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# REGULATION OF REDUCTIVE PROCESSES BY GLUTATHIONE

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Bio-reduction reactions in the activation of drugs are capable of consuming a significant fraction of the NADPH-reducing* equivalents in the cell. These reactions often utilize such reducing equivalents for bio-reduction to activate a drug to an unstable intermediate and to reduce, via glutathione-reducing equivalents, the hydrogen peroxide or hydroperoxide formed if the drug intermediate(s) reacts with molecular oxygen. Since the "resting" pool size of glutathione (GSH) in rat hepatocytes and in L-5178Y lymphoma cells is about 10-fold greater than that of NADPH [1, 2], the GSH pool has a major influence upon the status of the NADPH pool and its intracellular utilization during drug metabolism. Failure of GSH to provide the necessary reducing equivalents for cellular detoxication of drug-induced hydrogen peroxide is characterized by glutathione depletion as GSSG formation and efflux occurs. For reviews see [2–4]. Thus, glutathione can be given a regulatory role in drug bio-reduction due to the high demand that can be placed on cellular capacity to generate NADPH-reducing equivalents.

This paper will focus on the evidence that the reducing equivalents contained in NADPH and GSH can provide very dynamic responses during bio-reduction of drugs and that the glutathione redox cycle is the major pathway to provide the reducing equivalents for bio-reduction of drug-induced superoxide and hydrogen peroxide. Sustained (3–4 hr) production of high levels of GSH-reducing equivalents has been demonstrated by a steady state GSH oxidant challenge with diamide to L-5178Y murine lymphoma cells without loss of cell viability.

## NADPH GENERATION

The reducing equivalents needed for the NADPH-dependent reactions, including those by NADPH-dependent bio-reductions that are catalyzed by cytochrome P-450 reductase, glutathione reductase, as well as fatty acid biosynthesis, are derived from substrates to several dehydrogenases (Table 1). Similar data by Kauffman *et al.* [5] suggest that isocitrate dehydrogenase activity supports the predominant utilization of NADPH and that the two dehydrogenases of the hexane monophosphate pathway contribute an increasing amount of NADPH as the inhibition by the high NADPH:$NADP^+$ ratio is reversed [6].

Table 1. Generation of NADPH-reducing equivalents in rat liver [8]

| Enzyme | Enzyme activity ($\mu$mol/min/g wet wt liver, 25°) |
|---|---|
| Isocitrate dehydrogenase | 22.4 |
| Glucose-G phosphate dehydrogenase | 1.42 |
| 6-Phosphogluconate dehydrogenase | 2.84 |
| Malic enzyme | 1.27 |

Glucose-6-phosphate dehydrogenase, which catalyzes the first step in the hexose monophosphate pathway, is inhibited by the product, NADPH. The inhibition approaches 100% at ratios of NADPH to $NADP^+$ greater than 9 [7]. Regulation of the enzyme may occur by the presence of a cellular constituent that relieves the NADPH inhibition since the ratio of NADPH to $NADP^+$ in mammalian tissues is about 100 to 1 [8]. The maximum rate of $NADP^+$ reduction to NADPH by the hexose monophosphate pathway in the liver appears to be about 1–2 $\mu$mols/min/g liver [9–11].

It is interesting that of the major enzymes that utilize NADPH as a substrate, glutathione reductase, cytochrome P-450 reductase, and DT-diaphorase, all have $\mu$M affinities for NADPH (Table 2) and thus are capable of lowering cellular NADPH to levels that would relieve the NADPH inhibition. Efforts to understand the regulation of the hexose monophosphate pathway have led Eggleston and Krebs [6] to consider the ability of GSSG to counteract the inhibition of NADPH. Levy and Christoff [12] have critically appraised the effect of GSSG on glucose-6-phosphate dehydrogenase activity, and they conclude that reversal is largely an artifact and that no evidence exists for GSSG counteracting NADPH inhibition of this enzyme.

