Mechanism of Alloxan-induced Calcium Release from Rat Liver Mitochondria*

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The objective of the present work was to investigate the mechanism of alloxan-induced Ca²⁺ release from rat liver mitochondria. Transport of Ca²⁺, oxidation and hydrolysis of mitochondrial pyridine nucleotides, changes in the mitochondrial membrane potential, and oxygen consumption by mitochondria were investigated. Alloxan does not inhibit the uptake of Ca²⁺ but stimulates the release of Ca²⁺ from liver mitochondria, which is accompanied by oxidation and hydrolysis of pyridine nucleotides. Oxidation of mitochondrial pyridine nucleotides by alloxan is not mediated by glutathione peroxidase and glutathione reductase and may occur largely nonenzymatically. Measurements of the mitochondrial membrane potential in combination with inhibitors of Ca²⁺ reuptake indicate that Ca²⁺ release takes place from intact liver mitochondria via a distinct pathway. Limited redox cycling of alloxan by mitochondria is indicated by measurements of the membrane potential and O₂ consumption in the presence of cyanide.

It is concluded that alloxan can cause Ca²⁺ release from intact rat liver mitochondria. Redox cycling of alloxan is not significantly involved in the Ca²⁺ release mechanism. Oxidation and hydrolysis of pyridine nucleotides, possibly in conjunction with oxidation of critical sulfhydryl groups, seem to be key events in the alloxan-induced Ca²⁺ release. Disturbance of cellular Ca²⁺ homeostasis may partly explain alloxan toxicity.

Since the discovery that the injection of alloxan (2,4,5,6-tetraoxypyrimidine) into animals causes necrosis of pancreatic β-cells, insulin deficiency, and diabetes mellitus (1), this substance has been widely used to produce experimental diabetes. The reason for the particular sensitivity of β-cells to the cytotoxic action of alloxan is not known. To understand the mechanism of action of diabetogenic agents appears of importance for elucidating not only the causes of insulin-dependent diabetes but also its prevention. Recent evidence suggests that alloxan causes Ca²⁺ release from mouse liver mitochondria (2, 3). Since disturbance of Ca²⁺ homeostasis can lead to cell death this finding may give new perspectives for the etiology of diabetes. The objective of the present work was to investigate the mechanism of the alloxan-induced Ca²⁺ release from rat liver mitochondria.

Alloxan is a mildly oxidizing agent and is reduced to dialuric acid (5-OH barbituric acid), for example, by thiols. In the presence of glutathione or certain thiol proteins, alloxan forms an addition product of unknown structure (4). Modifications of critical sulfhydryl groups in proteins were suggested to be the cause of alloxan toxicity (5). Alternatively it was postulated that dialuric acid, formed by unknown mechanisms in vivo from alloxan, generates cytotoxic free radicals upon its autoxidation to alloxan by O₂. According to this hypothesis autoxidation of dialuric acid is accompanied by the formation of superoxide radicals and hydrogen peroxide (6–9). Highly reactive hydroxyl radicals (OH⁻) would then be formed by a metal-catalyzed "Haber-Weiss reaction" that is the summation of the reduction of ferric ions by superoxide radicals (O₂⁻ + Fe³⁺ ↔ O₂ + Fe²⁺) and the Fenton reaction (H₂O₂ + Fe²⁺ ↔ OH⁻ + OH⁻ + Fe³⁺). The extreme and indiscriminatory reactivity of OH⁻ could easily explain the killing of β-cells by alloxan. In support of this hypothesis alloxan toxicity is counteracted in vivo by scavengers of OH⁻ (7) and by superoxide dismutase (10) and in vitro by such scavengers (9, 11) as well as by catalase and chelators of iron ions (9, 12, 13).

An extension of or an alternative to the free radical hypothesis of alloxan toxicity can be suggested on the basis of recent reports by Boquist (2, 3) that alloxan induces efflux of Ca²⁺ from mitochondria, since disturbance of cellular Ca²⁺ homeostasis may eventually lead to cell death. How alloxan induces Ca²⁺ release from mitochondria is presently not known. Unspecific damage of mitochondria due to the formation of highly reactive oxygen species could result in Ca²⁺ release. On the other hand, mitochondria possess specific Ca²⁺ release pathways (14) which could conceivably be opened by alloxan. Their regulation is not fully understood. However, experiments from this and other laboratories indicate that the redox state and hydrolysis of mitochondrial pyridine nucleotides (15–21) and thiols (22–25) could be important in the regulation of Ca²⁺ release from mitochondria of nonexcitable tissue, notably liver.

