

The Effect of D-Glucosamine on the Adenine and Uridine Nucleotides of Sarcoma 180 Ascites Tumor Cells*

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SUMMARY

The effect of D-glucosamine on the metabolism of adenine and uridine nucleotides in Sarcoma 180 ascites tumor cells was investigated as part of an effort to determine the biochemical basis for the cytotoxic effects of glucosamine on tumor cells. Incubation of ascites tumor cells with exogenous glucosamine resulted in a 4-fold increase in the pool size of UDP-N-acetylhexosamine. A large fraction of ^{14}C -labeled uridine was accumulated as radioactive UDP-N-acetylhexosamine when the cells were incubated in the presence of D-glucosamine and labeled uridine. At the same time there was a significant reduction in radioactivity associated with UMP, UDP, UTP, and UDP-glucose, as well as a reduction of the UTP pool to half of its normal level. Glucosamine in the absence or in the presence of low (1 mM) concentrations of glucose caused a significant reduction of the intracellular ATP pool. However, no such decrease in the intracellular ATP level was observed in ascites cells exposed to glucosamine in the presence of 10 mM glucose.

the action of glucosamine on tumors. Harpur and Quastel (13) showed that glucosamine was phosphorylated by bovine brain extracts in the presence of ATP, and suggested that exogenous glucosamine might deplete the intracellular ATP levels by such phosphorylation.

Kornfeld *et al.* (14) have shown that UDP-N-acetylhexosamine inhibits the enzyme glutamine 6-phosphate transaminidase, which catalyzes the conversion of fructose 6-phosphate to glucosamine 6-phosphate, thus preventing the formation of excessive amounts of the amino sugar derivatives. However, the biosynthesis of such derivatives, including UDP-N-acetylhexosamine, from exogenous glucosamine bypasses this regulating step, and the administration of exogenous glucosamine results in an intracellular accumulation of this sugar nucleotide (15). Since this biosynthetic process involves the utilization of both ATP and UTP, it seemed possible that a substantial portion of adenine and uridine nucleotide pools in the cancer cell could be diverted to the synthesis of the amino sugar nucleotides.

The work to be reported here was designed to test these hypotheses.

MATERIALS AND METHODS

Animals—Adult, male Swiss-Webster mice, weighing 30 to 35 g, and Sprague-Dawley rats, weighing from 310 to 350 g, were used. Transplantation of Sarcoma 180 was carried out as described previously (3). Animals were fed with Purina chow and tap water *ad libitum* in a thermostatically controlled room at 20° with 12-hour light and dark cycles.

Isotopes—D-Glucosamine-1- ^{14}C (3.1 mCi per mmole) and uridine-2- ^{14}C (50 mCi per mmole) were obtained from New England Nuclear. Radiopurity of uridine-2- ^{14}C was tested by paper chromatography and of glucosamine with an ion exchange chromatography. Isotopic compounds were freshly prepared for each experiment by dissolving them at the indicated activities in Krebs-Ringer phosphate buffer.

Determination of UDP-N-Acetylhexosamine Pool—Sarcoma 180 ascites cells were incubated at 37° in Krebs-Ringer phosphate buffer, pH 7.4, containing 2 mM glucose and 20 mM glucosamine. Final volume was 15 ml. At predetermined times reaction flasks were removed, rapidly cooled, and centrifuged for 5 min at 1,000 $\times g$. The supernatant was discarded and the cells were washed twice with 10 ml of 0.9% NaCl containing 1 mg per ml of glucosamine. Finally, the cells were resuspended in 0.9% NaCl to which an equal volume of 20% ice-cold per-

An inhibitory effect of exogenous D-glucosamine on the growth of experimental tumors in mice, originally reported by Quastel and Cantero (1), has been confirmed and extended in this laboratory (2). Loss of viability and transplantability of several ascites tumor lines exposed *in vitro* to D-glucosamine has been demonstrated (3), and cytotoxic effects of glucosamine on various tumor cell lines in tissue culture have been observed (4, 5). Recently it has been shown that the incorporation of labeled lysine, uridine, and thymidine into the trichloroacetic acid-insoluble fraction of tumor cells is significantly inhibited by D-glucosamine (6). The biochemical bases for these inhibitory effects of D-glucosamine, however, are poorly understood.

