Br. J. Pharmacol. (1988), **95**, 851–859

Structural requirements of alloxan and ninhydrin for glucokinase inhibition and of glucose for protection against inhibition

[1]Sigurd Lenzen, Franz-Hermann Brand & Uwe Panten

Institute of Pharmacology and Toxicology, University of Göttingen, Robert-Koch-Str. 40, D-3400 Göttingen, Federal Republic of Germany

1 In order to elucidate the mechanism underlying the interactions between glucose and alloxan when competing for the sugar binding site of glucokinase from pancreatic B-cells or liver, the structural requirements of the enzyme for inhibition by alloxan and for protection by glucose were determined.

2 With a half-maximal inhibitory concentration of 5 μM, alloxan was the most potent pyrimidine derivative inhibitor of glucokinase. Uramil was a less potent enzyme inhibitor. A variety of other pyrimidine derivatives and related substances were ineffective.

3 Ninhydrin also inhibited glucokinase with a half-maximal inhibitory concentration of 5 μM. Isatin was a slightly less potent enzyme inhibitor. Several other indoline derivatives were ineffective.

4 Only glucose derivatives with a sufficiently bulky substituent in position C-2, such as the glucokinase substrates glucose and mannose and the inhibitors mannoheptulose, glucosamine, and N-acetylglucosamine, protected glucokinase against inhibition by alloxan by binding to the active site of the enzyme. Glucose epimers which differed in other positions did not protect the enzyme against alloxan inhibition.

5 DTT (dithiothreitol) protected glucokinase against inhibition by alloxan and reversed the inhibition of the enzyme induced by alloxan. Thus the mechanism of glucokinase inhibition by alloxan and other inhibitors, such as uramil and ninhydrin, is an oxidation of functionally essential SH groups of the enzyme, where the most reactive keto group of the inhibitor acts as the hydrogen acceptor. The protective action of glucose and several C-2 epimers demonstrates that these functionally essential SH groups are situated in the sugar binding site of the glucokinase.

6 The present results support our contention, that the pancreatic B-cell glucokinase is the major target mediating the inhibition of insulin secretion by alloxan.

Introduction

Glucokinase in pancreatic B-cells has all the characteristics to qualify it as the signal recognition enzyme which can couple changes in the blood glucose concentration to corresponding changes of the glycolytic flux rate and, hence, to the rate of insulin secretion (for review see Lenzen & Panten, 1988).

Recently we showed that alloxan (Wöhler & Liebig, 1838) inhibits this enzyme, apparently through an interaction with the sugar binding site of the enzyme, and that the characteristics of inhibition of glucokinase were identical with the characteristics of inhibition of glucose-induced insulin secretion by alloxan (Lenzen *et al.*, 1987a). Therefore, we proposed that this interaction of the B-cell toxic agent alloxan (Dunn & McLetchie, 1943; Dunn *et al.*, 1943; Cooperstein & Watkins, 1981) with the pancreatic B-cell glucokinase might contribute to the inhibitory action of alloxan on glucose-induced insulin secretion (Lenzen *et al.*, 1987a).

It was the aim of the present investigation to elucidate the mechanism underlying the interactions between glucose and alloxan when competing for the glucose binding site of the glucokinase. We therefore decided to examine the chemical properties of alloxan (Figure 1) and its derivatives (Wöhler & Liebig, 1838; Brown, 1962; Webb, 1966) which determine their ability to inhibit glucokinase, as well as the chemical properties of glucose (Figure 1) and a

[1] Author for correspondence.

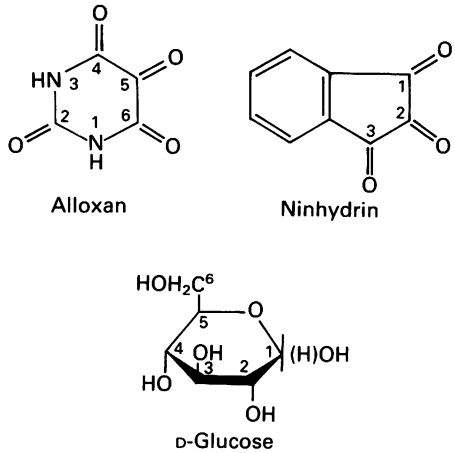

Figure 1 Chemical structures of alloxan, ninhydrin and D-glucose

variety of other sugars which determine their ability to protect glucokinase against inhibition by alloxan.

Due to the limited availability of pancreatic islet tissue for studies on pancreatic B-cell glucokinase, we also used liver glucokinase, which is functionally and immunologically identical to pancreatic B-cell glucokinase (Lenzen & Panten, 1988). Some of the results have been presented in abstract form (Lenzen *et al.*, 1987b,c).

