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ALLOXAN TOXICITY IN ISOLATED RAT HEPATOCYTES AND PROTECTION BY SUGARS

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Abstract—Suspensions of isolated rat hepatocytes incubated in the presence of the diabetogenic agent alloxan exhibit time- and concentration-dependent damage. At concentrations of 3.5 mM and above, alloxan caused an increase in lactate dehydrogenase (LDH), glutamate-pyruvate transaminase (GPT) and intracellular potassium (K^+) leakage, all of which are indices of plasma membrane damage, and decreased the intracellular reduced glutathione content (GSH) of the cells. Preincubation (10 min) in D-glucose (50 or 100 mM, but not 10 mM) partially protected the hepatocytes from LDH, GPT and K^+ leakage and the decrease in GSH produced by alloxan (7 mM) during a 60-min incubation period. Other sugars (D-galactose, 2-deoxy-D-glucose, D-fructose, D-mannoheptulose and D-mannitol) were also found to protect hepatocytes against damage caused by alloxan. D-Fructose was found to be the most potent protective sugar. These results indicate that alloxan is not selectively toxic to the pancreatic β-cell and that sugars can protect against alloxan-induced cytotoxicity in hepatocytes.

Pancreatic β-cells are particularly sensitive to the cytotoxic effects of alloxan, and administration of this agent to animals leads to the production of a diabetic state [1]. D-Glucose and certain other hexoses protect pancreatic β-cells from alloxan, and this has led to speculation that alloxan may act by binding at or near a specialized glucose receptor unique to insulin-producing cells [2, 3]. If alloxan and glucose were competing for a glucose receptor unique to β-cells, one would not expect glucose to protect other cells from cytotoxic effects produced by alloxan. The ability of glucose to protect other cell types from the effects of alloxan exposure has not been studied, and this lack of information prompted us to conduct the series of experiments reported here.

In the present study, the ability of alloxan to damage isolated hepatocytes was investigated and the protective role of sugars was assessed. Although alloxan-induced damage to the liver has not been reported previously, freshly isolated hepatocytes were chosen for this study because the measurements which reflect hepatocyte damage are well known [4]. Alloxan-induced damage to rat hepatocytes was measured by monitoring cytoplasmic enzyme and intracellular potassium leakage. The ability of alloxan to deplete reduced glutathione in hepatocytes was measured, and the possible role of sulfhydryl reduction in alloxan toxicity was assessed. The hexoses selected for study were based on their abilities to protect pancreatic β-cells from alloxan. D-Glucose is a very effective protectant, 2-deoxy-D-glucose and D-galactose are less effective, whereas D-fructose is a very poor protectant [3]. Mannoheptulose eliminates the protective effect of D-glucose from alloxan damage in the pancreatic islet cell [2, 3], and this sugar was examined for a similar effect using hepatocytes. The results obtained from our studies of alloxan damage to hepatocytes provide additional insight into the possible mechanism involved in its cytotoxic action.

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MATERIALS AND METHODS

All chemicals used were obtained from the Sigma Chemical Co. (St. Louis, MO). Male rats (Sprague–Dawley, Biolabs, Madison, WI), 200–300 g body weight, were maintained on rat chow (Ralston Purina, St. Louis, MO) and water *ad lib.* Isolation of rat liver cells was performed by perfusing rat liver with a collagenase-containing bicarbonate buffer solution as described elsewhere [5]. After isolation, the cells were suspended to produce a concentration of 30–40 mg wet weight/ml in the bicarbonate buffer, pH 7.3, containing 1.6 mM $CaCl_2$, 25 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) and 1.2% bovine serum albumin (Fraction V) and equilibrated with 95% O_2:5% CO_2. Initial viability estimates were determined on each cell preparation by exclusion of Trypan Blue (0.1%, w/v). Greater than 93% of cells excluded the dye in all preparations. Incubations of 2.5 ml volume were carried out in 25-ml Erlenmeyer flasks under a constant flow of humidified 95% O_2:5% CO_2 in a Dubnoff metabolic shaking bath at 37° and 80 oscillations/min. Alloxan was added to the required concentration in 25 μl of cold saline. In experiments involving sugars, the cells were preincubated for 10 min with the sugar before addition of alloxan. In some experiments, diethylmaleate (DEM) was used to deplete intracellular GSH. DEM was dissolved in ethanol and added (12.5 μl) to the incubation mixture to produce a concentration of 0.33 mM which was then preincubated for 10 min prior to addition of alloxan.

