

Disulfiram Inhibition of the Alternative Respiratory Pathway in Plant Mitochondria¹

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ABSTRACT

Disulfiram (tetraethylthiuram disulfide) was found to be a potent and selective inhibitor of the alternative respiratory path of plant mitochondria. The onset of inhibition by disulfiram takes several minutes and the inhibition is not readily reversed by washing, nor by metal ions. By contrast, thiols such as dithiothreitol not only reverse, but also prevent, disulfiram inhibition. Inhibition by disulfiram and by hydroxamic acids are not mutually exclusive. Structural analogs of disulfiram are far less potent inhibitors, with the exception of bisethyl xanthogen. Inhibition is due to disulfiram, *per se*, and not to its reduction product, diethyldithiocarbamate, a powerful chelator. Accordingly, the inhibitory effect of disulfiram is considered to involve the formation of mixed disulfides with one or more sulfhydryl groups in the alternative path. Disulfiram does not act as an electron sink diverting electron flow from oxygen.

Disulfiram inhibition was observed only with isolated mitochondria or submitochondrial particles. In intact cells or tissues either a failure to absorb disulfiram, or its dissipation in the cytosol, precludes inhibition. *In vitro*, bovine serum albumin reduces disulfiram inhibition by complexing free inhibitor.

The binding of ³⁵S-disulfiram by cyanide-resistant mitochondria displays the same kinetics as disulfiram inhibition. A comparison was made of ³⁵S-disulfiram binding by cyanide-sensitive and cyanide-resistant potato mitochondria. Cyanide-resistant mitochondria were obtained from ethylene-treated potato tubers. Incorporation of label proved essentially the same in both types of mitochondria, suggesting that the disulfiram-sensitive component of the alternative path is present in untreated potato tubers, and is not induced by ethylene.

Inhibitors of cytochrome-mediated electron transport, such as cyanide and antimycin, often fail effectively to block the O₂ consumption of plant mitochondria. The resistance of these mitochondria results from a branched respiratory chain comprising the conventional Cyt path and an alternative path leading to a cyanide-resistant terminal oxidase (29).

Early efforts to characterize the alternative path revealed its lack of optically detectable redox components and its sensitivity to inhibition by certain metal chelators (3). Although the chelators used in these preliminary studies were of limited experimental usefulness because of their considerable side effects on the Cyt path, discovery of their effectiveness prompted the speculation that a non-heme iron protein may be a component of the alter-

native path.

Schonbaum *et al.* (27) found substituted hydroxamic acids were potent and seemingly selective inhibitors of the alternative path. This discovery added a new dimension to research on cyanide resistance. For the first time it was possible to study either branch of the respiratory chain independently by specifically blocking the other pathway. This eventually allowed the development by Bahr and Bonner (2) of a system of analysis to determine the extent to which the alternative path contributes to the total respiration in the absence of cyanide. Hydroxamic acids are well known chelators of transition metal ions, and form particularly strong complexes with Fe³⁺. This fact added to the general support of Bendall and Bonner's suggestion that a non-heme iron protein was involved in the alternative path, even though Schonbaum *et al.* did not themselves particularly favor this interpretation. Rich and co-workers (25, 26) later presented evidence against the iron chelation mechanism of inhibition by hydroxamates and supported other possible mechanisms, including charge transfer complex formation and polyfunctional hydrogen bonding, a possibility originally suggested by Schonbaum *et al.* (27).

We initially examined the chelator sensitivity of the alternative path to seek evidence of a metal-containing regulatory site where ethylene and cyanide, putative activators of the alternative path (30), might bind. However, Arron *et al.* (1) have since shown that ethylene has no direct effect on the alternative path *in vitro*. Accordingly, chelator sensitivity probably has no relation to ethylene binding sites. Nevertheless, in the course of our survey of chelators, we found that disulfiram (tetraethylthiuram disulfide), a complexing agent thought to favor copper, rather specifically inhibits the alternative path in isolated mitochondria. We have subsequently probed its mechanism of action in light of the potential of this compound to act either as a metal-complexing agent or as a sulfhydryl poison.

MATERIALS AND METHODS

Plant Materials. Potato tubers (*S. tuberosum* var. Russet Burbank) were generously provided by Professor Herman Timm, University of California at Davis. During summer months when Russet potatoes were out of season, commercial supplies (chiefly *S. tuberosum* var. Norgold) were purchased from local markets. Red sweet potatoes (*Ipomea batatas*) were also obtained commercially. All tubers were stored at 7°C at 80 to 90% RH in the dark.

Cyanide-resistant mitochondria were prepared from tubers of Russet potatoes held for 10 days at room temperature, then treated for 24 h with a gas mixture composed of 10 μl/l C₂H₄, 10% CO₂, and 90% O₂ (7). The gas mixture was passed through a 3-liter treatment chamber containing two to four tubers at a rate of 30 ml/min.

Chemicals and Stock Solutions. Disulfiram was purchased from Sigma Chemical Co. and for routine purposes was used without further purification. As the solubility of disulfiram in water is low, stock solutions of disulfiram were prepared in ethanol (in concen-

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trations up to 50 mM). SHAM⁴ was obtained from Aldrich Chemical Co. and 1 M stock solutions prepared in 2-methoxyethanol. Stock solutions of various other metal chelators were prepared in acetone or ethanol. In all cases where nonaqueous solvents were employed in respiratory studies, the final solvent concentration in the reaction medium was restricted to levels that had no significant effect on respiration.