Methods

Pancreatic islets from ob/ob mice (50–60 g body wt.) were isolated from the pancreas following collagenase digestion (Lernmark, 1974). The pancreas from ob/ob mice has large islets which contain more than 90% B-cells. Liver was obtained from fed Wistar rats (200–250 g body wt.). Pancreatic islets and liver were homogenized in ice cold homogenization medium (20 mM HEPES, 210 mM mannitol, 70 mM sucrose, pH 7.4) (Lenzen *et al.*, 1985), supplemented with 0.5 mM L-dithiothreitol and 5% glycerol. Cytoplasmic supernatant was obtained by three successive centrifugation steps at 4°C, 700 *g*, 8000 *g* and 100,000 *g*, respectively (Lenzen *et al.*, 1985). Rates of glucose phosphorylation in 100,000 *g* soluble cytoplasmic fractions were assayed at 37°C and pH 7.4 by recording the increase in absorbance at 340 nm in 250 μl of a reaction mixture containing 20 mM HEPES (pH 7.4); 125 mM KCl, 7.5 mM $MgCl_2$, 5 mM ATP, 0.5 mM NADP, 0.7 u ml^{-1} glucose-6-P-dehydrogenase from Bakers' Yeast, 0.01 u ml^{-1} 6-P-gluconate-dehydrogenase plus soluble cytoplasmic supernatant from pancreatic islets (7–10 μg protein in 50 μl) or liver (13–15 μg protein in 10 μl) (Walker & Parry 1966; Lenzen *et al.*, 1987a). Glucokinase activity was assayed in this test medium in the presence of 100 mM D-glucose. Hexokinase activity was assayed in the presence of 1 mM D-glucose and subtracted from the total activity recorded at 100 mM D-glucose to give glucokinase activity. Blank values for the increases of absorbance were obtained for hexokinase and glucokinase activities in the absence of cytoplasmic supernatant and were subtracted from the increases of absorbance in the presence of cytoplasmic supernatant before calculation of enzyme activities. Protein was determined according to McKnight (1977). In the experiments on glucokinase inhibition by alloxan and other test agents, cytoplasmic fractions were preincubated for 5 min with the test agent in the absence or additional presence of sugars before measurement of the glucose phosphorylation rate.

When the protective effect of D-glucose, D-mannoheptulose, D-glucosamine, N-acetyl-D-glucosamine, 5-thio-D-glucose, 2-deoxy-D-glucose, or 3-O-methyl-D-glucose against inhibition of rat liver glucokinase by alloxan was investigated, soluble cytoplasmic fractions were pre-incubated in test medium with alloxan for 5 min in the presence of 20 or 50 mM of the respective sugar in a total reaction volume of 100 μl. The glucose phosphorylation rate was measured after addition of 1.9 ml test medium in the presence of 1 or 100 mM D-glucose, thereby diluting the concentration of the test sugar by a factor of 20. Stock solutions of alloxan and all other test agents in a 5 mM concentration were dissolved in 0.01 M HCl, kept on ice and added to the test medium at the beginning of the pre-incubation period. Only 5,5-diethylbarbituric acid, 2-thiobarbituric acid, pyrimidine, indoline, indole, oxindole, and allantoic acid were dissolved in water. Results are given as the means $\pm$ s.e.mean and tested for statistical significance by use of Student's *t* test.

Chemicals and drugs

ATP (adenosine 5'-triphosphate, disodium salt), NADP (nicotinamide adenine dinucleotide phosphate, disodium salt), 6-phosphogluconate-dehydrogenase, and collagenase were purchased from Boehringer Mannheim, Germany; oxalurate was from K & K Fine & Rare Chemicals, Plainview, NJ, U.S.A.; D-glucose, D-mannitol, alloxan (monohydrate), ninhydrin, 5,5-diethylbarbituric acid, and all other reagents of chemical grade were from Merck, Darmstadt, Germany; sucrose, glycerol, alloxantin, and 2-thiobarbituric acid were from Serva, Heidelberg, Germany; glucose-6-P-dehydro-

genase (type XV from Bakers' Yeast), HEPES, L-dithiothreitol (DTT), D-mannoheptulose, D-glucosamine, N-acetyl-D-glucosamine, 5-thio-D-glucose, 2-deoxy-D-glucose, 3-O-methyl-D-glucose, pyrimidine, barbituric acid, uramil, violuric acid, allantoic acid, imidazole, parabanic acid, indoline, indole, oxindole and isatin were from Sigma, St Louis, MO, U.S.A.

Results

Structural requirements of alloxan and ninhydrin for glucokinase inhibition

When cytoplasmic fractions from ob/ob mouse pancreatic B-cells or rat liver were pre-incubated for 5 min, in the absence of glucose, with the pyrimidine derivatives alloxan (Figure 1), or uramil, ninhydrin (Figure 1) or the indole derivative isatin, glucokinase activities were inhibited in a concentration-dependent manner (Figure 2). Glucokinase activities in these cytoplasmic fractions were measured thereafter in the presence of 100 mM D-glucose. The half-maximal inhibitory concentrations for glucokinase from pancreatic B-cells as well as from liver for alloxan ($5 \pm 0\,\mu$M; $n = 4$ and $5 \pm 0\,\mu$M; $n = 6$, respectively) and ninhydrin ($5 \pm 0\,\mu$M; $n = 4$ and $5 \pm 0\,\mu$M; $n = 6$, respectively) were not significantly different from each other. However, the half-maximal inhibitory concentration of uramil ($36 \pm 7\,\mu$M; $n = 3$ and $20 \pm 4\,\mu$M; $n = 6$, respectively) was several times higher ($P < 0.01$) than that for alloxan and the half-maximal inhibitory concentration of isatin ($10 \pm 1\,\mu$M; $n = 3$ and $9 \pm 1\,\mu$M; $n = 6$, respectively) was also significantly ($P < 0.01$) higher than for ninhydrin.

