Alloxan Cytotoxicity *in vitro*

INHIBITION OF RUBIDIUM ION PUMPING IN PANCREATIC β -CELLS

By LARS-ÅKE IDAHL, ÅKE LERNMARK, J. SEHLIN and INGE-BERT TÄLJEDAL

Department of Histology, University of Umeå, S-901 87 Umeå 6, Sweden

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Exposing micro-dissected pancreatic islets of non-inbred *ob/ob* mice to 2–5 mM-alloxan for 10 min decreased the ability of the islets to accumulate Rb^+ . Rb^+ accumulation in pieces of exocrine pancreas was unaffected by alloxan. When islets were treated with alloxan in the presence of 2–20 mM-D-glucose, the Rb^+ -accumulating ability was protected in a dose-dependent manner. The protective action of D-glucose was reproduced with 3-O-methyl-D-glucose but not with L-glucose or D-mannoheptulose; mannoheptulose prevented D-glucose from exerting its protective action. The inhibition of Rb^+ accumulation was due to a decreased inward pumping, since alloxan did not affect Rb^+ efflux from pre-loaded islets. The inhibitory effect of alloxan had a latency of about 1 min, as revealed by experiments with dispersed islet cells in suspension. Alloxan-treated islets showed only a marginal decrease in ATP and no change in glucose 6-phosphate concentration. Although alloxan slightly decreased the hydrolysis of ATP in a subcellular fraction enriched in plasma membranes, this effect could not be attributed to a ouabain-sensitive adenosine triphosphatase. The plasma membranes exhibited a K^+ -activated hydrolysis of *p*-nitrophenyl phosphate; this enzyme activity too was insensitive to alloxan. Glucose may protect the univalent-cation pump by preventing permeation of alloxan via a path coupled to the hexose-transport system. Inhibition of the pump may be fundamental to the induction of alloxan-diabetes.

Injection of alloxan kills the pancreatic β -cells in most mammals, resulting in insulin deficiency and diabetes (Rerup, 1970). The toxic action of alloxan is relatively specific for the β -cells. It can be prevented by prior injection of glucose (Bhattacharya, 1954; Scheynius & Täljedal, 1970), the major physiological stimulus of insulin secretion. Therefore alloxan is not only a useful tool for inducing experimental diabetes, but the drug may also be valuable as a probe of those cytophysiological characteristics that enable β -cells to function as glucostats.

Studying the interactions between alloxan, glucose and mannoheptulose *in vivo*, Scheynius & Täljedal (1970) suggested that glucose protects against alloxan by inducing a conformational change of an alloxan-reactive site in the β -cells, perhaps associated with the transmembrane hexose-transport system. To explore the mechanism of action of alloxan in more detail, sensitive and reliable methods are required for assaying the acute effects of alloxan on mammalian islets *in vitro*. Electrophysiological measurements of the β -cell membrane potential (Dean & Matthews, 1972) and immunoassay of insulin release from perfused islets (McDaniel *et al.*, 1975) have been used. We decided to try to exploit the finding that intact β -cells are

capable of concentrating Rb^+ severalfold by means of a univalent-cation pump (Sehlin & Täljedal, 1974a; Lernmark *et al.*, 1975). The measurements of Rb^+ pumping have a high degree of reliability and uniformity and, at least in our hands, are less laborious than those of membrane electric potentials or insulin release.

We report here that alloxan inhibits the Rb^+ pump in *ob/ob*-mouse islets. The effect is characterized with respect to dose-dependence, specificity for the endocrine pancreas, protection by sugars and latency. ATP, glucose 6-phosphate and membrane-associated phosphatase activities were also measured to see whether changes of these factors could explain the alloxan-induced inhibition of Rb^+ pumping.

Materials and Methods

Animals and isolation of islets

Adult non-inbred *ob/ob* mice were taken from the Umeå colony and starved overnight. These animals have islets with more than 90% β -cells, and the β -cells have been shown to be vulnerable to alloxan *in vivo* (Nordenström *et al.*, 1973). In most

experiments islets were isolated by free-hand micro-dissection without the use of collagenase. However, collagenase digestion of the pancreas (Lernmark, 1974) was used to obtain the large numbers of islets required for preparing suspensions of dispersed islet cells and subcellular fractions enriched in plasma membranes. For micro-dissection, the pancreas was suspended in a salt-balanced buffer (pH 7.4) of the same composition as Krebs-Ringer bicarbonate buffer (DeLuca & Cohen, 1964), except that the bicarbonate was replaced by 20 mM-Hepes [2-(*N*-2-hydroxyethyl)piperazin-*N'*-yl]ethanesulphonic acid]. Digestion with collagenase was carried out in Hanks buffer (Paul, 1973), supplemented with 5 mM-D-glucose. Both media were equilibrated with ambient air.