One reasonable hypothesis is that glucosamine might inhibit hexokinase (7-9), and thus glucose metabolism. However, the work of Kono and Quastel (10), of Cahill *et al.* (11), and of Martin (12) suggests that such inhibition is not a significant factor in

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chloric acid had been added and homogenized in a VirTis homogenizer at full speed for 2 min. Samples were centrifuged at $10,000 \times g$ at 2° for 30 min. The residue was resuspended in 15 ml of 10% ice-cold perchloric acid, centrifuged, and the supernatants were combined. The pooled supernatant fractions were neutralized with 50% KOH and the precipitate removed by centrifugation. The acid-soluble nucleotides were adsorbed on 5 g of acid-washed Norit A charcoal (16). The charcoal was collected by centrifugation and washed three times with 30 ml of 0.01 N ammonium hydroxide in 50% ethanol. All of these preparatory steps were carried out at 4° . The combined eluates were reduced in volume to 1.5 to 2.0 ml by a rotary evaporator. Initial separation of UDP-N-acetylhexosamine, obtained from neoplastic tissues, was carried out on Whatman No. 3MM paper developed in ethanol-1.0 M ammonium acetate (75:30, v/v), pH 7.6, solvent system for 22 hours. The radioactive spot corresponding to that of authentic UDP-N-acetylglucosamine was eluted with distilled water, and the optical density of UDP-N-acetylhexosamine was measured at $260 m\mu$.

Isolation and Characterization of Acid-soluble Nucleotides—The methods of Schmitz *et al.* (17), of Hurlbert *et al.* (18), and of Schmitz, Hurlbert, and Potter (19) were used with slight modifications. Sarcoma 180 ascites tumor cells (approximately 4.2×10^9 cells) were added to each reaction flask containing 60 ml of Krebs-Ringer phosphate buffer, pH 7.4, 90 μ moles of glucose, 10 μ Ci of uridine-2- 14 C, and the indicated amount of glucosamine. Final volume was 90 ml. At predetermined time intervals the flasks were rapidly cooled and centrifuged at low speed. The supernatant was discarded, and the cells were resuspended in 15 ml of 20% trichloroacetic acid, homogenized at full speed in a VirTis homogenizer for 2 min, and centrifuged at $10,000 \times g$ for 15 min. The supernatant was then collected. The residue was washed twice with 20 ml of 10% ice-cold trichloroacetic acid, centrifuged, and the supernatants combined. The trichloroacetic acid-soluble fraction was extracted four times with diethyl ether and was brought to pH 6.5 with ammonium hydroxide. Fractionation of the acid-soluble fraction

was achieved by stepwise gradient elution from a Dowex 1-X8 (formate) 200 to 400 mesh resin column (25×1 cm), using a 500-ml mixing volume, initially containing distilled water. The column was washed with 400 ml of distilled water followed by a four-step gradient elution from reservoirs containing successively 4.0 N formic acid, 0.2 M ammonium formate in 4.0 N formic acid, 0.4 M ammonium formate in 4.0 N formic acid, and 1.0 M ammonium formate in 4.0 N formic acid by changing reservoirs to contain the above solutions at tubes 0, 110, 210, and 285, respectively. Fractions (5 ml) were collected on a refrigerated fraction collector. The optical density at $260 m\mu$ and radioactivity in alternate tubes was determined. Effluents from the column contained more than 95% of the initially applied $260 m\mu$ absorbing material. Samples were pooled at the appropriate peaks, passed through a Dowex 50-H $^+$ column (30×2.5 cm), and lyophilized. All steps were carried out at 4° .

Purification of individual nucleotides was accomplished by paper chromatography. Samples were applied on Whatman No. 1 or No. 3MM paper, and were subjected to descending chromatography using solvent systems of isobutyric acid-concentrated ammonium hydroxide-water (57:4:39), pH 4.3, or isobutyric acid-ammonium hydroxide-water (66:1:33), pH 3.7. The R_F values of unknown nucleotides were compared with those of authentic ribonucleotides chromatographed simultaneously. Samples were eluted with water, and purine and pyrimidine bases were identified by their characteristic spectral shifts occurring at pH 2.0, 7.0, and 11.0, utilizing a Cary model 14 spectrophotometer.

Estimation of Intracellular Adenine Nucleotides—For determination of intracellular adenine nucleotides, Sarcoma 180 as-

TABLE I

Effect of exogenous glucosamine (20 mM) on UDP-N-acetylhexosamine pool size of Sarcoma 180 ascites tumor cells

Incubation and subsequent estimation of UDP-N-acetylhexosamine was carried out as described under "Materials and Methods."