Pre-incubation of the liver cytoplasmic fraction with alloxan (50 μM) for 0.6 ± 0.0 min ($n = 4$), in the absence of glucose, was sufficient time to obtain a 50% inhibition of the glucokinase activity.

Several other chemically related substances, e.g., pyrimidine, the pyrimidine derivatives violuric acid, barbituric acid, 2-thiobarbituric acid and oxalurate and allantoic acid, two agents with a structure similar to pyrimidine, imidazole and the imidazole derivative parabanic acid, as well as indole and the indole derivatives indoline and oxindole, did not inhibit glucokinase from pancreatic B-cells or liver at a concentration of 50 μM (data not shown).

Alloxantin, which decomposes in solution to yield alloxan and dialurate, also inhibited liver glucokinase activity with a half-maximal inhibitory concentration of $5 \pm 0\,\mu$M ($n = 6$); this was not significantly different from the half-maximal inhibitory concentra-

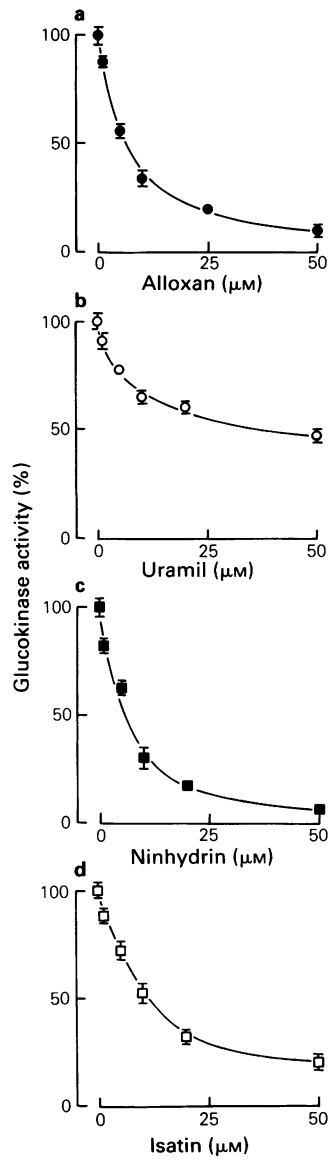

Figure 2 Concentration-dependent inhibition by (a) alloxan, (b) uramil, (c) ninhydrin and (d) isatin of glucokinase activities in cytoplasmic fractions of ob/ob mouse pancreatic B-cells. During a 5-min period the cytoplasmic fractions were exposed to serial concentrations of the inhibitor (0, 1, 5, 10, 20 and 50 μM) in the absence of D-glucose. Glucokinase activities were measured in the presence of 100 mM D-glucose. The 100% glucokinase activity was 5.9 ± 0.2 mu mg^{-1} protein. The hexokinase activity was 2.1 ± 0.1 mu mg^{-1} protein. Values shown are the means of 3 experiments and are expressed as % of the glucokinase activity measured in the absence of the inhibitor; vertical lines indicate s.e.mean.

Table 1 Stability of alloxan, uramil, ninhydrin, and isatin in reaction medium at pH 7.4 and 37°C

	Residual glucokinase activity (%) Delay before addition of cytoplasmic fraction to the pre-incubation medium	
Inhibitor	0 min	5 min
Alloxan	8.0 ± 1.8	98.3 ± 4.2*
Uramil	29.0 ± 5.4	62.7 ± 6.3*
Ninhydrin	8.0 ± 2.5	0.6 ± 0.6
Isatin	14.3 ± 4.5	13.3 ± 4.3

The rat liver cytoplasmic fractions were added to the reaction medium containing the inhibitor alloxan (50 μM), uramil (50 μM), ninhydrin (50 μM), or isatin (50 μM) either immediately or 5 min after addition of these inhibitors to the reaction medium. After pre-incubation of the cytoplasmic fraction to the reaction medium containing the respective inhibitor, glucose phosphorylation capacity was measured in the presence of 100 mM D-glucose; 100% glucokinase activities were 8.8 ± 0.6 mu mg^{-1} protein and 9.7 ± 0.8 mu mg^{-1} protein 0 min and 5 min values, respectively. Hexokinase activities were 1.9 ± 0.2 mu mg^{-1} protein and 1.9 ± 0.1 mu mg^{-1} protein 0 min and 5 min values, respectively. Values shown are the means ± s.e.mean of 6 experiments in each case. Statistical analysis was performed by use of Student's *t* test (*$P < 0.001$).

tion of alloxan. Glucose protected liver glucokinase against this inhibition with a half-maximal concentration of 19.5 ± 1.7 mM ($n = 4$).

Stability of alloxan and ninhydrin

Ninhydrin and isatin were stable in the glucokinase reaction mixture at pH 7.4, as shown by the identical degree of glucokinase inhibition when these agents were placed in contact with the glucokinase in the liver cytoplasmic fraction immediately or after a 5 min pre-incubation in the absence of enzyme in reaction mixture at pH 7.4 and 37°C (Table 1). However, alloxan decomposed rapidly, as shown by the complete inability of alloxan to inhibit glucokinase activity after a 5 min pre-incubation of this agent in the absence of enzyme in reaction mixture at pH 7.4 and 37°C (Table 1). The significantly reduced degree of inhibition of glucokinase activity by uramil after a 5 min pre-incubation in the absence of enzyme in reaction mixture at pH 7.4 and 37°C is also indicative of rapid decomposition of this agent. (Table 1).

