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## ESR STUDY OF ELECTRON TRANSFER REACTIONS BETWEEN $\gamma$ -IRRADIATED PYRIMIDINES, ADRIAMYCIN AND OXYGEN

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**Abstract**—Solid pyrimidine nucleic acid bases (cytosine, thymine, and uracil) were  $\gamma$ -irradiated (50 KGy) and dissolved in deaerated solutions of adriamycin in water and dimethylsulfoxide (DMSO). Analogous experiments using unirradiated pyrimidines as controls were also performed. In water only  $\gamma$ -irradiated cytosine showed a reaction with the adriamycin yielding a single ESR peak ( $g = 2.0033$ ) consistent with the adriamycin semiquinone radical. Since the unirradiated cytosine gave no reaction, the result suggests an electron transfer from cytosine radicals (generated by  $\gamma$ -radiolysis) to adriamycin. In DMSO the three  $\gamma$ -irradiated and unirradiated pyrimidines reacted with adriamycin yielding the adriamycin semiquinone radical observed by ESR. These results suggest that in DMSO an electron is transferred to adriamycin from the pyrimidine radicals and from the parent pyrimidine molecules. However, the process is on the order of  $10^3$  times more efficient for the pyrimidine radicals. Superoxide radicals ( $O_2^{\cdot-}$ ) were formed following addition of oxygen to the deaerated DMSO solutions containing adriamycin semiquinone radicals.  $O_2^{\cdot-}$  was spin trapped using 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO). The results show a possible reaction sequence in which an electron transferred to adriamycin, by pyrimidine radicals and parent pyrimidine molecules, is subsequently transferred to dissolved oxygen.

**Keywords**—ESR, Spin trapping,  $\gamma$ -Radiolysis, Pyrimidines, Adriamycin, Oxygen, Free radicals

### INTRODUCTION

The direct effect of ionizing radiation on DNA generates mainly guanine and thymine radicals.<sup>1-5</sup> These radicals have been observed in solid DNA samples using electron spin resonance (ESR) spectroscopy. However, the exact fate of the guanine and thymine radicals when DNA is in solution is still unclear. One possibility of interest to this work, is the reaction of these radicals with other molecules in the solution generating products which may be harmful to cells. For this reason and as a model, the reaction between  $\gamma$ -irradiated pyrimidine bases with adriamycin was studied.

Adriamycin was chosen for two reasons: i) it is one of the most widely used antitumor agents functioning primarily through its ability to intercalate into DNA disrupting DNA and RNA synthesis.<sup>6-8</sup> The intercalated adriamycin is in close proximity to the DNA

bases, therefore, knowledge about the direct interaction between bases and adriamycin is of interest. Furthermore, the direct interaction between  $\gamma$ -irradiated bases and adriamycin is of interest because of the possible enhancement of combined radiation and adriamycin therapies; ii) Adriamycin participates in oxidation reduction reactions via free radical mechanisms. Several reports have shown the formation of superoxide radicals ( $O_2^{\cdot-}$ ) when adriamycin is incubated with cellular components.<sup>9-12</sup> In these cases adriamycin is first reduced forming the semiquinone radical which rapidly reacts with oxygen to form  $O_2^{\cdot-}$ . Superoxide is also formed via the oxidation of photoexcited adriamycin in air-saturated aqueous solutions and in air-saturated aqueous solutions containing pyrimidine bases.<sup>13, 14</sup> However, in the absence of oxygen the pyrimidines are oxidized, thus reducing the photoexcited adriamycin.<sup>14</sup>

Superoxide and the adriamycin semiquinone radical are potentially lethal to cells. The disproportionation of  $O_2^{\cdot-}$  produces hydrogen peroxide which in the presence of trace amounts of metals generates hydroxyl radicals ( $\cdot OH$ ).<sup>15-18</sup> Hydroxyl radicals are powerful ox-

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idants with no known enzyme for their removal. On the other hand, the intercalation into DNA of the adriamycin semiquinone radical is known to cause DNA strand scission.<sup>19</sup>

Since intercalated adriamycin may be found in various environments, the reactions of adriamycin with pyrimidines (cytosine, thymine, and uracil) and with their radicals, formed by  $\gamma$ -radiolysis, were carried out by dissolution of the pyrimidines or their radicals in deaerated aqueous or dimethylsulfoxide solutions of adriamycin. In addition to providing an aprotic environment, DMSO allows the dissolution of larger amounts of pyrimidine bases. The reactions involving free radical intermediates were studied using ESR. The results show a possible reaction sequence in which an electron is transferred from the pyrimidines to adriamycin and subsequently to dissolved oxygen.

