Transport and Metabolism of Glucosamine by Cultured Novikoff Rat Hepatoma Cells and Effects on Nucleotide Pools

Peter G. W. Plagemann and John Erbe

Department of Microbiology, Medical School, University of Minnesota, Minneapolis, Minnesota 55455

SUMMARY

As estimated from initial uptake rates, the uptake of glucosamine-1-14C by cultured Novikoff rat hepatoma cells in glucose-free basal medium follows normal Michaelis-Menten kinetics, with an apparent K_m of about 20 mM. Since glucosamine is not accumulated by the cells against a concentration gradient, the main mode of uptake is by facilitated diffusion, but other evidence indicates that glucosamine also enters the cells by simple diffusion. Glucosamine is probably transported by the same system as glucose. Glucosamine transport is competitively inhibited by glucose and, to about the same extent as is glucose transport, by Persantine and Cytochalasin B. Since neither of the two inhibitors affects the phosphorylation of glucose or glucosamine by cell-free preparations, we suggest that transport is a reaction distinct from phosphorylation. At low concentrations of glucosamine in the medium (0.1 mM and below), the inhibition of glucosamine transport into the cell by glucose, Persantine, or Cytochalasin B results in a proportional decrease in glucosamine incorporation into macromolecules. When cells are incubated with low concentrations of glucosamine-1-14C, most of the acid-soluble intracellular radioactivity is associated with uridine diphospho-N-acetylglucosamine, and, after a 30- to 60-min lag period, glucosamine enters glycoproteins and glycolipids at a rapid and constant rate. With an increase in glucosamine concentration in the medium, an increasingly greater proportion of the intracellular radioactivity accumulates in N-acetylglucosamine 6-phosphate, glucosamine 6-phosphate, and free glucosamine (in that order), and a progressively smaller proportion is incorporated into macromolecules. This inhibition of glucosamine incorporation into macromolecules at high glucosamine concentrations is correlated with a lack of uridine diphospho-N-acetylglucosamine formation and an inhibition of macromolecular synthesis. Both effects seem to result from a rapid loss of adenosine triphosphate and uridine triphosphate during incubation of the cells with high concentrations of glucosamine, which loss is caused by the rapid phosphorylation of the glucosamine taken up by the cells. The nucleotides become degraded to nucleosides and bases and are released into the culture fluid. The inhibition of protein synthesis by treatment of the cells with puromycin or cycloheximide also results in an inhibition of glucosamine incorporation into macromolecules without affecting the uptake of glucosamine or its conversion to uridine diphospho-N-acetylglucosamine.

INTRODUCTION

Glycoproteins are involved in many important and diverse cellular processes (42). They represent the major structural proteins of cellular membranes (4, 5, 9, 12, 18, 39) and of the lipoprotein envelopes of many animal viruses (7, 10, 14, 22, 38).

A useful way to identify these glycoproteins is to label them specifically by allowing the cells to incorporate radioactively labeled glucosamine. Glucosamine is a specific precursor of GlcNAc, N-acetylgalactosamine, and sialic acid (9, 16, 17, 19, 20), which are major carbohydrate components of glycoproteins. It is not significantly incorporated into other types of macromolecules (4, 16, 21, 35) except, to a variable extent, into glycolipids (4, 9, 13). However, glucosamine has a toxic effect on tumor cells (1, 13, 32), and exposure of cells or animals to glucosamine can result in a depletion of their UTP pool, apparently due to an excessive formation of UDP-amino sugars (2, 3, 15, 16, 36, 40). The ATP pool may also become depleted (2, 40). To gain further insight into the relationship between the various effects of glucosamine, we have investigated the mode of uptake of glucosamine by cultured Novikoff rat hepatoma cells and the relationship between the rate of uptake of glucosamine, its incorporation into macromolecules, and its effect on levels of adenine and uracil nucleotides. Cell cultures have an advantage over tumor cells grown in animals and over whole animals (used in most previous studies), in that they represent growing homogeneous cell populations, and the various effects can be analyzed under physiological conditions.

MATERIALS AND METHODS

Materials were purchased as follows. D-Glucosamine- HCl-1-14C, adenosine-8-3H, uridine-5-3H, protein hydrolysate-3H, and unlabeled nucleosides were from Schwarz/Mann, Orangeburg, N. Y.; unlabeled D-glucosamine-HCl, UDP-Glc, UDP-GlcNAc, GlcN-6-P, and Glc6-P were from the Sigma

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Chemical Co., St. Louis, Mo.; puromycin was from the Nutritional Biochemical Corp., Cleveland, Ohio; and cycloheximide (Actidione) was from Calbiochem, Los Angeles, Calif. Solutions of labeled substances with lower concentrations of radioactivity and lower specific radioactivities were prepared by the addition of appropriate unlabeled substances and 0.14 M NaCl. No significant contamination of the glucosamine-1-14C by glucose-14C could be detected by analysis in a Beckman amino acid analyzer showed that 5 to 10% of the radioactivity was associated with 2 or 3 components distinct from glucosamine which eluted close together, and under about the same conditions as with glucose. These components have not been identified further but were prepared by the addition of appropriate unlabeled substances.

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Frequently tested for contamination by Mycoplasma with the medium by N1S1-67 cells at about the same time as was glucosamine-1-14C. Cytochalasin B was kindly provided by Dr. R. D. Estensen (6), and 2,6-bis(diethanolamino)-4,8-dipiperidinopyrimido(5,4-d)-pyrimidine (Persantine) was a gift from Geigy Pharmaceuticals, Yonkers, N. Y.

