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Mechanisms of DNA Strand Breakage and Interstrand Cross-Linking by Diaziridinylbenzoquinone (Diaziquone) in Isolated Nuclei from Human Cells

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ABSTRACT

AZQ had been found to produce DNA strand breaks and interstrand cross-links in intact cells; evidence had indicated that these two DNA lesions arise by different chemical mechanisms and vary independently in degree in different cell types. In the present work, the mechanisms of the production of DNA strand breaks and interstrand cross-links by AZQ were studied in isolated cell nuclei. This system avoided the problem of poor penetration of test substances into cells. The DNA lesions were measured by means of the alkaline elution technique. It was found that the production of DNA strand breaks by AZQ in isolated nuclei required the addition of a reducing agent such as NADPH and was almost completely prevented by superoxide dismutase. This indicates that the mechanism of DNA strand breakage involves transfer of an electron from a reduced form of AZQ to molecular oxygen. Unexpectedly, interstrand cross-linking also was enhanced greatly by previous reduction of AZQ by NADPH or $NaBH_4$. However, this reaction was not inhibited by superoxide dismutase. General alkylating activity of AZQ also was stimulated by reduction; the pH-dependence of this reaction was determined. The mechanism of DNA interstrand cross-linking by AZQ was surmised to stem from alkylation reactions of the two aziridine groups. The findings suggest the possibility that AZQ or related compounds may function as bioreductive alkylating agents which might be selectively toxic to hypoxic tissues.

INTRODUCTION

In the preceding paper (11), we found that AZQ[3] produces DNA strand breaks, interstrand cross-links, and DNA-protein cross-links in several mammalian cell strains. The strand breaks were hypothesized to arise by a free radical mechanism stemming from a reduction-oxidation cycle involving the AZQ quinone function. The cross-links were hypothesized to arise by a bifunctional alkylation mechanism involving the 2 aziridine groups. We observed marked differences among the cell strain in regard to the relative production of DNA strand breaks and cross-links, indicating that intracellular conditions can independently influence processes leading to these 2 types of DNA damage.

In the current work, we investigated the mechanisms by which AZQ produces DNA strand breaks and interstrand cross-links using isolated cell nuclei as the test system. Thus, the problem of poor penetration of test substances into cells is avoided. The role of a free radical mechanism in the production of strand breaks was confirmed.

An unexpected finding was that the alkylating and cross-linking activities of AZQ are enhanced by chemical reduction; this raises the possibility that the drug might be particularly effective in hypoxic tumor tissues. An abstract summarizing this work has been published (10).

MATERIALS AND METHODS

The SV40-transformed human embryo cell line VA-13 was grown and labeled with [^{14}C]thymidine as described in the preceding paper (11).

Preparation of Cell Nuclei. ^{14}C-labeled cells were gently scraped from the flask surface, washed, and resuspended in Buffer B [0.15 M NaCl:5 mM $MgCl_2$:2 mM KH_2PO_4:1 mM ethyleneglycolbis(β-aminoethyl ether)-*N*, *N'*-tetraacetic acid (Sigma Chemical Co.), pH 6.4]. Cells were lysed, and nuclei were isolated as described by Filipski and Kohn (3).

Drug Treatment. AZQ, obtained through the Drug Synthesis and Chemistry Branch, National Cancer Institute, Bethesda, MD, was dissolved in *N*,*N*-dimethylacetamide immediately before use. Maximum *N*, *N*-dimethylacetamide concentration during treatment of nuclei was 0.15% and did not alter the DNA elution patterns. Aliquots of nuclei were diluted into Buffer B prewarmed to 37°; then the drug was added, and the mixture was incubated for 1 hr at 37°. To terminate the treatment, cold Buffer B was added to each sample, and the nuclei were centrifuged for 5 min at 1500 × *g* and resuspended in cold Buffer B.

The superoxide dismutase preparations were purified by DNA-cellulose column (Sigma) equilibrated with Buffer B in order to remove nucleases. The nonadsorbing material containing superoxide dismutase was collected, and the protein concentration was estimated spectrophotometrically at 280 nm.

