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NON-ENZYMATIC REDUCTION OF ALLOXAN BY REDUCED NICOTINAMIDE NUCLEOTIDE

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Abstract—Much evidence has been reported that the diabetogenic action of alloxan is caused by the formation of cytotoxic free radicals during the autoxidation of dialuric acid, a reduction product of alloxan, to alloxan. The mechanism by which alloxan is reduced *in vivo* to dialuric acid, however, is unknown. The non-enzymatic reaction of alloxan with NAD(P)H was studied as a possible candidate for the reduction of alloxan. The reaction was carried out at 37° in 50 mM phosphate buffer (mostly at pH 7.0) and was followed by measuring the decrease in absorbance at 340 nm. NADH and NADPH were found to be stoichiometrically oxidized by alloxan to NAD and NADP respectively. When the alloxan concentration (1.0 mM) was kept constant and the concentration of NAD(P)H (0.05 to 0.2 mM) was varied, the rate of decrease in the relative concentration of NAD(P)H was almost constant, suggesting that the autoxidation of dialuric acid by O_2 was rapid enough to neglect its presence in the medium. The reaction between alloxan and NAD(P)H was accelerated by decreasing the pH. Both the rate of decrease in NAD(P)H concentration and the rate of O_2 consumption resulting from autoxidation of the dialuric acid formed by reduction of alloxan were not affected by the presence of 20 mM D-glucose. Ethylene formation by the reaction of methional with ·OH, one of the autoxidation products of dialuric acid, was clearly reduced by the presence of α- or β-D-glucose (20 mM), but there was no significant difference between the effects of the two anomers. These results with D-glucose ruled out the possibility that the protection of β-cells by D-glucose against the diabetogenicity of alloxan can be explained either by its inhibitory action on dialuric acid formation or by its scavenging effect on ·OH.

Alloxan [2,4,5,6(1H,3H)-pyrimidinetetrone] injected into animals destroys pancreatic β-cells, resulting in insulin deficiency and diabetes [1]. The diabetogenic action of alloxan is relatively specific for the β-cells [2]. The exact mechanism by which alloxan produces necrosis of the β-cells is unknown, although various hypotheses have been proposed.

One of the hypotheses is that alloxan directly reacts with certain protein thiols, on the β-cell membrane, which are necessary for the integrity of the β-cells [3, 4]. An alternative hypothesis is that the destruction of the β-cells is due to the reduction, by unknown mechanisms, of alloxan to dialuric acid (5-hydroxybarbituric acid) and its rapid autoxidation by O_2, yielding cytotoxic free radicals [5, 6].

Grankvist *et al.* [6] briefly reported that NADH† and NADPH [NAD(P)H] are rapidly oxidized non-enzymatically by alloxan, although they did not describe the possibility of the involvement of this reaction in the generation of dialuric acid in the β-cells. During the course of experiments designed to test the inhibition of D-glucose-metabolizing enzymes, e.g. glucose dehydrogenase (EC 1.1.1.47), hexokinase (EC 2.7.1.1), glucokinase (EC 2.7.1.2), and aldose reductase (EC 1.1.1.21), by various substances including alloxan, we happened to confirm their results. Since the concentration of NAD(P)H in the pancreatic islets was calculated to be as high as 100–200 μM from the amount of NAD(P)H per islet [7, 8], with the assumption of an intracellular islet water space of 2.17 nl/islet [9], we postulated that NAD(P)H may be responsible for the reduction of alloxan to dialuric acid in the β-cells. We thus investigated the reaction of alloxan with NAD(P)H in detail.

MATERIALS AND METHODS

Chemicals. Alloxan monohydrate was obtained from Katayama Chemical Industries (Osaka, Japan). NADH, NADPH, NADP, and glucose-6-phosphate dehydrogenase were from the Oriental Yeast Co., Ltd. (Osaka, Japan). Catalase (from bovine liver, 20,000 units/mg protein) and methional [3-(methylthio)propionaldehyde] were from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). α-D-Glucose and β-D-glucose were prepared as described previously [10]. Active alumina TR (adsorbent for gas chromatography) was from Gasukuro Kogyo (Tokyo, Japan).

A stock solution of alloxan was prepared in 10^{-3} N HCl to prevent its decomposition to alloxanic acid. The alloxan concentration was determined at 270 nm ($E = 980\ M^{-1}\ cm^{-1}$) in 50 mM phosphate buffer (pH 7.4) by the method of Patterson *et al.* [11].

