Effect of D-Mannosamine on the Metabolism of Sarcoma 180 Ascites Cells[1] (36014)

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The cytotoxic effect of hexosamines has been demonstrated by a number of studies. Quastel and Cantero (1) observed that repeated administration of D-glucosamine to mice bearing sarcoma 37 tumor inhibited tumor growth. These observations were extended by Bekesi *et al.* (2) who also found that D-galactosamine, D-mannosamine, and D-mannose inhibited the viability and transplantability of sarcoma 180 ascites cells. Keppler *et al.* (3) have shown that repeated injections of D-galactosamine to rats induced acute liver changes resembling human viral hepatitis.

The biochemical basis for the toxicity of amino sugars is still not understood. Bekesi *et al.* (4) has shown that the biosynthesis of proteins and nucleic acids in neoplastic cells is inhibited by the amino sugars. Since D-glucosamine and D-galactosamine are converted into a number of metabolic intermediates, it is difficult to identify the underlying biochemical cause of their toxicity. D-Mannosamine is equally cytotoxic to sarcoma 180 ascites cells, but its metabolism is very limited. It is rapidly taken up by the cells and is phosphorylated to mannosamine-6-phosphate but is not metabolized further (5). Therefore, it seemed possible that the mechanism of hexosamine toxicity might more readily be elucidated using this amino sugar. In the present work, the effect of D-mannosamine on a number of metabolic processes in sarcoma 180 ascites cells has been studied.

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Materials and Methods. Sarcoma 180 ascites cells, originally obtained from Dr. L. Weiss, Roswell Park Memorial Institute, Buffalo, NY were carried in male Swiss Webster mice by weekly intraperitoneal transplantation of about 1×10^7 cells. The ascites fluid was diluted with 4 vol of 0.9% NaCl solution (containing 10,000 units of penicillin/ml) and 0.5 ml was injected.

Incubation conditions. Freshly harvested cells, 8–10 days after transplantation were used in all experiments. In most experiments 1 vol of ascites fluid containing about 1×10^8 cells/ml was incubated with 2 vol of Krebs–Ringer phosphate buffer (pH 7.4), containing 12.5 m*M* glucose and mannosamine as indicated. D-Mannosamine (Sigma Chemical Co.) was freshly dissolved for each experiment and neutralized to pH 7 with sodium hydroxide. The incubations were carried out at 37° in a metabolic shaker equilibrated with air and oscillated at a rate of 40 cycles/min.

Protein and nucleic acid biosynthesis. Protein synthesis was assessed by following the incorporation of labeled amino acids into trichloroacetic acid-insoluble products. Cells (1.5×10^8) were incubated in a total volume of 7.5 ml of Krebs–Ringer phosphate buffer (pH 7.4), containing 3.3 m*M* glucose and 0.5 μCi of uniformly labeled ^{14}C-amino acid mixture (sp act 1 mCi/mg; International Chemical and Nuclear Corp.). In some experiments, uniformly labeled L-leucine-^{14}C (sp act 263 mCi/mmole, New England Nuclear Corp.) was used. The incubation was terminated after 30 min by addition of an equal volume of cold 20% trichloroacetic acid. The cells were homogenized in a VirTis 45 homogenizer, and then washed 5 times with 10% trichloroacetic acid, once with 95%

ethanol, and once with acetone. The acid-insoluble fraction was air dried, weighed aliquots were dissolved in 1 ml of NCS solubilizer (Nuclear Chicago), and the radioactivity was determined with a liquid scintillation counter. The samples were counted for 4 or 10 min using the channel ratio method, and disintegrations were calculated from quenched standards. The biosynthesis of RNA was studied using 0.5 μCi of uridine-2-^{14}C (sp act 50.0 mCi/mmole; New England Nuclear Corp.) following the procedure described above. The biosynthesis of DNA was determined in a similar way using 0.5 μCi of thymidine-2-^{14}C (sp act 30.1 mCi/mmole; New England Nuclear Corp.)

Determination of ATP. At the end of an incubation period, perchloric acid was added to a final concentration of 0.6 *N*. After homogenization in a VirTis 45 homogenizer the precipitated proteins were removed by centrifugation at 9000*g* for 15 min in the cold. The acid-soluble fraction was neutralized with saturated $KHCO_3$, and the ATP was determined fluorometrically as described by Lowry *et al.* (6)

Measurement of oxygen consumption. Sarcoma 180 cells (3×10^8) were incubated in 30 ml of 0.01 *M* phosphate buffered saline (pH 7.4) at 37°. Aliquots containing about 5×10^7 cells were removed at different times of incubation and placed in a 5 ml cell equipped with a magnetic stirrer and maintained at 35°. The rate of oxygen consumption was measured with a Clarke electrode.