## GLUTATHIONE REDUCTASE AND GLUTATHIONE PEROXIDASE

Glutathione reductase has a key role in the regulation by glutathione of the bio-reductive activation of drugs. Similar to other reductases such as nitrate, nitrite, and $NADP^+$ reductase, glutathione reductase

* Abbreviations used: NADPH, nicotinamide adenine dinucleotide phosphate, reduced; GSH, reduced glutathione; GSSG, oxidized glutathione; NAPQI, *N*-acetyl-*p*-benzoquinone imine; BCNU, *N*,*N*-bis(2-choloroethyl)-*N*-nitrosourea; DTT, dithiothreitol; ADR, Adriamycin; LDH, lactate dehydrogenase; DEM, diethylmaleate.

Table 2. Substrates affinities for related reducing enzymes

| Enzyme | Substrate | $K_m$ ($\mu$M) | $K_I$ ($\mu$M) | [Ref.] |
|---|---|---|---|---|
| Glucose-6-$PO_4$ | Glucose-6-$PO_4$ | 39 | | [35] |
| Dehydrogenase | $NADP^+$ | 21 | | [35] |
| | NADPH | | 27 | [36] |
| Glutathione | NADPH | 3 | | [37] |
| Reductase | GSSG | 50 | | [37] |
| Cytochrome | NADPH | 5 | | [38] |
| P-450 Reductase | $NADP^+$ | | 4 | [38] |
| DT-Diaphorase | NADPH | 44 | | [39] |
| | NADH | 83 | | [39] |
| | Dicoumarol | | 0.01 | [39] |
| Glutathione | GSH | 130 | | [16] |
| Peroxidase | $H_2O_2$ | 8 | | [16] |
| Catalase | $H_2O_2$ | 1,100,000 | | [40] |

is inactivated upon reduction by its own electron donor, NADPH. It is been proposed that this autoinactivation of glutathione reductase by NADPH and the protection as well as reactivation by GSSG have *in vivo* an important regulator role [13]. The activity of glutathione reductase may reflect the physiological needs of the cell. For example, 40–50 $\mu$M intracellular NADPH inactivates glutathione reductase in the absence of GSSG and decreases glucose metabolism via the hexose monophosphate pathway. The physiological GSSG:GSH ratio should provide sufficient GSSG at this level of NADPH to permit retention of significant glutathione reductase activity by preventing inactivation [13].

The maximum rate of GSSG reduction by glutathione reductase appears to be about 8–10 $\mu$moles/min/g liver [14]. Glutathione peroxidase, a selenium-dependent enzyme that is extremely specific for GSH, is capable of rapidly detoxifying hydrogen peroxide and certain hydroperoxides. The maximal rate of GSH oxidation by glutathione peroxidase is 40 $\mu$moles/min/g liver [15]. The chemical and physical properties of glutathione peroxidase have been reviewed [16]. The enzyme fails to display saturation with respect to GSH concentration, and thus the extrapolated $V_{max}$ is infinite [16]. The lack of a defined $K_m$ value agrees with the observation that the apparent maximum velocities for infinite peroxide concentration are a linear function of the concentration of GSH and thus support a ter uni ping-pong mechanism for this enzyme [16].

## GLUTATHIONE REDOX CYCLE

It is apparent that a major protective role against the reactive drug intermediates generated by bioreduction is provided by the ubiquitous glutathione redox cycle (Fig. 1). This cycle utilizes NADPH- and, indirectly, NADH-reducing equivalents in the mitochondrial matrix as well as the cytoplasm to provide a recycling supply of GSH by the glutathione reductase-catalyzed reduction of GSSG.

The rates of NADPH consumption in liver by the various NADPH-dependent enzymes indicate that glutathione reductase has by far the highest rate (Table 3). Therefore, when the glutathione redox cycle is functioning at maximum capacity, a major regulatory effect is imposed on fatty acid synthesis, mixed-function oxidase activity, and other NADPH cytochrome reductase activities.

