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EFFECTS OF MITOMYCIN C ON METABOLISM IN A RAT LIVER PREPARATION*

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Abstract—Quinone drugs are used extensively as anti-neoplastic agents. The mechanism of their actions and the reasons for their unfavorable side effects are not well understood. Mitomycin C (MC) is an N-heterocyclic quinone with chemotherapeutic action against solid tumors. Previous research has led to the development of a model for drug activation involving NADPH reduction of the drug via microsomal mixed-function oxidases. We tested the possibility that NADPH is provided from the hexose monophosphate shunt (HMPS). The MC did indeed increase HMPS activity aerobically, while not affecting Kreb's cycle activity. Anaerobic stimulation of the shunt is also predicted by the model. However, under hypoxic conditions no HMPS or Kreb's activity was observed in MC-treated or untreated samples. Other investigators have documented the involvement of reactive oxygen species in microsomal systems *in vitro*. The oxygen requirement for MC stimulation of HMPS suggests oxygen radical involvement. We carried out experiments using [^{14}C]-formate as a scavenger for hydrogen peroxide. There was no apparent change in H_2O_2 production when MC was added. Catalase is known to be involved in peroxide metabolism *in vivo*; however, addition of the catalase inhibitor sodium azide did not alter endogenous or MC-stimulated shunt activity. The microsomal inhibitor SKF-525A (10^{-3} M) prevented MC stimulation of the HMPS, which is consistent with the model implicating microsomal enzymes in MC metabolism. Overall, we have shown the oxygen dependence of endogenous and MC-stimulated shunt activity, and the results provide evidence for MC activation of oxidative metabolism by a mechanism which involves microsomes.

The quinone type anti-tumor drugs have become important agents in the treatment of patients with cancer. These drugs include the anthracyclines such as adriamycin and the N-heterocyclic quinone mitomycin. In addition to their antineoplastic effects, these drugs may also cause a dose-dependent cardiomyopathy [1], interstitial pneumonia [2–4], and renal toxicity [5, 6]. However, the exact mechanism of their action is still unclear. Recent evidence suggests that cellular microsomal systems may be essential for the activation of these drugs *in vivo* [7–9]. The metabolism of quinone drugs occurs *in vitro* when reconstituted enzyme systems with added NADPH are created. The enzyme systems have been made with whole microsomes, or purified cytochrome reductases (NADPH:cytochrome P-450 oxidoreductase, EC 1.6.2.4; NADPH:cytochrome *c* oxidoreductase, EC 1.6.2.4; NADH:cytochrome b_5 oxidoreductase, EC 1.6.2.2.).

Various authors report that microsomal pyridine nucleotide utilization, O_2 consumption, and free radical formation are greatly enhanced by the quinones [8–16]. Several groups have provided evidence that the drugs can act as electron carriers from NADPH:cytochrome *c* reductase to O_2 by cycling in a shuttle between the oxidized and semiquinone (half reduced) forms [9, 17]. Reaction of the reduced drug with oxygen leads to increased production of reactive oxygen species (ROS‡), including superoxide, hydrogen peroxide, hydroxyl radical, and lipid peroxides [7, 8, 11–13, 16, 18, 19]. The reactive oxygen species may be responsible for the therapeutic effects of the drugs and may have toxic activity against the cell itself. Several investigators [20, 21] have shown that adriamycin reduction by microsomes leads to DNA cleavage in an oxygen-mediated reaction. Nuclear electron transport systems may also carry out the same free radical formation reaction [7].

Mitomycin C (MC) is the only clinically available antitumor drug demonstrated to be a bioreductive alkylating agent. Like other quinone drugs, MC undergoes apparent oxidation–reduction cycling in the presence of oxygen. Under these conditions the MC is not degraded [22, 23]. However, when used as a chemotherapeutic agent, the efficacy, of MC is greatest against hypoxic tumors, suggesting that anaerobic activation occurs with subsequent damage to tumor cells [24]. Kennedy *et al.* [7] used an *in vitro* enzyme system to demonstrate that NADPH, NADPH–cytochrome P-450 reductase, cytochrome P-450 phospholipid and anaerobiosis are all required for the optimal activation of MC to an alkylating

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‡ Abbreviations: ROS, reactive oxygen species; MC, mitomycin C; HMPS, hexose monophosphate shunt; and SKF-525A, diethylaminoethyl diphenyl propylacetate.

species which they consider to be the active tumor inhibitory form of the drug.