In reaction mixture at pH 7.4 and 37°C the inhibitory effect on liver glucokinase activity was reduced by 50% after 2.7 min ($n = 6$) of pre-incubation in the

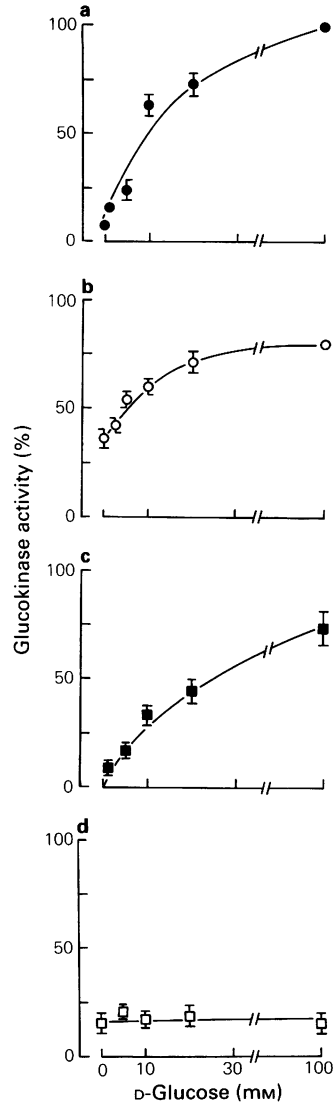

Figure 3 Concentration-dependent protection by D-glucose against inhibition of glucokinase activities in cytoplasmic fractions of ob/ob mouse pancreatic B-cells induced by (a) alloxan (50 μM), (b) uramil (50 μM), (c) ninhydrin (50 μM) and (d) isatin (50 μM). During a 5 min pre-incubation period the cytoplasmic fractions were exposed to alloxan (50 μM), uramil (50 μM), ninhydrin (50 μM), or isatin (50 μM) and serial concentrations of D-glucose (0, 1, 5, 10, 20 and 100 mM). Glucokinase activities were measured in the presence of 100 mM D-glucose. The 100% glucokinase activity was 5.9 ± 0.2 mu mg^{-1} protein. The hexokinase activity was 2.1 ± 0.1 mu mg^{-1} protein. Values shown are the means of 3 experiments and are expressed as % of the glucokinase activity measured in the absence of D-glucose; vertical lines indicate s.e.mean.

Table 2 Protective effect of various sugars against inhibition of glucokinase activities by alloxan (50 μM) in rat liver cytoplasmic fractions

Sugar	*Glucokinase activities* Control (mu mg^{-1} protein)	*Alloxan* (mu mg^{-1} protein)	*Residual glucokinase activity* (%)
None	6.0 ± 0.5	1.4 ± 0.3	23.3
D-Glucose (20 mM)	5.6 ± 0.3	4.5 ± 0.5**	80.4
D-Glucose (50 mM)	6.0 ± 0.6	5.6 ± 0.6**	93.3
D-Mannoheptulose (20 mM)	5.4 ± 0.5	3.2 ± 0.4**	59.3
D-Mannoheptulose (50 mM)	5.1 ± 0.5	4.4 ± 0.6**	86.3
D-Glucosamine (20 mM)	5.6 ± 0.6	4.3 ± 0.4**	76.8
D-Glucosamine (50 mM)	5.0 ± 0.7	4.0 ± 0.4**	80.0
N-Acetyl-D-glucosamine (20 mM)	3.5 ± 0.5	2.1 ± 0.2**	60.0
N-Acetyl-D-glucosamine (50 mM)	2.7 ± 0.3	1.9 ± 0.2**	70.4
5-Thio-D-glucose (20 mM)	5.2 ± 1.0	3.0 ± 1.0**	57.7
5-Thio-D-glucose (50 mM)	4.0 ± 1.0	2.6 ± 0.2**	65.0
2-Deoxy-D-glucose (20 mM)	4.9 ± 0.8	1.5 ± 0.2	30.6
2-Deoxy-D-glucose (50 mM)	4.4 ± 0.7	1.5 ± 0.1*	34.1
3-O-Methyl-D-glucose (20 mM)	4.8 ± 0.7	1.3 ± 0.4	26.5
3-O-Methyl-D-glucose (50 mM)	4.5 ± 1.4	1.2 ± 0.3	26.7

Rat liver cytoplasmic fractions were pre-incubated for 5 min with alloxan (50 μM) in a total reaction medium volume of 100 μl in the presence of 20 or 50 mM of the various sugars. Thereafter 1.9 ml of reaction medium was added thereby diluting the concentration of the tested sugar by a factor of 20. This dilution medium contained D-glucose in a concentration which raised the D-glucose concentration to 100 mM in the final volume of 2 ml of the reaction medium. In this solution the glucose phosphorylation by glucokinase was measured. Values shown are the means ± s.e.mean of 6 experiments in each case. Statistical comparison between control glucokinase activities and glucokinase activities in the presence of 50 μM alloxan was performed by use of Student's t test (**$P < 0.001$; *$P < 0.01$).

case of alloxan (50 μM) and after 11 min ($n = 6$) of pre-incubation in the case of uramil (50 μM).