Basic reaction system. All reactions were performed at 37° in this study unless otherwise stated.

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† Abbreviations: NADH, reduced form of nicotinamide adenine dinucleotide; NADPH, reduced form of nicotinamide adenine dinucleotide phosphate; NAD(P)H, NADH and NADPH; and KRB buffer, Krebs–Ringer bicarbonate buffer.

The basic reaction system contained 50 mM phosphate buffer (pH 7.0), 1.0 mM alloxan and 0.1 mM NADPH in a total volume of 1.0 ml. Reactions were initiated by addition of alloxan and were followed by recording the decrease in absorbance at 340 nm on a Gilford 250 spectrophotometer equipped with a thermostatted cuvette-holder. The basic reaction system was modified as the occasion demanded.

Measurement of the decomposition rate of alloxan. The rate of decomposition of alloxan was measured at 37° in 50 mM phosphate buffer at various pH values and in KRB buffer (pH 7.4) by following the decrease in absorbance at 270 nm [11].

Assay of NADP formed by oxidation of NADPH with alloxan. The reaction between NADPH and alloxan was performed in a cuvette with the system, in a total volume of 3.0 ml, which contained the same reactant concentrations as the basic reaction system did. The reaction was stopped by addition of 0.6 ml of 3 N $HClO_4$ after about 7 min of incubation. The absorbance at 340 nm at the instant just before the addition of $HClO_4$ was read to calculate the amount of NADPH consumed during the incubation period. Acidification of the reaction mixture by addition of $HClO_4$ decomposes NADPH, but not NADP [12]. To one of two test tubes containing a portion (1.2 ml) of the reaction mixture, 0.05 ml of 1.0 mM NADP was added as an internal standard. The same volume (0.05 ml) of H_2O was added to the other, instead of NADP. Then 0.2 ml of 1 M K_2HPO_4 and an appropriate amount of 3 N KOH were added to both test tubes with intensive shaking in an ice-cold bath until the pH was 7.2 to 7.4. The test tubes were warmed to room temperature and kept for about 25 min until alloxan decomposed completely. One ml of the supernatant fraction obtained from each of the two reaction mixtures was transferred to a microcuvette, and the amount of NADP was assayed by adding 0.04 ml of 100 units/ml glucose-6-phosphate dehydrogenase, 0.02 ml of 1 M $MgSO_4$ and 0.04 ml of 0.1 M glucose-6-phosphate. The amount, x, of NADP present in portions (1.2 ml) of the reaction mixture acidified with $HClO_4$ was calculated from the following equation

$$\frac{a+x}{x}=\frac{s}{t},$$

where a is the amount (50 nmoles) of NADP added as an internal standard, and s and t are the absorbance values obtained with and without added NADP respectively.

Reaction of alloxan with NADPH in oxygen-free medium. A specially devised gas-tight cuvette was used for measuring the absorbance in oxygen-free medium. Two millilitres of 0.1 mM NADPH in 50 mM phosphate buffer (pH 7.0) was placed in the cuvette, gassed with N_2 for 10 min in the cold, and then warmed to 37°. The reaction was initiated by addition of 0.02 ml of 100 mM alloxan gassed well with N_2 and the decrease in absorbance at 340 nm was followed.

Measurement of O_2 consumption. Oxygen consumption caused by the reaction of alloxan (1.0 or 2.0 mM) with NADPH (0.2 mM) was measured with a Clark oxygen electrode (Beckman Instruments, Fullerton, CA, U.S.A.) in 1.0 ml of 50 mM phosphate buffer (pH 7.0). In some cases, 20 mM α- or β-D-glucose was also present in the reaction mixture. The temperature of the system was kept at 37° by a circulating water pump.

