On the Mechanism of Glucose Protection against Alloxan Toxicity

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Summary. The modifying effects of some sugars and amino acids on the diabetogenic action of alloxan were studied in mice. The severity of diabetes was assessed by measuring the blood glucose level 48 h after the alloxan injection. In accordance with earlier reports, D-glucose and D-mannose, but not D-galactose, protected against alloxan diabetes. No such effect was observed with D-mannoheptulose, L-alanine, or L-leucine. The protective action of glucose was abolished by prior treatment with mannoheptulose. It is concluded that alloxan and glucose do not compete for a common biochemical site in the β -cell membrane. Protection against alloxan toxicity may, however, result from a conformational change in the β -cell membrane induced by glucose, glucose transport, or glucose metabolism.

Mécanisme de protection du glucose contre la toxicité de l'alloxane

Résumé. On a étudié chez la souris les effets modifiants de certains sucres et acides aminés sur l'action diabétogène de l'alloxane. La gravité du diabète est définie grâce à la mesure du taux de glucose dans le sang 48 h après l'injection d'alloxane. Selon les rapports antérieurs, le D-glucose et le D-mannose, mais pas le D-galactose, protègent contre le diabète provoqué par l'alloxane. Le même effet n'a pas été observé avec le D-mannoheptulose, la L-alanine ou la L-leucine. L'action protectrice du glucose a été annulée par un traitement préalable au mannoheptulose. En conclusion, l'alloxane et le glucose ne sont pas en compétition

pour le même site biochimique dans la membrane de la cellule β . La protection contre la toxicité de l'alloxane peut résulter, cependant, d'un changement de conformation sur la membrane de la cellule β provoqué par le glucose, le transport du glucose, ou le métabolisme du glucose.

Über den Mechanismus der Glukoseprotektion gegen die Alloxantoxizität

Zusammenfassung. Es wurde an Mäusen der modifizierende Effekt von einigen Zuckern und Aminosäuren auf die diabetogene Wirkung von Alloxan studiert. Die Schwere des Diabetes wurde an Hand von Blutzuckermessungen 48 Std. nach der Alloxaninjektion beurteilt. In Übereinstimmung mit früheren Berichten schützen D-Glukose und D-Mannose, jedoch nicht D-Galaktose gegen den Alloxandiabetes. Kein Effekt wurde mit D-Mannoheptulose, L-Alanin und L-Leuzin festgestellt. Der Schutzeffekt von Glukose wurde durch vorherige Behandlung mit Mannoheptulose aufgehoben. Daraus wurde geschlossen, daß Alloxan und Glukose nicht an der gleichen Stelle der Membran der β -Zelle angreifen. Jedoch kann der Schutz gegen die Alloxantoxizität auf einer konformationellen Änderung der Membran der β -Zelle, welche durch Glukose, den Glukosetransport oder den Glukosemetabolismus herbeigeführt wurde, beruhen.

Key-words: Alloxan, alanine, diabetes, galactose, glucose, glucose carrier, leucine, mannoheptulose, mannose, pancreatic β -cell.

Introduction

Alloxan has long been used to produce selective destruction of the pancreatic β -cells in laboratory animals, resulting in diabetes. However, the molecular basis of alloxan action is unknown. The β -cells are physiologically very sensitive to changes in the surrounding glucose concentration, and it is interesting that glucose is able to block the β -cytotoxic action of alloxan (Bhattacharya, 1954). It has been suggested that this protective effect of glucose indicates that the alloxan molecule may act via glucose receptors located at the β -cell membrane (Cooperstein and Lazarow, 1969; Matthews and Dean, 1970). Alternatively, it was early suggested that interference of glucose with alloxan occurs at the hexokinase level (Bhattacharya, 1954).

Mannoheptulose is a seven-carbon sugar that inhibits the secretory response of the β -cells to glucose. Like alloxan, mannoheptulose has been thought to act by inhibiting glucose phosphorylation (Ashcroft and Randle, 1970) and/or by binding to glucose receptors located at the β -cell membrane (Matschinsky *et al.*, 1970; Hellman *et al.*, 1971). Against this background, we have tested the capacity of mannoheptulose to prevent the β -cytotoxic action of alloxan and to modulate the protective effect of glucose. In view of the possibility that accumulation of alloxan in the β -cells (Hammarström *et al.*, 1966) may be due to the affinity of alloxan for some physiological transport mechanism, we have also studied the protective capacity of L-alanine and L-leucine.

