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Alloxan effects on mitochondria: study of oxygen consumption, fluxes of Mg^{2+}, Ca^{2+}, K^+ and adenine nucleotides, membrane potential and volume change in vitro

L. Boquist

Institute of Pathology, University of Umeå, Umeå, Sweden

Summary. Isolated mouse liver mitochondria incubated with alloxan showed stimulated resting (state 4) respiration with succinate, and inhibited resting respiration with pyridine-linked substrates, whereas active (state 3) respiration was decreased with both kinds of substrates. The effects were dependent on the concentration of alloxan, on the energy state, and on transport of inorganic phosphate and uptake of Ca^{2+}. Using succinate as substrate, the effects of alloxan on endogenous Mg^{2+}, K^+ and adenine nucleotides, uptake of K^+, accumulated Ca^{2+}, membrane potential and volume were studied in liver mitochondria, and in addition efflux of endogenous K^+ and accumulated Ca^{2+} were investigated in mouse islet mitochondria. High concentrations of alloxan ($\geqslant 3$ mmol/l) induced efflux of endogenous Mg^{2+}, K^+ and adenine nucleotides, efflux of accumulated Ca^{2+}, inhibition of uptake of K^+, loss of membrane potential, and swelling. Low concentrations of alloxan (< 3 mmol/l) had similar effects only in the presence of added Ca^{2+} and inorganic phosphate. The influence of potentially protective agents was studied mainly with regard to alloxan induced swelling. Complete or partial protection was offered by antimycin A, malonate, La^{3+}, Ni^{2+}, ruthenium red, mersalyl and N-ethylmaleimide, suggesting requirement for energized transport of Ca^{2+} and uptake of inorganic phosphate. The start of the respiratory changes, decrease of membrane potential and loss of Mg^{2+} preceded the release of accumulated Ca^{2+}, which occurred in parallel with efflux of K^+ and swelling. The loss of Ca^{2+} in association with swelling agrees with data previously obtained using qualitative and quantitative electron microscopy and X-ray microanalysis of islet β cells from alloxan-treated mice. Since preceding studies in vivo have shown that alloxan passes across plasma membranes and is taken up in mitochondria of islet β cells and hepatocytes, the combined data support the view that alloxan diabetes may be due to mitochondrial damage.

Key words: Alloxan, diabetes, mitochondria, liver, endocrine pancreas.

A preceding study demonstrated that alloxan induces release of Ca^{2+} from isolated mouse liver mitochondria [1]. An alloxan-induced loss of Ca^{2+} from mitochondria was suggested also by our observations in mouse islet β cells in vivo, where using the pyroantimonate technique and qualitative and quantitative electron microscopy, the loss was shown to be associated with mitochondrial swelling [2, 3].

There are reports of an association in isolated mitochondria between efflux of Ca^{2+} induced by different means, and acceleration [4] or inhibition [5] of respiration, collapse of membrane potential, and large amplitude swelling. In addition to release of Ca^{2+}, loss of K^+, Mg^{2+}, adenine nucleotides and pyridine nucleotides have been reported [5–8]. Movement of inorganic phosphate has been suggested to play a prominent role in the efflux of Ca^{2+} from isolated mitochondria [9–11], and calmodulin [12] and membrane phospholipase A_2 [13] have also been ascribed a role in Ca^{2+} release from mitochondria. Since alloxan is a thiol reagent, it is of interest that the ability of mitochondria to maintain membrane integrity and retain Ca^{2+} may be dependent on generation of thiol groups for the provision of [9, 14].

The oxidation of different substrates has been studied using the Warburg manometric technique on rat kidney and liver tissue exposed to alloxan in vitro [15], and the Cartesian diver technique on mouse islets isolated 10 min after injection of alloxan [16]. In those studies there was an inhibition of the oxidation of pyridine-linked substrates, but unaffected [16] or only slightly decreased [15] oxidation of succinate.

Against this background, the present work on isolated mitochondria was carried out in the presence and absence of added Ca^{2+} and inorganic phosphate to study

