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Alloxan and Streptozotocin Diabetes

Abstract

The cytotoxic glucose analogues alloxan and streptozotocin are the most prominent diabetogenic chemical agents in experimental diabetes research. While the mechanism of the selectivity of pancreatic beta cell toxicity is identical the mechanisms of the cytotoxic action of the two compounds are different. Both are selectively toxic to beta cells because they preferentially accumulate in beta cells as glucose analogues through uptake *via* the GLUT2 glucose transporter. Alloxan, in the presence of intracellular thiols, especially glutathione, generates reactive oxygen species (ROS) in a cyclic reaction with its reduction product, dialuric acid. The beta cell toxic action of alloxan is initiated by free radicals formed in this redox reaction. Autoxidation of dialuric acid generates superoxide radicals ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and, in a final iron catalysed reaction step, hydroxyl radicals ($OH^{\bullet}$). These hydroxyl radicals are ultimately responsible for the death of the beta cells with their particularly low antioxidative defence capacity and the ensuing state of insulin-dependent "alloxan diabetes". As a thiol reagent, alloxan selectively inhibits glucose-induced insulin secretion through its ability to specifically inhibit the glucokinase through oxidation of functionally essential thiol groups in this protein, thereby impairing oxidative metabolism and the glucose sensor function of this signalling enzyme of the beta cell. Streptozotocin, after uptake into the beta cells, is split up into its glucose and methylnitrosourea moiety. The latter, due to its alkylating properties, modifies biological macromolecules, fragments DNA and destroys the beta cells, causing a state of insulin-dependent "streptozotocin diabetes". The ability of streptozotocin to inhibit glucose-induced insulin secretion, likewise, can be explained with its alkylating potency, targeting mitochondrial DNA and thereby impairing the signalling function of beta cell mitochondrial metabolism.

Introduction

Alloxan and streptozotocin are the most prominent diabetogenic chemical compounds in experimental diabetes research. Both compounds are cytotoxic glucose analogues. While the mechanisms of cytotoxic action of the two compounds are different, the mechanism of the selectivity of the beta cell action is identical.

In 1838 Wöhler and Liebig synthesized a pyrimidine derivative (Wöhler and Liebig, 1838), which they later called alloxan (for review see: Lenzen and Panten, 1988a; Lenzen et al., 1996b). In 1943, alloxan became of interest in diabetes research when Shaw Dunn and McLetchie reported that this compound could induce diabetes (Dunn, 1943; Dunn and McLetchie, 1943) due to a specific necrosis of the pancreatic beta cells in experimental animals (Dunn et al., 1943a,b; Jörns et al., 1997; Peschke et al., 2000). The emerging insulinopenia causes a state of experimental diabetes mellitus called "alloxan diabetes" (Dunn and McLetchie, 1943; Goldner and Gomori, 1943; McLetchie, 1982). The reduction product of alloxan, dialuric acid (Wöhler and Liebig, 1838), has also been shown to be diabetogenic in experimental animals (Brückmann and Wertheimer, 1945, 1947; Bailey et al., 1946; Saviano and De Franciscis, 1946; Merlini, 1951), and to cause, like lipophilic alloxan derivatives such as butylalloxan, identical ultrastructural changes (Jörns et al., 1997).

Streptozotocin is an antimicrobial agent originally derived from the soil micro-organism *Streptomyces achromogenes* (White, 1963; Herr et al., 1967; Weiss, 1982). It has been used as a chemotherapeutic alkylating agent in the treatment of metastasizing pancreatic islet cell tumours and in other malignancies (Evans et al., 1965; Schein et al., 1974). In 1963 Rakieten and collaborators reported that streptozotocin is diabetogenic (Rakieten et al., 1963). Ever since streptozotocin has been the agent of choice to induce experimental diabetes mellitus in laboratory animals (Rerup, 1970). This insulinopenia syndrome, called "streptozotocin diabetes" (Schein et al., 1967), is due to the ability of this compound to induce a specific necrosis of the pancreatic beta cells (Arison et al., 1967; Lenzen et al., 1996b).

After decades of research a unifying explanation for the molecular mechanism of toxic action of these two most prominent diabetogenic agents can be provided. As the understanding of the chemical reactivity of these compounds is crucial for the understanding of their diabetogenicity, this review will provide an integrated view upon the chemical properties and the biological effects of alloxan and streptozotocin. It is not the aim to provide

an extensive review on the entire literature on these two diabetogenic agents, since this has been covered in previous reviews (Rerup, 1970; Cooperstein and Watkins, 1981; Weiss, 1982; Preston, 1985; Lenzen and Panten, 1988a; Szkudelski, 2001; Brömme and Peschke, 2003).

Alloxan diabetes and streptozotocin diabetes

Alloxan and streptozotocin, when injected into to an experimental animal, induce a multiphasic blood glucose response (Fig. 1), which is accompanied by corresponding inverse changes in the plasma insulin concentration (Goldner and Gomori, 1944; Shipley and Beyer, 1947; Wrenshall et al., 1950; Suchowsky, 1953; Lundquist and Rerup, 1967; Boquist, 1968a,b; Rerup and Tarding, 1969; Rerup, 1970; Losert et al., 1971; Schusdziarra et al., 1979; Tasaka et al., 1988; West et al., 1996; Szkudelski, 2001) as well as sequential ultrastructural beta cell changes finally leading to necrotic cell death (Wellmann et al., 1967; Boquist, 1968a,b, 1977; Richter et al., 1971; Tomita, 1972; Boquist and Lorentzon, 1979; Lorentzon and Boquist, 1979; Lenzen et al., 1996b; Jörns et al., 1997; Mythili et al., 2004).

A 1st transient hypoglycaemic phase lasting maximally 30 min starts within the first minutes after alloxan injection (Wrenshall et al., 1950; Suchowsky, 1953; Tasaka et al., 1988). This short-lived hypoglycaemic response is the result of a transient stimulation of insulin secretion (Pagliara et al., 1977; Weaver et al., 1978c; Goto et al., 1980; Kliber et al., 1996), as documented by an increase of the plasma insulin concentration (Tasaka et al., 1988). The underlying mechanism of this transient hyperinsulinaemia is a temporary increase of ATP availability due to inhibition of glucose phosphorylation through glucokinase inhibition. This initial transient hypoglycaemic phase is not observed in the case of a streptozotocin injection because, at variance from alloxan, streptozotocin does not inhibit glucokinase (Lenzen et al., 1987b). During these first five minutes after toxin exposure the beta cells show no morphological signs of damage (Jörns et al., 1997). Up to one hour after exposure to both alloxan and streptozotocin, at a time, when normoglycaemia and normoinsulinaemia are prevailing, morphological beta cell changes are still minimal. Discrete changes in the form of a pale cytoplasm and beginning intracellular microvacuolisation, dilatation of the cisternae of the rough endoplasmic reticulum and mildly swollen mitochondria become visible after 20–30 min, apparent in particular on the ultrastructural level (Wellmann et al., 1967; Boquist, 1968a,b, 1977; Richter et al., 1971; Tomita, 1972; Boquist and Lorentzon, 1979; Lorentzon and Boquist, 1979; Lenzen et al., 1996b; Jörns et al., 1997). At this stage the secretory granules are still normal in number and ultrastructure. These early signs can be considered potentially reversible intracellular lesions.

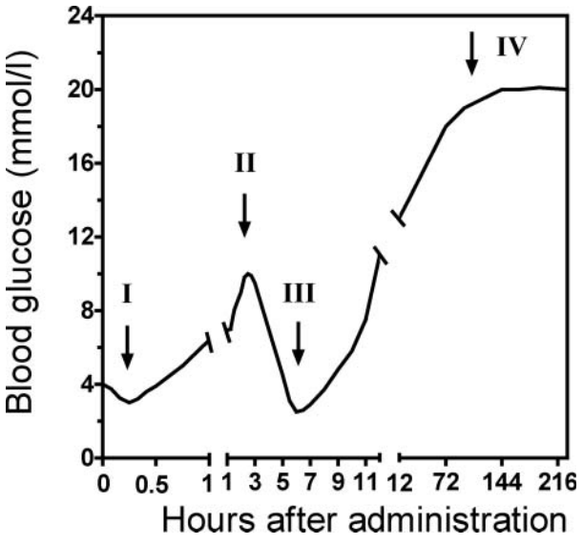

Fig. 1: Phasic blood glucose response to a diabetogenic dose of alloxan (tetraphasic; I–IV) or streptozotocin (triphasic; first phase does not develop in the case of streptozotocin; II–IV).