Sodium and potassium determination. At the end of an incubation, the cells were washed at room temperature with 0.25 *M* sucrose, suspended in deionized water, and homogenized with a VirTis 45 homogenizer. The homogenate was centrifuged for 30 min at 34,000*g* in the cold. Sodium and potassium in the supernatants were determined by flame photometry.

Chemical determination. Glucose was determined with glucose oxidase (Glucostat; Worthington Biochemical Corp.) as reported by McComb and Yushok (7). Lactic acid was determined by the method of Barker and Summerson (8). Inorganic phosphate and total phosphate was determined as reported by Bartlett (9).

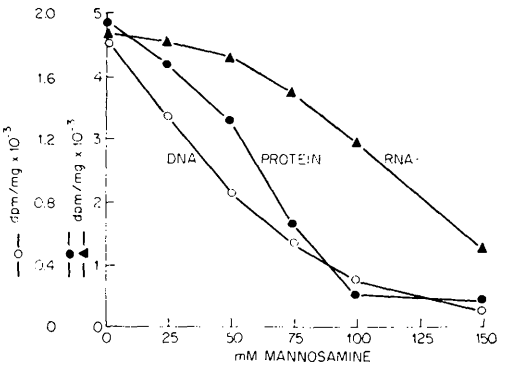

FIG. 1. Protein synthesis was determined using 1.5 $\times$ 10^5 cells incubated for 30 min in Krebs-Ringer phosphate buffer (pH 7.4) containing 0.5 μCi of ^{14}C-amino acid mixture, 3.3 m*M* glucose, and 0 to 150 m*M* D-mannosamine. Radioactivity was determined in washed, TCA-insoluble fractions. RNA synthesis was determined in the same system except that 0.5 μCi of uridine-2-^{14}C was used instead of ^{14}C-amino acid mixture. DNA synthesis was determined in same system but using 0.5 μCi of thymidine-2-^{14}C.

Results. Figure 1 shows the inhibition of protein RNA and DNA as a function of the concentration of mannosamine. Inhibition of the synthesis of all three macromolecular components was observed.

The possibility that mannosamine might interfere with the transport of the labeled precursors into the cells was tested by determining the appearance of ^{14}C-leucine in the acid-soluble and acid-insoluble fractions of cell in the presence and absence of exogenous mannosamine. Table I shows that the acid-soluble radioactivity from leucine was higher and the protein-bound radioactivity was lower in the cells incubated with mannosamine than in the controls. The sum of the acid-soluble and acid-insoluble radioactivity in the cells was not affected by mannosamine. The results are interpreted as an indication that the transport of ^{14}C-leucine into the cells is not inhibited by mannosamine and that the observed accumulation of the radioactivity in the acid-soluble fraction is due to the inhibition of protein biosynthesis.

The possibility that mannosamine inhibited synthesis of protein and nucleic acid by interfering with oxygen consumption was tested by measuring the consumption of ox-

TABLE I. Effect of D-Mannosamine on the Radioactivity from ^{14}C-Leucine in the TCA-Soluble and TCA-Insoluble Fractions.[a]

	(dpm/10^8 cells ± SD)		
	TCA		
Incubation	Soluble	Insoluble	Total
Control	125,600 ±3200	290,900 ±15,900	416,500 ±19,100
Mannosamine	248,000 ±9800	162,200 ±6400	410,200 ±16,200

[a] About 1×10^8 cells were incubated for 30 min in Krebs–Ringer phosphate buffer (pH 7.4) containing 8.3 m*M* glucose, 50 m*M* mannosamine, and 1 μCi of ^{14}C-leucine in a total volume of 3 ml. Control incubations did not contain mannosamine. At the end of the incubation peroid, the cells were washed and homogenized in 10% TCA. Radioactivity was determined in TCA-soluble fractions after separation from TCA-insoluble component by centrifugation. TCA-insoluble radioactivity was determined on washed air-dried samples.

ygen by sarcoma 180 cells in the presence and absence of mannosamine. Table II shows that 50 m*M* mannosamine did not affect the oxygen consumption even after a 2-hr incubation. This observation makes it unlikely that inhibition of oxygen consumption is responsible for the effects of this amino sugar on protein and nucleic acid biosynthesis.