Measurement of Rb⁺ fluxes

All incubations were carried out at 37°C. The basal incubation medium was the same as that used in micro-dissection of islets. To study the effect of alloxan on Rb⁺ accumulation, micro-dissected islets were incubated for 40 min in 1 ml of basal medium supplemented with D-glucose as required. After the first 30 min we added 10 µl of a freshly prepared stock solution of alloxan in 0.01 M-HCl and continued the incubation for 10 min; control media were supplemented with 10 µl of 0.01 M-HCl without alloxan. It was checked that the addition of HCl did not alter the pH of incubation media (pH 7.4). The islets were then incubated for 7 or 120 min in alloxan-free basal medium that had been labelled with 28 µM-⁸⁶RbCl (378–713 Ci/mol) and 8 µM-[6,6'-³H₂]sucrose (2000 Ci/mol). After incubation was completed, the islets were placed on 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, 0.1 Pa), weighed on a quartz-fibre balance, dissolved in Instafluor, and analysed for ⁸⁶Rb and ³H in a liquid-scintillation counter. Sucrose was used as an extracellular-space marker (Sehlin & Täljedal, 1974a); the islet content of Rb⁺ in excess of this marker was calculated by using samples of incubation medium as external references in the liquid-scintillation-counting procedure.

To study the effect of alloxan on Rb⁺ efflux, micro-dissected islets were loaded with the radioisotope by incubation for 120 min in basal medium labelled with 28 µM-⁸⁶RbCl (713 Ci/mol) and 8 µM-[6,6'-³H₂]sucrose (2000 Ci/mol). They were then briefly rinsed (approx. 2 s) in non-radioactive medium and incubated for various periods of time in basal medium with or without alloxan; alloxan was added from a fresh stock, and control media were prepared as in the accumulation experiments. After incubation,

the islets were processed as in the accumulation experiments described above.

The latency of onset of any effect of alloxan on Rb⁺ accumulation was studied with the aid of dispersed islet cells in suspension. The methods for preparing cell suspensions by shaking collagenase-isolated islets in Ca²⁺-free buffer, and for measuring Rb⁺ accumulation in dispersed cells, have been described in detail previously (Lernmark, 1974; Lernmark *et al.*, 1975). In the present study, the dispersed cells were incubated in tissue-culture medium 199 (Salk *et al.*, 1954) supplemented with 20 mM-Hepes for 30 min, after which period they were collected by low-speed centrifugation (2 min, 50g). Cells resuspended in tissue-culture medium were distributed to tubes containing or lacking 2 mM-alloxan in a total incubation volume of 105 µl. After 10, 30 or 60 s, or 10 min, 2 ml of alloxan-free tissue-culture medium was added. The cells were collected by low-speed centrifugation, resuspended, incubated with ⁸⁶RbCl for 120 min, and analysed for ⁸⁶Rb as described previously (Lernmark *et al.*, 1975).

Phosphatase measurements

Phosphohydrolase activities against ATP and *p*-nitrophenyl phosphate were measured in crude homogenates of islets as well as in various subcellular fractions obtained by centrifuging the homogenate through sucrose gradients. The protocols for homogenization by shaking islets in hypo-osmotic borate buffer and for density-gradient fractionation have been described in detail in a previous study on the preparation of rat islet-cell plasma membranes (Lernmark *et al.*, 1976). Adenosine triphosphatase activities against 0.1 mM- γ -³²P]ATP at 37°C in 25 mM-histidine buffer, adjusted to pH 7.4 with HCl, were also assayed as described previously (Lernmark *et al.*, 1976). Hydrolysis of *p*-nitrophenyl phosphate was spectrophotometrically measured as *A*₄₁₀ after incubating plasma membranes (1–4 µg of protein) at 30°C with 3.9 mM substrate in 30 mM-Tris/HCl buffer, pH 7.8, and stopping the reaction with NaOH. The presence of cations, ouabain and alloxan is described in the legends to Figures and Tables. A subcellular fraction enriched in plasma membranes was identified by the binding and distribution of ¹²⁵I-labelled wheat-germ agglutinin on the sucrose gradients (Lernmark *et al.*, 1976). Protein was assayed by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Measurements of ATP and glucose 6-phosphate

ATP was measured with firefly luciferin-luciferase (Ashcroft *et al.*, 1973) in islet extracts prepared as previously described (Hellman *et al.*, 1969). Glucose 6-phosphate was measured by enzymic cycling as described by Idahl (1971) and Matschinsky (1971).

Chemicals

Alloxan monohydrate was from Nutritional Biochemical Corp., Cleveland, OH, U.S.A.; tissue-culture medium 199 with or without calcium from the National Bacteriological Laboratories, Stockholm, Sweden; $^{86}\text{RbCl}$, $[6,6\text{'-}^3\text{H}_2]\text{sucrose}$ and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (sodium salt) were from The Radiochemical Centre, Amersham, Bucks., U.K.; ATP (tris salt), *p*-nitrophenyl phosphate (sodium salt), 3-*O*-methyl-D-glucose, L-glucose, D-mannoheptulose, ouabain, bovine serum albumin (fraction V) and firefly lantern extracts were from Sigma Chemical Co., St. Louis, MO, U.S.A.; D-glucose was from British Drug Houses, Poole, Dorset, U.K.; collagenase was from Worthington Biochemical Corp., Freehold, NJ, U.S.A.; ultrapure sucrose (special enzyme grade) was from Schwartz/Mann, Orangeburg, NY, U.S.A.; and Instafluor was from Packard Instruments, Warrenville, IL, U.S.A. Other reagents were of analytical grade.