The following solutions were used during the preparation and assay of activity of mitochondria and submitochondrial particles. Extraction medium: 350 mM mannitol, 25 mM Tes (pH 7.8), 250 mM sucrose, 1 mg/ml BSA, and 0.1 mg/ml Na-mercaptobenzothiozole (added just prior to homogenization). Wash and suspension medium: 350 mM mannitol, 250 mM sucrose, 25 mM Tes (pH 7.4), and 1 mg/ml BSA. Sonication medium: 250 mM sucrose, 1 mM ATP, 1 mM EDTA, 10 mM Tes (pH 7.5). Reaction medium: 400 mM mannitol, 25 mM Tes (pH 7.4), 5 mM KH₂PO₄, and 1 mg/ml BSA.

Preparation of Mitochondria. Tissue was cut into small blocks and homogenized in an Oster vegetable juicer with 2 volumes extraction buffer. The coarse debris was removed by filtration of the homogenate through a strip of Miracloth which lined the basket centrifuge of the juicer. The homogenate was centrifuged at 750g for 10 min. The resulting pellet, which contained starch, nuclei, unbroken cells, and cell wall material, was discarded. Mitochondria were sedimented from the supernatant by centrifugation at 14,000g for 20 min. This crude mitochondrial fraction was suspended in an excess of wash medium and recentrifuged at 14,000g for 20 min. The washed mitochondria were resuspended in wash medium.

When purified mitochondria were required, the washed mitochondria prepared by the above procedures were layered on a linear sucrose gradient (1 to 2 M) containing 10 mM Tes (pH 7.4), and centrifuged for 3 h at 25,000 rpm in a Beckman L5-50 centrifuge equipped with a SW 27.1 rotor. The mitochondria were located in a single band with an average density of 1.186. The mitochondria were removed from the gradient with a large bore syringe with a tip bent some 90°, then diluted to 0.4 M sucrose by dropwise addition of 10 mM Tes buffer (pH 7.4), plus 0.1% BSA. The dilution procedure was carried out over a period of at least 20 min, as faster dilution resulted in mitochondrial breakage. The diluted solution was centrifuged at 14,000g for 10 min to pellet the purified mitochondria, which were then taken up in a small volume of resuspension medium.

Preparation of Submitochondrial Particles. Submitochondrial particles were prepared by sonication of intact mitochondria at a protein concentration of approximately 10 mg/ml. Sonication was performed in an ice bath with a Bronson sonifier in two bursts of 25 s each at 40 w with 1 min between the bursts. Unbroken mitochondria were removed by centrifugation of the crude sonicate at 14,000g for 10 min. The supernatant was then centrifuged at 100,000g for 1 h to pellet the submitochondrial particles which were then taken up in a minimal volume of resuspension medium.

Respiratory Measurements. Mitochondrial respiration was measured with a Clark O₂ electrode in a 3-ml Plexiglas chamber maintained at 25 C with a circulating water bath. When succinate was the respiratory substrate, mitochondria were pretreated for 2 min with 0.1 mM ATP to activate succinate dehydrogenase prior to substrate presentation. When malate was used as a substrate, an equal concentration of glutamate was used to remove oxaloacetic acid by transamination. Respiration rates, when not given in nmol O₂ min⁻¹ mg⁻¹ protein are given in nmol O₂ min⁻¹, the rate of O₂ consumption for the entire reaction chamber. ADP/O ratios were calculated from polarographic traces as discussed by

Chance and Williams (6).

Protein Determination. Mitochondrial protein was determined by one of two methods: a modified Lowry procedure designed for membranous samples (20) or the Bio-Rad protein assay based on the dye-binding method of Bradford (5).

Synthesis of ³⁵S-Disulfiram. ³⁵S-Disulfiram was prepared from uniformly labeled ³⁵S-sodium DIECA (Amersham Corp.) by oxidation with potassium ferricyanide. One hundred two mg (0.596 mmol) of ³⁵S-DIECA with a nominal specific radioactivity of 6.8 mCi/mmol, was dissolved in 5 ml water and after 20 min of stirring was filtered through Whatman No. 1 filter paper to remove an insoluble residue. To the filtered solution, 2.98 ml (0.596 mmol) 0.2 M solution of K₃Fe(CN)₆ was added and the solution stirred for 5 min. The insoluble precipitate (disulfiram) was filtered out of the solution with an 0.8 μm Millipore filter and washed with approximately 10 ml ice-cold water. The white precipitate was then dried on the filter with suction for 15 min, transferred from the filter to a vial, and placed in a desiccator jar and dried to constant weight. The theoretical yield of disulfiram was 88.4 mg (0.298 mmol). The actual yield was 47.5 mg, 54% of the theoretical yield. The specific radioactivity of the product was found to be 20.5 mCi/mmol, whereas a value of 13.8 mCi/mmol was expected on the basis of the nominal specific radioactivity of the DIECA. The presence of the insoluble contaminant in the starting material may have contributed to both the low over-all yield and the seemingly high specific radioactivity of the disulfiram.

The identity of the product was confirmed by comparison of the melting point and IR spectrum of the synthesized material with recrystallized commercial samples of disulfiram. The melting point of the synthesized disulfiram (70.5–71.2 C) agreed well with that of recrystallized, authentic disulfiram (71.0–71.6 C) and with published values (21). Similarly, the IR spectrum of the synthesized disulfiram was virtually identical with that of authentic disulfiram and with published spectra (21).