This 2nd phase of the blood glucose response starts with a rise of the blood glucose concentration one hour after administration of the toxins, while at the same time the plasma insulin concentration decreases. This is the first hyperglycaemic phase after the first contact of the beta cells with the toxins (Goldner and Gomori, 1944; Shipley and Beyer, 1947; Wrenshall et al., 1950; Suchowsky, 1953; Lundquist and Rerup, 1967; Boquist, 1968a,b; Rerup and Tarding, 1969; Rerup, 1970; Losert et al., 1971; Schusdziarra et al., 1979; Tasaka et al., 1988; West et al., 1996). This pronounced hyperglycaemia usually lasts 2–4 hours and is accompanied by decreased plasma insulin concentrations (Lundquist and Rerup, 1967; Tasaka et al., 1988). These changes result from an inhibition of insulin secretion from the pancreatic beta cells, which both toxins induce due to their beta cell toxicity. Morphologically this phase is characterized in the beta cells by intracellular vacuolization, dilatation of the cisternae of the rough endoplasmic reticulum, a decreased Golgi area, a reduction of insulin content and of the number of secretory granules as well as swollen mitochondria with a loss of cristae of the inner membranes (Wellmann et al., 1967; Boquist, 1968a,b, 1977; Richter et al., 1971; Tomita, 1972; Boquist and Lorentzon, 1979; Lorentzon and Boquist, 1979; Lenzen et al., 1996b; Jörns et al., 1997). The observations are consistent with a defective mitochondrial energy production (Boquist, 1977; Boquist and Lorentzon, 1979; Lorentzon and Boquist, 1979) due to the toxic action of the diabetogens and an inhibition of pre-proinsulin biosynthesis as well as processing, packaging and storage of insulin in the secretory granules (Jain and Logothetopoulos, 1976; Niki et al., 1976). This is the underlying cause for the inhibition of insulin secretion (Lenzen and Panten, 1988a,b) and the

resultant hyperglycaemia and hypoinsulinaemia (Lundquist and Rerup, 1967; Tasaka et al., 1988) during this phase.

The 3rd phase of the blood glucose response is again a hypoglycaemic phase typically 4–8 hours after the injection of the toxins, which lasts several hours (Jacobs, 1937; Goldner and Gomori, 1944; Shipley and Beyer, 1947; Wrenshall et al., 1950; Suchowsky, 1953; Lundquist and Rerup, 1967; Boquist, 1968a,b; Rerup and Tarding, 1969; Rerup, 1970; Losert et al., 1971; Schusdziarra et al., 1979; Tasaka et al., 1988; West et al., 1996). It may be so severe that it causes convulsions (Goldner and Gomori, 1944) and may even be fatal without glucose administration (Shipley and Beyer, 1947; Losert et al., 1971), in particular when liver glycogen stores are depleted through starvation (Wrenshall et al., 1950). This severe transitional hypoglycaemia is the result of a flooding of the circulation with insulin as a result of the toxin-induced secretory granule and cell membrane rupture (Banerjee, 1945, 1948). Pancreatectomy prevents this phase (Griffiths, 1948). As documented through morphological and ultrastructural analyses (Wellmann et al., 1967; Boquist, 1968a,b, 1977; Richter et al., 1971; Tomita, 1972; Boquist and Lorentzon, 1979; Lorentzon and Boquist, 1979; Lenzen et al., 1996b; Jörns et al., 1997; Mythili et al., 2004), these changes comprise not only ruptures of the secretory granules with loss of their insulin content and of the plasma membrane but also of other subcellular organelles, including the cisternae of the rough endoplasmic reticulum and the Golgi complex. The outer and inner membranes of the mitochondria also loose their structural integrity in this phase. Expression of functionally essential proteins such as GLUT2 glucose transporter and glucokinase is lost and insulin protein is undetectable. The beta cell nuclei are shrunken with condensed chromatin as signs of pyknosis. Nuclei show no TUNEL positive staining. These changes are irreversible and highly characteristic for a necrotic type of beta cell death.

The 4th phase of the blood glucose response is the final permanent diabetic hyperglycaemic phase, which characterizes alloxan and streptozotocin diabetes (Goldner and Gomori, 1944; Shipley and Beyer, 1947; Wrenshall et al., 1950; Suchowsky, 1953; Lundquist and Rerup, 1967; Boquist, 1968a,b; Rerup and Tarding, 1969; Rerup, 1970; Losert et al., 1971; Schusdziarra et al., 1979; Tasaka et al., 1988; West et al., 1996). Morphological and ultrastructural analyses document a complete degranulation and loss of the integrity of the beta cells within 12–24–48 h after administration of the toxins (Wellmann et al., 1967; Boquist, 1968a,b, 1977; Richter et al., 1971; Tomita, 1972; Boquist and Lorentzon, 1979; Lorentzon and Boquist, 1979; Lenzen et al., 1996b; Jörns et al., 1997; Mythili et al., 2004). Non-beta cells such as alpha cells and other endocrine and non-endocrine islet cell types as well as the extrapancreatic parenchyma remain intact (Lenzen et al., 1996b; Jörns et al., 1997), proving the beta cell selective character of the toxic action of these diabetogenic compounds. Cell debris of the dying beta cells is removed by scavenger macrophages, which are not activated (Williamson and Lacy, 1959). After destruction of the beta cells, when survival through insulin supplementation is secured, so-called end stage islets reside in the pancreas, exclusively composed of non-beta cells (Jörns et al., 2001).

Thus, injections of alloxan and streptozotocin induce principally the same blood glucose and plasma insulin response and induce an insulin-dependent type 1 like diabetes syndrome. All morphological features of beta cell destruction are characteristic for a necrotic cell death (Lenzen et al., 1996b; Jörns et al., 1997; Peschke et al., 2000). This is clearly at variance from the situation of autoimmune type 1 diabetes mellitus, both in humans as well as in mouse and rat models of the disease, where beta cell demise is the result of apoptotic cell death without leakage of insulin from ruptured secretory granules (Lenzen et al., 2001; Lally and Bone, 2002; Jörns et al., 2004, 2005).

Alloxan mechanism of action

The diabetogenic agent alloxan (Fig. 2) has two distinct pathological effects interfering with the physiological function of the pancreatic beta cells. It selectively inhibits glucose-induced insulin secretion through its ability to specifically inhibit the glucokinase, the glucose sensor of the beta cell, and it causes a state of insulin-dependent diabetes mellitus through its ability to induce a selective necrosis of the beta cells. These two effects of alloxan can be assigned to specific chemical properties of alloxan. The common denominator of these effects is selective cellular uptake and accumulation of alloxan by the beta cell.

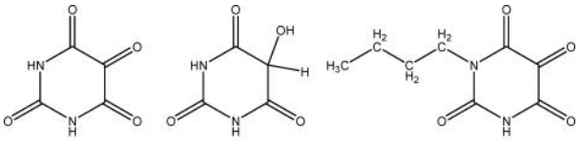

Fig. 2: Chemical formulas of alloxan, dialuric acid, and butylalloxan.

Chemical properties of alloxan

Alloxan (2,4,5,6-tetraoxypyrimidine; 2,4,5,6-pyrimidinetetrone) is:

- *An oxygenated pyrimidine derivative. It is also a barbituric acid derivative (5-ketobarbituric acid).*
- *Present as alloxan hydrate in aqueous solution.*

- *A beta cell toxic glucose analogue with a molecular shape similar to that of glucose (Weaver et al., 1979).*
- *A very hydrophilic compound (partition coefficient –1.8) (Lenzen and Munday, 1991).*
- *A week acid (Patterson et al., 1949).*
- *Chemically instable in buffer solutions with a half-life of 1.5 min at pH 7.4 and 37 °C decomposing to alloxanic acid (Patterson et al., 1949; Lenzen and Munday, 1991).*
- *Stable at acid pH (0.01 M HCl) (Lenzen and Munday, 1991).*
- *For experimentation concentrated stock solutions in 0.01 M HCl, kept on ice, should be used and added to test medium just prior to the start of the experiment in order to obtain the final concentration.*
- *For injection stock solution should be diluted with ice-cold saline (0.9 % NaCl) immediately prior to injection.*
- *A toxic thiol reagent (Lenzen and Munday, 1991).*
- *It is reduced to dialuric acid in the presence of GSH and other thiols (Winterbourn and Munday, 1989).*
- *During redox cycling between alloxan and dialuric acid, new alloxan is formed continuously, so that toxic ROS species can be generated intracellularly over a long time period (>1 h) (Lenzen and Munday, 1991).*
- *During each redox cycle a small amount of "Compound 305", an alloxan-GSH adduct of unknown structure with a characteristic absorbance at 305 nm that is not toxic, is formed (Patterson et al., 1949; Winterbourn and Munday, 1989; Lenzen and Munday, 1991; Brömme et al., 2002).*
- *A protoxin, which generates in its xenobiotic metabolism toxic ROS species (superoxide radicals ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and, in the presence of an iron catalyst, hydroxyl radicals ($^{\bullet}OH$), when it redox cycles with dialuric acid (Munday, 1988; Winterbourn and Munday, 1989; Elsner et al., 2006).*

Biological effects of alloxan due to hydrophilicity, chemical instability and glucose similarity of the alloxan molecule: Selective uptake and accumulation by the beta cell

Alloxan is a hydrophilic and very instable chemical compound (Lenzen and Munday, 1991) and the alloxan molecule has a molecular shape, which is very similar to that of glucose (Weaver et al., 1979; Gorus et al., 1982). Due to their hydrophilic character both alloxan and glucose do not penetrate the lipid bilayer of the plasma membrane. However, the molecular shape of alloxan is so similar to that of glucose, that the GLUT2 glucose transporter in the beta cell plasma membrane accepts this molecule as a glucose analogue (glucomimetic) and transports it into the cytosol (Weaver et al., 1978a; Gorus et al., 1982). Alloxan does not inhibit the function of the transporter within the biological lifetime of the chemical compound alloxan (Elsner et al., 2002), so that the alloxan molecule can enter the beta cells through this protein pore unrestricted, where it is accumulated selectively (Hammarström et al., 1967; Boquist et al., 1983; Malaisse et al., 2001) and executes its biological effects.

