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Extensive Ca^{2+} Release from Energized Mitochondria Induced by Disulfiram

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Abstract

The effect of the alcohol-deterrent drug, disulfiram, on mitochondrial Ca^{2+} content was studied. Addition of this drug (20 μM) to mitochondria induces a complete loss of accumulated Ca^{2+}. The calcium release is accompanied by a collapse of the transmembrane potential, mitochondrial swelling, and a diminution of the NAD(P)H/NAD(P) radio. These effects of disulfiram depend on Ca^{2+} accumulation; thus, ruthenium red reestablished the membrane $\Delta\psi$ and prevents the oxidation of pyridine nucleotides. The binding of disulfiram to the membrane sulfhydryls appeared to depend on the metabolic state of mitochondria, as well as on the mitochondrial configuration. In addition, it is shown that modification of 9 nmol -SH groups per mg protein suffices to induce the release of accumulated Ca^{2+}.

Key Words: Disulfiram; antabuse; Ca^{2+} release; mitochondria; kidney.

Introduction

Disulfiram [*bis*(diethylthiocarbamoyl)disulfide] is a drug that has been long used as an alcohol deterrent. Investigations on its toxic effects have established tht it is an inhibitor of several enzyme systems, i.e., liver aldehyde dehydrogenase (Graham 1951), microsomal cytochrome P_{450} (Strip *et al.*, 1969), and dopamine β-hydroxylase (Serio *et al.*, 1984). In addition, it has been reported that administration of high doses of disulfiram (Dis) results in the production of cardiac arrhythmias (Fossa *et al.*, 1982). It has been concluded that the toxic effects of Dis are essentially related to its interaction with protein sulfhydryl groups (Kelner and Alexander, 1986).

There is a close mechanistic relationship between cardiac arrhythmias and cytoplasmic Ca^{2+} concentration (Valenzuela and Vasalle, 1983, 1985). On the other hand, the significance of membrane thiol groups in the

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mitochondrial permeability to Ca^{2+} release has been well documented (Ramachandran and Bygrave, 1978; Pfeiffer *et al.*, 1979; Beatrice *et al.*, 1980; Palmer and Pfeiffer, 1981; Vercesi, 1984; Chávez *et l.*, 1987; Chávez and Holguín, 1988). Taking into account the above statements, the present work was performed to examine the effect of Dis on the ability of the mitochondrial membrane to maintain a low permeability to Ca^{2+} efflux.

The results presented show that addition of 20 μM Dis to Ca^{2+}-loaded mitochondria results in a fast release of the accumulated cation. As Ca^{2+} efflux proceeds, there is an accompanying oxidation of pyridine nucleotides, collapse of membrane $\Delta\psi$, and mitochondrial swelling. These effects of Dis correlate with the binding of its derivative diethyldithiocarbamate (DDC) to the membrane, and are abolished by the addition of ADP. It is proposed that the arrhythmogenic effect of Dis could be produced through its Ca^{2+}-releasing action on mitochondria, which in turn, increases the cytoplasmic Ca^{2+} concentration.

Materials and Methods

Rat kidney mitochondria were prepared as described previously (Chávez *et al.*, 1985). Calcium movement was followed spectrophotometrically at 685–674 nm using the calcium indicator Arsenazo III (Kendrick, 1976). In some experiments, $^{45}CaCl_2$ (sp. act. 1000 cpm/nmol) was used. Mitochondrial transmembrane potential was assayed, as previously described by Akerman and Wikström (1976), in a dual-wavelength spectrophotometer at 533–511 nm, by using the dye safranine. Mitochondrial swelling was analyzed by the method reported by Brierley *et al.* (1968). Pyridine nucleotide oxidation was followed by dual-wavelength spectroscopy at 370–340 nm. DDC residue from disulfiram was determined with Ellman's reagent, 5, 5′-dithio-*bis*-(2-nitrobenzoic acid), at 412 nm as previously decribed by (Ellman, 1959), using cysteine as the standard for the calibration curve. Proteins were determined by the method of Lowry *et al.* (1951).

The incubation media contained 250 mM sucrose; 10 mM HEPES–K, pH 7.3; 10 mM succinate–K, plus 10 μg rotenone; 10 mM acetate–K, pH 7.3; and 50 μM $CaCl_2$. Protein concentration was 2 mg/ml of the incubation medium. Departures from these conditions, or additions to the media, are described in the figure legends.