Structural requirements of glucose for protection of glucokinase

The concomitant presence of D-glucose during the 5 min pre-incubation period of cytoplasmic fractions from pancreatic B-cells or liver protected glucokinase enzyme activity against inhibition by alloxan (50 μM), uramil (50 μM) or ninhydrin (50 μM), in a manner dependent on the concentration of glucose (Figure 3). The protection by glucose against inhibition by alloxan (50 μM) was half-maximal at a D-glucose concentration of 7.7 ± 0.3 mM ($n = 3$) in the case of pancreatic B-cell glucokinase and 9.0 ± 0.4 mM ($n = 6$) in the case of liver glucokinase. In comparison, the protection by glucose against inhibition by ninhydrin (50 μM) was half-maximal at a significantly higher ($P < 0.01$) D-glucose concentration of 21.3 ± 1.3 mM ($n = 3$) in the case of pancreatic B-cell glucokinase (Figure 3) and 22.7 ± 1.0 mM ($n = 6$) in the case of liver glucokinase. However, D-glucose provided no protection whatsoever, even at a concentration 100 mM, against inhibition of pancreatic B-cell as well as liver glucokinase activity by isatin (50 mM) (Figure 3).

In addition to the two substrates of glucokinase, the aldohexoses D-glucose (Figure 1) and D-mannose (Lenzen & Panten, 1988), several other sugars inhibited phosphorylation of glucose by liver glucokinase due to binding to the glucose binding site of the enzyme. The half-maximal inhibitory concentrations were 9.0 ± 1.3 mM ($n = 4$) for D-mannoheptulose, 9.0 ± 0.5 mM ($n = 4$) for D-glucosamine, 5.3 ± 0.5 mM ($n = 4$) for N-acetyl-D-glucosamine, and 6.8 ± 0.8 mM ($n = 4$) for 5-thio-D-glucose. 2-Deoxy-D-glucose and 3-O-methyl-D-glucose did not significantly inhibit glucokinase activity.

All the sugars that inhibited glucokinase activity were also able to protect glucokinase significantly against inhibition by alloxan (50 μM), though the protective action was usually less effective than that of D-glucose (20 and 50 mM) (Table 2).

The greatest protective effect was obtained with D-mannoheptulose (20 and 50 mM) and D-glucosamine (20 and 50 mM), followed by N-acetyl-D-glucosamine

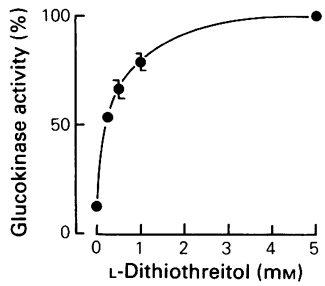

Figure 4 Concentration-dependent reversal by dithiothreitol (DTT) of inhibition of glucokinase activities in cytoplasmic fractions of ob/ob mouse pancreatic B-cells induced by alloxan (50 μM). During a 5 min pre-incubation period the cytoplasmic fractions were exposed to alloxan (50 μM). Thereafter serial concentrations of DTT (0, 0.25, 0.5, 1.0 and 5.0 mM) were added before measurement of glucokinase activities was started by addition of 100 mM D-glucose. The 100% glucokinase activity was 4.2 ± 0.2 mu mg^{-1} protein. The hexokinase activity was 2.1 ± 0.3 mu mg^{-1} protein. Values shown are the means of 4 experiments and are expressed as % of the glucokinase activity measured in the absence of DTT; vertical lines indicate s.e.mean.

(20 and 50 mM) and 5-thio-D-glucose (20 and 50 mM) (Table 2). 2-Deoxy-D-glucose provided very weak protection only at a concentration of 50 mM. 3-O-Methyl-D-glucose did not protect glucokinase against inhibition by alloxan (50 μM) at all (Table 2).

Interactions between alloxan and dithiothreitol

Addition of DTT (dithiothreitol), after a 5 min pre-incubation period of cytoplasmic fractions from ob/ob mouse pancreatic B-cells with alloxan (50 μM), resulted in a concentration-dependent reversal of the alloxan-induced inhibition of glucokinase activity, when glucokinase activities in these cytoplasmic fractions were measured thereafter in the presence of 100 mM D-glucose (Figure 4). The half-maximal concentration of DTT for reversal of pancreatic B-cell glucokinase inhibition was 0.31 ± 0.03 mM ($n = 4$).

Addition of DTT after a 5 min pre-incubation period of cytoplasmic fractions from rat liver with either alloxan (50 μM), uramil (50 μM) or ninhydrin (50 μM) also resulted in a concentration-dependent reversal of the inhibition of glucokinase activity induced by these agents, when glucokinase activities in these cytoplasmic fractions were measured thereafter in the presence of 100 mM D-glucose. The half-maximal concentrations of DTT for reversal of glucokinase inhibition were below 1 mM in each case (Table 3). Only when liver glucokinase was inhibited by isatin (50 μM) were significantly higher effective concentrations of DTT, in the half-maximal range above 5 mM, required for reversal of glucokinase inhibition (Table 3). Even when the pre-incubation period of liver cytoplasmic fractions with alloxan (50 μM) was extended up to 30 min, inhibition of glucokinase activity was completely reversible after addition of 10 mM DTT (data not shown).