One of the general characteristics of neoplastic cells is an increased rate of glycolysis (10). Since glucosamine can be a competitive inhibitor of hexokinase (11–15), the effect of mannosamine on glucose utilization was determined. Table III shows the effect on different amounts of mannosamine on the utilization of glucose and on lactic acid production. Glucose was utilized at the rate of 240 μmoles/hr/10^9 cells. D-Mannosamine up to 20 m*M* has no significant effect on glucose utilization or lactic acid production. Only moderate inhibition of glycolysis was observed even at 100 m*M* of mannosamine. Therefore it is unlikely that inhibition of glucose utilization or of glycolysis is responsible for the marked inhibition of protein and nucleic acid biosynthesis.

Quastel and Cantero (1) suggested that the inhibitory effect of D-glucosamine could be due to the depletion of intracellular ATP, due to a rapid phosphorylation of glucosamine. Since mannosamine is also rapidly phosphorylated to mannosamine-6-phosphate (5) it is possible that the rate of mannosamine phosphorylation might be faster than the rate at ATP regeneration, leading to a depletion of ATP.

Table IV shows that the ATP levels in cells incubated with 20 or 100 m*M* D-mannosamine over a 30 min time period are not reduced below the control values and in fact, may be somewhat elevated. This may reflect decreased utilization of ATP for protein and nucleic acid biosynthesis. The results presented in Table V show that neither the intracellular levels of inorganic phosphate nor total phosphate are altered by mannosamine. Therefore, interference with ATP generation or with the inorganic phosphate pool is not responsible for the inhibition of protein or nucleic acid biosynthesis.

The times used in the present studies on the effects of D-mannosamine on the ATP levels are longer than those used in the study of the effect of glucosamine on intracellular nucleotides (16) in which a brief decrease in ATP levels was noted. It is likely that a transient decrease in intracellular ATP levels also occurs in the presence of exogenous mannosamine, but this point was not investigated. Keppler *et al.* (17), in studying the effect of galactosamine on rat liver, did not see a decrease in the ATP levels. Bekesi and Winzler (16) and Keppler *et al.* (17) ob-

TABLE II. Rates of Oxygen Consumption in Sarcoma 180 Cells Incubated with Mannosamine.[a]

Incubation medium	Time of incubation (min)	O_2 Consumption (μmoles/min/10^9 cells)
Control	0	0.61
	120	0.59
Mannosamine	0	0.63
	120	0.58

[a] At indicated times of incubation with or without 50 m*M* mannosamine, ca. 5×10^7 cells were placed in a 5 ml cell at 35°, and the oxygen consumption was measured with a Clarke electrode.

TABLE III. Effect of Mannosamine on Glucose Consumption and Lactate Production by Sarcoma 180 Cells.[a]

Incubation medium (m*M*)	Time of incubation (min)	Glucose (μmoles) Remaining in medium	Glucose (μmoles) Utilized	Lactic acid produced (μmoles)
Krebs–Ringer phosphate buffer				
No mannosamine	0	23.9	1.1	0
	10	18.9	6.1	18.8
	30	12.2	12.8	28.4
	60	0.8	24.2	45.2
Mannosamine, 5	10	19.2	5.8	12.4
	30	11.8	13.2	26.0
	60	0.9	24.1	44.8
10	10	18.6	6.4	12.0
	30	12.4	12.6	22.0
	60	1.1	23.9	42.0
20	10	18.8	6.2	12.4
	30	14.2	10.8	22.4
	60	1.1	23.9	42.8
100	10	19.6	5.1	9.2
	30	15.1	9.9	14.4
	60	13.1	11.9	33.2

[a] About 1×10^8 cells were incubated up to 60 min with different amounts of mannosamine in Krebs–Ringer phosphate buffer (pH 7.4) containing 24 μmoles of glucose in a total volume of 3 ml. At the end of incubation the cells were centrifuged off and the remaining glucose and the lactic acid produced were determined.

served a sharp decrease in the uridine nucleotides and an increase in the uridine diphosphate hexosamine. However, this cannot be a factor in mannosamine toxicity since exogenous mannosamine in sarcoma 180 cells is phosphorylated to mannosamine-6-phosphate but is not converted to uridine diphosphate mannosamine (5).

