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Superoxide Dismutase, Catalase and Scavengers of Hydroxyl Radical Protect against the Toxic Action of Alloxan on Pancreatic Islet Cells *in vitro*

By Kjell GRANKVIST, Stefan MARKLUND, Janove SEHLIN and Inge-Bert TÄLJEDAL
Departments of Histology and Clinical Chemistry, University of Umeå, S-901 87 Umeå 6, Sweden

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Experiments with isolated pancreatic islets or dispersed islet cells from non-inbred *ob/ob* mice were performed to test the hypothesis that free radicals, notably OH·, mediate the diabetogenic toxicity of alloxan. Accumulation of $^{86}Rb^+$ by whole islets and exclusion of Trypan Blue by dispersed cells were used as previously validated criteria of islet-cell viability. Alloxan alone drastically inhibited the Rb^+ accumulation and significantly decreased the frequency of cells excluding Trypan Blue. Enzymic scavengers of $O_2^{\cdot-}$ and H_2O_2 or non-enzymic scavengers of OH· or singlet oxygen were added to the incubation medium and tested for their ability to protect against these effects of alloxan. Superoxide dismutase, catalase, dimethyl sulphoxide, benzoate, and mannitol counteracted the effects of alloxan in both cytotoxicity assays. Significant protection of the Rb^+-accumulating capacity was also afforded by butanol, caffeine, theophylline, NADH, NADPH and, to a small extent, NAD^+. Urea has a poor affinity for OH· and did not protect against alloxan. No effect was obtained with the singlet-oxygen scavenger, histidine. Except for the protection by NADH and NADPH, which may be due to a direct reaction with alloxan in the medium, the results strongly support the hypothesis. β-Cells may be particularly vulnerable to alloxan because their metabolic specialization facilitates reduction of the drug and perhaps of other substrates for $O_2^{\cdot-}$-yielding redox cycles.

The pancreatic β-cells, which produce insulin, are particularly sensitive to the cytotoxic action of alloxan. The reasons for this great sensitivity are poorly known, as are the chemical reactions mediating the toxicity. The pharmacological nature of alloxan notwithstanding, its mode of action may be sufficiently general for naturally occurring substances to be diabetogenic by a similar mechanism.

On the basis of experiments *in vivo*, Heikkila and co-workers (Heikkila *et al.*, 1974, 1976; Heikkila, 1977) proposed that the diabetogenic action of alloxan is mediated by hydroxyl radicals. A conceivable sequence of reactions generating OH· radicals involves reduction of alloxan to dialuric acid, and formation of $O_2^{\cdot-}$ by autoxidation back to alloxan (Deamer *et al.*, 1971; Cohen & Heikkila, 1974); formation of H_2O_2 from $O_2^{\cdot-}$ and H^+, spontaneously or by superoxide dismutase catalysis (McCord & Fridovich, 1969); and formation of OH· from $O_2^{\cdot-}$ and H_2O_2 according to the so-called Haber–Weiss reaction, as catalysed by iron (Halliwell, 1978; McCord & Day, 1978) or perhaps other transition-metal ions (Van Hemmen & Meuling, 1977).

The hypothetical involvement of OH· radicals in alloxan action is supported by the findings that a number of primary alcohols, thiourea, or dimethyl sulphoxide protect against alloxan-diabetes when injected into living animals (Heikkila *et al.*, 1974, 1976; Heikkila, 1977); all of these agents are scavengers of OH·. However, considering the many uncontrollable factors associated with whole-body experiments, the evidence obtained so far is not compelling. In particular, Schauberger *et al.* (1977) have presented results indicating that the protection afforded by butanol injections is non-specific and an indirect consequence of the hyperglycaemia after injection of alcohol.

Against this background it seems essential to use isolated pancreatic islets *in vitro* to test whether free radicals are involved in the β-cytotoxic action of alloxan. Such experiments are reported in the present paper. By measuring $^{86}Rb^+$ accumulation by whole islets, or Trypan Blue exclusion by dispersed islet cells, as indices of β-cell integrity, we have investigated whether alloxan can be protected against by superoxide dismutase, catalase, and non-enzymic scavengers of OH·.

Materials and Methods

Animals and isolation of islets and islet cells

Male adult mice homozygous for the gene *ob* were taken from a local colony (Umeå *ob/ob* mice) and starved overnight.

When studying Rb^+ uptake by whole islets, the islets were isolated by free-hand micro-dissection without the use of collagenase (Hellerström, 1964). Dissection was performed at about 14°C with the pancreas immersed in a salt-balanced medium with the same formula as Krebs–Ringer bicarbonate (De Luca & Cohen, 1964), except that the bicarbonate was replaced by 20mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], pH 7.4. This buffer was also used as the basal medium in all subsequent incubations of micro-dissected islets. The characteristics of Rb^+ uptake and release from thus isolated islets have been described previously (Sehlin & Täljedal, 1974, 1975; Idahl *et al.*, 1977).

