⁾ Mast cells were collected from the peritoneal fluids of normal Wistar rats (250–350 g) of both sexes as described elsewhere.¹⁶⁾

Assay of Synthesis of RNA, DNA, and Protein — About 2×10^6 cells were incubated at 37° for the indicated periods of time in 0.5 ml of Dulbecco's phosphate buffered saline (PBS)¹⁷⁾ containing 0.5 mg of glucose, 20 mg of bovine serum albumin (BSA) and 1 μ Ci of [5- 3 H]-uridine, [methyl- 3 H]thymidine or [4,5- 3 H]-L-lysine. The reaction was terminated by the addition of 0.5 ml of cold 10% trichloroacetic acid (TCA). After centrifuging at $10000 \times g$ for 20 min, the TCA-insoluble fraction was washed three times with 1 ml of cold 5% TCA, mixed with 0.3 ml of NCS tissue solubilizer and 10 ml of a toluene-based scintillation mixture [5 g of 2,5-diphenyloxazole (PPO) in 1 l of toluene], and counted in a liquid scintillation counter.

In some experiments, in which the effect of GlcN on RNA synthesis was examined, both [5- 3 H]uridine and [8- 14 C]adenine were used as precursors, since GlcN may affect the intracellular uridine pool and thereby cause the isotope dilution effect. About 4×10^6 cells were incubated at 37° for 5 and 30 min in 1 ml of PBS containing 1 μ Ci of 3 H-uridine or 14 C-adenine. After incubation, the cells were washed three times with 2 ml of cold PBS resuspended in 1 ml of 5% TCA, and freeze-thawed three times. After the TCA-soluble fraction was separated by centrifugation at $3000 \times g$ for 20 min, the RNA fraction was obtained by fractionating the TCA-insoluble fraction according to the method of Schmidt and Thannhauser.¹⁸⁾ Aliquots of each fraction were counted in 0.4 ml of NCS tissue solubilizer and 10 ml of the toluene-based scintillant described above.

Assay of Glucose Uptake and CO_2 Production — In order to examine the effect of GlcN on both sugar uptake and release simultaneously, about 5×10^6 cells suspended in 0.5 ml of PBS were first incubated at 37° for 15 min with 0.1 mM [1- 3 H]2-deoxyglucose (2 μ Ci), and then for another 10 min with 0.1 mM [U- 14 C]glucose (1 μ Ci) in the presence or absence of various doses of GlcN or its N-acetyl derivative (GlcNAc). After incubation, cells were washed three times with 2 ml of cold PBS, mixed with 0.4 ml of NCS tissue solubilizer and 10 ml of the toluene scintillant, and counted for 14 C to estimate sugar uptake and for 3 H to measure sugar release. To measure the CO_2 production from glucose, 6×10^6 cells were suspended in 1 ml of PBS containing 0.01 mM [U- 14 C]glucose (0.2 μ Ci) with or without indicated doses of GlcN or GlcNAc. The mixture was incubated in a 2-ml brown short necked ampoule which was connected through a double sleeved rubber stopper to a 20-ml scintillation vial containing 0.2 ml of 0.5 N KOH. After the incubation at 37° for 60 min with gentle shaking, the reaction was terminated by injecting 0.1 ml of 70% HClO_4 through the rubber sleeve into the reaction mixture. After incubating for another 60 min, [14 C] CO_2 trapped in 0.5 N KOH was mixed with 1 ml of water, and counted in 15 ml of a toluene-based scintillant containing 33% by volume of Triton X-100.

Measurement of ATP and cAMP — ATP was measured by the luciferine-luciferase method of Bihler and Jeanrenaud,¹⁹⁾ and cAMP by the radioimmunoassay method of Okabayashi *et al.*²⁰⁾

Assay of Adenylate Cyclase and Phosphodiesterase — Mastocytoma P-815 cells (about 5×10^8) were homogenized in 5 volumes of 20 mM Tris-HCl (pH 7.8) containing 3 mM MgCl_2 , and centrifuged at $800 \times g$ for 10 min. After the supernatant fraction was further centrifuged at $5000 \times g$ for 20 min, the precipitate (crude plasma membrane) was resuspended in 1 ml of the same buffer, and assayed for the adenylate cyclase activity as previously described.²¹⁾ The assay mixture (1 ml) containing 75 mM Tris-HCl