Results

Effects of various concentrations of alloxan on Rb^+ accumulation

Fig. 1 shows the accumulation of Rb^+ in islets pretreated for 10 min with various concentrations of

Fig. 1. Accumulation of Rb^+ by islets treated with various concentrations of alloxan in the presence of 2 mM (solid bars) or 20 mM (open bars) D-glucose

The indicated glucose concentrations were present during 40 min of the initial incubation and alloxan was present during the final 10 min of the same period. All islets were then incubated with 2 mM-D-glucose and $28\text{ }\mu\text{M-RbCl}$ in alloxan-free basal medium for 2 h. Mean values and s.e.m. are given for the following numbers of experiments (concn. of alloxan in parentheses): 22 (0 mM), 3 (0.5 mM), 4 (1, 3 and 5 mM) and 7 (2 mM). Controls not exposed to alloxan were run in parallel with each concentration of alloxan; they did not vary significantly from experiment to experiment and have been brought together in one bar. At 2 mM-alloxan, the values for 2 and 20 mM-glucose differ, with $0.05 < P < 0.1$ (*t* test on paired data).

Fig. 2. Protection against alloxan by various concentrations of D-glucose

Islets were incubated for 40 min with D-glucose as indicated. During the last 10 min of this period, 2 mM-alloxan was also present. All islets were then incubated for 2 h in basal medium lacking glucose or alloxan but containing $28\text{ }\mu\text{M-RbCl}$. The accumulation of Rb^+ is given as mean values $\pm$ s.e.m. for the following numbers of experiments (concn. of glucose in parentheses): 19 (0 mM), 14 (2 and 20 mM), 8 (5 and 10 mM) and 7 (15 mM). Controls not exposed to alloxan or glucose were run in parallel with each concentration of glucose. They did not differ significantly between experiments and on average accumulated 0.82 ± 0.08 mmol of Rb^+ /kg dry wt. of islets (mean $\pm$ s.e.m. of 19 experiments).

alloxan; 0.5–1 mM-alloxan had negligible effects, but 2–5 mM-alloxan caused a dose-dependent inhibition of the Rb^+ accumulation. The drastic effect of 5 mM-alloxan remained about the same whether the drug was added to a medium containing 20 or 2 mM-D-glucose. However, the effect of 2–3 mM-alloxan appeared to be decreased by the high glucose concentration.

Effects of various concentrations of D-glucose on the islet sensitivity to 2 mM-alloxan

The interaction between alloxan and D-glucose was further investigated by pretreating the islets with 2 mM-alloxan in the presence of various concentrations of glucose. D-Glucose protected against alloxan in a dose-dependent manner up to 20 mM (Fig. 2). This high glucose concentration fully prevented the inhibitory influence of 2 mM-alloxan on Rb^+ accumulation.

Interactions between alloxan and various sugars

The specificity of the protective effect of D-glucose was studied by treating islets with 2 mM-alloxan in

the presence of various sugars (Table 1). In the presence of 20mM-L-glucose the Rb^+ accumulation was as low as when alloxan had been added to a glucose-free medium; it was significantly lower than in islets not exposed to alloxan ($P < 0.001$). After combined treatment with alloxan and 20mM-3-O-methyl-D-glucose, the Rb^+ accumulation did not differ significantly from that in untreated control islets. That 3-O-methyl-D-glucose protected against alloxan is also indicated by the significant difference from islets exposed to alloxan alone ($P < 0.01$).

Mannoheptulose (20mM) did not in itself interfere with the effect of alloxan on Rb^+ accumulation. However, combined treatment with 20mM-mannoheptulose and 20mM-D-glucose counteracted the protective action of D-glucose alone ($P < 0.025$); after treatment with alloxan in the presence of mannoheptulose plus D-glucose, the accumulation of Rb^+ was not significantly different from that in islets exposed to alloxan in the absence of any sugar.

Effect of alloxan on Rb^+ uptake by exocrine pancreas

In vivo, the islet β -cells are especially vulnerable to alloxan, although other cells and organs may be damaged by injecting the drug in larger dosages than those required to produce diabetes (Rerup, 1970). To test whether the effect of alloxan *in vitro* on Rb^+ accumulation exhibits a similar specificity for the β -cells, islets were incubated in parallel with pieces of exocrine pancreas. Table 2 shows that 2mM-alloxan did not inhibit the accumulation of Rb^+ in exocrine pancreas, when under identical conditions the Rb^+ accumulation was markedly suppressed in the islets ($P < 0.02$). The exocrine pancreas showed the same concentrative uptake of Rb^+ as control islets not exposed to alloxan.

Table 1. *Interaction between alloxan and various sugars*

Islets were incubated for 40 min in the presence of one of the sugars listed. During the last 10 min of this period, test media also contained 2mM-alloxan. The islets were then incubated for 2 h in basal medium lacking sugar and alloxan but containing $28 \mu\text{M}$ -RbCl. The accumulation of Rb^+ is given as mean values $\pm$ S.E.M. for the numbers of experiments stated in parentheses. The outcome of statistical *t* testing is presented in the text.