Binding Studies with ³⁵S-Disulfiram. Mitochondria for label incorporation studies were prepared by sucrose gradient purification in the normal way, except that BSA was excluded from all solutions. Mitochondria (0.5–1.0 mg protein) were incubated in 3.0 ml standard reaction mix (-BSA) in the presence of various concentrations of ³⁵S-disulfiram, ±200 μM unlabeled disulfiram or other reagents. The binding reaction was allowed to proceed for 10 min and the reaction was then stopped by the addition of *N*-ethylmaleimide to a final concentration of 10 mM to stop mixed disulfide formation between protein thiols and disulfiram. The samples were immediately centrifuged at 12,000g for 15 min. The mitochondrial pellet was dissolved in minimal volume of 8 M urea and the protein was precipitated from this dissolved pellet by the addition of 4 ml mixture of 10 mM HCl in acetone. The solution was chilled for 5 min. The pellet was washed with the cold HCl-acetone mixture two more times to remove unreacted disulfiram and the final pellet was taken up in a small volume of 8 M urea. Samples were then taken for protein determination by the modified Lowry procedure and for liquid scintillation counting in 10 ml Aquasol.

RESULTS

Inhibition Constant. Disulfiram strongly inhibits cyanide-resistant respiration in isolated mitochondria (Fig. 1). The apparent *K_i* for this inhibition, as determined from Dixon plots, is typically in the range of 4 to 17 μM. However, the Dixon plots are sometimes concave upward rather than linear. Evidence presented in later sections shows that a significant fraction of the total disulfiram pool can be depleted by a combination of specific and nonspecific binding even in purified mitochondrial preparations. Such depletion, when it exceeds a certain limit, undermines the usual methods of inhibitor analysis (12) and may lessen the precision of *K_i* determinations.

⁴ Abbreviations: SHAM, salicyl hydroxamic acid; CLAM, *m*-chlorobenzhydroxamic acid; DIECA, diethyldithiocarbamate; RCR, respiratory control ratio.

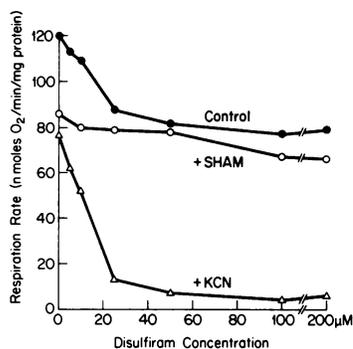


FIG. 1. Effect of disulfiram on the respiration of red sweet potato mitochondria in state 4. Substrate 25 mM succinate, with 0.2 mM ATP; 0.3 mM KCN and 2 mM SHAM given as indicated.

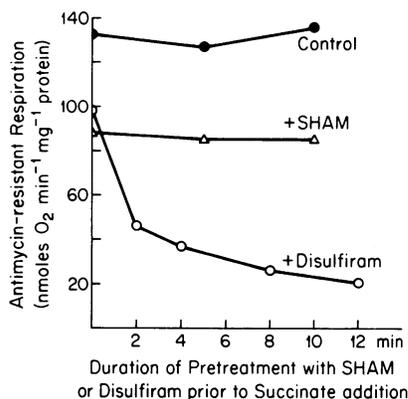


FIG. 2. The time course of inhibition of the alternative path by disulfiram. Mitochondria were isolated from ethylene-treated potato tubers. The 25 μ M disulfiram and 0.5 mM SHAM were given as pretreatments, as indicated, for various periods of time before the addition of 10 mM succinate and 0.2 mM ATP. In all cases, 0.2 μ g/ml antimycin was given 5 min prior to substrate, and initial rates were measured.

Time Course of Inhibition. The onset of inhibition by disulfiram is slow. Figure 2 compares the time dependency of disulfiram inhibition with inhibition caused by SHAM. In routine work, disulfiram was added to mitochondria suspensions several min prior to substrate presentation and respiration rates were followed till linear. Synergism between disulfiram and antimycin makes the further point that disulfiram inhibition is not linked to CN action *per se*, but rather to the inhibition of the cytochrome path.

Specificity of Disulfiram. In the absence of cyanide, disulfiram reduces the state 4 rate of red sweet potato mitochondria oxidizing succinate by 30% (Fig. 1), suggesting that the alternative oxidase normally contributes this fraction to the total state 4 rate. SHAM, which inhibits the alternative path without major side effects on the Cyt chain, also inhibits the state 4 rate by about 30% when given at near saturating levels. Disulfiram has but a small additional effect when given to mitochondria in the presence of 2 mM SHAM (Fig. 1). Whereas this marginal inhibition by disulfiram in the presence of SHAM may reflect some slight inhibition of the Cyt chain, the facts taken together indicate that disulfiram, like the hydroxamic acids, rather specifically blocks the alternative path when succinate is the substrate.

With malate as substrate, the nonspecific effects of disulfiram are larger. That is, disulfiram has a greater effect on malate oxidation through the Cyt path than on succinate oxidation through the Cyt path. A Hill plot of disulfiram effectiveness in the presence of CN yields a Hill coefficient of 1.8, suggesting two sites of inhibition, without necessarily indicating cooperativity of inhibitor binding (28). This is in contrast to Hill plots of substrate

binding, where Hill numbers greater than one indicate cooperative binding.

