

The Mechanism of Alloxan Toxicity: an Indication for Alloxan Complexes in Tissues and Alloxan Inhibition of 4-Acetamido-4'-Isothiocyanato-Stilbene-2,2'-Disulphonic Acid (SITS) Binding for the Liver Cell Membrane*

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Received: July 5, 1974, and in revised form: October 4, 1974

Summary. It is shown that alloxan inhibits binding of SITS to liver cells. This indicates the cell membrane as a site of alloxan action. Alloxan is found to react with tissues to form complexes that are detectable up to 3 hrs after alloxan treatment. On the basis of the present findings, an

assumption is made that alloxan inhibits a cell membrane processes by blockade of functionally important groups.

Key words: Alloxan, tissues, complexes, inhibition, binding SITS, assay, diphosphopyridine nucleotides, liver, kidney.

Although alloxan is widely used for induction of experimental diabetes, the mechanism of its diabetogenic activity and of its toxicity has so far remained unsettled.

Alloxan appears to be selectively toxic to the pancreatic beta-cells producing their necrosis [8]. At higher doses alloxan produces necrotic changes in other tissues as well [8, 10]. However, at subdiabetogenic doses alloxan produces ultrastructural beta-cell changes that are quite similar in character to those observed after large doses [14]. These studies suggest that alloxan is toxic to tissues at any given dose, and that necrotic changes are related to tissue sensitivity and to amounts of alloxan injected.

Although morphological studies provide evidence for cell membrane damage [14], no conclusion could be drawn in regard to the primary site of alloxan action. Alloxan was shown to react with sulfhydryl groups [12]. Recent studies with toad-fish islets suggest that alloxan acts at or near the sugar transport site [13]. A theory was put forward that alloxan toxicity is based on inactivation of some critical enzymes containing active sulfhydryl groups [8]. Selectivity of alloxan for the beta-cells was explained either by a selective accumulation of the agent [4], or by relative deficiency of protective sulfhydryl groups in the beta-cells [8].

The present results show that alloxan forms complexes with tissue constituents. To show this effect, it was necessary to develop a specific assay for alloxan in tissues. This is fully described. Furthermore, it is shown that alloxan inhibits binding of SITS for the liver cell membrane. This suggests the cell membrane

as a site of alloxan action. Also, it is shown that ratios of diphosphopyridine nucleotides in both liver and kidney remain constant over a few hours after alloxan treatment. This suggests that at least in these tissues energy metabolism is not primarily affected by alloxan.

Materials and Methods

Animals were albino rats of our random-bred Institute colony, that are descendents of Wistar rats. Animals were used at weights ranging from 180 to 250 g. Unless otherwise stated, all reagents were of analytical grade.

Alloxan Experiments

Double glass-redistilled water was used for preparation of buffer solutions. These were charcoal-treated and kept frozen when not in use. Concentrated citrate-phosphate buffer pH 7 was made by mixing 1 molar citric acid and 2 molar K_2HPO_4 . The acidity was adjusted using a glass electrode. Citrate-phosphate buffer pH 4 was prepared from 0.1 molar citric acid and 0.2 molar K_2HPO_4 . This was used for preparation of alloxan solutions and for washing of talc columns after sample applications.

Alloxan solutions were prepared daily by dissolving alloxan in cold citrate-phosphate buffer pH 4. orto-Phenylenediamine of chromatographic grade was dissolved in 0.1 normal HCl just prior to use at a concentration of 10 mg/ml.

Talc of medical grade was used. It was repeatedly suspended in water and fines removed by aspiration. Talc was dried in an oven and stored at room tem-

* Supported by the Federal Research Fund

perature. Florisil 200—400 mesh was prepared identically.

A day before use talc was suspended in water at a 10% concentration (w/v). While keeping talc in suspension with a magnetic stirrer, 2 ml of the suspension was transferred into a glass column, 6×100 mm, having a Whatman fiber-glass disk as support. The talc was allowed to settle by letting water run out. Florisil was packed as such by pouring 100 mg of the adsorbent into each column.

Under ether anesthesia, the right jugular vein was exposed and alloxan injected at a dose of 60 mg/kg body weight. Then a gastrocnemius muscle was exposed and the abdominal cavity opened by a medial incision. At times indicated in the experiment, tissues were sampled in the following order: kidney, pancreas, duodenal loop, liver, gastrocnemius muscle and lungs. A thread loop was placed around the left kidney hilus and tied. The upper pole was cut off and freeze-clamped with tongs precooled in liquid nitrogen. Then samples of other tissues were taken and cooled as well. Samples were kept in solid CO_2 , until weighing and homogenization were completed.