Concn. of alloxan (mM)	Sugar tested	Accumulation of Rb^+ (mmol/kg dry wt. of islets)
0	None	1.07 ± 0.07 (19)
2	None	0.44 ± 0.06 (19)
2	20mM-D-Glucose	0.91 ± 0.11 (15)
2	20mM-3-O-Methyl-D-glucose	0.82 ± 0.11 (10)
2	20mM-L-Glucose	0.42 ± 0.09 (10)
2	20mM-D-Mannoheptulose	0.40 ± 0.10 (8)
2	20mM-D-Glucose + 20mM-D-mannoheptulose	0.51 ± 0.11 (8)

Table 2. *Comparisons of islets and exocrine pancreas, of fresh and inactivated alloxan, and of alloxan effects in the presence or absence of albumin*

In experimental series I and II, islets or pieces of exocrine pancreas were incubated for 40 min in basal medium containing 2mM-D-glucose. During the last 10 min of this period, 2mM-alloxan was added to some groups. Other groups were only treated with a corresponding volume of the HCl used for dissolving alloxan (no alloxan) or of a 2mM-alloxan solution that had been kept at pH 10 overnight and then re-adjusted with HCl (inactivated alloxan). In all cases the incubation was continued in basal medium lacking alloxan but containing 2mM-D-glucose and $28 \mu\text{M}$ -RbCl. In experimental series III, islets were incubated as above, except that some of the media contained bovine serum albumin (10mg/ml) throughout. The accumulation of Rb^+ is given as mean values $\pm$ S.E.M. for the numbers of experiments stated in parentheses. The outcome of statistical *t* testing is presented in the text.

Tissue	Alloxan preparation	Accumulation of Rb^+ (mmol/kg dry wt. of islets)
Experimental series I		
Islets	None	1.43 ± 0.11 (5)
	Fresh	0.68 ± 0.11 (5)
Exocrine pancreas	None	1.33 ± 0.11 (5)
	Fresh	1.27 ± 0.07 (5)
Experimental series II		
Islets	None	1.45 ± 0.13 (8)
	Fresh	0.70 ± 0.12 (8)
	Inactivated	1.40 ± 0.17 (8)
Experimental series III		
Islets	None	1.60 ± 0.18 (4)
	(no albumin)	
	None	1.64 ± 0.20 (4)
	(albumin present)	
	Fresh	0.74 ± 0.19 (4)
	(no albumin)	
	Fresh	0.43 ± 0.05 (4)
	(albumin present)	

Effect of alkali-treated alloxan on Rb^+ accumulation in islets

At 37°C and pH 7.4, alloxan is unstable and rapidly converted into non-diabetogenic compounds (Rerup, 1970). To test whether the inhibition of Rb^+ accumulation is due to alloxan or a conversion product, islets were treated with alloxan solutions that had been kept at pH 10 at room temperature (about 22°C) overnight. Alloxan treated thus did not affect the islet accumulation of Rb^+ (Table 2); fresh alloxan was markedly inhibitory in the same experiments ($P < 0.001$) and differed significantly from the inactivated alloxan ($P < 0.01$).

Effect of alloxan on Rb^+ accumulation in the presence of albumin

Certain parameters of islet function, e.g. insulin release and intermediary-metabolite concentrations, for methodological reasons are best studied in albumin-containing media. To allow the effects on Rb^+ accumulation to be compared with effects on islet metabolites, it was checked that bovine serum albumin did not interfere with the inhibitory action of alloxan on Rb^+ uptake (Table 2). Inhibition by alloxan was significant in both the absence ($P < 0.02$) and the presence ($P < 0.02$) of albumin.

Effect of alloxan on Rb^+ efflux from pre-loaded islets

The above experiments have described the islet accumulation of $^{86}Rb^+$ during 2 h, an incubation period sufficiently long for the radioisotope to reach equilibrium (Sehlin & Täljedal, 1974a). The alloxan-induced decrease of accumulation could therefore

Fig. 3. Efflux of Rb^+ from islets in the absence (○) or presence (●) of 2mM-alloxan

After loading with $^{86}Rb^+$ during 2 h of incubation in basal medium containing $28 \mu M$ -RbCl, the islets were briefly rinsed (approx. 2 s) in non-radioactive basal medium and further incubated for the indicated periods of time in basal medium containing or lacking alloxan. Amounts of Rb^+ retained by the islets are given as the mean values of four different experiments, except for the zero-time point, which is based on eight experiments. Bars denote $\pm$ S.E.M. The inset is a semi-logarithmic plot of the same data.

be due to inhibition of the inward active transport or to an increased islet-cell permeability for Rb^+ ; conceivably, both of these changes may have occurred. To decide between the different mechanisms, islets were preloaded with $^{86}Rb^+$ for 2 h; then the unidirectional efflux into a non-radioactive medium was studied. As previously described (Sehlin & Täljedal, 1974a), the Rb^+ efflux approximated to first-order kinetics (Fig. 3). Since the efflux curve was the same whether or not 2mM-alloxan was included in the non-radioactive medium, alloxan did not demonstrably increase the β -cell permeability to Rb^+ .