DNA Damage Measurement. DNA single-strand breaks and DNA interstrand cross-links were measured as described previously (7, 11). The procedures utilized polycarbonate filters (pore size, 0.8 μm) (Nucleopore) and included proteinase K digestion to minimize protein adsorption to the filter.

Determination of Alkylating Activity. The procedure of Wheeler *et al.* (12) was used, with minor changes. AZQ at 0.7 mM was incubated at 65° with 30 mM 4-(*p*-nitrobenzylpyridine) (Sigma) for 1 hr in Buffer B adjusted with NaOH or HCl to the desired pH. In the experiments with AZQ hydroquinone, 0.05 M AZQ stock solutions were mixed with 0.05 M $NaBH_4$ (Fisher Scientific Co.) and immediately added to the alkylation test mixture. At the end of incubation, samples were chilled in ice water; then, 2 ml of acetone and 1 ml of 0.4 M NaOH were added. The blue product was extracted with 5 ml of ethyl acetate, and absorbance of this solution was determined at 540 nm. Alkylating activity was expressed as A_{540} units.

RESULTS

DNA Strand Scission. The procedure used to isolate cell nuclei introduced only an insignificant degree of DNA strand

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[3] The abbreviation used is: AZQ, 3,6-diaziridinyl-2,5-bis(carboethoxyamino)-1,4-benzoquinone (diaziquone).

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breakage. Isolated nuclei were treated with AZQ for 1 hr at 37° in Buffer B. AZQ concentration as high as 200 μM did not increase the small extent of DNA scission that was present in untreated isolated nuclei. However, addition of NADPH to the treatment mixture containing 75 μM AZQ resulted in marked increases in strand scission, depending on the NADPH concentration (Chart 1). NADPH alone did not produce strand scission.

The alkaline elution patterns produced by treatment of isolated nuclei with AZQ plus NADPH had the linearity expected for random DNA scission (Chart 1), as opposed to the curvature which was seen in the previous paper when a population of intact cells was treated with AZQ (11). This is in accord with the possibility that the curvature seen previously in intact cells was due to cell subpopulations with quantitatively different responses to AZQ.

NADPH may reduce the AZQ quinone group, either through the action of reductive enzymes which may be present in the nuclear preparation or by direct chemical reduction. In either case, an electron could be transferred from AZQ to molecular oxygen to generate superoxide. If superoxide formation is a required step in the production of DNA strand breaks, then the strand scission should be prevented by the addition of superoxide dismutase to the reaction mixture. In accord with this expectation, superoxide dismutase (50 μg/ml) almost completely prevented the NADPH-dependent DNA scission by AZQ (Chart 2).