When studying the exclusion of Trypan Blue, islets in greater numbers (about 100) were isolated by collagenase digestion, broken up to a milky suspension of cells by shaking in Ca^{2+}-free (1mM-EGTA) tissue-culture Medium 199 (Salk *et al.*, 1954), and cleansed of tissue debris by centrifuging through dense albumin (Lernmark, 1974; Grankvist *et al.*, 1977). Throughout this study, tissue-culture Medium 199 was buffered with 20mM-Hepes (pH 7.4) and lacked Phenol Red, D-glucose and albumin; unless otherwise stated, 2.6mM-Ca^{2+} was present. The viability and functional characteristics of dispersed islet cells have been described (Lernmark *et al.*, 1975; Idahl *et al.*, 1976; Grankvist *et al.*, 1977).

Protection against alloxan as assayed by Rb^+ accumulation

Quadruplicate or sextuplicate batches of three islets were incubated at 37°C for 40 (or sometimes 60) min in 1ml of basal medium. Duplicate batches were incubated with a substance to be tested for its ability to protect against alloxan, and duplicate batches were incubated without this test substance. After 30 (or 50) min we added 2μl of a freshly prepared stock solution of 250mM-alloxan in 10mM-HCl to one batch within each pair; 2μl of 10mM-HCl alone was added to the other member of the pair. The four treatments thus achieved in parallel incubations were: neither alloxan nor test substance; test substance alone; alloxan alone; test substance in combination with alloxan. Occasionally, sextuplicate batches of islets were run in parallel to study the effects of two different test substances in the same experiment. Incubation was continued for 10min. The presence of alloxan did not noticeably alter the pH of the incubation medium. All batches of islets were then incubated in 200μl of medium labelled with 28μM-^{86}RbCl (sp. radioactivity 396–668Ci/mol) and 8μM-[6,6′-$^3H_2$]sucrose (sp. radioactivity 2000Ci/mol). The radioactive medium contained no alloxan or test substance. After 120min the islets were transferred to small pieces of aluminium foil, rapidly freed of contaminating fluid with a micro-pipette, and plunged into melting isopentane. They were then freeze-dried overnight (−40°C at 0.1Pa), weighed on a quartz-fibre balance, dissolved in Hyamine and Instafluor and analysed for ^{86}Rb and ^{3}H radioactivity in a liquid-scintillation counter. The islet content of Rb^+ in excess of the extracellular-space marker, sucrose (Hellman *et al.*, 1971; Sehlin & Täljedal, 1974), was calculated by using samples of incubation medium as external standards in the liquid-scintillation-counting procedure.

Protection against alloxan as assayed by Trypan Blue exclusion

Quadruplicate or sextuplicate portions, generally 50μl, of islet-cell suspension were incubated for 30min at 37°C with 1.0ml of tissue-culture Medium 199 (no Phenol Red, D-glucose or albumin; 2.6mM-Ca^{2+}) buffered with 20mM-Hepes, pH 7.4; in duplicate tubes the medium also contained one or two substances to be tested for ability to protect against alloxan. After 15min, alloxan or HCl alone was added to the tubes in each pair as described above for measurements of Rb^+ uptake. After completed incubation, the tubes were centrifuged for 2min at 50*g*. The supernatant was removed to leave the cells in 50μl of medium to which was added 50μl of a 0.2% (w/v) solution of Trypan Blue in Hepes-buffered tissue-culture Medium 199. Drops of stained-cell suspensions were placed between object and cover glasses. The frequency of cells with Trypan Blue-stained nuclei was assessed by counting about 200 randomly selected cells under a 40× objective lens in a light microscope. The validity of this counting procedure has been demonstrated previously (Grankvist *et al.*, 1977).

Reactivity of alloxan with test substances in cell-free medium

To study whether alloxan reacted directly with test substances in the incubation medium, the decay of alloxan-dependent light absorbance was followed in Hepes-buffered Krebs–Ringer medium to which had been added 8–10mM-alloxan alone or in combination with equimolar (or more) test substance. Measurements were performed at 290nm, when the test substance itself exhibited little absorbance at this wavelength; methylxanthines mixed with alloxan were studied at the approximate isosbestic point, 303nm. The reaction between reduced nicotinamide nucleotides and alloxan was followed by measuring the decrease of A_{340} after mixing 10mM-alloxan with only 0.08mM-NADH or 0.08mM-NADPH. This approach was necessitated by the very strong absorbance of reduced nicotinamide nucleotides at 290nm.

Chemicals

Alloxan monohydrate was from United States Biochemical Corp., Cleveland, OH, U.S.A. The batch of alloxan used was more effective in inhibiting Rb^+ accumulation than was a batch from another source that we used in two previous studies (Grankvist *et al.*, 1977; Idahl *et al.*, 1977); lower concentrations of alloxan were therefore employed throughout the present study.