Materials and Methods

Chemicals. Alloxan monohydrate was obtained from Nutritional Biologicals Corporation, Cleveland,

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Ohio, U.S.A. It was dissolved in distilled water adjusted to pH 3.8 with HCl and, if not used immediately, kept at -20°C . D-Glucose was from British Drug Houses Ltd., England, and D-galactose, D-mannoheptulose, D-mannose, L-alanine, and L-leucine were from Sigma Chemical Company, St. Louis, Mo., U.S.A. ATP, NADP⁺, glucose 6-phosphate dehydrogenase, and hexokinase were supplied by Boehringer und Soehne GmbH, Mannheim, Germany. ¹²⁵I-labelled ox insulin and other reagents for the insulin radioimmunoassay were purchased from The Radiochemical Centre, Amersham, England. Crystalline mouse insulin was prepared by Novo Industri A/S, Copenhagen, Denmark. All reagents were of analytical grade.

Experimental procedure. Three-month-old mice of both sexes were obtained from the Umeå colony, which carries the gene *ob*. The homozygous occurrence of this gene gives rise to a syndrome of obesity and hyperglycaemia (Hellman, 1965). However, no obese-hyperglycaemic mice were used in the present study; the animals were all of normal phenotype and weighed 19–29 g. Within each experiment the animals were matched with respect to sex and weight. Unless otherwise stated, the mice had free access to food and water until about 15 h before the injection of alloxan and test substances into a tail vein. Alloxan (1% w/v solution) was regularly administered in a dose of 70 mg/kg body weight. The concentrations and dosages of NaCl, sugars and amino acids administered before or after alloxan are given in the legends to the tables. After the injection the animals were fed *ad lib*. for 45 h. After 2–3 h of food withdrawal duplicate samples of blood (5 µl each) were obtained from the orbital venous plexus (Riley, 1969), and assayed fluorophotometrically for glucose according to the coupled hexokinase/glucose 6-phosphate dehydrogenase method (Lowry *et al.*, 1964).

One experiment was performed to test the effect of mannoheptulose on glucose-stimulated insulin secretion in mice. In this experiment the mice were killed by decapitation. Mixed arterial and venous blood was obtained from the neck wound and used for the preparation of serum. The serum level of insulin was assayed radio-immunologically (Hales and Randle, 1963), using crystalline mouse insulin as reference.

Results

The blood glucose level of non-injected mice was 149 ± 6 mg/100 ml (mean value $\pm$ S.E.M. for 6 animals). Five min after an intravenous glucose load (6 mmol/kg body weight; 0.28 ml 10% aqueous solution), the blood glucose of 6 mice amounted to 288 ± 9 mg/100 ml. For 9 control animals injected with the same volume of NaCl (0.9% aqueous solution) the corresponding value was 150 ± 13 mg/100 ml, which is not different from that of the non-injected animals.

Forty-eight hours after intravenous injection of alloxan (70 mg/kg body weight) the blood glucose of six

mice was 416 ± 45 mg/100 ml (mean value $\pm$ S.E.M.). As shown in Table 1, NaCl failed to protect against the diabetogenic action of such an alloxan injection. Alloxan given 5 min after an intravenous load of glucose or mannose was significantly less diabetogenic, the differences from the NaCl-treated animals being statistically significant ($P < 0.01$). It can also be seen in Table 1 that glucose was more effective than

Table 1. *Effects of sugars and amino acids on the diabetogenic action of alloxan*

The data represent blood glucose levels 48 h after an intravenous injection of alloxan (70 mg/kg body weight) given a few min before or after a similarly administered dose of the listed compounds. The time interval between alloxan and test compounds was in all cases 5 min except when mannoheptulose was given prior to alloxan. In the latter case the time interval was 1 min in order to minimize the risk that mannoheptulose-induced hyperglycaemia would affect the result. The amount of sugar administered was 6 mmol/kg body weight (10% w/v aqueous solutions), whereas the dose of amino acids was 4 mmol/kg body weight (2–3% w/v aqueous solutions). Control animals received corresponding volumes of NaCl at a concentration of 0.9% (w/v). The results are given as mean values $\pm$ S.E.M. for the numbers of animals indicated within parentheses