When disulfiram titration is performed in the presence of SHAM, so that all electrons from malate oxidation are forced through the conventional respiratory chain, a K_i of 190 μ M is obtained and the Hill coefficient is 1.15, suggesting but one site of inhibition. There seem to be two disulfiram-sensitive sites involved in malate oxidation, an alternative path site and a second, lower affinity site, possibly in complex I of the respiratory chain.

To further circumscribe the locus of disulfiram action, disulfiram was tested on succinate oxidation in mitochondria that have no functional alternative path. CN-sensitive mitochondria from untreated white potatoes were chosen wherein any inhibition by disulfiram must be attributable to an effect on conventional mitochondrial respiration. In such mitochondria, 100 μ M disulfiram inhibits state 3 succinate oxidation only 17%, while state 4 is virtually unaffected. Figure 3 demonstrates that with succinate as substrate, disulfiram does not affect the ADP/O ratio, and diminishes respiratory control very little, much as does SHAM (2). In contrast to its effects in cyanide-sensitive white potato mitochondria, where disulfiram decreases the RCR, and fails to affect the ADP/O ratio, disulfiram increases both the RCR and the ADP/O values of cyanide resistant red sweet potato mitochondria (Fig. 3). The intrinsically low RCR and ADP/O values in sweet potato mitochondria are due in part to the participation of the alternative path in the absence of inhibitors. The effect of disulfiram is consistent with its inhibition of the nonphosphorylating alternative path, insofar as the increase in the ADP/O ratio reflects the exclusive participation of the cytochrome path in the presence of disulfiram.

Comparison with Other Inhibitors. Disulfiram, with a K_i of 7 μ M, is a more potent inhibitor of the alternative path than is SHAM or CLAM. The K_i values for the latter are 350 μ M and 195 μ M, respectively, in red sweet potato mitochondria. Disulfiram has the additional virtue, in distinction to the hydroximates, of not inhibiting lipoxygenase, an enzyme that may be found in mitochondria (M. G. Miller, personal communication). Most analogs of disulfiram are less effective than disulfiram, as seen in Table I. Tetramethylthiuram disulfide is much less effective than disulfiram (tetraethylthiuram disulfide). Whereas the tetrabutyl derivative shows little if any activity, the dicyclopentamethylene derivative and the methylhomopiperazinyll derivative show some potency. Bisethylxanthogen is the most potent of all the tested analogs and has virtually the same I_{50} value as disulfiram. Monosulfide derivatives and compounds not bridged by sulfur possess little or no activity. There is no obvious correlation between the size of substituent groups and activity in this series of analogs. Other factors, such as the tendency to partition into membranes or the redox potential of the disulfide-thiol couples, may be important in determining activity. The I_{50} value (18 mM) for DIECA, the sulfhydryl reduction product of disulfiram, was estimated with DIECA given at the same time as the substrate. It was

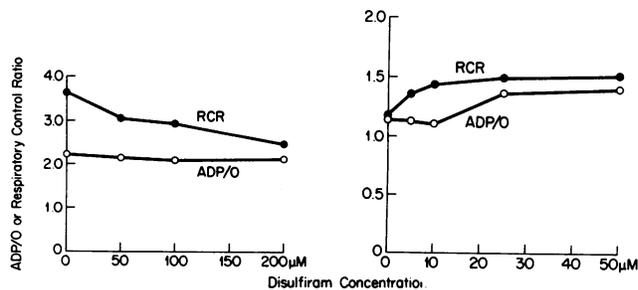


FIG. 3. Effect of disulfiram on the ADP/O and respiratory control ratio of mitochondria isolated from untreated (left) potato tubers, (right) sweet potatoes. Substrate 10 mM succinate, with 0.2 mM ATP.

TABLE I
Comparison of disulfiram with some analogs

| | Approximate I_{50} for alternative path | Structure |
|--|--|--|
| Disulfides | | |
| Tetramethylthiuram disulfide | >100 μM | <chem>CN(C)S(=S)N(C)C</chem> |
| Tetraethylthiuram disulfide (disulfiram) | 7 μM | <chem>CCN(CC)S(=S)N(CC)C</chem> |
| Tetrabutylthiuram disulfide | >100 μM | <chem>CCN(CCC)S(=S)N(CCC)C</chem> |
| Dicyclopentamethylenethiuram disulfide | 100 μM | <chem>C1CCN(C1)S(=S)N(C1CCN1)C</chem> |
| Bis(4 methyl-1-homoperazinyl-thiocarboxyl) disulfide | =100 μM | <chem>CN1CCN(C1)S(=S)N(C1CCN1)C</chem> |
| Bisethyl xanthogen | 10 μM | <chem>CCN(CC)S(=S)C(=O)O</chem> |
| Monosulfides | | |
| Tetramethylthiuram monosulfide | >200 μM | <chem>CN(C)S(=S)N(C)C</chem> |
| Tetrabutylthiuram monosulfide | 75 μM | <chem>CCN(CCC)S(=S)N(CCC)C</chem> |
| (N, N diethylthiocarbamoylthio) acetic acid | >200 μM | <chem>CCN(CC)S(=S)C(=O)O</chem> |
| Other compounds with related structures | | |
| Dithioamide (rubeanic acid) | 2 mM | <chem>CN(C)S(=S)N(C)C</chem> |
| 2,4 dithioburet | 100 μM | <chem>CN(C)S(=S)N(C)C</chem> |
| Sodium diethyldithiocarbamate | 18 mM | <chem>CCN(CC)S(=S)N(C)C</chem> |
| Potassium ethylxanthate | 20 mM | <chem>CCN(CC)S(=S)N(C)C</chem> |