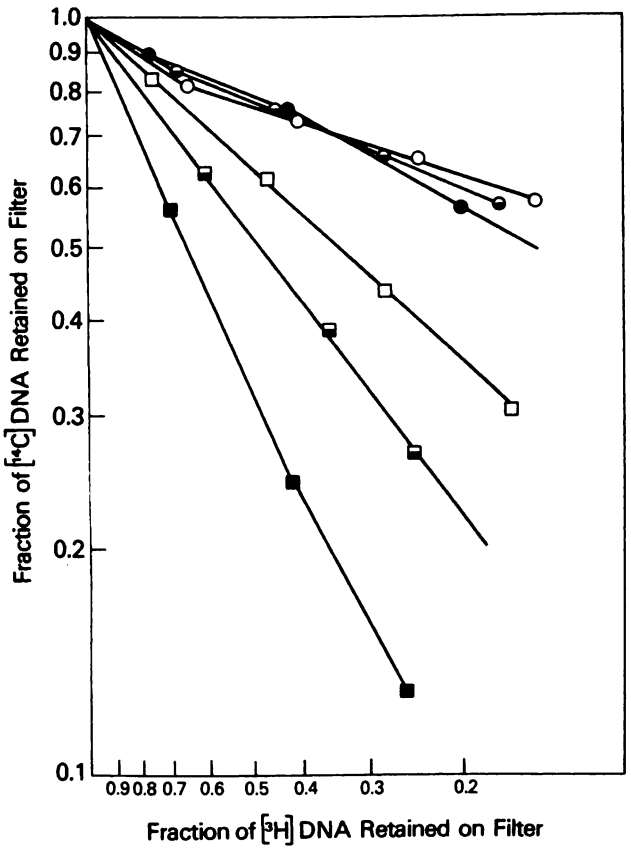

Chart 1. DNA alkaline elution kinetics for isolated nuclei treated with 75 μM AZQ in the presence of different NADPH concentrations. Nuclei were treated for 1 hr at 37° in Buffer B, lysed, and subjected to alkaline elution. ○, untreated control + 1.2 mM NADPH; ⊖, 75 μM AZQ without NADPH; ●, 75 μM AZQ + 0.1 mM NADPH; □, 75 μM AZQ + 0.3 mM NADPH; ⊟, 75 μM AZQ + 0.6 mM NADPH; ■, 75 μM AZQ + 1.2 mM NADPH.

DNA Interstrand Cross-Linking. Interstrand cross-linking was produced by AZQ in isolated nuclei, but only if a reducing agent such as NADPH was added (Chart 3). Superoxide dismutase was also added in order to prevent the production of DNA strand breaks. In the absence of NADPH, no interstrand cross-links

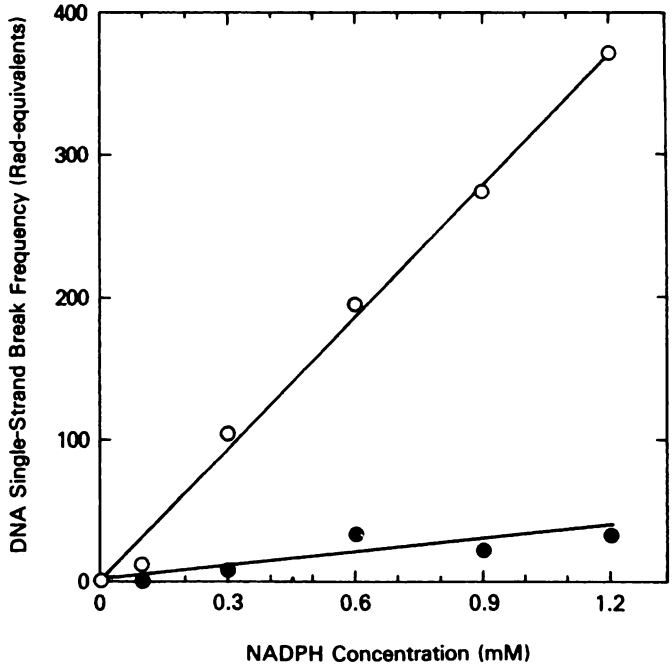

Chart 2. Effect of bovine superoxide dismutase on NADPH-dependent DNA strand break production by AZQ in isolated nuclei. Nuclei were treated with 75 μM AZQ at the indicated NADPH concentrations for 1 hr at 37° and then assayed by alkaline elution. ○, treatment without superoxide dismutase; ●, treatment in the presence of 50 μg of bovine superoxide dismutase per ml (140 units/ml).