Besides reacting with thiols, alloxan causes nonenzymatic oxidation of pyridine nucleotides (9). In addition, several enzymes such as NAD(P)H dehydrogenase (quinone) (26), the thioreductase system (27), and the glutathione system (16) could link alloxan or its metabolites in mitochondria to the redox state of pyridine nucleotides. We report here that in vitro alloxan causes Ca²⁺ release from intact rat liver mitochondria. Redox cycling of alloxan does occur but is not significantly involved in Ca²⁺ release. Oxidation of pyridine nucleotides and their hydrolysis, possibly in conjunction with oxidation of critical sulfhydryl groups, seem to be the key events of alloxan-dependent Ca²⁺ release. Disturbance of cellular Ca²⁺ homeostasis may partly explain alloxan toxicity.

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**EXPERIMENTAL PROCEDURES**

Isolation of Mitochondria—Female Wistar rats (180–250 g) were fasted overnight and killed by decapitation. Liver mitochondria were isolated by a conventional differential centrifugation method (28) using 210 mM mannitol, 70 mM sucrose, 5 mM Hepes, pH 7.4 (MSH buffer), and 1 mM EDTA as isolation medium. Mitochondria were washed twice in MSH buffer. The protein content of the final mitochondrial suspension was determined by the biuret method with bovine serum albumin as standard.

Labeling of Mitochondrial Pyridine Nucleotides in Vivo—Overnight fasted rats were injected intravenously with [carboxyl-14C]nicotinic acid (54 nmol/mg of protein) and 0.223 pmol. After 3 h, the animals were killed and liver mitochondria isolated (17).

**Standard Incubation Procedure**—Mitochondria, 2 mg of protein/ml, were incubated at 25 °C in 3 ml of MSH buffer under constant stirring. A fine jet of oxygen was blown onto the surface of the suspension to prevent anaerobiosis except during O2 consumption measurements. Reduction of mitochondrial pyridine nucleotides and release of endogenous Ca2+ was induced by addition of 5 mM rotenone. Thereafter, when appropriate, Ca2+ was added or, to prevent possible reuptake of Ca2+, EGTA was added. Mitochondria were then energized with 2.5 mM K+-succinate. When appropriate, Ca2+ uptake was allowed to proceed for 2 min. As determined spectrophotometrically, Ca2+ uptake at this time was complete at all loads. The final Ca2+ loads given under “Results” represent the sum of the endogenous and added Ca2+. Finally, alloxa and 100 µM t-butylhydroperoxide were added (time zero).

**Determination of Endogenous Ca2+**—Mitochondria, 2 mg of protein/ml, were incubated in MSH buffer containing 50 µM purified arsenazo III. Release of endogenous Ca2+ was induced with 5 mM rotenone or 1.5 mM carbonyl cyanide m-chlorophenylhydrazone. The resulting absorbance change of arsenazo III at 685-675 nm monitored by dual wavelength spectrophotometry was calibrated by addition of small amounts of Ca2+. The endogenous Ca2+ ranged from 14 to 20 nmol/mg of protein.

**Determination of Ca2+ Uptake and Release by Mitochondria**—Ca2+ movements across the inner mitochondrial membrane were followed by dual wavelength spectrophotometry (see above) or by the isotope technique (29). For the latter, mitochondria were incubated according to the standard procedure using “Ca2+” (700 dpm/nmol). At zero time, 0.5 mM EGTA alone or together with 2 mM alloxa was added. To determine the time course of the intramitochondrial [Ca2+] changes, 150-µl aliquots were withdrawn at the times indicated, filtered through Millipore filters (0.45-µm pore size), and rinsed twice with 150 µl of cold MSH buffer. The radioactivity remaining on the filters was determined in a liquid scintillation counter.

**Determination of Nicotinamide Release**—Pyridine nucleotides were labeled in vivo (17). Mitochondria from the livers of these animals were incubated according to the standard procedure, and at zero time alloxa alone or together with the indicated compounds was added. Release of nicotinamide was followed by determining the remaining intramitochondrial radioactivity with the above Millipore filtration technique.

