THE TOXICITY OF DISULPHIDES TO ISOLATED HEPATOCYTES AND MITOCHONDRIA

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Abbreviations used: *RSSR = drug disulfide; Prot-SH = protein thiol; GSSR = glutathione-drug mixed disulfide; Prot-SSG = protein-drug mixed disulfide.*

SUMMARY

The disulfide metabolites of thiono-sulfur drugs were found to be about 50 to 100 times more toxic to isolated rat hepatocytes than the corresponding parent drugs. The order of decreasing cytotoxicity for the disulfide metabolites was disulfiram > propylthiouracil disulfide > formamidine disulfide > phenylthiourea disulfide > thiobenzamide disulfide > cystamine. Depletion of intracellular GSH levels preceded cytotoxicity. GSH could be restored and cytotoxicity averted by adding the thiol reducing dithiothreitol. Depletion of GSH with diethylmaleate potentiated the toxicity of disulfides 3 to 4-fold confirming the protective role of GSH in disulfide toxicity. The toxicity of disulfiram was increased 4-fold in cells pretreated with ATP (0.8mM) to effect a transient increase in cytosolic $Ca^{2}+$ suggesting an impairment of $Ca^{2}+$ homeostasis by the toxicant. Disulfiram (200μM) rapidly depleted hepatocyte ATP levels within 15 minutes which suggests that ATP production is inhibited. The disulfide effectiveness at causing mitochondrial $Ca^{2}+$ release was similar to their effectiveness at inducing hepatocyte cytotoxicity. These results suggest that hepatocyte toxicity is the result of oxidative inactivation of membrane protein thiols that regulate intracellular $Ca^{2}+$ homeostasis.

INTRODUCTION

The use of thiono-sulfur ($\succ C=S$ or $\equiv P=S$) containing compounds e.g. drugs and pesticides is associated with various toxic effects in mammals including bone marrow depression, liver and lung damage, induction of neoplasia and inhibition of enzymes /1,2/. Therapeutic doses of thiono-sulfur-containing antithyroid drugs such as methimazole, propylthiouracil, methylthiouracil or thiouracil can induce agranulocytosis in some patients (<1%) or more rarely liver injury /3/. Disulfiram in therapeutic doses for the treatment of alcoholism is associated with hepatic toxicity and dysfunction in some patients /4/. Thiobenzamide induces centrilobular hepatotoxicity in the rat and the necrosis has been attributed to the S-oxide metabolites /5/.

It is generally thought that thiono-sulfur drugs require metabolic activation via S-oxygenations catalyzed by mixed function oxidases (6-8) or peroxidases /9/ to reactive metabolites for expression of

their toxicity. However, the molecular mechanisms of these toxic effects are not fully understood. A general sequence of reactions in the S-oxidations of thiol compounds has been proposed as in Figure 1.

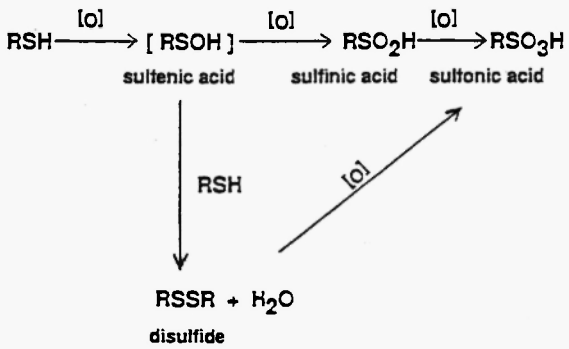

Fig. 1: Metabolic S-oxidation of thiols (from ref. 8).

Methimazole undergoes S-oxygenations by the flavin-containing enzyme to give sulfenic acid (RSOH) and sulfinic acid (RSO2H) intermediates /8/. The sulfenic acid is particularly very reactive and can react with excess thiol to generate a disulfide which can by metabolized further to a sulfinic acid or sulfonic acid (RSO3H). Although the sulfinic and sulfonic acid metabolites of thiono-sulfur drugs have been involved in toxicity studies the potential disulfide metabolites have received very little attention. The aim of the present research is to investigate if disulfide metabolites are of toxicological importance in the mechanism of toxicity of these drugs. Recently a disulfide cystamine has been shown to induce cytotoxicity in hepatocytes /10/. We now present data obtained with freshly isolated hepatocytes and liver mitochondria as model cell and cell organelle systems respectively.