(pH 7.4), 8 mM MgCl_2 , and 4 mM $[8\text{-}^3\text{H}]\text{ATP}$ (1 μCi) was incubated with enzyme at 37° for 15 min in the presence or absence of 5 mM GlcN, 10 mM NaF or 5 μM prostaglandin E_1 . The $5000\times g$ supernatant fraction was further centrifuged at $105000\times g$ for 60 min, and the supernatant fraction was assayed for the phosphodiesterase activity by a slight modification of the method of Boudreau and Drummond.²²⁾ The assay mixture (0.1 ml) containing 20 mM Tris-HCl (pH 7.8), 50 μM Ca^{2+} , 10 μM $[^3\text{H}]\text{cAMP}$ (0.05 μCi), and enzyme was incubated at 37° for 7 to 15 min. Protein was determined either with Folin-Ciocalteu reagent with bovine plasma albumin as standard or from the level of absorbance at 280 nm as described by Layne.²³⁾

Measurement of Histamine and Serotonin — Histamine and serotonin were measured by the fluorometric procedures described by Shore *et al.*²⁴⁾ and Snyder *et al.*,²⁵⁾ respectively. Histamine and serotonin formations were estimated by measuring the production of $[^{14}\text{C}]\text{histamine}$ from $[\text{ring-2-}^{14}\text{C}]\text{L-histidine}$ and that of $[^{14}\text{C}]\text{serotonin}$ from $[\text{methylene-}^{14}\text{C}]\text{L-tryptophan}$ as previously described.²¹⁾

Incorporation of $[^{35}\text{S}]\text{SO}_4^{2-}$ into Macromolecules — About 4×10^6 cells were incubated in 1 ml of PBS containing $[^{35}\text{S}]\text{SO}_4^{2-}$ (10 $\mu\text{Ci}/0.4$ mmol of H_2SO_4 , adjusted to pH 7.0 with 1 N NaOH) and 10 mg of glucose for 30 min at 37° . After incubation, cells were washed three times with 2 ml of cold PBS, homogenized in 2 ml of 0.04 M Tris-HCl (pH 7.4) in a glass homogenizer, and incubated with 10 mg of Pronase-M at 55° for 25 hours. After removing 5% TCA-insoluble fraction from the Pronase digest, ^{35}S -labeled acidic glycosaminoglycan was precipitated with cetylpyridinium chloride as previously described.²¹⁾ Washed precipitates were counted in 0.4 ml of NCS tissue solubilizer and 10 ml of the toluene-based scintillant.

Incorporation of Labeled Glucosamine — About 1.3×10^7 cells were incubated with $[1\text{-}^3\text{H}]\text{GlcN}$ (10 μCi) in 2 ml of the standard medium without glucose and serum at 37° for 60 min. Cells were washed three times with 5 ml of

cold PBS, and resuspended in 0.2 ml of distilled water. An aliquot (0.01 ml) was counted for the total radioactivity associated with whole cells in 10 ml of a dioxane-based scintillant (5 g of PPO and 100 g of naphthalene in 1 l of dioxane). Another aliquot (0.01 ml) was mixed with 200 mg of BSA in 0.05 ml of water and 1 ml of 7% TCA, and centrifuged at $700\times g$ for 2 min. The precipitates were counted in 10 ml of the dioxane-based scintillant for radioactivity associated with the acid-insoluble fraction. The radioactivity incorporated into the acid-soluble fraction was calculated by subtracting the value for the acid-insoluble fraction from that for whole cells.

For the isolation of GlcN and its derivatives in acid extracts, the remaining cell suspension (0.18 ml) was mixed with 0.01 ml of the carrier mixture solution containing 0.1 μmol each of GlcN, N-acetyl-D-glucosamine (GlcNAc), GlcN-6-phosphate (GlcN-6-P), GlcNAc-6-phosphate (GlcNAc-6-P), and uridine diphosphate-GlcN (UDP-GlcN), and then extracted with 2 ml of 70% ethanol acidified with HCl to pH 3.5 for 20 min at 4° . After centrifuging at $1000\times g$ for 15 min, the supernatant fraction was lyophilized, and subjected to descending chromatography on Whatman No. 1 paper for 14 hr in 1 M ammonium acetate (pH 5.0) : 95% ethanol (3 : 7, v/v).²⁶⁾ In order to separate GlcN-6-P from UDP-GlcNAc, a duplicate chromatogram was developed in saturated ammonium sulfate : 0.05 M phosphate (pH 6.0) : isopropanol (79 : 19 : 2, by volume).²⁶⁾ Developed chromatograms were cut into 1-cm wide pieces, and counted in 10 ml of the toluene-based scintillant.