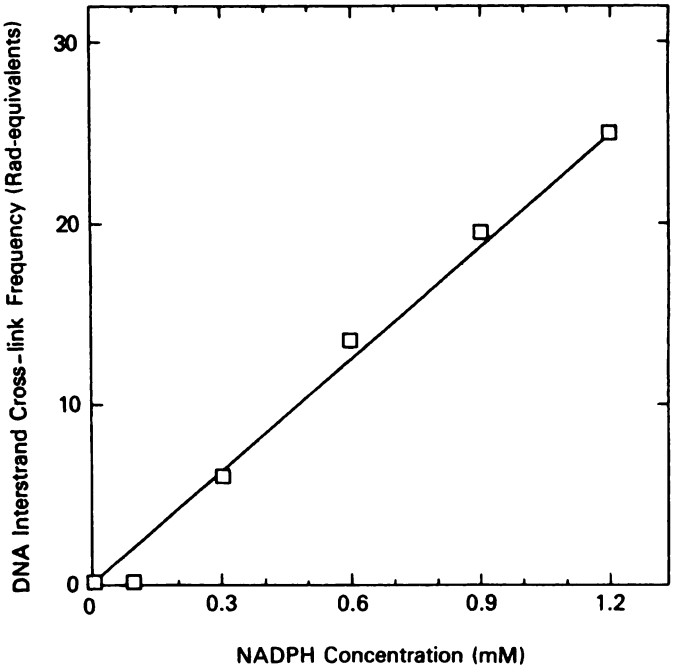

Chart 3. NADPH-dependent production of DNA interstrand cross-links in isolated VA-13 nuclei by AZQ. Nuclei were treated with 75 μM AZQ at various NADPH concentrations in the presence of 50 μg of bovine superoxide dismutase per ml (140 units/ml) for 1 hr at 37° and then irradiated with 300 rads and assayed by alkaline elution.

were formed even by AZQ concentrations as high as 200 μM (data not shown).

When AZQ plus NADPH were preincubated prior to mixing with nuclei, interstrand cross-linking increased with preincubation time up to 3 hr (Chart 4). By shortening the incubation time of nuclei with AZQ plus NADPH to 15 min, interstrand cross-linking in the nonpreincubated mixture was reduced to undetectable levels; interstrand cross-linking was then nearly proportional to the preincubation time (Chart 4). Thus, the reductive activation of AZQ by NADPH can occur nonenzymatically.

Chemical reduction of AZQ was also carried out by mixing AZQ with an equimolar quantity of $NaBH_4$. The reduction was almost instantaneous, as indicated by the loss of color. Reduced AZQ had a markedly enhanced interstrand cross-linking activity in isolated nuclei; significant cross-linking was observed within 5 min (Chart 5).

Alkylating Activity. Chemical alkylating activity was measured using a colorimetric assay for alkylation of 4-(*p*-nitrobenzyl)pyridine. Chart 5 (*inset*) shows the alkylating activity of AZQ tested at different molar ratios of $NaBH_4$ to AZQ. The alkylating activity of AZQ increased markedly in the presence of $NaBH_4$ at molar ratios of 1:1 to 4:1. The molar ratio of 1:1 corresponds to a stoichiometric ratio of 2:1 (reductant:AZQ), and it seems to be sufficient to ensure reduction of almost all AZQ molecules since, immediately after reduction, the characteristic AZQ quinone absorption at 340 nm disappeared. However, at higher molar ratios of 2:1 and 4:1, more alkylated product was found. The excess reducing agent was probably needed to prevent reoxidation of reduced AZQ under aerobic conditions during the 1-hr alkylation period. At a molar ratio of 8:1, the amount of alkylation activity decreased, perhaps due to changes in the 4-(*p*-nitrobenzyl)pyridine substrate or in the alkylation-active AZQ reduction product. Chart 6 shows the dependence of alkylation activity of AZQ and of its reduced form on pH. Both forms exhibited a minimum in the activity *versus* pH curve at pH 6 and loss of activity above pH 10. In the pH range of 6 to 7, the AZQ quinone had about 10 times less alkylating activity than did the reduced form.