**Determination of Δψ**—This was done as reported previously (30). Mitochondria were incubated under standard conditions in MSH buffer, 20 mM KCl, and 20 mM tetrathenephosphonium.

**Determination of Oxygen Consumption**—Mitochondria were incubated under standard conditions in MSH buffer containing 20 mM KCl. Total volume was 1 ml. O2 consumption was determined with a Clark-type electrode. The respiratory control index of alloxa-treated mitochondria was determined as follows. Mitochondria were incubated under standard conditions in the presence of Ca2+. 5 and 10 m after the addition of alloxa and EGTA, 3 ml of ice-cold MSH buffer were added. The mitochondria were immediately centrifuged at 10,000 × g for 10 min. The pellet was resuspended in MSH buffer containing 0.5 mM EDTA, 20 mM KCl, 0.5 mM phosphate, and 0.5 mg/ml bovine serum albumin to a final concentration of 0.5 mg mitochondrial protein/ml. The respiratory control index was then determined at 25 °C according to Estabrook (31).

**Alloxan-induced Ca2+ Release from Mitochondria**

Alloxan at 0.5, 1, and 5 mM induces Ca2+ release from energized mitochondria loaded with a fixed amount of Ca2+ (54 nmol/mg of protein); 2 mM alloxa is slightly more effective than 100 µM t-butylhydroperoxide (Fig. 1A). At a fixed amount of alloxa the rate and onset of Ca2+ release also depend on the amount of Ca2+ preaccumulated by mitochondria (Fig. 1B). At all Ca2+ loads the release is biphasic consisting of a clearly distinct slow and fast phase. In the absence of alloxa no net release of Ca2+ is observed under these conditions. When ruthenium red, an inhibitor of mitochondrial Ca2+ uptake is added to Ca2+-loaded mitochondria, an immediate and slow release of Ca2+ is observed (Fig. 2A). When 2 mM alloxa is added instead of ruthenium red, again significant net release of Ca2+ is observed only after some time lag. When alloxa is added together with ruthenium red, Fig. 1. Alloxan-induced release of Ca2+ from rat liver mitochondria. A, mitochondria were loaded with 54 nmol of Ca2+/mg of protein. At the arrow, Ca2+ release was initiated by the addition of 1, 2, or 5 mM alloxa (All) or 100 µM t-butylhydroperoxide (BuOOH). B, mitochondria were loaded with the indicated amounts of Ca2+ (numbers in parentheses indicate nmol/mg of protein), and, at the arrow, Ca2+ efflux was initiated with 2 mM alloxa.
Alloxan-induced Ca\(^{2+}\) Release from Mitochondria

Ca\(^{2+}\) release is both immediate and fast. The maximal release rate in this case is between that seen in the presence of ruthenium red or alloxan alone. Very similar results have been obtained for the t-butylhydroperoxide-induced Ca\(^{2+}\) release (33). These results suggest that, in the absence of ruthenium red, the alloxan-induced Ca\(^{2+}\) release is initially followed by a fast Ca\(^{2+}\) reuptake ("Ca\(^{2+}\) cycling") until at some critical point, depending on Ca\(^{2+}\) cycling and lowering of \(\Delta \phi\) (see below), release becomes faster than reuptake and consequently net Ca\(^{2+}\) release is observed.

Since there is disagreement on whether ruthenium red is an effective inhibitor of Ca\(^{2+}\) uptake at low \(\Delta \phi\) (34, 35), EGTA was also used in place of ruthenium red to minimize reuptake of Ca\(^{2+}\). The presence of EGTA required that \(^4\)Ca\(^{2+}\) be used for the determination of Ca\(^{2+}\) release. Again, release in the presence of EGTA alone is immediate and slow and that in the presence of EGTA and alloxan is faster (Fig. 2B).

Alloxan up to 5 mM, added to mitochondria 1 min before succinate, does not affect the initial rate of Ca\(^{2+}\) (54 nmol/mg of protein) uptake (results not shown).

