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Mitomycin C and Porfiromycin: Studies on Bioactivation and Cytotoxicity in Cultured Cell Lines

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MITOMYCIN C AND PORFIROMYCIN: STUDIES ON BIOACTIVATION AND CYTOTOXICITY IN CULTURED CELL LINES

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ABSTRACT

MITOMYCIN C AND PORFIROMYCIN: STUDIES ON BIOACTIVATION AND CYTOTOXICITY IN CULTURED CELL LINES

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Solid neoplasms are known to contain deficient or poorly functional vascular beds and as a result populations of cells in these solid tumors may contain hypoxic or poorly oxygenated tumor cells. These hypoxic tumor cells may form a therapeutically resistant cell population within the tumor difficult to eradicate by ionizing radiation and most existing chemotherapeutic agents. As a consequence, this dissertation has investigated the mechanism of bioactivation and cytotoxicity of mitomycin C and porfiromycin, structurally similar antibiotics which are selectively cytotoxic to hypoxic cells.

Mitomycin C was preferentially cytotoxic to hypoxic EMT6 and V79 cells in culture, but was not selectively cytotoxic to CHO cells in culture. Porfiromycin produced hypoxic cell cytotoxicity comparable to that of mitomycin C in EMT6 and V79 cells, but exhibited significantly less aerobic cytotoxicity. Porfiromycin was significantly more cytotoxic to hypoxic CHO cells than to aerobic CHO cells. This increase in hypoxic cell cytotoxicity for CHO cells treated with porfiromycin, however, was substantially less than the differential cytotoxicity seen with the EMT6 and V79 cells treated with this drug.

NADPH-Cytochrome c reductase, NADH-cytochrome b_5 reductase, cytochrome b_5, and DT-diaphorase were present in all three cell lines, whereas cytochrome P-450 was not detectable. The highest activity of NADPH-cytochrome c reductase and DT-diaphorase were observed in EMT6 cells. Sonicates of

EMT6, V79, and CHO cells enzymatically formed reactive metabolites of MC under hypoxic conditions as detected by the trapping agent 4-(p-nitrobenzyl)pyridine. EMT6 cells were able to generate reactive metabolites of mitomycin C to a greater extent than V79 and CHO cells. The formation of reactive metabolites occurred most efficiently in the presence of an NADPH regenerating system and to a lesser degree with added NADPH or NADH alone.

Carbon monoxide, an inhibitor of cytochrome P-450, did not decrease the formation of mitomycin C-derived reactive metabolites by sonicates of the three cell lines. $NADP^+$ and mersalyl inhibited NADPH cytochrome c reductase activity and the formation of reactive metabolites from mitomycin C. Dicoumarol inhibited DT-diaphorase activity completely, while the rate of formation of reactive metabolites of mitomycin C was enhanced. The results indicate that cytochrome P-450 and DT-diaphorase are not directly involved in the activation of mitomycin C, but appear to modulate the degree of activation of the antibiotic to reactive species, which are presumably responsible for cytotoxicity. Furthermore, NADPH-cytochrome c reductase was capable of activating mitomycin C to a reactive species, but did not appear to be the sole enzyme involved in the reductive activation of mitomycin C by these cell lines.

Alkaline elution methodology was utilized to study both the formation and repair of DNA single strand breaks, DNA interstrand cross-links, and DNA-protein cross-links produced by mitomycin C and porfiromycin in the EMT6 and CHO cell lines. DNA single strand breaks, DNA interstrand cross-links and DNA protein cross-links were negligible in hypoxic or aerobic CHO cells immediately after treatment with mitomycin C or porfiromycin. Although hypoxic and aerobic EMT6 cells demonstrated no single strand breaks immediately after treatment with these antitumor antibiotics under both hypoxic and aerobic conditions, DNA interstrand

cross-links were produced in this cell line. Substantially more DNA interstrand cross-linking occurred under hypoxic conditions than under aerobic conditions, with the same amount of cross-linking occurring after mitomycin C and porfiromycin treatment. These results are consistent with the degree of cytotoxicity produced by these agents to hypoxic and aerobic CHO and EMT6 cells. Further studies indicated that DNA interstrand cross-links persisted in hypoxic EMT6 cells over a 24 hr period following removal of mitomycin C and porfiromycin, with a decrease in DNA interstrand cross-links observed at 24 hr. Aerobic EMT6 cells treated with mitomycin C and porfiromycin demonstrated an increase in DNA interstrand cross-links hours after drug removal, with a decrease in these lesions being observed by 24 hrs, suggesting that the rate of formation of the cross-links may be slower under aerobic conditions. A low degree of DNA-protein cross-links occurred in both hypoxic and aerobic EMT6 cells treated with mitomycin C and porfiromycin. These results suggest that bioactivation and formation of DNA lesions may be important to cytotoxicity but are not the sole determinants of toxicity by mitomycin C and porfiromycin in hypoxic and aerobic cultured cell lines.

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ATP	adenosine 5'-triphosphate
CHO cells	Chinese hamster ovary cells
DCPIP	dichlorophenolindophenol
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EMT6 cells	EMT6 mouse mammary tumor cells
ESR	electron spin resonance
FACS	fluorescent activated cell sorter
HBSS	Hanks' balanced salt solution
MC	mitomycin C
NAD^+/NADH	nicotinamide adenine dinucleotide/reduced form
$NADP^+$/NADPH	nicotinamide adenine dinucleotide phosphate/reduced form
PBS	phosphate buffered saline
PM	porfiromycin
RNA	ribonucleic acid
SDS	sodium dodecyl sulfate
TRIS	tris(hydroxymethyl)aminomethane
UV/Vis	ultraviolet/visible spectrum
V79 cells	Chinese hamster V79 lung fibroblasts

CHAPTER I

Background

ORIGIN AND CHEMICAL STRUCTURE

The first naturally occurring mitomycins were isolated from Streptomyces caespitosus and described by Hata et al. (1956) in Japan. These workers isolated two fractions, A and B, which had activity against various microbes and Ehrlich ascites tumor cells in mice. Within the next few years, Wakaki et al. (1958) reported the isolation of mitomycin C (MC), also from Streptomyces caespitosus, and DeBoer et al. (1961) described the isolation and activity of porfiromycin (PM) produced by another actinomyces strain, Streptomyces ardus. Mitomycins A, B, C and PM were found to have similar bactericidal and antitumor activity, and Webb et al. (1962a, 1962b) demonstrated chemically that these antibiotics had a common structure to which they gave the trivial name, mitosane, and differed only in minor substituents as indicated in Table 1. These workers further showed that these antibiotics were the first naturally occurring compounds which contained an aziridine ring, a n-pyrrolo(1,2-a)indole ring system, an amino- or methoxybenzoquinone, and a pyrrolizine residue. Stevens et al. (1964) confirmed the structure of MC by chemical analysis. The absolute configurations of mitomycin A, B, and C were determined by x-ray crystallographic analysis (Tulinsky, 1962; Tulinsky and Van den Hende, 1967; Yahashi and Matsubara, 1976, 1978; Shirahata and Hirayama, 1983). In addition, the total synthesis of MC has been achieved (Nakatsubo et al., 1977).

Mitomycins A, B, C and PM, and many of their analogues exhibit useful antibacterial (Kinoshita et al., 1971a, 1971b) and antitumor properties (Crooke, 1979). The principal interest in the mitosanes is derived from their antitumor activity

(Crooke, 1979). MC, the most thoroughly studied mitosane antibiotic, is effective against a relatively wide range of neoplasms, including head and neck cancers, adenocarcinomas of the gastrointestinal tract, squamous cell carcinoma of the cervix, and ovarian carcinoma (Crooke, 1979). PM, the N-methyl homolog of MC, was developed in the United States for phase I clinical trials (Izbicki et al., 1972), but both the activity against solid tumors and the toxicity of PM were similar to MC and as a result, PM was never marketed.

THE MECHANISM OF ACTION OF MC AND PM

Early investigations on the mechanism of action of MC clearly demonstrated that this antibiotic inhibited the biosynthesis of DNA in bacteria (Sekiguchi and Takagi, 1959, 1960a; Shiba et al., 1959) and in mammalian cells (Sekiguchi and Takagi, 1960b; Shatkin et al., 1962), while RNA and protein synthesis continued relatively unaffected (Schwartz et al., 1963; Shiba et al., 1959). The preferential inhibition of DNA synthesis was accompanied by massive degradation of preexisting DNA, suggesting that DNA was the principal target of this drug. In 1963, Iyer and Szybalski demonstrated that MC-induced cell death occurred more rapidly than the rate of DNA breakdown (Iyer and Szybalski, 1963), and suggested that the effects on DNA synthesis and breakdown might be secondary to an earlier action of the antibiotic. Using thermal denaturation profiles and cesium chloride and cesium sulfate equilibrium density centrifugation, these investigators demonstrated cross-linking of DNA in MC treated microorganisms (Iyer and Szybalski, 1963). Furthermore, they reported that MC had no effect on purified bacterial DNA in vitro unless a cell extract was added (Iyer and Szybalski, 1963). Subsequently, they proposed that the inhibitory effects of MC on DNA synthesis was a consequence of the cross-linking of the complimentary strands of DNA, and they provided addi-

tional evidence that cross-linking occurred in human cells, although higher concentrations of MC were required to obtain comparable amounts of cross-linking (Szybalski, 1964; Szybalski and Iyer, 1964a). At the same time, Matsumoto and Lark (1963) reported that DNA from MC-treated bacteria demonstrated a low degree of spontaneous renaturation, as indicated by a decrease in the hyperchromicity of DNA at a higher temperature than that at which the hyperchromicity of the normal DNA decreases. They interpreted these results to indicate that bonds (or cross-links) were formed between the two strands of DNA in MC treated microorganisms. They were not able to demonstrate changes in cesium chloride equilibrium density centrifugation patterns indicative of DNA interstrand cross-linking, nor were they able to demonstrate changes in the renaturation kinetics with MC-treated purified bacterial DNA in vitro. However, further studies by Iyer and Szybalski (1964) indicated that the mitomycins and PM reacted with purified bacterial DNA in vitro when incubated in the presence of NADPH and a cell-free lysate; from these investigations it was concluded that a NADPH-dependent enzymatic reduction was necessary for DNA cross-linking. Additionally, they have shown that cross-linking of bacterial, viral, or mammalian DNA in vitro could be accomplished by chemical reduction of MC under anaerobiosis. These investigators also noted that a higher degree of cross-linking occurred in bacterial, viral, and mammalian DNAs of higher guanine and cytosine content (Iyer and Szybalski, 1964; Szybalski and Iyer, 1964a). Other studies indicated that the rate of cell death was correlated with the degree of DNA interstrand cross-linking: 10 min of exposure at 37°C to 0.1, 1, and 10 μg/ml of MC resulted in 90%, 5%, and 5×10^{-4}% survival of Bacillus subtilis cells and 1, 10, and 100 cross-links/10^9 daltons of DNA, i.e., per one cell genome, respectively (Szybalski and Iyer, 1964a). Using ^{14}C-labeled PM, these investigators demonstrated that chemically reduced PM covalently bound to purified Bacillus

subtilis DNA, with one out of every 5 to 10 antibiotic molecules participating in the cross-linking and the remaining molecules forming monoadducts with the DNA (Szybalski and Iyer, 1964b). These findings were confirmed by White and White (1965), who showed that after chemical reduction, ^{3}H-labeled PM strongly bound bacterial DNA, and that the PM-DNA adduct was resistant to denaturation, suggesting a cross-linking of DNA strands. Their work also demonstrated that some DNA, which did not renature, still retained label, implying that PM caused monoalkylations without interstrand cross-linking.

Further work by Szybalski and Iyer (1964a) demonstrated that it was not possible to cross-link in vivo more than 30-40% of the DNA molecules in the human cell line, D98S, and in rabbit kidney cells, although it was possible to cross-link 100% of bacterial DNA in vitro and in vivo with high concentrations of MC. Because these investigators felt that the incomplete cross-linking of mammalian DNA might reflect poor penetration of MC into the cell nucleus, they exposed rabbit kidney cells infected with pseudorabies virus (localized to the nucleus of the rabbit kidney cells) to various concentrations of MC and discovered that at a concentration of 50 µg/ml of MC all of the viral DNA was fully cross-linked while all of the mammalian DNA was not. This difference in the degree of cross-linking was not explained by a difference in the guanine-cytosine content between mammalian and viral DNA. However, after deproteinization, mammalian DNA became fully susceptible to in vitro cross-linking by the antibiotic (Szybalski, 1964; Szybalski and Iyer, 1964a), leading these investigators to conclude that the chromosomal structure itself must be implicated in the protection of the rabbit kidney cell from MC induced interstrand cross-links.

Weissbach and Lisio (1965) chemically reduced ^{3}H-MC and ^{14}C-PM and demonstrated covalent binding of these antibiotics to purified bacterial DNA and

RNA at a level of about one molecule of antibiotic per 500 nucleotide residues. Further studies showed that chemically reduced ^{14}C-PM bound preferentially to the homopolymer, poly-G (Lipsett and Weissbach, 1965). The isolation of not only monoguanylated but diguanylated PM as a product of the in vitro reaction of soluble RNA (t-RNA) with chemically reduced PM supported the hypothesis that these antibiotics are bifunctional alkylating agents (Lipsett and Weissbach, 1965). However, analogous experiments with DNA were not reported by these investigators.

In recent years, Lown and his coworkers have described covalent cross-linking of λ-phage DNA by MC using an ethidium fluorescence assay (Lown et al., 1976). In this methodology, λ-phage DNA was incubated with chemically reduced MC; following the incubation period, DNA was heat denatured at alkaline pH, cooled in the presence of ethidium, and measured for ethidium fluorescence enhancement, observed only if ethidium bromide intercalated between bihelical nucleic acid. This finding was confirmed by conducting a similar experiment employing S_1-endonuclease to digest single stranded DNA. Additionally, these investigators demonstrated that interstrand cross-linking increased with higher guanine-cytosine content. Furthermore, MC was able to cross-link DNA in the absence of reduction at low pH (pH 4), suggesting to these investigators that preferential activation of this antibiotic would occur in tumor cells which may tend to have a lower pH than normal cells, as well as a more reducing environment. Lastly, other investigators have demonstrated the presence of DNA cross-linking in mammalian cells. By alkaline sucrose sedimentation, hydroxyapatite chromatography, and S_1-nuclease digestion, the removal of interstrand cross-links in many mammalian cells in vitro has been correlated with their sensitivity to MC (Fugiwara, 1982; Fugiwara and Tatsumi, 1975, 1977; Kano and Fugiwara, 1981), while DNA interstrand and DNA-protein cross-links were demonstrated in normal human fibroblasts and in a

Fanconi's anemia fibroblast strain by alkaline elution (Fornace and Little, 1977; Fornace et al., 1979).

The exact site of attachment to DNA by these antitumor quinones is under intensive investigation. As mentioned previously, a correlation has been found between the guanine and cytosine contents of DNA and the degree of cross-linking in vitro and in vivo (Iyer and Szybalski, 1964; Lown et al., 1976), while others have reported that the monofunctional binding of MC to DNA and RNA is highly guanine specific (Lipsett and Weissbach, 1965), indicating that guanine is the major or perhaps the only linkage point of the drug to DNA. This is supported by the finding that poly(dG) binds MC well, while binding of the antibiotic to poly(dA), poly(dT) or poly(dC) is undetectable (Tomasz et al., 1974).

Lipsett and Weissbach (1965) hypothesized that MC might act like other bifunctional alkylating agents and, thereby, alkylate the N-7 of guanine. However, using space filling models, Szybalski and Iyer (1964a) suggested that the O^6 positions of the nearest guanine residues on opposite strands were the only possible points of attachment of MC which would allow interstrand cross-link without distortion of the double helix. Tomasz (1970) reasoned that MC did not covalently bind to the N-7 or C-8 positions of guanine in vitro because no tritium loss of the C-8 hydrogen or guanine occurred upon alkylation of this base by MC. She argued that methylation of the N-7 position of guanine renders the C-8 hydrogen extremely labile under physiological conditions, a property that would result in the release of tritium into the medium. Further evidence by Lown and his coworkers also suggested that MC did not covalently bind the N-7 position of guanine (Hsiung et al., 1976). In their work, they demonstrated a time dependent loss of ethidium fluorescence of DNA methylated by dimethyl sulfate or nitrogen mustard at high pH. The loss of fluorescence was thought to be secondary to slow base-catalyzed

imidazole ring opening, characteristic of N-7 alkylation of deoxyguanosine residues at high pH, and subsequent loss of ethidium binding sites. Because the loss of ethidium fluorescence was not observed to be pH dependent with MC bound DNA, they reasoned that MC did not alkylate the N-7 position of guanine.

Subsequent work has demonstrated MC binding to the phosphate group of a series of nucleotides, including 5'-uridylic acid and 5'-guanylic acid by either acid-catalyzed activation, or chemical or enzymatic reduction of the drug (Hashimoto et al., 1980; Tomasz and Lipman, 1979, 1981). Recently, Hashimoto et al. (1982) reported three adducts after a 10 min catalytic reduction of 1.5 μM MC in the presence of 1 mg/ml of calf thymus DNA. These adducts were identified by acid hydrolysis to be the modified nucleotides with adducts at the N^6 position of adenosine and the O^6 and N-2 positions of guanosine; the amount of MC bound to DNA was estimated to be 1 molecule per 200 to 300 nucleotides. Further work demonstrated these same three modified nucleotides in DNA from rat liver homogenates incubated with MC, as well as from livers of rats treated with MC in vivo (Hashimoto et al., 1983). In the livers from rats treated in vivo with MC, about 1 molecule MC was estimated to be bound per 1 to 2 x 10^4 nucleotides with preferential binding to guanine, especially at the O^6 atom. Unfortunately, details concerning the dose of MC administered and the duration of treatment of rats were not presented. In addition, Tomasz et al. (1983) identified the major adduct of chemically and enzymatically reduced MC with the deoxynucleotide, d(GpC), to be the O^6 adduct of deoxyguanosine.

BIOACTIVATION OF MC AND PM BY ENZYMATIC REDUCTION

Early in the studies on the action of MC and PM, investigators demonstrated the necessity for metabolic activation of these antibiotics. Thus, Schwartz and

Philips (1961) reported that the decrease in 363 nm absorption of MC by rat tissue homogenates in vitro was proportional to a decrease in antibiotic activity, and that rat liver homogenates caused the greatest decrease in 363 nm absorption of MC. Furthermore, they demonstrated the necessity for anaerobiosis (oxygen inhibiting the metabolism of MC by 90%) and reduced pyridine nucleotides for the metabolism of MC. They reported that alcohol dehydrogenase, glucose-6-phosphate dehydrogenase, xanthine oxidase, and a non-specific diaphorase did not decrease the absorption of this agent at 363 nm (Schwartz, 1961). Additional studies demonstrated two anaerobic systems by which MC was metabolized in rat liver (Schwartz, 1962). The more active system required either the entire rat liver homogenate or microsomes and NADPH or a NADPH regenerating system, while the less active system required the entire rat liver homogenate or the recombination of mitochondrial, microsomal, and supernatant fractions (for activity comparable to the whole homogenate) in addition to NAD^+, ATP, Mg^{++}, and a substrate such as malate. These investigators suggested that the proposed anaerobic, reductive activation of MC might explain the potent effects of MC against experimental tumors, as the ability of MC to inhibit the growth of a variety of the transplanted rodent tumors may be due to the relative anoxia under which such cells grow after being implanted (Schwartz et al., 1963).

In an analogous manner, Iyer and Szybalski (1963) and Matsumoto and Lark (1963) showed that interstrand cross-linking of purified bacterial DNA did not occur unless the DNA was incubated with bacterial cell lysates under anaerobic conditions (Iyer and Szybalski, 1963). However, aging, aeration, or dialysis against sodium chloride-sodium citrate buffer destroyed the activating capacity of the lysate. The activity of the dialyzed lysate could be restored by the addition of NADPH or a NADPH-generating system; however, NADPH alone was ineffective.

These investigators found that Clostridium kluveryi diaphorase, in the presence of NADPH, caused cross-linking of bacterial DNA by MC. However, they reported that the reductase activity resided in the lysate fraction which sedimented at 198,000 x g, and that this activity was not decreased by dicoumarol, an inhibitor of DT-diaphorase (Iyer and Szybalski, 1964). These workers concluded that a NADPH-dependent enzymatic reduction was necessary for DNA cross-linking.

In 1979, Kennedy and Sartorelli extended these earlier findings to mouse liver subcellular fractions (Kennedy and Sartorelli, 1979). They demonstrated that the rate of MC disappearance from incubations with mouse liver nuclei or microsomes required NADPH and anaerobiosis. Furthermore, using the trapping agent, 4-(p-nitrobenzyl)pyridine, they demonstrated a correlation between the disappearance of MC and the appearance of an alkylated species. Furthermore, two tumor cell lines, Sarcoma 180 and EMT6 carcinoma, were able to metabolize and activate MC to an alkylating agent under hypoxic conditions (Kennedy et al., 1980).

From these studies, it was clear that a NADPH-dependent enzyme(s) was necessary for the activation of the antibiotic. However, the enzyme(s) which was responsible for this activation was obscure. In 1976, Handa and Sato showed that MC stimulated rat liver microsomal oxidation of NADPH under aerobic conditions, a finding extended to partially purified rat liver NADPH-cytochrome c reductase (Handa and Sato, 1976). Since MC increased the NADPH oxidation by microsomes and by the enzyme preparation to almost the same degree, these investigators suggested that the NADPH oxidation produced by MC was catalyzed by NADPH-cytochrome c reductase. In addition, Bachur and coworkers have described NADPH oxidation and oxygen consumption by both rat liver microsomes and purified rat liver NADPH-cytochrome P-450 reductase (Bachur et al., 1978, 1979). Reductive activation of MC by rat liver NADPH-cytochrome P-450 and buttermilk xanthine

oxidase under anaerobic conditions has been reported by Komiyama et al. (1979a, 1982). Using these enzymes, they demonstrated loss of the quinone absorbance at 363 nm and antimicrobial activity under anaerobic conditions but not under aerobic conditions and, in addition, demonstrated that the reduction of MC by xanthine oxidase was at least 10-times less efficient than that produced by NADPH-cytochrome P-450 reductase. Furthermore, Kennedy et al. (1982) have described the metabolic disappearance of MC and the production of a reactive species with a reconstituted rat liver cytochrome P-450 enzyme system under anaerobiosis, and hypothesized that NADPH-cytochrome P-450 reductase, along with cytochrome P-450, was necessary for MC activation under hypoxic conditions. Most recently, Bachur and coworkers reported the anaerobic reduction of MC and the formation of MC metabolites by rat liver NADPH-cytochrome P-450 reductase and bovine milk xanthine oxidase (Pan et al. 1984).

PROPOSED MECHANISMS OF REDUCTION AND ACTIVATION OF MC AND PM

The enzymatic activation of MC and PM has been proposed to occur through either a one electron reduction to form the semiquinone or a two electron reduction to form the hydroquinone. As early as 1964, Patrick and coworkers reported the detection of a stable semiquinone of mitomycin B by ESR after chemical reduction; however, no data were presented to support this contention (Patrick et al., 1964). In 1966, a theoretical paper by Murakami (1966) postulated that a one electron reduction of the mitomycins to the semiquinone was important for DNA cross-linking. He suggested that the semiquinone form of the mitomycins was the active intermediate and that cross-linking included the O^6 position of guanine, the N-4 of cytosine, and the N-4 and O-5 of mitomycin. This proposed scheme of cross-linking resulted in a structural change in the bases corresponding

to their tautomeric isomers. He suggested further that the greater tendency towards isomerization of cytosine and guanine, as compared to thymine and adenine, provided the basis for the preferential contributions of guanine and cytosine to MC cross-linking. Bachur and coworkers hypothesized that one electron reduction of MC under anaerobiosis forms a MC radical anion which undergoes conversion to metabolites (Figure 1, adapted from Pan et al., 1984). After generation of the anion, the quinone moiety forms an aromatic ring which favors the release of the methoxy group at C-9a as methanol. They argue that nucleophilic attack at C-1 is facilitated in this indole intermediate as the aziridine ring cleaves. In support of this mechanism, these researchers produced the MC radical anion by electrochemical reduction and observed nucleophilic addition at the C-1 position of MC (Andrews et al., 1983). However, it is notable that single electron reduction may also be followed by reoxidation of MC in the presence of oxygen to give the parent compound and the superoxide radical as shown in Figure 1 (Bachur et al., 1978, 1979). This radical may dismute to hydrogen peroxide and to other radicals, such as the hydroxyl radical which are known to be cytotoxic (Fridovich, 1972). Such cyclic reduction and oxidation of the quinone may be responsible for the toxicity of this drug in air (Kennedy et al., 1980).

Additional evidence for the existence of a semiquinone intermediate was described by Nagata and Matsuyama (1969). These investigators demonstrated an ESR signal indicative of a stable semiquinone after reduction of MC by sodium borohydride under alkaline conditions in air or nitrogen. Lown and coworkers noted the MC semiquinone after chemical reduction by sodium borohydride using ESR and also observed superoxide and hydroxyl radical formation by spin trapping (Lown et al., 1978). Bachur and coworkers reported an ESR signal indicative of the formation of a semiquinone after reduction of MC with purified xanthine oxidase;

however, they were not able to generate a significant signal after reduction of MC with rat liver microsomes or NADPH-cytochrome P-450 reductase (Bachur et al., 1978, 1979; Pan et al., 1984). Kalyanaraman et al. (1980) incubated MC with rat liver microsomes anaerobically and generated an ESR signal, which they believed to be the semiquinone, though they could not exclude the possibility that the signal was produced by the NADPH-cytochrome c reductase semiquinone.

Although direct evidence for the formation of the semiquinone form of MC is limited, many investigators have found indirect evidence for the presence of MC semiquinone. Thus, MC stimulated the superoxide dismutase-sensitive oxidation of sulfite by microsomes, probably through the formation of superoxide from oxidation of the semiquinone (Handa and Sato, 1975). Tomasz (1976) demonstrated that either chemically reduced MC or antibiotic incubated with bacterial extracts and NADPH generated hydrogen peroxide, while Handa and Sato (1976) and Bachur et al. (1978, 1979) showed microsomal NADPH oxidation and oxygen uptake in microsomes and tumor cells by MC, suggesting the presence of the MC semiquinone.

The semiquinone of MC has been reported to bind DNA. Thus, after one electron reduction, Tomasz et al. (1974) showed that reduction of MC by sequential addition of substoichiometric amounts of sodium dithionite was more efficient in generating MC-DNA adducts than the addition of dithionite in excess. Therefore, they proposed that the semiquinone formed an initial noncovalent attachment to DNA, followed by further reduction of MC, and subsequent formation of a covalent linkage. Sinha and Chignell (1979) reported loss of the ESR hyperfine splitting of the MC semiquinone radical after addition of DNA, which they attributed to immobilization of the radical upon binding; however, subsequent work with the

anthracyclines suggested that the binding process may be more complex (Sinha and Gregory, 1981).