Results

Rat kidney mitchondria suspended in sucrose medium and respiring with succinate retain Ca^{2+} until the suspension becomes anaerobic. Figure 1

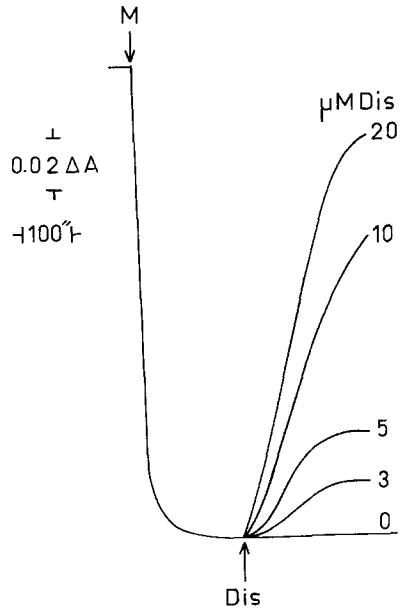

Fig. 1. The effect of increasing concentration of disulfiram on mitochondrial Ca^{2+} release. Mitochondrial protein (M), 2 mg, was added to a medium as described in *Materials and Methods*. In addition, the medium contained 50 μM Arsenazo III. The concentrations of disulfiram (Dis) were as shown. Final volume, 3 ml. Temperature, 25°C.

illustrates the effect of increasing concentrations of disulfiram (Dis) on Ca^{2+}-loaded mitochondria. Addition of 20 μM Dis promoted a fast and extensive release of the accumulated cation. A rapid loss of intramitochondrial Ca^{2+} is also seen on the addition of 10 μM Dis, but to a lesser extent 3 and 5 μM Dis having a slight effect on Ca^{2+} efflux.

The experiment shown in Fig. 2 indicates the effect of cysteine and ADP on the Ca^{2+}-releasing action of Dis. It can be seen that, when mitochondria were incubated in the presence of 200 μM cysteine, 20 μM Dis failed to liberate matrix Ca^{2+} (trace A). A similar result was obtained when 200 μM ADP was present in the incubation medium, although a final concentration as high as 30 μM Dis was added (Fig. 2B). In the experiment shown in Fig. 2C, mitochondria were added to a medium containing 10 μM Dis. Under these conditions, a partial uptake of Ca^{2+} was followed by its fast release. However, a rapid reuptake of the cation occurred after the addition of 200 μM ADP. The protective effect of cysteine establishes that the effect of Dis on mitochondrial calcium content occurs through its binding to external membrane -SH groups, which are implicated in the permeability to Ca^{2+} release (Ramachandran and Bygrave, 1978; Palmer and Pfeiffer 1981; Vercesi 1984; Chávez and Holguín, 1988). On the other hand, the ability of ADP to

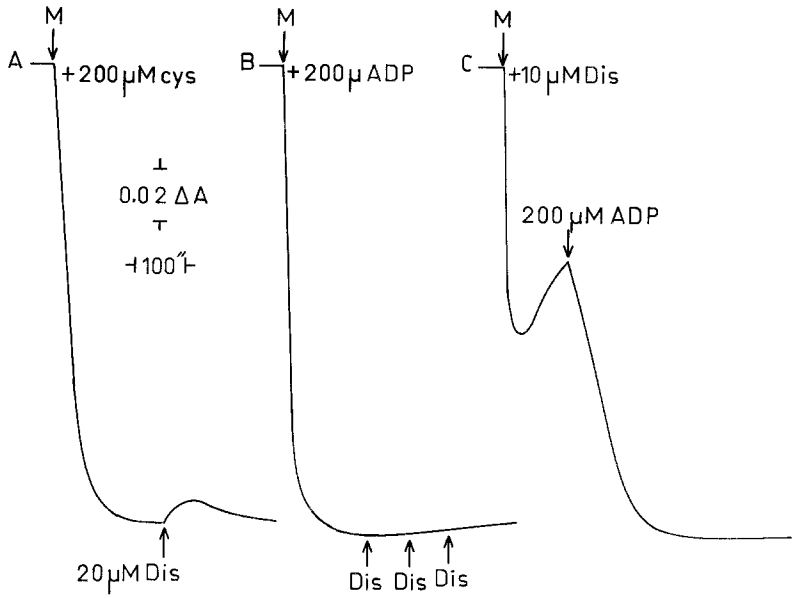

Fig. 2. The protective role of cysteine and ADP on the Ca^{2+}-releasing effect of disulfirm. Experimental conditions are as described for Fig. 1. In addition, the medium contained the indicated concentrations of cysteine, ADP, and disulfiram (Dis), in A, B, and C, respectively. In B, 10-μM additions of Dis are indicated.