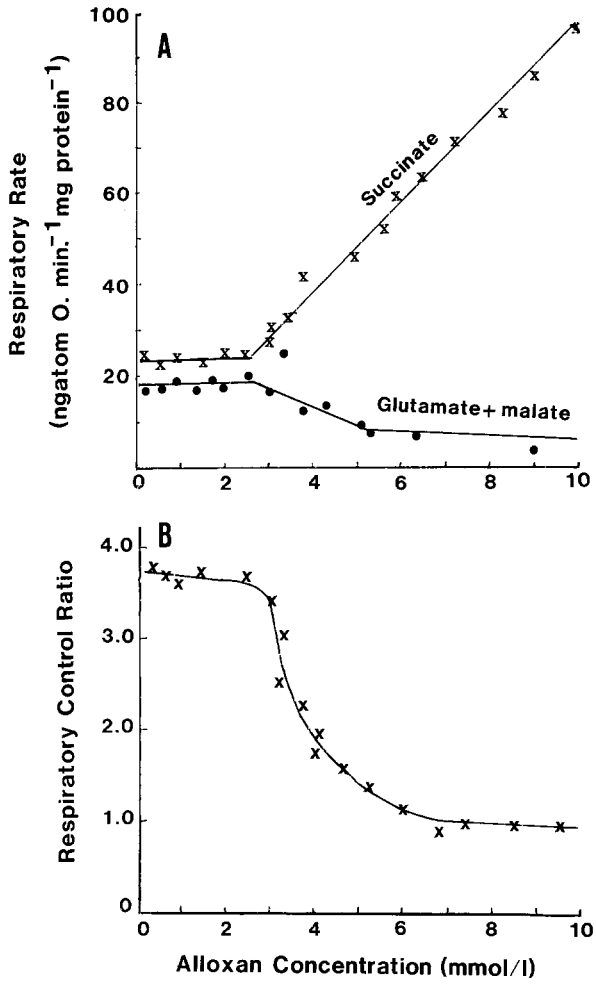

Fig. 1. **A** Effect of different concentrations of alloxan on resting respiration with either succinate or pyridine-linked substrates. Isolated mouse liver mitochondria (1 mg protein/ml) were incubated in the absence of added Ca^{2+} in a medium containing sucrose (200 mmol/l), Hepes (10 mmol/l), KCl (50 mmol/l) and $MgCl_2$ (4 mmol/l), pH 7.2. Additions were made of rotenone (1 μmol/l) and succinate (×—× 5 mmol/l), or of glutamate (●—● 5 mmol/l) and malate (0.5 mmol/l), followed by alloxan 1 min later. **B** Correlation between alloxan concentration and respiratory control ratio, with succinate as substrate. Alloxan was added 1 min after succinate (5 mmol/l), and ADP (100 μmol/l) was added 1 min following alloxan, after which the active respiration and the subsequent resting respiration were determined, and the respiratory control ratio was calculated. Medium and protein concentration as in Figure 1 A with addition of inorganic phosphate (3 mmol/l)

whether alloxan affects the oxygen consumption using different substrates, and whether the Ca^{2+}-releasing action of alloxan [1] is accompanied by efflux of other ions and by altered membrane potential and volume.

Liver mitochondria were used in these experiments since they are much more easily obtained in sufficient amount than mitochondria from the endocrine pancreas. Also, there is an uptake of alloxan in liver mitochondria both in vivo and in vitro [17], and our preceding work has demonstrated that alloxan affects ion transport in isolated liver mitochondria [18, 19]. Recent studies by others, not specifically directed to mitochondria, have verified a considerable, rapid accumulation [20] and cytotoxicity [21] of alloxan in hepatocytes. However, mitochondria from mouse endocrine pancreas can be isolated [17], and, to facilitate the interpretation of the data with regard to diabetogenicity of alloxan, some additional experiments were carried out on mitochondria from the endocrine pancreas.

Materials and methods

Animals

The animals used were C57BL-KsJ-+/+ and *ob/ob*-mice from local stocks kept under standard laboratory conditions with free access to a standard ration and water. The *ob/ob*-mice, which possess hyperplastic pancreatic islets composed mainly of β cells, were used for the experiments with islet mitochondria, whereas the C57BL-KsJ-+/+ mice were used for the experiments with liver mitochondria. Pilot studies did not demonstrate any significant differences between isolated liver mitochondria from the two kinds of mice with regard to the different parameters studied here. All animals were 3–4 months old, of both sexes, and were starved for 21 h before experimentation.

Methods

Hepatic mitochondria were isolated in medium containing sucrose (250 mmol/l), Tris-HCl (5 mmol/l) and EDTA (1 mmol/l), pH 7.4, which represents a conventional medium for isolation of mitochondria from liver. The pancreatic islets were isolated using the collagenase technique, and the islet mitochondria were isolated as described previously [17] in a medium containing mannitol (190 mmol/l), sucrose (60 nmol/l), N-tris(hydroxymethyl)methyl-2-aminoethane sulphonic acid (5 mmol/l), and EDTA (0.5 mmol/l), pH 7.4, with 0.5% bovine serum albumin. The same medium was used in pilot studies for isolation of liver mitochondria for comparison with those isolated in the conventional medium. No significant difference was found between liver mitochondria isolated with the two media with regard to the parameters studied. Resuspensions in EDTA-free medium were performed when Ca^{2+} fluxes were studied. All isolations were carried out at 0 °C. The mitochondrial protein concentration was determined spectrophotometrically (model 24, Beckman Instruments, Irvine, California, USA).