Table 3 Half-maximal effective concentrations of dithiothreitol (DTT) for protection against and reversal of inhibition of glucokinase activities by alloxan, uramil, ninhydrin and isatin

	Half-maximal effective concentrations of DTT for	
Inhibitor	*Protection* (mM)	*Reversal* (mM)
Alloxan	0.04 ± 0.01	0.6 ± 0.0
Uramil	0.03 ± 0.01	0.3 ± 0.1
Ninhydrin	0.8 ± 0.0	0.9 ± 0.1
Isatin	>5	5.1 ± 1.5

For determination of the half-maximal effective concentrations of DTT for protection against inhibition of glucokinase activities by alloxan (50 μM), uramil (50 μM), ninhydrin (50 μM) or isatin (50 μM) (left column), rat liver cytoplasmic fractions were pre-incubated for 5 min with increasing concentrations of DTT (0.01; 0.05; 0.1; 0.5; 1.0; 5.0 mM), added to the reaction medium containing 50 μM of the inhibitor. Thereafter glucokinase activities were measured in the presence of 100 mM D-glucose. For determination of the half-maximal effective concentrations of DTT for reversal of inhibition of glucokinase activities by alloxan (50 μM), uramil (50 μM), ninhydrin (50 μM) or isatin (50 μM) (right column), increasing concentrations of DTT (0.5; 1.0; 2.5; 5.0; 10.0 mM) were added to the reaction medium after a 5 min pre-incubation of rat liver cytoplasmic fractions in a reaction medium containing 50 μM of the inhibitor, before measurement of glucokinase activities was started by addition of 100 mM D-glucose. The 100% glucokinase activity was 6.2 ± 0.6 mu mg^{-1} protein. The hexokinase activity was 1.0 ± 0.1 mu mg^{-1} protein. Values shown are the means $\pm$ s.e.mean of 6 experiments in each case.

Addition of DTT before the beginning of the 5 min pre-incubation period of cytoplasmic fractions from rat liver with either alloxan (50 μM) or uramil (50 μM) provided a concentration-dependent protection of glucokinase activity against inhibition by these agents, when glucokinase activities in these cytoplasmic fractions were measured thereafter in the presence of 100 mM D-glucose. The half-maximal concentrations of DTT for protection of glucokinase against inhibition by alloxan (50 μM) or uramil (50 μM) were significantly lower than those required for reversal of inhibition (Table 3). When glucokinase was inhibited by ninhydrin (50 μM), significantly

higher half-maximal concentrations of DTT were required for protection of glucokinase against inhibition (Table 3), and even higher half-maximal concentrations of DTT (above 5 mM) were required for protection of glucokinase against inhibition by isatin (50 μM) (Table 3).

Discussion

Dithiothreitol inactivates the thiol reagent alloxan (Labes & Freisburger, 1930; Lazarow, 1949) (Figure 1), and thereby yields dialurate, through reduction of alloxan at the 5-CO group, its most reactive keto group (Patterson *et al.*, 1949; Brown, 1962; Webb, 1966). The ability of alloxantin to inhibit glucokinase can be explained by its rapid break down into alloxan and dialurate (Abderhalden, 1949; Brown, 1962), so that its inhibitory effect is actually due to the action of alloxan. Replacement of the 5-CO group of alloxan (Figure 1) by an amino group as in uramil (Brown, 1962) greatly reduced the ability to inhibit glucokinase through reaction with SH groups of the enzyme. Other derivatives such as violuric or barbituric acid (Brown, 1962) were ineffective.

Ninhydrin (Figure 1) was as potent an inhibitor as alloxan. The keto group in position 2 (Figure 1) is the most reactive keto group (McCaldin, 1960) and therefore responsible for inhibition of glucokinase through oxidation of SH groups of the enzyme. Only one structurally related substance, isatin, where the 3-CO group is replaced by an imino group, also inhibited glucokinase. However, isatin appears to inhibit glucokinase not only through the reversible oxidation of SH groups but also through an irreversible action (Webb, 1966; Connolly & Trayer, 1979b). This may even take place at a site other than the active site of the enzyme, as indicated by the inability of glucose to protect against isatin inhibition.

Thus alloxan, uramil and ninhydrin inhibit pancreatic B-cell (Lenzen *et al.*, 1987a) and liver (Hara *et al.*, 1986; Meglasson *et al.*, 1986; Lenzen *et al.*, 1987a) glucokinase as a result of their sulphhydryl reactivity (Labes & Freisburger, 1930). Alloxan has a quinoid structure (Webb, 1966). However, uramil, a diketone, cannot act as a quinoid system, even though this has been proposed by Ashcroft *et al.* (1986). Thus, a reversible redox process, which accounts for the biological effects of quinoid systems, cannot be responsible for inhibition of the glucokinase by uramil.

Alloxan, uramil and ninhydrin inhibit glucose-induced insulin secretion (Tomita *et al.*, 1974; McDaniel *et al.*, 1977; Tait *et al.*, 1983). However, only alloxan (Brückmann & Wertheimer, 1945; 1947; Brückmann & Isaacs, 1949), not ninhydrin (Thorogood, 1944), has been found to be diabetogenic. The most likely explanation for this difference is that ninhydrin, which is far more lipophilic and chemically stable (McCaldin, 1960) than alloxan (Patterson *et al.*, 1949; Brown, 1962; Webb, 1966), produces toxic effects *in vivo* before it can express a diabetogenic action due to selective pancreatic B-cell destruction.