Incorporation of $[^{14}\text{C}]\text{Uridine}$ into Acid-soluble Fraction — The method of Bekesi and Winzler³⁾ was used with a slight modification. Approximately 2.8×10^9 cells were incubated at 37° in 30 ml of PBS containing 5.4 mM glucose and 50 μCi of $[2\text{-}^{14}\text{C}]\text{uridine}$ with or without 20 mM GlcN. After 60-min incubation, cells were centrifuged at $110\times g$ for 3 min at 4° , immediately homogenized in 5 ml of 5% TCA in a Dounce homogenizer, and centrifuged at $20000\times g$ for 20 min. After the supernatant fraction was collected, the

residue was washed twice with 3 ml of 5% TCA, and washings were combined to the supernatant fraction. Combined TCA-soluble fractions were extracted five times with diethyl ether, if necessary, brought to pH 6.5 with 1 N NH_4OH , and then applied to a column (1 $\times$ 25 cm) of Dowex 1-X8 (formate form, 200 – 400 mesh). The column was eluted successively with 400 ml each of water, 4 N formic acid, 0.2 M ammonium formate in 4 N formic acid, 0.4 M ammonium formate in 4 N formic acid, and 1 M ammonium formate in 4 N formic acid. Fractions of 5 ml were collected, and the total optical density at 260 nm was determined. The radioactivity of 1-ml aliquot was counted in 10 ml of a Triton-based scintillant (5 g of PPO in 666 ml of Triton X-100 and 334 ml of toluene). Nucleotide fractions were combined and lyophilized. Individual nucleotides were purified by descending chromatography on Whatman No. 1 paper in isobutyric acid : concentrated ammonium hydroxide : water (57 : 4 : 39, by volume, pH 4.3; or 66 : 1 : 33, by volume, pH 3.7).¹³⁾ Nucleotides were identified by comparing their mobilities with those of co-chromatographed authentic compounds.

Materials — ATP, ADP, and adenosine were obtained from Kohjin Co., Hachiwoji, Tokyo, Japan; cAMP from Seishin Seiyaku Co., Noda, Chiba, Japan; Pronase M from Kakenyaku Co., Kyoto, Japan; firefly tail extract from Sigma Chemical Co., St. Louis, Mo., U.S.A.; fetal calf serum from Micobiological Associate, Bethesda, Maryland, U.S.A.; and NCS tissue solubilizer from Amersham Corporation, Arlington, Ill., U.S.A. [$1\text{-}^3\text{H}$]D-Glucosamine (20 Ci/mmol), [methyl- ^3H]thymidine (40 Ci/mmol), [$5\text{-}^3\text{H}$]uridine (26.7 Ci/mmol), [$4,5\text{-}^3\text{H}$]L-lysine (80 Ci/mmol), [$8\text{-}^3\text{H}$]cAMP (27 Ci/mmol), [$8\text{-}^3\text{H}$]ATP (26 Ci/mmol), [$1\text{-}^3\text{H}$]2-deoxyglucose (17 Ci/mmol), [$8\text{-}^{14}\text{C}$]adenine (54.2 mCi/mmol), [$2\text{-}^{14}\text{C}$]uridine (50 mCi/mmol), [$\text{U}\text{-}^{14}\text{C}$]D-glucose (260 mCi/mmol), [ring- $2\text{-}^{14}\text{C}$]L-histidine (50 mCi/mmol), and [methylene- ^{14}C]L-tryptophan (45 mCi/mmol) were purchased from Radiochemical Center, Amersham, England.

Carrier-free [^{35}S]H $_2\text{SO}_4$ was obtained from

Japan Atomic Energy Research Institute, Tokyo, Japan. Other chemicals of reagent grade were commercial products.