The specifity of microsomes for NADPH over NADH and other co-factors in the activation of quinone drugs has been shown by several groups [18, 25]. Further, these investigators have frequently utilized an NADPH-generating system, which is made up of components of the HMPS pathway (glucose 6-phosphate, glucose 6-phosphate dehydrogenase and NADP) and has greater activity than NADPH alone. This observation suggests that the HMPS may be necessary for activation of quinone drugs in the intact cell since this pathway is the major source of cellular NADPH (see Fig. 1). We have tested this possibility in rat liver preparations using ^{14}C-labeled glucose and a continuous ionization chamber-electrometer system to distinguish between Embden–Meyerhoff/Krebs activity and HMPS activity [26–28]. The minced liver preparation allows the normal cell to cell interactions as in whole liver, while allowing us to use a chamber apparatus.

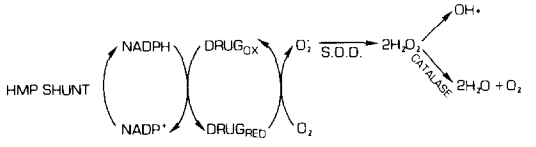

Fig. 1. Scheme for oxidation–reduction cycling of quinone drugs. MC and other quinone drugs have been shown to undergo reduction in the presence of microsomal enzymes, in a reaction dependent on NADPH. The NADPH may be provided by the HMP shunt, the cell's main source of NADPH. When oxygen is present it is reduced, and the drug appears to undergo redox cycling. Superoxide (O_2^-) and hydrogen peroxide (H_2O_2) would be formed as a result of the reduction of O_2. In microsomes, the H_2O_2 could be further metabolized to OH·, an organic radical, or potentially degraded by catalase. Alternatively, the H_2O_2 might also be degraded by the glutathione peroxidase pathway (not shown in scheme).

MATERIALS AND METHODS

Rat liver preparation. Unanesthesized rats were killed by decapitation. Livers were rapidly removed surgically and injected via the portal vein with Dulbecco's phosphate-buffered saline (DPBS) until the liver was blanched. The liver was kept on ice while 300-mg pieces were cut from the middle lobe. These pieces were placed in DPBS augmented with 50 mg/100 ml glucose.

A 25-ml triple-headed distilling flask (reaction chamber) containing 5 μCi of the appropriate [^{14}C]-glucose substrate, or 1 μCi of [^{14}C]-formate in 0.5 ml of normal saline was connected to an ionization chamber electrometer system as previously described [16, 26–31]. The center head of each flask was capped with a rubber stopper to seal the system. The solution was then stirred and gassed with humidified air (37°) at a rate of 65 ml/min. In some experiments, nitrogen rather than air was used as the gas mixture.

When the ionization system was completely set up, the liver pieces were minced into 1-mm pieces, washed once in DPBS with 50 mg/100 ml glucose, then resuspended to a total volume of 4.0 ml and transferred to the reaction chambers.

Older rats (251–300 g) were used in initial experiments on mitomycin stimulation and anaerobiosis (data given in Figs. 2–5), whereas rats of 200–250 g were used for formate and SKF experiments (data given in Figs. 6 and 7).

Ionization chamber. We utilized the continuous ionization chamber electrometer system to measure $^{14}CO_2$ evolved from the metabolism of labeled substrates. [^{14}C-6]-Glucose is metabolized to release $^{14}CO_2$ primarily by the glycolytic pathway. $^{14}CO_2$ released by catabolism of [^{14}C-1]-glucose is due to glycolysis and HMPS activity. Thus, the difference between [^{14}C-1]-glucose oxidation and [^{14}C-6]-glucose oxidation is approximately the HMPS activity [32–35]. [^{14}C]-Formate reacts with hydrogen peroxide in the presence of catalase to evolve $^{14}CO_2$, and H_2O [36]. Therefore, the oxidation of formate was used to estimate the production of H_2O_2. For these experiments, unlabeled formate was added to the incubation mixture in a final concentration of 10 mM. The rate of $^{14}CO_2$ production was calculated from the rate constant of each chamber using the specific activity of the ^{14}C-substrate as previously described [26].

Radioactive substrates. Radioactive substrates were obtained from the New England Nuclear Corp. (Boston, MA) and the Sigma Chemical Co. (St. Louis, MO). Specific activity of [^{14}C]-formate was 59 μCi/μmole. The specific activities of the glucose substrates were 3.9 and 6.8 for 1- and 6-labeled glucose respectively.