An essential feature of the inhibition of insulin secretion by alloxan is the ability of glucose and mannose to protect the pancreatic B-cell against its effects (Cooperstein & Watkins, 1981). Glucokinase is also protected against alloxan inhibition by its two substrates glucose (Figure 1) and mannose (Lenzen *et al.*, 1987a). Several other sugars which inhibit glucokinase activity also protected glucokinase against inhibition by alloxan (Table 2). Besides 5-thioglucose and mannoheptulose (Hara *et al.*, 1986) these were C-2-derived glucose analogues (Table 2). The extent of the protective action was dependent on the substituent. The C-3-derived analogue, 3-O-methylglucose (Table 2) and the C-4-derived analogue, galactose (Lenzen *et al.*, 1987a) did not protect the enzyme. Meglasson *et al.* (1986) missed observing a protective effect of N-acetylglucosamine, as they tested this sugar at a concentration too low to obtain a significant effect.

As the protective potency of mannose is not higher than that of mannoheptulose (Table 2; Lenzen *et al.*, 1987a; Lenzen, unpublished observations), a conformational change of the glucokinase upon substrate binding (Steitz *et al.*, 1977) does not apparently mediate the protective action of glucose or mannose. An increased generation of reducing equivalents in the cytosol during metabolism of glucose or mannose, to keep the SH groups in a reduced state and to inactivate alloxan (Sener *et al.*, 1982), also does not appear to participate in their protective action.

It is concluded that there is an open space in the sugar binding site of the glucokinase molecule beneath the C-2 atom of the sugar, as has been demonstrated for yeast hexokinase (Anderson *et al.*, 1978a). The functionally essential SH groups (Anderson *et al.*, 1978b) of the glucokinase (Connolly & Trayer, 1979b) are, apparently, located in this open space. These SH groups are oxidized by alloxan, thereby causing a reversible inactivation of the enzyme. Glucose binds to the sugar binding site through formation of hydrogen bonds (Steitz *et al.*, 1977; Connolly & Trayer, 1979b). Protection of the glucokinase against alloxan inhibition can be provided only when a sufficiently bulky substituent in position C-2 of the sugar (Table 2) (Connolly & Trayer, 1979a; Connolly & Trayer, 1979b) points into this open space of the sugar binding site of the enzyme, thereby hindering access of alloxan to the

SH groups. The present results exclude two alternative explanations, namely that alloxan binds to a structure not identical with the active site of the glucokinase, as proposed by Meglasson *et al.* (1986), and that glucose and alloxan both bind to the same sugar binding site of the pancreatic B-cell due to a common molecular configuration, as proposed by Weaver *et al.* (1979).

Some of the results were obtained during work for a medical thesis by F.-H.B. This investigation was supported by the Deutsche Forschungsgemeinschaft.

References

ABDERHALDEN, E. (1947). Beitrag zum Problem der Alloxan-, Alloxantin-, Ninhydrin- und Ninhydrinharnstoffwirkung auf den tierischen Organismus. *Z. Vit. Horm. Ferm. Forsch.*, **1**, 241–256.

ANDERSON, C.M., STENKAMP, R.E. & STEITZ, T.A. (1978a). Sequencing a protein by X-ray crystallography. II. Refinement of yeast hexokinase B co-ordinates and sequence at 2.1 Å resolution. *J. Mol. Biol.*, **123**, 15–33.

ANDERSON, C.M., STENKAMP, R.E., McDONALD, R. & STEITZ, T.A. (1978b). A refined model of the sugar binding site of yeast hexokinase B. *J. Mol. Biol.*, **123**, 207–219.

ASHCROFT, S.J.H., HARRISON, D.E., POJE, M. & ROCIC, B. (1986). Structure-activity relationships of alloxan-like compounds derived from uric acid. *Br. J. Pharmacol.*, **89**, 469–472.

BROWN, D.J. (1962) The pyrimidines. In *The Chemistry of Heterocyclic Compounds*. ed. Weissenberger, A. New York: Interscience Publishers.

BRÜCKMANN, G. & WERTHEIMER, E. (1945). Diabetogenic action of alloxan derivatives. *Nature*, **155**, 267–268.

BRÜCKMANN, G. & WERTHEIMER, E. (1947). Alloxan studies: the action of alloxan homologues and related compounds. *J. Biol. Chem.*, **168**, 241–256.

BRÜCKMANN, G. & ISAACS, S.D. (1949). Preparation and properties of new derivatives of alloxan. *J. Am. Med. Soc.*, **71**, 390–392.

CONNOLLY, B.A. & TRAYER, I.P. (1979a). Affinity labelling of rat-muscle hexokinase type II by a glucose-derived alkylating agent. *Eur. J. Biochem.*, **93**, 375–385.

CONNOLLY, B.A. & TRAYER, I.P. (1979b). Reaction of rat hepatic glucokinase with substrate-related and other alkylating agents. *Eur. J. Biochem.*, **99**, 299–308.

COOPERSTEIN, S.J. & WATKINS, D. (1981). Action of toxic drugs on islet cells. In *The Islets of Langerhans*. ed. Cooperstein, S.J. & Watkins, D. pp. 387–425. New York: Academic Press.

