Biochemical Pharmacology, Vol. 32, No. 8, pp. 1383–1388, 1983.
Printed in Great Britain.

0006-2952/83/081383-06 $03.00/0

DEPLETION *IN VITRO* OF MITOCHONDRIAL GLUTATHIONE IN RAT HEPATOCYTES AND ENHANCEMENT OF LIPID PEROXIDATION BY ADRIAMYCIN AND 1,3-BIS(2-CHLOROETHYL)-1-NITROSOUREA (BCNU)

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(*Received* 17 *May* 1982; *accepted* 8 *October* 1982)

Abstract—Treatment of isolated rat hepatocytes with 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) and adriamycin (ADR) produced a complete depletion of cellular glutathione accompanied by a significant increase in lactate dehydrogenase (LDH) leakage. Separation of the mitochondrial and cytoplasmic pools of glutathione by digitonin disruption showed that, although BCNU, a specific inhibitor of glutathione, completely depleted the cytoplasmic pool of glutathione, the mitochondrial supply was not entirely expended and LDH leakage was only moderately stimulated. Only after depletion of the mitochondrial supply of glutathione by ADR and BCNU did LDH leakage increase markedly. Measurement of lipid peroxidation, by monitoring malondialdehyde through the thiobarbituric acid procedure, showed that malondialdehyde accumulated more extensively and at a rate mirroring release of LDH from ADR/BCNU treated cells. The time of increase in LDH leakage and malondialdehyde production corresponded to the time of depletion of mitochondrial glutathione to less than 10% of the initial pool size. No such increase in LDH leakage was observed with BCNU or ADU treatment alone or when aminopyrine, an inhibitor of lipid peroxidation, was included. Aminopyrine was found to prevent, in a dose-dependent manner, both LDH leakage and malondialdehyde production stimulated by ADR/BCNU treatment. The protective effect peaked at 5 mM aminopyrine, and higher concentrations produced significant LDH leakage exhibiting LDH release kinetics different than those observed with ADR/BCNU. Although aminopyrine had no effect on the rate or extent of cytoplasmic glutathione depletion by ADR/BCNU treatment, the mitochondrial pool was conserved significantly in those cells protected by aminopyrine. These data suggest that enhanced hepatocyte damage observed after treatment with a combination of ADR and BCNU versus BCNU or ADR alone is due to the extensive depletion of mitochondrial glutathione supported by ADR after glutathione reductase inhibition. Further, enhancement of lipid peroxidation is strongly implicated in the mechanism of adriamycin toxicity.

The question of toxic oxygen species, such as H_2O_2 or O_2^-, the superoxide anion radical, has attracted much attention in recent years [1–3]. Considerable disagreement exists not only about the toxicity of these species [4] but also about the importance of activated oxygen related toxic processes, such as lipid peroxidation.

Previous studies in this laboratory and others [5–7] have shown that the production of lipid peroxides is closely coupled to membrane damage and cytotoxic events. The peroxidation of polyenoic fatty acids has been proposed as a mechanism of chemical toxicity for a wide variety of compounds, including carbon tetrachloride, and has been reviewed extensively [8, 9]. Among the compounds proposed to induce lipid peroxidation as a mechanism of cytotoxicity is adriamycin (ADR)‡, the antitumor benzanthroquinone, although some disagreement exists as to the actual importance of activated oxygen species and peroxidation in the toxicity of the compound [10]. Babson *et al.* [11] recently demonstrated that adriamycin causes a depletion of isolated rat hepatocyte glutathione (greater than 90%) when used in conjunction with BCNU, a specific inhibitor of glutathione reductase [12]. In addition, it was shown that malondialdehyde formation was enhanced as a result of ADR/BCNU treatment when compared to treatment with BCNU or ADR alone. It was concluded that ADR stimulated lipid peroxidation in the absence of functional glutathione reductase activity.

We have recently shown depletion of the supply of glutathione sequestered within the mitochondria in the isolated rat hepatocyte to be an obligate step in the hepatotoxicity of ethacrynic acid, as measured by lactate dehydrogenase leakage [13]. In the studies reported here, we have demonstrated that the stimulation of LDH leakage from rat hepatocytes induced *in vitro* by ADR in conjunction with BCNU also occurred only after depletion of the mitochondrial pool of glutathione. In addition, malondialdehyde formation was enhanced after mitochondrial glutathione depletion. The data suggest that, as a result of the oxidation challenge presented by ADR, lipid peroxidation is accelerated after depletion of mitochondrial glutathione and that membrane damage

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‡ Abbreviations: ADR, adriamycin; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; DMSO, dimethyl sulfoxide; LDH, lactate dehydrogenase; AP, aminopyrine; DEM, diethylmaleate; MDA, malondialdehyde; and MOPS, 3-(*N*-morpholino) propane sulfonic acid.

was a major factor in ADR induced hepatocyte damage.