RESULTS

Effect of Glucosamine on Cell Division

As shown in Fig. 1, increasing concentrations of GlcN up to 10 mM progressively inhibited the cell division of mastocytoma P-815 cells, while D-mannosamine and D-galactosamine were less inhibitory, and GlcNAc was not inhibitory at all. Among several other monosaccharides tested, only D-mannose, which was reported to decrease the viability and transplantability of sarcoma 180, sarcoma 37, and Ehrlich ascites carcinoma cells by *in vitro* incubation,⁵⁾ showed some inhibitory effect. Although at concentrations higher than 10 mM, GlcN became extremely cytotoxic, the growth-inhibitory effect of 5 mM GlcN was

FIG. 1. *Effect of D-Glucosamine and Various Related Sugars on Cell Division of Cultured Mastocytoma P-815 Cells*

Mastocytoma P-815 cells (2×10^5 cells/ml, 30 ml) in 100-ml Erlenmeyer flasks were incubated with GlcN (●), D-mannosamine (○), D-galactosamine (□), GlcNAc (△) and D-mannose (×) at 37° for 20 hr. The per cent inhibition was calculated from the cell number in the control flask without sugars (6.7×10^5 cells/ml after 20 hr) and that in individual experimental flasks with sugars.

reversed by transferring treated cells to the fresh medium containing no glucose after washing, or by the concomitant addition of a high dose (20 mM) of glucose or pyruvate (Fig. 2A).

Effect of Glucosamine on Uridine Nucleotide Biosynthesis

FIG. 2. Effect of Glucose and Uridine on Cell Division of D-Glucosamine-treated (A) and AMP-treated Cells (B)

(A) Suspensions of mastocytoma P-815 cells (2×10^5 cells/ml, 30 ml) were incubated at 37° with (Δ) or without ($\circ$) 5 mM GlcN, with 5 mM GlcN plus 20 mM glucose ($\bullet$), or with 5 mM GlcN plus 10 mM uridine ($\square$). After 20 hr, portions of GlcN-treated cells were washed, transferred to the fresh medium, and further incubated at 37° for 20 hr with ($\blacktriangle$) or without ($\bullet$) GlcN.

(B) Suspensions of mastocytoma P-815 cells (0.5×10^5 cells/ml, 30 ml) were incubated at 37° with ($\bullet$) or without ($\circ$) 0.2 mM AMP, or with 0.2 mM AMP plus 10 mM uridine ($\square$). After 20 hr, a portion of AMP-treated cells were washed, transferred to the fresh medium without AMP, and further incubated at 37° for 20 hr ($\circ$ --- $\circ$).

As previously reported,¹⁵⁾ AMP also inhibited the cell division (Fig. 2B) and suppressed the uridine nucleotide biosynthesis. The inhibitory effect of AMP was reversed by transferring washed cells to the fresh medium or by the concomitant addition of uridine (10 mM). In contrast, the growth inhibitory effect of GlcN was not overcome by uridine (Fig. 2A), although GlcN significantly suppressed the uridine nucleotide synthesis from uridine. As shown in Fig. 3, GlcN

FIG. 3. Separation of Acid-soluble Nucleotides from Mastocytoma P-815 Cells incubated with ^{14}C -Uridine in the Absence or Presence of D-Glucosamine

Mastocytoma P-815 cells (2.8×10^9) were incubated with $[2-^{14}\text{C}]$ uridine ($50 \mu\text{Ci}$) at 37° for 1 hr in the absence (A) or presence (B) of 20 mM GlcN. The acid-soluble nucleotide fractions were chromatographed and characterized as described under experimental procedures. $\circ$ — $\circ$, absorbance at 280 nm; $\bullet$ — $\bullet$, cpm ($\times 10^2$).

FIG. 4. Effect of D-Glucosamine on the Incorporation of ^3H -Thymidine, ^3H -Uridine, ^{14}C -Adenine, and ^3H -Lysine into Acid-insoluble Fractions of Mast Cells and Mastocytoma P-815 Cells

(A): Mast cells (---) and Mastocytoma P-815 cells (—) (2×10^6 each) were incubated at 37° for 30 min in 1 ml of PBS with [methyl- ^3H]thymidine ($1 \mu\text{Ci}$) ($\circ$) and [4,5- ^3H]L-lysine ($1 \mu\text{Ci}$) ($\bullet$) in the presence of indicated doses of GlcN. $\circ$ ^3H -thymidine, $\bullet$ ^3H -lysine.