Trypan Blue from BDH Chemicals, Poole, Dorset, U.K., was partially purified before being used for staining cells. Red impurities were extracted into the organic phase by shaking in a mixture of water and n-pentane (1:1, v/v). The water phase was applied to a column (40cm × 1cm) of Sephadex G-15 equilibrated with absolute ethanol. After washing the column with 90% (v/v) ethanol to remove violet impurities, Trypan Blue was eluted with water, dialysed against water and freeze-dried. The dye was dissolved in tissue-culture Medium 199 and ultrasonicated for 30s to disperse any clumps or particles. Sonication did not cause re-appearance of red or violet impurities.

Bovine blood superoxide dismutase was obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. It was quantified by the pyrogallol autoxidation method, assuming that 1 unit (50% inhibition) corresponds to 100ng of enzyme/ml (Marklund & Marklund, 1974). No catalase activity was detected in the preparations.

For control experiments, inactivated superoxide dismutase was prepared. The enzyme is very sensitive to H_2O_2, and inactivation is apparently due to destruction of histidine residues liganding copper ions. Only the number of histidine residues was significantly decreased in amino acid analysis of thus inactivated enzyme (Bray *et al.*, 1974). Superoxide dismutase (4.2mg/ml) was exposed to 20mM-H_2O_2 for 15min at 22°C in 60mM-Tris/HCl, pH9.50, containing 60mM-NaCl. The reaction was stopped by adding 0.1μM-catalase. The enzyme was inactivated by 98.8%. Inactivated superoxide dismutase was compared with native enzyme by electrophoresis in polyacrylamide gel at pH8.6 and by electrophoresis in the presence of sodium dodecyl sulphate. No significant differences were observed.

Bovine liver catalase from C.F. Boehringer Mannheim G.m.b.H., Mannheim, Germany, was purified by chromatography on Sephacryl S-200 (Pharmacia Fine Chemicals, Uppsala, Sweden) and contained no superoxide dismutase activity. Catalase was quantified by using the A_{405}, assuming that the molar absorptivity at this wavelength is 3.24×10^5 $M^{-1} \cdot cm^{-1}$ (Samejima & Yang, 1963) and the mol.wt. 232000 (Schroeder *et al.*, 1969). The apparent reaction-velocity constant with H_2O_2 was reasonably high ($k^{+1} = 2 \times 10^7 M^{-1} \cdot s^{-1}$).

Collagenase was from Worthington Biochemical Corp., Freehold, NJ, U.S.A. Tissue-culture Medium 199 without Ca^{2+} was from the National Bacteriological Laboratories, Stockholm, Sweden. $^{86}RbCl$ and [6,6′-$^3H_2$]sucrose were from The Radiochemical Centre, Amersham, Bucks., U.K. L-Histidine, caffeine, theophylline and Hepes were from Sigma Chemical Co., St. Louis, MO, U.S.A. Sodium benzoate and butanol were from Merck A.G., Darmstadt, Germany. D-Mannitol was from Difco Laboratories, Detroit, MI, U.S.A. Dimethyl sulphoxide was from Fischer Scientific Company, Fair Lawn, NJ, U.S.A. Nicotinamide nucleotides were from C. F. Boehringer Mannheim G.m.b.H. Human serum albumin was from AB Kabi, Stockholm, Sweden. Instafluor and Hyamine 10-X were from Packard Instruments, Warrenville, IL, U.S.A. Other reagents were of analytical grade.

Statistics

The *P* values given indicate the degree of significance obtained in two-tailed tests. The level of significance for rejecting the null hypothesis of zero treatment effect was taken to be $P = 0.05$. The Tables and instructions given by Diem (1962) were generally used.

Results

Effects of alloxan on Rb^+ accumulation and exclusion of Trypan Blue

The present study comprised 112 separate experiments in which we measured the Rb^+ accumulation by whole islets, and 70 separate experiments in which we counted the frequency of cells excluding Trypan Blue. Fig. 1 shows the distribution of Rb^+-uptake values for islets not exposed to alloxan as well as for those treated with 0.5mM-alloxan alone. Both the mean values (control, 0.94mmol Rb^+/kg dry wt. of islets, alloxan treatment, 0.11mmol/kg) and the variances (control, 0.08mmol/kg dry wt. of islets; alloxan treatment, 0.02mmol/kg) differed between the two groups.

The distributions of Trypan Blue-exclusion values are given in Fig. 2. With this cytotoxicity assay the difference in mean value (control, 72.5% unstained cells; alloxan treatment, 51.5%) was associated with less heterogeneity in variance (controls, 43.3%; alloxan treatment, 82.9%).