#### MATERIALS AND METHODS

The pyrimidine bases and adriamycin were obtained from Sigma (St. Louis, MO). The spin trap 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) was purchased from Aldrich (Milwaukee, WI). DMPO was purified using the method described by Buettner and Oberley.<sup>20</sup> In this method aqueous DMPO solutions are repeatedly treated with activated charcoal until free radical impurities are eliminated as verified by ESR. The DMPO concentration was measured spectrophotometrically ( $\lambda = 227$  nm,  $\epsilon = 8 \times 10^3$  M<sup>-1</sup> cm<sup>-1</sup>).<sup>21</sup> For experiments in DMSO, samples of pure DMPO were dissolved directly into dry DMSO and contained no free radicals also verified by ESR. The DMSO was dried over calcium hydride overnight.

Pyrimidine base radicals were obtained by room temperature  $\gamma$ -radiation of cytosine, thymine, and uracil powders at a dose rate of 51 Gy/min to a total dose of 50 KGy. As observed by ESR, the pyrimidine radicals formed most likely originate from  $\pi$ -anions. Sevilla has shown that upon warming of glasses containing a mixture of  $\pi$ -cations and  $\pi$ -anions of 5-methyluracil (thymine) the ESR spectrum of the  $\pi$ -cation is lost, however, the spectrum for the  $\pi$ -anion persists.<sup>22</sup> In addition, spin density calculations have shown that the highest spin density for the pyrimidine anions is at the C(6) carbon.<sup>22, 23</sup>

Experiments requiring the absence of oxygen were carried out in an apparatus described by Russell et al.<sup>24</sup> and Evans.<sup>25</sup> This apparatus consists of a "U" tube connected to an ESR flat cell (60  $\times$  10  $\times$  0.25 mm) via a ground glass joint. Adriamycin was placed in one stem of the "U" tube and any one of the  $\gamma$ -irradiated pyrimidines (cytosine, thymine, or uracil) was placed in the other stem. Nitrogen-saturated water or DMSO were added to the adriamycin and the "U" tube was

sealed. Nitrogen bubbling through the adriamycin solution was then continued for 20 min. Pure water was obtained from a Sybron/Barnstead NANO pure II water system and the DMSO was obtained from Aldrich (Gold Label or HPLC Grade). Therefore, unless the adriamycin contained trace metal impurities, which is unlikely as verified by the optical absorption spectrum of adriamycin solutions, there were virtually no trace metal ions in the solutions. Following deaeration with nitrogen, the adriamycin solution was transferred to the stem containing the  $\gamma$ -irradiated pyrimidine powder. The base was rapidly dissolved in the adriamycin solution by stirring with a magnetic stirring bar or a vortex mixer. After complete dissolution of the pyrimidine powder, the "U" tube was inverted and the reaction mixture was transferred into the ESR flat cell and its ESR spectrum recorded. Control experiments were done under identical conditions using nonirradiated pyrimidine bases. Control experiments were also carried out with adriamycin solutions alone and gave no ESR signals.

For reactions requiring oxygen, DMPO was added to the reaction mixture to a final concentration of 0.15 M prior to saturation with oxygen (1–2 min bubbling). The reaction mixture was then saturated with nitrogen (~2 min) to prevent the spin adduct ESR line broadening caused by dissolved oxygen.

The radical yields of adriamycin and of the solid  $\gamma$ -irradiated pyrimidines was determined by double integration of the first derivative ESR spectrum. For adriamycin radicals a solution of 3-carbamoyl-2,2,5,5-tetramethyl-1-pyrrolidone-1-yloxy free radical (3-CAR) was used as the standard. This stable nitroxide has been used previously as an ESR standard for determining unknown radical concentrations.<sup>21</sup> For the solid  $\gamma$ -irradiated pyrimidines, a homogeneous solid mixture of 3-CAR and KCl as described by Lion et al.<sup>26</sup> was used as the standard. Using the method described by Hall,<sup>27</sup> the accuracy of the double integrations was  $\pm 10\%$ .