Effect of alloxan on the rate of Rb^+ uptake

From the inhibition of Rb^+ accumulation and the lack of effect of alloxan on Rb^+ efflux, it is inferred that alloxan inhibits the active inward transport of Rb^+ . To test this conclusion further, eight experiments were performed in which the islets were incubated with $^{86}Rb^+$ for only 7 min. Such a brief incubation is insufficient to bring the isotope to equilibrium, and the Rb^+ taken up may predominantly reflect the inward transport rate (Sehlin & Täljedal, 1974a). Pretreatment of the islets with 2mM-alloxan for 10 min before incubation with $28 \mu M$ - $^{86}RbCl$ resulted in an uptake of 0.33 ± 0.02 mmol of Rb^+ /kg dry wt. of islets in 7 min (mean $\pm$ S.E.M.). This value is significantly lower ($P < 0.01$) than 0.50 ± 0.04 mmol/kg, the uptake observed in control islets incubated without alloxan.

Latency of the alloxan-induced inhibition of Rb^+ accumulation

To elucidate how long the β -cells must be exposed to alloxan for a demonstrable inhibition of Rb^+ accumulation to occur, we performed experiments with islet cells in suspension. Intact islets were considered unsuitable for these experiments, because diffusion in their extracellular space makes it difficult to control the concentration of the drug within seconds. The islet-cell suspensions are virtually free of a tissue extracellular space, and the cells have previously been shown to accumulate Rb^+ about as vigorously as do intact islets (Lernmark *et al.*, 1975). After incubation of cell suspensions with 2mM-alloxan for 10, 30 or 60 s or 10 min, excess of buffer was added to lower the effective alloxan concentration below 0.1 mM; this low concentration does not inhibit the Rb^+ accumulation (Fig. 1). Table 3 shows that the ability of the islet cells to accumulate Rb^+ decreased after 1 min of exposure to 2mM-alloxan. Shorter treatment had no demonstrable effect.

Effects of alloxan on the islet content of ATP and glucose 6-phosphate

In view of possible metabolic requirements in Rb^+ pumping, the effects of alloxan on islet ATP

Table 3. Latency of the effect of alloxan on Rb⁺ accumulation in islet cells

Suspensions of dispersed islet cells were incubated with 2 mM-alloxan, or a corresponding volume of HCl (control), in tissue-culture medium. After the brief periods of time indicated, the alloxan concentration was decreased below 0.1 mM by addition of more tissue-culture medium. The cells were then collected and incubated with 28 μ M-RbCl for 2 h. Their uptake of Rb⁺ is expressed in relation to the number of islets used for preparing the cell suspensions (means $\pm$ S.E.M.). Since test and control incubations were always run with each individual suspension, the values for the alloxan-treated groups are also given as a percentage of the corresponding control (means $\pm$ S.E.M.). The effect of alloxan is significant, with $P < 0.05$, after 60 s. In two other experiments it was checked that treating the cells with alloxan for as long as 10 min decreased the Rb⁺ accumulation to 41 and 19% of the control value (compare with results for intact islets in Tables 1 and 2).

Length of treatment (s)	Accumulation of Rb ⁺ (pmol/islet)		Accumulation by alloxan-treated cells (% of parallel control)
	Control	Alloxan	
10	1.53 $\pm$ 0.48 (3)	1.50 $\pm$ 0.41 (3)	101 $\pm$ 9 (3)
30	1.57 $\pm$ 0.49 (5)	1.36 $\pm$ 0.41 (5)	88 $\pm$ 11 (5)
60	1.61 $\pm$ 0.18 (5)	1.26 $\pm$ 0.25 (5)	76 $\pm$ 9 (5)

Table 4. Effect of alloxan on ATP and glucose 6-phosphate in islets

Islets were incubated in basal medium containing 2 mM-D-glucose and 1 mg of bovine serum albumin/ml. After 30 min, 2 mM-alloxan was added and the incubation continued for the periods of time indicated. The islets were then freeze-dried, weighed, extracted and assayed for metabolites. Results are mean values $\pm$ S.E.M. for the numbers of experiments stated in parentheses.

Time after exposure to alloxan (min)	ATP (mmol/kg dry wt.)	Glucose 6-phosphate (μ mol/kg dry wt.)
0	4.58 $\pm$ 0.54 (8)	126 $\pm$ 17 (7)
1	4.53 $\pm$ 0.45 (6)	108 $\pm$ 21 (7)
5	3.37 $\pm$ 0.46 (8)	118 $\pm$ 27 (8)
10	3.98 $\pm$ 0.46 (8)	111 $\pm$ 24 (8)
20	3.84 $\pm$ 0.63 (7)	126 $\pm$ 27 (8)

and glucose 6-phosphate were studied. Table 4 shows that treatment with 2 mM-alloxan for 10 min had a marginal effect on ATP concentration during subsequent observation periods of 1–20 min; the mean values were slightly lower after alloxan treatment, but at no time-point was the decrease statistically significant. Glucose 6-phosphate concentration remained unchanged after alloxan.