The mitochondrial glutathione redox cycle has a

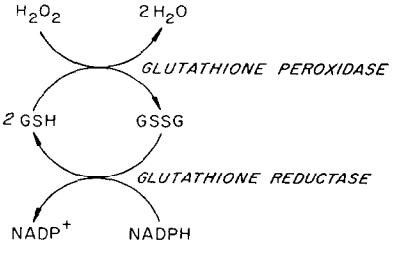


Fig. 1. Glutathione redox cycle.

Table 3. Estimation of the rates of NADPH consumption in liver

| Activity | Rate ($\mu$mol/min/g liver) | [Ref.] |
|---|---|---|
| Fatty acid synthesis | 1–2 | [9] |
| Mixed function oxidase | 1–2 | [10] |
| *t*-Butyl hydroperoxide reduction | 1.5 | [41] |
| Glutathione reductase* | 8–10 | [14] |

* The estimated maximal rate of glutathione peroxidase activity is 40 $\mu$mol/min/g of liver [15].

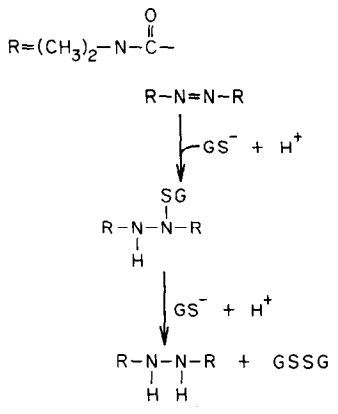


Fig. 2. Diamide oxidation of glutathione.

role in regulating mitochondrial oxidations in liver. Various oxidants decreased $O_2$ uptake by isolated mitochondria and caused a complete turnover of GSH via glutathione peroxidase every 10 min [17]. It appears that a continuous flow of reducing equivalents through the glutathione redox cycle is balanced by a continuous formation of mitochondrial NADPH, which is needed for glutathione reductase activity. In addition, hydrogen peroxide metabolism in mitochondria poses a regulating function in regard to the oxidation of substrates by lipoamide-dependent ketoacid oxidases [17], which generate NADPH-reducing equivalents. The entire NADPH:$NADP^+$ pool may turn over at least once every minute during a maximum oxidant challenge [17].

## DIAMIDE

Diazenedicarboxylic acid bis(*N*,*N*-dimethylamide) was given the trivial of diamide by Kosower *et al.* [18] when it was introduced as a new reagent for the intracellular oxidation of GSH to GSSG. This compound is stable, and its reduction product, a hydrazide, is stable and relatively nontoxic to cells. Although some nonspecific reactions occur with diamide [19, 20] the rate of GSH oxidation is nearly 100-fold faster than that of NADH [21]. The reaction sequence in the oxidation of GSH is shown in Fig. 2.

A steady-state elevation of the ratio of oxidized to reduced glutathione in L-5178Y lymphoma cells was achieved by infusion of diamide (diazenedicarboxylic acid bis[*N*,*N*-dimethylamide]) through semipermeable hollow fibers into high-density cell suspensions (Bump and Reed, unpublished results).

Initially, the reductive capacity of L-5178Y cells was determined by dripping a diamide solution at a constant rate into a stirring cell suspension (in Fischer's medium) at 37° and measuring the effect on the intracellular ratio of GSSG to GSH. These cells were found to be capable of reducing GSSG to GSH at a rate of 2–3 μmoles/min per ml packed cell volume, while maintaining a constant GSSG:GSH ratio. This is equivalent to a turnover of the entire cellular glutathione pool every minute (Bump and Reed, unpublished results).

The alteration of the glutathione redox state in L-5178Y cells after 2 hr of diamide infusion through hollow fibers was based on the total packed cell volume in each minibeaker. Although some variation of the GSSG:GSH ratio was noted, as would be expected for cells under stress to overcome an oxidative challenge, a linear relationship between diamide treatment (in the range of 0–30 μmoles per ml packed cell volume per min), and glutathione oxidation (GSSG:GSH ratios of 0.016 to 0.05) was observed. At diamide levels above 30 μmoles per ml packed cell volume per min, the reductive capacity of the cells was sharply exceeded, and glutathione became essentially 100% oxidized. The reductive capacity of L-5178Y cells under these conditions is sufficient to turn over the entire cellular glutathione pool ten times every minute. In turn, the level of diamide required to achieve a minimal effect on the intracellular GSSG:GSH ratio in this system was sufficient to turn over the entire cellular content of glutathione every 30 sec. Total glutathione levels (GSSG + GSH) were in the range of 2–3 μmoles