impede the development of a permeability increase for the efflux of increase for the efflux of intramitochondrial Ca^{2+} has been well established (Hunter and Haworth, 1974; Harris, 1979; Nicholls and Crompton, 1980; Jurkowitz and Brierley, 1984; Chávez and Jay, 1987).

The efficiency of Dis in inducing mitochondrial Ca^{2+} release could be related to a collapse of the internal membrane potential. Therefore, the effect of Dis on this parameter was analized. Figure 3A indicates that, when 50 μM Ca^{2+} was added, a partial depolarization took place (from 136 to 119 mV); however, a complete loss in $\Delta\psi$ was attained upon the subsequent addition of 20 μM Dis. That Dis did not produce permanent damage in mitochondrial membrane is demonstrated by the fact that addition of 0.16 μM ruthenium red caused rapid and complete reenergization. Figure 3B shows tht Dis by itself was unable to promote a decrease in membrane potential. However, after the addition of 50 μM Ca^{2+}, a rapid drop in $\Delta\Psi$ was attained. Figure 3C indicates the role of ADP in preventing membrane depolarization as induced by Dis–Ca^{2+}.

Previous studies showed that swelling of mitochondria occurs in parallel with a collapse of membrane potential and Ca^{2+} efflux (Beatrice *et al.*, 1980; Palmer and Pfeiffer, 1981; Jurkowitz and Brierley, 1984; Bellomo *et al.*,

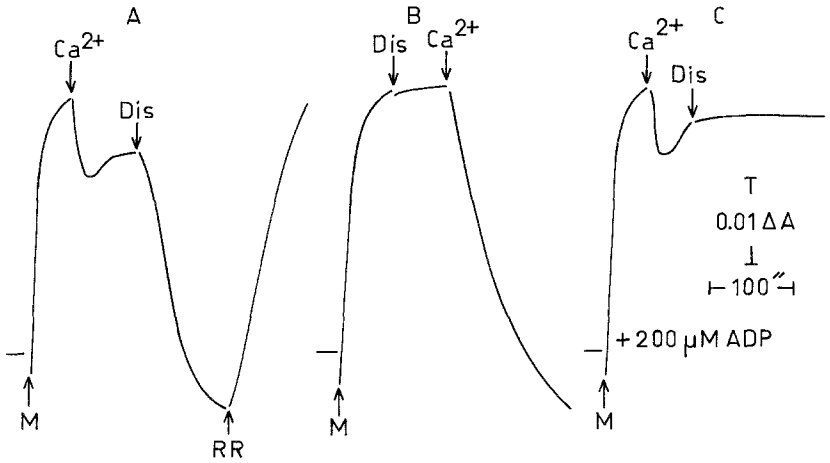

Fig. 3. The effect of disulfiram on the mitochondrial membrane potential. Mitochondrial protein (M), 2 mg, was added to a medium similar to that described in *Materials and Methods*. In addition, the medium contained 10 μM safranine. Where indicated, 20 μM disulfiram (Dis) and 50 μM $CaCl_2$ were added. In A, where indicated, 0.6 μM ruthenium red (RR) was added. in C, the medium also contained the indicated concentration of ADP. Final volume 3 ml. Temperature, 25°C.

1984). To determine whether the same event occurs when Dis is used as the Ca^{2+}-releasing agent, we investigated the effect of this drug on the mitochondrial volume. Figure 4 shows that, after addition of Dis (20 μM), mitochondria suffer large-amplitude swelling. Similar to that which occurs with the effect of Dis on membrane energization, this swelling reaction requires exogenous Ca^{2+}. The failure of Dis to promote mitochondrial swelling *per se* is emphasized in Fig. 4B. As observed, the swelling response of mitochondria to Dis took place only after the addition of 50 μM Ca^{2+}. Figure 4C indicates the protective effect of ADP on the action of Dis on mitochondrial volume, since, as indicated, the presence of 200 μM ADP in the incubation medium largely eliminates mitochondrial swelling. Figure 4D reinforces the statement that Ca^{2+} accumulation is an essential prerequisite for the effects of Dis. As is shown, mitochondria did not swell in the absence of an oxidizable substrate, even in the presence of 50 μM Ca^{2+}; however, mitochondrial swelling occurred after the addition of succinate—in other words, after Ca^{2+} accumulation.