Oxygen consumption was studied with a Clark type electrode (Hansatech, King's Lynn, Norfolk, UK) at room temperature with 1 mg mitochondrial protein/ml, in medium containing sucrose (200 mmol/l), N-2-hydroxyethyl-piperazine-N'-2-ethanesulphonic acid (Hepes; 10 mmol/l), KCl (50 mmol/l), $MgCl_2$ (4 mmol/l) and Pi (3 mmol/l), pH 7.2, in the presence or absence of rotenone (1 μmol/l), mersalyl (20 μmol/l), and N-ethylmeleimide (NEM; 40 or 100 μmol/l). Some experiments on resting respiration were carried out without added Pi; succinate (5 mmol/l), glutamate (5 mmol/l), malate (0.5 mmol/l), Ca^{2+} (35 or 70 nmol/mg mitochondrial protein), ADP (100 μmol/l), 2,4-dinitrophenol (DNP, 100 μmol/l) and different concentrations of alloxan were added.

In general, the studies of the ion fluxes, membrane potential and volume change were carried out at room temperature, usually with 1 mg mitochondrial protein/ml, in a basic medium containing sucrose (200 mmol/l), Hepes (10 mmol/l), KCl (50 mmol/l), $MgCl_2$ (4 mmol/l), Pi (3 mmol/l), rotenone (1 μmol/l) and succinate (5 mmol/l) with 35 or 70 nmol Ca^{2+}/mg mitochondrial protein.

The Ca^{2+} fluxes were monitored as absorbance changes of the metallochromic indicator antipyrylazo III (Fluka, Buchs, Switzerland) at the wave-length pair 720–790 nm in a dual wave-length spectrophotometer (Aminco DW-2; American Instruments, Silverspring, Maryland, USA), and alterations in the mitochondrial membrane potential were studied in the same spectrophotometer with 10 μmol/l safranin 0 (Sigma Chemicals, St. Louis, Missouri, USA) as indicator

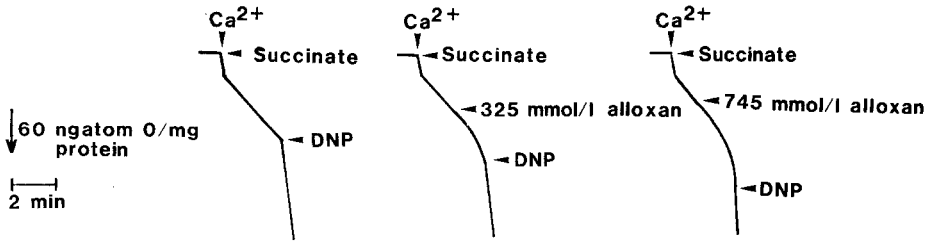

Fig. 2. Potentiation of the alloxan effect on succinate-resting respiration by addition of Ca^{2+} to medium containing Pi (3 mmol/l). Ca^{2+} (35 nmol/mg mitochondrial protein), succinate (5 mmol/l) plus rotenone (1 μmol/l), 2,4-dinitrophenol (DNP; 100 μmol/l) and different concentrations of alloxan were added as indicated. Medium and protein concentration as in Figure 1 A

dye, responding with spectral change upon induction of an electrical potential difference across the mitochondrial membranes [22]. The mitochondrial volume change was monitored by apparent absorbance measurements at 520 nm.

The release of endogenous K^+ was studied with a K^+ sensitive electrode and a reference electrode (Radiometer, Copenhagen, Denmark). These electrodes were also used for investigation of energized uptake of K^+ in the presence of valinomycin. The efflux of Mg^{2+} was determined by atomic absorption spectroscopy of the supernatant. Mitochondrial adenine nucleotides can be assayed by high pressure liquid chromatography [23], and here these nucleotides were determined using 4 mg mitochondrial protein/ml, with a reversed phase μBondapak C_{18} column, a model 440 absorbance detector, two model 6000 A pumps and a model 720 system controller (all of Waters Associates, Milford, Massachusetts, USA). Measurements were based on ultraviolet absorbance at 254 and 280 nm.

Results

Oxygen consumption

In the following text 'resting respiration' represents respiration in the absence of added ADP (state 4), and 'active respiration' corresponds to respiration in the presence of added ADP (state 3).

When added to the incubation medium, alloxan induced a slight concentration-dependent increase in oxygen consumption.