Hydroperoxides like t-butylhydroperoxide or H\(_2\)O\(_2\), which are known to release Ca\(^{2+}\) from rat liver mitochondria (16-18, 30, 33), are reduced in liver mitochondria by pyridine nucleotides via glutathione peroxidase, glutathione reductase, and the energy-linked transhydrogenase (16-18). If alloxan were to induce Ca\(^{2+}\) release by forming H\(_2\)O\(_2\) secondary to redox cycling, its effect should, similar to the hydroperoxide-induced release, be sensitive to alterations of the glutathione enzyme cascade. This is not the case (Fig. 3, A, B, and C). Incubation of mitochondria for 30 min in the presence of 100 \(\mu\)M BCNU, an inhibitor of glutathione reductase (35), lowered the activity of this enzyme from 27.3 to 15.0 nmol of NADPH oxidized/min and mg of protein. Such pretreatment of mitochondria with BCNU slows the t-butylhydroperoxide-induced Ca\(^{2+}\) release considerably, whereas the alloxan-dependent release is affected very little (Fig. 3A).

Selenium deficiency lowers the activity of mitochondrial glutathione peroxidase drastically (16). In mitochondria isolated from selenium-deficient rats (Fig. 3B), 2 mM alloxan is about as effective as in control mitochondria (see Fig. 1A) while the Ca\(^{2+}\) release induced by t-butylhydroperoxide is much delayed in selenium-deficient mitochondria. Since there is disagreement on whether ruthenium red is an effective inhibitor of Ca\(^{2+}\) uptake at low \(\Delta \phi\) (34, 35), EGTA was also used in place of ruthenium red to minimize reuptake of Ca\(^{2+}\). The presence of EGTA required that 45Ca\(^{2+}\) be used for the determination of Ca\(^{2+}\) release. Again, release in the presence of EGTA and alloxan is faster (Fig. 2B).

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Alloxan-induced Ca$^{2+}$ Release from Mitochondria

When selenium-deficient mitochondria are treated with BCNU (Fig. 3C), again alloxan is as effective in causing Ca$^{2+}$ release as in control mitochondria, whereas release induced by t-butylhydroperoxide is even further retarded.

Added superoxide dismutase (32 µg/ml) had no influence on the alloxan-induced Ca$^{2+}$ release. 200 µM ATP, known to prevent the t-butylhydroperoxide-induced Ca$^{2+}$ release (18), increased the time lag between alloxan addition and the onset of Ca$^{2+}$ release by a factor of two and decreased the Ca$^{2+}$ release rate to 40% of the control (not shown).

Oxidation of mitochondrial pyridine nucleotides can accompany Ca$^{2+}$ release from intact rat liver mitochondria, as first shown by Lehninger's group (15). Nonenzymatic oxidation of NADPH by alloxan has been reported previously (9). We confirm this and, in addition, conclude that NADP$^+$ is formed in this reaction since glucose 6-phosphate plus glucose-6-phosphate dehydrogenase caused reduction back to NADPH as measured spectrophotometrically (results not shown). When alloxan and NADPH were mixed at 80 µM each, NADPH was oxidized at a rate of 0.34 µM/min. A nonenzymatic oxidation of 80 µM NADPH by t-butylhydroperoxide (100 µM) could not be detected.

In Fig. 4 the alloxan-induced absorption changes at 340-370 nm, largely caused by oxidation of mitochondrial pyridine nucleotides, are shown. When mitochondria have been depleted of Ca$^{2+}$ (Fig. 4A), 3.5 and 5 mM but not 2 mM alloxan cause absorption decrease. In the presence of Ca$^{2+}$ (Fig. 4B), alloxan causes a decrease of absorption even at 2 mM. The rate and the extent of the absorption change increase when going from 2 to 5 mM. At a given concentration of alloxan, the absorption change is slowest when no Ca$^{2+}$ is added to mitochondria and becomes progressively faster and slightly larger with increasing Ca$^{2+}$ loads. When Ca$^{2+}$ cycling is prevented by EGTA, the absorption change is very slow and significantly smaller as compared to the corresponding trace under conditions of Ca$^{2+}$ cycling. In the absence of EGTA, with both 2 and 5 mM alloxan, the initial decrease in absorption is followed by an increase. This increase most likely does not reflect re-reduction of pyridine nucleotides since in these experiments pyridine nucleotides are virtually completely hydrolyzed (see below). The increase may reflect the formation of unknown reaction products with an absorption maximum at 350 nm formed from alloxan or its metabolites and sulfhydryl compounds (4). As compared to the controls, treatment of mitochondria with BCNU (Fig. 4C) or selenium deficiency (Fig. 4D) do not influence the alloxan-induced absorption change but slow down the t-butylhydroperoxide-induced oxidation of pyridine nucleotides drastically.