Cell Culture. The cultivation of Novikoff rat hepatoma cells (subline N1S1-67) in a suspension culture in Swim's Medium 67 has been described previously (30, 41). Cultures were frequently tested for contamination by Mycoplasma with negative results. Cells were harvested from exponential phase cultures (2.0 to 2.8 X 10^6 cells/ml) and suspended in glucose-free BM42 or glucose-free HEPES-BM42, in which the bicarbonate was replaced by 5 mM HEPES (30, 34). BM42 is of the same composition as Swim's Medium 67 except that the calf serum and pancreatic autolyzates (30) are omitted.

Incorporation of Labeled Glucosamine or Nucleosides. Suspensions of cells were supplemented with labeled precursors as indicated in the appropriate experiments, and were incubated on a gyratory shaker at 37°. Duplicate samples analyzed for radioactivity in total cell material (acid-soluble plus acid-insoluble) and in acid-insoluble material (macromolecules), as described previously (24, 29). We measured the conversion of glucosamine-1-14C to 14CO2 by incubating 10-ml samples of cell suspension in 14CO2 collection flasks (34). The acid-soluble pools were extracted from labeled cells with perchloric acid at 0° (24).

The lipids were extracted from perchloric acid-washed cell pellets with chloroform:methanol (2:1, v/v), and the extracts were evaporated to dryness. The residue and the lipid-extracted cell pellet each were suspended in a small volume of 1 N HCl. The suspensions were heated at 100° for 1 to 4 hr, neutralized by addition of 5 N KOH, and clarified by centrifugation.

Nucleotides, nucleosides, and glucosamine and its various derivatives in acid extracts or acid hydrolysates were separated by ascending chromatography on Whatman No. 3MM paper (24). The paper was developed with a solvent composed of 79 ml saturated ammonium sulfate, 19 ml 0.05 M phosphate buffer (pH 6.0), and 2 ml isopropyl alcohol at room temperature for 15 hr (Chart 1, G and H). Developed chromatograms were cut into 1-cm segments at right angles to the direction of migration. The segments were eluted with water, and the eluates, together with the paper, were analyzed for radioactivity.

Radioactivity Determinations. Radioactivity was measured by liquid scintillation counting with a modified Bray's solution (30).

RESULTS

Time Course of Glucosamine Incorporation into Acid-soluble Pool and Macromolecules as a Function of Glucosamine Concentration. As reported previously (8), glucosamine-14C was rapidly incorporated by N1S1-67 cells into the acid-soluble pool and was incorporated more slowly into macromolecules. The time courses of incorporation of glucosamine into total cell material (acid-soluble plus acid-insoluble) and into acid-insoluble material at 4 substrate concentrations are illustrated in Chart 1, A to D. Label appeared in acid-insoluble material only after a short lag period. The relative proportion of the total radioactivity incorporated into acid-insoluble material by the cells became progressively lower as the glucosamine concentration became higher. At higher glucosamine concentrations, the absolute amounts incorporated into acid-insoluble material were actually depressed. For example, after 180 min of labeling with 1 mM glucosamine, 10^6 cells had incorporated approximately 32 nmols into acid-insoluble material. The corresponding values were 10 and 5 nmols/10^6 cells with 5 and 50 mM glucosamine, respectively.

After 3 hr of labeling with 5 µM (8) or 100 µM glucosamine-14C (Chart 1A), 20 to 30% of the radioactivity in acid-insoluble material was extractable with chloroform:methanol. Over 80% of the radioactivity in the lipid extracts and over 90% of the radioactivity in the lipid-free residues was released in acid-soluble form after heating at 100° for 1 to 4 hr in 1 N HCl; 75 to 85% of the released radioactivity was associated with glucosamine and GlcNAc (mainly glucosamine). A progressively greater proportion of the released radioactivity was recovered in glucosamine with an increase in the period of hydrolysis from 1 to 4 hr. The remainder of the released radioactivity was associated with a Component (X), that migrated at about the same rate as GlcN-6-P in Solvent 28 but was not identified further. The results indicate that, during incubation with 5 to 100 µM glucosamine-14C, most of the glucosamine was incorporated into the GlcNAc moiety of glycoproteins and glycolipids; about 70 to 80% was incorporated into glycoproteins, and the rest was incorporated into glycolipids. On the other hand, with 1 mM and higher concentrations of glucosamine, up to 50% of the total radioactivity associated with acid-insoluble material was recovered in the lipid extract, and an increasingly greater proportion of the radioactivity was not released by hydrolysis in 1 N HCl. Furthermore, only about 50% of the radioactivity released by acid hydrolysis was associated with glucosamine, and the remainder was associated primarily with Component X. Thus, the incorporation of glucosamine into glycoproteins and glycolipids with high glucosamine concentrations in the medium was even more depressed, in total acid-insoluble material, than was indicated.
Chart 1. Time course of glucosamine-\(^{14}\)C incorporation by N1S1-67 cells as a function of glucosamine concentration in the medium. Samples of a suspension of 4 x 10^6 cells/ml of glucose-free HEPES-BM42 were supplemented with 2 \(\mu\)M glucosamine-\(^{14}\)C (200,000 cpm/ml) and unlabeled glucosamine to the indicated final concentrations. A 10-ml sample of each suspension was incubated in a \(^{14}\)CO\(_2\) collection flask at 37\(^\circ\) for 180 min and then was analyzed for radioactivity in CO\(_2\). The remainders of each suspension were incubated on a gyratory shaker at 37\(^\circ\), and duplicate 0.5-ml samples of each suspension were analyzed for radioactivity in total cell material or acid-insoluble material. Points, averages of the duplicate samples (A to D). At 90 and 180 min of incubation, acid extracts were prepared from samples of 2 x 10^7 cells and 50-\(\mu\)l samples of each acid extract were chromatographed with Solvents 28 and 9 (Table 1). E to H, composites of the radioactivity profiles of the 90-min acid extracts.

by the analysis of radioactivity total acid-soluble material (Chart 1, C and D). This inhibition probably resulted from both a lack of formation of UDP-GlcNAc and a general inhibition of macromolecular synthesis at high glucosamine concentrations (see below).