Succinate oxidation: under energized conditions (alloxan added after the substrate), alloxan induced enhanced resting respiration. This effect was dependent on the concentration of alloxan (Fig. 1 A); higher concentrations caused a rapid alteration of respiration and high rates of respiratory stimulation, whereas lower concentrations caused a less rapid change of oxygen consumption preceded by a lag with unaltered respiration, and lower respiratory rates. The minimum concentration of alloxan for observable effect was ~3 mmol/l in the absence of added Ca^{2+} and inorganic phosphate. Stimulated resting respiration also was found under non-energized conditions (alloxan added before the substrate) in which case the minimum effective concentration of alloxan was ~1 mmol/l (data not shown).

Active respiration (Pi added to the medium) was decreased in a concentration-dependent manner by $\geqslant$ 3 mmol/l concentrations of alloxan (data not shown). A markedly decreased respiratory control ratio was seen in mitochondria exposed to high concentration of alloxan (Fig. 1 B), and at ~7 mmol/l alloxan the mitochondria were unresponsive to respiratory stimulation by 2,4-dinitrophenol.

Oxidation of pyridine-linked substrates: using glutamate plus malate as substrates, inhibition of resting respiration was found in the presence of $\sim \geqslant 3$ mmol/l concentrations of alloxan under energized conditions (Fig. 1 A), and in the presence of $\sim \geqslant 1$ mmol/l concentrations of alloxan in the non-energized state (data not shown). These concentrations of alloxan also induced decreased active respiration (Pi present in the medium), and $\sim \geqslant 7$ mmol/l alloxan caused unresponsiveness of the mitochondria to 2,4-dinitrophenol-stimulation of respiration (data not shown).

Effect of external Ca^{2+} and Pi: it has been reported that inorganic phosphate induces a spontaneous release of Ca^{2+} accompanied by alterations in oxygen consumption in isolated rat liver mitochondria [4, 24], and it has been found in this laboratory that this holds true also

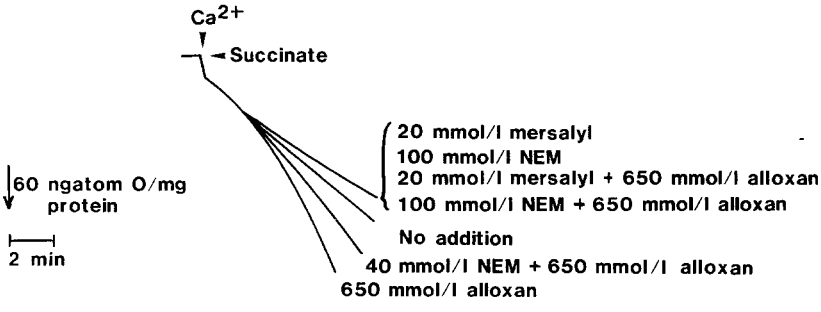

Fig. 3. Inhibition by mersalyl and N-ethylmaleimide (NEM) of the stimulation of succinate-resting respiration induced by alloxan in the presence of added Ca^{2+}, with Pi (3 mmol/l) in the medium. Additions were made as indicated of Ca^{2+} (35 nmol/mg mitochondrial protein), succinate (5 mmol/l) plus rotenone (1 μmol/l), mersalyl and NEM. Medium and protein concentration as in Figure 1 A

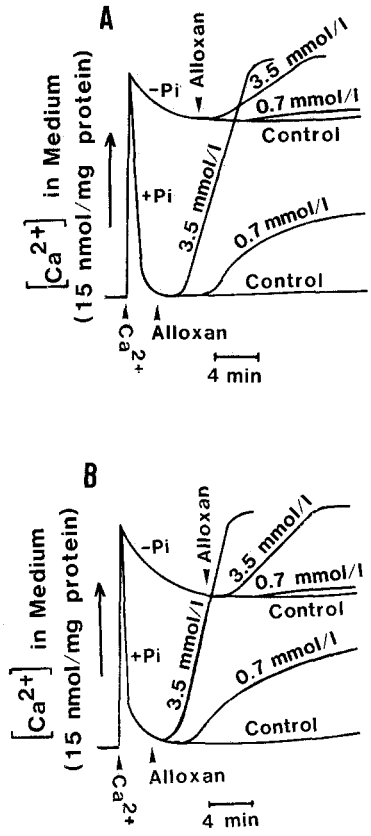

Fig. 4. Alloxan-induced release of accumulated Ca^{2+} in isolated (A) liver and (B) islet mitochondria (1 mg protein/ml) incubated in medium containing sucrose (200 mmol/l), Hepes (10 mmol/l), KCl (50 mmol/l), $MgCl_2$ (4 mmol/l), succinate (5 mmol/l) and rotenone (1 μmol/l), pH 7.2, with and without Pi (3 mmol/l). Additions: Ca^{2+} (35 nmol/mg mitochondrial protein) and alloxan (as indicated)