DUNN, J.S., SHEEHAN, H.L. & McLETCHIE, N.G.B. (1943). Necrosis of islets of Langerhans produced experimentally. *Lancet*, **244** (I), 484–487.

DUNN, J.S. & McLETCHIE, N.G.B. (1943). Experimental alloxan diabetes in the rat. *Lancet*, **245** (II), 384–387.

HARA, H., MIWA, I. & OKUDA, J. (1986). Inhibition of rat liver glucokinase by alloxan and ninhydrin. *Chem. Pharmacol. Bull.*, **34**, 4731–4737.

LABES, R. & FREISBURGER, H. (1930). Das Alloxan als Oxydationsmittel für Thiolgruppen, als Kapillargift und als Krampfgift. *Naunyn-Schmiedebergs Arch. Pharmakol. Exp. Pathol.*, **156**, 226–252.

LAZAROW, A. (1949). Factors controlling the development and progression of diabetes. *Physiol. Rev.*, **29**, 48–74.

LENZEN, S., SCHMIDT, W. & PANTEN, U. (1985). Transamination of neutral amino acids and 2-keto-acids in the pancreatic B-cell mitochondria. *J. Biol. Chem.*, **260**, 12629–12634.

LENZEN, S. & PANTEN, U. (1988). Signal recognition by pancreatic B-cells. *Biochem. Pharmacol.*, **37**, 371–378.

LENZEN, S., TIEDGE, M. & PANTEN, U. (1987a). Glucokinase in pancreatic B-cells and its inhibition by alloxan. *Acta Endocrinol.*, **115**, 21–29.

LENZEN, S., FREYTAG, S. & BRAND, F.-H. (1987b). Mechanismus der Hemmung der Glucokinase in B-Zellen des Pankreas durch Alloxan. *Akt. Endokrin, Stoffw.*, **8**, 96.

LENZEN, S., FREYTAG, S., BRAND, F.-H. & TIEDGE, M. (1987c). Alloxan inhibits glucokinase in pancreatic B cells. *Diabetologia*, **30**, 548A.

LERNMARK, A. (1974). The preparation of, and studies on free cell suspensions from mouse pancreatic islets. *Diabetologia*, **10**, 431–438.

McCALDIN, D.J. (1960). The chemistry of ninhydrin. *Chem. Rev.*, **60**, 39–51.

McDANIEL, M.L., ROTH, C.E., FINK, C.J., SWANSON, J.A. & LACY, P.E. (1977). Ninhydrin inhibition of glucose-induced insulin release. *Diabetologia*, **13**, 603–606.

McKNIGHT, G.S. (1977). A colorimetric method for the determination of submicrogram quantities of protein. *Anal. Biochem.*, **78**, 86–92.

MEGLASSON, M.D., BURCH, P.T., BERNER, D.K., NAJAFI, H. & MATSCHINSKY, F.M. (1986). Identification of glucokinase as an alloxan-sensitive glucose sensor of the pancreatic B-cell. *Diabetes*, **35**, 1163–1173.

PATTERSON, J.W., LAZAROW, A. & LEVEY, S. (1949). Alloxan and dialuric acid: their stabilities and ultraviolet absorption spectra. *J. Biol. Chem.*, **177**, 187–196.

SENER, A., MALAISSE-LAGAE, F. & MALAISSE, W.J. (1982). Noncarbohydrate nutrients protect against alloxan-induced inhibition of insulin release. *Endocrinology*, **110**, 2210–2212.

STEITZ, T.A., ANDERSON, W.F., FLETTERICK, R.J. & ANDERSON, C.M. (1977). High resolution crystal structures of yeast hexokinase complexes with substrates, activators, and inhibitors. Evidence for an allosteric control site. *J. Biol. Chem.*, **252**, 4494–4500.

TAIT, S.P.C., POJE, M., ROCIC, B. & ASHCROFT, S.J.H. (1983). Diabetogenic action of alloxan-like compounds, the effect of dehydrouramil hydrate hydrochloride on isolated islets of Langerhans of the rat. *Diabetologia*, **25**, 360–364.

THOROGOOD, E. (1944). Alloxan-induced diabetes in rats. *Fed. Proc.*, **3**, 48.

TOMITA, T., LACY, P.E., MATSCHINSKY, F.M. & McDANIEL, M.L. (1974). Effect of alloxan on insulin secretion in isolated rat islets perifused in vitro. *Diabetes*, **23**, 517–524.

WALKER, D.G. & PARRY, M.J. (1966). Glucokinase. *Methods Enzymol.*, **9**, 381–391.

WEAVER, D.C., BARRY, C.D., McDANIEL, M.L., MARSHALL, G.R. & LACY, P.E. (1979). Molecular requirements for recognition at a glucoreceptor for insulin release. *Mol. Pharmacol.*, **16**, 361–368.

WEBB, J.L. (1966). Alloxan. In *Enzyme and Metabolic Inhibitors*. ed. Webb, J.L. pp. 367–419. New York: Academic Press.

WÖHLER, F. & LIEBIG, J. (1838). Untersuchungen über die Natur der Harnsäure. *Ann. Pharm.*, **26**, 241–340.

(Received January 13, 1988
Revised May 3, 1988
Accepted May 31, 1988)