II. MATERIALS AND METHODS

2.1 Chemicals

The following chemicals were purchased from the commercial sources indicated. Collagenase (from *Clostridium histoliticum*),

4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid (Hepes) and bovine serum albumin were obtained from Boehringer-Mannheim (Montreal, Canada). Glutathione (GSH), glutathione disulfide (GSSG), Arsenazo III, dithiothreitol (DTT), diethylmaleate (DEM) were obtained from Aldrich Chemical Company, Inc. (Milwaukee, Wisconsin, USA). Trypan blue, rotenone, safranin O, succinate, dimethyl sulfoxide (DMSO), $CaCl_2$, ethylene glycol bis(β-aminoethyl ether)-N,N'-tetraacetic and (EGTA), diethyldithiocarbamate (DEDC), disulfiram, thiourea, formamidine disulfide dihydrochloride, cysteamine hydrochloride,cystamine dihydrochloride, phenylthiourea, propylthiouracil (PTU) and thiobenzamide were obtained from Sigma Chemical Co. (St. Louis, MO). The disulfide metabolites of phenylthiourea, propylthiouracil and thiobenzamide were prepared by oxidation of the parent drugs with iodine (11,12).

2.2 Animals

Male Sprague-Dawley rats (225-250g) fed a standard chow diet and tap water *ad libitum* were used for isolation of hepatocytes and liver mitochondria.

2.3 Isolation and incubation of hepatocytes

Hepatocytes were isolated from rats by collagenase perfusion of the liver as described by Moldeus et al. /13/. Isolated cells were suspended in Krebs-Henseleit buffer supplemented with 12.5mM Hepes, PH7.4. Cell viability was assessed by the Trypan blue dye exclusion test using a Neubauer chamber and a light microscope. Only cells with initial viability > 85% were used for all experiments. Cells were incubated (1.0×10^6 cells/ml) in round bottomed flasks rotating in a water bath at 37°C under a constant flow of 95% O_2/5% CO_2. Stock solutions of componds were made in buffer or DMSO for addition to the incubation mixtures after approximately 30min preincubation. The final concentration of DMSO added to cells did not exceed 0.5% (v/v) which was found to be nontoxic to cells, and did not affect GSH levels.

Total GSH and GSSG levels in cells were determined using the

derivatisation and HPLC method of Reed et al. /14/. Levels of ATP in hepatocytes were measured as described by Stochi et al. /15/.

2.4 Isolation and incubation of liver mitochondria

Liver mitochondria were prepared according to the procedure described by Moore et al. /16/. The mitochondria were suspended in a medium containing 210mM mannitol, 70mM sucrose and 10mM Hepes at final pH7.4 and kept on ice for use immediately. Protein concentration was determined by the Bradford method /17/ using bovine serum albumin as a standard. Mitochondria were incubated (1mg protein/ml) at room temperature in the suspending medium supplemented with 3μM rotenone and 5mM succinate (preadjusted to pH7.4).

The incubations were carried out in cuvettes and were constantly stirred while other additions to the mixtures were made as shown in the figure legends. All spectrophotometric assays were carried out in an Aminco DW 2000 dual wavelength spectrophotometer.

Ca^{2+} fluxes through mitochondrial membrane were followed by measuring the absorbance changes of the metallochromic dye Arsenazo III (40μM) using a wavelength pair 654-685nm /18/. Mitochondrial transmembrane potential was measured using the dye safranin O (10μM, final concentration) with the wavelength pair 533-511nm /19/. Mitochondrial NAD(P)H oxidation was followed spectrophotometrically using the wavelength pair 340-375nm/20/.

III. RESULTS

The disulfide metabolites induced a dose and time-dependent decrease in cell viability at concentrations lower than the corresponding parent drugs (Table 1). The factor of increased cytotoxic effectiveness of these disulfides was about 50 to 100-fold depending on the disulfide. The thiol compound cysteamine was however equally as toxic as its disulfide metabolite probably due to its rapid oxidation to cystamine /8/. The cytotoxicity of disulfides was preceded by a depletion of intracellular GSH levels without a stoichiometric formation of GSSG.