Mannosamine is cationic and when a molecule enters the cell, it either must be accompanied by an anion or must exchange with an intracellular cation. Since mannosamine is rapidly taken up by the cells (5), the effect of mannosamine on the levels of intracellular sodium and potassium were determined. Figure 2 shows that intracellular concentration of sodium and potassium is somewhat lower in the cells incubated with 100 mM D-mannosamine. The decrease in sodium and potassium (20 mEq/liter of water) is about the same as the increase in total mannosamine in cells incubated with 100 m*M*

TABLE IV. ATP Levels in Cells.[a]

Incubation	Time of incubation (min)	ATP (μmoles/10^9 cells $\pm$ SD)
Control	0	6.02 $\pm$ 0.42
(no amino sugar)	5	6.54 $\pm$ 0.65
	15	6.96 $\pm$ 0.12
	30	7.26 $\pm$ 0.19
Mannosamine		
20 m*M*	5	7.49 $\pm$ 0.79
	15	7.58 $\pm$ 0.58
	30	8.51 $\pm$ 0.97
100 m*M*	30	7.18 $\pm$ 0.23

[a] About 1×10^8 cells were incubated up to 30 min with different amounts of D-Mannosamine in Krebs–Ringer phosphate buffer (pH 7.4) containing 8.3 m*M* glucose in a total volume of 3 ml. The incubation was terminated by addition of perchloric acid. ATP was determined fluorometrically on the neutralized perchloric acid-soluble homogenates.

TABLE V. Phosphate Levels in Sarcoma 180 Cells Incubated with Mannosamine.[a]

Incubation medium	Inorganic PO_4 (μmoles/10^9 cells)	Alcohol-soluble PO_4 (μmoles/10^9 cells)	
		Total	After charcoal adsorption
Krebs–Ringer PO_4			
No mannosamine	6.4 ± 0.5	62.7 ± 3.5	16.6 ± 0.3
Mannosamine, 100 m*M*	6.4 ± 0.2	62.5 ± 5.2	17.0 ± 0.4

[a] About 2 × 10^8 cells were incubated for 30 min with, or without, 100 m*M* D-Mannosamine in Krebs–Ringer phosphate buffer (pH 7.4) containing 8.3 m*M* glucose in a total volume of 6 ml. At the end of incubation, the cells were washed with 0.25 *M* sucrose and homogenized in water–ethanol [1:8]. Inorganic phosphate was determined on the charcoal-treated solution. Total alcohol-soluble phosphate was determined before and after treatment with charcoal.

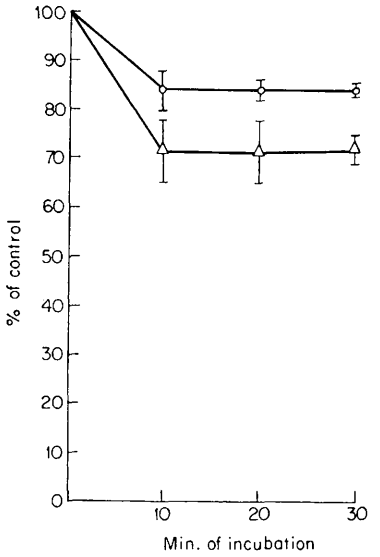

FIG. 2. Five milliliters of ascites fluid (ca. 5 × 10^8 cells) were incubated in Krebs–Ringer phosphate buffer (pH 7.4) containing 8.3 m*M* glucose and 100 m*M* mannosamine for times indicated. At the end of incubation the cells were washed with 0.25 *M* sucrose and homogenized. Na^+ and K^+ were determined by flame photometry in the supernatants after clarification by centrifugation. Control levels of Na^+ were 95 meq/liter and of K^+, 19 mEq/liter.
K^+ (O—); Na^+ (Δ—).

mannosamine (15 mmoles/liter of intracellular water) (5).

Discussion. The observation that D-mannosamine is cytotoxic to neoplastic cells (2), and the fact that it is not extensively metabolized by sarcoma 180 cells (5) led to the present attempt to ascertain the biochemical basis for the toxicity of amino sugars. This study has not provided the answer to the problem, but it has eliminated a number of possible causes for mannosamine toxicity. The fact that such different biochemical processes as protein, RNA, and DNA biosynthesis are affected by mannosamine suggests that a vital metabolic step in energy metabolism or utilization is affected by this amino sugar. However, no effects on respiration, glycolysis, ATP, or inorganic phosphate levels were seen at mannosamine concentrations which strongly inhibited protein and nucleic acid biosynthesis. It is hard to assess the significance of the slight decrease in intracellular Na^+ and K^+ in cells incubated with mannosamine.

Summary. Mannosamine at concentrations above 25 m*M* inhibit protein and nucleic acid biosynthesis in sarcoma 180 ascites cells during 30 min incubation. This inhibition could not be attributed to effects of the amino sugar on oxygen consumption, glycolysis, glucose utilization, or the intracellular levels of ATP or inorganic phosphate. A small reduction in the intracellular sodium and potassium concentrations occurred in cells incubated with mannosamine.

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