Table 4. L-5178Y cell viability and metabolic activity after diamide treatment by hollow fiber perfusion*

| Diamide treatment (μmol/ml PCV/min) | Cell viability (%) | Lactate production (μmol/min) 60 min | 120 min | $O_2$ uptake (120 min) (μml/ml PCV/min) | Leucine incorp. into protein† (% of control) |
|---|---|---|---|---|---|
| Untreated cells | 99 | | | 515 | 104 |
| Control | 93 | 0.34 | 0.25 | 512 | 100 |
| 3–10 | 93 | 0.30 | 0.28 | 590 | 46 |
| 13–27 | 78 | 0.39 | 0.13 | 330 | 41 |
| 35–55 | 5 | 0.28 | 0.13 | | < 1 |

* Unpublished data of E. A. Bump and D. J. Reed.

† [1-$^{14}$C]Leucine for 20 min after 3 hr of infusion of diamide.

Cells harvested from tumor-bearing mice were treated with diamide by hollow fiber perfusion. A Bio-Fiber® 50 minibeaker was filled with Fischer's medium (containing 10% horse serum and antibiotics), and a solution of 1 mg/ml diamide in Fischer's medium (without horse serum) was pumped through the hollow fibers at a rate of 100 ml/hr. Samples of medium inside and outside the fibers were taken at 5-min intervals. The steady state concentration of diamide outside the fibers without L-5178Y lymphoma cells was calculated from the difference between diamide levels in the effluent and in the original solution. Under these conditions, 27% (± 2%) of the diamide that was pumped through the hollow fibers passed through the fiber pores into the medium contained in the minibeaker.

per ml packed cell volume and did not decrease appreciably during the course of diamide treatment.

Marked inhibition of [1-$^{14}$C]leucine incorporation was observed after exposure of L-5178Y lymphoma cells to diamide by the hollow fiber technique (Table 4). Even at the lowest levels of diamide (3–10 μmoles diamide per ml packed cell volume per min), which resulted in steady-state GSSG levels from 1.6 to 3% of the total glutathione content, the rate of $^{14}$C incorporation into cellular protein was of the order of 50% of that of controls. At these levels of GSSG, cell viability remained high (greater than 93% of vital staining), and no deficiency in amino acid transport could be detected. Intracellular leucine concentration and [1-$^{14}$C]leucine specific activity, as determined by high-performance liquid chromatography of the double-labeled dinitrophenyl methyl esters, were comparable to those of controls at the end of the 20 min [1-$^{14}$C]leucine incorporation period.

Two concentrations of reduced diamide, the hydrazide product of the reaction of diamide with GSH, were utilized in a control experiment to determine the extent that diamide could be causing inhibition of protein synthesis indirectly, through hydrazide formation. The extent of inhibition that was observed was very slight, compared to that seen with equivalent amounts of diamide.

Lactate production is a major metabolic pathway in L-5178Y lymphoma cells, accounting for as much as 90% of the pyruvate produced from glucose. Lactate production from glucose is reported to be 1.5 μmoles per mg protein per hour under aerobic conditions, compared to 2.5 μmoles per mg protein per hour under anaerobic conditions [22]. Although the rate of lactate production at any given time is of limited diagnostic value, the ability of cells to maintain a constant rate of production over the 4-hr treatment period indicates their metabolic capabilities are not impaired during this period. The ability of cells to supply reducing equivalents for glutathione reduction in the face of massive challenge with diamide throughout the 3-hr diamide infusion is, in itself, evidence of viability.