Several studies support the proposal that the Ca^{2+} efflux pathway is regulated by changes in the pyridine nucleotide redox state (Lehninger *et al.*, 1978; Pfeiffer *et al.*, 1979; Beatrice *et al.*, 1980; Palmer and Pfeiffer, 1981; Vercesi, 1987; Chávez and Jay, 1987). The data reported in Fig. 5A show that, as with other sulfhydryl reagents (Pfeiffer *et al.*, 1979; Chávez *et al.*,

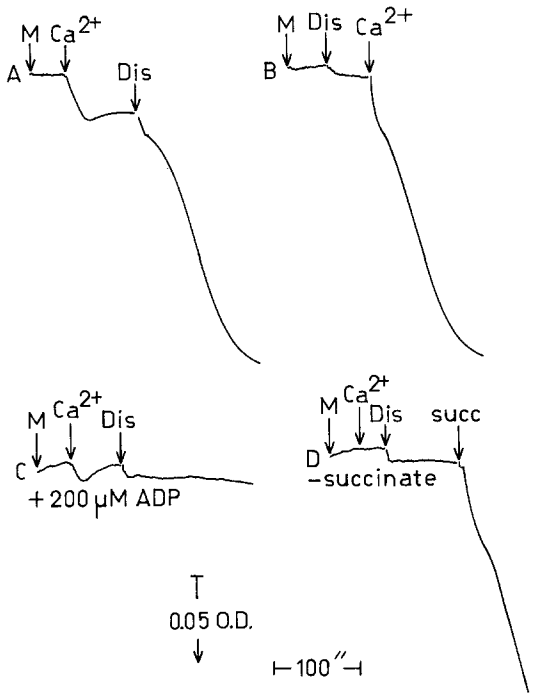

Fig. 4. Effect of disulfiram on mitochondrial volume. Mitochondria protein (M), 2 mg, was added to the incubation medium similar to that described in *Materials and Methods*. Where indicated, 50 μM $CaCl_2$, 20 μM disulfiram (Dis), and 10 mM succinate (succ) were added. In C, the medium also contained the indicated concentration of ADP. Final volume 3 ml. Temperture, 25°C.

1987; Chávez and Holguín, 1988), inclusion of 20 μM Dis in the incubation medium initiates a fast oxidation of pyridine nucleotides (12 nmol/min/mg) in the presence of Ca^{2+}. In the presence of ruthenium red (0.16 μM), the oxidation rate was diminished to lower levels (Fig. 5B). Hence, Ca^{2+} uptake was also required for Dis to affect the intramitochondrial NAD(P)H/NAD(P) ratio. Figure 5C indicates that ADP also protected against the oxidation of NAD(P)H by Dis. The latter indicates that, in spite of the Ca^{2+} uptake, the maintenance of a highly reduced state of pyridine nucleotides by ADP suffices to protect against the effect of Dis.

The magnitude of Ca^{2+} efflux, as induced by Dis, is presented quantitatively in Fig. 6. Within 1 min of the addition of Dis, 33 nmol Ca^{2+}/mg was released. This efflux rate is similar to that obtained by using other -SH reagents (Beatrice *et al.*, 1980; Chávez and Holguín, 1988).