All four data samples were reasonably symmetric and appeared to approximate to a normal distribution fairly well. However, the true frequency distributions cannot be deduced within each limited series of experiments devoted to testing substances for protection against alloxan. In the following, therefore, the significance of protection will be evaluated by

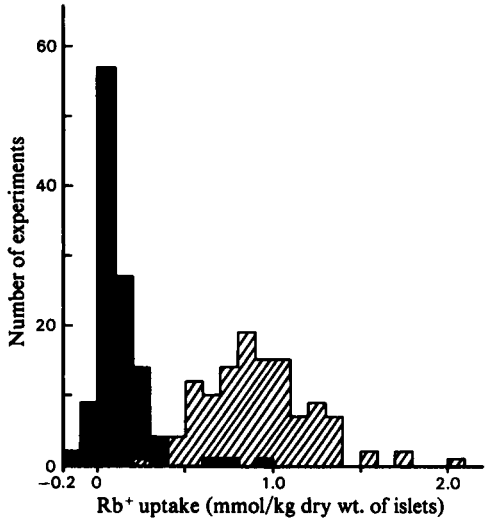

Fig. 1. *Frequency distribution of values recorded for the islet uptake of Rb^+ in 112 separate experiments*
Uptake in basal medium is indicated by hatched bars, whereas uptake after treatment with 0.5 mM-alloxan is indicated by solid bars. Negative values mean that the distribution volume for sucrose occasionally exceeded that for Rb^+ in alloxan-treated islets.

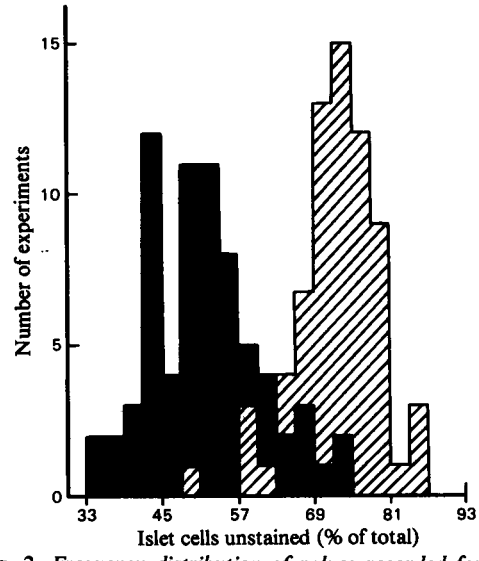

Fig. 2. *Frequency distribution of values recorded for the percentage of islet cells excluding Trypan Blue in 70 separate experiments*
Explanation of the bars is as in Fig. 1.

both Student's *t* test and the distribution-independent Wilcoxon's rank-sum test.

Protection against alloxan by catalase and superoxide dismutase

When whole pancreatic islets were exposed to high concentrations of catalase or superoxide dismutase or both, the inhibitory effect of alloxan on Rb^+ accumulation was partially prevented (Table 1). This partial protection was not a non-specific protein effect, since the same concentration of serum albumin completely failed to protect against alloxan. Neither the enzymes nor albumin had any effect on Rb^+ accumulation by control islets not treated with alloxan.

Catalase and superoxide dismutase also counteracted the toxicity of alloxan against dispersed islet cells (Fig. 3). In this system, which permits a more efficient contact between islet cells and big molecules, protection by either catalase or superoxide dismutase was significant at a concentration as low as 20 mg/litre; it was complete at 200 mg/litre. Again, the protective effect could not be reproduced with a corresponding concentration of serum albumin. In a separate series of six experiments we also checked whether protection could be reproduced with enzymically inactive superoxide dismutase. In comparison with cells not exposed to alloxan or other test substances, the frequency of unstained cells decreased by the following percentage units: 24.0± 3.1 in response to 0.5 mM-alloxan alone, 10.7±4.6 in response to 0.5 mM-alloxan plus 158 mg of active superoxide dismutase/litre (*t* test of difference from alloxan alone: $P<0.02$), and 22.7±6.7 in response to 0.5 mM-alloxan plus 168 mg of inactivated superoxide dismutase/litre (mean values ±S.E.M.).

Protection by non-enzyme scavengers of hydroxyl radicals

Table 1 shows that 22 mM-butanol, 50–70 mM-mannitol, 50 mM-sodium benzoate, and 28 mM-dimethyl sulphoxide counteracted the effect of alloxan on Rb^+ accumulation; 10 mM-histidine and 50 mM-urea had no effect. All of these substances except urea and histidine were studied because they are established $OH^\cdot$ scavengers (Anbar & Neta, 1967; Dorfman & Adams, 1973). Urea was tested because of its poor reactivity with $OH^\cdot$ radicals. Histidine at a comparatively low concentration was tested as a scavenger of singlet oxygen (Matheson *et al.*, 1975); scavengers of singlet oxygen, rather than of $OH^\cdot$ radicals, protect against the toxicity of $O_2^{\cdot-}+H_2O_2$ in some systems (Kellogg & Fridovich, 1975, 1977; Lynch & Fridovich, 1978), suggesting that the O_2 formed ($O_2^{\cdot-}+H_2O_2 \rightarrow OH^\cdot+O_2+OH^-$) may be in a highly reactive singlet state. Within this whole