Gas chromatography for measuring ethylene formation. Generation of ·OH in the reaction of alloxan with NADPH was detected using the reaction of ·OH with methional to form ethylene [13]. Ethylene was determined on a Shimadzu GC-5A gas chromatograph (Shimadzu Seisakusho, Kyoto, Japan) with a flame ionization detector. The reaction was carried out at 37° in 10-ml test tubes stoppered with silicone rubbers. The reaction mixture was at a final volume of 1.0 ml and contained 50 mM phosphate buffer (pH 7.0), 2.0 mM alloxan, 0.2 mM NADPH, and 1 mM methional. In some cases, 20 mM α- or β-D-glucose was also present in the reaction mixture. The reaction was initiated by injection of 50 μl of 40 mM alloxan through the silicone rubber. Samples (1.0 ml) of head gas were removed by means of a 1.0-ml gas-tight syringe. Gas samples were injected immediately into the gas chromatograph equipped with a column (3 mm × 2 m) of active alumina. The retention time of ethylene was 1.7 min when the instrument was operated at 50° and at a flow rate of 40 ml/min for N_2 (carrier gas).

RESULTS AND DISCUSSION

A certain amount of evidence has been accumulated [5, 6, 14, 15] that free radicals (·OH and O_2^-) and H_2O_2 generated by autoxidation of dialuric acid, a reduction product of alloxan, may play a primary role in the diabetogenic action of alloxan, although the mechanism by which alloxan is reduced to dialuric acid is not known. Holmgren and Lyckeborg [16] have very recently reported that bovine thioredoxin reductase catalyzes the reduction of alloxan by NADPH to dialuric acid and have suggested that the enzyme is responsible for rapid generation of

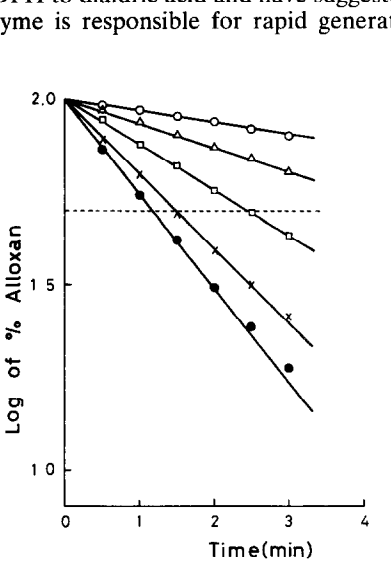

Fig. 1. Rate of decomposition of alloxan (1.0 mM) at 37°. The decrease in absorption at 270 nm was followed in 50 mM phosphate buffer [(—○—), pH 6.0; (—△—), pH 6.5; (—□—), pH 7.0; (—×—), pH 7.4] and in KRB buffer [(—●—), pH 7.4)]. The dotted line represents the point of 50% decomposition.

dialuric acid *in vivo*. However, there is neither evidence that thioredoxin reductase is present in the β-cells of pancreas nor proof that enzymatically catalyzed rapid generation of dialuric acid is necessary for production of enough free radicals and H_2O_2 to cause the diabetogenicity of alloxan. Therefore, non-enzymatic reduction of alloxan by NAD(P)H is a candidate for the true mechanism of generation of dialuric acid *in vivo*. On the basis of this thought, the present study was conducted.

Almost all of the experiments were done in 50 mM phosphate buffer. It seemed important, however, to consider whether the reactions in 50 mM phosphate buffer can take place *in vivo* also. To test this, some of the experiments were performed in KRB buffer, which is more physiological than 50 mM phosphate buffer. Although we describe here only the experiments performed with NADPH, the same results were obtained also with NADH.

Spontaneous decomposition of alloxan. The rates of decomposition of alloxan in 50 mM phosphate buffer and in KRB buffer are shown in Fig. 1. Decomposition of alloxan markedly slowed down with decreasing pH. The half-lives of alloxan in 50 mM phosphate buffer were 1.5 and 10 min at pH 7.4 and 6.0 respectively. These values were higher than those (0.9 and 8.7 min) reported by Patterson *et al.* [11] although the conditions used by them were almost the same as ours except for the use of 85 mM phosphate buffer. Spontaneous decomposition of alloxan in KRB buffer (pH 7.4) was a little faster than that in 50 mM phosphate buffer (pH 7.4), suggesting that some components in KRB buffer may accelerate decomposition of alloxan. The decomposition rate of alloxan in KRB buffer (pH 7.4) containing 0.5% bovine serum albumin could not be determined because of the extremely high absorbance at 270 nm due to albumin. Spontaneous decomposition of NADPH was negligibly slow even at pH 6.0 (data not shown).