The ESR spectra were recorded on a Varian E-9 X-band spectrometer at 100 KHz magnetic field modulation. *g*-Value measurements were carried out using  $\alpha, \alpha'$ -diphenyl- $\beta$ -picrylhydrazyl (DPPH) as a standard ( $g = 2.0036$ ).

#### RESULTS AND DISCUSSION

Room temperature  $\gamma$ -irradiation of cytosine, thymine, and uracil powders generates pyrimidine radicals which most likely originate mainly from  $\pi$ -anions. Spin density calculations have shown that the highest spin density for pyrimidine anions is at the C(6) carbon.<sup>22, 23</sup> Sevilla has shown that upon warming of glasses containing a mixture of  $\pi$ -cations and  $\pi$ -anions

of 5-methyluracil (thymine) the ESR spectrum of the  $\pi$ -cation is lost, however, the spectrum of the  $\pi$ -anion persists.<sup>22</sup>

When solid  $\gamma$ -irradiated pyrimidine bases are dissolved in oxygen-free adriamycin solutions, an electron is transferred from the pyrimidine to adriamycin (Fig. 1). Figure 1a shows the ESR spectrum obtained following dissolution of  $\gamma$ -irradiated cytosine (0.025 M) in deaerated aqueous adriamycin (7 mM) solutions. The single ESR line with  $g = 2.0033$  is consistent with the previously observed adriamycin semiquinone radical in water.<sup>11, 12</sup> The semiquinone radical was observed only in the reaction involving  $\gamma$ -irradiated cytosine but not for  $\gamma$ -irradiated thymine or uracil. It is possible that the reaction in water may be more efficient for  $\gamma$ -irradiated cytosine. Since the control experiments involving adriamycin and nonirradiated cytosine (0.025 M) gave no ESR signals, it is concluded that the semiquinone adriamycin radical (Fig. 1a) is formed in the reaction between cytosine radicals (generated by  $\gamma$ -radiolysis) and adriamycin. The result of similar experiments carried out in deaerated adriamycin solutions in DMSO is shown in Fig. 1b. In this case the reaction occurs following dissolution of any one of the  $\gamma$ -irradiated pyrimidine bases (cytosine, thymine, or uracil) in the adriamycin (3.5 nM) solution. Although an ESR spectrum for the reduced adriamycin radical in DMSO has not been reported, the hyperfine structure observed in Fig. 1b is similar to that of the chemically reduced adriamycin analog, daunomycin, semiquinone radical.<sup>28, 29</sup> In addition to the ESR spectrum of the adriamycin semiquinone radical, a single ESR line with  $g = 2.00$  is observed in Fig. 1b. This additional ESR spectrum is typical of charge transfer processes between organic molecules.<sup>30</sup> It must be noted that control experiments under the same conditions using nonirradiated pyrimidines also yield the adriamycin semiquinone radical. Therefore, the result shown in Fig. 1b suggests that in DMSO an electron is transferred to adriamycin from pyrimidine radicals (produced by  $\gamma$ -radiolysis) and from the parent pyrimidine molecules. However, as will be shown in other experiments in this work, the electron transfer process is on the order of  $10^5$  times more efficient for the pyrimidine radicals than for the parent pyrimidine molecules. When DMSO solutions containing the adriamycin semiquinone radical are saturated with oxygen in the presence of added spin trap DMPO (0.15 M), the semiquinone ESR spectrum rapidly disappears and a new ESR spectrum is obtained (Fig. 1c). This twelve-line ESR spectrum has hyperfine coupling constants,  $a_N = 0.127$  mT,  $a_H^\beta = 0.103$  mT and  $a_H^\alpha = 0.013$  mT, corresponding to the  $O_2\cdot^-$  spin adduct of DMPO (DMPO- $O_2\cdot^-$ ).<sup>31</sup> This result suggests that an electron