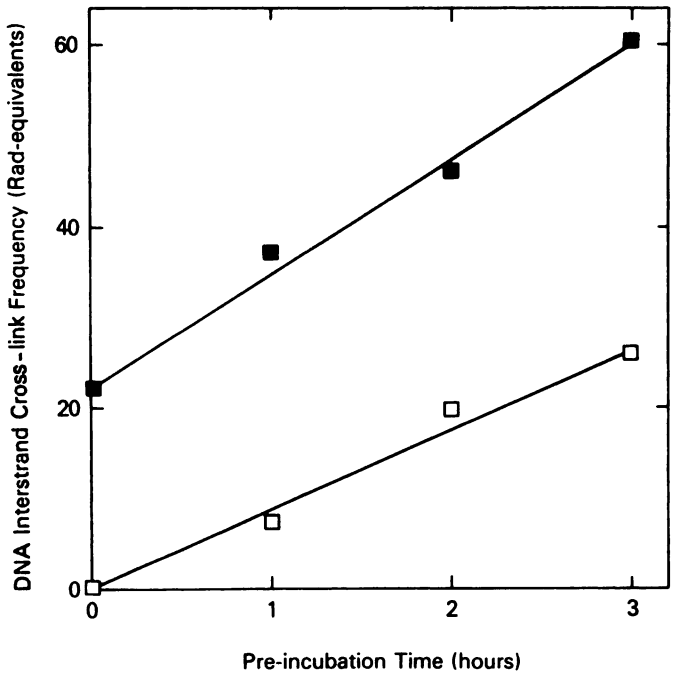

Chart 4. DNA cross-linking by AZQ in VA-13 isolated nuclei following preincubation of the drug with NADPH. AZQ at 95 μM was preincubated in Buffer B with 1.5 mM NADPH for indicated times at 37°, and superoxide dismutase and nuclei were then added. The complete nuclei-incubated mixture, containing 75 μM AZQ, 1.2 mM NADPH, and 50 μg of bovine superoxide dismutase per ml, was incubated for 15 min (□) or 60 min (■) and then irradiated with 300 rads and assayed by DNA alkaline elution.

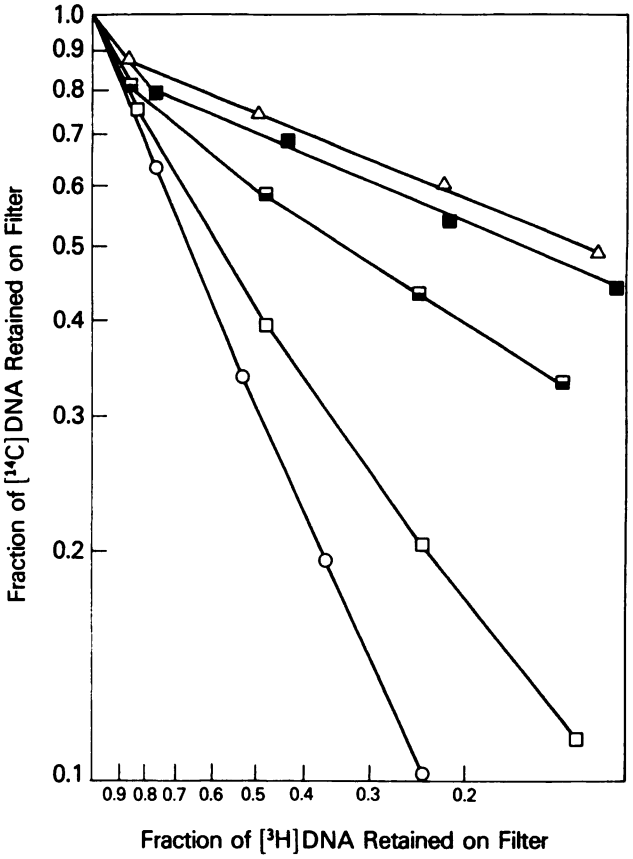

Chart 5. DNA alkaline elution kinetics of VA-13 isolated nuclei exposed to AZQ hydroquinone. Nuclei were treated with 75 μM AZQ reduced with an equimolar amount of $NaBH_4$ for different times at 37°. The incubation mixture contained 50 μg of bovine superoxidase dismutase per ml. ○, untreated control + 300 rads; □, nuclei treated for 5 min + 300 rads; ◪, treated for 15 min + 300 rads; ■, treated for 30 min + 300 rads; △, treated for 45 min + 300 rads.

DISCUSSION

Free-Radical Mechanism of DNA Strand Breakage. The DNA strand breaks produced by AZQ in cells and in isolated nuclei can be attributed to a free radical mechanism that may be stimulated by reductase enzymes but which may also occur by direct chemical action. The addition of a reducing agent such as NADPH was absolutely required for the production of DNA strand breaks by AZQ in isolated nuclei. The reduction of AZQ by NADPH can occur directly, without enzymes. In cells, however, reductive enzymes may speed the reaction.