Thus, with an increase in glucosamine concentration in the medium, a progressively larger proportion of the intracellular radioactivity accumulated in the intracellular acid-soluble pool (difference between total radioactivity in the cell and radioactivity in acid-insoluble material; Chart 1, A to D). The pools were extracted with perchloric acid, and the acid-soluble components were separated by paper chromatography with Solvents 28 and 9. Representative chromatograms are illustrated in Chart 1, E to H. With Solvent 28, GlcNAc-6-P, glucosamine, GlcNAc, and an additional component close to the origin were well separated. The latter has not been identified unequivocally, but it may be mainly ATP, since its migration correlated with that of ATP in both Solvent 28 and 9, and it was adsorbed by activated charcoal. Furthermore, when N1S1-67 cells are incubated with 10 to 50 \(\mu\)M-glucose-\(^{14}\)C, about 50% of the radioactivity in the acid-soluble pool is associated with the ribose moiety of nucleoside triphosphates, mainly ATP (34). UDP-GlcNAc and GlcN-6-P, however, migrated together in Solvent 28 (Chart 1, E and F) and were separated by chromatography with Solvent 9 (Chart 1, G and H). From the combined chromatographic
analyses of each acid extract with Solvent 28 and 9, we have estimated the relative proportion of radioactivity associated with these intracellular components. The results were similar for acid extracts prepared from cells after 90 and 180 min of incubation with glucosamine-\(^{14}\)C, and the observed ranges of values are summarized in Table 1. The data show that the relative distribution of radioactivity among the acid-soluble intracellular components varied markedly with the glucosamine concentration in the medium. With the use of 0.1 mM glucosamine (Table 1) and lower concentrations (not shown), most of the radioactivity in the acid-soluble pool was associated with UDP-GlcNAc. Little, if any, free labeled glucosamine was present intracellularly, indicating that the glucosamine became phosphorylated as rapidly as it entered the cells. At 1 mM, the greatest proportion of radioactivity was associated with GlcNAc-6-P and, at 5 mM, it was associated with GlcN-6-P, whereas at 50 mM most of the intracellular radioactivity represented free glucosamine. Little, if any, label was present in UDP-GlcNAc when the cells were incubated with 50 mM glucosamine, probably because incubation of the cells with these high concentrations of glucosamine resulted in a rapid depletion of the nucleoside triphosphate pools (see below).

From the combined data in Chart 1 and Table 1, we estimate that the cells incubated with 5 or 50 mM glucosamine accumulated 40 and 70 nmoles of phosphorylated glucosamine per 10\(^6\) cells during 180 min of incubation. This is far in excess of the total amount of nucleoside triphosphates present in exponentially growing N1S1-67 cells [10 to 15 nmoles/10\(^6\) cells (27)].

**Glucosamine Conversion to CO\(_2\) and Lactate.** It has been reported that various tumor cells convert glucosamine to lactate, CO\(_2\), and glycogen at about one-half the rate at which they metabolize glucose (40), whereas, in liver, glucosamine serves almost exclusively for the formation of glycoproteins (20, 35). On the other hand, the production of \(^{14}\)CO\(_2\) by N1S1-67 cells incubated with glucosamine-\(^{1-14}\)C depended greatly on the glucosamine concentration in the medium. At concentrations of 2 µM (Chart 7) or 4 µM (not shown), the amount of \(^{14}\)CO\(_2\) produced was equivalent to only about 2 to 3% of the amount of glucosamine incorporated into total cell material. This \(^{14}\)CO\(_2\) could have been derived from the small amounts of glucose-\(^{14}\)C that probably contaminated the glucosamine-\(^{14}\)C. The \(^{14}\)CO\(_2\) produced at 100 µM glucosamine, on the other hand, was probably not derived solely from contaminating glucose-\(^{14}\)C, since it was produced at 60 to 70% of the rate at which glucosamine was incorporated into total cell material (Chart 1A). At higher concentrations of glucosamine, CO\(_2\) production again became depressed (Chart 1, B to D). We have not determined whether labeled CO\(_2\) was released from glucosamine-\(^{1-14}\)C in the pentose pathway or in the tricarboxylic acid cycle. The former is probably the case, since chromatographic analysis of the cell culture fluid showed that a significant conversion of glucosamine-\(^{1-14}\)C to lactate did not occur at any of the glucosamine concentrations investigated. Approximately 100% of the glucosamine-\(^{14}\)C lost from the culture fluid during 180 min of incubation was accounted for by the radioactivity in total cell material plus CO\(_2\).

**Kinetics of Glucosamine Transport and Uptake by Simple Diffusion.** The results in Chart 1, A to D, also indicate that the rates of glucosamine incorporation into total cell material were approximately constant for at least the first 5 to 10 min of incubation. At low concentrations, glucosamine incorporation continued at a relatively constant rate for a prolonged period (Charts 1A and 5), apparently because glucosamine was rapidly phosphorylated as it entered the cells and thereby trapped. This is indicated by the finding that cells incubated with up to 1 mM glucosamine did not contain significant amounts of free glucosamine (Chart 1E). With higher glucosamine concentrations in the medium at which some free glucosamine accumulated intracellularly, the initial rates of glucosamine incorporation into total cell material began to decrease rapidly (Chart 1, C and D).