EXPERIMENTAL PROCEDURES

Rat hepatocytes were prepared as previously described [14] and resuspended, 3×10^6 cells/ml, in Fischer's medium prepared without sulfur-containing amino acids and supplemented with 10 mM HEPES, pH 7.4. Cells were incubated under a stream of water-saturated 95% O_2/5% CO_2 at 37° in a gyratory shaker. Cell viability was measured by Trypan blue exclusion [11] and lactate dehydrogenase leakage [15] by a Beckman TR analyzer. Glutathione was measured by the high performance liquid chromatography method of Reed *et al.* [16]. Separation of cytoplasmic and mitochondrial components was done by digitonin disruption and centrifugation through dibutylphthalate, as described elsewhere [13]. Malondialdehyde levels were measured by the thiobarbituric acid procedure of Fong *et al.* [17]. Adriamycin and aminopyrine were added to hepatocyte cultures from 50-fold concentrated stock solutions (in 0.9% NaCl) prepared immediately prior to use. BCNU was added from a freshly prepared 1000-fold concentrated solution in DMSO.

All data presented represent the results of at least three experiments. Values were derived from duplicate assays of replicate treatments; therefore, each point is the mean of at least twelve determinations. Unless otherwise noted, standard deviation was less than 5%.

RESULTS

The studies of Babson *et al.* [11] have demonstrated that the treatment of rat hepatocytes *in vitro* with ADR and BCNU leads to an extensive depletion of glutathione. As shown in Fig. 1A, while control levels remained essentially constant, cytoplasmic glutathione was decreased by ADR and BCNU to less than 10% of initial levels after 1 hr and less than 2% by 3 hr. In addition, by 3 hr less than 10% of zero time mitochondrial GSH remained (Fig. 1B). Initial glutathione levels were 38 ± 5 and 3.2 ± 0.7 nmoles/10^6 cells for the cytoplasmic and mitochondrial pools respectively. Although ADR and BCNU produced, individually, significant diminution of glutathione in the cytoplasmic compartment, and BCNU caused some depletion of mitochondrial GSH, no single treatment depleted glutathione in the mitochondrial pool to less than 15% of the zero time level. Depletion of GSH by BCNU was found to be dose dependent up to 150 μM. Previously, 75 μM BCNU had been shown to completely inactivate both mitochondrial and cytoplasmic glutathione reductase [13].

As a criterion of viability during the experiments described, lactate dehydrogenase leakage was measured; and the results are shown in Fig. 2. Increases in LDH leakage were noted only for the higher concentration of BCNU (150 μM) and for ADR plus BCNU, with the combination treatment producing about 65% leakage by 4 hr. Between 3 and 4 hr, a rapid increase in the rate of LDH leakage was observed, correlating well with the timing of mitochondrial glutathione depletion (Fig. 1B).

The involvement of lipid peroxidation in ADR/BCNU induced LDH leakage was investigated in experiments using aminopyrine, an inhibitor of lipid peroxidation in microsomes *in vitro* [18, 19] as well as mice [20] and rat isolated hepatocytes [5]. As seen in Fig. 3, when cells were treated with ADR/BCNU, as in Figs. 1 and 2, lactate dehydrogenase leakage was unaffected by 0.1 and 1.0 mM aminopyrine, completely suppressed by 2.5 and 5.0 mM aminopyrine and increased in a dose-dependent manner by 7.5, 10, and 20 mM aminopyrine. The protection provided by 5 mM aminopyrine was expended after 4 hr (Fig. 4A), and LDH leakage occurred at a rate comparable to that observed in cultures treated with ADR plus BCNU but not aminopyrine. Additional aminopyrine (5 μmoles/ml) was added after 3 hr in