Table 1. *Alloxan-induced inhibition of Rb^+ accumulation in whole islets and its partial prevention by various agents*

During preliminary incubation for 40min (all test compounds except enzymes and albumin) or 60min (enzymes and albumin) islets were incubated in basal medium (control) or in basal medium supplemented with test compound as indicated (test). Duplicate batches of islets were incubated with each type of medium. During the final 10min of the preliminary incubation, 0.5mM-alloxan was added to one of the duplicate batches, whereas a corresponding volume of HCl alone was added to the other. The islets were then transferred to new test tubes containing medium labelled with 28μM-^{86}RbCl and 8μM-[6,6′-$^3H_2$]sucrose, but free from alloxan or test substance. Accumulation of Rb^+ by islet cells in 120min is given as mean values ± S.E.M. for the numbers of experiments stated in parentheses. The degree of significance for effects in the presence of alloxan was evaluated by Student's *t* test and Wilcoxon's rank-sum test for differences between paired data. When the two tests gave the same result, only one *P* value is given. Otherwise the outcome of *t* testing is presented first and separated from the outcome of rank testing by a solidus. NS means $P > 0.05$ with both tests. No substance significantly influenced Rb^+ accumulation in the absence of alloxan. Because few experiments were performed with each mannitol concentration, comparison was also made with all the 22 observations for alloxan alone in the mannitol series. On such comparison the protective effect of 70mM-mannitol was highly significant (*t* test: $P < 0.001$).

	Accumulation of Rb^+ (mmol/kg dry wt. of islets)				
	No alloxan		0.5mM-Alloxan		*P* value for protection
Test substance	Control	Test	Control	Test	against alloxan
Catalase (220mg/litre) (12)	0.79 ± 0.05	0.87 ± 0.08	0.09 ± 0.03	0.30 ± 0.08	<0.05
Superoxide dismutase (220mg/litre) (11)	0.78 ± 0.05	0.77 ± 0.04	0.08 ± 0.03	0.23 ± 0.07	<0.1/ = 0.05
Catalase (220mg/litre) plus superoxide dismutase (230mg/litre) (9)	0.83 ± 0.07	0.90 ± 0.08	0.14 ± 0.04	0.26 ± 0.04	<0.02
Human serum albumin (250mg/litre) (10)	0.98 ± 0.06	1.05 ± 0.07	0.15 ± 0.02	0.14 ± 0.02	NS
22mM-Butanol (11)	1.10 ± 0.11	1.02 ± 0.12	0.24 ± 0.08	0.47 ± 0.11	<0.05/ = 0.01
50mM-Benzoate (9)	1.04 ± 0.12	1.29 ± 0.08	0.01 ± 0.02	0.61 ± 0.18	<0.02/<0.01
28mM-Dimethyl sulphoxide (10)	0.99 ± 0.07	0.88 ± 0.08	0.03 ± 0.03	0.39 ± 0.17	<0.05
10mM-Mannitol (6)	0.84 ± 0.13	0.90 ± 0.17	0.03 ± 0.03	0.37 ± 0.23	NS
30mM-Mannitol (5)	0.82 ± 0.11	0.95 ± 0.13	0.15 ± 0.03	0.23 ± 0.07	NS
50mM-Mannitol (6)	1.03 ± 0.05	0.67 ± 0.11	0.07 ± 0.04	0.69 ± 0.09	<0.01/ = 0.05
70mM-Mannitol (5)	0.76 ± 0.06	0.81 ± 0.19	0.13 ± 0.04	0.52 ± 0.17	NS
50mM-Urea (6)	1.03 ± 0.05	0.75 ± 0.04	0.07 ± 0.04	0.13 ± 0.02	NS
10mM-Histidine (8)	0.82 ± 0.11	0.66 ± 0.07	0.12 ± 0.03	0.17 ± 0.07	NS
10mM-Caffeine (6)	0.95 ± 0.11*	0.82 ± 0.05*	0.14 ± 0.04	0.72 ± 0.13	<0.01/<0.05
10mM-Theophylline (6)	0.95 ± 0.11*	0.97 ± 0.06	0.14 ± 0.04	0.72 ± 0.16	<0.02/<0.05
1mM-NAD^+ (12)	0.89 ± 0.09	0.98 ± 0.10	0.04 ± 0.02	0.18 ± 0.04	<0.01
1mM-NADH (11)	0.95 ± 0.07	0.87 ± 0.11	0.03 ± 0.02	0.29 ± 0.10	<0.02/<0.01
1mM-$NADP^+$ (12)	1.01 ± 0.07	0.94 ± 0.11	0.09 ± 0.02	0.33 ± 0.13	NS
1mM-NADPH (12)	0.94 ± 0.09	0.88 ± 0.09	0.11 ± 0.03	0.39 ± 0.11	<0.05/<0.05

* Owing to the accidental loss of one observation, this mean ± S.E.M. is for five observations.

group of compounds, none of which have previously been demonstrated to have any specific effect on islet cells, a reasonable correlation appeared to exist between the degree of protection against alloxan and the expected reactivity with OH· radicals (Fig. 4).