The Lineweaver-Burk plot (Chart 2B) of the initial rates of incorporation of glucosamine into total cell material as a function of glucosamine concentration in the medium (Chart 2A) indicates that glucosamine incorporation followed normal Michaelis-Menten kinetics. This implicates the involvement of a catalyzed reaction. The failure of glucosamine to accumulate intracellularly, when present at low concentrations in the medium, suggests that the rate-limiting step in glucosamine

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**Table 1**

**Distribution of radioactivity among intracellular acid-soluble components after incubation of cells with various concentrations of glucosamine-\(^{14}\)C**

Details of the experiment are given in the legend to Chart 1. Acid extracts were prepared from samples of 2 x 10\(^7\) cells after 90 and 180 min of incubation with the indicated concentrations of glucosamine-\(^{14}\)C. Samples of 50 µl of each acid extract were chromatographed with both Solvent 28 and 9 (see Chart 1E to H). The relative proportion of radioactivity associated with the indicated components was estimated from the combined chromatographic profiles obtained with the 2 solvent systems. The radioactivity was similarly distributed in acid extracts from 90- and 180-min-labeled cells, and the range of values observed in duplicate chromatographic analyses of each acid extract is presented.

<table>
<thead>
<tr>
<th>Glucosamine (mM)</th>
<th>% total acid-soluble (^{14})C in</th>
<th>ATP</th>
<th>UDP-GlcNAc</th>
<th>GlcNAc-6-P</th>
<th>GlcN-6-P</th>
<th>GlcN</th>
<th>GlcNAc</th>
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<tr>
<td>0.1</td>
<td>10-15</td>
<td>65-70</td>
<td>6-8</td>
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<td>14-18</td>
<td>45-50</td>
<td>28-33</td>
<td>2-4</td>
<td>2-4</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0-2</td>
<td>3-4</td>
<td>25-30</td>
<td>45-50</td>
<td>13-16</td>
<td>4-8</td>
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<td>50</td>
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<td>0-2</td>
<td>12-15</td>
<td>25-30</td>
<td>48-55</td>
<td>3-6</td>
<td></td>
</tr>
</tbody>
</table>
Chart 2. Initial rates of glucosamine incorporation at 37° as a function of concentration. In A, portions of a suspension of 1 X 10^7 cells/ml of glucose-free BM42 were supplemented with 2 μM glucosamine-1^14C (200,000 cpm/ml) and unlabeled glucosamine to 5, 7, 10, 20, 50, and 100 mM. After 5 min of incubation at 37°, duplicate 1-ml samples of suspension were analyzed for radioactivity in total cell material. The initial velocities (V) of uptake were estimated from these values. B, Lineweaver-Burk plot of the initial velocities estimated in A.

incorporation was its transport into the cell. Additional evidence presented subsequently and studies with KCN-pretreated cells support these conclusions. Since KCN-treated cells were depleted of nucleoside triphosphates (28), they failed to phosphorylate glucosamine, and glucosamine uptake could thus be measured directly. By incubation of KCN-pretreated cells at 18°, approximate initial rates of uptake could be obtained with various concentrations of glucosamine in the medium (Chart 3A). Chromatographic analysis of acid extracts showed that over 90% of the intracellular radioactivity was associated with free glucosamine (not shown). The Lineweaver-Burk plot of the 1-min uptake values (Chart 3C) shows that glucosamine uptake by the KCN-pretreated cells also followed normal Michaelis-Menten kinetics and that the V_max and K_m values were about the same as those based on the incorporation of glucosamine into total cell material (Chart 2B).

The data also show that glucosamine was not transported against a concentration gradient, indicating that uptake was by facilitated diffusion. Glucosamine uptake by KCN-pretreated cells ceased rapidly (Chart 3A), and the maximal intracellular concentrations of free glucosamine were well below those in the medium. For instance, based on an average overall cell volume of about 20 μl/10^7 cells (31), the total concentration of glucosamine inside the cells reached a maximum of about 1.5 mM when the cells were incubated in medium that contained 5 mM glucosamine. The maximal levels of glucosamine inside the cells increased linearly with an increase in the extracellular concentration, but in less than direct proportionality (Chart 3B). These results resemble those obtained with uridine (28). The maximal intracellular amounts of free glucosamine that accumulated intracellularly upon incubation of cells with 5 or 50 mM glucosamine at 37° (Chart 1; Table 1) were also appreciably less than the extracellular concentrations.

Although at low concentrations the kinetics of glucosamine uptake indicated entrance by facilitated diffusion, at concentrations above 50 mM, glucosamine entered the cells more rapidly than was expected from the transport rates (Chart 2A), and the Lineweaver-Burk lines showed deflections towards the origin (Charts 2B and 3C). In view of our previous findings with various nucleosides (23, 24), glucose (34), and choline (26), this result is consistent with the view that glucosamine also entered the cells by simple diffusion. Because of the very high apparent K_m for glucosamine transport (about 20 mM), the exact contribution of facilitated and simple diffusion to the overall rate of glucosamine uptake is difficult to determine. The broken line through the origin and parallel to the linear portion of the rate curve in Chart 2A represents a rough estimate of the rate of uptake due to simple diffusion. Furthermore, because simple diffusion occurred at a significant rate at the concentrations used for the kinetic analysis of glucosamine transport, the V_max and K_m values are probably somewhat overestimated (34).