Evidence to support a two electron reduction of the mitomycins, with the formation of a hydroquinone was first reported by Patrick et al. (1964). They demonstrated that under anaerobic conditions, chemically reduced mitomycin B yielded an ultraviolet spectrum consistent with the loss of the hydroxyl group on position 9a, and hypothesized that the reduction of mitomycin B to its hydroquinone released a free electron pair on N-4 from its involvement in a resonance with the quinone carbonyl group, thereby facilitating the elimination of the group at 9a. Iyer and Szybalski (1964) published a similar, more extensive discussion. They called attention to the fact that, although these antibiotics contain an aziridine ring which is usually highly reactive, the oxidized form of the antibiotics exhibited little alkylating ability when reacted thiosulfate with γ-(4-nitrobenzyl)-pyridine due to the withdrawal of electrons from the nitrogen atom at position 4 into the quinone ring. However, upon reduction of the quinone to the hydroquinone, loss of the 9a-methoxy occurred because the regaining of electrons by the N-4 atom coupled with the high driving force for the formation of a fully aromatic indole system, facilitated fission of the aziridine ring because of the stabilization of the positive charge by the indole ring. This resonant system provided stabilization for an activated carbonium ion at C-1, the first alkylating center. The release of the methoxy group as methanol during chemical reduction of MC was quantitative, as determined by a microdiffusion technique. Furthermore, they predicted activation of a second alkylating center to form a carbonium ion at C-10, because the carbamate anion was a good leaving group and a positive charge at C-10 would be stabilized by the indole nitrogen. Finally, these investigators postulated a third reactive site at the C-7 position of MC. In more recent years,

this proposed mechanism was modified slightly to include the following sequence of events: reduction to the hydroquinone with elimination of methoxy group as methanol, opening of the aziridine ring by an elimination process to give a C-1 quinone methide, nucleophilic addition of DNA to the quinone methide to give the monoalkylated product, and finally, intramolecular SN_2 displacement of the carbamate to form a second quinone methide at C-9 that leads to the cross-linked adduct (Figure 2, Moore, 1977; Moore and Czernik, 1981). Supportive evidence for this mechanism in vitro was provided by Mercado and Tomasz (1972) and Otsuji and Murayama (1972) who demonstrated that mitomycin derivatives lacking the aziridine ring or the carbamoyl group were inferior antibacterial agents and were considerably less active than MC in the cross-linking of bacterial DNA. More direct evidence has involved the electrochemical reduction of mitomycin by Rao et al. (1977). These investigators presented evidence that MC was reduced to the hydroquinone with subsequent loss of the methoxy group at C-9a and fission of the aziridine ring.

After the reduction of MC, there is substantial evidence in support of the reactivity of the C-1 position of MC. Thus, following either acid-catalyzed activation, or chemical or enzymatic reduction of MC, a variety of 1-substituted phosphate products were formed (Hashimoto et al., 1980; Pan et al., 1984; Tomasz and Lipman, 1979, 1981). Furthermore, deoxyribonucleotide adducts from the deoxyribonucleotide dimer d(GpC), calf thymus DNA, or rat liver DNA bound to the C-1 position of MC have been characterized (Hashimoto et al., 1982, 1983; Tomasz et al., 1983). The evidence in support of the reactivity of the C-10 position of MC is less substantial. In a chemical system, Hornemann et al. (1979, 1983) reported the isolation and characterization of 1,10-diethylxanthyl-2,7-diaminodecarbomoyl-mitosene after reduction of MC with sodium dithionite or palladium on charcoal in

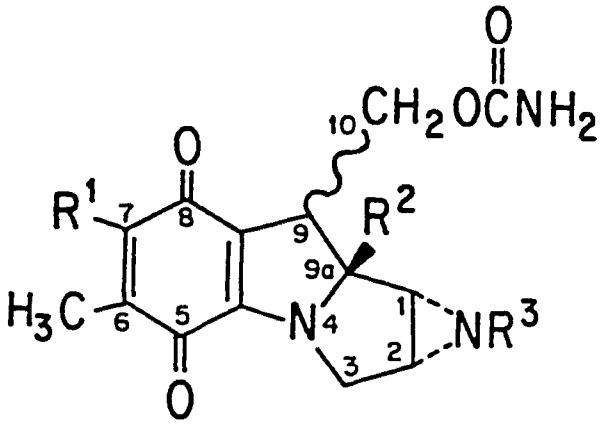
the presence of potassium ethyl xanthate. Similarly, Bean and Kohn (1983) demonstrated that reduction of MC by sodium dithionite in the presence of the nucleophile, potassium ethyl monothiocarbonate, at room temperature led to the formation of aziridine ring-opened disubstituted mitosene adducts at the C-1 and C-10 positions of MC. In addition, they reported that reduction of MC at 0 to 5°C led to preferential substitution at the C-1 position of this antibiotic. However, despite the intensive investigation for an adduct on the C-10 position of MC in a biological system, at present, no evidence exists for such a complex.

The purpose of this thesis is three-fold: (a) to examine the cytotoxicity of MC and PM to aerobic and hypoxic cultured cell lines; (b) to determine whether enzyme(s) proposed to activate these drugs are present in these cell lines and if present, whether a correlation exists between the activity of these enzymes and the cytotoxicity of these antitumor agents; and finally, (c) to ascertain the kinds of DNA lesions caused by these antitumor antibiotics and to determine whether they correlate with the cytotoxicity of these agents observed in the cell systems employed. In Chapter II of this thesis, data are presented which demonstrate that these antitumor quinones are more cytotoxic to hypoxic EMT6 mouse mammary tumor cells and Chinese hamster V79 cells than to their oxygenated counterparts, but are equitoxic to Chinese hamster ovary cells under these conditions of oxygenation. In order to ascertain the reason(s) for differences in the degree of cytotoxicity to MC and PM in these cultured cell lines, the microsomal electron transport enzymes and DT-diaphorase, enzymes proposed to bioactivate the anti-cancer quinones, were examined in sonicates of these cultured cells, and the results of these investigations are presented in Chapter III. In addition, data are presented which demonstrate the ability of cell sonicates to reduce and activate MC to a

reactive product(s). Finally, in Chapter IV, alkaline elution methodology was utilized to assess the importance of DNA lesions to the cytotoxicity of these drugs under conditions of hypoxia and normal aeration.

Table I

STRUCTURES OF MITOMYCINS A, B, C AND PORFIROMYCIN



Antibiotic	R^1	R^2	R^3
Mitomycin A	CH_3O	OCH_3	H
Mitomycin B	CH_3O	OH	CH_3
Mitomycin C	H_2N	OCH_3	H
Porfiromycin	H_2N	OCH_3	CH_3

FIGURE I. One Electron Reduction and Activation of MC. One electron reduction of MC forms the radical anion. In the absence of oxygen, elimination of the methoxy group and attack of DNA at C-1 is facilitated as the aziridine ring cleaves (adapted from Pan et al., 1984). In the presence of oxygen, the quinone is oxidized to give the parent compound and the superoxide radical (Bachur et al., 1978, 1979).

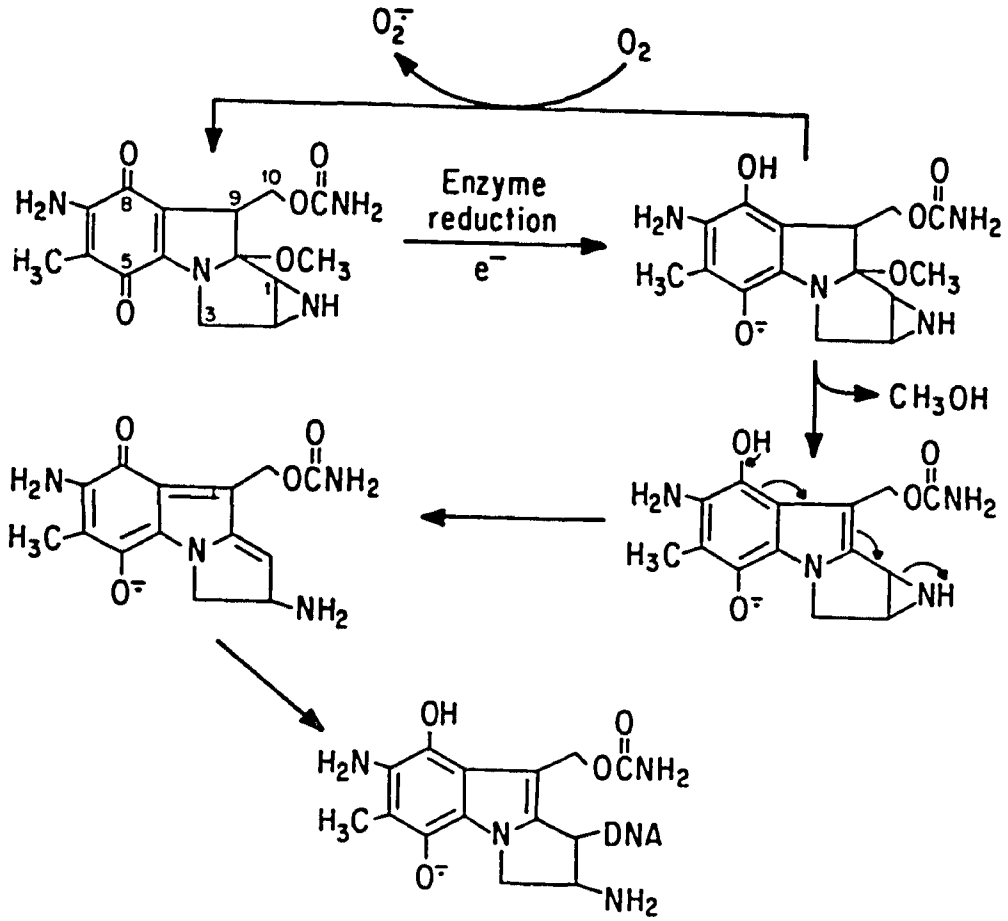
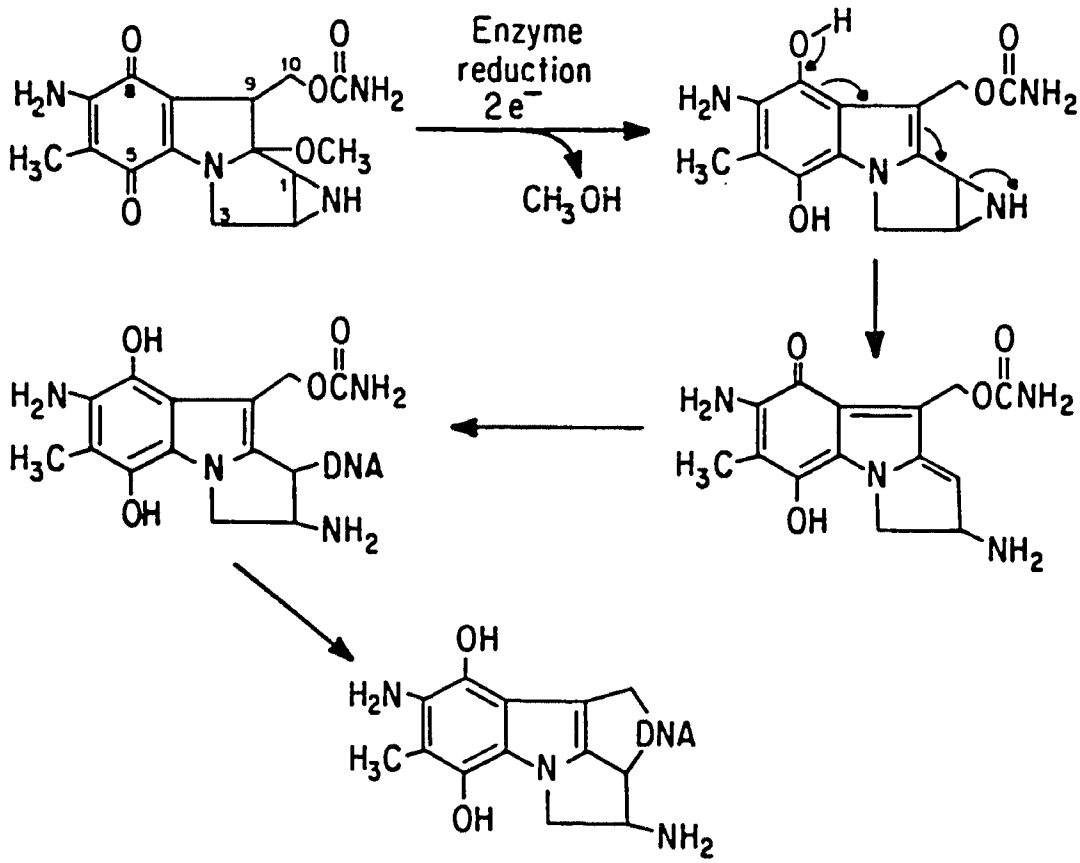


FIGURE 2. Two Electron Reduction and Activation of MC. Two electron reduction of MC to the hydroquinone facilitates the elimination of the methoxy group, opening of the aziridine ring, and the formation of a quinone methide at C-1. Attack of DNA at this position gives the monoalkylated product, and subsequent SN_2 displacement of the carbamate to form a second quinone methide at C-9 results in the cross-linked adduct (Moore, 1977; Moore and Czerniak, 1981).



CHAPTER II

Cell Characteristics and Cytotoxicity Studies

INTRODUCTION

Solid neoplasms are known to contain deficient vascular beds and areas of severe vascular insufficiency, and, as a result, such tumors may develop regions containing hypoxic, or less well-oxygenated tumor cells (Thomlinson and Gray, 1955; Vaupel, 1977; Vaupel and Thews, 1974). These tumor cells, which frequently constitute 5-30% of the total viable tumor cell population, may form a therapeutically resistant cell population within solid tumors. Protected from radiotherapy and chemotherapy by hypoxia, these malignant cells may be capable of proliferating and causing tumor regrowth after treatment. Investigations in our laboratory hypothesized that hypoxic cells in solid tumors probably exist in an environment conducive to reductive processes. This environment might be exploited by chemotherapeutic agents that become cytotoxic after reductive activation. The bioreductive alkylating agents are a class of antitumor antibiotics which require metabolic reduction to form a species capable of alkylating critical cellular macromolecules. Hypoxic tumor cells would be expected to activate and be susceptible preferentially to drugs of this class.

The prototypic bioreductive alkylating agents are MC, an antineoplastic antibiotic in clinical use for a variety of solid tumors (Crooke, 1979; Crooke and Bradner, 1976), and PM, a structurally similar antibiotic. Previous studies in our laboratory, as well as in Dr. Sara Rockwell's laboratory, have demonstrated that EMT6 and Sarcoma 180 tumor cells are more sensitive to MC under hypoxic conditions than in the presence of air (Kennedy et al., 1980; Rockwell et al., 1982; Teicher et al., 1981). Initial work by Dr. Sara Rockwell in collaboration with our

laboratory has extended these findings to other cell lines. Chinese hamster lung fibroblasts (V79) and Chinese hamster ovary (CHO) cells were used for comparative purposes, as these cell lines are commonly used for investigations on the effects of radiosensitizing agents and cytotoxic drugs with specificity for hypoxic cells. After treatment of these cell lines with a single concentration of MC, our laboratories demonstrated that MC is essentially equitoxic to aerobic and hypoxic CHO cells, whereas it is preferentially cytotoxic to V79 under hypoxic conditions.

In this chapter, the EMT6, CHO, and V79 cell lines have been examined and data are presented on the generation times, cell volumes, and cell cycle distributions. This brief characterization was conducted in order to establish the conditions under which these cell lines would be employed for the experiments conducted throughout this dissertation. More important, this chapter presents more extensive data on the cytotoxicity of MC to aerobic and hypoxic EMT6, CHO, and V79 cells. In addition, it compares these results with the cytotoxicity data obtained after treatment of the three cell lines with the structurally similar antibiotic, PM.

METHODS

Cultured Cell Lines--EMT6 mouse mammary tumor cells (subline EMT6-Rw, supplied by Dr. Sara Rockwell, Department of Therapeutic Radiology, Yale University); Chinese hamster ovary cells (subline HA-1, supplied by Dr. Daniel S. Kapp, Department of Therapeutic Radiology, Yale University); and Chinese hamster V79 cells (subline V79-8, supplied by Dr. R. Michael Liskay, Department of Therapeutic Radiology, Yale University) were grown at 37°C in Waymouth's medium (Grand Island Biological Company, Grand Island, NY) supplemented with 15% fetal bovine serum (Grand Island Biological Company) and antibiotics in a humidified atmosphere of 95% air/5% CO_2. Stock cultures of EMT6, CHO, and V79 cells were seeded in 25 cm^2 plastic flasks at 5 to 10 x 10^5, 1 to 2 x 10^6, 3 to 5 x 10^5 cells per ml medium, respectively, with subculturing being required every 3 to 4 days. Other characteristics of these cell lines have been described in detail previously (Kapp and Hahn, 1979; Liskay, 1978; Rockwell, 1977).

Generation Times and Median Cell Volumes of the Cultured Cell Lines--Growth characteristics of the three cell lines were established by seeding 2.0 x 10^5 cells per 10 ml medium in glass milk dilution bottles. Cell numbers were obtained using a Coulter Model Z_{BI} Counter (Coulter Electronics, Inc., Hialeah, FL) and counting duplicate samples every day for one week. The mean doubling times were determined from the linear portion of the logarithmic growth curves. Using aliquots of cells from the bottles used for the growth curves, the cell volumes were determined for each cell line using a Coulter Channelyzer calibrated with mulberry and ragweed particle standards of 12-13 μm and 19-20 μm diameters, respectively. Aliquots of approximately 10^6 cells were also obtained at intervals throughout the growth curve for each cell line for determination of the DNA histograms and cell cycle phase distributions (see below).

DNA Distributions and Cell Cycle Analyses of Cultured Cell Lines--The DNA and cell cycle distributions were determined for each cell line throughout the growth curve. Approximately 10^6 cells were collected by centrifugation at 600 x g for 5 min and resuspended in 2.0 ml phosphate buffered saline (PBS; 0.2 g KCl, 0.2 g KH_2PO_4, 8 g NaCl, and 1.15 g Na_2HPO_4 per liter, pH 7.4). At that time, 6 ml of ice-cold 95% ethanol was added in three 2 ml aliquots. Fixed cells were refrigerated until stained with 0.01% mithramycin using the methodology of Chrissman and Tobey (1974). The cellular DNA content was measured using a Becton-Dickinson FACS IV flow cytometer, and cell cycle distributions were estimated by computer analysis of DNA histograms by the method of Dean and Jett (1974).

Cell Survival Studies--For cell survival studies, 2 x 10^5 EMT6 cells, 3 to 4 x 10^5 CHO cells, and 1 to 2 x 10^5 V79 cells per 10 ml of Waymouth's medium supplemented with 15% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin were seeded in glass milk dilution bottles. Cultures were allowed to grow for 3 days at which time the cells were in mid-exponential growth. At that time, the medium was removed and replaced with 5 ml of fresh medium. The bottles were then sealed with sterile rubber sleeve stoppers (Aldrich Chemical Company, Milwaukee, WN) and fitted with 13 gauge needles for gas inflow and 18 gauge needles for gas outflow; tubing connected to the outflow needles was immersed in water to allow visible monitoring of gas flow and to prevent back flow of air into cultures. To produce hypoxia, cultures were gassed continuously for three hours at 37°C with a humidified mixture of 95% N_2/5% CO_2 (oxygen content, <10 ppm; Presto Welding Service Centers, North Haven, CT). This incubation technique produces full radiobiologic hypoxia after 2 hr of exposure to N_2/CO_2 (Rockwell and Kennedy, 1979). Normally aerated cultures were incubated in a humidified

atmosphere of 95% air/5% CO_2 (Presto Welding Service Centers). During the third hour, the appropriate dilution of either MC or PM (the generous gifts of Dr. Maxwell Gordon, Bristol-Myers Company, Syracuse, NY) dissolved in 70% ethanol was added directly to cultures without breaking the hypoxia, by injecting a small volume of drug through the rubber sleeve. After exposure to each drug for 1 hr under hypoxia or air, the cells were washed twice with 5 ml of sterile PBS, and suspended by treatment with 0.05% trypsin (Grand Island Biological Company) in PBS for 10 min. Cell numbers were obtained using a Coulter Model Z_{BI} counter (Coulter Electronics, Inc.). After the appropriate dilutions were made, cells were plated in replicate dishes and allowed to form colonies for 10 to 14 days. At the end of this time, the cell colonies were stained with Gram's crystal violet (Fischer Scientific Co., Fairlawn, NJ) and counted. Both hypoxic and aerobic vehicle (0.35% ethanol) controls were included in each experiment; the surviving fractions for hypoxic cultures were calculated using the hypoxic controls.

RESULTS

Generation Times and Cell Volumes of the Cultured Cell Lines--Generation times of 9.6, 12, and 9.6 hr for the EMT6, CHO, and V79 cell lines, respectively, were determined from the linear portion of the logarithmic growth curves (Table 1). Cell volumes of the EMT6, CHO, and V79 cell lines were measured throughout the growth curve and because most experimental studies were done on day 3, only volume measurements for day 3 are reported in Table 2.

DNA Distributions and Cell Cycle Analyses--The DNA content of the EMT6, CHO, and V79 cell lines was measured during the entire growth curve. Days 2 and 3, which represented the early and mid-logarithmic portion of the growth curve for the three cell lines, had similar cell cycle profiles. The profiles for day 4 for the CHO and V79 cells were similar to days 2 and 3; however, the profile for day 4 in the EMT6 cells demonstrated a larger proportion of G_1 phase cells, indicating the beginning of a confluent culture and depletion of nutrients in the medium. The cell cycle distributions for the EMT6, CHO, and V79 cell lines on day 2 to day 4 are presented in Table 3.

Cell Survival Studies--Measurements of the surviving fraction of hypoxic and aerobic vehicle-treated controls demonstrated a slight cytotoxicity with all three cell lines after hypoxic treatment. The surviving fraction of the hypoxic vehicle-treated control cells was 0.472, 0.534, and 0.343, while the aerobic vehicle-treated controls was 0.738, 0.794, and 0.614 for EMT6, CHO, and V79 cells, respectively. The survival curves for aerobic and hypoxic cells treated with various concentrations of MC or PM are shown in Figures 3-8. These graphs represent the geometric mean $\pm$ the standard error of the mean of 3 to 5 experiments. The findings with

the EMT6 cells treated with MC (Figure 3) agreed with those published previously (Kennedy et al., 1980; Rockwell et al., 1982; Teicher et al., 1981). The survival curves for the EMT6 and V79 cells treated with various concentrations of MC for 1 hr were similar, with the survival of the hypoxic cells being significantly lower than that of aerobic cells, and with the slopes of the hypoxic curves being steeper than those of the aerobic curves (Figures 3 and 5). For CHO cells, there was little difference between aerobic and hypoxic cell survival with MC treatment (Figure 4).

Interestingly, the differential cytotoxicity of PM to hypoxic versus aerobic EMT6 and V79 cells was significantly greater than that of MC (Figures 6 and 8). These results demonstrate that PM and MC are essentially equitoxic to hypoxic tumor cells, while PM is considerably less cytotoxic than MC to aerobic cells. For CHO cells, the results demonstrated significantly greater hypoxic cell cytotoxicity than aerobic cell cytotoxicity at 2 and 4 μM PM (Figure 7). However, this increase in hypoxic cell cytotoxicity for CHO cells treated with PM was substantially less than the differential cytotoxicity seen with EMT6 and V79 cells treated with this drug.

Table 2

MEAN GENERATION TIME AND VOLUME OF CULTURED CELL LINES

Cell line	Generation time (hr)	Cell Volume (μm^3)	Cell Diameter (μm)
EMT6	9.6	3575	19
CHO	12.0	2724	17
V79	9.6	2043	10

The mean generation time and medium cell volume were determined in exponentially growing cells (day 3) as described in the Methods. The volume and diameter measurements were calculated based on the volumes of particle standards, assuming a spherical shape for cells.

Table 3

CELL CYCLE DISTRIBUTIONS IN CULTURED CELL LINES

Cell line	Day	G_1	S	G_2-M
EMT6	2	30.2	56.5	13.2
	3	43.4	36.4	20.2
	4	67.6	17.5	14.9
CHO	2	40.5	39.5	20.2
	3	25.5	59.2	15.3
	4	28.8	64.6	6.7
V79	2	31.0	46.6	22.3
	3	27.2	48.3	24.6
	4	42.8	39.3	17.8

The cell cycle distributions for each of the cultured cell lines were determined by computer analysis of the DNA histograms obtained on exponentially growing cells using a Becton-Dickinson FACS IV flow cytometer as described in the Methods.

FIGURE 3. Survival of Aerobic and Hypoxic EMT6 Cells after Exposure to MC. Exponentially growing cells were exposed to varying concentrations of drug for 1 hr under conditions of normal aeration or hypoxia, and cytotoxicity was estimated by the ability of cells to form colonies as described in the Methods. The data are expressed as a fraction of control survival of aerobic (O) or hypoxic (●) cells and represent the means and standard errors of the means for $n \geq 3$.

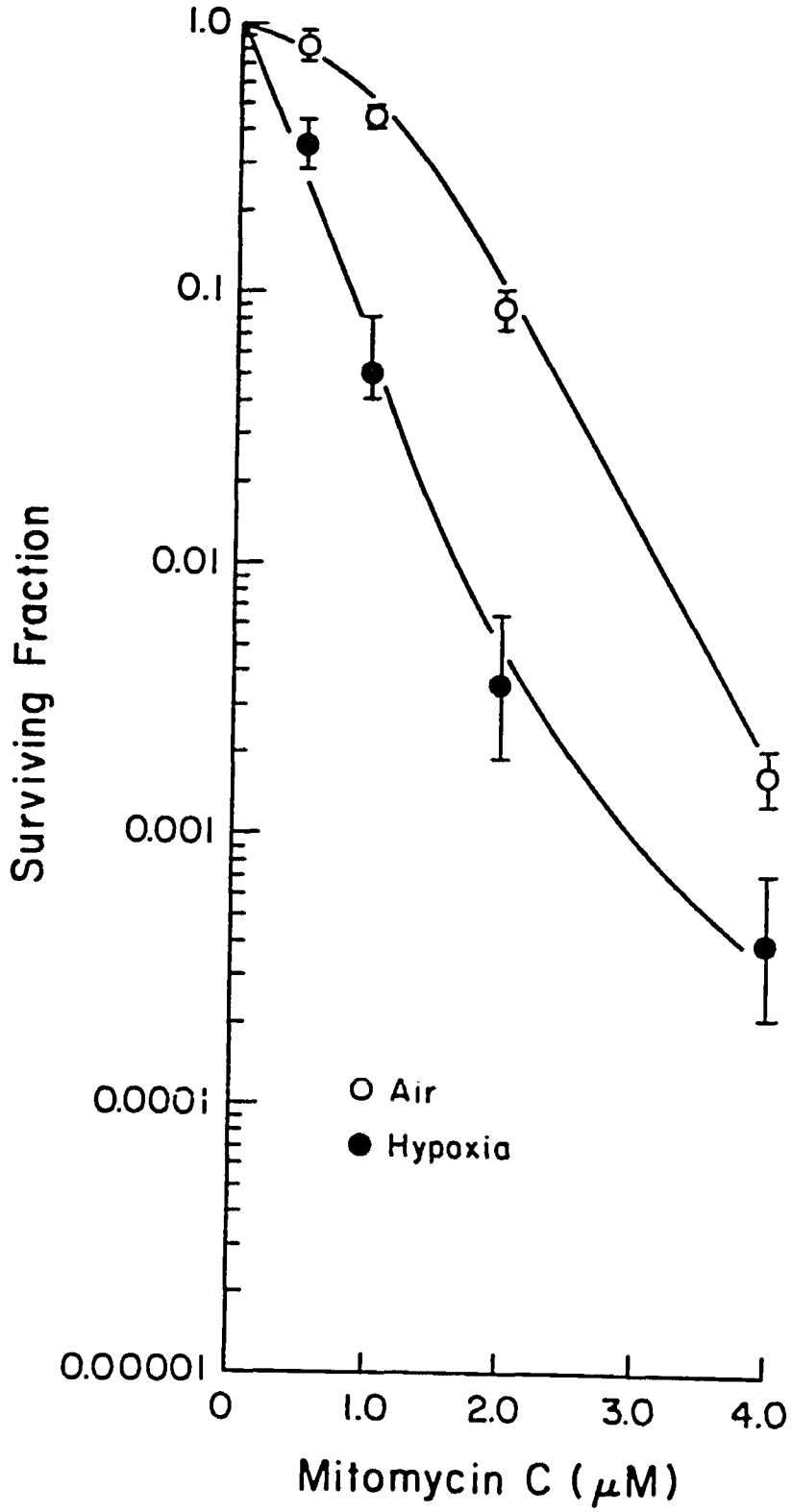


FIGURE 4. Survival of Aerobic and Hypoxic CHO Cells after Exposure to MC. Exponentially growing cells were exposed to varying concentrations of drug for 1 hr under conditions of normal aeration or hypoxia, and cytotoxicity was estimated by the ability of cells to form colonies as described in the Methods. The data are expressed as a fraction of control survival of aerobic (O) or hypoxic (●) cells and represent the means and standard errors of the means for $n \geq 3$.