Each sample was homogenized in ice-cold water at a 5% tissue concentration (w/v) using a glass-glass homogenizer. Homogenates were deproteinized with perchloric acid at a final concentration of 0.3 Normal. After centrifugation at 3000 rpm, each supernatant was passed through a Florisil column in order to reduce native tissue fluorescence. Acidity was adjusted to pH 4 by adding a predetermined volume of concentrated citrate-phosphate buffer pH 7. To this extract ortho-phenylenediamine in a 0.01 ml volume per ml was added. After 2 hrs of incubation at room temperature in dark, extracts derived from alloxan-treated tissues showed blue-greenish fluorescence when exposed to 365 nm light. In order to make measurements specific for alloxan, the alloxan condensation product (alloxan quinoxaline) was isolated by passing each incubate through a talc column. A band of blue-greenish fluorescence appeared on the top of adsorbent, whereas nonspecific condensation products showing blue fluorescence passed through. The band was rapidly eluated using 3 ml of slightly alkaline media, for instance 20 mM K_2HPO_4 . A blank for each sample was provided by subsequently eluting the column with additional 3 ml of the eluent.

To test the reliability of this method of measuring alloxan in tissues, alkaline eluates were examined by chromatography on both Silica Gel G (Merck) and Chromar Sheet 500 (Mallinckrodt), which is 70% silicic acid and 30% glass fibers. Developing solvents were buthanol, ethanol, acetic acid (50:20:10 v/v) for the former and 0.1 molar acetate buffer pH 4.6,

methanol (50:50 v/v) for the latter system. Eluates derived from alloxan-treated homogenates or from tissues of alloxan-treated rats gave a single fluorescent spot in either chromatography, which fully corresponded to R_f values of standard alloxan quinoxaline. R_f values were 0.62 and 0.94 on Silica Gel G and Chromar Sheet, respectively.

SITS Experiments

Fluorescence measurements of this dye is based on observations that the dye is practically nonfluorescent at pH values below 12, and highly fluorescent in 2 Normal KOH (Marinetti and Gray 1967). Preliminary tests were made using liver tissue homogenized in 20 mM phosphate buffer pH 7.5. To this the dye was added, and after 5 min of incubation precipitation was performed with perchloric acid. The precipitate showed weak fluorescence when dissolved in glycine-NaOH buffer pH 12 (50 mM in respect to glycine), and high fluorescence when dissolved in 2 Normal KOH.

Rats were decapitated and their abdominal cavities opened; 10 mls of ice-cold Krebs-Ringer-Phosphate buffer containing 100 mg/100 ml glucose was injected into the liver through the portal vein. The middle lobe was removed, washed in Krebs-Ringer-Phosphate, blotted on filter paper and weighed. The tissue was cut into small pieces and pressed through a nylon mesh (pore size 1×1 mm) stretched over a 50 ml beaker. The tissue concentration was adjusted to 5% (w/v) with the buffer. The tissue suspension was centrifuged at 1000 rpm for 1 minute. The supernatant was transferred and centrifuged again at 3000 rpm at 4° for 5 min. The pellet obtained was suspended in the buffer to give a 10% tissue concentration on the basis of initial tissue weight. The cell suspension was distributed in 2 ml volumes into plastic vials for incubation. Further details are given under the experiment.

Binding of SITS for liver cells was followed by transferring samples at times indicated in the experiment into 5 ml volumes of 0.3 normal perchloric acid. After centrifugation the pellet was suspended in 5 ml of the acid and centrifuged again. This pellet was dissolved in 3 ml of the glycine-NaOH buffer. A 0.5 ml volume was transferred into a fluorometer cell and made up to 1 ml with the glycine-NaOH buffer. This served as a blank. A 0.5 ml volume was transferred into another cell and made up to 1 ml with 5 Normal KOH. This served as a sample.

Diphosphopyridine Nucleotide Measurements

These were measured by the enzymatic cycling procedure. Under light ether anesthesia, at times in-

licated in the experiment, tissues were frozen in situ using a clamp precooled in liquid nitrogen, and processed as recommended. Enzymes used were of Boehringer, Mannheim, Germany.

Fluorometry

A Farrand ratio fluorometer was used. Filter selections were as follows: In all measurements, excitation light was isolated using a 365 nm narrow-band inter-

ference filter. Fluorescence light was isolated using a 511 nm narrow-band interference filter for alloxan quinoxaline, a Wratten 58 filter (peaks at 525 nm) for SITS and a Wratten 3 filter (sharp cut-off, passes wavelengths above 455 nm) for pyridine nucleotides. Fluorescence readings were made using 1 ml pyrex cells.