Effects of alloxan on membrane-located phosphatase activities

Hydrolysis of ATP and K⁺-activated hydrolysis of *p*-nitrophenyl phosphate represent enzyme activities that have been implicated with univalent-cation pumping in other cells (Bonting, 1970; Woodin & Wieneke, 1970). Attempts to demonstrate a ouabain-inhibitable adenosine triphosphatase in whole homogenates of *ob/ob*-mouse islets have failed (I.-B. Täljedal, unpublished work). Table 5 shows rates of ATP hydrolysis in a plasma-membrane fraction isolated by centrifuging homogenates of *ob/ob*-mouse islets through sucrose gradients as described by Lernmark *et al.* (1976). The portion of the membrane-located adenosine triphosphatase activity that could be inhibited with 0.9 mM-ouabain

Table 5. Effects of ouabain and alloxan on adenosine 5'-triphosphatase activity in islet cell membranes

Islet homogenates were centrifuged through sucrose gradients and a fraction enriched in plasma membranes was obtained at a sucrose density of 1.1 g/cm³; the preferential binding to this fraction of wheat-germ agglutinin is shown in Fig. 4. Samples of the membrane fraction were incubated for 10, 20 or 60 min at 37°C in 25 mM-histidine buffer (pH 7.4) with 0.9 mM-MgCl₂, 0.1 mM-EDTA, 108 mM-NaCl, 4.2 mM-KCl and 0.1 mM-ATP. To half of the tubes, 0.9 mM-ouabain was also added. Liberation of the terminal phosphate in the absence of ouabain is given as the 'total activity', that in presence of ouabain as the 'ouabain-insensitive activity' and the difference due to ouabain as the 'ouabain-inhibitable activity'. In both the presence and the absence of ouabain, parallel incubations were performed with and without 2 mM-alloxan in the medium. Results are mean values $\pm$ S.E.M. for three experiments (three plasma-membrane preparations assayed on three different occasions). The effect of alloxan was *t*-tested on paired observations: * $P < 0.01$.

Group	Adenosine triphosphatase activity (pmol of P _i liberated/ μ g of protein)		
	Incubation time ... 10min	20min	60min
Total activity			
Control	466 $\pm$ 145	705 $\pm$ 221	1390 $\pm$ 459
Alloxan	471 $\pm$ 180	742 $\pm$ 272	1272 $\pm$ 420
Difference	6 $\pm$ 40	37 $\pm$ 52	-118 $\pm$ 87
Ouabain-insensitive activity			
Control	438 $\pm$ 124	629 $\pm$ 142	1459 $\pm$ 319
Alloxan	470 $\pm$ 114	553 $\pm$ 147	1328 $\pm$ 315
Difference	32 $\pm$ 11	-76 $\pm$ 5*	-131 $\pm$ 39
Ouabain-inhibitable activity			
Control	27 $\pm$ 22	77 $\pm$ 79	-69 $\pm$ 153
Alloxan	1 $\pm$ 66	189 $\pm$ 126	-56 $\pm$ 110
Difference	-26 $\pm$ 47	113 $\pm$ 47	13 $\pm$ 50

Fig. 4. Distribution of K^+ -dependent *p*-nitrophenyl phosphatase activity on the final sucrose gradient used for isolating islet-cell plasma membranes

Samples from the gradient were incubated for 60 min at 30°C with or without 7.7 mM-KCl in 30 mM-Tris/HCl buffer (pH 7.8) containing 3.9 mM-*p*-nitrophenyl phosphate and 3.9 mM- MgCl_2 . Enhancement of substrate hydrolysis by KCl (●) is plotted, together with the binding of wheat-germ agglutinin (○) and sucrose density (—) in the same gradient. Repeated experiments consistently showed a coincidence of enzyme activity and wheat-germ-agglutinin binding. However, the proportion of the different enzyme peaks varied such that the one coinciding with wheat-germ agglutinin was not regularly the highest. There is no indication as to whether this variation is biological or artificial.

was not statistically significant and represented less than one-tenth of the total activity in the presence of Na^+ , K^+ and Mg^{2+} . Alloxan (2 mM) could not be shown to inhibit the ouabain-sensitive portion, and

Table 6. K^+ -activated *p*-nitrophenyl phosphatase activity in islet-cell plasma membranes

Islet-cell plasma membranes were isolated as in Table 5 and incubated for 60 min at 30°C in 30 mM-Tris/HCl buffer (pH 7.8) with 3.9 mM- MgCl_2 and 3.9 mM-*p*-nitrophenyl phosphate. Some tubes also contained 7.7 mM-KCl or 2 mM-alloxan or both. The results of three different experiments are given. In each experiment equal amounts of membrane were incubated in parallel in all media. The amount of protein added to each tube was $0.97\text{ }\mu\text{g}$ in Expt. I, $3.77\text{ }\mu\text{g}$ in Expt. II, and not measured in Expt. III.