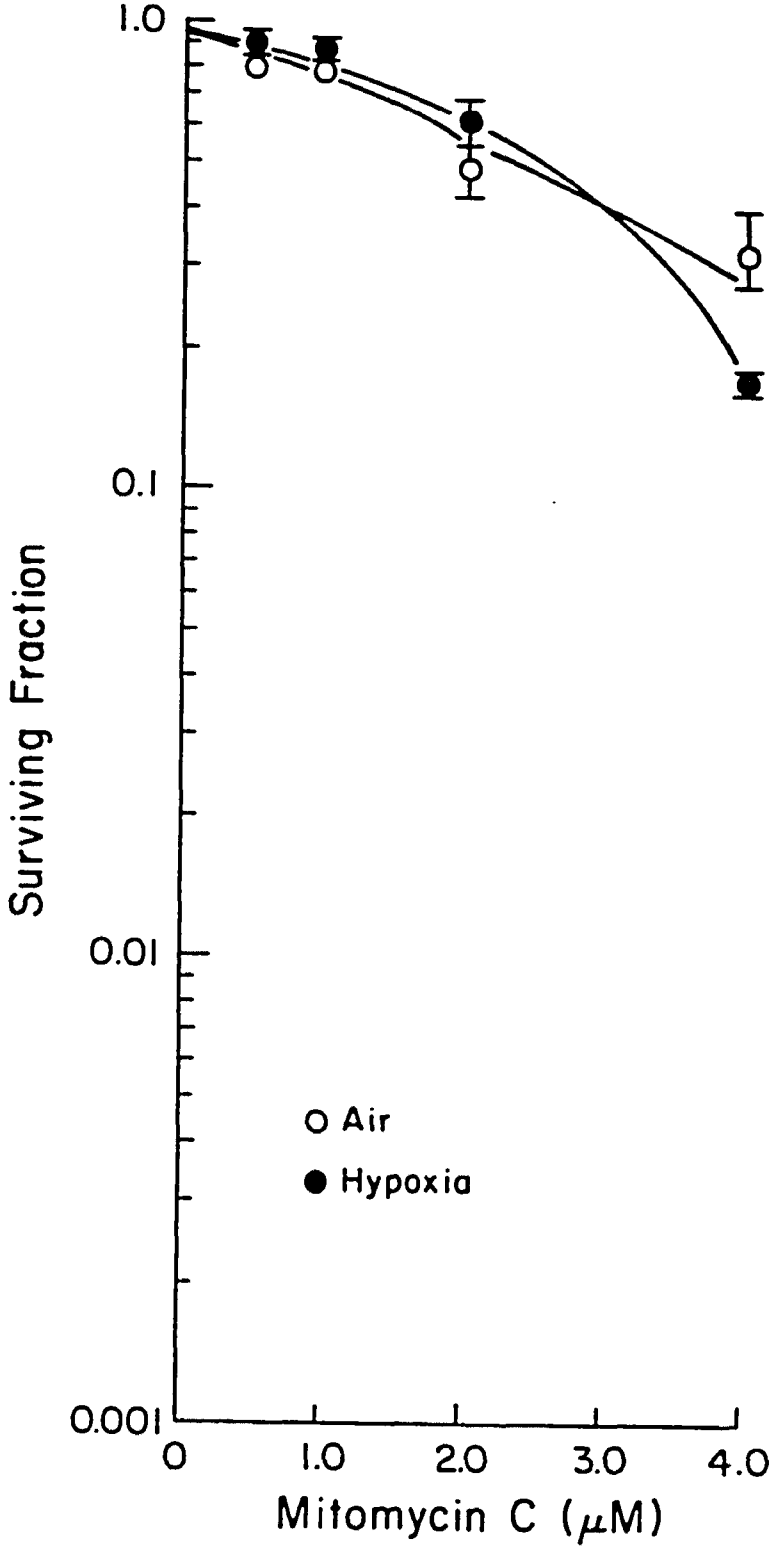


FIGURE 5. Survival of Aerobic and Hypoxic V79 Cells after Exposure to MC. Exponentially growing cells were exposed to varying concentrations of drug for 1 hr under conditions of normal aeration or hypoxia, and cytotoxicity was estimated by the ability of cells to form colonies as described in the Methods. The data are expressed as a fraction of control survival of aerobic (O) or hypoxic (●) cells and represent the means and standard errors of the means for $n \geq 3$.

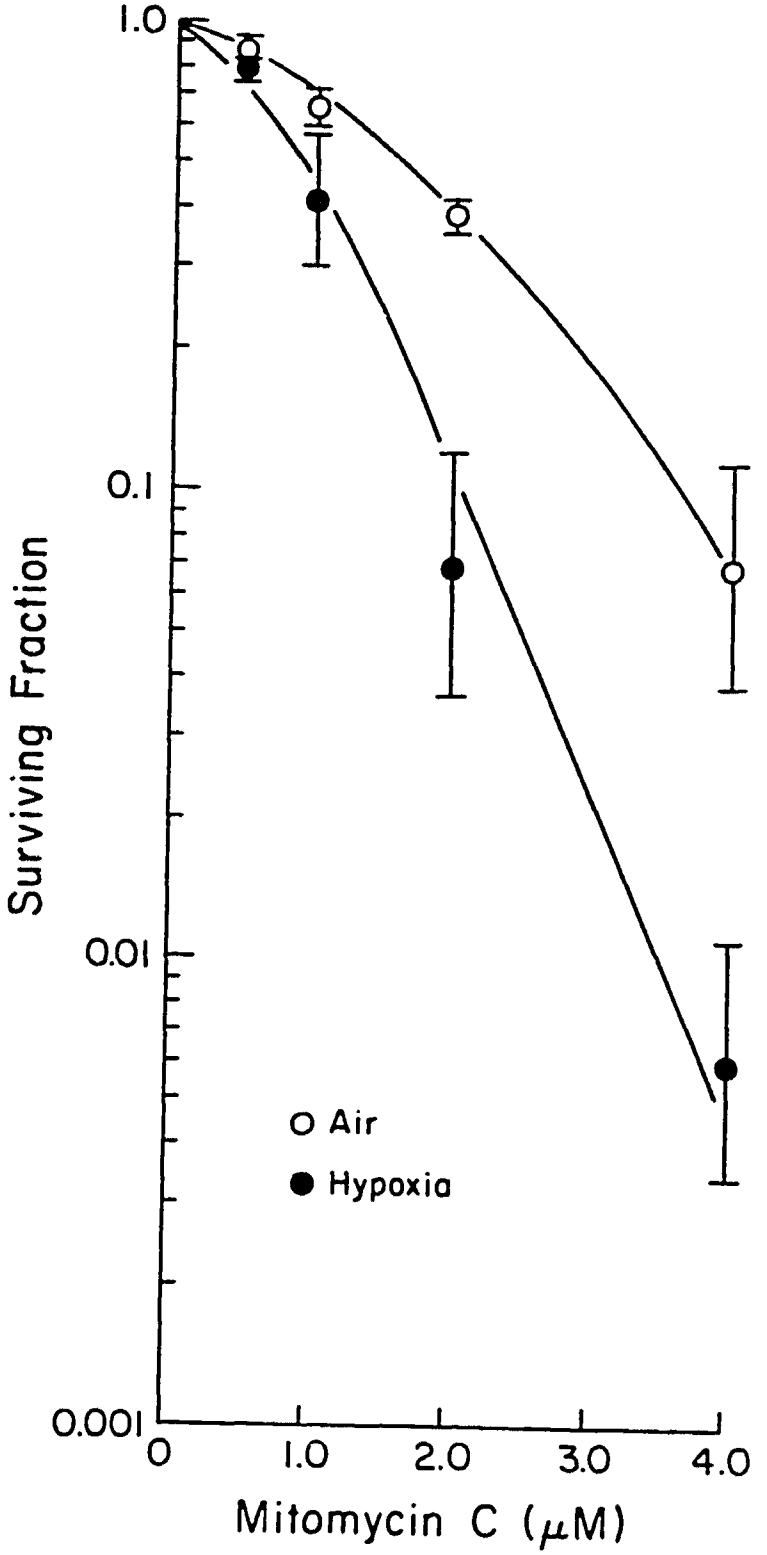


FIGURE 6. Survival of Aerobic and Hypoxic EMT6 Cells after Exposure to PM. Exponentially growing cells were exposed to varying concentrations of drug for 1 hr under conditions of normal aeration or hypoxia, and cytotoxicity was estimated by the ability of cells to form colonies as described in the Methods. The data are expressed as a fraction of control survival of aerobic (O) or hypoxic (●) cells and represent the means and standard errors of the means for $n \geq 3$.

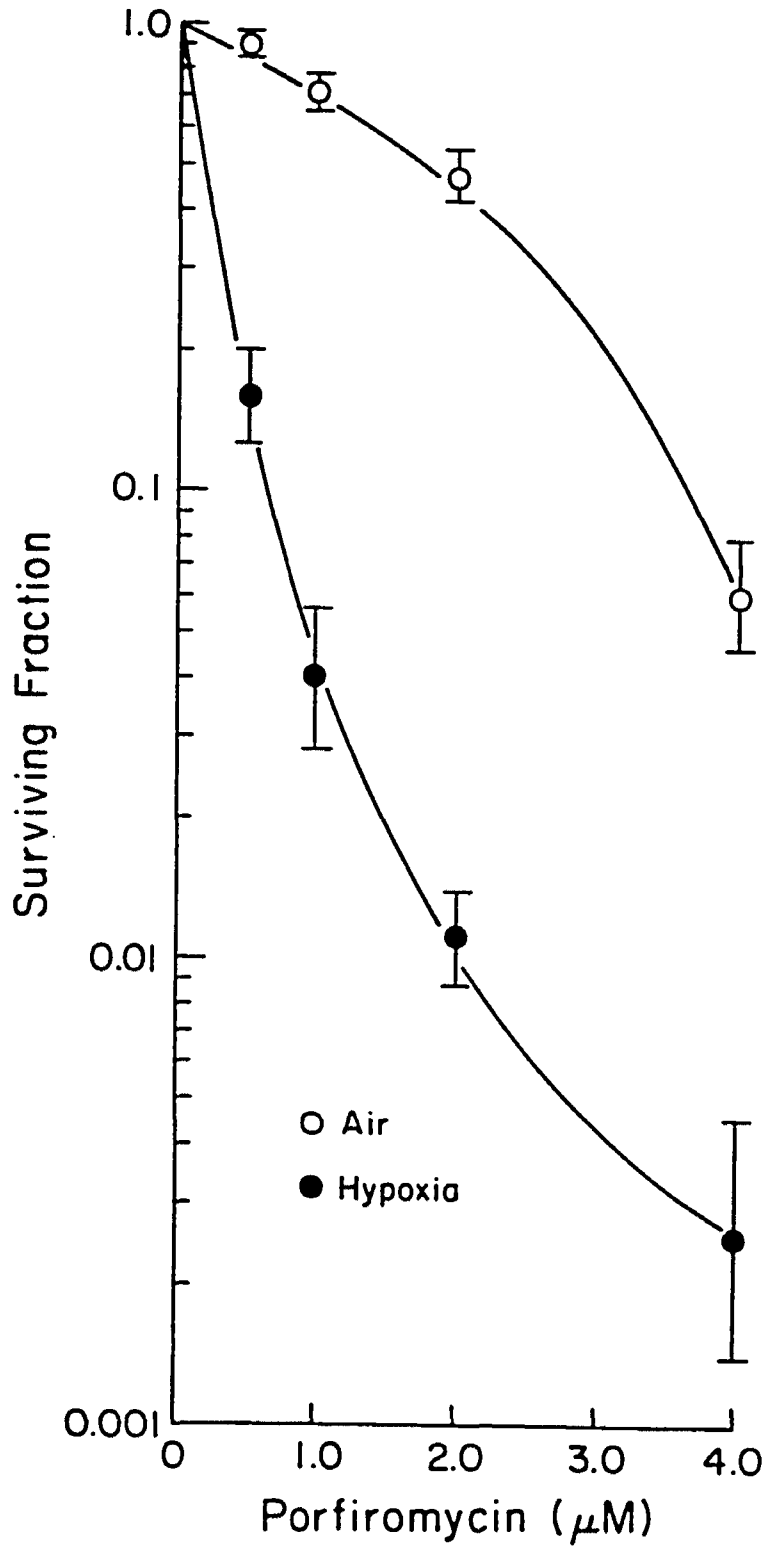


FIGURE 7. Survival of Aerobic and Hypoxic CHO Cells after Exposure to PM. Exponentially growing cells were exposed to varying concentrations of drug for 1 hr under conditions of normal aeration or hypoxia, and cytotoxicity was estimated by the ability of cells to form colonies as described in the Methods. The data are expressed as a fraction of control survival of aerobic (O) or hypoxic (●) cells and represent the means and standard errors of the means for $n \geq 3$.

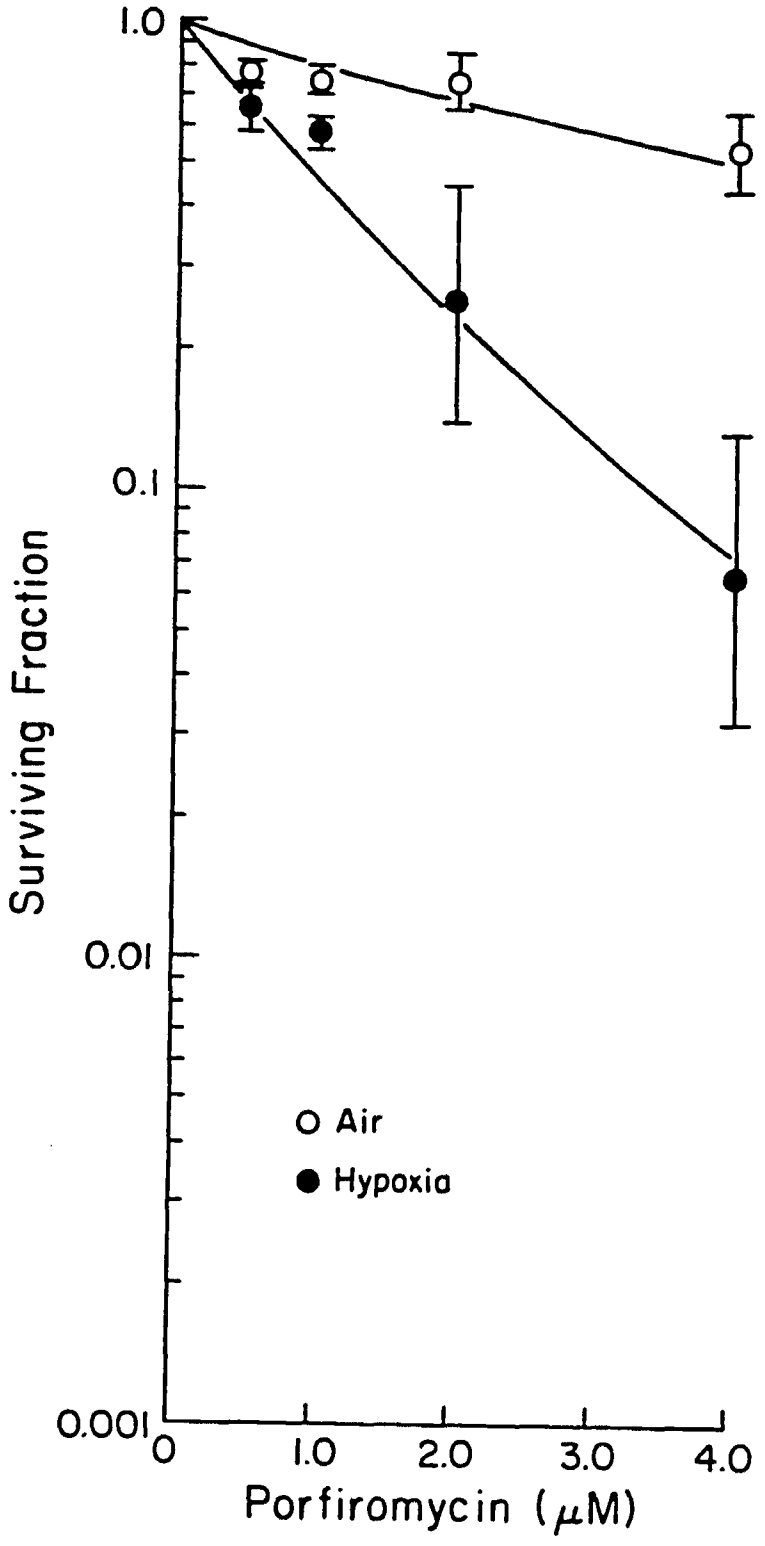
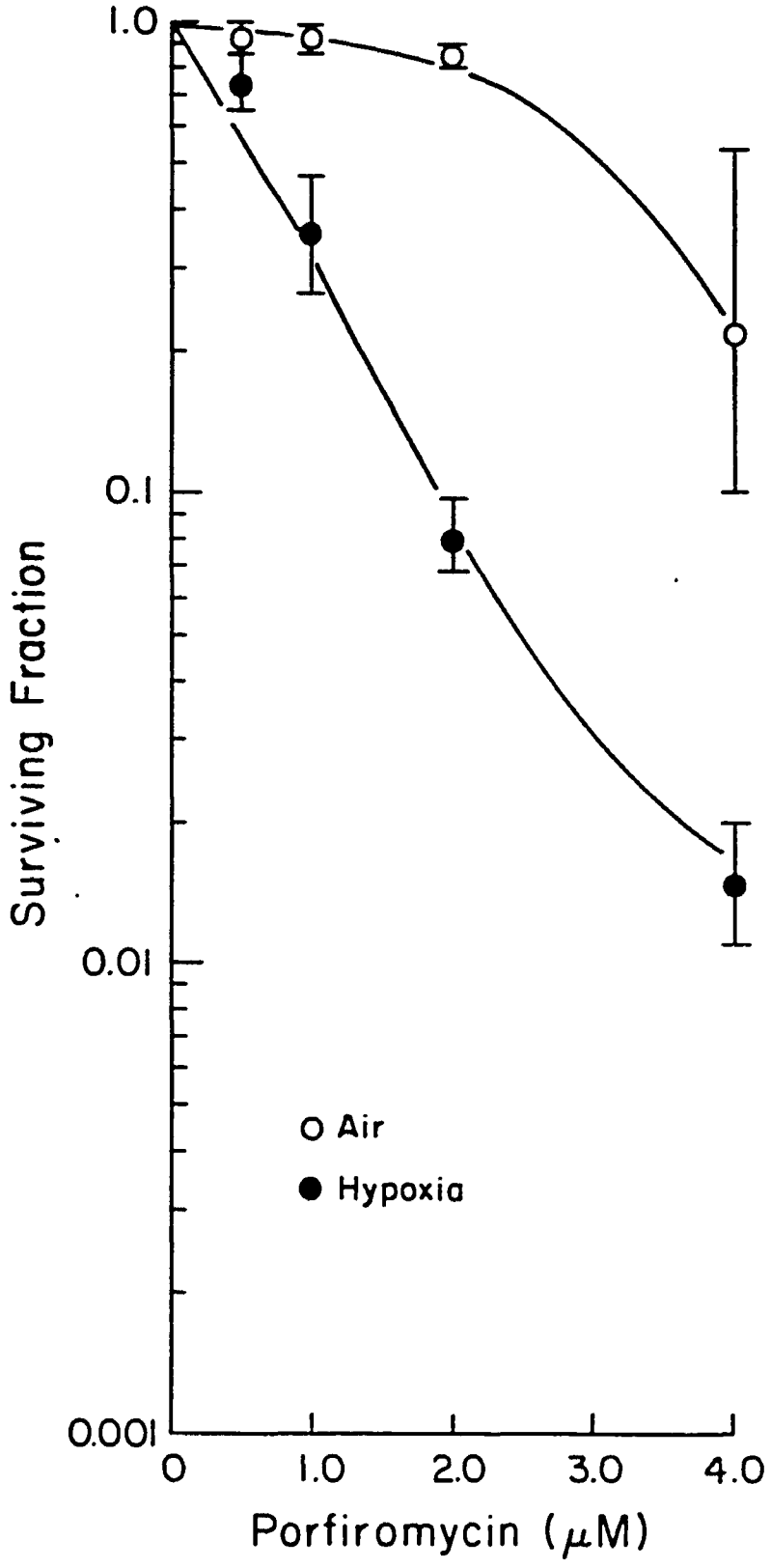


FIGURE 8. Survival of Aerobic and Hypoxic V79 Cells after Exposure to PM. Exponentially growing cells were exposed to varying concentrations of drug for 1 hr under conditions of normal aeration or hypoxia, and cytotoxicity was estimated by the ability of cells to form colonies as described in the Methods. The data are expressed as a fraction of control survival of aerobic (O) or hypoxic (●) cells and represent the means and standard errors of the means for $n \geq 3$.



DISCUSSION

Significant advances toward the cure of human cancer have been achieved by chemotherapy and radiotherapy, primarily in certain rapidly growing cancers such as the childhood leukemias and Hodgkin's disease. However, these neoplasms represent only a small proportion of human cancers. Unfortunately, the majority of cancers are slow-growing solid tumors, such as colon, lung, or breast cancer, which frequently are not cured by existing treatment modalities.

One of the more interesting explanations for the poor curability of some solid tumors by existing treatment protocols is the presence of hypoxic, or less well-oxygenated tumor cells. Slow-growing solid neoplasms are known to contain cellular subpopulations remote from functional blood vessels; as a consequence, these subpopulations are heterogeneous with respect to degree of oxygenation and rate of proliferation. In a recent review which compiled data on the hypoxic fractions of almost 100 rodent tumors, it was concluded that the vast majority of macroscopic rodent tumors appear to contain between 5 to 30% hypoxic cells regardless of tumor histology, growth rate, or size (Moulder and Rockwell, 1984). More important, there is indirect evidence that hypoxic cells exist in human tumors. Histologic studies have shown architecture and vascular patterns suggestive of regions of hypoxia (Thomlinson and Gray, 1955). Measurements of oxygen concentrations with oxygen electrodes have demonstrated that there are lower average oxygen tensions in human tumors than in normal tissues (Cater and Silver, 1960; Evans and Naylor, 1963). Bush et al. (1978) have published data indicating that patients with high hemoglobin levels (>12 g%) have a greater probability of radiotherapy cure for Stages IIb and III carcinoma of the cervix than individuals with relatively low hemoglobin levels. They proposed that the increased cure rate with higher hemoglobin levels was the result of better oxygen delivery

and reduced tumor hypoxia. Analyses of dose-response curves for therapy of superficial skin carcinoma with large single doses of radiation also suggest the presence of hypoxic cells (Bush and Hill, 1975). Furthermore, improved responses of certain malignancies with radiotherapy in combination with hyperbaric oxygen or hypoxic cell sensitizers (Churchill-Davidson et al., 1955; Duncan, 1973) give further credence to the importance of hypoxic cells in limiting the responsiveness of human solid tumors to conventional therapy.

Because hypoxic cells are relatively resistant to the cytotoxic effects of radiation and cell cycle specific chemotherapeutic agents, it is not unlikely that the hypoxic cell population has the capacity to limit the curability of solid tumors. Therefore, approaches have been undertaken to develop agents which will eradicate these populations of cells. These agents, with specificity for hypoxic cells are divided into two classes: the hypoxic cell radiosensitizers and the hypoxic cell cytotoxic agents.

The hypoxic cell radiosensitizers, including metronidazole and misonidazole, increase the sensitivity of hypoxic cells to ionizing radiation. These compounds are thought to sensitize hypoxic cells to radiation by interacting with electron excess or free radical to form a nitro radical anion (Mason and Holtzman, 1975; Perez-Reyes et al., 1980). As a consequence, these compounds sensitize hypoxic cells to radiation (Fowler et al., 1976) without significantly affecting the already radiosensitive aerobic cells. In addition, these radiosensitizers have been shown to be selectively toxic to hypoxic cells in vitro (Hall and Roizin-Towle, 1975; Mohindra and Rauth, 1976; Moore et al., 1976). This selective cytotoxicity of misonidazole to hypoxic cells appears to be the result of enzymatic reduction of the drug to reactive species capable of alkylating DNA (Chapman et al., 1983; Varghese et al., 1976; Varghese and Whitmore, 1980). Because high concentrations of most of the

radiosensitizers are required for either significant radiosensitization or selective toxicity to hypoxic cells and because these concentrations cause dose-limiting central nervous system toxicity (Urtasun et al., 1976), these compounds have been disappointing as clinically useful drugs.

The hypoxic cell specific agents primarily include agents which require reductive activation for cytotoxicity. In 1972, Sartorelli and coworkers hypothesized that since anaerobic microbial cultures had a greater capacity for reduction than aerobic cultures, then hypoxic cells, remote from the vascular supply of the tumor, may also have a greater capacity for reductive processes (Lin et al., 1972). Furthermore, it was thought that agents which became cytotoxic after reductive activation might be useful in the eradication of hypoxic cells. Recognizing MC as a naturally occurring prototype, these workers designed and synthesized many "bioreductive alkylating agents" including a series of benzo-, naphtho-, and anthraquinones (Lin et al., 1972, 1973, 1974, 1975, 1976; Lin and Sartorelli, 1973, 1976a, 1976b; Lin et al., 1980) and a group of o-nitrobenzyl halides and carbamates (Teicher and Sartorelli, 1980), and they have demonstrated that representative compounds of these classes were significantly more cytotoxic toward hypoxic cells than oxygenated cells in culture. As indicated previously, the nitroheterocyclic radiosensitizers are not only cytotoxic to hypoxic cells because of their ability to sensitize these cells to the actions of ionizing radiation, but also because hypoxic cells are able to reductively activate these drugs to toxic metabolites (Chapman et al., 1983; Varghese et al., 1976; Varghese and Whitmore, 1980).

Adriamycin and MC have been extensively studied as agents which require reductive activation for cytotoxicity. Our laboratory has found Adriamycin to be selectively cytotoxic to hypoxic EMT6 and Sarcoma 180 cells (Kennedy et al., 1983; Teicher et al., 1981). While the preferential sensitivity of hypoxic cells to

adriamycin also has been demonstrated in less well-oxygenated melanoma cells (Gupta and Krishan, 1982), other laboratories have reported that oxygenated cells have greater or equal sensitivity to this drug (Born and Eichholtz-Wirth, 1981; Harris and Shrieve, 1979; Martin and McNally, 1979, 1980; Smith et al., 1980; Tannock and Guttman, 1981).