NADP formation in the reaction of NADPH with alloxan. The amount of NADP formed by the reaction of NADPH with alloxan was determined by converting NADP to NADPH in the presence of excess glucose-6-phosphate and excess glucose-6-phosphate dehydrogenase. Prior to NADP determination, NADPH and alloxan remaining in the reaction mixture were destroyed by treating with $HClO_4$ and by allowing the mixture to stand for a time at room temperature at neutral pH.

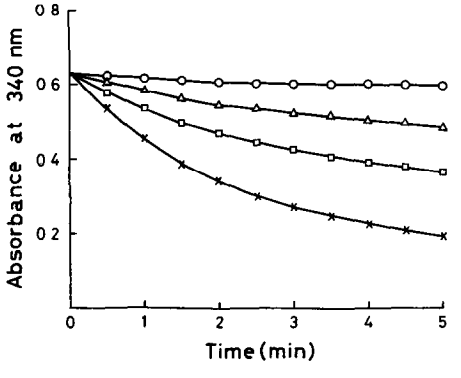

Fig. 2. Rate of decrease in NADPH concentration in the reaction of alloxan (0.1 to 2.0 mM) with NADPH (0.1 mM). The reaction was performed in 50 mM phosphate buffer (pH 7.0) at 37°. Alloxan concentration: (—○—), 0.1 mM; (—△—), 0.5 mM; (—□—), 1.0 mM and (—×—), 2.0 mM.

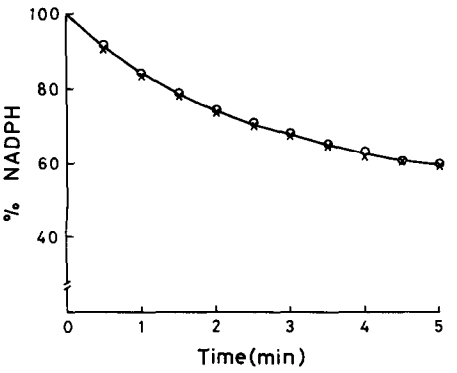

Fig. 3. Rate of decrease in relative concentration of NADPH in the reaction of alloxan (1.0 mM) with NADPH (0.05 to 0.2 mM). The reaction was performed in 50 mM phosphate buffer (pH 7.0) at 37°. NADPH concentration: (—○—), 0.05 mM and (—×—), 0.2 mM. Since the curves obtained with 0.1 and 0.15 mM NADPH were completely superimposable on that depicted in this figure, the marks for both concentrations (0.1 and 0.15 mM) were omitted from this figure.

The proportion of the amount of NADP formed in the reaction to that of NADPH consumed in the reaction was 95.0 ± 3.7% (mean ± S.D. for four experiments). This result indicates that NADPH was stoichiometrically oxidized to NADP by the alloxan, i.e. that NADPH acted only as a reducing agent and did not form any addition product with alloxan.

Concentration dependence of the reaction. When NADPH (0.1 mM) was kept constant and the concentration of alloxan was varied, the rate of decrease in NADPH concentration varied dose-dependently (Fig. 2). This seemed reasonable because the oxidation of NADPH by alloxan proceeded according to a second-order reaction mechanism unless the decomposition of alloxan was taken into consideration. When alloxan (1.0 mM) was kept constant and the concentration of NADPH was varied, however, the rate of decrease in relative concentration of NADPH did not vary substantially (Fig. 3). This may be explained as follows. Given that the decomposition of alloxan is not taken into consideration, the reaction velocity (dx/dt) is expressed by equation 1 for a second-order reaction,

$$\frac{dx}{dt} = k\,(a-x)\,(b-x) \qquad (1)$$

where k is the rate constant, a and b are the initial concentrations of NADPH and alloxan, and x is the concentration of reactants consumed during time t. Supposing that the reoxidation of dialuric acid to alloxan by O_2 is rapid enough for $(b-x)$ to approach b, equation 1 can be rewritten as:

$$\frac{dx}{dt} = k'\,(a-x) \qquad (2)$$

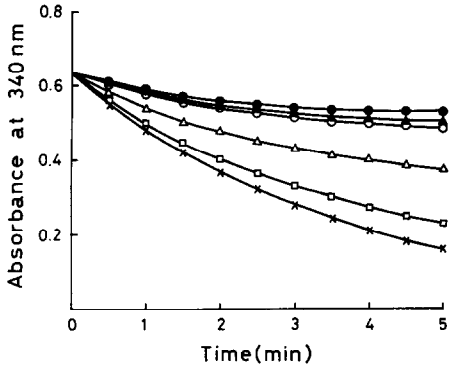

Fig. 4. Effects of pH and buffer on the rate of decrease in NADPH concentration in the reaction of alloxan (1.0 mM) with NADPH (0.1 mM). The reaction was performed at 37° in 50 mM phosphate buffer [(—○—), pH 7.4; (—△—), pH 7.0; (—□—), pH 6.5 and (—×—), pH 6.0)], in KRB buffer [(—●—), pH 7.4], and in KRB buffer containing 0.5% bovine serum albumin [(—▲—), pH 7.4].

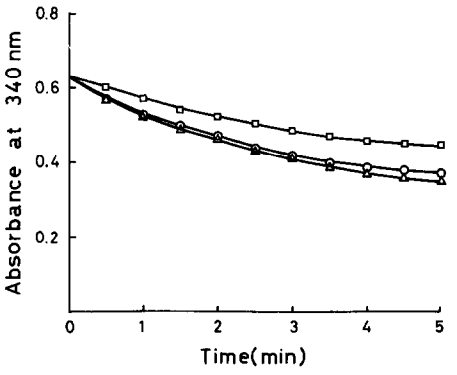

Fig. 5. Effects of D-glucose and oxygen on the rate of decrease in NADPH concentration in the reaction of alloxan (1.0 mM) with NADPH (0.1 mM). The reaction was performed in 50 mM phosphate buffer (pH 7.0) at 37°. Both the control experiment (—○—) and the experiment in the presence of 20 mM D-glucose (—△—) were carried out in medium saturated with air. The experiment in oxygen-free medium (—□—) was carried out as described in Materials and Methods.

where k' is $k \times b$. Equation 2 can be transformed to:

$$-\ln\frac{a-x}{a} = k't \qquad (3)$$

This equation means that the rate of decrease in relative concentration of NADPH is constant independently of the initial concentration of NADPH. The results shown in Fig. 3 thus indicate that dialuric acid was instantaneously reoxidized to alloxan without any appreciable time lag.

Effects of pH and buffer on the reaction rate. The rate of the reaction of alloxan with NADPH was found to be severely influenced by pH, i.e. to increase with decreasing pH (Fig. 4). This may have resulted partly from the stability of alloxan at acid pH. Because the pK of alloxan is reported to be 6.6 [17], 6.8 [11], or 7.2 [18], the non-ionized form of alloxan predominates over the ionized forms at pH values below about 7. Hence, it also seemed likely that the pH dependence of the reaction could be accounted for by high reactivity of the non-ionized form of alloxan as compared with the ionized forms.

Boquist [19] reported that the metabolic alkalosis induced by treatment with $NaHCO_3$ protected mice against the β-cell toxicity of alloxan. Klebanoff and Greenbaum [20] reported that systemic acidosis (caused either by $CaCl_2$ or NH_4Cl ingestion, or by respiration of gas mixtures rich in CO_2) increased the vulnerability of rats to alloxan and suggested that relative acidity in the β-cells could contribute to the regulation of the vulnerability of these cells to alloxan. Our present results on pH dependence are compatible with the results and suggestions of these two reports.

The reaction rate in 50 mM phosphate buffer (pH 7.4) was a little higher than that in KRB buffer (pH 7.4) or in KRB buffer (pH 7.4) containing 0.5% bovine serum albumin (Fig. 4), though not statistically analyzed. These results seem to be compatible with the faster decomposition of alloxan (Fig. 1) in KRB buffer (pH 7.4) than in 50 mM phosphate buffer (pH 7.4).

Effects of D-glucose and O_2 on the reaction rate. Since administration of D-glucose has been shown to protect β-cells against the diabetogenic action of alloxan [21, 22], the effect of D-glucose on the reaction rate was examined. As shown in Fig. 5, the inclusion of 20 mM D-glucose in the basic reaction system scarcely affected the reaction between alloxan and NADPH.

The reaction under anaerobic conditions was expected to be slower than that under aerobic conditions because of the impossibility of reoxidation of dialuric acid to alloxan, and this was ascertained in fact as shown in Fig. 5.