All of the reported experiments were carried out with mitomycin C prepared in normal saline and used at a final concentration of 100 μg/ml. Mitomycin C was ordered from the Sigma Chemical Co. Sodium azide was obtained from the Sigma Chemical Co. and used at a 10^{-4} M concentration. SKF-525A (diethylaminoethyl diphenyl propylacetate) was the gift of Smith Klein-Beckman Laboratories. SKF-525A is a known microsomal inhibitor. For most experiments described in Results we used this compound at a final concentration of 10^{-3} M.

Statistical analysis. Data were analyzed according to the *t*-test for independent or dependent samples.

RESULTS

The liver preparation metabolized glucose readily, producing $^{14}CO_2$ at a steady rate from both 1- and 6-labeled [^{14}C]-glucose. This steady state was well established after 60 min of incubation (Fig. 2). [^{14}C-1]-Glucose oxidation was significantly higher than [^{14}C-6]-glucose oxidation. These data indicate that liver has an active HMPS pathway as well as Kreb's cycle. As shown in a typical experiment in Fig. 3, once steady-state conditions were established, the addition of MC to the incubation chamber enhanced [^{14}C-1]-glucose oxidation. Figure 4 shows the results of several experiments in which both C-1 and C-6 metabolism were studied. [^{14}C-1]-Glucose oxidation was increased almost 2-fold while [^{14}C-6]-glucose oxidation was enhanced only slightly. Thus, HMP shunt activity was increased by the presence of the drug.

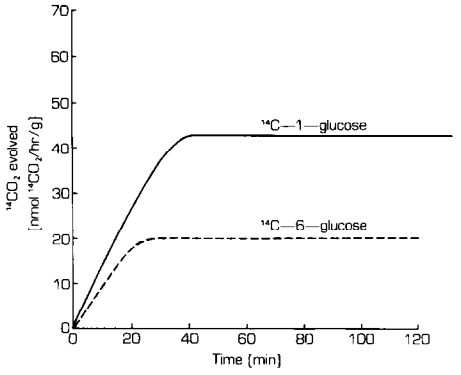

Fig. 2. Comparison of $^{14}CO_2$ evolution from [^{14}C-1]-glucose and [^{14}C-6]-glucose by untreated rat liver. Representative curves from a single experiment are shown. The difference in the rates of oxidation of the two substrates approximately equals HMP shunt activity. Steady-state data from four such experiments are summarized in Fig. 4.

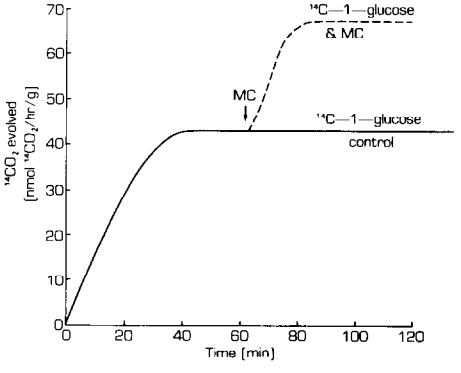

Fig. 3. Effect of MC (100 μg/ml) on oxidation of [^{14}C-1]-glucose by a rat liver mince. A representative curve from a single experiment illustrates the increase in $^{14}CO_2$ evolution from [^{14}C-1]-glucose. Incubation chambers were set up as described in the text. As shown here, $^{14}CO_2$ evolution maintained a steady state for at least 15 min before MC was added. Data from four such experiments are summarized in Fig. 4.

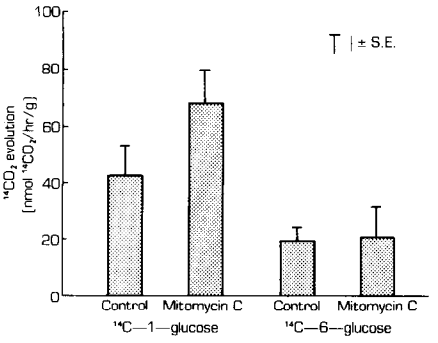

Fig. 4. Effect of mitomycin C on oxidation of 1- and 6-labeled glucose by rat liver. Mitomycin C (100 μg/ml) was added to chambers containing minced liver, ^{14}C-labeled glucose, and buffered saline solution as described in the text. The addition was made after a steady state of $^{14}CO_2$ evolution had been achieved and was maintained for 15 min (see Fig. 3). The reported measurements are steady-state values attained after MC was added. A significant change ($P < 0.05$, $N = 3$) in [^{14}C-1]-glucose oxidation occurred when mitomycin C was present. No significant change ($P < 0.10$, $N = 3$) in C-6-glucose oxidation is observed. The reported values are from results of paired experiments using older animals.