The effect of alloxan on Trypan Blue exclusion was also counteracted by 50mM-sodium benzoate, 28mM-dimethyl sulphoxide and 50mM-mannitol, whereas 10mM-histidine or 22mM-butanol had no significant effect (Table 2). The failure of butanol to protect against alloxan in this system may be apparent only, because butanol in itself decreased the frequency of unstained cells ($P = 0.05$; Wilcoxon's rank-sum test, two-tailed).

Protection by compounds known to influence the function of β-cells and that may or may not act as radical scavengers

Rb^+ accumulation was significantly protected against alloxan by 10mM-caffeine, 10mM-theophylline, 1mM-NADH, 1mM-NADPH, or, to a lesser extent, 1mM-NAD^+ (Table 1). These compounds were tested because reduced nicotinamide nucleotides, caffeine and theophylline reportedly can both influence insulin release and protect against alloxan in other systems of endocrine pancreas (Cooperstein & Watkins, 1977; Lacy *et al.*, 1975). In comparison with the above OH· scavengers, the methylxanthines and

the reduced nicotinamide nucleotides appeared to afford greater protection than would be predicted solely by the likely rate constants for their $OH^{\bullet}$-trapping activity (Fig. 4).

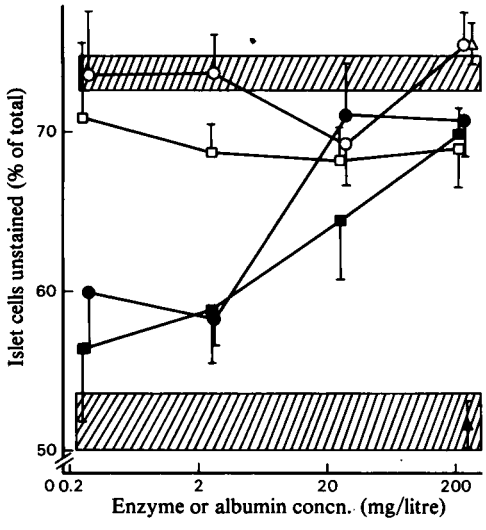

Fig. 3. *Effects of various concentrations of catalase* (○, ●) *and superoxide dismutase* (□, ■) *on the frequency of islet cells excluding Trypan Blue after treatment with no alloxan* (○, □) *or 0.5 mM-alloxan* (●, ■)

Dispersed islet cells were incubated for 30 min with or without enzyme. During the final 15 min 0.5 mM-alloxan or HCl alone was also added to the medium. In addition to the enzymes, one concentration of human serum albumin was similarly tested (no alloxan, △; 0.5 mM-alloxan, ▲). Each point indicates the mean value for six to ten different experiments. Vertical bars denote S.E.M. Hatched areas indicate the means ± S.E.M. for exclusion of Trypan Blue in the absence of both alloxan and test enzyme (upper area, $n = 34$) or after exposure to 0.5 mM-alloxan alone (lower area, $n = 34$).

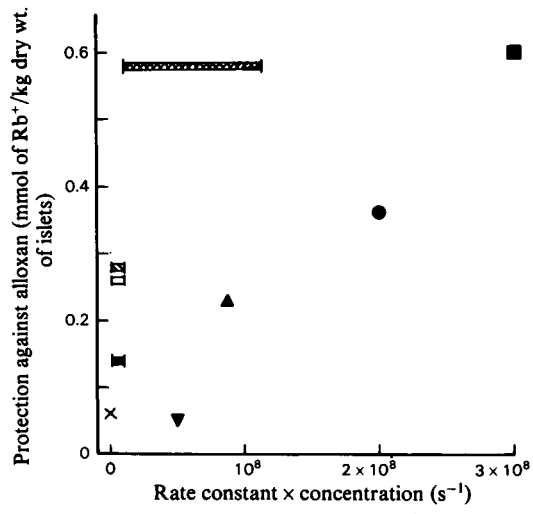

Fig. 4. *Relationship between the protection of Rb^+-accumulating capacity of islets and the theoretical $OH^{\bullet}$-scavenging activity of various test substances*

Rate constants ($M^{-1} \cdot s^{-1}$) for the reaction of test substances with the hydroxyl radical were taken from Dorfman & Adams (1973) and were: histidine (▼), 5.0×10^9; butanol (▲), 4.0×10^9; dimethyl sulphoxide (●), 7.1×10^9; benzoate (■), 6.0×10^9. The rate constant for urea (X) was taken as the upper limit given by Anbar & Neta (1967), a value of 7×10^5. For caffeine and theophylline (cross-hatched bar), NADPH (hatched bar), NADH (open bar), or NAD^+ (solid bar), exact rate constants were not found. Considering that constants in the range 10^9–$10^{10} M^{-1} \cdot s^{-1}$ are given for various adenine and guanine derivatives (Anbar & Neta, 1967; Dorfman & Adams, 1973), this range has been used to approximate the rate constants for methylxanthines and nicotinamide nucleotides. For each test compound the rate constant was multiplied with the concentration used in the present study and plotted along the abscissa. The ordinate shows the increase of Rb^+ accumulation in alloxan-treated islets as determined from Table 1. Mannitol is not included in the diagram because its rate constant was not found.