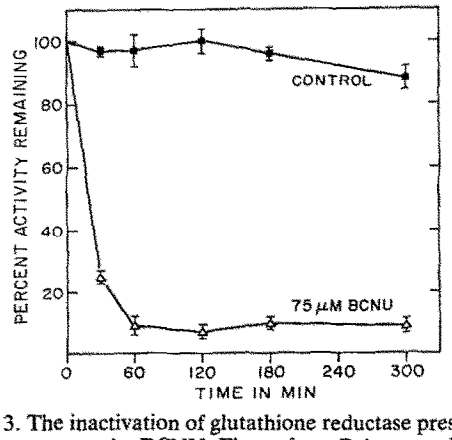


Fig. 3. The inactivation of glutathione reductase present in rat hepatocytes by BCNU. Figure from Babson *et al.* [25].

## BCNU INACTIVATION OF GLUTATHIONE REDUCTASE

Many studies have employed the inactivation of glutathione reductase to assess the key importance of the glutathione redox cycle during oxidant stress generated by intracellular bio-reductive processes. Frischer and Ahmad [23] observed that only glutathione reductase, out of 19 erythrocyte enzymes assayed, is inactivated *in vivo* during chemotherapy of patients with BCNU. Further studies by Babson and Reed [24] found that inactivation of glutathione reductase occurs only when the enzyme is in reduced state (i.e. NADPH reduction) and two thiol groups are present at the active site. The inactivation appears to occur as a thiocarbamate adduct, presumably with the distal thiol group. Inactivation of glutathione reductase activity in freshly isolated hepatocytes is about 90% complete within an hour [25] (Fig. 3). Both cytoplasmic and mitochondrial glutathione reductase activity are inactivated by BCNU [26].

Studies of the BCNU effects on freshly isolated hepatocytes [25, 26] have been supported by the work of [27] in which it was concluded that the glutathione redox cycle is very important in the intracellular metabolism of $H_2O_2$ formed by the cytochrome P-450 electron transport system. Furthermore, the rapid utilization of GSH causes GSSG efflux because of a decreased NADPH:NADP$^+$ redox status that is unable to provide sufficient reducing equivalents to maximize GSH formation from GSSG.

## BIO-REDUCTION REACTIONS

Bio-reduction in the activation of drugs appears to incur the potential hazards of pro-drugs. Pro-drugs undergo conversion to active drugs essentially by two mechanisms: either by direct conversion or the formation of an unstable intermediate that undergoes a reaction, usually spontaneous, to yield the drug [28]. These processes are not exclusive, and the intermediates may have the potential for greater toxicity then the drug itself.

*N*-acetyl-*p*-benzoquinone imine (NAPQI), a reactive metabolite of acetaminophen, undergoes a nonenzymatic two-electron reduction in the presence of GSH to yield stoichiometric amounts of acetaminophen and GSSG [29]. A competing reaction at physiological pH is the formation of an acetaminophen-glutathione conjugate. Incubation of NAPQI with hepatocytes yields the same reaction products in control and BCNU-treated hepatocytes. BCNU inactivation of glutathione reductase prevents the reduction of NAPQI-generated GSSG and enhances cytotoxicity but has no effect on the covalent binding of NAPQI to cellular proteins. A thiol, DTT, provides protection from cytotoxicity even after covalent binding has occurred. It has been concluded that the toxicity of NAPQI to isolated hepatocytes may result primarily from its oxidative effects on cellular proteins [29].

Bio-reductive activation of aromatic and heterocyclic nitro compounds occurs by reduction of the nitro moiety to unstable intermediates including free radical species (Fig. 4). For reviews see [30, 31].