TABLE 1

Cytotoxicity and GSH depletion induced by disulfides in isolated hepatocytes

Compounds	Conc. (mM)	LD50 + time (min)	% initial GSH at 60 min
No addition		ND	95
Cysteamine	2.0	130	10
Cystamine	2.0	160	22
Cystamine	0.5	>240	33
Cystamine + DEM	0.5	75	ND
DEDC	10.0	>240	90
Disulfiram	0.3	30	12
Disulfiram	0.2	90	60
Disulfiram + DEM	0.2	15	ND
Thiourea	30.0	>240	93
Formamidine disulfide	0.5	60	17
Formamidine disulfide	0.1	140	51
Phenylthiourea	10.0	210	40
Phenylthiourea disulfide	0.5	90	31
Phenylthiourea disulfide	0.2	150	53
Propylthiouracil	10.0	>240	ND
Propylthiouracil disulfide	0.5	75	26
Propylthiouracil disulfide	0.1	180	47
Thiobenzamide	30.0	>240	ND
Thiobenzamide disulfide	1.0	120	68
Thiobenzamide disulfide	0.5	200	64

Time at which 50% cell death occurred as judged by Trypan blue uptake
DEM, diethylmaleate (1 mM) was used.
ND = Not determined.

At subtoxic dose levels however disulfiram caused a stoichiometric oxidation of GSH to GSSG which remained oxidized for a long time (data not shown). When cells were preincubated with DEM to effect the depletion of GSH the toxicity of disulfides was increased 3 to 4-fold resulting in loss of cell viability at lower doses of the toxicant.

TABLE 2

Modulation of disulfide-induced cytotoxicity by DTT

Disulfide	Concn. (mM)	LD50[+] at 60 min	% initial GSH
No addition		ND	95
Cystamine	2.0	160	22
Cystamine + DTT*	2.0	240	100
Disulfiram	0.3	30	12
Disulfiram + DTT	0.3	60	100
Formanidine disulfide	0.5	60	17
Formanidine disulfide + DTT	0.5	120	100
Phenylthiourea dislfide	0.5	90	31
Phenylthiourea dislfide + DTT	0.5	120	100
Propylthiouracil disulfide	0.5	75	26
Propylthiouracil disulfide + DTT	0.5	120	100
Thiobenzamide disulfide	1.0	120	38
Thiobenzamide disulfide + DTT	1.0	210	100

+ Time at which 50% cell death occurred as judged by Trypan blue uptake.

* DTT, dithiothreitol (1 mM) added 20 min after the disulfide was added to the hepatocytes.

ND = not determined.

This suggests that GSH plays a protective role in the disulfide toxicity.

3.1 Effect of dithiothreitol on disulfide cytotoxicity

To study further the role of GSH on the toxicity of disulfides, the thiol reducing agent dithiothreitol (DTT) was added to the incubate 20 minutes after addition of the disulfides. Table 2 indicates that the presence of DTT decreased 2 to 3 fold the toxicity of disulfides.

Furthermore DTT caused a full recovery of the GSH levels of cells suggesting that the GSH depletion was due to mixed disulfide formation with the drug disulfide.

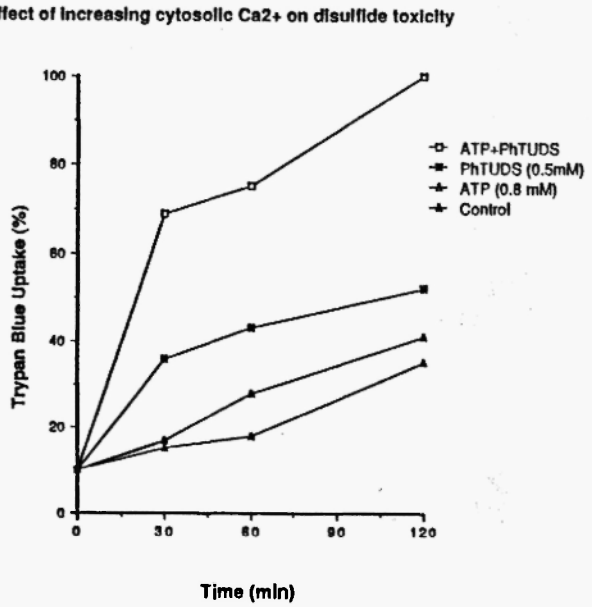

Fig. 2: Effect of increasing cytosolic Ca^{2+} on disulfide toxicity to hepatocytes. Hepatocytes were preincubated in absence or presence of ATP (0.8mM) as described in Materials and Methods. Phenylthiourea disulfide (PhTUDS) was added after about 5 minutes and cell viability was monitored by trypan blue uptake. Each point represents the mean of two separate experiments.