Enzyme activity (nmol of *p*-nitrophenol liberated/h)

Expt. no.	No K^+		7.7 mM- K^+	
	Control	Alloxan	Control	Alloxan
I	0.18	0.22	0.53	0.58
II	0.58	0.66	1.19	0.94
III	0.73	0.55	1.75	1.54

at most decreased the predominant ouabain-insensitive portion by 12%.

Although *p*-nitrophenyl phosphate has previously been used as a substrate for phosphatases in islets (Hellerström *et al.*, 1964; Täljedal *et al.*, 1966a,b), the K^+ -sensitivity of hydrolysis has not been reported. In the present study, whole islet homogenates were incubated with 3.9 mM substrate and 3.9 mM- MgCl_2 in Tris/HCl buffer, pH 7.8. The release of *p*-nitrophenol (in nmol/ μg of protein) was 0.43 ± 0.13 in 10 min, 0.73 ± 0.23 in 20 min and 0.89 ± 0.32 in 60 min (mean $\pm$ S.E.M. of three experiments). Including 7.7 mM-KCl in the medium resulted in the following increments of enzyme activity: 0.16 ± 0.06 (20 min), 0.19 ± 0.06 (40 min) and 0.28 ± 0.07 (60 min) nmol/ μg of protein. On fractionation of the homogenates by density-gradient centrifugation, more than one peak of K^+ -activated *p*-nitrophenyl phosphatase was observed. One of these coincided with the binding of wheat-germ agglutinin (Fig. 4). The *p*-nitrophenyl phosphatase in this plasma-membrane fraction was unaffected by 2 mM-alloxan (Table 6).

Discussion

Rb⁺ accumulation as a model *in vitro* for studying the diabetogenic action of alloxan

The results allow two basic conclusions: alloxan *in vitro* inhibits the active Rb^+ uptake in islet cells, and this effect most strikingly parallels the diabetogenic action of alloxan *in vivo*. The effect on Rb^+ uptake resembled the diabetogenic action in being prevented by sugars in a similar way, in affecting islets but not the acinar pancreas, and in being due to alloxan

rather than a conversion product. The accumulation of Rb^+ is therefore suggested to be a valuable model *in vitro* for elucidating the diabetogenicity of alloxan. The inhibition of Rb^+ accumulation explains the previously observed disruption of the membrane electric potential (Dean & Matthews, 1972). Blockade of the univalent-cation pump is such a fundamental change that it may well lead to other cytophysiological derangements, and ultimately to the death of the β -cells. The viability of islet cells exposed to alloxan *in vitro* is examined in the following paper (Grankvist *et al.*, 1977).

Nature of the Rb^+ pump and its inhibition by alloxan

Arresting the univalent-cation pump need not be the most basic action of alloxan in the β -cells. It is unclear whether the Rb^+ accumulation was inhibited because alloxan reacted with, and thus directly damaged, a membrane-located vectorial catalyst, or the pump was indirectly crippled owing to shortage of some necessary cofactor. This uncertainty is inescapable as long as the precise nature of the Rb^+ pump is unknown.

Rb^+ can substitute for K^+ in the function of both the Na^+/K^+ -pump and the associated ATP hydrolase in other cells (Bonting, 1970). Since β -cells accumulate Rb^+ in a Na^+ -dependent and ouabain-inhibitable manner (Sehlin & Täljedal, 1974a), their univalent-cation pump may be identical with the usual Na^+/K^+ -pump. However, this inference can be challenged until both the vectorial and scalar catalytic functions of a typical Na^+/K^+ -pump have been shown to exist in the islets. Although the demonstration of a vectorial function, i.e. Rb^+ accumulation, is straightforward (Sehlin & Täljedal, 1974a), considerable difficulties attend the demonstration of the scalar catalysis, i.e. a specific $\text{Na}^+ + \text{K}^+$ -activated adenosine triphosphatase. Although this enzyme is readily observed in whole homogenates of *ob/ob*-mouse brain, parallel incubations of *ob/ob*-mouse islet homogenates revealed no ATP-splitting activity that was dependent on K^+ or inhibitable with ouabain (I.-B. Täljedal, unpublished work). Microsomal fraction from normal mouse islets showed no activity unless they were treated with urea, and even then the activity was less than 1% of that in brain microsomal preparations (Formby *et al.*, 1976). To decrease the activity of other adenosine triphosphatases in the present study, we isolated a fraction enriched in plasma membranes; the technique has permitted a ouabain-inhibited adenosine triphosphatase to be observed in rat islets (Lernmark *et al.*, 1976). The ouabain-sensitive adenosine triphosphatase activity in the *ob/ob*-mouse islet membranes was so low that its sensitivity to alloxan could hardly be ascertained; no hint of an inhibition was obtained, however.