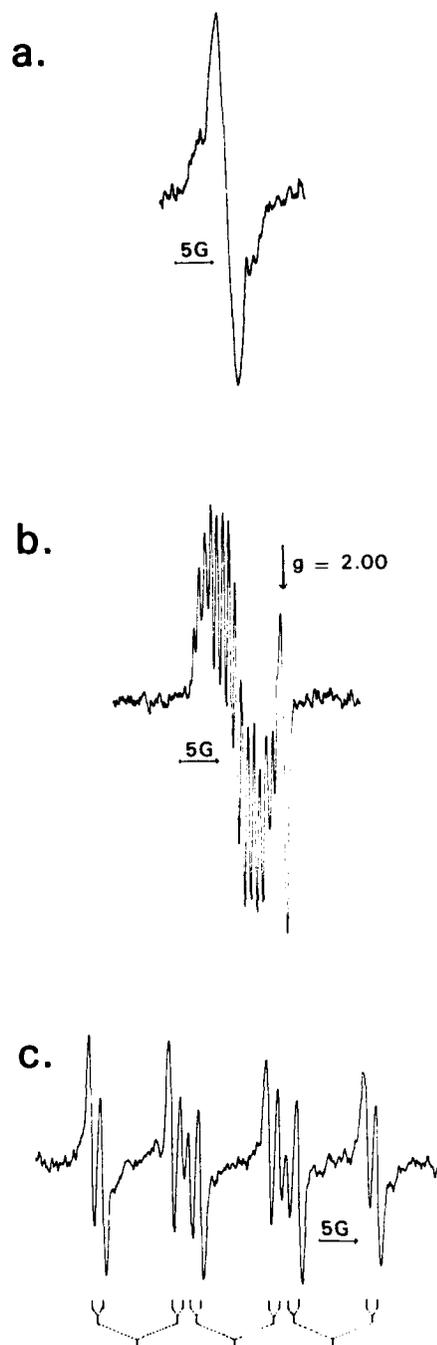


Fig. 1. (a) ESR spectrum of the adriamycin semiquinone radical in water. Spectrum obtained following dissolution of  $\gamma$ -irradiated cytosine (0.025 M) in deaerated aqueous adriamycin solutions. (b) ESR spectrum of the adriamycin semiquinone radical in deaerated DMSO. Spectrum obtained following dissolution of any one of the  $\gamma$ -irradiated pyrimidine bases in deaerated adriamycin solutions. Cytosine, 0.09 M; thymine, 0.4 M; uracil, 0.4 M. (c) ESR spectrum of the DMPO- $O_2\cdot^-$  spin adduct in DMSO. Spectrum obtained following the addition of DMPO (0.15 M) to the deaerated DMSO solution of adriamycin containing the semiquinone radical formed in (b). After the addition of DMPO the solution was saturated with oxygen. Instrument settings: magnetic field, 340.0 mT; modulation amplitude, 0.1 mT for (a), 0.05 mT for (b) and (c); microwave power, 4 mW for (a), 0.4 mW for (b) and 10 mW for (c); receiver gain,  $3.2 \times 10^7$  for (a),  $5 \times 10^7$  for (b) and  $6.3 \times 10^8$  for (c).

was transferred from the semiquinone radical to dissolved oxygen yielding  $O_2^{\cdot -}$ . In an analogous experiment in water using  $\gamma$ -irradiated cytosine to generate the semiquinone radicals, the ESR spectrum of the  $DMPO-O_2^{\cdot -}$  was not observed. However, it is known that  $DMPO-O_2^{\cdot -}$  is unstable in water and rapidly decomposes forming  $DMPO-OH$ .<sup>32, 33</sup> The  $DMPO-OH$  ESR spectrum consists of a 1:2:2:1 quartet ( $a_N = a_H^{\beta} = 1.49$  mT) and is different from the  $DMPO-O_2^{\cdot -}$  ESR spectrum.<sup>29</sup> Because the yield of adriamycin semiquinone radicals was low when produced in water in the reaction between cytosine radicals and adriamycin, it is possible that the yield of  $DMPO-O_2^{\cdot -}$  and of its decomposition product,  $DMPO-OH$ , are too low in the reaction mixture to be observed by ESR. It is also possible that the  $DMPO-O_2^{\cdot -}$  was not observed in the aqueous reaction mixture due to the decomposition of  $DMPO-O_2^{\cdot -}$  by  $O_2$ .<sup>34</sup>