Gutierrez and Bachur (4, 5) observed an AZQ free radical as a 5-line electron spin resonance spectrum during the enzymatic aerobic reduction of the drug. The reaction appeared to require both NADPH and cytochrome *c* reductase and was accompanied by consumption of oxygen. Further studies, however, indicated that the reaction can occur in the absence of enzyme when

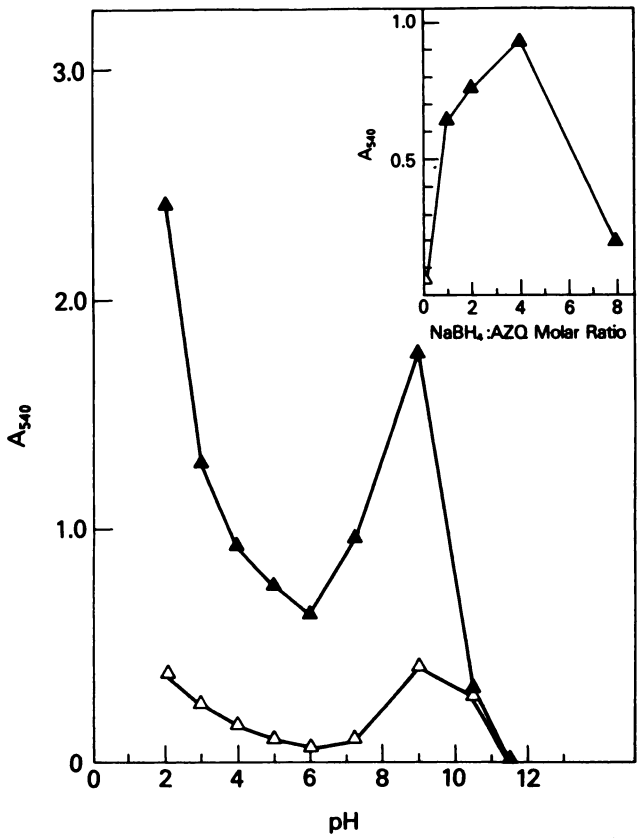

Chart 6. Alkylating activity by AZQ as assayed by measuring the reaction of AZQ with 4-(*p*-nitrobenzyl)pyridine. The test at different pH values was carried out in the absence (Δ) or presence of $NaBH_4$ at molar ratio 1:1 (▲) as described in "Materials and Methods." In the *inset*, the drug alkylating activity was tested at pH 6.3.

higher concentrations of NADPH are used.[4] In the enzymatic or nonenzymatic reduction, an electron is transferred from NADPH or other reducing agent to AZQ to yield a free radical anion which, in turn, is reoxidized by transfer of the electron to O_2, yielding the superoxide radical, $O_2^{\cdot-}$. Superoxide is rapidly destroyed by superoxide dismutase. Since superoxide dismutase almost completely prevented the formation of DNA strand breaks by AZQ plus NADPH in isolated nuclei (Chart 4), superoxide must be involved in the reaction. The superoxide radical is relatively stable and has not been implicated directly in reaction mechanisms such as might produce DNA strand breakage. However, its protonated form, $HO_2^{\cdot}$, ($pK_a = 4.88$) is more reactive (9) and is not excluded as a source of DNA strand breakage.

A much more reactive radical, OH·, can be formed from superoxide in the presence of traces of iron or copper ions (even in chelated form) in a reaction sequence which proceeds via the production of H_2O_2 (2). This mechanism for the production of DNA strand breaks in isolated nuclei by AZQ plus NADPH, however, is excluded by the finding that the strand breakage was abolished by superoxide dismutase, since superoxide dismutase converts superoxide to H_2O_2 which would still be available for the iron-catalyzed formation of OH·.

In intact cells, superoxide production may be greatly accelerated by reductase enzymes. This, together with a greater availability of Fe or Cu, might greatly enhance the production of OH· as well as $HO_2^{\cdot}$ radicals. The marked differences that were observed in the production of DNA strand breaks by AZQ in different cell lines (11) may thus be due to possible differences in the cellular contents of reducing agents, superoxide dismutase, catalase, and/or available iron or copper ions.

Chemical Mechanism of DNA Interstrand Cross-Linking. Akhtar *et al.* (1) reported the interstrand cross-linking of purified phage λ DNA by some diaziridinylbenzoquinone derivatives. In our studies with isolated nuclei, however, we found that AZQ does not produce detectable interstrand cross-linking unless it is chemically reduced, *e.g.*, by NADPH or $NaBH_4$. Unlike the production of DNA strand breaks, the production of interstrand cross-links by reduced AZQ was not prevented by superoxide dismutase. Interstrand cross-linking therefore is not due to superoxide or to its free radical products.