The accumulation of Rb^+ in β -cells occurs in the absence of an impressive Na^+ gradient; the intra-

cellular Na^+ is about 95 mM in islets incubated with 144 mM- Na^+ (Sehlin & Täljedal, 1974b). In view of this fact and the very low ouabain-sensitive adenosine triphosphatase activity, one may ask whether the Rb^+ pump is really identical with the classical $\text{Na}^+ + \text{K}^+$ -activated adenosine triphosphatase (Bonting, 1970). Polymorphonuclear leucocytes are reported to have a defective Na^+ pump and to lack a specific $\text{Na}^+ + \text{K}^+$ -activated adenosine triphosphatase (Woodin & Wieneke, 1970). They pump K^+ , however, and a K^+ -activated and ouabain-inhibitable *p*-nitrophenyl phosphatase has been attributed significance in this process (Woodin & Wieneke, 1970). The present experiments give the first indication that the same enzyme is present in β -cells. Its properties and possible relevance for univalent cation pumping requires further study. The results reported here show that the K^+ -stimulated aryl phosphatase in islet-cell plasma membranes is insensitive to alloxan at a concentration causing marked inhibition of the Rb^+ accumulation.

Islets from alloxan-injected *ob/ob* mice exhibit decreased O_2 consumption and glucose oxidation (Hellerström & Gunnarsson, 1970), as well as abolition of proinsulin synthesis (Gunnarsson, 1975). Therefore it was essential to study how alloxan *in vitro* affected the islet ATP concentration; a marked depletion of this key metabolite could mean that the Rb^+ accumulation was inhibited in consequence of some basic metabolic derangement whether or not ATP is directly used as a substrate for the pump. Because alloxan is a thiol blocker (Rerup, 1970), and some such agents can impede the glycolytic flow with a piling-up of early glycolytic intermediates (Hellman *et al.*, 1973a), we also checked the islet content of glucose 6-phosphate. The fact that alloxan did not influence the glucose 6-phosphate and only marginally decreased the ATP concentration encourages further search for direct effects of alloxan on the Rb^+ pump. However, it is not known how much the ATP must decrease for marked inhibition of the pump to occur. So the results cannot completely rule out the possibility that alloxan inhibits the Rb^+ accumulation in an indirect way.

Mechanism of glucose protection against alloxan

Experiments *in vivo* led to the hypothesis that glucose protects against alloxan-diabetes by altering an alloxan-reactive site in the β -cells, rather than by competing for the same site (Scheynius & Täljedal, 1970). The present data from work *in vitro* agree with those of Scheynius & Täljedal (1970) in showing that mannoheptulose cannot mimic the protective action of D-glucose, although mannoheptulose prevents D-glucose from exerting its protective effect. The results also conform with other experiments *in vivo*, which have shown that the protective ability of D-glucose is shared by 3-O-methyl-D-glucose but not by

L-glucose or mannoheptulose (Zawalich & Beidler, 1973; Rossini *et al.*, 1975a,b).

The protective effect of D-glucose increased with concentration in the range 2–20 mM. Since this dose-dependence is reminiscent of insulin release and islet glucose metabolism (Hellman *et al.*, 1974), one is tempted to assume that the protection against alloxan relates to the recognition of D-glucose as an insulin secretagogue. Such an assumption is also promoted by reports that 3-O-methyl- α -D-glucose offers better protection than 3-O-methyl- β -D-glucose when injected into rats (Rossini *et al.*, 1975a,b); this anomeric difference resembles the fact that α -D-glucose is a more potent insulin secretagogue than β -D-glucose (Niki *et al.*, 1974; Grodsky *et al.*, 1974; Rossini *et al.*, 1974; Idahl *et al.*, 1975, 1976). However, the meaning of these correlations appears obscure when one considers a striking difference between the protection against alloxan and the induction of insulin release: 3-O-methyl-D-glucose is a good protector, but neither stimulates nor inhibits insulin release (Hellman *et al.*, 1974; Matschinsky & Ellerman, 1973).

So far, the only sugar-binding site in β -cells that is known to accept D-glucose, 3-O-methyl-D-glucose (Hellman *et al.*, 1973b) and probably mannoheptulose (Hellman *et al.*, 1971, 1972) and to exclude L-glucose (Hellman *et al.*, 1971) is the membrane-located glucose-transport system. Scheynius & Täljedal (1970) suggested that glucose may protect against alloxan-diabetes by preventing the drug from entering the β -cells via a path coupled to the sugar carrier. This idea is corroborated by the present data *in vitro*. It is also supported by the observation in rat islets that only one of two cytochalasins inhibited glucose transport and protected against alloxan (McDaniel *et al.*, 1975).

The uptake of an organic mercurial indicated that β -cells are well equipped with superficial thiol groups, and their possible involvement in alloxan-sensitivity has been discussed (Hellman *et al.*, 1973c). However, since the effect of alloxan on Rb⁺ pumping (Table 3), as well as on the membrane electric potential (Dean & Matthews, 1972), exhibits a noticeable latency, it is plausible that the diabetogenic effect requires penetration of the drug beyond the outermost surface of the β -cells. Whether glucose can inhibit the penetration of alloxan remains to be directly tested. In experiments performed so far (J. Sehlin & I.-B. Täljedal, unpublished work), the islet uptake of radioactive alloxan did not yield sufficient labelling of the β -cells for quantitative studies of the effect of glucose to be performed.

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