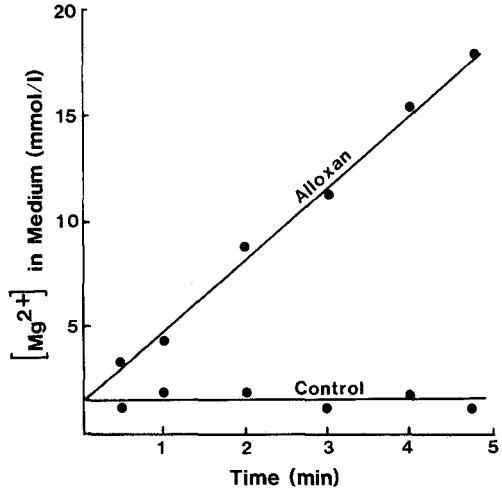

Fig. 5. Release of endogenous Mg^{2+} from isolated mouse liver mitochondria (4 mg protein/ml) incubated with alloxan in a medium containing sucrose (200 mmol/l), Hepes (10 mmol/l), succinate (5 mmol/l) and rotenone (1 μmol/l), pH 7.2, with or without addition of alloxan (3 mmol/l). Each point represents one determination of Mg^{2+} in the supernatant

for isolated mouse liver mitochondria (unpublished data). Addition to the media of Mg^{2+} and adenine nucleotides has been suggested to prevent Pi-induced release of Ca^{2+} [7, 24]. Since addition of adenine nucleotides to the basic medium would affect the respiration, such addition was not used in the present study, with the aim to inhibit specifically the efflux of Ca^{2+} induced by Pi. However, Mg^{2+} was included at a physiological concentration in the basic medium, since our unpublished experiments have shown that Mg^{2+} markedly inhibits the efflux of Ca^{2+} from isolated mouse liver mitochondria incubated with Pi in the absence of external adenine nucleotides.

Both with succinate (Fig. 2) and pyridine-linked substrates (data not shown), the sensitivity of the mitochondria to the respiratory effects of alloxan was markedly increased by addition of Ca^{2+}, in the presence in the basic medium of 3 mmol/l Pi, which approximately corresponds to the cytosolic concentration. The usual jump in oxygen consumption was found following addition of Ca^{2+}, and stimulation of resting respiration with succinate, and inhibition of resting respiration with pyridine-linked substrates were seen after addition of alloxan. As described above, in the absence of external Ca^{2+} and Pi, higher concentrations of alloxan caused a more rapid alteration of respiration and, with succinate, a more marked respiratory stimulation than at lower concentrations. Active respiration was inhibited by alloxan. This effect was increased with increasing concentrations of alloxan. At the lowest concentrations of alloxan, the stimulation of resting respiration with succinate was preceded by a slight, transient decrease in oxygen consumption. This effect was not seen with higher concentrations of alloxan.

The potentiating effect of external Pi was demonstrated further by abolishment of the alloxan-induced alterations of resting respiration in the presence of mersalyl and N-ethylmaleimide. Mersalyl was fully efficient at a concentration of 20 μmol/l, whereas 40 μmol/l N-ethylmaleimide caused partial inhibition, and 100 μmol/l N-ethylmaleimide caused complete inhibition of the alloxan effect both, with succinate (Fig. 3) and pyridine-linked substrates (data not shown).

Efflux of accumulated Ca^{2+}

Our preceding studies have demonstrated that the Ca^{2+}-releasing effect of alloxan in isolated mouse liver mitochondria is potentiated by exogenous Pi [1, 25]. To study the influence of inorganic phosphate further, Ca^{2+} release experiments were carried out, both with and without a physiological concentration of Pi in the incubation medium. In control experiments with added Pi, a slow release of accumulated Ca^{2+} was observed after a lag of several minutes in the absence of added Mg^{2+}. Addition of a physiological concentration of Mg^{2+}, however, resulted in retention of accumulated Ca^{2+} in control mitochondria during the period investi-

Table 1. Efflux of adenine nucleotides in isolated mouse liver mitochondria incubated with alloxan

	Incubation time (min)	*n*	Adenine nucleotides (nmol/mg mitochondrial protein)			
			ATP	ADP	AMP	Total
Control		6	3.53 + 0.41	5.14 ± 0.49	3.70 ± 0.62	12.37 + 0.59
Alloxan (0.8 mmol/l) Ca^{2+} + Pi added	1	4	3.45 ± 0.53	5.01 ± 0.63	3.73 ± 0.50	12.19 ± 0.55
Alloxan (0.8 mmol/l) Ca^{2+} + Pi added	3	5	2.05 ± 0.43	3.09 ± 0.48[a]	2.79 ± 0.51	7.93 ± 0.41[c]
Alloxan (0.8 mmol/l) Ca^{2+} + Pi added	5	6	1.14 ± 0.40[b]	0.71 + 0.21[c]	0.96 ± 0.21[b]	2.81 ± 0.42[c]
Alloxan (3 mmol/l)	1	5	2.94 ± 0.80	4.08 ± 0.63	3.40 ± 0.81	10.42 ± 0.70[a]
Alloxan (3 mmol/l)	3	6	0.75 ± 0.15[c]	0.86 ± 0.21[c]	0.92 ± 0.17[b]	2.53 ± 0.18[c]
Alloxan (3 mmol/l)	5	6	0.20 ± 0.10[c]	0.07 ± 0.04[c]	0.18 ± 0.09[b]	0.45 ± 0.14[c]