Our laboratory, in collaboration with Dr. Sara Rockwell's laboratory, has demonstrated that MC is more cytotoxic to hypoxic EMT6 and Sarcoma 180 cells in culture (Kennedy et al., 1980; Rockwell et al., 1982; Teicher et al., 1981). In this chapter, results have been presented which extend earlier findings on the cytotoxicity of MC to other cultured cell lines. Thus, the results demonstrate that MC is preferentially cytotoxic to hypoxic EMT6 and V79 cells, while it is not preferentially cytotoxic to hypoxic CHO cells. This work is in agreement with the findings of Rauth et al. (1983) which demonstrated that MC is more active against hypoxic V79 cells, but it is not in agreement with their data which demonstrated that MC is also preferentially cytotoxic to hypoxic CHO cells. The reasons for the differences between the results presented in this chapter and those of Rauth and his coworkers are not clear, but it is possible that the sublines of CHO cells employed differ in their response to hypoxia. It is notable that the studies performed in this dissertation were conducted on cells in monolayer, whereas the studies performed by Rauth et al. (1983) were carried out in suspension culture.

The cytotoxicity of PM in hypoxic and aerobic cultured cells yielded results different from those of MC. As expected, PM produced hypoxic cell cytotoxicity comparable to that of MC in the three cultured cell lines, but exhibited significantly less aerobic toxicity in the three cell lines. These findings agree with earlier studies which reported that PM was an active anticancer drug but with less potency and less toxicity than MC (Driscoll et al., 1974). That this may be related to its

susceptibility to reduction was suggested by the findings of Patrick et al. (1964), that hydrogenation of mitomycin B over a platinum catalyst at atmospheric pressure in dimethylformamide produced an aziridinomitosene; whereas, hydrogenation of PM under the same conditions gave no product. Since both of these antibiotics have identical redox potentials of -0.40V, it is not clear why differences should exist between these agents in their susceptibility to reduction.

Although PM was discarded clinically in favor of MC because it was not as potent, it appears from these results that it has greater potential as an agent with specificity for hypoxic cells of solid tumors, in that it produces the greatest differential kill of hypoxic cells relative to oxygenated cells of any known quinone. Therefore, throughout this dissertation, comparisons of MC and PM have been conducted in an effort to ascertain the mechanism involved in the differential toxicities of these agents to oxygenated cells.

CHAPTER III

The Role of Oxidoreductases in the Bioactivation of Mitomycin C and Porfiromycin

Part A: Studies on the Enzymes Implicated in the Activation of MC and PM in Cultured Cell Lines.

INTRODUCTION

MC and PM are closely related in structure and in mechanism of action, which is thought to be monofunctional and bifunctional alkylation of DNA. Early investigators have demonstrated that enzymatic bioactivation of MC results in cytotoxicity associated with cross-linking of DNA (Iyer and Szybalski, 1963). Furthermore, these early studies demonstrated that these antitumor antibiotics require reducing equivalents and anaerobiosis as a prerequisite for the production of DNA adducts in both bacteria and rat liver homogenates (Iyer and Szybalski, 1964; Schwartz, 1962). Experiments performed by our laboratory and by others have shown both rodent liver microsomes and nuclei, as well as by purified cytosolic xanthine oxidase and purified NADPH-cytochrome c (P-450) reductase, can catalyze the anaerobic reduction of MC (Kennedy et al., 1982; Komiyama et al., 1979a; Pan et al., 1984) with the concomitant generation of a reactive electrophile (Pan et al., 1984). Our laboratory in collaboration with Dr. Stephen G. Sligar has suggested that cytochrome P-450 might be involved in the anaerobic metabolism of MC (Kennedy et al, 1982), while work by others on DT-diaphorase, an enzyme active in the reduction of quinones, including menadione and Adriamycin (Komiyama et al., 1979b; Lind et al., 1982; Thor et al., 1982), suggests that this enzyme should also be considered a possible activator of MC.

Although much work has demonstrated that MC can be metabolically activated by bacteria, rodent liver microsomes and nuclei, and various purified enzymes, little is known about the activation of MC and PM in cultured cell lines. Therefore, in this part of Chapter III, the microsomal electron transport enzymes and DT-diaphorase, enzymes proposed to activate the anticancer quinones, were examined in the EMT6, CHO, and V79 cells.

METHODS

Cultured Cell Lines--EMT6, CHO, and V79 cells were grown at 37°C in Waymouth's medium supplemented with 15% fetal bovine serum and antibiotics in a humidified atmosphere of 95% air/5% CO_2, as previously described in Chapter II, Methods. Cells were grown both in monolayer and in suspension culture. For both monolayer and suspension cultures, 1 to 4 x 10^4 cells/ml of Waymouth's medium plus 15% fetal bovine serum and antibiotics were seeded in plastic flasks or in glass spinner bottles (Bellco Glass, Inc., Vineland, NJ). From the suspension cultures, a large number of cells were attainable for the preparation of cell sonicates and microsomes.

Preparation of Cell Sonicates--Exponentially growing monolayer cells (suspended after incubation with 0.05% trypsin in PBS--see Chapter II, Methods) or suspension cells were pelleted by sedimentation at 200 x g for 5 min. The cell pellets were washed with 10 ml of ice-cold PBS, combined into one tube and centrifuged at 200 x g for 5 min. The cells were resuspended in approximately 6 volumes of water. After a 10 min incubation on ice, an equal volume of 1.8% NaCl was added and cells were disrupted by three 6-sec bursts with a Branson Model 160 sonicator set at 25% of maximum intensity. Measurement of the amount of protein in the cell sonicates was conducted by the method of Lowry et al. (1951) using bovine serum albumin as the standard. Cell sonicates were used for enzyme assays and for preparation of subcellular fractions.

Isolation of Microsomes from Cultured Cell Lines--After cell sonicates were adjusted to a concentration of 0.25 M sucrose by addition of 1.25 M sucrose, microsomes were prepared by differential centrifugation and calcium precipitation

according to a modification of the method of Cinti et al. (1972). Briefly, the cell sonicates in 0.25 M sucrose were centrifuged for 15 min at 600 x g in a Sorval Model RC-5 centrifuge to remove nuclei, large cellular debris, and unbroken cells. The resultant supernatant was decanted and retained. The precipitate was resuspended in approximately 2 volumes of 0.25 M sucrose and centrifuged again at 600 x g for 15 min. The precipitate was retained and the supernatant was combined with the earlier 600 x g supernatant and centrifuged at 12,500 g for 15 min to remove mitochondrial and lysosomal fragments. Calcium chloride was added to the supernatant to give an 8 mM solution followed by centrifugation at 27,000 x g for 15 min to pellet the microsomes. The pellet was then washed with an equal volume of 150 mM KCl-50 mM Tris-HCl (pH 7.4) and resedimented at 27,000 x g for 15 min. This wash removed any absorbed protein and residual calcium. The resultant pellet of microsomes was resuspended in 150 mM KCl-50 mM Tris-HCl (pH 7.4).

All of the subcellular fractions obtained from the preparation of microsomes were assayed for activity of various enzymes. Microsomal yield was measured by comparing NADPH-cytochrome c reductase activity (see below) in the sonicate with the activity of the microsomes. Succinate-cytochrome c reductase activity (see below) and lactate dehydrogenase activity (see below) were measured in all subcellular fractions to determine the degree of mitochondrial and cytosolic contamination in the microsomal fraction. Protein recovery was determined by the method of Lowry et al. (1951), using bovine serum albumin as the standard.

Measurement of Succinate-Cytochrome c Activity--A modification of the method of Sottocasa et al. (1967) was used to assay succinate-cytochrome c activity in the subcellular fractions obtained from the preparation of microsomes. The reaction mixture was assayed in a Beckman Model 25 UV/Vis spectrophotometer at 550 nm

and 30°C and contained 100 mM Tris-HCl (pH 7.5), 0.04 mM cytochrome c (Type III, Sigma Chemical Co., St. Louis, MO), 0.6 mM KCN (Fischer Scientific Co., Fairlawn NJ), and 0.05-0.5 mg of subcellular or cell sonicate protein in total volume of 1 ml. After recording the base-line spectrum, 3 mM succinate (disodium salt, Sigma Chemical Co.) was added to initiate the reaction. The enzymatic activity was expressed as nmol reduced cytochrome c/min/mg protein, using an extinction coefficient of 27.7 $mM^{-} cm^{-1}$.

Measurement of Lactate Dehydrogenase Activity--Lactate dehydrogenase activity in subcellular fractions was determined by a slight modification of the method of Henry et al. (1960), using a Beckman Model 25 UV/Vis Spectrophotometer at 340 nm and 30°C. The reaction mixture contained 300 mM potassium phosphate buffer (20.4 g K_2HPO_4 per 500 ml and 26.1 g KH_2PO_4 per 500 ml, combined to obtain pH 7.7) containing 100 mM Na_2EDTA (J. T. Baker Chemical Co., Philipsburg, NJ), 0.2 mM NADH (Grade III, Sigma Chemical Co.) and 0.01 to 0.5 mg of subcellular or cell sonicate protein in a total volume of 1 ml. After recording the base-line spectrum, the reaction was initiated by the addition of 0.6 mM pyruvate (sodium salt, Type II, Sigma Chemical Co.); the activity of lactate dehydrogenase was expressed as the µmol of oxidized NADH/min/mg protein, using an extinction coefficient of 6.2 $mM^{-1} cm^{-1}$.

Measurement of Microsomal Electron Transport Proteins in Cultured Cell Lines--NADPH-cytochrome c reductase activity was determined by the method of Masters et al. (1967), as modified by Vermilion and Coon (1974) using a Beckman Model 25 UV/Vis spectrophotometer at 550 nm and 30°C. Briefly, the reaction mixture contained 300 mM of potassium phosphate buffer (pH 7.7) containing 100 mM

Na_2EDTA, 0.04 mM cytochrome c, and 0.05 to 0.5 mg of subcellular protein or cell sonicate protein in a total volume of 1.0 ml. After the base-line spectrum was obtained, 0.1 mM of NADPH (Type X, Sigma Chemical Co.) and 0.6 mM of KCN were added to begin the reaction. The activity of the enzyme was expressed as nmol of reduced cytochrome c/min/mg protein, using an extinction coefficient of 27.7 mM^{-1} cm^{-1}.

NADH-cytochrome b_5 reductase (measured as ferricyanide reductase) activity was assayed by the method of Sottocasa et al. (1967) using a Beckman Model 25 UV/Vis spectrophotometer at 420 nm and 30°C. The assay mixture contained 300 mM potassium phosphate buffer (pH 7.7), 0.15 mM NADH, and 0.25 mM potassium ferricyanide (J. T. Baker Chemical Co.) in a total volume of 1 ml. After the base-line spectrum was obtained, the reaction was started by addition of 0.1 to 0.5 mg of cell sonicate protein. The activity was measured as µmol reduced potassium ferricyanide/min/mg protein, using the extinction coefficient of 1.02 mM^{-1} cm^{-1}.

The method of Sottocasa et al. (1967) was used to assay NADH-cytochrome c reductase activity. Briefly, the assay mixture contained 300 mM potassium phosphate buffer (pH 7.7), 0.05 mM cytochrome c, 0.3 mM KCN, 0.5 µM rotenone (Sigma Chemical Co.), and 0.1 mM NADH in a total volume of 1 ml. After obtaining a base-line spectrum, the assay was initiated by the addition of 0.1 to 0.5 mg of cell sonicate protein, and activity was recorded as nmol reduced cytochrome c/min/mg protein using the extinction coefficient of 27.7 mM^{-1} cm^{-1}.

Cytochrome b_5 and cytochrome P-450 levels were measured in microsomes by the method of Omura and Sato (1964a, 1964b) using an Aminco DW-2 UV/Vis spectrophotometer in the split beam mode at room temperature. A 2.5 ml microsomal suspension containing 1 to 4 mg protein in 150 mM KCl-50 mM Tris-HCl (pH 7.4) was placed in both the sample and reference cells. After recording the

base-line spectrum from 350 nm to 500 nm, a saturating amount of NADH was added to the sample cuvette to reduce cytochrome b_5, and the spectral difference was measured and recorded. Carbon monoxide (Presto Welding Service Centers, North Haven, CT), deoxygenated by passing the gas through a vanadous sulfate solution (Meites and Meites, 1948), was bubbled through the microsomal suspension in the reference cuvette for approximately 1 min; this was sufficient to saturate the sample and to allow complete binding of the cytochrome P-450 with the gas. A saturating amount of sodium dithionite was added to both reference and sample cuvettes, and after switching the sample and reference cuvettes, the difference spectrum of the reduced carbon monoxide complex of cytochrome P-450 was recorded.

The cytochrome b_5 content (nmol/mg microsomal protein) was calculated from the difference in absorbance of the reduced cytochrome between the 424 nm peak and 410 nm trough, using an extinction coefficient of 185 mM^{-1} cm^{-1} (Omura and Sato, 1964a, 1964b). The cytochrome P-450 content (pmol/mg microsomal protein) was calculated from the difference in absorbance of the reduced carbon monoxide complex of the protein between the 450 nm peak and 490 nm using an extinction coefficient of 91 mM^{-1} cm^{-1} (Omura and Sato, 1964a, 1964b). These studies were performed at the University of Connecticut Health Center, Farmington, CT with the help of Dr. Dominick L. Cinti.

Measurement of DT-Diaphorase Activity--DT-Diaphorase activity was assayed using a Beckman Model 25 UV/Vis spectrophotometer at 600 nm and 30°C by the method of Ernster (1967) as modified by Lind et al. (1982). The reaction mixture contained 50 mM Tris-HCl containing 0.08% Triton X-100 (New England Nuclear Corp., Boston, MA) (pH 7.5), 0.04 mM 2,6-dichlorophenolindophenol (DCPIP; Sigma

Chemical Co.) and 0.05 to 0.5 mg of cell sonicate protein in a total volume of 1 ml. After obtaining a base-line spectrum, the reaction was initiated by the addition of 0.3 mM NADPH. Because the assay mixture was not a purified enzyme preparation but a cell sonicate, and other diaphorases were present, 0.1 mM of dicoumarol (Aldrich Chemical Co., Milwaukee, WI), a potent inhibitor of DT-diaphorase, was added to the reaction mixture. The dicoumarol-sensitive part of the activity was used as a reflection of the DT-diaphorase activity of the preparation. The activity was recorded as nmol reduced DCPIP/min/mg cell sonicate protein, using the extinction coefficient of 21 mM^{-1} cm^{-1}.

RESULTS

Isolation of Microsomes from Cultured Cell Lines--Microsomes were prepared for each of the three cultured cell lines by the calcium precipitation method of Cinti et al. (1972). Using this method for obtaining microsomes, the yield, as represented by NADPH-cytochrome c reductase activity of the microsomal fraction compared with that of the total cell sonicate was 19%, 18%, and 20% for the EMT6, CHO, and V79 cell lines, respectively (Table 4). Mitochondrial contamination, as monitored by succinate cytochrome c activity, was <5% in all cell lines, while cytosolic contamination, as monitored by lactate dehydrogenase activity, was 1% in all cell lines. Protein recovery was approximately 90% or more of the protein measured in the cell sonicate remaining after the isolation of the subcellular fraction.

Microsomal Electron Transport Proteins in Cultured Cell Lines--NADPH-cytochrome c reductase activity was present in all three cell lines, while the hemoprotein, cytochrome P-450 was not detected (Table 5). NADPH-cytochrome c reductase activity was higher in the EMT6 cells than in the CHO and V79 cells. As expected, the specific activity of this enzyme in microsomes was higher than in the sonicates. NADPH-cytochrome c reductase activity in cell sonicates was measured in the cell lines throughout the growth curve and the activity was essentially unchanged in exponentially growing and plateau phase cells. The amount of cytochrome P-450, if present, was not detected within the limits of instrument sensitivity (<5 pmol/mg protein).

NADH-cytochrome b_5, NADH-cytochrome c reductase activity (a measurement of the total activity of NADH-cytochrome b_5 reductase and cytochrome b_5), and cytochrome b_5 were observed in all three cell lines, with no significant differences occurring between the cell lines in the activities/amounts of the enzymes (Table 6).

The activities of NADH-cytochrome c reductase and NADH-cytochrome b_5 reductase were variable, as evidenced by the relatively large standard errors. This variation reflected increases in the two enzyme activities in the cells as the exponential cultures entered plateau phase.

DT-Diaphorase Activity in Cultured Cell Lines--DT-Diaphorase was measured by dichlorophenolindophenol reduction with and without dicoumarol in the cultured cell lines (Table 7). Cytosolic diaphorases were present as indicated by reduction of DCPIP in the presence of dicoumarol, a potent competitive inhibitor of DT-diaphorase. The enzyme activity was higher in EMT6 cells than in CHO and V79 cells.

Table 4

ISOLATION OF MICROSOMES IN CULTURED CELL LINES

	Cell Line		
	EMT6	CHO	V79
Microsomal Yield, %	19	20	18
Mitochondrial Contamination, %	3	4	4
Cytosolic Contamination, %	1	1	1
Protein Recovery, %	96	88	92

Microsomes were obtained from cell sonicates by the calcium precipitation method of Cinti et al. (1972) as described in the Methods. Microsomal yield represents the activity of NADPH-cytochrome c reductase in the microsomal fraction as a percent of the total activity of the enzyme in the cell sonicate. The percent mitochondrial and cytosolic contamination was measured by the activities of succinate cytochrome c reductase and lactate dehydrogenase, respectively. Percent protein recovery represents the summation of the protein in all of the subcellular fractions compared with the total protein in the cell sonicates. The data represent the average percentages from three experiments.

Table 5

MICROSOMAL ELECTRON TRANSPORT PROTEINS IN CULTURED CELL LINES

CYTOCHROME P-450 PATHWAY

Cell line	NADPH-Cytochrome c Reductase (nmol/min/mg protein) Sonicates	NADPH-Cytochrome c Reductase (nmol/min/mg protein) Microsomes	Cytochrome P-450 (pmol/mg protein) Microsomes
EMT6	4.6 ± 0.6	14.0 ± 1.6	<0.5
CHO	0.9 ± 0.1	2.0 ± 0.6	<0.5
V79	1.1 ± 0.1	3.6 ± 1.0	<0.5

The enzyme activity or concentration was determined by standard assays described in the Methods. The NADPH-cytochrome c reductase in cell sonicates was the mean of ≥8 determinations ± the standard error of the mean. NADPH-cytochrome c reductase activity and cytochrome P-450 concentration in microsomes were the average of 3 and 1 determinations, respectively.

Table 6

MICROSOMAL ELECTRON TRANSPORT PROTEINS IN CULTURED CELL LINES

CYTOCHROME b_5 PATHWAY

Cell line	NADH-Cytochrome b_5 Reductase (μmol/min/mg protein) Sonicates	Rotenone-Insensitive NADH-Cytochrome c Reductase (nmol/min/mg protein) Sonicates	Cytochrome b_5 (pmol/mg protein) Microsomes
EMT6	1.5 ± 0.3	60.1 ± 20.3	31.1
CHO	1.6 ± 0.2	42.6 ± 10.7	34.2
V79	1.0 ± 0.1	34.8 ± 5.6	37.5

The enzyme activity or concentration was determined by standard assays described in the Methods. The NADH-cytochrome b_5 reductase and the rotenone-insensitive NADH-cytochrome c reductase activities in cell sonicates were the means of ≥8 determinations ± the standard errors of the means. The cytochrome b_5 concentrations in microsomes was the average of two determinations.

Table 7

DT-DIAPHORASE ACTIVITY IN CULTURED CELL LINES

Cell line	DT-Diaphorase Activity (nmol/min/mg protein) Sonicates
EMT6	151.4 ± 48.7
CHO	10.0 ± 3.2
V79	13.7 ± 2.1

DT-Diaphorase activity was determined by standard assays as described in the Methods. The data are the means of 7 determinations ± the standard errors of the means.

Part B: Studies on the Formation of Reactive Metabolites by MC in Cultured Cell Lines.

INTRODUCTION

Studies on the enzymes proposed to activate MC and PM in cultured cell lines (Chapter III, Part A) demonstrated that all three cell lines contained the oxidoreductases NADPH-cytochrome c reductase, NADH-cytochrome b_5 reductase, cytochrome b_5, and DT-diaphorase, but none contained detectable levels of cytochrome P-450. These studies indicated that the EMT6 cells contained more NADPH-cytochrome c reductase activity and DT-diaphorase activity than the V79 and CHO cells. However, the absolute amounts of activity of NADPH-cytochrome c reductase and DT-diaphorase in the three cell lines did not correlate with measurements of the cytotoxicity of MC and PM to EMT6, V79 and CHO cells (Chapter II). These latter studies demonstrated that both antitumor antibiotics are more cytotoxic to the EMT6 and V79 cells under hypoxic conditions than in the presence of air, while these antibiotics demonstrate less differential cytotoxicity under hypoxic and aerobic conditions with the CHO cells.

Previous investigations in our laboratory have shown that anaerobic sonicates of EMT6 and Sarcoma 180 tumor cells are capable of generating a reactive species from MC (Kennedy et al., 1980). In this part of Chapter III, experiments have been designed to extend these findings by comparing the ability of V79 and CHO cell sonicates to that of EMT6 cells in generating a reactive product(s) from MC under anaerobic and aerobic conditions. In addition, experiments were done to examine the efficiency of the pyridine nucleotides, NADPH and NADH, as electron donors in the generation of the reactive product(s) of MC by the cell sonicates. Finally, to determine the relative importance of NADPH-cytochrome c reductase and DT-

diaphorase in the formation of reactive species of MC, inhibitors of these enzyme activities were used. Through these studies, a correlation was sought between the cellular enzyme(s) responsible for the generation of toxic alkylating species and the biochemical basis for the cytoxicity observed in these cell lines after MC or PM treatment under aerobic and hypoxic conditions.

METHODS

Cultured Cell Lines and Preparation of Cell Sonicates--EMT6, V79 and CHO cells were grown at 37°C in Waymouth's medium supplemented with 15% fetal bovine serum and antibiotics in a humidified atmosphere of 95% air/5% CO_2 (see Chapter II, Methods). Cells were seeded at 1 to 4 x 10^4 cells/ml in Waymouth's medium and 15% fetal bovine serum and antibiotics in glass spinner bottles (Bellco Glass Inc., Vineland, NJ). Cell sonicates were prepared as previously described (Chapter III, Part A, Methods) and used for enzyme assays and for the measurement of reactive metabolites.

Measurement of the Effects of Inhibitors on NADPH-Cytochrome c Reductase and DT-Diaphorase Activity in Cultured Cell Lines--NADPH-cytochrome c reductase activity and DT-diaphorase activity were measured as previously described (Chapter III, Part A, Methods). After the activity of the enzymes was measured, the inhibitors were added and the residual activity was monitored. $NADP^+$ (sodium salt, Sigma grade, Sigma Chemical Co.), dissolved in 100 mM Tris-HCl (pH 7.5), was added to the enzyme assay at final concentrations of 1.0, 5.0, and 10.0 mM, mersalyl acid (Sigma Chemical Co.), dissolved in 0.5 N NaOH, was added to the reaction mixture at final concentration of 0.1, 0.25, and 0.05 mM, and dicoumarol, dissolved in 0.5 N NaOH, was added to the enzyme assay at final concentrations of 0.1, 0.5, and 1.0 mM. Neither vehicle affected the reaction rate.

Measurement of Reactive Metabolites--Reactive metabolites of MC were estimated by a modification of the method of Wheeler and Chumley (1967). The incubation mixture contained a NADPH regenerating system (0.72 mM $NADP^+$ (sodium salt, Sigma grade, Sigma Chemical Co.), 5 mM $MgCl_2$ (J. T. Baker Chemical Co.), 5 mM

glucose-6-phosphate (disodium salt, Sigma grade, Sigma Chemical Co.), 1.25 U glucose-6-phosphate dehydrogenase (Type XII, Sigma Chemical Co.), 0.47 mM 4-(p-nitrobenzyl)pyridine (Aldrich Chemical Co., Milwaukee, WI), 2 to 6 mg of cell sonicate protein, and 100 mM Tris-HCl buffer (pH 7.4) in a total volume of 1.0 ml at $0^{\circ}C$. The reaction mixture also included the pyridine nucleotides, NADPH and NADH, in place of the NADPH regenerating system, and the inhibitors, $NADP^{+}$, mersalyl, and dicoumarol, as indicated in the Results. Hypoxia was produced by pregassing the reaction mixture with prepurified N_2 (Presto Welding Service Centers) for 10 min. After warming at $37^{\circ}C$ for 5 min in a shaking water bath, the reaction was initiated by addition of 300 nmol MC dissolved in acetone. This concentration of acetone did not affect the enzymatic activation of MC. The incubation was terminated after 20 min by addition of 2 ml of acetone (J. T. Baker Chemical Co.) and 1 ml of 1N NaOH. Following extraction with 4 ml of ethyl acetate (J. T. Baker Chemical Co.), the phases were separated by centrifugation for 2 min at 1000 x g and the absorbance of the organic layer was measured at 540 nm and $25^{\circ}C$ using a Beckman Model 25 UV/Vis spectrophotometer. The incubations and subsequent extractions used for estimating the presence of an alkylated product were carried out under subdued light.

RESULTS

Formation of Reactive Metabolites of MC by Cultured Cell Lines--Under the standard reaction conditions, which employed a NADPH regenerating system and anaerobiosis, the formation of reactive products from MC in the EMT6, CHO, and V79 cell lines as measured by the trapping reagent 4-(p-nitrobenzyl)pyridine, was dependent upon both time and protein concentration. The reaction rate was linear for all three cell lines for 30 min at protein concentrations ranging between 2 to 6 mg/ml depending upon the cell line (Figures 9, 10, and 11). The greatest monoalkylating activity was obtained with the EMT6 cell sonicates; substantially lower activities were observed with CHO and V79 cell sonicates. The generation of reactive metabolites from MC was inhibited in all three cell lines by boiling sonicates, indicating that the production of a reactive species was catalyzed by a cellular enzyme(s) (Table 8). No activation of MC was observed in the presence of air (Table 8). This inhibition was observed over a range of protein concentrations for extended time periods. As expected, the formation of reactive metabolites by PM in hypoxic EMT6 cell sonicates equalled that demonstrated with MC (S. R. Keyes, personal communication). The rate of generation of a reactive species in the absence of cofactors was markedly faster than the rate in the presence of oxygen, indicating that low levels of endogenous reducing equivalents were present in the sonicates (Table 8). These results are in agreement with earlier data from the laboratory using EMT6 and Sarcoma 180 cell sonicates (Kennedy et al., 1980; Rockwell et al., 1982).

Both NADPH and NADH supported the formation of reactive metabolites from MC, with greater rates of alkylation occurring in the presence of NADPH; however, neither cofactor was as efficient as the NADPH-regenerating system (Table 9). A rate limiting supply of NADPH did not account for the lower

alkylating activity observed with this pyridine nucleotide, since increasing the concentration of NADPH did not increase the rate of formation of reactive metabolites above the levels reported in Table 9.

Effect of Enzymatic Inhibitors on the Formation of Reactive Metabolites of MC-- Inhibitors of various enzymes implicated in the biotransformation of MC were used as probes to estimate the contribution of various oxidoreductases to the reductive activation of MC. In agreement with the data which demonstrated that cytochrome P-450 could not be detected in the microsomal fraction of the cultured cells, the formation of a reactive product(s) from MC was not significantly decreased by CO in all three cell lines (Table 10), implying that cytochrome P-450 was not an important catalyst for this reaction.