Compound tested	Blood glucose concn. (mg/100 ml)	
	Compound given before alloxan	Compound given after alloxan
<i>Experimental series I</i>		
NaCl	357 ± 32 (15)	—
D-glucose	121 ± 7 (4)	480 ± 30 (3)
D-mannose	200 ± 16 (6)	467 ± 28 (4)
D-galactose	302 ± 44 (19)	411 ± 41 (8)
D-mannoheptulose	379 ± 7 (7)	489 ± 10 (3)
<i>Experimental series II</i>		
NaCl	635 ± 47 (9)	—
L-alanine	558 ± 40 (6)	436 ± 38 (4)
L-leucine	555 ± 10 (5)	609 ± 103 (5)

mannose ($P < 0.01$) in reducing alloxan toxicity. No protective effect was observed with a similar dose of galactose or mannoheptulose. None of the sugars tested had any protective action when given 5 min after the alloxan injection.

The possibility that mannoheptulose might interfere with the protective action of glucose was tested in a separate experiment. When mannoheptulose (18 mmol/kg body weight) was injected 1 min before glucose (6 mmol/kg body weight), a subsequent dose of alloxan caused significantly higher ($P < 0.001$) blood glucose levels than noted for mice given glucose alone prior to alloxan (Table 2). The blood glucose level of the mannoheptulose-injected mice was, in fact, no lower than that of control animals given NaCl before the alloxan injection.

Mannoheptulose and glucose were also investigated for effects on the serum insulin level in mice, using the same dosages as in the above alloxan experiment. It appears from Table 3 that the serum insulin level rose significantly ($P < 0.03$) within 15 min after the injection of glucose alone. This effect of glucose was completely abolished by mannoheptulose.

Neither alanine nor leucine (4 mmol/kg body weight) protected against alloxan toxicity when injected 5 min before the alloxan (Table 1).

Table 2. *Inhibition by mannoheptulose of glucose-protection against alloxan diabetes*

The data represent blood glucose levels 48 h after an intravenous injection of alloxan (70 mg/kg body weight) given 5–6 min after a similarly administered dose of the listed compounds. The dose of D-glucose was 6 mmol/kg body weight (28% w/v aqueous solution). In one group of mice D-mannoheptulose (18 mmol/kg body weight; 32% w/v aqueous solution) was given 1 min before the glucose load. Control animals received 0.33 ml NaCl (4.5% w/v aqueous solution). The concentration of NaCl was isotonic with the sugar solutions, and the volume of NaCl injected corresponded to the total volume of sugar given to the mannoheptulose-treated animals. For the sugar-injected mice the results are given as mean values $\pm$ S.E.M., whereas for 3 NaCl-injected controls the individual values are shown

Compound tested	No. of mice	Blood glucose concn. (mg/100 ml)
NaCl	3	482, 482, 500
D-glucose	5	258 ± 35
D-mannoheptulose plus D-glucose	6	668 ± 25

Table 3. *Effects of glucose and mannoheptulose on the serum insulin level*

The data represent serum levels of immunoreactive insulin (IRI) 15 min after intravenous injections of the listed compounds into non-fasted mice. Apart from the fact that no alloxan was given in this experiment, the concentrations and administration of injected compounds were the same as in Table 2

Compound tested	No. of mice	Serum IRI (ng/ml)
NaCl	6	1.05 ± 0.17
D-glucose	5	1.74 ± 0.19
D-mannoheptulose plus D-glucose	6	0.60 ± 0.17

Discussion

Interactions between alloxan and other compounds with respect to their effects on the β -cells should ideally be studied using isolated islets *in vitro*. However, since there is so far no suitable way to measure the diabetogenic action of alloxan in such a system, we used a technique *in vivo*, in spite of the obvious disadvantages that may arise from uncontrollable factors in the intact animal.