Time	UDP-N-acetylhexosamine			
	0.215 mM glucosamine		20 mM glucosamine	
	Pool size	Specific activity ^a	Pool size	Specific activity ^b
hrs	$\mu\text{mole}/1 \times 10^9$ cells	$\text{dpm} \times 10^{-5}/$ μmole	$\mu\text{moles}/1 \times 10^9$ cells	$\text{dpm} \times 10^{-4}/$ μmole
Control	0.42		0.42	
$\frac{1}{2}$	0.44	16.9	0.69	5.5
1.0	0.41	14.4	0.90	6.8
2.0	0.47	4.2	1.09	6.4
3.0	0.46	2.1	1.28	6.8
4.0	0.44	1.2	1.57	7.3

^a Specific activity of D-glucosamine- 14 C in the incubation media was 6.88×10^6 dpm per μmole .

^b Specific activity of D-glucosamine- 14 C in the incubation media was 7.32×10^4 dpm per μmole .

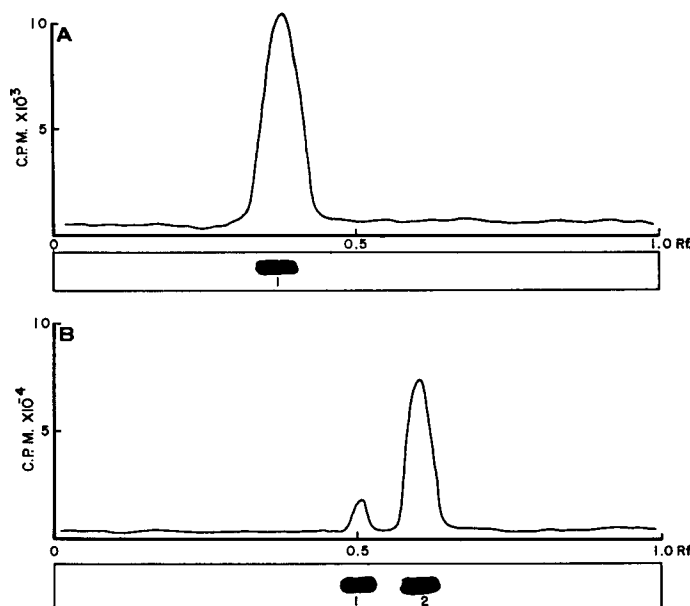


FIG. 1. Paper chromatographic separation of UDP-N-acetylhexosamines from Sarcoma 180 ascites tumor cells after 30 min of incubation with 20 mM radioactive glucosamine. A, paper chromatographic separation in 95% ethanol-1.0 M ammonium acetate (75:30, v/v), pH 7.6. The standard shown in *guide strip 1* is UDP-N-acetylhexosamine. B, chromatography of hydrolysates of UDP-N-acetylhexosamine in ethyl acetate-pyridine-water (13:5:4). The standards shown in *guide strip* are D-galactosamine (1) and D-glucosamine (2). Radioactive areas on the paper chromatograms were determined by using a Packard automatic paper chromatogram scanner.

cites tumor cells were incubated in Krebs-Ringer phosphate buffer (no calcium) in the presence or absence of glucose and glucosamine at 37° under aerobic conditions. Final volume was 45 ml. Cell concentration was 4.7×10^7 cells per ml. After incubation, the cell suspensions were cooled rapidly and centrifuged, and the cell residue was resuspended in 6% perchloric acid. The acid-soluble fraction was then obtained as described by Lamprecht and Trautschold (20). The level of ATP from the neutralized perchloric acid-soluble extract was determined by the glucose 6-phosphate dehydrogenase and hexokinase assay (20), whereas ADP and AMP were measured by the methods of Adam (21), using successive addition of lactic dehydrogenase, pyruvate kinase, and myokinase, respectively.

Other Analyses—Glucose was determined by the glucose oxidase method of Saifer and Gerstenfeld (22). Acid-labile phosphate was determined by the method of Chen, Toribara, and Warner (23), and total phosphorus according to the method of Lowry *et al.* (24). Reducing sugar was measured by the method of Park and Johnson (25). Pentose in nucleotides was determined by the orcinol reaction (26). Protein was estimated by the biuret method (27), with human serum albumin as a standard.