For the t-butylhydroperoxide-induced Ca$^{2+}$ release, oxidation of pyridine nucleotides is a necessary but not sufficient event. Rather, only if oxidized pyridine nucleotides are hydrolyzed, an event which is inhibited by ATP, is Ca$^{2+}$ release observed (18). A possible alloxan-dependent intramitochon-

![Fig. 4. Changes of redox level of mitochondrial pyridine nucleotides.](image-url)
mitochondrial pyridine nucleotide hydrolysis was, therefore, investigated by labeling pyridine nucleotides in vivo at the nicotinamide moiety and determining the release of nicotinamide from mitochondria, indicating hydrolysis (17). With 2 mM alloxan, the absence of Ca\(^{2+}\) no loss of radioactivity from mitochondria is observed. Virtually complete release of radioactivity is seen from mitochondria containing 16 or 56 nmol Ca\(^{2+}\)/mg of protein, the onset and rate of release being faster at the higher Ca\(^{2+}\) load. EGTA or ATP slow down the release of radioactivity (Fig. 5). In the presence of ruthenium red, release is somewhat more pronounced than in the presence of EGTA (not shown). Ca\(^{2+}\) cycling, therefore, favors release of radioactivity (Fig. 5). In the presence of ruthenium red, release is somewhat more pronounced than in the presence of EGTA (not shown). Ca\(^{2+}\) cycling, therefore, favors either directly or indirectly pyridine nucleotide hydrolysis. With 5 mM alloxan a faster but quantitatively similar release of radioactivity is observed except that hydrolysis takes place even in Ca\(^{2+}\)-depleted mitochondria.

The determination of \(\Delta\psi\) on the basis of movements of the lipid soluble cation tetrathenylphosphonium has proven useful to measure the energy state of mitochondria and to assess the intactness of mitochondria during and after the hydroperoxide-induced Ca\(^{2+}\) release (17, 30, 33). Addition of alloxan to energized mitochondria (Fig. 6) leads to an instantaneous small drop of \(\Delta\psi\), possibly indicating active uptake of alloxan by mitochondria. In Ca\(^{2+}\)-depleted mitochondria, this drop is followed by a transient decrease of \(\Delta\psi\), the rate and extent of which are dependent on the amount of alloxan added (Fig. 6A), reaching a minimal value of 174 and 161 mV for 2 and 5 mM alloxan, respectively. The decrease is not affected by 0.2 mM ATP (not shown). A very similar response to 2 mM alloxan is seen in mitochondria loaded with 16 nmol of Ca\(^{2+}\)/mg of protein, provided EGTA is given simultaneously with alloxan to prevent Ca\(^{2+}\) cycling (Fig. 6B). At a load of 55 nmol of Ca\(^{2+}\)/mg of protein, in the presence of EGTA the decrease induced by alloxan is more pronounced, \(\Delta\psi\) reaching a minimum of about 120 mV. The decrease is again transient, and mitochondria are fully energized after about 9 min as shown by their sensitivity to the uncoupler carbonyl cyanide m-chlorophenylhydrazone (Fig. 6C). Fig. 6, D and E, shows the influence of Ca\(^{2+}\) cycling on the energy state of mitochondria. After loading with 55 nmol of Ca\(^{2+}\)/mg of protein, release is induced with 2 mM alloxan, and EGTA is added to stop Ca\(^{2+}\) cycling. Mitochondria that cycle Ca\(^{2+}\) for up to 5 min are still capable of rebuilding a \(\Delta\psi\) of 180 mV upon addition of EGTA, whereas cycling for 10 min damages mitochondria completely as judged by their inability to restore \(\Delta\psi\) upon addition of EGTA.