Biological effects of alloxan due to thiol group reactivity: Selective inhibition of glucose-induced insulin secretion through inhibition of glucokinase

The selective inhibition of glucose-induced insulin secretion is the major pathophysiological effect of alloxan, which results from the thiol group reactivity of alloxan. The reactive 5-carbonyl group of the alloxan molecule reacts very avidly with thiol groups. The thiol groups of the low affinity glucose phosphorylating enzyme glucokinase, which functions in the beta cell as the glucose sensor for initiation of glucose-induced insulin secretion (Lenzen and Panten, 1988b), are particularly sensitive to oxidation by alloxan. With a half maximal inhibitory concentration in the range between 1 and 10 µmol/l the glucokinase protein is the most sensitive thiol enzyme known in the beta cell (Lenzen and Panten, 1988a; Tiedge et al., 2000).

There is a great overall parallelism between the characteristics of alloxan-induced inhibition of glucose-stimulated insulin secretion and of alloxan inhibition of pancreatic beta cell glucokinase.

i) Alloxan (Miwa et al., 1984; Hara et al., 1986; Meglasson et al., 1986; Lenzen et al., 1987b) inhibits glucokinase because the reactive 5-carbonyl group reacts with thiols. The exceptional reactivity of the 5-carbonyl group of alloxan is reflected in the facile reduction to the corresponding alcohol (dialuric acid) with reducing agents such as the thiols glutathione, cysteine and dithiothreitol (Munday, 1988; Lenzen and Munday, 1991). Dialuric acid, the reduction product of alloxan, is not thiol reactive and therefore does not inhibit glucokinase as long as it is kept in its reduced form through reducing agents (Lenzen et al., 1988b; Lenzen and Munday, 1991). This underlines the importance of the free reactive 5-carbonyl group of alloxan for its reaction with the thiol groups of the enzyme.

ii) The selectivity of the inhibition of insulin secretion after exposure to alloxan (Ishibashi et al., 1978; Weaver et al., 1978b,c; Tait et al., 1983; Miwa et al., 1986) is restricted to that induced by glucose and its epimer mannose, both of which induce insulin secretion through binding to the glucose sensor glucokinase (Lenzen and Panten, 1988b). This selective inhibition is due to the exclusive sensitivity towards oxidation of the thiol groups of this enzyme by alloxan (Lenzen and Panten, 1988a). The inhibition of glucokinase reduces glucose oxidation and ATP generation (Gunnarsson and Hellerström, 1973; Borg et al., 1979), thereby suppressing the signal generating metabolic flux and the generation of the ATP signal for glucose-induced insulin secretion (Lenzen and Panten,

1988a). Inhibition of glucokinase is achieved within one minute of exposure to alloxan and the result is a selective inhibition of glucose-induced insulin secretion within minutes (Weaver et al., 1978c; Lenzen et al., 1988a). Insulin biosynthesis is also inhibited by alloxan (Jain and Logothetopoulos, 1976; Niki et al., 1976), most likely through the same mechanism.

iii) The inhibition of glucose-induced insulin secretion is preceded by a very transient (1–2 min) stimulation of insulin secretion from the beta cells immediately after the exposure to alloxan (Weaver et al., 1978c). This effect of alloxan can be explained by a reduction of ATP consumption due to blockade of glucose phosphorylation by glucokinase (Lenzen and Panten, 1988b), resulting from the instantaneous oxidation of catalytically important thiol groups of this enzyme (Lenzen et al., 1988a; Tiedge et al., 2000). This results in a transient increase of ATP in the beta cell, which, through interaction with the ATP-sensitive potassium channel, causes this transient release of insulin, before insulin secretion in response to glucose stimulation is halted permanently through suppression of the mitochondrial metabolic flux and the ATP signal generated in this process.

iv) The insulin secretory response to other nutrient secretagogues such as 2-ketoisocaproic acid and leucine or non-nutrient secretagogues such as the sulfonylurea drug tolbutamide remains initially intact, because it is not mediated *via* glucokinase (Lenzen et al., 1988a). The secretory response to these secretagogues is lost only after a delay of up to one hour after alloxan exposure (Borg, 1981). This is a consequence of gradual deterioration of beta cell function due to oxidation of functionally essential proteins as well as of the toxicity of free oxygen radicals generated during persisting continuous intracellular redox cycling between alloxan and dialuric acid. These toxic actions affect in particular enzymes of mitochondrial metabolism.

v) Thiols such as GSH, cysteine and dithiotreitol protect glucokinase against alloxan inhibition because they reduce alloxan to dialuric acid (Lenzen et al., 1988b; Lenzen and Mirzaie-Petri, 1991). By keeping dialuric acid reduced, a formation of the thiol reactive alloxan molecule is prevented (Lenzen and Munday, 1991).

vi) However, only dithiols such as dithiotreitol but not monothiols such as GSH or cysteine (Lenzen et al., 1988b; Lenzen and Mirzaie-Petri, 1991) can readily reverse alloxan-induced glucokinase inhibition. They achieve this effect by reducing certain functionally essential cysteine residues of the glucokinase enzyme (Tiedge et al., 2000), which are oxidized through alloxan action (Lenzen et al., 1988b; Lenzen and Mirzaie-Petri, 1991).

At high concentrations, alloxan can inhibit many cellular functions. To this effect contributes significantly the ability to oxidize thiol groups of many functionally important enzymes e. g. hexokinase (Lenzen et al., 1990), phosphofructokinase (Miwa et al., 1984), the Ca^{2+}- and calmodulin-dependent protein kinase (Colca et al., 1983), and aconitase (Lenzen and Mirzaie-Petri, 1992) as well as other proteins.

Thus, it can be concluded that the inhibition of glucose-induced insulin secretion by alloxan is the result of the exquisite thiol reactivity of the glucose sensor glucokinase. ROS are not involved in this effect of alloxan.

Biological effects of alloxan due to redox cycling and generation of toxic reactive oxygen species (ROS): Beta cell toxicity and diabetogenicity

Alloxan diabetes (Dunn and McLetchie, 1943; Goldner and Gomori, 1943; McLetchie, 1982) is a form of insulin-dependent diabetes mellitus caused by a direct toxic effect upon the endocrine pancreas; occlusion of the arterial blood supply to the pancreas prevents access of the injected alloxan to the beta cells and thus diabetes (Gomori, 1945; Bailey et al., 1950; Hellman and Diderholm, 1955). Diabetes is the result of the selective pancreatic beta cell toxicity of this compound which induces a necrosis of the beta cells (Dunn et al., 1943a,b; Jörns et al., 1997).

i) In order to destroy insulin-producing cells, alloxan must enter the cell. Due to its hydrophilicity (Lenzen and Munday, 1991) it does not permeate the lipid barrier of the plasma membrane. However, due to its similarity with the glucose molecule, it can enter the cell *via* the low affinity GLUT2 glucose transporter in the plasma membrane (Elsner et al., 2002, 2003). This has been proven by the fact that alloxan is not toxic to insulin-producing cells which do not express the GLUT2 glucose transporter (Bloch et al., 2000; Elsner et al., 2002). Alloxan has no immediate and direct inhibitory effect upon glucose transport (Elsner et al., 2002).

ii) Since the half-life of alloxan is short (Patterson et al., 1949; Lenzen and Munday, 1991), it must be taken up and accumulated quickly in the beta cell (Hammarström et al., 1967). After a few minutes, in aqueous solution, alloxan has been converted into non-diabetogenic alloxanic acid through spontaneous decomposition (Lenzen and Munday, 1991). Thus, alloxan is ineffective, when blood flow to the pancreas has been interrupted for a few minutes after alloxan injection (Gomori, 1945; Bailey et al., 1950; Hellman and Diderholm, 1955).

iii) N-substituted alloxan derivatives with a long carbon side chain such as butylalloxan differ chemically in one respect from alloxan. In contrast to the hydrophilic alloxan, they are lipophilic (Munday et al., 1993). Butylalloxan (Fig. 1), like alloxan, is thiol reactive and generates ROS (Munday et al., 1993) and therefore can act in a similar manner. In particular beta cells are damaged preferentially, when isolated pancreatic islets are exposed to lipophilic alloxan derivatives (Brückmann and Wertheimer, 1947; Jörns et al., 1997). But as derivatives such as butylalloxan are lipophilic they can penetrate a plasma

membrane without GLUT2 glucose transporter expression (Elsner et al., 2002). Thus, these derivatives can enter all cell types and are systemically toxic rather then diabetogenic (Brückmann and Wertheimer, 1947). Nephrotoxicity is a dominating feature of the toxicity of lipophilic derivatives after systemic administration (Brückmann and Wertheimer, 1947). This nephrotoxity, which develops in rats after injection, is so severe that it causes fatal renal failure in the animals before diabetes can develop (Brückmann and Wertheimer, 1947). Though at the time of these early studies not known (Brückmann and Wertheimer, 1947), the particular susceptibility of the kidney to the toxic action of these lipophilic alloxan derivatives can nowadays be explained as a result of a preferential accumulation of these lipophilic toxins in the tubular cells of the kidney, which, like the pancreatic beta cells, express the GLUT2 glucose transporter (Thorens et al., 1988). Thus, experiments with lipophilic alloxan derivatives have added much experimental support to the concept that the hydrophilic character of the alloxan molecule is the decisive chemical property of this compound, which is responsible for selective accumulation in the beta cells and thus for its diabetogenicity (Munday et al., 1993; Jörns et al., 1997).