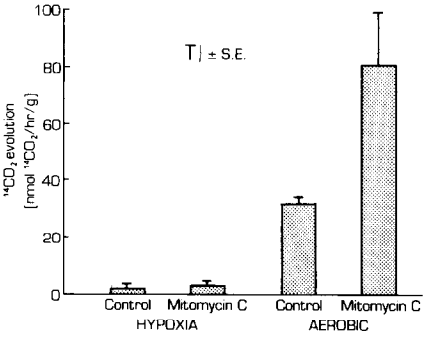

Fig. 5. Effect of hypoxia on [^{14}C-1]-glucose oxidation by rat liver. The standard incubation chambers were run under air and under nitrogen atmospheres. The values are derived from the results of five sets of paired experiments.

We used anaerobic treatment to test the oxygen requirement for MC stimulation of the HMP shunt. As shown in Fig. 5, essentially no glucose oxidation occurred under hypoxic conditions. [^{14}C-1]- and [^{14}C-6]-Glucose were not metabolized whether untreated or treated with MC. Our cells were shown to be undamaged by the hypoxia because they recovered normal levels of glucose oxidation when air was let back into the system.

[^{14}C]-Formate oxidation was used as a measure of hydrogen peroxide production by the tissue. There was no detectable oxidation of [^{14}C-1]-formate in experiments done using nitrogen, indicating the requirement of oxygen for the reaction (data not shown). Addition of liver tissue to aerobic flasks with [^{14}C]-formate resulted in an initial burst in $^{14}CO_2$ evolution, followed by establishment of a steady-state rate of $^{14}CO_2$ production (Fig. 6). Addition of the heme inhibitor sodium azide (10^{-4} M) to MC-treated tissue preparations oxidizing [^{14}C]-formate

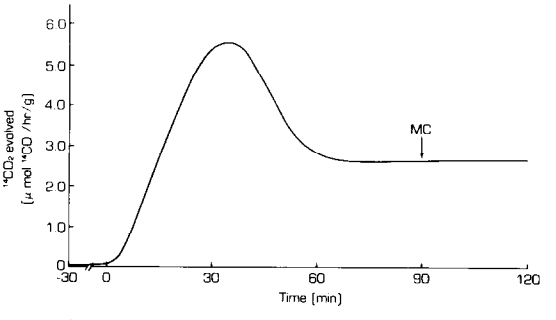

Fig. 6. [^{14}C]-Formate oxidation by liver mince and the effect of mitomycin C. This graph shows the curve from a single experiment, typical of five experiments. Results from steady-state measurements of these experiments are discussed in the text. [^{14}C]-Formate was used as a probe for hydrogen peroxide production. Total formate in the incubation chamber was in a concentration of 10 mM. One microcurie of labeled formate was used. The untreated liver mince showed a high level of endogenous peroxide production.

resulted in decreased $^{14}CO_2$ evolution (data not shown).

MC was added to incubations once the $^{14}CO_2$ from [^{14}C]-formate was being evolved at a steady rate. As shown in a typical experiment in Fig. 6, no significant change in the rate of the reaction occurred. The time of addition of MC to these incubations corresponded to the time of addition of MC to incubations of liver slices containing [^{14}C-1]-glucose. From these data we conclude that enhanced formate oxidation was not associated with the MC stimulation of HMPS. When azide was added to an aerobic sample with endogenous or MC-stimulated C-1-glucose oxidation, the $^{14}CO_2$ evolution was not affected significantly. In four experiments measuring [^{14}C-1]-glucose oxidation, we found that control and azide-treated samples were not significantly different, having values of 86.8 ± 32.0 (S.D.) and 99.0 ± 61.2 (S.D.) nmoles CO_2/hr/g respectively (P, NS). In two similar experiments with MC present, values were 272.5 ± 114.9 (S.D.) and 326.2 ± 85.4 (S.D.) nmoles CO_2/hr/g for samples treated with MC only, and MC plus azide, respectively.