Table 2. *Effects of alloxan and scavengers of hydroxyl radicals on the frequency of islet cells excluding Trypan Blue*

Suspensions of dispersed islet cells were incubated for 30 min with (test) or without (control) test compounds as indicated. During the final 15 min 0.5 mM-alloxan or HCl alone was also added to the medium. The frequency of cells excluding Trypan Blue during subsequent exposure to the dye is given as mean value ± S.E.M. for the number of experiments stated in parentheses. Statistical testing was performed as in Table 1.

	Percentage of islet cells unstained				
	No alloxan		0.5 mM-Alloxan		*P* value for protection
Test substance	Control	Test	Control	Test	against alloxan
22 mM-Butanol (6)	72.0 ± 1.9	63.2 ± 3.2	52.4 ± 3.8	53.9 ± 2.9	NS
50 mM-Sodium benzoate (6)	72.1 ± 2.4	68.0 ± 2.5	50.1 ± 2.5	61.9 ± 2.6	<0.01/<0.05
28 mM-Dimethyl sulphoxide (8)	70.8 ± 1.8	73.5 ± 1.8	50.8 ± 1.6	58.3 ± 2.7	<0.05
50 mM-Mannitol (5)	73.9 ± 2.4	67.1 ± 3.3	47.8 ± 3.6	63.2 ± 6.6	<0.05/<0.1
10 mM-Histidine (6)	71.7 ± 4.0	75.4 ± 1.2	56.2 ± 5.3	58.4 ± 3.2	NS

Control studies of the reaction between alloxan and test substances in the absence of islet cells

Mixing alloxan with equimolar (or more) butanol, sodium benzoate, mannitol, dimethyl sulphoxide, caffeine, or theophylline did not noticeably influence the rate of alloxan disappearance (A_{290} or A_{303}) in cell-free basal medium. However, by measuring A_{340} it was evident that both 0.08mM-NADH and 0.08mM-NADPH were rapidly oxidized by 10mM-alloxan in the absence of islet cells (Fig. 5).

Discussion

The present results support the free-radical hypothesis of alloxan action. All but one of the non-enzyme OH· scavengers protected against alloxan in both of our cytotoxicity assays; butanol failed to do so in the Trypan Blue-exclusion test, but counteracted the inhibition of Rb^+ accumulation. Moreover, clear-cut evidence was obtained for protection by superoxide dismutase and catalase.

Apart from the fact that $O_2^{\cdot -}$ and H_2O_2 may be harmful in themselves to the β-cells, these reactants are postulated intermediates in the alloxan-induced generation of OH· radicals (Heikkila *et al.*, 1976; Heikkila, 1977). If after formation of $O_2^{\cdot -}$ in the alloxan–dialuric acid redox cycle, OH· radical is generated by a metal-catalysed reaction between $O_2^{\cdot -}$ and H_2O_2 (Van Hemmen & Meuling, 1977; Halliwell, 1978; McCord & Day, 1978), the enzyme-catalysed removal of $O_2^{\cdot -}$ (superoxide dismutase) or H_2O_2 (catalase) or both should protect against alloxan as effectively as the direct trapping of OH· radicals. This prediction is verified in the present paper, but may be difficult to test *in vivo* or with the perfused pancreas *in vitro*, because of the diffusion barriers to big molecules. Here the enzymes were much more potent when tested on dispersed islet cells compared with whole islets. The likelihood that both H_2O_2 and $O_2^{\cdot -}$ are required to produce the OH· radical is supported by the fact that the combination of catalase and superoxide dismutase did not seem to be more effective than either enzyme alone.

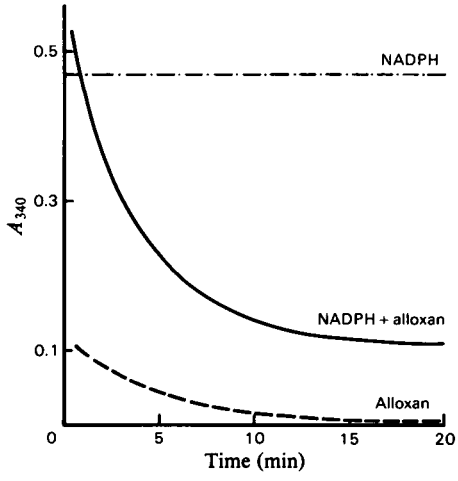

Fig. 5. *Oxidation of NADPH by alloxan in cell-free solution*

The curves show the change of A_{340} with time after adding 10mM-alloxan alone, 0.08mM-NADPH alone, or alloxan in combination with NADPH to Hepes-buffered Krebs–Ringer medium, pH7.4, at room temperature. Similar results were obtained with NADH.