iv) Alloxan (Fig. 1) can generate "reactive oxygen species" (ROS) in a cyclic reaction between this substance and its reduction product, dialuric acid (Cohen and Heikkila, 1974; Munday, 1988; Winterbourn et al., 1989; Winterbourn and Munday, 1989) (Reactions i–ii). The beta cell toxic action of alloxan (A) is initiated by free radicals formed in this redox reaction (Cohen and Heikkila, 1974; Munday, 1988; Oberley, 1988; Winterbourn et al., 1989; Winterbourn and Munday, 1989). Autoxidation of dialuric acid (AH_2) generates superoxide radicals ($O_2^{\bullet -}$) (Reactions iii–iv) and hydrogen peroxide (H_2O_2) (Reactions iii–iv) and, in the Fenton reaction (Reaction v), in the presence of a suitable metal catalyst (typically iron) (Reaction vi), hydroxyl radicals ($^{\bullet}OH$) (Reactions v–vii). The autoxidation of dialuric acid involves the intermediate formation of the alloxan radical ($^{\bullet}AH$) (Reactions i–iv) (Munday, 1988; Winterbourn et al., 1989; Winterbourn and Munday, 1989).

$$AH_2 + O_2 \rightarrow AH^{\bullet} + O_2^{\bullet -} + H^+ \quad \text{(i)}$$

$$AH^{\bullet} + O_2 \rightarrow A + O_2^{\bullet -} + H^+ \quad \text{(ii)}$$

$$AH_2 + O_2^{\bullet -} + H^+ \rightarrow AH^{\bullet} + H_2O_2 \quad \text{(iii)}$$

$$AH^{\bullet} + O_2^{\bullet -} + H^+ \rightarrow A + H_2O_2 \quad \text{(iv)}$$

$$H_2O_2 + e^- \rightarrow HO^{\bullet} + HO^- \quad \text{(v)}$$

$$Fe^{II} + H_2O_2 \rightarrow Fe^{III} + OH^{\bullet} + OH^- \quad \text{(vi)}$$

$$\text{Net} : O_2^{\bullet -} + H_2O_2 \overset{\text{metal}}{\underset{\text{catalyst}}{\rightarrow}} O_2 + OH^{\bullet} + OH^- \quad \text{(vii)}$$

v) Reduction of alloxan (A) to dialuric acid (AH_2) in the cell requires the presence of a suitable thiol, typically the tripeptide glutathione (GSH) to generate the redox cycling partner (dialuric acid), while glutathione (GSSG) is oxidized (Reaction viii) (Winterbourn and Munday, 1989; Brömme et al., 2000).

$$A + 2GSH \rightarrow AH_2 + GSSG \quad \text{(viii)}$$

The reaction takes place in two steps with the alloxan radical ($^{\bullet}AH$) as an intermediate product (Reactions ix–x).

$$A + GSH \rightarrow AH^{\bullet} + GS^{\bullet} \quad \text{(ix)}$$

$$AH^{\bullet} + GSH \rightarrow AH_2 + GS^{\bullet} \quad \text{(x)}$$

vi) Other intracellular thiols, present at lower concentrations in the cell, such as the monothiol cysteine and other thiols and dithiols as well as ascorbic acid, are also suitable reducing agents and thus may contribute, even though to a lesser extent, to alloxan reduction (Elsner et al., 2006). Reaction with thiols and thus generation of ROS is also possible with proteins such as enzymes (Lenzen et al., 1990; Lenzen and Mirzaie-Petri, 1991, 1992) and albumin (Sakurai and Miura, 1989). During each redox cycle a small amount of "Compound 305", an alloxan-GSH adduct (Patterson et al., 1949; Winterbourn and Munday, 1989; Lenzen and Munday, 1991; Brömme et al., 2002), is formed through reaction between alloxan and GSH. While the intracellular concentration of "Compound 305" increases in a time-dependent manner, the amount of reduced GSH available in the cell for redox cycling diminishes gradually and thus fosters a lower prooxidative ratio between alloxan and GSH, rather than a higher antioxidative ratio (Munday, 1988; Winterbourn et al., 1989; Winterbourn and Munday, 1989).

vii) Thiols, cysteine as well as GSH, have been reported long ago already to protect rats against development of alloxan diabetes, when injected together with alloxan (Lazarow, 1946; Lazarow et al., 1948; Sen and Bhattacharya, 1952). This, at a first glance, paradoxical observation can now be explained in the light of the understanding of the molecular mechanism of alloxan action. When concentrations of reducing agents in the blood stream or in the extracellular space are significantly increased through injection of a thiol, more alloxan is reduced extracellularly so that less is available for accumulation in the beta cells, thereby ameliorating the beta cell toxic and diabetogenic action of alloxan. Normally the capacity for reduction of alloxan, redox cycling and generation of ROS in the circulation (Sakurai and Miura, 1989) is not sufficient to prevent the alloxan molecule to reach and enter the pancreatic beta cell. On the other hand, through fostering of redox cycling in the organism, the general systemic toxicity of alloxan is increased.

viii) The major oxidation pathway of dialuric acid, an $O_2^{\bullet -}$-dependent chain reaction, is inhibited by superoxide dismutase (SOD) (Reaction xi). In the presence of SOD,

an autocatalytic process involving the interaction between dialuric acid and alloxan becomes important (Munday, 1988), while in the presence of a transition metal, a third oxidation mechanism, dependent upon H_2O_2, has been identified (Munday, 1988). This latter step is inhibited by the hydrogen peroxide inactivating enzyme catalase (Munday, 1988) (Reaction xii). The other hydrogen peroxide inactivating enzyme, glutathione peroxidase (GPx), can principally act in a similar manner. But this enzyme requires GSH, which is oxidized in this reaction to GSSG (Reaction xiii).

$$2H^+ + 2O_2^{\bullet -} \xrightarrow[SOD]{} H_2O_2 + O_2 \qquad \text{(xi)}$$

$$2H_2O_2 \xrightarrow[Catalase]{} O_2 + 2H_2O \qquad \text{(xii)}$$

$$2GSH + H_2O_2 \xrightarrow{GPx} 2H_2O + GSSG \qquad \text{(xiii)}$$

ix) The alloxan molecule itself is not cytotoxic. When kept in the oxidized form and when reduction and/or redox cycling are prevented, alloxan does not generate ROS (Elsner et al., 2006). Without ROS generation alloxan is not toxic to insulin-producing cells (Elsner et al., 2006).

x) The reduction product dialuric acid itself is also not toxic, when kept in the reduced form and when oxidation and redox cycling and/or generation of ROS is prevented (Munday, 1988; Winterbourn and Munday, 1989; Elsner et al., 2006). SOD and catalase prevent dialuric acid toxicity as documented by the protection against death of insulin-producing cells (Elsner et al., 2006).

xi) At variance from alloxan, dialuric acid autooxidizes spontaneously in the presence of O_2, thus generating cytotoxic ROS in the absence of a thiol (Munday, 1988; Winterbourn et al., 1989), even when not taken up into the cell. In contrast, alloxan, when restricted to the extracellular space, is not toxic in the absence of thiols such as GSH (Elsner et al., 2006). Thus it is not surprising that dialuric acid can also induce diabetes (Brückmann and Wertheimer, 1945, 1947; Bailey et al., 1946; Saviano and De Franciscis, 1946; Merlini, 1951) and causes the same beta cell lesions as alloxan (Jörns et al., 1997). Thiols in the plasma membrane, with which alloxan could interact and in the consequence could be reduced and generate ROS in a redox cycle, are apparently not present or not accessible to an extent, which would allow generation of ROS sufficient to damage the cells (Elsner et al., 2006). Thus, a former hypothesis that alloxan might be cytotoxic due to interaction with thiol groups in the beta cell membrane (Watkins et al., 1970) is not valid.

xii) The antioxidative enzyme SOD greatly attenuates the toxicity of both alloxan and dialuric acid to insulin-producing cells in the presence of GSH. The reason for this cytoprotective effect is the ability of SOD to scavenge superoxide radicals, which are generated in the O_2^- dependent chain reaction between dialuric acid and alloxan (Munday and Winterbourn, 1989). The resultant suppression of dialuric acid autoxidation prevents ROS generation (Munday, 1988; Winterbourn and Munday, 1989) and through this mechanism counteracts the toxicity to insulin-producing cells (Elsner et al., 2006). Increasing the concentrations of the toxins, however, can reinstall the toxic action of both compounds. This is due to the fact that an autocatalytic reaction between the oxidized and reduced pyrimidine comes into action, which also generates ROS, when the chain reaction is suppressed by SOD (Munday, 1988; Munday and Winterbourn, 1989; Winterbourn and Munday, 1989).

xiii) The superoxide radical, however, is apparently not the species responsible for the cytotoxicity of alloxan and dialuric acid, because the hydrogen peroxide (H_2O_2) inactivating enzyme catalase provided significantly better protection against the toxicity to insulin-producing cells than the superoxide radical ($O_2^{\bullet -}$) inactivating enzyme SOD, though catalase does not prevent redox cycling and therefore does not prevent superoxide radical formation (Munday, 1988; Winterbourn and Munday, 1989). This proves convincingly that not the superoxide radical ($O_2^{\bullet -}$) but rather the hydroxyl radical ($^{\bullet}OH$) is the ultimate toxic ROS species, whose formation from hydrogen peroxide (H_2O_2) is prevented through destruction of hydrogen peroxide by catalase (Munday, 1988; Winterbourn and Munday, 1989).