subsequently observed that the onset of inhibition of the alternative path by DIECA is slow, and that concentrations as low as 5 mM cause nearly complete inhibition of the alternative path after pretreatments of 30 to 40 min. Such time-dependent inhibition might stem from an inability of the polar, water-soluble DIECA to penetrate the mitochondrial membrane. In fact, the prospect was considered that DIECA is the inhibitory species, arising from disulfiram within the mitochondrion. This possibility was tested by comparing the effects of disulfiram and DIECA on sub-mitochondrial particles, where penetration is not at issue. In short times, DIECA has but a minor effect (13% inhibition) on succinate oxidation by sub-mitochondrial particles in the presence of CN, whereas 50 μM disulfiram causes 77% inhibition. Thus, some other explanation must be sought for the time dependency of DIECA inhibition. The explanation may lie in the slow oxidation of DIECA to disulfiram by mitochondrial components.

Binding Site Comparisons. Hydroxamic acids and disulfiram bind at separate sites, as determined by the analysis of Yonetani and Theorell (36). When the reciprocal of the alternative path flux is plotted against SHAM in the presence and absence of fixed levels of disulfiram, the family of lines should intersect on the horizontal axis at a point equal to the apparent $-K_1$ for SHAM alone when the two inhibitors bind independently. If the two inhibitors bind in a mutually exclusive manner, the lines should be parallel. Titration with SHAM in the presence of disulfiram indicates independent and simultaneous binding of both inhibitors (Fig. 4), whereas titration with SHAM in the presence of another hydroxamic acid (namely CLAM) indicates mutually exclusive binding (Fig. 5).

Reversibility of Disulfiram Inhibition. The percentage inhibition caused by a fixed level of disulfiram is roughly independent of the

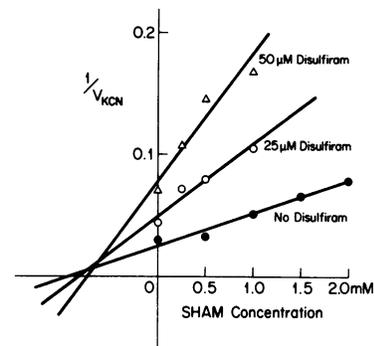


FIG. 4. Dixon plot of SHAM binding in the presence of disulfiram. Red sweet potato mitochondria with 10 mM succinate and 0.2 mM ATP. State 3 was initiated by the addition of 2 μM ADP. Disulfiram and SHAM were present as indicated. The 0.3 mM KCN present in all assays. Respiration rates were expressed as nmol O_2 per min per reaction chamber.

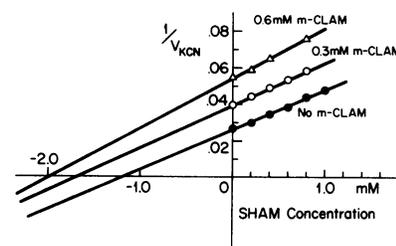


FIG. 5. Dixon plot of SHAM binding in the presence of *m*-CLAM. Red sweet potato mitochondria with 10 mM succinate and 0.2 mM ATP. State 3 initiated by the addition of 2.5 μM ADP. *m*-CLAM and SHAM present as indicated. The 0.3 mM KCN was present in all assays. Respiration rates expressed as nmol O_2 per min per reaction chamber.

quantity of mitochondrial protein, as would be expected of a reversible inhibitor. When disulfiram-treated mitochondria are washed with buffer, however, the inhibition is not readily relieved. For example, the respiration of CN-resistant sweet potato mitochondria is inhibited some 86% by 25 μM disulfiram in the presence of CN. After disulfiram-treated mitochondria are washed twice, inhibition drops to 57%. Although continued washing may fully relieve inhibition, the difficulty with which the inhibition is relieved contrasts sharply with hydroxamate inhibition, which is easily reversed by washing (27).

Inhibition caused by disulfiram is not relieved by Cu^{2+} , Fe^{3+} , Ni^{2+} , or Co^{2+} in concentrations up to 1 mM (data not shown). By contrast, Henry *et al.* (13) found that the inhibition caused by benzhydroxamic acid is almost completely reversed by the addition of Fe^{3+} . On the other hand, thiols such as DTT or mercaptoethanol completely reverse (and prevent) inhibition by disulfiram (Fig. 6). Although the effect of thiols may be due to the destruction of unbound disulfiram (with the formation of DIECA) in conjunction with reversible disulfiram binding, a more likely view is that reversal by thiols is due to the regeneration of enzyme sulfhydryl groups bound up in mixed disulfide formation with disulfiram. Other enzymes, such as D-amino acid oxidase (11, 23) and hexokinase (31), are inhibited by disulfiram in this way. This model for the mechanism of action of disulfiram would account for the meager reversal observed when disulfiram-treated mitochondria are washed in buffer.