The contributions of NADPH-cytochrome c reductase and DT-diaphorase to the activation of MC were estimated using inhibitors of these enzymes in EMT6 cell sonicates, since sonicates from this cell line exhibited the greatest monoalkylating activity and, therefore, effects of these inhibitors on enzymes implicated in the bioactivation of MC would be more easily observed. Dicoumarol, a potent competitive inhibitor of DT-diaphorase, was employed to examine the role of this enzyme in the bioactivation of MC. This inhibitor effectively blocked the activity of DT-diaphorase at all concentrations studied, with little effect on the NADPH-cytochrome c reductase activity (Table 11). In addition, dicoumarol did not decrease, but instead enhanced the formation of reactive product from MC. In the absence of MC, the reaction mixture containing dicoumarol was not able to generate alkylating species, indicating that dicoumarol itself was not converted into a reactive species by the cell sonicate.

The role of NADPH-cytochrome c reductase in the activation of MC was evaluated using $NADP^+$ and mersalyl, competitive inhibitors of the NADPH-cytochrome c reductase activity. At concentrations of 1 to 10 mM, $NADP^+$ significantly lowered both NADPH-cytochrome c reductase activity and DT-diaphorase activity and decreased the formation of reactive metabolites of MC (Table II). Mersalyl, likewise, inhibited NADPH-cytochrome c reductase and DT-diaphorase activities and decreased the formation of reactive MC metabolites (Table II). Although $NADP^+$ and mersalyl inhibited DT-diaphorase, studies using the more specific inhibitor, dicoumarol, to competitively inhibit this enzyme demonstrated that DT-diaphorase did not catalyze the production of reactive metabolites of MC. Therefore, the results of the studies with $NADP^+$ and mersalyl can be used to assess the role of NADPH-cytochrome c reductase in the formation of alkylated species of MC. Although there was no absolute correlation between the degree of inhibition of NADPH-cytochrome c reductase activity by $NADP^+$ or mersalyl and the rate of production of reactive species over the range of inhibitor concentrations used, in general, inhibition of NADPH-cytochrome c reductase and concomitant inhibition of the formation of reactive metabolites was observed.

FIGURE 9. Formation of Reactive Metabolites of MC by EMT6 Cell Sonicates. The reactive metabolites of MC by EMT6 cell sonicates were estimated by a modification of the method of Wheeler and Chumley (1967) as described in the Methods. The reaction rate for hypoxic EMT6 cell sonicates was linear for 30 min with a protein concentration between 2 to 5 mg of cell sonicate. The data represent the means $\pm$ standard errors of the means with $n \geq 3$.

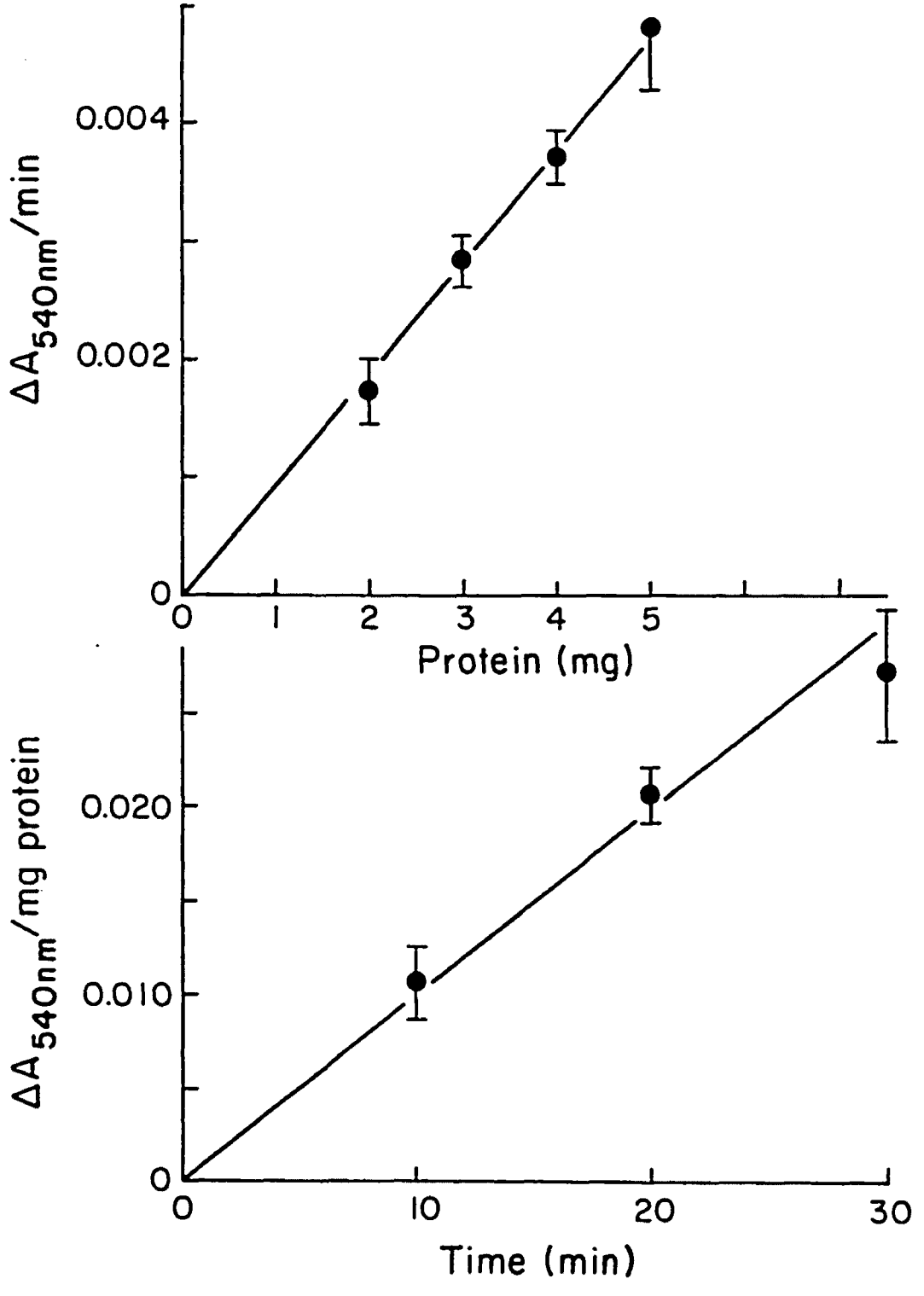


FIGURE 10. Formation of Reactive Metabolites of MC by CHO Cell Sonicates. The reactive metabolites of MC by CHO cell sonicates were estimated by a modification of the method of Wheeler and Chumley (1967) as described in the Methods. The reaction rate for hypoxic CHO cell sonicates was linear for 30 min with a protein concentration between 4 to 6 mg of cell sonicate. The data represent the means $\pm$ standard errors of the means with $n \geq 3$.

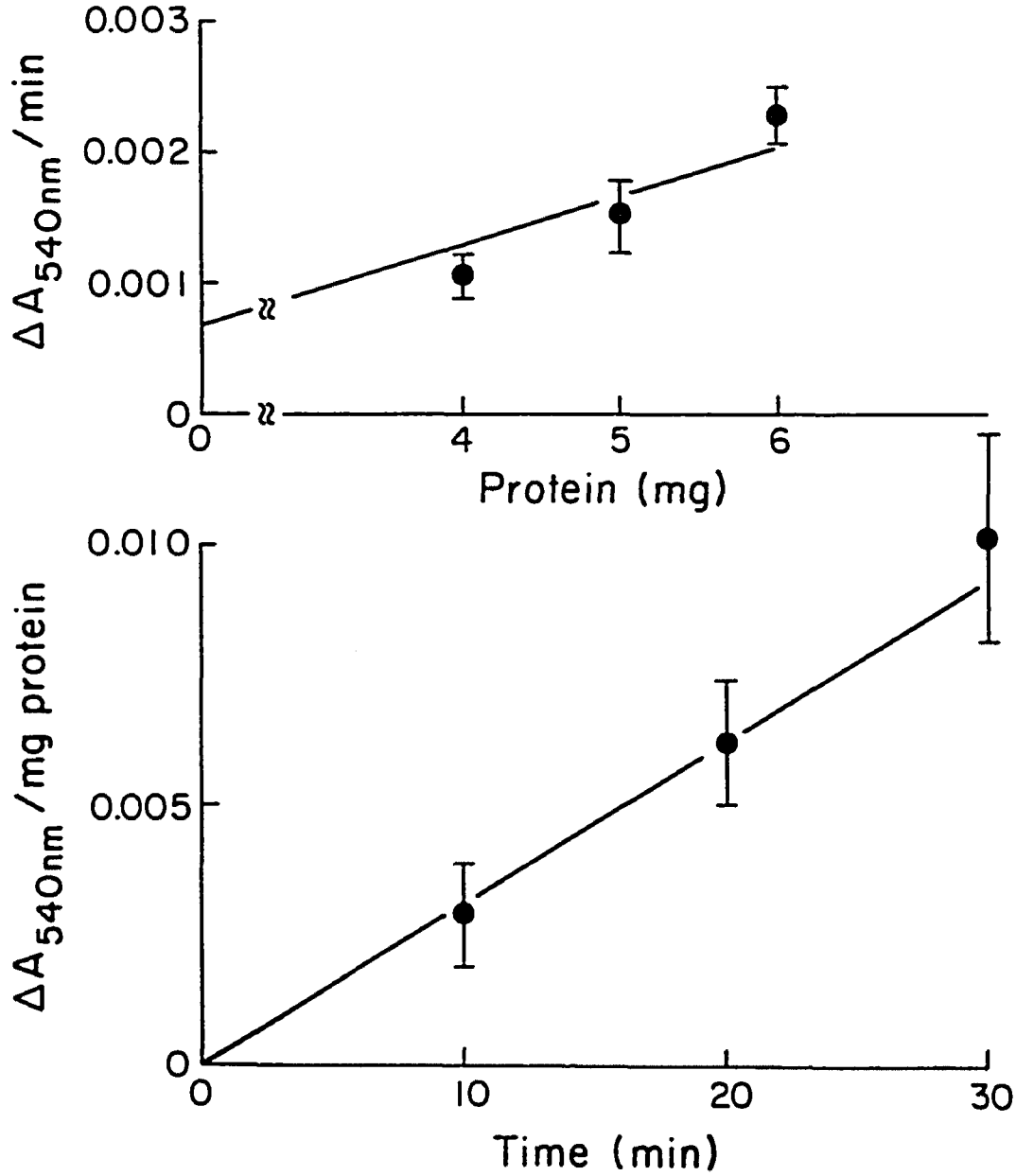


FIGURE 11. Formation of Reactive Metabolites of MC by V79 Cell Sonicates. The reactive metabolites of MC by V79 cell sonicates were estimated by a modification of the method of Wheeler and Chumley (1967) as described in the Methods. The reaction rate for hypoxic V79 cell sonicates was linear for 30 min with a protein concentration between 4 to 6 mg of cell sonicate. The data represent the means $\pm$ standard errors of the means with $n \geq 3$.

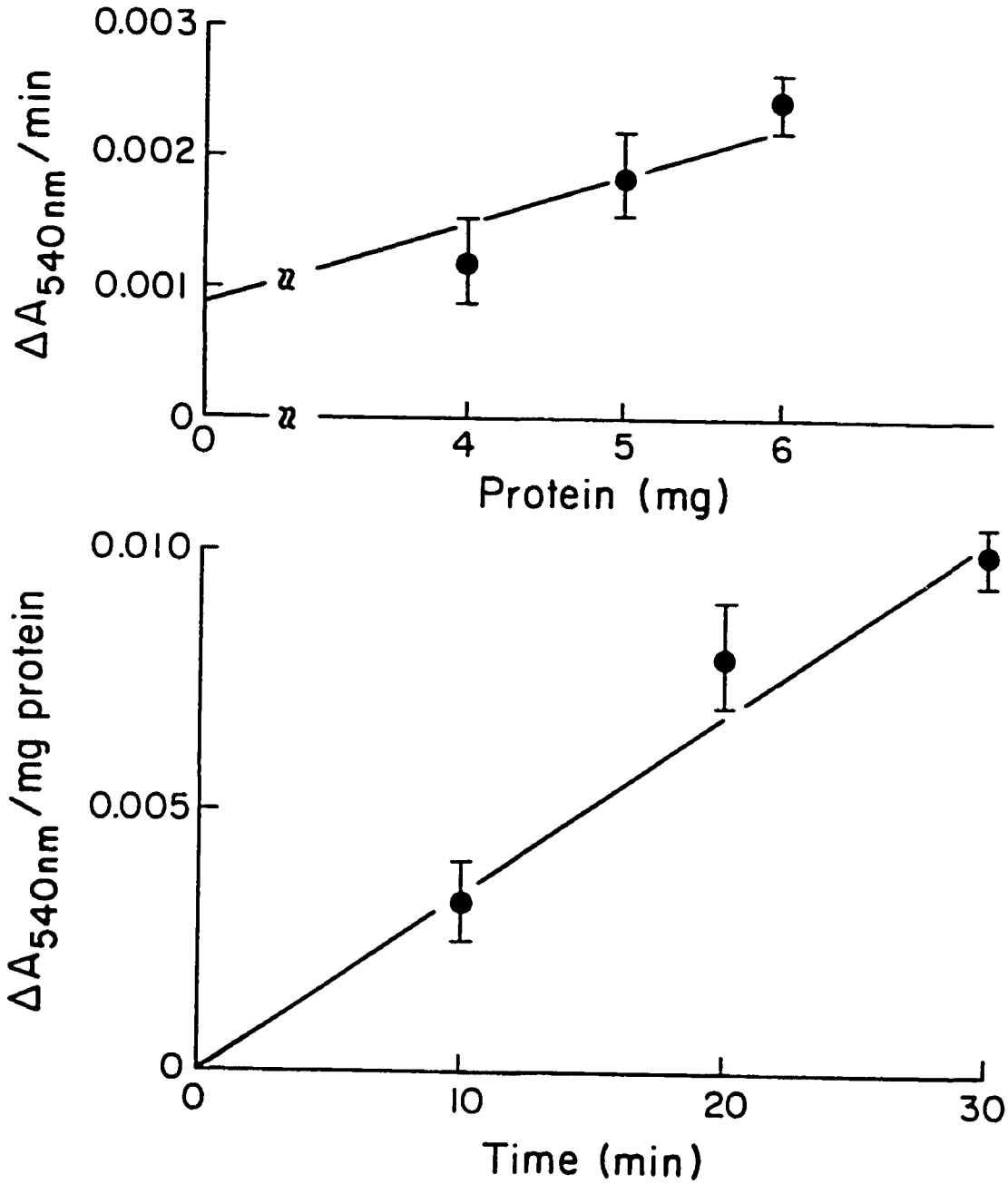


Table 8

FORMATION OF REACTIVE METABOLITES OF MITOMYCIN C IN CULTURED CELL LINES

Cell line	NADPH regenerating system + Hypoxia	+ Air	+ Boiled Sonicates	No cofactors + Hypoxia
	($A_{540} \times 10^3$/min/mg protein)			
EMT6	1.04 ± 0.05	0.05 ± 0.01	0.02 ± 0.01	0.17 ± 0.02
CHO	0.33 ± 0.06	0.03 ± 0.01	0	0.11 ± 0.02
V79	0.39 ± 0.04	0.03 ± 0.01	0.01 ± 0.01	0.12 ± 0.05

The formation of reactive metabolites of MC were estimated by a modification of the method of Wheeler and Chumley (1967) as described in the Methods. The reaction mixture contained an NADPH regenerating system (0.72 mM $NADP^+$, 5 mM $MgCl_2$, 5 mM glucose-6-phosphate and 1.25 U glucose-6-phosphate dehydrogenase), 0.47 mM 4-(p-nitrobenzyl)pyridine and 3 to 5 mg of cell sonicate in a total volume of 1 ml of 100 mM Tris-HCl (pH 7.4). The reaction was initiated by the addition of 300 nmol MC. The data represent the means ± standard errors of the means with $n \geq 4$.

Table 9

FORMATION OF REACTIVE METABOLITES OF MITOMYCIN C BY PYRIDINE NUCLEOTIDES IN CULTURED CELL LINES

Cell line	NADPH regenerating system + Hypoxia	NADPH + Hypoxia	NADH + Hypoxia
	($A_{540} \times 10^3$/min/mg protein)		
EMT6	1.04 ± 0.05	0.77 ± 0.13	0.36 ± 0.11
CHO	0.33 ± 0.06	0.18 ± 0.03	0.15 ± 0.03
V79	0.39 ± 0.04	0.30 ± 0.04	0.20 ± 0.02

The formation of reactive metabolites of MC were estimated by a modification of the method of Wheeler and Chumley (1967) as described in the Methods. The reaction mixture contained an NADPH regenerating system (0.72 mM $NADP^+$, 5 mM $MgCl_2$, 5 mM glucose-6-phosphate and 1.25 U glucose-6-phosphate dehydrogenase), 0.47 mM 4-(p-nitrobenzyl)pyridine and 3 to 5 mg of cell sonicate in a total volume of 1 ml of 100 mM Tris-HCl (pH 7.4). The reaction was initiated by the addition of 300 nmol MC. The NADPH regenerating system was omitted or replaced with 1 mM reduced pyridine nucleotide as indicated. The data represent means ± standard errors of the means with $n \geq 4$.

Table 10

EFFECT OF CARBON MONOXIDE ON REACTIVE METABOLITES OF MITOMYCIN C IN CULTURED CELL LINES

Cell line	N_2	CO
	($A_{540} \times 10^3$/min/mg protein)	
EMT6	1.10 ± 0.08	1.02 ± 0.11
CHO	0.45 ± 0.13	0.38 ± 0.06
V79	0.41 ± 0.05	0.29 ± 0.02

The formation of reactive metabolites of MC were estimated by a modification of the method of Wheeler and Chumley (1967) as described in the Methods. The reaction mixture contained an NADPH regenerating system (0.72 mM $NADP^+$, 5 mM $MgCl_2$, 5 mM glucose-6-phosphate and 1.25 U glucose-6-phosphate dehydrogenase), 0.47 mM 4-(p-nitrobenzyl)pyridine and 3 to 5 mg of cell sonicate in a total volume of 1 ml of 100 mM Tris-HCl (pH 7.4). The reaction was initiated by the addition of 300 nmol MC. Flasks were gassed with either nitrogen (N_2) or carbon monoxide (CO) throughout the reaction period. The data represent means of 3 to 5 experiments ± standard errors of the means.

Table II

EFFECTS OF INHIBITORS OF NADPH-CYTOCHROME c REDUCTASE ACTIVITY AND DT-DIAPHORASE ACTIVITY ON THE FORMATION OF REACTIVE METABOLITES OF MITOMYCIN C IN EMT6 CELLS

Inhibitor (mM)	NADPH-cytochrome c Reductase Activity	DT-Diaphorase Activity	Reactive Metabolites
	(Percent control activity)		
DICOUMAROL			
0.1	100 ± 1	2 ± 1	98 ± 13
0.5	100	0	143 ± 13
1.0	83 ± 10	0	185 ± 22
$NADP^+$			
1.0	21 ± 8	18 ± 9	74 ± 10
5.0	3 ± 3	4	69 ± 12
10.0	0	9	33 ± 2
MERSALYL			
0.1	88 ± 12	45 ± 3	71 ± 9
0.25	65 ± 10	19 ± 4	22 ± 10
0.5	33 ± 13	13 ± 13	6 ± 3

The formation of reactive metabolites of MC were estimated by a modification of the method of Wheeler and Chumley (1967) as described in the Methods. The reaction mixture contained an NADPH regenerating system (0.72 mM $NADP^+$, 5 mM $MgCl_2$, 5 mM glucose-6-phosphate and 1.25 U glucose-6-phosphate dehydrogenase), 0.47 mM 4-(p-nitrobenzyl)pyridine and 3 to 5 mg of EMT6 cell sonicate in a total volume of 1 ml of 100 mM Tris-HCl (pH 7.4). Inhibitors were added as indicated and the reaction was initiated by the addition of 300 nmol MC. The effects of inhibitors are expressed as percentages of the control activities which are given in previous tables. The data represent the means of at least 3 determinations ± standard errors of the means where applicable.

DISCUSSION

The biochemical mechanism of MC and PM activation has been widely investigated using bacterial, liver, and purified enzyme systems. Few studies, however, have employed tumors or other mammalian cell lines. In bacteria and liver, studies have demonstrated that MC is activated under anaerobic conditions to a reactive product that is capable of cross-linking DNA (Iyer and Szybalski, 1963; 1964; Schwartz, 1962) and that the reduced pyridine nucleotide NADPH is required to support this reaction. In rodent liver, enzymatic activity resides predominantly in the endoplasmic reticulum and nuclear subcellular fractions. With both purified NADPH-cytochrome P-450 and milk xanthine oxidase, MC has shown to be capable of consuming the appropriate substrate, NADPH or xanthine under hypoxic conditions (Bachur et al., 1979; Komiyama et al., 1979a), and enzymatic activity is accompanied by the formation of MC adducts (Pan et al., 1984). In addition, it is conceivable that several other enzymes, including cytochrome P-450 and DT-diaphorase, may be involved in the activation of MC under hypoxic conditions.

Previous work by our laboratory has employed mammalian tumor cell lines and has demonstrated that EMT6 mouse mammary tumor cells and Sarcoma 180 ascites cells are more sensitive to the cytotoxic action of MC under hypoxic conditions than in air (Kennedy et al., 1980; Rockwell et al., 1982; Teicher et al., 1981), and that sonicates of these cell lines are capable of consuming MC to generate a reactive species. Extensions of these findings to other cell lines demonstrated that MC is essentially equitoxic to aerobic and hypoxic CHO cells; whereas, it is preferentially cytotoxic to V79 cells under hypoxic conditions (see Chapter II). These studies suggested that MC is enzymatically activated by cultured cells. Therefore, it was of interest to determine which enzyme(s) are important in the bioactivation of MC and PM to cytotoxic species in EMT6, CHO,

and V79 cells. In Part A of this Chapter, the microsomal electron transport enzymes and DT-diaphorase, enzymes proposed to activate anticancer quinones, were investigated in the EMT6 mouse mammary tumor cell line and in the two Chinese hamster cell lines, CHO and V79. Enzyme activities characteristic of NADPH-cytochrome c reductase and DT-diaphorase were present in all three cell lines, but cytochrome P-450 was undetectable (Tables 4 and 6). The enzymes of the cytochrome b_5 pathway were also present in the three cell lines with no significant differences occurring among the cell lines (Table 3). These results indicate that two of the enzymes implicated in the activation of MC were present in all three cell lines.

In Part B of Chapter III, the ability to generate a reactive species from MC as measured by the trapping agent 4-(p-nitrobenzyl)pyridine under hypoxic conditions was demonstrated in EMT6 (in agreement with previous data, Kennedy et al., 1980), CHO, and V79 sonicates, with the greatest alkylating activity occurring in EMT6 sonicates (Table 3). Furthermore, data have demonstrated similar activity with PM (S. R. Keyes, personal communication). The pyridine nucleotide, NADPH, was more efficient as an electron donor than NADH for the generation of the reactive product(s) (Table 9). These findings are in agreement with data derived with liver homogenates (Kennedy et al., 1982; Schwartz, 1962). Although enzymes of the cytochrome b_5 pathway were present in the three cell lines, these enzymes require NADH as an electron donor. Because NADH is not as efficient an electron donor as NADPH, further studies with NADH-cytochrome b_5 reductase and with cytochrome b_5 were not conducted. Instead, studies were carried out with NADPH-cytochrome c reductase, cytochrome P-450, and DT-diaphorase, enzymes which utilize NADPH as a cofactor. To gain a better assessment of the involvement of these enzymes in the bioactivation of MC, we investigated the

effects of inhibitors of cytochrome P-450, NADPH-cytochrome c reductase, and DT-diaphorase on both enzyme activities and the generation of reactive metabolites from MC.

Cytochrome P-450 was not present in any of the cell lines at levels within the limits of detectability (<0.5 pmol/mg); moreover, CO did not inhibit the formation of MC-derived reactive metabolites by cell sonicates (Table 10). These findings were in contrast to earlier studies from this laboratory (Kennedy et al., 1980, 1982), which reported inhibition of reactive metabolite formation by CO. The contradiction with the present studies appears to be attributable to an oxygen leak in the experimental apparatus used for the earlier investigation. Furthermore, work in our laboratory has demonstrated that CO neither inhibited the formation of MC derived reactive metabolites by purified NADPH-cytochrome c reductase and P-450, nor protected EMT6 cells from MC induced cytotoxicity (Keyes et al., 1984a). In addition, utilization of purified NADPH-cytochrome c reductase and cytochrome P-450 has demonstrated that cytochrome P-450 appears to modulate NADPH-cytochrome c reductase activity by increasing the efficiency of electron transfer from NADPH-cytochrome c reductase to MC (Keyes et al., 1984a). Potter and Reed (1983) have recently reported a similar observation for the anaerobic reduction of 1-(2-chloroethyl)-3-(cyclohexyl)-1-nitrosourea.

The nonspecific NADPH-cytochrome c reductase inhibitors, $NADP^+$ and mersalyl, also yielded interesting results (Table 11). $NADP^+$ completely inhibited NADPH-cytochrome c reductase activity, and although it did not completely inhibit the formation of reactive metabolites from MC, it was able to substantially reduce the rate of formation of reactive products. Mersalyl inhibited the generation of reactive metabolites of MC to a greater extent than NADPH-cytochrome c reductase activity. The different degrees of inhibition of alkylating

activity and NADPH-cytochrome c reductase activity by $NADP^+$ and mersalyl supports the concept that NADPH-cytochrome c reductase is responsible for some of the bioactivation of MC in EMT6 cells, but that at least one other enzyme is also involved.

Dicoumarol markedly inhibited DT-diaphorase without decreasing either NADPH-cytochrome c reductase activity or the generation of reactive metabolites (Table II). Indeed, the reactive product(s) generated from MC increased significantly in the presence of dicoumarol, suggesting that DT-diaphorase may metabolize MC to a non-toxic substance. Further work in our laboratory with whole cells has demonstrated that both MC and dicoumarol in combination increased the toxicity of MC to hypoxic cells (Keyes et al., 1984b).

The results obtained, particularly with the inhibitor dicoumarol, suggest that the activation of MC under hypoxic conditions proceeds through an intermediate semiquinone radical or through a hydroquinone (Moore, 1977; Moore and Czerniak, 1981). The semiquinone radical intermediate has been suggested by Bachur and his coworkers (Andrews et al., 1983), who reported that MC reduced electrochemically to the semiquinone was capable of forming covalent adducts. This conclusion is supported by the findings of Tomasz et al. (1974) that partially reduced MC bound more avidly to DNA than the fully reduced antibiotic. These conclusions, however, appear to be incompatible with the results of the chemical reduction of the model compound 2,3-dimethyl-5,6-bis(methylene)-1,4-benzoquinone to the hydroquinone level, a process leading to the generation of a reactive species, presumably through the formation of a quinone methide (Lin et al., 1973).

In conclusion, the results from this Chapter indicate that two enzymes postulated to activate anticancer quinones, NADPH-cytochrome c reductase and DT-diaphorase, were present in all three cell lines. These studies demonstrated

that all three cell types generate an electrophile from MC, which can be measured with 4-(p-nitrobenzyl)pyridine. Experiments with enzyme inhibitors suggested that DT-diaphorase and cytochrome P-450 are not involved in the bioactivation of MC, although they may modulate its activation. On the other hand, NADPH-cytochrome c reductase was capable of activating MC, but may not be the sole enzyme involved in the reductive activation of the antibiotic by these cell lines.

Although there was a loose correlation between enzyme activity and the generation of reactive metabolites from MC, these parameters did not correlate quantitatively with the extent of MC-induced cytotoxicity to these cell lines. These findings do not preclude bioactivation of MC as a critical event responsible for cytotoxicity, because studies with sonicates cannot include the effects of such factors as the rate of transport of drug into cells and the repair of lethal and sublethal lesions. However, the lack of correlation between the rate at which sonicated cells generate reactive species and the cytotoxicity of MC to cells indicates that either the rate of bioactivation of MC is not the sole determinant of cytotoxicity or that the interaction between activated MC and 4-(p-nitrobenzyl)-pyridine does not completely reflect reactivity leading to cytotoxicity.