xiv) Optimal protection against the cytotoxic action of alloxan and dialuric acid towards insulin-producing cells, however, is provided only by a combination of SOD plus catalase. Only this combination completely prevents redox cycling between alloxan and dialuric acid and thus the generation of all ROS species in this chain reaction, namely superoxide radicals ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($^{\bullet}OH$), which are ultimately responsible for cell death (Munday, 1988; Winterbourn and Munday, 1989).

xv) A multitude of metal and in particular specific iron chelators as well as hydroxyl scavengers have been tested for their protective action both *in vitro* and *in vivo*. But though metal chelators such as EDTA, diethylenetriamine pentaacetic acid, desferrioxamine and phenanthroline and ROS scavengers such as the alcohols butanol and dimethylsulfoxide and the urea derivatives dimethylurea and thiourea have been shown in many experimental situations to provide protection against alloxan toxicity, the results obtained have never been entirely unequivocal (Heikkila et al., 1974, 1976; Heikkila, 1977; Heikkila and Cabbat, 1978, 1982; Grankvist et al., 1979a; Tibaldi et al., 1979; Fischer and Hamburger, 1980a,b; Fischer and Harman, 1982; Jörns et al., 1999). Due to the extremely short half-life and the extraordinary chemical reactivity of the hydroxyl radical, it is not surprising that a complete protection against alloxan toxicity cannot be achieved, because hydroxyl radicals interact with biological targets before they can be inactivated through hydroxyl scavengers. In the same way, it is not surprising that it

has been extremely difficult to efficiently suppress metal catalysed hydroxyl radical formation through metal chelators (Grankvist et al., 1979b; Fischer and Hamburger, 1980b; Fischer and Harman, 1982; Heikkila and Cabbat, 1982; Jörns et al., 1999), since it is crucial that metal chelation is achieved before hydroxyl radical formation is initiated. Due to the chemical properties of the chelators and scavengers, this cannot always be achieved completely, unless experimental conditions are optimized. This conclusion is supported by the observation that the toxicity of alloxan and dialuric acid to insulin-producing cells *in vitro* is suppressed through the iron chelator desferrioxamine (Elsner et al., 2006), which prevents the generation of the very toxic hydroxyl radicals in the iron catalyzed Fenton reaction (Munday, 1988; Winterbourn and Munday, 1989). Overall, the results with radical scavengers and metal chelators support the contention, that the hydroxyl radical is the ROS species ultimately responsible for alloxan-induced cell death.

xvi) The intracellular GSH concentrations are in the same millimolar concentration range in insulin-producing cells as in liver cells. As both cell types express the GLUT2 glucose transporter a difference in the intracellular GSH concentration cannot explain the much greater susceptibility of insulin-producing cells than of liver cells to the toxicity of alloxan *in vivo* (Rerup, 1970). However, liver cells are much better endowed with the hydrogen peroxide inactivating enzyme catalase than insulin-producing cells (Grankvist et al., 1979a; Lenzen et al., 1996a; Tiedge et al., 1997). When the low intracellular levels of catalase protein expression are raised in insulin-producing cells through overexpression, these cells are protected equally well (Elsner et al., 2006). This proves convincingly that the low hydrogen peroxide (H_2O_2) inactivating enzyme capacity is crucially responsible for the exquisite susceptibility of insulin-producing beta cells towards alloxan toxicity.

xvii) Thus the mechanism underlying the cytotoxic action of alloxan to insulin-producing cells is due to reduction by interaction with intracellular thiols such as GSH and the resultant formation of cytotoxic ROS in a cyclic reaction between alloxan and its reduction product, dialuric acid (Munday, 1988; Winterbourn and Munday, 1989). The autoxidation of the latter generates superoxide radicals ($O_2^{\bullet -}$), and hydrogen peroxide (H_2O_2) (Munday, 1988; Winterbourn and Munday, 1989) and ultimately, from the latter, the hydroxyl radical ($^{\bullet}OH$) (Munday, 1988; Winterbourn and Munday, 1989). Alloxan diabetes, induced in laboratory animals through injection of this diabetogenic compound, is therefore the result of selective uptake of alloxan *via* the low affinity GLUT2 glucose transporter (Elsner et al., 2002) into a pancreatic beta cell, which due to its weak expression of hydrogen peroxide inactivating enzymes (Lenzen et al., 1996a; Tiedge et al., 1997) is badly protected against hydroxyl radical ($^{\bullet}OH$) mediated cytotoxicity (Munday, 1988; Winterbourn and Munday, 1989). Not surprisingly, therefore, effective prevention of redox cycling and generation of ROS or efficient inactivation of ROS can prevent beta cell death (Jörns et al., 1999; Elsner et al., 2006) and counteract the development of alloxan diabetes *in vivo* (Heikkila et al., 1976).

xviii) Thus, it can be concluded that the pancreatic beta cell toxicity and the resultant diabetogenicity of alloxan is due to the redox cycling and the generation of toxic reactive oxygen species (ROS) in combination with the hydrophilicity and the glucose similarity of the molecular shape of alloxan.

Streptozotocin mechanism of action

The diabetogenic agent streptozotocin (STZ) (Fig. 3) inhibits insulin secretion and causes a state of insulin-dependent diabetes mellitus through its ability to induce a selective necrosis of the pancreatic beta cells. Both effects can be assorted to the alkylating potency of streptozotocin. The common chemical denominator of the two effects is the selective cellular uptake and the accumulation of streptozotocin by the beta cell.

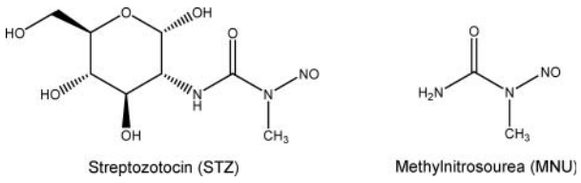

Fig. 3: Chemical formulas of streptozotocin (STZ) and methylnitrosourea (MNU).

Chemical properties of streptozotocin

Streptozotocin (2-deoxy-2-(((methylnitrosoamino)carbonyl)amino)-D-glucopyranose) is:

- *A cytotoxic methylnitrosourea moiety (N-methyl-N-nitrosourea) attached to the glucose (2-deoxyglucose) molecule. It is also a glucosamine derivative.*
- *A beta cell toxic glucose analogue.*
- *A hydrophilic compound.*
- *An alkylating agent (Bennett and Pegg, 1981).*
- *Relatively stable at pH 7.4 and 37 °C (at least for up to one hour) (Axler, 1982; Lee et al., 1993; Povoski et al., 1993).*
- *For in vitro experimentation concentrated stock solutions in 0.01 M HCl, kept on ice, should be used and added to test medium just prior to the start of the experiment in order to obtain the final concentration.*
- *For injection a stable solution in citrate buffer (pH 4.5) is most suited.*

Biological effects of streptozotocin due to hydrophilicity and glucose similarity of the streptozotocin molecule: Beta cell selectivity of biological effects

The toxic action of streptozotocin and chemically related alkylating compounds requires their uptake into the cells. Typically nitrosoureas are lipophilic and tissue uptake is quick. However, due to the hexose substitution streptozotocin is less lipophilic and this significantly affects the cellular distribution. The greater hydrophilicity of streptozotocin reduces its chance to enter cells without support and also prevents access to the brain *via* the blood-brain barrier. Streptozotocin, in contrast to other nitrosoureas including *N*-methyl-*N*-nitrosourea (MNU) (Fig. 3), causes little myelosuppression or carbamoylation of lysine residues of proteins (Bennett and Pegg, 1981; Weiss, 1982). On the other hand, through the attachment of the methylnitrosourea moiety to the 2 carbon of glucose as a carrier molecule streptozotocin is selectively accumulated in pancreatic beta cells (Karunanayake et al., 1976; Tjälve et al., 1976) and becomes a beta cell toxic and diabetogenic compound, though it also exhibits significant renal and hepatic toxicity (Rerup, 1970; Weiss, 1982).

Streptozotocin has also been employed in the treatment of human islet-cell carcinomas and malignant carcinoid tumours (Schein et al., 1974). However, clinical streptozotocin cancer treatment typically involves repetitive, miniscule doses significantly lower than necessary to induce diabetes in experimental animals. This together with the resistance of human beta cells to streptozotocin toxicity explains why diabetes is not a typical side effect of streptozotocin treatment though renal and hepatic toxicity are often encountered in these patients (Schein et al., 1974; Weiss, 1982).

The selective pancreatic beta cell toxicity of streptozotocin and the resulting diabetic metabolic state are clearly related to the glucose moiety in its chemical structure, which enables streptozotocin to enter the beta cell *via* the low affinity GLUT2 glucose transporter in the plasma membrane (Elsner et al., 2000). This hypothesis is supported by the observation that insulin-producing cells that do not express this glucose transporter, are resistant to streptozotocin toxicity (Ledoux and Wilson, 1984; Elsner et al., 2000) and become sensitive to the toxic action of this compound only after expression of the GLUT2 glucose transporter protein in the plasma membrane (Schnedl et al., 1994; Elsner et al., 2000). This observation explains the greater toxicity of streptozotocin, when compared with *N*-methyl-*N*-nitrosourea (MNU) in GLUT2 expressing beta cells even though both substances alkylate DNA to a similar extent (Ledoux et al., 1986; Wilson et al., 1988; Elsner et al., 2000). The importance of the GLUT2 glucose transporter for the toxic action of streptozotocin is also confirmed by the observation of streptozotocin damage to other cells expressing this transporter such as hepatocytes and renal tubular cells (Thorens et al., 1988). Therefore any streptozotocin treatment of animals leads not only to diabetes but can also cause liver and kidney damage of variable degree (Rerup, 1970). A number of chemically very different alkylating agents, which have been studied for their beta cell toxicity (Delaney et al., 1995), are not dependent for their toxic action upon the expression of the GLUT2 glucose transporter (Elsner et al., 2000). Therefore it is not surprising that these compounds are not selectively toxic to beta cells and not diabetogenic (Delaney et al., 1995; Elsner et al., 2000).