To test the involvement of microsomal enzymes in the glucose oxidation, we treated samples with the microsomal inhibitor SKF-525A at a concentration of 10^{-3} M, a concentration which others have used to inhibit microsomal activity [8, 37]. This concentration of SKF-525A produced some stimulation of endogenous glucose oxidation (52%) compared to the control ($P < 0.05$) (Fig. 7). However, this stimulation was significantly lower than that observed with mitomycin. In this series of paired experiments, mitomycin produced almost a 4-fold increment in [^{14}C-1]-glucose oxidation. The degree of MC stimulation in these experiments was greater than observed in the earlier experiments (see Fig. 4). Although the reasons for this difference are not clear, it is most likely related to the age of the animals used in the experiments (see Methods). As seen in Fig. 7, SKF-525A inhibited the mitomycin stimulation of [^{14}C-1]-glucose oxidation. The [^{14}C-6]-glucose oxidation decreased in the presence of both MC and SKF-525A, but the decrease was not enough to account for the suppression of stimulated [^{14}C-1]-glucose oxidation. The decrease in [^{14}C-6]-glucose oxidation would only account for 38% of MC stimulation above control (57 μmoles CO_2/hr/g) for [^{14}C-1]-glucose oxidation (data not shown). We conclude that the microsomal electron transport enzymes are important in MC stimulation of [^{14}C-1]-glucose oxidation. We also studied the effects of a higher concentration of SKF and found that a 10^{-2} M concentration was toxic.

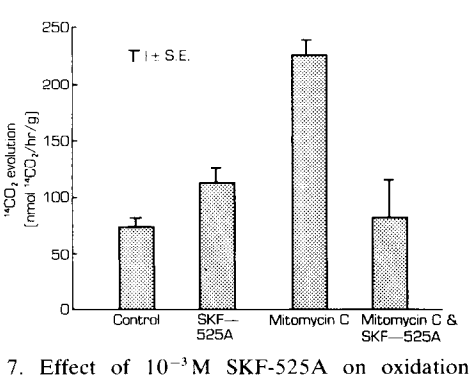

Fig. 7. Effect of 10^{-3} M SKF-525A on oxidation of [^{14}C-1]-glucose by rat liver. The microsomal inhibitor SKF-525A was added to untreated and MC-treated samples of liver mince. The increase in [^{14}C-1]-glucose oxidation with SKF alone was statistically significant ($P < 0.05$). Glucose oxidation in the presence of SKF and mitomycin C was at control levels. The values reported are the results of four paired experiments. For this series of experiments, livers were obtained from animals weighing 200–250 g.

DISCUSSION

Our results confirm the reports of others which indicate that rat liver slices actively metabolize both [^{14}C-1]- and [^{14}C-6]-glucose. The enhanced oxidation of [^{14}C-1]-glucose compared to [^{14}C-6]-glucose indicates that this tissue has an active hexose monophosphate shunt pathway as well as an active Kreb's cycle [32–35]. Under anaerobic conditions, liver slices did not oxidize substantial amounts of either of these substrates. The results with the [^{14}C-6]-glucose were not unexpected since the requirements of oxygen for optimal mitochondrial activity is well established. The observations with [^{14}C-1]-glucose indicate that the biochemical reactions involved in the hexose monophosphate shunt activity in liver are also highly dependent on oxygen.

We also demonstrated that liver slices actively oxidize formate to CO_2. This reaction is also oxygen dependent and could be largely inhibited by azide in a concentration which does not appreciably alter glucose oxidation. Therefore, the oxidation of formate by liver slices appears to be mediated primarily by the classic reaction observed in other cells involving hydrogen peroxide and catalase [36]. The capacity of liver to produce hydrogen peroxide was anticipated. Studies by others indicate that the liver is actively producing hydrogen peroxide *in vivo* and that a substantial amount of H_2O_2 produced is degraded by catalase [38].

MC was added to the liver slices once steady-state glucose oxidation was established. MC addition was associated with a stimulation of [^{14}C-1]-glucose oxidation and only a slight increase in [^{14}C-6]-glucose oxidation. These data indicate that mitomycin does not impair the Kreb's cycle activity and that the metabolism of mitomycin by liver slices is associated with a stimulation of the HMP shunt pathway. There was no obvious stimulation of hydrogen peroxide production since there was no notable change in the resting [^{14}C]-formate oxidation when MC was added.