CHAPTER IV

The Role of DNA Lesions Caused by Mitomycin C and Porfiromycin in Cultured Cell Lines

INTRODUCTION

Early studies have demonstrated cross-linking of bacterial DNA in situ and cross-linking of purified bacterial DNA in the presence of cell lysates in vitro by MC (Iyer and Szybalski, 1963; Matsumoto and Lark, 1963). These findings have been extended to include studies on the production of cross-links in DNA using a cultured human cell line (Szybalski, 1964; Szybalski and Iyer, 1964a). In addition, after chemical reduction of MC, Lown et al. (1976) observed DNA-interstrand cross-links in purified λ-phage DNA by the ethidium fluorescence assay. Fujiwara and coworkers observed DNA interstrand cross-links with MC treated normal human fibroblasts using alkaline sucrose sedimentation (Fujiwara, 1982; Fugiwara and Tatsumi, 1975, 1977; Kano and Fugiwara, 1981), hydroxyapatite chromatography, and S_1-nuclease digestion methodologies, while Fornace and coworkers observed DNA interstrand and DNA-protein cross-links with MC treated human fibroblasts by alkaline elution methodology (Fornace and Little, 1977; Fornace et al., 1979). While production of DNA interstrand cross-links and DNA-protein cross-links are believed to be part of the mechanism by which MC and PM exert their cytotoxicities, no direct evidence exists that these lesions are responsible for the antineoplastic activity of these antibiotics.

In this chapter, the alkaline elution methodology developed by Kohn and coworkers (Kohn et al., 1976, 1981; Kohn and Ewig, 1979) was utilized to study both the formation and repair of DNA single strand breaks, DNA interstrand cross-links, and DNA-protein cross-links produced by these antibiotics in the EMT6 and CHO cell lines. A concentration of 2 μM MC or PM was chosen, because at this

concentration EMT6 cells exhibited preferential sensitivity to both MC and PM under hypoxic conditions. These cells demonstrated less aerobic cytotoxicity towards PM than towards MC, whereas the antibiotics were essentially equitoxic to these neoplastic cells under hypoxic conditions. Consequently, if DNA interstrand cross-links and DNA-protein cross-links are important, these antibiotics would be expected to form more DNA interstrand cross-links and DNA-protein cross-links under hypoxic conditions than aerobic conditions. CHO cells were also chosen as a test system because they exhibited slight but equal cytotoxicity with MC under both hypoxic and aerobic conditions, and slight but significant increases in cytotoxicity with PM under hypoxic conditions. Consequently, these antibiotics would be expected to form few cross-links in these cells under both hypoxic and aerobic conditions.

METHODS

Cultured Cell Lines--EMT6 and CHO cells were grown at 37°C in Waymouth's medium supplemented with 15% fetal bovine serum and antibiotics in a humidified atmosphere of 95% air/5% CO_2, as previously described in Chapter II, Methods. Cells were seeded in glass milk dilution bottles at a density of 1 to 2 x 10^5 cells per 10 ml of Waymouth's medium supplemented with 15% fetal bovine serum and antibiotics. After a 24 hr incubation period, sample cells were labeled with 0.02 μCi/ml [2-^{14}C]thymidine (55 mCi/mmol; Amersham Corp., Arlington Heights, IL) and reference cells were labeled with 0.05 μCi/ml [methyl-^{3}H]thymidine (2 Ci/mmol; Amersham Corp.). The radioactive medium was removed after 24 hrs and replaced with fresh medium until the start of experiments. This procedure enabled the labeled material to be incorporated into high molecular weight DNA.

In order to ascertain whether the radioisotopes cause any cytotoxicity in EMT6 or CHO cells, the surviving fraction determined by colony formation (see Chapter II, Methods) and DNA cell cycle distributions (see Chapter II, Methods) were ascertained after labeling the cells for 24 hrs.

Drug Treatment--MC and PM, at a concentration of 2 μM, were dissolved in 70% ethanol. The concentration of ethanol in treated and control cells was never greater than 0.35%, and this concentration of ethanol did not affect DNA elution profiles. Drug-treatments were terminated after 1 hr by removing the drug containing medium, washing the cell monolayer with Hanks' balanced salt solution containing glucose but no Ca^{++} and Mg^{++} (HBSS; Grand Island Biological Company, Grand Island, NY), and adding fresh medium to the glass milk dilution bottles.

Assay of Single Strand Breaks and DNA Interstrand Cross-Links by Alkaline Elution--The alkaline elution procedure has been described in detail (Kohn et al., 1976; 1981). Briefly, EMT6 or CHO sample cells prelabeled with ^{14}C-thymidine were treated with 2 μM MC or PM for 1 hr under hypoxia or air. Hypoxia was achieved by a 2 hr pregassing with 95% N_2/5% CO_2 (oxygen content, < 10 ppm) and continuous gassing throughout the period of drug treatment. Immediately following drug treatment, the drug containing medium was removed, the cell monolayer was washed with HBSS and fresh medium was added. After the appropriate incubation period, cells were washed with ice-cold HBSS and irradiated in 5 ml of HBSS with O or 300 R of x-irradiation (Siemans Stabilipan 250 kV, 15 mA, 2 mm Al filter, dose rate 153 R/min) for the single-strand breaks and DNA interstrand cross-linking assays, respectively. Untreated EMT6 reference cells prelabeled with ^{3}H-thymidine were also washed and irradiated with 300 R when used in the assays to measure single strand breaks and DNA interstrand cross-links. These reference cells provided DNA within each experiment with consistent elution kinetics which were not influenced by the elution rate of the experimental ^{14}C-labeled DNA. After x-irradiation, the cells were collected from the milk dilution bottles using a T-flask scraper (Bellco Glass, Inc., Vineland, NJ), 5 ml of ice-cold medium was added to each bottle, and the cells were dispersed by gentle pipetting. Approximately 2.5 x 10^5 ^{14}C-labeled sample cells and a similar number of ^{3}H-labeled reference cells were mixed, diluted in ice-cold PBS, and collected on a 0.8 μm pore size, 2 mm diameter, polycarbonate filter (Nucleopore Corp., Pleasanton, CA), which was placed on a 25 mm polyethylene filter holder (Swinnex, Millipore Corp., Bedford, MA) connected to a 50 ml polyethylene Luer-lok syringe. These cells were then lysed on the filter with 5 ml of a lysis solution containing 2% sodium dodecyl sulfate (SDS, 99% purity, BDH Chemicals, Ltd., Poole, England),

0.025 M Na_2EDTA (Fischer Scientific Co., Fair Lawn, NJ) and 0.1 M glycine (Bethesda Research Laboratories, Inc., Gaithersburg, MD), pH 10.0, which was allowed to flow through the filter by gravity. The lysate was deproteinized by placing 2 ml of 2% SDS, 0.025 M Na_2EDTA, and 0.1 M glycine (pH 10.0) containing 0.5 mg/ml of proteinase K (20 mAnson units/mg; E. Merck, Darmstadt, West Germany) on the filters in the upper chamber of the filter holder. Following the addition of proteinase K, 40 ml of a solution containing tetrapropylammonium hydroxide (RSA Corp., Elmsford, NY), 0.02 M H_2EDTA (Sigma Chemical Co., St. Louis, MO), and 0.1% SDS (pH 12.1) was placed in the syringe. Both solutions were pumped through the filter in the dark at a rate of 0.035 ml/min, allowing the proteinase digestion time to be approximately 2 hr. Eluted fractions were collected at 3 hr intervals for 15 hr. When all of the fractions were collected, they were processed as follows. The eluting solution remaining in the funnel reservoir was gently poured off and discarded. The solution remaining in the pump tubing and filter holder was pumped at maximum speed into an empty scintillation vial. The filter was removed and placed in a scintillation vial containing 0.4 ml of 1 N HCl. Subsequently, the filter was heated at 60°C for 1 hr to depurinate the DNA. After heating the filter, it was cooled to room temperature and 2.5 ml of 0.4 N NaOH (which converts the apurinic sites to strand breaks) was added for one additional hr. Finally, 10 ml of 0.4 N NaOH was added to the funnel to flush the filter holder and pump tubing and a 2.5 ml aliquot of this solution was assayed for its content of radioactivity. A 2 ml aliquot of lysis solution was obtained and water was added to this fraction and to the fractions containing 0.4 N NaOH to give a final volume of 6 ml. All fractions, except the fraction which contained the filter, were mixed with 10 ml Aquassure (New England Nuclear Corp., Boston, MA) with 0.7% glacial acetic acid. The fraction containing the filter was mixed with 5

ml Aquassure with 0.7% glacial acetic acid. Samples were counted in a Beckman LS 7500 scintillation counter (^{14}C efficiency of 63% and ^{3}H efficiency of 13%).

DNA interstrand cross-linking indices were computed using the formula:

$$\text{cross-link index} = (1-r_o/1-r)^{1/2} - 1$$

where r_o and r were the fractions of the ^{3}H-labeled and ^{14}C-labeled DNAs remaining on the filter after approximately 10 hr of elution (Kohn et al., 1976, 1981).

Assay of DNA-Protein Cross-Linking by Alkaline Elution--The assay for drug induced DNA-protein cross-links utilized EMT6 or CHO cells prelabeled with ^{14}C-thymidine and treated with 2 μM MC or PM for 1 hr under hypoxia or air. Following drug treatment, the medium containing drug was removed, the cell monolayer was washed with HBSS, and fresh medium was added. After the appropriate incubation period, the ^{14}C-labeled sample cells and the untreated ^{3}H-labeled EMT6 reference cells were washed with ice-cold HBSS and irradiated in 5 ml HBSS with 3000 R. These reference cells provided DNA within each experiment with consistent elution kinetics, which were not influenced by the elution rate of the experimental ^{14}C-labeled DNA. After x-irradiation, cells were removed from the milk dilution bottles using a T-flask scraper, 5 ml of ice-cold medium was added, and cells were dispersed by gentle pipetting. Approximately 2.5×10^{5} ^{14}C-labeled sample cells and a similar number of ^{3}H-labeled reference cells were mixed, diluted in ice-cold PBS, and collected onto a 2 μm pore size, 25 mm diameter, polyvinyl chloride filter (Millipore Corp.), which was placed on a modified Swinnex filter holder connected to polyvinyl chloride alkaline elution funnel (Millipore Corp.). Cells were then lysed on the filter with 5 ml of the lysis solution used in the previous assay. This solution was allowed to flow through the filter by gravity. Following lysis, the filter was washed with 0.02 M Na_2EDTA (pH

10.0) to remove the remaining lysis solution. Finally, 40 ml of tetrapropylammonium hydroxide and 0.02 M H_2EDTA (pH 12.1) was placed in the elution funnel. The solution was pumped through the filter in the dark at a rate of 0.035 ml/min for 15 hrs with fractions collected every 3 hrs. When all of the fractions were collected, the fractions were processed as indicated in the previous assay.

DNA-protein cross-linking indices were computed using the formula:

$$\text{cross-link index} = (1-r)^{-1/2} - (1-r_o)^{-1/2}$$

where r and r_o were the extrapolated fractions of slow-eluting DNA in drug-treated and control cells, respectively (Kohn and Ewig, 1979).

Statistical Analyses--All statistical tests were performed by an Apple IIe microcomputer using the ALLFIT computer program. The data was analyzed by non-parametric analysis which included the Mann-Whitney test, analysis of variance, and constraint curve fitting. Significance was defined at the level of 95% confidence or better ($p<0.05$).

RESULTS

Cell Survival and Cell Cycle Distributions after Labeling with Radioisotopes--Both EMT6 and CHO cells were labeled with ^{14}C-thymidine, while only EMT6 cells were labeled with ^{3}H-thymidine and cell survival and cell cycle distributions were measured to ensure that radioactive labeling did not affect these parameters. These radioisotopes did not decrease cell survival as measured by the surviving fraction after cloning, nor did this prelabeling with radioisotopes affect the cell cycle distributions (Table 12). As there were no effects on cell survival or cell cycle distributions with the concentrations of ^{3}H-and ^{14}C-labeled thymidine used in these studies, the elution profiles obtained were assumed to be the result of drug treatment.

Formation of Single Strand Breaks and DNA Interstrand Cross-Links Detected Immediately Following Drug Treatment in Cultured Cell Lines--Initial experiments using both hypoxic and aerobic EMT6 cells treated with 2 μM MC or PM for 1 hr demonstrated differences dependent upon the degree of oxygenation (Figure 12). Thus, although the alkaline elution profiles of aerobic and hypoxic EMT6 cells treated with either MC or PM for 1 hr demonstrated no DNA single strand breaks, under both hypoxic and aerobic conditions, this cell line exhibited DNA interstrand cross-links, with substantially more DNA interstrand cross-links occurring under hypoxic conditions than with oxygenation. Interestingly, both drugs demonstrated a comparable amount of cross-linking with these antibiotics under hypoxic conditions, a finding which is consistent with the degree of cytotoxicity produced by these agents (see Chapter II). Aerobic EMT6 cells treated with MC demonstrated slightly greater interstrand cross-linking than those exposed to PM, which is also consistent with the cytotoxicity of these drugs in air (see Chapter II).

Both MC and PM did not form detectable single strand breaks in the CHO cell line under aerobic and hypoxic conditions (Figure 13). Contrary to the results observed with EMT6 cells, negligible DNA interstrand cross-linking was produced by MC or PM under hypoxic or aerobic conditions. DNA-protein cross link indices for both aerobic and hypoxic CHO cells treated with MC or PM demonstrate little DNA-protein cross-linking (Table 13). These findings are consistent with studies which demonstrated only slight cytotoxicity to CHO cells by these antibiotics (see Chapter II).

Formation and Removal of Single Strand Breaks, DNA Interstrand Cross-Links, and DNA-Protein Cross-Links Following Removal of Drug in EMT6 Cells--Further studies were done to examine whether the formation of single strand breaks or DNA-protein cross-links occurred in EMT6 cells as a function of time after removal of drug. These studies also examined whether these antitumor antibiotics continued to form DNA interstrand cross-links in EMT6 cells and/or whether these cells were able to remove these cross-links with time after drug exposure.

After exposure to 2 μM MC for 1 hr, no single strand breaks could be demonstrated in EMT6 cells under either hypoxia or air from 0 to 24 hr after drug removal (Figures 14 and 15). Under hypoxic conditions, MC-treated cells contained DNA interstrand cross-links throughout the 24 hr period after drug exposure. A slight increase in the amount of cross-linking over that occurring at 0 hr was observed at 6 and 12 hr, with almost complete repair of these lesions by 24 hr (Figure 14). The dependence of interstrand cross-linking on time of exposure to MC in aerobic EMT6 cells was similar to that seen with the hypoxic EMT6 cells; however, less cross-linking was observed immediately following drug treatment. In a manner analogous to that observed for hypoxic cells, an increase in the degree of

cross-linking occurred during the 6 to 12 hr post incubation period with a reduction in the number of cross-links occurring during the 12 to 24 hr period following drug exposure (Figure 15). A comparison of the DNA interstrand cross-link indices for both hypoxic and aerobic EMT6 cells treated with MC is shown in Figure 16. There were significantly more DNA interstrand cross-links observed immediately following drug treatment in hypoxic EMT6 cells ($p<0.05$). There was, however, no significant difference in the amount of DNA interstrand cross-links occurring 6, 12, and 24 hr after drug removal in hypoxic and aerobic EMT6 cells.

DNA-protein cross-link indices were calculated and the results are shown in Figure 17. Approximately the same amount of DNA-protein cross-links was present in hypoxic and aerobic EMT6 cells throughout the 24 hr period after drug exposure.

No single strand breaks were demonstrable in hypoxic or aerobic EMT6 cells over the 0 to 24 hr period following exposure to 2 μM PM for 1 hr (Figures 18 and 19). DNA interstrand cross-links occurred with this agent in hypoxic EMT6 cells which were maintained over the 24 hr post incubation period, with approximately an equivalent amount of cross-linking being maintained over the O to 12 hr period and fewer cross-links at 24 hr (Figure 18). Barely detectable DNA interstrand cross-links were present immediately after PM treatment of aerobic EMT6 cells, however, an increase in DNA interstrand cross-links occurred at 6 hr post incubation period with a reduction in number of cross-links occurring during the 12 to 24 hr period following drug exposure (Figure 19). A comparison of the DNA interstrand cross-link indices for both hypoxic and aerobic EMT6 cells treated with PM is shown in Figure 20. There were significantly more DNA interstrand cross-links in hypoxic EMT6 cells than aerobic EMT6 cells at 0 hr ($p<0.05$) and 6 hr

($p<0.03$) after drug removal. There was no significant difference in cross-links observed 12 and 24 hr after drug removal.

The DNA-protein cross-linking indices for both aerobic and hypoxic EMT6 cells following treatment with PM are shown in Figure 21. It is notable that hypoxic EMT6 cells treated with PM have significantly greater DNA-protein cross-link indices than aerobic EMT6 cells throughout the 24 hr period following drug treatment ($p<0.01$). The amount of DNA-protein cross-links present in hypoxic and aerobic EMT6 cells was not significantly different with time following drug exposure.

Table 12

SURVIVING FRACTIONS AND CELL CYCLE DISTRIBUTIONS IN CULTURED CELL LINES AFTER RADIOISOTOPE LABELING

Cell line	Surviving Fraction	Cell Cycle Distributions (%) G_1	S	G_2-M
CHO	0.639	27.5	58.2	14.3
^{14}C-labeled CHO	0.684	22.2	52.4	25.4
EMT6	0.622	33.1	48.6	18.2
^{14}C-labeled EMT6	0.575	24.1	59.6	16.3
^{3}H-labeled EMT6	0.629	36.2	49.2	14.6

Exponentially growing cells were exposed to either ^{14}C-thymidine or ^{3}H-thymidine for 24 hr, and the surviving fraction estimated by the ability of cells to form colonies as described in the Methods. Cell cycle distributions were determined using a Becton-Dickinson FACS IV flow cytometer as described in the Methods.

FIGURE 12. Alkaline Elution Profiles of Aerobic and Hypoxic EMT6 Cells Immediately Following MC or PM Treatment. Exponentially growing cells were treated with 2 μM MC or PM for 1 hr under conditions of normal aeration or hypoxia. Subsequently, the drug-containing medium was removed, and the cells were irradiated with O R or 300 R and assayed for single strand breaks or DNA interstrand cross-links, respectively, by the alkaline elution assay as described in Methods. The alkaline elution profiles for the measurement of single strand breaks and DNA interstrand cross-links are shown by open and closed symbols, respectively. O, ●, control cells;□,■, MC treated cells; Δ, ▲, PM treated cells.

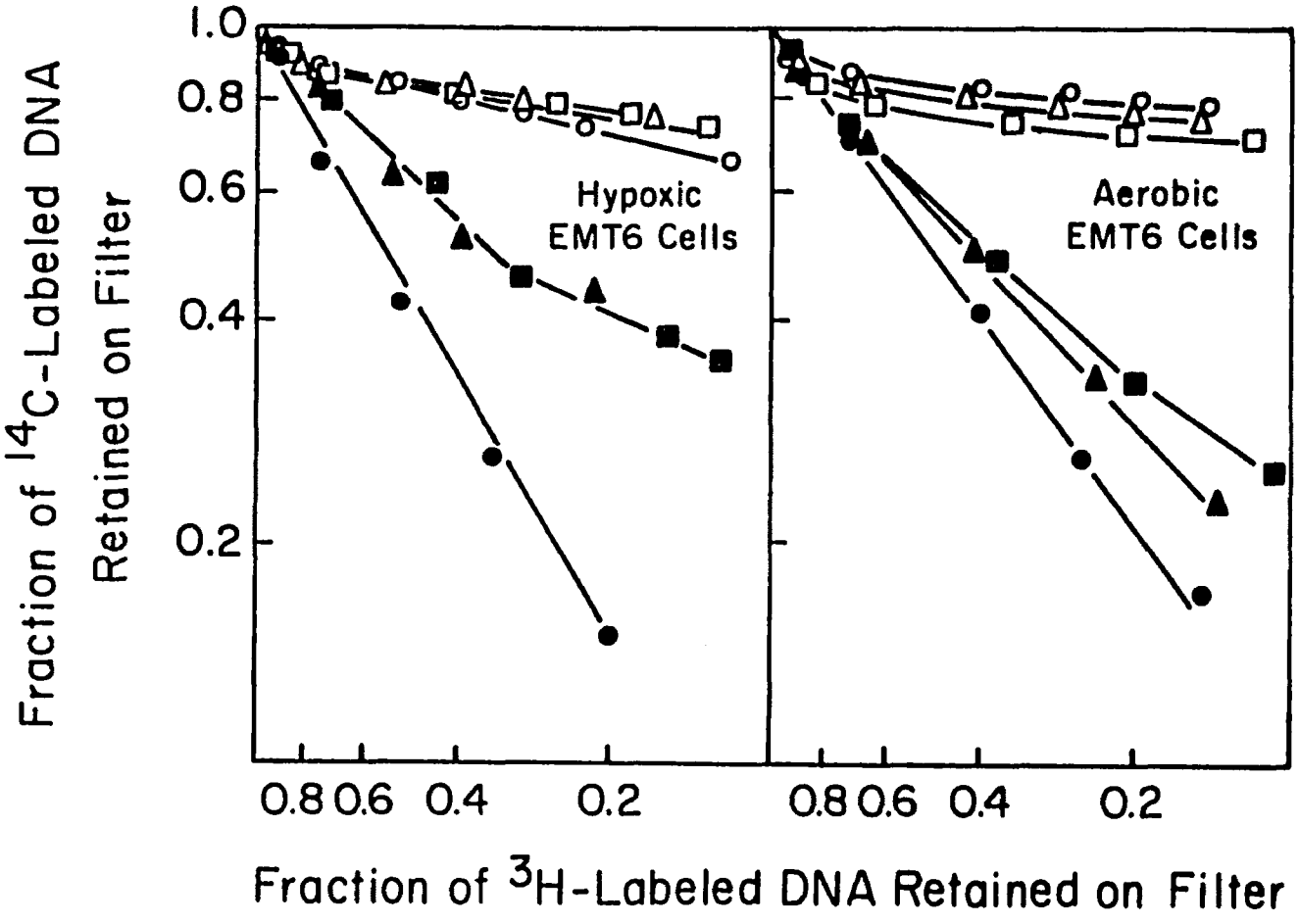


FIGURE 13. Alkaline Elution Profiles of Aerobic and Hypoxic CHO Cells Immediately Following MC or PM Treatment. Exponentially growing cells were treated with 2 μM MC or PM for 1 hr under conditions of normal aeration or hypoxia. Subsequently, the drug-containing medium was removed, and the cells were irradiated with O R or 300 R and assayed for single strand breaks or DNA interstrand cross-links, respectively, by the alkaline elution assay as described in the Methods. The alkaline elution profiles for the measurement of single strand breaks and DNA interstrand cross-links are shown by open and closed symbols, respectively. O, ●, control cells;□,■, MC treated cells; Δ, ▲, PM treated cells.

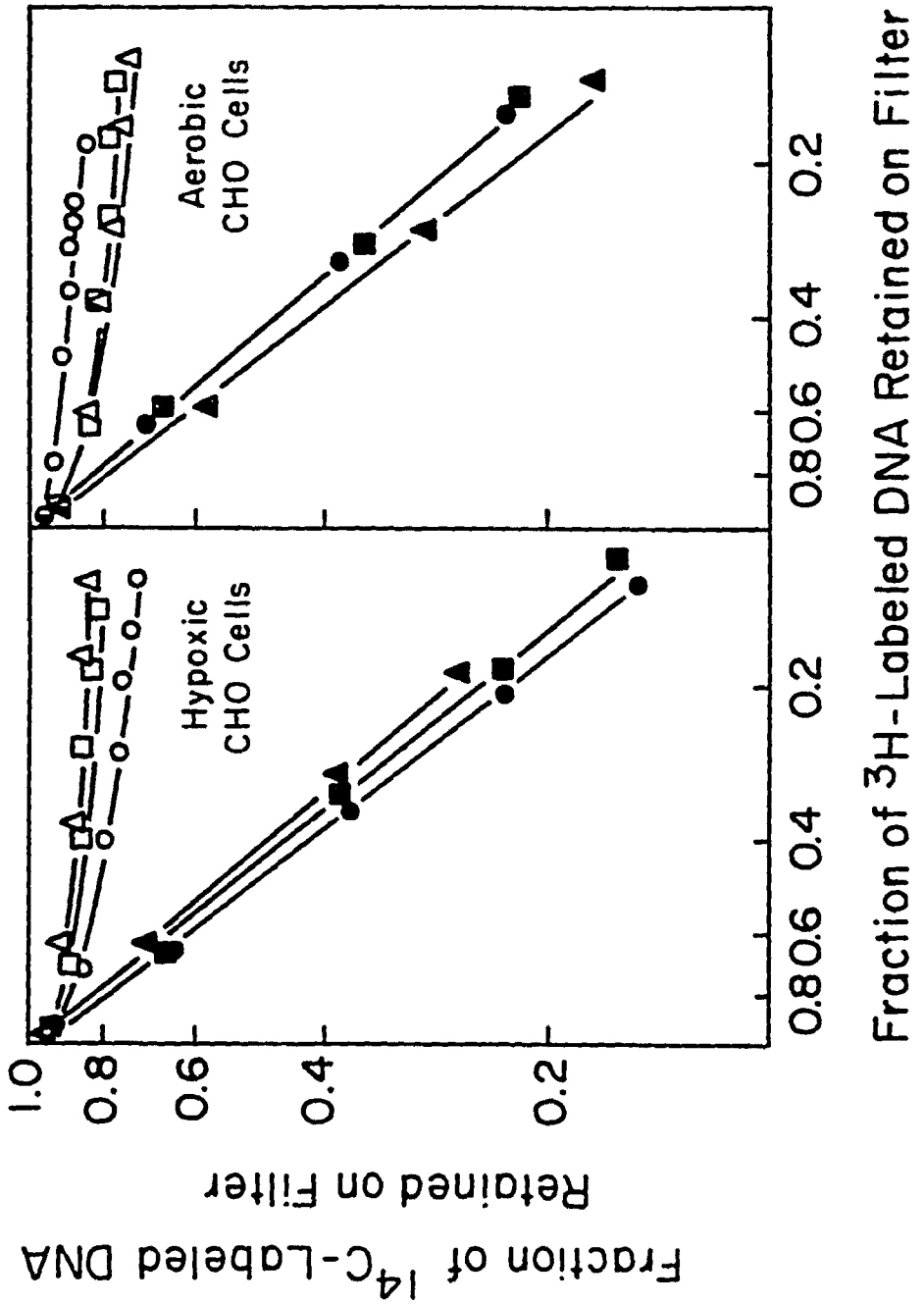


Table 13

DNA-PROTEIN CROSS-LINK INDEX IN CHO CELLS AFTER DRUG TREATMENT

Drug	Hypoxia	Air
Mitomycin C	0.019	0.027
Porfiromycin	0.041	0.021

Exponentially growing cells were treated with 2 μM MC or PM for 1 hr under conditions of normal aeration or hypoxia. Subsequently, the drug containing medium was removed, the cells were irradiated with 3000 R and assayed for DNA-protein cross-linking by the alkaline elution assay as described in the Methods. The DNA-protein cross-linking index was determined using the formula, cross-link index $= (1-r)^{-1/2} - (1-r_o)^{-1/2}$ where r and r_o were the extrapolated fractions of the slow eluting DNA on drug treated and control cells, respectively (Kohn and Ewig, 1979). The data represent results from one or two experiments.