The electron transfer in DMSO from pyrimidine radicals and parent pyrimidine molecules to adriamycin is not immediate and can be followed over a period of time. Therefore, it is important to determine whether there are differences between the ability of cytosine, thymine, and uracil radicals to reduce adriamycin and also if there are differences between the parent pyrimidines' ability to reduce adriamycin. For this purpose, powder samples of  $\gamma$ -irradiated cytosine, thymine, and uracil adjusted to contain an equal quantity of radicals ( $1.5 \times 10^{15}$ ), were each dissolved in different adriamycin (3.5 nM) in DMSO. Analogous control experiments using nonirradiated cytosine, thymine, and uracil were also studied. Figure 2 shows, as a function of time, the formation of adriamycin semiquinone radicals originating from the pyrimidine radicals and parent pyrimidine molecules. The adriamycin semiquinone radicals originating only from the reactions involving the pyrimidine radicals was determined as the difference in the results obtained from the experiments using nonirradiated pyrimidines (controls) and those using  $\gamma$ -irradiated pyrimidines. Control experiments using deaerated solutions containing only adriamycin gave no ESR signals. The results in Figure 2 also indicate that the difference between the reduction of adriamycin by thymine or uracil radicals is small and that the process is more efficient for cytosine radicals. It must be noted that the initial concentration of pyrimidine radicals ( $2.5 \mu M$ ) dissolved in the deaerated adriamycin solutions, is slightly larger than the adriamycin semiquinone radical yield at 30 min originating from cytosine radicals, therefore, explaining the curvature observed in the data obtained from the experiment involving cytosine radicals (Fig. 2).

Although in DMSO the electron transfer to adriamycin occurs from pyrimidine radicals and parent py-

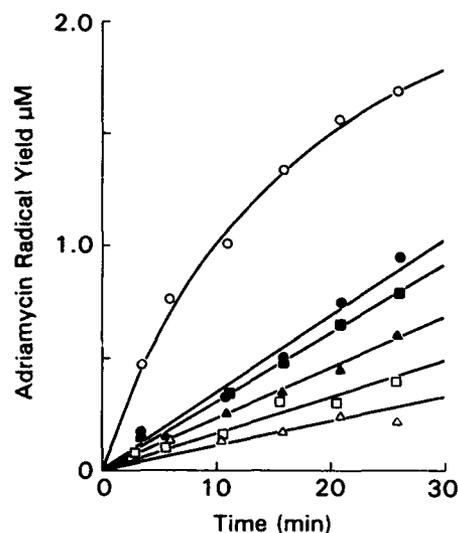


Fig. 2. Adriamycin radical yield as a function of time.  $\gamma$ -Irradiated pyrimidine bases, containing parent pyrimidine molecules and pyrimidine radicals, were dissolved in deaerated adriamycin (3.5 mM) solutions in DMSO. Open symbols correspond to reactions between pyrimidine radicals and adriamycin. Closed symbols to the reaction between neutral pyrimidines and adriamycin. The amount of  $\gamma$ -irradiated pyrimidines was adjusted to contain equal number of pyrimidine radicals ( $1.5 \times 10^{15}$ ). (○) cytosine radicals; (□) thymine radicals; (△) uracil radicals; (●) cytosine; (■) thymine; (▲) uracil.

rimidine molecules, the quantity of pyrimidine radicals ( $1.5 \times 10^{15}$ ) dissolved is smaller than the quantity of parent pyrimidine molecules ( $5.4 \times 10^{19}$  for cytosine and uracil;  $8.1 \times 10^{19}$  for thymine). Figure 3a shows the adriamycin semiquinone yield normalized for the initial concentration of pyrimidine radicals dissolved in the deaerated adriamycin solution. Likewise, Fig. 3b (plotted on the same scale as Fig. 3a) shows the adriamycin semiquinone radical yield in the controls (Fig. 2) normalized for the pyrimidine molecules present. The results indicate that the electron transfer to adriamycin is approximately  $10^5$  times more efficient from pyrimidine radicals than from parent pyrimidine molecules.