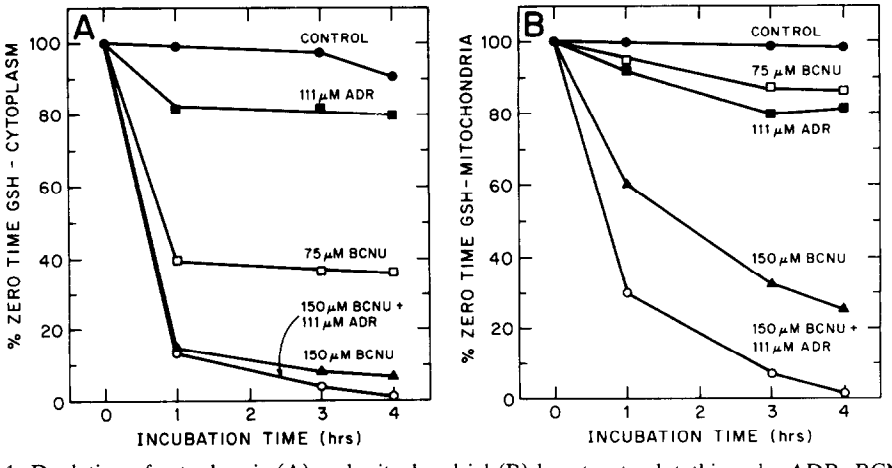

Fig. 1. Depletion of cytoplasmic (A) and mitochondrial (B) hepatocyte glutathione by ADR, BCNU and a combination. Hepatocytes, 3×10^6 cells/ml in Fischer's medium with 10 mM HEPES, pH 7.4, were incubated with 111 μM ADR (■), 75 (□) or 150 (▲) μM BCNU, or 111 μM ADR plus 150 μM BCNU (○). Samples (1 ml) were taken and, after removal centrifugation, hepatocytes were resuspended in buffer containing 0.8 mg/ml digitonin, 250 mM mannitol, 2.5 mM EDTA and 17 mM MOPS, pH 7.4. Compartments were separated, and glutathione was analyzed as described in Ref. 13.

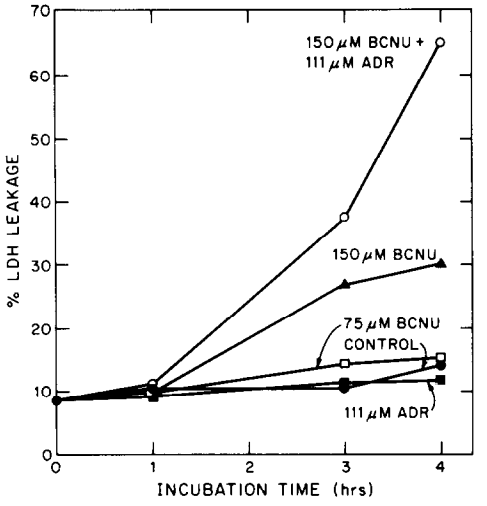

Fig. 2. ADR/BCNU induced lactate dehydrogenase leakage. Hepatocytes were incubated as described in Fig. 1, and percent LDH leakage was determined by direct assay of the culture medium after sedimentation of cells or sonication of medium and cells in the presence of 0.1% (v/v) Triton X-100. Key: 111 μM ADR (■), 75 (□) or 150 (▲) μM BCNU, and 111 μM ADR plus 150 μM BCNU (○).

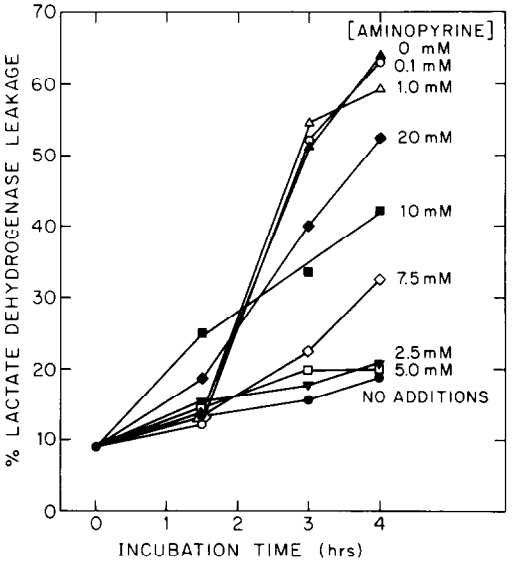

Fig. 3. Inhibition by aminopyrine of ADR/BCNU-induced lactate dehydrogenase leakage. Hepatocytes were incubated with no additions (control, ●) and with 150 μM BCNU and 111 μM ADR plus 0 (▲), 0.1 (○), 1.0 (△), 2.5 (▼), 5.0 (□), 7.5 (◇), 10 (■) or 20 (◆) mM aminopyrine added 5 min prior to ADR/BCNU. Lactate dehydrogenase was measured as described in Experimental Procedures.

culture, preventing the rise in LDH leakage observed from 4 to 6 hr in culture.