As discussed previously, the reports from several laboratories which characterized the metabolism of mitomycin in microsomal systems served as background for our experiments [8–17, 23, 25]. These studies suggest that two distinct mechanisms for mitomycin C activation occur in: (1) aerobic cells, and (2) hypoxic cells. Under aerobic conditions, the mitomycin seems to be cycled with the simultaneous production of reactive oxygen species. MC was one of the anticancer chemicals which Handa and Sato [9] found stimulated O_2 uptake by tumor cells in the presence of glucose. In contrast, under anaerobic conditions the mitomycin is metabolized to an alkylating compound which may have antitumor

properties [14, 23, 25]. In these microsomal enzyme systems, NADPH appears to be an essential co-factor for these reactions under both conditions [7, 13, 25].

Bachur *et al.* [13] have reported that SKF-525A impairs the metabolism of mitomycin in microsomal systems. Kennedy *et al.* [7] have demonstrated that azide and cyanide, which inhibit heme enzymes, have no effect on the reaction. Similarly, in our experiments we found that azide did not greatly alter the mitomycin stimulation of the HMP shunt while SKF appeared to be inhibitory at a 10^{-3} M concentration. However, effects of SKF on liver metabolism were much more complex than predicted by microsomal data. SKF also stimulated glucose oxidation, an observation which makes interpretation of MC experiments more difficult. The MC stimulation of [^{14}C-1]-glucose oxidation was far greater, and addition of MC to an SKF-treated sample resulted in [^{14}C-1]-glucose oxidation which was not significantly different from control. Therefore, SKF impairs the capacity of mitomycin to activate [^{14}C-1]-glucose oxidation. Presently, the only known activity of SKF-525A is inhibition of microsomal enzymes so it is logical to conclude that the effects of SKF in the whole cell preparation are related to its effects on microsomal function. Consequently, the mechanism of mitomycin stimulation of the HMPS pathway under aerobic conditions is consistent with the model proposed in the microsomal system (Fig. 1). The capacity of SKF to stimulate the oxidative metabolism of liver requires additional study and does raise the question whether this drug can affect cellular pathways other than microsomes.

Unexpectedly, we found that mitomycin stimulation of glucose metabolism in liver was highly oxygen dependent. It is not clear why mitomycin failed to stimulate the hexose monophosphate shunt pathway of liver under anaerobic conditions. As discussed above, this reaction is also predicted by the microsomal system. In addition, studies by Kennedy *et al.* [22] indicate that activation of mitomycin occurs in hypoxic tumor cells by this mechanism. There are several reasons why this reaction may not have occurred in liver. One obvious answer is that extrapolation of the results from the isolated microsomal systems to the intact cell may not be valid because alternate enzyme systems and regulatory mechanisms are present in the intact liver cell. Phenobarbital induction of liver microsomes for isolation may cause a preferential induction of certain enzymes. Under anaerobic conditions, these alternate enzyme pathways may be more important in the metabolism of mitomycin than the NADPH-dependent microsomal mechanism or may, in some way, suppress microsomal activity. Further, the metabolism of cancer cells may differ significantly from liver or other normal tissues. These metabolic alterations may allow preferential activation of mitomycin in cancer cells under anaerobic conditions. This may explain the apparent contradictions between our results with normal liver and those of Kennedy *et al.* with tumor cells [24]. We did not test directly whether mitomycin was actively metabolized by hypoxic liver slices. Whether mitomycin is activated anaerobically via the mechanism proposed by Kennedy requires further study.

Finally, we have not excluded the possibility that the stimulation of the HMP shunt pathway induced by mitomycin under aerobic conditions is related to the degradation of cellular peroxide or an organic peroxide. In this circumstance, the activation of mitomycin generates hydrogen peroxide which ultimately stimulates the HMP shunt as a consequence of degradation of H_2O_2 or an organic peroxide by glutathione peroxidase. This possibility requires additional study and could be an explanation for our results.

In summary, our observations indicate that mitomycin resulted in an oxygen-dependent stimulation of the HMP shunt pathway. This observation is consistent with the metabolism of mitomycin in the intact liver by the mechanism suggested by other experiments using isolated microsomal preparations. Unexpectedly, the reaction did not occur under anerobic conditions even though this reaction was predicted by the microsomal model. In any event, our results provide evidence that the aerobic activation of quinone drugs occurred in the intact cell by the mechanism suggested by isolated microsomes. Presumably, this microsomal activation was associated with the simultaneous generation of ROS. In the liver this probably was not associated with significant damage to the tissue because of the capacity of this cell to rapidly degrade these compounds. Under other circumstances, the generation of these compounds may be associated with cellular damage in tissue less able to degrade these reactive oxygen species. More careful choice of a specific chemotherapy drug and co-therapy with anti-oxidants may help reduce the toxic effects of these drugs.

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