Inhibition of Glucosamine Transport by Glucose, Persantine, and Cytochalasin B. It has been observed that the

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Inhibition of Glucosamine Transport by Glucose, Persantine, and Cytochalasin B. It has been observed that the
toxic effect of glucosamine on tumor cells (2, 40) and the incorporation of glucosamine-\(^{14}\)C into glycoproteins (20) are prevented by the presence of glucose in the medium. The effect of glucose has been attributed to a competition between glucose and glucosamine for hexokinase (20). In contrast, our data indicate that glucose inhibits glucosamine incorporation by N1S1-67 cells (Chart 4) by interfering with its transport into the cell rather than with its intracellular phosphorylation and further metabolism. A comparison of the data in Chart 4, A and B, shows that the incorporation of glucosamine into both total cell material and acid-insoluble material was inhibited about equally. The data in Chart 4C show that glucose inhibited the formation of UDP-GlcNAc without causing an intracellular accumulation of free glucosamine, and the data in Chart 5B indicate that glucosamine incorporation into total cell material was inhibited by glucose in an apparently competitive manner. The \(k_i\) of the inhibition (about 7 mM) was similar to the \(K_m\) for the transport of glucose by N1S1-67 cells (about 2 mM (34)).

It was demonstrated previously (8) that Cytochalasin B and glucose inhibit the incorporation of glucosamine about equally into the acid-soluble pool and into macromolecules. Our results show that the inhibition of glucosamine incorporation by Cytochalasin B was of the simple competitive type (Chart 5B) and that Persantine also competitively inhibited glucosamine incorporation (Chart 5B). The very low \(k_i\) for the inhibition of glucosamine incorporation by Cytochalasin B (about 1 \(\mu\)M) was the same as that for the inhibition of glucose transport by this drug (8). This finding, together with previous observations that neither Cytochalasin B (8) nor Persantine (34) affects the hexokinase reaction, indicates that the inhibition of glucosamine incorporation by either of these compounds was due to an inhibition of the transport of glucosamine into the cell rather than of its intracellular phosphorylation. In agreement is the finding that the presence of either Cytochalasin B (8) or Persantine (not shown) resulted in a decreased formation of phosphorylated derivatives of glucosamine without a concomitant intracellular accumulation of free glucosamine. The kinetic analysis of the effects of glucose, Cytochalasin B, and Persantine on glucosamine transport (Chart 5B) was conducted with substrate concentrations below the \(K_m\) of the transport reaction, because the \(K_m\) for glucosamine transport is relatively high and because, at concentrations around and above the \(K_m\), the initial rates of uptake decrease rapidly and progressively (Chart 1). More accurate initial uptake rates could be obtained at the lower substrate concentrations (Chart 5A). Nevertheless, in view of this procedure, the apparent \(K_i\) values estimated from the Lineweaver-Burk plots in Chart 5B must be considered approximations.

**Effect of Glucosamine on the Levels of ATP and UTP Pools.**

Samples of cells were prelabeled with adenosine-\(^{3}\)H or uridine-\(^{3}\)H and then freed of extracellular labeled precursors and incubated further in fresh glucose-free BM42 with and without 1 or 5 mM glucosamine. Cell samples were analyzed for radioactivity in total cell material and in acid-insoluble material (not shown), and the acid-soluble pools were analyzed chromatographically (Table 2). As was demonstrated previously (25), when N1S1-67 cells are prelabeled under these conditions, few of the accumulated nucleotides are chased into nucleic acids, because they are probably compartmentalized in the cytoplasm and thus are not readily available for nucleic acid synthesis in the nucleus (25, 27). There is only a small loss of nucleotide triphosphates during a chase in glucose-containing media (25). Even during a chase in glucose-free BM42, the levels of ATP and UTP decreased only slowly (Table 2; Ref. 28). In contrast, in the presence of 5 mM glucosamine, the chase resulted in a rapid loss of labeled ATP and UTP (Table 2). About 90% of the ATP and UTP was lost within 20 min of chase. A large proportion of the ATP was degraded to adenosine, inosine, adenine, and hypoxanthine, and the UTP was degraded to uridine and uracil. The release of these degradation products into the medium was indicated both by the loss of total radioactivity from the cells (Table 2) and by the identification of these products in the culture fluid by chromatographic analysis (not shown; see Ref. 28). Only about 20% of the UTP in glucosamine-treated cells was used for the synthesis of UDP-amino sugars, as indicated by the increase in radioactivity in this fraction during the chase (Table 2). Furthermore, upon continued incubation, label was lost from UDP-GlcNAc and UDP-Glc. A similar loss was observed in untreated control cells. This loss of label was probably due to the degradation of the UDP-GlcNAc and UDP-Glc rather than to a transfer of the sugar moieties to macromolecules, since little radioactivity was incorporated into acid-insoluble material during the chase (not shown). The presence of 1 mM glucosamine had relatively little effect on the levels of ATP and UTP (not shown).
Chart 5. Apparent competitive inhibition of glucosamine transport by glucose, Persantine, and Cytochalasin B. Samples of a suspension of $1 \times 10^7$ cells/ml of glucose-free BM42 were supplemented, where indicated, first with the various inhibitors and, immediately thereafter, with $2 \mu M$ glucosamine-\(^{14}\)C (200,000 cpm/ml) and unlabeled glucosamine to 0.5, 0.8, 1.25, 2.5, or 10 mM. At 5, 10, and 20 min of incubation at 37°, duplicate 1.0-ml samples of each suspension were analyzed for radioactivity in total cell material. The initial rates of incorporation were estimated from the initial linear portions of the incorporation curves (see A for cells without inhibitor), and the values were analyzed in a Lineweaver-Burk plot (B). For graphical reasons, the 0.5 mM points were not included for the Cytochalasin B and glucose curves in B, but we considered the values when drawing the lines. The $K_i$ values were estimated from the slopes of the lines.