To confirm the presumed effect on lipid peroxidation produced by aminopyrine, malondialdehyde formation was measured. In the absence of treatment, as with ADR or BCNU alone, no significant rise in malondialdehyde was observed. Cultures treated with the combination of ADR and BCNU exhibited, after a 1.5-hr delay, a rapid and linear increase in malondialdehyde concentration. As with LDH leakage, treatment with 5 mM aminopyrine prevented the increased malondialdehyde formation supported by ADR/BCNU treatment. In addition, protection against lipid peroxidation ceased after 4 hr, as seen in Fig. 4B by the rise in malondialdehyde content. The continued inhibition of malondialdehyde formation, observed after a second addition of aminopyrine, is consistent with the effect of this treatment on LDH leakage.

When cytoplasmic and mitochondrial glutathione levels were measured in ADR/BCNU treated hepa-

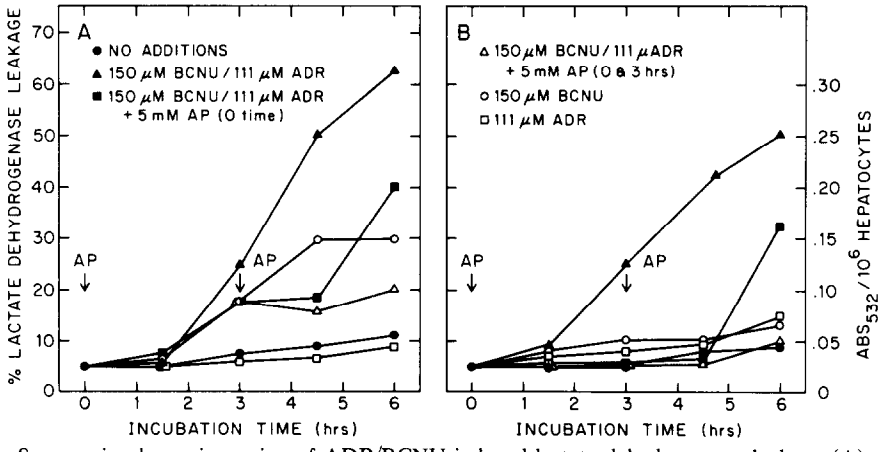

Fig. 4. Suppression by aminopyrine of ADR/BCNU induced lactate dehydrogenase leakage (A) and malondialdehyde formation (B). Hepatocytes were incubated at 3×10^6/ml in the presence of 111 μM ADR (□), 150 μM BCNU (○), and 111 μM ADR + 150 μM BCNU with (■) or without (▲) 5 mM aminopyrine added at time zero. An amount equal to the original aminopyrine addition was added at 3 hr (△); the control (●) received no addition. Cells for malondialdehyde determination were pelleted by centrifugation and resuspended in 1 ml of 10% trichloroacetic acid (TCA) prior to extraction with 2 ml $HCCl_3$. 2-Thiobarbituric acid reactive material was determined as described in the text, and the results represent the data from two experiments.