Cell viability was assessed at the end of the incubation period using indices of plasma membrane integrity (LDH, GPT and K^+ leakage). The GSH content of the cells was also determined. A 1.0-ml aliquot of cell suspension was centrifuged for 2 min at 500 rpm to separate the cells from incubation medium. The cell pellet was resuspended in 1.0 ml of 5% trichloroacetic acid (TCA) and centrifuged (15 min, 5000 rpm, 4°) to sediment the protein precipitate. The clear supernatant fraction was assayed for K^+ by flame photometry (Instrumentation Laboratory Inc., model 343) and GSH with the Ellman reagent [6] using the following procedure. A 0.4-ml aliquot of the protein-free supernatant fluid was added to 3.4 ml of 0.1 M phosphate buffer, pH 8, followed by the addition of 0.2 ml of 0.4 mg/ml 5,5'-dithiobis-2-nitrobenzoic acid in 1% sodium citrate solution. The resulting color was measured in a spectrophotometer at 412 nm. Intracellular GSH and K^+ contents were expressed per gram wet weight of cells.

The cell-free incubation medium was assayed for LDH activity [7] and GPT activity [8]. Total LDH and GPT activities in the cell suspension were determined by solubilizing a 0.1-ml aliquot overnight in a solution containing 0.1 ml of 5% Triton X-100 and 0.8 ml water. LDH and GPT activities in the solubilized cell suspension were measured by the same procedures used for the cell-free supernatant fraction. Enzyme leakage was measured as the activity in the cell-free supernatant fraction expressed as a percentage of the total activity found in the solubilized cell suspension.

Percent protection of hepatocytes afforded by a sugar was defined as the extent to which the sugar reduced toxicity, i.e. reduced the alloxan-related decrease in intracellular GSH or K^+ levels or reduced the alloxan-induced increase in enzyme leakage. More precisely, this was defined for GPT and LDH leakage as:

$$\text{Percent protection} = 100\left(1 - \frac{P_{s+a} - P_c}{P_a - P_c}\right)$$

and for K^+, GSH as:

$$\text{Percent protection} = 100\left(1 - \frac{P_c - P_{s+a}}{P_c - P_a}\right)$$

where P_a = value due to alloxan exposure, P_c = control value, and P_{s+a} = value due to alloxan exposure in the presence of the sugar.

Statistical analysis of the data was performed by analysis of variance with a randomized block design [9], and comparisons between individual treatment groups with controls were made using Dunnett's procedure [10] with the level of significance set at $P < 0.05$. Analysis was performed on the data before they were transformed to values given as "Percent protection". To determine if a sugar provided significant protection, values of variables in cells treated with alloxan plus sugar were compared with those values obtained in cells treated with alloxan alone.

RESULTS

Isolated rat hepatocytes incubated as suspensions in the presence of alloxan showed both time- and concentration-dependent damage. Figure 1 shows that alloxan concentrations of 1.4 mM and below did not produce evidence for membrane damage in the cells nor was there any reduction of intracellular GSH levels over periods of incubation up to 120 min. However, at concentrations of 3.5 mM and above there was damage as measured by the leakage of LDH and K^+ from the cells as well as a decrease in GSH. The damage increased with increasing alloxan concentrations and with longer incubation times. Results shown in Fig. 2 indicate that, with concentrations of 3.5 and 7.0 mM alloxan, the intracellular GSH levels fell rapidly during the first 10 min but then changed very little with further incubation. Leakage of LDH and K^+ was not pronounced at 10 min, but damage became increasingly evident after 20 min of exposure to alloxan. A concentration of 7.0 mM alloxan was chosen for subsequent experiments investigating protective agents as this seemed to be a threshold dose for producing more marked hepatocyte toxicity.