Table 2

Effect of glucosamine on the distribution of \(^3\)H among nucleotides during a chase of adenosine-\(^3\)H- and uridine-\(^3\)H-prelabeled cells

<table>
<thead>
<tr>
<th>Nucleoside</th>
<th>Glucosamine (mM)</th>
<th>Time (min)</th>
<th>ATP</th>
<th>ADP</th>
<th>AMP</th>
<th>Adenosine</th>
<th>Total</th>
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<td>1,500</td>
<td>360</td>
<td>40</td>
<td>9,900</td>
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<td>380</td>
<td>7,040</td>
<td></td>
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<tr>
<td></td>
<td>20</td>
<td>890</td>
<td>2,600</td>
<td>2,140</td>
<td>380</td>
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<td>380</td>
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<tr>
<td></td>
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<td>1,230</td>
<td>100</td>
<td>8,240</td>
<td></td>
</tr>
</tbody>
</table>

Distribution (cpm) of \(^3\)H/50 µl acid extract in

| Nucleoside | Glucosamine (mM) | Time (min) + UTP UDP-GlcNAc +UDP-Glc UMP Uridine Total |
|-----------|-----------------|-----------|-----------|-----------|----------|--------|
| Adenosine-\(^3\)H | 5 | 0 | 9,200 | 3,260 | 530 | 90 | 13,080 |
| | 5 | 4,790 | 3,970 | 860 | 200 | 9,820 |
| | 25 | 840 | 4,970 | 420 | 80 | 6,310 |
| | 85 | 360 | 3,620 | 100 | 80 | 4,160 |
| | 0 | 5 | 6,790 | 3,360 | 980 | 70 | 11,200 |
| | 25 | 5,770 | 3,180 | 640 | 90 | 9,680 |
| | 85 | 4,230 | 2,280 | 490 | 30 | 7,030 |

Effect of Glucosamine on Macromolecular Synthesis. The results shown in Chart 6 demonstrate that the presence of 10 mM glucosamine in glucose-free BM42 caused a rapid 90% inhibition of the incorporation of labeled amino acids into hot acid-precipitable material, whereas 1 mM glucosamine had no significant effect. The incorporation of adenosine-\(^3\)H and uridine-\(^3\)H into acid-insoluble material was inhibited by 10 mM glucosamine to an extent similar to that of amino acids (not shown). The inhibition of macromolecular synthesis by glucosamine, which has been observed with other cell systems.
Glucosamine Transport and Metabolism

probably was due mainly to a failure of the substrate to become phosphorylated because of the depletion of the ATP pool caused by the phosphorylation of the excess glucosamine and the increased formation of UDP-amino sugars. This seemed probable because the incorporation of adenosine-3H and uridine-3H into the acid-soluble pool was markedly reduced in glucosamine-treated cells, and little of the radioactivity was associated with ATP or UTP, respectively (Table 3). Most of the adenosine label was associated with ADP and AMP rather than ATP, whereas most of the uridine label was recovered in the UDP-GlcNAc plus UDP-Glc fraction (Table 3). About twice as much label was associated with this fraction in glucosamine-treated than in untreated cells, and the increase was due solely to an increase in labeled UDP-GlcNAc.

However, the incorporation of N1S1-67 cells in glucose-free BM42 containing 10 mM glucosamine resulted in loss of viability only after a relatively long period of incubation. After 8 hr of incubation, less than 5% of the treated cells were stainable by trypan blue, although about 90% were stainable by 20 hr. At that time, less than 5% of the untreated control cells were stained. Furthermore, the effect of glucosamine on the nucleotide pools and macromolecular synthesis was readily reversible. When cells were suspended in fresh growth medium (Swim's Medium 67) after 5 hr of incubation in glucose-free BM42 containing 20 mM glucosamine, the nucleotide triphosphate pools re-formed within 10 to 20 min of incubation, as measured by the incorporation of uridine-3H and adenosine-3H. These cells resumed growth at about the same rate as did cells that had been incubated in glucose-free BM42 without glucosamine for 5 hr. The number of cells in both types of suspension approximately tripled during 18 hr of incubation after the cells were resuspended in fresh growth medium.

Effect of Inhibition of Protein Synthesis on Glucosamine Incorporation. Both puromycin and cycloheximide almost completely inhibited the incorporation of glucosamine-14C into acid-insoluble material (Chart 7A). These results are similar to those reported for liver (13, 21) and activated lymphocytes (12), whereas inhibition of protein synthesis has little effect on the incorporation of glucosamine into glycoproteins by Ehrlich ascites cells (6, 13, 20). Glucosamine incorporation by these cells was inhibited only at very high concentrations of cycloheximide (5 mg/ml), which caused a rapid degradation of UDP-GlcNAc (13). In contrast, our data show that the inhibition of glucosamine incorporation by puromycin or cycloheximide in NIS1-67 cells was not due to a failure of the cells to take up glucosamine or to convert it to UDP-GlcNAc. The incorporation of glucosamine into total cell material was not affected by either inhibitor (Chart 7A), and in treated and untreated cells alike, over 90% of the acid-soluble intracellular label was associated with UDP-GlcNAc (not shown). Results from other experiments have shown that the incorporation of glucosamine into glycoproteins and glycolipids was about equally inhibited. On the other hand, the formation of CO2 from glucosamine was