Disulfiram is not an electron sink for the alternative path. If disulfiram were actively reduced by mitochondria *in vitro* it would divert electrons from O_2 and thus inhibit respiration. This type of inhibition would be transient and would end when all disulfiram was reduced. However, the inhibition caused by 100 μM disulfiram, an amount, if reduced, permitting adequate definition on the O_2

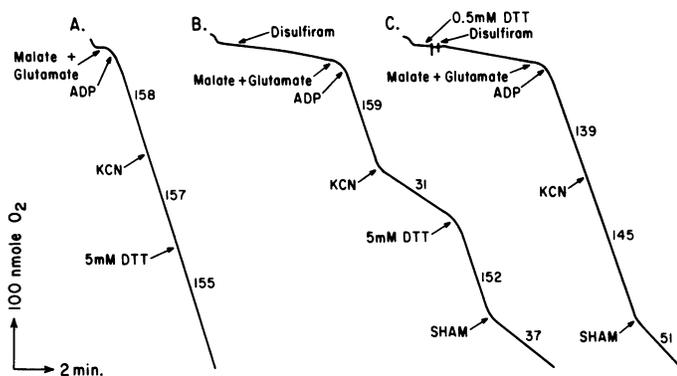


FIG. 6. Effect of DTT on disulfiram inhibition of mitochondrial respiration. Mitochondria isolated from ethylene-treated potato tubers. Additions, as indicated, are 10 mM malate, 10 mM glutamate, 2.5 μ mol ADP, 0.3 mM KCN, 25 μ M disulfiram, and 1 mM SHAM. Either 0.5 or 5 mM DTT was given as indicated. O_2 consumption rates on traces in nmol O_2 per min per mg protein.

electrode, showed no decrease over a period 5 times longer than that necessary to convert all of the disulfiram to DIECA, assuming that the decreased O_2 uptake had resulted from preferential reduction of disulfiram.

Interference by BSA. Although a certain level of BSA is necessary to obtain well coupled mitochondria and to allow full functioning of the alternative path, high concentrations have an adverse effect on disulfiram inhibition. Table II shows the effect of BSA on the respiratory rate and its effect on disulfiram potency. BSA reduces the effectiveness of disulfiram strikingly. The free sulfhydryl content of typical samples of BSA ranges from 0.65 to 0.70 mol SH/mol protein (14). As the standard level of BSA in the reaction mix is 1 mg/ml, this amounts to 10 μ M BSA-SH. Given the propensity of disulfiram to form mixed disulfides with protein SH groups in general, and the demonstrated ability of disulfiram to react in this way with plasma proteins (32), it is highly likely that the BSA effect on disulfiram potency stems, at least in part, from mixed disulfide formation. The presence of this relatively high concentration of sulfhydryl-bearing protein in the reaction medium undoubtedly contributes to the nonlinear trends often observed in Dixon plots of disulfiram inhibition. Nevertheless, nonspecific lipophilic binding of disulfiram by BSA has not been ruled out.

Disulfiram Effect in Whole Tissues. Disulfiram is ineffective against the cyanide-resistant respiration of whole tissue, including

disks of red sweet potato tissue. The same is true of 24-h aged disks of Russet potatoes and intact cells of *Chlorella protothecoides* and *Candida albicans*. The ineffectiveness of disulfiram *in vivo* may be caused by the reduction of disulfiram to DIECA in the cytoplasm. Rapid *in vivo* destruction of disulfiram has been observed in animal systems (8, 33).

^{35}S -Disulfiram Binding Studies. As a prelude to binding studies, the inhibitory effectiveness of synthesized ^{35}S -disulfiram was compared with that of commercially obtained, unlabeled disulfiram. Figure 7 shows that the inhibitor titrations for the synthesized and commercial disulfiram are superimposable.

The binding studies with labeled disulfiram were carried out under the standard assay conditions used for respiratory measurements, with the exception that BSA was excluded from the assay medium and from all solutions used in the preparation of the mitochondria. Accordingly, the mitochondria used in binding studies were tested separately for respiratory competency and cyanide resistance with reaction mix containing BSA. Gradient-purified mitochondria were used in all binding studies.

In conjunction with titrations with labeled disulfiram, parallel titrations were performed with ^{35}S -disulfiram and an excess of unlabeled disulfiram, following a generally accepted strategy for estimating specific binding of labeled hormones and drugs (24). The high level of unlabeled inhibitor is added competitively to prevent ^{35}S -disulfiram binding at the specific high affinity binding sites involved in alternative path activity. Radioactivity retained in the pellet under these conditions is considered to represent nonspecific binding due to occlusion or to nonspecific adsorption at low affinity sites. Specific binding is obtained by subtracting nonspecific binding from total binding. In almost all cases nonspecific binding represented less than half of the total binding.

Potato tubers treated with ethylene yield CN-resistant fresh slices (7). To address the question of whether ethylene induces the synthesis of the alternative path in potato tubers, we compared the number of specific disulfiram binding sites in mitochondria from untreated and ethylene-treated potato tubers. Figure 8 shows the concentration dependency of specific ^{35}S -disulfiram-saturable binding to mitochondria isolated from ethylene-treated potato tubers. The concentration of specific disulfiram reactive sites appears to be 4 to 6 nmol/mg protein, assuming that one-half of the disulfiram molecule is present on the protein in mixed disulfide form. Half-maximal saturation of disulfiram binding occurs between 5 and 20 μ M. These values are in reasonable agreement with the K_i for disulfiram inhibition of the alternative path.