(B): As in (A), mast cells (----) and mastocytoma P-815 cells (—) were incubated with [5- ^3H]uridine ($1 \mu\text{Ci}$) ($\circ$) and [8- ^{14}C]adenine ($1 \mu\text{Ci}$) ($\bullet$) in the presence of indicated doses of GlcN. Incorporation of labeled compounds into TCA-insoluble fractions were determined as described under experimental procedures. Values obtained in the absence of GlcN were taken as 100% (control). Radioactivities (cpm/ 10^6 cells) incorporated into mastocytoma cells and mast cells in control experiments were: 3820 and 275 for ^3H -thymidine, 9820 and 1890 for ^3H -lysine, 2970 and 510 for ^3H -uridine, and 895 and 208 for ^{14}C -adenine, respectively. $\circ$ ^3H -uridine, $\bullet$ ^{14}C -adenine.

(20 mM) markedly suppressed the incorporation of ^{14}C -uridine into UMP, UDP, and UTP during 1-hr incubation at 37° , and increased the radioactivity associated with UDP-GlcNAc.

Effect of Glucosamine on Synthesis of Nucleic Acids and Protein

GlcN inhibited the incorporation of labeled thymidine and uridine (or adenine) into DNA and RNA fractions of mastocytoma P-815 cells, respectively, and also suppressed the incorporation of labeled lysine into the protein fraction (Fig. 4). Although two RNA precursors were used to avoid a possible dilution effect of GlcN on the uridine pool (Fig. 3), GlcN suppressed the incorporation of both precursors into RNA to a similar extent (Fig. 4B). In contrast, the macromolecule synthesis in normal mast cells of rats was not affected by 20 mM GlcN.

In the presence of increasing concentrations of GlcN, the total cellular uptake of ^3H -uridine during 5-min incubation at 37° was slightly elevated, and the radioactivity was about equally distributed between TCA-soluble and TCA-insoluble fractions (Table I). After a longer incubation (30 min) in the presence of increasing concentrations of GlcN, the incorporation of labeled uridine into TCA-insoluble fraction decreased more drastically than that into TCA-soluble fraction. The total incorporation also decreased.

Effect of Glucosamine on Glucose Transport and ATP Level

As shown in Fig. 5, the sugar release, as estimated by liberation of preincorporated [^3H]2-deoxyglucose, was completely blocked by 5 mM GlcN after about 40% release. No more release was observed in the presence of higher concentrations of GlcN. The sugar uptake was more markedly hindered than the sugar release by GlcN. The uptake was reduced to about 25% of the control level by 1 mM GlcN, and completely blocked by 5 mM GlcN.

GlcN also dose-dependently inhibited CO_2 -production from glucose in mastocytoma P-815 cells. At 5 mM, GlcN caused about 50% reduction of [^{14}C] CO_2 production from [U- ^{14}C]glucose (Fig. 6A). Furthermore, GlcN also dose

TABLE I. Effect of D-Glucosamine on the Incorporation of [^3H]Uridine into TCA-soluble and -insoluble Fractions of Mastocytoma P-815 Cells

D-Glucosamine concentration (mM)	TCA-soluble fraction		TCA-insoluble fraction	
	5 min	30 min	5 min (%) ^a	30 min (%) ^a
	($\times 10^4$ cpm/ 10^6 cells)			
0	0.30	0.45	0.28 (48.2)	1.10 (70.8)
1	0.98	1.03	0.90 (47.8)	0.95 (48.0)
5	0.42	0.75	0.35 (46.2)	0.64 (46.3)
10	0.41	0.50	0.41 (50.0)	0.38 (43.2)
20	0.52	0.50	0.40 (43.4)	0.32 (39.0)

Mastocytoma P-815 cells (2×10^6) were incubated with $1 \mu\text{Ci}$ of [^3H]uridine in 1 ml of PBS containing the indicated doses of GlcN for 5 and 30 min at 37° . Values were the mean of duplicate determinations. a) Percentage of the count in TCA-insoluble fraction over the total (TCA-soluble + -insoluble) count.

independently reduced the ATP level, and this reduction was completely prevented by the addition of 20 mM glucose (Fig. 6B). In contrast, GlcNAc had no ATP-depleting activity.

Incorporation of Glucosamine into Acid-soluble Components

When cells were incubated with increasing concentrations of [^3H]GlcN, the radioactivity associated with GlcN increased, and those with GlcN-6-P, GlcNAc-6-P, UDP-GlcNAc, and GlcNAc decreased (Table II). The net synthesis of each intermediate calculated from the specific activity also increased in the presence of increasing concentrations of GlcN. However, GlcN at 10 mM slightly suppressed the synthesis of GlcN-6-P and GlcNAc-6-P.