AZQ radicals (such as the semiquinone) may be present but may not be reactive enough to produce covalent bonds with DNA. The more likely possibility is that the cross-links are due to bifunctional alkylation by the 2 aziridine groups, as originally supposed.

The marked enhancement of alkylating activity by reduction of the quinone was also observed in direct chemical assays (Chart 6). The alkylating activity of reduced AZQ was found to be a minimum near pH 6 and to rise markedly when the pH was either lowered or elevated from this value.

These results are in accord with recent independent studies by King and Loo (6), who found, using calf thymus DNA, that the covalent binding of AZQ to DNA, as well as interstrand cross-linking, are markedly enhanced by $NaBH_4$. A pH dependence with a minimum at pH 6 to 7 was observed in the cross-linking of calf thymus DNA by reduced AZQ.

Possible Explanations for the Effects of pH and Reduction on Alkylating Activity. The enhancement of the alkylating activity of AZQ by reduction was unexpected, since it had been assumed that the electron-withdrawing effect of the quinone nucleus on the aziridine nitrogen would suffice to activate the azaridine ring towards nucleophilic attack (8). Indeed, Akhtar *et al.* (1) obtained interstrand cross-linking of purified phage λ DNA by nonreduced diaziridinylbenoquinone derivatives. These derivatives differed from AZQ in that they lacked the carboethoxyamino groups and usually had either alkoxy groups or no substitutions at these positions. A marked enhancement in cross-linking activity was noted when the pH was decreased from 7 to 5. The effects of reduction and of high pH, however, were not reported.

The increased alkylating activity at low pH is as expected for aziridines, due to the good leaving group character of the protonated quaternary nitrogen. It might have been thought that the unshared electron pair of the aziridine nitrogen could be conjugated into the quinone nucleus, thereby activating the aziridine for alkylation. However, this would require the aziridine ring to become coplanar with the quinone, and this may be impossible because the 3-membered ring may be incompatible with a planar sp^2 geometry. The increased alkylating activity of the aziridinylhydroquinone formed by reduction of AZQ might be due to hydrogen bonding of the hydroquinone hydroxy group to the adjacent aziridine nitrogen. The increased alkylating activity at high pH is difficult to explain but might be due to ionization of a hydroquinone hydroxy group, perhaps causing an increase in the hydrogen bond strength between the other hydroquinone hydroxy group and its adjacent aziridine nitrogen.

[4] P. L. Gutierrez, personal communication.

Is AZQ a Bioreductive Alkylating Agent? The preceding paper (11) indicated that DNA interstrand cross-linking may be a significant factor in cell killing by AZQ. Cell strains were found to differ markedly in their responses to AZQ in regard to the extents of interstrand cross-linking and DNA strand breakage. These 2 types of DNA lesions varied independently of each other, indicating that they are influenced by different aspects of the intracellular environment. Cell killing appeared to be related to interstrand cross-linking and not to DNA scission. In the current work, we find that both interstrand cross-linking and DNA strand scission are stimulated by reducing conditions. The reaction mechanisms differ, however, in that strand scission involves the production of superoxide, whereas interstrand cross-linking does not. Strand scission may depend on reduction-oxidation cycling of the AZQ quinone moiety, which could occur enzymatically or nonenzymatically but which would, in either case, be stimulated by molecular oxygen. Interstrand cross-linking, on the other hand, may be favored by reduction of AZQ to the hydroquinone and may therefore be inhibited by molecular oxygen. In view of these considerations, AZQ or chemically related drugs may be bioreductive alkylating agents which may have enhanced cytocidal action in hypoxic tissues. The effect of hypoxia on the cytocidal activity remains to be determined.

The types and extent of DNA damage produced by AZQ in cells would depend on: (*a*) the activity of reductase enzymes; (*b*) the concentrations of thiols and other reducing compounds; (*c*) the concentration of molecular oxygen; (*d*) the availability iron and copper ions; (*e*) the concentrations of free radical quenching compounds; and (*f*) the activity of superoxide dismutase.

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