Titration of membrane sulfhydryl groups by Dis, depending on the metabolic state of mitochondria, is presented in Table I. The addition of 20 μM Dis to mitochondria incubated in a sucrose-buffered medium,

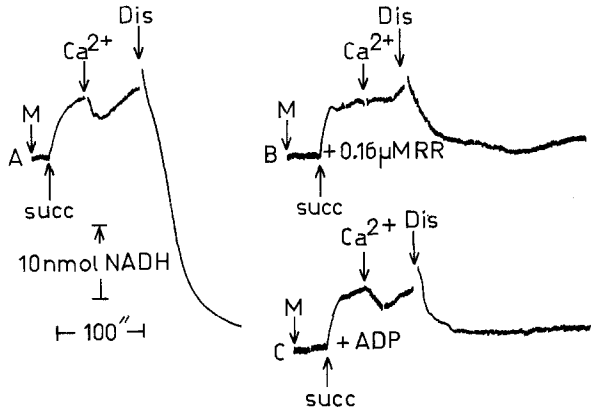

Fig. 5. Effect of disulfiram on mitochondrial pyridine nucleotide oxidation. Experimental conditions are as described for Fig. 4. In B, the medium contained, in addition, the indicated concentration of ruthenium red (RR). In C, the medium contained 200 μM ADP. Where indicated, 10 mM succinate (succ), 50 μM $CaCl_2$, or 20 μM disulfiram (Dis) was added.

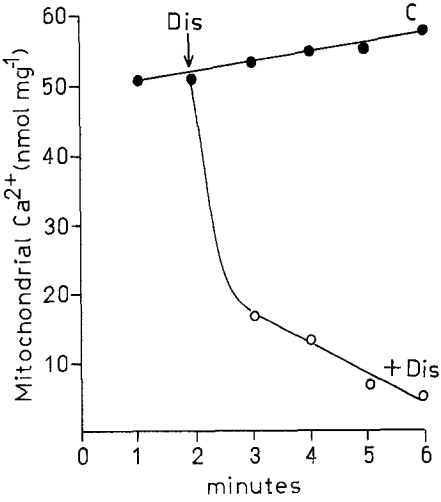

Fig. 6. Release of endogenous Ca^{2+} as induced by disulfiram. Mitochondrial protein 2 mg, was incubated in a medium similar to that described for Fig. 1, except that no Arsenazo III was added and calcium movement was followed by using $^{45}CaCl_2$ (sp. act. 1000 cpm/min/nmol). At the times indicated, 0.2-ml aliquots were withdrawn and filtered through a Millipore filter (0.45-μm pore size). At the indicated time, 20 μM disulfiram (Dis) was added. Final volume, 3 ml. Temperature, 25°C.

Table I. Membrane Binding of Disulfiram Depending on the Metabolic State of Mitochondria[a]

Conditions	Modified −SH ($nmol\,mg^{-1}$)
Complete mixture	8.5 ± 0.80
− Succinate + antimycin A	4.0 ± 0.89
$-Ca^{2+}$ + EGTA	12.8 ± 1.32
+ Ruthenium red	11.6 ± 0.49
+ 200 μM ADP	13.2 ± 0.98
+ ADP + atractyloside	8.6 ± 1.50

[a]Mitochondria (2 mg protein) were incubated as described in *Materials and Methods*. Where indicated, 10 μg antimycin A, 50 μM EGTA, 0.16 μM ruthenium red, or 20 μM atractyloside was added. Following 2 min of preincubation, 20 μM disulfiram was added. After 2 min of incubation, the mixture was spun down and the concentration of diethyldithiocarbamate was determined in the supernatant by using Ellman's reagent. Cysteine was used as the standard. Values are mean ± standard deviation of five experiments.

containing succinate and 50 μM $CaCl_2$, titrated 8.5 nmol −SH/mg protein. This amount of Dis, bound to the membrane, proved to be energy dependent since, upon the addition of antimycin A, the titration of sulfhydryl groups decreased considerably (from 8.5 to 4 nmol/mg). On the other hand, when Ca^{2+} ions were not added to the incubation medium, or when its uptake was inhibited by ruthenium red, the number of −SH groups, as modified by Dis, increased (from 8.5 to 12.8 nmol/mg). A similar result was obtained when the incubation medium was supplemented with 200 μM ADP. It is noteworthy that ADP abolishes the effects of Dis on Ca^{2+} release, membrane potential, and mitochondrial swelling. In the same token, Ca^{2+} uptake is required for both the collapse of $\Delta\psi$ and the increase of mitochondrial volume induced by Dis. Accordingly, it would appear that the effects of Dis in the studied mitochondrial functions depend on the binding of the regent to specific −SH groups, which are exposed in the absence of ADP or after Ca^{2+} accumulation. These latter results together with those reported in Fig. 6 support the theory that titration of 9 nmol sulfhydryl groups per mg protein by Dis is sufficient to release the accumulated Ca^{2+}. Here it should be noted that Dis is not reduced by mitochondrial electron transport or glutathione reductase (not shown).