FIGURE 14. Alkaline Elution Profiles of Hypoxic EMT6 Cells Various Times after the Removal of MC. Exponentially growing cells were treated with MC for 1 hr under hypoxic conditions. Subsequently, the drug containing medium was removed, and the cells were incubated in fresh medium for 0, 6, 12, or 24 hr. After incubation, cells were irradiated with 0 or 300 R and assayed for single strand breaks or DNA interstrand cross-links, respectively, by the alkaline elution assay as described in the Methods. The alkaline elution profiles for the measurement of single strand breaks and DNA interstrand cross-links are shown by open and closed symbols, respectively. ▽, ▼, control cells; O, ●, 0 hr; △, ▲, 6 hr; □, ■, 12 hr; and ◇, ◆, 24 hr after drug removal.

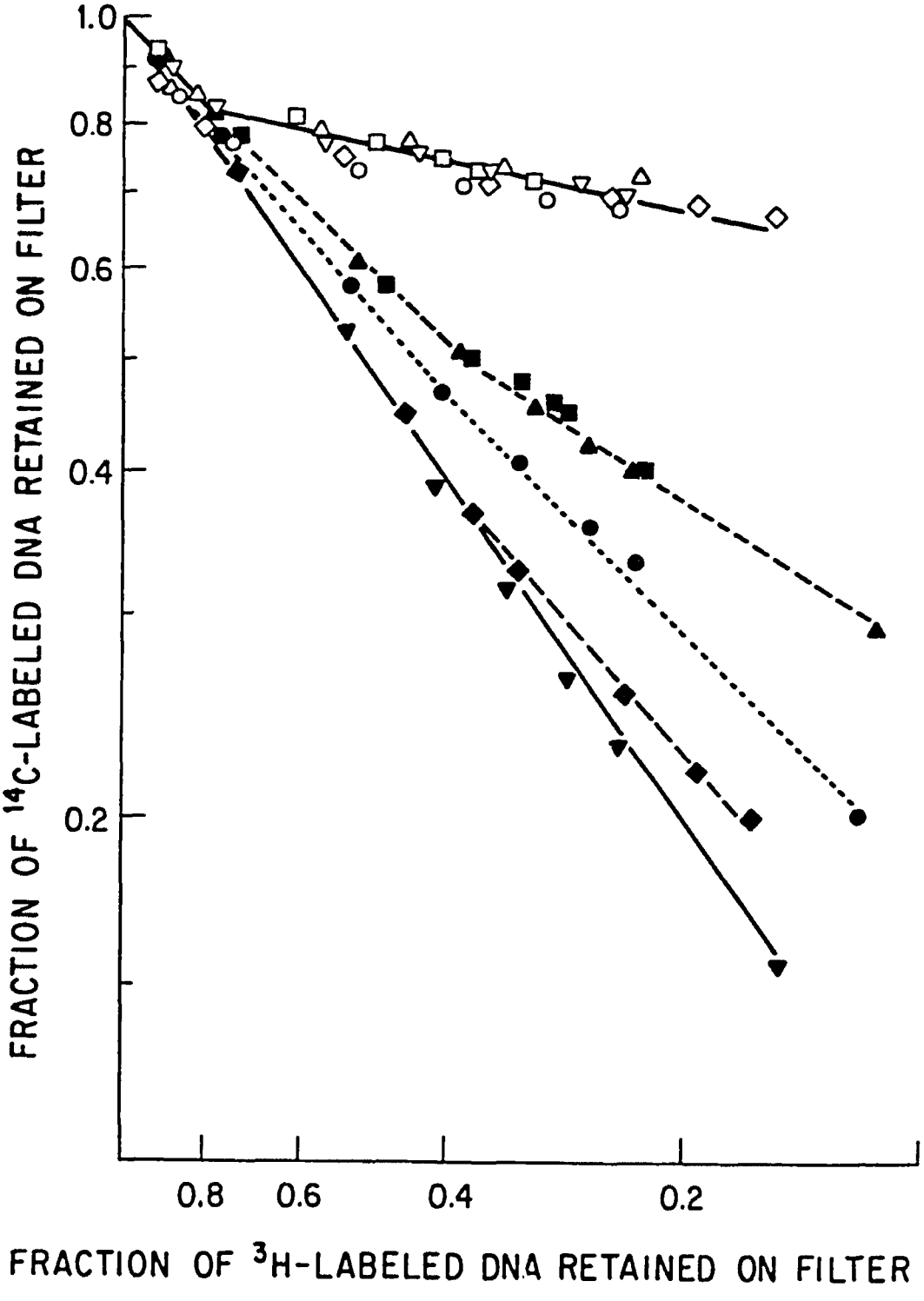


FIGURE 15. Alkaline Elution Profiles of Aerobic EMT6 Cells Various Times after the Removal of MC. Exponentially growing cells were treated with MC for 1 hr under conditions of normal aeration. Subsequently, the drug containing medium was removed, and the cells were incubated in fresh medium for 0, 6, 12, or 24 hr. After incubation, cells were irradiated with 0 or 300 R and assayed for single strand breaks or DNA interstrand cross-links, respectively, by the alkaline elution assay as described in the Methods. The alkaline elution profiles for the measurement of single strand breaks and DNA interstrand cross-links are shown by open and closed symbols, respectively. ▽, ▼, control cells; ○, ●, 0 hr; △, ▲, 6 hr; □, ■, 12 hr; and ◇, ◆, 24 hr after drug removal.

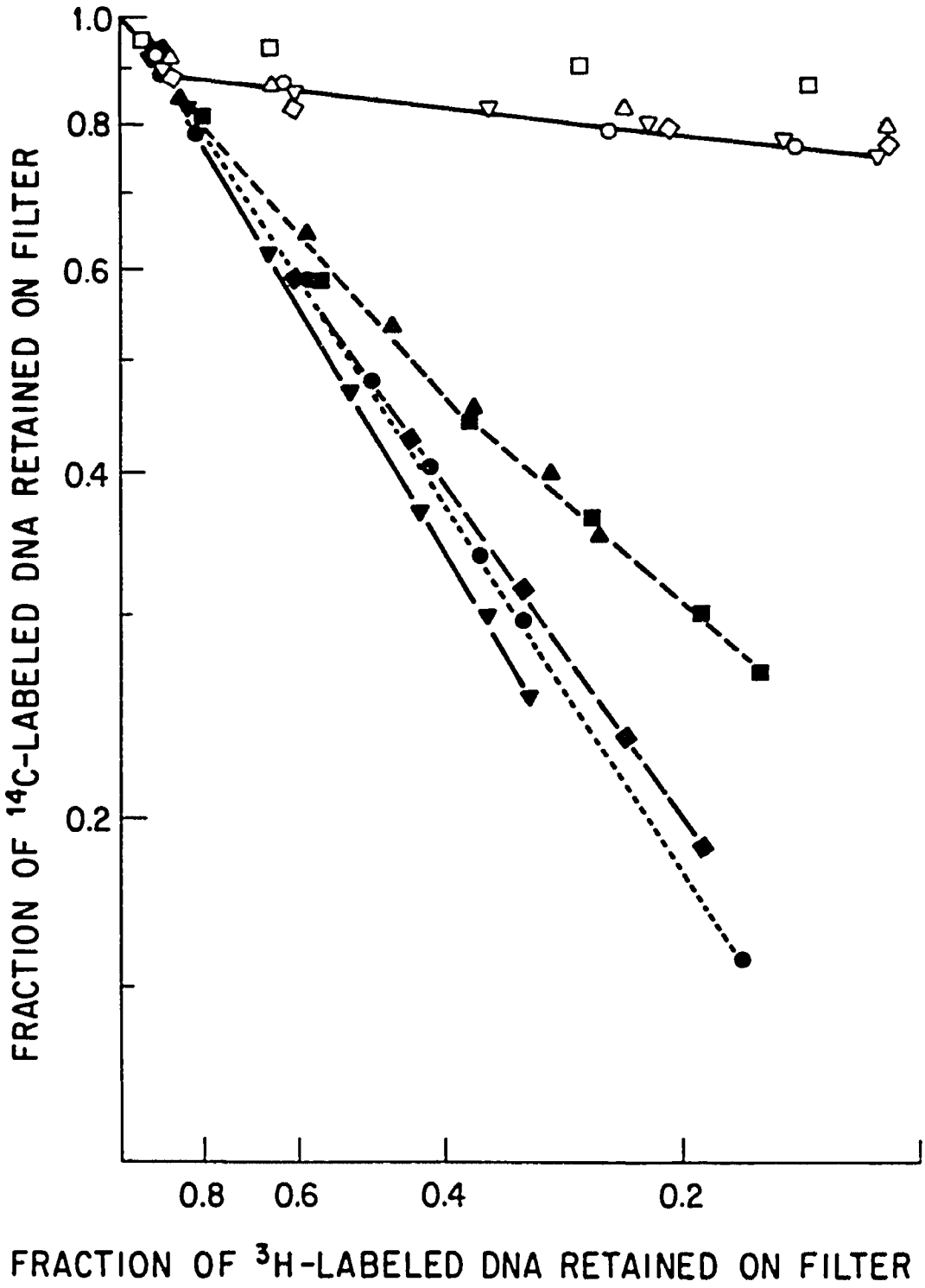


FIGURE 16. DNA Interstrand Cross-Link Index in EMT6 Cells after MC Treatment. Exponentially growing cells were treated with 2 μM MC for 1 hr under conditions of normal aeration or hypoxia. Subsequently, the drug containing medium was removed, the cells were irradiated with 300 R and assayed for DNA interstrand cross-linking by the alkaline elution assay, as described in the Methods. The DNA interstrand cross-linking index was determined using the formula, cross-link index = $(1-r_o/1-r)^{1/2} - 1$, where r_o and r were the fractions of control and drug treated DNA remaining on the filter after approximately 10 hr of elution (Kohn et al., 1976; 1981). The DNA interstrand cross-link indices for hypoxic and aerobic EMT6 cells are represented by the hatched and open bars, respectively. The data represent the means and standard errors of the means of three experiments. There were significantly more DNA interstrand cross-links ($p<0.05$) immediately following MC treatment in hypoxic EMT6 cells.

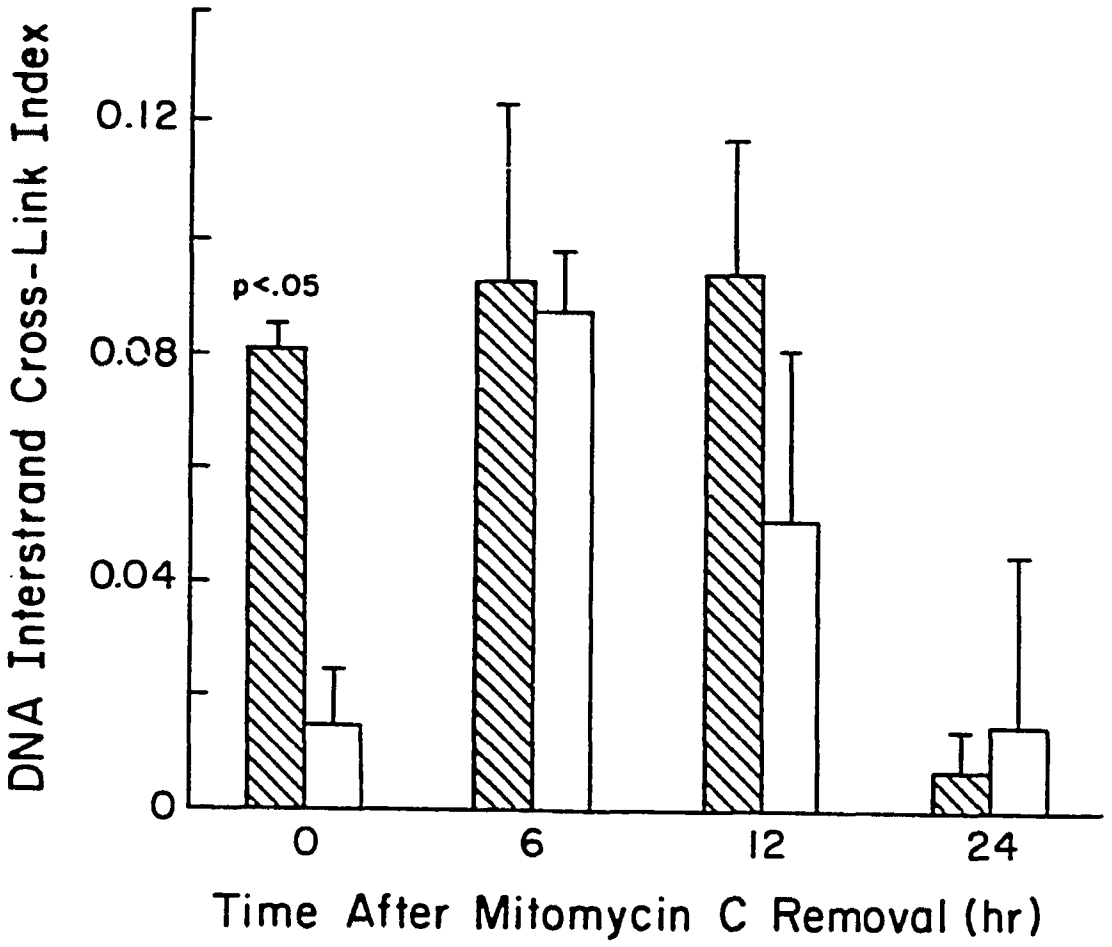


FIGURE 17. DNA-Protein Cross-Link Index in EMT6 Cells after MC Treatment. Exponentially growing cells were treated with 2 μM MC for 1 hr under conditions of normal aeration or hypoxia. Subsequently, the drug containing medium was removed, and the cells were incubated in fresh medium for 0, 6, 12, and 24 hr. After incubation, cells were irradiated with 3000 R and assayed for DNA-protein cross-linking by the alkaline elution assay as described in the Methods. The DNA-protein cross-linking index was determined using the formula, cross-link index = $(1-r)^{-1/2} - (1-r_o)^{-1/2}$ where r and r_o were the extrapolated fractions of the slow eluting DNA in drug treated and control cells, respectively (Kohn and Ewig, 1979). Each symbol represents a different experiment: O,□, drug treatment under conditions of normal aeration; ●,■, drug treatment under hypoxic conditions.

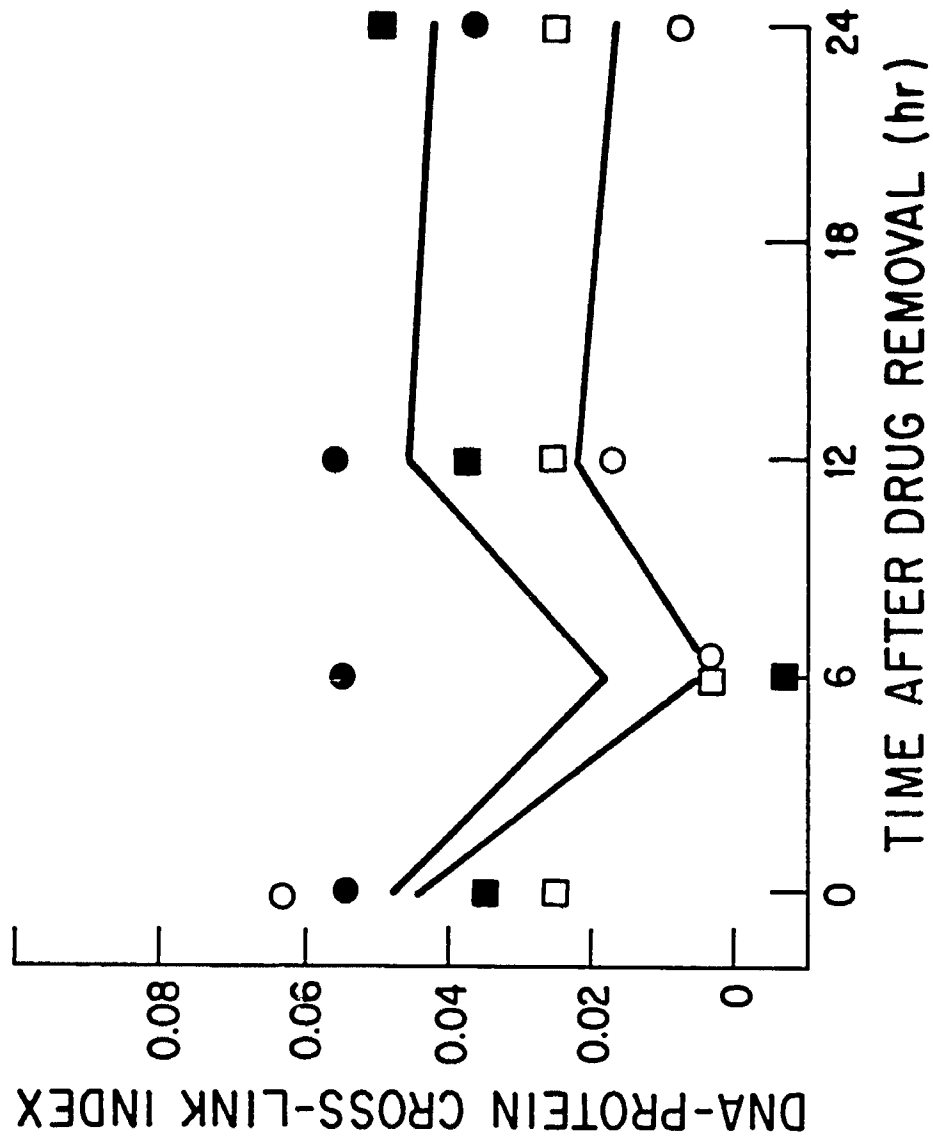


FIGURE 18. Alkaline Elution Profiles of Hypoxic EMT6 Cells Various Times after the Removal of PM. Exponentially growing cells were treated with PM for 1 hr under hypoxic conditions. Subsequently, the drug containing medium was removed, and the cells were incubated in fresh medium for 0, 6, 12, or 24 hr. After incubation, cells were irradiated with 0 or 300 R and assayed for single strand breaks or DNA interstrand cross-links, respectively, by the alkaline elution assay as described in the Methods. The alkaline elution profiles for the measurement of single strand breaks and DNA interstrand cross-links are shown by open and closed symbols, respectively. ▽, ▼, control cells; ○, ●, 0 hr; △, ▲, 6 hr; □, ■, 12 hr; and ◇, ◆, 24 hr after drug removal.

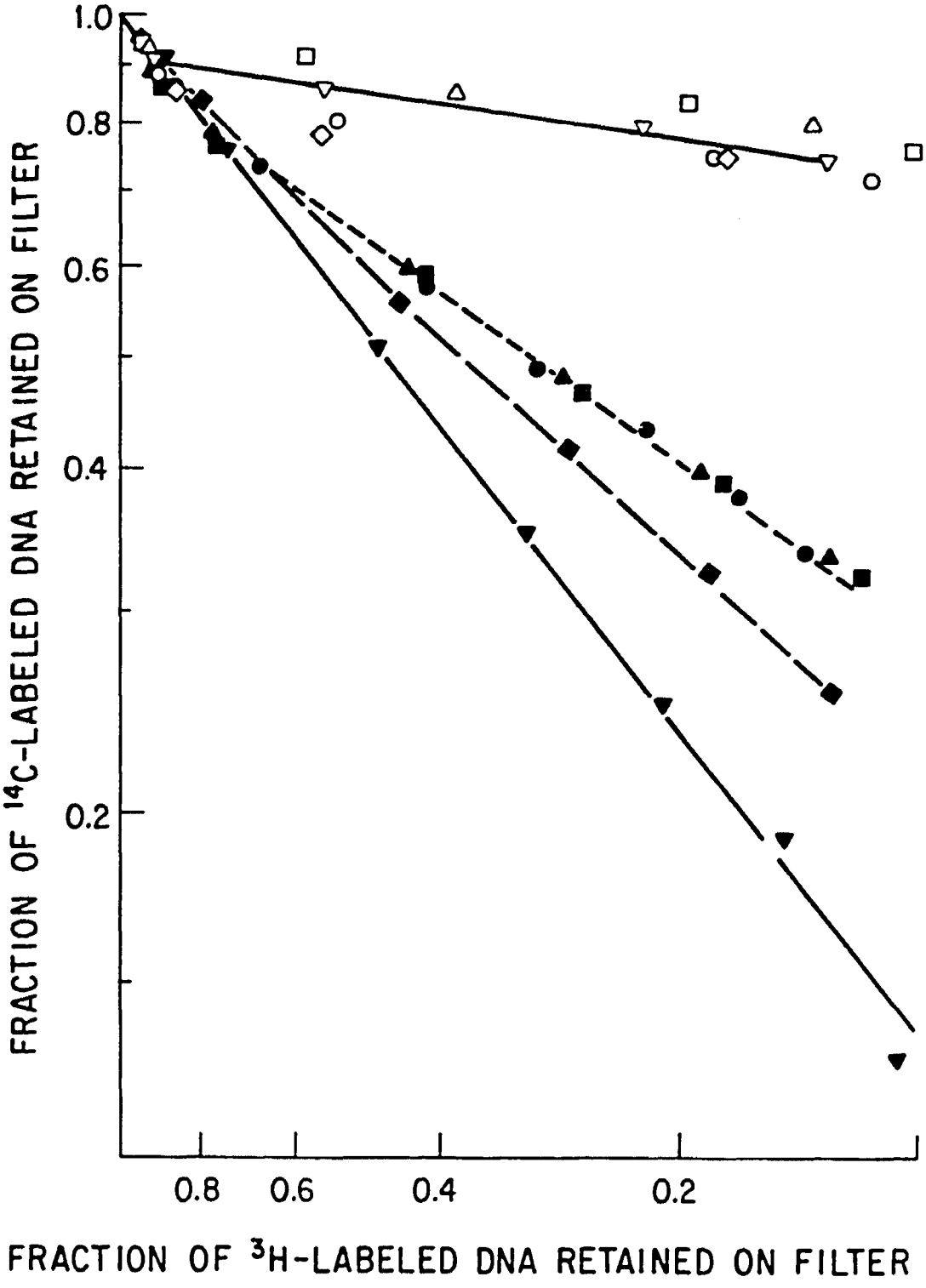


FIGURE 19. Alkaline Elution Profiles of Aerobic EMT6 Cells Various Times after the Removal of PM. Exponentially growing cells were treated with PM for 1 hr under conditions of normal aeration. Subsequently, the drug containing medium was removed, and the cells were incubated in fresh medium for 0, 6, 12, or 24 hr. After incubation, cells were irradiated with 0 or 300 R and assayed for single strand breaks or DNA interstrand cross-links, respectively, by the alkaline elution assay as described in the Methods. The alkaline elution profiles for the measurement of single strand breaks and DNA interstrand cross-links are shown by open and closed symbols, respectively. ▽, ▼, control cells; ○, ●, 0 hr; △, ▲, 6 hr; □, ■, 12 hr; and ◇, ◆, 24 hr after drug removal.

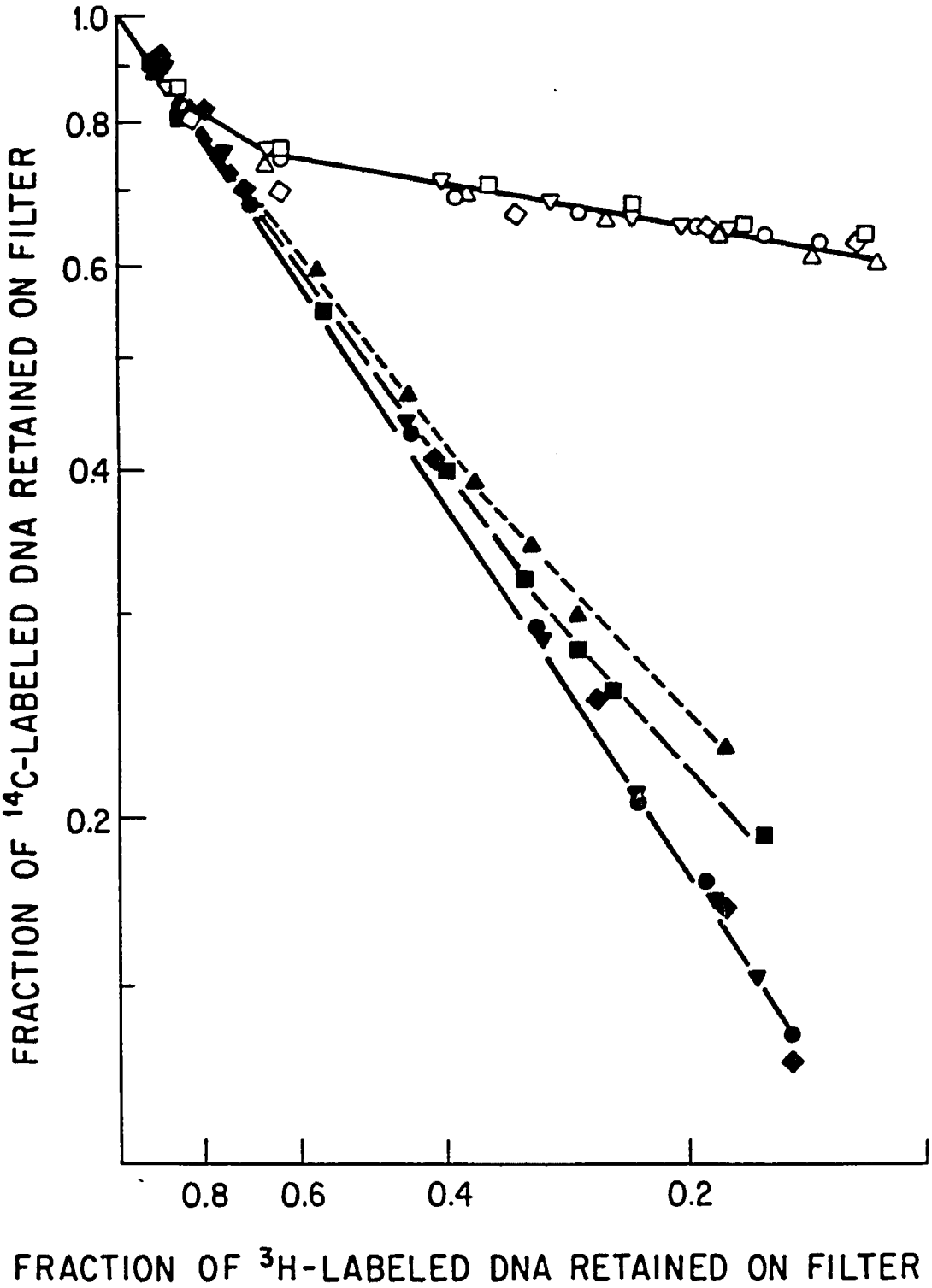


FIGURE 20. DNA Interstrand Cross-Link Index in EMT6 Cells after PM Treatment. Exponentially growing cells were treated with 2 μM PM for 1 hr under conditions of normal aeration or hypoxia. Subsequently, the drug containing medium was removed, the cells were irradiated with 300 R and assayed for DNA interstrand cross-linking by the alkaline elution assay, as described in the Methods. The DNA interstrand cross-linking index was determined using the formula, cross-link index = $(1-r_o/1-r)^{1/2} -1$, where r_o and r were the fractions of control and drug treated DNA remaining on the filter after approximately 10 hr of elution (Kohn et al., 1976; 1981). The DNA interstrand cross-link indices for hypoxic and aerobic EMT6 cells are represented by hatched and open bars, respectively. The data represent the means and standard errors of the means of three experiments. There were significantly more DNA interstrand cross-links immediately following PM treatment ($p<0.05$) and 6 hr after PM removal ($p<0.03$) in hypoxic EMT6 cells than in aerobic EMT6 cells.