Comparison of Figs. 2 and 6 shows that the alloxan-induced Ca\(^{2+}\) release is not due to unspecific damage of mitochondria as long as excessive Ca\(^{2+}\) cycling is prevented. Yet, alteration of mitochondrial functions in the presence of alloxan and Ca\(^{2+}\) cannot be totally excluded as shown by the following observation. Mitochondria loaded with 54 nmol of Ca\(^{2+}\)/mg of protein and exposed to 2 mM alloxan together with EGTA lost their ability to synthesize ATP. The respiratory control index dropped from a control value of 2.42 at time zero min to 1.54 and 1.00 within 5 and 10 min, respectively. In contrast,
Ca\textsuperscript{2+}-depleted mitochondria fully maintained for at least 10 min their ability to synthesize ATP when exposed to 2 mM alloxan.

In the presence of 5 mM alloxan, rat liver mitochondria depleted of Ca\textsuperscript{2+} consume slightly less O\textsubscript{2} than in the absence of alloxan (Fig. 7A). When mitochondria were first loaded with Ca\textsuperscript{2+} (54 nmol/mg of protein), alloxan causes a greatly increased O\textsubscript{2} consumption both in the presence and absence of EGTA (Fig. 7A). Therefore, and in agreement with the results of Boquist (2), mitochondrial respiration is clearly not inhibited by alloxan.

Reduction of alloxan to dialuric acid and subsequent autoxidation should result in redox cycles and O\textsubscript{2} consumption other than that by cytochrome oxidase. To assess possible redox cycling of alloxan in mitochondria, O\textsubscript{2} consumption by the respiratory chain was blocked by 200 \mu M cyanide. Results are shown in Fig. 7B. 5 mM alloxan, when given briefly after cyanide, causes significant O\textsubscript{2} consumption by mitochondria. The extent and rate of extra O\textsubscript{2} consumption are the same when alloxan is added to mitochondria at an O\textsubscript{2} concentration of 200 and 50 \mu M indicating no limitation of redox cycling by O\textsubscript{2} (results not shown). The alloxan-dependent O\textsubscript{2} consumption is only transient. This can be shown (Fig. 7B) by addition of cyanide at various intervals after alloxan. For example, with 5 mM alloxan and cyanide given simultaneously, about 45 nmol of O\textsubscript{2} per mg of protein is consumed whereas very little extra O\textsubscript{2} is consumed when cyanide is added about 7 min after alloxan.

**DISCUSSION**

Many compounds induce Ca\textsuperscript{2+} release from rat liver mitochondria. With a few exceptions they do so by de-energizing mitochondria. Examples are uncouplers, respiratory inhibitors, and substances that cause nonspecific damage to mitochondria. In contrast, release of Ca\textsuperscript{2+} induced by acetocetate or oxaloacetate (15, 21, 25, 37), by hydroperoxides (16-18, 33), and by menadione (2-methyl-1,4-naphthoquinone) (19, 20) is paralleled by oxidation of mitochondrial pyridine nucleotides and leaves mitochondria intact. Upon exposure to alloxan, rat liver mitochondria release Ca\textsuperscript{2+} and, when Ca\textsuperscript{2+} cycling is prevented by EGTA, retain their ability to build up a high Δψ. In the absence of EGTA, restoration of Δψ is no longer achieved after 10 min. From these results we conclude that alloxan, similar to the above-mentioned compounds, induces Ca\textsuperscript{2+} release from intact mitochondria and that an eventually observed damage of mitochondria in vitro is due to excessive Ca\textsuperscript{2+} cycling, i.e. damage is the consequence of and not the cause for Ca\textsuperscript{2+} release.

Some specific changes do, however, take place in mitochondria in the presence of both alloxan and Ca\textsuperscript{2+}, as shown by the decrease of the respiratory control index. Alloxan alone does not influence the respiratory control index. The site of inhibition of ATP synthetase during the combined action of alloxan and Ca\textsuperscript{2+} is not known. It may be speculated that sulfhydryl groups are involved, since alloxan is a sulfhydryl reagent (4). This could also explain the Ca\textsuperscript{2+} requirement for the inhibition of ATP synthetase, since the transport of Ca\textsuperscript{2+} is energy dependent, and energy-dependent variations of the reactivity of sulfhydryl groups occur in mitochondria (38). It is worth noting here that the reaction of alloxan with sulfhydryl groups can be highly specific. For example, alloxan reacts with the thioredoxin system but not with NADPH-glutathione reductase (27).