Effect of Glucosamine on Synthesis of Histamine, Serotonin, and Acidic Glycosaminoglycan

Pretreatment of mastocytoma P-815 cells with GlcN (5 mM, 37° for 20 hr) resulted in the elevation of cellular contents of histamine and serotonin as well as their formations (Table III). In addition, the incorporation of [^{35}S]SO $_4^{2-}$ into acidic glycosaminoglycan was also elevated 2- to 3-fold by GlcN. In a separate experiment, the stimulatory effect of GlcN on the sulfate uptake was observed within 1 hr of GlcN treatment. None of these stimulatory effects were observed with GlcN.

FIG. 5. Effect of D-Glucosamine on Cellular Uptake and Release of Glucose

Mastocytoma cells (2×10^6) were incubated with 0.1 mM [$1\text{-}^3\text{H}$]2-deoxyglucose ($2 \mu\text{Ci}$) at 37° for 15 min, and then with 0.1 mM [$\text{U-}^{14}\text{C}$] glucose ($1 \mu\text{Ci}$) and indicated doses of GlcN for another 10 min. Glucose release (● — ●) and uptake (○ — ○) were determined as described under experimental procedures. Values obtained in the absence of GlcN were taken as 100% (control).

FIG. 6. Effect of D-Glucosamine and Related Compounds on $^{14}\text{CO}_2$ Production from ^{14}C -Glucose (A) and on ATP Content (B)

(A): Mastocytoma P-815 cells (5.7×10^6) were incubated with 0.01 mM [^{14}C]glucose (0.2 μCi) and indicated doses of GlcN ($\circ$ — $\circ$) or GlcNAc ($\times$ — $\times$) for 60 min at 37°.

(B): Mastocytoma P-815 cells (3×10^6) were incubated with indicated doses of GlcN with ($\bullet$ — $\bullet$) or without ($\circ$ — $\circ$) 20 mM glucose, or with ($\times$ — $\times$) 20 mM GlcNAc for 30 min at 37°. $^{14}\text{CO}_2$ -Production and ATP content were determined as described under experimental procedures.

Effect of Glucosamine on cAMP Level and Activities of Adenylate Cyclase and Phosphodiesterase

Intracellular cAMP levels were elevated 3-fold over the control level by incubating the cells with GlcN for 10 min at 37° in the presence of 1-methyl-3-isobutylxanthine (MIX) (Table IV). In the absence of MIX, GlcN was ineffective. GlcNAc was ineffective even in the presence of MIX. Growth-suppressed cells cultured for 20 hr in the presence of 5 mM GlcN also contained much higher levels of cAMP than control cells. MIX also augmented the GlcN effect during this longer incubation.

However, 5 mM GlcN did not affect the adenylate cyclase activity in the crude plasma membrane preparation *in vitro*, although the enzyme activity was significantly stimulated by 10 mM NaF and 5 μM prostaglandin E_1 (Table V). Similarly, GlcN was not effective on the phosphodiesterase activity in the cytosol fraction in the presence of Ca^{2+} *in vitro*.

DISCUSSION

The present data obtained with mastocytoma P-815 cells mostly confirmed the demonstration by other investigators that GlcN was cytotoxic to neoplastic cells,¹⁻⁵ reduced the cellular pool size of uridine nucleotides^{3,5,12,13} and inhibited the synthesis of macromolecules^{3,5,11,12} in these cells.

Although the mechanism of selective cytotoxicity of GlcN toward neoplastic cells has not been fully elucidated yet, GlcN has been reported to affect the cellular utilization of glucose in various ways. GlcN is known to compete with glucose for the entry into cells of insulin-sensitive extrahepatic tissues^{6,7,27} as well as cultured Novikoff rat hepatoma cells,²⁸ inhibit glucose phosphorylation by non-specific hexokinases,⁸ suppress glycogen synthesis,⁹ reduce glycolysis in tumor cells, reverse the Crabtree effect induced by glucose or induce the Crabtree effect like glucose,^{29,30} and decrease glucose oxidation.⁶ These results as well as our demonstration that GlcN suppressed glucose uptake by mastocytoma P-815 cells and accelerated glucose release (Fig. 5), all indicated that the inhibition of glucose metabolism induced

by GlcN resulted in the reduction of cellular ATP levels (Fig. 6) and the subsequent decrease of macromolecular synthesis (Fig. 4, Tables I and III). However, Bosmann¹²⁾ reported that ATP at all levels tested (10 to 100 mM) was extremely inhibitory to macromolecular synthesis in mouse leukemic cells with "leaky" plasma membranes. Therefore, the decrease in ATP levels alone cannot fully explain the mechanism of the cytotoxic effect of GlcN on tumor cells. Furthermore, both

glucose and pyruvate are reported to reduce only slightly the cytotoxic effect of GlcN on Ehrlich ascites carcinoma cells⁵⁾ and not to reverse the inhibition by GlcN of macromolecular synthesis in Walker carcinosarcoma 256, sarcoma 180 and C3H spontaneous mammary tumor.¹¹⁾