---

**Table 3**

*Effect of glucosamine on the incorporation of adenosine-3H or uridine-3H into the nucleotide pool*

<table>
<thead>
<tr>
<th>Nucleoside</th>
<th>Glucosamine (mM)</th>
<th>ATP (cpm)</th>
<th>ADP (cpm)</th>
<th>AMP (cpm)</th>
<th>Total (cpm)</th>
</tr>
</thead>
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<td>53,020</td>
<td>7,560</td>
<td>3,160</td>
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<td>5</td>
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<td>17,150</td>
<td>9,570</td>
<td>28,790</td>
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<td>UTP + UDP</td>
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<td>14,360</td>
<td>8,980</td>
<td>850</td>
<td>24,190</td>
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<tr>
<td>UMP</td>
<td>630</td>
<td>17,730</td>
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</tbody>
</table>

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an apparent Km similar to the Km of its own transport (1 to 2 acts as a competitive inhibitor of glucosamine transport with and glucosamine transport. It seems probable that glucosamine are 2 to 3 orders of magnitude lower than those for glucose

nucleosides (23, 24), choline (26), and glucose (34). The apparent Km's for nucleoside and choline transport, however, simple diffusion becomes the main mode of entry. Similar

by both facilitated and simple diffusion. With low glucosamine

mM). Glucosamine also competitively inhibits the transport

reaction, whereas, at concentrations above 50 mM, glucosamine is phosphorylated as rapidly as it enters the cells, and it does not accumulate intracellularly. The results also indicate that, under these conditions, transport is the rate-limiting step in glucosamine metabolism. Similar observations have been made on the incorporation of glucose and other low-molecular-weight substances (26, 29, 34).

On the other hand, with high concentrations of glucosamine in the medium, additional factors limit the incorporation of glucosamine into macromolecules. Incubation of NIS1-67 cells with 5 to 50 mM glucosamine in glucose-free medium results in rapid loss of ATP, UTP, and other nucleoside phosphates. As previously suggested by Sukeno et al. (40), the loss of ATP seems to be a consequence of the rapid phosphorylation of the glucosamine, which enters the cells at a progressively greater rate concurrent with an increase in glucosamine concentration in the medium (Chart 1). A 2nd factor is probably the limitation of energy production by the cells caused by the absence of glucose in the medium and the relatively low capacity of the NIS1-67 cells to oxidize or ferment glucosamine. Any ATP that may be generated probably is utilized for the phosphorylation of the excess glucosamine as rapidly as it becomes available. It is also probable that this process is largely responsible for the rapid depletion of UTP and other nucleotides and for the inhibition of macromolecular synthesis in cells (Refs. 1 and 3; Chart 6). The nucleotides become degraded to nucleosides and bases and are released into the culture fluid. This also occurs when energy production is blocked by KCN (28), iodoacetate, or oxamate; Racker (33) indicates that this degradation occurs whenever the dephosphorylation of ATP exceeds its regeneration. Although glucosamine stimulates the production of UDP-GlcNAc by NIS1-67 cells, as well as in other cell systems (2, 3, 15, 16, 36, 40), only about 20% of the UTP lost during the pulse-chase experiment in the presence of glucosamine can be accounted for by the formation of UDP-GlcNAc; the remainder becomes degraded.

The loss of intracellular ATP as well as UTP caused by glucosamine is similar to results obtained with suspensions of and metabolism of glucose (34, 37). Furthermore, the apparent K_i's for the inhibition of glucosamine transport by Cytochalasin B and Persantine are similar to those for the inhibition of glucose transport by these substances (8, 34). The apparent affinity of the glucose transport system for Cytochalasin B is at least 3 orders of magnitude greater than that for the natural substrates.

Glucosamine seems to be transported by the glucose transport system less efficiently than is glucose, since the apparent K_m for glucose transport is about 10-fold lower than that for glucosamine, whereas the apparent V_max (about 100 nmoles/10^7 cells/min) is about the same for both substrates. Similarly, the apparent K_m for the phosphorylation of glucose by cell-free preparations (0.1 mM) is about 5-fold lower than that for the phosphorylation of glucosamine (11). Transport, however, is a reaction distinct from phosphorylation, as is indicated by studies with KCN-pretreated cells (Chart 3), by the fact that Persantine and Cytochalasin B competitively inhibit the incorporation of glucosamine and glucose by whole cells without affecting the hexokinase reaction (8, 34), and by other evidence previously presented (34). When present in low concentrations in the medium, glucosamine is phosphorylated as rapidly as it enters the cells, and it does not accumulate intracellularly. The results also indicate that, under these conditions, transport is the rate-limiting step in glucosamine metabolism. Similar observations have been made on the incorporation of glucose and other low-molecular-weight substances (26, 29, 34).

On the other hand, with high concentrations of glucosamine in the medium, additional factors limit the incorporation of glucosamine into macromolecules. Incubation of NIS1-67 cells with 5 to 50 mM glucosamine in glucose-free medium results in rapid loss of ATP, UTP, and other nucleoside phosphates. As previously suggested by Sukeno et al. (40), the loss of ATP seems to be a consequence of the rapid phosphorylation of the glucosamine, which enters the cells at a progressively greater rate concurrent with an increase in glucosamine concentration in the medium (Chart 1). A 2nd factor is probably the limitation of energy production by the cells caused by the absence of glucose in the medium and the relatively low capacity of the NIS1-67 cells to oxidize or ferment glucosamine. Any ATP that may be generated probably is utilized for the phosphorylation of the excess glucosamine as rapidly as it becomes available. It is also probable that this process is largely responsible for the rapid depletion of UTP and other nucleotides and for the inhibition of macromolecular synthesis in cells (Refs. 1 and 3; Chart 6). The nucleotides become degraded to nucleosides and bases and are released into the culture fluid. This also occurs when energy production is blocked by KCN (28), iodoacetate, or oxamate; Racker (33) indicates that this degradation occurs whenever the dephosphorylation of ATP exceeds its regeneration. Although glucosamine stimulates the production of UDP-GlcNAc by NIS1-67 cells, as well as in other cell systems (2, 3, 15, 16, 36, 40), only about 20% of the UTP lost during the pulse-chase experiment in the presence of glucosamine can be accounted for by the formation of UDP-GlcNAc; the remainder becomes degraded.