For mitochondrial Ca\textsuperscript{2+} release, two pathways must be considered. The different kinetics of release in the presence or absence of EGTA or ruthenium red as well as the quanti-
tative determination of $\Delta \psi$ allow us to distinguish between release via reversal of the electrogenic uptake route ("uniport") and via a distinct electroneutral "antiport" (39). A demonstration of a distinct carrier for Ca$^\text{2+}$ efflux is that selective inhibition of the uniport causes a net efflux of accumulated Ca$^\text{2+}$ (40). For acetocetate, oxaloacetate, and hydroperoxides Ca$^\text{2+}$ release has been indicated to occur via the antiport. According to Nichols (41) release of Ca$^\text{2+}$ via the other pathway, namely by reversal of the uniport (thermodynamic control), may become important at $\Delta \psi$ values of about 130 mV and lower. In the absence of EGTA and ruthenium red, i.e. when Ca$^\text{2+}$ cycling is possible, alloxan addition leads to a decrease of $\Delta \psi$ with time, and, after some lag, to a fast net release of Ca$^\text{2+}$. In contrast, when alloxan is added together with EGTA or ruthenium red to prevent Ca$^\text{2+}$ cycling, Ca$^\text{2+}$ is released at a slower and linear rate and decreases only transiently with a minimal value of 120 mV. Since Ca$^\text{2+}$ release occurs in the presence of ruthenium red and since $\Delta \psi$ does not fall below 120 mV under noncycling conditions we conclude that most of the alloxan-induced Ca$^\text{2+}$ release indeed occurs via the antiport as long as Ca$^\text{2+}$ cycling is inhibited. Interestingly, not a transient decrease but rather an instantaneous increase of $\Delta \psi$ had been observed upon simultaneous addition of EGTA and t-butylhydroperoxide (instead of alloxan) to Ca$^\text{2+}$-loaded mitochondria (33) due to the sudden relief of mitochondria from Ca$^\text{2+}$ cycling and subsequent Ca$^\text{2+}$ release. The transient decrease of $\Delta \psi$ in the presence of alloxan and EGTA may be due to redox cycling of this compound as indicated by the extra O$_2$ consumption in the presence of cyanide under these conditions (see below).

Similarly to the Ca$^\text{2+}$ release induced by hydroperoxides (16, 18, 30) or menadione (19, 20), the alloxan-induced Ca$^\text{2+}$ release is accompanied by oxidation and hydrolysis of mitochondrial pyridine nucleotides. However, the mechanisms by which the hydroperoxide- and alloxan-induced oxidation of pyridine nucleotides occurs are different. Oxidation of pyridine nucleotides by alloxan in the test tube also makes nonenzymatic oxidation of intramitochondrial pyridine nucleotides likely. Evidence in favor of enzyme-catalyzed pyridine nucleotide oxidation in mitochondria by alloxan or metabolites derived thereof was not obtained in the present study. In contrast to hydroperoxides, oxidation induced by alloxan does not involve glutathione peroxidase or glutathione reductase to a significant extent since lowering of the activity of these enzymes has no influence on pyridine nucleotide oxidation by alloxan. This indicates that alloxan does not oxidize pyridine nucleotides indirectly by formation of hydroperoxides.

Enzymatic hydrolysis of pyridine nucleotides in Ca$^\text{2+}$- loaded mitochondria has recently been reported by us (16-18). Also in the presence of alloxan, mitochondrial pyridine nucleotides are hydrolyzed. Interestingly, 5 mM alloxan causes oxidation and hydrolysis also in Ca$^\text{2+}$-depleted mitochondria. This observation is in line with the Ca$^\text{2+}$ insensitivity of the NAD$^+$ glycohydrolase in submitochondrial particles (42).

Both the hydrolysis of pyridine nucleotides and Ca$^\text{2+}$ release induced by alloxan are strongly inhibited by ATP. Similarly, pyridine nucleotide hydrolysis and Ca$^\text{2+}$ release but not pyridine nucleotide oxidation induced by t-butylhydroperoxide in mitochondria are prevented by ATP (18). ATP also prevents Ca$^\text{2+}$ release induced by menadione (19). Since ATP also inhibits the NAD$^+$ glycohydrolase activity and (ADP)-ribosylation in submitochondrial particles (42), a close interrelationship between Ca$^\text{2+}$ release, ATP, and (ADP)-ribosylation emerges.