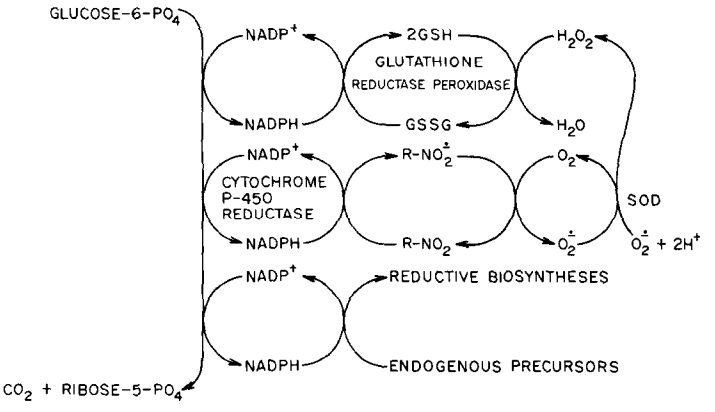


Fig. 4. Nitro aromatic reduction and redox cycling.

The production of superoxide and nitro anion free radicals during the metabolism of nitrazepam [32] is a typical example of the NADPH cytochrome P-450 reductase-dependent secondary formation of hydrogen peroxide that is capable of stimulating the hexose monophosphate pathway [33].

### ADRIAMYCIN

The antitumor benzanthraquinone, Adriamycin (ADR), undergoes rapid bio-reduction by NADPH-dependent cytochrome P-450 reductase with concomitant consumption of $O_2$ [34]. These findings suggest that ADR cytotoxicity may be the result of bio-reduction to form free radicals that overwhelm the cellular antioxidant capability including that proportion provided by the glutathione redox cycle. Inactivation of glutathione reductase with BCNU has permitted the demonstration of the protective role of the glutathione redox cycle against an ADR-mediated challenge in isolated rat hepatocytes [25, 26]. Depletion of GSH concurrently with glutathione reductase inactivation enhances the cellular injury mediated by the ADR-generated radicals [26].

As Fig. 3 indicates, 75 $\mu$M BCNU inactivated more than 90% of the hepatic glutathione reductase within 60 min. This level of inactivation was maintained throughout the remainder of the 5-hr incubation. BCNU (75 $\mu$M) had no significant effect on the hepatocytes as determined by either Trypan blue staining, LDH leakage, or lipid peroxidation. However, in the absence of supplemental sulfur-containing amino acids, the intracellular GSH level was decreased 60% by BCNU when compared to the initial GSH level in control cells (Fig. 5).

ADR was used to challenge both normal and BCNU-treated hepatocytes [25]. Figure 5 illustrates the effects of ADR and BCNU alone or in combination on the intracellular GSH levels of isolated hepatocytes. ADR (100 $\mu$M) only slightly reduced intracellular GSH levels when compared to corresponding control values. BCNU (75 $\mu$M) rapidly decreased GSH levels to 45% of initial values within 60 min. The level of GSH decreased to, and

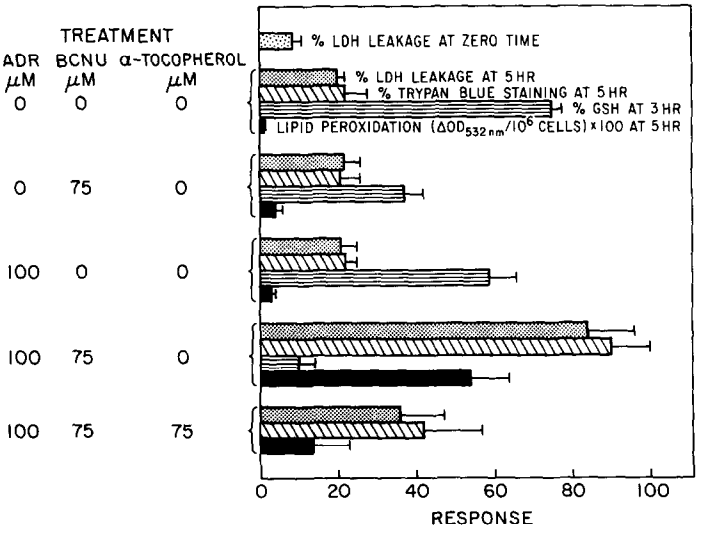


Fig. 5. BCNU-ADR mediated effects in hepatocytes. The methods and conditions used have been described [25].

remained at, 35% of initial control values for the rest of the incubation. In combination, 75 μM BCNU and 100 μM ADR decreased GSH levels to the same extent as 75 μM BCNU alone by 60 min. However, the level dropped markedly to 5% of initial values by 180 min with no GSH detectable at 300 min. Absence of methionine and cystine in the medium limited GSH synthesis and prevented recovery of GSH content in all of these experiments.