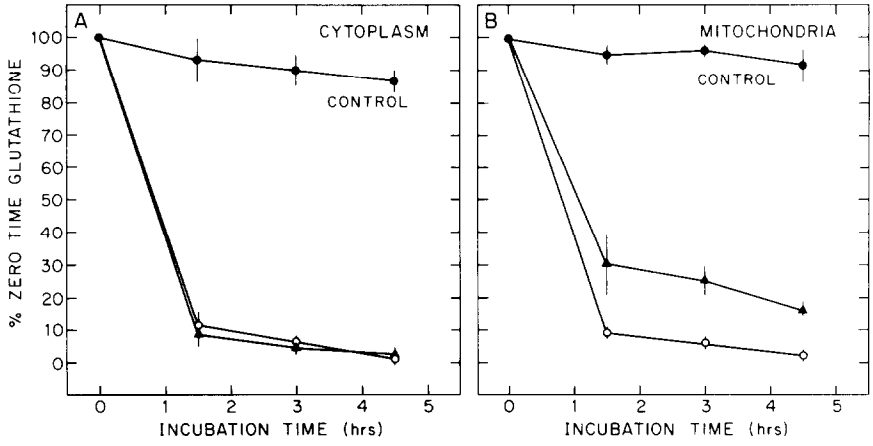

Fig. 5. Effect of aminopyrine on depletion of cytoplasmic (A) and mitochondrial (B) glutathione by ADR/BCNU. Hepatocytes, 3 × 10⁶/ml, were incubated as described in Experimental Procedures in the presence (▲) or absence (○) of 5 mM aminopyrine 10 min prior to addition of 111 μM ADR plus 150 μM BCNU. Control cells (●) received no additions. Mitochondrial and cytoplasmic pools of glutathione were separated by digitonin disruption and analyzed by HPLC as described in Refs. 13 and 16.

tocytes, 5 mM aminopyrine had no effect on the extent of cytoplasmic glutathione depletion (Fig. 5A). Within 1.5 hr, GSH levels were reduced to about 10% of control levels with or without aminopyrine. However, the mitochondrial pool was conserved to a significant extent (Student's *t*-test, $P > 0.05$) in the presence of 5 mM aminopyrine (Fig. 5B). Without aminopyrine addition, the mitochondrial pool was reduced to about 10% of initial levels after 1.5 hr and continued to decline over the experimental period. Aminopyrine treatment slowed the initial depletion phase, resulting in a decline from 30% of zero time concentration at 1.5 hr to 17% at 4.5 hr. As previously observed, LDH leakage and malondialdehyde formation did not exceed control values in cultures treated only with 5 mM aminopyrine (data not presented).

DISCUSSION

These experiments demonstrate the importance of the mitochondrial pool of glutathione. Previous experiments [13] had shown the mitochondrial pool to be a stable, physiologically distinct glutathione reservoir exerting a critical influence on hepatotoxicity. Although diethylmaleate was found to reduce cytoplasmic glutathione levels below detectable limits, the mitochondrial pool was unaffected and no increase in LDH leakage was detected; addition of BCNU and DEM together did not produce sufficient GSH depletion to induce cell damage. However, ethacrynic acid, a sulfhydryl modifying agent known to penetrate the mitochondrial membrane, was shown to deplete both cytoplasmic and mitochondrial glutathione [13], producing marked LDH leakage only after the mitochondrial pool of GSH was eliminated. The observation by Babson *et al.* [11] that ADR, in conjunction with BCNU, could produce extensive cellular damage after extensive GSH depletion suggested effects of these drugs on the mitochondrial pool.

In the present experiments we have demonstrated a relationship between the depletion of the mitochondrial glutathione pool by ADR/BCNU and the onset of LDH leakage as previously observed with ethacrynic acid treated hepatocytes [13]. Comparison of the timing of GSH depletion in the cytoplasmic and mitochondrial compartments (Fig. 1, A and B) with the time of increased LDH leakage (Fig. 2) revealed that depletion of the mitochondrial GSH pool must precede increased LDH leakage. Conservation of the mitochondrial pool of GSH, as observed with ADR/BCNU treatment in the presence of aminopyrine (Fig. 5B) was accompanied by greater retention of membrane integrity (Fig. 4A).

A major concern in measurement of lipid peroxidation by the thiobarbituric acid method [17] is the metabolism of malondialdehyde in the mitochondria [21]. When chloralhydrate, an inhibitor of aldehyde dehydrogenase, is added to isolated hepatocytes treated with ADP · Fe^{3+} to stimulate peroxidation, approximately 40% more malondialdehyde is detected [21]. Taking this as a good estimate of the metabolism of malondialdehyde and correcting Fig. 4B accordingly (not shown), significant lipid peroxidation occurred prior to the 1-hr point, therefore preceding or occurring concurrently with the enhanced LDH leakage (Figs. 2 and 4A). This suggested sequence of events provides even stronger support for the hypothesis that lipid peroxidation is a significant part of the toxicity of ADR.