Biological effects of streptozotocin due to alkylation: Inhibition of beta cell function and insulin secretion

The effects of streptozotocin on glucose and insulin homeostasis reflect toxin-induced abnormalities in pancreatic beta cell function. Initially an inhibition of insulin biosynthesis and glucose-induced secretion as well as an impairment of glucose metabolism, both glucose oxidation and oxygen consumption (Strandell et al., 1988; Nukatsuka et al., 1990; Bedoya et al., 1996), become evident. On the other hand, streptozotocin has no immediate and direct inhibitory effect upon glucose transport (Elsner et al., 2000) or upon glucose phosphorylation through glucokinase (Lenzen et al., 1987b). However, at later stages of functional beta cell impairment, deficiencies at the level of gene and protein expression and function of these structures become apparent (Wang and Gleichmann, 1998).

Even before negative effects due to mitochondrial DNA and protein alkylation become evident, a streptozotocin-induced depletion of NAD^+ may result in an inhibition of insulin biosynthesis and secretion (Yamamoto et al., 1981a,b; Uchigata et al., 1982; Strandell et al., 1988). Later, inhibition of glucose-induced and also of amino acid-induced (Eizirik et al., 1988a,b) insulin secretion, due to dysfunction of mitochondrial enzymes (Rasschaert et al., 1992) and damage to the mitochondrial genome (Eizirik et al., 1991) become apparent. This impairment is more marked for nutrient than for non-nutrient induced insulin secretion. This interpretation has been confirmed through studies which have shown that pretreatment of isolated pancreatic islets with the poly (ADP-ribose) polymerase (PARP) inhibitor nicotinamide prevents early inhibition of beta cell function during the first day after streptozotocin exposure, while long-term inhibition of insulin secretion six days after streptozotocin exposure was not counteracted by nicotinamide (Strandell et al., 1989).

When exposed to high cytotoxic streptozotocin concentrations, these initial circumscribed functional defects gradually turn into more severe functional deficiencies. These changes are of a more general and unspecific nature and result from the progressive deterioration of the

function of a dying beta cell. Of note, at lower streptozotocin concentrations, many beta cells are able to survive the initial insult, but keep a long-term functional defect characterized by a preferential deficiency of mitochondrial oxidative metabolism (Eizirik et al., 1988a,b, 1991; Rasschaert et al., 1992).

Biological effects of streptozotocin due to alkylation: Beta cell toxicity and diabetogenicity

Toxicity of streptozotocin and related compounds resides in their ability to alkylate biological macromolecules (Bennett and Pegg, 1981). It is generally assumed that the toxic activity of streptozotocin relates to the DNA alkylating activity of its methylnitrosourea moiety (Bennett and Pegg, 1981; Uchigata et al., 1982; Ledoux et al., 1986; Wilson et al., 1988; Murata et al., 1999), especially at the O^6 position of guanine (Goldmacher et al., 1986; Green et al., 1989; Karran and Bignami, 1992). This DNA damage results, along a defined chain of events (Pieper et al., 1999b), in a fragmentation of the DNA (Yamamoto et al., 1981a,b; Morgan et al., 1994) and ultimately in the necrosis of the pancreatic beta cells (Lenzen et al., 1996b) through depletion of cellular energy stores. It is the resultant activation of PARP that, in an attempt to repair the damaged DNA, depletes the cellular NAD^+ and subsequently the ATP stores (Schein and Loftus, 1968; Yamamoto et al., 1981a,b; Uchigata et al., 1982; Sandler and Swenne, 1983) due to overstimulation of the DNA repair mechanisms (Pieper et al., 1999b). Though streptozotocin also methylates proteins (Bennett and Pegg, 1981; Wilson et al., 1988) DNA methylation is ultimately responsible for beta cell death. But it can be anticipated that the methylation of beta cell proteins also contributes to the functional defects of the beta cells after exposition to streptozotocin *in vitro* or *in vivo*.

Inhibitors of poly ADP-ribosylation suppress this process of DNA methylation. Thus, injection of nicotinamide and other poly (ADP-ribose) polymerase inhibitors in parallel with or prior to the administration of streptozotocin is well known to protect beta cells against the toxic action of streptozotocin and to prevent the development of a diabetic state (Schein et al., 1967; Dulin and Wyse, 1969; Stauffacher et al., 1970), however, on account of the later development of insulin-producing pancreatic tumours (Rakieten et al., 1971; Preston, 1985; Lenzen et al., 1987a). Poly (ADP-ribose) polymerase inhibitors protect islets also *in vitro* against streptozotocin-induced inhibition of insulin biosynthesis and secretion through the same mechanism of action (Bedoya et al., 1996). This concept has been convincingly confirmed by the observation that mice deficient in PARP are resistant to beta cell death mediated by streptozotocin in spite of DNA fragmentation. The absence of PARP, which is activated by DNA damage, prevents the depletion of the cofactor NAD^+ and the subsequent loss of ATP (Burkart et al., 1999; Masutani et al., 1999; Pieper et al., 1999a,b) and thus cell death.

The role of alkylation in beta cell damage has also been examined by the use of the ethylating agents *N*-ethyl-*N*-nitrosourea and ethyl methanesulphonate. These ethylating agents are known to be less toxic than their methylating counterparts (Karran and Bignami, 1992; Delaney et al., 1995). This has been attributed to O^6-ethylguanine being less toxic than O^6-methylguanine (Karran and Bignami, 1992). The lower toxicity of the ethylating agents has been interpreted as evidence for a role of O^6-alkylguanine in the mechanism of the toxic action of this group of alkylating compounds. The fact that *N*-ethyl-*N*-nitrosourea and ethyl methanesulphonate are significantly less toxic to insulin-producing cells than MNU and methyl methanesulphonate (Delaney et al., 1995; Elsner et al., 2000) has been taken as support for the contention that in insulin-producing cells, like in other cell types, the mechanism of toxic action is due to alkylation (Bennett and Pegg, 1981), with the methylation of DNA bases being more toxic than the ethylation (Karran and Bignami, 1992).

An alternative hypothesis proposes that part of the diabetogenic effect of streptozotocin may relate not to its alkylating ability but to its potential to act as an intracellular nitric oxide (NO) donor (Turk et al., 1993). Both streptozotocin and MNU contain a nitroso group (Fig. 3) and can liberate nitric oxide (Rogers and Ignarro, 1992; Kwon et al., 1994; Kaneto et al., 1995; Kröncke et al., 1995; Tanaka et al., 1995; Bedoya et al., 1996; Murata et al., 1999) similar to that of other nitric oxide donors such as sodium nitroprusside or 3-morpholinosydnonimine (Tiedge et al., 1999). In fact streptozotocin has been shown to increase the activity of guanylyl cyclase and the formation of cGMP, which are characteristic effects of NO. However, the alkylating agent methyl methanesulphonate is the most toxic compound though, unlike MNU, it is not a nitric oxide donor (Delaney et al., 1995), indicating that NO is not an indispensable prerequisite for the toxic action of this group of alkylating agents including the diabetogenic compound streptozotocin. This view is convincingly supported by the observation that mice deficient in PARP are resistant to beta cell death mediated by streptozotocin in spite of DNA fragmentation. The absence of PARP, which is activated by DNA damage, prevents the depletion of the cofactor NAD^+ and the subsequent loss of ATP (Burkart et al., 1999; Masutani et al., 1999; Pieper et al., 1999a,b). Thus NO and free nitrous radicals (i. e. peroxinitrite) may be an aggravating factor in the toxic action of streptozotocin but NO is apparently not crucial for its beta cell toxic action (Delaney et al., 1995; Yamamoto et al., 1981a,b; Elsner et al., 2000).

An involvement of reactive oxygen species in the toxic action of streptozotocin, which may be produced during

uric acid generation as the final product of ATP degradation by xanthine oxidase from hypoxanthine, has also been considered (Nukatsuka et al., 1990). And indeed, some indirect evidence for a participation of ROS has been obtained in experiments with scavengers (Sandler and Swenne, 1983; Okamoto, 1996). So some minor generation of ROS including superoxide and hydroxyl radicals originating from hydrogen peroxide dismutation during hypoxanthine metabolism may accompany the effect of streptozotocin and accelerate the process of beta cell destruction. However, it is the alkylating potency of streptozotocin, which causes through ATP depletion a state of energy deficiency similar to that, which results from other states of hypoxia and ischemia and is thus crucial for its beta cell toxicity.

Alloxan causes not only protein but also DNA damage (Takasu et al., 1991; Okamoto, 1996) through ROS toxicity. In the case of alloxan toxicity nicotinamide may provide protection through free radical scavenging (Ledoux et al., 1988). Such a capacity for free radical scavenging may also be a minor complementary but not crucial component in the protective action of nicotinamide against alloxan toxicity.