Values are expressed as mean ± SEM of adenine nucleotide concentrations determined in perchloric acid extracts by high pressure liquid chromatography. Statistical difference from corresponding control values by Student's t-test: [a] $p < 0.05$, [b] $p < 0.005$ and [c] $p < 0.001$

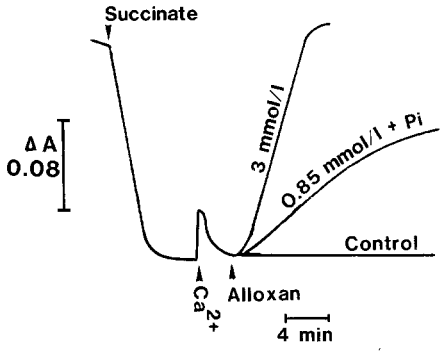

Fig. 6. Effect of alloxan on the membrane potential of isolated liver mitochondria (1 mg protein/ml) incubated in medium containing sucrose (200 mmol/l), Hepes (10 mmol/l), KCl (50 mmol/l), $MgCl_2$ (4 mmol/l) and rotenone (1 μmol/l), pH 7.2. When present the concentration of Pi was 3 mmol/l. Additions: succinate (5 mmol/l), Ca^{2+} (35 nmol/mg mitochondrial protein) and alloxan as indicated. Downward deflection represents increase in membrane potential

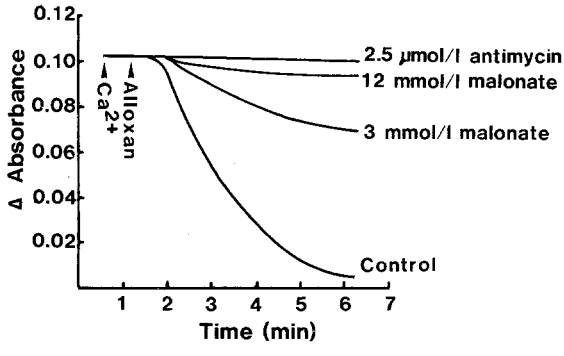

Fig. 7. Alloxan-induced swelling of isolated liver mitochondria, and protection against this swelling by antimycin A and malonate. Additions: Ca^{2+} (35 nmol/mg mitochondrial protein) and alloxan (0.9 mmol/l). Medium as in Figure 4 with 3 mmol/l Pi

gated. The rate and extent of Ca^{2+} uptake were slightly decreased in the presence of Mg^{2+}.

Without added Pi, high concentrations of alloxan induced a slow release of accumulated Ca^{2+}, beginning after a lag and occurring at a rate dependent on the concentration of alloxan (Fig. 4A). In the presence of added Pi, the mitochondrial uptake of Ca^{2+} was significantly increased, and high alloxan concentrations added after the uptake induced an immediate efflux of all the accumulated Ca^{2+}. Endogenous Ca^{2+} also seemed to be released by this kind of treatment. Low concentrations of alloxan induced no release at all in the absence of added Pi, whereas a release of Ca^{2+} was seen when Pi had been added to the incubation medium. As described above for high concentrations of alloxan, this release began after a lag and occurred at a rate and an extent which were dependent on the concentration of alloxan.

Alloxan also induced release of Ca^{2+} from isolated islet mitochondria (Fig. 4B), similar to that described above for isolated liver mitochondria incubated with alloxan.

Fluxes of Mg^{2+}, K^+ and adenine nucleotides

The effects of alloxan on endogenous Mg^{2+}, K^+ and adenine nucleotides were studied in the absence of added Mg^{2+}, K^+ and adenine nucleotides, and, since exogenous Ca^{2+} and Pi might affect the mitochondrial contents of Mg^{2+} and K^+ [7, 26], these determinations were performed in the absence of added Ca^{2+} and Pi.