The ability of D-glucose to protect hepatocytes from alloxan damage was investigated, and the results are shown in Fig. 3. When hepatocyte suspensions were preincubated for 10 min in 50 or 100 mM D-glucose, the damage produced by alloxan (7.0 mM) during a 60-min incubation was reduced significantly. These concentrations reduced the leakage of LDH and GPT into the incubation medium and decreased the loss of K^+ from the cells. They

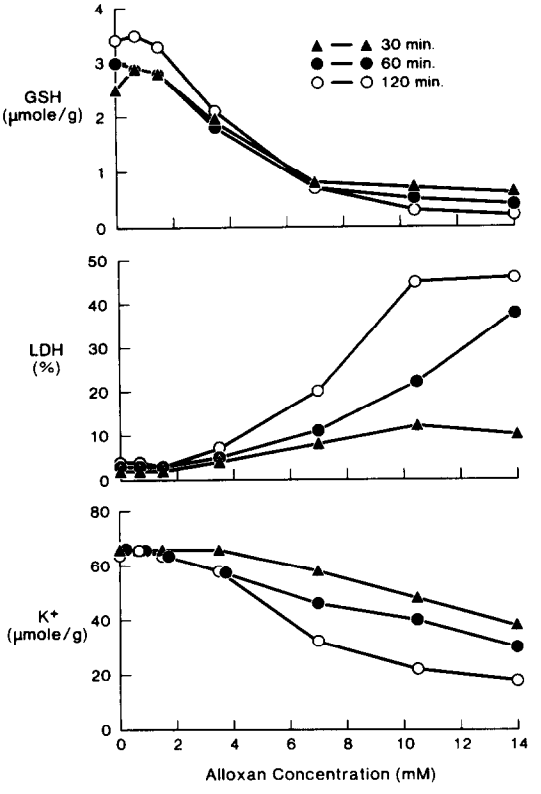

Fig. 1. Effect of different initial concentrations of alloxan on hepatocyte GSH and K^+ content as well as LDH leakage after 30, 60 and 120 min of incubation. For clarity, results from one experiment typical of three are presented.

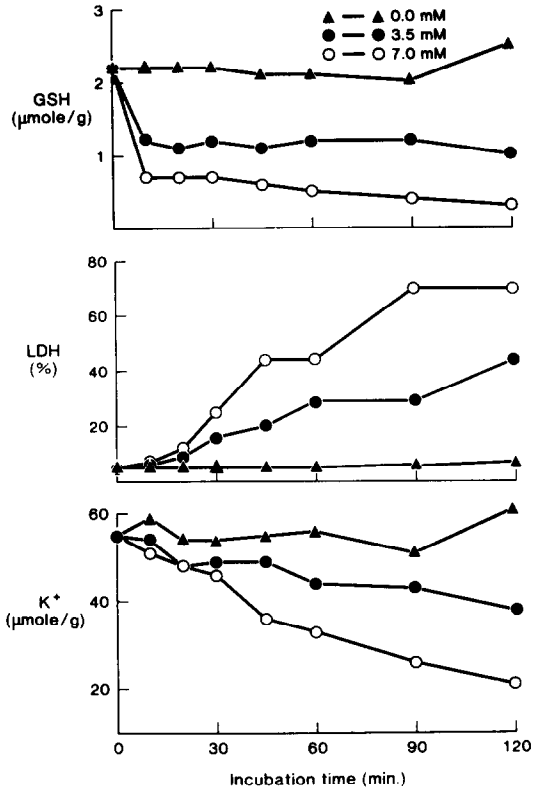

Fig. 2. Effect of incubation time on hepatocyte GSH and K^+ content as well as LDH leakage using alloxan concentrations of 0, 3.5 and 7.0 mM. For clarity, results from one experiment typical of three are presented.

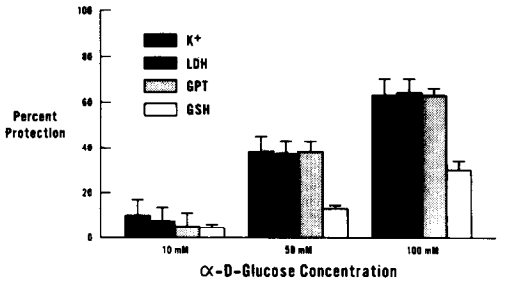

Fig. 3. D-Glucose protection from alloxan-induced damage to isolated hepatocytes. Hepatocyte suspensions were preincubated for 10 min in D-glucose (0, 10, 50 and 100 mM) prior to the addition of alloxan (7 mM), and incubation was continued for a further 60 min. Control values were obtained from cells treated with vehicle. At a concentration of 10 mM, D-glucose did not protect from alloxan damage as measured by alterations in K^+, LDH, GPT and GSH values ($P > 0.05$). D-Glucose did protect from alloxan damage at 50 and 100 mM concentrations ($P < 0.01$). Values are means ± S.E.M. for nine experiments.

also reduced the loss of GSH in the cells. However, 10 mM D-glucose did not protect from alloxan damage as it failed to alter the leakage of enzymes and K^+, nor did it alter depletion of intracellular GSH in the presence of alloxan. D-Glucose (10–100 mM) itself did not alter hepatocyte viability (data not shown).