RESULTS

Effect of Exogenous Glucosamine on Pool Size of UDP-N-Acetylhexosamine in Sarcoma 180 Ascites Cells—Table I shows that

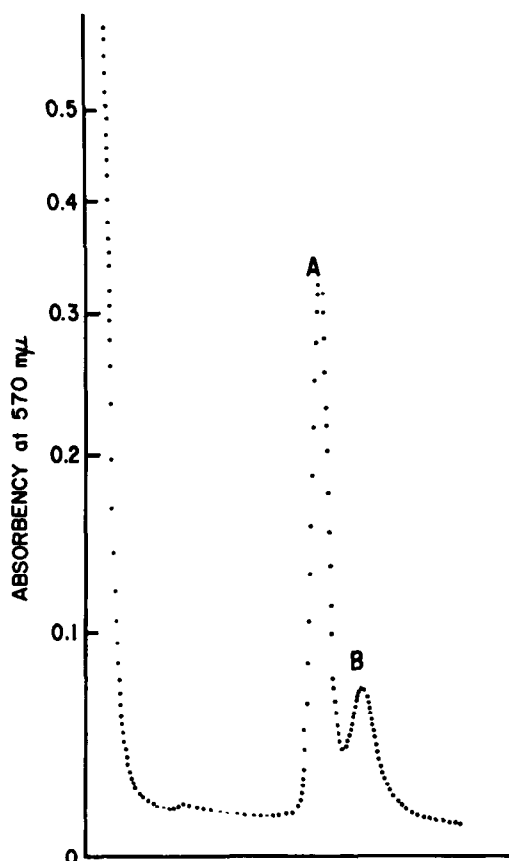


FIG. 2. Amino sugar composition of UDP-N-acetylhexosamine obtained from Sarcoma 180 ascites tumor cells incubated with 20 μ moles per ml of D-glucosamine. After paper chromatographic separation, the sugar nucleotide was eluted and hydrolyzed in 2.0 N HCl at 100° for 2 hours. After hydrolysis, the sample was assayed for amino sugar composition on an amino acid analyzer (Beckman-Spinco model 120). Peak A corresponds to D-glucosamine and Peak B to D-galactosamine.

appreciable accumulation of UDP-N-acetylhexosamine over the control levels occurs in Sarcoma 180 cells with exposure to 20 mM glucosamine for a period as short as 30 min, and that a 400% increase occurred in 4 hours. The specific activity of the intracellular UDP-N-acetylhexosamine of cells incubated with a tracer dose of glucosamine reached a maximum of 1.69×10^6 dpm per μ mole (23% of the activity of the exogenous glucosamine) within 30 min, and then fell off rapidly, with a biological half-life of 65 min. The pool size was not changed by the tracer amount of glucosamine. These results indicate that the synthesis of endogenous glucosamine serves to dilute out the specific activity of the radioactive exogenous glucosamine in cells incubated with tracer amounts of glucosamine. In incubations with 20 mM glucosamine, however, the pool of UDP-N-acetylglucosamine increased, and its specific activity rapidly reached the level of the exogenous glucosamine, suggesting that endogenous glucosamine synthesis had been inhibited.