3.2 Effect of increasing cytosolic Ca^{2+} on disulfide toxicity

Depletion of intracellular thiols by certain toxicants has been reported to cause a disruption of Ca^{2+} homeostasis which results in a sustained increase in cytosolic Ca^{2+} concentration and subsequent cytotoxicity /10,21,22/. The effect of increasing cytosolic Ca^{2+} on disulfide toxicity by has been studied by preincubation of the cells with ATP (0.8mM) to effect a transient increase in cytosolic Ca^{2+} /23/. Figure 2 shows that the toxicity of phenylthiourea disulfide was

potentiated 2 to 3-fold in the ATP-treated cells. This suggests the disulfide may prevent the redistribution of Ca^{2+} in the cytosol during the transient Ca^{2+} increase effected by ATP treatment. The disulphides cystamine and disulfiram cause a decrease in ATP levels in hepatocytes (Figure 3).

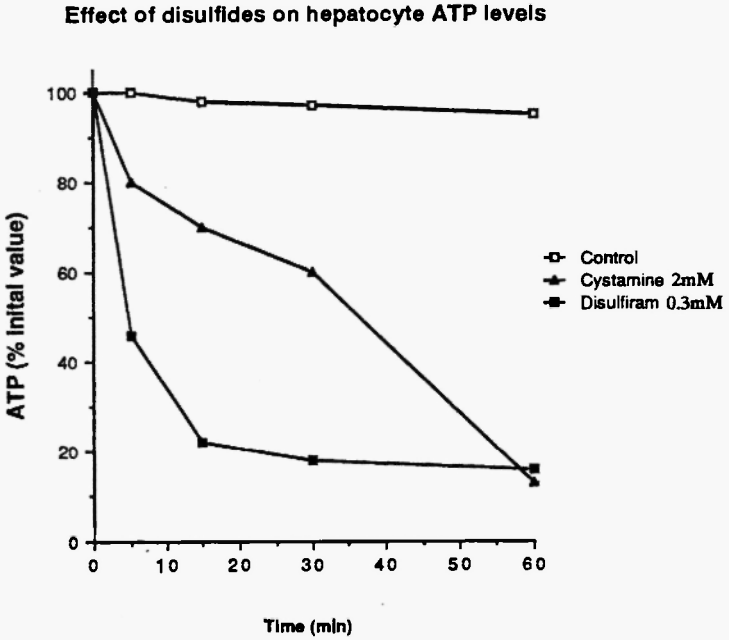

Fig. 3: Effect of disulfides on hepatocyte ATP levels. Hepatocytes were incubated with cystamine (2mM) or disulfiram (0.3mM) and aliquots were withdrawn at the times indicated for determination of ATP levels. Control value for ATP was 14.7 nmols/10^6 cells. Each point represents the mean from two separate experiments.

3.3 Studies with isolated liver mitochondria

Mitochondria play an important buffering role in the control of cytosolic Ca^{2+} levels by taking up Ca^{2+} when excessive Ca^{2+} levels are reached in the cytosol and releasing it when excess Ca^{2+} leaves the cell /22/. To test further the hypothesis that disulfides disrupt intracellular Ca^{2+} homeostasis experiments were carried out using

liver mitochondria. The addition of disulfides to Ca^{2+}-loaded mitochondria caused a dose dependent release of the sequestered Ca^{2+} as shown for disulfiram and cystamine (Fig. 4A). The disulfides when added first to mitochondria for 2 minutes before addition of Ca^{2+} did not prevent the Ca^{2+} uptake process which suggests that the Ca^{2+} uptake route was not blocked by disulfides.