Efflux of endogenous Mg^{2+}: there was no release of Mg^{2+} from control mitochondria, whereas alloxan treatment induced a rapid release of endogenous Mg^{2+}, which steadily increased with time during the period investigated. This release was dependent on the concen-

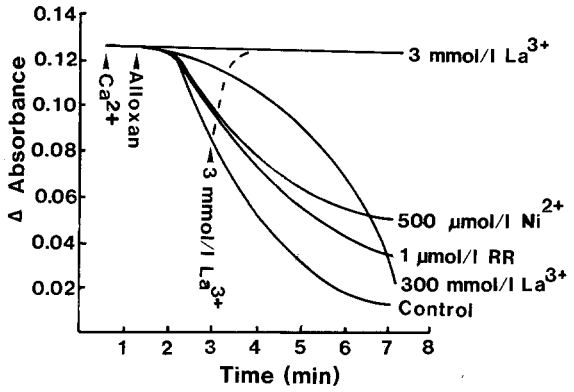

Fig. 8. Protection against alloxan-induced swelling of isolated liver mitochondria by Ca^{2+} transport inhibitors: La^{3+}, Ni^{2+} and ruthenium red (RR). Additions: Ca^{2+} (35 nmol/mg mitochondrial protein) and alloxan (0.75 mmol/l). Medium as in Figure 4 with 3 mmol/l Pi

tration of alloxan. Data for 3 mmol/l alloxan are shown in Figure 5.

Efflux of endogenous K^+ and uptake of K^+: alloxan induced a similar release of endogenous K^+ from both liver and islet mitochondria (data not shown). After a lag there was an efflux of K^+, and as observed above, the duration of the lag and the rate and extent of efflux were dependent on the concentration of alloxan. Both the rate and extent of the K^+ uptake induced by succinate and valinomycin were decreased when alloxan was added to energized and non-energized liver mitochondria (data not shown).

Efflux of endogenous adenine nucleotides: isolated liver mitochondria incubated with high concentrations of alloxan in the absence of added Ca^{2+} and Pi, or incubated with low concentrations of alloxan in the presence of added Ca^{2+} and Pi, released endogenous adenine nucleotides, whereas no release occurred from control mitochondria (Table 1).

Membrane potential

In isolated liver mitochondria incubated with alloxan, the membrane potential was lost after a lag and at a rate dependent on the concentration of alloxan and on the presence or absence of added Ca^{2+} and Pi (Fig. 6). Added Ca^{2+} potentiated the membrane potential decreasing action of alloxan in a manner similar to that described above for the effect of alloxan on mitochondrial respiration.

Volume change

Isolated liver mitochondria incubated with alloxan showed swelling which coincided with, and occurred at a similar rate to the release of Ca^{2+}. Because of the relationship which seemed to exist between swelling and efflux of Ca^{2+}, particular swelling experiments were performed to verify the relationship further. It is well known that the uptake of Ca^{2+} is energy-dependent, and since the substrate was succinate, antimycin A and malonate might inhibit the swelling. Figure 7 demonstrates that this was the case. Antimycin A caused a complete inhibition, and malonate induced a concentration-dependent inhibition of the swelling induced by alloxan.

Ruthenium red is known to inhibit the uptake of Ca^{2+} in isolated mitochondria, and a similar effect has been reported for Ni^{2+} [27], whereas La^{3+} inhibits both uptake and release of Ca^{2+} from isolated mitochondria. Figure 8 shows that 1 μmol/l ruthenium red partially inhibited the swelling induced by alloxan. Higher concentrations of ruthenium red were not used, since concentrations ≥ 10 μmol/l of this compound may cause uncoupling. A partial inhibition of alloxan-induced swelling was seen also in the presence of Ni^{2+}. La^{3+} protected against alloxan-induced swelling in a concentration-dependent manner, and contraction was seen when La^{3+} was added to already swelling mitochondria.

In an attempt to differentiate temporally the effect of alloxan on the different parameters studied, it was found that the efflux of Ca^{2+} was closely accompanied by efflux of K^+ and swelling, but preceded by the start of a decrease of membrane potential. The effect of alloxan on the oxygen consumption preceded the efflux of Ca^{2+}, and seemingly also the start of decrease of membrane potential. The relationship of Mg^{2+} release to change in respiration and membrane potential could not be revealed, but the efflux of Mg^{2+} preceded the release of Ca^{2+}. The relationship of efflux of adenine nucleotides to the other parameters studied could not be established clearly.

Discussion

The data show that alloxan alters the oxygen consumption of isolated mouse liver mitochondria, and that the respiratory response to alloxan is dependent on the type of oxidizable substrate, the concentration of alloxan, and addition of Ca^{2+} and inorganic phosphate. Alloxan inhibited active respiration with both kinds of substrates used, whereas resting respiration with succinate was stimulated, but pyridine-linked resting respiration was inhibited.