The binding of ^{35}S -disulfiram to cyanide-sensitive potato mitochondria yields a saturation curve similar to that obtained for

Table II. Effect of BSA on Disulfiram Inhibition

The standard reaction medium was modified with respect to BSA content as noted. State 3 was initiated by addition of 2.5 μ mol ADP, and 0.3 mM KCN and 25 μ M disulfiram were added as indicated.

| | BSA Concentration mg/ml | Respiration Rate | | | Inhibition Caused by Disulfiram % |
|--|-------------------------------|------------------|-----|---------------------|--|
| | | Control | KCN | KCN + disulfiram | |
| <i>nmol O₂ min⁻¹ mg⁻¹ protein</i> | | | | | |
| Experiment I | | | | | |
| 10 mM Succinate | 0.03 | 135 | 30 | 12 | 60 |
| | 1 | 138 | 89 | 37 | 58 |
| | 5 | 136 | 82 | 58 | 29 |
| | 10 | 144 | 95 | 77 | 19 |
| Experiment II | | | | | |
| 10 mM Malate | | | | | |
| 10 mM Glutamate | 0.4 | 103 | 66 | 6 | 91 |
| | 0.8 | 94 | 58 | 11 | 81 |
| | 2 | 93 | 63 | 24 | 63 |
| | 4 | 90 | 60 | 32 | 47 |

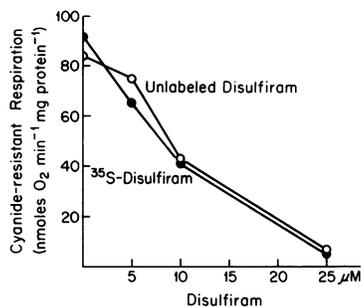


FIG. 7. Inhibition of alternative path activity caused by ^{35}S -disulfiram and unlabeled disulfiram. Mitochondria isolated from ethylene-treated potato tubers. Substrate 10 mM succinate with 0.2 mM ATP. State 3 was initiated by the addition of 2.5 μmol ADP. 0.3 mM KCN present in all assays. ^{35}S -Disulfiram or commercial, unlabeled disulfiram given as indicated.

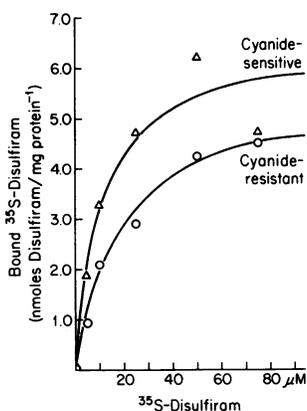


FIG. 8. The concentration dependence of specific ^{35}S -disulfiram binding to cyanide-sensitive and cyanide-resistant potato mitochondria. Cyanide-sensitive mitochondria isolated from untreated tubers; cyanide-insensitive mitochondria isolated from ethylene-treated tubers. So-called bound disulfiram in fact represents bound half-molecules of disulfiram (DIECA), since binding denotes mixed disulfide formation as depicted in equation 2 under "Discussion."

Table III. Effect of SHAM and DTT on Total ^{35}S -Disulfiram Binding in Cyanide-Resistant Potato Mitochondria

| Components of Binding Assay | Total Label Bound <i>nCi/mg protein</i> |
|---|--|
| A. 25 μM ^{35}S -disulfiram | 41.8 |
| B. As A, plus 200 μM unlabeled disulfiram | 11 |
| C. As A, plus 2 mM SHAM | 38.1 |
| D. As A, plus 1 mM DTT (given after 8 min treatment with ^{35}S -disulfiram) | 19.7 |

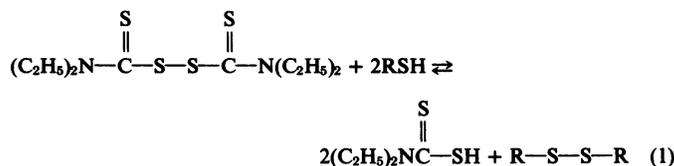
cyanide-resistant mitochondria (Fig. 8). No substantial difference in the number of specific disulfiram binding sites per mg protein was observed. The small differences that do exist are probably not significant, as there is slightly more binding in the cyanide-sensitive mitochondria. Thus, ethylene treatment does not increase the number of disulfiram binding sites in potato tuber mitochondria, as measured in this type of assay.

As a check on the physiological significance of label binding, the effect of SHAM and DTT was tested on ^{35}S -disulfiram binding to cyanide-resistant potato mitochondria. Two min of treatment with 1 mM DTT after 8 min of labeling with ^{35}S -disulfiram reversed most of the label incorporation (Table III), consistent with the ability of DTT to reverse disulfiram inhibition of the

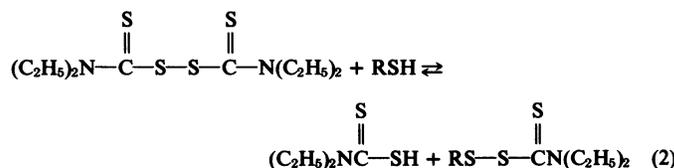
alternative path. Also in agreement with the results of kinetic studies, saturating levels of SHAM failed significantly to block ^{35}S -disulfiram binding (Table III).

DISCUSSION

Although the chemistry of disulfiram has been exploited in different ways in its diverse range of applications (9, 16, 19), the ability of disulfiram to interact with thiol groups is perhaps its most thoroughly understood characteristic. Disulfiram can oxidize a sulfhydryl containing compound to form a symmetrical disulfide:



or alternatively, a mixed disulfide:



Whether the reaction proceeds via equation 1 or 2 depends on the nature of the thiol. The symmetrical disulfide products are formed in reaction with low molecular weight thiols, whereas mixed disulfides result from reaction with protein sulfhydryl groups (22).