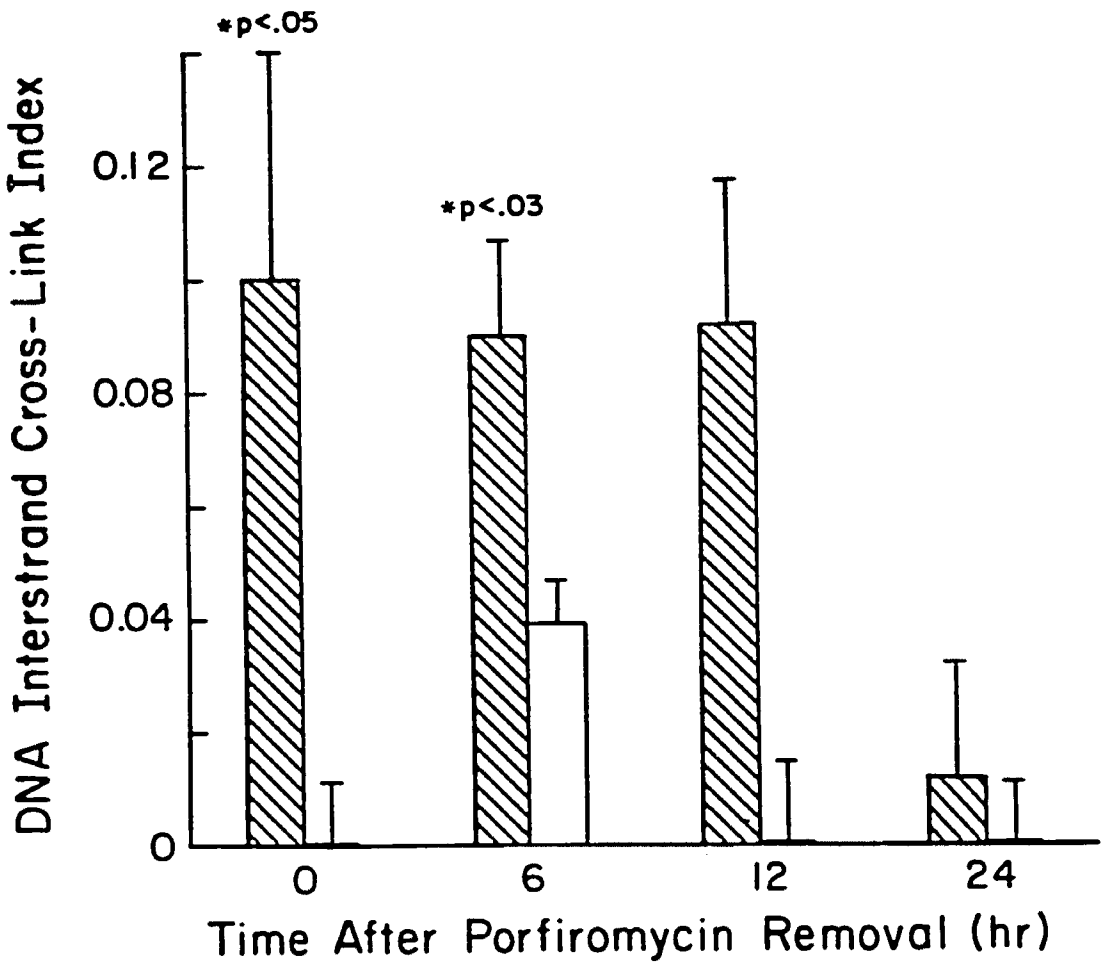
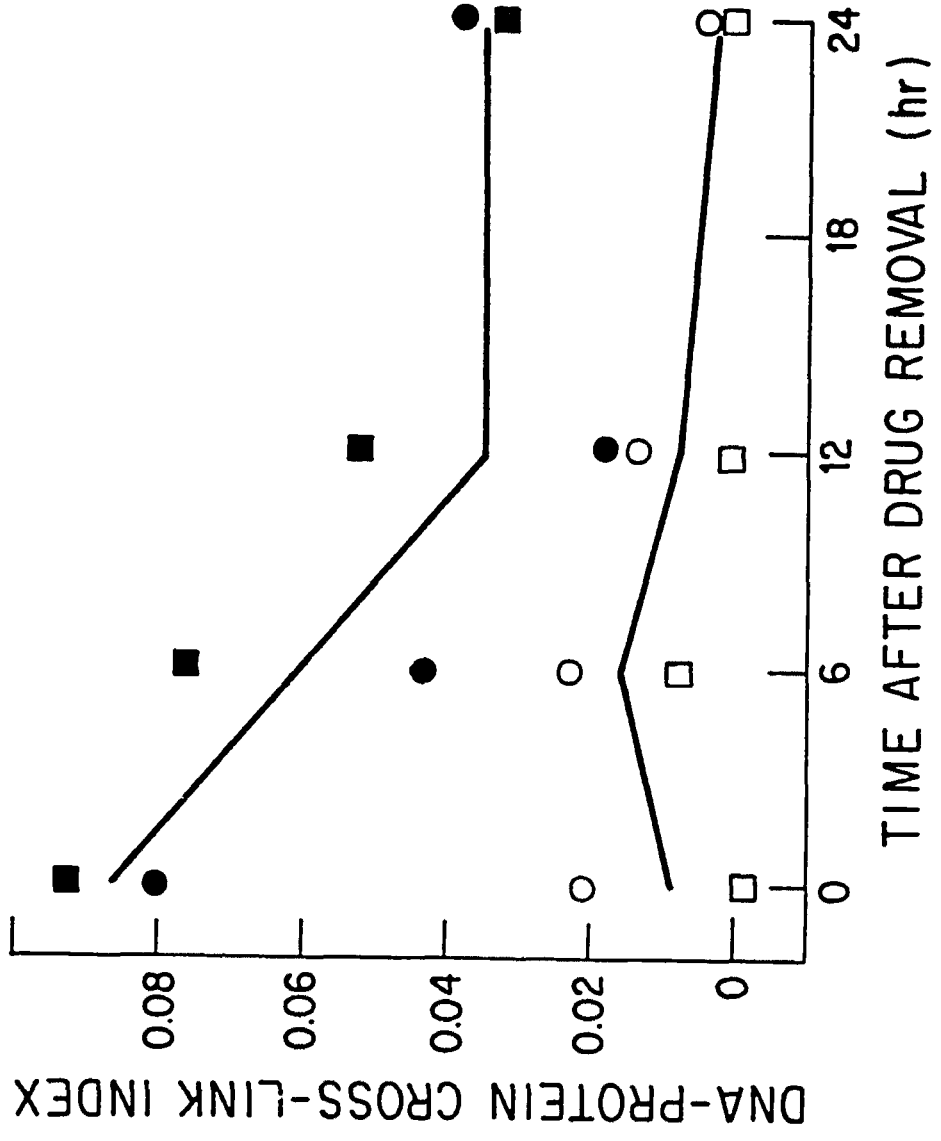


FIGURE 21. DNA-Protein Cross-Link Index in EMT6 Cells after PM Treatment. Exponentially growing cells were treated with 2 μM PM for 1 hr under conditions of normal aeration or hypoxia. Subsequently, the drug containing medium was removed, and the cells were incubated in fresh medium for 0, 6, 12, and 24 hr. After incubation, cells were irradiated with 3000 R and assayed for DNA-protein cross-linking by the alkaline elution assay as described in the Methods. The DNA-protein cross-linking index was determined using the formula, cross-link index = $(1-r)^{-1/2} - (1-r_o)^{-1/2}$ where r and r_o were the extrapolated fractions of the slow eluting DNA in drug treated and control cells, respectively (Kohn and Ewig, 1979). Each symbol represents a different experiment: O,□, drug treatment under conditions of normal aeration; ●,■, drug treatment under hypoxic conditions.



DISCUSSION

The alkaline elution technique developed by Kohn and coworkers (Kohn et al., 1976, 1981; Kohn and Ewig, 1979) has been utilized to study the relationship of DNA single strand breaks, DNA interstrand cross-links, and DNA-protein cross-links to cytotoxicity in hypoxic and aerobic cultured cells after treatment with either MC or PM. This technique is a sensitive method for the measurement of DNA lesions and is based on the behavior of large DNA strands released from mammalian cells by lysis on membrane filters (Kohn, 1979). The detergent containing lysis removes most of the cell protein and RNA but retains double-stranded DNA, and, if desired, DNA-protein cross-links can be removed by a proteinase digestion. The DNA is eluted through the filter by pumping an alkaline solution (which disrupts the DNA helix) slowly through the filter and collecting fractions containing single-stranded DNA. DNA is thought to be eluted from the filter as a function of strand length, with smaller strands eluting initially and larger stranded DNA eluting at later times. This technique has been used to measure a variety of lesions including DNA single-and double-strand breaks, alkali-labile sites, DNA interstrand cross-links and DNA-protein cross-links (Kohn, 1979; Kohn et al., 1981).

MC and PM are believed to exert their antineoplastic activity by the formation of DNA interstrand cross-links. By isopycnic sedimentation, many investigators have correlated MC-induced DNA cross-linking in bacteria and in a human cell line with cytotoxicity (Iyer and Szybalski, 1963; Matsumoto and Lark, 1963; Szybalski, 1964; Szybalski and Iyer, 1964a). By the ethidium fluorescence assay, Lown et al. (1976) confirmed the formation of DNA cross-links in purified λ-phage DNA. By alkaline sucrose sedimentation, hydroxyapatite chromatography, and S_1 nuclease digestion, other investigators were able to correlate the removal of cross-links in many mammalian cells in vitro with their sensitivity to MC

(Fujiwara, 1982; Fugiwara and Tatsumi, 1975, 1977; Kano and Fugiwara, 1981). These authors observed that the removal of interstrand cross-links in Fanconi's anemia strains sensitive to MC occurred at a slower rate than the rate of removal of cross-links in less sensitive normal human fibroblasts and other mammalian cells. In contrast, using alkaline elution methodology, Fornace et al. (1979) were not able to demonstrate a correlation between the removal of interstrand cross-links in normal human fibroblasts and a Fanconi's anemia human fibroblast strain (HG 261). In this study, the HG 261 strain was significantly more sensitive to MC than the normal fibroblasts; however, there were no differences in the degree of cross-linking occurring over a 24 hr period after drug treatment in either the normal fibroblasts or the HG 261 strain.

In this chapter, the results of alkaline elution studies on hypoxic and aerobic EMT6 and CHO cells treated with MC and PM suggest a correlation between the cytotoxicity of these drugs and the formation of DNA cross-links under hypoxic and aerobic conditions. In both cell lines, single strand breaks were not detected (Figures 12 and 13); however, the levels of DNA interstrand cross-links present in antibiotic treated EMT6 cells may have masked single strand breaks which may have occurred during or after drug treatments by retaining the DNA on the filter. Furthermore, even though MC is known to form hydrogen peroxide and hydroxyl radicals after reductive activation (Komiyama et al., 1982; Lown and Chen, 1981; Tomasz, 1976), and these radicals may cause single strand breaks, Bradley and Erickson (1981) have reported rapid repair rates for single strand breaks induced by hydrogen peroxide in V79 cells.

MC is more cytotoxic to hypoxic EMT6 cells than to corresponding aerobic cells (see Chapter II) and more DNA interstrand cross-linking was observed under hypoxic conditions than under aerobic conditions immediately following drug

treatment (Figure 12). A similar pattern was also observed in PM treated EMT6 cells (Figure 12). Interestingly, the aerobic cytotoxicity occurring with MC in EMT6 cells is greater than that produced by PM (see Chapter II), and the degree of DNA cross-linking under these conditions corresponds with these data (Figure 12).

Both MC and PM produced interstrand cross-linking in EMT6 cells that persisted over a 24 hr period following drug removal (Figures 14, 15, 18, and 19). Interestingly, the formation of DNA interstrand cross-links in aerobic EMT6 cells increased hours after drug removal following both MC or PM treatment. With MC treatment, the amount of cross-linking observed in aerobic EMT6 cells was comparable to the amount of cross-linking observed in hypoxic EMT6 cells 6 and 12 hr after drug removal. While this may suggest that the rate of formation of cross-links in EMT6 cells is slower under air, it is also possible that a greater amount of cross-linking under hypoxia may have been demonstrated at earlier times after drug removal. Further studies on the amount of DNA interstrand cross-links in hypoxic and aerobic EMT6 cells between 0 and 6 hr after drug removal would examine this possibility.

Substantially less DNA interstrand cross-links were observed at 24 hr after MC or PM removal in hypoxic and aerobic EMT6 cells. These results may indicate removal and repair of cross-links by these cells, although it is probable that these cells divided and that the decrease in cross-links reflects the fact that the original population of cells is diluted 24 hr after drug exposure. It would be of interest to compare the amount of radioactivity in the medium at various times after drug treatment. An increase in the amount of radioactivity at 24 hr from that measured at 0 hr may reflect degraded DNA in the medium secondary to cell death or excision repair of alkylated DNA. On the other hand, if the amount of radioactivity in the medium at 24 hr was the same or less than that measured at 0

hr, the decrease in the cross-link index may indicate repopulation with daughter cells. It is probable that cell death, repair of cross-links, and repopulation with daughter cells contributed to the decrease in cross-link index observed at 24 hr.

In conclusion, these studies demonstrated a relationship between the cytotoxicity of these quinone antibiotics in hypoxic and aerobic cell lines and the degree of DNA interstrand and DNA-protein cross-linking. In these studies, CHO cells have appeared "resistant" to the cytotoxic effects of these drugs and to the formation of DNA interstrand cross-links, while EMT6 cells are more sensitive to these parameters. Further studies are warranted to ascertain the reason(s) for the differential cytotoxicity between hypoxic and aerobic EMT6 cells and the very slight cytotoxicity observed in CHO cells. It may be argued that uptake, activation, and/or subsequent reaction with DNA is different in the two cell lines, since DNA-protein cross-links are formed to a lesser degree in CHO cells than in EMT6 cells. Finally, work by Erickson et al. (1980a, 1980b, 1981) and Gibson et al. (1984a, 1984b) has shown that normal (IMR-90) and simian virus transformed (VA-13) human embryo cells differed in their response to many cross-linking agents, possibly as a result of their ability to repair O^6-guanine lesions. These investigators believe that the capacity of IMR-90 cells to repair methylated lesions enable these cells to remove an O^6-monoadduct, and thereby prevent subsequent cross-linking and cell death. In contrast, the inability to remove the O^6-monoadduct in VA-13 cells would account for the preferential cytotoxicity of cross-linking agents to these cells. Since Hashimoto et al. (1983) have identified an O^6-guanine-MC adduct after chemical reduction of MC in the presence of calf thymus DNA, and Tomasz et al. (1983) have identified an O^6-guanine-MC after chemical and enzymatic reduction of MC in the presence of deoxyribonucleotide dimer, d(GpC), it is possible that CHO cells may be able to repair the initial

monoalkylation before cross-linking occurs and that EMT6 cells may be deficient in this repair capability. Furthermore, it is known that MC and PM are more easily reduced under anaerobic conditions (Iyer and Szybalski, 1964; Kennedy et al., 1982; Komiyama et al., 1979a; Pan et al., 1984; Schwartz, 1962; Tomasz et al., 1983), which would account for the additional cross-linking observed under hypoxic conditions in the EMT6 cell line immediately after drug treatment.

CHAPTER V

Conclusions

Investigations on the antitumor antibiotics MC and PM have demonstrated that these agents require bioactivation to cause DNA interstrand cross-links which may be responsible for the cytotoxicity of these drugs. Previous work by our laboratory has employed mammalian tumor cell lines and has demonstrated that EMT6 mouse mammary tumor cells and Sarcoma 180 ascites cells are more sensitive to the cytotoxic action of MC under hypoxic conditions than in air, and that sonicates of these cell lines are capable of consuming MC to generate a reactive species. Studies presented in this dissertation have extended these findings to other cultured cell lines and have demonstrated that MC is essentially equitoxic to aerobic and hypoxic CHO cells, whereas it is preferentially cytotoxic to V79 cells under hypoxic conditions. PM is preferentially cytotoxic to hypoxic EMT6, V79, and CHO cells, although the cytotoxicity to hypoxic CHO cells is considerably less than that to EMT6 and V79 cells.

The reasons for acquired resistance to alkylating agents were reviewed by Connors (1974). In this review, he stated that a) reduced activation or increased deactivation of the drugs, b) reduced formation or increased repair of cytotoxic lesions, c) reduced uptake of the drugs, and d) increased production of other target nucleophiles may explain resistance of tumors to alkylating agents. These reasons may also explain the decreased sensitivity observed by the CHO cells compared to EMT6 and V79 cells after MC and PM treatment. In this dissertation, studies were conducted to investigate the activation of these drugs in EMT6, CHO, and V79 cells and to examine lesions created by these drugs on a potential target molecule, DNA.

In order to assess the importance of MC and PM activation to cytotoxicity,

the activities and amounts of certain enzymes in EMT6, CHO, and V79 cell lines were measured. All three cell lines contained the oxidoreductases: NADPH-cytochrome c reductase, NADH-cytochrome b_5 reductase, cytochrome b_5, and DT-diaphorase, but none contained detectable levels of cytochrome P-450. The EMT6 cells contained the greatest activity of NADPH-cytochrome c reductase and DT-diaphorase, and all three cell lines had comparable activities/amounts of the enzymes of the cytochrome b_5 pathway.

To gain a better assessment of the involvement of the oxidoreductase enzymes in the bioactivation of MC, the ability of sonicates of these cell lines to generate a monoalkylated species from MC was measured using the trapping agent 4-(p-nitrobenzyl)pyridine. All three cell sonicates were able to generate an alkylated species, with the greatest alkylating activity occurring in EMT6 sonicates. The pyridine nucleotide, NADPH, was more efficient as an electron donor than NADH for the generation of the reactive products. Although enzymes of the cytochrome b_5 pathway were present in all three cell lines, these enzymes require NADH as an electron donor. Since NADH was not as efficient an electron donor as NADPH, further studies with NADH-cytochrome b_5 reductase and with cytochrome b_5 were not conducted. Instead, studies were carried out with NADPH-cytochrome c reductase, cytochrome P-450, and DT-diaphorase, enzymes which utilize NADPH as a cofactor. Inhibitors were used to assess the contribution of each of these enzymes in the bioactivation of MC. Cytochrome P-450 was not present in any of the cell lines within the limits of detectability; moreover, carbon monoxide, an inhibitor of cytochrome P-450, did not inhibit the formation of MC-derived metabolites. These results, in conjunction with other work in our laboratory using purified enzyme and whole cells, demonstrated that cytochrome P-450 is not directly involved in the transfer of electrons to MC.

$NADP^+$, a competitive inhibitor of NADPH-cytochrome c reductase, decreased the activity of NADPH-cytochrome c reductase activity completely but did not completely inhibit the formation of alkylating metabolites in EMT6 cell sonicates. On the other hand, mersalyl, an organic mercurial and a nonspecific inhibitor of NADPH cytochrome c reductase, did not decrease activity of NADPH-cytochrome c reductase completely, but totally inhibited the formation of reactive metabolites in EMT6 cell sonicates. It is possible that mersalyl did not inhibit the formation of reactive metabolites of MC but instead the active MC intermediates may have bound to the sulfhydryl groups in mersalyl and limited the availability of MC to alkylate the trapping agent. The $NADP^+$ and mersalyl data, however, support the concept that NADPH-cytochrome c reductase activity is responsible for some of the bioactivation of EMT6 cells, but that at least one other enzyme is also involved.

Studies involving dicoumarol, a potent inhibitor of DT-diaphorase, demonstrated inhibition of the activity of this enzyme in EMT6 cell sonicates without decreasing the generation of reactive metabolites. In fact, the reactive product(s) generated from MC increased significantly in the presence of dicoumarol, suggesting that DT-diaphorase may metabolize MC to a non-toxic substance. Further work in our laboratory with whole cells has demonstrated that both MC and dicoumarol in combination increased the toxicity of MC to hypoxic cells. These results suggest that DT-diaphorase is not involved in the activation of MC to a reactive species in EMT6 cell sonicates.

These studies do not suggest a relationship between the enzyme activity and the generation of reactive metabolites from MC and the extent of MC-induced cytotoxicity observed in EMT6, CHO, and V79 cells. These studies were done, however, using cell sonicates and, therefore, may not be directly comparable to

what is occurring in whole cells. The lack of correlation between the rate at which sonicated cells generate reactive species and the degree of cytotoxicity of MC to cells indicates that other events may be contributing to the bioactivation of MC. Thus, xanthine oxidase or other cellular reductases may also be involved in the bioactivation of MC, and contribute to the increase in cytotoxicity observed in EMT6 and V79 cells. Alternatively, it is possible that there is an enzyme(s) present in the CHO cells which deactivates MC and this may be responsible for the decreased cytotoxicity observed in CHO cells. More fundamental than enzyme activities/amounts in these cells is the possibility that differing levels of the pyridine nucleotide NADPH or other cofactors needed for reduction of these drugs exist in these cell lines. Finally, the interaction between activated MC and 4-(p-nitrobenzyl)pyridine may not completely reflect reactivity leading to cytotoxicity. This may be due to the fact that this assay largely measures monoalkylations produced by these drugs. It remains possible, however, that the rate of bioactivation of MC and PM may not be the sole determinant of cytotoxicity.

In order to examine the formation and removal of the lesions created by these drugs on a potential target molecule such as DNA, alkaline elution methodology was utilized to study the relationships between DNA interstrand and DNA-protein cross-links to cytotoxicity in hypoxic and aerobic cultured cells after treatment with either MC or PM. This technique has the advantage of also measuring DNA single strand breaks, which may be important in the toxicity of these drugs in air, since reduction and reoxidation of the quinone ring under these conditions generates superoxide, hydrogen peroxide, and hydroxyl radicals.

The results of alkaline elution studies on hypoxic and aerobic EMT6 and CHO cells treated with MC and PM suggest a relationship between cytotoxicity and the formation of DNA interstrand cross-links. In both cell lines, single strand breaks

were not detected; however, the levels of DNA interstrand cross-links present in antibiotic treated EMT6 cells were sufficient to mask single strand breaks which may have occurred during or after drug treatment by causing retention of DNA on the filter. Negligible DNA interstrand and DNA-protein cross-links were observed immediately following MC or PM treatment in CHO cells. In contrast, DNA interstrand cross-links were observed in EMT6 cells after MC and PM treatment under both hypoxic and aerobic conditions. Significantly more DNA interstrand cross-linking was observed under hypoxic conditions than under aerobic conditions immediately following MC or PM treatment.

These studies demonstrate a relationship between the cytotoxicity of these quinone antibiotics to hypoxic and aerobic cells and the degree of DNA interstrand cross-links immediately following drug treatment. Repair of the alkylated lesions may also be important in the cytotoxicity exerted by MC and PM in EMT6, CHO, and V79 cells. A possible explanation for the differential in both cytotoxicity and DNA interstrand cross-links between EMT6 and CHO cells after MC and PM treatment may be that CHO cells are able to repair initial monoalkylations which occur before cross-linking is induced and that EMT6 cells may be deficient in this repair capability. This would be analogous to the ability of certain cells, thought to contain O^6-methyltransferase, to repair the initial monoalkylations of simple alkylating agents. Future studies with these cell lines must include further work on the repair of alkylated DNA.

It is possible that the bioactivation and the DNA interstrand cross-linking observed by these drugs in EMT6, CHO, and V79 cells are only partially responsible for the cytotoxicity observed with these drugs. Future studies are required to determine DNA interstrand cross-links in V79 cells as this cell line demonstrates a differential kill to hypoxic and aerobic cells but does not bioactivate MC to a

reactive species to the same extent as the EMT6 cells. Substantial formation of DNA interstrand cross-links in hypoxic V79 cells in contrast to aerobic V79 cells immediately following MC or PM treatment would indicate that bioactivation by cell sonicates is not the sole determinant in the cytotoxicity observed by these drugs. In contrast, the absence of DNA interstrand cross-links in hypoxic V79 cells relative to aerobic V79 cells would indicate that the formation of DNA interstrand cross-links may be only partially responsible for the cytotoxicity observed by these drugs.

This dissertation has focused on DNA as a potential target molecule and cross-linking as the cytotoxic lesion. In the absence of good evidence that DNA damage is directly responsible for cytotoxicity, it remains possible that cross-linking of other cellular macromolecules including cell membrane components, amino acids, lipids, structural proteins, coenzymes, enzymes, as well as nucleic acids may occur at highly sensitive sites in these molecules and result in cytotoxicity. At the level of the cell membrane, a recent review described reports of alkylating agents not only inhibiting ion and amino acid flux across murine tumor cell lines but also causing elevations of cAMP concentrations thought to be secondary to a cross-linked, inactivated, membrane-bound cAMP phosphodiesterase (Hickman et al., 1984). As these authors indicate, the cell membrane plays a critical role in the control of cell proliferation and differentiation, therefore, it may be important to observe the role of these drugs at the level of the cell surface in EMT6, CHO, and V79 cells. Other studies have demonstrated that DNA precursors are more rapidly alkylated than residues in the DNA duplex (Topal and Baker, 1983). These alkylated precursors may be incorporated into the DNA daughter strand and have significant biological consequences. It is possible that the availability of different DNA precursors to alkylation by MC and PM may be

different in the three cell lines and it would be interesting to analyze the DNA precursors and their alkylated products in these cell lines after MC or PM treatment.

Differential uptake of these drugs may explain the differences in cytotoxicity between hypoxic and aerobic EMT6 cells and also between EMT6 and CHO cells, although previous studies in this laboratory, in collaboration with Dr. Thomas Tritton, have shown that there were no significant differences in the uptake of another quinone antibiotic, Adriamycin, in hypoxic and aerobic S180 cells. Resistance of animal tumors to alkylating agents has been associated with a reduced uptake of the drug, however, the reduction in uptake is often small compared with the degree of resistance (Connors, 1974). Therefore, while it is possible that the CHO cells do take up less drug than EMT6 cells, again this may not be the only factor involved in the decreased sensitivity of these cells to MC and PM. Nonetheless, investigation of the uptake of radiolabeled drugs needs to be accomplished in these cell lines.

Another interesting explanation for the decrease in sensitivity of CHO cells may be that MC and PM form many more non-lethal alkylations in these cells than in EMT6 and V79 cells. Non-lethal alkylations may occur in cells with a higher content of certain nucleophiles, including thiols such as glutathione, which are able to reduce the amount of activated drugs in the cell and, therefore, may play an important role in decreasing the cytotoxicity of these drugs to the cell. It would be important to measure the glutathione levels in these cell lines to determine whether these levels are important in MC and PM cytotoxicity.

In summary, the decreased sensitivity of CHO cells compared to EMT6 and V79 cells to MC and PM may not be due to a single biochemical event. Instead, diminished drug uptake, reduced activation or increased catabolism of the drug,

differences in the rate of formation and repair of critical lesions, and increased production of target nucleophiles may all play a part in the sensitivity or resistance of these cell lines to MC and PM. This dissertation has demonstrated that bioactivation of MC is important in these cell lines for monoalkylation. An important enzyme needed to accomplish this activation is NADPH-cytochrome c reductase, although it is probably not the only enzyme involved. DNA, as a target nucleophile for these drugs, may be important in cytotoxicity as more interstrand cross-links were observed in EMT6 cells than in CHO cells and this correlates well with the cytotoxicity studies. Whether or not other factors are involved in the cytotoxicity observed in aerobic and hypoxic EMT6 and V79 cells and the decreased cytotoxicity observed in aerobic and hypoxic CHO cells will only be determined after future experimentation.

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