Species differences of alloxan and streptozotocin toxicity

Alloxan and streptozotocin induce insulin deficiency due to their selective pancreatic beta cell toxicity, but there are significant species differences in the diabetogenicity of alloxan and streptozotocin (Rerup, 1970). Rodents are particularly prone to the diabetogenic action of these two diabetogens, while humans are resistant to the toxicity (Rerup, 1970). Isolated human pancreatic beta cells, in contrast to rodent beta cells, are resistant to the toxic action of alloxan and streptozotocin *in vitro* (Eizirik et al., 1994; Tyrberg et al., 2001; Yang and Wright, 2002). Several studies have shown that after transplantation of human pancreatic islets into nude mice, beta cells were not damaged even after injection of high doses of streptozotocin (Yang and Wright, 2002) or alloxan (Eizirik et al., 1994; Tyrberg et al., 2001), while the beta cells of rat islets transplanted concomitantly into the same animal were destroyed (Eizirik et al., 1994; Tyrberg et al., 2001; Yang and Wright, 2002).

Rat insulin-producing tissue culture cells, which do not constitutively express the GLUT2 glucose transporter, are also resistant while expression of the rat GLUT2 glucose transporter isoform renders such cells sensitive to the toxicity of both toxins (Elsner et al., 2000, 2002). In a study comparing insulin-producing cells, in which either the rat or the human GLUT2 glucose transporter isoform had been expressed (Elsner et al., 2003), it could be proven that it is apparently the very low level of constitutive GLUT2 glucose transporter expression in the human beta cell (De Vos et al., 1995; Ferrer et al., 1995) rather than the inability of the human GLUT2 glucose transporter isoform to provide uptake of alloxan and streptozotocin into the intracellular compartment which is responsible for the extraordinarily high resistance of humans against the diabetogenic action of alloxan and streptozotocin. However, even if the human GLUT2 glucose transporter isoform would be more abundant in human pancreatic beta cells the lower capacity for uptake of the toxins through the human GLUT2 glucose transporter isoform as compared to the rat transporter isoform would limit the diabetogenicity of both alloxan and streptozotocin in humans (Elsner et al., 2003). Therefore, it is also not surprising that development of diabetes is not known to be a typical side effect when streptozotocin is used as a chemotherapeutic agent in human cancer treatment and that it is not a particularly efficient anticancer drug when used in the treatment of human insulinomas which usually do not express the GLUT2 glucose transporter (Yang and Wright, 2002).

On the other hand there is no evidence for any difference in antioxidative equipment between rodent and human beta cells that would be sufficiently large (Welsh et al., 1995) to explain these observed species differences in susceptibility towards the toxicity of the two agents (Elsner et al., 2003). But, as shown in studies comparing different strains differences in antioxidative equipment may contribute to differences in sensitivity to alloxan toxicity in the same species (Mathews and Leiter, 1999a,b).

The exclusion of the toxins from the intracellular space due to a lack of GLUT2 glucose transporter protein expression in the plasma membrane can provide a common explanation for the species differences in toxicity of both compounds. A unifying concept on the basis of a difference in the intracellular milieu, on the other hand, would, in view of the different mechanisms of cytotoxic action of alloxan and streptozotocin, require a separate explanation for each compound for these species differences in beta cell toxicity and diabetogenicity. However, there is no convincing experimental basis for such an alternative unifying concept. It is thus the exclusion of the toxins from the intracellular space due to a lack of GLUT2 glucose transporter expression, which provides the common explanation for the resistance of human insulin-producing cells against toxicity of these two beta cell toxins.

Rodents but also other species such as rabbits and dogs are sensitive to the diabetogenic action of alloxan and streptozotocin, even though there are significant species differences, so that different doses of the toxins are required in different animal species in order to obtain the same result (Rerup, 1970). The guinea pig is an example of a particularly insensitive species when the toxins are administered systemically. Injection of alloxan into the circulation of the pancreas has been shown to cause beta cell necrosis and diabetes in the guinea, while systemic

injection was ineffective (Griffiths, 1948). Therefore, it is not the resistance of the guinea pig beta cell, which is responsible for resistance of guinea pigs to the diabetogenic action of alloxan. This interpretation could be confirmed by the observation that isolated pancreatic islets are sensitive to the beta cell toxic action of alloxan and streptozotocin (unpublished observation). At variance from human beta cells guinea pig beta cells, like beta cells of rodents, express the GLUT2 glucose transporter protein (unpublished observation), which enables alloxan and streptozotocin to enter the guinea pig beta cell. Glutathione injection has been shown to suppress alloxan beta cell toxicity and diabetogenicity in rats (Lazarow, 1946; Lazarow et al., 1948) and reduction of glutathione levels in the blood made guinea pigs sensitive to the diabetogenic action of injected alloxan (Griffiths, 1948). Thus, alloxan might be reduced to dialuric acid by interaction with glutathione in the guinea pig circulation, before the alloxan molecule can enter the beta cell. Through this extracellular inactivation, intracellular redox cycling and ROS generation and consequently beta cell death can be prevented.

Likewise, guinea pigs have often (Kushner et al., 1969; Losert et al., 1971) but not consistently (Brosky and Logothetopoulos, 1969) been reported to be less sensitive to streptozotocin diabetogenicity than other species. Because glutathione does not play a crucial role in the toxicity of streptozotocin, the resistance of the guinea pig to streptozotocin diabetogenicity must have another reason than in the case of alloxan. It is known that starvation prior to injection (Brosky and Logothetopoulos, 1969) and insulin-induced hypoglycaemia (Losert et al., 1971) increase the diabetogenic action of streptozotocin, a phenomenon that is well-known for alloxan. Otherwise, there is no convincing explanation available at present for the greater resistance of guinea pig beta cells against the diabetogenicity of streptozotocin. But there may be other reasons such as differences in the blood supply to the pancreatic islets, which may contribute to a greater resistance of this animal species, even if such reasons have not been documented.

In the case of alloxan it is generally accepted that the diabetogenic potency of the toxin is very much lower in fed than in starved animals, which can be explained easily by the protective action of glucose against the diabetogenic effect of alloxan. This is also true for streptozotocin, even though to a lesser extent. Therefore it is generally accepted, that after a short period of starvation before the injection of alloxan or streptozotocin, it is easier to induce a reproducible diabetic metabolic state.

Overall, in many experimental studies over decades in a wide range of animal species, streptozotocin has proven to be the diabetogenic agent with the wider species effectiveness and greater reproducibility than alloxan. One major reason for this may be the lesser dependence upon the feeding status. Another reason may be the fact that streptozotocin is more stable in solution before injection and after injection in the animals than alloxan.

Glucose protection against toxic effects of alloxan and streptozotocin

Alloxan

Glucose: Glucose protection is a key characteristic of the toxic action of alloxan (Fischer and Harman, 1982; Lenzen and Panten, 1988a). Glucose provides a concentration-dependent universal protection against all toxic effects of alloxan, including beta cell dysfunction and alloxan diabetes, both *in vivo* (Maske et al., 1953; Arteta et al., 1954; Bhattacharya, 1954; Kaneko and Logothetopoulos, 1963; Bansal et al., 1980) and *in vitro* (Jörns et al., 1997; Elsner et al., 2002). This also explains why alloxan is less toxic and less diabetogenic in fed than in starved animals (Arteta et al., 1954; Boquist, 1977) and why a decrease of the plasma glucose concentration through insulin injection increases the diabetogenicity of alloxan (Arteta et al., 1954; Kaneko and Logothetopoulos, 1963). Glucose and mannose, with a preference for the α-anomer when compared with the β-anomer (Rossini et al., 1974; Tomita et al., 1974; McDaniel et al., 1976; Niki et al., 1976; Tomita and Kobayashi, 1976; Meglasson et al., 1986; Lenzen et al., 1987b) also protect glucose-induced insulin secretion and pancreatic beta cell glucokinase (Meglasson et al., 1986; Lenzen et al., 1987b; Tiedge et al., 2000) against inhibition by alloxan, because they bind to the sugar binding site of the enzyme. Thereby the hexoses prevent access of alloxan to functionally essential thiol groups of the enzyme, so that they are protected against oxidation (Lenzen and Panten, 1988a). It is not easy to provide a comprehensive explanation for the mechanism of glucose protection against alloxan toxicity. The reason is that glucose can potentially interfere at different sites with the action of alloxan and thereby antagonize through different mechanisms the action of this beta cell toxic glucose analogue.

3-O-Methylglucose: Through its ability to prevent uptake of alloxan into the beta cell *via* the GLUT2 glucose transporter, the non-metabolizable glucose analogue 3-O-methylglucose can also provide protection against all effects of alloxan. These comprise the protection by 3-O-methylglucose against inhibition of glucokinase (Munday et al., 1993) and glucose metabolism (Henquin et al., 1979) as well as against inhibition of alloxan sensitive mitochondrial enzymes such as aconitase (Lenzen and Mirzaie-Petri, 1992), and glucose-induced insulin secretion (Tomita et al., 1974; Pagliara et al., 1977; Goto et al., 1980). Uptake suppression of alloxan also prevents the toxic and diabetogenic effects (Carter and Younathan, 1962; Zawalich and Beidler, 1973; Rossini et al., 1975a,b; Jansson and Sandler, 1988) due to redox cycling

and ROS generation (Elsner et al., 2006). Other glucose analogues such as 2-deoxyglucose and 5-thioglucose do not show this protective effect but cytochalasin B can mimic the protective effect of 3-O-methylglucose through interference with GLUT2 mediated uptake of alloxan (McDaniel et al., 1975; Ishibashi et al., 1976).