Mannoheptulose did not alter the protective effect of D-glucose (Table 1). When hepatocytes were incubated in the presence of alloxan (7.0 mM) and glucose (100 mM) or glucose (100 mM) plus mannoheptulose (100 mM), there was no difference in leakage of LDH. In fact, mannoheptulose (100 mM) alone produced significant reduction in LDH leakage in cells treated with alloxan compared to cells receiving alloxan alone.

The non-metabolizable analogue of D-glucose, 2-deoxy-D-glucose, as well as D-galactose and D-fructose, were studied to determine their abilities to protect the hepatocyte from alloxan damage. Concentrations of 50 and 100 mM D-galactose and 2-deoxy-D-glucose partially protected the cells from leakage of LDH produced by alloxan (7.0 mM) but concentrations of 10 mM of these two hexoses had no effect on LDH leakage (Fig. 4). On the other hand, 10 mM D-fructose almost completely protected the hepatocytes from alloxan-induced LDH leakage (Fig. 4). Similarly, all the sugars were able to protect from the decrease in GSH levels produced by alloxan (7.0 mM) at concentrations of 50 and 100 mM, but only D-fructose was able to protect at 10 mM (Fig. 5). D-Fructose was also found to be a more potent protectant for GPT and K^+ leakage from the hepatocytes (data not shown). None of the sugars themselves altered hepatocyte viability (data not shown).

Further studies were conducted to determine the protective effect of sugars that are not actively transported or metabolized by the hepatocyte. The L-isomer of glucose at a concentration of 100 mM was found not to alter the toxicity of alloxan (7.0 mM) as measured by LDH leakage (Table 2). However,

Table 1. Effect of mannoheptulose on D-glucose protection from LDH leakage produced by alloxan*

Treatment	LDH (% leakage)
Alloxan	65 ± 12
Glucose + alloxan	37 ± 6†
Mannoheptulose + alloxan	47 ± 9†
Glucose + mannoheptulose + alloxan	38 ± 7†

* Hepatocytes were preincubated with the sugars at a concentration of 100 mM 10 min prior to the addition of alloxan (7 mM) and then incubated for 60 min. Each value is the mean ± S.E. of four experiments.

† Significantly different from alloxan alone ($P \leq 0.01$).

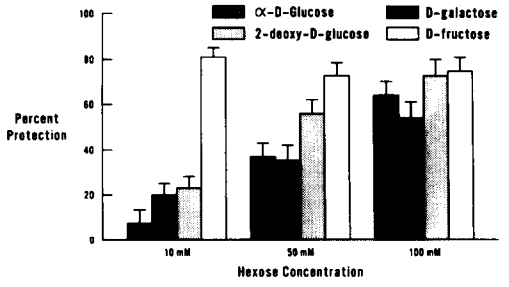

Fig. 4. Protection by hexose sugars from alloxan-induced LDH leakage from hepatocytes. Hepatocytes were preincubated for 10 min in either D-glucose (N = 9), D-galactose (N = 6), 2-deoxy-D-glucose (N = 6) or D-fructose (N = 5) at concentrations of 0, 10, 50 and 100 mM prior to the addition of alloxan (7 mM) and incubation was continued for 60 min. At 50 and 100 mM, all sugars protected against leakage of LDH produced by alloxan ($P < 0.05$). Only D-fructose significantly protected at 10 mM. Values are means ± S.E.M. of five to nine experiments (as indicated).

the extracellular space marker, D-mannitol, was equieffective with D-glucose as a protective agent.

The possible protective role of GSH against the toxic action of alloxan was investigated by prior depletion of intracellular GSH with the alkylating agent DEM. It was found in preliminary experiments that DEM, in concentrations of 0.067 to 0.67 mM, rapidly reduced GSH levels in the hepatocytes without producing significant toxicity over a 60-min period. At concentrations above these, DEM did produce evidence of plasma membrane damage. When cells were preincubated with 0.33 mM DEM for 10 min prior to the addition of 3.5 mM alloxan and incubated for a further 60 min, it was found that this concentration of alloxan, previously shown to be a threshold concentration for toxicity in normal hepatocytes, produced marked damage in cells depleted of GSH (Table 3). These results show that cells treated with a non-toxic concentration of DEM that produced a marked decrease in intracellular GSH were rendered more susceptible to alloxan damage.