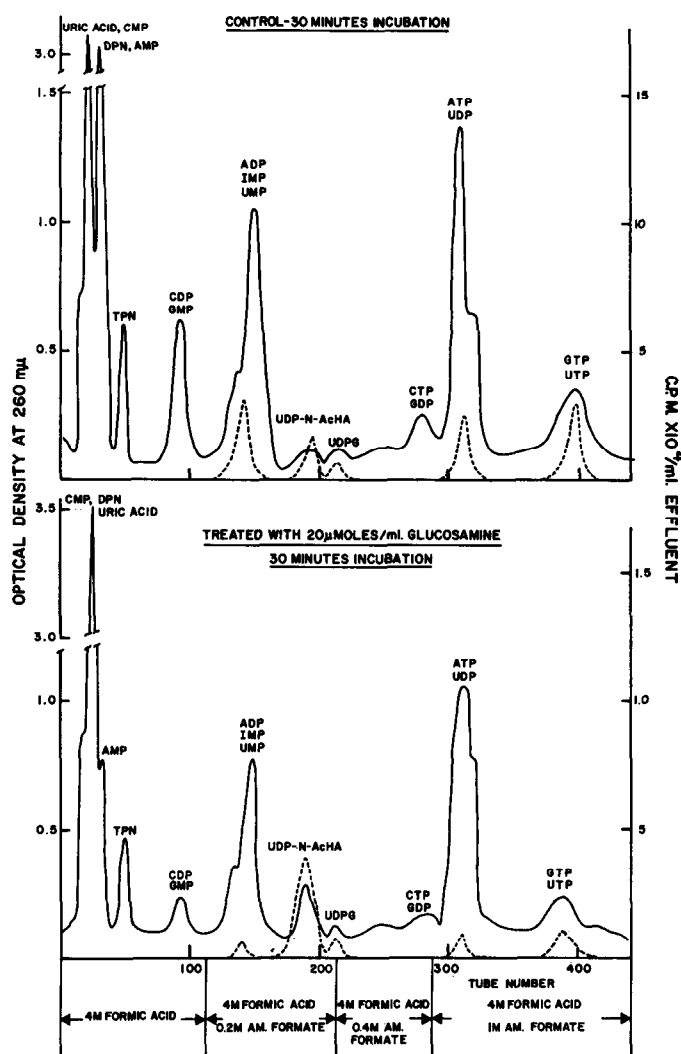


FIG. 3. Separation of acid-soluble nucleotides from Sarcoma 180 ascites tumor cells incubated for 30 min in the presence or absence of exogenous D-glucosamine. The acid-soluble fraction of Sarcoma 180 tumor was obtained after incubation of approximately 4.2×10^9 cells for 30 min in Krebs-Ringer phosphate buffer containing 10 μ Ci of uridine-2- 14 C, no added glucosamine (*upper*) or 20 mM glucosamine (*lower*). The isolation and characterization of acid-soluble nucleotides was as described under "Materials and Methods." Absorbance at 260 μ , —; radioactivity, ----.

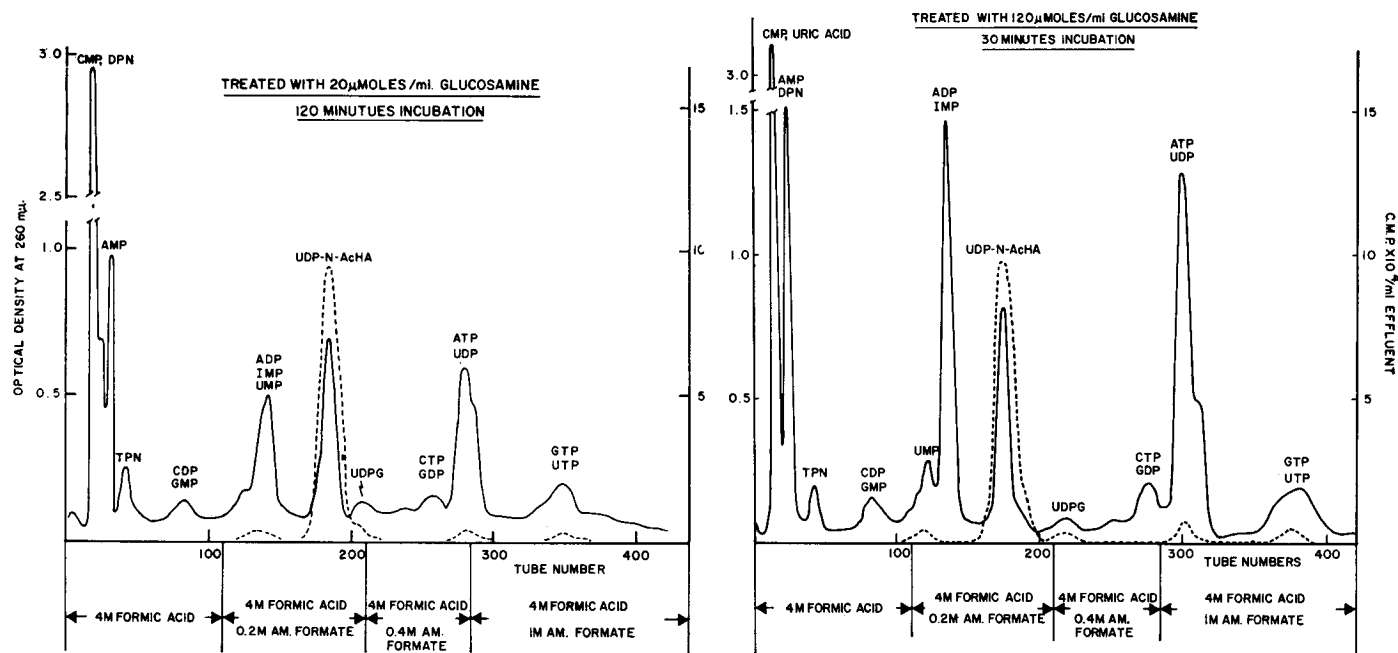


FIG. 4. Separation of acid-soluble nucleotides from Sarcoma 180 ascites tumor cells. *Left*, incubated for 120 min in the presence of 20 mM D-glucosamine; *right*, incubated for 30 min in the presence of 120 mM D-glucosamine. Absorbance at 260 m μ , —; radioactivity, - - -.