TABLE 3

Disulfide-induced Ca^{2+} release by mitochondria

Additions	Concn. (μM)	Time of 50% Ca^{2+} release (min)	
		-DTT	+ DTT at 2 min
No addition		>15.0	
Disulfiram	25	7.0 ± 0.4	>15
Disulfiram	50	4.5 ± 1.0	>15
Formanidine disulfide	100	4.0 ± 0.4	ND
Formanidine disulfide	200	2.5 ± 0.3	ND
Phenylthiourea dislfide	500	6.5 ± 1.0	(.5 ± 1.2
Propylthiouracil disulfide	500	5.0 ± 0.7	8.0 ± 1.4
Thiobenzamide disulfide	500	6.0 ± 0.5	10.0 ± 1.5
Cystamine	5000	5.5 ± 0.5	10.5 ± 0.8

Note: Mitochondria were preloaded with 50 nmol Ca^{2+}. After 1.5 min, the disulfides were added at the concentrations shown. Release of Ca^{2+} was followed as described under Experimental Procedures. Values represent ±S.E., where n=3.

N.D. = not determined.

Table 3 presents the times for 50% Ca^{2+} release by various concentrations of the different disulfides. The effectiveness of the different disulfides at causing Ca^{2+} release correlated with their effectiveness at inducing hepatocyte cytotoxicity. However, adding less than physiological concentrations of ATP-Mg^{2+} (200μM) to the incubation medium prevented the disulfide-induced Ca^{2+} release (data not shown).

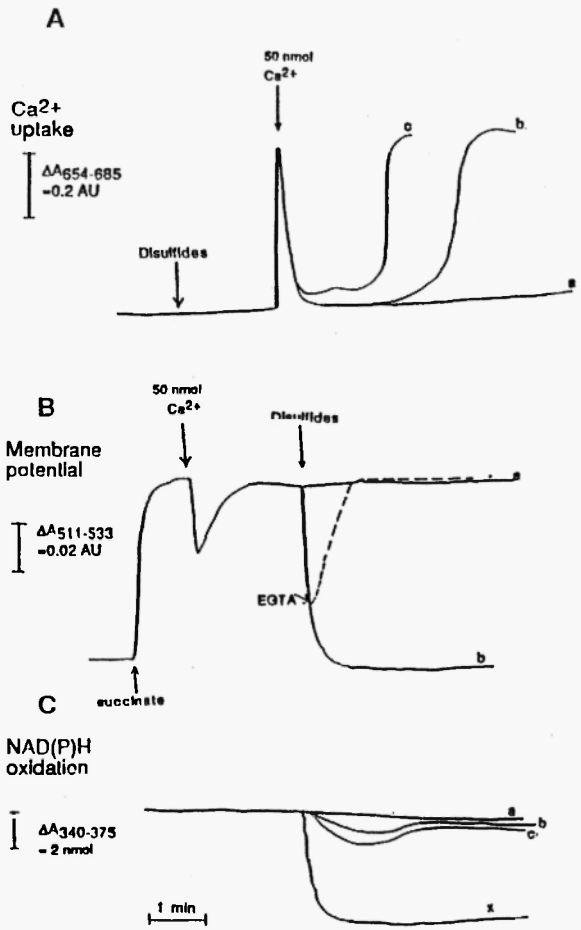

Fig. 4: Effect of disulfides on mitochondrial functions. Mitochondria (1 mg/ml) were preincubated at 25°C.

A) Disulfides were added to the above incubation mixture after which 50nmol of Ca^{2+} was added and Ca^{2+} uptake and release were followed. (a) No disulfides present, (b) cystamine (3mM) (c) disulfiram (50μM) as described in Materials and Methods.

B) Succinate-energised mitochondria were first loaded with Ca^{2+} (50nmol) for 1.5min followed by the addition of disulfides. Membrane potential was measured as described in Materials and Methods. (a) No disulfide added (b) Disulfide added.

C) Mitochondria were preloaded with 50nmol Ca^{2+} protein. Disulfides were added as indicated by the arrow. NAD(P)H oxidation was monitored as described in the Materials and Methods.

Addition of the reductant DTT to isolated mitochondria 2 minutes after reaction with the disulfides prevented Ca^{2+} release or delayed the onset of release. This was probably due to reduction of the disulfides to the parent drugs and regeneration of membrane thiols.

3.4 Effect of disulfides on mitochondrial transmembrane potential

The effective uptake and retention of Ca^{2+} by mitochondria is dependent on a membrane potential. Fig 4B shows that when disulfides were added to mitochondria which were energized with succinate to maintain a membrane potential, there was a rapid decline of the membrane potential. Addition of EGTA (20μM) to chelate the released Ca^{2+} prevented Ca^{2+} reuptake and caused a recovery of the membrane potential to the original level. This indicates that the collapse in membrane potential caused by disulfides was not due to damage of the mitochondrial membrane but was probably due to energy draining Ca^{2+} recycling /24/.