Hydroxyl radicals can induce lipid peroxidation in membranes as well as attack DNA, RNA, proteins and other cell components (Anbar & Neta, 1967; Dorfman & Adams, 1973). This indiscriminatory reactivity can explain why alloxan rapidly affects as wide a range of phenomena in the β-cells as insulin synthesis (Gunnarsson, 1975), insulin release (Lacy *et al.*, 1975), univalent-cation pumping (Idahl *et al.*, 1977), glucose oxidation and O_2 consumption (Hellerström & Gunnarsson, 1970), and Trypan Blue exclusion (Grankvist *et al.*, 1977). Confronted with this assortment of effects one is tempted to try and identify the most basic damage from which the others would follow. Evidently, however, the most basic reaction of alloxan need not in itself represent an injury to the β-cells. In view of the present and previous (Heikkila *et al.*, 1976; Heikkila, 1977) results, the common denominator of all the alloxan-induced changes may simply be the initial reduction of alloxan to dialuric acid and its $O_2^{\cdot -}$-yielding autoxidation.

The great sensitivity of β-cells to alloxan may thus be due to their physiological specialization in a direction that also facilitates reduction of the drug, and perhaps of other substrates for analogous $O_2^{\cdot -}$-yielding redox cycles. Several authors have proposed that nicotinamide nucleotide-linked redox processes are important mediators of glucose-induced insulin release. The possible location of such mechanisms within the β-cell plasma membrane has been discussed in view of the insulin-releasing action of exogenous NADH, NADPH (Watkins *et al.*, 1968, 1971; Ammon *et al.*, 1973), thiol-blocking reagents (Hellman *et al.*, 1973*a*, 1974), or thiol compounds (Hellman *et al.*, 1975). Detailed speculation along this line is not appropriate here. However, it must be stressed that the β-cells are not particularly poor in reduced thiol groups, as was once hypothesized (Lazarow, 1949). It is, rather, a relative abundance of reducing groups in these cells, perhaps reflected in the relatively high concentration of reduced glutathione (Havu, 1969; Falkmer, 1970) and high activity of glutathione reductase (Berne, 1975) in endocrine pancreas, that would seem helpful in explaining their vulnerability to alloxan.

If the β-cells are sensitive to alloxan because they easily reduce the drug, protection by D-glucose may seem paradoxical. D-Glucose at high concentration renders the β-cells even more reduced, as indicated by measurements of nicotinamide nucleotides (Panten & Christians, 1973; Ammon, 1975; Malaisse *et al.*, 1978). However, protection is also afforded by 3-*O*-methyl-D-glucose (Tomita *et al.*, 1974; Rossini *et al.*, 1975; Idahl *et al.*, 1977; Pagliara *et al.*, 1977), which is not oxidized by the β-cells (Hellman *et al.*, 1973*b*). The protective action of sugars, perhaps related to their ability to traverse or otherwise interact with the β-cell plasma membrane (Cooperstein & Lazarow, 1969; Scheynius & Täljedal, 1971; Idahl *et al.*, 1977; Weaver *et al.*, 1978), or to their direct $OH^{\bullet}$-trapping capacity (Anbar & Neta, 1967; Dorfman & Adams, 1973) or both, apparently can outweigh any negative effect of β-cell reduction due to D-glucose.

That alloxan can interfere with nicotinamide nucleotide-linked processes is shown here by the rapid oxidation of NADH and NADPH in solution. Against this background the protection against alloxan by NADH or NADPH does not necessarily involve an interaction between nucleotide and islet cells. Because protection by nicotinamide nucleotides in our hands was not entirely specific for the reduced forms (cf. Cooperstein & Watkins, 1977), attention is also drawn to the capacity of adenine nucleotides to function as $OH^{\bullet}$ scavengers (Anbar & Neta, 1967; Dorfman & Adams, 1973).

Lacy *et al.* (1975) reported that caffeine and theophylline protect against alloxan in the rat pancreas and that the effect was independent of cyclic nucleotides. Our results confirm the protective capacity of these methylxanthines. Further studies are required to decide whether they owe their comparatively great efficiency to a combination of $OH^{\bullet}$-trapping ability and rapid uptake by the β-cells (cf. McDaniel *et al.*, 1977).

Nothing is known about the activity in β-cells of such enzymes, which may determine the liability of cells to engage in toxic autoxidation processes, e.g. catalase and superoxide dismutase. Such measurements would seem highly desirable in view of the results reported in the present paper. Any marked deviation from the typical would provide an alternative basis for explaining the great vulnerability of β-cells to alloxan.

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