Results

Detection of Alloxan in Tissues

Using the present procedure alloxan was found for 3 hrs after alloxan administration in all tissues tested (Fig. 1). At half an hour after alloxan tissue concentrations ranged between 0.1 and 0.3 mg/kg of tissue, except in kidneys. In other words, it amounts to less than 1% of the dose given. Although these values seem to be low, they may be significant in regard to the mechanism of alloxan action. These results indicate that alloxan enters into reactions with tissues, otherwise it would not be possible to detect any because free alloxan undergoes fast decomposition with a half-life of less than one minute.

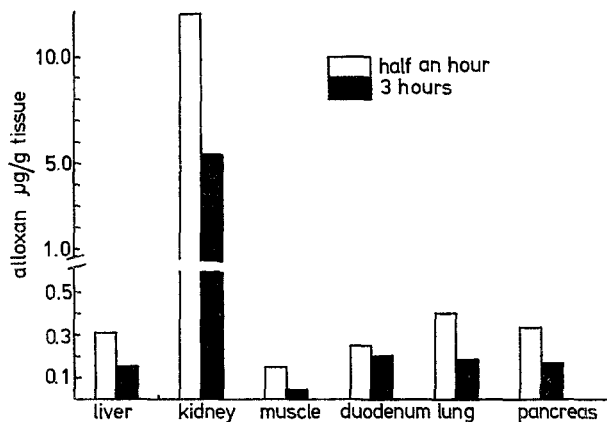


Fig. 1. Alloxan in rat tissues. Each value is a mean of 5 determinations

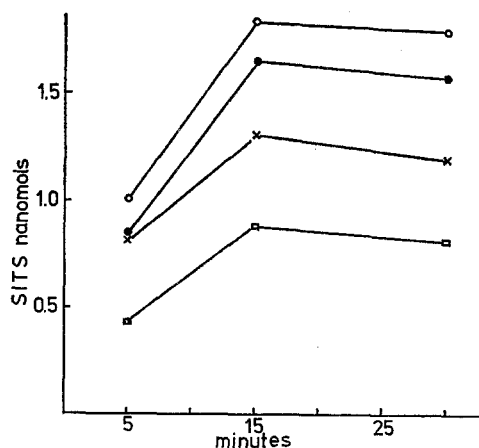


Fig. 2. Alloxan inhibition of SITS binding to liver cells. The different curves are: (o) control, (●) cells reacted with 0.01 mM alloxan, (x) cells reacted with 0.1 mM alloxan, (□) cells reacted with 1.0 mM alloxan. To 2 ml of the liver cell suspension in Krebs-Ringer-Phosphate buffer containing glucose 100 mg/100 ml, alloxan in 0.025 ml volumes was added to give concentrations as indicated. Incubation continued for 15 minutes at 37° with a shaking rate of 100 cycles per minute. Then SITS in a 0.025 ml volume was added to each vial to give a 0.1 mM concentration. Addition of SITS indicates time zero. Using a constriction pipette, a 0.1 ml volume of the cell suspension was transferred from each vial into 5 ml of 0.3 normal perchloric acid for assay of bound SITS. Each value is a mean from 3 incubation vials. The ordinate represents quantities of bound SITS per 0.1 ml cell suspension

Effect of Alloxan on Binding of SITS by Liver Cells

Both with and without prior reaction of liver cells with alloxan, binding of SITS reached saturation within the initial 15 min of incubation. When liver cells were reacted with different concentrations of alloxan, binding of SITS was inhibited depending on the alloxan levels used (Fig. 2). When liver cells were reacted with 1 mM alloxan, a 50% decrease in binding of SITS was observed.

Although the mechanism of SITS binding is unclear, there are indications that about 10% is bound covalently and the remainder noncovalently [3]. Further, it was suggested that SITS inhibits transport of anions through the cell membrane by reacting with positively charged groups, possibly with amino groups having high pK values. How alloxan inhibits binding of SITS, is not known. It is possible that alloxan reacts rapidly with these amino groups, thus transforming them chemically into unreactive ones, or alloxan reacts with these groups to form complexes, thus making them inaccessible to SITS.

Effect of Alloxan on Diphosphopyridine Nucleotides Concentration in Both Liver and Kidney Tissues

Results are given in Tab. 1. Although the number of observations is insufficient for statistical treatment, it is clearly seen that a ratio of these nucleotides in

both tissues remained more or less constant after alloxan treatment. This suggests that at least in these tissues energy metabolism is not primarily affected by alloxan.

However, in liver tissue, 10 min after alloxan treatment, there was a 20% decrease in total nucleotide levels. In kidneys no change was observed. Reasons for this inconsistency are unknown, although both tissues were shown to be sensitive to higher doses of alloxan.