Discussion

This study analyzes the effect of disulfiram (Dis) on mitochondrial calcium balance. As has been shown, after Dis binds to membrane sulfhydryl groups, mitochondria lose their ability to retain Ca^{2+}. The increased

permeability correlated with a diminution in the NAD(P)H/NAD(P) ratio, extensive swelling, and collapse of the membrane $\Delta\psi$. This mechanism is apparently common to other chemically diverse sets of sylfhydryl reagents (Pfeiffer *et al.*, 1979; Beatrice *et al.*, 1980; Palmer and Pfeiffer 1981; Jurkowitz *et al.*, 1983; Vercesi, 1984; Jurkowitz and Brierley 1984; Chávez and Holquín, 1988).

For compounds such as diamide and *N*-ethylmaleimide, it has been suggested that the collapse of the transmembrane potential occurs by an increased proton conductance of the inner membrane (Beatrice *et al.*, 1980; Vercesi, 1984). However, from our results, we cannot use this argument to explain the apparent uncoupling produced by Dis. Considering that ruthenium red restores the collapsed membrane potential (Fig. 3), it seems instead that the energy drain of the system must be caused by Ca^{2+} cycling, which can be interrupted by inhibition of the calcium uniport. This alternative explanation is in close agreement with that addressed to explain the drop in membrane $\Delta\psi$ that occurred when *t*-buthylhydroperoxide (Lötscher *et al.*, 1980; Bellomo *et al.*, 1982) or mercuric ions (Chávez and Holquín, 1988) are used as Ca^{2+}-releasing agents.

In addition to the modification of membrane $-$SG groups, there is an apparent requirement for Ca^{2+} to increase the permeability of the mitochondrial membrane. Beatrice *et al.* (1980), using *N*-ethylmeleimide, suggested tht the role of Ca^{2+}, in combination with the sulfhydryl reagent, is to promote lysophospholipid accumulation in mitochondria, with consequent membrane damage. However, since the permeability change induced by the binding of Dis to the membrane was reversible (see Fig. 3), it appears reasonable to discard mitochondrial damage as the underlying event in the mechanism that leads to Ca^{2+} efflux. An alternative explanation for the Ca^{2+} requirement has been suggested by Hunter and Haworth (1974), who propose that mitochondria contain endogenous hydrophylic channels that are opened by Ca^{2+} binding. In addition, it is interesting to note that the binding of Dis to mitochondrial membrane is modified by Ca^{2+} uptake (Table I). The latter appears to agree with data reported (Hunter and Haworth, 1974) indicating that Ca^{2+} induces changes in mitochondrial configuration, these changes resulting in a modification in the number of exposed membrane $-$SH groups.

It has been also demonstrated that ADP promotes conformational changes in mitochondria (Stoner and Sirak, 1973; Klingenberg, 1985). The latter must be accounted for to explain the increased binding of Dis to mitochondria as induced by 200 μM ADP (see Table I). Interestingly, in such conditions, regardless of the high amount of modified $-$SH, mitochondria retained accumulated Ca^{2+} (see Fig. 2). In regard to the function of ADP in mitochondrial Ca^{2+} homeostasis, Harris (1979), proposed that ADP bound

to mitochondria causes occlusion of a pathway for Ca^{2+} efflux. Toninello *et al.* (1983) showed that ADP is necessary for the maintenance of $\Delta\psi$. In addition, Nicholls and Crompton (1980) and Zoccarato *et al.* (1981) reported that adenine nucleotides stabilize the membrane against the deleterious effects of some Ca^{2+}-releasing agents—even sulfhydryl-blocking reagents. Nevertheless, since ADP maintains the steady state of pyridine nucleotides in a more reduced state, even after the addition of 20 μM Dis (Fig. 5), these findings would indicate that the effect of ADP on Ca^{2+} retention may be through activation of NAD-dependent dehydrogenase, as proposed in a previous study (Chávez and Jay, 1987).

The site to which Dis binds in order to exert its Ca^{2+}-releasing action would seem to be located on the external side of the inner membrane. The above conclusion is based on the hydrophilic character of the drug and on the protective effect of cysteine (Fig. 2).

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