Quinones serve as substrates for many flavoenzymes, including NADH-ubiquinone oxidoreductase, and can undergo either a direct 2-electron reduction to the hydroquinone or a 1-electron reduction to the semiquinone radical (26, 43-45). In the presence of O$_2$ most semiquinonones rapidly autoxidize with the formation of the superoxide anion radical ($O_2^-$) and thus regenerate the quinone (46). Superoxide radicals dismutate to yield H$_2$O$_2$ and O$_2$. Along this line, redox cycling of alloxan has been reported to cause alloxan toxicity in vivo (cf. introduction). That alloxan can undergo redox cycling to some extent also in isolated rat liver mitochondria is indicated by the extra alloxan-induced O$_2$ consumption in mitochondria, the respiratory chain of which is blocked by cyanide. Yet, hydroperoxides formed by redox cycling of alloxan do not play a significant role in Ca$^\text{2+}$ release as indicated by the insensitivity of alloxan-induced release to decreased activities of glutathione peroxidase and reductase. Also OH$^-$ radicals, thought to be highly damaging due to their extreme and indiscriminate reactivity, are not responsible for Ca$^\text{2+}$ release since, as shown here, release takes place from intact mitochondria. Interestingly, both the alloxan-dependent extra O$_2$ consumption and the decrease of $\Delta \psi$ in Ca$^\text{2+}$-depleted mitochondria are transient and of similar duration, possibly indicating a transient diversion of electrons by alloxan from the respiratory chain to $O_2$ with the formation of $H_2O_2$.

At variance to the report of Nelson and Boquist (2), measurements of $\Delta \psi$ and O$_2$ consumption rule out uncoupling unspecific damage or inhibition of succinate utilization as the cause of Ca$^\text{2+}$ release. Rather, release occurs via the specific antiport. How could this antiport be opened by alloxan? The studies by the groups of Lehninger, Orenius, and ourselves (15-20, 37) indicate that oxidation of pyridine nucleotides, possibly in conjunction with the oxidation of critical sulfhydryl groups, and hydrolysis of pyridine nucleotides are the key events that allow Ca$^\text{2+}$ release from rat liver mitochondria. Here it is shown that alloxan causes oxidation and hydrolysis of mitochondrial pyridine nucleotides. Alloxan can also lower the glutathione content in isolated mitochondria (47). However, lowering of the mitochondrial glutathione alone cannot be responsible for Ca$^\text{2+}$ release, since 12.5 nmol of N-ethylmaleimide/mg of protein, known to lower mitochondrial glutathione to about 11% of the control value (48), does not induce Ca$^\text{2+}$ release from rat liver mitochondria. Furthermore, inhibition of intramitochondrial glutathione reductase enhances the shift of glutathione from the reduced to the oxidized form upon addition of t-butylhydroperoxide. Under these conditions Ca$^\text{2+}$ release is retarded.

This and previous studies allow us to propose a mechanism of the alloxan-induced Ca$^\text{2+}$ release from rat liver mitochondria which involves pyridine nucleotides and possibly sulfhydryl groups. The common denominator of the chemically unrelated substances acetocetate, oxaloacetate, hydroperoxides, menadione, and alloxan is their ability to cause intramitochondrial oxidation and hydrolysis of pyridine nucleotides and release of Ca$^\text{2+}$. In addition, both pyridine nucleotide hydrolysis and Ca$^\text{2+}$ release are similarly inhibited by ATP. This gives strong circumstantial evidence that these substances cause Ca$^\text{2+}$ release by ultimately the same mechanism and that pyridine nucleotide hydrolysis is part of this mechanism. The present findings are compatible with our working hypothesis (18, 42) that protein (ADP)-ribosylation, which is observed upon hydrolysis of NAD$^+$ by inner mitochondrial membranes and which is inhibited by ATP (42), could ultimately trigger Ca$^\text{2+}$ release.

Disturbance of the cellular Ca$^\text{2+}$ homeostasis may eventu-

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S. Baumhüter and C. Richter, unpublished observation.

 Ally lead to cell death. Alloxan is taken up by mitochondria in vivo (49) and induces Ca\(^{2+}\) release from mitochondria in vitro. Further studies are needed to test the attractive hypothesis that diabetes can be caused in at least some instances by a disturbed Ca\(^{2+}\) homeostasis in the pancreatic \(\beta\)-cell.

REFERENCES

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