Discussion

Alloxan was shown to react with free sulphhydryl groups of proteins and with both reduced glutathione and cystein [12]. Although the mechanism of this reaction has not been fully investigated, it seems that oxi-

in tissues over at least a 3 hr period after alloxan treatment. This is in contrast with what one might expect on the basis of alloxan behavior in buffers [11]. However, the present results are in agreement with the interpretation that alloxan reacts with tissues to form complexes. Hence it is recovered by acid treatment of tissue samples in the course of analytical procedure. An assumption is made that alloxan toxicity is based probably on blockade of functionally important groups.

The present results show that alloxan inhibits binding of SITS to liver cells. Since the dye does not penetrate the cell membrane [3, 7, 9], it is reasonable to conclude that alloxan acts at least at the level of cell membrane. The dye inhibits transport of anions through the cell membrane by reacting presumably

Table 1. *Diphosphopyridine nucleotides concentration (micromoles/kg tissue)*

Time after alloxan treatment	liver				kidney			
	DPN+	DPNH	Total	Ratio of DPN to DPNH	DPN+	DPNH	Total	Ratio of DPN to DPNH
control	680	108	788	6.2	520	22	542	23.0
	620	164	784	3.7	420	52	472	8.0
	612	68	680	9.0	496	34	530	14.0
	658	82	740	8.0	437	54	491	8.0
	630	70	700	8.7	460	70	510	6.6
10 minutes	520	78	598	6.6	480	60	540	8.0
	510	88	598	5.8	500	52	552	9.6
	538	75	613	7.1	520	60	580	8.6
30 minutes	520	84	604	6.2	482	32	514	15.0
	556	106	662	5.2	472	52	524	9.0
360 minutes	630	84	714	7.5	452	40	492	11.3
	620	88	708	7.0	434	46	480	9.4

dation of sulphhydryl groups takes place. In the course of this reaction, depending on incubation conditions, alloxan was shown to form complexes with reacting substances having absorption maxima either at 305 or 280 nm [12, 15]. Evidence was presented that a nitrogen base is involved in the reaction with alloxan [15].

It is of interest that, when using ortho-phenylenediamine as a reagent, alloxan could be recovered at least in part from alloxan-glutathione complexes¹. This suggests that alloxan has not undergone a marked chemical transformation while in complex form. In agreement with this interpretation are findings that prior glutathione treatment increased blood alloxan levels in alloxan-treated rats [1].

The present results show the existence of alloxan

with positively charged groups [3, 7]. Further, the dye inhibits binding of chloromercuribenzenep-sulphonic acid for exposed sulphhydryl groups on the cell membrane [6]. Therefore, these observations support an assumption that alloxan inhibits binding of SITS for the cell membrane by a mechanism that involves reactions with both amino and sulphhydryl groups.

It was shown that alloxan increased permeability of toad-fish islets, whereas various sugars may prevent or even reverse this effect [13]. Furthermore, it was shown that glucose given prior to alloxan protects rats against diabetes [16]. These studies suggest that alloxan acts at the sugar transport site. Since sulphhydryl, amino and imidazole groups were shown to be essential for sugar transport [2], an involvement of these groups in the reaction with alloxan is therefore presumed.

¹ unpublished observations

In the present studies a decrease in total levels of diphosphopyridine nucleotides in liver tissue was observed 10 min after alloxan treatment. This effect might be due to tissue edema including cell swelling. This is in agreement with the interpretation that alloxan affects the cell membrane functions including the transport processes, and therefore may induce disturbances in movements of both fluid and solutes through the membrane. However, both leakage of nucleotides due to cell membrane damage and increased degradation for some unknown reasons may account for this effect as well.

The mechanism of high toxicity of alloxan to the beta-cells is unknown. As already discussed, the present results support the concept that alloxan reacts with important chemical groups on the beta-cell membrane. High density of these groups on the surface of the beta-cells may account for their high sensitivity to alloxan. In agreement with this view are demonstrations that the beta-cell plasma membrane is rich in thiol groups [6], and that the beta-cells contain a carrier system for very rapid transport of glucose [5]. This assumes high density of glucose "channels" whose structure involves chemical groups capable of reacting with alloxan. Furthermore, as shown by microautoradiography, mouse islets in vivo concentrate labelled alloxan [4]. This finding may be interpreted either in terms of high density of alloxan reacting groups on the surface of the beta-cell or in terms of alloxan accumulation within the beta-cell. The latter possibility assumes that alloxan shares a carrier system operating long enough to produce alloxan accumulation. However, this seems to be unlikely because alloxan reacting with carriers' functional groups may well inactivate their function.

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