Previous reports that glucose (Bhattacharya, 1954; Carter and Younathan, 1962; Matthews and Dean, 1970) and mannose (Bhattacharya, 1953) but not

galactose (Bhattacharya, 1954), prevent alloxan toxicity were clearly confirmed in the present study. In the light of this, the fact that mannoheptulose alone did not affect the diabetogenic action of alloxan is highly significant. Mannoheptulose is assumed to compete with glucose at the level of transmembrane transport (Matschinsky *et al.*, 1970; Hellman *et al.*, 1971) and/or phosphorylation (Ashcroft and Randle, 1970). We must therefore conclude either that glucose does not bind to the same site as alloxan, or that the interaction between alloxan and glucose occurs at a site other than the site of interaction between mannoheptulose and glucose. The former alternative receives strong support from the observation that mannoheptulose prevented the protective action of glucose.

The reasoning on which the above conclusion is based may be clarified by considering three hypothetical possibilities. First suppose that alloxan binds to a glucose receptor site in the β -cell membrane, as has previously been suggested (Cooperstein and Lazarow, 1969; Matthews and Dean, 1970). The protective action of glucose might then be thought to be due to its having a greater affinity than alloxan for this receptor site. However, the fact that mannoheptulose inhibits the protective action of glucose would mean that mannoheptulose must have an even greater affinity than glucose for this hypothetical receptor site. Were this so, mannoheptulose would be expected to protect against alloxan diabetes, which was not found to be the case. If the possibility of a common site for all three compounds (glucose, mannoheptulose, alloxan) is rejected, one may envision mannoheptulose preventing the protective action of glucose by interfering with some event distal to the entry of glucose. It is, however, implicit in this view that competition between glucose and alloxan at a membrane-located receptor site does not occur.

A third alternative, based on the present results, is that glucose, glucose transport, or glucose metabolism induces some molecular alteration in the β -cell so as to render it insensitive to alloxan. Although our data do not indicate what the nature of such an alteration might be, these and previous observations make it tempting to consider a novel model of alloxan interaction with the β -cell. It has been shown by radioautography that in the adult mouse the β -cells differ from other cells in being able to concentrate alloxan. By contrast, the β -cells of young, alloxan-resistant mice do not perform such a concentration (Hammarström *et al.*, 1966). This suggests that the rather specific β -cytotoxic action of alloxan may be due to its affinity for some physiological transport mechanism in the β -cells. Neither alanine nor leucine protected the β -cells against alloxan in the present study. So, the "A" and "L" systems (Oxender and Christensen, 1963) for transport of neutral amino acids in these cells (Hellman, Sehlin and Täljedal, unpublished) are probably not responsible for the apparent uptake of alloxan.

The question arises whether alloxan may utilize the same carrier as glucose. Glucose influx into the β -cells is an extremely rapid process that has not been considered carrier-mediated in the past. However, recent studies of the uptake of D-glucose and L-glucose *in vitro* by isolated pancreatic islets indicate the existence of a stereospecific D-glucose carrier in the islets of toadfish (Cooperstein and Lazarow, 1969) as well as

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Fig. 1. Schematic illustration of a hypothetical model for the interactions of alloxan, D-glucose and D-mannoheptulose with the β -cell. Alloxan binding to a biochemical site at the β -cell membrane (perhaps on a D-glucose carrier) is prevented by a conformational change induced by the binding of glucose to another biochemical site. Because of its high affinity for the glucose-site, mannoheptulose prevents glucose from inducing the protective conformational change at the alloxan-site. However, mannoheptulose is not thought to alter the affinity of the alloxan-site for alloxan

in the β -cells of mice (Hellman *et al.*, 1971). In the latter species D-glucose entry into the β -cells appeared to be inhibited by mannoheptulose. If the binding of glucose, but not that of mannoheptulose, is associated with a conformational change in the carrier, this might reduce the carrier's affinity for alloxan as schematically outlined in Fig. 1. Our results are, of course, also compatible with the assumption that glucose, glucose transport, or glucose metabolism induces a protective conformational change at some other site in the β -cell membrane. It is less likely that the effect of glucose is mediated by enhanced insulin release, since 3-O-methyl glucose has also been found to protect against alloxan diabetes (Carter and Younathan, 1962). This observation is of considerable interest since there is some evidence that 3-O-methyl glucose and D-glucose share a common transport mechanism in the β -cells of mice (Hellman *et al.*, 1971).

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