3.5 Disulfide-induced oxidation of NAD(P)H in Mitochondria

The induction of mitochondrial Ca^{2+} release by certain toxicants has been reported to be initiated by the oxidation of NAD(P)H resulting form oxidative stress /21/. Disulfides were found to cause the oxidation of only some of the NAD(P)H in mitochondria as compared to complete oxidation produced by oxalacetate (Fig. 4C). This suggests that the disulfide-induced mitochondrial Ca^{2+} release does not require NAD(P)H oxidation.

VI. DISCUSSION

The results presented show that disulfide metabolites of thiono-sulfur drugs are about two orders of magnitude more toxic to hepatocytes than the corresponding parent drugs. An exception was the physiological thiol compound, cysteamine, which was found to be slightly more toxic than its disulfide metabolite cystamine. Catalase partially prevented cysteamine toxicity (data not presented) indicating

that hydrogen peroxide (H_2O_2) formed by autoxidation of the cystamine in the medium contributed to the cytotoxicity. However, hepatocytes are not normally susceptible to H_2O_2 /25/ and it is likely that cystamine formed by the hepatocyte flavin-containing monooxygenase /8/ made the hepatocytes highly susceptible to H_2O_2 by inactivating enzymes involved in defending the hepatocyte against H_2O_2

The cytotoxicity of disulfides observed in this study agree with the reports that disulfiram reacts with thiol groups to form mixed disulfides and cause inhibition of many enzymes including glyceraldehyde-3-phosphate dehydrogenase, aldehyde dehydrogenase and xanthine oxidase /26/. Formamidine disulfide also undergoes mixed disulfide formation with the thiol groups of the enzymes papain, ureas and arginine-glycine transmidinase which results in inactivation of these enzymes /27/.

Thiol-containing drugs for example penicillamine and captopril /28/ have been shown to be converted to their disulfide metabolites as well as form mixed disulfide derivatives with cysteine and GSH both in vitro and in vivo. Whether autoxidation of thiol-containing drugs occurs in vivo is not known. Evidence that thiono-sulfur drugs are metabolized to disulfides in vivo is lacking.

However, microsomal S-oxygenation of thiourea derivatives has been reported to be catalyzed by FAD-containing monooxygenase /8/. The oxidative metabolites of [2-^{14}C]propylthiouracil in rat thyroid have been studied by Lindsay et al. /9/ who reported an unidentified metabolite having characteristics consistent with PTU thiosulfonic ester. They concluded that this metabolite was probably formed via an intermediate disulfide of PTU.

The present results confirm the observation of Nicotera et al. /10/ that cystamine causes cytotoxicity in isolated hepatocytes. These authors found that cystamine formed mixed disulfides with plasma membrane thiols, the plasma membrane Ca^{2+}-ATPase was inactivated and the cytosolic Ca^{2+} concentration was increased before cytotoxicity ensued. They found no GSH depletion and they therefore concluded that cystamine did not enter the hepatocytes so that only the plasma membrane was modified. However, our present data shows that all disulfides including cystamine at subtoxic depleted intracellular GSH. Furthermore the reductant, dithiothreitol, added later restores intracellular GSH and prevented cytotoxicity.

This shows that disulfides penetrated the plasma membrane to form mixed disulfides with GSH and probably other intracellular thiols. However, the rate of penetration of these disulfides into the cells was probably a factor in their toxicity since cystamine was the least toxic of the compounds tested and this disulfide has been shown to be poor penetrant of mitochondrial membrane /29/. Even so, toxic doses of cystamine depleted intracellular GSH and it is not clear why our results are different from those reported /10/.

Pretreatment of hepatocytes with ATP markedly increased their susceptibility to disulfides (Fig. 2) and is in agreement with other reports that the cytotoxicity of other toxicants is preceded by a sustained increase in Ca^{2+} level in the cytosol /10,24/. Treatment of hepatocytes with ATP causes a transient increase of cytosolic Ca^{2} presumably as a result of mobilisation of calcium from storage sites. However, the ATP treated hepatocytes quickly redistribute the Ca^{2+} by homeostasic changes and release the Ca^{2+} to prevent toxic effects /23/.