The reaction between pyrimidine radicals and adriamycin in DMSO is far more efficient than the reaction using parent pyrimidines. In order to determine the optimal adriamycin concentration for the electron transfer process, from pyrimidine radicals to adriamycin, a fixed quantity of pyrimidine radicals was dissolved in several deaerated adriamycin solutions (0.8–7 mM). The increase in intensity of the adriamycin semiquinone radical ESR spectrum was measured at various time intervals. The intensity of the ESR spectrum is directly proportional to the adriamycin semiquinone radical yield. Figure 4 shows the semiquinone radical yield in time following the dissolution of  $\gamma$ -

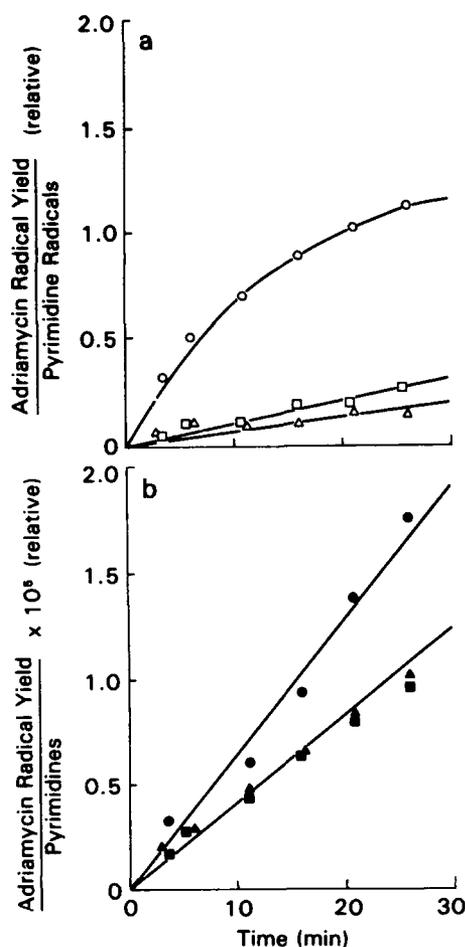


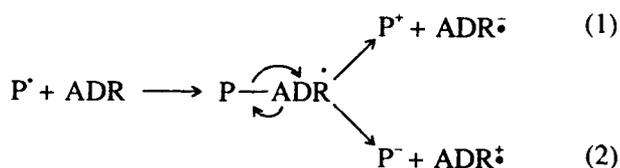
Fig. 3. Adriamycin radical yield (normalized for the quantity of pyrimidine radicals or neutral pyrimidines) vs. time. Reaction conditions are the same as in Fig. 2. (a) Normalized for pyrimidine radicals: (○) cytosine; (□) thymine; (△) uracil. (b) Normalized for neutral pyrimidines: (●) cytosine; (■) thymine; (▲) uracil.

irradiated thymine (0.4 M) in the deaerated adriamycin solutions. The results indicate that the maximum interaction between thymine radicals and adriamycin occurs using 3.5 mM adriamycin (Fig. 4, Insert). Similar results were obtained in experiments using  $\gamma$ -irradiated cytosine and uracil.

Pyrimidine radicals generated by  $\gamma$ -radiolysis of pyrimidine powders are unstable when dissolved and rapidly disappear. This property, in addition to the observed results (Figs. 2 and 3) showing that the reduction of adriamycin occurs over a period of time and most likely via a direct interaction between the pyrimidine radicals and adriamycin, suggests that a pyrimidine–adriamycin complex may be formed prior to the electron transfer to adriamycin. It is conceivable that such a complex could stabilize the pyrimidine radicals sufficiently for their reaction with adriamycin to occur.

Since the reaction between cytosine radicals and adriamycin yields adriamycin radicals approaching the initial concentration of cytosine radicals dissolved (Fig. 2), it is probable that most of the pyrimidine radicals rapidly interact with adriamycin prior to their decomposition. In addition, the results shown in Fig. 3 suggest that if the formation of such a complex were possible, this interaction would have to occur approximately  $10^5$  times more efficiently for pyrimidine radicals than for parent pyrimidine molecules.

A possible mechanism for the electron transfer from pyrimidine radicals ( $P^\cdot$ ) to adriamycin (ADR) can be explained following the reaction scheme for the oxidation or reduction of organic molecules by free radicals described by Steenken<sup>15</sup>:



In general, the mechanism first involves the covalent bonding between  $P^\cdot$  and ADR to form an inter-