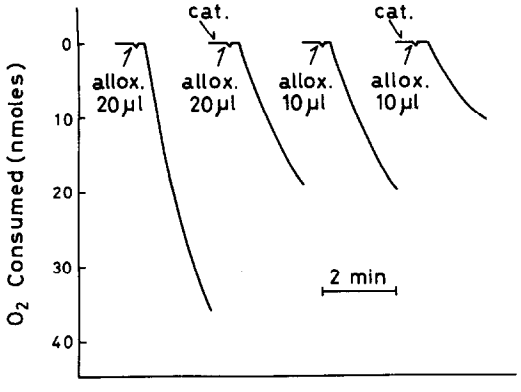

Fig. 6. Consumption of O_2 in the reaction of alloxan (1.0 or 2.0 mM) with NADPH (0.2 mM) in the presence and absence of catalase. Oxygen consumption was measured with an oxygen electrode in 1.0 ml of 50 mM phosphate buffer (pH 7.0) at 37° as described in Materials and Methods. Concentrations of alloxan (allox.) and catalase (cat.) were 100 mM and 30 mg/ml, respectively, and the volume of the catalase solution added was 5 μl. Tracings of O_2 consumption in the absence and presence of 20 mM D-glucose were almost the same in all cases shown in this figure.

Oxygen consumption and H_2O_2 *generation.* Catalase at 150 μg/ml reduced the rate of O_2 consumption by nearly half in the reaction of alloxan (1.0 or 2.0 mM) with NADPH (2.0 mM), thus verifying the accumulation of H_2O_2 (Fig. 6). It seemed that the O_2 consumption and H_2O_2 generation could be ascribed to the reoxidation of dialuric acid by O_2 to alloxan. The amount (51 nmoles/ml) of NADPH oxidized for the first 2 min in the reaction of 1.0 mM alloxan and 0.2 mM NADPH (Fig. 3), however, was about 2.5 times that (20 nmoles/ml) of O_2 consumed for the same period of time under the same conditions, whereas the oxidation of 1 mole of NADPH was expected to result in the consumption of 1 mole of O_2. In combination with results that the amount of NADPH consumed was nearly equal to the amount of NADP produced and that the amount of dialuric acid in the medium seemed to be negligibly small, the present results strongly suggest that the reaction was reversible and that only part of the dialuric acid produced was reoxidized to alloxan. An alternative concept is that several oxy groups of alloxan may have been reduced as suggested by Holmgren and Lyckeborg [16] and that some, but not all, of the reduced oxy groups were reoxidizable to an alloxan-like compound(s).

The inclusion of 20 mM D-glucose in the medium little affected the O_2 consumption and H_2O_2 generation. This result was consistent with the inability of D-glucose (20 mM) to suppress the reaction of alloxan with NADPH, suggesting that the protection of the β-cells against the diabetogenic action of alloxan by D-glucose cannot be explained by its inhibitory action on the formation of dialuric acid.

Scavenging effects of D-*glucose anomers on hydroxyl radicals.* The reaction of alloxan with NADPH was found, by detection of ethylene formation in reaction with methional, to generate ·OH. Ethylene was determined by gas chromatography. Of the active oxygens (O_2^-, ·OH, and H_2O_2) known to be produced by oxidation of dialuric acid to alloxan [23], the hydroxyl radical has strongly been suggested to be responsible for the damage to the β-cells [5, 15]. D-Glucose was reported to have the ability to scavange ·OH [23]. Besides, the ability of D-glucose to protect the β-cells from alloxan is known to be highly stereospecific for the α anomer of D-glucose both *in vivo* [24] and *in vitro* [25]. Hence, it seemed worthwhile to compare the inhibitory effect of α-D-glucose on ethylene formation with that of β-D-glucose. We found that α- and β-D-glucose (20 mM each) were not different in effectiveness (36% inhibition for α, 39% for β) in preventing ethylene formation, i.e. in scavenging ·OH. This result indicates that the α-specific protection of the β-cells cannot be explained by the difference between α- and β-D-glucose in reactivity with ·OH. One possibility for the stereospecific protection of the β-cells by D-glucose, yet to be explored, is that there are some proteins or some membrane sites, which are stereospecific for the α anomer of D-glucose as well as necessary for maintaining the β-cells viable, in the β-cells, and the binding of α-D-glucose to those components protects the β-cells from the damage by ·OH.

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