TABLE II

Effect of 20 mM D-glucosamine on pool size of various uridine nucleotides in Sarcoma 180 cells in vitro

Incubation time	UMP	UDP	UTP	UDP-N-acetylhexosamine
<i>hrs</i>	<i>μmoles/1 × 10⁹ cells</i>			
Control (no glucosamine)	0.46 ± 0.06	0.36 ± 0.01	0.38 ± 0.01	0.39 ± 0.05
1/4	0.46	0.36	0.18	0.53
1/2	0.46	0.36	0.16	0.65
1.00	0.29	0.37	0.18	0.82
2.00	0.29	0.29	0.19	0.96
4.00	0.25	0.30	0.16	1.42

The identity of the radioactive nucleotide amino sugar peaks as a mixture of UDP-N-acetylglucosamine and UDP-N-acetylgalactosamine was established as follows. The perchloric acid-soluble fraction was isolated from 5 ml of freshly harvested cells incubated in Krebs-Ringer phosphate buffer, pH 7.4, for 30 min at 37° in a final volume of 15 ml with 10 μ Ci of D-glucosamine-¹⁴C and 20 mM nonradioactive glucosamine. Paper chromatography of the fraction resulted in a single radioactive peak with an R_F corresponding to that of an authentic UDP-N-acetylglucosamine standard (Fig. 1A). When this peak was eluted, it gave a negative test for reducing sugar and had an absorption spectrum identical with that of UDP-N-acetylglucosamine. The composition of eluted sample, expressed as micromoles per μ moles of uridine was: uridine, 1.00; hexosamine, 0.98; pentose, 0.91; acid-labile phosphate, 1.01; and total phosphate, 1.94. These results indicate that the radioactive nucleotide was UDP-N-acetylhexosamine. In order to establish the nature of the hexosamine component, a sample eluted from filter paper was hydrolyzed in 2.0 N HCl for 2 hours at 100° and subjected to paper chromatographic separation using an ethyl acetate-pyridine-water (13:5:4) solvent system. Two radioactive spots corresponding to stand-

ard D-glucosamine and D-galactosamine were observed (Fig. 1B). This observation was confirmed by quantitative analysis on the same hydrolyzed sample using an amino acid analyzer system (28). Data obtained from this analysis (Fig. 2) show that the sugar nucleotide mixture had a composition of 75% glucosamine and 25% galactosamine. This same ratio of the amino sugars was found if large or if tracer doses of glucosamine were used in the incubations. Glucosamine and galactosamine moieties had the same specific activities in a number of separate experiments.

Effect of D-Glucosamine on Acid-soluble Nucleotides in Sarcoma 180 Ascites Tumor—Since the formation of UDP-N-acetylhexosamine requires UTP, it was considered possible that a portion of the UTP pool in the tumor cells might be diverted to the synthesis of the amino sugar nucleotides, and that the UTP pool might be depleted. To test this possibility, Sarcoma 180 ascites tumor cells (approximately 4.2×10^9 cells) were incubated in 60 ml of Krebs-Ringer phosphate buffer, pH 7.4, containing 10 μ Ci of uridine-2-¹⁴C, 90 μ moles of glucose, and D-glucosamine at several concentrations. Incubation was carried out at 37° for 1/4 to 4 hours, followed by the isolation of acid-soluble fraction from the tumor samples and fractionation of acid-soluble nucleotides in Dowex 1-formate columns as described under "Materials and Methods."

The *upper portion* of Fig. 3 shows elution pattern of ultra-violet-absorbing material and radioactivity from 4.2×10^9 tumor cells incubated with uridine-2-¹⁴C in the absence of exogenous D-glucosamine for 1/4 and 2 hours, respectively. Radioactivity was present in five peaks which were identified by chromatographic methods in the order of their elution as uridine monophosphate, UDP-N-acetylhexosamine, uridine diphosphate glucose, uridine diphosphate, and uridine triphosphate.