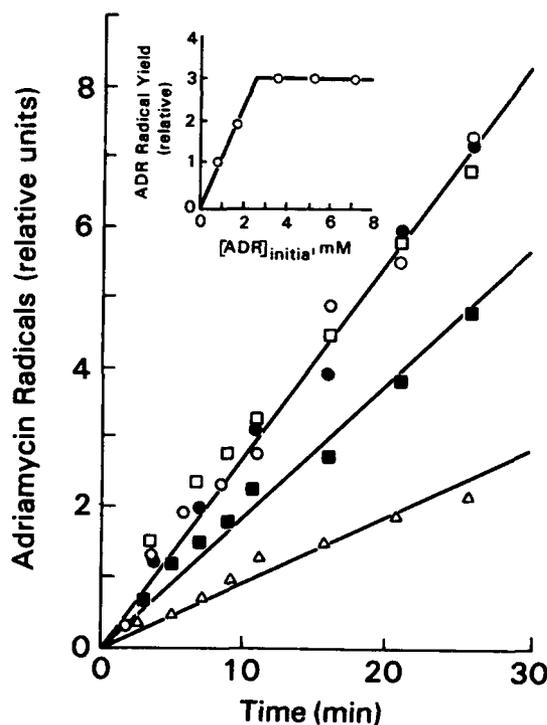


Fig. 4. Adriamycin radical yield in deaerated DMSO solutions as a function of time. A constant amount of  $\gamma$ -irradiated thymine was dissolved in various deaerated adriamycin solutions in DMSO. Thymine concentration: 0.4 M. Adriamycin concentrations: (●) 6.9 mM; (○) 5.2 mM; (□) 3.5 mM; (■) 1.7 mM; (△) 0.86 mM. Insert: adriamycin radical yield at any time ( $t$ ) versus initial concentration of adriamycin.

mediate, P-ADR<sup>•</sup>, which subsequently undergoes heterolysis leading to the products in reaction (1) or (2) depending on whether the electron pair shared by P-ADR<sup>•</sup> goes to ADR [reaction (1)] or to the pyrimidine [reaction (2)]. The experimental results shown in this work favor the mechanism given in reaction (1) in which adriamycin is reduced forming the semiquinone radical. Pyrimidine radicals in which the highest spin density is at the C(6) carbon are considered reducing species.<sup>35</sup> Figure 1b shows the ESR spectrum of the adriamycin semiquinone radical indicating that adriamycin is reduced by the pyrimidine radicals. In addition, Fig. 1b also shows an ESR spectrum ( $g = 2.00$ ) upfield from the semiquinone radical ESR spectrum which is characteristic of a charge-transfer type complex between organic molecules.<sup>30</sup> This suggests that a species similar to P-ADR<sup>•</sup> is formed. It is conceivable that a similar mechanism could apply for the reaction between the parent pyrimidine molecules and adriamycin. However, as shown in Figs. 2 and 3 this process is less efficient than the process involving pyrimidine radicals.

Fluorescence-quenching experiments were attempted to confirm the presence of an intermediary pyrimidine adriamycin complex in deaerated DMSO solutions. The results of these experiments showed no appreciable quenching of the adriamycin emission spectrum, even at pyrimidine concentrations 100 times larger than the adriamycin concentration. However, the ESR spectrum ( $g = 2.00$ ) in Fig. 1b upfield from the semiquinone radical ESR spectrum supports the formation of such a complex.

Although the experiments in this work have shown that in an aprotic environment (DMSO) the reduction of adriamycin by the parent pyrimidine molecules is not an efficient process, it is possible that this may not be the case when adriamycin is intercalated into hydrophobic regions of DNA. Whereas the pyrimidine and adriamycin molecules in solution are in rapid motion possibly making strong complex formation more difficult, the adriamycin intercalated into DNA is locked into position nearby the nucleic acid bases. This proximity could facilitate a direct interaction between the bases and adriamycin. A similar argument could be made for the pyrimidine radicals formed by the effects of ionizing radiation on DNA. However, the results suggest that the subsequent reactions following a base-adriamycin interaction is far more efficient for the pyrimidine radicals than for the parent pyrimidine molecules.

Finally, the results obtained in this work show that it may be possible at least for pyrimidine radicals formed by the effects of ionizing radiation on DNA, to react with molecules other than oxygen in solution.<sup>36</sup>

In this case the pyrimidine radicals react with a DNA intercalating agent, adriamycin, forming the adriamycin semiquinone radical. This radical intercalated into DNA causes DNA strand scission.<sup>19</sup> Furthermore, the adriamycin semiquinone radical was shown to react with dissolved oxygen. This reaction generates O<sub>2</sub><sup>•-</sup> which may disproportionate and lead to the formation of hydroxyl radicals which are known to cause irreversible DNA damage and are also lethal to cells.

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