Figure 5 shows that the combination of 75 μM BCNU and ADR (100 μM) increased hepatocyte lipid peroxidation as monitored by the absorbance at 532 nm of 2-thiobarbiturate reaction products. Neither BCNU nor ADR alone caused any measurable increase in lipid peroxidation. Figure 5 indicates that BCNU and ADR in combination induced membrane damage as evidenced by the increased LDH leakage. This increase corresponded in time with the observed increase in lipid peroxidation (Fig. 5). Again, ADR (100 μM) or BCNU (75 μM) alone did not increase LDH leakage over that of control incubations.

To determine whether the increase in cell damage and lipid peroxidation was actually due to ADR, ADR at several concentrations was incubated with hepatocytes in the presence of 75 μM BCNU. The increase in lipid peroxidation trypan blue staining, and LDH leakage proved to be concentration-dependent with respect to ADR [25]. The increase in lipid peroxidation corresponded well with the cell viability criteria. Both cell damage and lipid peroxidation due to 100 μM ADR plus 75 μM BCNU were decreased significantly by 75 μM α-tocopherol (Fig. 5).

An attempt was made to determine if the BCNU-mediated inactivation of glutathione reductase *per se* was primarily responsible for the increased hepatic sensitivity to ADR and not simply to the decreased intracellular GSH level. When 340 μM DEM was used to deplete intracellular GSH to 25% of initial values at 180 min, no lipid peroxidation or cell damage was observed with either 50 μM or 100 μM ADR after 5 hr (Fig. 6). ADR (50 μM) in the presence of 75 μM BCNU, however, caused a similar decrease of GSH content (75%) by 180 min, which

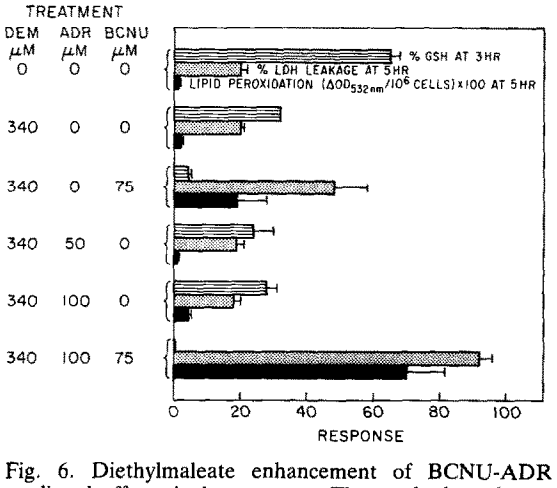


Fig. 6. Diethylmaleate enhancement of BCNU-ADR mediated effects in hepatocytes. The methods and conditions used have been described [25].

was accompanied by significant lipid peroxidation and cellular damage after 5 hr. A combination of DEM and BCNU caused about the same degree of GSH depletion as the combination of BCNU and ADR but failed to cause a similar degree of LDH leakage or lipid peroxidation. DEM did not prevent the extensive cellular damage caused by BCNU and ADR but instead increased both LDH leakage and lipid peroxidation (Fig. 6). The overall effects of depletion of glutathione and inactivation of glutathione reductase give strong evidence for the vital protective role of glutathione during oxidative stress.

## CONCLUSIONS

The evidence is overwhelming that the glutathione redox cycle has a vital role in the cellular response to bio-reduction and activation of various classes of compounds. The regulatory role of glutathione is very evident in the utilization of reducing equivalents for the reductive elimination of hydrogen peroxide and related hydroperoxides. In many instances, only after the inactivation of glutathione reductase or elimination of the activity of glutathione peroxidase by selenium deficiency is it possible to decrease the function of the glutathione redox cycle to observe the extreme degree of cell injury possible by the absence of this protective system.

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