Mannoheptulose: The seven carbon sugar mannoheptulose, which competitively inhibits glucokinase enzyme activity through interacting with the binding of the substrate glucose to its binding site (Lenzen and Panten, 1988b; Lenzen et al., 1996b), can prevent the inhibition of this enzyme through alloxan (Lenzen et al., 1988a; Lenzen and Panten, 1988b). But mannoheptulose cannot protect against any of the other toxic effects of alloxan. Thus thiol reactivity of alloxan, which causes glucokinase inhibition, is not responsible for alloxan mediated cytotoxicity and diabetogenicity.

Glucose plus 3-O-methylglucose: 3-O-methylglucose antagonizes the glucose-mediated protection (Jörns et al., 1997). An explanation for this at a first glance surprising observation is an unfavourable shift of the balance between protective and antiprotective elements of the action of these sugars. 3-O-methylglucose reduces the uptake of alloxan. But the extent of the alloxan uptake reduction by 3-O-methylglucose is not sufficient to counteract the increase of alloxan uptake mediated by glucose (Weaver et al., 1978a), so that many alloxan molecules gain access to the interior of the beta cell. On the other hand, however, due to the competition with 3-O-methylglucose, less glucose is taken up into the cell, and this provides less protection intracellularly through prevention of ROS formation. Thus relatively more alloxan molecules and relatively fewer glucose molecules in the cell shift the balance from protection towards toxicity. Taken together, therefore, the conclusion is allowed that the protective effect of glucose against alloxan toxicity is not mediated *via* uptake suppression through the GLUT2 glucose transporter into the beta cell.

Glucose plus mannoheptulose: Interestingly, glucose-mediated protection is also antagonized by mannoheptulose (Scheynius and Taljedal, 1971; Zawalich and Beidler, 1973; Tomita et al., 1974; Rossini et al., 1975a; Idahl et al., 1977; Jörns et al., 1997). This indicates that mannoheptulose, like glucose, and at variance from 3-O-methylglucose (Weaver et al., 1978a; Malaisse-Lagae et al., 1983), does not suppress alloxan uptake through the GLUT2 glucose transporter. It also shows that glucose provides protection *via* metabolism. When the phosphorylation of glucose is suppressed though glucokinase inhibition by mannoheptulose (Lenzen et al., 1996b), the ability to generate reducing equivalents in glucose metabolism is suppressed and thus the ability of glucose to protect against alloxan toxicity is abolished.

3-O-methylglucose plus mannoheptulose: The observation that mannoheptulose also antagonizes the 3-O-methylglucose mediated protection (Zawalich and Beidler, 1973; Rossini et al., 1975a) indicates that mannoheptulose competes with the access of 3-O-methylglucose to the GLUT2 glucose transporter of the beta cell. But mannoheptulose does not, at variance from 3-O-methylglucose, prevent or reduce alloxan uptake through this transporter. This is apparently the reason why mannoheptulose abolishes the 3-O-methylglucose mediated protection against alloxan toxicity.

Comparison of the different sugars: Glucose differs from the sugars 3-O-methylglucose and mannoheptulose insofar as it provides protection against the toxic effects of alloxan independently from its ability to prevent access of alloxan to the interior of the cell through the GLUT2 glucose transporter and independently from the protection against glucokinase inhibition. Glucose, as a beta cell substrate, can protect through reduction of the beta cell, thereby increasing the antioxidative capacity of the beta cell, which helps to detoxify the ROS (in particular hydrogen peroxide) generated in the alloxan xenobiotic metabolism. This protection is provided though glucose increases alloxan uptake (Weaver et al., 1978a) or at least does not decrease it (Malaisse-Lagae et al., 1983). This conclusion is also possible because glucose protects against the beta cell toxic action of the alloxan derivative butylalloxan (Jörns et al., 1997; Elsner et al., 2002). Since the lipophilic butylalloxan, in contrast to alloxan, does not depend upon the GLUT2 glucose transporter for uptake into the cell (Munday et al., 1993), glucose protection due to uptake inhibition of the toxin into the cell cannot account for the protective action of glucose against ROS mediated toxicity (Jörns et al., 1997; Elsner et al., 2002).

Support for the idea that the pentose phosphate pathway may significantly contribute to the glucose protection through its ability to provide reducing equivalents in the form of NADPH for the reduction of oxidized glutathione comes from various observations. Glucose, in its protective capacity against beta cell toxicity, cannot be replaced by other nutrient insulin secretagogues such as leucine (Scheynius and Taljedal, 1971) and 2-ketoisocaproate (Jörns et al., 1997). Leucine and 2-ketoisocaproate provide only a very minor protection against alloxan-induced inhibition of glucose-induced insulin release (Sener et al., 1982; Malaisse-Lagae et al., 1983). Thus, the mechanism of protection cannot simply be a question of keeping the beta cell in a more reduced metabolic state. Providing NADPH though malic enzyme *via* oxidation of malate is apparently also not sufficient to provide significant protection.

Thus the wealth of the experimental evidence provided throughout the decades of alloxan research can be interpreted only in the sense that glucose protection against the different toxic actions of alloxan is not mediated at a single site of interaction but rather through a concerted action of glucose interfering with different mechanisms and molecular sites of interaction with deleterious effects of alloxan. Crucial is in this context ap-

parently the ability of glucose to provide NADPH to keep glutathione in its reduced form (Elsner et al., 2006). Recent evidence from experimentation with other model systems supports this contention of an important role of glutathione reduction systems for protecting beta cells from oxidative stress mediated damage (Rydstrom, 2006; Zraika et al., 2006).

In view of the very low content of glutathione peroxidase in the beta cell (Lenzen et al., 1996a; Tiedge et al., 1997), an ample availability of NADPH to keep a vital amount of glutathione in its reduced form and thereby maintain the capability of glutathione peroxidase to oxidize hydrogen peroxide generated in alloxan xenobiotic metabolism is crucial. This is all the more important since the beta cell is virtually completely devoid of catalase and glutathione peroxidase (Lenzen et al., 1996a; Tiedge et al., 1997), which, in principle, due to its kinetic characteristics; would be most suited to inactivate large amounts of hydrogen peroxide generated in alloxan xenobiotic metabolism (Elsner et al., 2006). Thus, in a sense, glucose metabolism in the pancreatic beta cell with its special enzymatic equipment enabling ample glucose phosphorylation and oxidation and thereby allowing handling of high glucose concentrations (Lenzen and Panten, 1988b), is the option which the beta cell has chosen to protect itself against alloxan-mediated oxidative damage.

The alternative option, which the liver cell has chosen to protect itself against peroxide-mediated damage such as that caused by alloxan, namely a plentiful equipment with the hydrogen peroxide inactivating enzymes glutathione peroxidase and catalase (Lenzen et al., 1996a; Tiedge et al., 1997), is not a realistic option for the beta cell, since a high expression level of these antioxidative enzymes would dissipate the mitochondrial membrane potential through uncoupling protein 2 overexpression (unpublished observation), thereby imparing the proper transmission of the metabolic signal for glucose-induced insulin secretion (Chan et al., 2001, 2004).

Streptozotocin

Several non-metabolizable glucose analogues, 3-O-methylglucose (Ganda et al., 1976; Rossini et al., 1977), 2-deoxyglucose (Ganda et al., 1976), and 5-thioglucose (Wang et al., 1993), are known to protect the pancreatic beta cell against streptozotocin diabetogenicity, both *in vivo* and *in vitro*. This protection is likely to be a result from an interference of these non-metabolizable sugars with streptozotocin uptake into the beta cell. But in contrast to the situation after alloxan administration, these non-metabolizable sugars protect not only when given prior to the toxin but also when administered after streptozotocin (Ganda et al., 1976). The reason for this difference may be the greater chemical stability of streptozotocin (Axler, 1982; Lee et al., 1993; Povoski et al., 1993). However, interestingly, glucose itself does not provide protection against streptozotocin toxicity (Ganda et al., 1976; Elsner et al., 2000). Thus, at variance from the situation of alloxan diabetes, glucose protection is not a key characteristic of the action of streptozotocin.

3-O-methylglucose-streptozotocin, the 3-O-methyl-2-deoxy analogue of streptozotocin, in which the glucose moiety of the streptozotocin molecule has been replaced by the 3-O-methylglucose molecule, has been shown to be equally toxic and diabetogenic (Kawada et al., 1986). This proves that the glucose molecule, which is split off from the methylnitrosourea moiety, is, like the 3-O-methylglucose in this analogue, crucial for directing the toxin preferentially into the pancreatic beta cell *via* its GLUT2 glucose transporter but that the sugar moiety is not relevant for the induction of the beta cell death. It is the split off nitrosourea moiety of the streptozotocin molecule, which causes the death of the cell, while the split off hexose moiety is toxicologically inert.

Even though not immediately evident, a lesson regarding the mechanism of protection of glucose against alloxan toxicity can be learned from the streptozotocin situation. Thus, in the case of streptozotocin, and at variance from alloxan, there is only one potential site for interaction with a protective sugar molecule, namely the uptake into the cell *via* the GLUT2 glucose transporter. Since glucose does not interfere with the uptake of the glucose analogue streptozotocin *via* the GLUT2 glucose transporter, it is likely that an interference of the glucose molecule with the uptake of the toxic glucose analogue alloxan does also not significantly contribute to the protective action of glucose against alloxan toxicity.

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