The effect of exogenous glucosamine on the uridine nucleotides in Sarcoma 180 ascites cells was striking. This is shown in Figs. 3 and 4 where the cells were incubated in the presence of 20 mM glucosamine for 1/4 to 2 hours, respectively, and in Fig. 4 (*right*)

where the cells were incubated in the presence of 120 mM glucosamine for 30 min. The most noticeable effect of exogenous glucosamine was the very marked increase in radioactivity and absorbance in the UDP-*N*-acetylhexosamine peak. At the same time there was a marked reduction in radioactivity associated with UMP, UDP, UTP, and UDP-glucose.

Data presented in Table II show that incubation of cells with 20 mM glucosamine for 4 hours caused an increase from 0.39 to 1.42 μ moles/ 1×10^9 cells in the UDP-*N*-acetylhexosamine pool, in good agreement with data obtained by the charcoal-absorption method (Table I). The level of UTP in the glucosamine-treated cells was reduced to about one-half of the control level within 15 min of incubation with 20 mM *D*-glucosamine, but it was not reduced in further incubation up to 4 hours. The UMP level was also found to be reduced by 35% after 60 min of incubation.

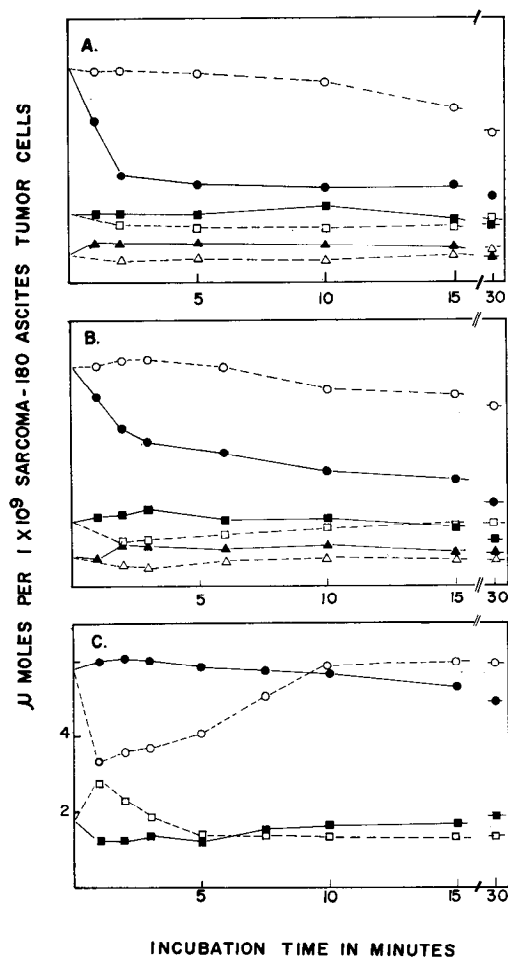


FIG. 5. Intracellular adenine nucleotides in Sarcoma 180 ascites tumor cells in the presence or absence of exogenous *D*-glucosamine. Sarcoma 180 cells (2.1×10^9 cells) were incubated at 37° in Krebs-Ringer phosphate buffer (no calcium present) at pH 7.4, containing 20 mM glucosamine. At the indicated time intervals, samples were removed and the adenine nucleotides assayed as described under "Materials and Methods." Control samples were treated in the same manner, except that glucosamine was omitted from the incubation media. *B* and *C*, incubation was carried out as described above, except that 1 mM or 10 mM of glucose was added to the incubation media, respectively. Control (no glucose, or 1 mM or 10 mM glucose): ATP, ○---○; ADP, □---□; AMP, △---△. Treated (20 mM glucosamine, or 1 mM glucose plus 20 mM glucosamine, or 10 mM glucose plus 20 mM glucosamine): ATP, ●---●; ADP, ■---■; AMP, ▲---▲.

Effect of D-Glucosamine on Levels of Intracellular Adenine Nucleotides—Changes in the levels of adenine nucleotides of Sarcoma 180 cells provoked by *D*-glucosamine in the absence or in the presence of low (1 mM) and high (10 mM) concentrations of glucose are shown in Fig. 5, *A*, *B*, and *C*, respectively. At low glucose levels, addition of exogenous glucosamine caused a rapid decrease in the cellular ATP levels to about 50% of the control levels (Fig. 5, *A* and *B*). This was accompanied by a moderate increase of ADP and AMP which may reflect the active phosphorylation of glucosamine and glucose. The total adenine nucleotides in the glucosamine-treated ascites cells was always decreased in comparison with the corresponding controls. Assay for glucose showed that the glucose was substantially depleted in 5 min in the control, and in 7 min in the glucosamine-treated cell suspension.