In contrast to the cytotoxicity of adenosine or AMP,¹⁵⁾ the inhibition induced by GlcN of cell division of mastocytoma P-815 cells (Fig. 2) was not reversed by exogenous uridine. According to

TABLE II. *Distribution of Glucosamine Derivatives in Mastocytoma P-815 Cells Incubated with Various Concentrations of [³H]D-glucosmine*

D-Glucosamine concentration (mM)	GlcN	GlcN-6-P	GlcNAc-6-P	UDP-GlcNAc	GlcNAc
		(nmol/10 ⁶ cells)			
0	0.0042	0.0057	0.005	0.0177	0.0008
0.1	0.35	1.36	0.9	1.7	0.343
1.0	4.5	13.4	27.3	3.0	2.1
5.0	31.5	71.5	51.0	7.0	9.5
10.0	295.5	43.5	41.3	17.6	—

Cells (1.3×10^7) were incubated with 10 μ Ci of [³H]GlcN in 1 ml of PBS containing the indicated doses of GlcN for 60 min at 37°. GlcN derivatives in acid-soluble fraction of the cells were separated by paper chromatography and counted as described under experimental procedures. Net amounts (nmol/10⁶ cells) were calculated from their specific activities.

TABLE III. *Effect of D-Glucosamine on Content and Synthesis of Histamine, Serotonin, and Acidic Glycosaminoglycan in Mastocytoma P-815 Cells*

Treatment	Histamine		Serotonin		Acidic glycosaminoglycan
	Content (nmol)	Synthesis (pmol)	Content (nmol)	Synthesis (pmol)	Synthesis (cpm)
				per 10 ⁷ cells	
None	8.3	114	1.78	13.5	3200
D-Glucosamine (5 mM)	19.5	385	2.45	46.5	5800

Mastocytoma P-815 cells in the logarithmic phase of growth were incubated with 5 mM GlcN for 20 hr at 37°. After washing with cold PBS, cells (2×10^7) were incubated with [ring-2-¹⁴C]L-histidine (5 μ Ci) or [methylene-¹⁴C]L-tryptophan (2.5 μ Ci) for 60 min at 37° in 3 ml of PBS containing 5.4 mM glucose as described under experimental procedures. The incorporation of ³⁵S into acidic glycosaminoglycan was estimated by incubating the cells (4×10^6) with [³⁵S]SO₄²⁻ (10 μ Ci) in 1 ml of PBS containing 5.4 mM glucose for 30 min at 37°. Values are the mean of duplicate determinations.

Bosmann,¹²⁾ the inhibition of macromolecular synthesis by GlcN in mouse leukemic cells could be reversed by the addition of uridine nucleotides, but not uracil or uridine. These results indicate that the selective cytotoxicity of GlcN to tumor cells may be attributed to its inhibitory effect on the salvage pathway of pyrimidine synthesis. It is well known that the salvage pathway operates frequently at an accelerated rate in rapidly growing normal and malignant tissues, while this pathway is of secondary importance in normal tissues. The involvement of the inhibition of the

pyrimidine salvage pathway in GlcN cytotoxicity was also suggested by Friedman *et al.*^{32,33)} They observed that the activity of thymidine kinase, the initial enzyme in the thymidine salvage pathway in rat C6 glioma cells was considerably inhibited by preincubation with GlcN.³²⁾ Furthermore, exogenous GlcN apparently suppressed the utilization of thymidine for DNA synthesis by reducing the cellular uptake of thymidine and by elevating its release from the cell.³³⁾ The decrease of thymidine phosphorylation (Fig. 3) and thymidine incorporation into