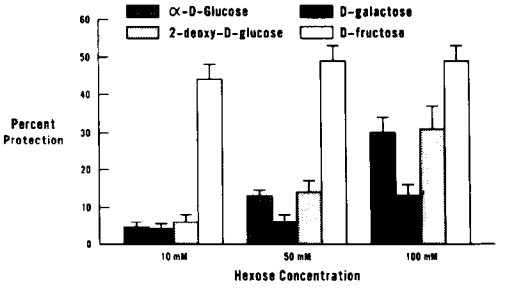

Fig. 5. Protection by hexose sugars from alloxan-induced GSH decrease in the hepatocytes. Conditions are as described in the legend to Fig. 3. D-Fructose significantly protected ($P < 0.05$) against the alloxan-induced reduction at all concentrations studied, whereas D-glucose, D-galactose and 2-deoxy-D-glucose only provided protection at 50 and 100 mM concentrations.

Table 2. Comparison of the abilities of D-glucose, L-glucose and D-mannitol to protect hepatocytes from alloxan*

Treatment	LDH (% leakage)
Alloxan	31 ± 6
D-Glucose + alloxan	15 ± 1†
L-Glucose + alloxan	31 ± 8
D-Mannitol + alloxan	13 ± 1†

* Hepatocytes were preincubated with the sugars (100 mM) for 10 min prior to addition of alloxan (7 mM) and then incubated for 60 min. Each value is the mean ± S.E. of four experiments.

† Significantly different from alloxan alone ($P < 0.05$).

DISCUSSION

Alloxan was shown in this study to produce concentration-dependent damage to isolated hepatocytes. This effect on hepatocytes was expected because alloxan has been shown to produce free radical species of oxygen (e.g. superoxide anions and hydroxyl free radicals) in oxygenated biological systems [11]. Insulin-secreting β-cells of the endocrine pancreas are particularly susceptible to the cytotoxic effects of alloxan *in vivo* [12] and *in vitro* [1, 3]. Exposure of isolated rat pancreatic islets to 1 mM alloxan produces functional deficits in insulin-secreting cells, and this effect can be eliminated by pretreating cells with certain hexoses [3]. Somewhat higher concentrations of alloxan (i.e. 3.5 mM) were needed to damage hepatocytes, and it was found that hexoses also inhibited alloxan-induced toxic effects in this isolated cell system.

The sugars, D-glucose, D-galactose, D-fructose, 2-deoxy-D-glucose and D-mannitol, were all effective in reducing the damaging effects of alloxan on hepatocytes. In isolated pancreatic islets, these sugars have been found to protect from alloxan-induced damage as assessed by various criteria for cell integrity and function [2, 13]. L-Glucose, which has no protective effect in β-cells [3], did not alter alloxan-induced hepatocyte damage in the present study. Hence, there are similarities between hepatocytes and pancreatic β-cells in the types of sugars which will protect isolated cells from alloxan damage.

Some of the results from experiments employing sugars as protectants from alloxan damage to hepatocytes were inconsistent with those found by others using the isolated pancreatic islet system. Tomita *et al.* [3] showed that D-glucose was far more potent in protecting against alloxan damage to pancreatic islets than either D-galactose or 2-deoxy-D-glucose, and D-fructose had very little protective effect. In contrast, D-fructose, at a concentration of 10 mM, provided almost complete protection against plasma membrane damage in isolated hepatocytes, whereas the other three sugars were not effective at that concentration. Another difference between the islet cell and hepatocyte systems is that mannoheptulose, a seven-carbon sugar which antagonizes the protective effect of glucose in the pancreatic β-cell [2, 3], does not have this property in the hepatocyte preparation. In fact, mannoheptulose itself protected

Table 3. Effect of diethylmaleate (DEM) pretreatment on intracellular K^+ and GSH and on LDH leakage produced by alloxan*