Since decrease in the concentration of adenine nucleotides in liver mitochondria is accompanied by lowered phosphorylating respiration [28, 29], the finding that alloxan inhibits active respiration might agree with efflux of adenine nucleotides from mitochondria incubated with alloxan. The inhibition of resting respiration with pyridine-linked substrates may be due to a direct alloxan-induced respiratory inhibition, or may be a consequence of loss of pyridine nucleotides in mitochondria incubated with alloxan, either as a result of leakage

across the mitochondrial membranes because of alloxan-induced permeability changes, or as a result of hydrolysis of pyridine nucleotides induced by alloxan inside the mitochondria, like that caused by hydroperoxides in rat liver mitochondria [6]. Moreover, a direct respiratory inhibition caused by alloxan, may lead to a secondary loss of pyridine nucleotides. Although pyridine nucleotides were not determined in the present study, it is conceivable that the observed efflux of Mg^{2+}, Ca^{2+}, K^+ and adenine nucleotides was accompanied by loss of pyridine nucleotides.

The relationship between efflux of Ca^{2+} and respiratory activity, and the importance of external inorganic phosphate for the release of Ca^{2+}have been emphasized in the Introduction. Consequently it is not unexpected that the respiratory changes induced by alloxan were potentiated by addition of Ca^{2+}, with inorganic phosphate present in the medium. The rôle played by inorganic phosphate was demonstrated by the observation that the potentiation was abolished by the inorganic phosphate transport inhibitors mersalyl and N-ethylmaleimide. The partial inhibition of the uptake of inorganic phosphate [18, 19] induced in isolated mouse liver mitochondria by millimolar concentrations of alloxan is not expected to affect inorganic phosphate uptake significantly under the present experimental conditions.

The findings also suggest a relationship between efflux of Ca^{2+} and volume change. Ca^{2+} uptake in mitochondria is energy-dependent, and can be blocked by the respiratory inhibitor antimycin A and the competitive inhibitor, malonate, when succinate is the substrate. In the present study both antimycin A and malonate protected against alloxan-induced swelling of isolated liver mitochondria. Moreover, ruthenium red inhibited mitochondrial uptake of Ca^{2+}, and Ni^{2+} had an inhibitory effect mainly on the uptake of Ca^{2+}[27], whereas La^{3+} inhibited both influx and efflux of Ca^{2+} in mitochondria. All these inhibitors were found to protect, partially or completely, against swelling of mitochondria incubated with alloxan. In the case of La^{3+}, a contraction could be induced even when the inhibitor was added to already swelling mitochondria. All these data emphasize the relationship between fluxes of Ca^{2+} and volume changes in isolated mitochondria incubated with alloxan.

Other thiol reagents, namely N-ethylmaleimide and diamide, have been reported to induce loss of Ca^{2+}, collapse of membrane potential and large amplitude swelling of isolated liver mitochondria [4, 30–32], whereas Ramachandran and Bygrave [33] found release of Ca^{2+} but no swelling in rat liver mitochondria incubated with N-ethylmaleimide, possibly due to their use of potassium chloride as osmotic support instead of mannitol/sucrose [34]. A requirement of external Ca^{2+} for the release of Ca^{2+} and the other events induced by N-ethylmaleimide has also been suggested [31].

The finding that the efflux of Ca^{2+} in alloxan-treated mitochondria is preceded by efflux of Mg^{2+} and decreased membrane potential conforms to observations in other studies of Ca^{2+} release from isolated liver mitochondria [35]. A potentiation by inorganic phosphate with shortening of the delay period before Ca^{2+} release from mitochondria has been reported also [7].

The reason why Ca^{2+} potentiates the alloxan effects described above is, so far, not clearly known. However, since alloxan probably exists mainly in anionic form at physiological pH [36], the rôle played by Ca^{2+} may be to facilitate the passage of alloxan across the mitochondrial membranes through the transient decrease in membrane potential, which is demonstrated in Figure 8. This interpretation may be supported by the present finding that the alloxan effects were more marked under non-energized than under energized conditions, since the mitochondrial membrane potential is affected by the energetic state.

For reasons given above, the effect of alloxan on islet mitochondria was studied with regard to the efflux of endogenous K^+ and accumulated Ca^{2+}. The data obtained were similar to those for liver mitochondria. It is of interest that loss of Ca^{2+} and swelling are induced by alloxan in isolated mitochondria, since qualitative and quantitative electron microscopy and X-ray microanalysis of islet β cells from alloxan-treated mice show dislocation of Ca^{2+} from mitochondria to cytosol, and volume increases in mitochondria very early during the development of alloxan diabetes [2, 3]. Furthermore, study in vivo has demonstrated that alloxan passes across plasma membranes and is taken up in mitochondria *inter alia* of islet β cells and hepatocytes [17].

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Professor L. Boquist
Institute of Pathology
University of Umeå
S-901 87 Umeå
Sweden