TABLE IV. *Effect of D-Glucosamine on cAMP Level of Mastocytoma P-815 Cells*

Treatment		cAMP (pmol/10 ⁶ cells)	
		– MIX	+ MIX
Experiment A (10 min)			
None		0.39±0.03	0.54±0.04
D-Glucosamine	5mM	0.29±0.04	1.08±0.07
	10mM	0.29±0.03	1.69±0.06
N-Acetyl D-Glucosamine	5mM	0.24±0.06	0.59±0.03
	10 mM	0.26±0.02	0.56±0.08
Experiment B (20 hr)			
None		0.42±0.04	0.66±0.07
D-Glucosamine	5mM	1.98±0.10	3.78±0.05

A: Cells (5.5×10^6) were incubated with indicated doses of GlcN and GlcNAc, with or without 0.2 mM MIX at 37° for 10 min. cAMP was assayed as described under experimental procedures. Values are the mean of triplicate determinations ± S.E.

B: Cells (3×10^6) incubated for 20 hr with 5 mM GlcN were harvested, washed with cold PBS, and then assayed for cAMP in the presence or absence of MIX. Values are the mean of triplicate determinations ± S.E.

TABLE V. *Effect of D-Glucosamine on Adenylate Cyclase and Phosphodiesterase from Mastocytoma P-815 Cells in Vitro*

Addition		Adenylate cyclase	Phosphodiesterase
		μmol cAMP/mg/min	μmol cAMP/mg/min
None (Control)		0.881±0.072	0.42 ±0.036
D-Glucosamine	(5 mM)	1.02 ±0.054 (N.S.)	0.452±0.047 (N.S.)
NaF	(10 mM)	8.25 ±0.21 (<i>p</i> <0.05)	—
PGE ₁	(5 μM)	5.03 ±0.79 (<i>p</i> <0.05)	

The activities of adenylate cyclase of crude plasma membrane preparation and cAMP phosphodiesterase in cytosol were assayed as described under experimental procedures. Values are the mean of triplicate determinations ± S.E.

DNA fraction (Fig. 4) of mastocytoma P-815 cells also indicated the importance of the salvage pathway for the selective cytotoxicity of GlcN, supporting the suggestion by Friedman *et al.*

In contrast to GlcN, GlcNAc did not affect the cell division, the cellular levels of ATP and cAMP, or the formation of histamine, serotonin, and acidic glycosaminoglycan of mastocytoma P-815 cells. This is probably partially due to a difference in the permeability of the cells to these two compounds, as suggested by Bekesi *et al.* for non-cytotoxicity of GlcNAc to ascites tumor cells.⁵⁾ The difference in the metabolism of GlcN and GlcNAc, *i.e.*, higher rate of oxidation, incorporation into glycoprotein, and excretion of GlcNAc than that of GlcN reported in rats,³⁴⁾ and preferential formation of GlcNAc instead of GlcN-6-P from GlcN in the presence of glucose observed in rat liver³⁵⁾ do not seem to sufficiently explain the selective cytotoxicity of GlcN.

As in the case of growth inhibition by adenosine and AMP,¹⁵⁾ and by Bt₂cAMP, prostaglandins and other agents,²¹⁾ the suppression of cell division induced by GlcN was accompanied by the alteration of several functions of mastocytoma P-815 cells, including the accumulation of cAMP, histamine, serotonin, and UDP-GlcNAc, a substrate for the synthesis of heparin-like mucopolysaccharide.³⁶⁾ GlcN also stimulated the incorporation of [³⁵S]SO₄²⁻ into acidic glycosaminoglycan of mastocytoma cells (Table III). A similar stimulation of [³⁵S]SO₄²⁻ incorporation into acidic mucopolysaccharides by Bt₂cAMP was also reported in SV40 transformed fibroblasts by Goggins *et al.*³⁷⁾ Of these functional changes, the elevation of cellular cAMP levels was one of the early detectable effects of GlcN in mastocytoma P-815 cells (Table IV). GlcN may act directly upon adenylate cyclase, since the stimulatory effect of GlcN on cAMP levels in intact cells was detectable only in the presence of MIX. However, GlcN neither affected activity of adenylate cyclase of the crude plasma membrane preparation nor the activity of phosphodiesterase of cytosol *in vitro* (Table V). These results indicate that GlcN may affect the cAMP synthesis

either by activating adenylate cyclase only when the enzyme is present in intact cells, as observed for the action of histamine and norepinephrine on cAMP accumulation in brain tissues,^{38,39)} or by altering the level or turnover rate of ATP available for cAMP synthesis after its incorporation into the cell. This point certainly requires further investigation.

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