As has been observed in several laboratories (29–33), ascites tumor cells exposed to high concentration of glucose show an initial drop of the ATP level associated with a rise in ADP (Fig. 5*C*). Both ATP and ADP return to their initial concentrations in about 10 min. This reduction of ATP in the presence of 10 mM glucose was prevented by 20 mM glucosamine (Fig. 5*C*).

DISCUSSION

The UDP-*N*-acetylhexosamine pool of Sarcoma 180 cells consists of a mixture of UDP-*N*-acetylglucosamine and UDP-*N*-acetylgalactosamine in a ratio of about 3:1. Incubation of the cells with 20 mM glucosamine resulted in a progressive increase in the pool of UDP-*N*-acetylhexosamine with no change in the ratio of glucosamine to galactosamine. This finding is in accord with the observation of Kornfeld and Ginsburg (15), who showed an accumulation of UDP-*N*-acetylhexosamine in HeLa cells incubated with glucosamine.

Since the formation of amino sugar nucleotides involves the utilization of UTP, it is possible that a portion of UTP pool could be diverted into the formation of this amino sugar nucleotide. Glucosamine significantly changed the distribution of the uridine nucleotides in the neoplastic cells even in a period as brief as 15 min. There was a significant diversion of tracer uridine-2-¹⁴C to UDP-acetylhexosamine synthesis, and an accumulation of UDP-acetylhexosamine. At the same time, there was a reduction in radioactivity associated with other uridine nucleotides, particularly UTP, which fell to half of the control level. It is not possible to conclude from these data that the reduction of intracellular UTP is the cause of glucosamine toxicity, but neither can the possibility be excluded.

There are also significant changes in the intracellular adenine nucleotide pools associated with incubation of Sarcoma 180 ascites cells with glucosamine when no glucose or 1 mM glucose was present. There was a rapid reduction of intracellular ATP after the addition of glucosamine with slight increase of ADP. This corresponds with the very rapid entry and phosphorylation of glucosamine by the tumor cells (13). Under these conditions, the total adenine nucleotide content was gradually decreased upon continued incubation. However, incubation of the cells with glucosamine in the presence of 10 mM glucose resulted in no significant reduction of the intracellular ATP pool. These results are in accord with those of Creaser and Scholefield (34), who found that glucosamine, in the absence of glucose, reduced the amount of intracellular ATP and prevented the resynthesis of ATP in Ehrlich ascites carcinoma, and that the effect of glu-

cosamine was almost completely reversed in the presence of a high concentration of glucose.

It is most interesting that glucose alone, or glucosamine alone or in the presence of low concentration of glucose, caused a rapid reduction of intracellular ATP, whereas, together, at high concentration, they did not. The explanation for this peculiar behavior is not apparent and is presently under investigation.

2-Deoxy-D-glucose has been shown to inhibit the anaerobic glycolysis (35) and oxygen consumption in a variety of neoplastic tissues (30, 36, 37). Recently, Letnansky (38) has shown that changes in the metabolism of various acid-soluble nucleotides accompanying the phosphorylation of 2-deoxy-D-glucose in ascites tumor cells. This effect was especially manifested by a diminution of the cellular ATP, GTP, and UTP content, which was associated with an increase in the level of the corresponding nucleoside mono- and diphosphates (37). These observations are relevant since, in contrast to glucosamine, 2-deoxyglucose is not metabolized appreciably beyond 2-deoxyglucose 6-phosphate by the neoplastic tissue (38) and does not cause loss of cell viability (3). Therefore the two compounds would appear to inhibit the growth of neoplastic cells by different mechanisms.

In the present studies no attempt was made to estimate the pool size of the several glucosamine phosphate esters (glucosamine-6-P, glucosamine-1-P, N-acetylglucosamine-6-P, N-acetylglucosamine-1-P). It is likely that these intermediates also accumulate in cells exposed to high concentrations of exogenous glucosamine, and it is possible that one or more of these phosphate esters may be intrinsically cytotoxic. Studies on these questions are currently in progress.

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