Although the mechanism by which disulfiram acts in isolated plant mitochondria is not known with certainty, it is clear that disulfiram is a reasonably specific inhibitor of the alternative path. While there is some evidence of a second site of inhibition outside the alternative path (perhaps in complex I), and a slight uncoupling effect, these secondary effects are minor in the concentration range required for alternative path inhibition.

Earlier workers have found inhibitory effects of disulfiram on mitochondrial oxidations. Whereas Keilin and Hartree (15) found succinate dehydrogenase in heart muscle preparations to be strongly inhibited by disulfiram, Hassinen (10) found that disulfiram had no effect on the succinoxidase activity of isolated rat liver mitochondria. In contrast, the oxidation of pyruvate and malate by these mitochondria was found to be inhibited 26% by 270 μM disulfiram, while the oxidation of β -hydroxybutyric acid was totally inhibited by 140 μM disulfiram (10). This inhibition of the oxidation of NAD-linked substrates is reminiscent of the effects of disulfiram on malate oxidation through the conventional respiratory chain of potato mitochondria.

The potency of disulfiram is much greater than that of hydroxamic acids, and disulfiram binds at a different site than the hydroxamates. Although both the hydroxamic acids and disulfiram are capable of complexing metal ions, the evidence for separate binding sites, and the fact that metal ions fail to reverse disulfiram inhibition both suggest that the two inhibitors have distinct mechanisms of action.

The facile reversal of disulfiram inhibition by thiols such as mercaptoethanol and DTT suggests that the disulfide nature of disulfiram is critical to its ability to inhibit the alternative path. While the reduction of disulfiram to DIECA by thiols destroys the ability of disulfiram to form mixed disulfides with protein SH groups, it creates a chelator (DIECA) more potent than disulfiram itself. It therefore seems likely that disulfiram is attacking a crucial sulfhydryl group of the alternative path rather than acting through metal complexation. Sulfhydryl group attack has been implicated

in disulfiram inhibition of D-amino acid oxidase (11, 23) and hexokinase (31).

There is no obvious explanation of the noneffectiveness of most of the close structural analogs of disulfiram. These other disulfides, and even the monosulfides, are capable of covalent interaction with free sulfhydryl groups (17). There are no apparent trends linking inhibitory activity to the size of the substituent groups attached to the nitrogen atom. The potency of bisethylxanthogen shows that the nitrogen itself can be replaced without loss of activity. One possible explanation of the differential activity could lie in the difference in lipophilicity (19) among disulfiram analogs. It could be that the polarity of the microenvironment surrounding the essential SH group selectively favors disulfiram partition.

The ³⁵S-disulfiram incorporation studies suggest that there is little difference in the number of disulfiram reactive sites in cyanide-resistant and cyanide-sensitive mitochondria. This unexpected result may be interpreted in several ways. First, disulfiram may react with a species that is a normal component of all mitochondria but which plays a special role in the shunt to the alternative path when the latter is present. This model requires that reaction of this component with disulfiram have but a minor effect on the operation of the main respiratory chain while causing complete inhibition of the alternative path. Although not impossible, such a model has no supporting evidence.

Secondly, the equal number of disulfiram binding sites observed in cyanide-resistant and cyanide-sensitive mitochondria may be attributable to a nonfunctional, alternative path in cyanide-sensitive mitochondria. There is evidence to support the notion that the cyanide sensitivity of mitochondria from some untreated storage organs is an artifact, the result of extensive phospholipase activity that follows cutting (35). Whereas fresh potato slices are cyanide-sensitive, they possess a SHAM-sensitive bypass of the antimycin-sensitive site. This bypass acts as a loop around the antimycin block, with electron flow returning to the Cyt chain and ending in Cyt oxidase (34). The position of this bypass in the electron transport chain and its SHAM sensitivity suggest that a portion of the alternative path is used in the bypass, even though the alternative path in its entirety is not functional in this tissue.

In the worst view, equal labeling in cyanide-sensitive and -resistant tissues may indicate that the binding reaction does not involve a component of the alternative path. Although this idea cannot be completely discounted, two observations offer indirect evidence against it. First, label incorporation saturates in the same concentration range in which physiological inhibition becomes complete, and secondly, both label incorporation and respiratory inhibition are reversed by DTT.

It should be noted that this assay selectively measures one particular type of interaction of disulfiram with mitochondria. As disulfiram is quite hydrophobic, some means of preventing non-specific retention of labeled disulfiram in membrane lipid was required. Acetone extraction of the mitochondrial pellet was used for this purpose. The potential drawback of this procedure is that it undoubtedly disrupts noncovalent complexes of disulfiram with protein, leaving only covalently bound label. However, as the evidence suggests that disulfiram inhibition involves mixed disulfide formation, the chosen protocol seems appropriate.

Assuming that all the label is incorporated into mixed disulfides, the binding studies suggest that 4 to 6 nmol disulfiram-sensitive protein sulfhydryls are present per mg mitochondrial protein. This matches closely the prevalence of coenzyme Q in plant mitochondria (4) and is an order of magnitude greater than the concentration of individual Cyt (18). Preliminary experiments suggest that a significant fraction of the bound disulfiram migrates with a low molecular weight protein (mol wt ≤ 13,000) in SDS gel electrophoresis.

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