The loss of intracellular ATP as well as UTP caused by glucosamine is similar to results obtained with suspensions of

only slightly inhibited by puromycin and cycloheximide. Within 5 min of their addition, the drugs (at the concentrations used) inhibited the incorporation of labeled amino acids into protein by over 95%.

**DISCUSSION**

The results indicate that glucosamine enters NIS1-67 cells by both facilitated and simple diffusion. With low glucosamine concentrations in the medium, uptake occurs mostly by the transport reaction, whereas, at concentrations above 50 mM, simple diffusion becomes the main mode of entry. Similar conclusions were reached regarding the uptake of various nucleosides (23, 24), choline (26), and glucose (34). The apparent K_m's for nucleoside and choline transport, however, are 2 to 3 orders of magnitude lower than those for glucose and glucosamine transport. It seems probable that glucosamine is transported by the glucose transport system, since glucose acts as a competitive inhibitor of glucosamine transport with an apparent K_i similar to the K_m of its own transport (1 to 2 mM). Glucosamine also competitively inhibits the transport...
other tumor cells (2, 40), but these results differ from those obtained with whole animals (15) or cultures of chick fibroblasts (36). The incubation of chick fibroblasts with 20 to 40 mM glucosamine has no effect on the incorporation of adenosine into the nucleotide pool or into RNA in these cells, and the loss of UTP is accounted for largely by its conversion to UDP-aminosugars (36). This difference is probably due to the presence of glucose in the minimal medium used in the study with chick fibroblasts. Glucose competitively inhibits the transport of glucosamine into the cell (Chart 5) and would enhance the capacity of the cells to produce ATP by either oxidative or substrate phosphorylation. Thus, the differential effect of glucosamine on the uracil and adenine nucleotide pools may depend on the relative concentrations of glucosamine and glucose in the medium (2). Treatment of N1S1-67 cells with KCN in glucose-free medium also results in a more rapid and extensive depletion of uracil than of adenine nucleotide pools (28).

The inhibition of macromolecular synthesis by high concentrations of glucosamine is probably partly responsible for the failure of glucosamine-14C to be incorporated into cellular glycoproteins and glycolipids by N1S1-67 cells (Chart 1D). The preferential inhibition of synthesis of certain viral glycoproteins by high concentrations of glucosamine (10, 14) may have other causes. It seems that glycosylation of proteins occurs concomitant with synthesis of the polypeptide chain, since the inhibition of protein synthesis by puromycin or cycloheximide results in the rapid inhibition of glucosamine incorporation into glycoproteins (Chart 7; Refs. 12, 13, and 21). It is not clear why the incorporation of glucosamine into glycolipids by N1S1-67 cells is also inhibited by inhibitors of protein synthesis. In N1S1-67 cells, as with HeLa cells (4) and cultured hamster fibroblasts (9), a relatively high proportion of glucosamine is incorporated into glycolipids, whereas, in liver, less than 2% of incorporated glucosamine is recovered in glycolipids (13). This low level of incorporation by liver, which is believed to represent the synthesis of lipid carrier molecules, is not inhibited by cycloheximide (13).

Another factor that may prevent the incorporation of glucosamine-14C into macromolecules is the failure of UDP-GlcNAc to be formed when the concentration of glucosamine in the medium is high (Table 1). Under these conditions, the glucosamine taken up by the cells accumulates as oxidative or substrate phosphorylation. Thus, the differential effect of glucosamine on the uracil and adenine nucleotide pools may depend on the relative concentrations of glucosamine and glucose in the medium (2). Treatment of N1S1-67 cells with KCN in glucose-free medium also results in a more rapid and extensive depletion of uracil than of adenine nucleotide pools (28).

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Another factor that may prevent the incorporation of glucosamine-14C into macromolecules is the failure of UDP-GlcNAc to be formed when the concentration of glucosamine in the medium is high (Table 1). Under these conditions, the glucosamine taken up by the cells accumulates intracellularly as GlcN-6-P and GlcNAc-6-P without being converted further, probably because the available ATP is preferentially used for the phosphorylation of glucosamine rather than for the regeneration of UTP required for the synthesis of UDP-GlcNAc. It is clear then that, for the purpose of labeling glycoproteins or glycolipids with glucosamine-14C, it is advisable to use glucose-free media supplemented with glucosamine in concentrations below 0.1 mM. As indicated in Charts 1 and 7 and Table 1, under these conditions, most of the glucosamine taken up by the cells accumulates as UDP-GlcNAc and, after a lag period, is rapidly incorporated into macromolecules.

ACKNOWLEDGMENTS

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# Transport and Metabolism of Glucosamine by Cultured Novikoff Rat Hepatoma Cells and Effects on Nucleotide Pools

Peter G. W. Plagemann and John Erbe

*Cancer Res* 1973;33:482-492.

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