Limited accumulation of malondialdehyde (Fig. 4B) was possible in the presence of substantial mitochondrial glutathione pools (Fig. 5B), as shown in Fig. 4B for control as well as BCNU, ADR, and ADR/BCNU and AP (0 and 3 hr) treated cells. This slight increase in MDA concentration in these samples indicated that lipid peroxidation was not a nonspecific result of glutathione depletion, and the similarity in the increases suggests that ADR and BCNU may not have damaged the hepatic mitochondrial ability to metabolize MDA. Impairment

by adriamycin of mitochondrial function, O_2 uptake and respiratory control, has, however, been noted in heart muscle [22, 23], as well as histological changes [24] attributed to formations of complexes between the drug and membrane lipids, particularly cardiolipin [25, 26]. Further, inhibition of coenzyme Q dependent enzymes [26], biosynthesis of coenzyme Q [27] and a protective effect of exogenous coenzyme Q during adriamycin treatment of isolated perfused rat hearts [28] present additional evidence that adriamycin exerts a cytotoxic effect at the mitochondrial membrane.

The results presented in this study provide additional evidence of the mitochondrial toxicity of adriamycin. Only in those hepatocyte cultures where the mitochondrial pool of glutathione was depleted by more than 80% (Figs. 1B and 5) was lipid peroxidation induced (Fig. 4B), strongly suggesting that the location of lipid peroxide generation was at or adjacent to the mitochondrial membrane.

Suppression of the ADR/BCNU effect on LDH leakage and MDA formation by aminopyrine, interpreted in light of the common opinion that aminopyrine inhibits lipid peroxidation [5, 20], seems to demonstrate conclusively the participation of lipid peroxides in the cytoxicity of ADR. The mechanism by which aminopyrine exerts the effect is not clear. The cytochrome P-450 dependent *N*-demethylation requires NADPH, and active metabolism of AP by hepatocytes increases the NADPH/$NADP^+$ and citrate/α-ketoglutarate ratios by 2-fold. Cellular citrate and isocitrate concentrations are also reduced by one-half, due to increased flux through $NADP^+$-dependent isocitrate dehydrogenase [29], a major NADPH-generating path. Reduction (activation) of ADR by cytochrome P-450 reductase requires NADPH [6]. It is therefore possible that AP inhibits ADR activation by competing for NADPH. However, this must be considered unlikely, since rates of AP *N*-demethylation are unchanged in hepatocytes treated with 10 mM NH_4Cl to support urea formation, an NADPH-dependent process consuming, under maximum rate conditions, five times more reducing equivalents than cytochrome P-450 dependent reactions [29]. As seen in Fig. 3, protection was complete in 2.5 and 5 mM aminopyrine and was expended with time (Fig. 4, A and B). Though suggested by the data, it is not clear whether metabolism of AP is required to provide a protective effect or by what route metabolism occurs.

The recent work of Lasker *et al.* [30], showing the prostaglandin synthetase dependent *N*-demethylation of aminopyrine in ram seminal vesicle microsomes, suggests an alternative explanation to cytochrome P-450 dependent metabolism. Proceeding by way of the peroxidase activity of prostaglandin synthetase, these workers found that lipid hydroperoxides could be reduced to the corresponding alcohols with the co-oxidation of aminopyrine to the radical cation. Reduction was followed by a disproportionation reaction and hydrolysis of the resulting iminium cation to yield formaldehyde and demethylated AP. By this scheme, AP could provide protection by consuming lipid hydroperoxides generated after GSH depletion by ADR/BCNU treatment.

The mechanism of mitochondrial adriamycin toxicity and the causative agent of the observed lipid peroxidation were not clearly identified by available data. Mimnaugh *et al.* [6] found that both heart and liver microsomes are capable of activating adriamycin and supporting lipid peroxidation through cytochrome P-450 reductase. They also showed that addition of reduced glutathione could inhibit adriamycin-induced peroxidation of microsomal lipids by more than 90%. Participation of a mitochondrial P-450 reductase in similar types of reactions can only be speculated upon at present. Although considerable study has been made of the mitochondrial cytochrome P-450 dependent reactions of steroid synthesis [31–33] and Jones *et al.* [34] found that the majority of the cytochrome P-450 of intestinal cells is found in the mitochondria, reactions of liver and heart mitochondrial cytochrome P-450 are largely unknown.

The results presented in this paper provide additional information on the biochemical mechanism of adriamycin toxicity. Considering the large volume of the cardiac myocyte occupied by mitochondria, observation of cardiomyopathy in clinical applications of ADR [35, 36] further supports the contention that the mitochondrion is the site of initial toxicity.

Acknowledgements—This work was supported by Grant ES-10978 awarded by the National Institute of Environmental Health Sciences. The authors wish to recognize the technical assistance of Mrs. Marda Brown.

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