Further evidence for the disruption of Ca^{2+} homeostasis by disulfides was obtained from experiments using liver mitochondria. Disulfides were found to inhibit mitochondrial Ca^{2+} retention and decrease the mitochondrial membrane potential (Fig. 4B). Release of Ca^{2+} from mitochondria can be induced by agents which collapse the transmembrane potential or specifically inhibit the uptake route /24/. Although the disulfides did not block the mitochondrial uptake of Ca^{2+}, they prevented its retention by the mitochondria. It therefore seems that the induction of Ca^{2+} release was a result of inhibition of the membrane potential by disulfides. This was confirmed when it was shown that disulfides cause a collapse in membrane potential as in (Fig. 4B). Furthermore both Ca^{2+} release and the inhibition of membrane potential were prevented by addition of dithiothreitol indicating that mixed disulfide formation with membrane thiols was responsible for inhibition of these mitochondrial functions. The induction of mitochondrial Ca^{2+} release by disulfides was also found to be prevented by less than physiological concentrations of ATP-Mg^{2+} to mitochondria. This suggests that ATP in the hepatocyte would prevent disulfides interfering with mitochondrial role in helping to maintain cellular Ca^{2+} homeostasis by ATP depletion. However, disulfiram was found to cause a marked depletion of ATP levels in hepatocytes (Fig. 3) which was probably due to the inhibition of glycolysis and

mitochondrial phosphorylation. Disulfiram cytotoxicity could therefore involve mitochondrial damage. Cystamine on the other hand did not deplete ATP so that the cytotoxic site could be the plasma membrane Ca^{2+}-ATPase.

There is evidence that the induction of Ca^{2+} release from mitochondria by oxidant toxicants involves ADP-ribosylation of the $Ca^{2+}/2H+$ antiporter initiated by NAD(P)H oxidation and depletion /24/. However, our results (Fig. 2C) suggest that mitochondrial Ca^{2+} release does not involve NAD(P)H depletion. Indeed it is likely that disulfides poorly oxidize mitochondrial NAD(P)H because of poor permeability of the inner membrane to disulfides /29/. Because of the reversal of Ca^{2+} release by dithiothreitol it is likely that the disulfides form mixed protein disulfides with membrane protein thiols involved in Ca^{2+} transport and that this causes Ca^{2+} release. It is possible that the disulfides activate phospholipase, another mechanism suggested for mitochondrial Ca^{2+} release /30/.

From the present data the mechanisms for cytotoxicity by disulfides on isolated hepatocytes can be illustrated as in Figure 5.

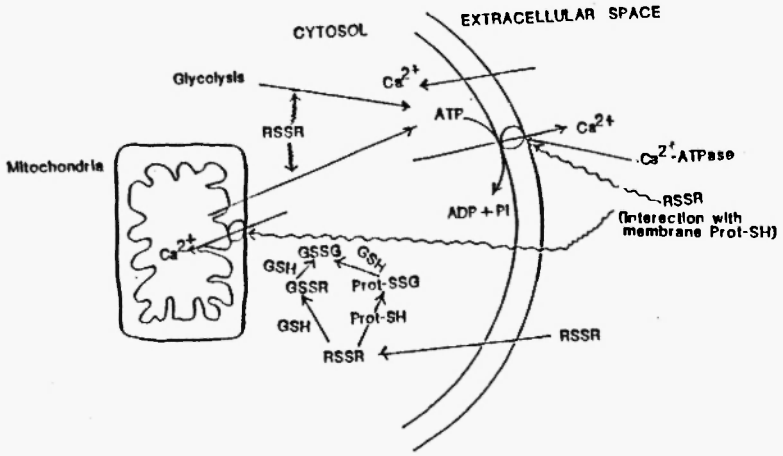

Fig. 5: Proposed mechanisms of disulfide-induced cytotoxicity.

The primary target would be oxidation of membrane thiols necessary for Ca^{2+}-ATPase to extrude Ca^{2+} from the cell and necessary for mitochondria to act as a Ca^{2+} buffer which results in cytotoxicity. Disulfides on entry into the cell become inactivated by GSH.

However, when the GSH is used up protein thiol groups would be extensively depleted which could result in inhibition of cellular respiration and prevention of ATP production.

It can therefore be concluded that the hepatocyte toxicity is the result of the oxidative inactivation of membrane proteins that regulate intracellular Ca^{2+} homeostasis.

V. ACKNOWLEDGEMENTS

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