Treatment	GSH (μmoles/g)	LDH (% leakage)	K^+ (μmoles/g)
Control	2.48 ± 0.25	10 ± 3	59 ± 1
DEM	0.37 ± 0.01†‡	11 ± 3	58 ± 1
Alloxan (3.5 mM)	0.48 ± 0.15†	15 ± 3†	45 ± 3†
DEM + alloxan (3.5 mM)	0.16 ± 0.01†‡	51 ± 6§	27 ± 5†‡
Alloxan (7 mM)	0.46 ± 0.03†	43 ± 4†§	26 ± 3†‡

* Hepatocytes were preincubated for 10 min with DEM (0.33 mM) or vehicle (control) prior to the addition of alloxan. Incubation was continued for 60 min, after which time measurements for GSH, LDH and K^+ were made. Each value is the mean ± S.E. of four experiments.

† Significantly different from control ($P < 0.05$).

‡ Significantly less than alloxan (3.5 mM) alone ($P < 0.05$) (for K^+, GSH).

§ Significantly greater than alloxan (3.5 mM) alone ($P < 0.05$) (for LDH).

hepatocytes from alloxan damage. While some similarities exist, the hepatocyte and pancreatic islet systems exhibit differences in the potency with which certain sugars act to inhibit alloxan damage.

The damage caused by alloxan to the hepatocytes is associated with a decrease in GSH content. Preliminary experiments demonstrated that this decrease could not be accounted for by leakage of GSH into the media (data not shown). It is known that alloxan can react directly with GSH to form an adduct which has not been fully identified [14]. There is evidence that depletion of GSH, by itself, may lead to lipid peroxidation and subsequent hepatocyte toxicity [15]. The direct interaction between alloxan and GSH in the cell may lead, therefore, to a reduced GSH content and ultimately cell damage. Results from experiments using DEM to deplete GSH in this study show that hepatocyte levels of GSH can be lower than that produced by alloxan exposure without producing plasma membrane damage (see Table 3). Hence, it appears that depletion of GSH by direct interaction with alloxan does not explain completely the hepatocyte damage caused by alloxan. When GSH was depleted with DEM prior to the addition of a marginally toxic concentration of alloxan (3.5 mM), toxicity was markedly greater than that observed in cells treated with this concentration of alloxan alone. It appears that, while the depletion of GSH caused by alloxan does not lead to hepatocyte damage, the presence of GSH in the cell acts to protect against the damaging events initiated by alloxan.

The onset of hepatocyte damage caused by alloxan appears to be rapid. Whereas intracellular GSH levels were unchanged in cells exposed to initial alloxan concentrations of less than 3.5 mM, at alloxan concentrations that did produce toxicity, the fall in GSH was rapid, occurring within the first 10 min of alloxan exposure. Preliminary experiments (data not shown) indicated that when hepatocytes were exposed to alloxan for 5 min, then washed to remove alloxan and incubated in fresh media for a further 60 min, the cells developed damage similar to that seen in experiments in which alloxan was not removed. Hence, the damage produced by alloxan appears to occur in the first few minutes of exposure, and this rapid onset is associated with a sharp fall in GSH. This rapid onset of hepatocyte damage is consistent with findings in pancreatic β-cells which show that exposure to alloxan for 5 min is sufficient to cause a permanent inhibition of glucose-stimulated insulin release [3]. The early biochemical events, in addition to a rapid fall in intracellular GSH, that occur in cells exposed to alloxan are currently under investigation.

It has been postulated that alloxan produces its toxic effects through the generation of hydroxyl free radicals, and that these reactive substances cause cellular damage [16, 17]. In the pancreatic β-cell, it has been suggested that alloxan acts near or at a glucose receptor or transport site and that sugars protect by not allowing alloxan free access to this site [18]. It was thought that this susceptible site, unique to the pancreatic β-cell, makes alloxan selectively cytotoxic to this cell type [2]. However, data indicating that alloxan can damage the hepatocyte and that D-glucose and other sugars can protect from this damage are consistent with a more general mechanism of alloxan toxicity, i.e. damage caused by the generation of free radicals. It is known that D-mannitol is a hydroxyl radical scavenger [13], and it is possible that the other protective sugars act in a similar manner. In this case, it might be expected that each sugar would have a similar protective effect in any cell system, and that this effect would be related to the ability of the sugar to scavenge free radicals. However, since some sugars are relatively more effective in protecting hepatocytes when compared to pancreatic β-cells, it may be that sugars have more than one mode of protecting cells from alloxan toxicity.

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